



Role of type-2 innate lymphoid cells (ILC2s) in type-2 asthma

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Purpose of review

The purpose of this review is to provide a synthesis of recent discoveries about type-2 innate lymphoid cells, especially, as they relate to the pathogenesis of asthma.

Recent findings

We focused on features and characteristics of type-2 innate lymphoid cells (ILC2s) that distinguish them from other type-2 cells, especially Th2 cells. We collected and reviewed data related to human asthma and airway ILC2s. We examined the concept of ILC2 memory and trained immunity. We also analyzed steroid resistance of ILC2s, which is relevant for steroid-resistant asthma.

Summary

The implications of the findings include an understanding of ILC2 inflammation, and pathways and molecules that can be targeted by biologics and other therapeutic agents for management severe and steroid-resistant asthma

Keywords

bronchoalveolar lavage, GATA nucleotide sequence binding protein 3, type-2 innate lymphoid cell, suppressor of tumorigenesis-2, T helper 2

INTRODUCTION

Basic and translational research related to type-2 innate lymphoid cells (ILC2s) is a rapidly growing field. A growing body of evidence suggests that despite its low abundance this newly identified type-2 immune cell plays a critical role in asthma and other type-2 illnesses. In this review, we concentrate on recent literature that critically examines the features of ILC2s that make them so important for pathogenesis of type-2 asthma.

TYPE-2 ASTHMA

Asthma is a heterogeneous disease. The vast majority of asthma patients are allergic to environmental allergens, which can be detected by skin testing [1^{••}]. These patients are categorized as having allergic asthma due to the type-2 immune response [2^{••},3]. Some skin test-negative 'nonallergic' asthma patients have increased eosinophils in the blood and airways. Eosinophils are another outcome of the type-2 immune response. Hence, type-2 immune cells are generally considered responsible for allergic (eosinophilic and noneosinophilic) and nonallergic eosinophilic asthma [4,5]. It should be noted that despite the skin test positivity and +/- eosinophilia

a significant proportion of allergic patients do not show a dominant type-2 transcriptomic profile in the lung. These patients are classified as having non-T2 (nontype-2) asthma [6^{••}]. Some non-T2 asthma patients have neutrophilic and rarely lymphocytic inflammation but a majority of non-T2 asthma patients have negligible airway inflammation and are classified as having pauci-inflammatory asthma [7].

IMMUNE CELLS IN TYPE-2 ASTHMA

Many immune cells produce the core type-2 cytokines—interleukin (IL)4, IL5 and IL13 and express the major type-2 transcription factor GATA3 [8,9^{••}]. These cells include Th2 cells, ILC2s, natural killer

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KEY POINTS

- ILC2s are the most potent source of type-2 cytokines on a per cell basis.
- ILC2-associated molecules are top asthma susceptibility genes.
- ILC2s are highly responsive to mucosal, chemosensory epithelial, neuroendocrine and neural mediators, which distinguishes them from Th2 cells.
- ILC2s are relatively less sensitive to inhibition by steroids, which is relevant for steroid-resistant asthma.

(NK) cells, NK T cells (NKT cells), cluster of differentiation (CD) 8 T cells, mast cells, basophils, eosinophils and rarely macrophages [10–16]. Th2 cells, ILC2s and the type-2 subtype of NK cells and NKT cells consistently produce all core type-2 cytokines. Other cells require special conditions for production of type-2 cytokines. For example, CD8 T cells require IL4 [17]. Mast cells and basophils require allergen cross-linking of the membrane immunoglobulin E (IgE) receptor. Genetic and/or antibody-mediated depletion of T cells and ILC2s frequently resulted in either a null phenotype or a significantly diminished asthma phenotype in mice. Germline genetic manipulation of type-2 cells in human is currently not applicable for ethical reasons. However, GATA3 knockdown through a DNzyme inhibited allergen-induced asthma (early and late decline in forced expiratory volume in 1 s [FEV1]), and sputum eosinophils and tryptase [18] suggesting a pivotal role of GATA3-expressing cells in allergic asthma. Interestingly, the anti-CD4 antibody keliximab that was designed to eliminate Th2 cells did affect asthma in a small study cohort [19]. On the other hand, Daclizumab, an anti-CD25 (the high affinity IL2 receptor) antibody, significantly improved asthma when compared to placebo in a different study cohort [20]. The latter finding suggests that IL2 dependent immune cells such as ILC2s and NK cells were likely to mediate the effect of Daclizumab. At this point there are no human studies that specifically targeted ILC2s for depletion, primarily because of the incomplete knowledge about ILC2-specific cell surface markers. Most ILC2 surface markers are also expressed by other cells. Genetic deletion of mast cells in the mouse model did not eliminate experimental asthma or other type-2 pathologies. The elimination of eosinophils through the use of antibodies against IL5 or IL5 receptor in humans helped a subgroup of eosinophil-high asthma but did not meaningfully affect the type-2 response. One of the major challenges of the field is to

generate a comprehensive understanding of the specific role played by each type-2 cell in various phenotypes of asthma.

CHARACTERISTICS OF TYPE-2 INNATE LYMPHOID CELLS

ILC2s are defined as lymphoid cells that do not express cell surface markers ('lineage markers') associated with T cells (including NKT and mucosal-associated invariant T cells), B cells, NK cells, macrophages and other myeloid cells [21–25]. Human ILC2s are commonly identified as lineage negative (lin-) chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2)+ interleukin-7 receptor subunit alpha (IL7Ra)+ cells or lin-CRTH2+ CD161+ cells [21,25]. Human lung ILC2s are also identified as lin-ST2+IL7Ra+ cells [26]. The expression of CRTH2, IL7Ra and ST2 on ILC2s is quite dynamic. Their expression level changes in a context-dependent manner. We recently reported the identification two CRTH2-negative nonconventional ILC2 subpopulations—lin-CRTH2-IL7Ra+ and lin-CRTH2-IL7Ra- ILC2s [27*]. Their ILC2 characteristics were documented by the expression of GATA3 and type-2 cytokines (IL5 and IL13). All ILC2s characteristically expressed the transcription factors RAR-related orphan receptor alpha (ROR-α) and B-cell lymphoma/leukemia 11B (Bcl11b). We identified novel ILC2 markers—CD30 (also known as TNFRSF8, is a cell membrane protein of the tumor necrosis factor receptor family and tumor marker) and TNFR2 (tumor necrosis factor receptor 2) that detected both conventional and nonconventional ILC2s [27*]. Similar to human ILC2s, mouse ILC2s were reported to be heterogeneous and contained both ST2+IL7Ra+ and ST2-IL7Ra- populations [28].

DATA SUPPORTING A ROLE FOR TYPE-2 INNATE LYMPHOID CELL IN TYPE-2 ASTHMA

ILC2s produce cytokines and biomolecules that are known to be increased in and important for asthma:

The cytokine profile of ILC2s is largely very similar to that of Th2 cells. However, ILC2s produce some of the core type-2 cytokines (IL4, IL5 and IL13) 5–100-fold higher on a per cell basis when compared with Th2 cells [29]. ILC2s uniquely produce certain biomolecules that are either not produced or produced in negligible quantities by Th2 cells. One of these molecules is prostaglandin D2 (PGD2). The production of PGD2 is of major interest as the PGD2 receptor CRTH2 is expressed at a high level on ILC2s. Thus, the PGD2-CRTH2 axis creates an auto-crine/paracrine pathway for ILC2 activation. CRTH2

is a major therapeutic target. Fevipirant, an oral CRTH2 antagonists inhibited sputum eosinophils in a controlled trial [30]. Of note, tumor-derived PGD2, activating ILC2 and releasing IL13, stimulates monocytic myeloid-derived suppressive cells (M-MDSC) and suppresses the type-1 immune response and tumor immunity [31]. The foregoing may explain the beneficial effect of aspirin in some cancers (e.g. colon cancer). ILC2s express higher levels of CCR1 (C-C chemokine receptor type 1), CXCR3 (C-X-C motif chemokine receptor 3), LTBR (lymphotoxin beta receptor), IL13RA1 (interleukin 13 receptor subunit alpha 1) and IL18R1 (interleukin 18 receptor 1) as compared to Th2 cells [32]. The expression of CXCR3 and IL18R1, which are typically associated with type-1 immune cells, points to plasticity of the ILC2 lineage. Indeed, mouse studies have demonstrated significant interconversion and plasticity of all three ILC lineages [33²²].

THE FREQUENCY OF TYPE-2 INNATE LYMPHOID CELL IS INCREASED IN ASTHMA

Blood studies

Multiple studies reported increased frequency of blood ILC2s in allergic asthma patients as compared to healthy controls [27²,34,35,36²²,37,38,39²,40]. Interestingly, the ILC2 number increased in the airways not only after exposure to allergens in the pollen season but also after infection with rhinovirus [41] in an IL33-dependent manner. The circulating ILC2 frequency was prospectively evaluated in asthmatic patients after treatment with inhaled budesonide and formoterol, which was significantly decreased at 1 and 3 months [42].

Sputum studies

Chen *et al.* reported that total ILC2s and type-2 cytokine+ ILC2s (as well as type-2 cytokine+ CD4T cells) increased in sputum from asthmatic patients after an allergen challenge [43]. The same group [44²²] recently reported an increase in DR3 (death receptor 3)+ ILC2s in sputum from asthma patients. Further, they showed that ILC2s, not Th2 cells, were the primary source of type-2 cytokines in sputum from severe asthma patients with eosinophilia [45].

Bronchoalveolar lavage studies

We demonstrated that both conventional (lin-CRTH2+IL7Ra+) and nonconventional (lin-CRTH2-IL7Ra+ and lin-CRTH2-IL7Ra-) ILC2s were

increased in bronchoalveolar lavage (BAL) from asthma patients as compared to disease controls [27²,46]. The frequency of ILC2s correlated positively with BAL eosinophils and lung function (FEV1). Winkler *et al.* found that the blood ILC2 numbers decreased but the BAL ILC2 numbers increased after an allergen challenge in asthmatic patients. This was associated with an increase in PGD2 and CXCL12 (C-X-C Motif Chemokine Ligand 12) in BAL [47]. The results suggested PGD2- and CXCL12-mediated recruitment of ILC2s from the blood to the airways. Recently, Dhariwal *et al.* reported that asthma patients had elevated levels of BAL ILC2s as compared to controls. The levels were further increased after a viral infection and infection-associated asthma exacerbation [48].

Nasal tissue

Lee *et al.* reported an increased number of ILC2 cells and the ILC2 promoting cytokines IL25, IL 33 and thymic stromal lymphopoietin (TSLP) in nasal tissue derived from patients with severe asthma as compared to nonsevere asthma [49].

Thus, a variety of airway tissue sample (BAL, sputum, nasal tissue) and blood studies demonstrated an increase in ILC2s at baseline, a further increase after an allergen challenge, and a reduction after clinically effective therapeutic interventions in asthma patients. ILC2s correlated with airway eosinophils and the lung function. The foregoing studies provided compelling data suggesting a strong mechanistic association between ILC2s and asthma.

SELECT TYPE-2 INNATE LYMPHOID CELL SURFACE MARKERS ARE TOP ASTHMA SUSCEPTIBILITY GENES

ILC2s express receptors for the mucosal cytokines—IL25, IL33, TL1A (tumor necrosis factor-like cytokine 1A) and TSLP [50–53]. IL25 is primarily produced by gut and lung chemosensory cells—tuft and brush cells, respectively [54,55]. IL33, TL1A and TSLP are produced by many cells including epithelial cells, endothelial cells and myeloid cells (reviewed in [8]). IL33 binds to ST2 (Il1rl1) and potently induces differentiation, maturation and activation of ILC2s. TL1A and TSLP activate ILC2s through TSLPR and DR3, respectively. IL33, ST2 and TSLP are top asthma susceptibility genes. Knockout studies validated the critical role for IL25, IL33, TSLP and TL1A in the mouse model of asthma. Antibodies against TSLP, IL33 and its receptor ST2 underwent clinical trials in asthma. The anti-TSLP antibody Tezepelumab, the anti-IL33 antibody Itepikeimab [56²²] and the anti-ST2 antibody Astegolimab

[57^{***}] showed significant clinical efficacy in controlled trials in asthma. Interestingly, both Tezepelumab and Astegolimab were equally effective in blood eosinophil-high and eosinophil-low asthma. It would be important to know if the blood eosinophil-low asthma patients had eosinophils or other signs of type-2 inflammation in the airways.

NEURAL REGULATION OF TYPE-2 INNATE LYMPHOID CELLS

An increasing body of evidence suggests that there is an intense network of interactions between ILCs and the nervous/neuroendocrine systems. ILC2s uniquely express NMUR, the receptor for neuromedin U (NMU), especially, after stimulation with IL25 [58,59]. NMUR activates ILC2s and potentiates the function of IL33. NMUR is another asthma susceptibility gene. In addition to NMUR, ILC2s express the receptor for CGRP (calcitonin gene-related peptide) and VIP (vasoactive intestinal peptide). CGRP negatively regulates ILC2s. Interestingly, ILC2s produce CGRP, which establishes an autocrine negative regulatory circuit [60]. IL5, a product of type-2 lymphoid cells, stimulates sensory nociceptors to release VIP [61,62]. VIP positively regulates ILC2s and establishes a feed-forward mechanism for type-2 inflammation. The neuropeptide neuromedin B (NMB) is a negative regulator of ILC2s, which is controlled by basophils. In the absence of basophils ILC2s are not inhibited by exogenous NMB [63^{***}]. Basophils induce the expression of the NMB receptors on ILC2s. Many of the foregoing neuropeptides are produced by pulmonary neuroendocrine cells (PNECs) that are located in close proximity to ILC2s near airway branch points [64]. The number of PNECs is increased in asthma.

Adrenergic and cholinergic neurotransmitters are highly relevant for airway function. They control the function of a variety of airway cells including the function of smooth muscles, blood vessels and goblet cells. Recent studies have demonstrated that they also control ILC2s. ILC2s express beta-adrenergic receptors. Beta-adrenergic agonists (both short- and long-acting) negatively regulate ILC2 development and cytokine production [65]. In regard to the cholinergic pathway, ILC2s not only express acetylcholine receptors but also choline acetyltransferase. The latter allows ILC2s to generate their own acetylcholine and maintain an autocrine positive regulation. IL33 and other ILC2 growth factors upregulate choline acetyltransferase. ILC2s that are deficient in choline acetyltransferase poorly respond to parasitic infection [65–67]. The foregoing findings suggest that the currently used beta-adrenergic agonists and anticholinergic agents benefit asthma not only

though their effect on structural cells but also through ILC2s.

TYPE-2 INNATE LYMPHOID CELL MEMORY AND TRAINED IMMUNITY

Memory is a fundamental property of all living entities as it allows adaptation to the ever-changing environment. Persistence of asthma (chronic asthma) is a memory-driven process. The memory of allergen sensitivity is generated and maintained by the adaptive immune cells- T and B cells. Since an increasing body of evidence supports a role for ILC2s in asthma, the question arises if ILC2s form memory. Two groups examined ILC2 memory formation [39^{*,}68]. Martinez-Gonzalez *et al.* reported the generation of memory ILC2s [68]. The authors showed that allergen and IL33-experienced ILC2s persisted in the airway for months, and responded to a different allergen more robustly than nonexperienced ILC2s. Their results suggested an antigen nonspecific memory of prior activation. This is more akin to acquisition of fitness through training. This has also been called trained immunity [69]. The authors examined differential gene expression in their IL33-elicited memory ILC2s. There was an increased expression of the IL25 receptor that persisted for 4 months. We demonstrated that repetitive exposure of Rag1^{-/-} mice to the *Alternaria* allergen extract generated a form of memory that elicited an asthma-like response upon a subthreshold recall challenge 3–15 weeks later [39^{*}]. This memory was associated with lung ICOS (inducible T cell costimulator)+ST2+ILC2s. Genetic, pharmacologic and antibody-mediated inhibition, and adoptive transfer established an essential role for ILC2s in memory-driven asthma. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) demonstrated a distinct epigenetic landscape of memory ILC2s, and identified Bach2 (BTB domain and CNC homolog 2) and AP1 (Activator protein 1: Fos11; FOS Like 1, Fos12; FOS Like 2 and JunD) motifs as major drivers of altered gene accessibility. scRNA-seq, gene knockout and signaling studies suggested that repetitive allergenic stress induced a gene repression program involving Fos11, Fos12, JunD, Nr4a2 (nuclear receptor subfamily 4 group a member 2) and Bach2, and a preparedness program involving Fhl2 (Four and a half LIM domains 2), FosB, Stat6 (signal transducer and activator of transcription 6), Srebf2 (sterol regulatory element-binding protein 2) and MPP7 (membrane palmitoylated protein 7) in memory ILC2s. A mutually regulated balance between these two programs established and maintained memory. The preparedness program (e.g. Fhl2) could be activated with a subthreshold cognate stimulation, which downregulated repressors and activated effector pathways to elicit the memory-

driven phenotype. Of note, the foregoing allergen elicited memory-driven asthma was dependent on IL33. The foregoing study identified ICOS, Fhl2 and Fosl1 as markers of memory ILC2s. We observed increased frequency of ICOS⁺ and Fhl2⁺ ILC2s in the blood from asthma patients suggesting a potential role for memory ILC2s in human asthma [39[¶]]. A recent study identified Fosl1 as the most upregulated transcription factor in BAL ILC2s from asthma patients in an RNA-seq analysis [47,70]. Another study identified Fosl2 and Nr4a2 as top upregulated genes in total BAL cells from severe asthma patients (the National heart, lung, and blood institute Severe asthma research program) cohort [71]. The foregoing studies validated the ATAC-seq findings from mouse memory ILC2s. The data also indicated the presence of memory ILC2s in the airways from asthma patients.

TYPE-2 INNATE LYMPHOID CELLS AND STEROID RESISTANCE

We and other groups reported that airway ILC2s contributed to steroid resistance in asthma [42,44^{¶¶},37]. Dexamethasone inhibited type 2 cytokine production by blood ILC2s stimulated with IL25 and IL33. However, it failed to do so when

ILC2s were stimulated with IL7 and TSLP, two ligands of IL7R- α . Unlike blood ILC2s, BAL ILC2s from asthmatic patients were resistant to dexamethasone. BAL from the asthmatic patients had elevated TSLP, which correlated with steroid resistance of ILC2s. Dexamethasone induced IL7R- α , which amplified STAT5 (Signal transducer and activator of transcription 5) signaling by TSLP. TSLP upregulated steroid resistance-related molecules—MEK (Mitogen-activated protein kinase), c-Fos, ID3 (inhibitor of DNA binding 3), pSTAT3 and pSTAT5. Dexamethasone inhibited c-Fos, ID3 and pSTAT3, but not pSTAT5 and MEK. The MEK inhibitor Trametinib, the JAK-STAT inhibitor Tofacitinib and the STAT5 inhibitor Pimozide reversed steroid resistance of BAL ILC2s. Machida *et al.* recently reported increased number of DR3⁺ ILC2s in sputum from asthma patients [44^{¶¶}]. DR3 is a receptor for the cytokine TL1A. Interestingly, TL1A induced ILC2 cytokine production was inhibited by dexamethasone. This inhibition was lost in presence of TSLP, which was similar to our findings [37]. ILC2s were found to be more resistant to steroids when compared to Th2 cells [39[¶]].

CD45RO is a marker of memory T cells. Ploeg *et al.* observed that nasal ILC2s from asthma patients

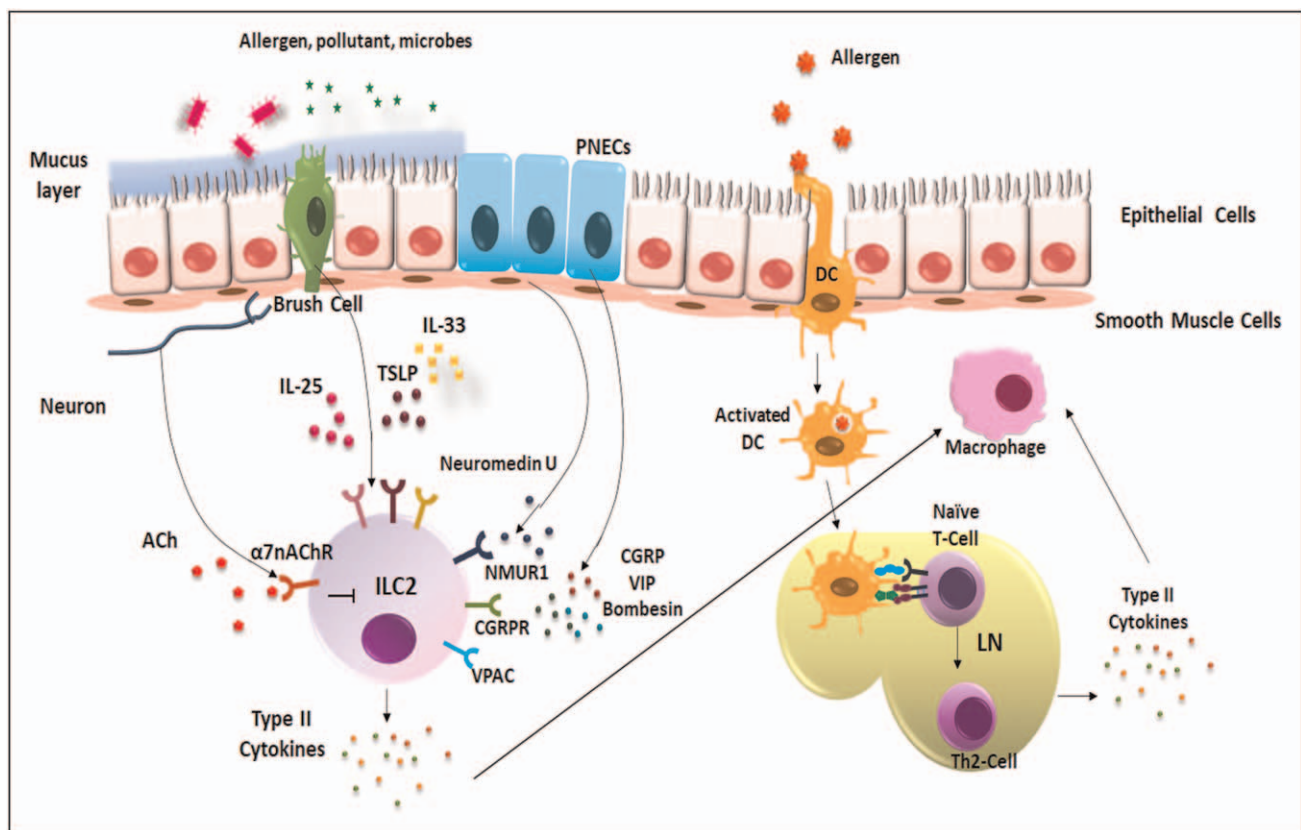


FIGURE 1. Activating intermediaries for ILC2s and Th2 cells and their cellular sources. ILC2s, type-2 innate lymphoid cells; Th2, T helper 2.

expressed CD45RO and CD45RA markers. Interestingly, CD45RO+ memory ILC2s were resistant to dexamethasone whereas CD45RA+ILC2s and T cells were sensitive to dexamethasone [72]. This is similar to our finding of Fhl2+ memory ILC2s being more steroid resistant than Fhl2- ILC2s and Th2 (Lin+ cells) cells [39[¶]]. In aggregate, the foregoing studies suggest that ILC2s, in general, and airway ILC2s, in particular, are relatively steroid resistant. This is not unanticipated as they are innate immune cells, which along with glucocorticoids constitute the stress response of the host. Their mutual antagonism would be detrimental to stress response.

In summary, ILC2s are a potent source of type-2 cytokines. ILC2s and Th2 cells have overlapping functions. There are a few distinguishing features: Unlike Th2 cells, ILC2s are primarily tissue-based and localized to the interface between the environment and the mucosa. Hence, they are ideally positioned to respond to environmental toxicants. Environmental insults including pattern-associated molecular patterns-containing allergens activate mucosal cells, mucosa-associated neuroendocrine cells, and chemosensory epithelial cells. This causes the release of damage-associated molecular patterns [IL33, HMGB1], mucosal cytokines (TSLP, TL1A), neuropeptides (NMU, CGRP, VIP) and neurotransmitters (acetylcholine, adrenergic agonists). ILC2s are specialized to respond to the foregoing mediators. On the other hand, Th2 cells are specialized to respond to inhaled allergens that are engulfed by mucosa associated antigen-presenting cells (dendritic cells and macrophages) and presented in the context of MHC molecules. Hence, the nature of the activating intermediaries and their cellular sources are different for ILC2s and Th2 cells (Fig. 1). This difference ensures maximal capture of the signals generated by a variety of cells at the interface with the environment. Th2 cells produce IL2, which is important for growth and differentiation of ILC2s. ILC2-derived IL13 promotes migration of mucosal dendritic cells to the draining lymph nodes for antigen-presentation to T cells [73]. Both these cell types co-operate to initiate a 'rejection and regeneration' response. The rejection of the environmental intruders and the regeneration of the injured/ altered tissue are designed to restore homeostasis. An overzealous rejection (e.g. smooth muscle constriction) and regeneration (e.g. goblet cell hyperplasia) results in asthma.

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Conflicts of interest

There are no conflicts of interest.

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- of outstanding interest

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