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# Amphiregulin producing ILC2s as a therapeutic target in allergic asthma: For better or for worse

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## Abstract

**Allergic asthma is a common disease characterized by mucus hyper secretion, eosinophil and mast cell recruitment, smooth muscle contraction and airway remodeling. Although allergic asthma was first described as a disease of the adaptive immune system, the focus nowadays is switched towards researching bronchial epithelial cells (BECs) and type 2 innate lymphoid cells (ILC2s). The airway epithelium is the first barrier to allergens, and regulates further immune responses. ILC2s are stimulated by IL-33, IL-25 and TSLP produced by damaged airway epithelial cells, and play a role in inflammation and tissue repair. ILC2s have 2 main phenotypes, a pro-inflammatory phenotype mediated by the production of IL-5, IL-9 and IL-13 on one hand and a tissue repair phenotype mediated by amphiregulin on the other hand. A therapeutic intervention that induces a shift from the IL-5/IL-13 producing phenotype towards an amphiregulin producing phenotype might both inhibit inflammation and stimulate tissue repair in allergic asthma patients. This essay reviews the function of BECs, ILC2s, and amphiregulin in order to investigate whether amphiregulin producing ILCs could function as a new therapeutic target. In addition we will discuss whether more amphiregulin is beneficial for allergic asthma patients due to its role in tissue fibrosis and cancer.**

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## 1. Introduction

Allergic asthma is a common chronic inflammatory disease of the airways in which a patient mounts an inappropriate response to allergens. Asthma is characterized by mucus hyper secretion, eosinophil and mast cell recruitment, smooth muscle contraction and airway remodelling, all of which contribute to bronchoconstriction (1). The symptoms of asthma are reversible, however, when asthma persists over years the inflammation becomes chronic and the lung gets irreversibly damaged. Therapies for asthma, like corticosteroids and β2-adrenoreceptor agonists, do focus on decrease the symptoms. Therapeutics that enhance tissue repair or cure asthma to give tissue a chance to recover are not yet available. Also, the reason why some individuals get allergic asthma and others do not is still not fully understood.

Inhaled allergens such as house dust mites (HDMs), animal dander, plant and tree pollen, and fungal spores are able to activate dendritic cells (DCs) in the lungs. The DCs migrate to lymph nodes, where Th2 cells are induced to differentiate. IL-4 producing Th2 cells induce class switching in B cells leading to IgE production. This IgE can bind to the FceRI receptor on the membrane of mast cells. When the individual is exposed to the allergen again, allergens will bind to the IgE antibodies. Crosslinking of FceRI receptors will activate a signal cascade in the mast cell. The mast cells immediately release histamine and other compounds causing an immediate hypersensitivity reaction. This first, IgE dependent reaction, is rapid and mostly local; histamine from the mast cells induces vasodilatation, vascular leakage, mucus secretion and contraction of smooth muscle cells of the bronchi. Histamine is not the only mediator produced by mast cells: proteases cause tissue damage, lipid mediators like leukotrienes, prostaglandin D2 and platelet-activating factor (PAF) have histamine-like actions and cytokines like TNF , IL-4 and IL-13 play a role too. TNF recruits leukocytes, IL-4 amplifies the Th2 response and IL-13 enhances IgE production of B-cells and stimulates epithelial cells to secrete mucus. Besides the early response, a second late-phase reaction will occur 2-24 hours after the exposure to the allergen. This second late response relays on Th2 cells. The late-phase reaction is characterized by infiltration of eosinophils, neutrophils, basophiles, monocytes and Th cells. Th2 cells produce cytokines such as IL-4 and IL-13 {{58 Kumar, V. 2010}} (2) Prolonged allergic inflammation results in irreversible lung tissue damage characterized by fibroblast proliferation and fibrosis (3).

While allergic asthma is classically considered to be a problem of the adaptive immune system, recently the focus is more on the role of innate immunity in the pathogenesis of allergic asthma. The epithelial barrier was first seen as a physiological barrier only, but is now recognized as an important mediator in regulating tolerance and inflammation(3). The epithelium 'senses' allergens and is able to recruit and activate dendritic cells and ILC2 cells. ILC2 cells have two phenotypes, one able to induce tissue repair (via amphiregulin) and one able to induce further tissue damage by increasing the inflammation (via IL-5 and IL-13). The ILC2s seem to be a heterogeneic population, although discrete subpopulations with different phenotypes have not yet been undisputedly identified. ILC2s are now widely studied, but their role in tissue repair is not investigated well and remains unclear. Which factors influence the phenotype of ILC2 cells, which mechanisms induce ILC2s to shift to the repair phenotype and the role of amphiregulin in this picture are still unknown. The research question of this essay will focus on the question if ILC2s can be shifted towards an amphiregulin producing phenotype and if this shift would induce tissue repair during allergic asthma.

## 2. Role of epithelial cells in allergic asthma

Allergic asthma has been seen as a disease of the adaptive immune system for years, caused by over-active Th2 lymphocytes and a disturbed Th1/Th2 balance. Th2 cells were seen as the main producers of IL-4, IL-5, IL-9 and IL-13. Studies were particularly focusing of the regulation of Th2 by DCs, and not on the activation of DCs (by the epithelium) itself. This view has changed over the last years, and the innate immune system is now known to play a major role in allergic asthma too. The physical barrier of the bronchial epithelium is part of the innate immune system and protects the body from invasive inhaled pathogens. However, the function of the epithelium goes further than being a barrier alone. Bronchial epithelial cells (BECs) are activated by inhaled antigens triggering pattern recognition receptors like protease activated receptors (PARs) and toll like receptors (TLRs) on the cell-membrane. The TLR triggering leads to a Nf-κB signaling cascade, which drives both the production of chemokines and cytokines and activation and recruitment of DCs and ILC2s. This could mean that the interactions between bronchial epithelial cells and DCs or ILC2s are the origin of allergic sensitization putting

epithelial cells center stage in the pathogenesis of allergic asthma (4).

### *2.1 Epithelial cells show loss of barrier function due to loss of functional junctions*

Asthma is associated with airway remodeling and fibrosis. The asthmatic airway shows structural changes in the epithelium like epithelial fragility, goblet cell hyperplasia, increased smooth muscle cells and thickening of the basement membrane (5). Asthma patients have a compromised barrier function of the bronchial epithelium and allergens can get in direct contact with antigen presenting cells like DCs (4, 6, 7). The physiological barrier of the epithelium is dependent on apical tight junctions and adherens junctions, which can be dysfunctional in allergic asthma patients. The involvement of junction proteins in asthma is also confirmed by genome wide association studies.

Genome wide association studies (GWAS) are used to compare frequencies of single nucleotide polymorphisms (SNPs) between a disease group and a healthy control group. SNPs that are more common with a disease might be linked to genes involved with the disease pathogenesis. Those studies help with finding important pathways in the disease. GWAS reported the involvement of CDHR3 (encoding cadherin-related family member 3) in early childhood asthma with severe exacerbations (6). CDHR3 is highly expressed in airway epithelium and belongs to the cadherin family of transmembrane proteins involved in homologous cell adhesion and important for several cellular processes, including epithelial polarity, cell-cell interaction and differentiation (6). A disturbance of this process might have in role in asthma. A second susceptibility gene for asthma found in GWAS is protocadherin-1 (PCDH1) (11,12). PCDH1 is involved with epithelial junctions and expressed in apical adhesion complexes in bronchial epithelial cells. PCDH1 is associated with an increase of bronchial hyperresponsiveness (11). A third asthma associated gene found by GWAS is Orosomucoid like 3 (ORMDL3) (13). ORMDL3 is mainly expressed in bronchial epithelial cells. In mice ORMDL3 expression is known to be induced by allergens (14), however, the role of ORMDL3 in the pathogenesis of asthma is unknown.

Other studies identified reduced expression of E-cadherin and zonula occludens-1 (ZO-1), both important proteins for the stability of cell-cell junctions, in the bronchial epithelium of asthma patients (7-9). In vitro cultures of epithelial cells from asthmatic patients show that down regulation of ZO-1 and E-cadherin is retained ex vivo, indicating the down regulation is caused by a cell-intrinsic mechanism. However, mRNA levels of E-cadherin and ZO-1 are not down regulated compared to healthy controls, indicating that the problem appears after translation(10). This could be problems with post-translational modification, protein stability, or the subcellular location of the proteins. The association between asthma and the proteins ZO-1 and E-cadherin is not confirmed in genome wide association studies (GWAS). Tissues at surface areas that are in contact with the environment, should react fast on environmental changes. Epigenetic changes appear much faster than genetic changes. Therefore might the down regulation of E-cadherin and ZO-1 be caused by epigenetic changes, which is plausible given the location of epithelial cells(10).

Epithelial cells with a delayed barrier function have problems with the prevention of allergens to invade. This epithelial cells get damaged and enhance an inflammatory response by interacting with DCs and ILC2s.

### *2.2 Damaged epithelial cells produce IL-33, IL-25 and TSLP*

The interaction between epithelial cells and immune cells is mainly established by the production of the alarmins IL-25, IL-33 and TSLP. These molecules are released from necrotic epithelial cells, or are secreted after epithelial damage and allergen exposure without cell death (15). Allergens are able to cause breakdown of the epithelial barrier and trigger mostly IL-33 secretion. The cytokines IL-25, IL-33 and TSLP are of great interest because of their ability to activate DCs and ILC2s and stimulate proliferation, survival and cytokine production in ILC2s.

Asthma patients have increased levels of IL-33, IL-25 and TSLP compared to healthy controls (16, 17). The level of IL-33 in serum and sputum is increased in young asthmatic children treated with glucocorticoids (18), and in serum of asthmatic patients without treatment (sputum was not measured) (19) compared to non-asthmatic individuals.

The importance of IL-33 in asthma has also been confirmed in GWAS studies. Both IL-33 and its receptor IL1RL1 show a strong association with asthma and allergic diseases (6, 20, 21). TSLP (20) and the IL-25 receptor (3) are also associated with asthma in GWAS studies however the involvement of those genes was not reproducible in all GWAS studies. Another GWAS identified epithelial gene involved in asthma is GSDMB (6,21). GSDMB belongs to the gasdermin protein family.

Gasdermin genes are implicated in the regulation of apoptosis in epithelial cells. Their involvement in allergic asthma could indicate a disturbed apoptotic cell regulation. Increased apoptosis could generate more anti-inflammatory cytokine production, caused by the engulfment of apoptotic cells by surrounding epithelial cells, as shown by Juncadella and colleagues in 2013 (22). Polymorphisms in the GSDMB gene could cause delayed apoptosis, resulting in less anti-inflammatory cytokine production by surrounding epithelial cells. Damaged epithelial cells could show enhanced necrosis instead, and this would lead to more inflammatory cytokine release. However, the exact mechanism of GSDMB contributing in allergic asthma is not understood.

In conclusion, Epithelial cells play a key role in allergic asthma not only by the physiological barrier they maintain, but also by the release of IL-25, IL-33 and TSLP after allergen exposure. Those cytokines are very important due to their ability to activate mainly ILC2s. With the possibility of activating ILC2s, the epithelium has an important function in regulation of tolerance to allergens.

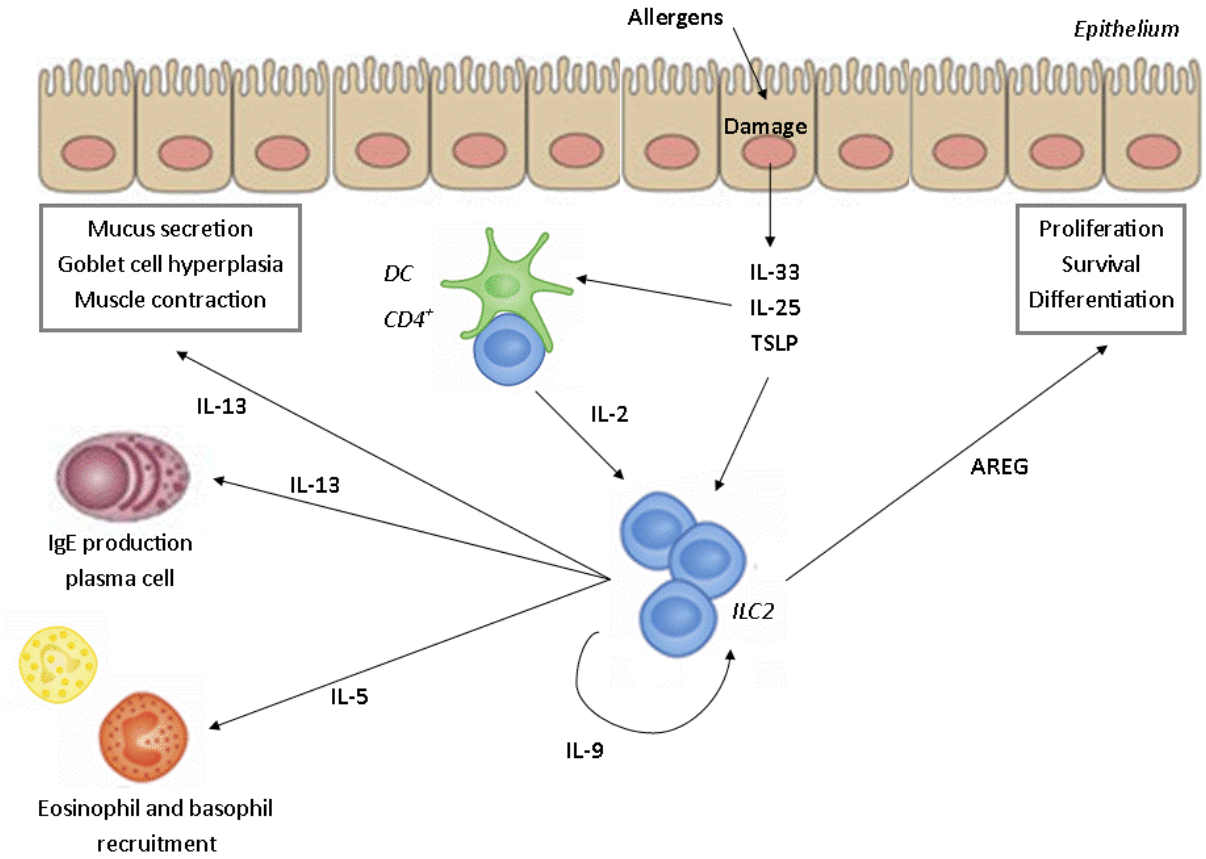


**Figure 1, Role of ILC2s in allergic asthma. Allergens stimulate the epithelial cells to produce IL-33, IL-25 and TSLP which activate dendritic cells (DCs) and type 2 innate lymphoid cells (ILC2s). DCs can activate CD4+ cells that produce IL-2. IL-2 can also stimulate ILC2s. ILC2s produce IL-5 and IL-13 that contribute to inflammation and asthmatic symptoms due to eosinophil and basophil recruitment, IgE production, mucus secretion, goblet cell hyperplasia and muscle contraction. ILC2 produce autocrine IL-9 to stimulate cell survival, and amphiregulin (AREG) that stimulates proliferation, survival and differentiation in epithelial cells, smooth muscle cells and fibroblasts. Picture composed from** (1, 3, 16).

## 3. Role of type 2 innate lymphoid cells in allergic asthma

Where earlier the T cells (especially the Th2 cells) were considered as the center of immune regulation, the perception changed towards dendritic cells and nowadays towards innate lymphoid cells (ILC). The ILC family consists of a group of cytokine producing cells with a lymphoid morphology and the lack of antigen-specific receptors. There are three subcategories of ILC identified: type 1 ILCs, type 2 ILCs and type 3 ILCs with all a different function and cytokine expression. Here, we will only focus on type 2 ILCs (ILC2s) because of their prominent role in asthma and the type 2 response. ILC2s mature under control of RORα and GATA3 from progenitors in the bone marrow. ILC2s play a role in immunity, inflammation, tissue remodeling and the pathogenesis of allergy and asthma (1, 23). ILC2s are present at mucosal surfaces like the bronchial

epithelium and are defined by their ability to produce type-2 cytokines (IL-5, IL-9, IL-13) during allergic airway inflammation. (15, 24, 25). This type 2 immune response is necessary to facilitate B-cell class switching to IgE production and to recruit and activate mast cells (MCs), basophils, and eosinophils. Type 2 cytokines are also able to cause goblet-cell hyperplasia, mucus production and contribute to smooth muscle contraction (17). A more unknown compound also produced by ILC2s is the EGFR ligand amphiregulin, a growth factor involved in tissue repair. See figure 1.

ILC2s can contribute to both tissue damage and tissue repair after the clearance of parasites, like helminths, from the epithelium. Parasites cause significant damage to bronchial epithelial cells. ILC2s are able to enhance epithelial proliferation via amphiregulin thereby restoring the epithelial barrier. ILCs are also able to enhance type-2 inflammation via IL-5, IL-9 and IL-13, thereby attracting and activating eosinophils (IL-5) and enhancing mucus secretion (IL-13), draining the parasites from the mucosal surfaces (1). The functions of ILC2s can thus be divided in two phenotypes; one characterized by pro-inflammatory activity mediated by IL-5, IL-9 and IL-13 and one characterized by promoting epithelial repair mediated by amphiregulin.

### *3.1 ILC2s contribute to allergic inflammation*

ILC2s contribute to allergic inflammation by the production of pro-inflammatory cytokines as a reaction on IL-33, IL-25 and TSLP produced by damaged epithelial cells (see figure 1 and 2). The pro-inflammatory cytokines IL-5 and IL-13 contribute to allergic inflammation and levels of the cytokines are both elevated in sputum and serum of asthma patients (26). IL-5 recruits eosinophils to the side of inflammation and stimulates eosinophil maturation and differentiation (27). Eosinophils are not present in healthy lungs, and the presence of eosinophils is a hallmark of asthma. Eosinophils enhance inflammation by the release of inflammatory mediators like reactive oxygen species and cytokines. Eosinophils also contribute to tissue remodeling during asthma (2).

IL-13 has several inflammatory actions, including disruption of the epithelial barrier and airway remodelling leading to fibrosis (4). IL-13 acts on smooth muscle cells leading to enhanced sensitivity for bronchoconstriction and on fibroblasts inducing the production of extracellular matrix. IL-13 stimulates goblet cells to produce mucus, induces goblet cell hyperplasia and induces airway hyperresponsiveness (29). IL-13 also facilitates IgE production in B-cells (3). The importance of IL-13 is further established by the fact that IL-13 deficient mice do not develop bronchial hyperreactivity reactions nor goblet cell hyperplasia. (30)

Another process induced by IL-13 is the activation of alternative activated macrophages (AAMs). Classically activated macrophages (CAMs) are activated by interferon-γ (IFN-γ). AAMs are activated by IL-13 and IL-4. Whereas CAMs have a pro-inflammatory phenotype, AAMs produce more anti-inflammatory and tissue repair factors (31). On the other hand, AAMs also produce chemokines that recruit and activate eosinophils, mast cells, and basophils. Furthermore, prolonged IL-13 expression promotes excessive fibrosis (32). AAMs also produce IL-4 and IL-13 (33). IL-13 activates AAMs, that produce more IL-13, and will activate even more AAMs thereby generating a positive feedback loop that further enhances inflammation and tissue damage mediated by the other pro-inflammatory actions of IL-4 and IL-13.

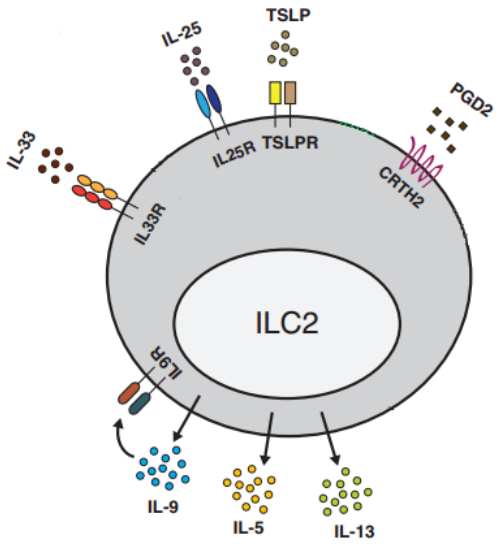


**Figure 2, source: Activation of ILC2s by IL-33, IL-25, TSLP and PGD2 stimulated the production of IL-9, IL-5 and IL-13. Modified from:** (28)

### *3.2 ILC2s are able to stimulate tissue repair*

The tissue repair function of ILC2s was first described by Monticelli *et al.,* in 2011. These authors reported that the loss of epithelial barrier function induced by viral infection in healthy mice was exaggerated after depletion of ILC2 cells or after IL-33 receptor blockade. Adding ILC2 cells back to mice with influenza induced epithelial damage restored the barrier function of the airway epithelium as measured by a higher mean body temperature, a restored oxygen saturation level and regions with epithelial proliferation. These data indicate that ILC2s and the IL-33 pathway can induce an epithelial repair mechanism in mice after severe

influenza induced epithelial damage. The authors investigated by which mechanism the airway epithelium was restored by ILC2s and identified an upregulation of amphiregulin in ILC2 cells after influenza infection. Exogenous amphiregulin was able to restore the epithelial barrier after the influenza induced damage as well and was found to induce this repair via the EGFR receptor on epithelial cells. This means that repair of a healthy airway epithelial tissue upon infection with an influenza virus inducing severe epithelial damage is likely mediated by amphiregulin-producing ILC2 cells activated by IL-33 released from damaged bronchial epithelial cells (34). Later the importance of autocrine production of IL-9 for ILC2 cell survival was demonstrated. In the absence of IL-9, ILC2s showed reduced IL-5 and IL-13 expression and reduced viability, reduced amphiregulin expression and impaired tissue repair (25).

## 4. The different faces of amphiregulin

Amphiregulin is an epidermal growth factor receptor (EGFR) ligand. Amphiregulin can act as an autocrine or paracrine factor after secretion. The expression and secretion of amphiregulin is induced by many stimuli including inflammatory lipids, cytokines, hormones and growth factors. Amphiregulin is expressed in many tissues, Including the colon, lungs, hearth and ovaries. Amphiregulin is expressed by several immune cells and epithelial cells. Amphiregulin stimulates cell survival, cell proliferation and cell motility in different cell types including epithelial cells, fibroblasts and immune cells like dendritic cells, neutrophils, mast cells and T-lymphocytes (35, 36). Amphiregulin activates a complex network of intracellular pathways, including Ras/MAPK, PI3K/AKT, PLCγ and STAT signaling (37).

Amphiregulin is synthesized as a type I trans-membrane protein (Pro-AREG). Amphiregulin becomes soluble after ectodomain shedding of pro-AREG by a membrane enzyme known as ADAM-17 (35). Since amphiregulin is released from the cell surface after cleavage, ADAM17 is responsible for the release of amphiregulin (39). ADAM-17 regulates the shedding of a wide variety of matrix-bound or cell surface proteins such as cytokines, cytokine receptors and adhesion proteins (38). ADAM-17 is expressed in a variety of lung cells such as bronchial epithelial cells, vascular smooth muscle cells, and macrophages. However, the exact role of ADAM17 in regulation of amphiregulin cleavage during asthma or other inflammatory diseases has not been evaluated into any detail.

### *4.1 Amphiregulin stimulates tissue repair*

As mentioned before, Monticelli *et al.*, were the first to link amphiregulin to a tissue repair function after an influenza induced lung infection (34). J. Fukumoto and colleagues showed an increased expression of amphiregulin during bleomycin-induced pneumopathy in mice. After administration of amphiregulin the survival of the mice improved and inflammation and fibrosis decreased in lung tissue (40). Several studies have also observed a role for amphireregulin in epithelial repair, although this function was not always recognized. Amphiregulin stimulation induces proliferation of bronchial epithelial cells and smooth muscle cells (41). Lemjabbar *et al.*, showed an amphiregulin dependent bronchial epithelial cell proliferation after cigarette smoke stimulation (42) which the authors linked to lung cancer, not considering a function in tissue repair. Amphiregulin is known to play a role in wound healing in other tissues as well. In dermatitis amphiregulin stimulates keratinocyte proliferation. (17).

Large quantities of amphiregulin can also activate Treg cells, which contribute to an immune-suppressive environment (17, 35) caused by the immune suppressive function of Tregs. Tregs have a major role in regulation of tolerance and allergic reactions by direct inhibiting the activation of Th2 cells and their cytokines, and suppress the production IgE (43). Tregs are also able to directly suppress mast cells, eosinophils and basophils and therefore inhibit the whole inflammatory response. The effect of Tregs on ILC2s is unknown.

### *4.2 Amphiregulin contributes to tissue fibrosis and plays a role in cancer.*

Amphiregulin is not only involved in tissue repair mechanisms, but also in tissue fibrosis and even in cancer. When a repair response is very intense or chronic, fibrotic tissue might develop. Amphiregulin levels are significantly increased in sputum and the bronchial epithelia from asthmatic patients (patients with exacerbation in the preceding month were excluded from the study)(44), and this excessive amount of amphiregulin might contribute to fibrosis (35). The source of the amphiregulin is unclear, it could be produced by ILC2s, but also by other amphiregulin producing cells like the epithelium. Amphiregulin expression is up-regulated in a wide variety of tumors including epithelial tumors in the lungs. Due to the cell survival and proliferation inducing capacity, amphiregulin can act as a proto-oncogene (35). In two human ovarian cancer cell lines, amphiregulin acted as

a down regulator for E-cadherin expression by interfering with ERK1/2 and AKT pathways (45). Several studies have confirmed the role of amphiregulin in tumorigenesis, such as self-sufficiency in generating growth signals, limitless replicative potential, tissue invasion and metastasis, angiogenesis, and resistance to apoptosis (37).

## 5. Discussion: amphiregulin producing ILC2 as a therapeutic target

Patients with allergic asthma are now treated with therapeutics developed in the 1970s; corticosteroids to decrease inflammation and β2-adrenoreceptor agonists to induce smooth muscle relaxation (46). Those treatments help most patients to control their symptoms, but does not cure the disease. Asthma in particular is challenging to treat due to the heterogeneous character of the disease (26).

Allergens cause epithelial stress and damage, and allergic asthmatic patients with an impaired epithelial barrier function and enhanced apoptosis suffer from more epithelial damage compared to healthy controls. The more epithelial damage, the more IL-33, IL-25 and TSLP is produced. IL-33, IL-25 and TSLP activate ILC2s, which have a function in both inflammation (mediated by IL-5, IL-13) and tissue repair (mediated by amphiregulin). IL-33 is the main ILC2 activator and evokes the strongest cytokine response. When considering ILC2s as a therapeutic target, the phenotype of ILC2s should be shifted from an inflammatory towards a repair function in order to diminish inflammatory symptoms and enhance tissue repair.

### *5.1 Regulation of the ILC2 phenotype is not well understood*

The regulation of the phenotype of ILC2s is not well understood. There are studies trying to unravel the mechanisms behind the production of IL-5 and IL-13 by ILC2s, but those studies mostly lack measures of the production of amphiregulin. For instance, inhibition of prostaglandin D2 (PGD2) decreases the production of IL-4, IL-9, IL-5 and IL-13 (25);IL-2 and IL-7 induce IL-5, IL-9 and IL-13 expression (17, 26) and the tumor necrosis factor (TNF) superfamily member TL1A is able to induce type 2 cytokine production in ILC2s. However, the influence of PGD2, IL-2, IL-7 and TL1A on amphiregulin production was not tested. Whether those factors can cause a shift in the phenotype of ILC2 is therefore unknown.

Not only cytokines are able to regulate ILC2s. ILCs express the killer cell lectin-like receptor G1 (KLRG1), which can bind to cadherins including E-cadherin. The interaction between E-cadherin and the KLRG1 on ILC2s down regulates both cytokine expression and the production of amphiregulin (17, 25). This interaction is also present in mature NK cells and differentiated T cells, which leads to an inhibition of functional responses in those lymphocytes as well (47). The interaction of KLRG1 with cadherins leading to a delayed lymphocyte response has been suggested to function as a mechanism towards epithelia repair in order to prevent autoimmunity (47, 48). This mechanism could play a role during allergic asthma too. During asthma the barrier function of the epithelium is compromised and E-cadherin expression is reduced. This could lead to a decreased interaction between KLRG1 and E-cadherin and a decreased inhibition of bound lymphocytes. The specific role of KLRG1—E-cadherin interaction during allergic asthma is unknown.

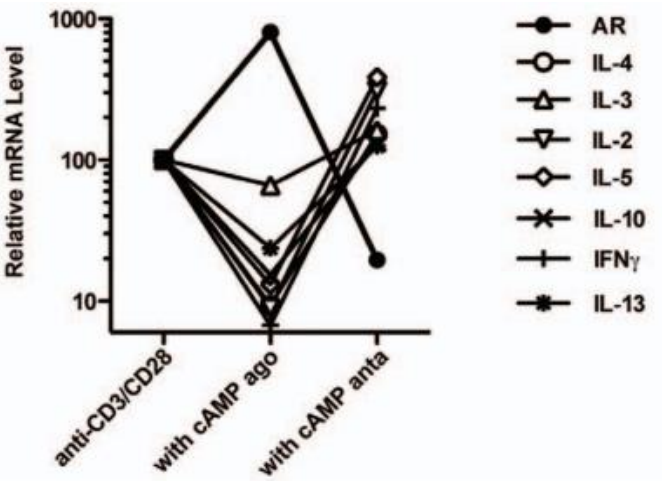


**Figure 3. Regulation of amphiregulin (AR) and cytokine expression in T-cell subsets by cAMP. cAPM ago – cAMP aginist to induce cAMP signaling, cAMP anta = cAMP antagonist to inhibit cAMP signaling. Figure shows a shift towards amphiregulin production after cAMP stimulation, and a shift towards cytokine expression after inhibiting cAMP signaling. Source:** (36)

Although studies about the regulation of the production of amphiregulin in ILC2s are limited, Yilin Qi *et al.*, report about the regulation of amphiregulin in human T cell subsets. They report expression of amphiregulin in naive and memory CD4 and CD8 T cells, Th1 and Th2 in vitro T cell lines, and subsets of memory CD4 T cells. Amphiregulin expression was induced by T cell receptor stimulation, enhanced by cAMP and protein kinase A (PKA) increasing compounds like prostaglandin E2 (PGE2) and adenosine and inhibited by blocking the cAMP/PKA pathway. Enhancing

cAMP/PKA signaling led to more amphiregulin expression while reducing synthesis of IL-4, IL-5 and IL-13. See figure 3. These findings suggest that amphiregulin synthesis is regulated by local environmental signals and not by pre-commitment of the T cell subset.

The cAMP elevation by PGE2 and adenosine was established by respectively G protein-coupled receptors E2 and E4 and A2A receptor signaling (36). PGE2 is produced by activated macrophages and adenosine is released from necrotic cells, so both compounds are present during allergic inflammation. Neither the E2, E4 nor the A2A receptor signaling is studied yet in ILC2s. It would be very interesting to investigate whether this signaling pathway works the same for ILC2s, and if PGE2 and adenosine can induce a phenotypic shift towards amphiregulin production and away from IL-5/IL-13 production.

### *5.2 Other therapeutic targets interfering with amphiregulin production*

Amphiregulin production is not only dependent on the expression level in ILC2s. This means that there are more therapeutic targets available to interfere with the available amount of amphiregulin on the site of inflammation Besides ILC2s there are more cell types able to produce amphiregulin. T-cell subsets, mast cells, basophils and bronchial epithelial cells are also known to produce amphiregulin in asthma patients. Here, the production of amphiregulin is regulated by asthma-related compounds including fine particulate matter IgE, IL-3, IL-5 and histamine (35, 36, 49-51). Allergic asthma patients have higher numbers of basophils able to produce amphiregulin compared with controls (52) and mast cells of asthma patients have a upregulated expression of amphiregulin compared to healthy controls (51). For the upregulation of amphiregulin as a therapeutic intervention more cell types than ILC2s can be considered. Attention should be paid to the effect of amphiregulin produced by other cell types. Cell types where the production of amphiregulin is coupled to pro-inflammatory cytokine release could induce more inflammation and fibrosis than actual tissue repair.

Another possible therapeutic target is ADAM-17. By the ectodomain shedding of pro-amphiregulin to amphiregulin, ADAM-17 plays a key role in regulation the amount of bioavailable amphiregulin. ADAM-17 is expressed on a variety of lung cells such as bronchial epithelial cells, vascular smooth muscle cells, and macrophages, but the expression in ILC2s is unknown. To evaluate ADAM-17 as a therapeutic target, the mechanisms behind ADAM-17 expression and function need to be fully understood. ADAM-17 cleaves more cell surface proteins such as cytokines, cytokine receptors and adhesion proteins and interfering with this process could induce negative side-effects (38).

Inhibition of IL-5 and IL-13 by lebrikizumab (anti- IL-13) and mepolizumab (anti-IL-5) show promising results in humans (26, 53) and blocking IL-33 signaling results in a dampening of type 2 bronchial inflammation in mice (26). Attenuation of ILC2s might also be established by KLRG1 agonists. Combination therapies of inhibiting the pro-inflammatory cytokine release from ILC2s and a upregulation of amphiregulin in cell types other than ILC2s can also be considered as a new therapeutic intervention.

Another therapeutic target could be found in the development of ILC2s. The expression of RORα and GATA3 is essential for the development of functional ILC2s. RORα expression is found in ILC2s only, and could therefore function as a biologic marker and a therapeutic target. (26)

### *5.3 Dealing with the different faces of amphiregulin*

Since amphiregulin is involved in tissue repair after epithelial damage, enhancing the amount of amphiregulin in the bronchial epithelial could be beneficial for asthmatic patients. However, the answer is not that simple. Amphiregulin levels are already significantly increased in sputum and the bronchial epithelia from asthmatic patients. So, the amount of amphiregulin does not seem to be the problem, but studies confirming this hypothesis lack. On the other side, J. Fukumoto and collaeges showed that administration of amphiregulin improved the inflammation and decreased fibrosis in lung tissue after bleomycin-induced pneumopathy in mice (40). So, maybe amphiregulin is only able to repair tissue in certain circumstances. Which circumstances these are, is not clear.

When the amount of amphiregulin is not the problem, then what is? Amphiregulin can be produced by epithelial cells an dILC2s, however, the source of amphiregulin during asthma related tissue damage is not studied. There could be a difference in ILC2 derived amphiregulin and epithelial cell derived amphiregulin. This difference would not be the molecular structure, but the place and time the amphiregulin is secreted. It would be very interesting to investigate whether asthmatic patients suffer from an amphiregulin response at the wrong time and at the wrong place. In asthmatic patients ILC2s could function as a source of mainly IL-5 and IL-13, while the epithelium is secreting

amphiregulin in order to enhance tissue repair. This situation might not be very well regulated, resulting in both inflammation and fibrosis. Another option is that the epithelial cells are not able to respond in a way of tissue repair in asthmatic patients. And when myofibroblasts and other cell types involved in tissue remodeling are, amphiregulin might only induce extracellular matrix deposition and smooth muscle cell proliferation leading to tissue fibrosis. As mentioned before, excessive amphiregulin is linked to fibrosis and can act as a proto-oncogene. Amphiregulin is also involved in cancer treatment resistance (37). From this point of view, enhancing the amount of amphiregulin might not be a good idea after all.

Studies comparing the response of allergic asthmatic patients versus healthy controls on epithelial damage are necessary to unravel this questions.

## 6. Conclusion

After the discovery of ILC2s the view of regulation of type-2 inflammations has changed remarkably. ILC2s are stimulated by IL-33, IL-25 and TSLP produced by damaged epithelial cells, and play a role in inflammation and tissue repair. ILC2s have 2 main phenotypes, pro-inflammatory mediated by the production of IL-5, IL-9 and IL-13 on one hand and tissue repair mediated by amphiregulin on the other hand. Studies assessing the regulation of the phenotype of ILC2s are limited, focus mainly on IL-5, IL-9 and IL-13 cytokine production and do not include amphiregulin measurements. Therefore it is unknown whether ILC2s can be stimulated towards a more amphiregulin producing phenotype. Yilin Qi *et al*., report that amphiregulin in human T cell subsets can be upregulated by enhancing cAMP and PKA, while reducing the synthesis of IL-4, IL-5 and IL-13. This mechanism is not studied in ILC2s yet, but might yield the same results. More research needs to be done to unravel the phenotypic regulation of ILC2s and possible therapeutic targets interfering with this phenotype. However, it can be questioned whether more amphiregulin is beneficial for allergic asthma patients, especially when not well regulated, due to its role in tissue fibrosis and cancer.

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