EARLY BIRDS OR NIGHT OWLS

GROUP 2 INNATE LYMPHOID CELLS IN ALLERGIC AIRWAY INFLAMMATION

Bobby W.S. Li

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Allergische Luchtweginflammatie
Vroege Vogels of Nachtuilen

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INTRODUCTION

CHAPTER 1

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Bobby W.S. Li, Rudi W. Hendriks
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Asthma phenotypes

Asthma is a chronic inflammation of the airways caused by a combination of genetic predisposition and environmental factors, affecting some 235 to 300 million people worldwide (1-3). Symptoms vary between individuals, but are often characterized by episodes of coughing, wheezing and shortness of breath. The term "asthma" encompasses a group of clinical symptoms and for a long time it was widely believed that asthma represented an allergic, eosinophilic and T helper type 2 (Th2)-mediated disease. However, mechanistic studies show that it is a heterogeneous condition with multiple subtypes that require distinct treatment modalities (reviewed in ref. 3). A hallmark of allergic asthma is airway hyperresponsiveness (AHR), which can be triggered by inhalation of allergens such as house dust mite (HDM), animal dander, pollen or fungal spores (1-3). This is typically associated with eosinophilic inflammation in the airways and increased numbers of eosinophils in the circulation that correlate with AHR in the clinic (4, 5). Persistent inflammation eventually leads to airway remodeling due to repair processes, most notably subepithelial fibrosis, smooth muscle hyperplasia, mucous cell metaplasia and increased angiogenesis (6).

To date, our knowledge on the pathophysiology of many asthma phenotypes is incomplete, but ongoing research efforts are expected to result in more targeted and personalized therapeutic approaches. This thesis will focus on type 2 immunity in allergic asthma, which is the most common and best studied form of asthma.

Key players in the initiation of allergic asthma

Airway epithelial cells make up the frontline defense that separates the host and the environment and are therefore essential in the control of inflammatory responses to allergens that induce asthma (reviewed in ref. 7). Epithelial cells express a wide variety of pattern recognition receptors that recognize pathogen-associated or damage-associated molecular patterns. For example, HDM allergens such as Der p II and Der p VII activate toll-like receptor 4 (TLR4) signaling that in turn promotes nuclear translocation of nuclear factor-κB (NF-κB), which controls a wide range of inflammatory genes (7). Using irradiated chimeric mice, it has been shown that HDM-induced asthma requires the presence of TLR4 on radioresistant lung structural cells and not on hematopoietic cells such as dendritic cells (DC) (8). Triggering of TLR4 on epithelial cells by HDM induces the production of various cytokines including thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1a (IL-1a), IL-1β, IL-25 and IL-33. *In vivo* experiments as well as air-liquid interface cultures of bronchial epithelial cells demonstrated that TLR signals induce the release of IL-1a, which then initiates an autocrine feedback loop to trigger production of other cytokines, including GM-CSF and IL-33 (9). A common effect of these cytokines is the activation of DCs towards a phenotype that promotes Th2 immunity (7). Additionally, DCs can be directly activated as they continuously

sample the airway lumen by forming dendritic extensions. Activated DCs are able to initiate sensitization in concert with the epithelium via antigen presentation to naïve T cells in the draining lymph nodes. It appears that activated DCs have intrinsic capacities to drive Th1 or Th2 responses. When DCs recognize bacterial or viral products via TLRs, they produce IL-12 and induce Th1 polarization. In contrast, DCs may sample inhaled allergens and initiate adaptive Th2 responses in asthma (7).

Th2 differentiation

Central to the initiation of Th2 differentiation is IL-4, which induces the Th2 master regulator GATA binding protein 3 (GATA3) through signal transducer and activator of transcription 6 (STAT6) (10, 11). The transcription factor GATA3, which is necessary and sufficient to instruct Th2 differentiation, acts in cooperation with various other nuclear proteins to induce the production of IL-4, IL-5 and IL-13 and to suppress Th1 development. A suggested mechanism for this activity is that GATA3 causes chromatin remodeling in the Th2 cytokine locus, in which the genes encoding IL-4, IL-5 and IL-13 are clustered. Intriguingly, the induction of GATA3 by the IL-4/STAT6 axis in differentiating Th2 cells raises the paradox that IL-4 is required for the generation of the cell type that is its major producer. Although IL-4 has long been thought to control Th2 cell development, the initial events resulting in IL-4 release *in vivo* and the initial source of IL-4 under physiological conditions remain to be identified. While innate immune cells might provide a source of IL-4, Th2 responses can be generated (i) when only T cells can make IL-4, and (ii) in mice lacking a functional IL-4 receptor signaling pathway, arguing against a requisite role for an external source of IL-4 (12). Inflammatory DCs are essential for the induction of Th2 immunity and features of asthma whereas basophils, which have the capacity to produce IL-4, are not required and do not take up inhaled antigen to present it to T cells (13). Although remarkably little is known about the initial pathways that induce IL-4 or GATA3 in activated T cells *in vivo*, elegant experiments by Amsen *et al*. provide evidence that DCs use the Notch signaling pathway in T cells to instruct their differentiation (14, 15). DCs expressing the Notch ligand Jagged induce Th2 differentiation independently of IL-4, whereas DCs expressing Delta-like ligand induce the alternative Th1 cell fate. Notch signaling in T cells leads to activation of the nuclear effector recombination signal binding protein for immunoglobulin kappa J region (RBPJκ), which binds to regulatory elements inducing *Gata3* and *Il4* gene expression. Interestingly, blocking Notch activity through intranasal administration of γ-secretase inhibitor reduced allergic airway inflammation in mice (16).

Once an individual has been sensitized to a specific allergen, re-exposure to the allergen activates primed Th2 cells, which are thought to play a central role in orchestrating an allergic immune response through secretion of the type 2 cytokines IL-4, IL-5 and IL-13. In this setting, IL-4 stimulates B cells to produce antigen-specific IgE, which then binds to the high-affinity FceRI on mast cells, enabling them to be fully activated and to release histamine,

leukotrienes and prostaglandins (17, 18). IL-5 is able to influence eosinophil survival, activation, differentiation and recruitment from the bone marrow into the tissues. It has been shown that when activated, eosinophils also release leukotrienes similar to mast cells, which act as potent bronchoconstrictors and this combination of leukotrienes and histamines further enhances AHR and airflow obstruction (17). IL-13 impacts airway epithelial and smooth muscle cells, where it mediates AHR, mucus hypersecretion and subepithelial fibrosis. In addition, IL-13 induces production of several matrix metalloproteinases, demonstrating its importance for airway remodeling in typical asthma (19). Th2 lymphocytes are regarded as the central cell type that orchestrates and amplifies allergic inflammatory events however, this pathway fails to explain why asthmatics experience increased frequency and severity of exacerbations during viral infections of the airways, such as respiratory syncytial virus, rhinovirus or influenza, which typically evoke a type 1 response.

Identification of group 2 innate lymphoid cells

Alternative ways to induce a type 2 response have been discovered and could be the reason therapies aimed at depletion of T cells have shown limited success in asthma patients (20). In fact, the first observation of a non-B/non-T cell population in *Rag2*$^{-/-}$ mice that had the capacity to produce IL-5 and IL-13, but not IL-4, was made by Fort *et al.* (21). They found that intraperitoneal injection of IL-25 induced type 2 cytokines and a Th2-like response, characterized by increased serum IgE, IgG1 and IgA, blood eosinophilia and pathological changes in the lungs and digestive tract. Subsequently, a previously unrecognized cell population staining positive for intracellular IL-5 was identified when IL-25 was given intranasally in *Rag2*$^{-/-}$ mice, but not in *Rag2*$^{-/-}$ mice lacking the common γ (γ_c) chain (22). A few years later these cells were found to provide an important source of type 2 cytokines critically involved in *Nippostrongylus brasiliensis* expulsion (23). Moreover, administration of IL-33 in *Rag2*$^{-/-}$ mice efficiently induced AHR, goblet cell hyperplasia and eosinophilic infiltration in the lungs via IL-4, IL-5 and IL-13 (24). In 2010, four independent groups almost simultaneously described Th2 cytokine producing non-B/non-T cells in detail. Moro *et al.* characterized a lineage-negative cell population that expresses Sca-1, c-Kit (CD117), IL-2Ra (CD25), IL-7Ra (CD127) and IL-33R (T1/ST2) in fat-associated lymphoid clusters and coined them natural helper cells. These natural helper cells were able to produce large amounts of typical Th2 cytokines like IL-5 and IL-13 in response to IL-2 and mediated protection against parasitic worms (25). Neill *et al.* reported nuocytes in the mesenteric lymph nodes that expanded *in vivo* in response to IL-25 and IL-33 and were an early source of IL-13 before T cell induction during helminth infection. Mice deficient in IL-25 and IL-33 manifested a severely impaired ability to expel *Nippostrongylus brasiliensis*, which could effectively be rescued by adoptive transfer of isolated nuocytes (26). Similar cells were described by Price *et al.* who also found them in the spleen and liver and named them innate helper type 2 cells (27). Although most

molecular surface markers are shared between natural helper cells, nuocytes and innate helper type 2 cells, subtle differences may exist (28, 29). However, now it is generally agreed that they can be categorized under group 2 innate lymphoid cells (ILC2) (29, 30). In addition, Saenz *et al*. observed that IL-25 also promotes the accumulation of a lineage-negative Sca-1^{+} CD117^{int} multipotent progenitor cell population in gut-associated lymphoid tissue that induces Th2 responses. As these multipotent progenitor cells have the capacity to differentiate into monocyte/macrophage and granulocyte lineages, they appear to be distinct from ILC2s (31).

Development of ILC2s

The ILC2s belong to a novel family of developmentally related ILCs (**Fig. 1**). A feature that these lymphocytes have in common is the absence of RAG-dependent rearranged antigen receptors and the lack of classic lineage markers on their cell surface. ILCs are classified into three groups based on their signature cytokines and the transcription factors that regulate their development and function (29, 32). Group 1 ILCs (ILC1) are characterized by the production of interferon gamma (IFN-γ) and highly express the transcription factor T-bet. The well-known natural killer (NK) cell is a prototypical member of this group, but other ILC1 subsets that are phenotypically and developmentally distinct from NK cells have been recognized (33). Group 3 ILCs (ILC3) produce IL-17 and/or IL-22 and are dependent on transcription factor retinoic acid receptor-related orphan receptor gamma (RORγt). A prominent member of this heterogeneous group of cells is the lymphoid tissue inducer cell, which plays a critical role in the formation of secondary lymphoid organs during embryogenesis (34). Several other ILC3 subsets have been discovered, including RORγt^{+}NKp46^{+} cells secreting IL-22 and RORγt^{+}NKp46^{-} cells producing both IFN-γ and IL-17 (35-38). Whether ILC1s and ILC3s are distinct and stable cell populations or whether they are different forms of the same plastic cell type remains to be elucidated (29). Especially since ILC3s may switch from IL-22 to IFN-γ production, whereby RORγt expression is progressively lost and the transcription factor T-bet is upregulated and essential for IFN-γ expression (39-41). It is thought that ILCs arise from common lymphoid progenitors in the bone marrow, which are Lin^{-}IL-7Rα^{+}Flt3^{+}. ILC2s require inhibitor of DNA binding 2 (ID2) for their development, which functions as an inhibitor of transcriptional activity of basic helix-loop-helix E proteins, such as E12, E47, HEB and E2-2. Deficiency studies indicate that lack of ID2 results in an absence of NK cells, RORγt^{+} ILCs and ILC2s (25, 42-44). *Rorc*-deficient mice have normal ILC2 numbers (25, 26), but the structurally related transcription factor RORa was shown to be important for ILC2 development in the bone marrow (45, 46). Although *Rora*-deficient mice appear to be able to develop low numbers of cells with an ILC2 phenotype, these are unable to proliferate in response to IL-25 stimulation (45). Interestingly, Notch signaling which is known to be a master regulator of T cell lineage commitment in the thymus, is also of particular importance in ILC2 development *in vitro* to block B cell potential in addition to repressing differentiation of several other lineages (45, 47).

Moreover, ILC2 development also requires T cell factor 1 (TCF-1) encoded by the *Tcf7* gene, a transcription factor that is implicated in T cell lineage specification; *Tcf7*$^{-/-}$ mice lack ILC2s and are unable to mount ILC2-mediated type 2 immune responses (48). The physiological role of Notch in ILC2 development *in vivo* remains to be determined, but these findings indicate that ILC2s may be closely related to T cells. This is also reported by the identification of GATA3 as a critical early regulator of ILC2 development (49, 50). Conditional deletion of the GATA3 gene in established Th2 cells showed that GATA3 is critical for the expression of IL-5 and IL-13, but not of IL-4 (51). Likewise, GATA3 deletion in ILC2s abolished the expression of IL-5 and IL-13 in the mouse and Mjosberg *et al.* showed that GATA3 is crucial for function of human ILC2s (52, 53). Using an inducible GATA3 ablation strategy it was shown by Hoyler *et al.* that intestinal ILC2 development and homeostasis required GATA3 expression (49). Analysis of chimeric mice, as well as mice overexpressing GATA3 at the common lymphoid progenitor stage, demonstrated an essential and dose-dependent role for GATA3 in ILC2 development (50). Collectively, these results identify GATA3 as a critical early regulator of ILC2 development, thereby extending the paradigm of GATA3-dependent control of type 2 immunity to include both innate and adaptive lymphocytes.

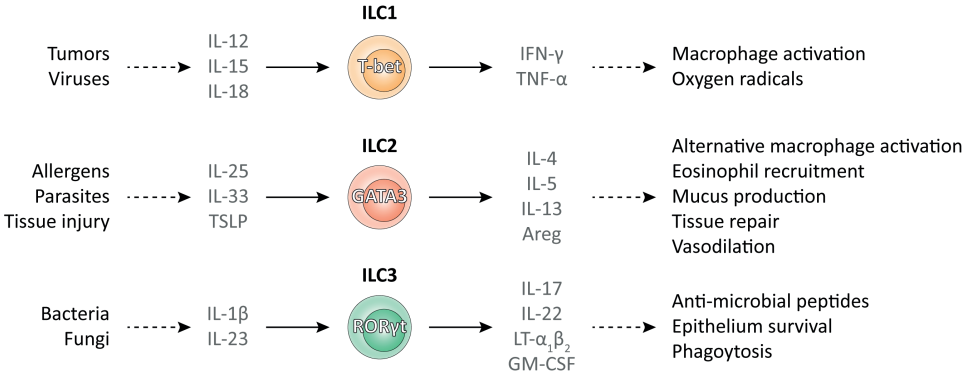

Figure 1. Activation and function of the ILC family.

An overview of signals that activate ILC1s, ILC2s and ILC3s and the effector function of these cells. The cytokines produced as well as the transcription factors that regulate ILC1s, ILC2s and ILC3s mirror that of Th1, Th2 and Th17 cells, respectively. Therefore, ILCs are considered innate counterparts of T cells. Adapted from (54).

ILC2s in allergic lung inflammation

After the identification of ILC2s in the gut, a similar population of type 2 cytokine-producing cells was described in the respiratory tract in the context of influenza virus infection in mice and allergic rhinitis in humans (55-57). Furthermore, it was shown that local or systemic administration of IL-25 or IL-33 induced proliferation of ILC2s as well as production of IL-5 and IL-13 effector cytokines by these cells (58-63). *In vivo* transfer experiments demonstrated

that IL-13 produced by ILC2s was sufficient to mediate IL-33-induced airway inflammation (27, 61, 62). These findings are relevant for asthma in humans as well, because polymorphisms in the *Il17rb* gene encoding one of the chains of the IL-25 receptor have been associated with asthma (64). Additionally, IL-33 levels correlate with asthma severity and both the *Il33* gene and the *Il1rl1* gene (encoding the IL-33 receptor chain T1/ST2) have been associated with asthma susceptibility in humans in large-scale genome-wide association studies (GWAS) (65-69). In these studies, the *Il1rl1* locus was also associated with atopic dermatitis and allergic rhinitis. Nevertheless, provoking type 2 immunity by intranasal administration of IL-25 or IL-33 generates an acute response and does not reflect the complex process of allergic sensitization and response in a physiological situation. Therefore, several other mouse models have been employed to study the role of ILC2s in allergic airway inflammation, including asthma induced by the ovalbumin (OVA) protein, fungal allergens derived from *Alternaria alternata*, glycolipid antigens from *Sphingomonas* bacteria that can stimulate natural killer T cells and the protease papain (70). Papain has proteolytic functions by cleaving tight junctions between epithelial cells, thereby gaining access to underlying dendritic cells and possibly promoting the production of endogenous danger signals by the epithelium. It induces asthma symptoms in mice mediated by ILC2s, independent of B or T lymphocytes and is known to cause occupational asthma (59, 71). Taken together, these findings showed that ILC2s have a critical role in particular mouse models for asthma in which allergic lung inflammation was studied in *Rag*-deficient mice, in the absence of functional B and T lymphocytes. However, the contribution of ILC2s in allergy in the context of an intact adaptive immune system is less well studied. We have shown in wildtype mice that in HDM-induced allergic asthma, the ILC2 population in lung and bronchoalveolar lavage (BAL) fluid increased significantly in size and that ILC2s were a major source of IL-5 or IL-13. Also in OVA-induced asthma, the contribution of ILC2s and Th2 cells to the total production of IL-5 or IL-13 appeared in the same range (60). The ILC2s may therefore be critical for the induction of allergic airway inflammation in the lung, even in models where T cells were previously thought to be the main producers of IL-5 and IL-13 (**Fig. 2**). In contrast, upon induction of asthma through ovalbumin or HDM only a minor proportion of IL-4$^+$ cells were ILC2s. Nevertheless, it has been reported that ILC2s have the capacity to produce IL-4 in response to TSLP or leukotriene D4 (53, 72, 73). Much less in known about ILC2s in lung inflammation in humans. Cells with an ILC2 phenotype were described in healthy human lung parenchymal tissue and bronchoalveolar lavage fluid from lung transplant recipients (56). Although ILC2s were found to be enriched in nasal polyps from patients with chronic rhinosinusitis and in skin lesions from patients with atopic dermatitis, the involvement of ILC2s in the pathogenesis of asthma in humans remains undefined (57, 74).

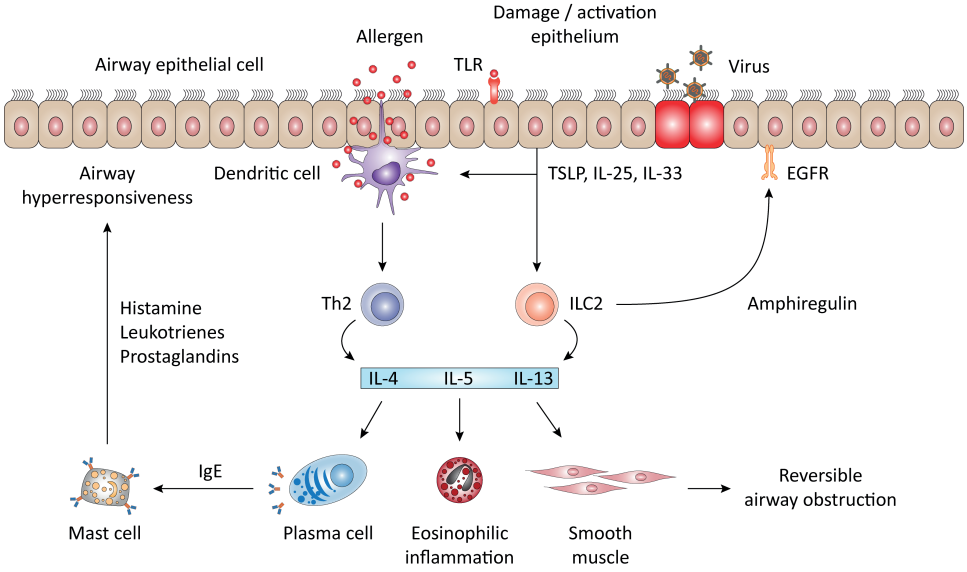

Figure 2. The role of ILC2s in allergic airway inflammation.

Allergens or viral infections trigger the epithelium to express the stress signals TSLP, IL-25 and IL-33. In response to these cytokines, ILC2s undergo proliferation and produce large amounts of IL-4, IL-5 and IL-13. Additionally, DCs also stimulate naïve T cells towards a Th2 phenotype. IL-4 and IL-13 cooperate to activate B cells that produce allergen-specific IgE, which binds to the high affinity FcεRI on mast cells. Upon secondary allergen exposure, primed mast cells degranulate and release histamine, leukotrienes and prostaglandins that are responsible for airway hyperresponsiveness. Furthermore, IL-5 is a chemoattractant for eosinophils, which are well-equipped to cause inflammation and IL-13 enhances smooth muscle cell contractility resulting in airway obstruction. The ILC2s may also produce amphiregulin and can therefore play a role in restoring epithelial integrity after viral infections.

ILC2s in pulmonary infection

In 2011, Chang *et al.* provided evidence for a critical role of ILC2s in the development of AHR induced by influenza in mice. They demonstrated that H3N1 influenza virus infection acutely induced AHR, independently of Th2 cells and adaptive immunity. The AHR response required IL-13 and IL-33 and was associated with airway neutrophils and macrophages, but not with eosinophils. By gating on lineage-negative T1/ST2^{+}c-Kit^{+}Sca-1^{+}CD25^{+}CD90.2^{+} lymphocytes, ILC2 numbers were shown to increase in the lungs and peak on day 5–6 when production of IL-5 and IL-13 and AHR was also strongest. Depleting ILC2s using a monoclonal antibody against Thy-1/CD90.2 in *Rag2*$^{-/-}$ mice, abolished the H3N1-induced AHR response. Conversely, AHR was fully reconstituted when purified ILC2s were adoptively transferred back into the recipient mice (55). The relationship between influenza virus infection and increased ILC2 numbers in the lung was confirmed by Monticelli *et al.*, who employed a H1N1 PR8 strain of influenza virus. Strikingly, in this study IL-33 receptor blockade or depletion of ILC2s with anti-Thy-1/CD90.2 antibodies in *Rag1*$^{-/-}$ mice during influenza virus infection resulted in

decreased lung function, lower blood oxygen saturation levels and loss of epithelial integrity, which suggests a previously unknown restorative role of ILC2s. These effects were effectively countered upon adoptive transfer of lung ILC2s, but appeared independent of IL-13. The authors further investigated the role of ILC2s in the maintenance of epithelial integrity by performing genome-wide transcriptional profiling of lung-resident ILC2s. From this analysis multiple genes emerged that were differentially expressed and were associated with wound repair. It was then suggested that amphiregulin, a member of the epithelial growth factor family, may be a key cytokine produced by ILC2s which mediates restoration of lung function (**Fig. 2**). Indeed, direct delivery of amphiregulin resulted in a significantly improved outcome for influenza virus-infected *Rag1*$^{-/-}$ mice depleted of ILC2s (56). The finding that ILC2s are recruited and activated during viral infection to promote local repair may be relevant to explain the phenomenon that asthma exacerbations can be triggered by viral respiratory tract infections. Moreover, it is conceivable that repeated infection may result in sustained activation of ILC2s and type 2 immunity.

Interactions of ILC2s with other immune cells

ILC2s are gaining increasing recognition and the evidence gathered implicates them in the pathogenesis of allergic asthma and provides clues to their contribution to fighting viral infections and restoration of the airway epithelium. Interestingly, ILC2s were also found in the skin, where they carry out a dual role as an immune regulator and a pro-inflammatory effector cell and were shown to functionally interact with mast cells. Stimulated dermal ILC2s promote an eosinophil influx and mast cell activation and lead to spontaneous dermatitis in areas routinely exposed to body fluids (72, 74). It remains unknown whether ILC2s also interact with mast cells in the context of allergic airway inflammation. In response to allergens or viruses, ILC2s are activated by a number of cytokines produced by epithelial cells and DCs, of which IL-25, IL-33 and TSLP are most studied. However, ILC2s in the lungs and bone marrow also express the cysteinyl leukotriene receptor 1 (CysLT1R) and challenge with leukotriene D_4, which binds to CysLT1R, was shown to increase the proportions of IL-5$^+$ ILC2s in the lungs (73). On the other hand, ILC2s in peripheral blood of humans express the pro-resolving G protein-coupled receptors, N-formyl peptide receptor ALX/FPR2 and chemokine receptor-like 1 (CMKLR1). Their ligands, such as lipoxin A_4 (LXA_4), have anti-inflammatory functions by inhibiting neutrophil activation and regulation of epithelial cytokine release. Importantly, LXA4 was found to effectively decrease the release of IL-13 by ILC2s (75). These findings therefore highlight a potential therapeutic strategy to control asthma in patients who do not respond to corticosteroids. Nevertheless, a large number of questions still need to be answered, especially regarding the interactions between ILC2s and the adaptive immune system. Knowledge on interaction between ILC2s and Th2 cells may be important for controlling allergic asthma. It has been reported that ILC2s can express MHC class II however, interaction between ILC2s and

Th2 cells is largely unexplored (31, 63). Interestingly, functional analyses revealed that RORγt$^+$ ILCs can process and present antigen in the context of MHC class II and thereby limit commensal bacteria-specific CD4$^+$ T cell responses (76). Despite the obvious central role of type 2 immunity in allergic asthma, the efficacy of humanized antibodies directed against the individual cytokines IL-4, IL-5 and IL-13 appears disappointing (77). Because of the function of GATA3 as a master regulator in both Th2 cells and ILC2s, inhibiting its function and thereby targeting all Th2 cytokines may be an attractive treatment option for asthma in humans. In this context, the observed capacity of the glucocorticoid fluticasone to inhibit GATA3 translocation from the cytosol to the nucleus would be an excellent starting point for drug discovery strategies (78).

AIMS AND OUTLINE OF THE THESIS

ILC2s are essential for the immune response to helminth infections in the gut and in the lung and have been implicated in the immunopathology of several atopic diseases including asthma. They enhance type 2 immune responses due to their ability to secrete high levels of IL-5 and IL-13 as described above.

In the experiments described in this thesis we aim to define the phenotype and to characterize the role and function of ILC2s in asthma using both robust HDM-induced allergic airway inflammation mouse models and clinical data derived from asthma patients. The field of ILC2s has not standardized the surface markers required to identify ILC2s and approaches often vary between research groups. Therefore, in **Chapter 2** we outline our definition of ILC2s in our mouse models and provide a detailed description of the procedures required for the analysis of these cells.

The innate nature of ILC2s in combination with evidence from particular mouse models lacking functional B and T cells have suggested that ILC2s are an early source of type 2 cytokines that can act independently of adaptive immunity. However, the behavior of ILC2s in asthma induced by the physiological allergen HDM is unclear, particularly because in HDM-induced allergic airway inflammation Th2 cells are thought to play a key role. Thus, in **Chapter 3** we aim to identify the relationship between ILC2 and T cell activation in acute asthma in HDM-driven allergic airway inflammation.

ILC2s not only arise rapidly, e.g. in response to IL-33 *in vivo*, but also in chronic HDM-mediated allergic airway inflammation in a completely different microenvironment, characterized by B and T lymphocyte infiltration and organization as well as tissue remodeling. We therefore aim to identify differences in phenotype and function of ILC2s in acute and chronic airway inflammation. We use genome-wide expression profiling to provide evidence for phenotypic and functional heterogeneity of ILC2s in **Chapter 4** by highlighting the presence of CD25^{low} and CD25^{high} ILC2s in the airways.

Influenza is one of the major causes of asthma exacerbation and ILC2s have been proposed to be the missing link between a Th1-mediated antiviral response and a prototypical Th2 disease.

In **Chapter 5**, we investigate this phenomenon *in vivo* in a mouse model combining influenza virus infection and chronic HDM-driven asthma.
In **Chapter 6** we use genome-wide analyses of gene expression profiles as well as epigenome to address two different questions in the field of ILC2 biology. First, the issue of ILC heterogeneity and plasticity: multiple ILC1 and ILC3 subsets are known to exist and have been well described in the literature. In contrast, ILC2s are considered more stable and their heterogeneity has been sparsely examined. Second, we aim to obtain epigenetic evidence, both in mouse and in human, for a role of ILC2s in human asthma, by integrating epigenetic analyses and genetic GWAS data.
Furthermore, the composition of peripheral blood immune cells as well as cells in induced sputum, reflecting the local immune response, in asthmatic individuals is illustrated in **Chapter 7.** In particular, we aim to define the ILC2 presence in controlled, partially controlled and uncontrolled asthma patients.
Finally, the implications of our findings concerning the role of ILC2s in airway inflammation in mouse as well as human studies are put into perspective in **Chapter 8**.

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CHARACTERIZATION OF GROUP 2 INNATE LYMPHOID CELLS IN ALLERGIC AIRWAY INFLAMMATION MODELS IN THE MOUSE

CHAPTER 2

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Characterization of group 2 innate lymphoid cells in allergic airway inflammation models in the mouse
Bobby W.S. Li, Dior M.J.M. Beerens, Maarten D. Brem, Rudi W. Hendriks
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ABSTRACT

Allergic asthma is a chronic inflammatory lung disease mediated by type 2 cytokines produced by T helper 2 (Th2) cells as well as the recently discovered group 2 innate lymphoid cells (ILC2). Due to a lack of unique markers, the accurate phenotypic characterization and quantification of ILC2 requires a comprehensive panel of fluorescently labeled antibodies. The markers that are currently used to characterize ILC2 have not been standardized and often vary between research groups, which poses significant challenges when comparing data. Intranasal administration of the pro-inflammatory cytokine IL-33 in mice is associated with strong, Th2 cell-independent ILC2 activation. ILC2 are also activated in mouse models of allergic asthma based on the physiologically relevant house dust mite (HDM) allergen, which parallel eosinophilic airway inflammation observed in asthma patients. Here, we describe the analysis of ILC2 by flow cytometry in these two commonly used allergic airway inflammation models in the mouse.

INTRODUCTION

Asthma is a disease of the airways involving chronic inflammation and remodeling and is characterized by episodes of coughing, wheezing, and shortness of breath. Patients can be clustered into several endotypes based on symptoms, disease mechanisms and immunological profile, the most common being allergic asthma (1). The T helper 2 (Th2) cell is classically placed in the center of the pathophysiology of allergic asthma. The Th2 signature cytokines IL-4, IL-5, and IL-13 are key orchestrators of the hallmarks of asthma. These include persistent inflammation, smooth muscle cell hyperplasia, mucous cell metaplasia, and airway hyperresponsiveness and remodeling (2). Recently, innate counterparts of Th2 cells referred to as group 2 innate lymphoid cells (ILC2) have been identified as an innate source of type 2 cytokines and have been hypothesized to contribute to the pathogenesis of allergic asthma (3, 4).

Observations that a non-B/non-T cell population was capable of producing IL-5 and IL-13 in response to IL-25 were first made by Fort et al. in 2001 (5). It was reported some years later that this IL-25-dependent population is an important source of type 2 cytokines and that these cells are critically involved in helminth expulsion (6). In 2010, several independent research groups characterized this novel non-B/non-T lymphocyte population in fat associated lymphoid clusters, mesenteric lymph nodes, spleen, and liver and found them to be highly responsive to both IL-25 and IL-33, although different names were assigned to the cells at the time (7-9). Cells with a similar phenotype and cytokine profile were also discovered in the lung in the context of influenza infection (10, 11). A universal nomenclature was proposed and it is now generally accepted that in the mouse ILC2 are negative for classic hematopoietic lineage markers, express Sca-1, CD117 (c-kit), CD25 (IL-2Ra), CD127 (IL-7Ra), and T1/ST2 (IL-33R) on their cell surface, and are dependent on the transcription factor GATA3 (7-9, 12-15).

IL-25/IL-33-responsive ILC2 have also been found in human lungs and are enriched in the nasal polyps of patients suffering from chronic rhinosinusitis, a typical type 2 inflammatory disease (16). These human ILC2 are defined by the expression of the seven-transmembrane prostaglandin D2 receptor CRTH2/CD294 and the natural killer marker CD161 and also require GATA3 (16, 17). More recently, studies in asthmatic patients revealed increased numbers of ILC2 in peripheral blood and sputum, compared with healthy controls (18-20). Moreover, this population appeared to be steroid insensitive, which is in agreement with in vitro studies showing that TSLP is a major determining factor in steroid sensitivity of ILC2 (20).

To explore the role of ILC2 in the complex inflammatory processes of allergic asthma, a number of mouse models have been developed that mimic allergy and asthmatic responses to physiological allergens. Intranasal administration of the protease allergen papain leads to activation of ILC2 and induces eosinophilic airway inflammation in wild-type as well as T and B cell-deficient *Rag1*$^{-/-}$ mice. However, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice that additionally lack ILC2 do not mount an inflammatory response to papain and adoptive transfer of wild-type ILC2 into

these mice restored inflammation (21). In addition, ILC2-derived IL-13 promotes migration of activated lung dendritic cells towards the lymph nodes where subsequent T cell priming occurs, further suggesting a central role for ILC2 as early initiators of inflammation (22). Experimental asthma models using the fungal allergen *Alternaria alternata* have led to similar results, showing an IL-33-mediated inflammatory response orchestrated by activated ILC2 rather than Th2 cells (23, 24). Together with data showing that ILC2 are capable of enhancing Th2 cell differentiation and function, these findings support a model in which ILC2 can become rapidly activated to provide an early source of type 2 cytokines and bridges the gap between innate and adaptive immunity (25, 26). In contrast, *Rag1*$^{-/-}$ mice exposed to *Aspergillus fumigatus*, another fungal allergen, fail to develop airway inflammation and hyperresponsiveness. Moreover, ovalbumin-driven airway inflammation is attenuated upon depletion of CD4^{+} T cells (27, 28). In house dust mite (HDM)-induced allergic airway inflammation, blockade of CD28 signaling strongly diminishes airway hyperresponsiveness and pulmonary infiltrates of inflammatory cells in the lung (29). Interestingly, chronic exposure to a cocktail of *Alternaria*, *Aspergillus*, and HDM synergistically induces airway inflammation, hyperresponsiveness, and remodeling that is dependent on adaptive immunity (30). Taken together, these models instead indicate a critical role for T cells in mediating and maintaining allergic airway inflammation. Therefore, the contribution of ILC2 in the induction of allergic inflammation appears highly dependent on the allergen model.

Here, we describe in detail a T cell-independent and a T cell-dependent murine model for airway inflammation via intranasal administration of IL-33 or HDM, respectively. The alarmin cytokine IL-33 is constitutively expressed in the nucleus of airway epithelial cells and is released upon cellular stress or injury that occurs during exposure to a number of different environmental allergens (31). IL-33 is a potent stimulator of ILC2 proliferation and cytokine production and intranasal administration of IL-33 leads to eosinophilic airway inflammation reminiscent of allergic asthma, which provides a unique tool to specifically investigate ILC2 function during inflammation (32-34). A more physiologically relevant mouse model for allergic asthma employs HDM extract. Intranasal sensitization followed by provocation challenge with HDM produces eosinophilic infiltrates in the lung and airway hyperresponsiveness that closely resembles clinical observations in patients with allergic asthma (2). Although there is abundant literature on mouse ILC2 in a variety of inflammatory diseases, the markers used to characterize ILC2 have not been standardized, which poses difficulties when comparing data from various publications. In this report, we provide a detailed insight into the induction of airway inflammation in mouse models based on exposure to IL-33 and HDM, focusing on the characterization of the ILC2 phenotype in bronchoalveolar lavage (BAL) fluid, lungs, and mediastinal lymph nodes (MLN) using flow cytometry.

MATERIALS

Standard laboratory equipment including plates, tubes, syringes, pipettes, and centrifuges is not listed.

Induction of IL-33 and HDM-Induced Airway Inflammation

1. 8–16-week-old C57BL/6 mice housed in a specific-pathogen-free facility, fed *ad libitum*, and age- and gender-matched (see **Note 1**).
2. Recombinant mouse IL-33 (BioLegend, USA; 0.5 μg/40 μL; see **Note 2**).
3. HDM extract from *Dermatophagoides pteronyssinus* (Greer, USA; 1 μg/40 μL for sensitization and 10 μg/40 μL for challenge; see **Note 3**).
4. PBS.
5. Isoflurane anesthesia device.

Collection of BAL Fluid, Lungs, and MLN

1. Anesthetic and tools suitable for sacrificing mice (see **Note 4**).
2. Cannula: 23-gauge × 1¼ in. needle with the tip sheathed by ~10 mm of polyethylene tubing (inner diameter 0.58 mm/0.023 in.) to prevent puncturing of the airways (**Fig. 1a**).
3. Suture thread.
4. PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA).
5. Hank's balanced salt solution (HBSS).

Preparation of Single-Cell Suspension

1. PBS containing 0.5 mM EDTA supplemented with 0.5 % bovine serum albumin (BSA).
2. Osmotic lysis buffer: 8.3 % NH4Cl, 1 % KHCO3, and 0.04 % Na2EDTA in Milli-Q.
3. 100 μm nylon cell strainers.
4. RPMI 1640 Cell culture medium containing 10 % fetal bovine serum (FBS).

Flow Cytometry

1. FACS-buffer containing 0.25 % BSA, 0.5 mM EDTA, and 0.05 % NaN3 in PBS with a final pH of 7.2.
2. For intracellular transcription factor detection: eBioscience FoxP3/transcription factor staining kit containing (1) fixation and permeabilization concentrate and (2) diluent and (3) 10x wash buffer to be diluted in Milli-Q.
3. For intracellular cytokine detection: phorbol myristate acetate (PMA), ionomycin, and GolgiStop (BD Biosciences, USA). 4 % paraformaldehyde (PFA) in PBS as stock solution, diluted 1:2 in PBS to a 2 % working solution. Permeabilization and wash buffer of 0.5 % saponin (Sigma-Aldrich, USA) in FACS-buffer.

4. Purified and unlabeled CD16/CD32 antibody (Fc-block, clone 2.4G2).
5. Fixable live/dead cell stain (Fixable Viability Dye eFluor® 506, eBioscience, USA).
6. Antibodies used for flow cytometry, listed in **Table 1**.
7. Data acquisition is performed using an LSR II flow cytometer (Beckton Dickinson, USA) equipped with three lasers and FACSDiva™ software (Beckton Dickinson, USA) and analyzed by FlowJo v10 (Tree Star Inc., USA) software.

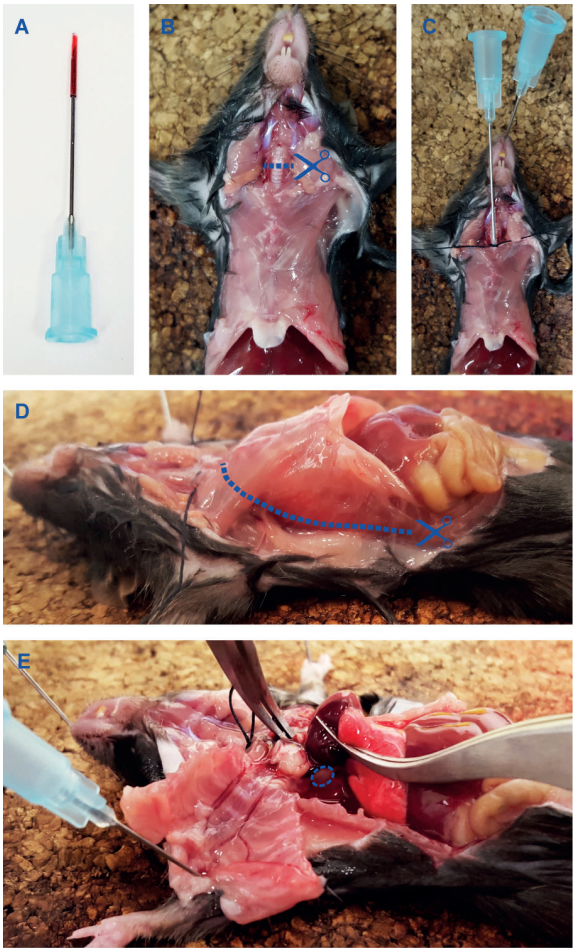

Figure 1. Stepwise procedure for the collection of BAL fluid, lungs and MLN.

(A) A cannula used for the collection of BAL fluid. The polyethylene sheath prevents puncturing of the airways and is coloured red to indicate its placement. (B) Preparation of the trachea for cannula insertion. The connective tissue is removed and a small incision is made between the tracheal cartilage along the dotted blue line. (C) Inserted cannula secured by suture thread. (D) The thoracic cavity can be opened by removing the diaphragm and cutting the ribs on both sides along the dotted blue line. (E) MLN can be exposed by lifting the right lung lobes and is situated below a blood vessel.

Table 1. Antibodies used for flow cytometric analysis of ILC2, eosinophils and neutrophils.

Antibody	Conjugate	Clone	Company
ILC2 lineage mix			
B220	PE	RA3-6B2	eBioscience
CD3e	PE	145-2c11	eBioscience
CD4	PE	GK1.5	eBioscience
CD5	PE	53-7.3	eBioscience
CD11b	PE	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD19	PE	1D3	BD Biosciences
CD8a	PE	53-6.7	eBioscience
FcεRIa	PE	MAR-1	eBioscience
Gr-1	PE	RB6-8C5	BD Biosciences
NK1.1	PE	PK136	eBiosciences
TER-119	PE	TER-119	eBioscience
ILC2 surface markers			
CD45	PE-CF594	I3/2.3	Abcam
ICOS	APC	C398.4A	eBioscience
KLRG1	Biotin	2F1/KLRG1	BioLegend
MHCII	Brilliant Violet 650	M5/114.15.2	BD Biosciences
Sca-1	Brilliant Violet 786	D7	BD Biosciences
T1/ST2	Biotin	DJ8	MD Bioproducts
T1/ST2	FITC	DJ8	MD Bioproducts
Streptavidin	APC-eFluor 780		eBioscience
ILC2 transcription factors			
Gata3	eFluor 660	TWAJ-14	eBioscience
ILC2 cytokines			
Amphiregulin	Biotin	Polyclonal	R&D Systems
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
IL-13	eFluor 450	eBio13A	eBioscience
Eosinophil and neutrophil surface markers			
Gr-1	APC-eFluor 780	RB6-8C5	eBioscience
Siglec-F	PE	E50-2440	BD Biosciences

METHODS

Intranasal Injection

1. Mice are placed in a small gas chamber and are exposed to 2.5 % isoflurane anesthesia with an oxygen airflow of 1 L/min (VerEquip).
2. When the breathing rate has dropped to approximately two breaths per second, hold the mouse by the scruff of the neck with the abdomen facing you and tilt the body to a 45° angle.

3. Carefully place a drop of liquid on the tip of the nose using a pipette and wait for the mouse to inhale (see **Note 5**).
4. Once the droplet has been successfully inhaled and the breathing rate of the mouse starts to increase, place it on its back in the cage.

IL-33-Induced Airway Inflammation

1. Mice are administered 0.5 µg recombinant IL-33 in 40 µL PBS via intranasal injection under isoflurane anesthesia, as described above, three times with 1 day of rest between each injection.
2. Organs of interest are harvested 1 day after the final injection.
3. Control mice are treated with PBS.

HDM-Induced Airway Inflammation

1. Mice are sensitized with 1 µg HDM extract dissolved in 40 µL PBS via intranasal injection under isoflurane anesthesia as described above.
2. After a resting period of 7 days, mice are challenged daily to 10 µg HDM extract dissolved in 40 µL PBS by intranasal injection for 5 consecutive days.
3. Organs of interest are harvested 1 day after the final challenge.
4. Control mice are sensitized with PBS and are challenged with HDM.

Collection of BAL Fluid, Lungs, and MLN

1. Mice are sacrificed, using 75 mg/kg ketamine combined with 1 mg/kg medetomidine intraperitoneally (i.p.).
2. A midline incision is made to retract the skin and the connective tissue surrounding the trachea is carefully removed.
3. A suture thread is placed underneath the trachea by carefully lifting it with tweezers. This will be used to secure the cannula in the next step.
4. A small incision between the tracheal cartilage is made to insert a cannula, be careful not to sever the entire trachea (**Fig. 1b**). The cannula is secured in place using the suture thread in step 3 by tying a knot around the portion of the trachea housing the cannula (**Fig. 1c**).
5. A syringe containing 1 mL PBS supplemented with 0.5 mM EDTA is attached to the cannula. The lungs are subsequently washed by gently flushing. Afterwards, the syringe is uncoupled and the aspirated BAL fluid is collected in a tube.
6. Step 5 is repeated two more times for a total of ~3 mL BAL fluid.
7. The thoracic cavity is then opened by removing the diaphragm and cutting the ribs on both sides along the dorsal plane. The connective tissue between the thymus and the ribcage is removed and a transversal cut is made above the heart to completely expose the lungs (**Fig. 1d**).

8. The MLN is situated underneath the right lung lobes below a blood vessel and can be carefully extracted using tweezers and placed in HBSS for further processing (**Fig. 1e**).
9. Lungs lobes are separated from the bronchi and placed in HBSS for further processing.

Preparation of Single-Cell Suspensions

1. Lung and MLN tissue are mechanically disrupted on a 100 µm cell strainer using the plunger of a syringe. The strainers are flushed with 5 mL PBS containing 0.5 mM EDTA and 0.5 % BSA to extract an optimal number of cells.
2. BAL fluid, lung, and MLN cell suspensions are centrifuged at 4 °C 400 × g for 7 min and the supernatant from the BAL fluid may be collected for the detection of cytokine levels by ELISA (not discussed here). Lung and MLN supernatants are discarded.
3. BAL and MLN cell pellets are resuspended in an appropriate volume of cell culture medium for cell counting and are ready for flow cytometric applications.
4. The cell pellet from the lung is resuspended in 1 mL osmotic lysis buffer and incubated at room temperature for 2 min. Lysis is stopped by adding 10 mL cold PBS and the suspension is centrifuged at 4 °C 400 × g for 7 min and the supernatant is removed.
5. Cells are resuspended in cold cell culture medium and after a sample is taken to determine absolute cell numbers, they are ready for flow cytometric applications.

Intracellular Flow Cytometry Staining Protocol for ILC2 Detection

1. To detect cytokine production, one million cells per well in a 96-well plate are stimulated with PMA (50 ng/mL; diluted from a 1000× stock solution) and ionomycin (300 ng/mL diluted from a 1000× stock solution), and GolgiStop (1:1500) diluted in cell culture medium for 4 h at 37 °C prior to antibody staining.
2. For surface marker staining, one million cells are placed in each well of a 96-well plate and are incubated with 40 µL primary antibody mix in FACS buffer containing Fc-block for 30 min at 4 °C.
3. The samples are then washed once with FACS buffer and once with PBS.
4. Following this, the cells are incubated with fixable live/dead stain with 40 µL secondary antibody mix in PBS and incubated for 15 min at 4 °C.
5. For intracellular transcription factor detection: cells are washed in PBS, fixed and permeabilized using eBioscience fixation and permeabilization buffer for 30 min at 4 °C, followed by two washing steps using eBioscience wash buffer. Next, the pellet is incubated with 40 µL tertiary antibody mix in wash buffer for 60 min at 4 °C. Finally, the samples are washed, once with wash buffer and once with FACS buffer, before being resuspended in FACS buffer and measured by a flow cytometer within 1 day (see Note 6).
6. For intracellular cytokine detection: cells are washed in PBS and fixed using 2 % PFA for 15 min at 4 °C followed by two washes with PBS. After fixation, the cells are permeabilized

with permeabilization and wash buffer containing 0.5 % saponin for 15 min at 4 °C. Next, the cell pellet is incubated with 40 µL tertiary antibody mix in perm/wash buffer for 60 min at 4 °C. Finally, the samples are washed once with perm/wash buffer and once with FACS buffer before being resuspended in FACS buffer and acquired within 1 day (see **Note 6**).

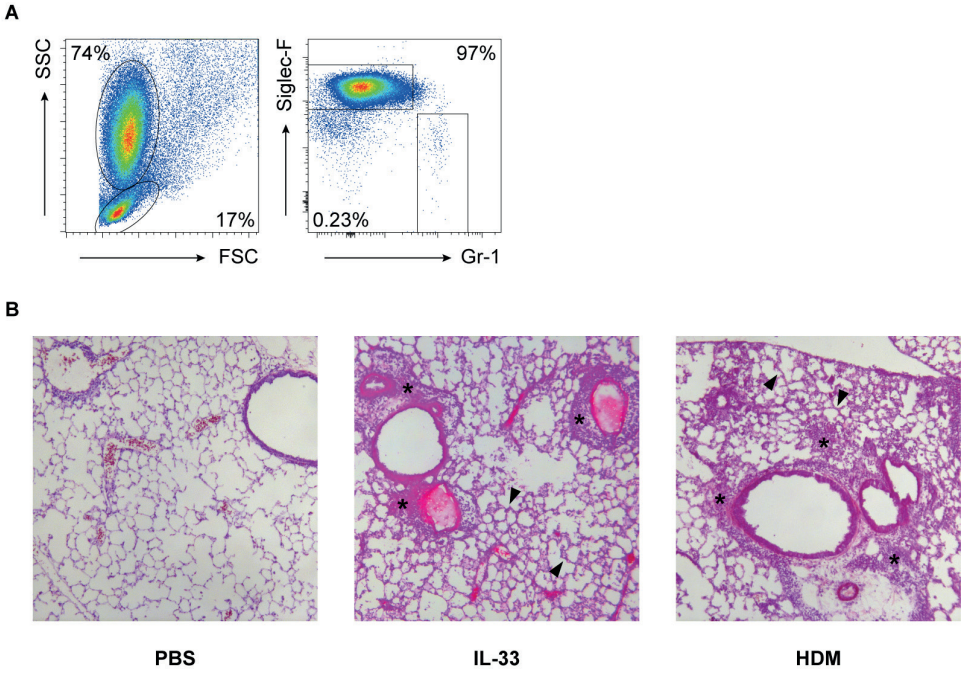

Figure 2. Extent of eosinophilic inflammation in BAL fluid and lungs after IL-33 or HDM treatment.

(A) Flow cytometric identification of eosinophils and neutrophils in the BAL fluid of IL-33-treated mice. Granulocytes are gated on the basis of high SSC and low FSC values and discriminated as eosinophils or neutrophils using Siglec-F and Gr-1, respectively. (B) Hematoxylin and eosin (H&E) staining of lung sections from PBS, IL-33, and HDM-treated mice, showing perivascular infiltrates (indicated by asterisks) and thickening of alveolar walls (indicated by arrow heads).

Analysis of Allergic Airway Inflammation by Flow Cytometry

The number of eosinophils present in the BAL fluid can be used as an indicator for the severity of allergic inflammation. Successful induction of airway inflammation yields large numbers of granulocytes, characterized by high side scatter (SSC) and relative low forward scatter (FSC) values, of which 90 % or more consist of Siglec-F$^+$ eosinophils (**Fig. 2a**). These percentages generally translate to between ~0.5 and ~1.0 million eosinophils in the BAL fluid. Cellular infiltrates can also be seen in the lungs and typically accumulate around the bronchioles and blood vessels. In addition, thickened alveolar septa are also observed (**Fig. 2b**).

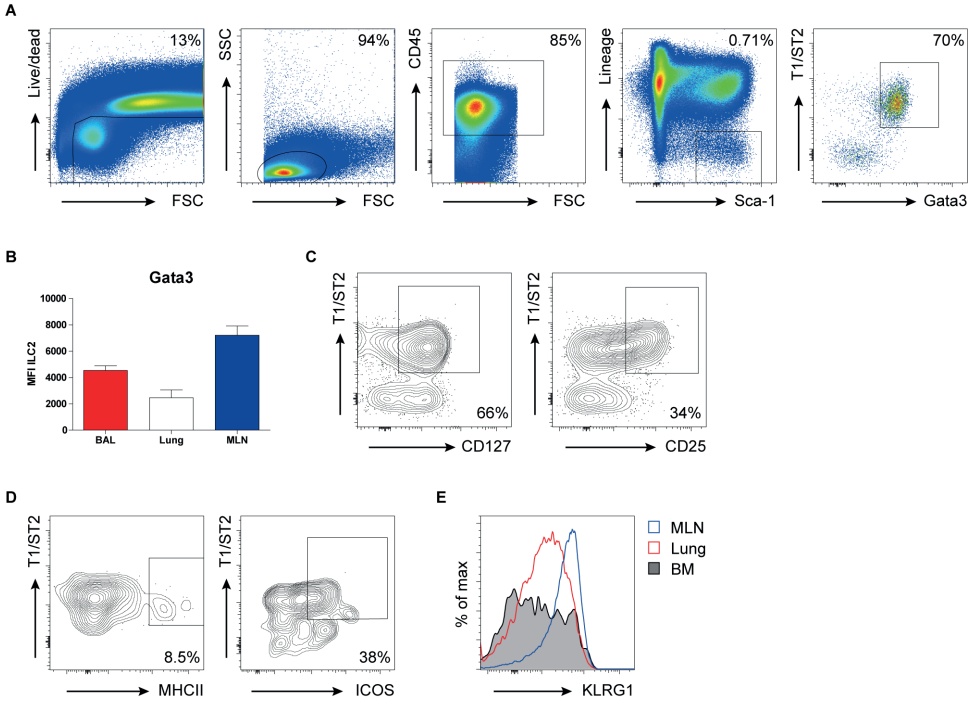

Figure 3. Gating strategy for the identification of ILC2 in BAL fluid of HDM-treated mice.

(A) After exclusion of dead cells and debris and gating for CD45$^+$ lymphocytes, ILC2 are lineage-negative and express high levels of Sca-1, T1/ST2, and intracellular GATA3. (B) GATA3 expression level by ILC2 is variable between tissues, as shown by mean fluorescence intensity (MFI) values. (C) Alternative gating strategy for ILC2 from Lineage$^-$Sca-1$^+$ cells without intracellular staining, using CD127 as an additional marker. CD25 expression is variable within this population. (D) Analysis of MHC-II and ICOS expression in ILC2. (E) Comparative analysis of KLRG1 expression in ILC2 derived from bone marrow (BM), lung, and MLN. Data are shown as mean ± SEM of n = 5 mice per group. All flow cytometry panels are pooled data from >4 mice.

Analysis of ILC2 by Flow Cytometry

To quantify ILC2 in the BAL fluid, living cells that are negative for the live/dead stain are selected. However, it should be kept in mind that macrophages are autofluorescent and therefore positive in the live/dead staining, irrespective of their viability, and are therefore also excluded. Furthermore, it is possible to remove any debris in this step that may interfere with subsequent gating. ILC2 are relatively small cells and fall within the general lymphocyte gate in the FSC/SSC plot. CD45 expression is used to exclude all non-leukocyte populations and the lineage markers are compiled in a "dump" channel to readily exclude major hematopoietic cell populations. ILC2 do not express lineage markers and express high levels of surface Sca-1. Furthermore, they also express the IL-33R component T1/ST2 and intracellular GATA3 (**Fig. 3a**). Because GATA3, T1/ST2 and Sca-1 are also expressed by Th2 cells, it is critically important to optimize the lineage mix and to conservatively gate for lineage-negative cells to

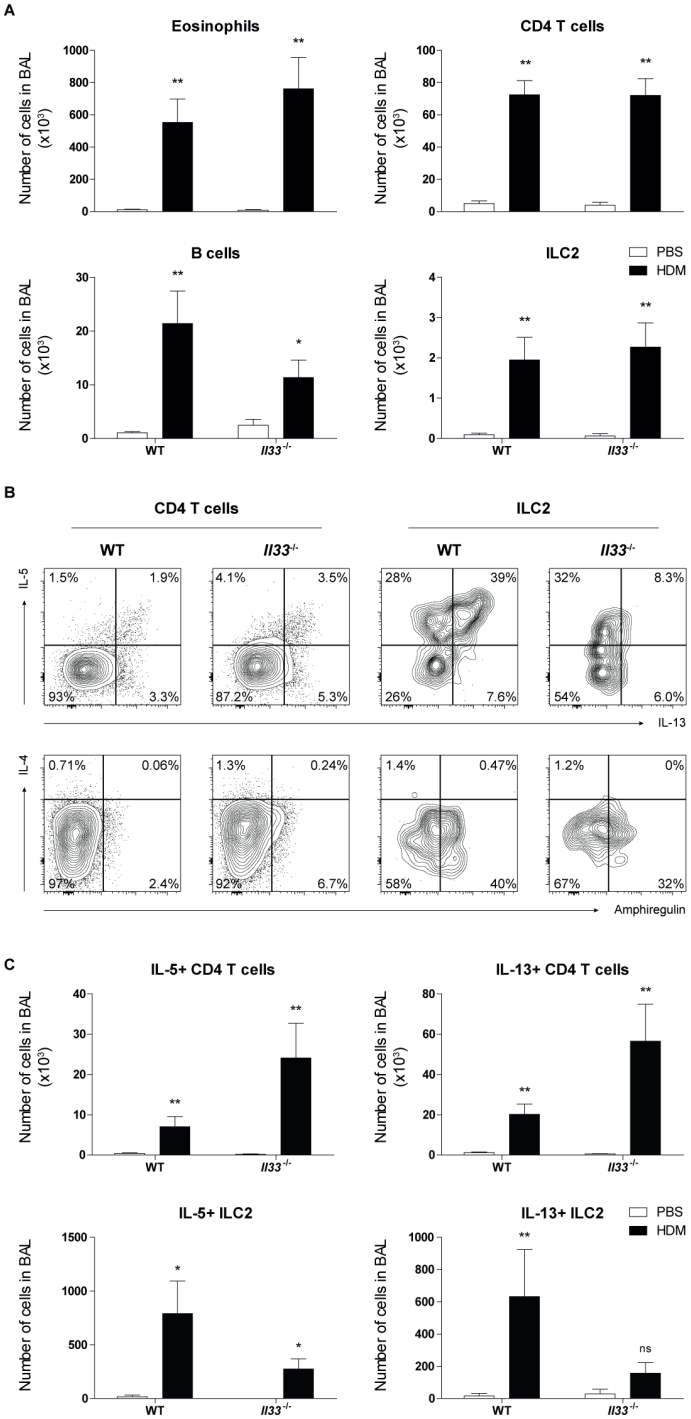
A
Eosinophils
CD4 T cells
B cells
ILC2
Number of cells in BAL (x10³)
PBS
HDM
WT
Il33-/-
B
CD4 T cells
ILC2
WT
Il33-/-
IL-5
IL-13
IL-4
Amphiregulin
1.5%
1.9%
93%
3.3%
4.1%
3.5%
87.2%
5.3%
28%
39%
26%
7.6%
32%
8.3%
54%
6.0%
0.71%
0.06%
97%
2.4%
1.3%
0.24%
92%
6.7%
1.4%
0.47%
58%
40%
1.2%
0%
67%
32%
C
IL-5+ CD4 T cells
IL-13+ CD4 T cells
IL-5+ ILC2
IL-13+ ILC2
ns

Figure 4. CD25$^-$ and CD25$^+$ ILC2 secrete similar amounts of cytokines in HDM-treated mice.

(A) Flow cytometric analysis of intracellular cytokine production of BAL fluid ILC2 in PBS, IL-33, or HDM-treated mice after stimulation of single-cell suspensions with PMA/ionomycin and GolgiStop for 4 h. (B) Expression of CD25 in BAL fluid derived ILC2 is dependent on the type of stimulation used. (C) Analysis of cytokine profiles of CD25$^-$ and CD25$^+$ ILC2 from BAL fluid of HDM-treated mice. (D) Relative contribution of various cell populations to amphiregulin (Areg), IL-4, IL-5, and IL-13 producing cells in BAL fluid of HDM-treated mice. Single-cell suspensions were stimulated with PMA/ionomycin in the presence of GolgiStop for 4 h and intracellular cytokine expression was quantified. Proportions of cytokine producing cells (expressed as percentages of the total number of cytokine-producing cells) were determined by flow cytometry in ILC2 and CD4 T cells. Data are shown as mean ± SEM of n = 4–6 mice per group. All flow cytometry panels are pooled data from >4 four mice. A Mann–Whitney U test was used to determine statistical significance between CD25$^-$ and CD25$^+$ ILC2 in HDM-treated mice.

ensure no T cells are included within the ILC2 gate. The level of GATA3 expression within the ILC2 population is variable between different tissues (**Fig. 3b**). This likely reflects the activation state, as we observed that GATA3 levels increase when ILC2 are activated by IL-33 stimulation. Although GATA3 is a consistent marker for ILC2, it is a nuclear protein that requires fixation and permeabilization steps for its detection. For applications where this is not desirable, such as live cell sorting, surface CD127 may be used as a replacement in conjunction with Sca-1 and T1/ST2. Although CD25 is also frequently associated with ILC2, its expression is variable (**Fig. 3c**). The observed differential expression on ILC2, depending on the type of stimulus applied, precludes the usage of CD25 as a surface marker for accurate quantification of ILC2 numbers. A fraction of ILC2 express MHCII and ICOS (**Fig. 3d**) and have sparked investigations into the interaction of ILC2 with other immune cells (25, 35). Expression of the co-inhibitory killer-cell lectin like receptor G1 (KLRG1) has been proposed as a marker acquired by mature ILC2 under inflammatory conditions (15, 36) and is differentially expressed between tissues (**Fig. 3e**).

ILC2 produce IL-5 and IL-13 in steady state and rapidly expand and upregulate their cytokine production upon stimulation. Amphiregulin production is low in resting ILC2 and is significantly upregulated when exposed to IL-33 or HDM (**Fig. 4a**). CD25 is expressed by ~50 % of ILC2 in the lung in steady state. Administration of IL-33 results in a homogeneous ILC2 population that consistently expresses high levels of CD25. However, HDM stimulation leads to the expansion of CD25$^{lo/-}$ ILC2 that still possess the same cytokine profile as "classical" CD25$^+$ ILC2 (**Fig. 4b, c**). Additionally, these CD25$^{lo/-}$ ILC2 are present in higher numbers in this model and significantly contribute to the total population of type 2 cytokine producing cells (**Fig. 4d**). It is conceivable that the CD25$^{lo/-}$ ILC2 population is also prominent in other allergen-based mouse models of lung inflammation. We therefore suggest the use of this marker as a method to discriminate between subpopulations of ILC2 rather than a characteristic to quantify ILC2 numbers.

Notes

1. Our observation is that female C57BL/6 mice tend to develop more severe eosinophilic lung inflammation than male mice and therefore it is crucial to gender match between experimental groups. Difference in the severity of inflammation also exists between various mouse strains.
2. The potency of recombinant mouse IL-33 diminishes over time and has an effective shelf life of 3 months if stored at −80 °C and 1 week if stored at 4 °C. Avoid repeated freeze–thaw cycles.
3. HDM extract is a natural product and has a variable composition between batches. It is important to note the endotoxin content of each batch and to compare the immune response generated between new and old batches in order to ensure similar levels of inflammation. If necessary, perform titrations of different amounts of HDM during challenge.
4. Cervical dislocation is not a viable option due to a high chance of tearing the trachea, thus preventing the placement of a cannula for BAL fluid extraction. Euthanasia by inhalation of CO2 gas potentially damages the immune cells in the lungs, and thus we recommend methods employing intraperitoneal overdose of anesthetic agents like ketamine/medetomidine.
5. If the mice are placed in the isoflurane chamber for extended periods of time (>1 min), they may become too deeply anesthetized and have trouble adequately inhaling the droplet. This is characterized by an extremely slow breathing rate of less than once per second. To avoid this, do not anesthetize more than four mice simultaneously to ensure optimal anesthesia. Additionally, it is normal for some mouse strains such as the C57BL/6 mice to hold their breath for a short amount of time during intranasal administration. We recommend not to exceed a volume of 80 µL for intranasal injections.
6. It is possible to leave the cells in the fixation step overnight at 4 °C and continue the FACS stain the next day if time is an issue.

Acknowledgments

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T CELLS ARE NECESSARY FOR ILC2 ACTIVATION IN HOUSE DUST MITE-INDUCED ALLERGIC AIRWAY INFLAMMATION IN MICE

CHAPTER 3

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T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice
Bobby W.S. Li, Marjolein J.W. de Bruijn, Irma Tindemans, Melanie Lukkes, Alex KleinJan, Henk C. Hoogsteden, Rudi W. Hendriks
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ABSTRACT

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.

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 hyperresponsiveness 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-2Ra), CD127 (IL-7Ra), 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 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 pro-inflammatory 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.

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 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**).

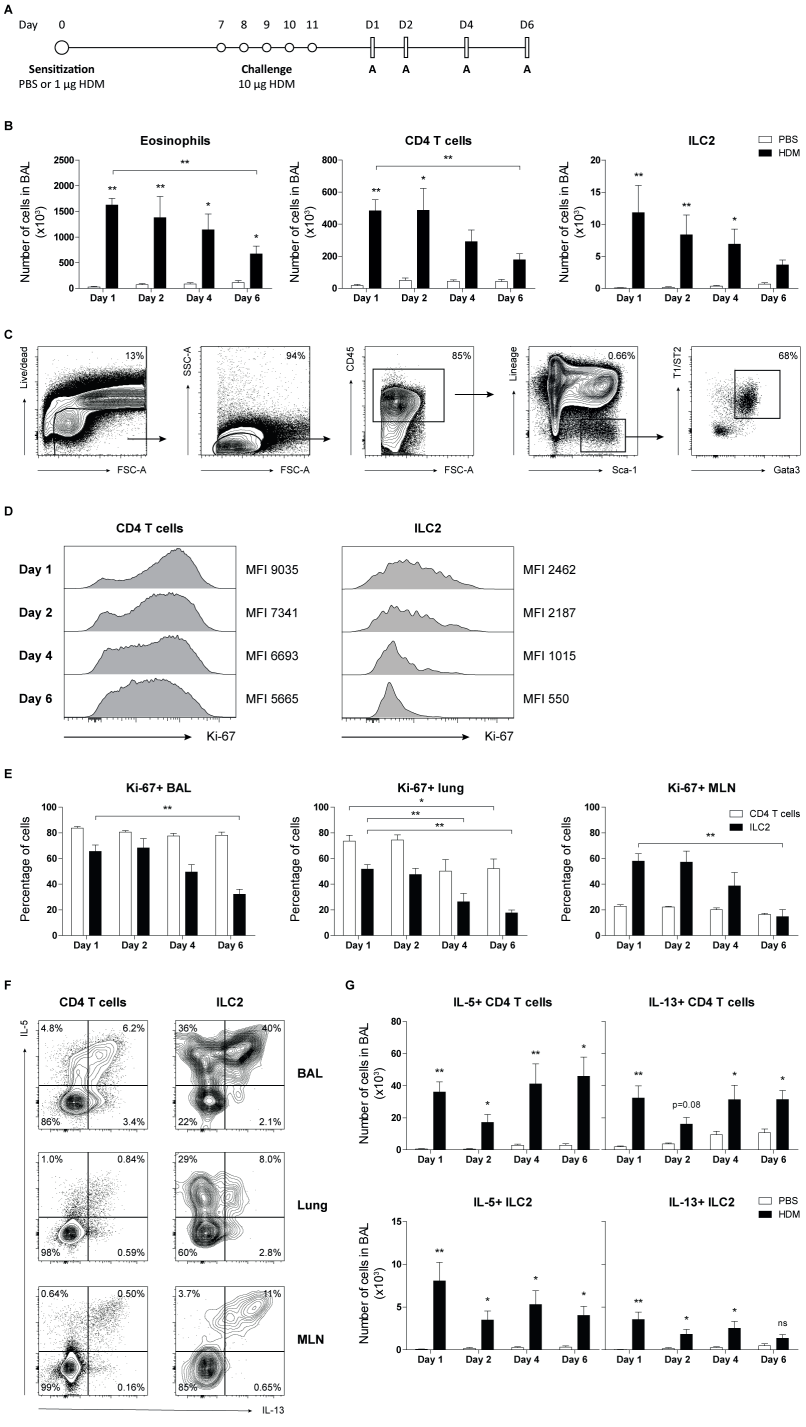

A
Day 0 7 8 9 10 11 D1 D2 D4 D6
Sensitization
PBS or 1 µg HDM
Challenge
10 µg HDM
A A A A
B
Eosinophils
CD4 T cells
ILC2
PBS
HDM
Number of cells in BAL (x10³)
Day 1 Day 2 Day 4 Day 6
C
Live/dead
13%
FSC-A
SSC-A
94%
CD45
85%
Lineage
0.66%
Sca-1
T1/ST2
68%
Gata3
D
CD4 T cells
ILC2
Day 1 MFI 9035 MFI 2462
Day 2 MFI 7341 MFI 2187
Day 4 MFI 6693 MFI 1015
Day 6 MFI 5665 MFI 550
Ki-67
E
Ki-67+ BAL
Ki-67+ lung
Ki-67+ MLN
Percentage of cells
CD4 T cells
ILC2
F
CD4 T cells
ILC2
IL-5
IL-13
BAL
Lung
MLN
4.8% 6.2% 86% 3.4%
36% 40% 22% 2.1%
1.0% 0.84% 98% 0.59%
29% 8.0% 60% 2.8%
0.64% 0.50% 99% 0.16%
3.7% 11% 85% 0.65%
G
IL-5+ CD4 T cells
IL-13+ CD4 T cells
IL-5+ ILC2
IL-13+ ILC2
p=0.08
ns

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 $CD45^+Lineage^-Gata3^+T1/ST2^+Sca\text{-}1^+$ 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.

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 $IL\text{-}5^+$ and $IL\text{-}13^+$ 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. Thus, the influx of $IL\text{-}5^+IL\text{-}13^+$ 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 $Ctcf^{\Delta T/\Delta T}$ 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 were not impaired in *Ctcf*$^{\Delta T/\Delta T}$ (**Supporting Information Fig. 3**). When we subjected *Ctcf*$^{\Delta T/\Delta T}$ 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**).

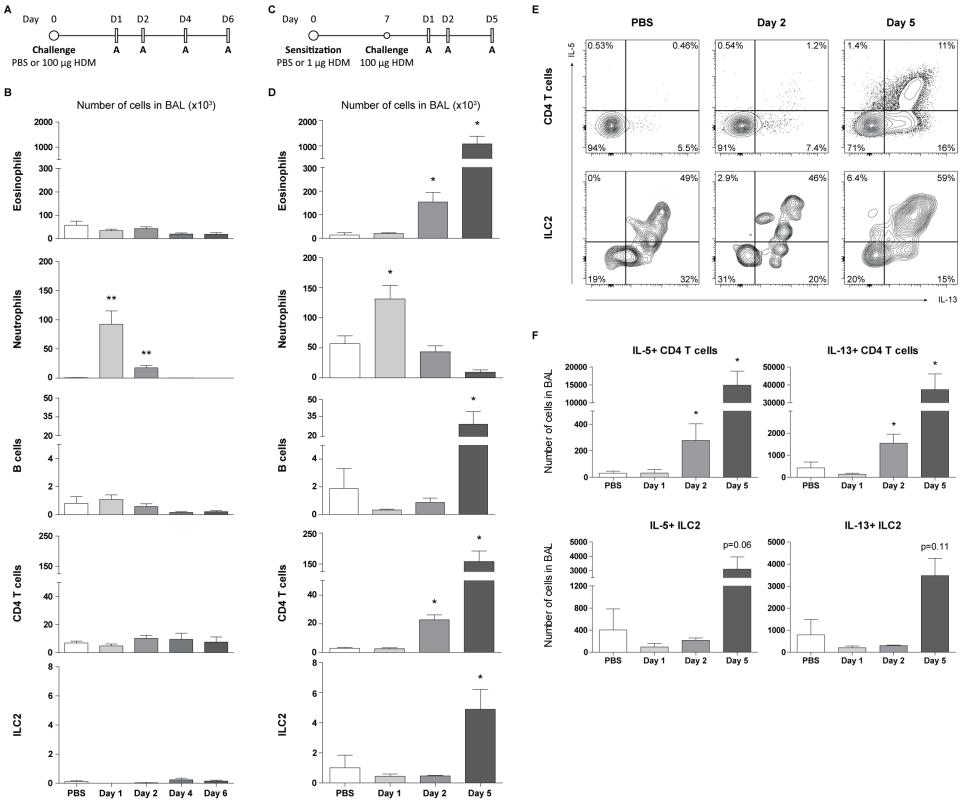


Figure 2. T-cell activation precedes ILC2 induction.

(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.

Mb1$^{-/-}$ mice that are deficient in the expression of Iga/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).

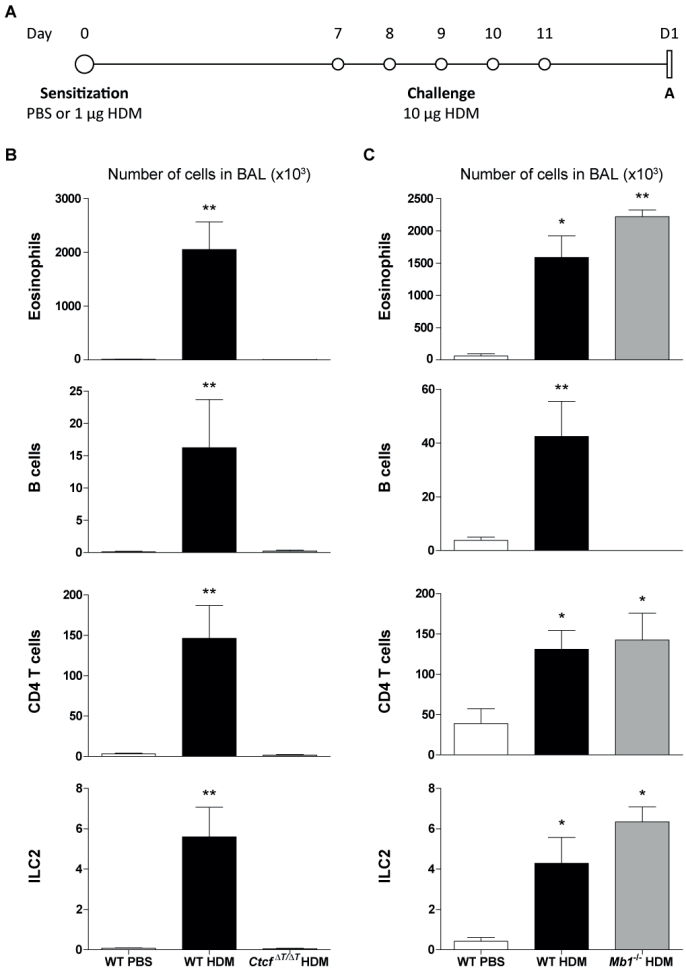


Figure 3. T-cell activation is required for ILC2 induction in HDM-driven allergic inflammation.

(A-C) C57BL/6, *Ctcf*$^{\Delta T/\Delta T}$ 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. * $p \leq 0.05$, ** $p \leq 0.01$; Mann-Whitney U test.

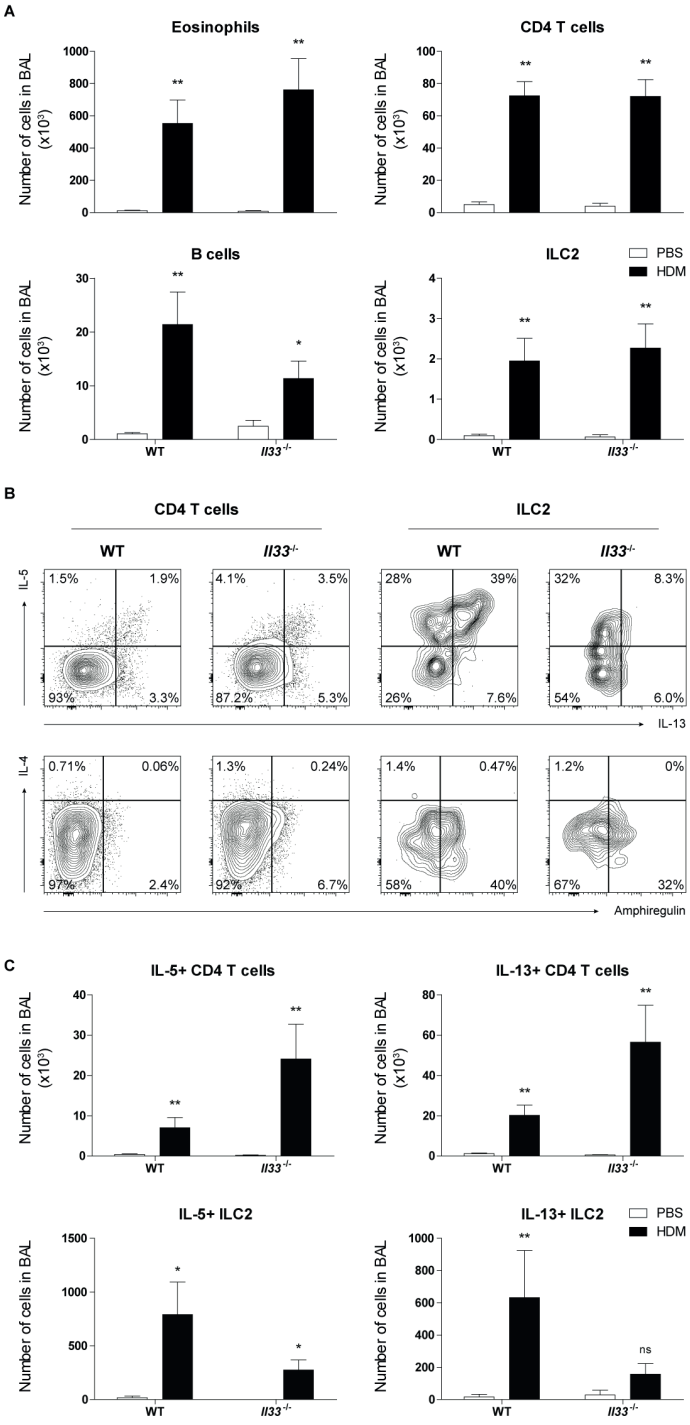

A
Eosinophils
CD4 T cells
B cells
ILC2
PBS
HDM
Number of cells in BAL (x10³)
WT
Il33-/-
B
CD4 T cells
ILC2
WT
Il33-/-
IL-5
IL-13
IL-4
Amphiregulin
1.5%
1.9%
93%
3.3%
4.1%
3.5%
87.2%
5.3%
28%
39%
26%
7.6%
32%
8.3%
54%
6.0%
0.71%
0.06%
97%
2.4%
1.3%
0.24%
92%
6.7%
1.4%
0.47%
58%
40%
1.2%
0%
67%
32%
C
IL-5+ CD4 T cells
IL-13+ CD4 T cells
IL-5+ ILC2
IL-13+ ILC2
ns

Figure 4. ILC2 influx into the BALF is not impaired by the absence of IL-33.

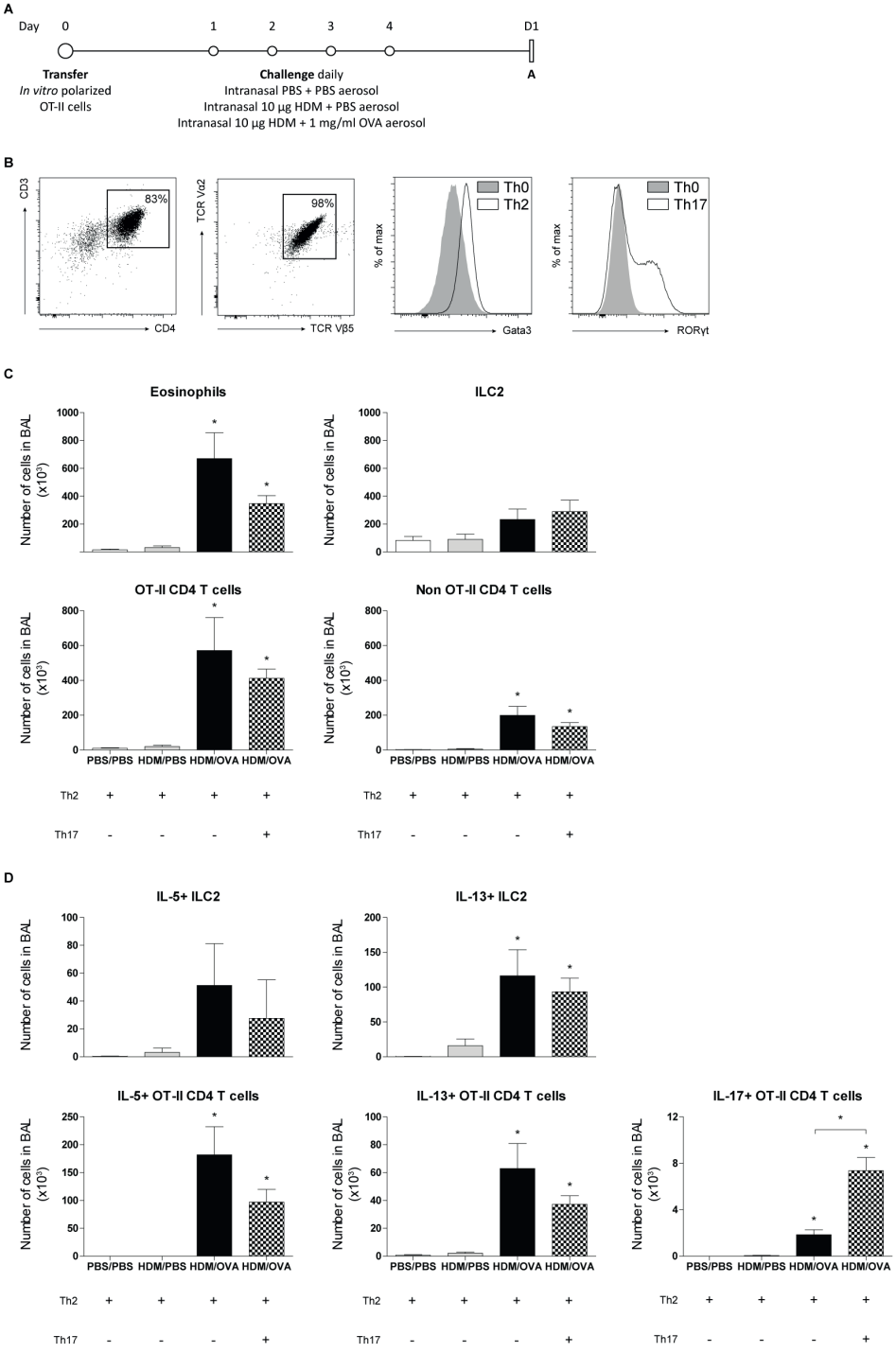
(A-C) WT and *Il33*$^{-/-}$ mice were treated intranasally with PBS or HDM as described in Fig. 3A and analysed one day after the final challenge. (A) Quantification of the indicated cell populations in the BALF of WT and *Il33*$^{-/-}$ mice. (B) Flow cytometric analysis of IL-5 (upper panels) and IL-4 (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) 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 + 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.

The pro-inflammatory 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**).


A
Day 0 1 2 3 4 D1
Transfer
In vitro polarized
OT-II cells
Challenge daily
Intranasal PBS + PBS aerosol
Intranasal 10 µg HDM + PBS aerosol
Intranasal 10 µg HDM + 1 mg/ml OVA aerosol
A
B
CD3
83%
CD4
TCR Vα2
98%
TCR Vβ5
% of max
Th0
Th2
Gata3
% of max
Th0
Th17
RORγt
C
Eosinophils
ILC2
Number of cells in BAL (x10³)
OT-II CD4 T cells
Non OT-II CD4 T cells
PBS/PBS HDM/PBS HDM/OVA HDM/OVA
Th2 + + + +
Th17 - - - +
D
IL-5+ ILC2
IL-13+ ILC2
IL-5+ OT-II CD4 T cells
IL-13+ OT-II CD4 T cells
IL-17+ OT-II CD4 T cells

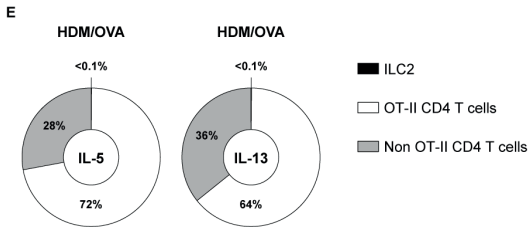


Figure 5. Activation and stimulation of T cells alone is not sufficient to induce ILC2s.

(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$^+$TCRVa2$^+$TCRVβ5$^+$) were analysed for intracellular Gata3 and RORγt 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 + 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.

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 pro-inflammatory cytokines and T cell-derived IL-2. In this context, it is however 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 pro-fibrotic 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 responsible 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 pro-inflammatory 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.

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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 µg HDM (Greer, USA) or PBS (GIBCO Life Technologies, USA). Seven days later, mice were challenged daily for five consecutive days with 10 µg 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 µg HDM or PBS and challenged once with 100 µg 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 µg 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, CD8a, CD11b, CD11c, CD19, B220, NK1.1, FcεRIa, 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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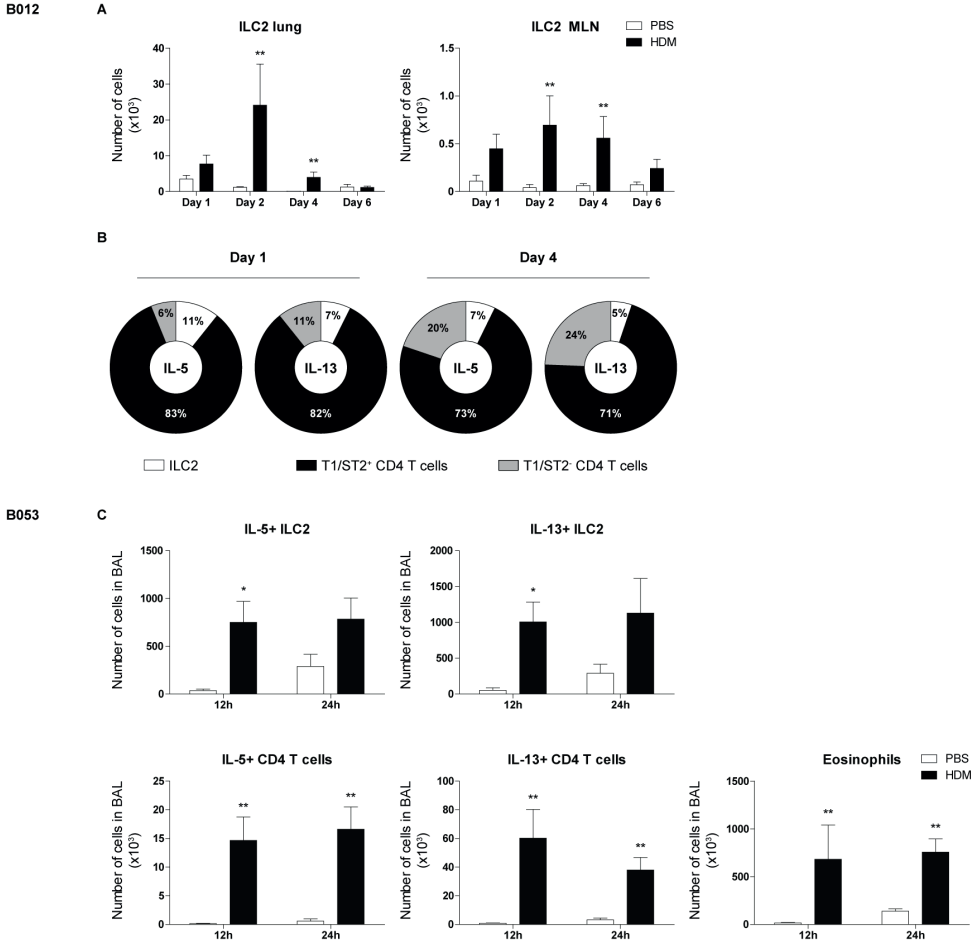
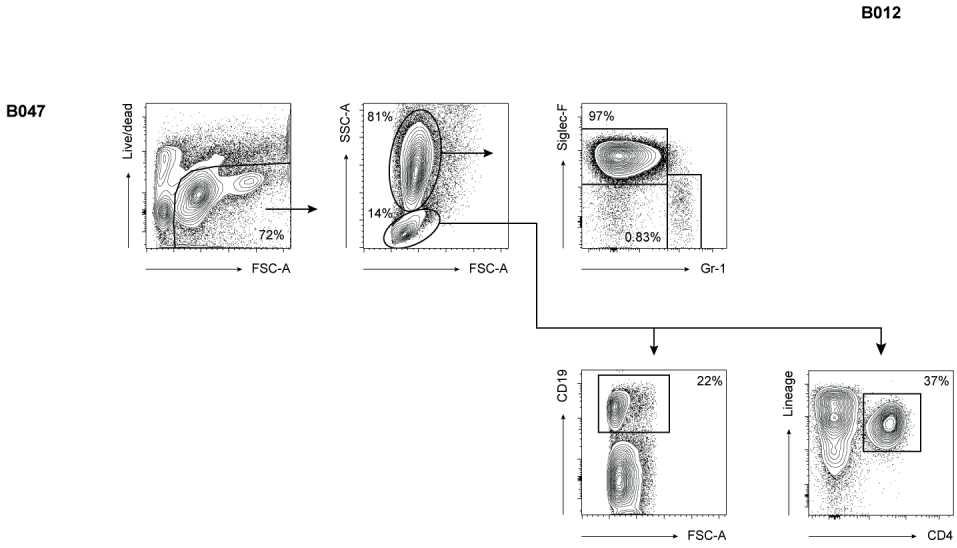
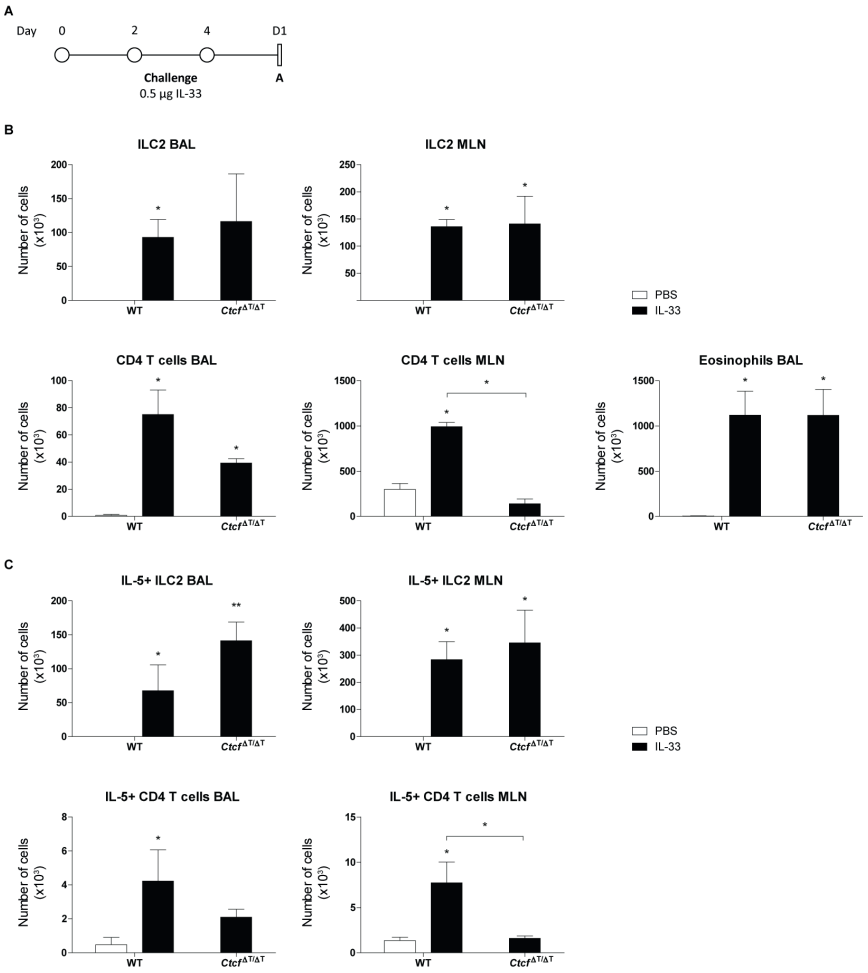


Supporting Information Figure 1. Concomitant induction of ILC2 and CD4⁺ T cells in lung and MLN of HDM-sensitized mice.

(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. 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 Ctcf$^{\Delta T/\Delta T}$ 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.

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	TWAJ-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

GROUP 2 INNATE LYMPHOID CELLS EXHIBIT A DYNAMIC PHENOTYPE IN ALLERGIC AIRWAY INFLAMMATION

CHAPTER 4

This chapter is published in Frontiers in Immunology:

Group 2 innate lymphoid cells exhibit a dynamic phenotype in allergic airway inflammation

Bobby W.S. Li, Ralph Stadhouders, Marjolein J.W. de Bruijn, Melanie Lukkes, Dior M.J.M. Beerens, Maarten D. Brem, Alex KleinJan, Ingrid M. Bergen, Heleen Vroman, Mirjam Kool, Wilfred F.J. van IJcken, Tata Nagreswara Rao, Hans Jörg Fehling, Rudi W. Hendriks

ABSTRACT

Group 2 innate lymphoid cells (ILC2) are implicated in allergic asthma as an early innate source of the type 2 cytokines IL-5 and IL-13. However, their induction in house dust mite (HDM)-mediated airway inflammation additionally requires T cell activation. It is currently unknown whether phenotypic differences exist between ILC2s that are activated in a T cell-dependent or T cell–independent fashion. Here, we compared ILC2s in IL-33- and HDM-driven airway inflammation. Using flow cytometry, we found that surface expression levels of various markers frequently used to identify ILC2s were dependent on their mode of activation, highly variable over time, and differed between tissue compartments including bronchoalveolar lavage (BAL) fluid, lung, draining lymph nodes and spleen. Whereas *in vivo* IL-33-activated BAL fluid ILC2s exhibited an almost uniform $CD25^{+}CD127^{+}T1/ST2^{+}ICOS^{+}KLRG1^{+}$ phenotype, at a comparable time point after HDM exposure BAL fluid ILC2s had a very heterogeneous surface marker phenotype. A major fraction of HDM-activated ILC2s were $CD25^{low}CD127^{+}T1/ST2^{low}$ $ICOS^{low}KLRG1^{low}$, but nevertheless had the capacity to produce large amounts of type 2 cytokines. HDM-activated $CD25^{low}$ ILC2s in BAL fluid and lung rapidly reverted to $CD25^{high}$ ILC2s upon *in vivo* stimulation with IL-33. Genome-wide transcriptional profiling of BAL ILC2s revealed ~1600 differentially expressed genes: HDM-stimulated ILC2s specifically expressed genes involved in the regulation of adaptive immunity through B and T cell interactions, whereas IL-33-stimulated ILC2s expressed high levels of proliferation-related and cytokine genes. In both airway inflammation models ILC2s were present in the lung submucosa close to epithelial cells, as identified by confocal microscopy. In chronic HDM-driven airway inflammation ILC2s were also found inside organized cellular infiltrates near T cells. Collectively, our findings show that ILC2s are phenotypically more heterogeneous than previously thought, whereby their surface marker and gene expression profile are highly dynamic.

INTRODUCTION

The capability of group 2 innate lymphoid cells (ILC2) to secrete large amounts of IL-5 and IL-13 has led to the investigation of their role in the pathogenesis of allergic diseases in recent years (1, 2). ILCs are a family of effector lymphocytes that do not express antigen receptors. They are classified according to their transcription factor requirements and distinct cytokine secreting patterns that mirror the profiles of T helper (Th) subsets (3, 4). ILC2s were originally characterized as an IL-25- and IL-33-responsive cell population that provides a critical early source of type 2 cytokines for the expulsion of parasitic worms (5-8). Like other ILC family members, ILC2s develop from the common lymphoid progenitors (CLP) and lack classic hematopoietic lineage markers and are thus defined as Lineage negative (9, 10). They express Thy-1 (CD90), c-Kit (CD117) and Sca-1 as well as a broad range of cytokine receptors including IL-7Ra (CD127), IL-2Ra (CD25), IL-25R (IL-17RB) and IL-33R (T1/ST2), leading to frequent use of these markers to identify and isolate ILC2s (11-13).

When stimulated by epithelial cell-derived innate cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), prostaglandin D2 from mast cells or cysteinyl leukotrienes secreted by activated hematopoietic cells, ILC2s rapidly expand and secrete large amounts of type 2 cytokines (1, 2, 14). Accordingly, intranasal administration of IL-25 or IL-33 induces eosinophilic airway inflammation and expansion of ILC2s, independently of B or T cells (15-17). Mouse models of allergic airway inflammation induced by papain and *Alternaria* have shown rapid release of IL-25 and IL-33 followed by robust ILC2 induction prior to T cell activation, suggesting an early sentinel function (16, 18-20). In contrast to these studies, exposure to other allergens such as *Aspergillus* and house dust mite (HDM) indicate a prominent role of T cells in the initiation of allergic inflammation (21, 22). We have previously shown that in HDM-induced allergic inflammation ILC2 induction requires T cell activation. Although accumulation of ILC2s in the broncho-alveolar lavage (BAL) fluid is independent of IL-33, cytokine production by ILC2s is markedly reduced in IL-33 knockout mice (22). Additionally, T cell-derived IL-21 promotes type 2 immunity to HDM and blockade of CD28 signaling during HDM exposure represses airway hyperreactivity and lung inflammation (23, 24), further supporting that both IL-33 and T cells are necessary for full ILC2 responses. Evidence for direct interactions between T cells and ILC2s includes the expression of MHC class II and co-stimulatory molecules such as CD86 and ICOS/ICOS-L by ILC2s (25-27). Taken together, these studies indicate the involvement of a complex array of signals and interactions for the activation of ILC2s in allergy. Importantly, ILC2s have mainly been studied in models in which they are strongly and rapidly activated in a T cell-independent fashion, but the phenotypic characteristics of ILC2s induced in T cell-dependent inflammation, including HDM-mediated allergic airway inflammation models, is currently not clear. Studies using IL-5 and IL-13 reporter mice have shown that in unstimulated conditions or upon IL-33 stimulation pulmonary ILC2s are mainly localized in

the lung submucosa close to epithelial cells in collagen-rich regions near blood vessels and airways (28, 29). However, ILC2 localization within a more physiological airway inflammation and their localization relative to Th2 cells remain unknown.

Plasticity of ILCs has first been reported in intestinal group 3 innate lymphoid cells (ILC3), which downregulate RORγt expression and simultaneously upregulate T-bet to transform into a group 1 innate lymphoid cell (ILC1)-like phenotype depending on IL-12, IL-18 and IL-7 (30). Conversely, ILC1s can transdifferentiate into ILC3s in the presence of IL-1β and IL-23 (31). ILC2s are also able to upregulate T-bet under influence of IL-33 and IL-1β and can produce IFN-γ, whereby retention of IL-13 producing capabilities resulting in a hybrid ILC1/ILC2 phenotype has been reported (32-35). Heterogeneity and plasticity in relation to environmental signals have recently been substantiated by single-cell transcriptome analyses (36-38). Taken together, these publications demonstrate the importance of micro-environmental cues for the function of ILC2s. As a result, the expression of cytokines and cytokine receptors by ILC2s may depend on their manner of activation and may differ between tissues. Thus, we relied on transcription factor GATA3 as a key ILC2 marker, which is central to ILC2 development and function and is constitutively expressed at high levels (39). We have previously reported dose-dependent effects of GATA3 both on ILC2 development from CLPs and on ILC2 function in allergic airway inflammation (40, 41). GATA3 additionally plays a major role as a master regulator of Th2 cell differentiation and drives the early development of other ILC subsets from the common ILC progenitor (42-44).

Although plasticity of ILC2 is studied in the context of their capacity to trans-differentiate into other types of ILCs, it remains unknown how the ILC2 phenotype is dependent on activation status, how it develops over time, what the differences are between various tissue compartments and how stable or reversible the ILC2 phenotype is. In this report, we aimed to compare the dynamics and kinetics of the ILC2 phenotype in the context of T cell-independent and T cell-dependent airway inflammation using IL-33 and HDM, respectively. We employed a novel *Gata3*$^{YFP/YFP}$ mouse strain, named GATA3 IRES Reporter (GATIR), which allows for synchronous transcription of GATA3 and yellow fluorescent protein (YFP) as separate proteins without affecting GATA3 protein levels or function [T.N.R and H.J.F., manuscript in preparation]. These mice enabled us to characterize and compare the phenotype of ILC2s in detail in acute and chronic mouse models of airway inflammation and to evaluate their localization in the lungs during an inflammatory response relative to other immune cells.

MATERIALS AND METHODS

Mice

Wildtype C57BL/6 mice were purchased from Envigo (United Kingdom). The GATIR mouse strain, in which an IRES-YFP sequence was inserted into the 3′ untranslated region of the *Gata3*

gene resulting in concomitant production of GATA3 and YFP protein, was on a C57BL/6 background [T.N.R and H.J.F., manuscript in preparation]. Only homozygous GATIR knock-in mice were used for analysis. *Gata3*$^{+/-}$ mice (C57BL/6), in which one of the *Gata3* alleles is targeted by a lacZ reporter, have been described previously (45). Mice were ~8-16 weeks old at time of analysis. All animals were housed at the Erasmus MC Animal Center under specific pathogen-free conditions. All experiments were approved by the Erasmus MC Animal Ethics Committee.

Induction of airway inflammation

For IL-33-induced airway inflammation, mice were anesthetized using isoflurane and treated three times intranasally or intratracheally every other day with 0.5 µg IL-33 (BioLegend, USA) or PBS (GIBCO Life Technologies, USA) as described previously (17). Mice were sacrificed at the indicated time points after the final IL-33 administration.

For acute HDM-induced allergic airway inflammation, mice were anesthetized using isoflurane and sensitized by intranasal or intratracheal injection with 1 or 10 µg HDM extract (Greer, USA) or PBS. After 7 days, animals were challenged daily on days 0-4 with 10 µg HDM intranasally or intratracheally and sacrificed at the indicated time points after the final treatment as described previously (46). For chronic HDM-induced allergic airway inflammation, mice were anesthetized using isoflurane and treated intranasally three times weekly for five weeks with 25 µg HDM or PBS and sacrificed at the indicated time points after the final treatment (adapted from (47)).

Flow cytometry

Single cell suspensions were prepared from lungs, lymph nodes and spleens by mechanical disruption of the tissues without digestive enzymes using a 100 µm cell strainer (BD Biosciences, USA) in PBS containing 0.5% bovine serum albumin and 5 mM EDTA (Sigma-Aldrich, USA). Bone marrow cells were extracted from the femur and BAL fluid was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA. Intracellular cytokine production was measured upon stimulation of cells at 37 °C with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, USA), supplemented with GolgiStop (BD Biosciences, USA) for 4 hours prior to staining. Flow cytometry surface and intracellular staining procedures have been described previously (17, 41). A comprehensive list of antibodies used for flow cytometry is presented in Supplementary Table S1. Lineage negative cells were defined as cells not expressing CD3, CD4, CD5, CD8a, CD11b, CD11c, CD19, B220, NK1.1, FcεRIa, TER-119 and Gr-1. Data were acquired using a LSR II flow cytometer equipped with three lasers and FACSDiva (BD Biosciences, USA) and analyzed by FlowJo (Tree Star Inc., USA).

ILC2 cell sorting and RNA-Seq

For FACS sorting, BAL fluid cells were stained with fluorescently labeled monoclonal antibodies. ILC2 fractions were sorted as Lineage$^-$Sca-1$^+$YFP$^+$ cells using a FACSAria flow cytometer equipped with three lasers and FACSDiva software (Beckton Dickinson). Data analysis was performed with FlowJo software (Tree Star Inc.).

RNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. Library preparation was performed using the Smart-seq2 methodology (48) and sequenced according to the Illumina TruSeq Rapid v2 protocol on an Illumina HiSeq2500 (single read mode, 51 bp read length). Reads were aligned to the mouse genome (mm10 build) using HISAT2 (49). Sample scaling and statistical analysis were performed using the R package DESeq2 (50) as implemented in HOMER (51); genes with >1 absolute log2 fold change and adjusted $P < 0.01$ (Wald test) were considered differentially expressed. Standard RPKM values were used as an absolute measure of gene expression. Genes with average RPKM < 2 were considered not expressed. Pathway analyses on differentially expressed genes were performed using Metascape (http://metascape.org) (52).

Confocal microscopic imaging

Lungs from GATIR mice were inflated with OCT embedding medium containing 2% PFA (Thermo Fisher Scientific, USA) and snap frozen in liquid nitrogen to preserve morphology and YFP fluorescence. Lymph nodes were fixed in 2% PFA, placed in 30% sucrose in PBS overnight and embedded in OCT and stored at -80 °C. 7 µm thick cryosections were cut at -20 °C using a cryostat (Thermo Fisher Scientific) and stained at room temperature with primary antibodies for 1 hour and secondary antibodies for 30 minutes. A comprehensive list of antibodies used for confocal microscopy is presented in Supplementary Table S2. Slides were stained with DAPI (Invitrogen, USA) for 5 minutes and sealed with Vectashield (Vector Laboratories, USA) and examined with an LSM 510 Meta confocal microscope equipped with a 405 nm, 488 nm, 543 nm and 633 nm laser (Zeiss, Germany). Images were processed and analyzed in Fiji, an open source scientific image processing application based on ImageJ (http://fiji.sc/).

Statistical analysis

Statistical comparisons were performed by Mann-Whitney U tests and a p-value of <0.05 was considered statistically significant. All analyses were performed using Prism (GraphPad Software, USA).

RESULTS

The GATIR knock-in does not affect GATA3 expression levels

We aimed to make use of expression of the *Gata3* gene as a central ILC2 marker that is independent of cell surface proteins on ILC2s. We have previously shown that GATA3 is critical for ILC2 development in a dose-dependent manner (40). Reduction of GATA3 levels as in *Gata3*$^{+/-}$ mice did not detectably affect splenic or thymic CD4$^+$ and CD8$^+$ T cells in steady state (**Suppl. Fig. S1A**). However, a negative impact was observed on IL-33-induced ILC2 accumulation and cytokine production and the associated eosinophilia in the BAL fluid of mice (**Suppl. Fig. S1B**)**.** Similar effects were found in an HDM-induced mouse model for airway inflammation, whereby reduced GATA3 levels also affected CD4$^+$ T cell accumulation and their cytokine content in the BAL fluid (**Suppl. Fig. S1C**). Despite normal development of CD4$^+$ and CD8$^+$ T cells and normal *in vitro* proliferation of naïve CD4$^+$ T cells following aCD3/aCD28-mediated stimulation, *Gata3*$^{+/-}$ T cells showed a significant decline in type 2 cytokine production *in vitro*, indicating impaired Th2 differentiation (**Suppl. Fig. S1D, S1E**).

It was therefore critical that the *Gata3* reporter mice to be used for visualization and phenotypic characterization of ILC2s and Th2 cells had unaltered GATA3 expression. GATIR mice harbor an IRES-YFP sequence that was inserted into the 3' untranslated region of the *Gata3* gene, allowing for the production of YFP as a separate protein concomitant with *Gata3* transcription [T.N.R and H.J.F., manuscript in preparation]. We administered either IL-33 or HDM intranasally to GATIR mice to induce ILC2 activation and airway inflammation in a T cell-independent and T cell-dependent manner, respectively (**Fig. 1A, 1B**).

ILC2s were defined by flow cytometry as Lineage$^-$ lymphocytes expressing YFP and Sca-1 (**Fig. 1C**). Analysis of BAL fluid obtained one day after the final challenge showed that IL-33 triggered expansion of eosinophils, ILC2s and to a lesser extent CD4$^+$ T cells (**Fig. 1D**). The YFP signals paralleled the expression of GATA3 protein, as detected by intracellular flow cytometry using GATA3-specific antibodies, both within Lineage$^-$ and Lineage$^+$ lymphocyte fractions (**Fig. 1E**).

In HDM-mediated allergic airway inflammation B cells were also expanded, in addition to eosinophils, CD4$^+$ T cells and ILC2s (**Fig. 1F**), and a similar correlation between GATA3 expression and YFP signals was found in both Lineage$^-$ and Lineage$^+$ lymphocyte fractions (**Fig. 1G**). Furthermore, the influx of eosinophils and ILC2s in BAL fluid after IL-33 administration did not differ between GATIR mice and wildtype controls. Upon IL-33 exposure, the mean fluorescence intensity (MFI) values of intracellular GATA3 in ILC2s were comparable between the two groups of mice in BAL fluid, lung and mediastinal lymph node (MLN) (**Suppl. Fig. S2A**). Likewise, GATIR mice paralleled wildtype counterparts when exposed to HDM and no differences in the induction of eosinophils and CD4$^+$ T cells and GATA3 expression within Th2 cells were observed (**Suppl. Fig. S2B**).

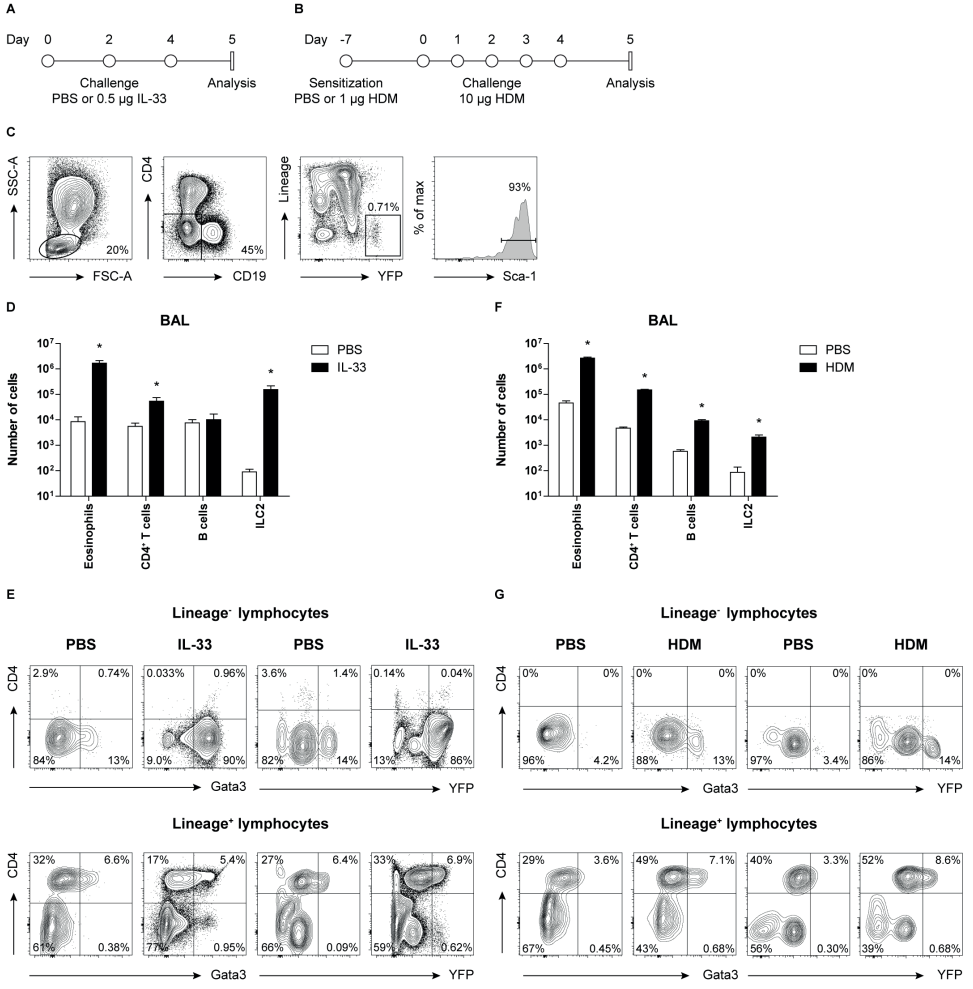


Figure 1. Concomitant GATA3 and YFP expression in ILC2s after IL-33 and HDM stimulation.

(A, B) Schemes for intranasal (A) IL-33 and (B) HDM treatment of GATIR mice for induction of acute airway inflammation. Mice were treated with PBS, IL-33 or HDM at indicated time points and analysis was performed one day after the final challenge. (C) Flow cytometric identification of Lineage$^-$YFP$^+$Sca-1$^+$ ILC2s in BAL fluid. (D, F) Quantification of eosinophils, CD4$^+$ T cells, B cells and ILC2s in the BAL fluid of IL-33 and HDM-treated mice. Data are shown as mean + SEM of n = 3-5 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$ compared to PBS control unless otherwise indicated. (E, G) Analysis of intracellular GATA3 and YFP expression by flow cytometry in Lineage$^-$ and Lineage$^+$ lymphocytes from BAL fluid of IL-33 and HDM-treated mice. Plots represent combined data using the concatenate option in FlowJo (n = 3-5), representative of two independent experiments.

Taken together, these data demonstrate that YFP provides an accurate reflection of intracellular GATA3 expression, although a direct comparison between intracellular GATA3 protein and YFP levels could not be accomplished due to the loss of YFP signal during cell fixation required

for GATA3 detection. We conclude that GATA3 protein levels and ILC2 and Th2 induction are not affected by the inserted IRES-YFP sequence, enabling us to use YFP expression in GATIR mice for the identification of ILC2s.

IL-33 and HDM-stimulated ILC2s display a variable, unstable and heterogeneous surface phenotype

Next, we compared the cell surface marker expression of ILC2s in steady state, in IL-33-mediated airway inflammation, as well as in HDM-induced allergic airway inflammation. Whereas IL-33 induced a robust expansion of ILC2s in BAL fluid, lung, MLN and spleen, HDM exposure resulted in a significant but more modest ILC2 response in BAL fluid, lung and MLN but not in spleen (**Fig. 2A**), as previously reported (17, 22).

Stimulation by IL-33 resulted in high surface expression of CD25, ICOS, KLRG1 and T1/ST2 on BAL fluid ILC2s, which are markers frequently used to define these cells (1, 2). In contrast, HDM-activated ILC2s in BAL fluid displayed large phenotypic heterogeneity and showed substantially reduced expression levels of CD25, ICOS, KLRG1 and T1/ST2, which were even lower than on ILC2s from naïve mice. ILC2s in lung and MLN showed a similar picture in that surface expression of CD25, ICOS and KLRG1 was lower on HDM-activated ILC2s than on IL-33-stimulated ILC2s. Cell surface expression of these three markers on spleen and bone marrow ILC2s were not different between mice with HDM-mediated inflammation and naïve mice, consistent with the expected localized effects of intranasal HDM exposure. T1/ST2 on ILC2s showed low expression in lung and spleen in both naïve mice and in mice with airway inflammation. On MLN and bone marrow ILC2s the expression of T1/ST2 was upregulated following IL-33 but not following HDM exposure. IL-33-stimulated ILC2s in the bone marrow were also high in KLRG1, typically expressed by inflammatory ILC2s in peripheral tissues, suggesting systemic ILC2 activation (1). In all compartments analyzed, the expression of CD117 and CD127 on ILC2s showed only minor differences between naïve and HDM-challenged mice. Whereas the effects of IL-33 on CD117 expression on ILC2s were modest, IL-33-stimulated ILC2s in lung, MLN and spleen featured a detectable downregulation of CD127 expression (**Fig. 2B**).

Next, we further investigated whether the ILC2 phenotype alters over time after exposure to IL-33. GATIR mice were challenged with IL-33 and sacrificed at several time points after the final challenge. As expected, eosinophil counts gradually decreased, but ILC2 numbers appeared to peak on day 3 (**Fig. 2C**). ICOS and KLRG1 expression followed a similar pattern to eosinophils, while CD117 was upregulated at later time points. Remarkably, we found that while CD25 was initially highly expressed on IL-33-stimulated ILC2s, the expression was significantly decreased from day 3 after the final challenge and onwards with similar trends in both BAL fluid and lung tissue (**Fig. 2D**). Only minor changes were observed in T1/ST2 expression and Thy1.2 (CD90.2) was mildly upregulated by day 10 in a similar pattern

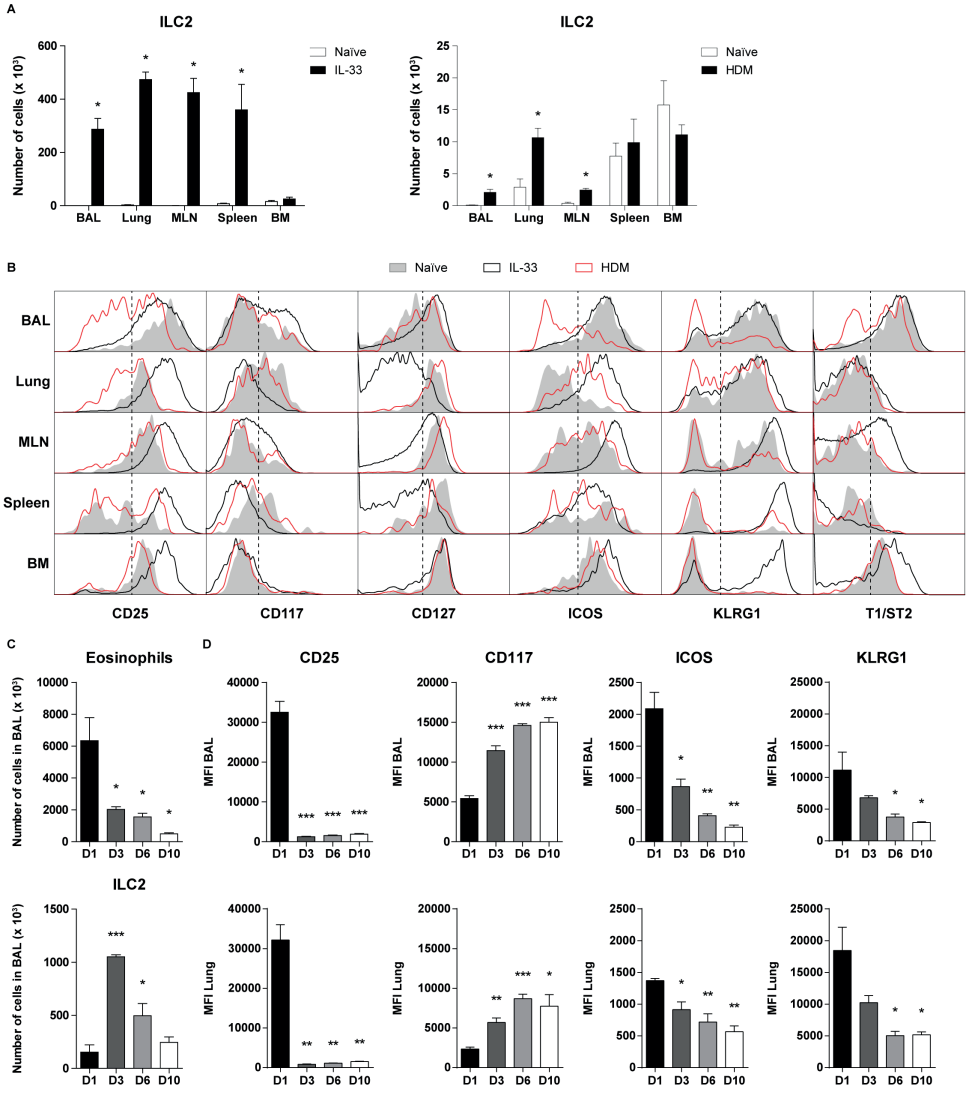


Figure 2. Dynamics of phenotypic ILC2 heterogeneity in airway inflammation.

(A) Quantification of ILC2s in BAL fluid, lung, MLN, spleen and bone marrow (BM) of GATIR mice treated with IL-33 and HDM. (B) Comparison of ILC2 phenotype in the indicated tissues derived from naïve (*shaded*), IL-33 (*black*) or HDM-treated (*red*) mice. Plots represent combined data using the concatenate option in FlowJo (n = 4-6), representative of two independent experiments. (C) Eosinophil and ILC numbers in BAL fluid of IL-33-treated mice at day 1, day 3, day 6 and day 10 after the final challenge. (D) Quantification of BAL fluid and lung ILC2 surface markers expressed as mean fluorescence intensity (MFI) values. (A, C, D) Data are shown as mean + SEM of n = 4-6 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to (A) naïve control or (C, D) D1 unless otherwise indicated.

as CD117 (**Suppl. Fig. S3**). Furthermore, the percentage of ILC2s expressing proliferation marker Ki-67 significantly decreased at later time points, suggesting a decreased activation state (**Suppl. Fig. S3**).

The characteristic downregulation of CD25 expression revealed a type of ILC2 phenotypic heterogeneity that to the best of our knowledge has not been described previously. Particularly in BAL fluid, but also in lung and MLN, IL-33-stimulated ILC2s showed a $CD25^{high}CD127^{low}$ phenotype on day 1 after challenge while IL-33-exposed ILC2s on day 6 and HDM-activated ILC2s showed an inversed $CD25^{low}CD127^{high}$ phenotype (**Fig. 3A**). When we specifically gated $CD25^{low}$ ILC2s present in BAL fluid and lung from mice with HDM-driven inflammation, we found that these cells displayed low surface expression of ICOS, KLRG1 and T1/ST2 compared to gated $CD25^{high}$ ILC2s. However, both $CD25^{high}$ and $CD25^{low}$ ILC2s highly expressed CD90.2 on the cell surface (data not shown). Additionally, $CD25^{high}$ ILC2s present in HDM-driven and IL-33-driven inflammation showed differences as well, e.g. in surface CD127 expression in the lung and in ICOS expression in BAL fluid and lung (**Suppl. Fig. S4**). Consistent with our previous finding that ILC2 induction in BAL fluid in HDM-driven allergic airway inflammation is IL-33-independent (22), we noticed that the absence of IL-33 in HDM-exposed *Il33*$^{-/-}$ mice did not change CD25 expression on ILC2s compared to wildtype control mice (**Fig. 3B**). Stimulation of ILC2s with IL-25 also did not result in altered CD25 expression compared to IL-33-stimulated mice (**Fig. 3C**). However, CD25 expression did decrease further over time in HDM-challenged mice, in particular in the BAL fluid, although not as drastically as the kinetics seen in IL-33-challenged mice. Consistent with this finding, T1/ST2 and Ki-67 were also downregulated over time while CD127 expression remained relatively stable (**Fig. 3D**). We confirmed these findings in wildtype mice and found that, as expected, GATIR mice did not significantly differ from wildtype counterparts in both surface marker expression of ILC2s (**Suppl. Fig. S5A**) or cytokine production (**Suppl. Fig. S5B**) in BAL fluid, lung or MLN. Taken together, these findings show that in BAL fluid IL-33-stimulated ILC2s had an almost uniform $CD25^{+}CD127^{+}T1/ST2^{+}ICOS^{+}KLRG1^{+}$ phenotype, while HDM-induced ILC2s displayed a heterogeneous surface phenotype characterized by substantially lower levels of CD25, ICOS and KLRG1. In both models of airway inflammation ILC2s exhibited variable expression of many surface markers commonly used to identify ILC2s with substantial differences across tissues and dependent on the time of analysis, suggesting that the microenvironment and the stage of inflammation are important factors in determining ILC2 phenotype.

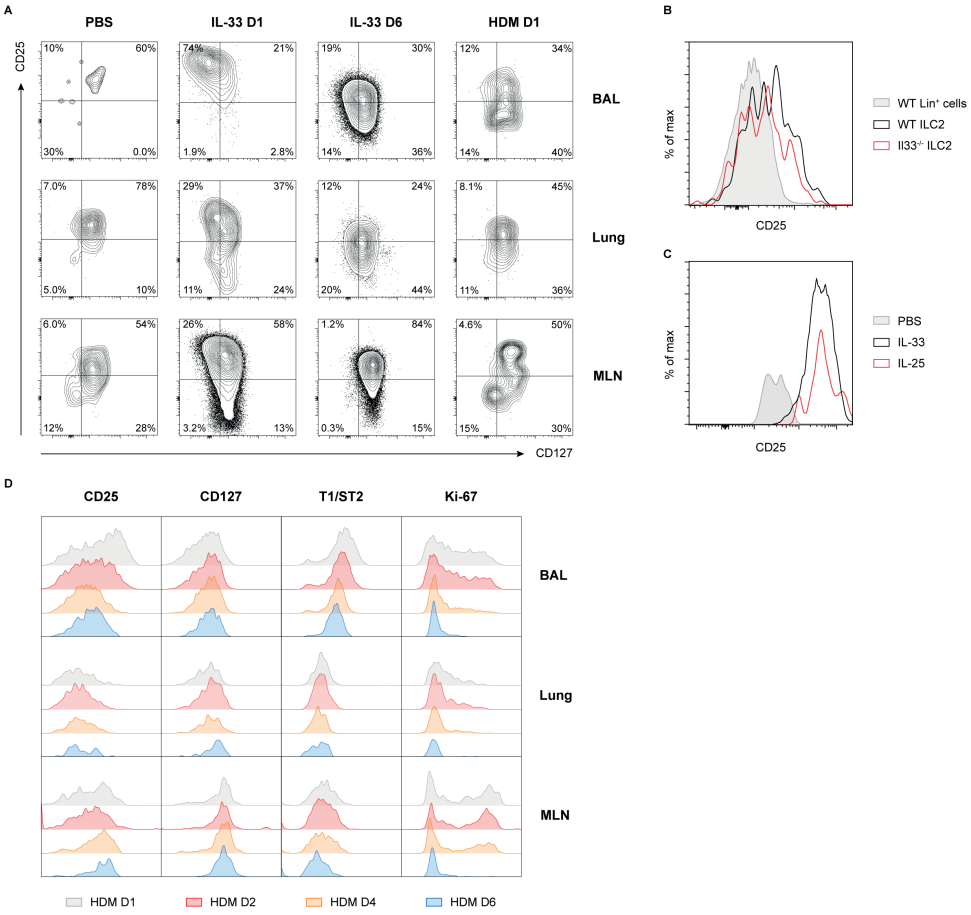


Figure 3. Expression of CD25 on ILC2s is highly variable and time dependent.

(A) Expression profiles of CD25 and CD127 on ILC2s from BAL fluid, lung and MLN in PBS, IL-33 and HDM-treated GATIR mice. (B) Flow cytometric analysis of CD25 expression on gated BAL fluid ILC2s in HDM-treated wildtype and *Il33*$^{-/-}$ mice, shown as histogram overlays, compared to control lineage marker positive cells. (C) Comparison of CD25 expression on gated BAL fluid ILC2s in PBS, IL-25 and IL-33-treated mice, shown as histogram overlays. (D) Expression profiles of CD25, CD127, T1/ST2 and Ki-67 over time on ILC2s from BAL fluid, lung and MLN in HDM-treated mice. (A-D) Plots represent combined data using the concatenate option in FlowJo (n = 3-6), representative of two independent experiments.

Both CD25^{high} and CD25^{low} ILC2s have the capacity to produce type 2 cytokines

Next, we used intracellular flow cytometry following *in vitro* stimulation with PMA and ionomycin for 4 hours to determine the capacity of ILC2 to product type 2 cytokines. Both CD25^{high} and CD25^{low} ILC2s present in BAL fluid from HDM-challenged mice had a similar capacity to produce IL-5, IL-13 and amphiregulin (**Fig. 4A, 4B**). The proportions of the BAL fluid ILC2s

that were IL-5^{+} and IL-13^{+} in HDM-driven inflammation were ~75% and ~55%, respectively, which was significantly lower than the proportions observed upon IL-33 exposure (~95% and ~85%, respectively) (17). We found marginal levels of IL-4 production (data not shown), but IL-17 production was detectable in both CD25^{high} and CD25^{low} ILC2s, albeit at low levels (**Fig. 4A, 4B**). In these experiments we did not detect expression of IFN-γ. IL-17^{+} ILC2s were more clearly visible after IL-33 stimulation and decreased quite rapidly together with amphiregulin, in contrast to IL-5 and IL-13, which remained highly expressed (**Suppl. Fig. 6A**). Importantly, all IL-17 producing ILC2s also secreted IL-5, hinting at functional plasticity (**Suppl. Fig. 6B**). As analyzed by Ki-67 expression, CD25^{low} ILC2s were slightly more proliferative than CD25^{high} ILC2s in BAL fluid, but not in lung or MLN (**Fig. 4C**).

In summary, low surface expression of CD25 did not appear to impact the capacity of ILC2 to produce cytokines. In addition, a proportion of CD25^{high} and CD25^{low} ILC2s were able to secrete IL-5 and IL-17 simultaneously, suggesting a hybrid ILC2/ILC3 function.

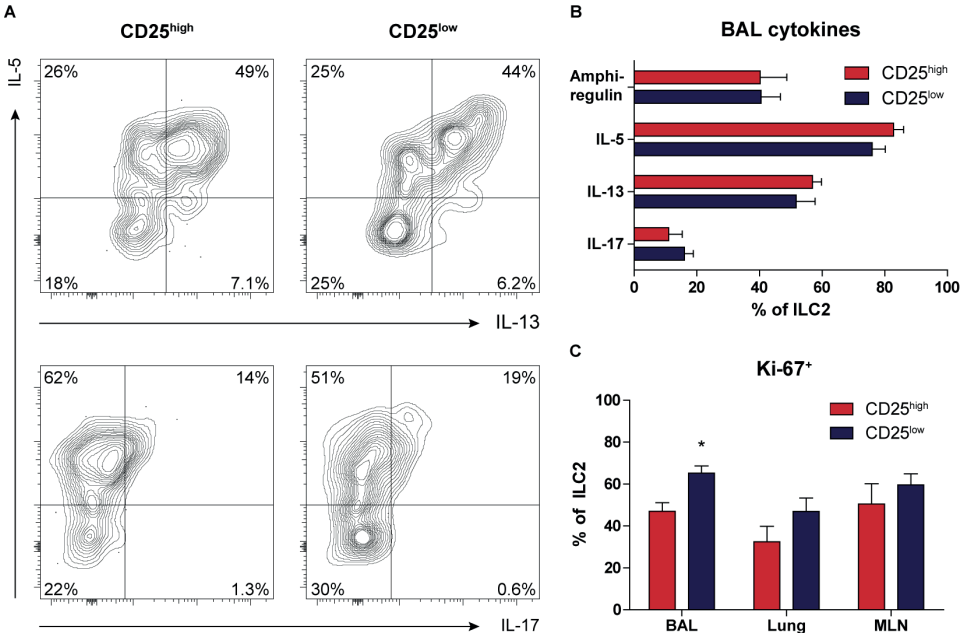


Figure 4. Both CD25^{high} and CD25^{low} ILC2s in HDM-driven airway inflammation have the capacity to produce type 2 cytokines.

(A) Flow cytometric analysis of IL-5, IL-13 and IL-17 production in BAL fluid by CD25^{high} and CD25^{low} ILC2s of HDM-treated mice. Plots represent combined data using the concatenate option in FlowJo (n = 6), representative of two independent experiments. (B) Proportions of cytokine producing ILC2s in the BAL fluid after HDM stimulation, stratified by CD25 expression. (C) Comparison of proliferative capacity between CD25^{high} and CD25^{low} ILC2s in BAL fluid, lung and MLN of mice after HDM treatment as indicated by Ki-67 expression. (B, C) Data are shown as mean + SEM of n = 6 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$ compared to CD25^{high} ILC2s unless otherwise indicated.

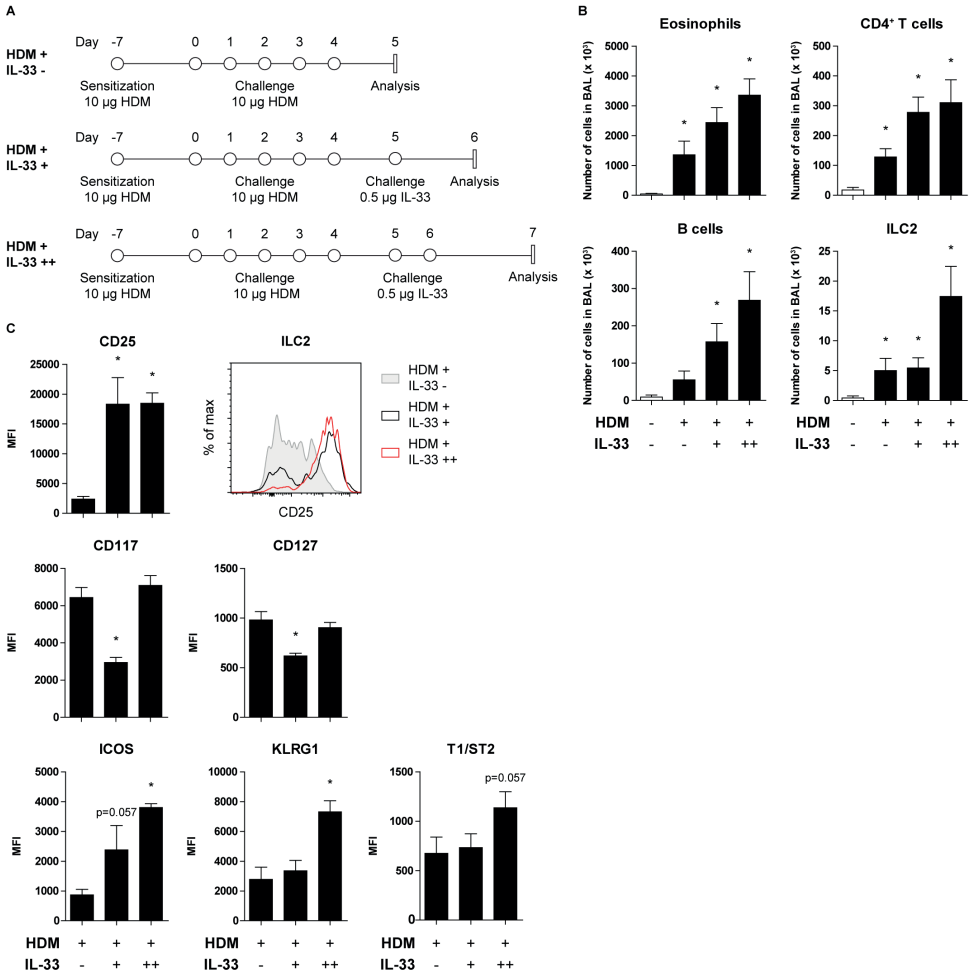


Figure 5. CD25 expression on HDM-stimulated ILC2s is restored by acute IL-33 exposure.

(A) Schemes for administration of HDM with and without subsequent IL-33 exposure in GATIR mice. (B) Quantification of BAL fluid eosinophils, CD4+ T cells, B cells and ILC2s in animals treated with PBS, HDM alone or in combination with IL-33. (C) Surface marker expression profile of BAL fluid ILC2s. (B, C) Data are shown as mean + SEM of n = 4 mice per group of a single experiment. * $p \leq 0.05$ compared to PBS control unless otherwise indicated. (C) Plot represents combined data using the concatenate option in FlowJo (n = 4).

CD25^{low} ILC2s in HDM-treated mice are reversed to a CD25^{high} phenotype upon subsequent stimulation with IL-33

Recent publications describing the plasticity and heterogeneity of ILC2s (32-35) prompted us to investigate the reversibility of the downregulation of CD25 on ILC2s as a result of HDM-induced allergic airway inflammation. GATIR mice were sensitized and challenged with

HDM and subsequently treated with a single or double exposure to IL-33 (**Fig. 5A**). Control mice were only treated with PBS. BAL fluid eosinophil, $CD4^+$ T cell and B cell counts were significantly elevated after HDM treatment and further exacerbated in a dose-dependent manner upon IL-33 exposure. ILC2 numbers did not increase with a single IL-33 treatment in HDM-exposed mice but required two consecutive IL-33 doses to expand (**Fig. 5B**). However, CD25 expression on HDM-activated ILC2s was readily upregulated after a single IL-33 dose while numbers remained stable, indicating upregulation of CD25 expression on existing cells as opposed to influx or generation of new $CD25^{high}$ ILC2s (**Fig. 5C**). Other ILC2 activation markers such as ICOS, KLRG1 and T1/ST2 were also upregulated, although less rapidly than CD25. Interestingly, the MFI values of CD117 and CD127 were initially decreased and returned to previously levels after a second IL-33 treatment, again highlighting the adaptability of ILC2 phenotype depending on the microenvironment (**Fig. 5C**).

Chronic HDM exposure predominantly induces $CD25^{low}$ ILC2s, which substantially contribute to type 2 cytokine production in the airways

Because ILC2s have been implicated in the maintenance of chronic asthma (53), we next investigated accumulation and phenotype of ILC2s in GATIR mice chronically exposed to HDM for a period of five weeks (**Fig. 6A**). We have recently shown that chronic HDM exposure induces allergic airway inflammation, in addition to the formation of inducible bronchus-associated lymphoid tissue and tissue remodeling in the lungs (54). At day 4 after the final challenge, we detected an eosinophilic infiltrate in the BAL fluid, indicative of active inflammation (**Fig. 6B**). Compared with our acute HDM-driven lung inflammation model, eosinophil numbers in BAL fluid were modest and the contribution of B cells was larger and in the same range as $CD4^+$ T cells (**Fig. 6C**). In comparison to PBS controls, the numbers of total ILC2s were significantly increased, of which the majority was $CD25^{low}$ (**Fig. 6D**) and both $CD25^{high}$ and $CD25^{low}$ populations of ILC2s in the BAL fluid expressed IL-5 and IL-13 (**Fig. 6E**). Compared with the acute HDM-driven inflammation model, a more sizeable contribution of ILC2s to the total number of cytokine producing cells was observed in chronic HDM airway inflammation exposure, of which the $CD25^{low}$ subset represented a larger proportion than the classic $CD25^{high}$ subset (**Fig. 6F**). Next, we investigated whether the longevity of $CD25^{high}$ and $CD25^{low}$ ILC2s was different in the resolution phase of chronic HDM-mediated allergic airway inflammation. We quantified $CD25^{high}$ and $CD25^{low}$ ILC2s at 3, 6 and 10 days after the last allergen challenge and found that total numbers of $CD25^{low}$ ILC2s appeared to be more persistent than $CD25^{high}$ ILC2s (**Fig. 6G**).

Taken together, these findings show that ILC2s that are present in chronic HDM-driven allergic airway inflammation are prominent producers of IL-5 and IL-13 and have a $CD25^{low}$ phenotype, particularly in the resolution phase of inflammation.

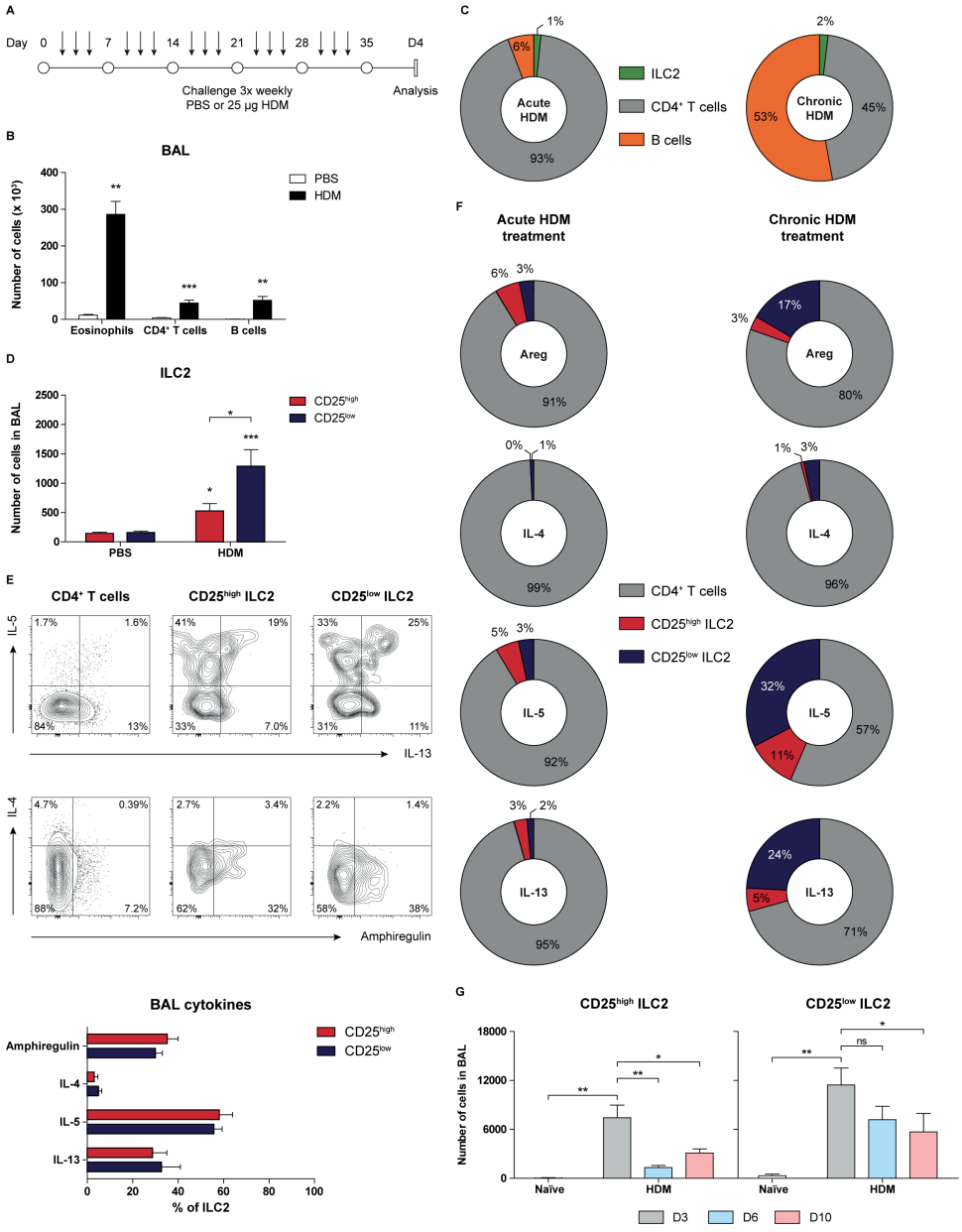


Figure 6. ILC2s with CD25^{low} phenotype are important cytokine producers in chronic HDM-induced airway inflammation.

(A) Scheme for intranasal HDM treatment of GATIR mice for induction of chronic allergic airway inflammation. (B) Quantification of eosinophils, CD4$^+$ T cells and B cells in the BAL fluid of PBS and HDM-treated mice. (C) Relative contribution of CD4$^+$ T cells, B cells and ILC2s in the BAL fluid of acute and chronic HDM-treated mice. (D) Quantification of CD25^{high} and CD25^{low} ILC2s in the BAL fluid of PBS and HDM-treated mice. (E) Flow cytometric

analysis and quantification of amphiregulin, IL-4, IL-5 and IL-13 production by $CD4^+$ T cells, $CD25^{low}$ and $CD25^{high}$ ILC2s in the BAL fluid of HDM-treated mice. Plots represent combined data using the concatenate option in FlowJo (n = 7), representative of two independent experiments. (F) Relative contribution of $CD4^+$ T cells, $CD25^{low}$ and $CD25^{high}$ ILC2s to the total number of amphiregulin (areg), IL-4, IL-5 and IL-13 producing cells in the BAL fluid of acute and chronic HDM-treated mice. (G) Numbers of $CD25^{high}$ and $CD25^{low}$ ILC2s in the BAL fluid after chronic HDM exposure at day 3, 6 and 10 after the final challenge. (B, D, E, G) Data are shown as mean + SEM of n = 5-7 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to PBS control unless otherwise indicated.

Transcriptome profiling indicates that acutely activated ILC2s are functionally different from ILC2s in chronic airway inflammation

As outlined above, we found that the rapid and robust activation of ILC2s by IL-33 induces high levels of type 2 cytokine production by these cells (17) and an almost uniform $CD25^+CD127^+T1/ST2^+ICOS^+KLRG1^+$ phenotype. In contrast, $CD25^{low}$ ILC2s, exhibiting lower surface expression of T1/ST2, ICOS and KLRG1 and a more modest cytokine production capacity dominated in our chronic HDM airway inflammation model.

To explore differences between acutely and chronically activated ILC2 populations in an unbiased and comprehensive fashion, we FACS-sorted BAL fluid ILC2s from GATIR mice one day after the final challenge following IL-33 or chronic HDM exposure, respectively, and analyzed gene expression in these cell fractions by RNA-Seq. We identified a total number of 1623 differentially expressed genes, of which 915 were upregulated in IL-33-stimulated ILC2s and 708 in chronic HDM-stimulated ILC2s. Indeed, we found that the most significantly upregulated gene in IL-33-stimulated ILC2s was *Il2ra* encoding CD25 (**Fig. 7A**), confirming our flow cytometry data. Pathway analysis of these genes indicated that processes related to cell proliferation and division (*Cenpe, Top2a, Ccnd2*) are highly active after IL-33 stimulation (**Fig. 7A, 7B**). In contrast, HDM-exposed ILC2s showed a transcriptional signature associated with regulation of the immune system and immune cell activation, including 'innate immune system' genes such as *Cd33, Ccr2, Ctsd and Ctss* (encoding Cathepsins D and S) and *Csf2* (encoding GM-CSF) (**Fig. 7C**). Interestingly, chronic HDM exposure generated ILC2s with increased expression of genes implicated in the modulation of T cell activity (*Flt3l, Icosl, Pdl1*) and chemo-attraction of T and B cells (*Ccl6, Cxcl10*) (**Fig. 7D**). Single nucleotide polymorphisms in *Rgs2,* and IRF5 activity have been associated with asthma (55, 56) and our data highlighted upregulated transcription of these genes in HDM-stimulated ILC2s (**Fig. 7A, 7D**). Acutely IL-33-activated ILC2s produced high levels of cytokine (*Il5, Il13, Il9*) and tissue-migratory chemokine receptor (*Ccr4, Ccr7*) genes, indicating an activated effector cell phenotype. Furthermore, mediators of innate signals were also prominent, including the TLR-signaling protein Myd88 and IRF4, which is known to respond to IL-33 and TSLP (57). Surprisingly, IL-33-activated ILC2s showed low but detectable *Tbx21* (encoding T-bet) transcripts, whereas we detected increased *Rorc* (encoding RORγt) levels in HDM-activated

ILC2s, indicative of ILC2 plasticity towards group 1 and group 3 ILCs, respectively (**Fig. 7D**). Taken together, these data suggest distinct roles of ILC2s dependent on the type and the duration of stimulus. While acutely activated ILC2s by IL-33 displayed a strong effector cell phenotype, chronically HDM-stimulated ILC2s appear to regulate and assist adaptive immunity.

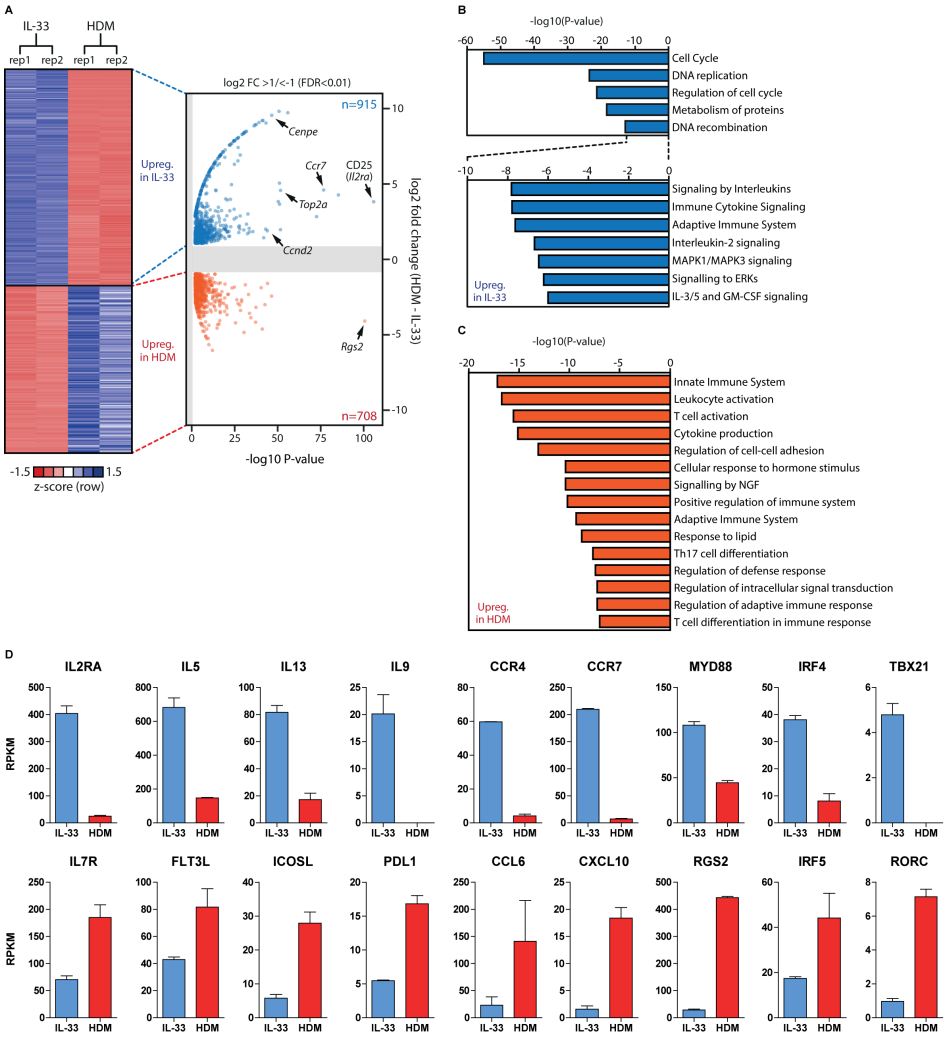


Figure 7. Gene expression signatures of ILC2s are dependent on type and duration of stimulus.

(A) Heat map and volcano plot of differentially expressed genes between IL-33 (n = 915) and chronic HDM-stimulated (n = 708) ILC2s derived from BAL fluid one day after the final challenge. (B, C) Metascape pathway analysis of genes upregulated in (B) IL-33-activated and (C) chronic HDM-stimulated ILC2s. (D) Comparison of Reads Per Kilobase Million (RPKM) values of selected differentially expressed genes in IL-33-activated (*blue*) and chronic HDM-stimulated (*red*) ILC2s.

ILC2s are situated below the lung epithelium and inside cellular infiltrates induced by acute and chronic airway inflammation

The GATIR knock-in allowed us to visualize Th2 cells and ILC2s as YFP$^+$ cells *in situ* using confocal microscopy. Our RNA-Seq data suggest contrasting functions between rapidly and chronically activated ILC2s, thus we examined whether IL-33 and HDM-stimulated ILC2s differed in localization in the lungs. Exposure to IL-33, acute and chronic HDM according to the protocols described in **Figures 1A**, **1B** and **6A** generated lung inflammation outlined by perivascular and peribronchial cellular infiltrates and thickened alveolar walls compared to PBS treated mice (**Fig. 8A**). Lung cryosections were counterstained with a combination of CD3 and B220 to label T and B cells, respectively. YFP$^+$ ILC2s were readily discriminated from YFP$^+$ Th2 cells by the absence of positivity for CD3 counterstaining (**Fig. 8B**).

Following IL-33 stimulation many CD3$^-$YFP$^+$ ILC2s were identified in cellular infiltrates directly underneath the lung epithelium, with T and B cells being largely absent (**Fig. 8C**), corroborating previously reported data and our flow cytometric data (28, 29). *In situ* hybridization of GATA3 revealed signals in similar locations as YFP$^+$ cells (**Suppl. Fig. S7**), supporting that YFP signals reflect *Gata3* transcription. In contrast, in acute HDM-induced airway inflammation T and B cells were present in higher numbers in the cellular infiltrates. Hereby, also CD3$^-$YFP$^+$ ILC2s were identified, although less abundantly present than after IL-33 stimulation (**Fig. 8C**). The ILC2s were not present in clusters and we did not find evidence for frequent interaction with B or T cells.

Chronically HDM-treated GATIR mice displayed cellular infiltrates surrounding blood vessels and near the airways in similar locations as acute lung inflammation models. However, the infiltrates appeared denser in a hematoxylin and eosin stain (**Fig. 8A**) and confocal microscopy revealed a well-defined organization within these infiltrates (**Fig. 8D**). The infiltrates were composed of separated B and T cell zones with striking similarity to inducible bronchus-associated lymphoid tissue (iBALT) structures found in influenza-infected lungs (58). CD3$^-$YFP$^+$ ILC2s were located at two distinct sites: in the submucosa close to epithelial cells, comparable to their location in IL-33-induced airway inflammation, as well as within the cellular infiltrates close to the lung epithelium and in proximity of T cell clusters (**Fig. 8D**). Interestingly, B cell areas were devoid of ILC2s suggesting preferential interaction of ILC2s with T cells. Neither in the submucosa nor within the cellular infiltrates did we find evidence for clustering or organization of ILC2s. Abundant numbers of Th2 cells, identified as CD3$^+$YFP$^+$ cells were found in the enlarged MLNs of chronically HDM-treated animals; CD3$^-$YFP$^+$ ILC2s were present near the border of the B and T cell zones (**Fig. 8D**).

In summary, these data indicate that ILC2s accumulate in cellular infiltrates underneath the lung epithelium after activation. In chronic HDM-driven inflammation ILC2s are mainly located in the submucosa close to epithelial cells, but are also found inside organized cellular infiltrates in close proximity to both epithelial cells and T cells, but not B cells.

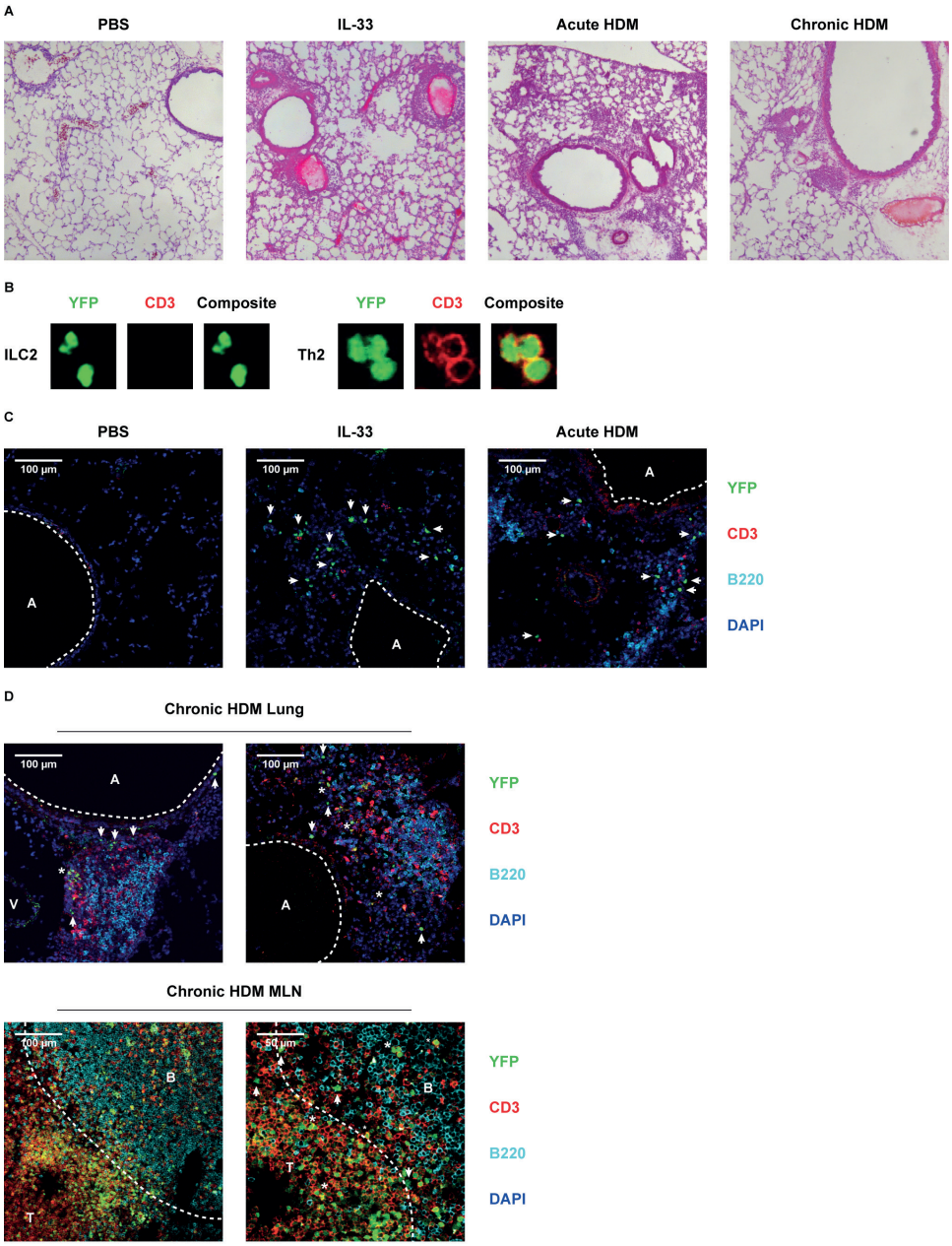


Figure 8. Pulmonary ILC2s are located within cellular infiltrates underneath the lung epithelium after IL-33, acute and chronic HDM exposure.

(A) Hematoxylin and eosin stained cryosections of lungs from PBS, IL-33, acute HDM and chronic HDM-treated GATIR mice. (B) Discrimination between ILC2s and Th2 cells by counterstain with CD3 and detection by confocal microscopy. (C) Lung cryosections from PBS, IL-33 and acute HDM-treated GATIR mice counterstained with CD3 and B220.

(D) Lung and MLN cryosections from chronic HDM-treated GATIR mice counterstained with CD3 and B220. In the confocal microscopy conditions, GATA3^{low} cells (such as non-Th2 CD4$^+$ T cells and basophils) are not YFP$^+$. It is of note that in chronic HDM-driven airway inflammation only ~10% of all CD4$^+$ T cells in the lung consists of GATA3^{high} Th2 cells (54). [A] indicates airway lumen. [V] indicates lumen of blood vessel. ILC2s are indicated by [arrows] and [*] indicates Th2 cells. [B] and [T] indicate B and T cell zones, respectively, separated by the dashed line. (A-D) Images are representative of two independent experiments.

DISCUSSION

In type 2 airway inflammation ILC2s act as an early innate source of IL-5 and IL-13 when strong allergenic stimuli induce epithelial pro-inflammatory cytokines, including IL-33, that have the capacity to directly activate ILC2s independently of T cells (1, 2). However, because we previously found that in HDM-mediated allergic airway inflammation ILC2s are activated by different T cell-dependent pathways and IL-33 is not required for the HDM-driven induction of ILC2s in the BAL fluid (22), we hypothesized that ILC2 phenotype, function and localization in HDM- and IL-33-driven inflammation may be different.

When using GATIR mice in an acute HDM-driven airway inflammation model, we detected the induction of a much more heterogeneous ILC2 population in BAL fluid, lungs and draining lymph nodes than upon robust IL-33-induced ILC2 activation. We found that a large proportion of the ILC2s expressed low levels of CD25, and that these CD25^{low} cells were generally low for KLRG1, T1/ST2 and ICOS on the cell surface as well, although we observed compartmental differences (**Fig. 2B** and **Suppl. Fig. S4**). Nevertheless, they displayed a type 2 cytokine profile that was similar to their classic CD25^{high} counterparts. The CD25^{low} ILC2s in HDM-treated mice could be reverted back to a CD25^{high} phenotype by subsequent exposure to IL-33 and ILC2s lost high CD25 expression by day 3 after IL-33 treatment, demonstrating the adaptability of ILC2s. We also observed that the levels of cell surface marker expression are highly dynamic over time, as e.g. shown by our analysis of CD25, CD117, ICOS and KLRG1 surface expression at different time points after the last IL-33 exposure (**Fig. 2C**).

Our IL-33 and HDM-treatment models are similar in terms of the activation period of ILC2s and the timing of the analysis: one day after the last challenge (**Fig. 1A, 1B**). In this context, it is of note that we have previously shown that in the HDM-driven model ILC2s are only activated in the allergen challenge phase when memory T cells are present and not upon sensitization, even when a single dose of 100 μg of HDM is given (22). It is therefore conceivable that certain differences in ILC2 phenotype between IL-33 and HDM-stimulated ILC2s are related to changes in cell-cell interactions or cytokine environment, given that activation of ILC2s by IL-33 and HDM is T cell-independent and T cell-dependent, respectively (17, 22). Collectively, our findings show that the cell surface marker composition of ILC2s is highly dynamic: markers follow a specific kinetic pattern after activation and are dependent on the mechanisms by which these cells are activated, e.g. a requirement of T cells for their activation. It is of note that quite some heterogeneity in the expression levels of cell surface molecules, including

CD25, CD69, CD117, ICOS and T1/ST2 on resident ILC2s in the lungs of naïve mice has been observed among published studies, which is likely due to differences in the experimental models and housing conditions of the mice (59). On the other hand, our finding of e.g. lower expression of T1/ST2 on lung ILC2s than on BAL ILC2s upon IL-33 or HDM-stimulation clearly parallels observations in a *Nippostrongylus brasiliensis* infection model (60).
In chronic HDM-driven inflammation the $CD25^{low}$ ILC2s outnumbered $CD25^{high}$ ILC2s, remained elevated at least 10 days after the last antigen challenge in the resolution phase of inflammation and produced substantial amounts of IL-5 and IL-13, thus contributing significantly to the total Th2 cytokine production. Transcriptome comparisons of BAL fluid ILC2 populations from mice with acute IL-33-driven and chronic HDM-driven allergic airway inflammation revealed distinct gene expression profiles. Rapid and robust activation of ILC2s by IL-33 resulted in elevated transcript levels of genes involved in cellular proliferation and cytokine production. In contrast, in chronic HDM-mediated airway inflammation ILC2s showed a transcriptional signature consistent with a capacity to modulate adaptive immunity by interacting with B and T cells. Visualization of ILC2s by confocal microscopy showed their submucosal accumulation upon IL-33 exposure and their presence in cellular infiltrates upon HDM exposure. Chronic exposure to HDM was associated with the formation of organized cellular clusters with defined T and B cells zones where ILC2s were located in close proximity to epithelial cells and T cells but not B cells and did not appear to form clusters.
Development and function of ILC2s critically depends on high levels of unperturbed GATA3 expression (39-41). To facilitate identification of ILC2 populations, potentially differing with respect to the expression levels of commonly used ILC2-associated surface markers, e.g. CD25 or KLRG1, we employed a novel strain of *Gata3* reporter mice, termed GATIR, carrying an IRES-YFP reporter construct within the 3' untranslated region of the endogenous *Gata3* alleles [T.N.R and H.J.F., manuscript in preparation]. Knock-in reporter mice are not without pitfalls and should not perturb the target gene expression itself. Therefore, we provided evidence that the presence of the GATIR reporter affected neither GATA3 protein levels (by intracellular flow cytometry with GATA3-specific antibodies), nor ILC2 surface marker profile or cytokine production. Although the IRES-driven reporter construct monitors gene expression at the transcript level, our intracellular flow cytometric analysis with GATA3-specific antibodies revealed a strong correlation between YFP signals and GATA3 protein in ILC2s and T cells.

ILC2s were considered to be relatively homogeneous and less plastic compared to the other ILC family members. This point of view may have been triggered by the use of robust ILC2 induction models such as administration of IL-25, IL-33, papain and *Alternaria* to study these cells in the context of allergic asthma or dermatitis (15, 16, 18, 20, 61, 62). Our finding that in allergic airway inflammation ILC2s are a phenotypically and functionally more heterogeneous and phenotypically dynamic cell population than previously thought adds to the modified picture of ILC2s that includes their functional plasticity driven by IL-12, IL-18 or viral infection

(32-35). In line with this notion, we detected simultaneous IL-5 and IL-17 production in HDM- and in particular IL-33-stimulated ILC2s and our results may support that ILC2s can upregulate T-bet or RORγt mRNA levels in a stimulus-specific manner. Phenotypical heterogeneity within the ILC2s induced in type 2 inflammation together with their functional plasticity allows ILC2s to adapt to changes in their microenvironment. Therefore, it is conceivable that these characteristics allow ILC2s to be involved in different human asthma endotypes, ranging from classic eosinophilic allergic airway inflammation to non-allergic airway hyperreactivity, as well as in infection-associated exacerbations.

By investigating the expression of several markers commonly used to define ILC2s, we identified a yet undescribed $CD25^{low}$ ILC2 surface phenotype. CD25 appeared to correlate well with the expression of T1/ST2 and in particular KLRG1, which is known as a marker for mature and inflammatory ILC2s (39, 63). This might suggest that the $CD25^{low}$ ILC2 population reflects an immature and non-inflammatory resident ILC2 population. However, in contrast to ILC2 progenitors in the bone marrow, these $CD25^{low}$ ILC2s are able to produce effector cytokines and expand upon stimulation with HDM, which would argue against their immature nature (39, 64). In further support of this concept, we found that $CD25^{low}$ ILC2s (i) are typically present after IL-33 exposure when the total numbers of ILC2s in the BAL are highest (**Fig. 2C, 2D**) and (ii) show prolonged cytokine production and contribute to the type 2 cytokine environment in our chronic HDM-driven allergic airway inflammation model. Indeed, the more pronounced role of ILC2s in chronic inflammation could be attributed to the acquisition of "memory-like" properties, as recently described (65). Such a memory phenotype allows experienced ILC2s to persist for extended periods of time and respond more potently than naïve ILC2s. Cytokine signaling through the common gamma chain is required for the survival of ILC2s and it has been demonstrated that IL-7 and in particular IL-2 synergistically augment ILC2 expansion in the presence of IL-33, which in turn promotes expression of CD25 in both adipose and lung tissue, thus forming a positive feedback loop by modulating sensitivity to IL-2 (6, 17, 66-69). Accordingly, our study also demonstrates upregulation of CD25 after IL-33 exposure of HDM-experienced ILC2s. The source of IL-2 has earlier been proposed to be T cell-derived, but recent data using IL-2 fate reporter mice has revealed that pulmonary ILC3 could be an innate producer of IL-2 (26, 69).

We found that high CD25 expression on the cell surface of IL-33-activated ILC2s was associated with a decrease in CD127 expression and conversely that $CD25^{high}$ and $CD25^{low}$ HDM-activated ILC2 showed persistent CD127 expression. This may imply that the $CD25^{low}$ ILC2s are more reliant on IL-7 for their survival and function. Therefore, it could be speculated that these ILC2s can partly escape cross-regulation by Tregs, which are reported to control ILC2 responses by competition for IL-2 uptake (70). In a recent study, co-culture of ILC2s with Tregs was shown to downregulate ICOS and CD25 expression (71). It is conceivable that $CD25^{low}$ ILC2s in HDM-mediated airway inflammation represent ILC2s that have interacted with Tregs *in*

vivo, because IL-5 and IL-13 levels are substantially decreased in ILC2s co-cultured *in vitro* with Tregs (71), and our expression analyses demonstrate that the $CD25^{low}$ ILC2s in HDM-driven inflammation have lower levels of cytokine transcripts than IL-33 activated ILC2s. However, it cannot be formally excluded that $CD25^{low}$ IlC2s are cells that just received an IL-2 stimulus leading to downregulation of the receptor. Based on our findings in *Il33*$^{-/-}$ mice, we conclude that IL-33 is unlikely to play a key role in the regulation of CD25 in HDM-mediated allergic airway inflammation. The combination of signals that specifically triggers CD25 up- and downregulation on ILC2s remains to be elucidated. Although expression of CD25 has been reported in human ILC2s from skin biopsies, adipose tissue and *in vitro* cultured ILC2s derived from peripheral blood, it is unknown whether pulmonary ILC2s also express this marker and its role in disease pathology has not been elucidated (25, 33, 61, 62).

Mice that were chronically exposed to HDM displayed well-organized and dense cellular clusters in the lungs reminiscent of iBALT structures seen after influenza infection (58). These structures harbored clear B and T cell zones and we observed ILC2s in proximity to T cells and not B cells. Indeed, ILC2s can express molecules that allow them to interact with T cells such as MHC class II, CD86 and ICOS/ICOS-L in addition to their cytokine repertoire (25-27). In the lungs, their positioning would allow them to act as an intermediate messenger to shuttle signals from the epithelium to T cells. For example, activation of ILC2s by epithelial signals can help reinforce a Th2 phenotype through direct cell contact. Conversely, $CD25^{high}$ ILC2s may become more activated in the presence of T cell-derived IL-2 and promote alternatively activated macrophages through IL-13, which in turn assist with repair of the epithelium and resolution of inflammation. In the lymph nodes, we found that ILC2s were situated along the border of B and T cells, an optimal site to communicate with both cell types. In this context, our RNA-Seq data supports the notion that ILC2s have the ability to interact with adaptive immune cells and revealed that HDM-stimulated ILC2s showed robust expression of FLT3L, ICOSL and PDL1, which would allow ILC2s to regulate B and T cell functions. Additionally, ILC2-derived factors such as IL-5 can enhance Ig production (72).

In summary, in addition to the reported plasticity of ILC2 towards other ILC subsets, we found that the ILC2 phenotype is highly dynamic. This phenotype is dependent on the mode of cellular activation, changes over time during stimulation, exhibits differences across tissues and is reversible. In particular, we identified previously undescribed ILC2s with a $GATA3^{+}CD25^{low}$ phenotype and a type 2 cytokine profile indistinguishable from their classic $GATA3^{+}CD25^{high}$ counterparts. These $CD25^{low}$ ILC2s contributed substantially to type 2 cytokine production and are able to readily revert to a $CD25^{high}$ phenotype upon stimulation with IL-33. Although it cannot be excluded that $CD25^{high}$ and $CD25^{low}$ ILC2s represent separate subsets, the observed changes of CD25, KLRG1, ICOS and T1/ST2 over time and differences between IL-33-activated and HDM-driven $CD25^{high}$ ILC2s point to a different activation status of a single ILC2 type. Our data suggest a more complex ILC2 phenotype than is currently appreciated. Hereby,

distinct functional capabilities of ILC2s, including responsiveness to IL-2, IL-33 or ICOSL, are tissue-specific and dependent on the type and duration of the stimulus used. The observed phenotypic heterogeneity should however not hamper the identification and quantification of ILC2s as long as GATA3, which is invariably expressed by ILC2s, Sca-1 and CD90 are included in flow cytometric analysis strategies.

Ethics Statement

All animal care and experimental procedures were performed in accordance to guidelines approved by the Erasmus MC Animal Ethics Committee.

Author Contributions

BL, RS, MdB, ML, DB, MB, AK, IB, performed the experiments; HV and MK provided methodologies (chronic HDM mouse model); WvIJ provided methodologies (RNA-Seq); TNR and HJF provided essential materials (GATIR mouse). BL and RS analyzed the data. BL, RS and RH conceived the study and wrote the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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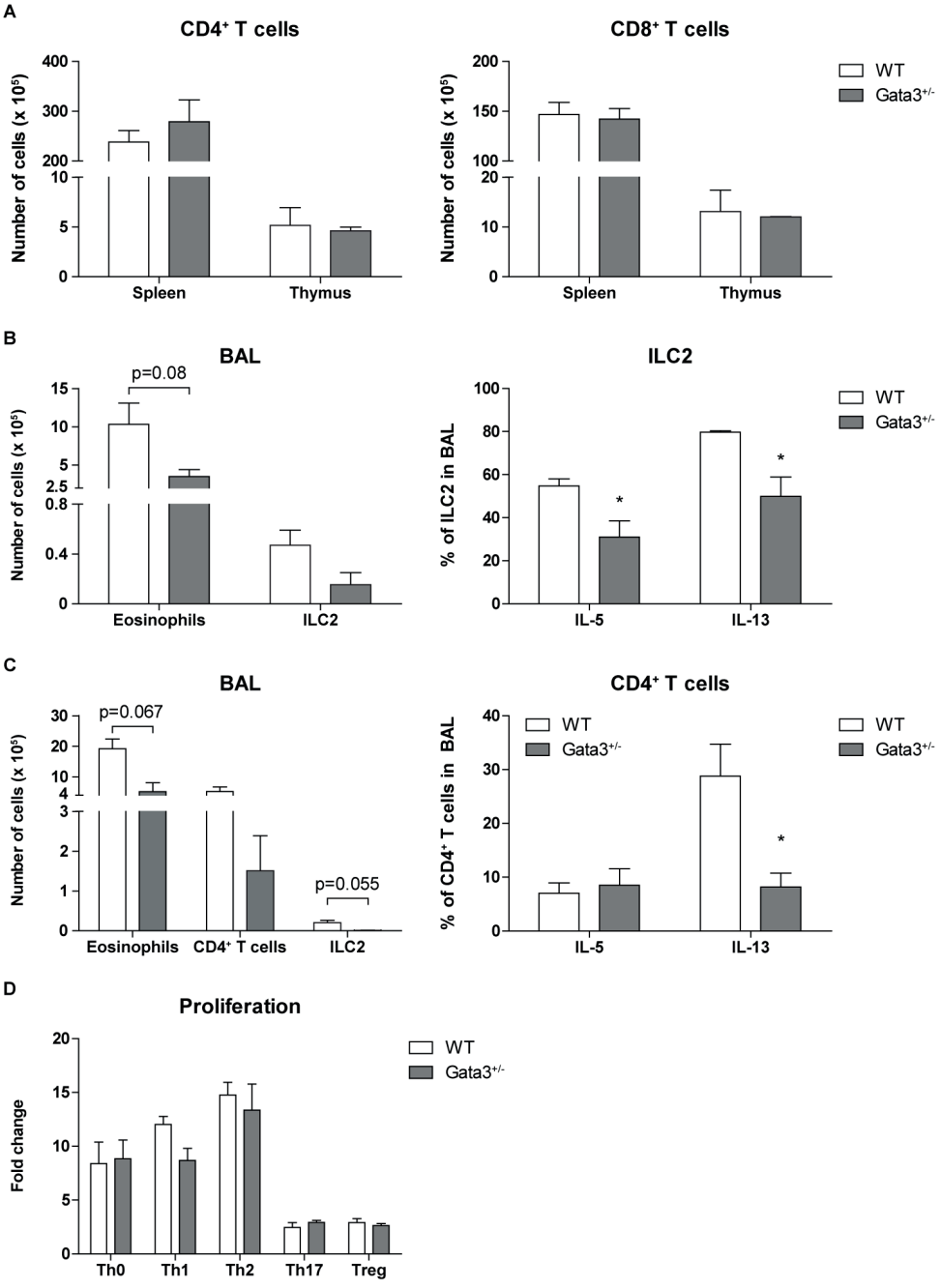
A
CD4+ T cells
Number of cells (x 10^5)
400
300
200
10
5
0
Spleen
Thymus
CD8+ T cells
200
150
100
20
10
0
WT
Gata3+/-
B
BAL
p=0.08
15
10
5
2.5
0.8
0.4
0
Eosinophils
ILC2
ILC2
% of ILC2 in BAL
100
80
60
40
20
0
*
IL-5
IL-13
C
BAL
p=0.067
30
20
10
4
3
2
1
p=0.055
Eosinophils
CD4+ T cells
ILC2
CD4+ T cells
% of CD4+ T cells in BAL
40
30
20
10
IL-5
IL-13
D
Proliferation
Fold change
20
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Th0
Th1
Th2
Th17
Treg

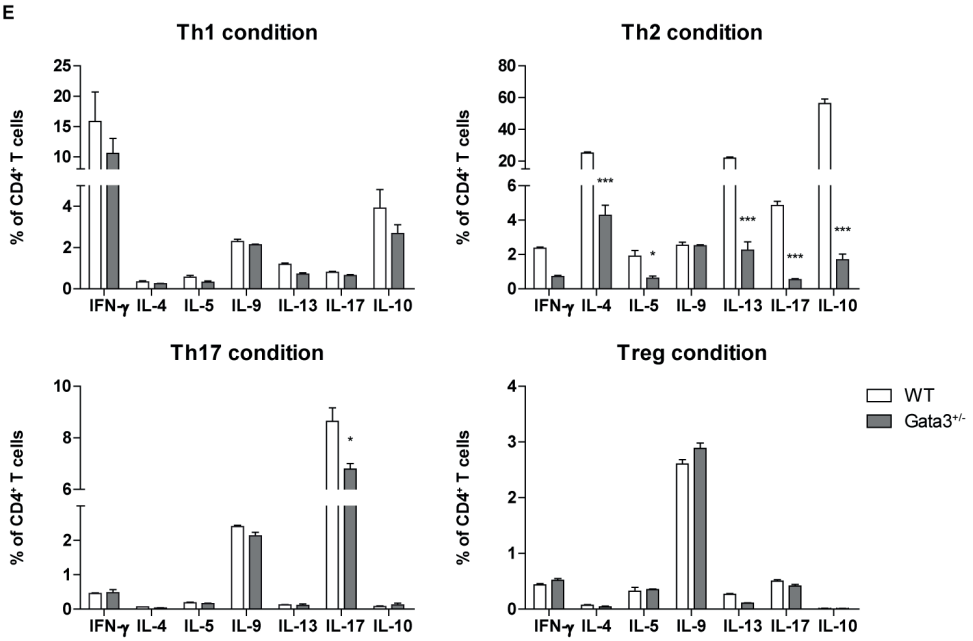

Figure S1. Reduction of GATA3 levels impairs ILC2 and Th2 induction and cytokine production.

(A) Quantification of $CD4^+$ and $CD8^+$ T cells in the spleen and thymus of naïve wildtype (WT) and *Gata3*$^{+/-}$ mice.
(B) Quantification of eosinophils, ILC2s and ILC2 cytokine production in broncho-alveolar lavage (BAL) fluid of WT and *Gata3*$^{+/-}$ mice stimulated with IL-33.
(C) Quantification of eosinophils, $CD4^+$ T cells, ILC2s and $CD4^+$ T cell cytokine production in BAL fluid of WT and *Gata3*$^{+/-}$ mice stimulated with house dust mite (HDM).
(D) *In vitro* proliferation of naïve $CD4^+$ T cells from WT and *Gata3*$^{+/-}$ mice cultured under Th0, Th1, Th2, Th17 and Treg polarizing conditions.
(E) Cytokine profiles of $CD4^+$ T cells from WT and *Gata3*$^{+/-}$ mice cultured under the indicated polarizing conditions.
(A-C) Data are shown as mean + SEM of n = 3-5 mice per group of a single experiment and are representative of two independent experiments.
(D, E) Data are shown as mean + SEM of n = 3 mice per group of a single experiment and are representative of three independent experiments.
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to WT control unless otherwise indicated.

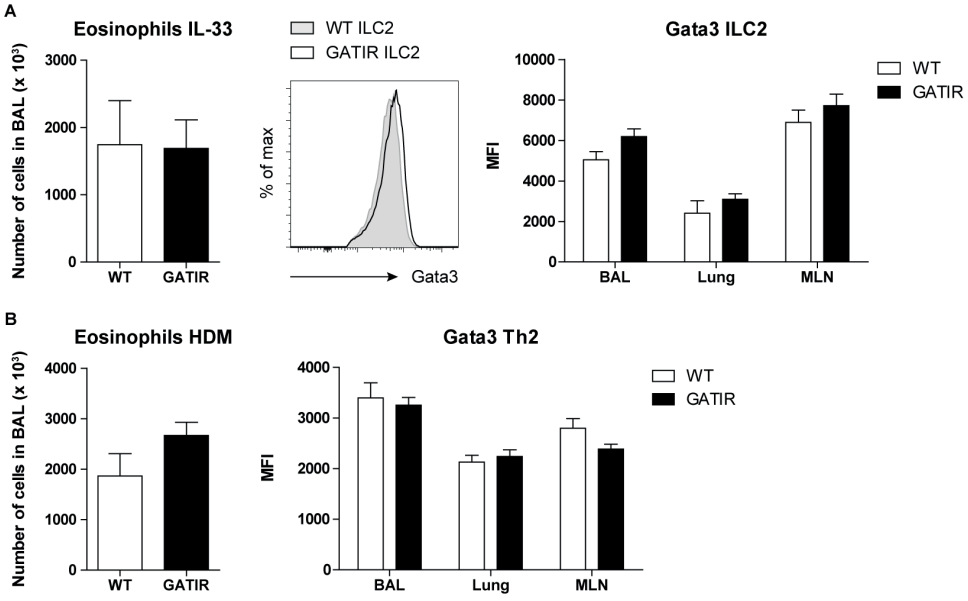

Figure S2. GATIR knock-in does not affect GATA3 expression in ILC2s and T cells.

(A) Number of BAL fluid eosinophils (*left*) and histogram overlay of intracellular GATA3 expression, as determined by flow cytometry in ILC2s from BAL fluid (*middle*) and comparison of GATA3 mean fluorescence intensity (MFI) values in ILC2s between IL-33-treated GATIR mice and WT controls (*right*). Plot represents combined data using the concatenate option in FlowJo (n = 3-5), representative of two independent experiments.
(B) Number of BAL fluid eosinophils (*left*) and comparison of GATA3 MFI values in $CD4^+$ T cells between HDM-treated GATIR mice and WT controls (*right*). (A, B) Data are shown as mean + SEM of n = 3-5 mice per group of a single experiment and are representative of two independent experiments.

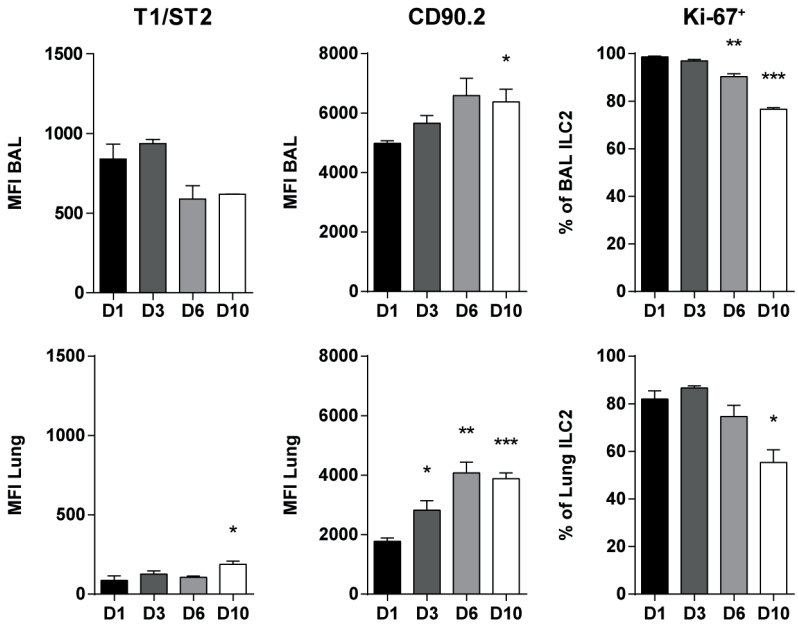

Figure S3. Expression of T1/ST2, CD90.2 and Ki-67 in IL-33-stimulated ILC2s.

MFI values of T1/ST2 and CD90.2 in ILC2s and percentage of Ki-67 expressing ILC2s in BAL fluid and lung at the indicated time points after IL-33 stimulation. Data are shown as mean + SEM of n = 3 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to D1 unless otherwise indicated.

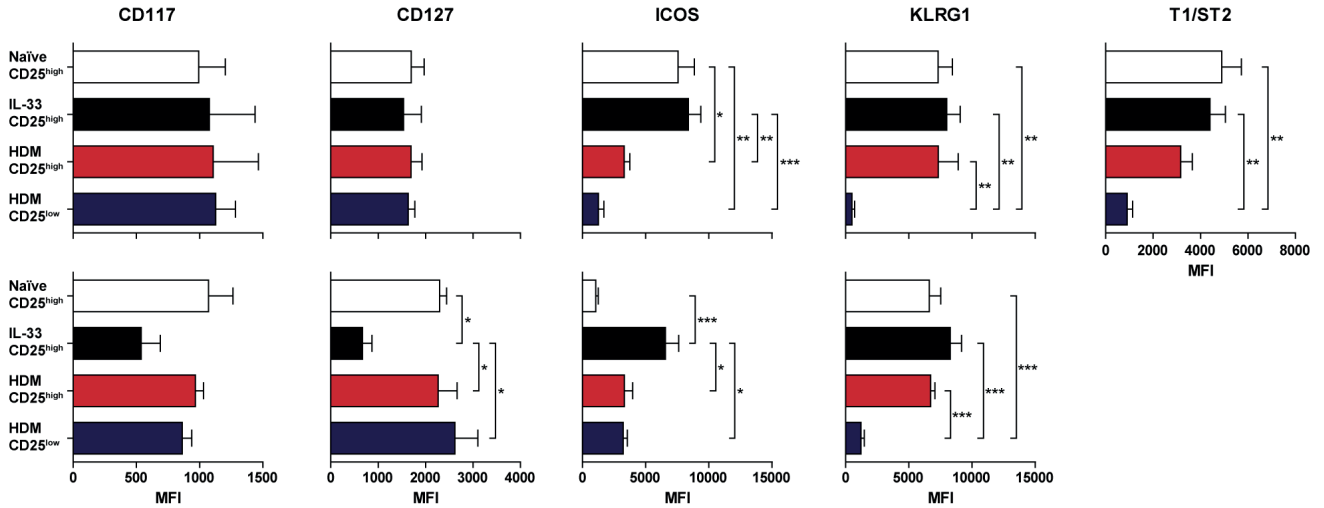

Figure S4. Characterization of phenotypic heterogeneity of ILC2s in BAL and lung in airway inflammation.

Analysis of the MFI values of CD117, IL-7R (CD127), ICOS, KLRG1 and IL-33R (T1/ST2), comparing naïve ILC2s (*white bars*) and IL-33-activated ILC2s (*black bars*) with $CD25^{high}$ (*red bars*) and $CD25^{low}$ (*blue bars*) ILC2s in HDM-driven airway inflammation in BAL fluid and lung from GATIR mice. T1/ST2 expression on lung ILC2s was very low in all conditions. Data are shown as mean + SEM of n = 4-6 mice per group of a single experiment and are representative of two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

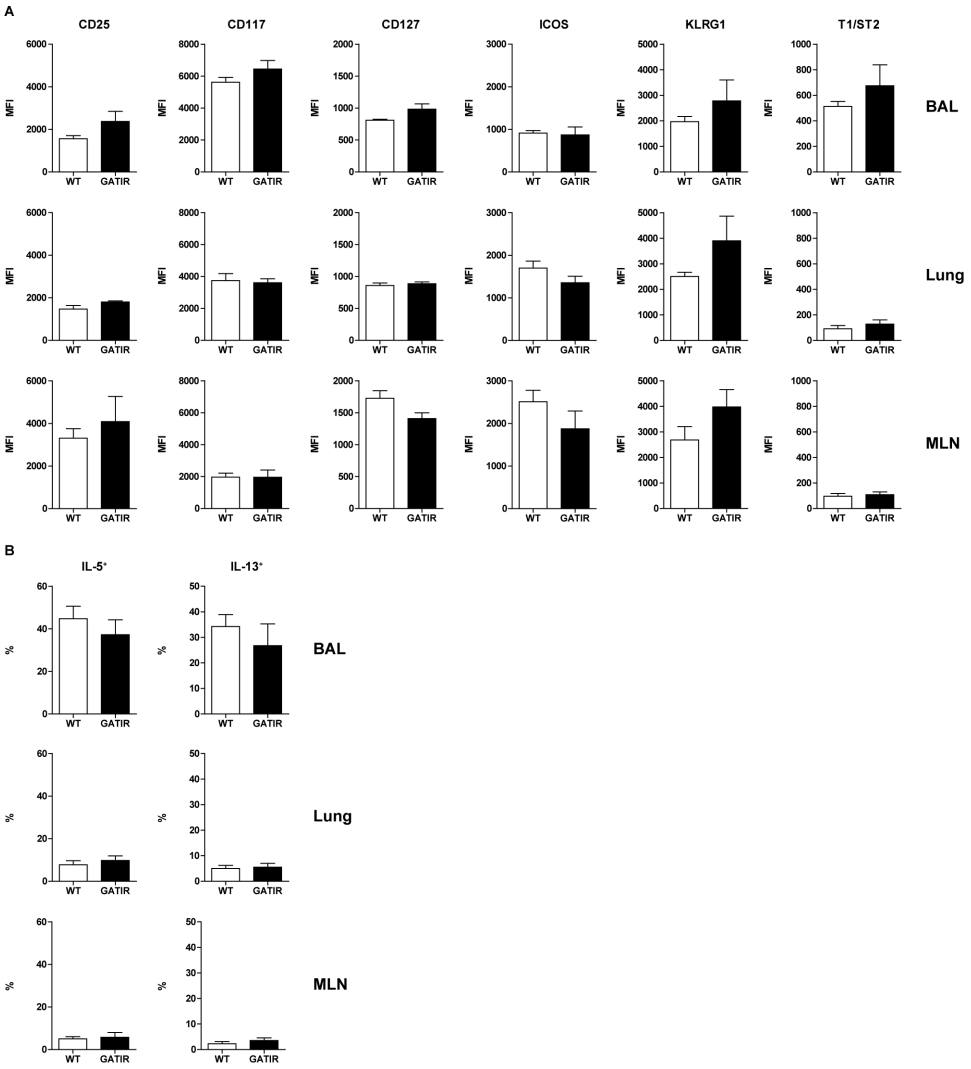

Figure S5. Comparison of surface marker expression profile on ILC2s from WT and GATIR mice.

Quantification of (A) MFI values of ILC2 surface markers and (B) IL-5 and IL-13 production by ILC2s in BAL fluid, lung and mediastinal lymph node (MLN) of HDM-treated GATIR mice and WT controls. Data are shown as mean + SEM of n = 4 mice per group of a single experiment and are representative of two independent experiments.

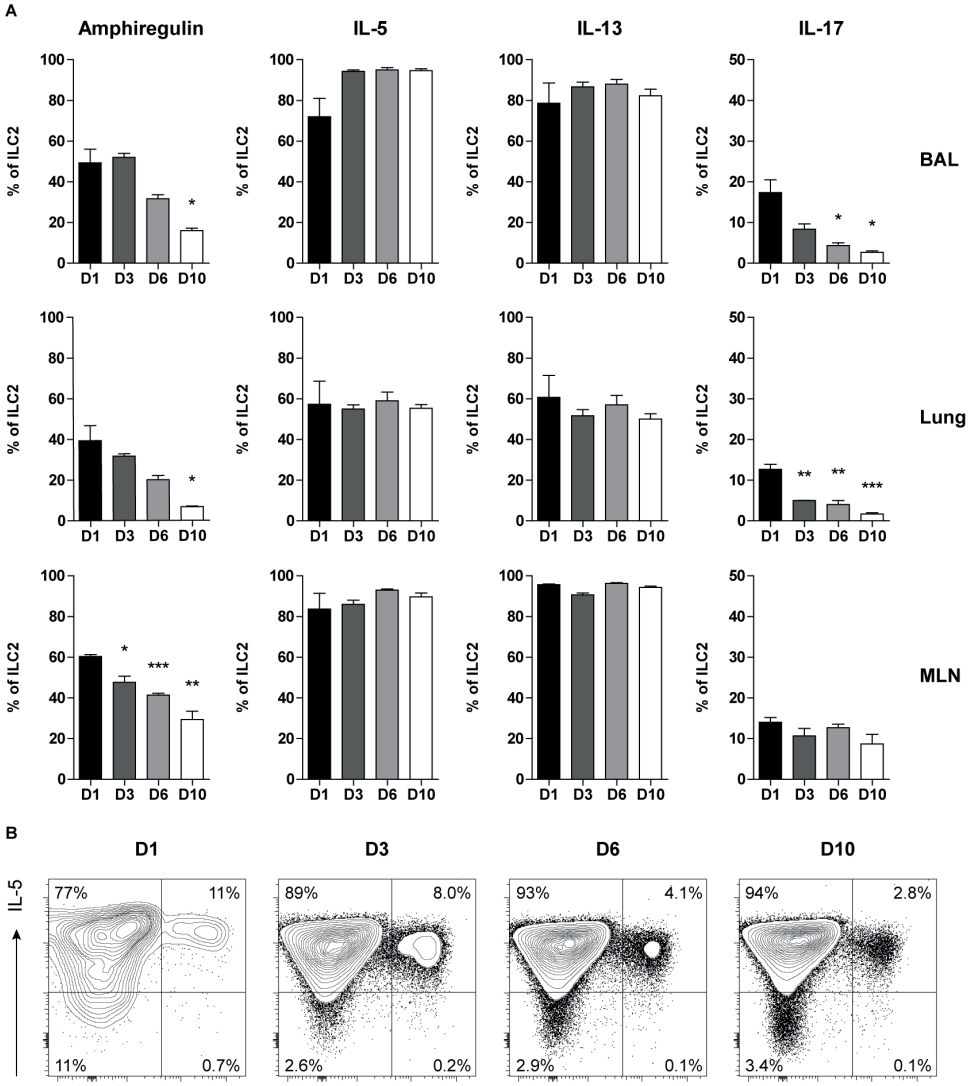

Figure S6. IL-33-activated ILC2s co-express IL-5, IL-13 and IL-17.

(A) Quantification of ILC2 cytokine production over time in BAL fluid, lung and MLN of IL-33-treated GATIR mice. Data are shown as mean + SEM of n = 3 mice per group of a single experiment. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to D1 unless otherwise indicated.

(B) Flow cytometric analysis of IL-5 and IL-17 production over time in BAL fluid ILC2s of IL-33-treated GATIR mice. Plots represent combined data using the concatenate option in FlowJo (n = 3).

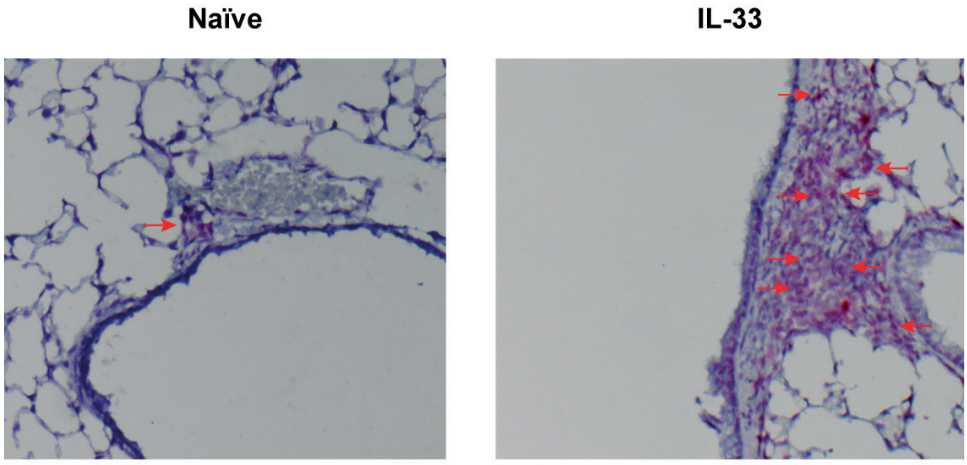

Figure S7. Accumulation of *Gata3*-expressing cells after IL-33 stimulation.

In situ hybridization assay of *Gata3* mRNA on lungs from naïve and IL-33-treated WT mice. Red arrows indicate *Gata3* mRNA expression. Images are representative of two independent experiments.

Table S1. Antibodies used for flow cytometry.

Antibody	Conjugate	Clone	Company
Amphiregulin	Biotin	Polyclonal	R&D Systems
B220	PE	RA3-6B2	eBioscience
CD117	Brilliant Violet 650	2B8	BD Biosciences
CD11b	PE	M1/70	eBioscience
CD11b	PerCP-Cy5.5	M1/70	BD Biosciences
CD11b	Alexa Fluor 700	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD11c	PE-Texas Red	N418	Invitrogen
CD11c	eFluor 450	N418	eBioscience
CD127	PE-Cy7	A7R34	eBioscience
CD127	eFluor 450	A7R34	eBioscience
CD19	PE	1D3	BD Biosciences
CD19	PerCP-Cy5.5	eBio1D3	eBioscience
CD19	Biotin	1D3	BD Biosciences
CD19	Alexa Fluor 700	eBio1D3	eBioscience
CD25	PerCP-Cy5.5	PC61.5	eBioscience
CD25	Brilliant Violet 605	PC61	BioLegend
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
CD4	Brilliant Violet 711	RM4-5	BD Biosciences
CD45	PE-CF594	I3/2.3	Abcam
CD45	Pe-Cy7	30-F11	eBioscience
CD5	PE	53-7.3	eBioscience
CD8	APC	53-7.3	eBioscience
CD8	APC-EF780	53-7.3	eBioscience
CD8	PE	53-6.7	eBioscience
CD86	PE-Cy7	GL1	BD Biosciences
CD90.2	FITC	53-2.1	BD Biosciences
FcεRIα	PE	MAR-1	eBioscience
FoxP3	Alexa Fluor 700	FJK-16s	eBioscience
FoxP3	PE-Cy7	FJK-16s	eBioscience
Gata3	eFluor 660	TWAJ-14	eBioscience
Gr-1	PE	RB6-8C5	BD Biosciences
Gr-1	APC-eFluor 780	RB6-8C5	eBioscience
ICOS	APC	C398.4A	eBioscience
ICOS	PE-Cy7	7E.17G9	eBioscience
IFN-γ	APC	XMG1.2	BD Biosciences
IFN-γ	Brilliant Violet 650	XMG1.2	BD Biosciences
IL-10	PerCP-Cy5.5	JES5-16E3	eBioscience

Table S1. Antibodies used for flow cytometry. (Continued)

Antibody	Conjugate	Clone	Company
IL-13	eFluor 450	eBio13A	eBioscience
IL-13	eFluor 660	eBio13A	eBioscience
IL-17	Alexa Fluor 700	TC11-18H10.1	BD Biosciences
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-4	Biotin	BVD4-1D11	eBioscience
IL-4	PE-Cy7	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
IL-5	Biotin	TRFK4	BD Biosciences
IL-9	PerCP-Cy5.5	D9302C12	BD Biosciences
IL-9	PE	D9302C12	BD Biosciences
Ki-67	FITC	SolA15	eBioscience
Ki-67	Alexa Fluor 700	SolA15	eBioscience
KLRG1	APC	2F1	BD Biosciences
KLRG1	PE-CF594	2F1	BD Biosciences
MHCII	Alexa Fluor 700	M5/114.15.3	eBioscience
MHCII	Brilliant Violet 650	M5/114.15.2	BD Biosciences
NK1.1	PE	PK136	eBiosciences
NK1.1	APC	PK136	BD Biosciences
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
Siglec-F	PE-CF594	E50-2440	BD Biosciences
Streptavidin	APC-eFluor 780		eBioscience
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
TER-119	PE	TER-119	eBioscience

Table S2. Antibodies used for confocal microscopy.

Antibody	Conjugate	Clone	Company
B220	Biotin	TY25	eBioscience
CD3	Biotin	145-2C11	BD Biosciences
αrat	Cy5	Polyclonal	Jackson ImmunoResearch
αhamster	Cy3	Polyclonal	Jackson ImmunoResearch

T CELLS AND ILC2S ARE MAJOR EFFECTOR CELLS IN INFLUENZA-INDUCED ASTHMA EXACERBATION

CHAPTER 5

Manuscript submitted to European Journal of Immunology:

T cells and ILC2s are major effector cells in influenza-induced asthma exacerbation

Bobby W.S. Li, Marjolein J.W. de Bruijn, Melanie Lukkes, Menno van Nimwegen, Ingrid M. Bergen, Corine H. GeurtsvanKessel, Arno Andeweg, Guus F. Rimmelzwaan, Rudi W. Hendriks

ABSTRACT

Influenza virus infection is an important cause of severe asthma exacerbations, but it remains unclear how a Th1-mediated antiviral response triggers a prototypical Th2 disease. We investigated $CD4^+$ T cells and group 2 innate lymphoid cells (ILC2s) in influenza virus-infected mice. We found that ILC2s accumulated in the lung rapidly after influenza virus infection, but the induction of IL-5 and IL-13 secretion was delayed and concomitant with T cell activation. In an influenza-induced asthma exacerbation model we noticed an initial reduction of ILC2 numbers and cytokine production in broncho-alveolar lavage compared to chronic house dust mite (HDM)-mediated airway inflammation alone. ILC2s had a phenotype characterized by low T1/ST2, ICOS, KLRG1 and CD25 expression, resembling naïve ILC2s. Consequently, the contribution of ILC2s to type 2 cytokine production in the early stage of the influenza-induced asthma exacerbation was limited. In contrast, T cells showed increased IL-4 and IL-5 production when exposed to both HDM and influenza virus. Nevertheless, upon clearance of the virus, ILC2s regained an activated $T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Collectively, our data indicate that both T cells and ILC2s contribute to influenza-induced asthma exacerbation, but with different kinetics.

INTRODUCTION

Asthma exacerbations are often provoked by respiratory viruses, particularly rhinovirus and respiratory syncytial virus (RSV) (1, 2). While influenza virus infections occur less often, it is able to cause the most severe forms of asthma exacerbations (3, 4). Adult asthma patients have a higher incidence of influenza virus infection than children, suggesting that adults are more susceptible (5). Influenza virus causes severe epithelial cell damage (6) and induces NF-κB-mediated release of cytokines from these cells such as IL-1β, IL-6 and IL-8 (7, 8). This compromises the epithelial barrier function and additionally increases vascular endothelial permeability (9), resulting in a highly robust inflammatory response. In conjunction with the pre-existing inflammatory environment in asthma patients, loss of barrier function provides a partial explanation for the clinical observations that indicate an association between influenza virus infection and asthma exacerbation. However, the precise underlying pathways remain elusive.

Allergic asthma is centrally mediated by T helper 2 (Th2) cells that secrete the key effector cytokines IL-4, IL-5 and IL-13 resulting in characteristic asthma pathology that includes persistent inflammation, airway hyperresponsiveness and remodeling, smooth muscle cell hyperplasia, goblet cell metaplasia and increased angiogenesis (10, 11). Group 2 innate lymphoid cells (ILC2) also secrete IL-5 and IL-13 when stimulated by epithelium-derived IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) as well as IL-4 under particular conditions (12). Several studies have stressed the potency of ILC2s as a crucial early T cell-independent source of type 2 cytokines in papain- or *Alternaria*-induced asthma (13-16). However, we and others have demonstrated that the contribution of T cells is critical in other allergen-based models of asthma such as house dust mite (HDM), ovalbumin and *Aspergillus* (17-19). Importantly, the groups of Chang *et al.* and Monticelli *et al.* have found increased ILC2 numbers in the lungs of influenza virus-infected mice. Hereby, ILC2s can contribute to airway hyperreactivity, but can also be protective because of their capacity to produce amphiregulin, a member of the epidermal growth factor family that restores epithelial cell integrity (20, 21).

These findings – together with evidence that during influenza virus infection NKT cells and alveolar macrophages can be important endogenous sources of IL-33 that enhance IL-5 production from ILC2s (22) – highlight a possible role for ILC2s as the causative agent between influenza virus infection and asthma exacerbation. Moreover, they open up new avenues of research into treatment of severe asthma exacerbations, as the current standard treatment with high doses of inhaled or system corticosteroids is not always effective and has a high risk of side-effects (23, 24).

When the effect of influenza virus infection on an acute model of HDM asthma was studied in mice, simultaneous activation of eosinophils and neutrophils and a synergistic effect of influenza and HDM on airway resistance and cell influx into the broncho-alveolar lavage (BAL) fluid was found (25). In addition, a marked innate response followed by an accumulation of

eosinophils, neutrophils, dendritic cells and T cells was reported in an influenza virus infection model preceded by chronic exposure to HDM (26). In these experiments, anti-IL-5 treatment prevented eosinophil influx and influenza-induced exacerbation of the airway inflammation. However, the involvement of ILC2s in influenza-mediated asthma exacerbations remains unknown.

Here, we studied the kinetics and function of ILC2s in influenza virus infection and their contribution to influenza-mediated exacerbation in a robust, physiologically relevant, chronic model of HDM-induced asthma. We found that although ILC2s accumulated earlier in the lungs than T cells after influenza virus infection, their cytokine production was delayed and only initiated after the peak of T cell activation. The contribution of ILC2s to the total type 2 cytokine milieu in influenza-mediated asthma exacerbation was modest in the early stages of infection, but increased to significant levels at day 11 post infection when the virus was cleared. Because in these experiments also Th2 cytokine production by $CD4^+$ T cells was increased, we conclude that both T cells and ILC2s play a role in influenza-induced asthma exacerbation, but with different kinetics.

RESULTS

Influenza virus infection induces major changes in expressed genes in the lungs

We aimed to identify the activation patterns of T cells and ILC2s in mice infected with the X31 H3N2 strain of influenza virus. Gene expression profiling of total lung cells over time revealed distinct patterns for genes associated with innate and adaptive immunity (**Fig. 1**). Compared to PBS-treated animals, several pro-inflammatory mediators exhibited a response that peaked at day 2-4 post infection, indicating initiation of viral immunity. Notably, IL-1β and IL-6 were increased ~4 and ~12-fold, respectively. Chemokines such as CCL11 and CXCL1 were also upregulated, which attract eosinophils and neutrophils, respectively. Likewise, factors secreted by innate immune cells, including IFN-β and TNFa were elevated early after infection (**Fig. 1A**). As expected, upregulation of genes that signify an adaptive immune response such as IgM and T cell associated markers (CD4, CD8a, CD28 and CTLA-4) occurred later in the infection at day 8 (**Fig. 1A, 1B**). Hereby, genes associated with Th1 immunity, IFN-γ, T-bet and IL-27Ra also showed maximal induction at day 8.

Chang *et al.* have demonstrated rapid increase of IL-33, a potent activator of ILC2s, in the lungs after influenza virus infection and have shown alveolar macrophages as a potential source (20). Our gene expression analysis confirmed this and we also found an upregulation of IL-33 at day 4 after inoculation. However, other genes implicated in ILC2 activation including IL-25, TSLP, IL-2 and IL-7, did not follow this pattern. Amphiregulin and arginase-1, cytokines known to be produced by ILC2s (21, 27), arose concomitantly with the increase in IL-33 expression. Several signature ILC2 genes, including IL-5 and IL-13 cytokines, and the transcription factors GATA3 and RORa remained remarkably unaltered (**Fig. 1C**). Although these findings may suggest that expression changes of these genes are difficult to detect in total lung, alternatively IL-33-driven Th2 cytokine production may be suppressed during influenza infection.

Taken together, this expression analysis shows that influenza virus infection induces major changes in gene expression within the lungs, reflecting induction of innate and adaptive immune responses. Changes in ILC2-associated genes were not readily detected, but several cytokines that were reported to suppress ILC2s including type I interferons and IFN-γ (28-32), were clearly induced.

T cells and ILC2s display distinct activation kinetics in influenza virus infection

To study the various innate and adaptive immune cell populations during influenza virus infection in detail, we infected mice with 10^4 PFU viral particles and followed the composition of the immune response over a period of 35 days. Mice rapidly lost weight in the first four days, after which their weight stabilized and gradually recovered from day 7 onwards (**Fig. 2A**).

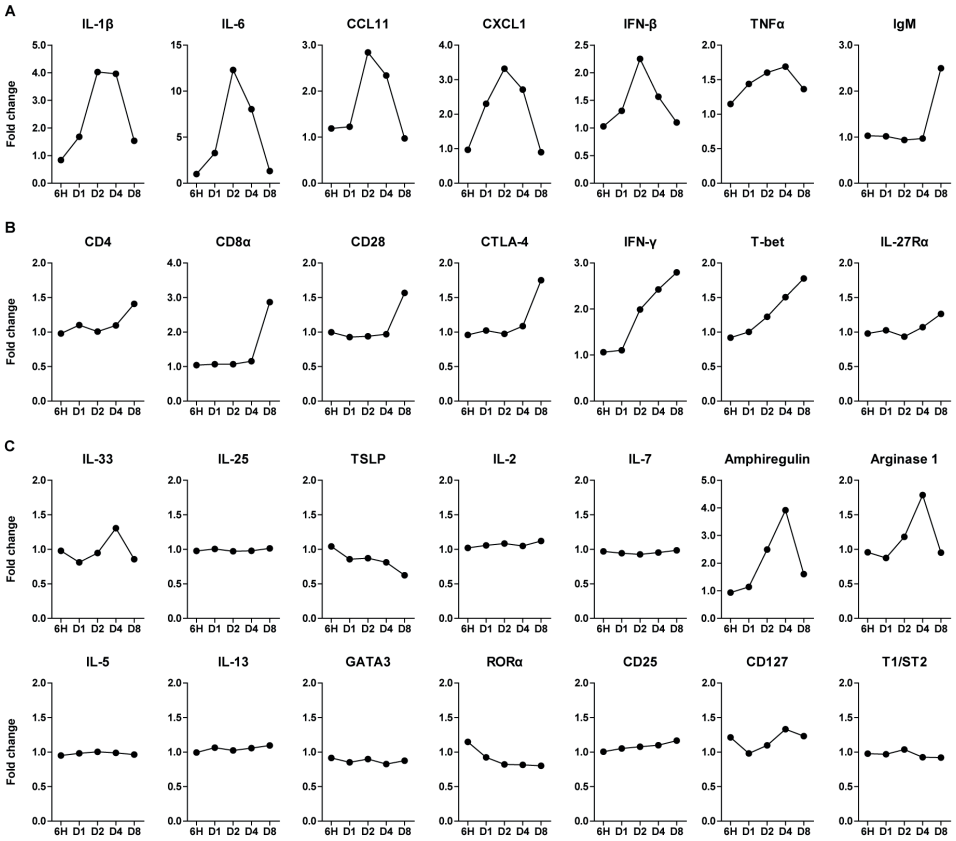

Figure 1. Expression profiles of genes associated with ILC2s and T cells show distinct temporal patterns in influenza virus infection.

Quantification of the expression levels of (A) inflammatory mediators (B) T cell-related genes and (C) ILC2-related genes at 6 hours, 1 day, 2 days, 4 days and 8 days after X31 influenza virus inoculation, expressed as fold change compared to PBS-treated mice. Expression was determined by microarray analysis.

Virus titers significantly decreased by day 7 and were almost undetectable by day 10 (**Fig. 2A**). ILC2s were characterized by flow cytometry as Lineage$^-$ lymphocytes that expressed Sca-1, IL-7R (CD127) and intracellular GATA3, as described previously (**Fig. 2B**) (17). In agreement with the gene expression data in Fig. 1, increased numbers of CD4$^+$ and CD8$^+$ T cells were present in the lungs at day 7, after which a slow decline to baseline was reached at day 17. In contrast, ILC2 accumulation was already evident at day 4 post infection and remained significantly elevated in the lungs at day 7 and day 10 and returned to baseline values by day 17 (**Fig. 2C**). Subsequently, we zoomed in on the initiation phase of the response to influenza virus infection and found that whereas the apex of ILC2 influx into the lungs occurred at day 3, T cell numbers remained unchanged. We observed a slight, but significant reduction in B

cell numbers in the lung (**Fig. 2D**). By employing a *Gata3* reporter mouse, which exhibits concomitant expression of GATA3 and yellow fluorescent protein (YFP) (33) [T.N.R and H.J.F., manuscript in preparation], we were able to visualize the location of ILC2s in the lung during influenza virus infection. ILC2s appeared underneath the airway epithelium, often in close proximity to CD3$^+$ T cells (**Fig. 2E**), also in severe cases where a strong influx of B220$^+$ cells (plasmacytoid dendritic cells or B cells) was present (**Suppl. Fig. S1**).
Taken together these data indicate that upon influenza virus infection ILC2s in the lung increase more rapidly in number than CD4$^+$ or CD8$^+$ T cells.

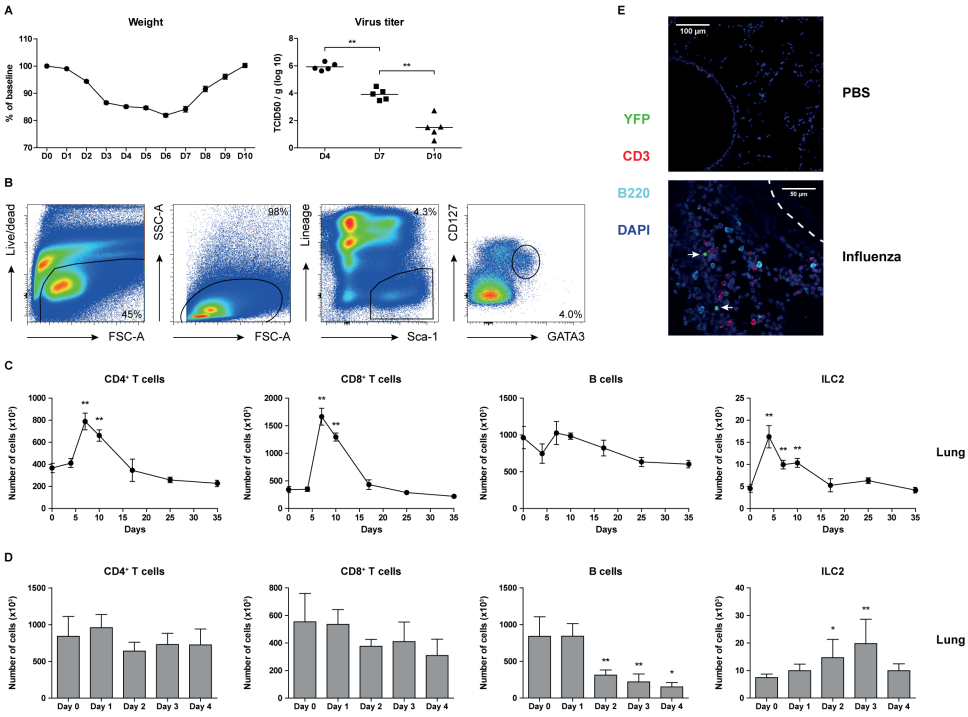

Figure 2. Accumulation of ILC2s in the lungs precedes T cell recruitment after inoculation with influenza virus.

(A) Percentage of weight loss in mice infected with 10^4 PFU X31 influenza virus particles and measurement of virus titers in the lungs at the indicated days post infection. (B) Flow cytometric identification of ILC2s. Dot plots showing subsequent gating represent combined data using the concatenate option in FlowJo (n = 5), representative of two independent experiments. (C) Kinetics of CD4$^+$ T cells, CD8$^+$ T cells, B cells and ILC2s in the lungs after X31 influenza virus infection. (D) Detailed kinetics of CD4$^+$ T cells, CD8$^+$ T cells, B cells and ILC2s in the lungs up to day 4 post infection. (E) Lung cryosection from PBS and X31 virus-infected *Gata3* reporter (GATIR) mice, counterstained with CD3 (T cells) and B220 (B cells and plasmacytoid dendritic cells). Airway epithelium is outlined by the dashed line. ILC2s, indicated with arrows, are identified as CD3$^-$YFP$^+$ cells. In the confocal microscopy conditions, GATA3^{low} cells (such as non-Th2 CD4$^+$ T cells and basophils) are not detectable as YFP$^+$ cells. (A, C-D) Data are shown as mean values ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

ILC2 cytokine response to influenza virus follows accumulation in BAL fluid and CD25 upregulation

Given that Th1-associated cytokines may drive plasticity of ILC2s (34-37), we examined the expression of cytokines as well as a number of cell surface markers of ILC2s over time in influenza virus-infected mice.

While the numbers of IL-5$^+$ and IL-13$^+$ T cells present in BAL fluid significantly increased at day 7 and 10, only a small proportion of T cells produced these Th2 cytokines (**Fig. 3A, 3B**). The numbers of ILC2s in BAL fluid increased as early as day 4 after infection and reached their highest values at day 7, however, type 2 cytokine production by this wave of ILC2s was remarkably limited. Rather, cytokine production by ILC2s was delayed and only initiated in a later phase of influenza virus infection at day 10, when the total number of ILC2s in the BAL fluid was declining (**Fig. 3A, 3B**). A comparable pattern, reflecting a peak of IL-5/IL-13-expressing T cells at day 7 and IL-5/IL-13-expressing ILC2s at day 10, was also observed in the lungs and the mediastinal lymph nodes (MLN) (**Suppl. Fig. S2**).

We observed that on the cell surface of ILC2s in the BAL fluid IL-33R (T1/ST2), KLRG1 and CD117 were upregulated at day 4 and 7, and remained highly expressed at day 11 (**Fig 3C**). Interestingly, high IL-2R (CD25) expression on ILC2s was only observed on day 7, coinciding with the peak of T cell activation, after which it was downregulated. Compared with naïve mice, ICOS expression was downregulated during influenza infection, particularly at day 4 after inoculation (**Fig. 3C**).

Taken together, these data show that during influenza virus infection the capacity of ILC2s to produce IL-5/IL-13 is delayed and is only acquired after their accumulation in the BAL fluid, after upregulation of T1/ST2 and CD25 and after the accumulation of activated T cells.

Influenza virus infection does not increase ILC2s in the BAL fluid in HDM-mediated inflammation

We previously found in chronic HDM-driven allergic airway inflammation that the numbers of both T cells and ILC2s were significantly increased in the BAL fluid (~50.000-100.000 and ~1.000-3.000, respectively) (33, 38). Hereby, ILC2s constituted 40% of the IL-5$^+$ lymphocytes and 30% of IL-13$^+$ lymphocytes and were mainly CD25^{low} (33, 38). To explore the contribution of ILC2s in influenza-induced asthma exacerbations, we developed a mouse model in which we first chronically exposed mice to HDM and subsequently infected them with X31 influenza virus. In these experiments, we included groups of control mice that were either chronically exposed to HDM only or that were infected with influenza virus without prior exposure to HDM. The immune response was analyzed by flow cytometry at day 4, day 7 and day 11 post infection (**Fig. 4A**).

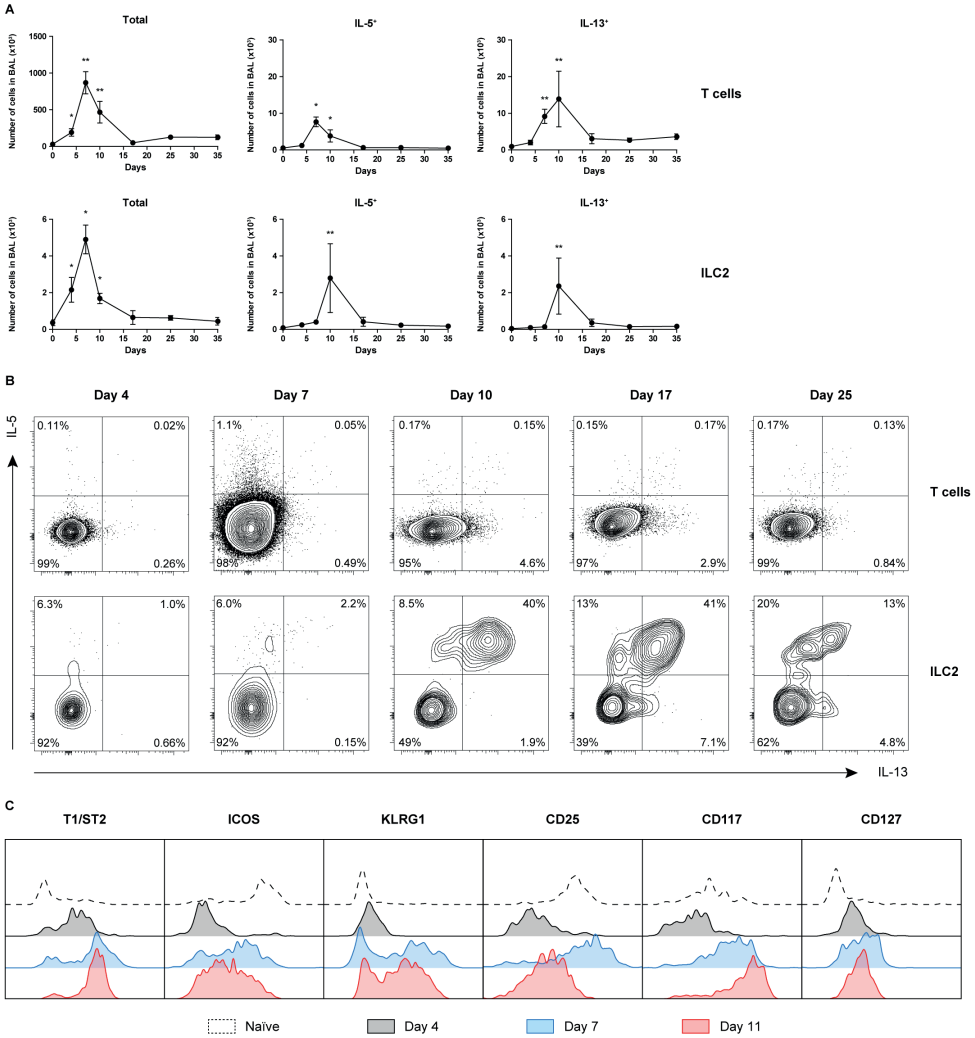

Figure 3. Delayed ILC2 cytokine production in influenza virus infection.

(A) Quantification of total IL-5 and IL-13 producing T cells and ILC2s in BAL fluid over time after X31 virus infection. Data are shown as mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$. (B) Flow cytometric analysis of IL-5 and IL-13 expression in BAL fluid T cells and ILC2s at day 4, day 7, day 10, day 17 and day 25 after X31 influenza virus infection. (C) Expression of ILC2 surface markers at day 4, day 7 and day 11. (B, C) Plots represent combined data using the concatenate option in FlowJo (n = 5 – 7), representative of two independent experiments.

Mice that received both HDM and X31 virus treatment lost marginally more weight than those only infected with X31 virus (**Fig. 4B**). Compared to influenza-only mice, the combination treated animals had similar virus-specific antibody titers in serum, but showed a decrease in the numbers of $CD8^+$ T cells that carried a T cell receptor specific for the X31 epitope $NP_{366-374}$

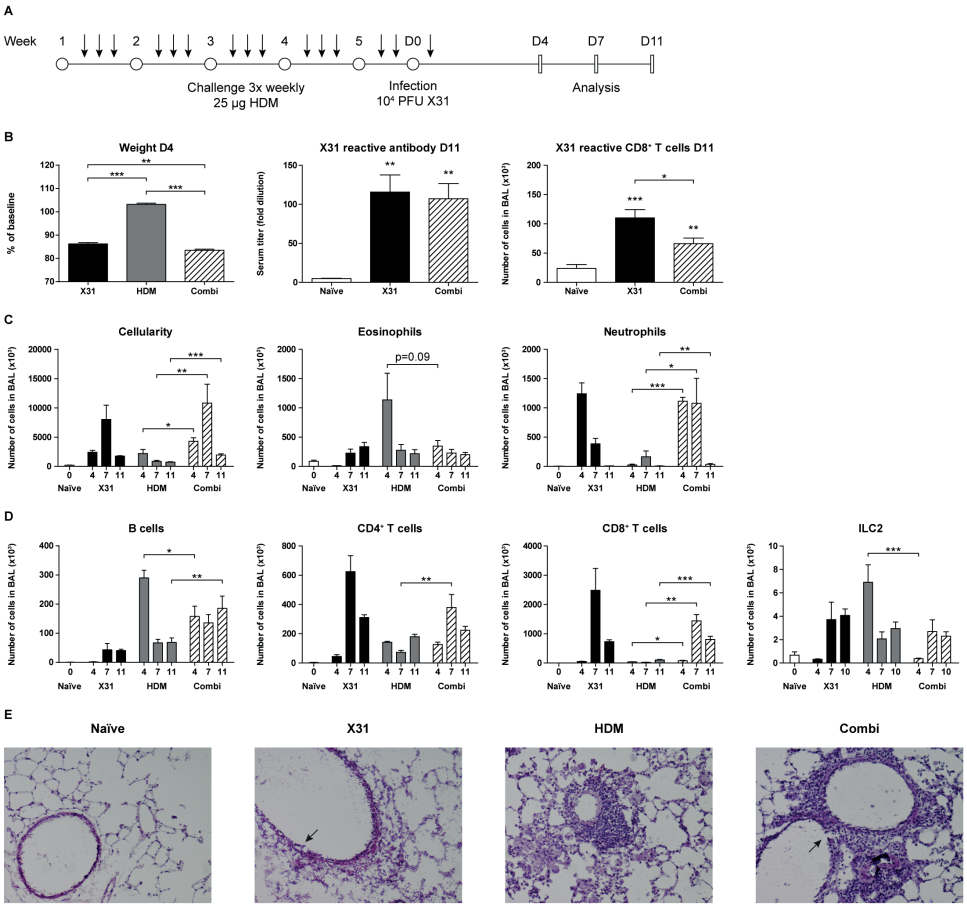

Figure 4. Influenza-induced asthma exacerbations are primarily neutrophilic and lead to increased T cell numbers in BAL fluid.

(A) Scheme for administration of HDM (arrows) for induction of chronic asthma followed by X31 influenza virus inoculation and analyses at days 4, 7 and 11 post infection. (B) Weight loss of mice inoculated with X31 virus, compared to HDM-treated mice with or without X31 virus (*left*). X31 influenza specific antibody titer in serum (*middle*) and influenza virus-specific CD8⁺ T cells in BAL fluid (*right*). (C) Enumeration of total cells, eosinophils and neutrophils infiltrating in the BAL fluid at day 4, day 7 and day 11 post infection. (D) Quantification of B cells, CD4⁺ T cells, CD8⁺ T cells and ILC2s in the BAL fluid. (E) Hematoxylin and eosin stained cryosections of lungs from naïve, HDM only, X31 virus only and combination-treated mice at day 4 post infection. Arrows indicate epithelial damage. (B-D) Data are shown as mean values ± SEM from 7 mice per group. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

(ASNENMETM) in the BAL fluid (**Fig. 4B**). Importantly, the combination treatment resulted in a significantly higher influx of immune cells into the BAL fluid than HDM alone at all time points investigated. The infiltrating granulocytes in the combination group consisted predominantly of neutrophils, but a significant eosinophil presence was also observed. The total influx of cells in the BAL fluid peaked at day 7, although both eosinophil and neutrophil numbers remained

comparable to the levels seen at day 4. By day 11, neutrophils returned to baseline levels and the inflammation reached its resolution phase (**Fig. 4C**). The combination of HDM exposure and X31 virus infection led to increased numbers of B cells, $CD4^+$ and $CD8^+$ T cells compared to HDM only, particularly at day 7 or 11 (**Fig. 4D**).

Notably, X31 virus infection significantly reduced ILC2 numbers in HDM-treated mice at day 4 to <1000 cells, despite a strong $CD4^+$ T cell presence, and recovered to a similar range as HDM-treated group by day 7 (**Fig. 4D**). Examination of lung cryosections stained with H&E confirmed a more severe inflammatory response in the combination treatment around the airways, compared to HDM alone and showed signs of epithelial damage similar to X31 virus infection alone (**Fig. 4E**).

In summary, influenza virus infection following HDM-mediated allergic airway inflammation differentially affects $CD4^+$ T cells and ILC2s and induces a transient reduction of ILC2 numbers in the BAL fluid at day 4, compared to HDM-mediated inflammation only.

Different kinetics of type 2 cytokine expression by T cells and ILC2s in influenza-induced asthma exacerbation

Next, we used intracellular flow cytometry to evaluate the capacity of Th2 cells and ILC2 to produce cytokines. These analyses revealed that the numbers of IL-4^+ and IL-5^+ $CD4^+$ T cells were significantly enhanced at day 4 and 7 of the X31-induced exacerbation of HDM-mediated allergic airway inflammation, when compared to influenza virus infection or HDM-challenge alone (**Fig. 5A**). Interestingly, the numbers of IL-5^+, IL-13^+ and amphiregulin-expressing ILC2s was increased at day 7 and day 11 but not at day 4, compared to HDM alone (**Fig. 5A**). The additive effect of X31 virus on IL-4^+ $CD4^+$ T cells and IL-5^+ ILC2s was also observed in the lungs (**Suppl. Fig. S3**).

Overall, when we compared the relative contribution of $CD4^+$ T cells and ILC2s to the population of type 2 cytokine expressing cells, it was apparent that $CD4^+$ T cells were the major source of IL-5 or IL-13 in the early phase of influenza-induced asthma exacerbation (**Fig 5B**). At later time points following influenza virus infection however, ILC2s contributed more to the total number of IL-5 and IL-13 producing cells, although $CD4^+$ T cells remained dominant (**Fig. 5B**). Moreover, the MFI values for IL-5 and IL-13 content in ILC2s at day 11 was significantly higher in combination-treated mice, indicating a higher cytokine production capacity per cell (**Fig. 5C**).

Taken together, these data imply that both T cells and ILC2s are major effector cells that contribute to the type 2 cytokine milieu in influenza-induced asthma exacerbation, albeit with different kinetics.

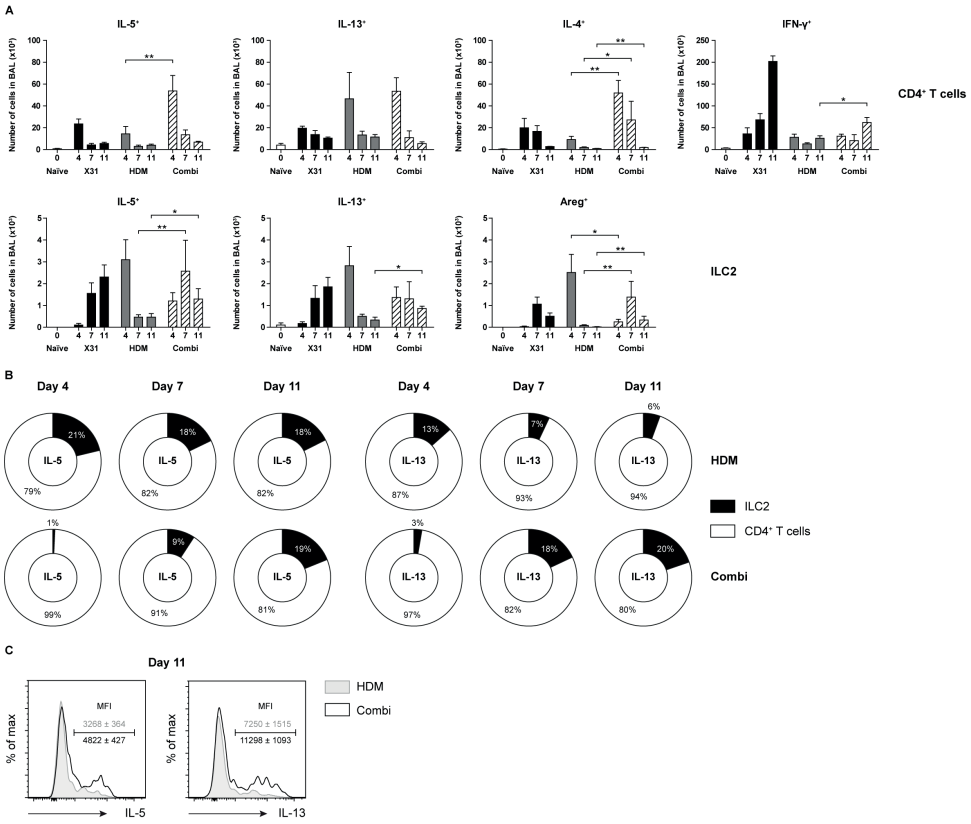

Figure 5. ILC2s are important contributors to type 2 cytokine producers in the late phase of influenza-induced asthma exacerbations.

(A) Quantification of the absolute number of cytokine producing CD4⁺ T cells (*top*) and ILC2s (*bottom*). Data are shown as mean ± SEM (n = 7); * p ≤ 0.05, ** p ≤ 0.01. (B) Ratio of IL-5 and IL-13 producing ILC2s and CD4⁺ T cells over time in BAL fluid. (C) Flow cytometric comparison of IL-5 and IL-13 production by BAL fluid ILC2s at day 11 post infection. Histogram overlay plots represent combined data using the concatenate option in FlowJo (n = 7) from a single experiment. MFI values shown are mean values ± SEM.

ILC2s display an activated phenotype only after virus clearance

Finally, we compared the surface phenotype of ILC2s as an indicator of the activation status of ILC2s in chronic HDM-driven airway inflammation, with and without X31 influenza virus-induced exacerbation, as described in **Fig. 4A**.

We noticed upregulation of T1/ST2 and KLRG1 paired with a downregulation of ICOS and CD25 in HDM-treated mice compared to naïve mice at day 4 (**Fig. 6;** quantified in **Suppl. Fig. S4**). As expected, the activated phenotype appeared to diminish slowly over time, as T1/ST2 and KLRG1 surface expression decreased, but did not yet return to baseline levels at day 11. In the combination group however, surface T1/ST2 and KLRG1 expression remained

close the levels found in naïve mice, suggesting that X31 influenza virus infection resulted in suppression of ILC2s that were activated by the HDM exposure (**Fig. 6;** quantified in **Suppl. Fig. S4**).

However, at day 7-11 ILC2s in combination-treated mice regained expression of various surface markers. Most interestingly, this resulted in an activated T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} surface phenotype of BAL fluid ILC2s, which was significantly different from the phenotype in mice that were not infected with influenza virus (**Fig. 6**; quantified in **Suppl. Fig. S4**).

Taken together, these findings show that after clearance of the virus by day 11, ILC2s present in the BAL fluid manifest – concomitant with a high capacity to produce type 2 cytokines - an activated surface phenotype.

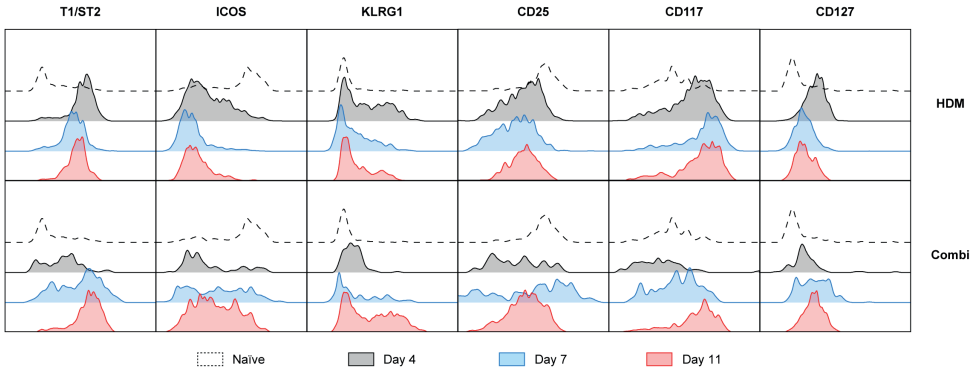

Figure 6. Influenza virus infection initially suppresses HDM-activated ILC2s but induces enhanced activation at day 11.

Flow cytometric comparison of T1/ST2, ICOS, KLRG1, CD25, CD117 and CD127 on ILC2s derived from BAL fluid at day 0, day 4, day 7 and day 11. Histogram overlay plots represent combined data using the concatenate option in FlowJo (n = 7) from a single experiment. Quantification of these data is given in **Suppl. Fig S6**.

DISCUSSION

Virus infection-induced exacerbations account for a major portion in healthcare costs associated with severe asthma (39). However, the current knowledge of how a Th1 response is able to trigger a Th2-mediated disease is fragmented. IL-33 and the type 2 cytokines IL-4, IL-5 and IL-13 have been shown to be induced by rhinovirus infection with a correlation between disease severity and cytokine levels. Furthermore, *in vitro* data show that supernatant from rhinovirus-infected bronchial epithelial cells induces type 2 cytokine production in both T cells and ILC2s (40). ILC2s have also been hypothesized to be the missing link in the underlying mechanisms between influenza virus infection and asthma exacerbation due to their accumulation in the lung after influenza virus infection and their ability to secrete type 2 cytokines to promote asthma symptoms (21).

Influenza-induced asthma exacerbation models have been reported, but the contribution of ILC2s is still unclear (25, 26). In our study we present a model for influenza-induced asthma exacerbation in which mice were chronically exposed to HDM (38) and subsequently infected with X31 influenza virus to induce exacerbation of the airway inflammation. Using multi-color flow cytometry, we observed an increased cellularity of the BAL fluid consisting of a mixed eosinophilic and neutrophilic infiltrate in influenza-infected HDM-exposed mice, compared to major eosinophilic inflammation present in mice that were only exposed to HDM. Concomitantly, at day 4 of the influenza virus infection, ILC2 numbers and their cytokine production capacity in BAL fluid were suppressed. However, once the virus was cleared, BAL fluid ILC2s resumed production of type 2 cytokines and gained an activated T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} surface phenotype on day 11, which was significantly different from the phenotype in mice that were exposed to HDM only. In contrast, T cells in the BAL fluid and MLN of HDM-exposed mice showed particularly at day 4 of the influenza virus infection increased IL-4 and IL-5 production, compared to mice that were not infected with influenza virus. Therefore, CD4^{+} T cells in this virus-induced HDM-driven inflammation exacerbation model were a more prominent contributor to the type 2 cytokine environment than ILC2s early after influenza virus infection. We conclude that both T cells and ILC2s contribute to influenza-induced asthma exacerbation, but with remarkably different kinetics.

Irrespective of the presence of chronic HDM-driven inflammation, we found that during influenza virus infection ILC2s are induced in the BAL fluid with remarkable kinetics: numbers of ILC2s peak at day 4-7 but substantial IL-5 and IL-13 cytokine production capacity was only detected later during infection at around day 10-11 in the resolution phase of influenza virus infection when virus particles had been eradicated and the type 1 immune response was reaching its conclusion. This would be in agreement with the proposed restorative functions of ILC2s (21), but also indicates that an ongoing influenza virus infection significantly suppresses ILC2 activation. This was also evident in our combination treatment in which the numbers of HDM-activated ILC2s were very low at day 4 after influenza virus infection. A likely explanation for this is that type I interferon, IFN-γ and IL-27, which are both abundantly present in influenza virus infections (Fig. 1), are able to inhibit ILC2s in a dose-dependent manner (28, 29, 31, 32). In particular, it was very recently reported that virus-induced IFN-γ restricts protective ILC2 functions in the lung following challenge with the pandemic H1N1 CA04 influenza virus, whereby neutralizing IFN-γ protected mice from lethal infection (30). Our finding that at day 4 of infection both IFN-β and IFN-γ are increased supports the hypothesis that these cytokines inhibit ILC2 induction. Other known cytokines that have an inhibitory effect on ILC2 function where either not significantly induced by the influenza virus infection (IFN-α) or not represented in the Affymetrix mouse microarray 430.2 (IL-27). It is very well possible that these cytokines act in concert to suppress ILC2 activity.

Nevertheless, upon virus clearance, ILC2s regained a T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Several lines of evidence support the notion that this surface phenotype signifies strong activation of ILC2s. Firstly, high cell surface expression of IL-33R (T1/ST2) on ILC2s will increase their activation status because IL-33, produced by epithelial cells e.g. upon allergen activation, is one of the most potent activators of ILC2s (41-43). In this context, we previously showed that although in HDM-mediated airway inflammation the accumulation of ILC2s in BAL fluid is IL-33 independent, IL-33 is critical for cytokine production by ILC2s (17). In our combination treatment model, next to HDM-activated epithelial cells, NKT cells might be an additional source of IL-33, given that NKT cells were shown to secrete IL-33 on day 7-12 following infection with A/PR/8/34 H1N1 influenza virus (22).

Secondly, IL-2 and IL-7, which alone are not sufficient for ILC2 activation, are thought to enhance the *in vivo* effect of IL-33 (13, 44). Therefore, high expression of IL-2R (CD25) and IL-7R (CD127) – together with high T1/ST2 expression would optimally prime ILC2s for receiving activating signals from NKT cells or epithelial cells via IL-33. Rapid downregulation of CD25 on ILC2s by day 11 is evidence for tight regulation of this pathway and was followed by a diminishing IL-5/IL-13 profile.

Thirdly, ICOS:ICOS-ligand interaction promotes cytokine production, proliferation and survival of ILC2s both under homeostatic and inflammatory conditions (45, 46) and moreover, ICOS on ILC2s may interact with B7RP-1-expressing dendritic cells in allergic inflammatory lung, which increases ILC2 activation (47).

Finally, KLRG1 has been reported as an ILC2 activation marker and is intermediate to highly expressed by mature ILC2s (48, 49). Heterogeneous surface expression of CD117 on ILC2s during an influenza virus infection has been previously described. Because the authors did not observe functional differences between CD117^{-} and CD117^{+} ILC2s, they concluded that the marked accumulation of CD117^{+} ILC2s during the recovery phase of influenza virus infection most likely reflects recruitment of these cells from the bone marrow (22). In this context, it is important to note that our T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} cells are different from the KLRG1^{high} inflammatory ILC2 population that is responsive to IL-25 and was proposed to be transient progenitors mobilized by inflammation (48). In contrast to these cells, which do not express T1/ST2, our activated ILC2s are T1/ST2^{high}.

In summary, our influenza-induced asthma exacerbation model presents a mixed eosinophilic and neutrophilic infiltrate in the BAL fluid and an initial suppression of ILC2 accumulation. After virus clearance however, ILC2s are significant cytokine producers and show an activated T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} state, which would prime ILC2s for receiving activating signals from allergen-activated epithelial cells. It is attractive to speculate that these activated ILC2s share characteristics with the allergen-experienced ILC2s that persist after resolution of inflammation and were shown to respond more potently to unrelated allergens than naïve

ILC2s did (50). It was proposed that memory-like properties of allergen-experienced ILC2s may explain why asthma patients are often sensitized to multiple allergens. Likewise, it is conceivable that - next to T cells - memory-like properties of ILC2s that are induced upon viral infections may contribute to asthma exacerbations upon allergen exposure.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

Author contributions

BWSL conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. MJWdB, ML, MvN, IMB and CHG performed experiments, AA performed data analyses. CHG and GFR designed and characterized the X31 influenza virus infection mouse model. RWH conceived the study and designed experiments and wrote the manuscript.

MATERIALS AND METHODS

Mice

Wildtype C57BL/6 mice were purchased from Envigo (United Kingdom). Homozygous *Gata3* reporter (GATIR) mice, which harbor an IRES-YFP sequence within the 3' untranslated region (3'-UT) of the *Gata3* gene resulting in concomitant production of GATA3 and YFP protein, were bred on a C57BL/6 background [T.N.R. and H.J.F., manuscript in preparation; Stadhouders et al., manuscript in revision]. Mice were ~8-16 weeks old at the time of analysis. All animals were housed at the Erasmus MC Animal Facility under SPF conditions. All experiments were approved by the Erasmus MC Animal Ethics Committee.

Induction of chronic allergic airway inflammation

To induce chronic allergic airway inflammation, mice were anesthetized using isoflurane and treated intranasally three times per week for five consecutive weeks with 25 µg HDM extract (Greer, USA) or PBS (GIBCO Life Technologies, USA) as previously described (38).

X31 influenza virus infection and gene expression profiling

Inoculation and harvest of the X31 H3N2 strain of influenza virus (Medical Research Council, UK) has been described previously (51). Mice were anesthetized using isoflurane and infected with 10^4 PFU X31 particles diluted in PBS via intranasal inoculation. Animals were sacrificed after infection at the indicated time points. Virus titers in lung tissue were determined as previously described (51, 52) X31-reactive antibody titers in serum was detected by hemagglutination inhibition assay as reported in the World Health Organization manual for laboratory diagnosis and virologic surveillance of influenza (53).
Gene expression profiling was performed in mice infected with 10^5 PFU X31 virus particles as described elsewhere (54). In brief, the lungs were excised and stored in RLT buffer (Qiagen, Germany) at -80 °C. Total RNA was then isolated using an RNeasy kit (Qiagen, Germany) and biotin labeled and hybridized to mouse DNA microarrays (Affymetrix, USA).

Flow cytometry

Single cell suspensions were prepared from lungs and lymph nodes by mechanical disruption using a 100 µm cell strainer (BD Biosciences, USA) in PBS supplemented with 0.5% bovine serum albumin and 5 mM EDTA (Sigma-Aldrich, USA). For determination of intracellular cytokine content, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Sigma-Aldrich, USA) at 37 °C for 4 hours prior to staining. Flow cytometry surface and intracellular staining was performed as described previously (43, 55). Lineage markers included CD3, CD4, CD5, CD8a, CD11b, CD11c, CD19, B220, NK1.1, FcεRIa, TER-119 and Gr-1. X31-reactive $CD8^+$ T cells were detected using MHC dextramer against epitope $NP_{366-374}$ (ASNENMETM). Detailed list of antibodies used available upon request. Samples were acquired using a LSR II flow cytometer and FACSDiva (BD Biosciences, USA) and analyzed by FlowJo X (Tree Star Inc., USA).

Histology

Lungs were inflated with OCT embedding medium (Sakura, the Netherlands) diluted in equal volume of PBS prior to being snap frozen in liquid nitrogen. Lungs from *Gata3* reporter mice were inflated with a 1:1 mixture of OCT embedding medium and PFA (Thermo Fisher Scientific, USA) to preserve YFP fluorescence. 7 µm thick cryosections were cut at -20 °C using a cryostat (Thermo Fisher Scientific, USA) and fixed in 4% PFA for 10 minutes. Then slides were stained with

primary antibodies for 1 hour and secondary antibodies for 30 minutes at room temperature. Subsequently, slides were incubated with DAPI (Invitrogen, USA) for 5 minutes and sealed with Vectashield (Vector Laboratories, USA). An LSM510Meta confocal microscope equipped with 405 nm, 488 nm, 543 nm and 633 nm lasers (Zeiss, Germany) was used to examine the slides. Images were processed and analyzed in Fiji, an open source scientific image processing application based on ImageJ.
For H&E stain, cryosections were fixed by dipping in 4% PFA followed by 30 seconds in Gill's Haematoxylin and 1 minute in 4% Eosin B acetified with glacial acetic acid (Sigma-Aldrich, USA). Samples were dehydrated by sequential rinsing in ethanol up to 100% and cleared in xylene for 2 minutes (Sigma-Aldrich, USA). Sections were mounted in Entallan (Merck, USA) and examined under a light microscope (Zeiss, Germany).

Statistical analysis

Mann-Whitney U tests were used for statistical comparisons between groups and a p-value of <0.05 was considered statistically significant. All analyses were performed using Prism 5 (GraphPad Software, USA).

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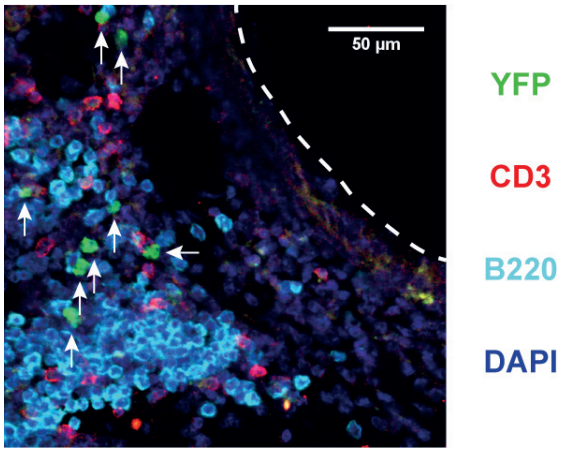

Figure S1. Severe inflammatory response to influenza recruits T cells, B220⁺ cells and ILC2s at day 4 post infection.

Lung cryosection from X31 influenza virus infected *Gata3* reporter mice counterstained with CD3 (T cells) and B220 (B cells and plasmacytoid dendritic cells). ILC2s are indicated with arrows and airway epithelium is outlined by the dashed line.

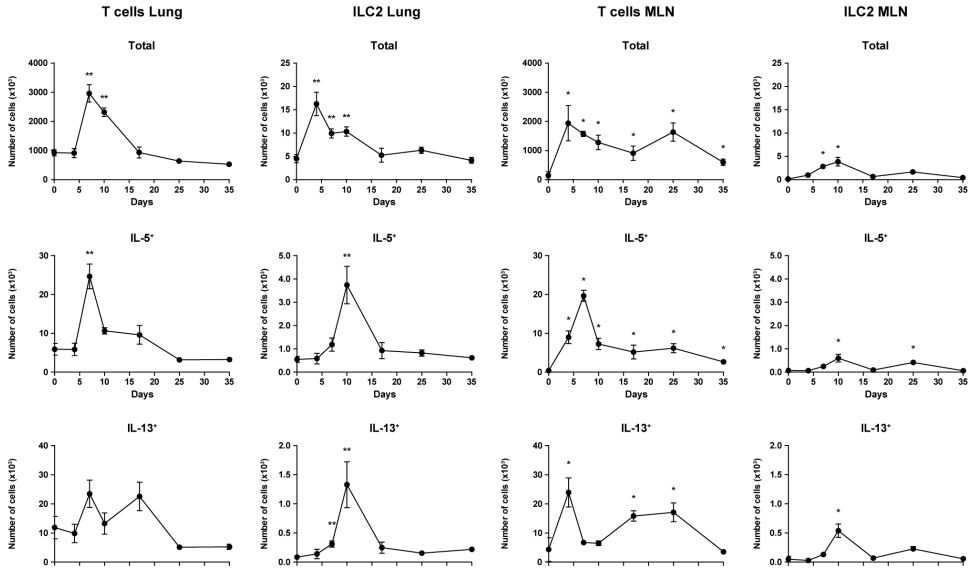

Figure S2. Kinetics of type 2 cytokine production by ILC2s and T cells after influenza virus infection.

Quantification of the number of IL-5 and IL-13-producing T cells and ILC2s in the lungs and MLN of mice infected with X31 influenza virus. Data are shown as mean values ± SEM (n = 5); * $p \leq 0.05$, ** $p \leq 0.01$.

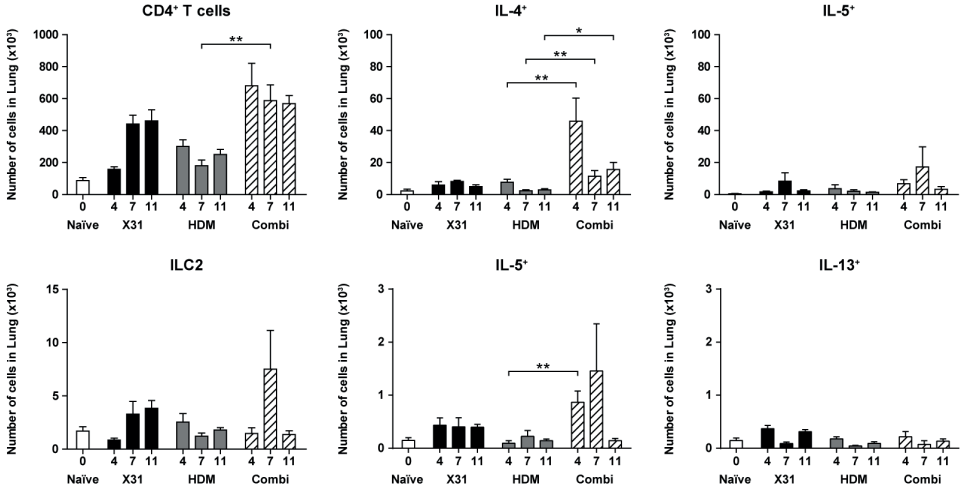

Figure S3. Synergistic effect of influenza virus infection in lungs of HDM-treated mice.

Quantification of the number of type 2 cytokine producing CD4$^+$ T cells (*top*) and ILC2s (*bottom*) in lungs. Not that there are significant synergistic effects of influenza virus infection on the induction of IL-4$^+$ T cells and IL-5$^+$ ILC2s in the lung. Data are shown as mean values ± SEM (n = 7); * $p \leq 0.05$, ** $p \leq 0.01$.

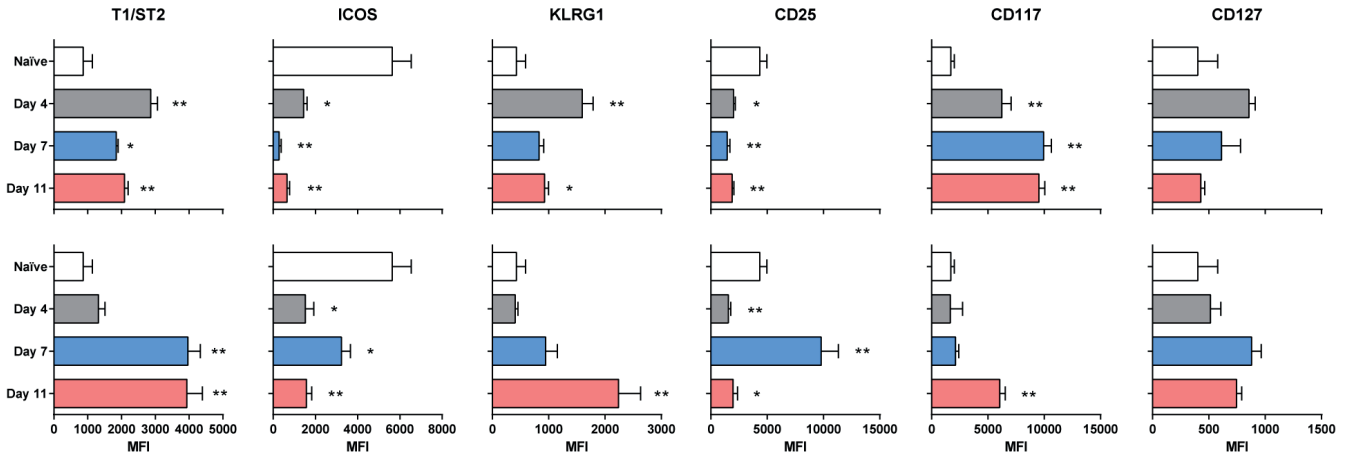

Figure S4. Quantification of surface expression of T1/ST2, ICOS, KLRG1, CD25, CD117 and CD127 on ILC2s in BAL fluid at day 0, day 4, day 7 and day 11 post influenza infection.

Mean fluorescence intensity (MFI) values corresponding to histograms shown in Figure 6. Data are shown as mean values ± SEM (n = 7). * $p \leq 0.05$, ** $p \leq 0.01$.

EPIGENOME ANALYSIS LINKS GENE REGULATORY ELEMENTS IN GROUP 2 INNATE LYMPHOCYTES TO ASTHMA SUSCEPTIBILITY

CHAPTER 6

This chapter is published in Journal of Allergy and Clinical Immunology:

Epigenome analysis links gene regulatory elements in group 2 innate lymphocytes to asthma susceptibility
Ralph Stadhouders, Bobby W.S. Li, Marjolein J.W. de Bruijn, Antonio Gomez, Tata Nageswara Rao, Hans Jörg Fehling, Wilfred F.J. van IJcken, Ai Ing Lim, James P. Di Santo, Thomas Graf, Rudi W. Hendriks
Journal of Allergy and Clinical Immunology (in press)

ABSTRACT

Background

Group 2 innate lymphoid cells (ILC2s) are major producers of cytokines driving allergic asthma and elevated numbers of ILC2s have been detected in blood and sputum of asthma patients. Asthma susceptibility has a strong genetic component, but the underlying mechanisms and whether asthma genetics impact ILC2 biology remains unclear.

Objective

To study the ILC2 transcriptome and epigenome during airway inflammation (AI) in order to couple these to genes and genetic variants associated with asthma pathogenesis.

Methods

Mice harboring a reporter for the key ILC2 transcription factor GATA3 were subjected to IL-33-driven AI and ILC2s were isolated from bronchoalveolar lavage fluid and mediastinal lymph nodes. Human ILC2s were purified from peripheral blood and activated *in vitro*. We employed RNA-Seq, genome-wide identification of histone-3 lysine-4 dimethylation (H3K4me2) marked chromatin and computational approaches to study the ILC2 transcriptome and epigenome.

Results

Activated ILC2s in mice displayed a tissue-specific gene expression signature that emerged from remarkably similar epigenomes. We identified superenhancers implicated in controlling ILC2 identity and asthma-associated genes. Over 300 asthma-associated genetic polymorphisms identified in genome-wide association studies localized to $H3K4Me2^{+}$ gene regulatory elements in ILC2s. A refined set of candidate causal asthma-associated variants was uniquely enriched in ILC2 - but not Th2 cell - regulatory regions.

Conclusions

ILC2s in AI employ a flexible epigenome that couples adaptation to new microenvironments with functional plasticity. Importantly, we reveal strong correlations between gene regulatory mechanisms in ILC2s and the genetic basis of asthma, supporting a pathogenic role for ILC2s in allergic asthma.

Key Messages

- Activated ILC2s utilize a broadly permissive epigenome that allows tissue-specific gene expression changes and provides opportunities for cellular plasticity
- ILC2 (super)enhancers control genes important for cellular identity, effector function and airway inflammation
- Many asthma-associated genetic variants, including candidate causal SNPs, localize to ILC2-specific regulatory elements, supporting a pathogenic role for ILC2s in allergic asthma

Capsule Summary

Asthma susceptibility has strong genetic components that are still poorly understood. We show that many asthma-associated genetic variants reside in regions that regulate gene expression in ILC2s, supporting a pathogenic role for ILC2s in asthma.

INTRODUCTION

Allergic asthma is a chronic disorder characterized by reversible airflow limitation, bronchial hyperreactivity, increased mucus production and airway inflammation (AI) (1). Over 50% of patients have allergic eosinophilic asthma mediated by excessive type-2 immune responses to inhaled allergens including house dust mite (HDM). Enhanced activation of T helper-2 (Th2) cells and the type-2 cytokines they produce explain many hallmarks of allergic asthma: interleukin-4 (IL-4) induces class switch recombination to allergen-specific IgE; IL-5 supports eosinophil differentiation, activation and recruitment into the lungs; and IL-13 mediates bronchial hyperreactivity, mucus hypersecretion and airway remodeling (1).

The recent identification of group 2 innate lymphoid cells (ILC2s) as major producers of type-2 cytokines has challenged the central role of Th2 cells in asthma etiology (1-3). ILCs do not express antigen-specific receptors but play key roles in inflammation, host defense and tissue homeostasis (3). ILCs are categorized based on selective cytokine production and their transcription factor (TF) requirements, which mirrors that of their adaptive T cell counterparts (3, 4). ILC1s produce IFNγ and express the TF T-bet; ILC3s are able to produce IL-17/IL-22 and depend on RORγt. ILC2s parallel Th2 cells in that their development and expression of type-2 cytokines is crucially dependent on GATA3 (5). When exposed to IL-25, IL-33, thymic stromal lymphopoietin (TSLP), prostaglandin D2 or leukotrienes, ILC2s rapidly expand and secrete large amounts of IL-5, IL-13 and under certain conditions also IL-4 (3, 6, 7). ILC2s are essential for protective immunity against helminths and drive AI mediated by various allergens including papain, chitin or *Alternaria alternata* spores (3, 8). Also in HDM-driven AI ILC2s are major producers of IL-5 and IL-13, but their induction depends on prior allergen sensitization and T cell activation (9, 10). Moreover, increased ILC2 numbers and/or type-2 cytokine production by ILC2s in blood and sputum of asthmatics have been reported (11-16).

Strong environmental and genetic components influence asthma susceptibility, persistence and response to medication. Numerous genes have been associated with asthma through genome-wide association studies (GWAS) (17, 18), but these provide limited insight in the underlying mechanisms and cell types affected. Recent studies have also uncovered regions of differential DNA methylation associated with allergic inflammation (19, 20), but the molecular underpinnings of asthma-associated genetic and epigenetic variation remain largely unclear (17, 21). Epigenomic profiling is uniquely suited to study the molecular basis of cellular identity and disease-associating genetic or epigenetic variation (17, 22, 23). Supporting this strategy is a recent study of human circulating memory Th2 cells reporting epigenetic differences between asthmatic patients and healthy controls, as well as an enrichment of asthma-associated single-nucleotide polymorphisms (SNPs) in gene regulatory elements (GREs) of Th2 cells (24). The epigenome of murine ILC2s from the lung in helminth-induced inflammation (25, 26), or the small intestine (27) have recently been explored. However, an in-depth analysis of the epigenetic basis of ILC2 cellular identity and activation is still lacking, precluding a better understanding of environmental adaptation and plasticity in ILC2s driving AI. Moreover, human ILC2 biology has remained unexplored at the epigenetic level. As a consequence, it is currently unknown whether genetic variants implicated in human asthma affect gene regulatory processes in ILC2s.

Here we addressed gene activation in ILC2s mediating AI in a genome-wide fashion using an epigenomic approach. We found that ILC2s activated *in vivo* by IL-33 in mice employ an extensively primed epigenome that allows for rapid adaptation of the ILC2 gene expression program to different tissue microenvironments. Genes that safeguard ILC2 identity and effector function are controlled by superenhancer elements, often bound by TFs GATA3 and GFI1. Importantly, analysis of human peripheral blood-derived ILC2s showed that the majority of asthma-associated genes could be linked to active or primed GREs in ILC2s, with >300 asthma-associated genetic variants - including candidate causal SNPs - residing in ILC2-specific GREs.

METHODS

A more detailed description of all methods can be found in the Supplementary Material.

Mice and lymphocyte isolation

ILC2s were isolated from IL-33 treated GATIR mice (*Gata3*$^{YFP/YFP}$; T.N.R and H.J.F., manuscript in preparation). Human peripheral blood Th2 cells and ILC2s were isolated and activated *in vitro* as previously described (24, 28). Experiments were approved by relevant ethical committees.

RNA-Sequencing and ChIPmentation

RNA extraction and sequencing library preparation were previously described (29, 30). ChIPmentation was performed as described (31). Tagmented DNA was amplified and sequenced on a NextSeq500 (Illumina). Downstream analyses were performed using HOMER (32).

Accession numbers

All sequencing datasets were deposited in the Gene Expression Omnibus (GEO), accession number GSE98843.

RESULTS

Isolation of ILC2s driving AI using Gata3 reporter mice

To isolate murine ILC2s, we employed *Gata3*$^{YFP/YFP}$ reporter mice harboring an IRES-YFP sequence knocked into the *Gata3* locus ('GATIR' mice). GATA3 protein levels or function in IL-33/HDM-driven models of AI were not affected by the IRES-YFP insertion (**Supplementary Fig. 1** and (33)). Since all ILC2s are critically dependent on GATA3 and robustly express the protein (5), GATIR mice greatly facilitated ILC2 purification as Lin$^-$T1/ST2(IL-33R)$^+$Sca-1$^+$YFP$^+$ cells (**Fig. 1A-B**). AI was induced by intranasal exposure to IL-33, resulting in the robust accumulation of eosinophils, CD4$^+$ T cells and ILC2s in bronchoalveolar lavage (BAL) and lung-draining mediastinal lymph nodes (MLN) (**Fig. 1C**). Both BAL and MLN ILC2 populations consisted predominantly of ICOS$^+$KLRG1$^+$CD25$^+$ activated effector cells with the capacity to produce IL-5 and/or IL-13 (**Fig.1D**, **Supplementary Fig. 1D**). Although surface marker profiles of BAL and MLN ILC2 populations were similar, we did notice tissue-specific differences in levels of e.g. KLRG1, T1/ST2 (IL-33R), ICOS and CD117 (**Fig. 1D**). To obtain further insight into the molecular underpinnings of ILC2s driving IL-33-induced AI, we set out to characterize their transcriptome (using RNA-Sequencing; RNA-Seq) and epigenome (using histone-3 lysine-4 dimethylation or H3K4Me2 ChIPmentation (31)) (**Fig. 1E**).

Tissue microenvironments modulate ILC2 gene expression programs

RNA-Seq revealed substantial gene expression differences between ILC2s from BAL or MLN (n=1201 genes, >2-fold difference, adjusted P<0.05) (**Fig. 2A-B, Supplementary Table 1**), with excellent purity and reproducibility between biological replicates (R^2>0.97, **Supplementary Fig. 2A-B**). In agreement with our flow cytometry analysis, *Klrg1* expression was higher in MLN ILC2s while *Il1rl1* encoding T1/ST2 (IL-33R), *Il2ra* (CD25) and *Kit* (CD117) were upregulated in BAL ILC2s (**Fig. 2B-E**). *Icos* and *Il7r* (CD127) were expressed at similar levels, suggesting that ICOS protein levels are differentially regulated at the post-transcriptional level (**Supplementary Table 1**).

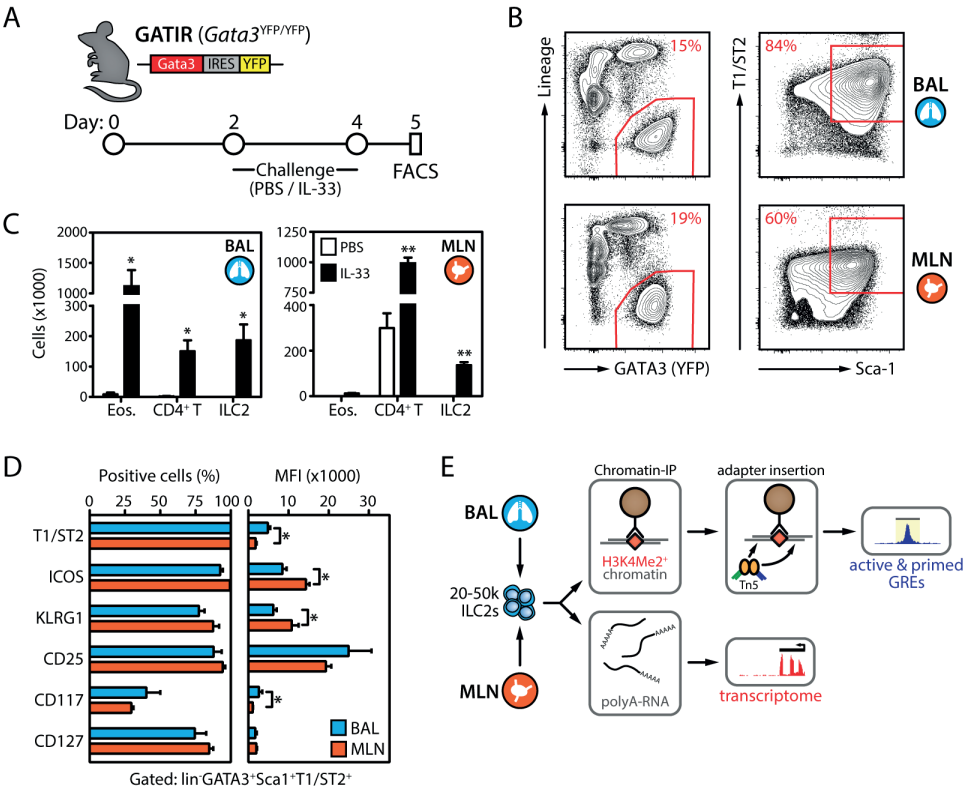

Figure 1. Isolation of ILC2s from GATA3 reporter mice with IL-33-induced AI. (

A) An in-frame IRES-YFP sequence was inserted just upstream of the *Gata3* stop codon to create *Gata3*$^{YFP/YFP}$ (GATIR) reporter mice. (B) Gating strategy for isolating Lin$^-$GATA3$^+$Sca1$^+$T1/ST2$^+$ ILC2s from bronchoalveolar lavage (BAL) fluid and lung-draining mediastinal lymph nodes (MLN). Percentages of cells gated are indicated in red. (C) Flowcytometric quantification of eosinophils (Eos.), CD4$^+$ T cells and ILC2s in BAL fluid and the MLN after *in vivo* treatment with PBS or IL-33 (D) Percentage of cells positive for a panel of ILC2 activation markers (left) and their surface expression (right; MFI: mean fluorescence intensity) for ILC2s from BAL fluid or MLN. (E) Next-generation sequencing (NGS) strategy for analyzing the H3K4Me2 epigenome (top) and mRNA transcriptome (bottom) of small numbers of freshly isolated ILC2s. ChIPmentation (31) utilizing an adapter-loaded Tn5 transposase was used to map active and primed gene regulatory elements (GREs) in ILC2s. Bar graphs in figure represent mean values + SEM of 3-6 mice per group (representative of three independent experiments). *$P \leq 0.05$; Mann-Whitney U test.

Compared to ILC2s from MLNs, BAL ILC2s exhibited a more inflammatory phenotype expressing high levels of IL-33R and cysteinyl leukotriene activation receptors (*Cysltr1/2*), pro-inflammatory cytokines (e.g. *Il9*, *Il21*, *Il5*, *Il13*), chemoattractants (e.g. *Ccl6*, *Ccl7*, *Ccl9*, *Eotaxin2*) and co-stimulatory receptors (e.g. *Gitr*, *Ox40*) (**Fig. 2B-E**). Surprisingly, *Ctla4* and *Il10* expression was also markedly higher in BAL ILC2s, hinting at a possible regulatory function of (a subset of) ILC2s specifically in the lungs (**Fig. 2E**).

Besides *Klrg1*, MLN ILC2s also expressed higher levels of *Ox40l* and MHC class II-like *H2-O* genes, suggesting MLN ILC2s are actively involved in cell-to-cell interactions with dendritic cells or Th2 cells (34-38). Interestingly, MLN ILC2s upregulated *Nod1*, a pattern recognition receptor that drives Th2 polarization and has been implicated in asthma (39, 40). MLN ILC2s expressed high levels of *Alox5*, which encodes the 5-lipoxygenase enzyme involved in the production of leukotrienes - potent activators of ILC2s and allergic asthma (41, 42). MLN ILC2s also expressed higher levels of the neuropeptide receptor gene *Nmur1*, recently implicated in promoting allergic inflammatory ILC2 responses via neuro-immune crosstalk (43) (**Fig. 2B-E**). In line with these observations, confocal microscopy analysis of IL-33-treated GATIR mice revealed differences in the ILC2 microenvironment in lung (underneath the epithelium) and MLN (near T and B cells) (**Supplementary Fig. 2C**).

We observed tissue-specific expression differences in metabolism and survival/proliferation-associated pathways. While BAL ILC2s upregulated genes involved in the lipid response (e.g. the free fatty acid receptor gene *Ffar2*) and cell survival (e.g. *Bcl2*), MLN ILC2s showed enhanced glycolysis and cell division (**Fig. 2B-E**). Upregulation of an aerobic glycolytic metabolic program is a hallmark of effector lymphocyte activation (44-46) and therefore consistent with increased ILC2 proliferation in the MLN. This is further illustrated by the upregulation of *Mki67* encoding the Ki67 proliferation marker, which we validated by flowcytometry analysis (**Fig. 2F**). Nevertheless, the absolute increase in ILC2 cellularity was comparable for BAL and MLN samples (**Fig. 1C**), suggesting that ILC2 expansion in the MLN occurred later or with slower kinetics than local proliferation in the lung. Alternatively, ILC2s from the MLN might be actively recruited to the lung.

Tissue-specific gene expression profiles in ILC2s arise from highly similar epigenomes

To assess whether the observed transcriptional differences were also reflected in epigenetic alterations, we profiled H3K4Me2^{+} chromatin in ILC2s. H3K4Me2 accurately marks both active and primed GREs, providing a comprehensive catalogue of transcription-permissive chromatin (47, 48). We employed ChIPmentation, a genome-wide chromatin immunoprecipitation (ChIP) protocol adapted to low numbers of starting cells (31), to analyze ~20,000 freshly isolated ILC2s (**Fig. 1E**). H3K4Me2 enrichments correlated with gene expression and showed high quality signal-to-noise ratios (**Supplementary Fig. 3** and Supplementary Methods). Results from two biological replicates consistently identified ~30,000 H3K4Me2^{+} GREs (**Supplementary Fig. 4A**). GRE distribution was identical between BAL and MLN ILC2s and, as expected, signature ILC2 loci (e.g. *Bcl11b*, *Gata3*, *Th2 locus*) were embedded in active chromatin (**Fig. 3A-B**). ChIPmentation also identified H3K4Me2 signatures at loci not previous implicated in ILC2 biology, e.g. the TCRγ locus, *Cd320* (encoding the vitamin B12 receptor) and the vascular endothelial growth factor C (*Vegfc*) locus (**Fig. 3C**).

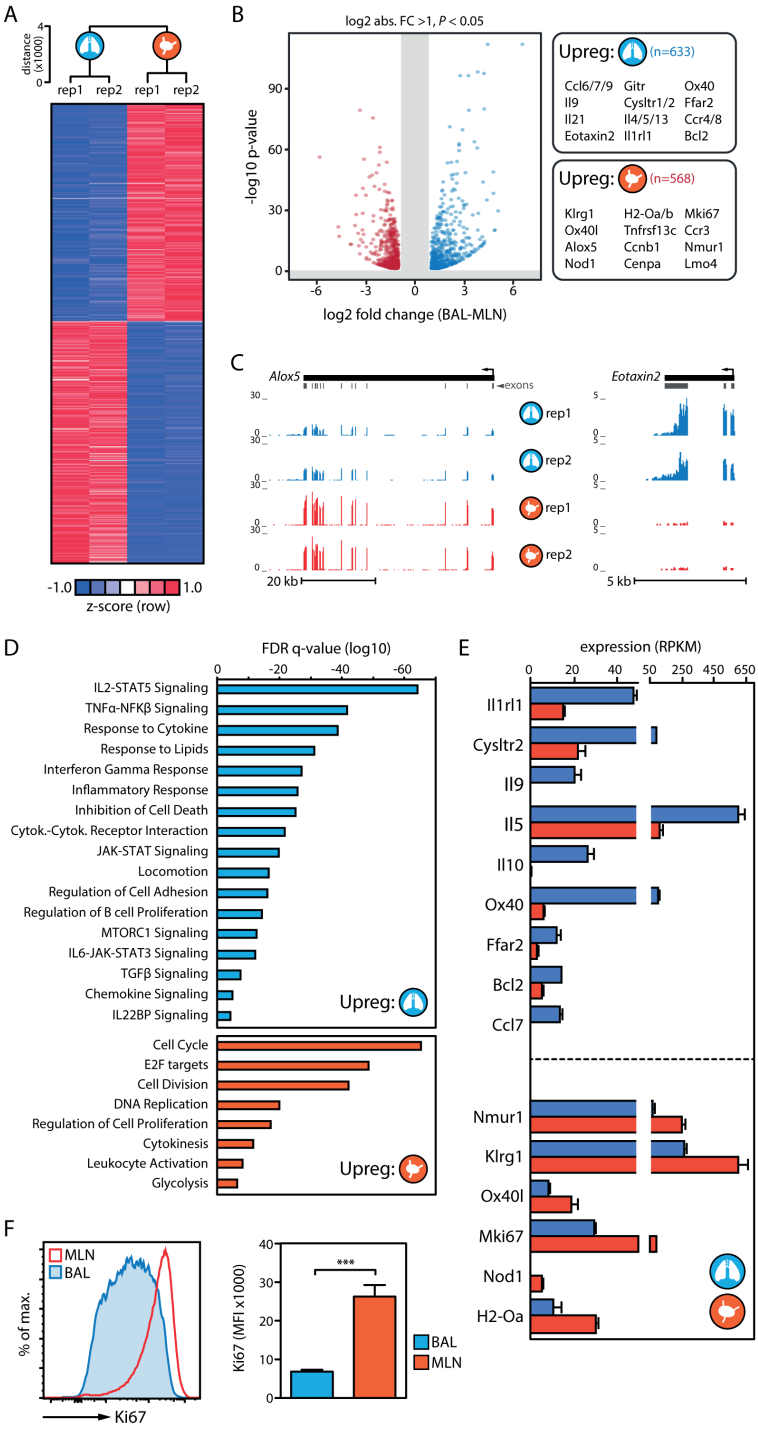
A
distance (x1000)
rep1 rep2 rep1 rep2
-1.0 1.0
z-score (row)
B
log2 abs. FC >1, P < 0.05
-log10 p-value
log2 fold change (BAL-MLN)
Upreg: (n=633)
Ccl6/7/9 Gitr Ox40
Il9 Cysltr1/2 Ffar2
Il21 Il4/5/13 Ccr4/8
Eotaxin2 Il1rl1 Bcl2
Upreg: (n=568)
Klrg1 H2-Oa/b Mki67
Ox40l Tnfrsf13c Ccr3
Alox5 Ccnb1 Nmur1
Nod1 Cenpa Lmo4
C
Alox5
exons
Eotaxin2
rep1
rep2
rep1
rep2
20 kb
5 kb
D
FDR q-value (log10)
IL2-STAT5 Signaling
TNFα-NFKβ Signaling
Response to Cytokine
Response to Lipids
Interferon Gamma Response
Inflammatory Response
Inhibition of Cell Death
Cytok.-Cytok. Receptor Interaction
JAK-STAT Signaling
Locomotion
Regulation of Cell Adhesion
Regulation of B cell Proliferation
MTORC1 Signaling
IL6-JAK-STAT3 Signaling
TGFβ Signaling
Chemokine Signaling
IL22BP Signaling
Upreg:
Cell Cycle
E2F targets
Cell Division
DNA Replication
Regulation of Cell Proliferation
Cytokinesis
Leukocyte Activation
Glycolysis
Upreg:
E
expression (RPKM)
Il1rl1
Cysltr2
Il9
Il5
Il10
Ox40
Ffar2
Bcl2
Ccl7
Nmur1
Klrg1
Ox40l
Mki67
Nod1
H2-Oa
F
MLN
BAL
% of max.
Ki67
Ki67 (MFI x1000)
***
BAL
MLN

Figure 2. Tissue-specific gene expression signatures of ILC2s driving AI.

(**A**) Heatmap visualizing gene expression levels (shown as row z-scores of RPKM levels) for genes detected as differentially expressed (n=1201) between ILC2s isolated from BAL fluid or the MLN. Signals from both biological replicates (each representing a pool of 5-6 mice) are depicted. (**B**) Volcano plot with gene expression changes (log2 scale, BAL minus MLN) plotted versus *P* values (-log10). Dots represent differentially expressed genes (>1 absolute log2 fold change and adjusted $P < 0.05$; red: upregulated in MLN, blue: upregulated in BAL). Boxes highlight selected genes from both groups. (**C**) Normalized expression counts for *Alox5* and *Eotaxin2* (*Ccl24*) shown in a genome browser view. Genes are plotted on top as black bars with an arrow indicating the transcription start site and directionality; exons are indicated below. (**D**) Molecular pathway enrichment (MSigDB) amongst genes upregulated in BAL (blue bars) or MLN (red bars) ILC2s. (**E**) Gene expression levels of selected genes differentially expressed between BAL and MLN ILC2s (mean values of n=2 pools of 5-6 mice + SEM). (**F**) Ki67 MFI in ILC2s isolated from BAL or MLN (mean values of n=3 groups of 3-6 mice + SEM). $***P \leq 0.001$; Mann-Whitney U test.

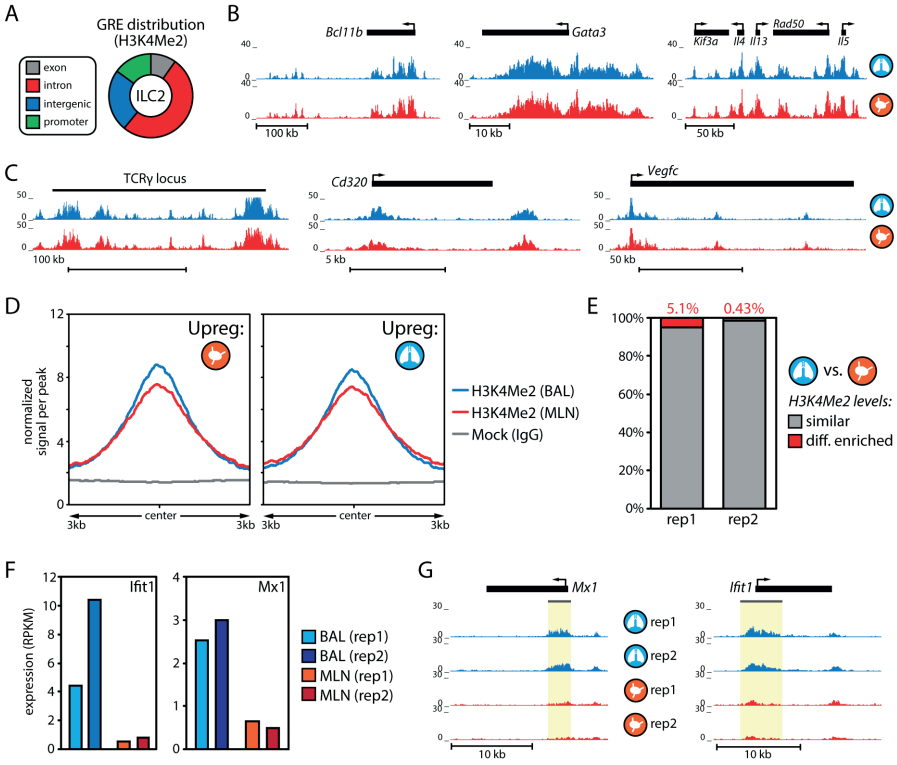

Figure 3. ILC2s residing in different tissues employ a highly similar H3K4Me2 epigenome.

(**A**) Distribution of H3K4Me2$^+$ gene regulatory elements (GREs) across the ILC2 genome. (**B,C**) Genome browser views of the H3K4Me2 chromatin landscape at selected loci known to be essential for ILC2 function (panel B) or newly identified as harboring an active chromatin signature in ILC2s (panel C). (**D**) Meta-profile depicting average enrichment levels for all H3K4Me2$^+$ GREs associated with genes upregulated in MLN (left) or BAL (right) ILC2s. Signal measured in a mock experiment (using an aspecific IgG) is shown in grey. (**E**) Identification of differentially H3K4Me2-enriched regions (>2 fold change) between BAL and MLN ILC2s in two independent experiments (each representing a pool of 5-6 mice). (**F,G**) Gene expression levels (panel F) and H3K4Me2 landscapes (panel G) of the interferon signaling-induced genes *Ifit1* and *Mx1* in ILC2s.

Surprisingly, H3K4Me2 levels at GREs near differentially expressed genes were highly similar in BAL and MLN ILC2s (**Fig. 3D**, **Supplementary Fig. 4B**). Unbiased genome-wide comparisons between the epigenomes of the two ILC2s populations across replicate experiments yielded very few reproducibly differentially enriched regions (n=43 out of >27,000 GREs, >2-fold difference) (**Fig. 3E**, **Supplementary Fig. 4C**). Only a single group of genes, involved in interferon signaling displayed consistently higher H3K4Me2 enrichment and gene expression in BAL ILC2s (**Fig. 3F-G**, **Supplementary Fig. 4D**). Thus, tissue-specific gene expression profiles in ILC2s driving AI arise from highly similar epigenomes.

Extensive epigenetic priming of ILC2s in AI

ILCs provide a sentinel function as early responders to immunological challenges (3) and would thus benefit from a broadly permissive epigenome that allows for rapid gene activation and functional plasticity. In IL-33 driven AI, a substantial fraction (~37%) of all H3K4Me2$^+$ GREs was associated with genes not or very lowly expressed (RPKM<2). These GREs displayed lower average H3K4Me2 levels when compared with GREs linked to active genes (**Fig. 4A**). Repressed genes associated with H3K4Me1/2 are considered 'primed' for rapid activation upon receiving an appropriate stimulus (49, 50). Pathway analysis of primed genes in ILC2s revealed a significant overrepresentation of genes involved in cytokine and chemokine signaling, in contrast to the many general housekeeping functions enriched among active genes (**Supplementary Fig. 5A**). For example, we detected primed chromatin environments at loci linked to airway remodelling (**Supplementary Fig. 5B-C**), including *Bmp2* (51), *Acvr1b* (encoding the receptor for TGFβ-family member Activin A, an allergen-responsive cytokine elevated in asthma (52)), the *Cxcl3* chemokine cluster that induces smooth muscle cell migration and airway remodeling (53) and *Has3* encoding an enzyme involved in the synthesis of hyaluronan, a major extracellular matrix component that accumulates during asthma (54). Primed genes in airway ILC2s also included many that were previously reported as specifically expressed in either ILC2s or ILC3s isolated from the small intestine (55), such as the *Rorc* locus that encodes RORγt (**Fig. 4B**). Moreover, key ILC1 genes *Tbx21* and *Ifng* (encoding T-bet and IFNγ respectively) were primed or expressed at low levels in ILC2s (**Fig. 4C-D**). Together, these findings reveal extensive priming of GREs and hallmarks of functional plasticity in ILC2s during AI.

We next explored the molecular mechanisms underlying epigenetic priming in ILC2s. The TFs GATA3 and GFI1 bind to thousands of GREs in ILC2s (56, 57), often representing H3K4Me2$^+$ enhancers (**Supplementary Fig. 6**). Co-binding by both TFs was infrequent (<20% of binding sites), although >50% of their target genes were shared, suggesting co-regulation using different GREs (**Supplementary Fig. 6F**). Genes regulated by both TFs or only by GATA3 were involved in various immune signaling pathways (e.g. Th2 cytokine expression), whereas genes specifically targeted by GFI1 predominantly displayed housekeeping functions (**Supplementary Fig. 6G**).

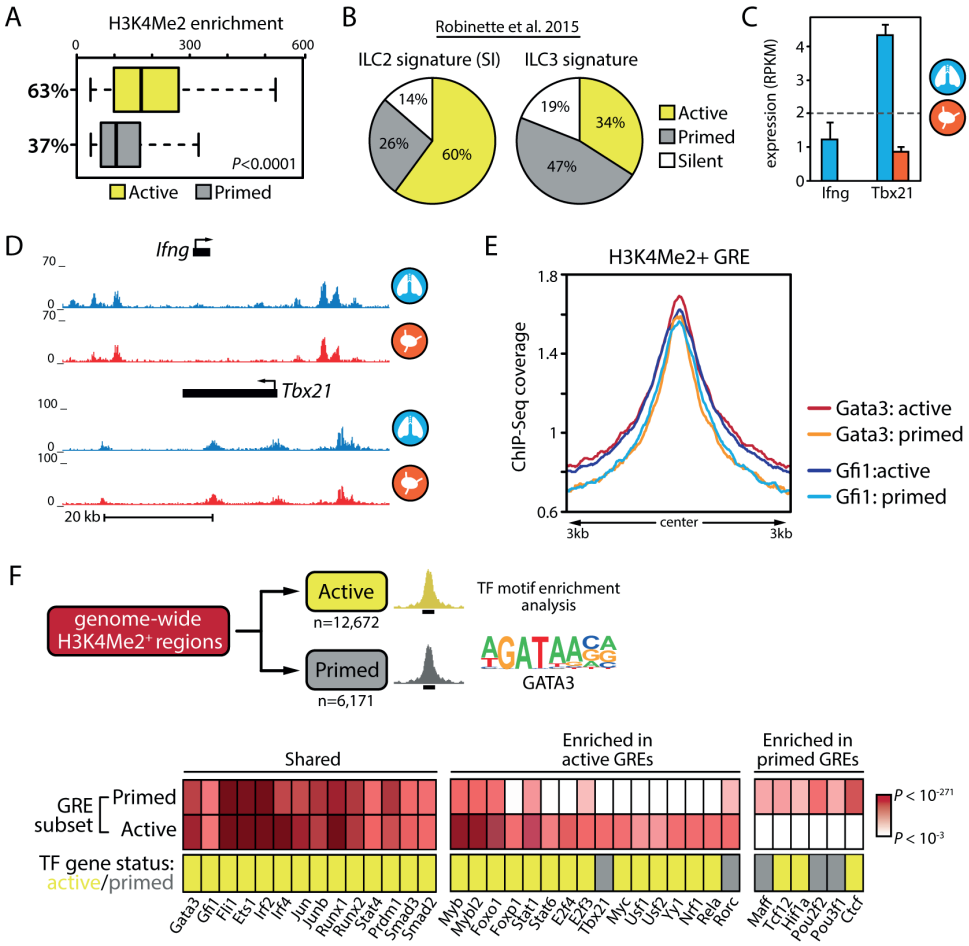

Figure 4. Extensive epigenetic priming in ILC2s is associated with a specific transcription factor binding landscape.

(**A**) Boxplot showing H3K4Me2 levels of gene regulatory elements (GREs) associated with active or primed genes. Genes were considered primed if associated with an H3K4Me2$^+$ GRE but expressed at very low levels (RPKM < 2). *P* value was determined using a Wilcoxon rank-sum test; percentages indicate proportion of all GREs belonging to either category. (**B**) Proportions of active, primed or silent genes (as determined using AI ILC2 H3K4Me2 and transcriptome data) belonging to previously defined (55) gene signatures of ILC2s (from the small intestine, SI) or ILC3s. (**C,D**) Gene expression levels (panel C) and H3K4Me2 landscapes (panel D) of core ILC1 genes *Ifng* and *Tbx21* in ILC2s (mean values of n=2 pools of 5-6 mice + SEM). Dashed line indicates the 2 RPKM cut-off used to define primed genes. (**E**) Meta-profile depicting average GATA3 or GFI1 enrichment levels at H3K4Me2$^+$ GREs associated with active or primed genes. (**F**) Unbiased transcription factor (TF) binding motif search within active or primed GREs. Heatmap indicates for each TF its expression status in ILC2s (yellow: active; primed: grey) and motif enrichment score (white-to-red gradient of adjusted p-values) for the two GRE subsets.

However, both factors bound with near equal affinities to active and primed GREs (**Fig. 4E**). A more unbiased approach for investigating differential TF binding is to scan regions of interest for

the presence of TF binding motifs (32). We detected a strong enrichment of GATA3 and GFI1 motifs in both categories of GREs, validating our approach (**Fig. 4F**). Other TFs expressed in ILC2s potentially operating at both primed and active GREs included ETS, IRF, PRDM and RUNX TFs - most of which have not been previously reported as ILC2 regulators. This group included the SMAD2/3 TFs that relay signals of the TGFβ pathway, known to enhance ILC2 function (58). We also found several differentially enriched motifs, indicating that factors such as FOXP1, MYC, YY1, STAT1 (activated in response to interferon signaling) and STAT6 (activated in response to IL-4/IL-13 signaling) are involved in creating and/or maintaining a chromatin state in ILC2s that facilitates gene activation. In contrast, TCF12, HIF1A and CTCF might play a role in preventing full gene activation by maintaining a primed environment (**Fig. 4F**).

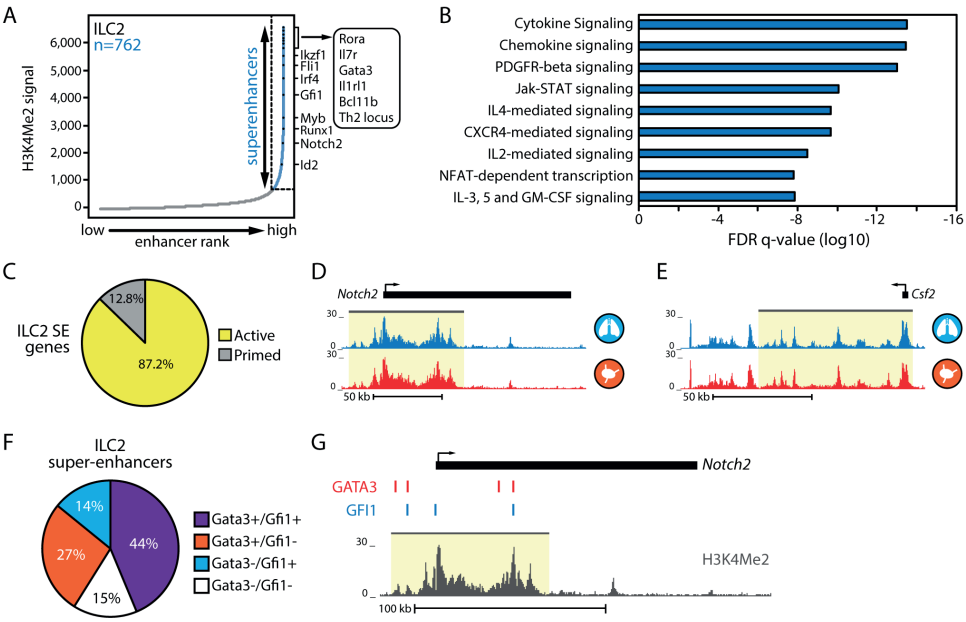

Figure 5. Superenhancer characterization reveals core ILC2 identity genes.

(**A**) Ranking stitched enhancer regions (59) based on their H3K4Me2 levels identifies superenhancers (SEs) in ILC2s. Selected SEs and their associated genes are indicated, with known key ILC2 genes (boxed) at the top. (**B**) Molecular pathway enrichment (MSigDB) amongst genes associated with SEs. (**C**) Proportion of ILC2 SE-associated genes residing in an active or primed state. (**D,E**) Examples of SE regions (marked with yellow shading) at the active *Notch2* gene (panel D) and the primed *Csf2* gene (encoding GM-CSF, panel E). (**F**) Proportion of ILC2 SEs bound by GATA3 and/or GFI1. (**G**) Example of a SE region bound by both GATA3 (red rectangles) and GFI1 (blue rectangles).

Superenhancers bound by Gata3 and Gfi1 control ILC2 identity

A special class of clustered GREs called superenhancers (SEs) regulate the expression of cell identity genes and harbor disease-associated genetic variants (59, 60). As ILC2-associated SEs have not yet been described, we used our H3K4Me2 datasets to identify 762 ILC2 SEs according to previously defined criteria (59) (**Supplementary Table 2**). ILC2 SEs were located in regions of open, transcription-permissive chromatin, as expected (**Supplementary Fig. 7A-B**). Top SEs were found at loci known to be critical for ILC2 biology, e.g. *Rora*, *Il7r*, *Gata3*, *Bcl11b* and the Th2 cytokine locus (**Fig. 5A, Supplementary Fig. 7C**). Importantly, this analysis identified many SE at loci encoding TFs that play important roles in T cell biology, but that have not been implicated in ILC2 function before, e.g. *Ikzf1* (encoding Ikaros), *Fli1* and *Myb* (**Fig. 5A**). SE-associated genes also controlled key signaling pathways in ILC2s (**Fig. 5B**).

Although most SE-associated genes resided in an active state (e.g. *Notch2*, **Fig. 5C-D**), a small proportion (~13%) was primed including the *Csf2* locus encoding GM-CSF (**Fig. 5E**). As ILC2s from blood or nasal polyps are able to produce GM-CSF (6), these observations are in line with tissue-specific gene expression programs in ILC2s emerging from a primed epigenome. Importantly, the majority (~85%) of SEs was bound by GATA3, GFI1 or both factors (**Fig. 5F-G, Supplementary Fig. 6B-C**), implicating these proteins as central regulators of ILC2 SEs.

Human asthma susceptibility genes reside in an active or primed state in murine ILC2s

Whether ILC2s contribute to the genetic basis and molecular mechanisms underlying allergic asthma remains unclear. We found H3K4Me2^{+} GREs in ILC2s near several active and primed genes associated with AI and asthma. Besides classic type-2 immunity genes (e.g. *Gata3, Ilrl1, Il5)* these included: 1) leukotriene metabolism genes (61), e.g. *Alox5* and *Cysltr1/2;* 2) cyclic nucleotide second messenger metabolic genes such as *Gucy1a3* (guanylate cyclase 1 soluble subunit alpha-3) and *Calcrl,* which encodes a receptor for the calcitonin gene-related peptide CGRP that acts as an inflammatory mediator in asthma (62); 3) the lipid inflammatory mediator prostaglandin E synthase (*Ptges*) (63); and 4) the serpin peptidase inhibitor clade E (*Serpine2*) (64) (**Fig. 6A-B**).

An unbiased analysis using a curated asthma-associated gene signature (Disease Ontology DOID:2841) (65) revealed highly significant associations of various epigenomic and transcriptomic ILC2 features with asthma (**Fig. 6C**). We next focused on genetic variants implicated in asthma susceptibility by GWAS. We assigned asthma-associated SNPs (17, 66) (see Methods) to 665 putative target genes (**Supplementary Table 3**). Over 90% of variants are located >5kb away from promoter regions (**Fig. 6D**) and therefore likely modulate gene expression via enhancers or post-transcriptional mechanisms. Intersecting these asthma-

associated genes with their mouse orthologues near H3K4Me2$^+$ GREs allowed us to link 494 (~74%) asthma-associated genes to murine ILC2 GREs (within 1 Mb, median distance of 21.3kb), of which 280 were active and 214 represented primed genes in the IL-33 activated ILC2s (**Fig. 6E**). Importantly, the expression profile of asthma-associated genes was similar for IL-33 activated ILC2s and ILC2s activated by chronic exposure to HDM allergen *in vivo* (Pearson R=0.85, **Supplementary Fig. 8**), indicating that epigenomic analysis of IL-33 activated ILC2s has relevance for allergen-driven airway inflammation.

The genetic basis of asthma is strongly linked to GREs in human ILC2s

To extend our findings to humans cells, we profiled H3K4Me2 levels in naïve ILC2s (nILC2) freshly isolated from human peripheral blood (67) of healthy individuals and after *in vitro* activation with the physiologically relevant alarmins IL-25/IL-33 (12, 28, 68) (referred to as 'aILC2') (n=3-4; **Fig. 7A, Supplementary Fig. 9A**). Activation of human nILC2s evoked substantial H3K4Me2$^+$ epigenome expansion around genes involved in lipid metabolism, cytokine/chemokine signaling and asthma (**Fig. 7B-C, Supplementary Fig. 9B-C**). As a consequence, nILC2 and aILC2 displayed markedly different SE compositions (**Fig. 7D-E**). While several hallmark ILC2 genes (e.g. *GATA3*, *RORA*, *PTGDR2*) were SE-associated in both conditions, nILC2s acquired SEs at e.g. the *IL5/IL13*, *BATF* and *DENND1B* loci (all implicated in AI or asthma (69, 70)) upon activation (**Fig. 7D-E; Supplementary Table 2**). Integrating our human ILC2 epigenome data with GWAS findings increased the number of asthma-associated genes linked to H3K4Me2$^+$ ILC2 GREs to ~91% (605/665), including almost all major asthma loci (**Fig. 7F**). The majority of these associations occurred with GREs marked with H3K4Me2 in both nILC2s and aILC2s ('shared' GREs) or only aILC2s ('aILC2-specific' GREs, **Fig. 7F**). Epigenome comparison of mouse and human ILC2s revealed substantial conservation of SE composition, epigenetic signatures of plasticity and permissive chromatin surrounding asthma-associated genes (**Supplementary Fig. 10**).

A more precise intersection of the 3.405 known asthma-associated GWAS SNPs with H3K4Me2$^+$ GREs revealed that >300 common genetic variants associated with asthma localized to active chromatin in ILC2s, a highly significant enrichment compared to random co-localization (P<5.1e-25; **Fig. 7G**, **Supplementary Table 5**). Many asthma-variants were detected in GREs present in both nILC2s and aILC2s. Strikingly, a substantial number of SNPs (125/308) were found in regions that gain H3K4Me2 upon activation (**Fig. 7G**). In addition, up to 52% of differentially methylated DNA elements linked to asthma through epiGWAS (19, 20) resided in an ILC2 H3K4Me2$^+$ GRE (**Supplementary Fig. 9D**).

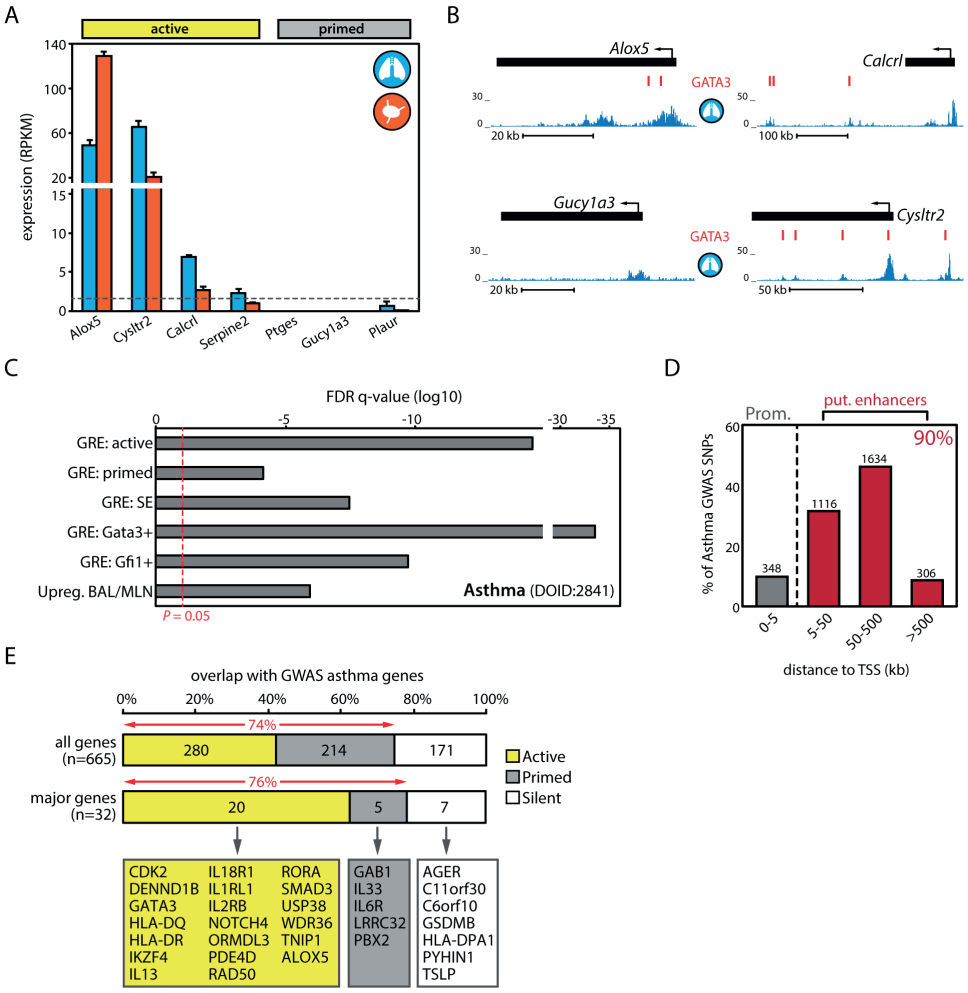

Figure 6. Asthma-associated genes frequently reside in an active or primed state in murine ILC2s.

(**A**) Gene expression levels in ILC2s of genes previously implicated in asthma (mean values of n=2 pools of 5-6 mice + SEM). Dashed line indicates the 2 RPKM cut-off used to define primed genes. (**B**) H3K4Me2 chromatin landscapes in BAL ILC2s at selected asthma-associated loci. Gata3 binding sites are indicated in red. (**C**) Enrichment for asthma-associated genes (curated set; DOID:2841) in various sets of epigenomic and transcriptomic features of ILC2s defined in this study (GRE: gene regulatory element, Upreg.: upregulated). (**D**) Bar graph showing the distance distribution of asthma-associated SNPs from GWAS to the nearest transcription start site (TSS). Note that 90% of all variants are located in putative enhancer regions. (**E**) Stacked bar graph indicating the proportions and numbers of GWAS asthma-associated genes (either all 665 genes or the 32 major (17) genes) residing in an active, primed or silent state in ILC2s driving AI. The individual 32 major asthma genes are shown in coloured boxes below.

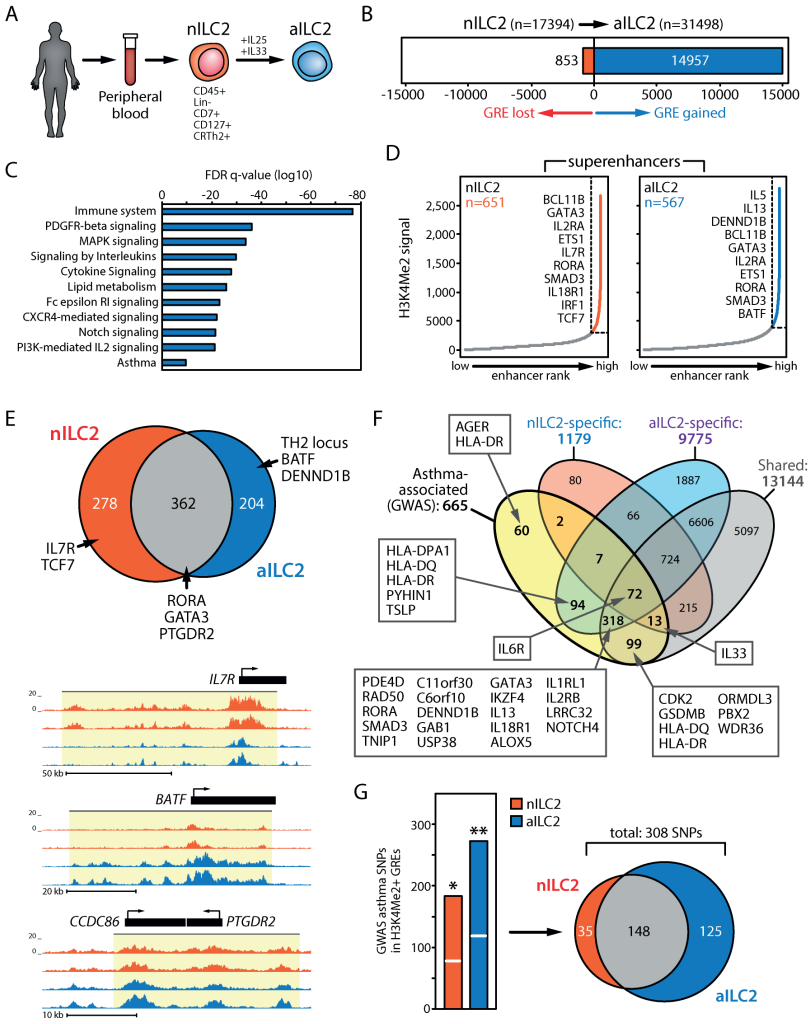

Figure 7. H3K4Me2 epigenome expansion during human ILC2 activation is linked to asthma genetics.

(**A**) Naïve ILC2s (Lin$^-$CD45$^+$CD7$^+$CD127$^+$CRTh2$^+$; nILC2 (67)) were isolated from human peripheral blood and activated *in vitro* with IL-25 and IL-33 (aILC2). (**B**) Number of H3K4Me2$^+$ gene regulatory elements (GREs) lost (red) or gained (blue) upon nILC2 activation. Only GREs reproducibly found in all 3-4 donors were used for downstream analysis. (**C**) Molecular pathway enrichment (MSigDB) amongst genes associated with H3K4Me2$^+$ GREs specific to aILC2s. (**D**) Superenhancer (SE) identification based on H3K4Me2 profiles in human ILC2s. Selected SEs and their associated genes are indicated. (**E**) Comparison of SE landscapes in nILC2s and aILC2s. Selected SE-associated genes are highlighted and genome browser views shown below (yellow shading: SE). (**F**) Venn diagram depicting the overlap between genes linked to asthma-associated SNPs (yellow) and nILC2-specific (red), aILC2-specific (blue) and shared GREs (grey). Major asthma genes (17) are highlighted. (**G**) Number and percentage of asthma GWAS SNPs overlapping with nILC2 or aILC2-specific GREs (*P = 5.1e-25, **P = 2.8e-34; Fisher's exact test). White markers denote random colocalization levels. Venn diagram depicts the overlap between SNPs located within GREs in nILC2s or aILC2s.

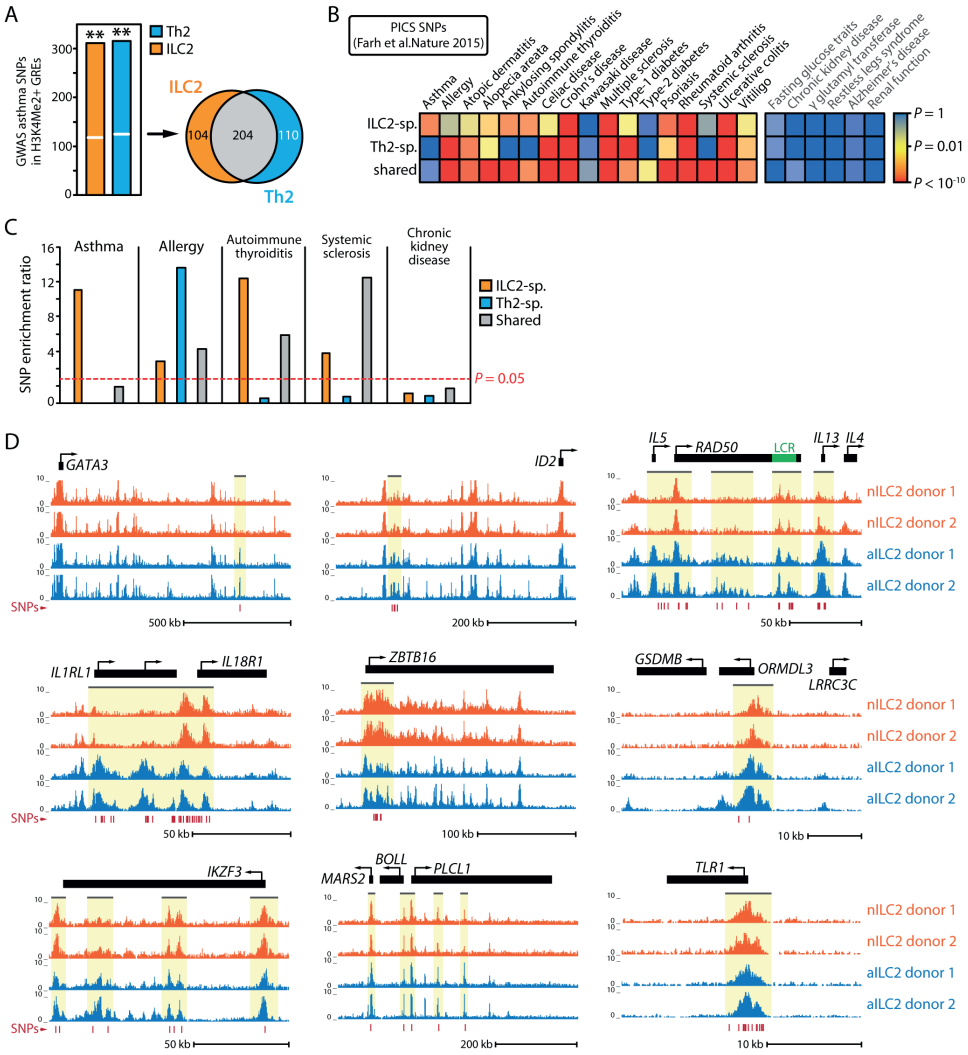

Figure 8. Asthma-associated genetic variation is concentrated in H3K4Me2⁺ regulatory elements of activated ILC2s.

(**A**) Number and percentage of all asthma GWAS SNPs overlapping with H3K4Me2⁺ GREs in ILC2s or Th2 cells (* * *P* = 3.0e-38; Fisher's exact test). White markers denote random colocalization levels. Venn diagram depicts the overlap between SNPs located within GREs in ILC2s or Th2 cells. (**B**) Enrichment (shown as a heatmap of *P* values from Fisher's exact test) of candidate causal SNPs (71) associated with various human disorders in ILC2-specific GREs, Th2-specific GREs and shared GREs. Non-immune diseases (grey names) served as controls. (**C**) SNP enrichment ratios (as defined by Fisher's exact test) of selected human disease from panel B for the three groups of GREs. Red dashed line indicated significance threshold of *P* = 0.05. (**D**) Genome browser views of loci where asthma-associated GWAS SNPs coincide with ILC2 GREs. Colocalization occurs within distal (e.g. *GATA3*, *ID2*), intronic (e.g. *ZBTB16*, *IKZF3*) and promoter (e.g. *IL1RL1*, *TLR1*) GREs. Representative tracks from two donors are shown for naïve (red, nILC2) and activated (blue, aILC2) ILC2s. SNPs are indicated by red lines, regions of SNP-GRE colocalization are highlighted with yellow shading.

GREs in human Th2 cells were previously shown to harbor asthma-associated SNPs (24). To directly compare variant enrichment between ILC2s with Th2 cells, we profiled H3K4Me2 levels in circulating memory Th2 cells from three healthy individuals. We found a similar number of asthma-associated SNPs residing in Th2 cells or ILC2 H3K4Me2$^+$ GREs (314 and 308, respectively, P<3.0e-38; **Fig. 8A**). Interestingly, similar proportions of SNPs localized to GREs shared between ILC2s and Th2 cells as to cell type-specific GREs. We next extended these findings by analyzing a comprehensive set of refined candidate causal variants associated with various human disorders (including asthma and allergy; n=236 SNPs on average per disease (71)), while separating shared, ILC2-specific and Th2 cell-specific H3K4Me2$^+$ GREs. Polymorphisms linked to immune disorders were highly enriched, often irrespective of the subset of GREs (e.g. rheumatoid arthritis, **Fig. 8B**). SNPs associated with non-immune related disorders (e.g. chronic kidney disease) did not show significant enrichments, underscoring the specificity of our approach. However, ILC2s and Th2 cells showed surprising differences in their disease associations. For example, while candidate causal SNPs for asthma were uniquely enriched in ILC2-specific GREs, allergy-associated SNPs were predominantly enriched in Th2-specific H3K4Me2$^+$ regions (**Fig. 8B-C**).

Our approach directly coupled ILC2 H3K4Me2$^+$ GREs to asthma-associated SNPs present in numerous loci implicated in human asthma pathogenesis, including distal enhancer elements and promoters (**Fig. 8D**). Most asthma-associated SNPs co-localized with H3K4Me2$^+$ regions present in both nILC2s and aILC2s or exclusively in aILC2, including several genes not previously implicated in ILC2 biology (e.g. *IKZF3*, *PLCL1*) (**Fig. 8D**).

DISCUSSION

Studies in mouse models and samples from human asthmatics have implicated ILC2s as potential drivers of allergic asthma (68). Here we studied the ILC2 epigenome to gain insight into tissue-specific functions, plasticity and activation of ILC2s in AI, as well as to assess whether ILC2 biology can be linked to the genetic basis of asthma susceptibility.

Our combined transcriptome and epigenome analyses demonstrate that ILC2s exposed to different tissue microenvironments have different gene expression profiles, while maintaining a virtually identical H3K4Me2 epigenome. Together with the observed widespread epigenetic priming of genes, these findings indicate that ILC2s use a permissive epigenome for rapid adaptation to new microenvironments and external signals. This concept of 'epigenetic flexibility' explains how activated ILC2s achieve functional plasticity in response to specific cytokines or viral infections (28, 72-74). Although a recent study reported chromatin accessibility at the Th2 cytokine locus already in murine ILC2 precursors (25), this might be different for human ILC2s: our experiments show substantial H3K4Me2 epigenome expansion and SE changes when naïve human ILC2s are exposed to IL-25/IL-33, including SE establishment at the Th2 cytokine locus.

Our gene expression analysis points at interesting functional differences between ILC2s from the lung or draining lymph nodes in AI. In the MLN, ILC2s are highly proliferative and express genes involved in promoting a Th2 response and leukotriene production. BAL ILC2s in contrast display a more inflammatory nature (e.g. highly active IRF4/IL-9 axis (75)), express chemotactic (e.g. *Eotaxin2*) and co-stimulatory molecules and show signs of plasticity. In addition, location appears to be linked to metabolic differences: MLN ILC2s displayed signs of enhanced glycolysis, while BAL ILC2s upregulated lipid response genes. Differences in nutrient availability between the various microenvironments could force ILC2s to adjust their transcriptome accordingly (45, 46). Surprisingly, we detected elevated expression of the inhibitory genes *Il10* and *Ctla4* in BAL ILC2s, suggesting that as inflammation progresses a 'regulatory' gene expression program emerges in (a subset of) ILC2s. Overall, these observations add to the concept of ILC2s as a phenotypically heterogeneous (76, 77) and plastic (28, 72-74) cell population. A limitation of our study is that IL-33 driven eosinophilic AI only partly recapitulates allergen-driven AI. Although in our hands chronic HDM allergen-driven AI generated too few ILC2s for epigenome analysis, expression of asthma-associated genes was similar in ILC2s generated by IL-33 or HDM induction, indicating that IL-33-activated ILC2s are relevant for allergen-driven AI. Our epigenome and transcriptome maps therefore provide a resource for discovering new pathways and proteins important for ILC2 function in general and in the context of AI. SE composition, epigenetic hallmarks of plasticity and permissive chromatin landscapes surrounding asthma-associated genes were often conserved between activated murine and human ILC2s.

Previous work has suggested that non-coding disease variants primarily function by disrupting long-range gene regulation (24, 78, 79). Our analysis of human ILC2s for the first time links the genetics underlying asthma pathogenesis to the ILC2 epigenome. Most asthma-associated genes reside near GREs active in ILC2s, which harbored over >300 asthma GWAS SNPs. Similar enrichments were observed for Th2 cells, but 50% of polymorphisms localized to cell type-specific H3K4Me2^{+} regions. Strikingly, candidate causal asthma SNPs were uniquely enriched in ILC2-specific GREs. Such differential enrichment of disease-associated variants among Th2 cells and ILC2s extended to other immune disorders and warrants further exploration in subsequent studies.

The current study prioritizes a highly specific part of the ILC2 epigenome that consists of 127 enhancers and promoters (0.77% of all combined human ILC2 GREs, **Supplementary Table 5**) for functional follow-up studies. Associated with these regulatory regions are genes with well-known roles in (allergic) AI and ILC2 function (e.g. *IL13*, *IL1RL1*, *GATA3*), but also many genes not previously associated with ILC2s (e.g. *ORMDL3*, *IKZF1*). A plausible scenario at play here is that genetic or epigenetic variants perturb TF binding to these GREs, resulting in deregulated gene expression and ultimately phenotypic changes in ILC2s.

In conclusion, our study directs a substantial proportion of the genetic basis of asthma to the

ILC2 epigenome, further strengthening a role for ILC2s in asthma pathogenesis. Moreover, as recently developed epigenetic drugs (e.g. BET bromodomain inhibitors (80)) primarily inhibit SE activity, the ILC2-associated SEs identified here could provide novel therapeutic angles to control ILC2-mediated AI.

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SUPPLEMENTARY METHODS

Mice

Wildtype C57BL/6 mice were purchased from Harlan. *Gata3*$^{YFP/YFP}$ 'GATIR' mice, carrying a Venus YFP sequence inserted between the last *Gata3* exon and the 3' UTR (T.N.R., H.J.F., *et al.*, manuscript in preparation), were bred on a C57BL/6 background. We have extensively validated GATA3 levels and immunological parameters in untreated GATIR mice or animals treated with IL-33 or house dust mite: under all conditions GATIR mice were identical to wildtype mice (**Supplementary Fig.1** and (33)). Mice were housed at the Erasmus MC Animal Centre under specific pathogen-free conditions and were 8-10 weeks old at time of analysis. All experiments were approved by the Erasmus MC Animal Ethics Committee.

Isolation of in vivo activated murine ILC2s

GATIR mice were exposed to 0.5 µg recombinant IL-33 (BioLegend) three times intranasally with one day of rest in between each challenge. Mice were sacrificed one day after the final administration. BAL fluid was obtained by flushing the lungs with 3 x 1 mL PBS containing 0.5 mM EDTA. Single-cell suspensions were prepared from mediastinal lymph nodes (MLN) in PBS containing 0.5% bovine serum albumin (BSA) and 5 mM EDTA, using standard procedures (9). For flowcytometric analysis and FACS sorting cells were stained with fluorescently labelled monoclonal antibodies (listed in **Supplementary Table 4**). Lineage-negative cells were defined as cells not expressing CD3, CD4, CD5, CD8, CD11b, CD11c, CD19, CD20, B220, NK1.1, FcεRIα, TER-119 and Gr-1. ILC2s were sorted using a FACSAria flow cytometer equipped with three lasers and FACSDiva software (Beckton Dickinson). Data analysis was performed with FlowJo software (Tree Star Inc.).

Isolation and culture of human ILC2s and Th2 cells

Blood samples (buffy coats from 500 ml peripheral blood) from healthy donors for ILC2 isolation were obtained from Etablissement Français du Sang (Paris, France) in an agreement signed with the Institut Pasteur (Paris, France). FACS strategies for isolation of naïve ILC2s (viable CD45$^+$Lin$^-$CD7$^+$CD127$^+$CRTh2$^+$ cells) from peripheral blood and subsequent short-term culture (7 days) of ILC2s in the presence of IL-2, IL-7, IL-25 and IL-33 have been described (28, 67). Peripheral blood samples from healthy donors for T helper subset isolation were obtained after informed consent in procedures approved by the Medical Ethical committee of the Erasmus MC Rotterdam. Memory Th2 cells were FACS-sorted from peripheral blood mononuclear cell fractions, using antibodies specific for CD3, CD4, CCR6, CXCR3 and CCR4 and CD45RA (81). Antibodies used are listed in **Supplementary Table 4**.

RNA isolation and RNA sequencing (RNA-Seq)

RNA was extracted from two independent ILC2 pools (obtained from 5-6 GATIR mice) using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. Library preparation was performed using the Smart-seq2 methodology (29) and sequenced according to the Illumina TruSeq Rapid v2 protocol on an Illumina HiSeq2500 (single read mode, 51 bp read length). Reads were aligned to the mouse genome (mm10 build) using HISAT2 (82). Sample scaling and statistical analysis were performed using the R package DESeq2 (83); genes with >1 log2 fold change and $P<0.05$ (Wald test) were considered differentially expressed. Standard RPKM values were used as an absolute measure of gene expression. Genes with average RPKM < 2 were considered not expressed.

Confocal microscopy

Lungs from GATIR mice were inflated with OCT embedding medium containing 2% PFA (Thermo Fisher Scientific, USA) and snap frozen in liquid nitrogen to preserve morphology and YFP fluorescence. Lymph nodes were fixed in 2% PFA, placed in PBS containing 30% sucrose overnight, embedded in OCT and stored at -80°C. 7 µm thick cryosections were cut at -20°C using a cryostat (Thermo Fisher Scientific) and stained at room temperature with primary antibodies for 1 hour and secondary antibodies (**Supplementary Table 4**) for 30 minutes (84). Slides were counterstained with DAPI (Invitrogen, USA) for 5 minutes, sealed with Vectashield (Vector Laboratories, USA) and examined with an LSM 510-META confocal microscope equipped with a 405 nm, 488 nm, 543 nm and 633 nm laser (Zeiss, Germany). Images were processed and analyzed in Fiji, an open source scientific image processing application based on ImageJ (http://fiji.sc/).

Chromatin immunoprecipitation and sequencing using ChIPmentation

Epigenome analysis of murine ILC2s was performed on two independent ILC2 pools (obtained from 5-6 GATIR mice). For human peripheral blood ILC2s and Th2 cells, samples from 3-4 healthy donors were analyzed. After FACS isolation (or short-term culture for human ILC2s) cells were immediately crosslinked in PBS containing 1% formaldehyde (Sigma) for 10 min. at room temperature for ChIPmentation analysis (85). Crosslinking was quenched by adding glycine (0.125M final concentration) followed by 5 min. incubation at room temperature. Cells were placed on ice, washed with PBS and snap-frozen for storage at -80°C. Pellets (20-50k cells) were processed in parallel to minimize technical variation. Cells were resuspended in 100µl sonication buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH8 and 1x EDTA-free complete protease inhibitors; Roche) and transferred to a 0.65ml Bioruptor sonication tube (Diagenode). After a 15 min. incubation on ice, cells were sonicated for 30 cycles (30 sec ON - 30 sec OFF) using a Bioruptor Pico sonicator (Diagenode) to shear chromatin down to ±250 bp fragments.

Chromatin was equilibrated by adding 900µl 10x ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH8, 167mM NaCl) and incubated overnight at 4°C with 1µl of H3K4Me2-specific antibody (ab32356, Abcam; previously validated as highly sensitive and specific for H3K4Me2 (86)) or normal rabbit IgG as a negative control (sc-2027, Santa Cruz). In addition, 20µl of protein A Dynabeads (Thermo Fisher Scientific) per IP were blocked in PBS containing 0.1% BSA (Sigma) by incubation overnight at 4°C. The next day, beads were resuspended in the original volume with ChIP dilution buffer and added to the chromatin extracts. After 2 hours of incubation at 4°C, beads were collected and washed with Low Salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 150mM NaCl), High Salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, 500mM NaCl) and LiCl buffer (10mM Tris-HCl pH8, 1mM EDTA, 250mM LiCl, 0.5% NP-40, 0.5% deoxycholic acid). Chromatin-antibody immobilized on magnetic beads were then subjected to tagmentation as recently described (31). Eluted DNA was purified using MinElute spin columns (Qiagen) and amplified for 8-12 cycles using Nextera PCR primers. Libraries were purified using dual (0.5x-2.0x) SPRI Ampure XP beads (Beckman Coulter), pooled (up to 10 per sequencing run) and sequenced on a NextSeq500 (Illumina) running a single-read 75bp protocol.

Initial ChIPmentation data processing, quality assessment and visualization

Reads were demultiplexed using BaseSpace (Illumina) and aligned to the mouse genome (mm10 build) using Bowtie2 (87) with standard settings, removing reads that could not be uniquely mapped. Indexed and sorted bam files were parsed to HOMER (32) for further analysis. Data quality and signal-to-noise ratios for all samples were in line with published guidelines (88): fraction of reads in peaks (FRiP) was on average 49.4% with <7% duplicate reads. Over 95% of all expressed genes (>2 RPKM) showed above-background H3K4Me2 promoter enrichment levels, demonstrating robust sensitivity. GATA3 and GFI1 ILC2 ChIP-Seq data were downloaded from the Gene Expression Omnibus (GEO, series GSE71198 (56) for GATA3 and series GSE50806 (57) for GFI1). Tag directories were generated for each sample with removal of duplicate reads (*-tbp 1* option). BedGraph files displaying normalized counts (reads per million) were generated for direct visualization in the UCSC Genome Browser (https://genome.ucsc.edu/) using the *makeUCSCfile* HOMER script.

Peak calling, annotation and downstream analyses

H3K4Me2 enriched regions were identified using HOMER *findPeaks* with *-region -size 1000 -minDist 2500* options, utilizing signal obtained from a negative control IgG experiment as background. GATA3 or GFI1 peaks were called using the *-style factor* option. Annotation

of peaks as defined by their overlap with known genomic features was performed using the *annotatePeaks* script. Overlapping and non-overlapping regions/peaks between two samples were identified using the *intersect* function of BEDTools (89) or the HOMER *mergePeaks* script (*-d given* option) requiring a minimal overlap of 1bp. Regions/peaks of similar and differential enrichment (fold change >2) were determined using HOMER *getDifferentialPeaks* (*-F 2* option). Histograms of ChIP signal were generated using the *annotatePeaks* script (*size 5000 -hist 10 -d* options) and visualized as heatmaps with Java TreeView (90). Venn diagrams were drawn using the VennDiagram package (91) in RStudio (v0.99.484).

Peak-gene assignment, gene ontology and molecular signature analysis

Regions/peaks were assigned to putative target genes using the 'single nearest gene' method of GREAT (92). GREAT was subsequently used to calculate enrichments of these genes for known Gene Ontology (GO) terms and molecular signatures (from the MSigDB (93)) using the whole genome as background.

Transcription factor binding motif discovery

The HOMER *findMotifsGenome* script (*-size 1000 -mask -len 6,8,10,12 -S 20* options) was used to search for known transcription factor binding motifs (n=255) in H3K4Me2 enriched regions. Motifs with $P<0.001$ were considered enriched and visualized as a heatmap.

Superenhancer identification

The HOMER *findPeaks* script (*-style super -L 1* options) was used to identify superenhancers in ILC2s using the same criteria as previously defined by Whyte *et al.* (59) We utilized signals obtained from a negative control mock (IgG) experiment as background for superenhancer identification. To verify that our superenhancers localized to regions of open chromatin we downloaded previously published (25) ILC2 ATAC-Seq data (GSE77695). BigWig files were used to generate meta-profiles of ATAC-Seq signal across the ILC2 superenhancers as described above using HOMER software. Overlapping and non-overlapping superenhancers were identified using the *intersect* function of BEDTools as described above.

Integration of epigenomic data with asthma-associated human genetic variation

SNPs associated with human asthma phenotypes in genome-wide association studies ($P<1.0 \times 10^{-5}$) were downloaded from the NHGRI-EBI GWAS Catalog (94). SNAP (95) was used to retrieve SNPs in high pairwise linkage disequilibrium (CEU population panel, $r^2>0.8$, distance limit = 500) with the GWAS Asthma SNPs, yielding a total of 3405 unique SNPs.

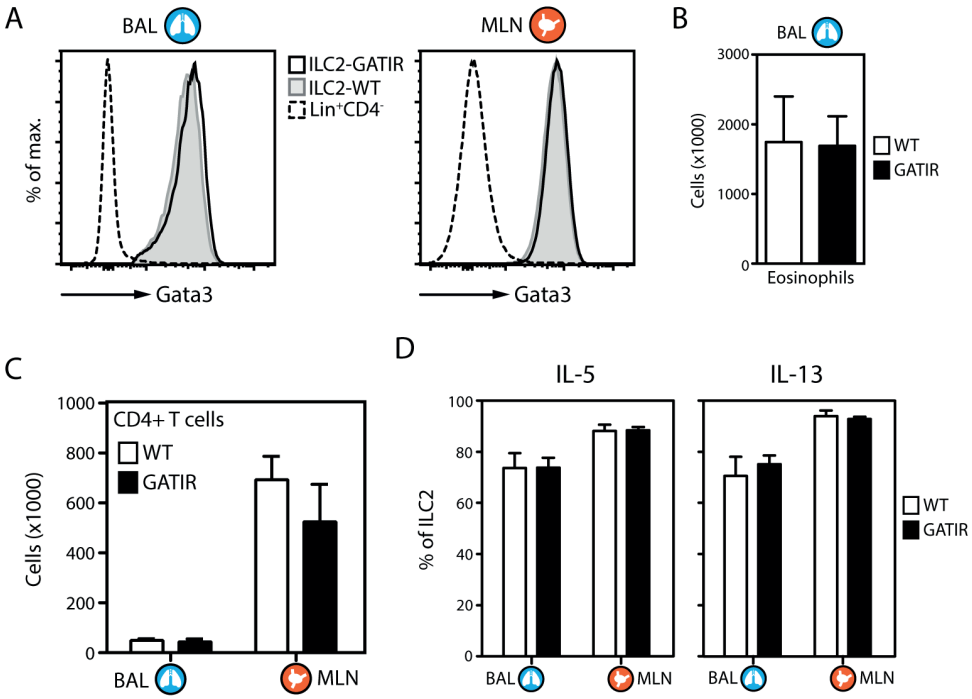
Putative causal SNPs for various human disorders were previously published (71). Differentially methylated regions associated with asthma were obtained from earlier epi-GWAS studies (19, 20). We used GREAT to generate a list of genes closest to these SNPs ('two nearest gene' association rule, n=664 genes) and added ALOX5 (66). Murine orthologues of human genes were retrieved using Ensembl BioMart. GREs containing Asthma-associated SNPs were identified using the *intersect* function of BEDTools. Statistical significance of overlap between SNP and GRE positions was assessed using the *fisher* function of BEDTools (Fisher's exact test). Random shuffling of the 3405 SNPs across the genome (n=10) consistently yielded non-significant enrichments using this test ($P>0.45$). SNP enrichment ratios were also obtained using BEDTools's *fisher* function ('ratio' parameter).

Statistical analysis

In the allergic airway inflammation experiments Mann-Whitney U tests were applied for comparison of the numbers of various immune cell populations between groups of mice. Fisher's exact test was performed using the BEDTools *fisher* function. All other tests for statistical significance were performed by the HOMER analysis suite or using RStudio. P values < 0.05 were considered statistically significant.

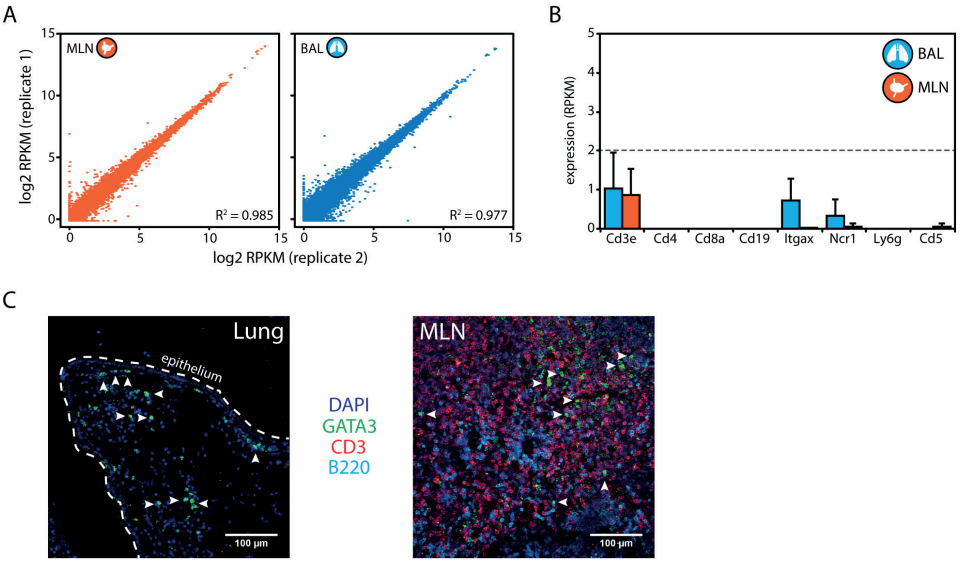
Accession numbers

All mouse and human ChIPmentation and RNA-Seq datasets were deposited in the Gene Expression Omnibus (GEO), accession number GSE98843.



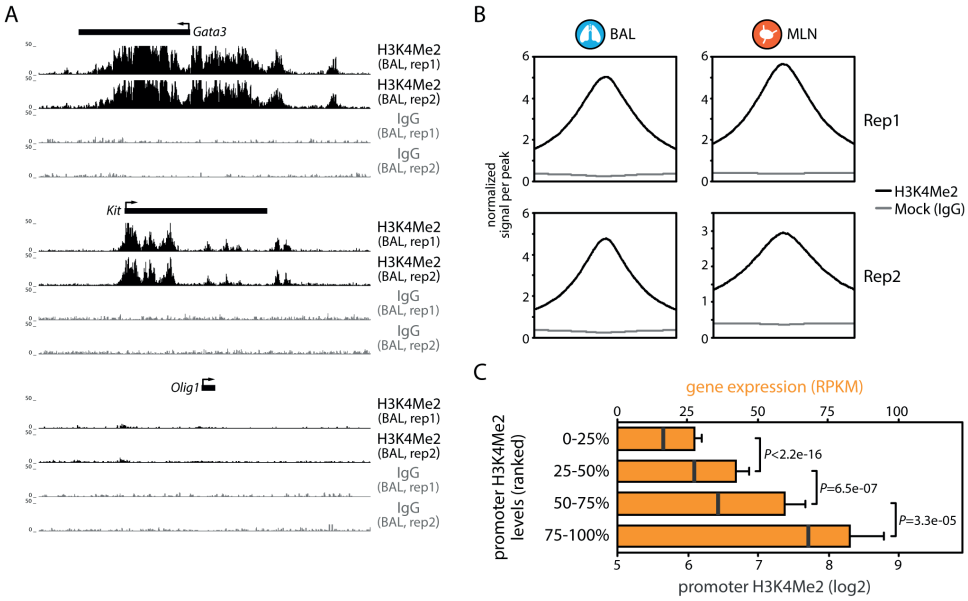
Supplementary Figure 1. GATIR mouse validation experiments.

(**A**) Histograms showing GATA3 mean fluorescent intensity levels (antibody staining) in ILC2s isolated from BAL or MLN of GATIR reporter or wildtype (WT) mice. Lineage(Lin)+ CD4- cells are shown (dashed line) as a GATA3-control. (**B**) Flowcytometric quantification of eosinophils in BAL fluid of wildtype (WT) and GATIR mice after *in vivo* treatment with IL-33. (**C**) Flowcytometric quantification of $CD4^+$ T cells in BAL fluid or MLN of wildtype (WT) and GATIR mice after *in vivo* treatment with IL-33. (**D**) Proportions of cytokine-producing ILC2s (after *in vitro* stimulation with PMA and ionomycin for 4 hours (9)) in BAL fluid or MLN of wildtype (WT) and GATIR mice after *in vivo* treatment with IL-33. All panels depict concatenated data from 3-5 mice; a representative of three independent experiments is shown.



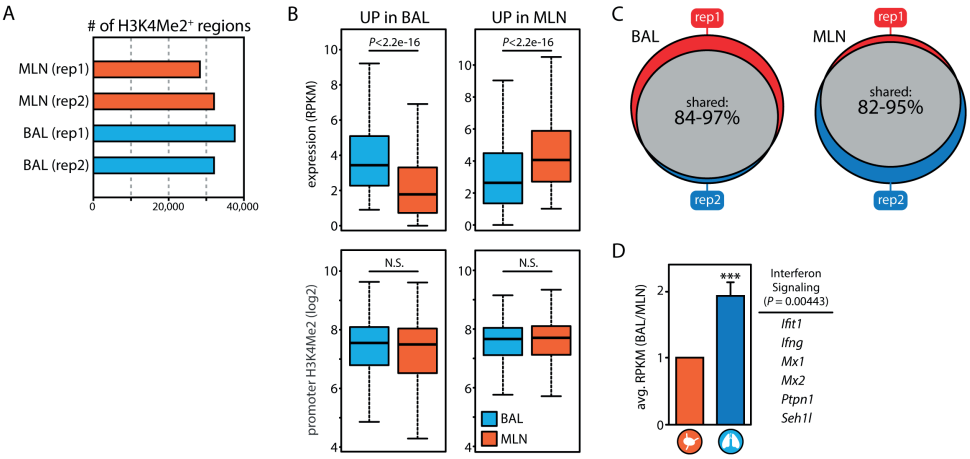
Supplementary Figure 2. Transcriptome, microscopy and epigenome analyses of ILC2s in AI.

(**A**) Scatterplot of log2 RPKM values for both RNA-Seq biological replicate experiments per tissue. Pearson correlation values (R^2) are indicated. (**B**) Gene expression levels (n=2 pools of 5-6 mice, mean values + SEM are shown) in purified ILC2s of archetypical marker genes specifically expressed in other hematopoietic cell types. Dashed line indicates the 2 RPKM cut-off used throughout this study to define actively expressed genes. (**C**) Lung or MLN cryosections from IL-33 treated GATIR mice counterstained with CD3 (T cell marker) and B220 (B cell marker). ILC2s (YFP^+CD3^-) are indicated by white arrowheads. Dashed line denotes airway epithelium.



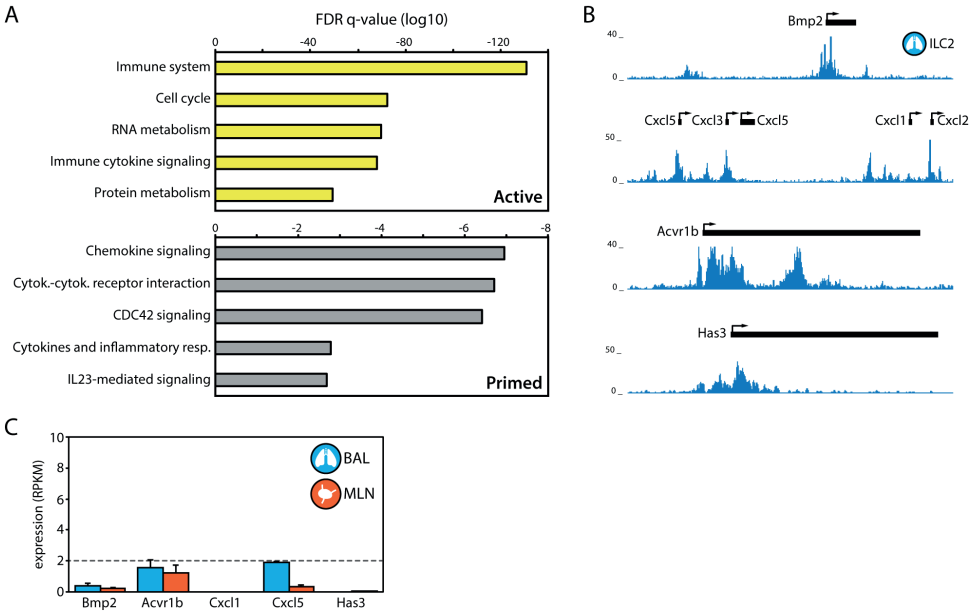
Supplementary Figure 3. Quality control metrics of murine ILC2 H3K4Me2+ epigenome maps.

(**A**) H3K4Me2 (black) and non-specific IgG (gray) signals at example loci that depict highly expressed genes (e.g. *Gata3*), moderately expressed genes (e.g. *Kit*) and lowly or non-expressed genes (e.g the neuronal *Olig1* gene) in BAL ILC2s. (**B**) Meta-profiles depicting average enrichment levels for all H3K4Me2^{+} GREs (black line) in BAL (left) or MLN (right) ILC2s for both replicate pools. Signal measured in a mock experiment (using an aspecific IgG) is shown in grey. (**C**) Orange bars denote average gene expression levels (errors bars showing 95% confidence interval) of genes ranked by promoter H3K4Me2 levels, which were grouped in four quartiles of increasing H3K4Me2 enrichment (average log2 normalized H3K4Me2 levels are indicated by black lines within the orange bars). A Wilcoxon rank-sum test was used to compute *P* values for quartile comparisons.



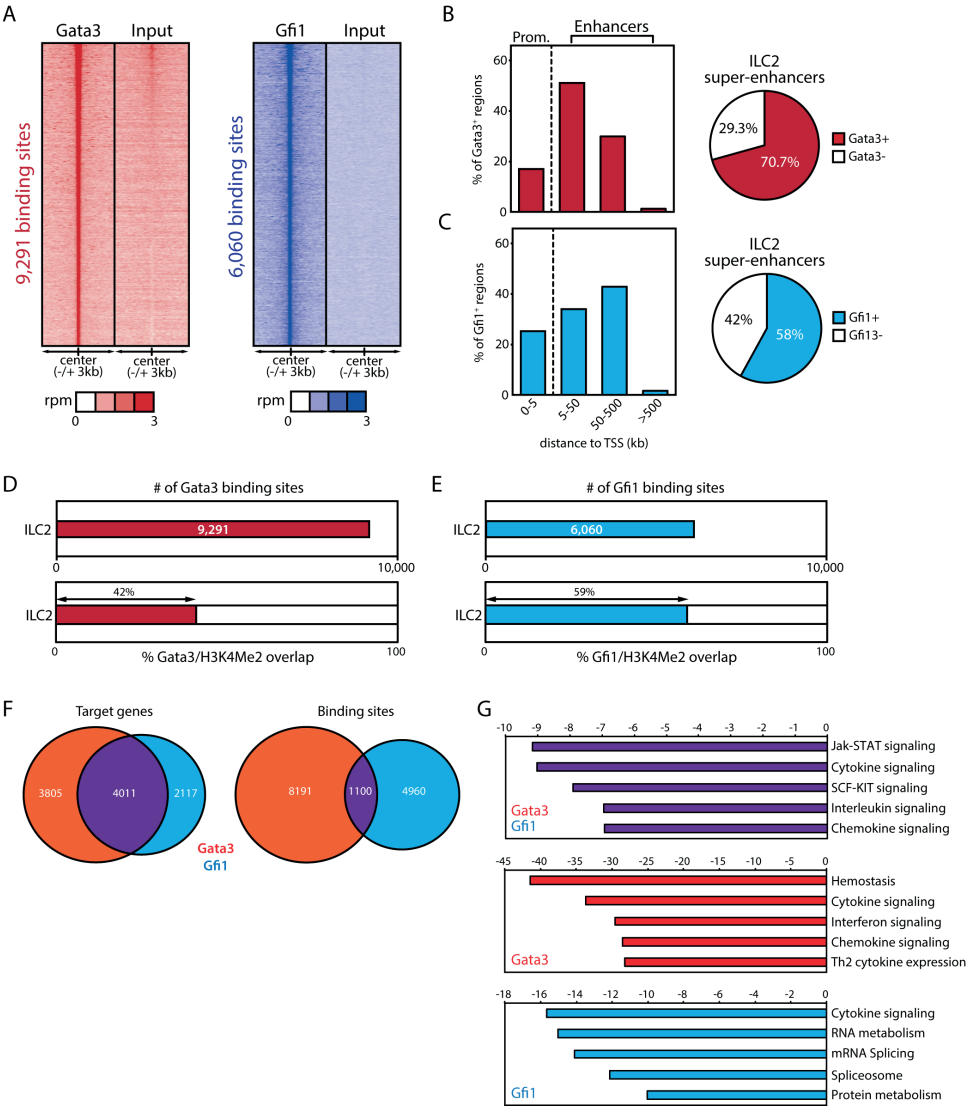
Supplementary Figure 4. Highly similar H3K4Me2+ epigenomes in transcriptionally divergent ILC2 populations.

(**A**) Number of H3K4Me2$^+$ regions detected in both ChIPmentation biological replicates per tissue. (**B**) Box plots depicting expression levels of genes differentially expressed between ILC2s from BAL or MLN (either upregulated in BAL ILC2s or upregulated in MLN ILC2s) are shown at the top, lower boxplots show normalized promoter H3K4Me2 levels of the same gene sets. Differences between BAL and MLN expression or H3K4Me2 levels were tested for statistical significance using a Wilcoxon rank-sum test (N.S. = not significant, $P > 0.05$). (**C**) Venn diagram showing the overlap between H3K4Me2$^+$ regions detected in paired ChIPmentation biological replicates per tissue. (**D**) Average RPKM expression values for all listed genes in the Interferon Signaling Gene Ontology term associated with BAL-specific H3K4Me2$^+$ regions ($P = 0.00443$, paired two-tailed Student's t-test).



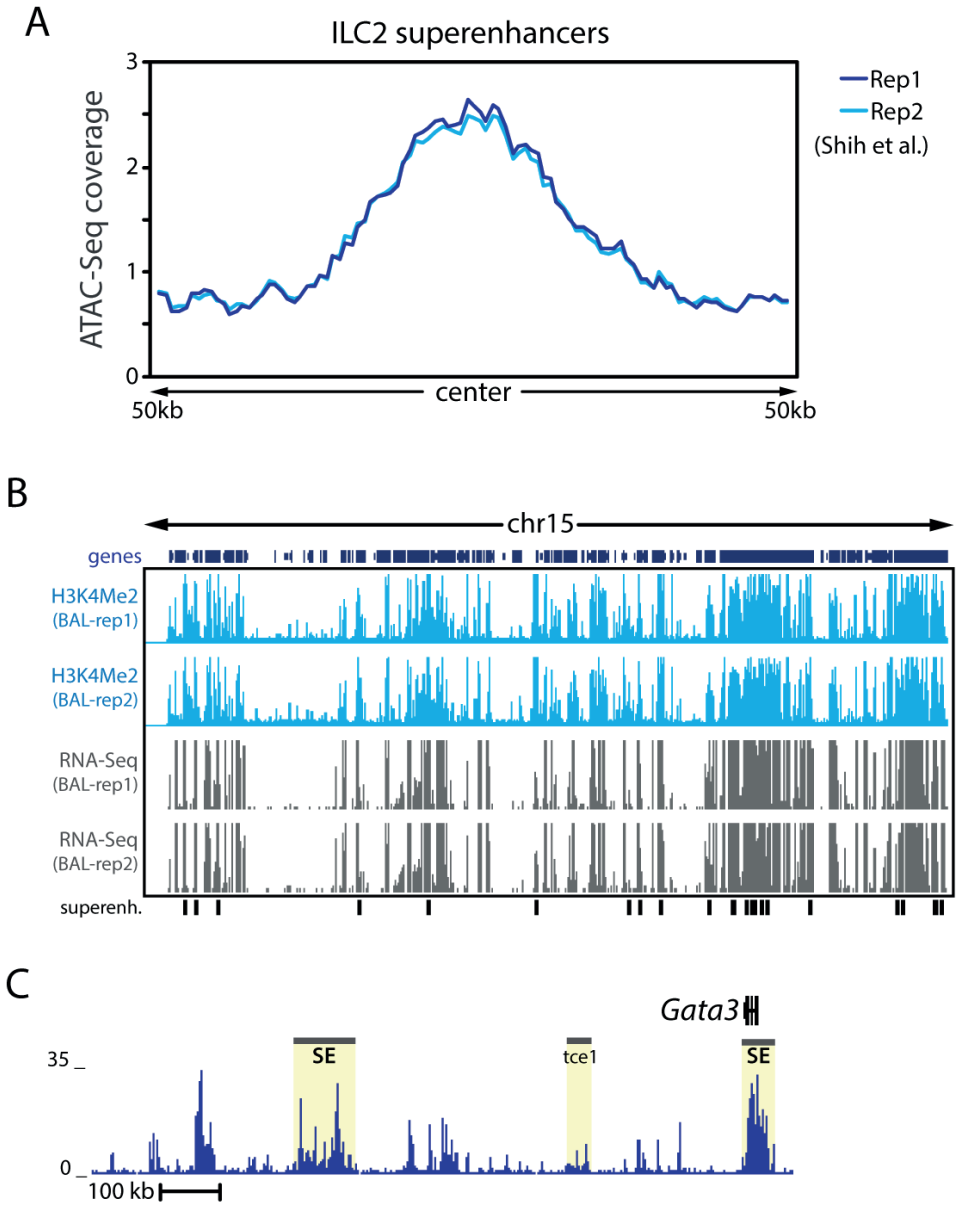
Supplementary Figure 5. Epigenetic priming in activated ILC2s.

(**A**) Molecular pathway enrichment (MSigDB) amongst genes associated with active (yellow bars) or primed (grey bars) GREs in ILC2s. (**B**) Genome browser views of the H3K4Me2 landscape around selected loci implicated in airway remodeling in BAL ILC2s. (**C**) Gene expression levels (n=2 pools of 5-6 mice, mean values + SEM are shown) in purified ILC2s of genes shown in panel B. Dashed line indicates the 2 RPKM cut-off used throughout this study to define actively expressed genes.



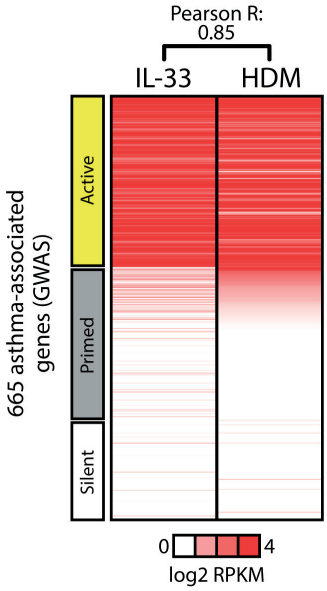
Supplementary Figure 6. The GATA3/GFI1 binding landscape in ILC2s.

(**A**) Heatmap showing GATA3 (left, in red) and GFI1 (right, in blue) normalized ChIP-sequencing signal (56, 57) (reads per million, RPM) for all genomic binding sites. Input genomic DNA signals are shown as negative control experiments. (**B**,**C**) Bar graph showing the distance distribution of GATA3 (panel B) or GFI1 (panel C) binding sites to the nearest transcription start site (TSS). Pie charts depict the proportion of ILC2 superenhancers bound by GATA3 (panel B) or GFI1 (panel C). (**D**,**E**) Number of binding sites (top) and percentage of overlap with H3K4Me2 enriched regions (bottom) for GATA3 (panel D) or GFI1 (panel E). (**F**) Venn diagrams showing overlap between GATA3 and GFI1 target genes (nearest gene within 1 Mb, left) or binding sites (at least 1 bp overlap, right). (**G**) Molecular pathway enrichment (MSigDB) amongst genes co-bound by GATA3 and GFI1 or bound specifically by one transcription factor.



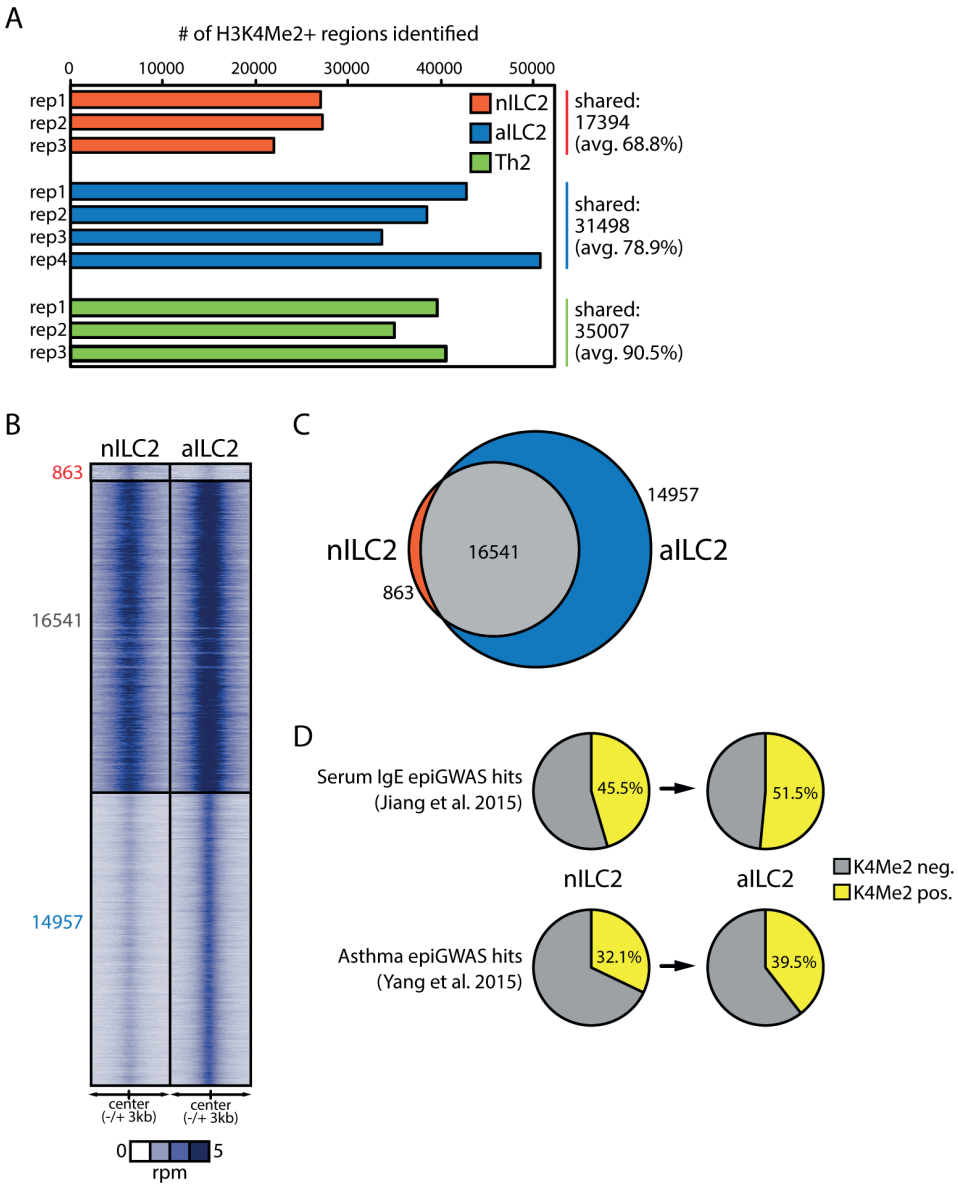
Supplementary Figure 7. ILC2 superenhancers reside in open, transcription-permissive chromatin.

(**A**) Meta-profiles of ILC2 ATAC-Seq signals (25) across all H3K4Me2-based ILC2 superenhancers (centered on superenhancer midpoints). (**B**) Genome browser view of BAL ILC2 H3K4Me2 (blue) and RNA-Seq (grey) profiles for chromosome 15. Superenhancer locations are shown below. (**C**) Genome browser view of the H3K4Me2 landscape around the *Gata3* gene in BAL ILC2s. Superenhancer (SE) elements are indicated by yellow shading, *tce1* is a previously identified (96) Gata3 enhancer in T cells.



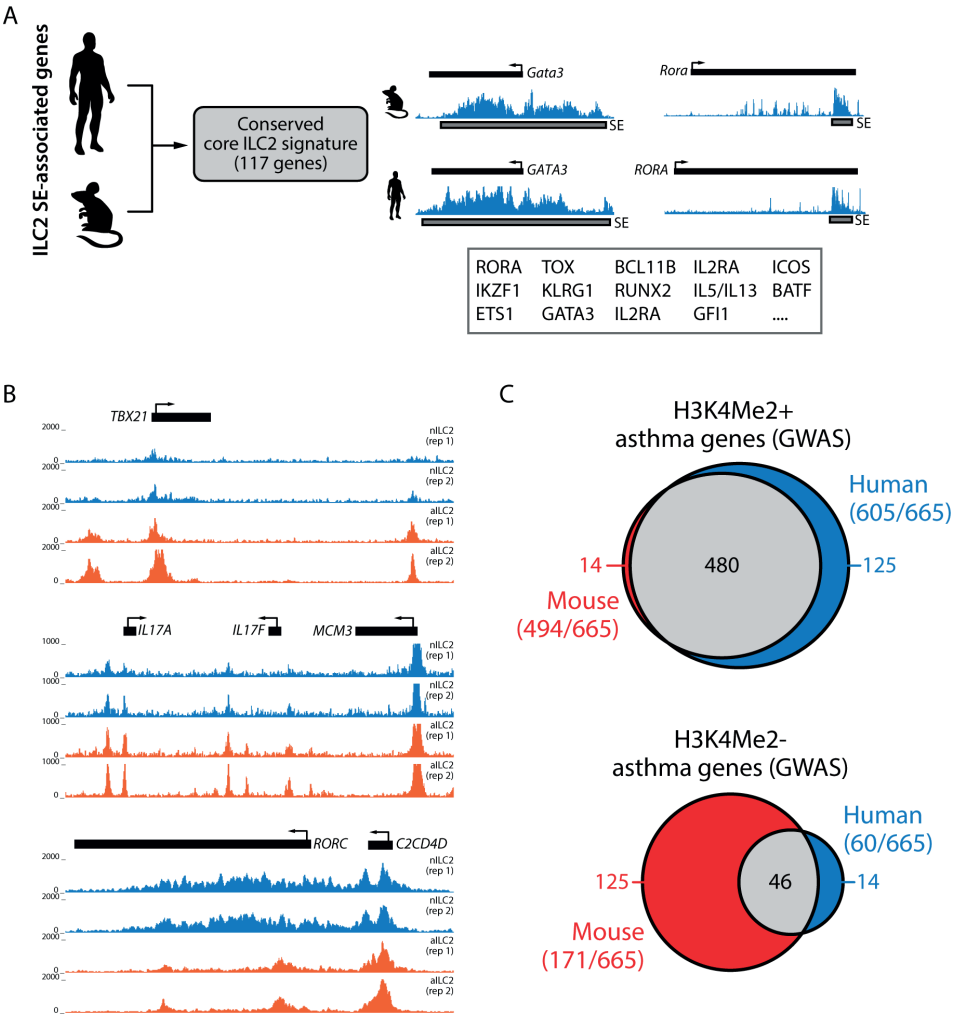
Supplementary Figure 8. Similar expression levels of asthma-associated genes in ILC2s generated by IL-33 or chronic allergen exposure.

Heatmap shows average gene expression levels in BAL ILC2s isolated from GATIR mice in which airway inflammation was induced by IL-33 or chronic allergen (house dust mite, HDM) exposure (mean of n=2 pools of 5-6 mice; log2 RPKM counts generated using RNA-Seq). Expression levels are shown for the 665 asthma-associated genes identified by GWAS, which were further divided into active, primed or silent genes based on their combined epigenome/transcriptome profile (see **Fig.4**) in IL-33 activated ILC2s.



Supplementary Figure 9. H3K4Me2 dynamics during human ILC2 activation.

(**A**) Number of H3K4Me2^{+} regions detected in all ChIPmentation biological replicates for naïve ILC2s (nILC2), activated ILC2s (aILC2) and circulating memory Th2 cells. The number and average percentage of shared enriched regions is indicated on the right. (**B**) Heatmap depicting normalized H3K4Me2 signal (in reads per million, RPM) for nILC2-specific (n=863), shared (n=16541) and aILC2-specific (n=14957) H3K4Me2^{+} gene regulatory elements (GREs). (**C**) Venn diagram indicating the overlap between H3K4Me2^{+} regions detected in nILC2s and aILC2s. (**D**) Pie charts showing the overlap between regions of differential DNA methylation associated with serum IgE or asthma from the indicated epigenome-wide association studies (epiGWAS) and H3K4Me2^{+} GREs in human ILC2s.



Supplementary Figure 10. Inter-species comparison of epigenomic features in activated ILC2s.

(**A**) Human and murine IL-33 activated ILC2s share a conserved core of 117 superenhancer (SE)-associated genes. Selected genes are shown boxed; genome browser views of the *Gata3/GATA3* and *Rora/RORA* loci are shown to indicate conserved ILC2 SE structure (denoted by a grey rectangle) between humans and mice. (**B**) Genome browser view of key ILC1 (*TBX21*) and ILC3 (*IL17, RORC*) loci in ILC2s. Note the extensive decoration of these loci with H3K4Me2, indicating a primed state in ILC2s. (**C**) Venn diagrams showing a comparison of GWAS asthma genes (n=665) associated with (top) or not associated with (bottom) H3K4Me2^{+} gene regulatory elements in murine (red) or human (blue) ILC2s.

Supplementary tables are included in the online version of this manuscript.

PERIPHERAL BLOOD ILC2S ARE NOT INCREASED IN ASTHMA PATIENTS BUT CORRELATE WITH TH2 AND TH17 FREQUENCIES

CHAPTER 7

Manuscript in preparation:

Peripheral blood ILC2s are not increased in asthma patients but correlate with Th2 and Th17 frequencies

Bobby W.S. Li, Heleen Vroman, Irma Tindemans, Melanie Lukkes, Menno van Nimwegen, Marjolein J.W. de Bruijn, Mirjam Kool, Gert-Jan Braunstahl, Rudi W. Hendriks

ABSTRACT

Group 2 innate lymphoid cells (ILC2) have been proposed to play an important role in the pathogenesis of asthma due to their ability to produce type 2 cytokines. It has been reported that peripheral blood ILC2 are increased or have enhanced activity in asthma patients. However, since asthma is a heterogeneous disease it is unclear whether ILC2s are important in all endotypes of asthma. In this study, we stratified asthma patients according to the Global Initiative for Asthma (GINA) 2014 classifications into the following subgroups: controlled, partly controlled and uncontrolled, based on asthma control questionnaire (ACQ) score. We used flow cytometry to compare peripheral blood and induced sputum immune profiles of asthma patients with healthy controls and correlated these to clinical parameters. Blood eosinophils were significantly elevated in all asthma groups and the proportions of Th2 and Th17 within $CD4^+$ T helper cells were increased as well. However, ILC2 frequency remained comparable to healthy controls. Interestingly, ILC2 frequency was significantly correlated with Th2 and Th17 frequency. For ILC2s the expression levels of the surface marker IL-2Ra (CD25) and the transcription factor GATA3 were also similar in asthma patients and controls. Our findings suggest that peripheral blood ILC2s are either not important in asthma pathogenesis or are not affected by an inflammatory status in the lung. Therefore, local ILC2s in the lung require further investigation as they may still contribute to asthma symptoms.

INTRODUCTION

Asthma is a chronic inflammation of the airways that is characterized by episodes of coughing, wheezing and shortness of breath. It is a heterogeneous disease that can be divided into endotypes based on the underlying disease mechanisms (1, 2). Group 2 innate lymphoid cells (ILC2) have emerged as non-B/non-T lymphocytes capable of secreting large amounts of type 2 cytokines and have led to research into their involvement in type 2 inflammatory diseases, such as allergic asthma (3, 4). They were originally described in mice as an early source of type 2 cytokines critical for the expulsion of parasitic worms upon activation by the innate epithelial cell-derived cytokines IL-25 and IL-33 (5-8). In contrast to Th2 cells, ILC2s do not express antigen receptors and also lack classic hematopoietic lineage markers, but do show surface expression of Thy-1 (CD90), c-Kit (CD117) and Sca-1, as well as a broad range of cytokine receptors including IL-7Ra (CD127), IL-2Ra (CD25), IL-25R (IL-17RB) and IL-33R (T1/ST2) (9-11). Consequently, ILC2s are primarily activated by epithelial cell-derived IL-25, IL-33 or thymic stromal lymphopoietin (TSLP) or by prostaglandin D2 produced by mast cells. In response to these signals, ILC2s rapidly expand and secrete large amounts of IL-5, IL-13 and under certain conditions also IL-4 (3, 12, 13). Several studies have shown the induction of eosinophilic airway inflammation in mice independent of adaptive immunity by stimulating ILC2s using IL-25, IL-33, *Alternaria* or papain (10, 14-17). However, other models using *Aspergillus* or house dust mite (HDM) as stimulus have highlighted a more T cell-centric response for the initiation of allergic inflammation (16, 18, 19).

Human ILC2s were first described in foetal and adult gut and lung tissues: they were reported as Lineage$^-$CD127$^+$CD161$^+$ cells expressing also the prostaglandin D2 receptor CRTH2, were responsive to IL-25 and IL-33, and produced type 2 cytokines in inflamed tissues (20). Furthermore, this population was found in broncho-alveolar lavage (BAL) fluid of lung transplant patients and expressed CD25 and T1/ST2 (21). CRTH2$^+$ ILC2s were enriched in nasal polyps of patients with chronic rhinosinusitis, a typical type 2-mediated disease (20). GATA3, potently regulated by TSLP, was identified as an essential transcription factor for the function of human ILC2s (12). Doherty and colleagues demonstrated that peripheral blood ILC2s from allergic individuals express transcription factor GATA3 and ETS-1, similar to mouse ILC2s (22). In addition, these ILC2s produce more IL-5 and IL-13 in response to IL-25 and IL-33 than ILC2s derived from healthy controls (23).

The involvement of ILC2s in asthma pathogenesis is supported by GWAS data that show associations of asthma with loci encoding for ILC2-related genes such as *Il1rl1*, *Il33*, *Il13* and *Rora* (24). The evidence is strengthened by findings of increased IL-33 and TSLP expression in the lungs of asthma patients, both of which are potent ILC2 inducers (25, 26). In recent studies investigating the role of ILC2s in asthma patients increased frequencies of ILC2s were observed in several tissues compared to healthy controls and IL-33 levels in BAL fluid were found to correlate with disease severity (27-30). Similarly, children with severe therapy-resistant asthma

displayed higher numbers of ILC2s in BAL fluid than children with recurrent lower respiratory tract infections, indicating that targeting these cells locally may prove to be beneficial (31). In peripheral blood and induced sputum, patients with severe eosinophilic asthma had a higher number of IL-5 and IL-13 producing ILC2s compared to patients with milder forms of asthma. Intracellular cytokine analysis demonstrated that ILC2s were the predominant source of type 2 cytokines, despite more abundant numbers of Th2 cells (29).
It has however become increasingly clear that the role of ILC2s likely differs in subgroups of asthma phenotypes, as is evident from the large variation of numbers of ILC2s found. This issue is further confounded by the use of different markers to define ILC2s in each study. We have previously used GATA3 as a main marker for ILC2s and here we propose to apply this strategy for characterizing human ILC2s (19). Furthermore, this study quantifies local and peripheral blood ILC2s in a wide range of asthma patients in order to elucidate the involvement of ILC2s in different asthma endotypes.

METHODS

Subjects

In this cross-sectional study, we recruited asthma patients (aged 18 – 50 years) from the Franciscus Gasthuis in Rotterdam, the Netherlands. The subjects were diagnosed with asthma and had a recent (<12 months) methacholine or histamine provocation test. Use of inhaled corticosteroids or β2-agonists was required for inclusion, however patients who received systemic corticosteroid therapy three months prior were excluded. Smokers (>10 pack years) and obese patients (BMI > 30) were also excluded. Healthy age- and gender-matched individuals were recruited as controls and had no history of atopic disease, FeNO <50 ppb and normal lung function. All asthma patients were categorized according to the Global Initiative for Asthma (GINA) 2014 classification into the following groups: controlled, partly controlled and uncontrolled. A standardized asthma control questionnaire (ACQ) was taken and patients who scored 0.75 or lower were placed in the controlled asthma group, 0.76 – 1.50 was considered partly controlled and >1.50 was characterized as uncontrolled asthma. All subjects provided a written informed consent and all experimental procedures were reviewed and approved by the medical ethics committees of the Franciscus Gasthuis and the Erasmus MC.

Peripheral blood collection

Peripheral blood was drawn by venipuncture into Vacutainers containing EDTA (Beckton Dickinson, USA). For the isolation of eosinophils and neutrophils, whole blood was lysed in osmotic lysis buffer (8.3% NH_4Cl, 1% $KHCO_3$ and 0.04% Na_2EDTA in Milli-Q). For the isolation of T cell and ILC subsets, whole blood was separated by Ficoll-Paque density gradient (Sigma-Aldrich, USA) and the mononuclear cell layer was extracted.

Sputum collection

Sputum was induced by inhalation of aerosolized hypotonic saline and collected and processed within 2 hours. Mucus plugs were extracted, incubated with Sputolysin (Sigma-Aldirch, USA) diluted in PBS (GIBCO Life Technologies, USA) and passed through a 100 µm nylon mesh filter (Beckton Dickinson, USA).

Flow cytometry procedures

Single cell suspensions were obtained from peripheral blood and induced sputum. Flow cytometry surface and intracellular staining procedures have been described previously (16, 32). Lineage negative cells were defined as cells not expressing CD2, CD3, CD4, CD8, CD11b, CD14, CD16, CD19, CD20, CD56, CD235a, FCεRI and TCRγδ. Details of monoclonal antibodies used are available on request. Data were acquired using a LSR II flow cytometer (Beckton Dickinson, USA) equipped with three lasers and FACSDiva software (Beckton Dickinson, USA) and analysed by FlowJo (Tree Star Inc., USA) software.

Statistical analysis

Statistical comparisons were performed by Mann-Whitney U tests and a p-value of <0.05 was considered statistically significant. Statistical significance of correlations between two parameters was tested by linear regression. All analyses were performed using Prism (GraphPad Software, USA).

RESULTS

Peripheral blood ILC2s are not elevated or activated in asthma patients compared to healthy controls

Enrolled asthma patients were recruited from the Franciscus Gasthuis in Rotterdam, the Netherlands and stratified into controlled, partially controlled and uncontrolled groups based on their ACQ score according to the GINA guidelines. Detailed characteristics and clinical parameters of the healthy controls and the three groups of asthma patients are listed in Table 1. Peripheral blood and sputum samples were collected and analysed by flow cytometry (**Fig. 1**). Peripheral blood ILCs were detected by sequential gating of $CD45^+$ and $Lineage^-$ lymphocytes that expressed CD161 (33). ILC3s were defined as ILCs that expressed RORγt and not CRTH2 (**Fig. 1A**). From the remaining $ROR\gamma t^-$ ILCs, ILC2s could then be distinguished as $CRTH2^+GATA3^+$ and the residual $GATA3^-CRTH2^-$ ILCs were mainly composed of the newly characterized ILC progenitor (ILCP) and very small numbers of ILC1s (referred to as ILC1/ILCP fraction; **Fig. 1A**) (34). Similarly, a distinction was made within $CD45^+CD3^+CD4^+$ T cells for $CRTH2^+GATA3^+$ Th2 cells and $CRTH2^-ROR\gamma t^+$ Th17 cells (**Fig. 1A**). To gain an impression

of the inflammatory phenotype of asthma patients, we assessed the eosinophil and neutrophil content in peripheral blood by gating for $CD45^+$ granulocytes that did not express CD33. Neutrophils were then characterized by CD16 expression, while eosinophils had high Siglec-8 expression and were negative for CD16 (**Fig. 1B**). An identical gating strategy was applied to detect these cells in sputum samples (**Fig. 1C, 1D**).

Table 1. Clinical characteristics of healthy controls and asthma patients enrolled in the study.

Characteristics	Healthy Control Subjects	Controlled Asthma Patients	Partly Controlled Asthma Patients	Uncontrolled Asthma Patients
Group size	23	10	10	16
Gender, (male/female)	5/18	2/8	2/8	1/15
Age, years	30.3 ± 1.8	34.6 ± 3.9	27.3 ± 3.9	29.2 ± 2.0
BMI, kg/m²	23.0 ± 0.5	24.6 ± 1.0	24.1 ± 1.3	26.4 ± 0.8**
FEV_1, %	107.5 ± 3.1	102.6 ± 5.3	84.0 ± 5.7*	96.4 ± 5.6
FeNO, ppb	13.0 ± 1.5	21.5 ± 4.4*	29.4 ± 7.2*	33.2 ± 7.9*

Data are presented as number of subjects or mean ± SEM.
* $p < 0.05$, ** $p < 0.01$ compared to healthy controls.

In agreement with previously published data (29), the proportions of blood eosinophils were significantly increased in all asthma patient groups, compared to healthy controls. In contrast, blood neutrophils were not significantly elevated in any of the three patient groups (**Fig. 2A**). While Th2 fractions in total $CD45^+$ lymphocytes were not significantly elevated in asthma patients, the proportion of Th2 cells within $CD4^+$ T cells did increase in controlled and partially controlled patients, suggestive of enhanced type 2 immunity (**Fig. 2B**). The proportions of ILC2s were however not expanded and ILC3s were only detected at very low levels and also did not differ between groups (**Fig. 2C**).

Next, we sought to determine the activation status of circulating ILC2s by assessing CD25 and GATA3 expression. We found a uniform, high expression of CD25 in peripheral blood ILC2s isolated from asthma patients and healthy controls (**Fig. 3A**). The mean fluorescent intensity (MFI) of GATA3 in ILC2s from asthma patients appeared to be somewhat higher than ILC2s from healthy controls, particularly in partially controlled and uncontrolled patients, however the observed differences did not reach significance. Additionally, GATA3 levels in Th2 cells did not show significant differences between healthy controls and asthma patients (**Fig. 3B**).

Taken together, these data show that peripheral blood eosinophils as well as Th2 cells were significantly elevated in asthma groups. However, ILC2 frequency remained comparable to healthy controls.

Analysis of sputum samples

In this study, we obtained sputum samples for 3 healthy controls and 11 asthma patients. As shown in **Figure 4A** many asthma patients showed increased proportions of eosinophils – but

not neutrophils – in their sputum, compared to the three control individuals. T cells and ILCs could only be evaluated by flow cytometry (**Fig. 1C**) in a low number of sputum samples from healthy controls (n=3) and asthma patients (n=4). In these small numbers of samples, no striking differences were found between healthy controls and asthma patients (**Fig. 4B, 4C**). However, the proportion of sputum ILC2s expressing CD25 was markedly reduced in healthy controls (~70%) and even more so in asthma patients (~30%) compared to ILC2s from peripheral blood (>90%) (**Fig. 4D**).

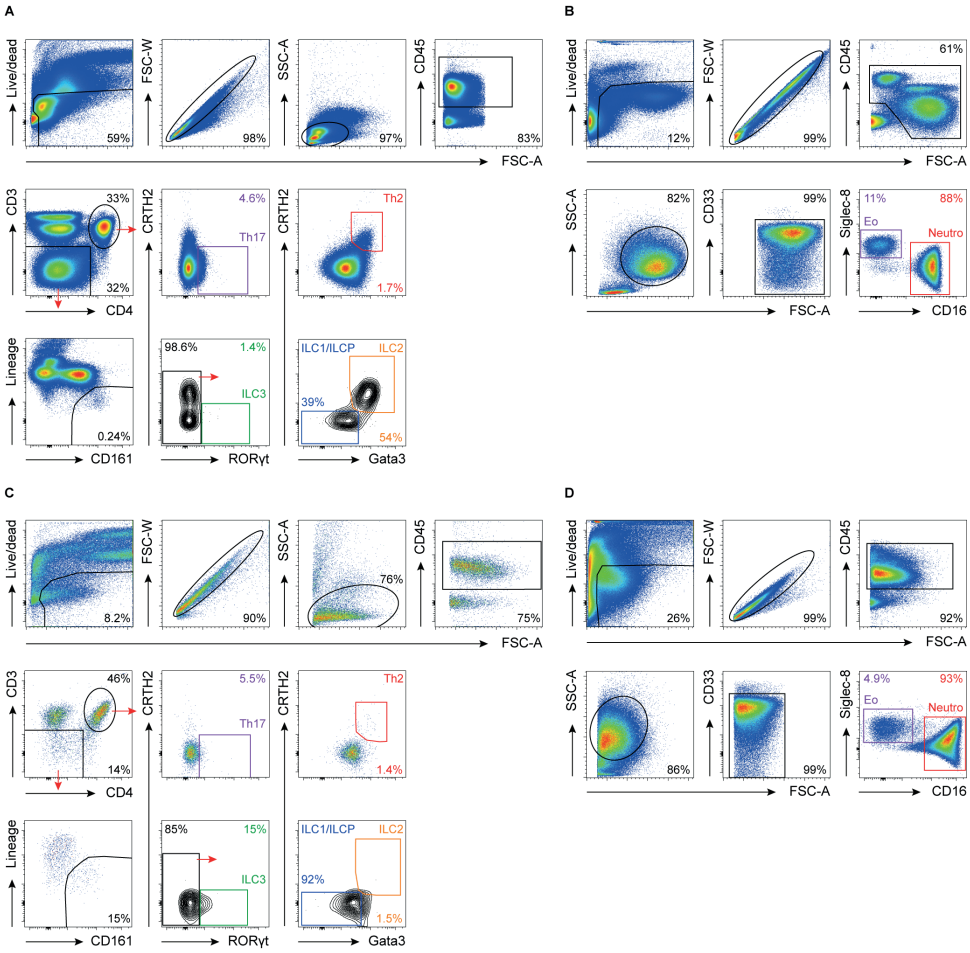


Figure 1. Flow cytometric gating strategy for the identification of (A) Th2, Th17, ILC2, ILC3, ILC1/ILCP and (B) eosinophils and neutrophils in (A, B) peripheral blood and (C, D) induced sputum.

(A-D) Plots are representatives of all samples.

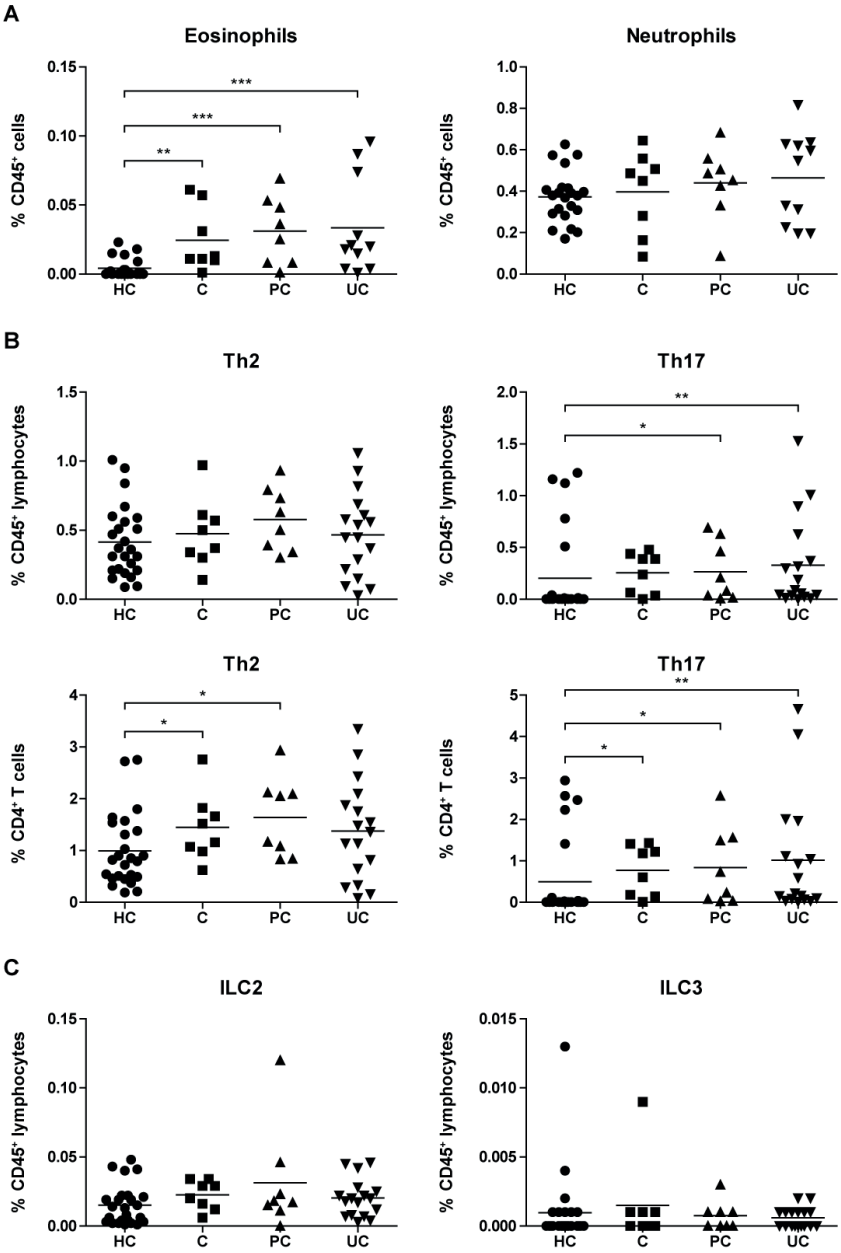


Figure 2. Peripheral blood from asthma patients show elevated proportions of eosinophil, but neutrophils, Th2 cells and ILC2s are comparable to healthy controls.

(A) Quantification of eosinophils and neutrophils in peripheral blood. (B, C) Comparison of (B) Th2 and Th17 and (C) ILC2 and ILC3 numbers in PBMCs from healthy controls and asthma patients. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. See Fig. 1 for gating strategy. HC = healthy control (n = 23), C = controlled asthma (n = 10), PC = partially controlled asthma (n = 10), UC = uncontrolled asthma (n = 16).

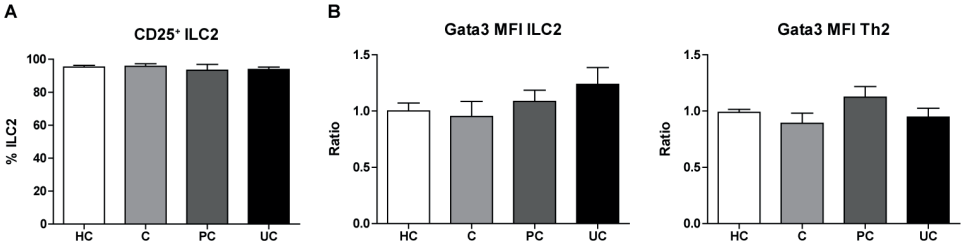


Figure 3. CD25 and GATA3 expression in ILC2s and Th2 cells are not significantly altered in asthmatics.

(A) Comparison of CD25$^+$ ILC2s isolated from PBMCs. (B) Quantification of the mean fluorescent intensity (MFI) of GATA3 in ILC2s and Th2 cells from asthma patients normalized to healthy controls, which were set to 1.0. Data are shown as mean ± SEM. See Fig. 1 for gating strategy. HC = healthy control (n = 23), C = controlled asthma (n = 10), PC = partially controlled asthma (n = 10), UC = uncontrolled asthma (n = 16).

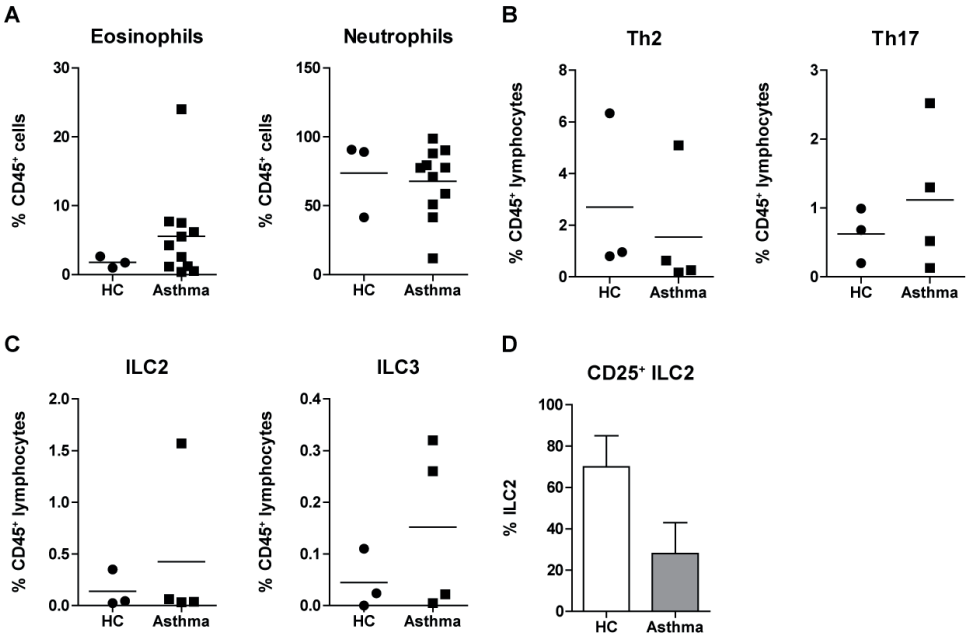
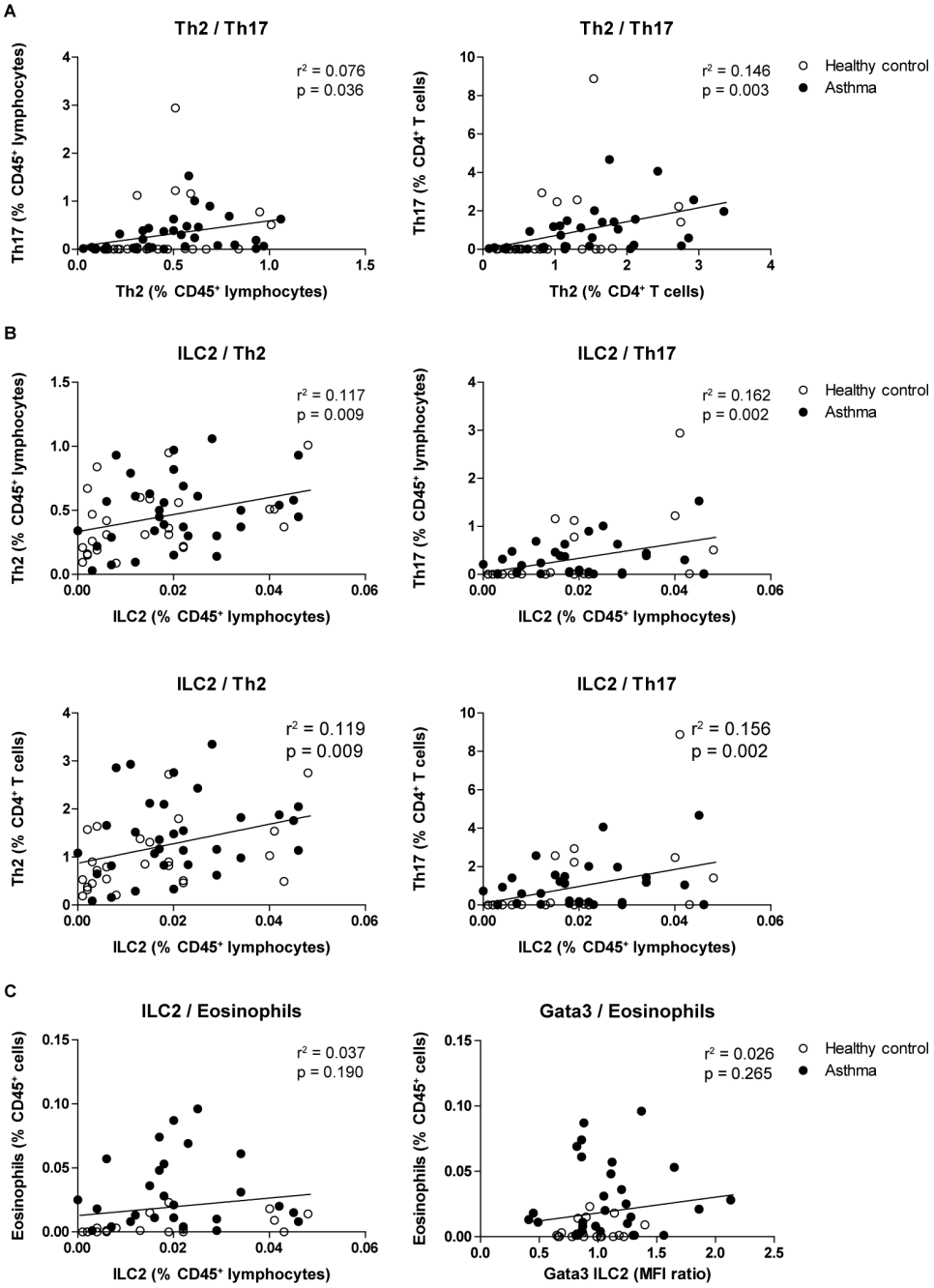


Figure 4. Immunological profiling of induced sputum shows minimal differences in T cells and ILCs between healthy controls and asthma patients.

(A-D) Quantification of (A) eosinophils, neutrophils, (B) Th2, Th17, (C) ILC2, ILC3 and (D) CD25$^+$ ILC2s in induced sputum from healthy controls and asthma patients. See Fig. 1 for gating strategy.


A
Th2 / Th17
r² = 0.076
p = 0.036
Th17 (% CD45⁺ lymphocytes)
Th2 (% CD45⁺ lymphocytes)
Th2 / Th17
r² = 0.146
p = 0.003
Th17 (% CD4⁺ T cells)
Th2 (% CD4⁺ T cells)
Healthy control
Asthma
B
ILC2 / Th2
r² = 0.117
p = 0.009
Th2 (% CD45⁺ lymphocytes)
ILC2 (% CD45⁺ lymphocytes)
ILC2 / Th17
r² = 0.162
p = 0.002
Th17 (% CD45⁺ lymphocytes)
ILC2 (% CD45⁺ lymphocytes)
ILC2 / Th2
r² = 0.119
p = 0.009
Th2 (% CD4⁺ T cells)
ILC2 (% CD45⁺ lymphocytes)
ILC2 / Th17
r² = 0.156
p = 0.002
Th17 (% CD4⁺ T cells)
ILC2 (% CD45⁺ lymphocytes)
C
ILC2 / Eosinophils
r² = 0.037
p = 0.190
Eosinophils (% CD45⁺ cells)
ILC2 (% CD45⁺ lymphocytes)
Gata3 / Eosinophils
r² = 0.026
p = 0.265
Eosinophils (% CD45⁺ cells)
Gata3 ILC2 (MFI ratio)
Healthy control
Asthma

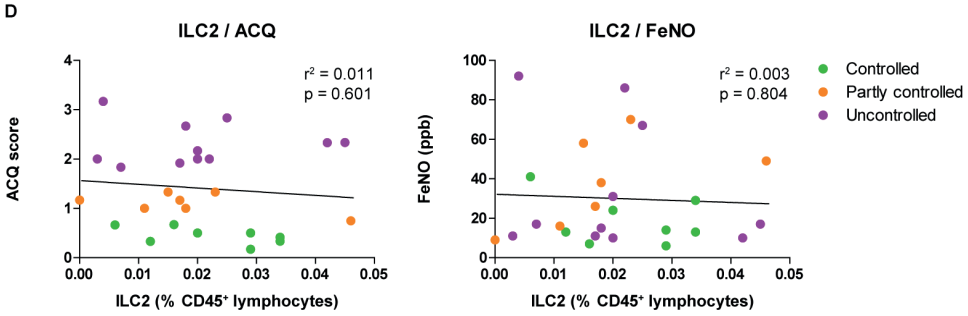


Figure 5. ILC2 frequencies correlates with Th2 and Th17 frequencies in peripheral blood.

(A-D) Correlations between (A) Th2 and Th17 frequency, (B) ILC2 frequency and Th2 or Th17 frequency, (C) eosinophil frequency and ILC2 frequency or GATA3 MFI values in ILC2s (D) ILC2 frequency and asthma control questionnaire (ACQ) score or FeNO.

Frequency of ILC2s in peripheral blood correlate with frequency of Th2 and Th17 cells

When we analysed peripheral blood samples from the three patient groups and healthy controls, a significantly positive correlation was observed between the proportions of Th2 and Th17 cells (**Fig. 5A**). A similarly pronounced correlation existed between proportions of ILC2 and Th2 cells (p=0.009) and ILC2s and Th17 cells (p=0.002), suggesting that these three cell types may be co-activated. This correlation remained true when ILC2s were compared to Th2 and Th17 frequencies within the $CD4^+$ T cell fraction (**Fig. 5B**). However, no correlation existed between eosinophils and ILC2 frequency or GATA3 MFI values within ILC2s (**Fig. 5C**).

In asthma patients, we performed further correlation analysis between ILC2 frequency and the key clinical parameters ACQ and FeNO. We observed that ILC2 frequencies did not correlate with clinical parameters (**Fig. 5D**), suggesting that peripheral blood ILC2s have limited influence on asthma phenotype and pathology.

DISCUSSION

ILC2s have been shown to contribute to asthma symptoms in mouse models and were detected at increased frequencies in asthma patients in a number of studies (10, 14, 15, 27-30). However, asthma is a heterogeneous disease and ILC2s could be important in only a subset of patients. Therefore, in our study we stratified 36 included asthma patients according to their control status obtained by a standardized ACQ score following the GINA guidelines. By multi-colour flow cytometry analyses we observed similar ILC2 frequencies between healthy controls and asthma patients, despite elevated eosinophil levels across all asthma groups. An important finding was that the frequencies of ILC2s followed the same pattern as T cells and were significantly correlated with both Th2 and Th17 cell frequencies. These results corroborate

our mice data in which ILC2 induction is critically dependent on T cell activity (19).
A notable issue in the literature on human ILC2s is that some groups have reported no or only modest differences in peripheral blood ILC2 numbers between healthy controls, mild and severe asthma patients (28, 35), while others state a 100-fold increase in severe asthmatics (29). This discrepancy could be explained by the different markers used to define ILC2s across various studies. To our knowledge, previous publications have only used surface markers to identify ILC2s and data from our house dust mite-driven and IL-33-driven allergic airway inflammation mouse models suggest that the presence of these markers may be highly dependent on localization and stimulus used to activate the ILC2s [B.W.L. and R.W.H., manuscript under revision]. Likewise, our data in this study demonstrate there is a marked heterogeneity in surface CD25 expression on ILC2s between the peripheral blood compartment and sputum PBMCs. Furthermore, some of the surface markers used are not ILC2-specific but rather expressed on numerous cell types, which may increase the risk of false-positives. We have found that intracellular GATA3 is a consistent marker that is only highly expressed in Th2 cells and ILC2s (36) and therefore we propose to include this to increase reliability of the identification of human ILC2s.
While the trend of increasing expression of GATA3 in ILC2s from controlled to uncontrolled asthma patients was not statistically significant for the number of samples we have collected, this could still be suggestive of a heightened activation state. Moreover, this finding would be in agreement with previously published results that peripheral blood ILC2s from asthma patients are capable of producing higher levels of IL-5 and IL-13 compared to healthy controls (23, 28). ILC2s from uncontrolled asthma patients appear to express the highest levels of GATA3 compared to less severe asthma groups and may be an indication of ineffective suppression by corticosteroids. Indeed, ILC2s have been associated with severe steroid-resistant asthma phenotypes in both humans and mice via IL-33 and TSLP, respectively (37, 38). Alternatively, other authors have emphasized that ILC2s are responsive to corticosteroid treatment in eosinophilic respiratory disease (39), highlighting the importance of a unified definition for ILC2s. Furthermore, we must entertain the possibility of multiple ILC2 subsets or activation states as evidence from both our own data and published studies suggests that ILC2s may be more heterogeneous than previously thought depending on the microenvironment (40). This would also be supported by recent single-cell RNA sequencing analyses of ILC subsets from intestine or human tonsils (41, 42). Therefore, it would be attractive to use such single-cell approaches also on ILC2s from lungs or lavages from asthma patients or asthma mouse models to define ILC2 heterogeneity.
In summary, our findings suggest that peripheral blood ILC2s do not significantly expand in human asthma regardless of control status. This is in agreement with the recent identification of systemic ILC precursors (34), which would support a model of local ILC2 differentiation and expansion from systemically distributed ILC precursors in response to local environmental

signals in the lung and thus would be very different between asthma patients and healthy controls. Nevertheless, our findings indicate that ILC2 frequencies are however, linked to T cell activity, reflecting results from our HDM-induced mouse models for allergic asthma that show that IIC2 activation is T-cell dependent. In depth analysis of the activation status of ILC2s in asthma patients may provide insights into their precise contribution to asthma pathogenesis. Furthermore, local roles of ILC2s in asthma remains to be elucidated when sufficient sputum samples have been collected. Investigation into the epigenome of healthy control and asthmatic ILC2s could also provide clues for their manner of activation and subsequent effector function, especially since it has been shown that peripheral blood Th2 memory cells display epigenetic differences between healthy and asthmatic individuals (43), which are thought to reflect disease-associated events that have occurred locally in the lung and draining lymph nodes.

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GENERAL DISCUSSION

CHAPTER 8

Asthma affects some 300 million people worldwide and the incidence is continuing to rise in developed countries. Our understanding of the underlying mechanisms in asthma has greatly advanced in the past 20 years and has led to improvements in therapeutic interventions. However, the primary treatment option has remained unchanged for 40 years and includes the use of adrenergic bronchodilators and corticosteroids (1), which are not able to effectively control all forms of asthma. Furthermore, a curative strategy is still to be discovered. Classically, T helper 2 (Th2) cells were considered to be the key orchestrators of allergic asthma pathology by production of the type 2 cytokines IL-4, IL-5 and IL-13. The discovery of group 2 innate lymphoid cells (ILC2s) in 2010 as an innate source of type 2 cytokines challenged this point of view and resulted in extensive research in recent years into the contribution of this novel cell population (2). ILC2s are activated by epithelial derived cytokines, in particular IL-33, and have been hypothesized to be the missing mechanistic link between viral infections of the respiratory tract and asthma exacerbations, a recurrent complication in the clinic. Further understanding of the role of ILC2s in such a scenario would give rise to new methods to combat allergic asthma and asthma exacerbations.

In this thesis, we addressed the role of ILC2s in the immunopathology of allergic asthma and asthma exacerbations resulting from influenza virus infections. We identified the critical importance of T cells in ILC2 activation and demonstrated that induction of ILC2s is independent of IL-33 in house dust mite (HDM)-induced asthma (**Chapter 3**). Using flow cytometry, we showed that ILC2s are a heterogeneous population that adapt their phenotype according to the microenvironment (**Chapter 4**). The importance of this finding is evident in Chapter 5, where we demonstrated the suppression of HDM-activated ILC2s by influenza virus, which are reactivated upon virus clearance (**Chapter 5**). Employing epigenomics allowed us to investigate the plasticity of ILC2s and we presented evidence that genome wide association study (GWAS) variants can be coupled to the ILC2 epigenome, further supporting an important role for ILC2s in human asthma (**Chapter 6**). Finally, we found an association between peripheral blood ILC2s and Th2 cells in a cross-sectional study of asthma patients (**Chapter 7**).

ILC2 as an early and late source of type 2 cytokines

Multiple animal models exist for studying allergen-induced asthma and consequently the contribution of ILC2s to asthma pathology has been investigated in ovalbumin, papain, *Alternaria*, *Sphingomonas* and our HDM-driven models (3-12). While both T cells and ILC2s produce IL-5 and IL-13 to generate type 2 inflammation in these models, a critical difference exists in the timing of cytokine expression. ILC2s are a potent early source of type 2 cytokines after intranasal administration of protease allergen papain or fungal allergen *Alternaria*, and are able to induce eosinophilic airway inflammation in absence of adaptive immunity (5, 6, 9, 10). A study using ICOS-T mice, which enables exclusive ILC2 deletion through diphtheria

toxin without affecting T cells (13), concluded that ILC2s act upstream of dendritic cells and are required for the orchestration of Th2 cell response to papain (14). From these studies it was concluded that ILC2s are crucially involved in memory Th2 cell recruitment by stimulating dendritic cells via IL-13 to produce CCL17. In contrast to these findings, we report in **chapter 3** that HDM induces ILC2s in a T cell dependent manner and that both T cells and ILC2s follow similar kinetics (12), suggesting that papain / *Alternaria* allergens employ different pathways to activate ILC2s than HDM (**Fig. 1**). Furthermore, preliminary data in ICOS-T mice that have been subjected to an acute HDM-driven asthma model suggest that ILC2 presence is dispensable and a reduction of ILC2s does not affect the severity of eosinophilic inflammation in mice [M. Schuijs, B. Lambrecht, personal communication]. ILC2s may however, play a larger role in chronic HDM asthma models.

The primary encounter of an inhaled allergen occurs at the airway epithelium and this interaction dictates the type of response mounted by the immune system. Papain harbours a strong proteolytic activity that not only triggers protease-activated receptors on epithelial cells (15, 16), but also breaches the integrity of the epithelial barrier. Similarly, *Alternaria* has been shown to disrupt epithelial permeability in cultured human bronchial epithelial cells (17). Although HDM consists of multiple allergens with proteolytic activity (18), its induction of asthma is strictly dependent on toll-like receptor 4 (TLR4) expressed on epithelial cells (19, 20). TLR4 stimulation is likely to be further enhanced by the naturally occurring lipopolysaccharides (LPS) in HDM extracts. Activation of the epithelium by papain and *Alternaria* results in secretion of IL-25, thymic stromal lymphopoietin (TSLP) and in particular IL-33 that initiates the recruitment of ILC2s (5, 10).

In comparison, HDM induces only minor levels of IL-33 *in vivo* and air-liquid interface (ALI) cultures of epithelial cells stimulated with HDM do not secrete IL-25 and TSLP (10, 21). Our data corroborate these findings and we demonstrated that IL-33 is dispensable for the recruitment of ILC2s in our HDM-induced asthma model, although cytokine production by ILC2s is significantly diminished in the absence of IL-33 (12). Instead, IL-1a is a crucial controller of HDM sensitization and asthma development and promotes granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion by epithelial cells in an autocrine feedback loop (21, 22) (**Fig. 1**). HDM-primed dendritic cells then migrate towards lymph nodes and initiate sensitization in which Th2 cells occupy a central role. In such a scenario, ILC2s are only mobilized when the T cell response is firmly established, although the signalling factors responsible for the accumulation of ILC2s remain to be determined. A viable candidate is IL-2, which is highly produced by activated T cells (23). Roediger *et al.* have demonstrated *in vivo* that IL-2 promotes ILC2 proliferation and enhances the production of type 2 cytokines in a mechanistically similar manner as in T cells (24). Mast cells are an alternate source of IL-2 and secrete this in response to IL-9 (25). While IL-9 is primarily linked to parasitic infections and cystic fibrosis, Th9 cells have also been found in murine models of asthma (26-28) as well as

enhanced IL-9 expression in lungs of asthmatic patients (29, 30). In our HDM-driven model, IL-9 production by BAL fluid T cells was detectable and significantly increased in HDM-treated mice (31). Furthermore, other mast cell mediators such as prostaglandin D_2 and leukotriene E_4 are reported to trigger ILC2 activation in humans (32, 33).

Therefore, an important step in the future is to establish the signalling molecules required for ILC2 induction in HDM-driven asthma and whether inhibition of these pathways could lead to reduced airway inflammation and novel treatment possibilities.

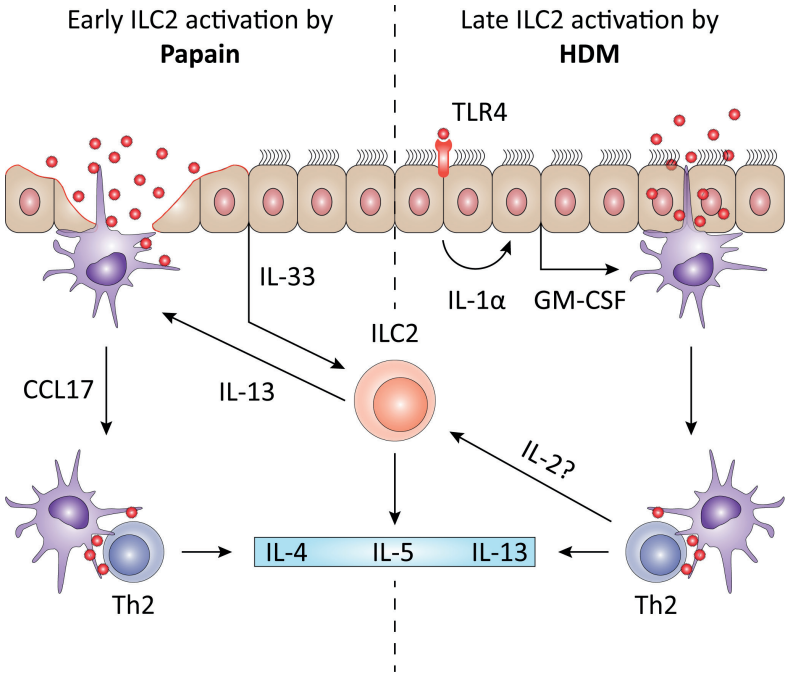

Figure 1. Mechanisms of ILC2 activation induced by papain compared to HDM.

Strong proteolytic activity by papain disrupts the epithelial barrier and induces secretion of IL-33 by the damaged epithelium, resulting in an early activation of ILC2s. IL-13 derived from ILC2s promotes Th2 immunity via dendritic cells. In contrast, HDM activates the epithelium via TLR4, which then produces GM-CSF to stimulate dendritic cells. A classic Th2 central pathway is initiated in this manner and ILC2 response is delayed.

Gata3 reporter mice as a tool for ILC2 characterization

ILC2s express a variety of surface markers that are often used in their identification in both mouse and human (34). Several markers are cytokine receptors such as IL-2R (CD25), IL-7R (CD127), IL-17RB and IL-33R (T1/ST2) and we hypothesized that these and perhaps others may be up or downregulated by feedback mechanisms and that the ILC2 phenotype could change depending on the type of stimulation received.

Therefore, we employed a novel *Gata3* yellow fluorescent protein (YFP) reporter (GATIR) mouse, generated in the lab of J. Fehling, Ulm, Germany, to investigate the expression profile of ILC2s derived from multiple tissues after different methods of stimulation. The GATIR knock-in construct consists of an IRES-YFP cassette inserted into the 3′ untranslated region of the *Gata3* locus, resulting in concomitant expression of GATA3 and YFP. Our group has previously shown that GATA3 levels affect ILC2 development in a dose-dependent manner (35) thus it was essential to ensure GATA3 protein expression was not altered by the IRES-YFP insertion. In **chapter 4**, we demonstrate that the expression of GATA3 protein measured with intracellular flow cytometry in GATIR mice is comparable to wild-type mice and is parallel to the detected YFP signals. Importantly, ILC2 and Th2 function are not adversely affected in the GATIR mice. Careful analysis of all stages of intra-thymic development, representative stages of hematopoietic stem and progenitor cell (HSPC) populations and cell subsets in lymphoid organs did not reveal any numerical or phenotypic differences between heterozygous or homozygous GATIR mice and wild-type controls [J. Fehling, personal communication]. Therefore, GATIR mice are the first *Gata3* reporter mice that do not suffer from impaired GATA3 expression. These findings allowed us to use the GATIR mice as a tool to investigate the phenotype of ILC2s with more accuracy by being less dependent on surface markers. An additional advantage is the easy detection of YFP by confocal microscopy and enabled the investigation into ILC2 localization *in situ*.

ILC2 adaptability to microenvironmental cues

Using GATIR mice, we report in **chapter 4** that ILC2s are a heterogeneous population and express unique combinations of markers in each tissue and that IL-33 stimulation produces a distinct ILC2 population from HDM exposure. This finding highlights the significance of the microenvironment for the ILC2 phenotype, which is conceivably linked to their function. KLRG1 is a well-known marker for mature ILC2s (36, 37) and as expected, we only found KLRG1 expression on ILC2s in effector sites such as broncho-alveolar lavage (BAL) fluid, lungs and mediastinal lymph nodes (MLN). Interaction of KLRG1 with its ligand E-cadherin has been revealed by several publications to generate an inhibitory response in NK cells and T cells (38-40). It is likely for KLRG1 to act in an analogous capacity in ILC2s to negatively regulate the potent type 2 cytokine production.

Another reason for ILC2s to adapt their surface marker expression is the interaction with other cell populations (**Fig. 2**). The first evidence of the ability of ILC2s to modulate $CD4^+$ T cell differentiation in a contact-dependent manner was presented by Mirchandani *et al*. These authors established that antigen presentation via MHC-II on ILC2s is able to induce T cell proliferation *in vitro* (41). Interestingly, stimulating T cells with anti-CD3/CD28 and simultaneously blocking MHC-II did not prevent Th2 differentiation by ILC2s, suggesting the existence of an alternate mechanism. In the context of *Nippostrongylus* expulsion, MHC-II expressing ILC2s have

been shown to interact with antigen-specific T cells, whereby T cell derived IL-2 promotes ILC2 proliferation and IL-13 production (13). More recently, Maazi and colleagues provided evidence that ILC2s express both functional inducible T cell costimulator ligand (ICOS-L) and ICOS, which are involved in ILC2 function and homeostasis (42). However, activated T cells highly express ICOS as well and this ICOS:ICOS-L interaction could be the missing alternate mechanism by which ILC2s stimulate Th2 differentiation. Importantly, Maazi *et al.* confirmed that ICOS-L is downregulated upon binding to ICOS implying a dynamic expression profile (42). Furthermore, ILC2s may potentially regulate germinal-center responses by binding to ICOS-L on B cells and it has been shown that ILC2s can enhance IgE production from B cells *in vitro* via IL-4 and CD40 signaling (43). Additionally, ILC2s present in fat-associated lymphoid clusters in the pleural cavity control B cells and local IgM secretion during pleural infection and lung inflammation through IL-5 and IL-6 production (44, 45). ILC2s are also involved in memory Th2 cell recruitment by stimulating dendritic cells via IL-13 to produce CCL17 (14). Interactions between ILC2s and mast cells are more complex and varying effects have been described in different contexts. Activated mast cells in intestinal helminth infection produce IL-33 in response to apoptotic intestinal epithelial cells, thereby inducing IL-13 secretion by ILC2s (46). In addition, serine proteases from mast cells transform IL-33 into mature forms with 30-fold increased potency on ILC2s (47). However, mast cells have also been found to contribute to immune suppression via IL-2 by expanding regulatory T cells, which may act as a sponge and reduce availability of free IL-2 in addition to secreting suppressive mediators like IL-10 (48).

In **chapter 5**, we further investigate the temporal expression of several ILC2 surface markers over the course of an influenza virus infection. From these data, it is clear that many classic ILC2 markers such as T1/ST2 and CD25 rapidly change over time, allowing ILC2s to respond adequately to the cytokine milieu. CD25 appears to be particularly diverse in this regard as it is rapidly downregulated upon influenza virus infection, but its expression is restored at day 7 post infection when the T cell response is established. Consequently, when the virus is cleared and the inflammatory response enters the contraction phase, CD25 expression decreases again in favor of T1/ST2. In addition, IL-5^{+} and IL-13^{+} ILC2s are also maximally induced during the peak of the T cell response. Taken together, this would suggest that ILC2s are sensitive to T cell activity and carry out their function in the resolution phase of the infection. As influenza is a notably destructive virus (49), these kinetics support findings in literature demonstrating the ability of ILC2s to directly assist restoration of the epithelium through production of growth factors such as amphiregulin (50). ILC2s may also indirectly contribute to repair through secretion of IL-13, which promotes alternatively activated macrophages that are responsible for resolving the aftermath of inflammation (51). IL-13 derived from ILC2s could however be a double-edged sword as it has been reported that it is sufficient to drive collagen deposition in the lungs of mice, leading to further exacerbated asthma conditions (52).

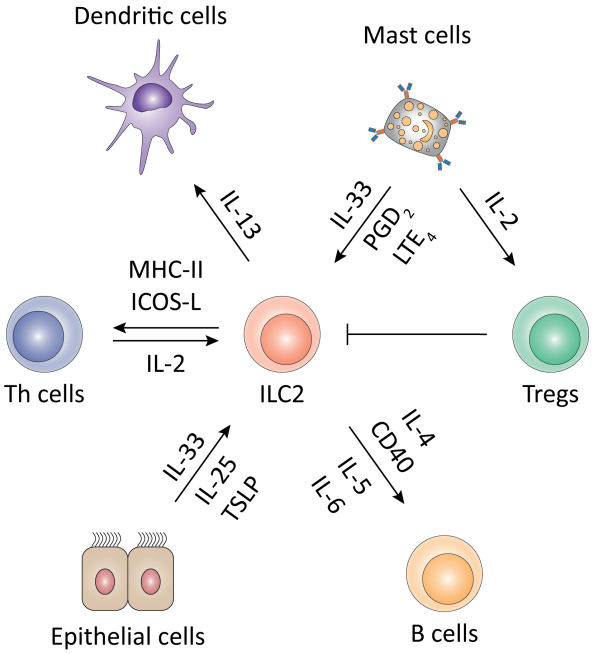

Figure 2. Integration of ILC2s and interaction with other cells of the immune system.

Abbreviations: inducible T cell costimulator ligand (ICOS-L), leukotriene E_4 (LTE_4), major histocompatibility complex class II (MHC-II), prostaglandin D_2 (PGD_2), regulatory T cell (Treg), T helper cell (Th), thymic stromal lymphopoietin (TSLP). See text for further details.

Asthma exacerbations caused by influenza virus infection are particularly severe (53, 54) thus in **chapter 5**, we developed a mouse model to simulate influenza-induced exacerbation in chronic HDM asthma. Recent literature describes that innate immune cells such as NK cells exhibit memory features that are mainly attributed to adaptive immunity (55). Mice that were exposed to allergens or IL-33 and subsequently challenged with another unrelated allergen displayed an enhanced ILC2 response compared to naïve mice (56). These findings can be regarded as examples of so-called "trained immunity" by innate cells, which is fundamentally different from classical immunological memory by T and B cells. Primarily, the increased responsiveness to secondary stimuli is not specific to a single pathogen and is mediated by epigenetic modifications, in contrast to gene rearrangement of the antigen receptor loci in T and B cells (57-59). ILC2s most likely achieve memory-like abilities via a similar mechanism involving changes in their epigenome. In addition to greater cytokine production, activated ILC2s appear to have a relatively long lifespan, because BrdU-labeled ILC2s were still detectable after 2 months (56). We hypothesized that ILC2s chronically activated by HDM would thus be primed to respond to a secondary stimulus and produce a significant type 2 response. However, in contrast to our expectations, the absolute number of HDM-activated

ILC2s in the BAL fluid is suppressed by the influenza virus infection and the vast majority of type 2 cytokines is derived from $CD4^+$ T cells. A likely cause for this phenomenon is the abundant presence of IFN-γ and IL-27 in an influenza virus infection, which are both known to suppress ILC2 activity (60-62) (**Fig. 3**). Similar to our previous observations with influenza virus infection alone, ILC2s are mainly active after clearance of the influenza virus and continue to produce IL-5, IL-13 and amphiregulin. Since the expression of T1/ST2 is also highest at day 7 and day 10 post infection, it is plausible that these ILC2s will be more sensitive to IL-33 and will be more readily activated upon stimulation. Because of the ongoing influenza virus infection pathology, mice are unable to tolerate isoflurane and intranasal treatments up to day 7 after infection. Nevertheless, it would be interesting to further expose asthmatic influenza virus-infected mice at day 10 post infection or later timepoints to IL-33 or HDM to investigate whether the T1/$ST2^{high}$ ILC2s will exhibit an enhanced response.

Depletion of ILC2s in the early phase of influenza virus infection could also be due to apoptosis or necrosis caused by the virus itself. Z-DNA binding protein 1 (ZBP1) is a cytoplasmic receptor that plays a role in the innate immune system against DNA from viral or bacterial origin and it has been shown recently that recognition of influenza virus particles by this receptor triggers cell death and promotes epithelial damage (63). However, it is unknown whether ILC2s carry the terminal α-sialic acid molecule on their cell surface that is required for influenza virus entry. Another explanation for the strongly reduced ILC2 induction is the migration of ILC2s to other tissues, although we have observed a similar phenomenon in lung tissue and the MLN. Expression of CCR2, CCR4, CCR5, CCR10 and CXCR4 have been detected on ILC2s, particularly after activation by IL-2, IL-25 or IL-33 (62, 64) and mature ILC2s are found circulating in peripheral blood (65). While ILC2s possess the necessary trafficking tools, the migration patterns have not been clearly defined and could provide insights into their function.

Functional plasticity of ILC2s

The evident heterogeneity in our ILC2 studies prompted the query of the existence of multiple ILC2 subsets and whether perhaps ILC2s can go one step further and achieve functional plasticity. Plasticity has been demonstrated in ILC3 when exposed to IL-12, IL-18 and IL-7, which leads to downregulation of RORγt and upregulation of T-bet resulting in an ILC1-like phenotype (66). Similarly, ILC1s can differentiate into ILC3 in the presence of IL-1β and IL-23 (67), once again demonstrating the impact of microenvironment on ILCs. Drastic changes in the microenvironment could thus potentially push ILC2s to transdifferentiate into other ILC populations. In **chapter 6**, we address this issue by analyzing the ILC2 transcriptome and show that different microenvironments effectively modulate ILC2 gene expression. Specifically, BAL fluid ILC2s show a more inflammatory phenotype than MLN ILC2s, evident from upregulation of pro-inflammatory cytokines, co-stimulatory receptors and chemoattractants. In contrast, MLN ILC2s expressed higher levels of *Ox40l* and genes associated with MHC class II, suggesting

active involvement in cell-cell interactions. Our confocal images of MLN ILC2s in **chapter 4** show that they are situated along the border of T and B cell zones, providing further evidence of interactions with both T and B cells. However, the active epigenome between BAL and MLN ILC2s is nearly identical and key ILC1 and ILC3 genes such as *Tbx21* (encoding for T-bet) and *Rorc* (encoding for RORγt) are also primed for activation by H3K4Me2 DNA methylation. This data suggests that ILC2s possess a flexible epigenome, which allows for functional plasticity and is in agreement with publications describing a hybrid ILC1/ILC2 phenotype developing under influence of IL-33, IL-12 and IL-1β in both mice and humans (68-71). Furthermore, current literature implicate Notch in the promotion of IL-17, a classic ILC3 cytokine, production in ILC2s (72). Taken together, ILC2s are more heterogeneous and less static than previously thought and are able to adapt their cytokine output. This could have implications for our understanding of influenza-induced asthma exacerbations and may be an explanation for the reduction of ILC2 numbers we observe in **chapter 5**.

Human genetic studies have identified numerous gene loci associated with asthma susceptibility and severity including several known to be expressed by ILC2s such as *Rora*, *Il1rl1* and *Il13* or linked to ILC2 activation such as *Il33* (73, 74). Recently, epigenome-wide association studies (epiGWAS) have pinpointed specific DNA methylation regions which are involved in regulating gene expression in allergic inflammation (75, 76). In **chapter 6**, we epigenetically profiled naïve and *in vitro* IL-33/IL-25-activated ILC2s from peripheral blood of healthy individuals and show that substantial differences exist in *Il5*, *Il13*, *Batf* and *Dennd1b* loci, which are all implicated in asthma (77, 78). Strikingly, over 90% of GWAS asthma associated genes displayed active chromatin in the ILC2 epigenome and strengthens a role for ILC2s in human asthma pathogenesis. It would be interesting to further explore epigenetic effects of stimulation of ILC2s by different activating cytokine combinations (IL-25, IL-33, TSLP, IL-2 or TL1A) compared to suppressive mediators (IFN-α, IFN-γ or IL-27) (**Fig. 3**).

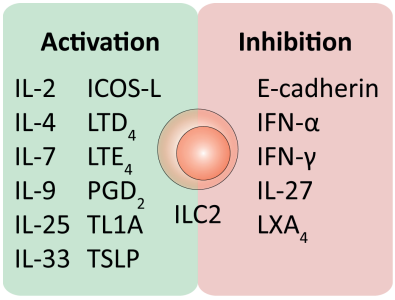

Figure 3. ILC2 activating and inhibiting factors.

Abbreviations: inducible T cell costimulator ligand (ICOS-L), leukotriene D_4 (LTD_4), leukotriene E_4 (LTE_4), Lipoxin A_4 (LXA_4), prostaglandin D_2 (PGD_2), thymic stromal lymphopoietin (TSLP), TNF-like ligand 1A (TL1A). See text for further details.

ILC2s in human asthma

Human ILC2s were identified soon after their discovery in mice and were reported as Lineage$^-$ cells that express CD127, KLRB1 (CD161) and CRTH2, a receptor for prostaglandin D_2 (65). The characterization of their phenotype has since expanded (34) and they have been shown to be present at increased frequencies in adult asthma patients compared to healthy controls (79-82). Evaluation of the BAL fluid of children with therapy-resistant asthma also indicate elevated ILC2s numbers (83) and patients presenting with severe eosinophilic asthma had higher numbers of IL-5$^+$ and IL-13$^+$ ILC2s than patients with milder forms of asthma (81). In contrast to these studies, our cross-sectional study in **chapter 7** and recently published study by Barnig *et al.* and Jia *et al.* reveal that ILC2s are not significantly increased in peripheral blood of asthma patients and there is no correlation with asthma severity (80, 84). In concurrence with our data in mice where ILC2s mimic T cell kinetics, we found correlations between ILC2 and T cell numbers, implying cooperation between these two cell populations. Furthermore, ILC2s from severe asthma patients may show higher GATA3 expression suggesting a more activated state in these patients. This is in agreement with previously published data that ILC2s from asthma patients produce higher levels of IL-5 and IL-13 (80, 85). **Table 1** summarizes the identification of ILC2s in current asthma studies and demonstrates a number of variations in the markers used by different research groups. The field of human ILC2s would benefit from a consensus on the preferred markers by enabling direct comparison between studies. Peripheral blood is the most commonly investigated compartment and understandably so, considering the difficulties of obtaining BAL fluid, lung tissue or sputum from asthma patients. However, as presented in **chapter 4**, mouse ILC2s are a heterogeneous population that adapt to their microenvironment and the same principle likely applies to human ILC2s as well. Thus, ILC2s isolated from peripheral blood are possibly phenotypically and functionally different from ILC2s present in the lungs. Our preliminary data supports this and shows diverse CD25 expression on ILC2s between peripheral blood and induced sputum. Recently, circulating ILC precursors (ILCP) have been discovered in peripheral blood that did not express the signature transcription factors of mature ILCs but did show CD7, CD117, CD127 and IL-1R1 on their surface (86). *In vitro* culture of ILCPs gave rise to mature ILC subsets capable of producing IFN-γ, IL-13, IL-17A or IL-22, depending on the cytokine environment. Interestingly, ILCPs are also detected in peripheral tissues and indicate that local differentiation into mature ILC subsets may supplement proliferation of resident ILCs on demand. The development of stable ILC culture systems by clonal expansion as well as single-cell transcriptome analysis as described by Lim *et al.* will be advantageous in elucidating the potential of ILCP in asthma patients and whether it is different from healthy controls (69).

Inhibition of ILC2s as a strategy to treat asthma

A key question that remains is whether ILC2s are a suitable therapeutic target to combat asthma and how to effectively achieve this. Corticosteroids have shown some efficacy in reducing ILC2 numbers in patients with chronic rhinosinusitis (87) although others have shown corticosteroid resistance by ILC2s that is regulated by TSLP (88). Therapies acting upstream of ILC2s could target molecules that activate ILC2s or induce ILC2 homing to the site of inflammation. ILC2s have been described to express the leukotriene and prostaglandin receptors CysLT1 and CRTH2, respectively. Montelukast, a leukotriene receptor antagonist, inhibits ILC2 activation and has shown promising effectiveness in obese asthmatics (33, 89) and a novel CRTH2 antagonist (OC000459) improves lung function in moderate and severe eosinophilic asthma patients (90, 91). CRTH2 is also expressed by effector Th2 cells thus the effect of the antagonist may be the result of a combined inhibition of both Th2 cells and ILC2s. Other potential candidates are the epithelial alarmins IL-25, IL-33 and TSLP. However, the function of these cytokines is not limited to type 2 inflammation and blocking these may have severe adverse effects. For example, IL-33 is required for the maintenance and suppressive function of regulatory T cells in adipose tissue and thereby contributes to the prevention of autoimmune disorders (60, 92, 93). Another approach is to inhibit downstream effects of ILC2s by blocking IL-5 with monoclonal antibodies such as mepolizumab, reslizumab and benralizumab. These have demonstrated improved disease outcome in patients with nasal polyps and refractory eosinophilic asthma (94-98). Notch signaling plays a major role in T cell differentiation (99) and has been shown to induce ILC2 development from thymic progenitors *in vitro* (100). Blocking Notch pathways with a γ-secretase inhibitor or excision of RBPJκ, a downstream effector of Notch, have already yielded promising results in mice by reducing acute airway inflammation (31, 101). In addition, targeting of the Notch transactivation complex by the SAHM1 peptide dampens eosinophilic inflammation and airway hyperreactivity in our HDM-induced asthma model (102).
A GATA3-specific DNA enzyme (DNAzyme), which leads to cleavage of GATA3 after binding, is another alternative to limiting ILC2 and Th2 function. This results in significantly reduced GATA3 mRNA and protein and the production of Th2 cytokines in T cells (103), in line with dose-dependent function of GATA3 previously reported by our group (35) and in **chapter 4.** Treatment with GATA3 DNAzyme (SB010) effectively attenuated asthmatic responses triggered by allergen provocation, although specific effects on ILC2s were not investigated (104). Taken together, these treatment strategies may be beneficial to asthma patients who are unresponsive to conventional therapies and exhibit strong Th2 and ILC2 immune responses.

Concluding remarks

The unveiling of ILC2s in 2010 placed a new contender in the field and challenged the dominating position that Th2 cells had occupied thus far in the pathogenesis of allergic asthma.

In mice ILC2s were convincingly shown to be a source of early type 2 cytokines that could induce severe inflammation in the absence of adaptive immunity. This thesis explored the role of ILC2s in a physiological allergen-induced model of type 2 airway inflammation by using HDM sensitization and challenge. Furthermore, we aimed to elucidate the contribution of ILC2s in influenza-induced asthma exacerbations in a novel mouse model. An important finding of these studies is that ILC2 induction is heavily dependent on T cell activation in HDM-induced asthma and therefore ILC2s can be a late type 2 cytokine producing cell population. Moreover, IL-33 is dispensable for ILC2 recruitment in this model, highlighting pathways to activate ILC2s beyond epithelial-derived innate cytokines. Future studies should therefore be directed at clarifying these activation pathways and should uncover which cells ILC2s interact with in HDM-driven asthma.

Furthermore, we show that ILC2s are a heterogeneous population and adapt their phenotype to their microenvironment after exposure to HDM and influenza virus. As a result, surface markers used for the identification of ILC2s must be carefully evaluated and we suggest to include intracellular GATA3 to further eliminate false-positives. Ideally, a consensus should be reached for the panel of markers most suited to the identification and isolation of ILC2s by flow cytometry. This would enable better comparisons between studies in both mouse and human. RNA sequencing and epigenetic analyses reveal the flexible genome of ILC2s, supporting a model of highly plastic ILC2s and may be of consequence in asthma exacerbations induced by (multiple) different agents. The scope of this thesis is focused on allergic asthma with a primarily eosinophilic nature and is just the tip of the iceberg. Further investigations into the function of ILC2s in other asthma endotypes such as neutrophilic, early or late onset, and obesity-related forms (105) would lead to a better understanding of the underlying mechanisms involved and provide valuable information for novel therapies that will take your breath away (and give it back to the asthma patients).

Table 1. Analysis of ILC2s in human asthma and rhinitis studies.

Ref.	Study population	Tissues investigated	ILC2 phenotype	Findings
(10)	Allergic rhinitis patients	Peripheral blood	Lin^- $CD127^+CRTH2^+$	High expression of GATA3 and ETS-1 in allergic individuals
(65)	Chronic rhinosinusitis patients	Nasal polyps, peripheral blood	Lin^-	Enriched ILC2 population in patients with nasal polyps
(79)	Mild to moderate asthma patients	Peripheral blood, induced sputum	Lin^- $CD127^+CRTH2^+$	Peripheral blood ILC2 numbers increased in eosinophilic asthma patients and correlates with sputum eosinophil counts
(80)	Well-controlled, party controlled and uncontrolled asthma patients	Peripheral blood	Lin^- $CD127^+CRTH2^+$	ILC2 frequency increased in asthma patients and IL-13^+ ILC2 frequency correlates with asthma severity
(81)	Patients with severe eosinophilic asthma or mild atopic asthma	Peripheral blood, induced sputum	Lin^-	Greater numbers of ILC2s in blood and sputum in severe asthma compared to mild asthma
(82)	Asthma patients, majority with allergic rhinitis	BAL fluid	$Lin^-CD127^+T1/ST2^+$	IL-33 levels and IL-5^+ ILC2 numbers increased in airways of asthma patients
(84)	Mild and severe asthma patients	Peripheral blood, BAL fluid	Lin^- $CD127^+CRTH2^+$	ILC2s are not elevated in asthma patients
(85)	Allergic rhinitis or allergic asthma patients	Peripheral blood	Lin^- $CD127^+CRTH2^+$	Greater prevalence of blood ILC2s in allergic asthma patients and also more responsive to IL-25 and IL-33 stimulation
(87)	Chronic rhinosinusitis patients	Nasal polyps	$Lin^-CD45^+CRTH2^+$	ILC2s elevated in patients with eosinophilic nasal polyps
(88)	Allergic asthma patients	Peripheral blood, BAL fluid	Lin^- $CD127^+CRTH2^+$	Airway ILC2s, but not blood ILC2s, are steroid resistant

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ENGLISH SUMMARY

CHAPTER 9

Asthma affects some 300 million people worldwide and the incidence is continuing to rise in developed countries. Allergen-specific T helper 2 (Th2) cells are thought to play a major role in asthma pathogenesis by producing the key cytokines IL-4, IL-5 and IL-13 that are involved in type 2 inflammation. This T cell-centric model is challenged by the recent discovery of group 2 innate lymphoid cells (ILC2) that represent a critical innate source of type 2 cytokines. ILC2s lack classic hematopoietic lineage markers and do not express antigen-specific receptors but are activated by epithelial cell-derived cytokines including IL-25, IL-33 and TSLP. They are important mediators in murine models for allergic airway inflammation and influenza virus infections and ILC2s play a critical role in the expulsion of helminth infections in mice, independent of T cell immunity. Human ILC2s have been found to be elevated in nasal polyps of chronic rhinosinusitis patients. In this thesis we focused on the role of ILC2s in HDM-driven allergic airway inflammation and asthma exacerbations induced by influenza virus infection. Furthermore, we investigated peripheral blood ILC2s in asthma patients. Due to a lack of unique markers, the accurate phenotypic characterization and quantification of ILC2s requires a comprehensive panel of fluorescently labeled antibodies. The markers that are currently used to characterize ILC2s have not been standardized and often vary between research groups, which poses significant challenges when comparing data. In **chapter 2**, we described the analysis of ILC2s by flow cytometry in our IL-33-induced and HDM-driven airway inflammation models in the mouse.

ILC2s can be rapidly activated and represent an early innate source of IL-5 and IL-13 that is independent of adaptive immunity after exposure to papain and *Alternaria*. In **chapter 3**, we used a HDM-driven asthma mouse model to study the induction of ILC2s in allergic airway inflammation. In broncho-alveolar lavage (BAL) fluid, lungs, and lymph nodes, ILC2 activation is critically dependent on prior sensitization with HDM. In contrast to papain and *Alternaria*-driven models, T cells are required for ILC2 induction in HDM-induced allergic airway inflammation, whereby T cell activation precedes ILC2 induction. Furthermore, the accumulation of ILC2s in BAL fluid 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 ILC2s, 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.

Heterogeneity of ILC2s was explored in **chapter 4** using T cell-dependent (HDM) and T cell-independent (IL-33) methods of ILC2 activation. We compared ILC2 surface phenotype, transcriptome and localization in IL-33 and HDM-driven airway inflammation. Using flow cytometry, we found that upon IL-33 stimulation, *in vivo* ILC2s in BAL fluid have an almost uniform CD25^{+}CD127^{+}T1/ST2^{+}ICOS^{+}KLRG1^{+} phenotype. In contrast, HDM-induced ILC2s

display a heterogeneous surface marker phenotype; a large fraction of ILC2s express low levels of CD25, ICOS and KLRG1, but nevertheless have the capacity to produce type 2 cytokines. In addition, both in IL-33-driven and in HDM-driven inflammation, ILC2s exhibit phenotypic differences across tissues. Transcriptional profiling reveals ~1600 differentially expressed genes: HDM-stimulated ILC2s specifically express genes involved in the regulation of adaptive immunity through B and T cell interactions, whereas IL-33-stimulated ILC2s express high levels of proliferation-related and cytokine genes. In both airway inflammation models ILC2s are present in the lung submucosa close to epithelial cells, as identified by confocal microscopy. In chronic HDM-driven airway inflammation ILC2s are also found inside organized cellular infiltrates near T cells. Collectively, our findings show that ILC2s are phenotypically more heterogeneous than previously thought, whereby their surface marker and expression profile is highly variable and dependent on the way they are activated.

Influenza virus infection is an important cause of severe asthma exacerbations, but it remains unclear how a Th1-mediated antiviral response triggers a prototypical Th2 disease. In **chapter 5**, we investigated $CD4^+$ T cells and ILC2s in influenza virus-infected mice. We observed that ILC2s accumulate in the lung rapidly after influenza virus infection, but the induction of IL-5 and IL-13 secretion is delayed and concomitant with T cell activation. In an influenza-induced asthma exacerbation model we noticed an initial reduction of ILC2 numbers and cytokine production in BAL fluid compared to chronic HDM-mediated airway inflammation alone. ILC2s have a phenotype characterized by low T1/ST2, ICOS, KLRG1 and CD25 expression, resembling naïve ILC2s. Consequently, the contribution of ILC2s to type 2 cytokine production in the early stage of the influenza-induced asthma exacerbation is limited. In contrast, T cells show increased IL-4 and IL-5 production when exposed to both HDM and influenza virus. Nevertheless, upon clearance of the virus, ILC2s regain an activated T1/$ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ phenotype paired with cytokine production and are major contributors to the type 2 cytokine milieu. Collectively, our data indicate that both T cells and ILC2s contribute to influenza-induced asthma exacerbation, but with different kinetics.

Asthma susceptibility has strong (epi)genetic components and elevated numbers of ILC2s have been detected in blood and sputum of asthma patients. In **chapter 6**, we employed RNA-Seq, genome-wide identification of histone-3 lysine-4 dimethylation (H3K4me2) marked chromatin and computational approaches to study the transcriptome and epigenome in the context of ILC2 activation and genetic susceptibility to asthma. Activated ILC2s in mice display a tissue-specific gene expression signature that emerges from a remarkably similar epigenome. We identify superenhancers – a class of key regulatory regions – controlling ILC2 identity and asthma-associated genes. Furthermore, ~90% of the genes implicated in asthma through genome-wide association studies can be linked to $H3K4me2^+$ gene regulatory elements in ILC2s. ILC2s in allergic airway inflammation employ a flexible epigenome that couples adaptation to new microenvironments with functional plasticity. Importantly, we reveal a strong link between

gene regulatory mechanisms in ILC2s and the (epi)genetic basis of asthma, supporting a key pathogenic role for ILC2s in allergic asthma.

In **chapter 7**, we stratified asthma patients according to the Global Initiative for Asthma (GINA) 2014 classifications into the following subgroups: controlled, partly controlled and uncontrolled, based on asthma control questionnaire (ACQ) score. Blood eosinophils are significantly elevated in all asthma groups and the proportions of Th2 and Th17 within $CD4^+$ T helper cells are increased as well. However, ILC2 frequency remains comparable to healthy controls. Interestingly, ILC2 frequency is significantly correlated with Th2 and Th17 frequency. For ILC2s, the expression levels of the surface marker IL-2Ra (CD25) and the transcription factor Gata3 are also similar in asthma patients and controls. Our findings suggest that peripheral blood ILC2s are either not important in asthma pathogenesis or are not affected by an inflammatory status in the lung. Therefore, local ILC2s in the lung require further investigation as they may still contribute to asthma symptoms.

The results described in this thesis provide new insights into the role of ILC2s in HDM-driven allergic airway inflammation and their contribution to influenza-induced asthma exacerbation. Further characterization of ILC2s and their functions in asthma will lead to a better understanding of the mechanisms involved. Targeting ILC2 function using GATA3 DNAzyme or CRTH2 antagonists may be a viable novel strategy for the treatment of therapy-resistant asthma patients.

NEDERLANDSE SAMENVATTING

CHAPTER 9

Astma is een chronische luchtwegaandoening gekarakteriseerd door ontsteking, overmatig slijmproductie en vorming van bindweefsel in de longen. Kenmerkend bij astmapatiënten zijn de astma-aanvallen, waarbij er een acute vernauwing van de luchtwegen optreedt die leidt tot kortademigheid, hoesten en een piepende ademhaling. Wereldwijd lijden ongeveer 300 miljoen mensen aan astma en dit aantal neemt jaarlijks toe, met name in ontwikkelde landen zoals Nederland. Bij veel patiënten met astma speelt allergie voor huisstofmijt of schimmels een belangrijke rol bij de ontwikkeling van de ziekte. Bij dit proces zijn een bepaalde soort witte bloedcellen, de zogenaamde T helper 2 (Th2) cellen, nauw betrokken. Deze cellen worden bij astmapatiënten geactiveerd doordat ze specifiek allergenen, stoffen waar mensen allergisch voor zijn, herkennen en vervolgens bepaalde factoren produceren die ontstekingen kunnen veroorzaken of versterken. Bij allergisch astma zijn dit met name de type 2 cytokines IL-4, IL-5 en IL-13.

Enkele jaren geleden is een nieuw celtype ontdekt, de groep 2 innate lymphoid cel (ILC2), die ook deze cytokines kan produceren (**Hoofdstuk 1**). Een cruciaal verschil tussen ILC2s en Th2 cellen is dat ILC2s geen allergeen-specifieke receptoren tot expressie brengen maar aangestuurd worden door signalen zoals IL-25, IL-33 en TSLP afkomstig van geactiveerde epitheelcellen. Er is aangetoond in een aantal muismodellen voor allergische luchtwegontsteking dat deze cellen hierdoor onafhankelijk van T helper cellen kunnen functioneren. ILC2s zijn ook in verhoogde aantallen gevonden in neuspoliepen van patiënten met chronische rhinosinusitis. Het is echter onbekend op welke manier precies de ILC2s bijdragen aan de pathogenese van allergisch astma. Daarnaast zorgt influenzavirus infectie van de luchtwegen ervoor dat astma-aanvallen vaker voorkomen en ernstiger van aard zijn. Deze verergering van astmatische klachten tijdens en na een virusinfectie wordt ook wel astma-exacerbatie genoemd. ILC2s zouden mogelijk de mechanistische link kunnen zijn tussen deze twee luchtwegaandoeningen. In dit proefschrift beschrijven wij de rol van ILC2s in huisstofmijt-gemedieerde allergische luchtwegontsteking en astma-exacerbaties die geïnduceerd zijn door influenzavirus infecties in diverse muismodellen. Daarnaast werden ILC2s uit perifeer bloed van astmapatiënten en gezonde controlepersonen onderzocht.

Doordat ILC2s geen enkele unieke marker op hun celoppervlak dragen, worden ILC2s gekarakteriseerd en gekwantificeerd met een uitgebreid panel van fluorescente antilichamen. De markers die gebruikt worden zijn echter niet gestandaardiseerd en variëren vaak tussen onderzoeksgroepen, waarmee het vergelijken en extrapoleren van data uit verschillende studies lastiger wordt. In **Hoofdstuk 2**, beschrijven wij de analyse van ILC2s met behulp van flow cytometrie in ons muismodel van IL-33- en huisstofmijt-geïnduceerde luchtwegontsteking. ILC2s kunnen snel geactiveerd worden door papaïne en *Alternaria* en zijn een vroege bron van IL-5 en IL-13 die onafhankelijk is van het adaptieve immuunsysteem, waar T en B cellen toe behoren. In **Hoofdstuk 3**, maken wij gebruik van een huisstofmijt-gemedieerd astma muismodel om de inductie van ILC2s te bestuderen in allergische luchtwegontsteking. In dit

model worden de dieren eerst gesensibiliseerd met een lage dosis huisstofmijt en vervolgens blootgesteld aan een hogere dosis huisstofmijt om luchtwegontsteking te induceren. In de broncho-alveolar lavage (BAL) vloeistof, longen en lymfeklieren is de activatie van ILC2s sterk afhankelijk van sensibilisatie met huisstofmijt. In tegenstelling tot modellen die gebruik maken van allergenen zoals papaïne en *Alternaria*, is in ons huisstofmijt model de aanwezigheid van T cellen noodzakelijk voor de inductie van ILC2s. Bovendien worden T cellen eerder geactiveerd dan ILC2s. Daarnaast is de accumulatie van ILC2s in de BAL vloeistof onafhankelijk van IL-33, hoewel de aanwezige ILC2s aanzienlijk minder cytokines produceren in muizen die geen IL-33 kunnen produceren. In dit huisstofmijt-gedreven astma model zijn ILC2s daarom geen vroege bron van type 2 cytokines en dragen ze bij aan een inflammatoire omgeving waarin Th2 cellen centraal staan.

Het expressiepatroon van ILC2 markers is niet uniform en is afhankelijk van de activerende stimulus, de directe omgeving van de ILC2 en de mogelijke interactie met andere cellen. Deze heterogeniteit is beschreven in **Hoofdstuk 4** met behulp van een T cel afhankelijke (huisstofmijt) en T cel onafhankelijke (IL-33) manier van ILC2 activatie, waarin we het ILC2 oppervlakte marker fenotype, de totale genexpressie (RNA transcriptoom) en de locatie in de long hebben vergeleken. We hebben gevonden dat de via IL-33 geactiveerde ILC2s een uniform fenotype vertonen. Daarentegen hebben de ILC2 die bij huisstofmijt-gedreven luchtwegontsteking worden geactiveerd een meer variabel fenotype, al lijkt deze variatie geen invloed te hebben op hun capaciteit om de type 2 cytokines IL-5 en IL-13 te produceren. Bovendien zijn er bij zowel IL-33- als huisstofmijt-gedreven stimulatie grote verschillen in de ILC2 fenotypes te vinden tussen diverse organen. Uit onze RNA transcriptoom analyses blijkt verder dat huisstofmijt-gestimuleerde ILC2s genen tot expressie brengen die betrokken zijn bij de regulatie van het adaptieve immuunsysteem, terwijl IL-33 stimulatie vooral leidt tot het aanzetten van celdeling en cytokineproductie. Beide manieren van ILC2 stimulatie resulteren in een toestroom van deze cellen onder het longepitheel, zoals we konden aantonen met behulp van confocale microscopie. Daarnaast lijken ILC2s bij voorkeur voor te komen in de directe omgeving van T cellen binnen georganiseerde cel infiltraten. Bij elkaar genomen, laten onze bevindingen zien dat ILC2s fenotypisch meer heterogeen zijn dan voorheen gedacht, waarbij hun expressie profiel zeer afhankelijk is van de manier waarop ze geactiveerd worden.

Een belangrijke oorzaak van astma-aanvallen is infectie van de luchtwegen met het influenzavirus. Het is echter onbekend hoe een antiviraal immuunrespons, waar voornamelijk T helper 1 (Th1) cellen bij betrokken zijn, een Th2-gemedieerde ziekte zoals astma kan verergeren. In **Hoofdstuk 5** onderzoeken wij de kinetiek van T cellen en ILC2s in influenzavirus geïnfecteerde muizen. De resultaten tonen aan dat ILC2s snel na influenzavirus infectie accumuleren in de long, maar dat de IL-5 en IL-13 cytokineproductie pas later op gang komt, nadat ook T cellen worden geactiveerd. In een influenza-geïnduceerd astma-exacerbatie muismodel, worden huisstofmijt-geactiveerde ILC2s in eerste instantie onderdrukt

en nemen ze in aantal af in de BAL vloeistof. Daarbij nemen ze een fenotype aan dat vergelijkbaar is met naïeve ILC2s. Daarom blijft de type 2 cytokineproductie door ILC2s in het vroege stadium van influenzavirus infectie beperkt. In tegenstelling tot ILC2s, produceren T cellen wel verhoogde hoeveelheden IL-4 en IL-5 wanneer ze blootgesteld zijn aan zowel huisstofmijt als influenzavirus. Echter op het tijdstip dat het virus bijna geklaard is krijgen ILC2s weer een geactiveerd fenotype, gekoppeld aan een hoge cytokineproductie. Deze resultaten betekenen dat zowel T cellen en ILC2s bijdragen aan een type 2 cytokine milieu, maar met een verschillende kinetiek.

Genetische componenten spelen een centrale rol in de ziektevatbaarheid en ontwikkeling van astma. In **Hoofdstuk 6**, maken wij gebruik van geavanceerde genetische technieken om het epigenoom (het geheel van overerfbare veranderingen in fenotype en genexpressiepatronen van cellen) van ILC2s te karakteriseren en om te bepalen hoe de genetica het ziektebeloop van astma kan beïnvloeden. We laten zien dat de genexpressie in geactiveerde ILC2s verschilt per weefsel, zoals verwacht gebaseerd op de resultaten in **Hoofdstuk 4**, maar dat het oorspronkelijke epigenoom van ILC2 uit BAL en lymfeklier zeer veel op elkaar lijkt. Dit betekent dat de expressie van genen in ILC2s zich kan aanpassen aan de omgeving. Verder blijkt ongeveer 90% van de genen waarvan bekend is dat ze betrokken zijn bij astma ook actief in ILC2s. Dit ondersteunt een mogelijke pathogene rol van ILC2s in allergisch astma.

In **hoofdstuk 7** onderzoeken wij ILC2s in het perifeer bloed van astmapatiënten. De patiënten zijn verdeeld in de volgende subgroepen volgens de Global Initiative for Asthma (GINA) 2014 classificatie: gecontroleerd, gedeeltelijk gecontroleerd en ongecontroleerd astma. Vergeleken met gezonde personen blijken in deze drie astma patiëntengroepen de percentages van zowel eosinofiele granulocyten, Th2 en Th17 cellen significant verhoogd. Echter is de ILC2 frequentie vergelijkbaar tussen alle patiëntengroepen, wat zou kunnen suggereren dat ILC2s geen goede afspiegeling zijn van de ontsteking in de long. Echter, we vinden wel een correlatie tussen ILC2 frequentie en Th2 en Th17 frequentie. Daarom zou het interessant kunnen zijn circulerende ILC2s van astmapatiënten diepgaander te karakteriseren en lokale ILC2s in de long te onderzoeken, omdat deze wellicht wel een sterke bijdrage kunnen leveren.

De resultaten die beschreven zijn dit proefschrift werpen een nieuwe blik op de rol van ILC2s in huisstofmijt-gedreven allergische luchtwegontsteking en hun bijdrage aan influenza-gemedieerde astma-exacerbaties. Het karakteriseren van ILC2s en hun functie in astma leidt tot een beter begrip van de onderliggende mechanismen. Ontwikkeling van nieuwe therapieën kan mogelijk worden gericht op het beïnvloeden van ILC2 functie. Er zou bijvoorbeeld gebruik gemaakt kunnen worden van specifieke remmers van de nucleaire factor GATA3, die noodzakelijk is voor de ontwikkeling en activiteit van ILC2s, of van remmers van CRTH2, een membraan receptor die ILC2s kan activeren. Dit is met name van belang voor astmapatiënten die niet adequaat reageren op huidige therapieën, bijvoorbeeld gebaseerd op corticosteroïden.

ABOUT THE AUTHOR

CHAPTER 10

"My life amounts to no more than one drop in a limitless ocean. Yet what is any ocean, but a multitude of drops?" – David C. Mitchell, Cloud Atlas

Bobby is our son and the only child. He was born on 30 August 1989 in Shanghai, China, where we lived until 1991. He was brought to Delft, the Netherlands when he was four years old in July 1993, because his father moved there for his studies.

He attended "Basis School" (Dutch primary school) in Delft immediately, while he had no Dutch knowledge at all. The only single Dutch word we could teach him was "plassen" (to go to the toilet). Fortunately, he managed to learn Dutch at school quickly and could start to speak in a few months. It was a huge challenge for him as we spoke Chinese at home because on one hand our Dutch language was very limited and on the other hand we would like to teach him Chinese in order for him to be able to communicate with his grandparents in China. In the summer of 2000, we moved to Singapore because his father was sent there to work as an expatriate for three years. He had to adapt himself to the big change a second time and to learn English intensively in order to be able to attend the international school (United World College of South East Asia) there. Once again, he showed his capability of dealing with change and of learning yet another foreign language. That was a very fruitful three years for him not only because he studied at the college with achievements, but he also made many international friends and mastered the English language, while enjoying the beautiful southeast Asian and Asia-Pacific countries, swimming as a member of Hollandse Club team and obtaining several trophies at competitions among international clubs in Singapore. In July 2003, we repatriated to the Netherlands and he attended the bilingual Kandinsky College in Nijmegen until his graduation in 2007. He showed his spirit of research already in this period, for example, making a thorough study of characters and listing their features, capability and equipment, etc. in an Excel sheet to determine the optimal approach in playing his favorite games.

He started to study Biomedical Sciences at Radboud University Nijmegen in 2007 and received his Bachelor degree in 2010 and Master of Science degree in 2012. From January to July 2012, he carried out a research internship with a focus on molecular epidemiology at Karolinska Institute, Sweden, when the living and travel expenses there were fully supported by several Dutch and European foundations, owing to his active searching and submission of study grant applications. In November 2012, he started his Ph.D. research at Erasmus Medical Centre Rotterdam, under the supervision of Professor Rudi Hendriks, in the field of immunology focusing on allergic asthma. He worked hard in the past 5 years aiming to obtain his Ph.D. degree and made full use of the facility in the open and stimulating academic environment created by the research group, the result of which is the dissertation presented here. Additionally, he published a number of papers in academic journals and presented his research in multiple international conferences, seminars and workshops, contributing to the scientific society of immunology. His Ph.D. research provided him not only the possibility to learn and broaden the cutting-edge knowledge of human biology in this very specific and challenging field, but also the opportunity to meet people studying the same subject around the world, enabling him to move further in his career development.

We are very proud of Bobby for his achievements and for receiving the doctor's degree and we are very happy and thankful that he is able to do what he likes to do, and most importantly, enjoys life while doing it.

Jing Wen and Yuan Xiong Li
Bobby's parents

LIST OF PUBLICATIONS

CHAPTER 10

- **Li BW**, Hendriks RW. Group 2 innate lymphoid cells in lung inflammation. Immunology. 2013 Nov;140(3):281-7.
- **Li BW**, de Bruijn MJ, Tindemans I, Lukkes M, KleinJan A, Hoogsteden HC, Hendriks RW. T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. Eur J Immunol. 2016 Jun;46(6):1392-403.
- Vroman H, Bergen IM, **Li BW**, van Hulst JA, Lukkes M, van Uden D, Hendriks RW, Kool M. Development of eosinophilic inflammation is independent of B-T cell interaction in a chronic house dust mite-driven asthma model. Clin Exp Allergy. 2017 Apr;47(4):551-564.
- **Li BWS**, Beerens DMJM, Brem MD, Hendriks RW. Characterization of Group 2 Innate Lymphoid Cells in Allergic Airway Inflammation Models in the Mouse. Methods Mol Biol. 2017;1559:169-183.
- Tindemans I, Lukkes M, de Bruijn MJW, **Li BWS**, van Nimwegen M, Amsen D, KleinJan A, Hendriks RW. Notch signaling in T cells is essential for allergic airway inflammation, but expression of the Notch ligands Jagged 1 and Jagged 2 on dendritic cells is dispensable. J Allergy Clin Immunol. 2017 Oct;140(4):1079-1089.
- Altenburg AF, van de Sandt CE, **Li BWS**, MacLoughlin RJ, Fouchier RAM, van Amerongen G, Volz A, Hendriks RW, de Swart RL, Sutter G, Rimmelzwaan GF, de Vries RD. Modified Vaccinia Virus Ankara Preferentially Targets Antigen Presenting Cells In Vitro, Ex Vivo and In Vivo. Sci Rep. 2017 Aug 17;7(1):8580.
- **Li BWS**, Stadhouders R, de Bruijn MJW, Lukkes M, Beerens DMJM, Brem MD, KleinJan A, Bergen I, Vroman H, Kool M, van IJcken WFJ, Nageswara Rao T, Fehling HJ, Hendriks RW. Group 2 innate lymphoid cells exhibit a dynamic phenotype in allergic airway inflammation. Front Immunol. (In press)
- Stadhouders R, **Li BWS**, de Bruijn MJW, Gomez A, Nageswara Rao T, Fehling HJ, van IJcken WFJ, Lim AI, Di Santo JP, Graf T, Hendriks RW. Epigenome analysis implicates ILC2s as key mediators of allergic airway inflammation. J Allergy Clin Immunol. (In press)
- **Li BWS**, de Bruijn MJW, Lukkes M, van Nimwegen M, Bergen IM, GeurtsvanKessel CH, Andeweg A, Rimmelzwaan GF, Hendriks RW. T cells and ILC2s are major effector cells in influenza-induced asthma exacerbation. (Submitted)

PHD PORTFOLIO

CHAPTER 10

Bobby W.S. Li

Erasmus MC department:	Pulmonary Medicine
PhD period:	November 2012 – February 2017
Thesis director:	Prof. Dr. Rudi W. Hendriks

PhD training

In-depth courses

Molecular Immunology (MolMed) – 3.0 ECTS	2013
Mucosal Imunnology (MolMed) – 0.5 ECTS	2013
Workshop on Adobe Photoshop and Illustrator CS6 (MolMed) – 0.3 ECTS	2013
Workshop on Adobe InDesign CS6 (MolMed) – 0.15 ECTS	2013
Microscopic Image Analysis (MolMed) – 0.8 ECTS	2015

(Inter)national conferences attended

MolMed Day (Rotterdam, the Netherlands)	2013
The Great Meeting (Ghent, Belgium)	2013
Asthma and Allergy Conference (Bruges, Belgium)	2013
International Lymphoid Tissue Meeting (Rotterdam, the Netherlands)	2013
NRS Animal Models Symposium (Utrecht, the Netherlands)	2013
Advances in Comparative Pathology (Rotterdam, the Netherlands)	2013
NVVI Lunteren Symposium (Lunteren, the Netherlands)	2014
NVVI Lunteren Symposium (Lunteren, the Netherlands)	2015
NVVI Lunteren Symposium (Lunteren, the Netherlands)	2016

Presentations and posters

NRS Young Investigator Symposium (Amsterdam, the Netherlands) – Poster	2013
NVVI Annual Meeting (Noordwijkerhout, the Netherlands) – Oral	2013
EAACI Winter School on Basic Research in Allergy and Clinical Immunology (Poiana Brasov, Romania) – Oral	2014
International Conference on Lymphocyte Activation & Immune Regulation (Irvine, USA) – Oral	2014
MolMed Day (Rotterdam, the Netherlands) – Poster	2014
RIKEN IMS Summer Program (Yokohama, Japan) – Poster and Oral	2014
Cell Symposium Type 2 Immunity (Bruges, Belgium) – Poster	2014
NVVI Annual Meeting (Kaatsheuvel, the Netherlands) – Oral	2014
MolMed Day (Rotterdam, the Netherlands) – Poster	2015

Coaching and teaching activities

Supervision of Maarten Brem (Master student)	October 2014 – March 2015
Supervision of Dior Beerens (Master student)	January 2015 – August 2015

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CHAPTER 10

The question "Is it finished yet?" is one that every doctoral student inevitably encounters (often frequently) in the final months of writing the thesis. It is with immense delight that I can now answer that question: YES, it is finished! This thesis is a compilation of the work and input of many people and I would like to thank everyone for their contributions to the research described here.

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