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Effects of type 2 inflammation on epithelial Wnt signaling and TGM2 mediated leukotriene production

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Changes of the Wnt signaling in bronchial epithelial cells can affect the immune response in asthma

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European Congress of Immunology, Vienna, Austria, September 2015

Abbreviations

5-hydroyperoxyeicosatetraenoic acid	5-HPETE
5-lipoxygenase	5-LO
Adenomatous polyposis coli protein	APC
Airway hyperresponsiveness	AHR
B cell-activating factor of TNF family	BAFF
β2-adrenergic receptor	β2AR
Bronchial epithelial basal medium	BEBM
Bronchial epithelial growth medium	BEGM
Bronchoalveolar lavage fluid	BALF
Calmodulin-dependent kinase II	CamKII
Casein kinase I	CKI
Chemokine ligand	CCL
c-Jun N-terminal kinase	JNK
Conditioned media	CM
Confidence interval	CI
Cycline adenosine monophosphate	cAMP
Cystamine	Cys
Cysteinyl leukotrienes	cysLT
Cytotoxicity detection kit	LDH assay
Dendritic cell	DC

Dishevelled	DVL
DVL associated activator of morphogenesis	Daam
Enzyme-linked immunosorbent assay	ELISA
Epidermal growth factor	EGF
Epithelial-mesenchymal transition	EMT
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Fluticasone propionate	FP
Frizzled receptors	FZD
GATA binding protein 3	GATA3
Glucocorticoid receptor	GR
Glucocorticoid responsive element	GRE
Glycogen synthase kinase-3β	GSK-3β
Granulocyte-macrophage colony-stimulating factor	GM-CSF
Group IVA cytosolic phospholipase A2	cPLA2α
Histone deacetylase-2	HDAC2
House dust mite extract	HDM
Immunofluorescence	IF
Immunoglobulin E	IgE
Immunohistochemistry	IHC

Innate lymphoid type 2 cell	ILC2
Interleukin	IL
Leukotriene	LT
Lipopolysaccharides	LPS
Lipoprotein receptor-related protein	LRP
Long-acting β2-agonist	LABA
Lymphoid enhancer-binding factor	LEF
Magnetic cell sorting	MACS
Major basic protein	MBP
Mitogen-activated protein kinase	MAPK
Monocyte derived macrophages	MDM
Monodansylcadaverin	MDC
Normal human bronchial epithelial cell	NHBE
Nuclear factor of activated T cells	NFAT
Nucleotide-binding oligomerization domain-like receptor	NOD-like receptor
Passage	p
Pattern recognition receptor	PRR
Peripheral blood mononuclear cell	PBMC
Peroxisome proliferator-activated receptor	PPAR
Phosphatidylinositol 3-kinase	PI3K

Phospholipase C	PLC
Planar cell polarity	PCP
Protease-activated receptor	PAR
Proteinkinase C	PKC
Pyrimidinergic nucleotide	P2Y
Quantitative real-time PCR	qPCR
Reactive oxygen species	ROS
Rho-associated kinase	ROCK
Secreted phospholipase A2 group X	sPLA2-X
Seven-transmembrane G protein-coupled receptor	GPCR
Signal transducer and activator of transcription	STAT
Smooth muscle cell	SMC
T cell factor	TCF
T helper type 2	Th2
Thymic stromal lymphopoietin	TSLP
Toll-like receptor	TLR
Transforming growth factor β	TGFβ
Transglutaminase 2	TGM2
Tumor necrosis factor	TNF
Vanilloid transient receptor potential v1 receptor	TRPV1

Vascular endothelial growth factor	VEGF

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1 Introduction

1.1 Inflammation

The term inflammation comes from the latin word *inflammatio*, meaning aflame, and describes a physiological process to protect the organism against injuries, infections or toxins. The five hallmarks of inflammation are pain, heat, redness, swelling and loss of function. If a harmful stimulus occurs, this can be sensed by immune cells or structural cells, which release inflammatory mediators responsible for the pain. These mediators lead to the recruitment of further immune cells. Therefore the blood flow towards the inflamed site is increased resulting in redness and heat of the affected area. Further the blood vessels get leaky to allow the influx of the recruited immune cells into the tissue, which also enables fluid to come in, resulting in swelling. Inflammation can be associated by the loss of function of affected areas either due to pain or exaggerated swelling. Normally the inflammation is terminated resulting in repair of the affected areas. However, in cases of recurring inflammation or chronic inflammation the failing repair mechanism can result in structural changes. Alterations of the structure or composition of tissues are referred to as tissue remodeling.

1.2 Allergic asthma

1.2.1 Prevalence and disease pattern of allergic asthma

Allergic asthma is a chronic disease with increasing prevalence. It is estimated that around 300 million people worldwide suffer from asthma and in about 50% of the asthmatic adults the disease is the result of allergic sensitization (WHO 2007, GINA 2011). Asthma patients suffer from recurrent episodes of wheezing, breathlessness, chest tightness and cough. These symptoms are caused by airway hyperresponsiveness (AHR), which is associated with a partly reversible airflow limitation, that either resolves spontaneously or under treatment (GINA 2011).

Allergic asthma is driven by an immune response within the lower airways to inhaled allergens, such as plant pollen, animal dander or house dust mite extract (HDM). In non-sensitized persons contact of the respiratory tract with allergens is well tolerated

by the immune system. However, in sensitized persons the subsequent chronic inflammation in the airways, particularly of the bronchi and the respiratory mucosa, results in the hyperreactivity of the airways. This hyperreactivity leads to stronger symptoms even to non-allergic stimuli. The inflammation also induces enhanced mucus production supporting the airflow limitation (Janeway et al. 2001). The bronchi of patients with asthma are characterized by immune cell infiltrates, mucus plugs and enhanced smooth muscle mass (Figure 1).

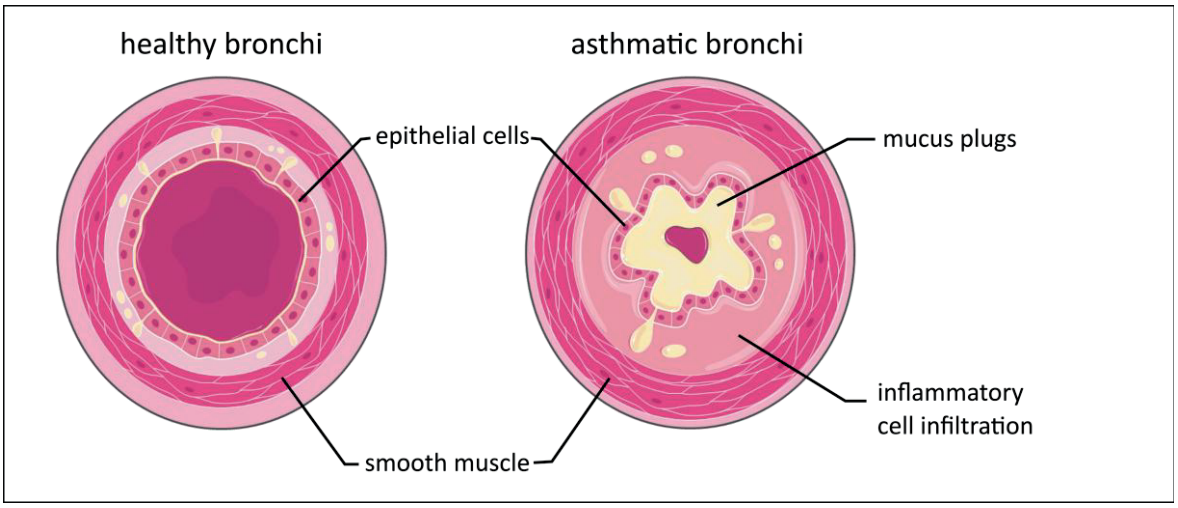

Figure 1: Pathophysiology of allergic asthma. Cross-section of bronchi from healthy and asthmatics as schematic. Asthmatic bronchi show signs of immune cell infiltrates, mucus plugs and smooth muscle hyperplasia. Graphics of bronchi were obtained and adapted from the powerpoint image bank from servier (Servier 2016).

1.2.2 Underlying immune mechanism of allergic asthma

The inflammatory process of allergic asthma is in most cases characterized by a T helper type 2 (Th2) cell dominated immune response, production of allergen-specific immunoglobulin E (IgE) and eosinophilia.

When an allergen encounters the airway mucosa, parts of it can trigger pattern recognition receptors on bronchial epithelial cells. These cells start to secrete chemokine ligand 20 (CCL20), granulocyte-macrophage colony-stimulating factor (GM-CSF), thymic stromal lymphopoietin (TSLP), interleukin (IL)-25 and IL-33 that recruit and activate antigen presenting cells, mainly dendritic cells (Hammad et al.

2009). Activated dendritic cells (DCs) take up allergens through the epithelial layer and migrate to the draining lymph nodes (Blank et al. 2007).

In the draining lymph nodes DCs present the allergen to naïve T cells. With the help of IL-4 secreting basophils, DCs induce allergen-specific Th2 cells (Yoshimoto et al. 2009, Hammad et al. 2010). While a part of the Th2 cells infiltrate the airway tissue, another part of the cells stay in the local lymph nodes and activate B cells to produce allergen-specific IgE (Del Prete et al. 1988, Punnonen et al. 1993). Th2 cells that infiltrated the airways have various functions. The Th2 cytokines IL-4, IL-5 and IL-13 in synergy with epithelial derived CCL11, CCL17, GM-CSF, TSLP, IL-8, IL-25 and IL-33 mediate recruitment and activation of basophils, Th2 cells, innate lymphoid type 2 cells (ILC2s), eosinophils, mast cells and neutrophils (see 1.2.3, 1.2.4 and Figure 2).

All these cells are able to infiltrate the airways and are responsible for prolonging the inflammation. Mast cells bind IgE to their cell surface by Fcε receptor. When the mast cell encounters an allergen, the allergen can bind to several IgE molecules on the mast cell surface. This crosslinking activates the mast cells to release granules and secrete proteases, histamine, lipid mediators, such as leukotrienes and prostaglandins, and several cytokines (Hart 2001). The activation of mast cells can occur independently of an inflammatory response in a sensitized person. There mast cells have already bound allergen specific IgE on their surface and the cells can then directly be activated by an allergen resulting in an immediate hypersensitivity response (Galli and Tsai 2012).

While mast cells are responsible for the acute response to an allergen encounter after sensitization, eosinophils are thought to mediate the more chronic inflammatory response. Like mast cells eosinophils can release granules containing highly basic proteins, such as the major basic protein (MBP), and secrete several cytokines and chemokines. They are an important source transforming growth factor β (TGFβ) and pro-inflammatory leukotrienes (Hogan et al. 2008).

If the inflammatory process is not resolved, infiltrated immune cells will drive further inflammatory cell infiltration and activation resulting in chronic inflammation. Histamine, from mast cells, as well as leukotrienes, from mast cells and eosinophils, induce the constriction of the airway. And proteases, from mast cells, and MPB, from

eosinophils, are leading to damage of the airways (Hart 2001, Hogan et al. 2008). Finally misguided repair processes and the ongoing inflammation lead to disease progression.

1.2.3 The role of lung epithelial cells in asthma – from blocker to playmaker

The epithelial layer of the bronchi consists of four major cell types: ciliated cells, goblet cells, club cells and basal cells. Ciliated cells make up 50% of the epithelial cells and their apical surface is covered with cilia for mucus transport. Goblet cells are secretory cells responsible for mucus production. Another secretory cell type is club cells that produce surfactant. Basal cells are not only progenitor cells of the other cells types, but are also responsible for attachment of the epithelium to the basement membrane and are able to secrete bioactive compounds at their basal side. The epithelial layer forms a tight physical barrier function, which normally should hinder pathogen or antigen uptake in the underlying tissue (Knight and Holgate 2003). This barrier was shown to be disrupted by proteolytic activity of allergens (Wan et al. 1999). Furthermore asthma is associated with a defective barrier, that is possibly caused by genetic predisposition (Koppelman et al. 2009, Xiao et al. 2011, Sweerus et al. 2016).

As it was shown that DCs are indispensable to induce a response to allergens and airway inflammation, it was thought that allergen recognition by DCs is essential for this response (van Rijt et al. 2005, Hammad et al. 2010). However, despite having an important barrier function, epithelial cells also seem to be integral for recognizing allergens and priming DC response.

Epithelial cells express a broad range of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), C-type lectin receptors and protease-activated receptors (PARs) (Asokananthan et al. 2002, Sha et al. 2004, Uehara et al. 2007, Nathan et al. 2009). In experimental allergic airway inflammation triggered by HDM the established inflammation was shown to be dependent on expression of epithelial TLR4 (Hammad et al. 2009). Also other allergens, containing lipopolysaccharides (LPS), are probably recognized by epithelial TLR4, as epithelial TLR4 signaling was responsible for inducing an allergic Th2 response to inhaled LPS containing ovalbumin (Tan et al. 2010). Despite inducing TLR4 signaling in a mouse model of

allergic airway inflammation, in human bronchial epithelial cells HDM recognition can further be mediated by a C-type lectin receptor binding to β-glucan motifs in HDM and ragweed (Nathan et al. 2009). Some allergens also exhibit protease activity and especially mucosal sensitization could be influenced by allergen recognition by PARs, as only mucosal sensitization in contrast to systemic sensitization to german cockroach required PAR-2 expression (Page et al. 2010). Together these data show, that allergens can be recognized by airway epithelial cells through diverse PRRs, resulting in an epithelial immune response leading to inflammation.

Epithelial HDM recognition by TLR4 or C-type lectin receptor leads to the expression of CCL2, CCL3 and CCL20, which attract monocytes and immature DCs (Nathan et al. 2009, Hammad et al. 2009). A fast CCL20 secretion by epithelial cells could lead to early DC recruitment and initiation of an allergic immune response (Nathan et al. 2009). Moreover, also activating cytokines are expressed by allergen-exposed epithelial cells, such as GM-CSF, TSLP, IL-25 and IL-33 (Hammad et al. 2009). These cytokines are clearly implicated in the asthmatic disease as shown by human epidemiologic studies (Ritz et al. 2002, Ying et al. 2005, Wang et al. 2007, Préfontaine et al. 2010). IL-33 was shown to activate DCs to induce a Th2 response and subsequent allergic airway inflammation (Besnard et al. 2011). Overexpression of IL-25 leads to development of a Th2 immune response and subsequent airway inflammation, while blocking of IL-25 reduces the Th2 immune response in allergic airway inflammation (Angkasekwinai et al. 2007). Despite having potentially direct effects on T cells, IL-25 was shown to promote DC polarization into Th2 inducing DCs (Angkasekwinai et al. 2007, Kaiko et al. 2010). If GM-CSF is expressed in the lung, this leads to allergic sensitization against otherwise tolerated ovalbumin by enhancing antigen-presenting capability of DCs (Stämpfli et al. 1998). Also TSLP expression in the lung is critical for inducing allergic airway inflammation and as an additional factor that was shown to be able to activate DCs (Zhou et al. 2005). Therefore epithelial cells do not only recruit DCs into the lung, but also activate DCs to induce an allergen specific Th2 response upon antigen presentation.

In addition to DCs, epithelial cells also recruit and activate other immune cells, such as basophils, macrophages, mast cells, ILC2s, Th2 cells and B cells. It was shown that IL-33 together with GM-CSF, as well as TSLP promotes basophil hematopoiesis and

activation (Schneider et al. 2009, Siracusa et al. 2011). IL-33 also promotes the alternative activation of macrophages (M2 macrophages), which contribute to allergic airway inflammation (Kurowska-Stolarska et al. 2009). Mast cells can be activated by epithelial TSLP to release cytokine and chemokines implicated in a Th2 immune response (Allakhverdi et al. 2007). IL-33 and IL-25 are involved in expansion of ILC2s that contribute to allergic airway inflammation (Neill et al. 2010, Klein Wolterink et al. 2012). As already mentioned above, IL-25 can have direct effects on T cells by promoting their differentiation to Th2 cells (Angkasekwinai et al. 2007). Furthermore airway epithelial cells were shown to produce the B cell-activating factor of TNF family (BAFF), thereby promoting expansion and survival of B cells (Kato et al. 2006).

As soon as the Th2 immune response is established, airway epithelial cells can be triggered to further drive the inflammation. In response to Th2 cytokines epithelial cells continue their expression of GM-CSF, TSLP and CCL20 (Lordan et al. 2002, Reibman et al. 2003, Kato et al. 2007). Human bronchial epithelial cells are shown to release IL-8, a neutrophil chemoattractant, upon stimulation with IL-4 and IL-13 (Stříž et al. 1999). CCL11, which is important in recruiting eosinophils, is upregulated by IL-13 in airway epithelial cells (Matsukura et al. 2001). Allergen and Th2 cytokines are shown to cooperatively induce CCL17 expression that mediates T cell trafficking and Th2 polarization (Andrew et al. 2001, Heijink et al. 2007, Post et al. 2012). With their response to Th2 cytokines epithelial cells contribute to the continuation of the disease. Thus, in allergic asthma airway epithelial cells do not only present a barrier to inhaled allergens, but also represent important immune cells, which actively contribute to the disease (Figure 2).

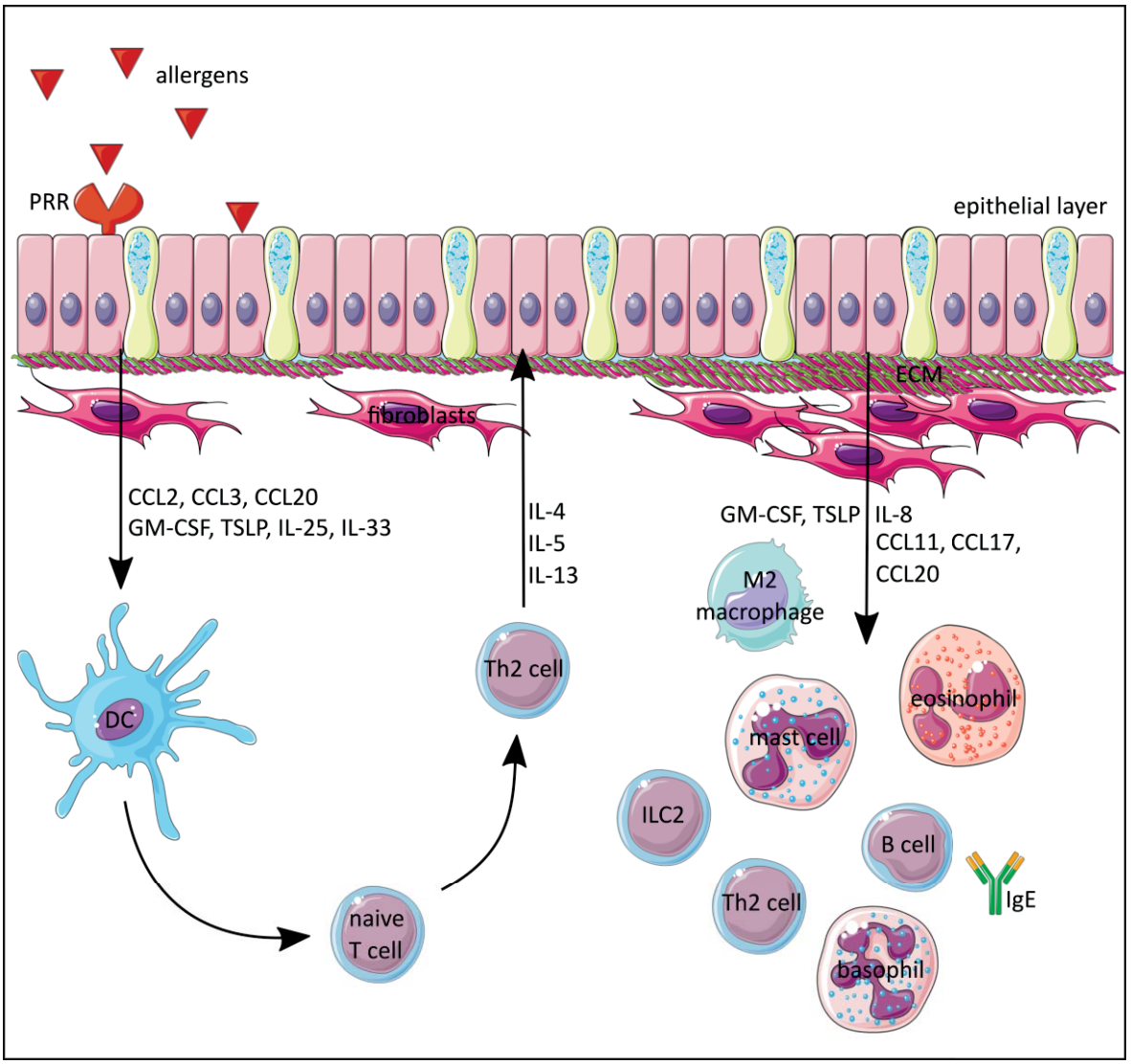

Figure 2: Lung epithelial cells drive the immune response after allergen recognition. Lung epithelial cells recognize allergens and produce chemokines, growth factors and cytokines that induce DC maturation and activation. DCs promote Th2 cell formation. Th2 cytokines induce release of further chemokines, growth factors and cytokines from epithelial cells driving inflammation. Established chronic inflammation results in lung remodeling with subepithelial fibrosis and thickening of the basement membrane. CCL, chemokine ligand; DC, dendritic cell; ECM, extracellular matrix; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; ILC2, innate lymphoid type 2 cell; IgE, immunoglobulin E; PRR, pattern recognition receptor; Th2 cell, T helper type 2 cell; TSLP, thymic stromal lymphopoietin.

1.2.4 The Th2 driving force

In allergic asthma predominant Th2 cells are found within the T cell infiltrates of the lung (Robinson et al. 1992). Th2 cells are signal transducer and activator of transcription (STAT) 6 and GATA binding protein 3 (GATA3) dependent and are

characterized by the expression of IL-4, IL-5 and IL-13, referred to as Th2 cytokines (Mosmann et al. 1986, Hou et al. 1994, Zheng and Flavell 1997).

B cell IgE switching is directed by IL-4 and IL-13 (Del Prete et al. 1988, Punnonen et al. 1993). While IL-4 was identified as mast cell growth factor, IL-13 can promote mast cell proliferation and activation (Smith and Rennick 1986, Kaur et al. 2006). IL-5 was shown to drive the differentiation, recruitment, activation and survival of eosinophils (Lopez et al. 1988, Clutterbuck et al. 1989, Walsh et al. 1990). Furthermore IL-4 and IL-13 support recruitment and activation of eosinophils (Patel 1998, Luttmann et al. 1999, Woltmann et al. 2000). IL-4 and IL-13 are both responsible for the polarization of macrophages to an M2 phenotype, whilst IL-5 is able to activate basophils (Hirai et al. 1990, Doyle et al. 1994).

Therefore the importance of Th2 cells and cytokines for the immune response in asthma is widely accepted. Further direct evidence for the key role of Th2 cells in allergy was provided by a study showing that adoptive transfer of allergen specific Th2 cells into unsensitized mice leads to allergic airway inflammation (Hansen et al. 1999). While the blockade of IL-4 and IL-5 resulted in a reduction of allergic airway inflammation, lung specific expression of IL-13 causes an inflammatory response similar to asthma (Corry et al. 1996, Foster et al. 1996, Zhu et al. 1999). The prominent role of Th2 cytokines in allergic inflammation has been translated into new treatments for human disease. The efficacy of monoclonal antibodies targeting the receptor of IL-4 and IL-13 or IL-5 has been demonstrated in recent clinical trials with asthma patients, who have high eosinophilia and a strong Th2 inflammation (Wenzel et al. 2013, Ortega et al. 2014).

However, not only Th2 cells contribute to the Th2 cytokine milieu in asthma. It was shown that Th2 cytokines are not exclusively expressed by infiltrating Th2 cells, but also by non-T cell populations (Bradding et al. 1994). Differentiated mast cells, basophils and eosinophils express and secrete IL-4 and IL-13 (Gessner et al. 2005). The cytokine IL-5 can be produced by mast cells, as well as eosinophils (Plaut et al. 1989, Dubucquoi et al. 1994). Macrophages are also possibly adding to Th2 cytokine production, as lung macrophages were shown to express IL-4 and IL-13 (Hancock et al. 1998, Pouliot et al. 2005).

Moreover ILC2s expand in the lung upon stimulation with epithelial derived IL-25 and IL-33 and become a major source of IL-5 and IL-13. In the same study it was shown that these cells are positive for IL-4, although not to a substantial amount (Klein Wolterink et al. 2012). Therefore a more appropriate nomenclature for these cytokines is "type 2 cytokines" rather than "Th2 cytokines". Interestingly even epithelial cells can contribute to the type 2 inflammation. Human bronchial epithelial cells of healthy and asthmatic donors release IL-13 and this release was further induced by TSLP treatment (Semlali et al. 2010).

Thus many cells drive the type 2 immune response that is not only implicated in eliciting and maintaining inflammation, but also in airway remodeling resulting in permanent loss of tissue function.

1.2.5 Structural alterations of the airways

Asthma is associated by airflow limitation due to AHR and narrowing of the airways. Airflow obstruction is partly mediated by airway remodeling. Remodeling in asthma, depicted in Figure 1 and Figure 2, is defined as smooth muscle hyperplasia and hypertrophy, basement membrane thickening, subepithelial fibrosis, epithelial shedding, goblet cell hyperplasia and increased mucus production, as well as angiogenesis (Roche et al. 1989, Carroll et al. 1993, Li and Wilson 1997, Ordoñez et al. 2001).

The main drivers of airway remodeling in asthma are thought to be TGFβ and type 2 cytokines, particularly IL-13. TGFβ is mainly responsible for fibrosis, as it enhances the deposition of extracellular matrix and collagen from fibroblasts and airway smooth muscle cells (SMCs) (Eickelberg et al. 1999, Kumawat et al. 2013). Furthermore it drives the differentiation of fibroblasts into myofibroblast, which are known to have enhanced collagen production and are able to support the contractile response in AHR (Michalik et al. 2009). TGFβ also increases the proliferation and migration of SMCs (Chen and Khalil 2006, Ito et al. 2009). Type 2 cytokines have several detrimental effects on bronchial epithelial cells. IL-13 and IL-4 promote goblet cell transdifferentiation and increase mucin expression of bronchial epithelial cells (Dabbagh et al. 1999, Zhen et al. 2007). Also proliferation of bronchial epithelial cells can be induced by IL-13 (Semlali et al. 2010).

TGFβ induces epithelial-mesenchymal transition (EMT) in airway epithelial cells, which potentially further contributes to fibrosis and basement membrane thickening (Hackett et al. 2009). Epithelial cells that undergo EMT lose their epithelial phenotype and develop a myofibroblast-like phenotype (Zhang et al. 2009).

However, also other growth factors, inflammatory mediators and signaling pathways are known to contribute to airway remodeling.

1.3 Leukotrienes

1.3.1 Leukotriene – biosynthesis and signaling of pro-inflammatory oxylipins

Leuktorienes are a family of lipid mediators, which are classified into two groups, cysteinyl leukotrienes (cysLT) and leukotriene B4 (LTB4). They are synthesized from arachidonic acid, an omega-6 polyunsaturated fatty acid that is part of the cell membrane (Figure 3). In a first step arachidonic acid is oxygenated into 5-hydroxyperoxyeicosatetraenoic acid (5-HPETE) by the enzyme 5-lipoxygenase (5-LO) (Borgeat et al. 1976). 5-HPETE is then further converted by 5-LO to LTA4, an unstable intermediate from which the two groups of leukotrienes arise (Rådmark et al. 1980). LTA4 is hydrolyzed by LTA4 hydrolase to generate LTB4 (Maycock et al. 1982). The cysteinyl leukotrienes are also synthesized from LTA4 by a stepwise enzymatic process. LTC4 is generated by the glutathione transferase LTC4 synthase by conjugation of glutathione to LTA4 (Jakschik et al. 1982). Then LTC4 is further converted into LTD4 by a γ-glutamyl transpeptidase and in a next step to LTE4 by a dipeptidase (Anderson et al. 1982, Lee et al. 1983).

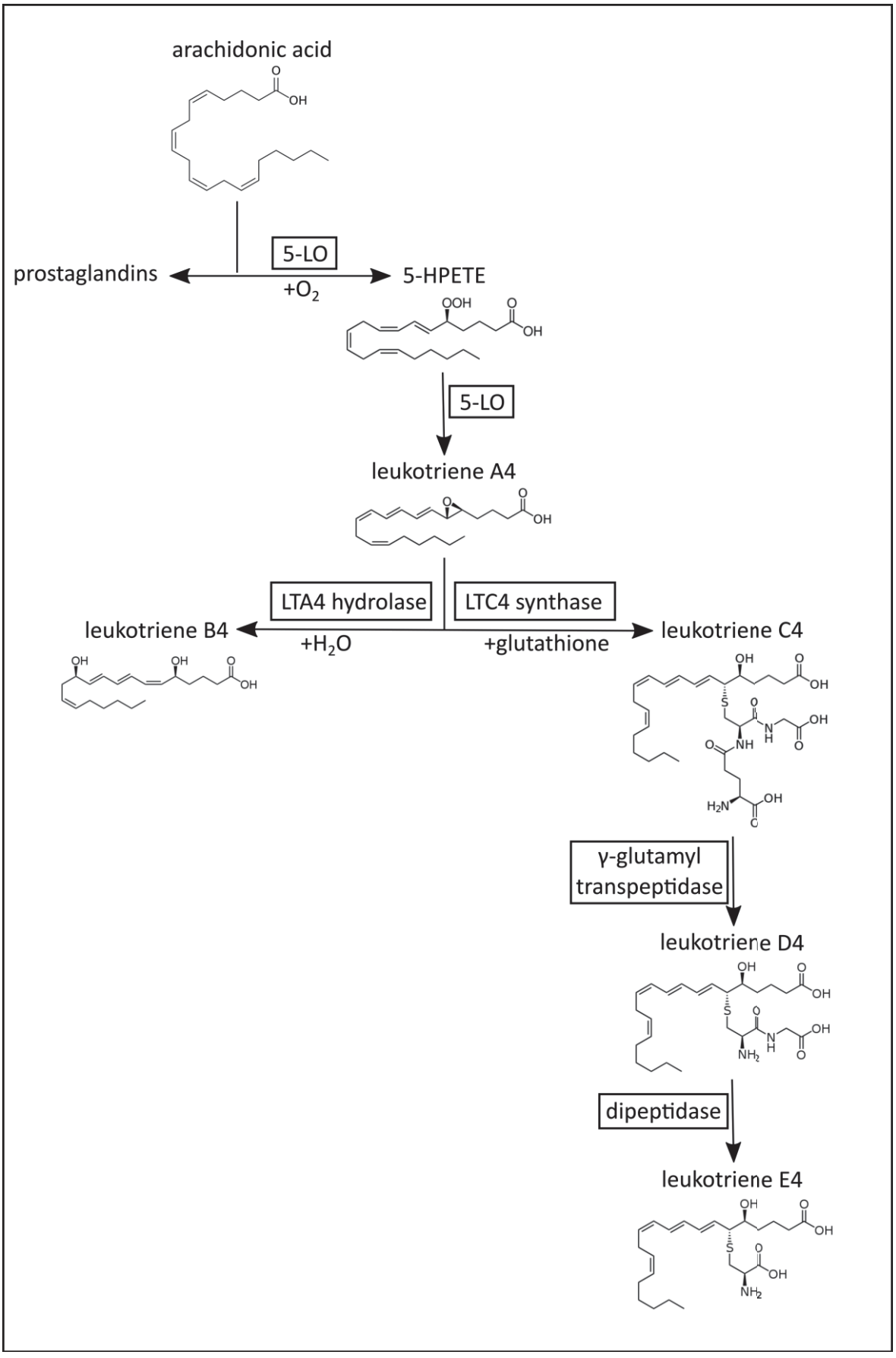
arachidonic acid
prostaglandins
5-LO
+O2
5-HPETE
5-LO
leukotriene A4
LTA4 hydrolase
+H2O
leukotriene B4
LTC4 synthase
+glutathione
leukotriene C4
γ-glutamyl transpeptidase
leukotriene D4
dipeptidase
leukotriene E4

Figure 3 : Pathway of leukotriene synthesis. Leukotrienes are step-wise synthesized from arachidonic acid. In a first step arachidonic acid is oxygenated into 5-HPETE by the enzyme 5-LO. 5-HPETE is then further converted by 5-LO to LTA4. LTA4 is hydrolyzed by LTA4 hydrolase to generate LTB4. LTC4 is generated by the glutathione transferase LTC4 synthase by conjugation of glutathione to LTA4. Then LTC4 is further converted into LTD4 by a γ-glutamyl transpeptidase and in a next step to LTE4 by a dipeptidase. 5-HPETE, 5-hydroxyperoxyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene.

Leukotrienes bind to seven-transmembrane G protein-coupled receptors (GPCRs) and for each leukotriene group two major receptors have been described. CysLTs bind to CysLT1 and CysLT2 receptor, while LTB4 binds to BLT1 and BLT2 receptor (Yokomizo et al. 1997, 2000, Lynch et al. 1999, Heise et al. 2000). Ligand binding to all receptors was shown to induce intracellular calcium signaling, phosphatidylinositol 3-kinase (PI3K) signaling and the mitogen-activated protein kinase (MAPK) pathway (Yokomizo et al. 2000, Heise et al. 2000, Nieves and Moreno 2006). MAPK pathway signaling is mediated by extracellular signal-regulated kinase (ERK) in cysLT and LTB4 stimulated cells (Mellor et al. 2002, Heller et al. 2005). CysLTs were also shown to be able to induce the p38 branch of the MAPK pathway (McMahon et al. 2000, Mellor et al. 2003). Furthermore the induction of the c-Jun N-terminal kinase (JNK) mediated MAPK signaling was reported for CysLT1 receptor activation (Poulin et al. 2011).

Besides the classical leukotriene receptors, cysLTs and LTB4 can bind to alternative receptors. LTB4 was shown to be able to activate peroxisome proliferator-activated receptors (PPARs) and vanilloid transient receptor potential V1 receptor (TRPV1) (Hwang et al. 2000, Narala et al. 2010). CysLTs potentially bind to pyrimidinergic nucleotide (P2Y) receptors and the GPCR GPR99 (Mamedova et al. 2005, Kanaoka et al. 2013).

1.3.2 Leukotrienes - mediators in allergic asthma and airway remodeling

CysLTs were first identified as bronchoconstrictors contributing to airway hyperresponsivness, but both groups of leukotrienes became recognized as important mediators of inflammation and remodeling in asthma (Dahlén et al. 1980, Bisgaard and Groth 1987).

In experimentally induced airway inflammation the expression of 5-LO was shown to be not only important for airway hyperresponsivness, but also favored development of eosinophilia and increased levels of immunoglobulins (Irvin et al. 1997). The cysLT1 receptor antagonist montelukast reduced airway eosinophil infiltration, mucus production, smooth muscle hyperplasia and subepithelial fibrosis in experimental airway inflammation (Henderson et al. 2002). Mice lacking the BLT1 receptor exhibit reduced airway hyperresponsivness, lower goblet cell hyperplasia and decreased IL-13 production in a model of asthma (Miyahara et al. 2005). Also BLT2 receptor function was shown to be important for developing an asthma-like inflammatory response (Cho et al. 2010).

Furthermore leukotrienes were shown to be enhanced in patients with asthma (Wardlaw et al. 1989). And treatment of patients with the cysLT1 receptor antagonist montelukast improved asthma control in several studies (Knorr et al. 1998, Noonan et al. 1998, Pizzichini et al. 1999, Volovitz et al. 1999). Therefore leukotrienes are clearly implicated in asthma, beyond their role as bronchoconstrictor.

CysLTs were shown to promote the activation of DCs by enhancing their cytokine production and antigen-presenting capacity (Okunishi et al. 2004). However, the production of IL-12 by DCs is reduced by cysLT, potentially helping to prevent Th1 induction (Machida et al. 2004). Expression of BLT1 receptor on DCs was shown to be important for DC migration into the draining lymph nodes in a model of airway inflammation (Miyahara et al. 2008). Furthermore LTs are also implicated in T cell recruitment during allergic inflammation. T cell recruitment into the airways was mediated by interaction of LTB4 with BLT1 receptor in an experimental asthma model (Tager et al. 2003). LTD4 mediated migration of T cells *in vitro* was shown to be dependent on CysLT1 receptor mediated calcium signaling (Prinz et al. 2005). Furthermore LTB4 can induce IL-5 release from T cells (Yamaoka and Kolb 1993). LTB4 can recruit eosinophils and mast cells to inflamed tissues (Tager et al. 2000, Lundeen et al. 2006). IL-4 induced production of IL-5 and tumor necrosis factor (TNF) α in mast cells is dependent on cysLTs, which likely act in an autocrine fashion (Mellor et al. 2002). Further it was shown that LTD4 enhanced migration and survival of basophils (Gauvreau et al. 2005). CysLTs can also enhance IL-4 induced IgE production of activated B cells (Lamoureux et al. 2006). Finally, cysLTs promote the

recruitment of ILCs and enhance the secretion of type 2 cytokines from these cells (Doherty et al. 2013).

In addition to airway smooth muscle contraction, cysLTs, as well as LTB4, can promote ASM proliferation and migration (Parameswaran et al. 2002, Watanabe et al. 2009). Together with epithelial derived epidermal growth factor (EGF), LTD4 induced the proliferation of bronchial fibroblast (Yoshisue et al. 2007). Furthermore TGFβ upregulates the expression of the cysLT1 receptor on fibroblast resulting in LTD4 dependent collagen production (Asakura et al. 2004). Of note, LTC4 induces the production of TGFβ by airway epithelial cells and together they enhance fibroblast proliferation (Perng et al. 2006). CysLTs were also shown to enhance mucus production in the lung epithelium (Marom et al. 1982). Thus, LTs are key mediators of chronic airway inflammation with perpetuating effects on the central processes of airway remodeling.

1.3.3 Transglutaminase 2 – a regulator of leukotriene production in asthma

Transglutaminase 2 (TGM2) is a ~80 kDa sized enzyme of the transglutaminase family, which catalyze calcium dependent posttranslational modification. TGM2 is also named tissue transglutaminase as it is the most abundant enzyme of the transglutaminase family (Odii and Coussons 2014). Besides its various biological functions, TGM2 was found to be able to increase secreted phospholipase A2 group X (sPLA2-X) enzymatic activity, which is acting upstream of the leukotriene synthesis (Hallstrand et al. 2010). The release of arachidonic acid from cellular membranes is the first rate-limiting step in generating leukotrienes and sPLA2-X was shown to directly release arachidonic acid from membrane phospholipids. Furthermore sPLA2-X induced group IVA cytosolic PLA2 (cPLA2α) activation that promotes endogenous arachidonic acid release and leukotriene synthesis resulting in an additive effect (McKew et al. 2008, Lai et al. 2010). The activation of cPLA2α was shown to be dependent on intracellular calcium signaling, as well as MAPK signaling via p38 and JNK (Lai et al. 2010). The role of TGM2 as regulator of leukotriene production is summarized in Figure 4.

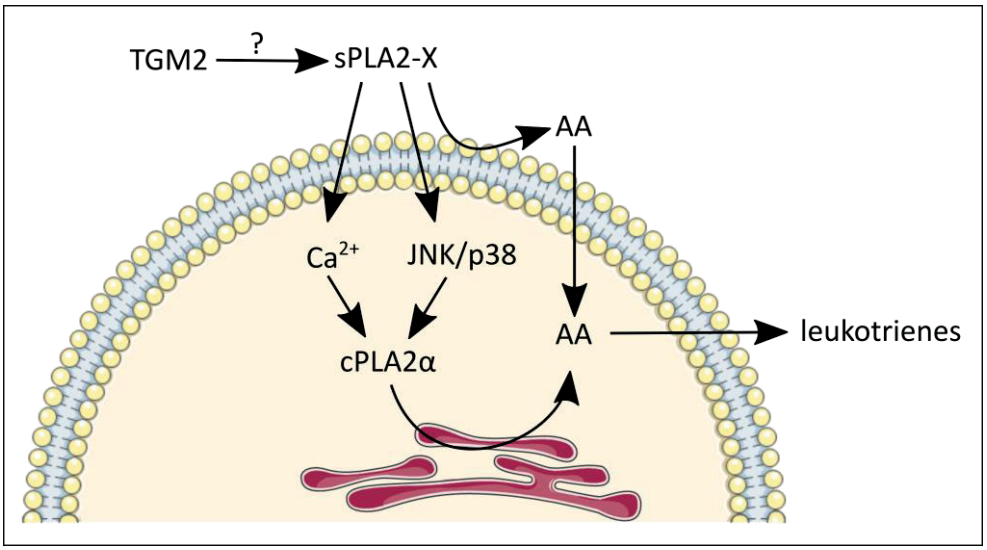

Figure 4 : Regulation of arachidonic acid release by TGM2. TGM2 activates sPLA2-X driving arachidonic acid release from the outer phospholipid membrane. Further sPLA2-X induces calcium and MAPK signaling to drive arachidonic acid release from inner membranes by cPLA2α. AA, arachidonic acid; cPLA2α, group IVA cytosolic phospholipase A2; JNK, c-Jun N-terminal kinase; sPLA2-X, secreted phospholipase A2 group X; TGM2, transglutaminase 2.

Leukotriene levels are enhanced in asthma patients, calling for higher need for arachidonic acid. In line with this, sPLA2-X is expressed at higher levels in asthmatics than in non-asthmatic controls with apparent localization in epithelial cells and macrophages (Hallstrand et al. 2007). Mice deficient in sPLA2-X show reduced signs of inflammation and remodeling in a model of allergic airway inflammation. Furthermore it was shown that these mice failed to produce leukotrienes in response to allergens (Henderson et al. 2007). TGM2 expression was further found to be elevated in asthma patients with a high expression in the bronchial epithelium (Hallstrand et al. 2010). While there is clear evidence that sPLA2-X regulates leukotriene production in asthma, a direct implication of TGM2 activity in enhanced leukotriene levels is unclear.

1.4 Wnt signaling

1.4.1 Introduction to the complex world of Wnt signaling

The Wnt signaling pathway is essential for developmental processes and is conserved across different species. Active signaling is initiated by the binding of Wnt ligands to frizzled receptors (FZD).

Wnt ligands are a family of 19 secreted cystein-rich and hydrophobic glycoproteins. In general the proteins consist of ~350 amino acids with a molecular weight of ~40 kDa (Nusse et al. 1991, Reichsman et al. 1996, Nusse 2016). They bind to FZD, a family of 10 seven-transmembrane receptors. Several Wnt ligands can bind to the same receptor and vice versa, yet with different binding affinities (Carmon and Loose 2010, Nusse 2016). Depending on the Wnt-FZD combination either canonical or non-canonical Wnt signaling is induced.

The canonical Wnt signaling signals through β-catenin and is the best described pathway activated by Wnt ligands (Figure 5). β-Catenin is a dual function protein being implicated in cell adhesion and gene transcription, which was first described as a component of a cadherin based cell-cell adhesion complex (Nagafuchi and Takeichi 1989). Activation of the pathway is initiated by binding of a Wnt ligand to FZD and the co-receptor lipoprotein receptor-related protein (LRP) 5/6 (Liu et al. 2005). Subsequently the downstream signaling protein dishevelled (DVL) is recruited, which directly interacts with FZD (Wong et al. 2003). LRP and DVL inhibit then either the formation or activity of the β-catenin destruction complex leading to the accumulation of β-catenin in the cytoplasm (Li et al. 1999a, Kofron et al. 2007, Piao et al. 2008). The β-catenin destruction complex consists of four core proteins: Axin, adenomatous polyposis coli protein (APC), glycogen synthase kinase-3β (GSK-3β) and casein kinase I (CKI). Upon binding to this destruction complex β-catenin is phosphorylated by GSK-3β and CKI and then actively released to be ubiquitinated and degraded by the proteasome (Xing et al. 2003). However, the degradation of β-catenin is disturbed by active canonical Wnt signaling and the accumulated non-phosphorylated β-catenin translocates into the nucleus. There β-catenin associates with transcription factors of the T cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family and initiates the transcription of a broad range of genes (Korinek

et al. 1998, Staal et al. 2002). The list of β-catenin target genes is still growing and comprises genes of diverse functions, such as transcription factors, cell-cycle regulators, growth factors, extracellular matrix proteins, cytokines, proteinases, as well as regulators of the pathway itself (Nusse 2016).

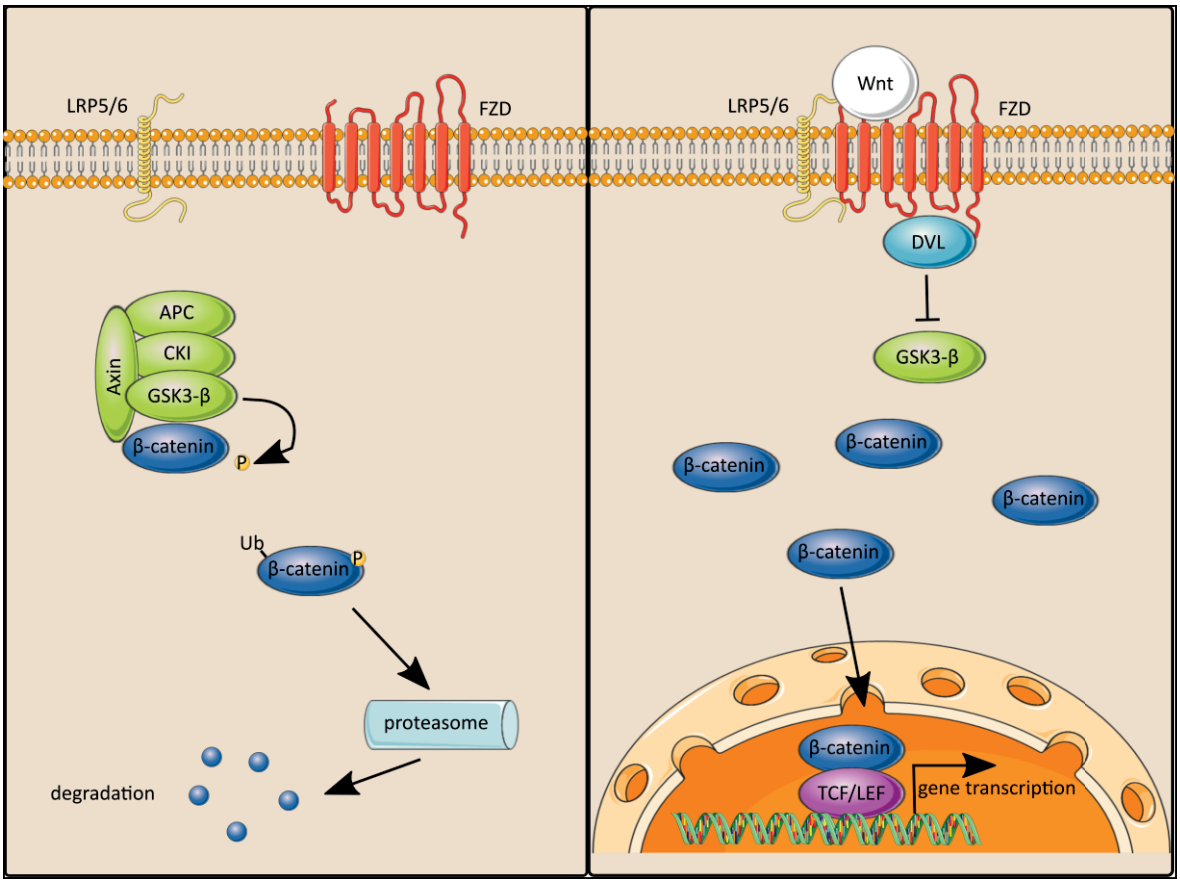

Figure 5 : Canonical Wnt signaling. The β-catenin destruction complex phosphorylates β-catenin, which gets ubiquitinated and degraded by the proteasome (left side). Wnt binding to FZD and LRP5/6 inhibits the β-catenin destruction complex with help of DVL leading to accumulation of β-catenin. Translocation of β-catenin to the nucleus leads to gene transcription (right side). APC, adenomatous polyposis coli protein; CKI, casein kinase I; DVL, dishevelled; FZD, frizzled; GSK3-β, glycogen synthase kinase-3β; LEF, lymphoid enhancer-binding factor; LRP, lipoprotein receptor-related protein; TCF, T cell factor.

The non-canonical Wnt signaling is far more complex and harbors several pathways that are initiated by Wnt ligands, but β-catenin independent. The best described are the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway (Figure 6 and Figure 7). To induce the PCP pathway a Wnt ligand binds to FZD and a co-receptor

different from LRP5/6. The signaling is also dependent on DVL, which binds to a DVL associated activator of morphogenesis 1 (Daam1). Daam1 binds and activates the small GTPase Rho (Habas et al. 2001). Subsequently Rho activation leads to the activation of the Rho-associated kinase (ROCK) resulting in the modification of the actin cytoskeleton (Marlow et al. 2002). Another branch of the PCP pathway signals through the small GTPase Rac. Rac is directly activated by DVL and in turn activates the MAP kinase JNK or p38 leading to modifications of the cytoskeleton and nuclear signaling (Paricio et al. 1999, Li et al. 1999b). Finally activation of PCP signaling can repress β-catenin dependent signaling by competitive binding of PCP inducing Wnt ligands to FZD (Sato et al. 2010).

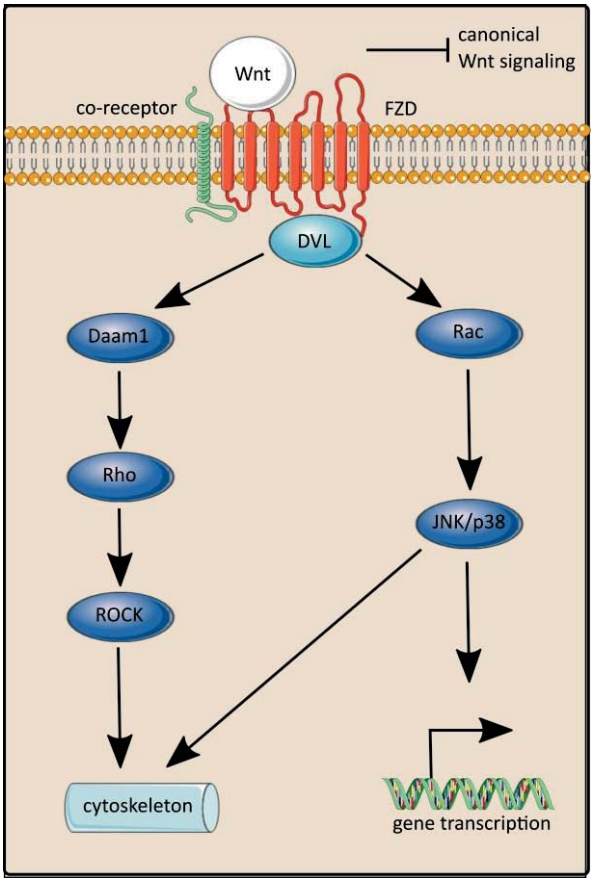

Figure 6 : Wnt signaling by the planar cell polarity pathway. Wnt binds FZD leading to activation of a series of kinases by DVL. The activated kinases act on the cytoskeleton or induce gene transcription. Daam1, DVL associated activator of morphogenesis 1; DVL, dishevelled; FZD, frizzled; JNK, c-Jun N-terminal kinase; ROCK, Rho-associated kinase.

The Wnt/Ca^{2+} pathway arised based on the finding that Wnt/FZD binding triggered the release of intracellular calcium. Calcium release follows G-protein dependent activation of phospholipase C (PLC) and phosphatidylinositol signaling (Slusarski, Corces, and Moon 1997). Accumulation of intracellular calcium is also DVL dependent and activates the calcium-responsive enzymes proteinkinase C (PKC), calmodulin-dependent protein kinase II (CamKII) and calmodulin-dependent protein phosphatase calcineurin (Sheldahl et al. 2003). While PKC can reorganize the cytoskeleton, calcineurin can induce nuclear factor of activated T-cells (NFAT) mediated transcription (Jönsson et al. 1998, Saneyoshi et al. 2002). Furthermore this pathway can also inhibit the β-catenin dependent pathway, as CamKII can directly inhibit active β-catenin (Flentke et al. 2014).

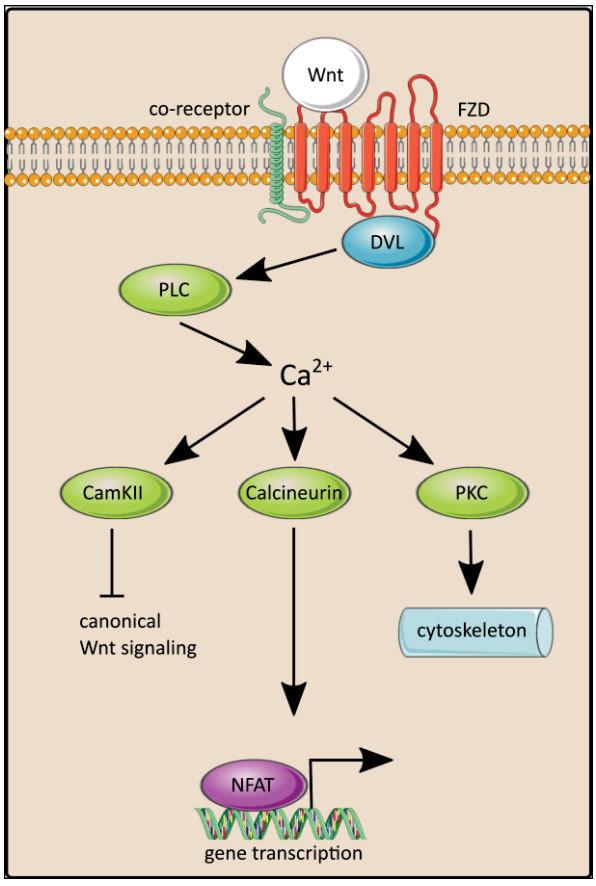

Figure 7 : Wnt/Ca2+ pathway. Wnt binding to FZD triggers the accumulation of intracellular calcium. Calcium activates PKC, CamKII and calcineurin modulating the cytoskeleton and gene transcription. CamKII, calmodulin-dependent protein kinase II; DVL, dishevelled; FZD, frizzled; NFAT, nuclear factor of activated T-cells; PLC, phospholipase C; PKC, proteinkinase C.

While the type of Wnt signaling pathway that is activated depends on the specific Wnt/FZD combination and the involvement of the co-receptor LRP5/6, there are some Wnt ligand, which are associated either with canonical (e.g. Wnt1 and Wnt3a) or non-canonical (e.g. Wnt5a) Wnt signaling (Liu et al. 2005).

1.4.2 New Wnt in allergic asthma pathogenesis

Wnt signaling is important for lung organogenesis, whereby both canonical and non-canonical Wnt signaling are required for proper lung development (Li et al. 2002, Harris-Johnson et al. 2009). However, in the last years the role of aberrant Wnt signaling in lung diseases, such as lung cancer and fibrotic lung diseases, became increasingly apparent.

In a mouse model of asthma an increased activation of canonical Wnt signaling was shown (Cohen et al. 2009). Another study confirmed the higher expression of β-catenin in the lung tissues of mice with experimental airway inflammation and downregulation of β-catenin resulted in reduced inflammation, subepithelial fibrosis and smooth muscle cell hyperplasia (Kwak et al. 2015). In contrast, lung specific Wnt1 expression and induction of β-catenin attenuated allergic airway inflammation and hyperresponsiveness (Reuter et al. 2014). Similar inhibition of GSK3β reduced airway inflammation, AHR and mucus production in mice (Bao et al. 2007). However, Wnt10b deficient mice showed an increase of type 2 inflammation in response to allergen sensitization (Trischler et al. 2015).

Wnt, FZD and genes related to canonical Wnt signaling are differentially expressed in peripheral blood cells from asthma patients compared to healthy controls (Lee et al. 2012). Furthermore mRNA expression of some Wnts and FZDs in lung tissue of asthmatic correlates with a type 2 driven immune response (Choy et al. 2011). Also expression of Wnt7a mRNA was shown to be elevated in sputum of asthma patients (Kwak et al. 2015). And the non-canonical Wnt5a is higher expressed in smooth muscle cells of asthma patients compared to non-asthmatic controls (Kumawat et al. 2013).

While Wnt3a stimulates DCs to produce TGFβ and vascular endothelial growth factor (VEGF) by induction of canonical Wnt signaling, Wnt5a induces IL-10 production in DCs by a β-catenin independent pathway. However, both Wnt3a and Wnt5a reduce

the production of pro-inflammatory cytokines by DCs and promote DC-mediated induction of regulatory T cells (Oderup et al. 2013). Furthermore it was shown that the induction of canonical Wnt signaling in DCs results in a reduced ability of DCs to activate T cells (Reuter et al. 2014). Also activation of canonical Wnt signaling by an GSK3β inhibitor or Wnt3a represses the differentiation of naïve T cell into effector T cells (Muralidharan et al. 2011). Though, active canonical Wnt signaling guides polarization of T cells into Th2 cells (Yu et al. 2009, Notani et al. 2010). Stable activation of canonical Wnt signaling enhances the survival of regulatory T cells (Ding et al. 2008). However, it was also shown that activation of canonical Wnt signaling was able to suppress Treg function (van Loosdregt et al. 2013). Basal expression of Wnt5a in macrophages ensures the expression of interferons, CD14 (part of monocyte PRR) and supports macrophage survival via non-canonical Wnt signaling (Naskar et al. 2014). While FZD1 is a marker of inflammatory macrophages, binding of Wnt3a to FZD1 induces canonical Wnt signaling and results in a reduced inflammatory response (Neumann et al. 2010). Wnt6 was shown to drive alternative macrophage polarization and Wnt5a to induce a tolerogenic phenotype of macrophages (Bergenfelz et al. 2012, Schaale et al. 2013).

Wnt signaling is clearly associated with fibrotic diseases. In this regard, Wnt5a was shown to induce the proliferation and extra cellular matrix (ECM) production of lung fibroblasts in a β-catenin independent manner (Vuga et al. 2009). However, activation of canonical Wnt signaling in lung fibroblast also increased ECM production (Baarsma et al. 2011). Furthermore Wnt3a was shown to induce myofibroblast differentiation by activating β-catenin (Carthy et al. 2011). Also SMCs produce enhanced ECM in response to Wnt5a induced non-canonical Wnt signaling (Kumawat et al. 2013). And active canonical Wnt signaling was shown to induce proliferation of SMCs (Gosens et al. 2010). Furthermore β-catenin was shown to be important for proliferation of bronchial epithelial cells (Zhu et al. 2007, Giangreco et al. 2012). Aeroallergens, such as HDM, were shown to enhance β-catenin dependent signaling within bronchial epithelial cells and active canonical Wnt signaling promotes EMT of these cells (Heijink et al. 2010, Giangreco et al. 2012).

1.5 Current therapeutic approaches in asthma

The current asthma treatment guidelines are mainly suggesting pharmacotherapy to control the diseases. The medications are divided in controllers, that are taken daily on long-term basis to keep the disease under control, and relievers, that are used to reverse bronchoconstriction when needed. The treatment starts with inhaled glucocorticoids, such as beclomethasone dipropionate, budenoside, ciclenoside, flunisolide, fluticasone propionate, mometasone furoate or triamcinolone acteonide, followed by an increase of either the glucocorticoid dose or addition of a different controller. Additional suggested controllers are leukotriene modifiers, such as montelukast, pranlukast, zafirlukast and zileuton, or long-acting inhaled β2-agonist (LABA), such as formoterol and salmeterol. If the disease can not be controlled by inhaled glucocorticoids and additional controllers, anti-IgE or oral glucocorticoids therapy is recommended (GINA 2011).

Inhaled glucocorticoids are the most widely used and still most effective medication to control asthma. Glucocorticoids were shown to decrease airway hyperresponsivness and airway inflammation, as well as to prevent exacerbations and mortality (Juniper et al. 1990, Jeffery et al. 1992, Pauwels et al. 1997, Suissa et al. 2000).

Glucocorticoids can diffuse through the cell membrane and bind to glucocorticoid receptors (GR) located in the cytoplasm. Glucocorticoid loaded GR translocate into the nucleus, where they form homodimers and bind to glucocorticoid response elements (GRE) resulting in transcription of glucocorticoid-responsive genes. These genes belong mainly to anti-inflammatory proteins, like the MAPK phosphatase 1, which inhibits the pro-inflammatory MAPK pathway (Lasa et al. 2002). Activated monomeric GR can further bind to histone deacetylase-2 (HDAC2) that allows association with the NF-κB complex, resulting in suppression of NF-κB mediated gene expression by histone acetylation (Ito et al. 2006). Thereby GR inhibits the formation of various inflammatory cytokines and chemokines (Ito et al. 2000). Additional to their direct effects on gene transcription of proinflammatory proteins, glucocorticoids were also shown to have an indirect effect on proinflammatory protein generation, as GR induced proteins include proteins that destabilize mRNA, for example mRNA of TNFα (Smoak and Cidlowski 2006). Despite their diverse effects on inflammation,

important features of glucocorticoids are the suppression of Th2 cells, by potentially inhibiting GATA3 translocation to the nucleus, and the induction of the anti-inflammatory cytokine IL-10 (Maneechotesuwan et al. 2008, 2009).

Usage of LABAs as controller is only suggested as add-on therapy to glucocorticoid treatment, as LABA monotherapy is inferior to inhaled glucocorticoids and increases the risk of asthma mortality (Lazarus et al. 2001, Cates and Cates 2008). As add-on therapy LABA treatment improves lung function and asthma symptoms and is favored compared to updosing of glucocorticoids (Greening et al. 1994, Wenzel et al. 1998).

LABA binding to β2-adrennergic receptors (β2AR) results in the activation of adenylyl cyclase leading to the production of cyclic adenosine monophosphate (cAMP). Increased levels of cAMP activate PKA, which in turn phosphorylates and activates the myosin light chain phosphatase. This phosphatase inhibits the myosin light chain kinase leading to the relaxation of airway smooth muscles. Furthermore LABA can inhibit SMC proliferation by cAMP activating a protein called EPAC, short for exchange protein activated by cAMP (Kassel et al. 2008). However, LABA can also induce an alternative β2AR signaling mediated by PLC or β-arrestin dependent MAPK signaling (McGraw et al. 2003, Gong et al. 2008). The alternative signaling is potentially the reason for adverse effects of long term LABA treatment as β-arrestin expression in hematopoietic cells is important for allergic airway inflammation and expression in structural cells is important for AHR (Hollingsworth et al. 2010).

As LABA, leukotriene modifiers are a beneficial add-on therapy for asthma patients to avoid updosing of glucocorticoids (Price et al. 2003). However, inhaled glucocorticoids are superior to leukotriene modifiers monotherapy (Bleecker et al. 2000). Nevertheless, a monotherapy with leukotriene modifiers is recommended in patients with asthma and seasonal allergic rhinitis (Philip et al. 2004). And clinical efficiency of leukotriene modifier treatment was shown in several studies (Knorr et al. 1998, Noonan et al. 1998, Pizzichini et al. 1999, Volovitz et al. 1999). The clinical approved leukotriene modifiers are the cysLT1 receptor antagonists montelukast, pranlukast and zafirlukast and the 5-LO inhibitor zileuton.

While glucocorticoids are highly effective in reducing airway inflammation, the effects on airway remodeling are less defined. By decreasing airway inflammation, glucocorticoids decrease mucus production and to some extent subepithelial fibrosis. Basement membrane thickening was shown to be reduced modestly in short-term low-dose treatment and more clearly in long-term high-dose treatment (Olivieri et al. 1997, Sont et al. 1999). However, expression of TGFβ and ECM proteins in the lung is not reduced despite systemic glucocorticoid treatment (Chakir et al. 2003). And high levels of glucocorticoid can induce apoptosis in lung epithelial cells (Dorscheid et al. 2001). As glucocorticoids only improve some features of airway remodeling upon long term treatment, the effectiveness of glucocorticoid treatment on airway remodeling is controversial.

There are still limited data on the effect of leukotriene modifiers on airway remodeling, however as described in 1.3.2 leukotrienes are implicated in features of airway remodeling and a study of montelukast treatment in a mouse model of allergic asthma shows clear improvement in airway remodeling. The beneficial effects of leukotriene modifiers on airway remodeling are supported by a study showing that montelukast treatment reduces myofibroblast numbers in the bronchi (Kelly et al. 2006).

Currently many new therapeutic interventions are under investigation, most of them targeting mediators of the type 2 airway inflammation, like IL-4, IL-5, IL-13, GATA3 and TSLP (Kim and Doherty 2016).

2 Aim of the study

Bronchial epithelial cells are important players of the immune system in allergic asthma. Upon allergen recognition they direct the initiation of a type 2 immune response. Furthermore in response to type 2 cytokines they drive continuation of the inflammation (see 1.2.3). However, bronchial epithelial cells can also contribute to airway remodeling as they express TGFβ, IL-13 and IL-33, which were shown to play an important role in remodeling (Kumar et al. 2004, Semlali et al. 2010, Saglani et al. 2013).

In a recent study Wnt5a and TGM2 were top hits in a gene expression analysis of IL-4 regulated genes in bronchial epithelial cells (Zissler et al. 2015). Changes in Wnt signaling are highly implicated in lung remodeling and fibrotic diseases. Differential regulation of Wnt signaling in asthma was already shown for smooth muscle cells (Kumawat et al. 2013). However, altered Wnt signaling in the bronchial epithelial cells in asthma by type 2 cytokines was not shown before.

TGM2 was identified as potential epithelial regulator of LT production in asthma, which play an important role in airway inflammation, AHR and lung remodeling (see 1.3.2 and 1.3.3). IL-4 induced changes in TGM2 expression is known for alternative activates macrophages (Martinez et al. 2013). Additional TGM2 was shown to be enhanced in the lung tissue of asthma patients (Hallstrand et al. 2010). A direct connection between type 2 cytokines, epithelial TGM2 expression and LT production is still missing.

Additional a link between TGM2 and canonical Wnt signaling was shown for a model of adipogenesis (Myneni et al. 2015). If potential regulatory loops between these pathways occur in the respiratory tract is still unknown.

Therefore aim of the study was to investigate the effects of type 2 cytokine on epithelial Wnt signaling and epithelial TGM2 expression, indirectly evaluating the contribution of bronchial epithelial cells to remodeling and inflammation in asthma. Then in this study a direct link between TGM2 expression and LT production was examined. Additional it was studied if there is a regulatory loop between Wnt signaling and TGM2.

In vitro experiments with bronchial epithelial cells were used to study the effects of type 2 cytokines on epithelial Wnt signaling and epithelial TGM2 expression. To mimic a type 2 cytokine milieu, IL-4 was used. The results were then confirmed in an *in vivo* model of allergic airway inflammation, with different strength of type 2 inflammation. Mice sensitized with HDM at different ages, day 3, day 15 and day 60 after birth, exhibited different strength of type 2 inflammation with mice sensitized at day 3 after birth showing the strongest type 2 inflammatory response (Gollwitzer et al. 2014). Furthermore using TGM2 inhibitors in *in vitro* experiments a direct link between TGM2 and leukotriene production was investigated. This was done for bronchial epithelial and macrophages mimicking both lung resident cells.

3 Material and methods

3.1 Material

3.1.1 Reagents

Reagent	Supplier
10x D-PBS w/o Ca/Mg	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
2-Propanol	Merck, Darmstadt, Germany
3,3'-Diaminobenzidine (DAB) Liquid Substrate System	Sigma-Aldrich, München, Germany
4',6-Diamidin-2-phenylindol (DAPI)	Thermo Fisher Scientific, Waltham, MA, USA
ABC Peroxidase Standard Staining Kit	Thermo Fisher Scientific, Waltham, MA, USA
Acetone	Merck, Darmstadt, Germany
β-Mercaptoethanol	Sigma-Aldrich, München, Germany
Bovine Serum Albumin (BSA)	Sigma-Aldrich, München, Germany
Citric acid monohydrate	Merck, Darmstadt, Germany
Cystamine dihydrochloride	Tocris Bioscience, Bristol, United Kingdom
Cytotoxicity Detection Kit (LDH)	Roche, Mannheim, Germany
Dansylcadaverine	Sigma-Aldrich, München, Germany
DEPC treated water	Thermo Fisher Scientific,

	Waltham, MA, USA
Dimethylsuolfoxid (DMSO) cell culture grade	Applichem, Darmstadt, Germany
DMEM/F12	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Donkey serum	Sigma-Aldrich, München, Germany
ECL Prime Western Blot Reagent	GE Healthcare, Freiburg, Germany
EDTA (0.05%, pH 8,0)	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
EDTA-free cOmplete Protease Inhibitor Cocktail	Roche, Mannheim, Germany
Ethanol absolute	Merck, Darmstadt, Germany
Fast Start Universal SYBR Green Master (Rox)	Roche, Mannheim, Germany
Fetal Calf Serum (FCS) Hyclone II	Perbio Science, Bonn, Germany
Fluticasone propionate	Sigma-Aldrich, München, Germany
Gentamycin	Thermo Fisher Scientific, Waltham, MA, USA
Hämalaun solution	Carl Roth, Karlsruhe, Germany
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA
Histokitt	Carl Roth, Karlsruhe, Germany
House dust mite extract (HDM)	Stallergenes, Antony, France

Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Hydrogen peroxide (H2O2)	Sigma-Aldrich, München, Germany
L-Glutamine	Thermo Fisher Scientific, Waltham, MA, USA
Methanol absolute	Sigma-Aldrich, München, Germany
Nonfat dried milk powder	Applichem, Darmstadt, Germany
NuPAGE LDS Sample Buffer (4x)	Thermo Fisher Scientific, Waltham, MA, USA
NuPAGE Sample Reducing Agent (10x)	Thermo Fisher Scientific, Waltham, MA, USA
Paraformaldehyde (PFA)	Sigma-Aldrich, München, Germany
Penicillin-Streptomycin	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
Polymorphoprep	Progen Biotechnik, Heidelberg, Germany
QIAshredder	Qiagen, Venlo, Netherlands
rh GM-CSF	Miltenyi Biotec, Bergisch Gladbach, Germany
rh IFNγ	Promokine, Promocell, Heidelberg, Germany
rh IL-4	Promokine, Promocell, Heidelberg, Germany
rh TGFβ1	Peprotech, Rocky Hill, NJ, USA

RIPA Lysis and Extraction Buffer	Pierce, Thermo Fisher Scientific, Waltham, MA, USA
Rnase-free DNase Set	Qiagen, Venlo, Netherlands
RNeasy Mini Kit	Qiagen, Venlo, Netherlands
Roticlear	Carl Roth, Karlsruhe, Germany
RPMI-1640 Medium	Gibco, Thermo Fisher Scientific, Waltham, MA, USA
SeeBlue Plus2 Pre-stained Protein Standard	Thermo Fisher Scientific, Waltham, MA, USA
Sodium azide (NaN3)	Merck, Darmstadt, Germany
Sodium carbonat (Na2CO3)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Carl Roth, Karlsruhe, Germany
Sodium hydrogen carbonate (NaHCO3)	Merck, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sulphuric acid (H2SO4)	Merck, Darmstadt, Germany
SuperSignal West Femto Maximum Sensitive Substrate	Pierce, Thermo Fisher Scientific, Waltham, MA, USA
Tetramethylbenzidine (TMB)	Sigma-Aldrich, München, Germany
Triton X-100	Sigma-Aldrich, München, Germany
Trizma base	Sigma-Aldrich, München, Germany
Trypanblue 0.4% solution	Thermo Fisher Scientific, Waltham, MA, USA

Trypsin 0.05% EDTA	Sigma-Aldrich, München, Germany
Tween 20 detergent	Merck, Darmstadt, Germany
Vectashield Mounting Medium	Vector Laboratories, Burlington, CA, USA

3.1.2 Media and Buffer

Media was prepared according to manufacturer, aliquoted and stored at -20°C.

Medium	Supplier
BEBM Basal Medium	Lonza, Basel, Switzerland
BEGM Bronchial Epithelial Cell Growth Medium	Lonza, Basel, Switzerland

Media were sterile filtered and either stored at 4°C or as aliquots at -20°C.

Medium	
Monocyte medium	RPMI-1640 Medium
	10% FCS
	1% Penicillin-Streptomycin
	1% L-Glutamine
	0.01% Gentamycin
Freezing medium	50% DMEM/F12
	40% FCS
	10% DMSO

Buffer	
MACS buffer	PBS
	0.5% BSA
	0.5 mM EDTA
10x Tris-buffered saline (TBS), pH 7.6	Trizma base 60.55 g
	NaCl 87.66 g
	distilled water 1000 ml
1x TBS	distilled water
	10% 10x TBS
Citrate buffer, pH 6.0	distilled water 1000 ml
	Citric acid 1.92 g
FACS buffer	PBS
	10% FCS
	0.02% NaN3

3.1.3 Primer

All Primers were ordered from metabion international (Planegg, Germany). After delivery primers were reconstituted in DEPC treated water to a concentration of 100 pmol/µl and stored at -20°C. The final concentration of a primer pair per qPCR reaction was 1.6 pmol/µl.

GeneName	FWD	RV
β-Actin	TGAGAGGGAAATCGTGCGTG	TGCTTGCTGATCCACATCTGC
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
TFRC	TGTGGGGAAGGGGCTGT	CCACCAAACAAGTTAGAGAATGC
GATA3	GCGGGCTCTATCACAAAATGA	GCTCTCCTGGCTGCAGACAGC
Wnt1	GGTTTCTGCTACGCTGCTG	TAAGCAGGTTCGTGGAGGAG
Wnt2	GGGCTGGCCTTTATCGCTC	GAGCCAGATTCCACCGAGAG
Wnt2b1	GATCCTTGAGGACGGCAGTA	GCTCACCAAACCAGGGATATT
Wnt2b2	TAGGTCTTGCCTGCCTTCTG	TTGTCACAGATCACTCGTGC
Wnt3	ACTTTTGTGAGCCCAACCCA	TTCTCCGTCCTCGTGTTGTG
Wnt3a	GTGGAACTGCACCACCGT	ATGAGCGTGTCACTGCAAAG
Wnt4	CTCGTCTTCGCCGTCTTCT	GATCAGGCCCTTGAGTTTCTC
Wnt5a	GCTCGGATTCCTCGGCT	CAAAGCAACTCCTGGGCTTA
Wnt5b	AACGCATCTGTCTTTGGGAG	GCTGATGGCGTTGACCA
Wnt6	TGCTGCTGCTGCTGCTC	CAGATGCTGGTAGGGTCCAT
Wnt7a	GAACTTGCACAACAACGAGG	AGTGTGGTCCAGCACGTCTT
Wnt7b	AGCCAACATCATCTGCAACA	CTGGTACTGGCACTCGTTGA
Wnt8b	CACCTGTGTCCTCCAACTCA	TGCCACACTGCTGGAGTAAA
Wnt9a	CTTCGGCCGCCTACTTC	GTCGCAGGCCTTGTAGTGC
Wnt9b	GTGCAGTGGTGCTGCTACG	GCACACATGCCGGTTTATGC
Wnt10a	AACACCAATTCAGGGACCAG	CAAAAGCGCTCTCTCGGAA

Wnt10b	GGTCCACGAGTGTCAGCAC	CCAGCATGGAGAAGGAAAAA
Wnt11	CGTGTGCTATGGCATCAAGT	GTGTGCATGAGCTCCAGGTT
Wnt16	CACGGGCAAAGAAAACAAAG	GCATGTTTTCACAGCACAGG
FZD1	AGCTTTGTGTGGGTTGGAAG	CGGTAAAATCTAAGCGCAGG
FZD2	ACATCGCCTACAACCAGACC	CCTTCACCAGCGGATAGAAC
FZD3	GCATCTGGGAAACAACGTG	TCAAGTCTGGACGACTCATTTG
FZD4	AACTTTCACACCGCTCATCC	TGACTGAAAGACACATGCCG
FZD5	TGGGGACTGTCTGCTCTTCT	CAAAGATAAACTGCTTCGGGA
FZD6	AAAATGGCCTACAACATGACG	ATTCCAGATTTGCGAGAGGA
FZD7	CGCCTCTGTTCGTCTACCTC	CCATGAGCTTCTCCAGCTTC
FZD8	AAGACAGGCCAGATCGCTAA	CGATAAGGAAGGTGGAGACG
FZD9	GAAGCTGGAGAAGCTCATGG	AAGTCCATGTTGAGGCGTTC
FZD10	TATGAGATCCCTGCCCAGTC	CAACCAAGAAAAGCACCACA

3.1.4 Antibodies and antibody-based kits

Isolation kit	Supplier
CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany

Antibody	Clone	Dilution	Supplier
5-LO Antibody	-	1:50 (IF/IHC)	In-house antibody, kind gift of Dr. Olof Rådmark (Karolinska Institute)

anti-Rabbit IgG (H+L) Cross Adsorbed Secondary Antibody, Biotin conjugate	polyclonal	1:500 (IHC)	Thermo Fisher Scientific, Waltham, MA, USA
Anti-WNT5A Antibody	polyclonal	1:50 (IF/IHC)	Lifespan Biosciences, Seattle, WA, USA
β-Actin	monoclonal	1:20000 (WB)	Santa Cruz Biotech, Dallas, Texas, USA
Donkey anti-Rabbit IgG Secondary Antibody, Alexa Fluor 488 conjugate	polyclonal	1:500 (IF)	Thermo Fisher Scientific, Waltham, MA, USA
FZD10 PolyAb Antibody	polyclonal	1:1000 (WB)	Proteintech, Rosemont, IL, USA
Goat anti-mouse IgG-HRP	polyclonal	1:2000 (WB)	Santa Cruz Biotech, Dallas, Texas, USA
Goat anti-rabbit IgG-HRP	ploYclonal	1:10000 (WB)	Santa Cruz Biotech, Dallas, Texas, USA
Group X sPLA2 Antibody	polyclonal	1:50 (IF/IHC)	Santa Cruz Biotech, Dallas, Texas, USA
HPRT Antibody	monoclonal	1:1000 (WB)	Santa Cruz Biotech, Dallas, Texas, USA
Human/ Mouse Wnt4 Antibody	clone #55025	1:1000 (WB)	R&D Systems, Minneapolis, MN USA
Human/ Mouse Wnt5a Antibody	clone #442625	1:1000 (WB) 1:400 (Cell culture)	R&D Systems, Minneapolis, MN USA

LTC4 synthase Antibody	polyclonal	1:50 (IF/IHC)	Santa Cruz Biotech, Dallas, Texas, USA
Phospho-Stat6 (Tyr641) Antibody	polyclonal	1:1000 (WB)	Cell Signaling Technology, Danvers, MA, USA
Rat IgG HRP-conjugated Antibody	polyclonal	1:5000 (WB)	R&D Systems, Minneapolis, MN USA
TGM2 XP Rabbit mAb	monoclonal	1:1000 (WB) 1:100 (IF/IHC)	Cell Signaling Technology, Danvers, MA, USA

ELISA kit	Supplier
BD OptEIA Human IL-8 ELISA Set	BD Biosciences, Heidelberg, Germany
CysLT EIA Kit	Cayman Chemical, Ann Arbor, MI, USA

3.1.5 Consumable material

Material	Supplier
Adhesion slides SuperFrost Plus	Thermo Fisher Scientific, Waltham, MA, USA
Amicon Ultra 0.5 ml centrifugal Filters 3K	EMD Millipore, Merck, Darmstadt Germany
Chamber slides (12-well)	Ibidi, Planegg/Martinsried, Germany
Corning Costar Tissue culture plates (6-well)	Thermo Fisher Scientific, Waltham, MA, USA
Coverslips	Thermo Fisher Scientific, Waltham, MA,

	USA
Cryotubes 1.8 ml	Nunc, Roskilde, Denmark
EDTA Monovettes	Sarstedt, Nümbrecht, Germany
Extra thick Western Blotting filter paper	Thermo Fisher Scientific, Waltham, MA, USA
FACS tubes	Thermo Fisher Scientific, Waltham, MA, USA
Falcon Conical Centrifuge Tubes	Thermo Fisher Scientific, Waltham, MA, USA
Filter tips	Starlab, Hamburg, Germany
Maxisorp plates (96 well)	Nunc, Roskilde, Denmark
NuPAGE Bis-Tris Gels	Thermo Fisher Scientific, Waltham, MA, USA
PCR foil- MicroAmp	Thermo Fisher Scientific, Waltham, MA, USA
PCR tubes	Eppendorf, Hamburg, Germany
Pipette tips	Eppendorf, Hamburg, Germany
Pipettes (1, 5, 10 and 25 ml)	Greiner Bio-One, Frickenhausen, Germany
PVDF Immobilon-P Membrane 0.45 µm	EMD Millipore, Merck, Darmstadt Germany
qPCR Plates 384-well	Thermo Fisher Scientific, Waltham, MA, USA
Reaction tubes	Eppendorf, Hamburg, Germany
Sterile filter device	Sarstedt, Nümbrecht, Germany

Tissue culture flask (75 cm^2)	Greiner Bio-One, Frickenhausen, Germany

3.1.6 Instruments

Instrument	Supplier
Bioanalyzer 2100	Agilent, Santa Clara, CA, USA
Centrifuge Megafuge 1.0 R	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge Multifuge 1L-R	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge Sigma 1-15 Microfuge	SIGMA, Osterode am Harz, Germany
Digital tube roller SRT9D	Bibby Scientific Limited, Staffordshire, United Kingdom
ECL ChemoCam Imager	Intas, Göttingen, Germany
Flow cytometer LSR Fortessa	BD Biosciences, Heidelberg, Germany
Incubator HeraCell	Thermo Fisher Scientific, Waltham, MA, USA
Laminar flow HeraSafe	Thermo Fisher Scientific, Waltham, MA, USA
Microplate Spectrophotometer Epoche	Bio Tek, Bad Friedrichshall, Germany
Microplate washer HydroSpeed	Tecan, Crailsheim, Germany
Microscope Axiovert 25	Zeiss, Oberkochen, Germany
Microscope Confocal Leica TCS SP5	Leica Microsystems, Wetzlar, Germany
Microscope EVOS FL Auto	Thermo Fisher Scientific, Waltham, MA, USA
Mini Blot Module	Thermo Fisher Scientific, Waltham, MA, USA
Mini Gel Tank	Thermo Fisher Scientific, Waltham, MA, USA

Multichannel pipettes	Branf, Wertheim, Germany
PCR machine TC-412	Techne Inc, Burlington, NJ, USA
pH meter SenTix 81	WTW GmbH, Weilheim, Germany
Pipettes with disposable tips	Eppendorf, Hamburg, Germany
Power Easy 90W	Thermo Fisher Scientific, Waltham, MA, USA
QuadroMACS Separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Spectrophotometer NanoDrop 2000	Thermo Fisher Scientific, Waltham, MA, USA
Thermo shaker TS100	Kisker Biotech, Steinfurt, Germany
ViiA 7 Real-Time PCR System	Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA
Waterbath	Julabo, Seelbach, Germany

3.2 Methods

3.2.1 Culture and stimulation of normal human bronchial epithelial cells

Normal human bronchial epithelial cells (NHBEs) from non-smoking and non-drinking individuals were obtained from Lonza (Basel, Switzerland) and expanded in Bronchial Epithelium Growth Medium (BEGM, Lonza), before they were stored in liquid nitrogen in passage (p) 2. Before experimentation cells were further expanded and then seeded in 6-well plates at a density of 1.5 – 2.5 x 10^5 cells/well. Cells were grown to 80-85% confluency and before stimulation serum-deprived in basal medium (BEBM, Lonza) overnight. Rested cells were then stimulated with 50 ng/ml human recombinant IL-4 (Promokine, Promocell), 15 ng/ml IFNγ (Promokine, Promocell), 5 ng/ml TGFβ1 (Peprotech) and/or 10 µg/ml HDM extract from *Dermatophagoides farina* (Stallergenes) or treated with 100 µM (in DMSO) cystamine dihydrochloride (Tocris Bioscience), 25 µM (in DMSO) dansylcadaverine (Sigma-Aldrich) or 1 µM (in DMSO) fluticasone propionate (Sigma-Aldrich) for the indicated

time points. Depending on the stimulation, control cells were either kept in BEBM or in BEBM with 0.01% or 0.1% dimethylsulfoxid (DMSO, Applichem) for the identical time. All experiments were performed at p 4. For the 72h time course experiment NHBEs were treated with 50 ng/ml IL-4 (Promokine, Promocell) every 24h.

3.2.2 Generation of epithelial derived conditioned media

Conditioned media (CM) was prepared by collecting cell-free supernatants of 24h stimulated NHBEs. Supernatants of 3 donors were combined and stored at -80°C before use.

3.2.3 Isolation of $CD14^+$ monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA anti-coagulated blood by density gradient centrifugation. For this purpose polymorphoprep was overlayed with an equal volume of fresh, undiluted blood and centrifuged for 35 min at 500g at 20°C without brake. After centrifugation the PBMC layer (Figure 8) was collected and transferred to a tube with 40 ml of PBS. Cells were washed once with MACS buffer and then monocytes were isolated from PBMCs by magnetic cell sorting (MACS) technology using human CD14 MicroBeads, LS columns and the QuadroMACS Separator (all Miltenyi Biotec) according to the manufacturer's instructions.

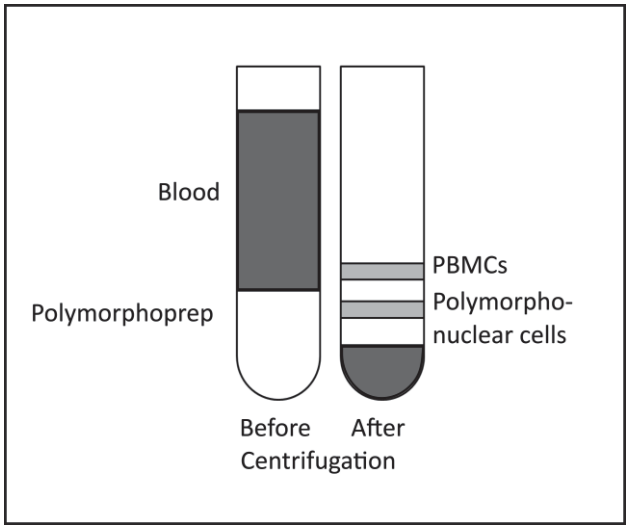

Figure 8 : Schematic representation of the different layers before and after centrifugation.

3.2.4 Generation and stimulation of monocyte derived macrophages

Monocyte derived macrophages (MDM) were generated from $CD14^+$ PBMCs. $CD14^+$ cells were differentiated for 7 days in monocyte medium supplemented with 10 ng/ml GM-CSF (Miltenyi Biotec) and 2 ng/ml TGFβ1 (Peprotech) at a density of 0.5 x 10^6 cells/ml. At day 3 and day 5 of culture supplements and half of the medium were exchanged. After differentiation cells were collected and seeded at a density of 2 x 10^5 cells/well in 12-well chamber slides (Ibidi). Cells were stimulated for 24h with epithelial derived CM and anti-Wnt5a antibody (R&D Systems) in a 1:400 dilution and with IL-4 supplemented monocyte medium, which was pre-incubated for 24h at 37°C, for immunofluorescence analysis. MDMs were also stimulated with 50 ng/ml human recombinant IL-4 (Promokine, Promocell) and treated with 100 µM (in DMSO) cystamine dihydrochloride (Tocris Bioscience) and 25 µM (in DMSO) dansylcadaverine (Sigma-Aldrich) in supplemented monocyte medium for 24h for analysis of cysteinyl leukotriene levels in culture supernatants, after stimulation of MDM with 5 µM ionophore A23187 for 10 min at 37°C. Depending on the stimulation control cells were either kept in control CM, in supplemented monocyte medium or in supplemented monocyte medium with 0.1% DMSO (Applichem) for the identical time.

3.2.5 Mouse model of allergic airway inflammation

Paraffin embedded lung lobes and bronchoalveolar lavage fluid (BALF) were kindly provided by Dr. Eva Gollwitzer (University of Lausanne, Service de Pneumologie) and analyzed in collaboration with Dr. Esser-von Bieren. Allergic airway sensitization was induced as published by Gollwitzer et al. (Gollwitzer et al. 2014). Briefly, BALB/c mice were sensitized intranasally with 5 µg of house dust mite extract from day 3, 15 and 60 after birth. Mice were treated with HDM every second day for a total of six exposures. Samples were collected four days after the last challenge.

3.2.6 RNA extraction

In brief, cells were directly lysed in 350 µl RLT buffer with 1% β-mercaptoethanol per well and homogenized using QIAshredder (Qiagen). Then RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. On-column DNase digestion was performed using an RNase-free DNase Set (Qiagen). The

RNA was eluted in 30 µl DEPC treated water and the RNA concentration was measured using a NanoDrop 2000 spectrophotometer.

3.2.7 cDNA synthesis

Isolated RNA was subjected to reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). A maximum of 2 µg of RNA was transcribed with a total volume of up to 14.2 µl, if needed a twofold reaction mixture was formulated to get a higher cDNA yield.

3.2.8 Quantitative real-time PCR (qPCR)

Expression of mRNA was quantified using the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). 3.4 µl of cDNA with a concentration of 5 ng/µl was mixed with 1.6 µl of a primer pair solution (see 3.1.3 for a list of primers) and 5 µl Fast Start Universal SYBR Green Master (Rox) (Roche) in a 384-well plate. The plate was then sealed and shortly centrifuged, before running the method shown in Figure 9. All samples were run in duplicates. Expression levels were determined by the comparative C_T method using the equation $2^{-\Delta\Delta CT}$. Expression levels were normalized to the house-keeping genes β-actin, HPRT and TFRC.

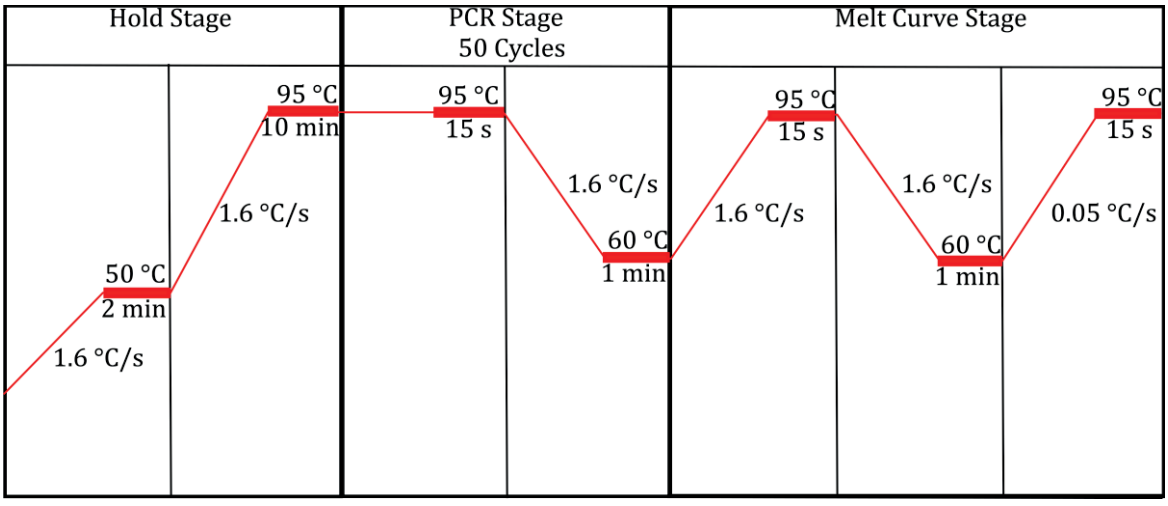

Figure 9 : qPCR Method.

3.2.9 Western Blot

Total cell lysates were obtained by direct lysis of cells in 250 µl RIPA buffer (Pierce, Thermo Fischer Scientific) per well with 2% EDTA-free complete protease inhibitor mixture (Roche) using a cell scraper. NuPAGE LDS sample buffer was added to total lysates, equal volumes of cell-free supernatants or equal volumes of BALF according to manufacturer's recommendation and heated at 70°C for 10 min before loading. Expression of pSTAT6 (Cell signaling technology), Wnt5a (R&D Systems), Wnt4 (R&D Systems), FZD10 (Proteintech) and TGM2 (Cell signaling technology) was analyzed by western blotting. HPRT or β–actin (both Santa Cruz Biotech) was used as loading control. Samples were loaded on 4-12% or 10% Bis-Tris protein gels (NuPAGE, Thermo Fisher Scientific) and proteins were separated at 125 Volt for 90 min. Proteins were then transferred to a PVDF membrane (EMD Millipore, Merck) using Mini Blot Module (Thermo Fisher Scientific) at 20 Volt for 90 min according to the manufacturer's instructions. After a short washing in 1x TBS containing 0.02% Tween, the PVDF membrane was blocked for 60 min in 5% nonfat dried milk in 1x TBS with 0.02% Tween to prevent unspecific binding. The primary antibodies were diluted in blocking solution (for dilution see 3.1.4) and incubated with the membrane overnight at 4°C in a 50 ml Falcon on a tube roller. After subsequent washing for at least 30 min at RT the membrane was incubated with the corresponding secondary HRP-conjugated antibody in blocking solution for 1h at RT. Following 30 min of washing, detection was performed using enhanced chemiluminescence (Amersham ECL Prime, GE Healthcare or SuperSignal West Femto Maximum Sensitive Substrate, Pierce, Thermo Fisher Scientific) according the manufacturer's instructions and recorded using the ECL ChemoCam Imager (Intas). Protein levels were quantified using LabImage 1D (Intas) by normalization to unstimulated control or mean expression and corrected for the amount of HPRT or β–actin in the samples.

3.2.10 Concentrating of supernatants for western blot

Equal volumes of cell-free supernatants were concentrated using Amicon Ultra 0.5 ml Centrifugal Filters 3K (EMD Millipore, Merck) according to the manufacturer's instructions. For each sample 500 µl of supernatant was 10 times concentrated, before analysis by western blot.

3.2.11 Immunofluorescence (IF)

For immunofluorescent staining, NHBEs were seeded in 12-well chamber slides (Ibidi) at a density of 7.0 – 10.0 x 10^4 cells/well. Cells were grown to 60-70% confluence before stimulation. After the stimulation cells were washed once with PBS, following fixation for 15 min with 4% PFA at RT. Then 3x 5 min washing steps with PBS were performed and cells were permeabiliezd with acetone for not more than 10 min at -20°C. Again 3x 5 min washing steps were performed with subsequent blocking in a 3% BSA solution with 10% donkey serum for 1h. Cells were incubated with rabbit primary antibody against 5-LO (a kind gift of Prof. Olof Rådmark, Karolinska Institute Stockholm), TGM2 (Cell signaling technology) or sPLA2-X (Santa Cruz Biotech) in a moisture chamber overnight at 4°C. After 3 washing steps (5 min, PBS), cells were incubated with a fluorescence (Alexa Fluor 488) conjugated donkey anti-rabbit secondary antibody (Thermo Fisher Scientific) for 1.5h at RT in the dark. Then, 3 washing steps were performed and the nucleus of cells was stained with DAPI (1µg/ml) for 10-15 min at RT. This was followed by a last washing for 2x 5 min. Slides were covered with mounting medium and a coverslip (Vectashield, Vector Laboratories). After drying for 1h at 4°C, slides were sealed with nail polish and stored at 4°C in the dark. Images were recorded on an EVOS system (Thermo Fisher Scientific) or a Leica SP5 confocal microscope (Leica Microsystems). Image analysis was performed using ImageJ and Cell Profiler software by Dr. Esser-von Bieren using previously published macros (Esser-von Bieren et al. 2013, 2015).

3.2.12 Enzyme-linked immunosorbent assay (ELISA)

Concentrations of IL-8 in cell free supernatants of NHBEs were measured using a commercially available ELISA kits (BD Biosciences) according to the manufacturer's instructions. Levels of cysteinyl leukotrienes in cell free supernatants or BALF were determined using commercially available EIA kits (Cayman Chemicals) according to the manufacturer's instructions.

3.2.13 LDH Assay

Cell death was quantified using the cytotoxicity detection Kit (LDH assay) from Roche according to manufacturer's instructions.

3.2.14 Immunohistochemistry (IHC)

Immunohistochemical staining was performed on sections (4 µm) of paraffin embedded left lobes of mice lungs. After dewaxing at 65°C for 30 min, tissues were re-hydrated in a descending alcohol series. Antigen-retrieval was performed by heating slides in citrate buffer with 0.05% Tween-20 in a microwave. Slides were washed 2x 3 min in distilled water and 1x 5 min in PBS. Then tissues were permeabilized in PBS with 0.2% Triton X-100 for 45 min at RT. Slides were washed 3x 5 min in PBS, before blocking of unspecific binding with a 3% BSA solution with 10% donkey serum for 1h at RT. Tissues were incubated with rabbit primary antibody against 5-LO (a kind gift of Prof. Olof Rådmark, Karolinska Institute Stockholm), LTC4 synthase (Santa Cruz Biotech), Wnt5a (Lifespan Bioscience), TGM2 (Cell signaling technology) or sPLA2-X (Santa Cruz Biotech) in a moisture chamber overnight at 4°C. The next day after a 3x 5min washing step, cells were incubated with a biotinylated donkey anti-rabbit secondary antibody for 1h at RT in a moisture chamber. Following a 3x 5 min washing step, detection was performed using an HRP-based detection kit (ABC, Thermo Fisher Scientific) with DAB detection reagents (Sigma-Aldrich). Hämalaun solution (Carl Roth) was used for nuclear counterstaining and tissues were dehydrated in ascending alcohol series. Slides were covered with a coverslip using Histokitt. Images were recorded on an EVOS system (Thermo Fisher Scientific). Image analysis was performed in ImageJ by Dr. Esser-von Bieren using previously published macros (Esser-von Bieren et al. 2013, 2015).

3.2.15 Statistics

Data are presented as medians with interquartile range, showing additional the individual data points. Time course data are presented as line, showing the individual data points. Statistical analysis was performed in cases of comparisons between two groups using Mann-Whitney test for unpaired data and Wilcoxon test for paired data. Gene expression data obtained by qPCR were normalized by using the logarithmic function and a one-sample t-test was performed. The effect size with the exact P-value and the 95% confidence interval (CI) is reported. For qPCR analysis only differences with a false discovery rate below 10% are reported. For all statistical analysis RStudio version 0.99.442 (RStudio, Boston, MA) with R version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria) was used.

4 Results

4.1 Normal human bronchial epithelial cells (NHBEs) are responsive to IL-4

Human bronchial epithelial cells are considered an important player in the asthmatic immune response and are therefore a widely used tool to study epithelial responses in allergic asthma. However, to study the response of the cells in settings of type 2 inflammation *in vitro*, the responsiveness to type 2 cytokines, especially IL-4, is required. It was shown that the bronchial epithelium *in vivo* as well as cultured cells from the bronchial epithelium *ex vivo* express the IL-4 receptor (van der Velden et al. 1998). In contrast a more recent study showed that the IL-4 receptor was only weakly expressed, when isolated airway epithelial cells were grown in submerged culture (White et al. 2010). As commercially available cells had to be used, the first experiments were to show the responsiveness of these NHBEs to the type 2 cytokine IL-4.

To test if the utilized NHBEs are responsive to IL-4, STAT6 phosphorylation was analyzed, as binding of IL-4 to the IL-4 receptor induces STAT6 phosphorylation and induction of the downstream transcription factor GATA3 (Hou et al. 1994, Zheng and Flavell 1997). NHBEs were stimulated with 50 ng/ml IL-4 for 2h and pSTAT6 expression was then determined by western blot. Control cells that were cultured in parallel, without the addition of IL-4, showed no pSTAT6 expression. In contrast, IL-4 stimulated cells showed a clear pSTAT6 expression even if the strength of pSTAT6 induction varied strongly between the different donors (Figure 10 A, B). Furthermore gene expression levels of the pSTAT6 target gene GATA3 were measured as a functional read-out of STAT6 phosphorylation. Therefore NHBEs were stimulated with 50 ng/ml IL-4 for 6h and GATA3 mRNA expression was analyzed by qPCR. GATA3 mRNA expression was 3.8 fold (95% CI_{log} 1.5-2.4; $P_{log}<0.001$) upregulated by IL-4 (Figure 10 C).

The release of the pro-inflammatory cytokine IL-8 by bronchial epithelial cells was shown to be increased by IL-4 (Stříž et al. 1999), thus IL-8 levels were measured in the supernatants of NHBEs stimulated with IL-4 for 24h. Higher levels of IL-8 were found in the supernatants of IL-4 stimulated cells than in unstimulated control cells.

While the IL-8 levels measured in the supernatants of unstimulated cells were 194.4-797.1 pg/ml, the IL-8 levels in the supernatants of the stimulated cells ranged from 756.3 to 6704.1 pg/ml (Figure 10 D).

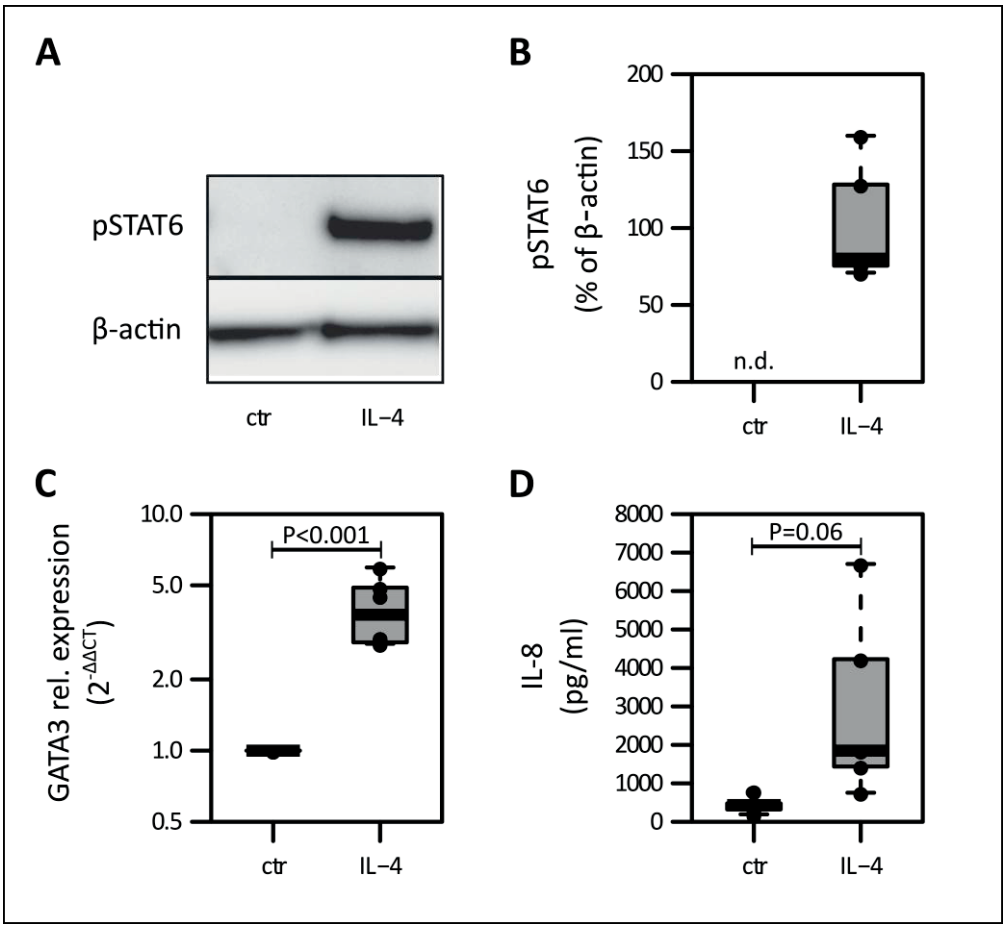

Figure 10 : NHBEs are IL-4 responsive. A, Representative western blot of pSTAT6 and housekeeper β-actin from IL-4 stimulated NHBEs and control NHBEs. B, Normalized expression of pSTAT6 determined by western blot of IL-4 stimulated NHBEs and control NHBEs as percentage of β-actin expression (n=5). C, Relative expression of GATA3 mRNA of IL-4 stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=6; one sample t-test). D, Levels of IL-8 measured in supernatants from IL-4 stimulated NHBEs and control NHBEs by ELISA (n=5; Wilcoxon test).

As the utilized NHBEs were responsive to IL-4, as shown by STAT6 phosphorylation with an according upregulation of GATA3 and induction of the release of the inflammatory cytokine IL-8, NHBEs from the respective donors were used for the following experiments.

4.2 IL-4, not IFNγ, specifically regulates expression of Wnt and frizzled mRNA

The Wnt ligand Wnt5a was one of the top hits of secreted proteins in a whole genome array of IL-4 stimulated NHBEs (Zissler et al. 2015). To see if further Wnt ligands or Wnt receptors FZD can be regulated by IL-4, the global Wnt and FZD mRNA expression was assessed in IL-4 stimulated NHBEs. Expression of Wnt and FZD mRNA was analyzed by qPCR in NHBEs stimulated with 50 ng/ml IL-4 for 6h. Even if some of the mRNA transcripts were only expressed at low levels (CT >30), for most Wnt ligands and all FZD receptors mRNA transcripts were detected, with Wnt7a, WNt7b and FZD6 being the most abundant (Figure 11 A, C). Comparison of the transcriptional levels for IL-4 stimulated NHBEs to those of unstimulated cells revealed that IL-4 selectively regulated particular Wnt ligands and FZD receptors. The expression of Wnt5a and Wnt11 mRNA was increased, while expression of Wnt7a, Wnt4, Wnt8b, Wnt5b and Wnt3a mRNA were decreased by IL-4 stimulation (Figure 11 B). Also the Wnt receptors FZD10 and FZD9 were upregulated, while the expression of the other receptors was unaffected by IL-4 (Figure 11 D).

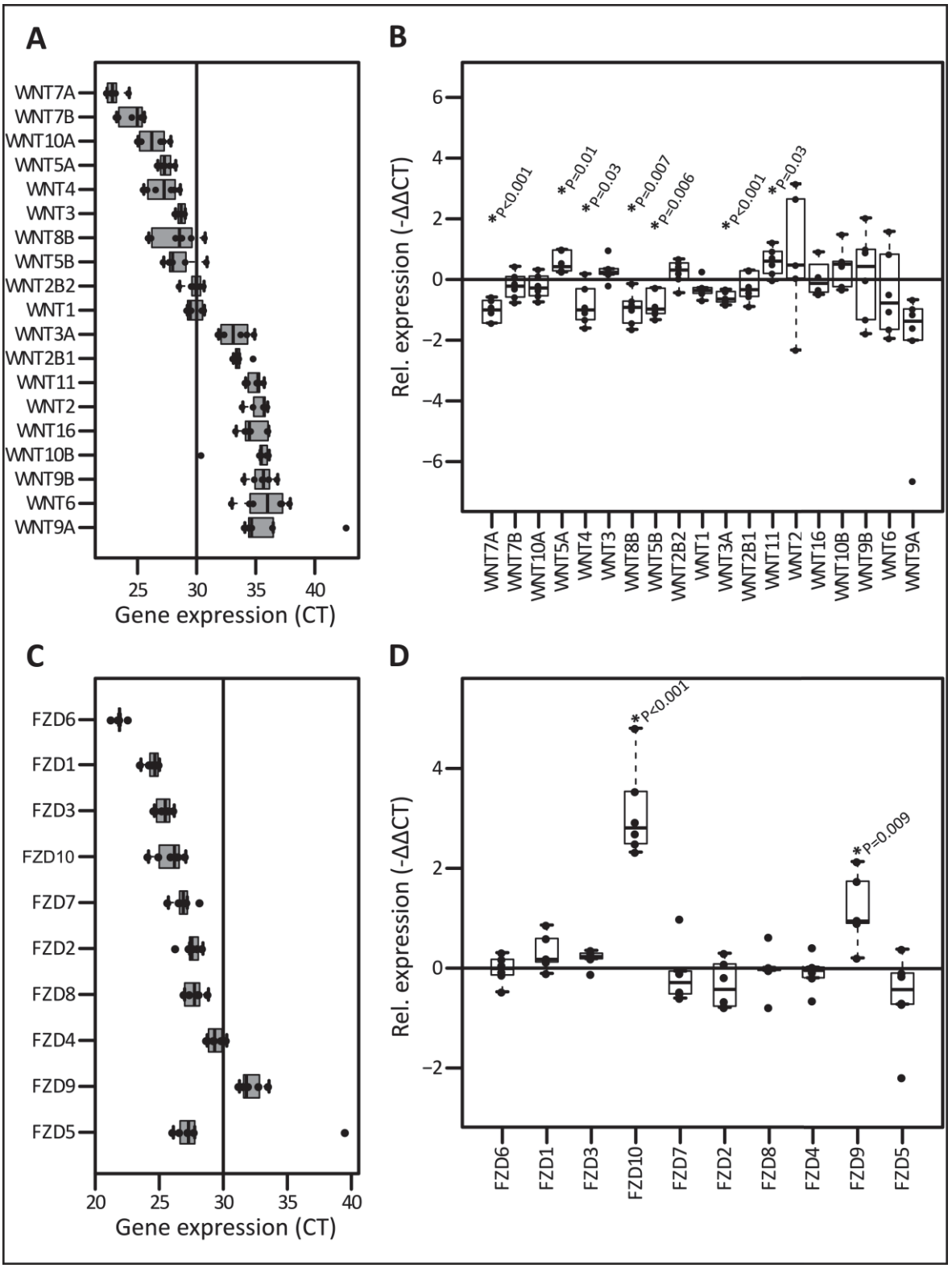

Figure 11 : IL-4 regulates gene expression of Wnt and FZD. A, Wnt mRNA expression was determined by qPCR and is shown as gene expression of Wnts in IL-4 stimulated NHBEs (n=6). B, Wnt mRNA expression was determined by qPCR and is shown as relative expression of Wnts in IL-4 stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=6; one-sample t-test). C, FZD mRNA expression was determined by qPCR and is shown as gene expression of FZD in IL-4 stimulated NHBEs (n=6). D, FZD mRNA expression was determined by qPCR and is shown as

relative expression of FZD in IL-4 stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=6; one-sample t-test).

To confirm that the transcriptional regulation of Wnt and FZD was specific for the type 2 cytokine IL-4 and thus potentially relevant for a type 2 immune response, the effect of the classical type 1 cytokine IFNγ on Wnt and FZD mRNA profiles was investigated. NHBEs were stimulated with 15 ng/ml IFNγ for 6h and Wnt and FZD transcription profiles were assessed. Again mRNA transcripts were detected for most Wnt ligands and all FZD receptors, also with Wnt7a, Wnt7b and FZD6 mRNA transcripts showing the highest expression level (Figure 12 A, C). However, IFNγ failed to transcriptionally regulate any of the Wnt or FZD family members (Figure 12 B, D).

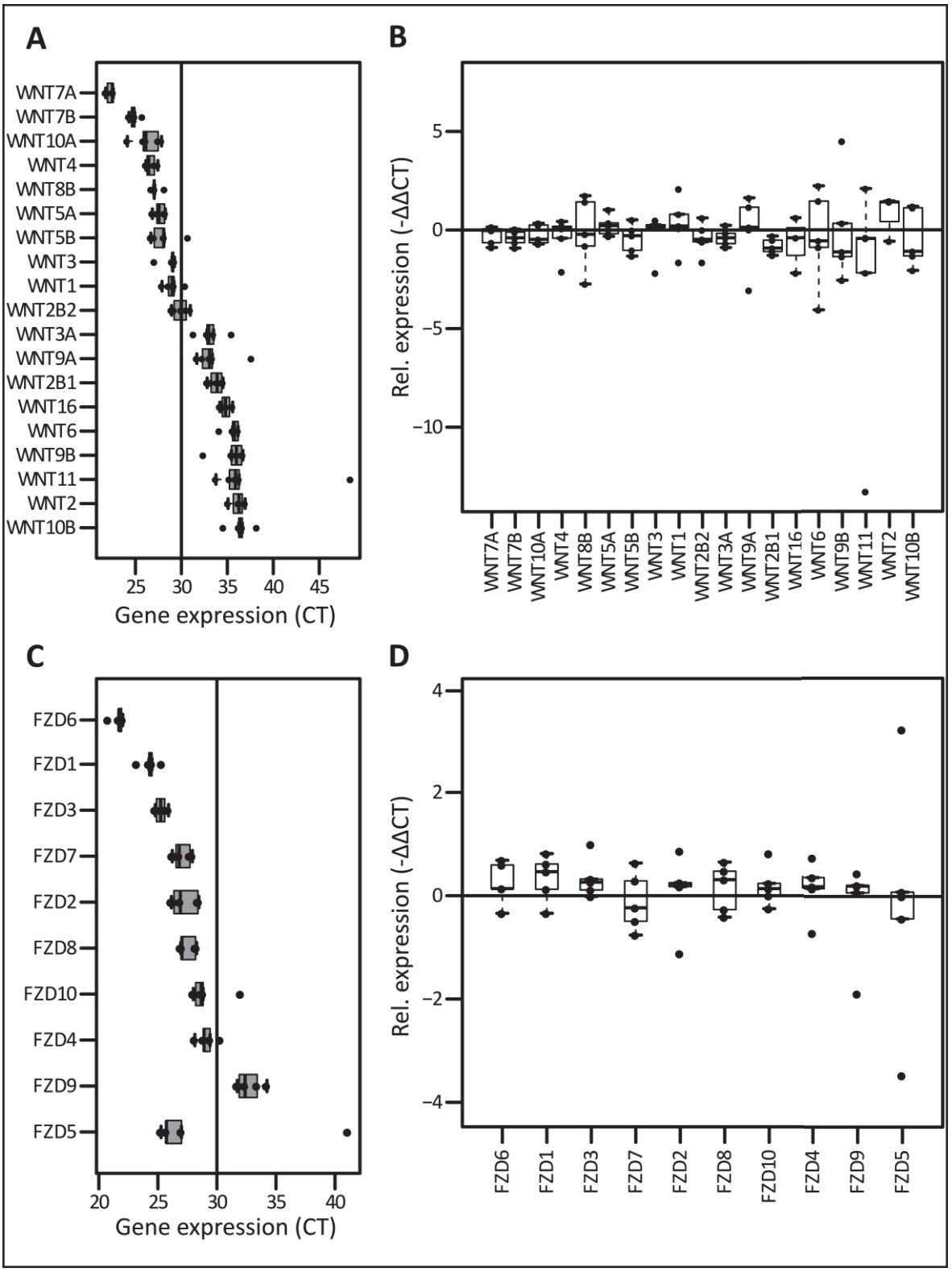

Figure 12 : IFNγ does not regulate gene expression of Wnt and FZD. A, Wnt mRNA expression was determined by qPCR and is shown as gene expression of Wnts in IFNγ stimulated NHBEs (n=6). B, Wnt mRNA expression was determined by qPCR and is shown as relative expression of Wnts in IFNγ stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=6; one-sample t-test). C, FZD mRNA expression was determined by qPCR and is shown as gene expression of FZD in IFNγ stimulated NHBEs (n=6). D, FZD mRNA expression was determined by qPCR and is

shown as relative expression of FZD in IFNγ stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=6; one-sample t-test).

In settings of allergic airway inflammation, bronchial epithelial cells are recurrently exposed to IL-4. To mimic these settings a 72h time course was performed with repetitive IL-4 stimulation. The expression of selected regulated Wnt and FZD mRNA transcripts was analyzed after 6h, 24h, 48h and 72h of IL-4 exposure. While Wnt4 was time dependently upregulated, IL-4 mediated up- or downregulation of Wnt5b, Wnt7a and FZD10 was stable over time (Figure 13 A-D). Depending on IL-4 restimulation after 24h Wnt5a and Wnt8b showed a periodic regulation, which could implicate that they are downregulated by a feedback mechanism (Figure 13 E, F).

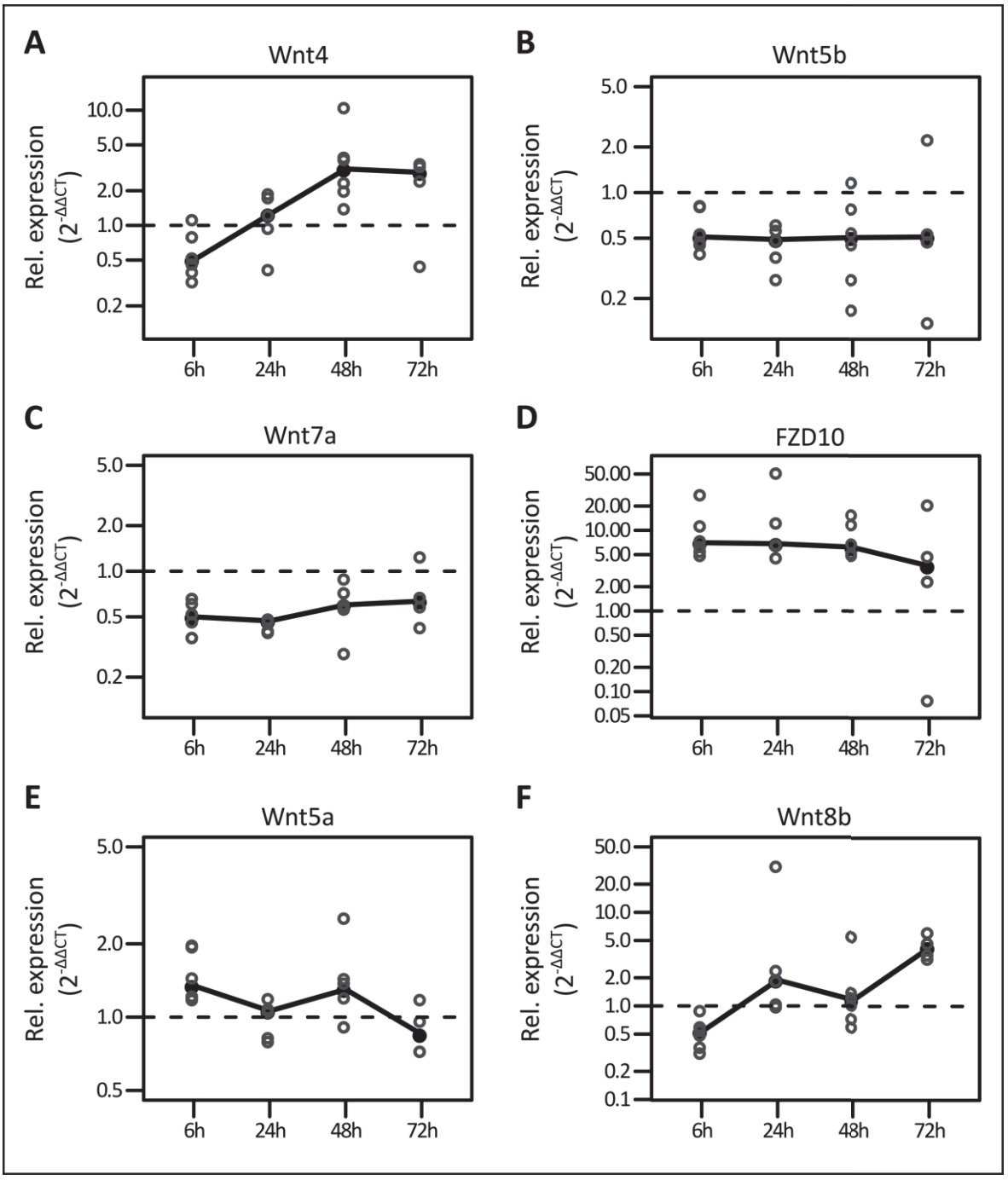

Figure 13 : Wnt expression changes during a timecourse of IL-4 treatment. A-F, Relative expression of Wnt and FZD mRNA during a timecourse of repetitive IL-4 stimulation over 72h (n=4-6).

4.3 Wnt4 secretion is enhanced by IL-4

As FZD10 mRNA was most prominently regulated by IL-4 and Wnt4 mRNA was time-dependently upregulated upon IL-4 treatment, the regulation of FZD10 and Wnt4 protein expression was further explored. While FZD10 mRNA was strongest

upregulated by IL-4, treatment of NHBEs with 50 ng/ml IL-4 for 24h failed to enhance FZD10 protein expression as determined by western blot analysis (Figure 14 A, B). Also Wnt4 expression in whole cell lysates was not altered by IL-4 (Figure 14 C, D). However, western blot analysis of supernatants from IL-4 treated cells revealed enhanced secretion of Wnt4 as compared to control cells (Figure 14 E, F).

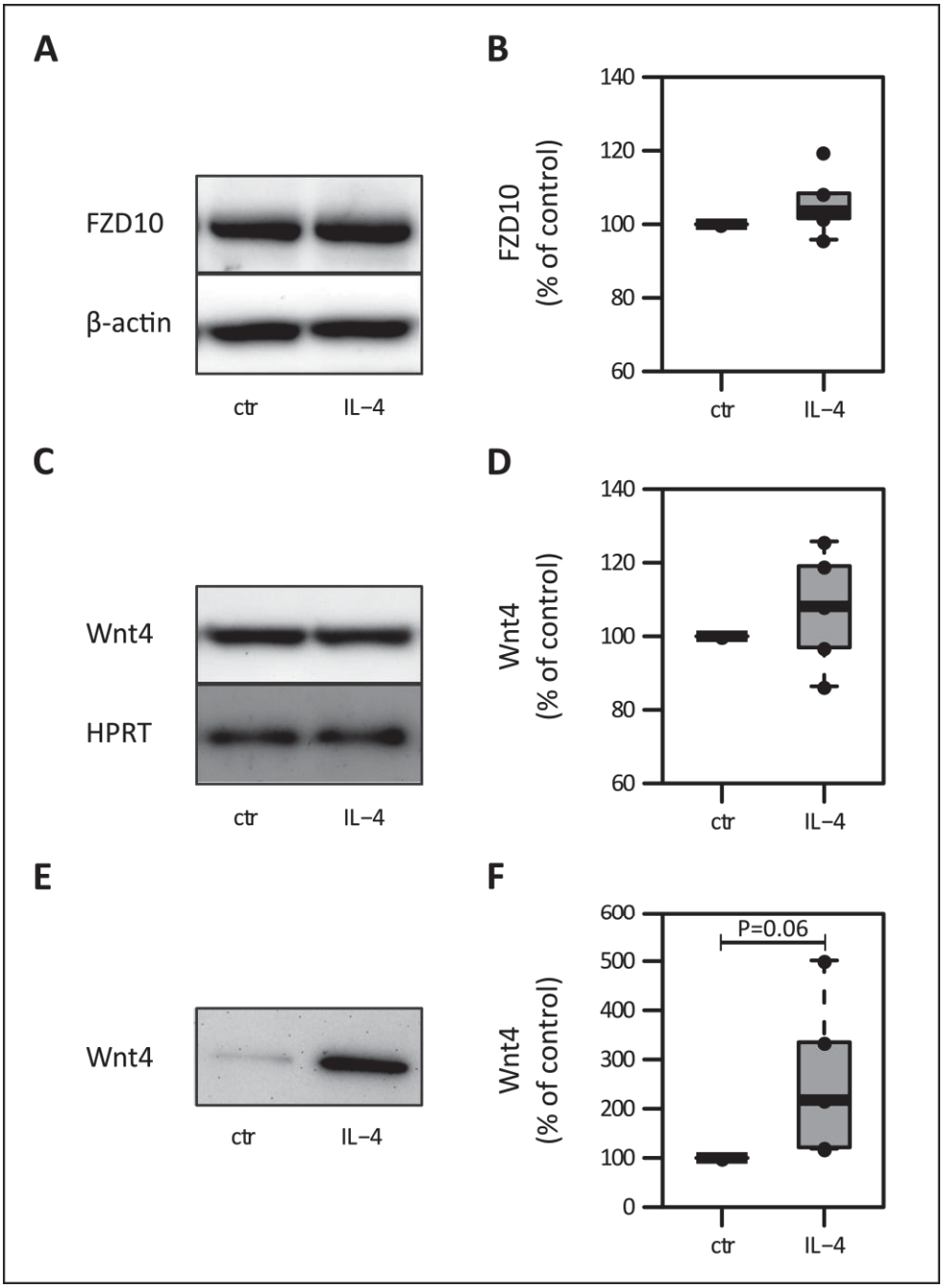

Figure 14 : Secretion of Wnt4 by NHBEs is enhanced by IL-4. A, Representative western blot of FZD10 and housekeeper β-actin from IL-4 stimulated NHBEs and control NHBEs. B, Normalized

expression of FZD10 determined by western blot of IL-4 stimulated NHBEs to control NHBEs corrected for β-actin (n=5). C, Representative western blot of Wnt4 and housekeeper HPRT from IL-4 stimulated NHBEs and control NHBEs. D, Normalized expression of Wnt4 determined by western blot of IL-4 stimulated NHBEs to control NHBEs corrected for HPRT (n=5). E, Representative western blot of Wnt4 in supernatants from IL-4 stimulated NHBEs and control NHBEs. F, Normalized expression of Wnt4 in supernatants determined by western blot from IL-4 stimulated NHBEs to control NHBEs (n=5, Wilcoxon test).

However, taken together the observed effects of IL-4 on Wnt4 and FZD10 were only moderate with questionable biological relevance. Thus, a different IL-4 induced target of the Wnt family was focused on.

4.4 Wnt5a can be upregulated by IL-4 or TGFβ

Wnt5a was recently shown to be induced by TGFβ in airway smooth muscle cells (Kumawat et al. 2013). Therefore the effect of TGFβ on Wnt5a expression in NHBEs was assessed in parallel and in combination with IL-4. NHBEs were either treated with 50 ng/ml IL-4 or with 5 ng/ml TGFβ for 6h and Wnt5a mRNA expression was analyzed by qPCR. IL-4 induced Wnt5a mRNA expression about 1.4 fold (95% CI_{log} 0.2-0.9, P_{log}=0.01), while TGFβ induced the mRNA expression about 1.6 fold (95% CI_{log} 0.3-0.9, P_{log}=0.005), thus demonstrating similar effects of both cytokines on Wnt5a production by NHBEs (Figure 15 A). Western blot analysis of Wnt5a protein expression after 24h of stimulation showed that IL-4 enhanced Wnt5a protein expression by 28% (median=128%, 95% CI 85%-155%, P=0.1) and TGFβ by 30% (median=130%, 95% CI 121%-274%, P=0.06). However, stimulation of the cells with both cytokines did not result in an additive upregulation (Figure 15 B, C). While the upregulation of Wnt5a expression was only moderate, IL-4 was able to clearly increase secretion of Wnt5a, as in supernatants of stimulated cells higher Wnt5a levels were detected by western blot. The levels of secreted Wnt5a were maximal 2.6 fold (median=128%, 95% CI 112%-262%, P=0.06) higher for IL-4 stimulated than for control cells (Figure 15 D, E).

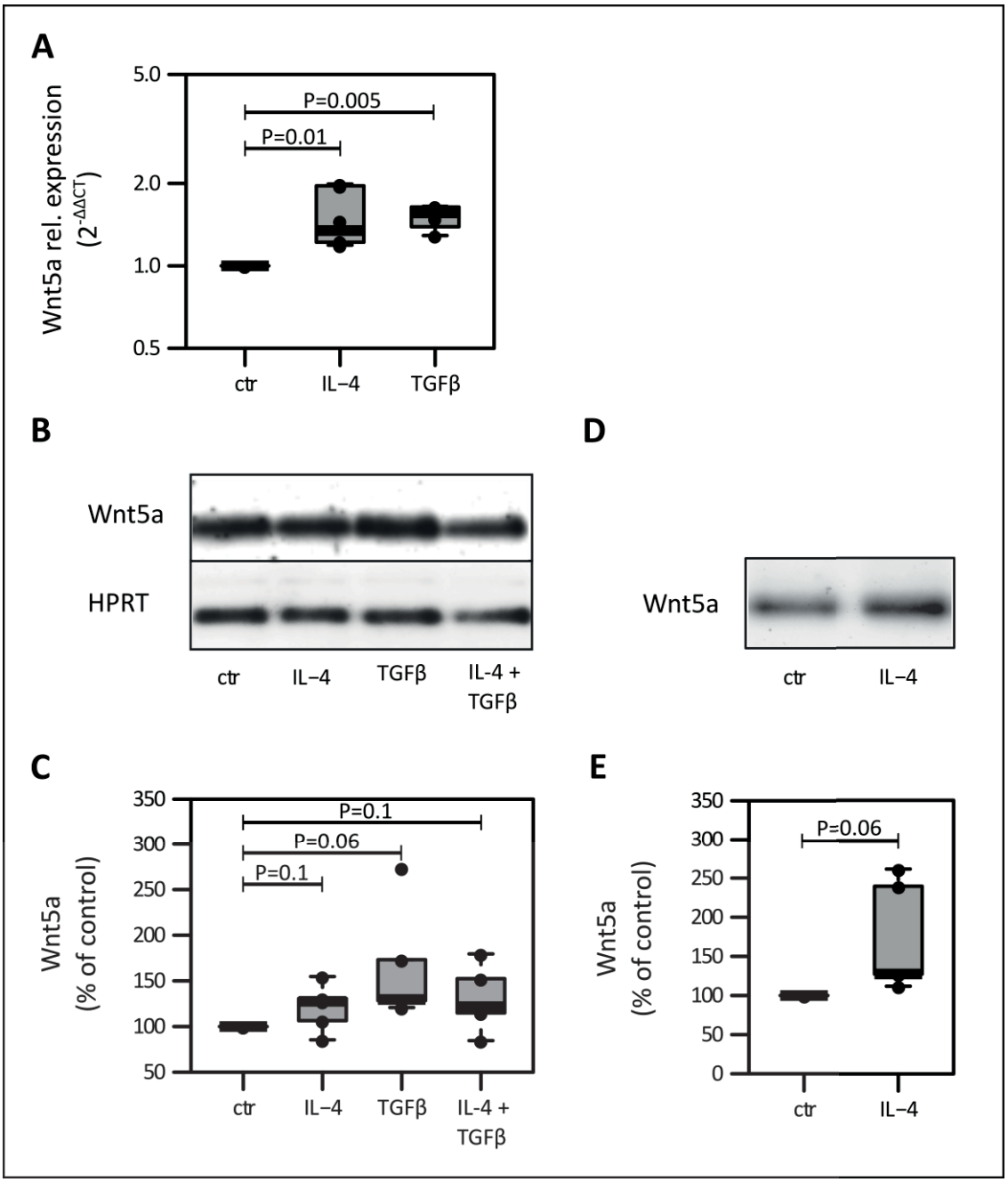

Figure 15 : Specifically Wnt5a is upregulated in NHBEs by IL-4 and TGFβ. A, Relative expression of Wnt5a mRNA of IL-4 and TGFβ stimulated NHBEs to control NHBEs, corrected for housekeeper genes (n=5-6, one sample t-test). B, Representative western blot of Wnt5a and housekeeper HPRT from IL-4 and TGFβ stimulated NHBEs and control NHBEs. C, Normalized expression of Wnt5a determined by western blot of IL-4 and TGFβ stimulated NHBEs to control NHBEs corrected for HPRT (n=5, Wilcoxon test). D, Representative western blot of Wnt5a in supernatants from IL-4 stimulated NHBEs and control NHBEs. F, Normalized expression of Wnt5a in supernatants determined by western blot from IL-4 stimulated NHBEs to control NHBEs (n=6, Wilcoxon test).

As these results showed that IL-4, as well as TGFβ, was able to induce Wnt5a secretion by NHBEs, potential effects of secreted Wnt5a on surrounding immune cells were further explored.

4.5 Epithelial derived Wnt5a enhances TGM2 expression in monocyte derived macrophages

During allergic inflammation the lung gets infiltrated with a large diversity of immune cells. However, some of them are even present in steady-state conditions, such as tissue resident macrophages. It was reported that epithelial derived Wnt5a can shift the polarization of macrophages from pro-inflammatory M1 to pro-fibrotic M2 macrophages (Bergenfelz et al. 2012). Therefore the effect of epithelial derived Wnt5a on the M2 macrophage marker TGM2 was investigated.

For this purpose conditioned media (CM) from unstimulated and IL-4 stimulated NHBEs was collected. To control for potential effects of remaining IL-4, medium containing 50 ng/ml IL-4 was pre-incubated at 37°C for 24h before adding it to the cells for 24h. MDMs in the performed experiments expressed high basal levels of TGM2 due to differentiation in the presence of TGFβ. However, the IL-4 containing medium did not further increase TGM2 expression (Figure 16 A. B).

Cultured MDMs were then stimulated with CM from IL-4 stimulated NHBEs in the presence or absence of a Wnt5a blocking antibody for 24h. TGM2 expression was assessed by immunofluorescence analysis. IL-4 CM enhanced TGM2 mean intensity in MDMs about $0{,}8x10^{-3}$ a.u. (95% CI $0{,}2x10^{-3}$-$1{,}7x10^{-3}$, P=0.02) compared to control CM (Figure 16 C, D). This effect was almost abrogated upon addition of a Wnt5a blocking antibody with a reduction of $0{,}6x10^{-3}$ a.u. (95% CI $0{,}2x10^{-3}$-$1{,}5x10^{-3}$, P=0.008) of TGM2 mean intensity (Figure 16 C, D).

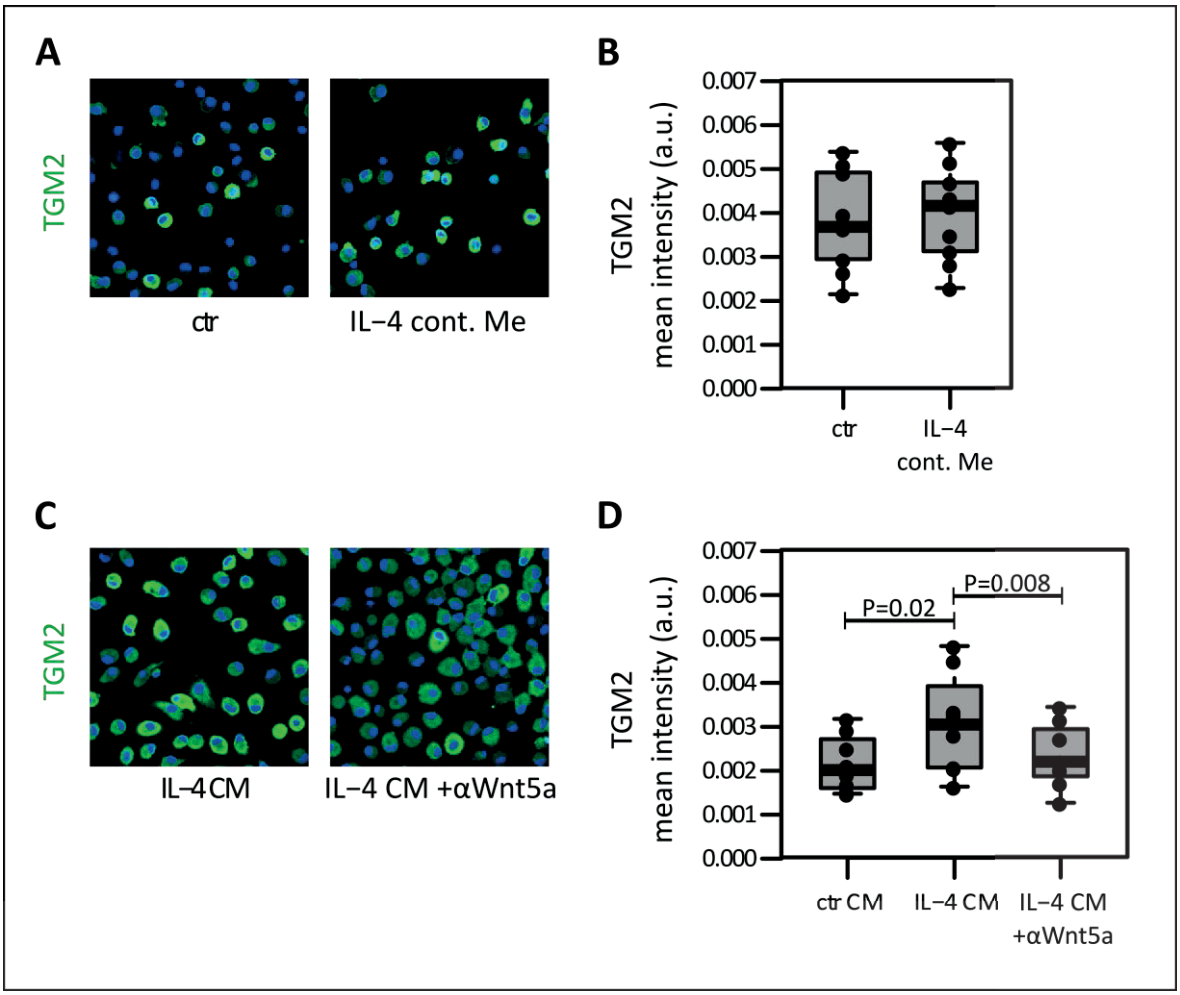

Figure 16 : Secretions from IL-4 stimulated NHBEs promote TGM2 in human macrophages in a Wnt5a dependent fashion. A, Representative immunofluorescence images of TGM2 expression in MDMs stimulated with IL-4 containing medium (IL-4 cont. Me) and control MDMs. B, Mean fluorescence intensity of TGM2 in MDM treated with IL-4 containing medium or control MDM (n=8-9). C, Representative immunofluorescence images of TGM2 expression in MDMs stimulated with CM from IL-4 stimulated NHBEs and a Wnt5a neutralizing antibody. D, Mean fluorescence intensity of TGM2 in MDM treated with control CM, IL-4 CM and a Wnt5a neutralizing antibody (n=8-9, Wilcoxon test).

Thus, TGM2 was upregulated by epithelial derived Wnt5a showing a potential relevance of Wnt5a secretion by IL-4 exposed bronchial epithelial cells during allergic inflammation.

4.6 IL-4 induces epithelial TGM2 resulting in enhanced leukotriene production

TGM2 could not only be upregulated by Wnt5a in MDMs, but was also one of the genes that were strongly upregulated by IL-4 as revealed by a whole genome array of IL-4 stimulated NHBEs (Zissler et al. 2015). To confirm upregulation of TGM2 on the protein level, NHBEs were stimulated with 50 ng/ml IL-4 and 5 ng/ml TGFβ and the combination of both cytokines for 24h and then TGM2 expression was analyzed by western blot. While IL-4 enhanced TGM2 protein levels by 76% (median=176%, 95% CI 106%-190%, P=0.06), TGFβ and the combined stimulation with both cytokines had a less strong effect with an induction of 41% (median=142%, 95% CI 75%-216%, P=0.3) and 53% (median=153%, 95% CI 89%-169%, P=0.3), respectively (Figure 17 A, B).

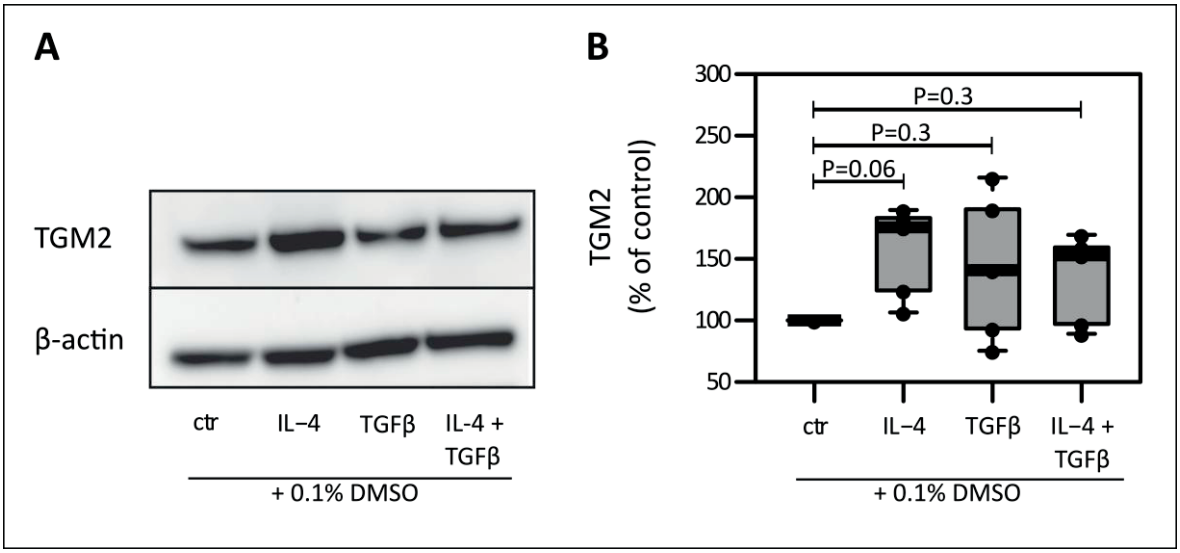

Figure 17 : Epithelial TGM2 expression is enhanced by IL-4. A, Representative western blot of TGM2 and housekeeper β-actin from IL-4 and TGFβ stimulated NHBEs and control NHBEs. B, Normalized expression of TGM2 determined by western blot of IL-4 and TGFβ stimulated NHBEs to control NHBEs corrected for β-actin (n=5, Wilcoxon test).

TGM2 was found to be overexpressed in the airways of asthmatics and to regulate production of cysLT via activating sPLA2-X (Hallstrand et al. 2010). Additional it was shown that extract of house dust mites is able to enhance the production of cysLT by bronchial epithelial cells by inducing expression of the enzyme 5-LO (Trian et al.

2015). To test if IL-4 is able to increase epithelial cysLT production by increasing TGM2 expression, first the expression of 5-LO was determined by immunofluorescence analysis. NHBEs were grown on microscope slides and stimulated with 50 ng/ml IL-4 in the presence or absence of 10 µg/ml HDM.

As the cells tended to detach from the microscope slides, especially when treated with HDM, quantification of the experiments did not properly display the observed effects. Therefore exemplary pictures of 3 donors are shown in Figure 18. Basal expression of 5-LO was strongly dependent on the donor of NHBEs and no changes were observed after IL-4 stimulation. In contrast, HDM clearly induced 5-LO expression in NHBEs (Figure 18).

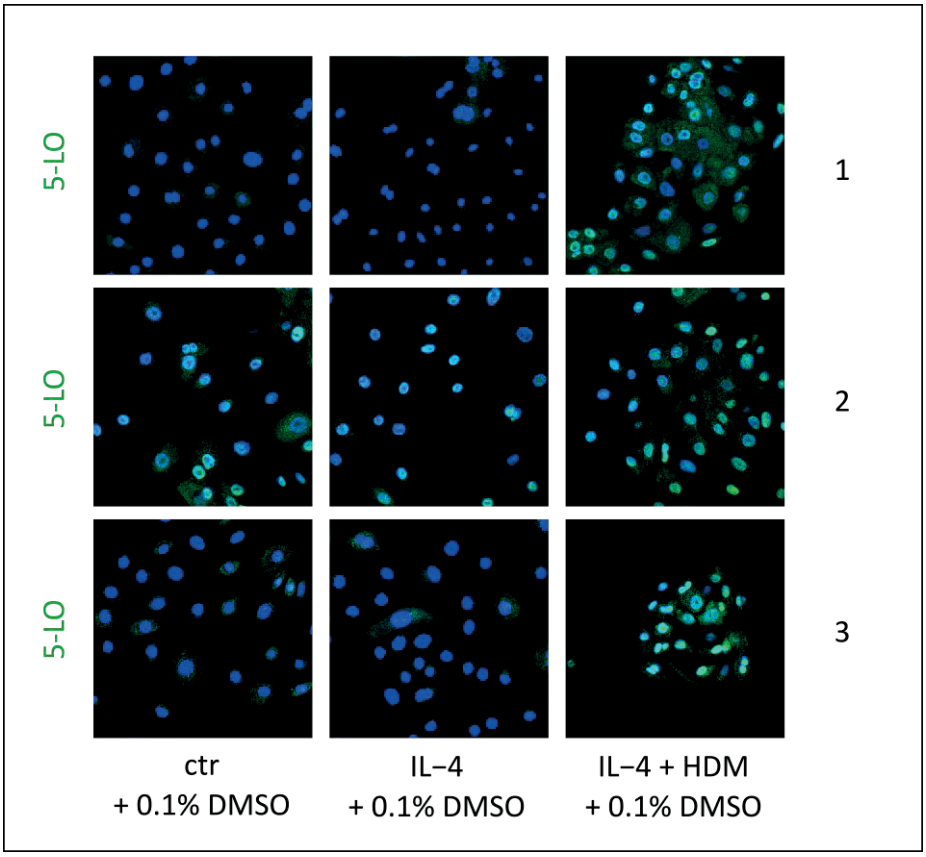

Figure 18 : 5-LO expression in NHBEs is selectively induced by HDM. Representative immunofluorescence images of 5-LO expression in NHBEs stimulated with IL-4 or HDM and control NHBEs of three different donors.

Before measuring the production of cysLT by the stimulated cells, TGM2 expression in HDM stimulated NHBEs was analyzed to ascertain if HDM had any effect on TGM induction by IL-4. NHBEs were stimulated with 50 ng/ml IL-4 or together with 10 µg/ml HDM for 24h and TGM2 expression was analyzed by western blot. However, HDM addition did not alter TGM2 expression in NHBEs (Figure 19 A, B).

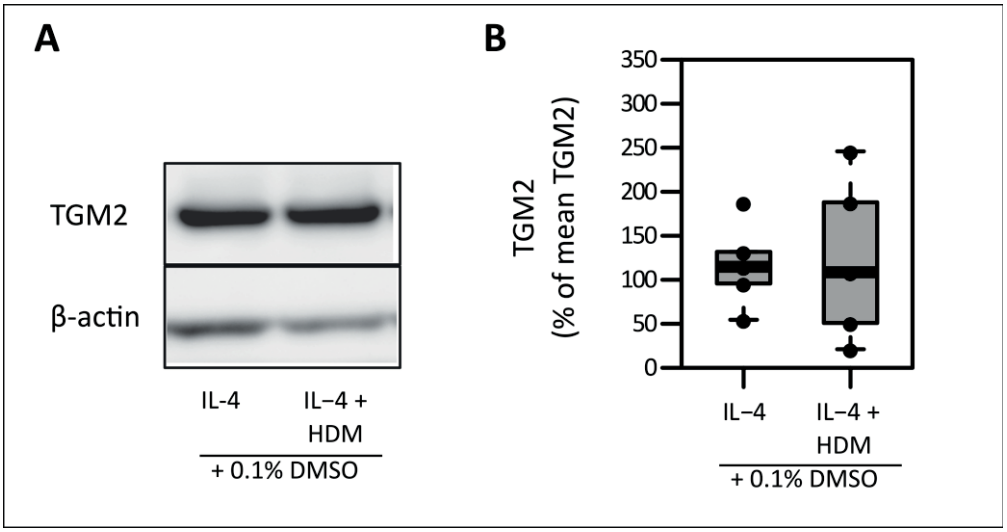

Figure 19 : HDM does not alter IL-4 induced epithelial TGM2 expression. A, Representative western blot of TGM2 and housekeeper β-actin from IL-4 and HDM stimulated NHBEs. B, Normalized expression of TGM2 determined by western blot of IL-4 and HDM stimulated NHBEs to mean TGM2 expression in all samples corrected for β-actin (n=5).

To confirm if TGM2 can regulate cysLT production, the effect of IL-4, HDM and the TGM2 inhibitors (monodansylcadaverine (MDC) and cystamine (Cys)) on cysLT production was assessed. Again NHBEs were treated with 50 ng/ml IL-4 with or without 10 µg/ml HDM for 24h in the presence or absence of either 25 µM MDC or 100 µM Cys. While IL-4 was able to induce TGM2, stimulation with IL-4 did not enhance cysLT production by NHBEs. However, by addition of HDM, cysLT production by NHBEs was enhanced by 109.1 pg/ml (95% CI -42.9-219.4 pg/ml, P=0.1). Treatment with the TGM2 inhibitors MDC and Cys resulted in a reduction of cysLT levels by 135.9 pg/ml (95% CI -66.3-343.0 pg/ml, P=0.3) and 116.7 pg/ml (95% CI -141.8-375.2 pg/ml, P=0.3), respectively (Figure 20).

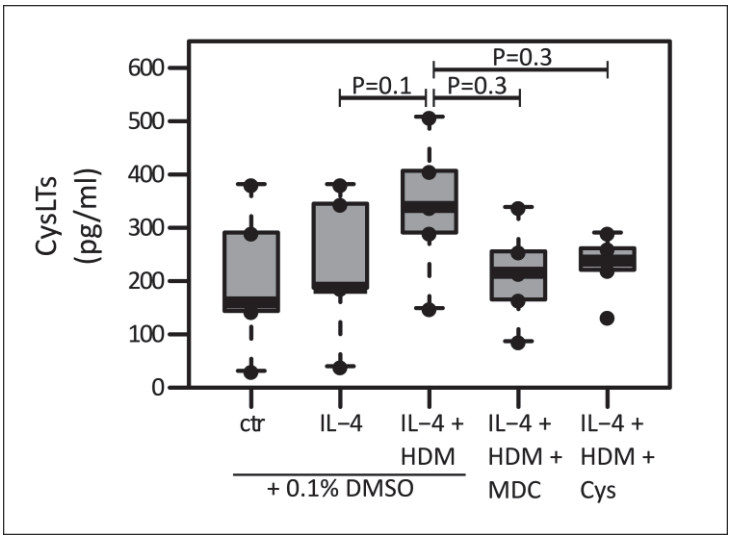

Figure 20 : TGM2 contributes to HDM-stimulated cysLT production in NHBEs. Levels of cysLTs measured in supernatants from IL-4 and HDM stimulated NHBEs treated with TGM2 inhibitors and control NHBEs by ELISA (n=5; Wilcoxon test).

These data show, if 5-LO expression is induced by an allergen as HDM, higher levels of active TGM2 lead to enhanced cysLT production.

4.7 Reduction of leukotriene levels by TGM2 inhibitors is not due to cell loss

Treatment of NHBEs with TGM2 inhibitors led to a tendency of decreased cysLT production. To exclude that this was due to loss of cells, a cytotoxicity assay was performed. The viability of NHBEs was determined by an LDH assay after stimulation with 50 ng/ml IL-4 together with 10 µg/ml HDM in the presence of either 25 µM MDC or 100 µM Cys. While stimulation with IL-4 and HDM enhanced the LDH release about 7% (95% CI -10%-47%, P=0.2), the treatment with MDC or Cys did not further enhance LDH release (Figure 21).

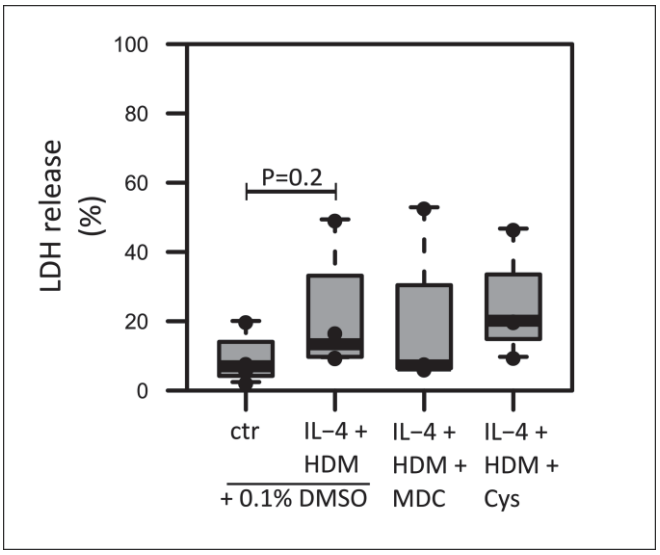

Figure 21 : Inhibitor treatment does not affect viability of NHBEs. Percentage of LDH release measured in supernatants from IL-4 and HDM stimulated NHBEs treated with TGM2 inhibitors and control NHBEs (n=3; Wilcoxon test).

Therefore treatment with the inhibitors did not cause any additional cytotoxicity compared to HDM stimulation.

4.8 Expression of epithelial TGM2 and sPLA2-X is steroid resistant

Inhaled glucocorticosteroids are currently the most effective treatment to control asthma. However, in case of resistance to high levels of inhaled glucocorticosteroids, leukotriene modifiers can help to regain control over the disease (Laviolette et al. 1999, Virchow et al. 2000). To evaluate the effect of glucocorticosteroids on TGM2 as well as sPLA2-X expression, NHBEs were stimulated with a combination of 50 ng/ml IL-4, 5 ng/ml TGFβ and 10 µg/ml HDM for 24h and treated with 1µM fluticasone propionate (FP), a commonly used glucocorticosteroid. While expression of TGM2 was not altered by fluticasone propionate (Figure 22 A, B), there was a tendency in most donors for a higher expression of sPLA2-X (Figure 22 C, D).

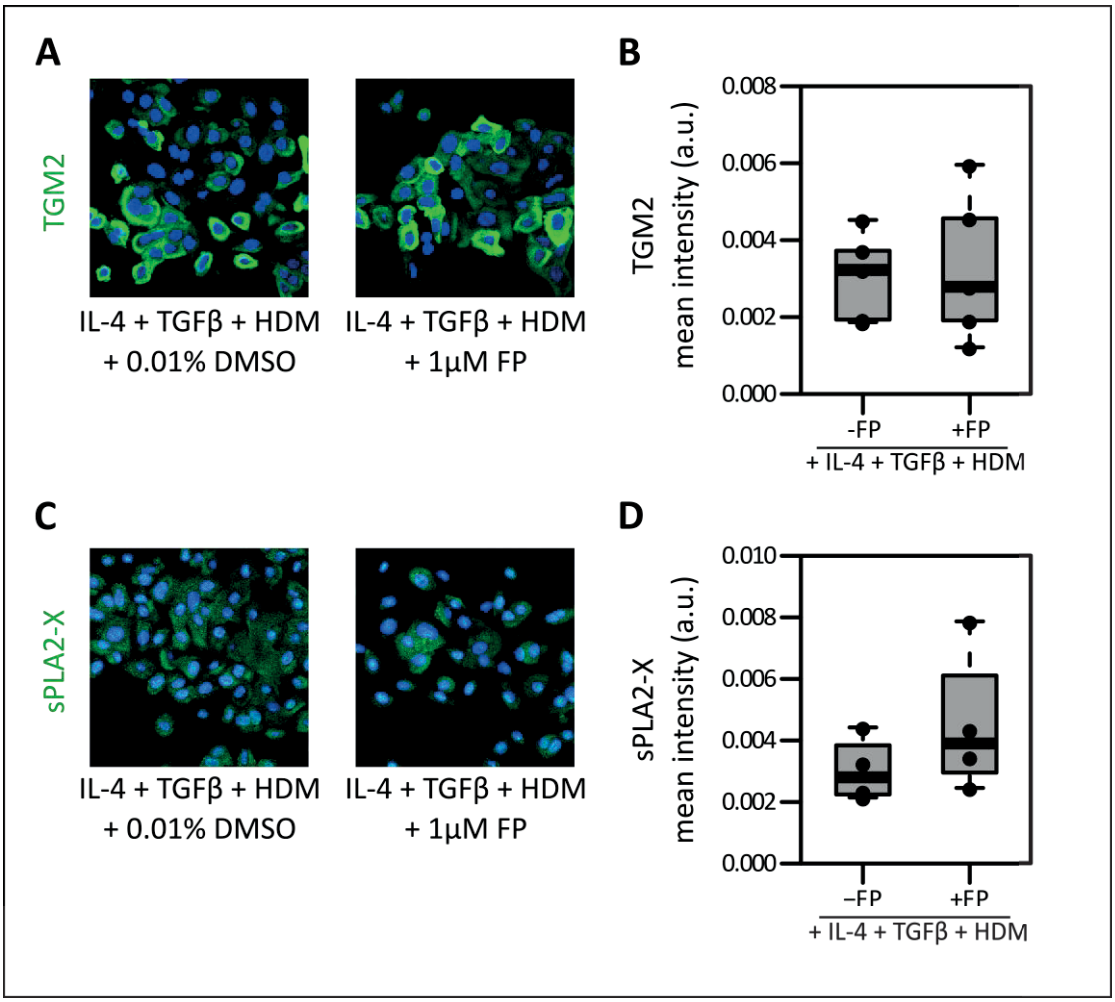

Figure 22 : TMG2 and sPLA2-X expression in NHBEs are steroid resistant. A, Representative immunofluorescence images of TGM2 expression in NHBEs stimulated with IL-4, TGFβ and HDM treated with or without FP. B, Mean fluorescence intensity of TGM2 in NHBEs stimulated with IL-4, TGFβ and HDM treated with or without FP (n=4). C, Representative immunofluorescence images of sPLA2-X expression in NHBEs stimulated with IL-4, TGFβ and HDM treated with or without FP. D, Mean fluorescence intensity of sPLA2-X in NHBEs stimulated with IL-4, TGFβ and HDM treated with or without FP (n=4).

As the expression of both proteins was not reduced by FP, TGM2 driven release of leukotrienes could play a role in steroid insensitive asthma.

4.9 Macrophages can contribute to TGM2 dependent cysLT production

As described above (see 4.6), NHBEs can release cysLT in a partially TGM2 dependent fashion. However, the production of cysLTs by NHBEs was quite low in comparison to the levels usually produced by myeloid cells (Lewis and Austen 1984). Additionally as shown in 4.5, epithelial derived Wnt5a can induce TGM2 in macrophages, which are a major source of LTs in the airways. Therefore the contribution of TGM2 to the cysLT production of MDMs was investigated. Contamination by epithelial derived products was avoided by stimulating MDMs with IL-4, which is known to induce TGM2 in macrophages (Martinez et al. 2013).

Cultured MDMs were stimulated with 50 ng/ml IL-4 and treated with 25 µM MDC or 100 µM Cys for 24h. Stimulation with IL-4 did not enhance cysLT production in MDMs. However, treatment of IL-4 stimulated MDMs with the TGM2 inhibitors MDC and Cys resulted in a reduction of cysLT levels about 9660.3 pg/ml (88% CI 3624.7-18410.7 pg/ml, P=0.1) and 5155.8 pg/ml (88% CI 926.3-8013.2 pg/ml, P=0.1), respectively (Figure 23).

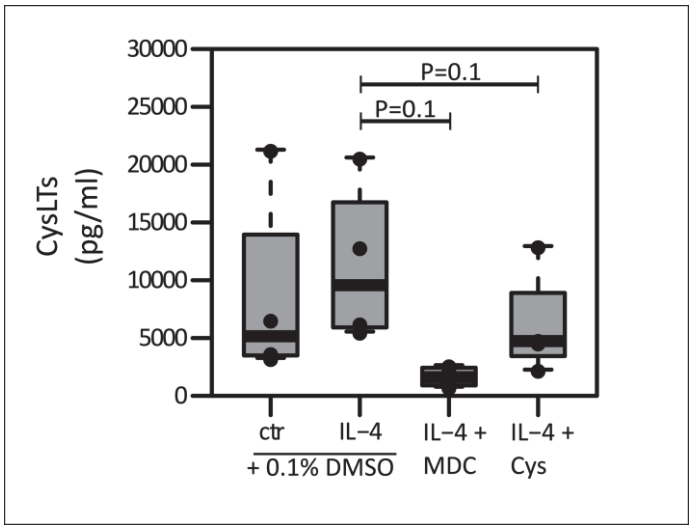

Figure 23 : IL-4 stimulated MDMs secrete cysLT in a TGM2 dependent fashion. Levels of cysLTs measured in supernatants from IL-4 stimulated MDMs treated with TGM2 inhibitors and control MDMs by ELISA (n=4; Wilcoxon test).

Thus, in comparison to NHBEs macrophages are producing higher amounts of cysLTs in a fashion that is also more sensitive to TGM2 inhibitors.

4.10 Wnt5a-TGM2-LT pathway could explain high levels of leukotrienes in a mouse model of allergic airway inflammation

The previous *in vitro* experiments with bronchial epithelial cells and monocytes suggested a contribution of epithelial derived Wnt5a and TGM2 to production of inflammatory cysLTs. As the experiments were performed under type 2 inflammatory conditions, the relevance of the Wnt5a-TGM2-LT pathway in a mouse model of allergic inflammation was investigated. Lung tissue samples and BALF from mice sensitized against HDM at day 3 (neonate), day 15 (preweanling) or day 60 (adult) after birth were kindly provided by Dr. Eva Gollwitzer (University of Lausanne) in cooperation with Dr. Esser-von Bieren. In this short model of allergic airway inflammation sensitized neonates showed enhanced airway eosinophilia and an exaggerated type 2 immune response as compared to sensitized preweanling and adult mice (Gollwitzer et al. 2014).

Further analysis revealed that mice sensitized at day 3 exhibited high levels of cysLTs in their BALF, which was in line with the strong eosinophilia in these mice (Figure 24 A). However, the highest content of cysLTs was measured in the BALF from adult mice, with 7715.1 pg/ml (95% CI -6754.6-18445.9 pg/ml, P=0.3) higher cysLT levels measured than neonatal mice and 20702.7 pg/ml (95% CI 14594.5-27725.1 pg/ml, P=0.03) higher cysLT levels than in preweanling mice (Figure 24 A). In line with the high eosinophilia in sensitized neonatal mice, immunohistochemistry analysis of lung tissue showed the highest numbers of 12/15-LO$^+$ cells in perivascular infiltrates of neonates (Figure 24 B, C). Surprisingly despite having the highest cysLT levels in the BALF, lung tissue from sensitized adult mice showed reduced expression of 5-LO, while expressing comparable levels of LTC4S as sensitized neonates (Figure 24 B, D, E). While levels of 5-LO were similar in adult sensitized mice and preweanling mice, LTC4S levels were only slightly higher in sensitized adults than preweanlings, though not explaining the strong difference in cysLT levels (Figure 24 A, B, D, E).

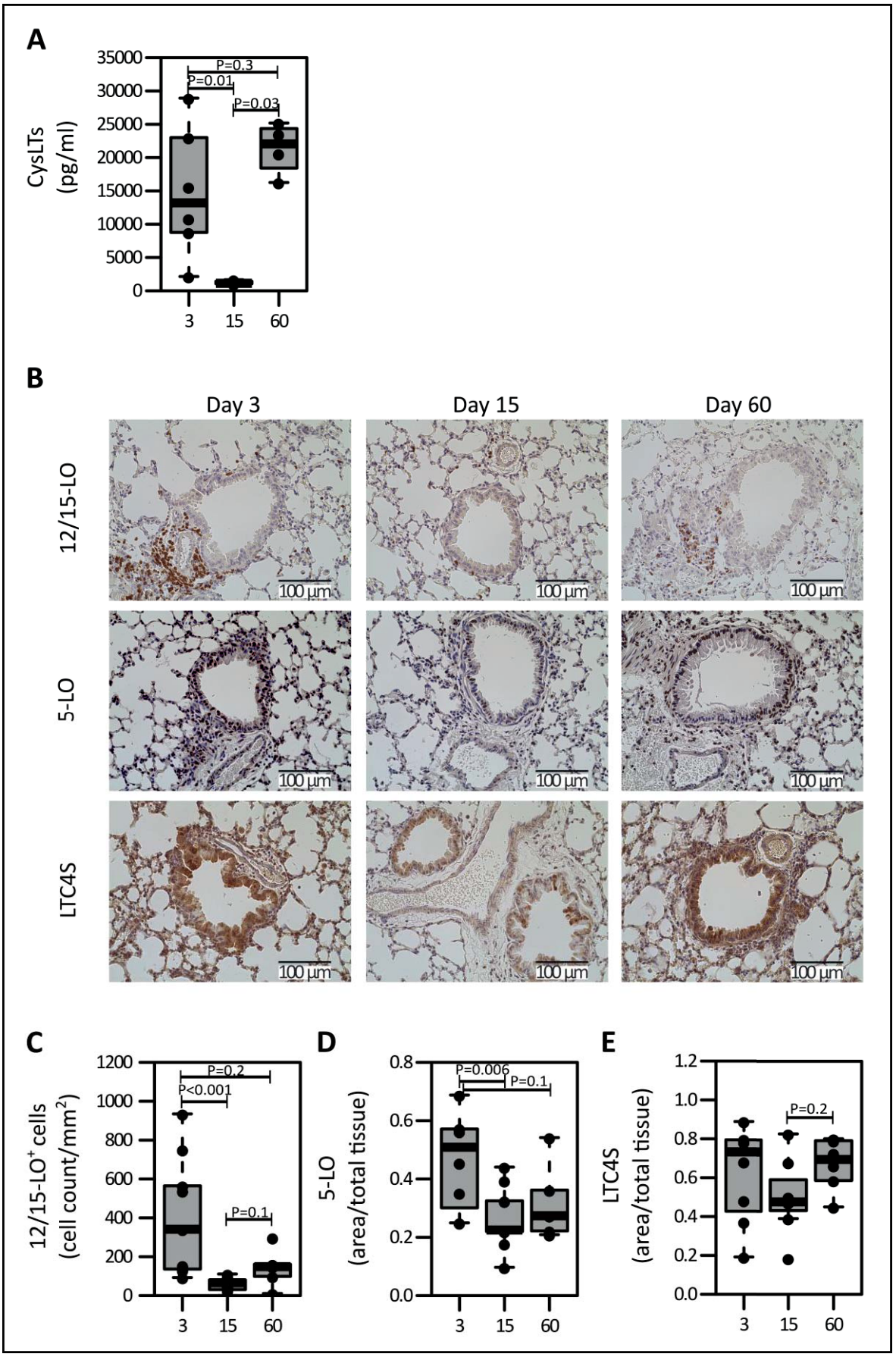
A
CysLTs (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
P=0.3
P=0.01
P=0.03
3
15
60
B
Day 3
Day 15
Day 60
12/15-LO
5-LO
LTC4S
100 µm
C
12/15-LO+ cells (cell count/mm2)
1200
1000
800
600
400
200
0
P=0.2
P<0.001
P=0.1
D
5-LO (area/total tissue)
0.8
0.6
0.4
0.2
0.0
P=0.006
P=0.1
E
LTC4S (area/total tissue)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P=0.2

Figure 24 : Leukotriene production is age dependent and LT biosynthetic enzymes are abundant in the bronchial epithelium during allergic airway inflammation *in vivo*. A, Levels of cysLTs measured in the BALF from sensitized mice by ELISA (n=4-6; Mann-Whitney test). B, Representative immunohistochemistry staining images of 12/15-LO, 5-LO, LTC4S expression in lung sections of sensitized mice. C, Counts of 12/15-LO positive cells per mm^2 lung section of sensitized mice (n=5-9; Mann-Whitney test). D, Quantification of 5-LO positive area of lung sections of sensitized mice (n=6-8; Mann-Whitney test). E, Quantification of LTC4S positive area of lung sections of sensitized mice (n=6-8; Mann-Whitney test).

To see if Wnt5a-TGM2-LT pathway could explain the high cysLT levels in adult mice, lung tissue samples of sensitized mice were analyzed by immunohistochemistry for the expression of Wnt5a and TGM2. Wnt5a was expressed in the lungs of all HDM-sensitized mice with a slightly increasing expression with age (Figure 25 A, B). While the expression of Wnt5a in the tissues of all mice was mainly located to SMCs, epithelial Wnt5a expression could only be detected in adult mice (Figure 25 A). In contrast to TGM2, which could not be detected in lung tissues by immunohistochemistry, sPLA2-X was detected in lung tissue of mice at every age. However, tissues of sensitized neonates showed only low expression of sPLA2-X (Figure 25 A). Already in preweanling mice sPLA2-X expression was higher and in adult sensitized mice the strongest expression of sPLA2-X was detected with apparent localization in SMCs, infiltrating leukocytes and epithelial cells (Figure 25 A, C).

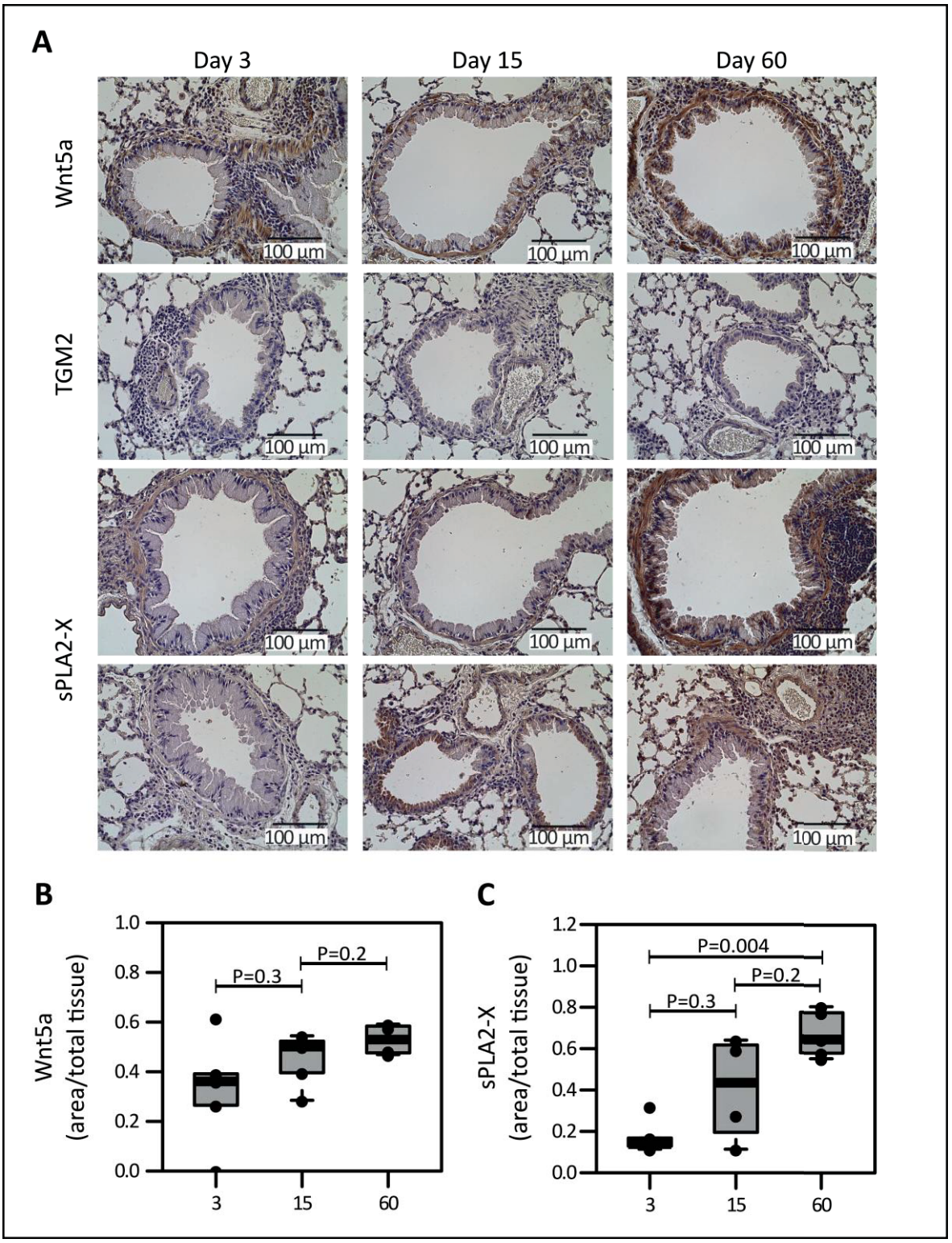

Figure 25 : Age-dependent increases of epithelial Wnt5a and sPLA2-X expression in the lung of sensitized mice. A, Representative immunohistochemistry staining images of Wnt5a, TGM2, sPLA2-X expression in lung sections of sensitized mice. B, Quantification of Wnt5a positive area of lung sections of sensitized mice (n=4-5; Mann-Whitney test). C, Quantification of sPLA2-X positive area of lung sections of sensitized mice (n=4-6; Mann-Whitney test).

As TGM2 could not be detected in lung tissues of sensitized mice, BALF was analyzed by western blot to test if TGM2 was secreted in the airways. The western blot analysis revealed that TGM2 was abundant in BALF and that TGM2 secretion increased with age (Figure 26 A). TGM2 production in adult sensitized mice was 78% (95% CI 46%-149%, P=0.06) higher than in neonates and 50% (95% CI 19%-109%, P=0.03) higher than in preweanling mice (Figure 26 B). As Wnt5a is also secreted by epithelial cells, BALF was additional analyzed for Wnt5a. Wnt5a was detected in the BALF from sensitized mice, where it was mainly present in its dimeric form (Figure 26 A). In BALF from neonatal sensitized mice the expression of Wnt5a was 26% (95% CI -4%-55%, P=0.1) higher than in preweanling mice (Figure 26 C). The highest expression of Wnt5a was detected in adult mice with being 50% (95% CI 14%-108%, P=0.03) higher than in preweanling mice and 24% (95% CI -11%-81%, P=0.1) higher than in neonatal mice (Figure 26 C).

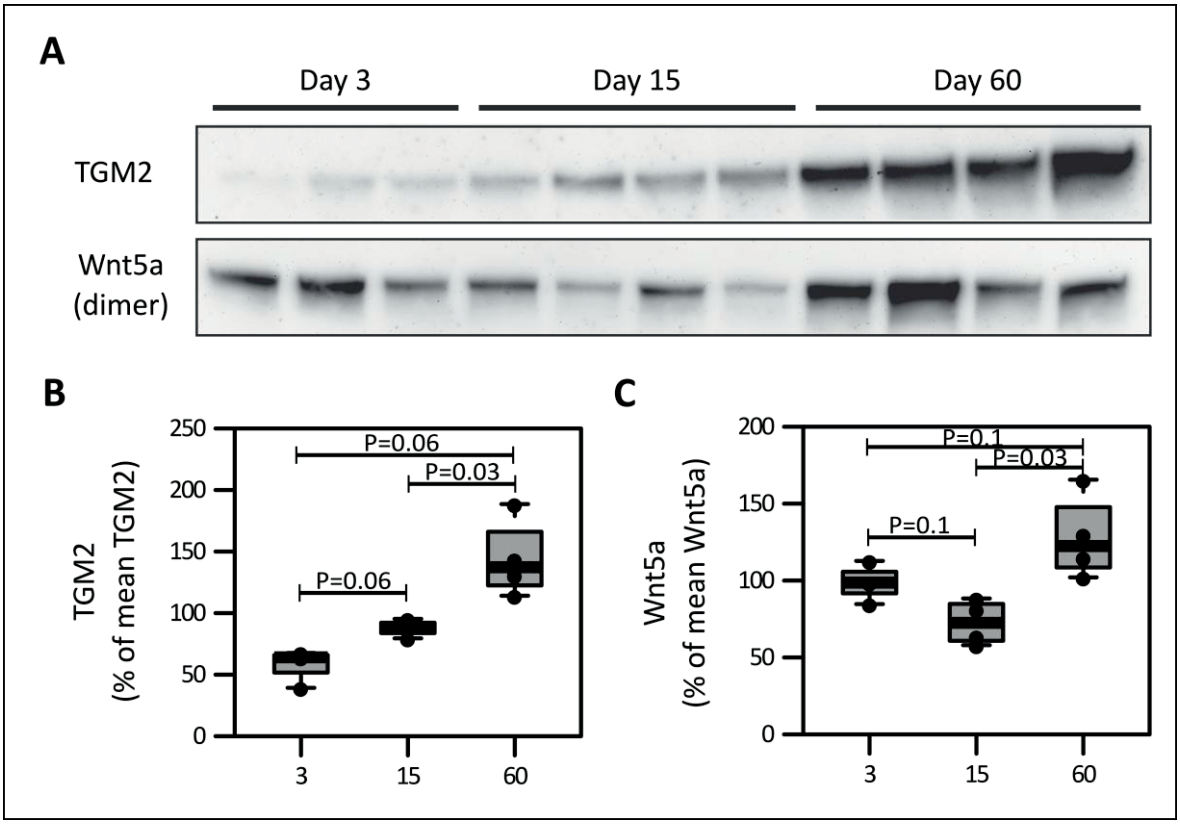

Figure 26 : Increased levels of secreted TGM2 and Wnt5a in the BALF from adult sensitized mice. A, Representative western blot of TGM2 and Wnt5a expression in BALF from sensitized mice. B, Normalized expression of TGM2 determined by western blot of BALF from sensitized mice to mean TGM2 expression (n=3-4, Mann-Whitney test). C, Normalized expression of Wnt5a

determined by western blot of BALF from sensitized mice to mean Wnt5a expression (n=3-4; Mann-Whitney test).

In summary the immunohistochemistry and western blot analysis showed that Wnt5a and TGM2, as well as the downstream enzyme sPLA2-X, were especially abundant in adult mice. Positive regulation by Wnt5a and TGM2 may thus promote the production of LTs by various cell types in inflamed airways, thereby contributing to the efficient production of cysLTs during allergic airway inflammation in adults.

5 Discussion

5.1 Identification of a novel cascade regulating cysLT production

Combination of human *in vitro* studies with a murine model of allergic airway inflammation induced by HDM-sensitization at different ages led to the identification of a new cascade of remodeling factors, which depends on type 2 inflammation and age. The identified cascade consists of Wnt5a, TGM2 and sPLA2-X and regulates cysLT production.

The current study revealed that epithelial derived Wnt5a enhances TGM2 expression in human macrophages, while TGM2 drives cysLT production in bronchial epithelial cells and macrophages likely by enhancing sPLA2-X activity.

It is known that TGM2 deficient mice exhibit reduced canonical Wnt signaling in the adipose tissue and exogenously added TGM2 is able to revert this effect (Myneni et al. 2015). Surprisingly epithelial derived non-canonical Wnt5a enhanced TGM2 expression of human macrophages. As TGM2 is an important marker of human and mouse M2 macrophages, the increased expression of TGM2 suggests that Wnt5a favors polarization of macrophages to an M2 phenotype (Martinez et al. 2013). Wnt ligands were shown to be able to induce M2 macrophage polarization in a mouse model of airway infection, however *in vitro* experiments with human macrophages showed that Wnt5a does not favor M2 polarization, but instead blocks M1 polarization (Bergenfelz et al. 2012, Schaale et al. 2013). Thus showing that Wnt5a might not directly drive M2 polarization, but indirectly promotes the expansion of M2 macrophages by preventing M1 polarization. However, in the study of Bergenfelz et al. macrophages were stimulated with IL-4 to obtain an M2 phenotype, which probably overrides the effects of Wnt5a (Bergenfelz et al. 2012). Therefore further studies have to show if Wnt5a has direct effects on TGM2 expression of macrophages as well as other myeloid cells, which could then contribute to LT production in the inflamed airways.

High TGM2 levels in the BALF of adult sensitized mice were associated with high cysLT levels in these mice and TGM2 inhibitors were able to decrease LT production of epithelial cells and macrophages thus linking TGM2 directly to LT production for

the first time. Mechanistically, TGM2 likely exerts its effect by activating sPLA2-X, which enhances bioavailability of arachidonic acid and enables arachidonic acid release by cPLA2 leading to increased production of cysLT (Hallstrand et al. 2010, 2016, Lai et al. 2010). Both enzymes, TGM2 and sPLA2-X, were highly expressed in the lung of adult sensitized mice, supporting their involvement in the production of cysLT in these mice.

TGM2 and sPLA2-X were thought to be epithelial regulators of cysLT production by leukocytes (Hallstrand et al. 2012). While sPLA2-X expression in bronchial epithelial cells was apparent in isolated cells and the airways of adult sensitized mice, the expression of TGM2 could only be shown for cultured bronchial epithelial cells. This is in contrast to data showing TGM2 expression in human endobronchial biopsies (Hallstrand et al. 2010). However, high levels of secreted TGM2 were found in the BALF from adult sensitized mice. TGM2 could therefore be rapidly secreted from bronchial epithelial cells. This is supported by additional experiments revealing that TGM2 was highly present in the basal membrane below the epithelial layer in human nasal polyp tissues of asthmatics (Dietz et al. 2016). In addition epithelial derived Wnt5a could enhance TGM2 expression and secretion by macrophages resulting in activation of epithelial or macrophage sPLA2-X leading to enhanced cysLT production by leukocytes.

Furthermore epithelial cells can be a direct source of cysLTs. The 3-week HDM model used in the current study involved both infiltration of eosinophils and upregulation of LT biosynthetic enzymes in the bronchial epithelium. Thus, it would have been difficult to determine the major source of cysLTs. However, this could be answered by using eosinophil deficient mice or conditional knock-outs for 5-LO in epithelial or myeloid cells. However, BALF cysLT levels were also increased in a shorter model of allergic airway inflammation, where no infiltrating eosinophils were present, which suggests that lung resident cells can contribute to cysLT production (Trian et al. 2015). Even if levels of NHBE secreted cysLTs were quite low, the amount of cysLT was comparable to secreted cysLT levels of reconstituted bronchial epithelium and to the concentration of cysLT in the BALF from the 3-day HDM model in the latter study (Trian et al. 2015). In the epithelial context inhibition of TGM2 only lead to a partial decrease of cysLT levels. However, this could be due to the fact, that the HDM

treatment increased stress and apoptosis in these cells probably resulting in the activation of pathways influencing cysLT production or in a change of the responsiveness of the cells to other stimuli. For instance it was shown that stress-responsive heat shock proteins can induce cysLT production in mast cells (Mortaz et al. 2007).

In summary the current study shows that bronchial epithelial cells can contribute to cysLT levels in the airways by expression of Wnt5a, TGM2, sPLA2-X and production of cysLTs.

5.2 Newly identified cascade of Wnt5a, TGM2 and sPLA2-X is dependent on a type 2 inflammatory response

As Wnt5a and TGM2 were found to be enhanced in gene expression profiles of IL-4 stimulated NHBEs, the influence of a type 2 immune response on epithelial Wnt signaling and TGM2 mediated cysLT production was investigated (Zissler et al. 2015). IL-4 responsiveness of NHBEs was evident for all donors, even though the magnitude of the response as shown by STAT6 phosphorylation was highly variable between the different donors. Accordingly, throughout the *in vitro* experiments high donor variations were observed regarding the expression of Wnt pathway proteins or the production of leukotrienes.

While IL-4 regulated mRNA expression of several Wnts and FZD, IFNy failed to regulate any of them. The link between Wnt signaling and type 2 immunity is well known as β-catenin is essential for Th2 cell polarization and GATA3 induction (Yu et al. 2009, Notani et al. 2010). Furthermore gene expression of some Wnts and FZDs were shown to correlate well with a type 2 cytokine signature in biopsies of asthma patients, indicating the relevance of type 2 cytokines in Wnt and FZD expression (Choy et al. 2011). The role of IFNγ driven FZD and Wnt expression is less clear. In the more type 1 prone disease COPD, Wnt5b and FZD8 are highly implicated. However, regulation seems to be mediated by TGFβ or environmental factors (Spanjer et al. 2016b, 2016a, Heijink et al. 2016). Nevertheless, there are some studies showing regulation of Wnts and FZDs by IFNγ, which is contrary to the data obtained in this study. In human endothelial cells IFNγ induced Wnt5b and reduced Wnt11 mRNA expression, when the cells were treated for 24h (Chrobak et al. 2013). Murine

macrophages infected with mycobacterium tuberculosis have elevated FZD1 expression that is further increased by IFNγ (Neumann et al. 2010). There is also evidence that IFNγ can regulate Wnt expression in human epithelial cells as 24h stimulation upregulates Wnt5a expression in human keratinocytes (Gudjonsson et al. 2010). However, in the latter studies, regulation of Wnt and FZD by IFNγ were either observed after stimulation for a longer time or together with an additional stimulus, supporting a rather indirect effect.

The upregulation of the Wnt pathway proteins Wnt4, Wnt5a and FZD10 on mRNA level was validated on the protein level by western blot. Surprisingly the highly upregulated FZD10 was not found to be regulated on protein level. FZD protein levels were shown to be controlled by ubiquitination and subsequent degradation in lysosomes (Jiang et al. 2015). Therefore transcriptional regulation of FZD10 might be less relevant.

IL-4 increased the expression of Wnt5a mRNA and protein as well as Wnt5a secretion of bronchial epithelial cells. A role for Wnt5a in type 2 inflammation mediated asthma was already implicated, as Wnt5a was shown to be highly expressed in asthmatic SMCs and to be involved in ECM deposition of SMCs (Kumawat et al. 2013). Of note, the expression of Wnt5a in SMCs was induced by the remodeling cytokine TGFβ and TGFβ also enhanced epithelial Wnt5a expression (Kumawat et al. 2013). Nevertheless, there is some evidence supporting the importance of a type 2 inflammation for Wnt5a induction. The expression of Wnt5a is strongly correlating with a type 2 cytokine signature in gene expression analysis of asthma biopsies (Choy et al. 2011). Recently it was shown that IL-4 enhanced Wnt5a expression of human endothelial cells, which in turn reduced barrier function of the endothelial layer (Skaria et al. 2016). Wnt5a was expressed in the lung tissues of HDM-sensitized mice at all ages. However, in the BALF from HDM-sensitized mice secreted levels of Wnt5a were higher in neonatal and adult mice as compared to juvenile, thus correlating with the magnitude of type 2 inflammation. Interestingly, epithelial expression of Wnt5a and highest levels of secretory Wnt5a were found in adult sensitized mice.

In contrary to Wnt5a, TGM2 is well known to be enhanced by IL-4. This knowledge mainly stems from studies investigating IL-4 polarized alternative macrophages, which were shown to express TGM2 (Gratchev et al. 2005, Martinez et al. 2013,

Gundra et al. 2014, Jiménez-Garcia et al. 2015). IL-4 was also able to induce TGM2 protein expression in bronchial epithelial cells. And as already mentioned TGM2 expression is enhanced in bronchial biopsies of asthma patients (Hallstrand et al. 2010). Additional experiments were performed to investigate TGM2 expression in nasal polyps of asthma patients showing highest TGM2 expression in tissues of patients suffering from HDM allergic asthma (Dietz et al. 2016). However, secreted TGM2 in the BALF from HDM-sensitized mice was highest present in BALF from adult sensitized mice, which had a less strong type 2 inflammation than neonatal mice, suggesting that mechanisms other than IL-4 may contribute to the induction of TGM2 in inflamed airways.

A similar expression pattern was observed for sPLA2-X. Still, there is some evidence for sPLA2-X expression being partly driven by a type 2 inflammation, as lung tissues of non-sensitized mice showed reduced expression of sPLA2-X and a different expression pattern than sensitized mice (Dietz et al. 2016).

While overall a type 2 inflammation seems to be important for expression of the Wnt5a, TGM2 and sPLA2-X cascade, the cascade seems to be dependent of additional factors as revealed by the *in vivo* experiments.

5.3 Age regulates Wnt5a, TGM2 and sPLA2-X mediated cysLT production

Gollwitzer et al. reported an exaggerated type 2 inflammation with a strong airway eosinophilia of neonatal HDM-sensitized mice (Gollwitzer et al. 2014). Therefore, finding higher cysLT levels in airways of adult sensitized mice was surprising. However, in the adult mice the novel identified cysLT regulating cascade components Wnt5a, TGM2 and sPLA2-X were highly expressed. Expression of these proteins was only partly dependent on type 2 inflammation and the *in vivo* data suggest a more age related expression.

Age-dependent expression of Wnt5a was striking for epithelial Wnt5a and secreted Wnt5a. Only in the adult HDM-sensitized mice Wnt5a was expressed in the epithelial layer of the bronchi and these mice also harbored the highest levels of secreted Wnt5a measured in the BALF. A change of Wnt ligands and Wnt signaling activity was described for aging in hematopoietic stem cells and muscle stem cells (Brack et al.

2007, Florian et al. 2013). Wnt5a expression increased age-dependent in hematopoietic stem cells (Florian et al. 2013). Contrary, canonical Wnt signaling increased with age in muscle stem cells leading to an altered fibrotic cell fate (Brack et al. 2007). Interestingly, the TGFβ induced Wnt5a expression in asthma smooth muscle cell was mediated by active β-catenin signaling (Kumawat et al. 2014). However, further studies are required to elucidate the age dependency of Wnt signaling and its underlying regulatory mechanisms in airway inflammatory disease.

Even more striking age differences were shown for TGM2 and sPLA2-X expression. Levels of secreted TGM2 detected in the BALF were strongly increasing with age in HDM-sensitized mice. And expression of sPLA2-X in the lung tissues of HDM-sensitized mice was strongest in adult mice, but also increased with age of sensitized mice. In contrast, no age fluctuations of sPLA2-X expression were observed in non-sensitized mice (Dietz et al. 2016). As most NHBE donors were middle-aged, investigation of age effects was limited to the *in vivo* experiments. However, it is noteworthy that NHBEs from a 14 years old donor showed almost no induction of TGM2 in response to IL-4. Age-dependent regulation of TGM2 and sPLA2-X expression in the lung has not been reported before. However, a recent study showed that the expression of another secreted phospholipase, sPLA2-IID, increased with age (Vijay et al. 2015). Interestingly, the increase of sPLA2-IID expression in elder mice was associated with higher levels of arachidonic acid in the lung upon viral infection (Vijay et al. 2015).

Investigating mechanisms underlying age-regulated Wnt5a, TGM2 and sPLA2-X expression in allergic airway inflammation was beyond the scope of this study. Nevertheless, there is literature evidence for epigenetic alterations or oxidative stress being a possible causative factor. The hallmarks of aging, as defined by López-Otín et al., are genomic instability, telomere attrition, epigenetic alterations (e.g. histone modifications and DNA methylation), loss of proteostasis (e.g. enhanced aggregation), deregulated nutrient sensing, mitochondrial dysfunction (e.g. increased oxidative stress), cellular senescence, stem cell exhaustion and altered intercellular communication (López-Otín et al. 2013). While there is no evidence of oxidative stress regulating Wnt5a so far, other Wnt ligands were shown to be enhanced by oxidative stress. In a bronchial epithelial cell line Wnt4 gene expression was induced

by hydrogen peroxide (Durham et al. 2013). Cigarette smoke, containing reactive oxygen species (ROS), induces expression of Wnt5b in bronchial epithelial cells from COPD patients (Heijink et al. 2016). TGM2 was also shown to be regulated and activated by oxidative stress (Shin et al. 2004). Histone modifications and DNA hypermethylation of Wnt5a were found in cancerous alterations (Jensen et al. 2009, Martín et al. 2010). And hypermethylation of Wnt5a induced by Epstein-Barr virus reduced its expression in gastric carcinoma cells (Liu et al. 2013). TGM2 was silenced in breast cancer cells by hypermethylation (Ai et al. 2008). Also sPLA2-X was shown to be epigenetically silenced in breast cancer cells, either by DNA hypermethylation or histone acetylation (Brglez et al. 2014).

This study shows a clear age-dependency of the Wnt5a-TGM2-sPLA2-X cascade in airway inflammation. If age differences of Wnt5a, TGM2 and sPLA2-X expression are dependent on either oxidative stress, epigenetic regulations or other age-related processes has to be further investigated.

5.4 Implications for disease progression and novel therapeutic approaches

The functional cascade of Wnt5a, TGM2 and sPLA2-X is not only implicated in regulating cysLT production, but its components can also directly contribute to airway remodeling. Wnt5a was shown to promote fibrosis as well as extracellular matrix production (Vuga et al. 2009, Kumawat et al. 2013). Similarly TGM2 contributes to fibrosis. TGM2 firstly enhances the ECM deposition, but also modulates ECM by cross-linking collagen fibers leading to stiffness of the tissue and scar formation (Jones et al. 2006, Fisher et al. 2009, Santhanam et al. 2010, Lee et al. 2013). Furthermore it was shown that TGM2 contributes to SMC proliferation and EMT (Kumar et al. 2010, Penumatsa et al. 2014). *In vivo* experiments revealed the importance of sPLA2-X in airway inflammation and remodeling (Henderson et al. 2007, 2011). However, effects were thought to be mediated by cysLTs and cysLTs were shown to induce SMC proliferation, fibrosis and ECM production (Henderson et al. 2007, Parameswaran et al. 2002, Asakura et al. 2004, Yoshisue et al. 2007).

For the current work an acute model of allergic airway inflammation was analyzed, where major structural changes were not observed. However, further in depth analysis of lung tissues showed a tendency of increased smooth muscle thickening and reduced expression of the tight junction protein ZO-1 in adult mice (Dietz et al. 2016). This suggests a role for the cascade of Wnt5a, TGM2, sPLA2-X and cysLT in airway remodeling. However, experiments using knock-out mice for each individual component would be needed to clarify the contribution of the respective factor. Epithelial- or macrophage-specific knock-outs would be of particular importance to elucidate whether Wnt-TGM2-mediated crosstalk between these cells occurs *in vivo*.

Furthermore this work showed the age dependency of the new identified remodeling cascade. Recent reports show a connection between age differences and the clinical picture of airway inflammation and remodeling (O'Reilly et al. 2013, Patel et al. 2014). Analysis of rodent models of experimental airway inflammation have shown increased airway eosinophilia in neonates, which is in line with data for human neonates (Frischer et al. 2000, Carnieli et al. 2011, Gollwitzer et al. 2014). However, while eosinophilia is increased at young age, signs of airway remodeling are more pronounced later in life (Carnieli et al. 2011, Uhlík et al. 2014).

These findings suggest an early drift for a type 2 inflammation, which is followed by an induction of epithelial remodeling factors initiated by aging and recurrent allergen exposure. This is supported by another study using a mouse model of HDM sensitization from day 3 after birth. In these mice levels of IL-33 in the lung increased with age, while lung IL-13 levels peaked early and declined with age (Saglani et al. 2013). In contrast to IL-13, IL-33 was shown to promote airway remodeling (Saglani et al. 2013).

Remodeling is an important driver of asthma severity and signs of remodeling correlate well with disease progression and worsening of asthma symptoms (Sont et al. 1999, Benayoun et al. 2003). However, current therapies, mainly inhaled glucocorticoids, have limited effects on airway remodeling and basically are used to control the inflammatory response (Chakir et al. 2003). Therapies currently under development are mainly targeting mediators of type 2 inflammation. The IL-13 antibody leprikizumab, the IL-4 receptor antibody dupilumab and the IL-5 antibody mepolizumab were shown to reduce symptoms, improve lung function and

inflammation in asthma patients with high eosinophilia and type 2 inflammation (Corren et al. 2011, Wenzel et al. 2013, Ortega et al. 2014). However, in severe asthma the strategy of targeting type 2 cytokines was less effective (Boever et al. 2014, Bel et al. 2014). In contrast, reducing the smooth muscle mass by bronchial thermoplasty was improving asthma in patients with severe persistent asthma (Wechsler et al. 2013).

This study shows that the identified remodeling cascade is to some extent dependent on type 2 inflammation, but also other age-dependent mechanisms, contribute. The age dependency of remodeling factors could explain why anti-inflammatory therapies targeting type 2 cytokines fail to stop disease progression and are often not sufficient to control severe asthma. Therefore besides targeting inflammation an anti-remodeling therapy is urgently needed for affected patients.

Studies on cystic fibrosis, idiopathic pulmonary fibrosis and lung cancer affirm the important role for TGM2 in aberrant wound healing and chronic inflammation and TGM2 inhibitors were developed as treatment modality (Maiuri et al. 2008, Park et al. 2010, Olsen et al. 2011, 2014). This study together with other studies shows a clear role for TGM2 in asthma and airway remodeling, thus it should be investigated if airway TGM2 levels correlate with disease severity and structural alterations. Hence, TGM2 could be deduced as target for an anti-remodeling therapy in asthma. TGM2 could be an attractive drug target particularly in steroid resistant forms of asthma, as it was shown that TGM2 activity and expression can be induced by glucocorticoids in brain and kidney cells (Campisi et al. 2008, Papasotiriou et al. 2012). The here performed experiments showed that TGM2 expression was not enhanced in bronchial epithelial cells by glucocorticoid treatment. However, its expression and the expression of the downstream protein sPLA2-X was resistant to glucocorticoid treatment, which was additionally shown for the expression in nasal polyps of asthma patients (Dietz et al. 2016).

Interestingly the steroid resistant sPLA2-X and Wnt5a activate similar signaling cascades, such as MAPK and calcium signaling, and homologies between these proteins are described (Reichsman et al. 1999, Doroudi et al. 2014). The present study identified sPLA2-X and Wnt5a as part of jointly regulated cascade in asthma.

This functional connection might be interesting for the development of a combined drug.

Moreover the newly identified cascade of Wnt5a, TGM2 and sPLA2-X regulates LT production by enhancing arachidonic acid bioavailability and the most commonly used drug targeting LTs is the cysLT1R antagonist montelukast. However, montelukast only prevents binding of cysLTs to the cysLT1R, so they can still bind to different receptors. It was recently shown that cysLT signaling through cysLT2R on platelets can promote allergic airway inflammation (Cummings et al. 2013). Therefore targeting the cascade of Wnt5a, TGM2 and sPLA2-X by topically administered compounds may not only serve as anti-remodeling therapy, but also anti-inflammatory by reducing LT levels.

In conclusion an age-dependent remodeling cascade of Wnt5a, TGM2 and sPLA2-X was identified in allergic airway inflammation. In allergic sensitization at young age a bias for a type 2 dominant inflammation exists. Following the strong type 2 inflammatory response the lung is infiltrated with high numbers of eosinophils, which produce accordingly high levels of cysLTs (Figure 27). After a phase of reduced susceptibility to allergic sensitization, the age dependent cascade of Wnt5a, TGM2 and sPLA2-X is induced and in cross-talk with resident myeloid cells epithelial cells contribute to cysLT production (Figure 27). This cascade is not only involved in cysLT production, but also contributes to airway remodeling. The age-dependent remodeling cascade is steroid-resistant and likely to be resistant to therapies targeting type 2 inflammatory mediators. Therefore targeting this newly identified remodeling cascade should be taken into account for patients with difficult to treat asthma as add-on therapy.

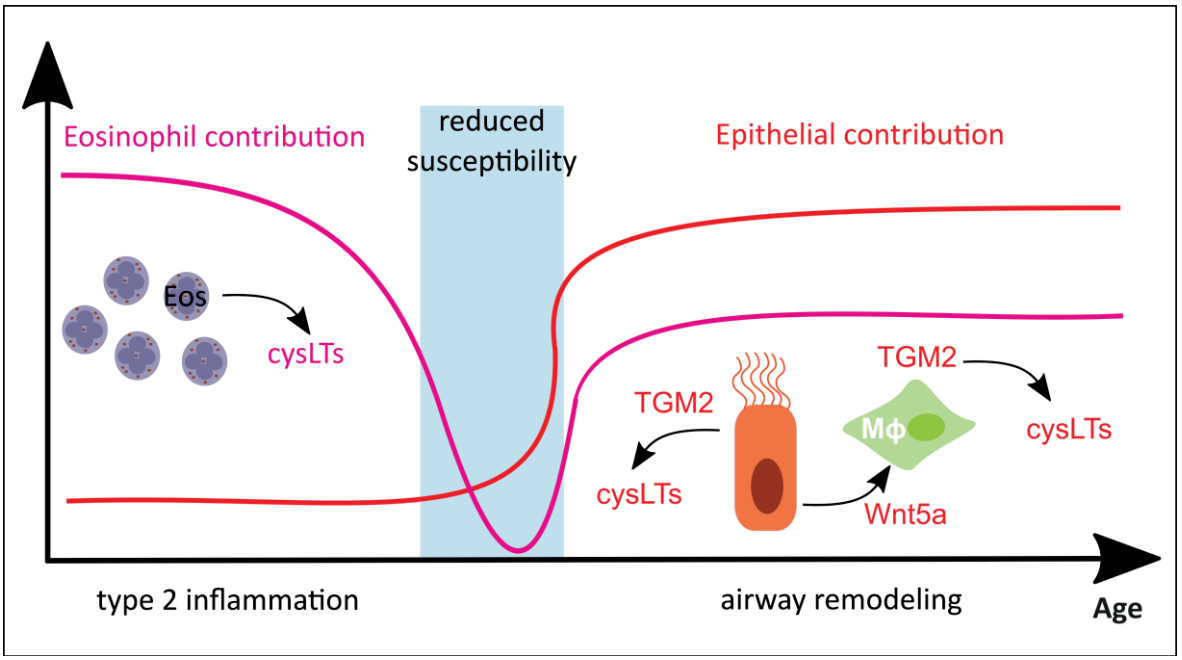

Figure 27 : Age dependent regulation of Wnt5a, TGM2, sPLA2-X and cysLTs as novel remodeling cascade during allergic airway inflammation. Following a strong type 2 inflammatory response at young age with high numbers of eosinophils, there are high levels of eosinophil derived cysLTs. CysLT levels are reduced during a phase of reduced susceptibility and increase with age, where Wnt5a, TGM2 and sPLA2-X regulate epithelial-myeloid cysLT production and airway remodeling. Figure is adapted from Dietz et al. (Dietz et al. 2016). cysLTs, cysteinyl leukotrienes; Eos, eosinophil; TGM2, transglutaminase 2.

6 Summary

Allergic asthma is a chronic disease with increasing prevalence and health costs. It is estimated that around 300 million people worldwide suffer from asthma and in about 50% of the asthmatic adults the disease is the result of allergic sensitization. The bronchial epithelium is integral for recognizing allergen and priming a type 2 immune response. Bronchial epithelial cells activate DCs and type 2 immune cells and in response to type 2 cytokines they further promote inflammation. In the last years these cells are more and more recognized to be important for the development of airway remodeling.

Leukotrienes are important inflammatory mediators and Wnt signaling is highly implicated in lung fibrosis. Recent evidences suggest that epithelial cells could contribute to leukotriene production by TGM2 and remodeling by altered epithelial Wnt signaling.

Therefore the aim of this study was to investigate the role of epithelial Wnt signaling and TGM2 expression in the context of an allergic airway inflammation. As experimental setup, bronchial epithelial cells were stimulated with IL-4, as type 2 cytokine mimic, and gene transcription of Wnt ligands and FZDs was analyzed by qPCR. Results were confirmed by western blot. Wnt5a was identified as IL-4 regulated epithelial Wnt ligand, which was able to induce TGM2 expression in macrophages. Epithelial and macrophage derived TGM2 was further able to induce LT production, as shown by *in vitro* experiments using TGM2 inhibitors, probably by activating sPLA2-X.

In a mouse model of allergic airway inflammation the relevance of these findings were confirmed. Mice sensitized with HDM starting from day 3, day 15 or day 60 after birth showed different strength in their type 2 inflammation, with mice sensitized at day 3 showing signs of the strongest type 2 inflammation. Surprisingly in these mice expression of Wnt5a, TGM2, sPLA2-X was lowest and expression increased with age. The levels of cysLT were high in mice sensitized at day 3, but even higher in mice sensitized at day 60. Elder mice exhibited also more signs of airway remodeling. Therefore in this study a novel epithelial derived cysLT regulating cascade of Wnt5a, TGM2 and sPLA2-X was identified, which is partly dependent on type 2 inflammation

or allergic sensitization. However, in the first place this cascade, which is further involved in remodeling, is age dependent.

As the newly identified remodeling cascade is steroid resistant and likely not affected by therapies targeting type 2 inflammation only, Wnt5a, TGM2 and sPLA2-X represent interesting novel drug targets for severe forms of chronic airway inflammation. This could present a first step into an anti-remodeling therapy, which could be effective especially in difficult to treat asthma.

Zusammenfassung

Allergisches Asthma ist eine chronische Erkrankung mit zunehmender Häufigkeit und immer höheren Kosten für das Gesundheitssystem. Es gibt Schätzungen, dass bereits 300 Millionen Menschen auf der Welt unter Asthma leiden. Die Hälfte der Asthmatiker leidet hierbei unter allergischem Asthma. Die Epithelzellen der Bronchien sind essentiell für die Erkennung von Allergenen und der Initiierung einer Typ 2 Immunantwort. Bronchiale Epithelzellen aktivieren Dendritische Zellen und Typ 2 Immunzellen, außerdem reagieren sie auf die von diesen sezernierten Typ 2 Zytokine und treiben damit die Entzündung weiter voran. In den letzten Jahren wurde immer mehr klar, dass bronchial Epithelzellen außerdem eine Rolle in der Lungenremodellierung spielen.

Leukotriene sind wichtige inflammatorische Substanzen und der Wnt Signalweg ist stark assoziiert mit fibrotischen Veränderungen in der Lunge. Kürzlich durchgeführte Studien lassen die Vermutung aufkommen, dass bronchiale Epithelzellen zur Leukotrienproduktion über das Enzym TGM2 beitragen und zur Remodellierung durch ein veränderten Wnt Signalweg.

Daher war das Ziel dieser Studie die Rolle des epithelialen Wnt Signalwegs und der Expression von TGM2 in der allergischen Atemwegsentzündung zu untersuchen. Hierfür wurden bronchiale Epithelzellen mit dem Typ 2 Zytokin IL-4 stimuliert und Gentranskription von Wnt Liganden und FZDs wurde mit qPCR untersucht. Die Ergebnisse wurden mit Western Blot bestätigt. Als Ergebnis wurde Wnt5a als IL-4 regulierter von Epithelzellen sezernierter Wnt Ligand identifiziert, welcher in Makrophagen die Expression von TGM2 erhöht. TGM2, welches von Epithelzellen oder Makrophagen sezerniert wird, beeinfluss direkt die Leukotrienproduktion, was über den Einsatz von TGM2 Inhibitoren in *in vitro* Experimenten nachgewiesen wurde. Die Beeinflussung der Leukotrienproduktion erfolgt vermutlich über die Aktivierung von sPLA2-X.

In einem Mausmodell der allergischen Atemwegserkrankung wurde die Relevanz der Ergebnisse bestätigt. Mäuse wurden sensibilisiert gegen HDM und zwar 3, 15 oder 60 Tage nach ihrer Geburt. Sie zeigten unterschiedlich starke Zeichen einer Typ 2 Entzündungsreaktion. Mäuse die 3 Tage nach ihrer Geburt sensibilisiert wurden,

hatte dabei die stärkste Entzündungsreaktion. Überaschenderweise war in genau diesen Mäusen die Expression von Wnt5a, TGM2 und sPLA2-X am geringsten. Die Expression nahm hingegen mit dem Alter der Mäuse zu. Mäuse die an Tag 3 sensibilisiert wurden, produzierten zwar große Mengen an cysLTs in ihren Lungen, aber Mäuse die an Tag 60 sensibilisiert wurden, hatten noch größere Mengen an cysLTs. Diese älteren Mäuse zeigten außerdem stärkere Anzeichen für eine Lungenremodellierung. Zusammenfassend wurde in dieser Studie eine neue Kaskade aus Wnt5a, TGM2 und sPLA2-X gefunden, welche die cysLT Produktion reguliert und nur teilweise abhängig von einer Typ 2 Entzündung oder allergischen Sensibilisierung ist. Denn in erster Linie ist diese Kaskade, welche auch eine Rolle in der Lungenremodellierung spielt, abhängig vom Alter.

Diese neu identifizierte Remodellierungskaskade ist resistent gegenüber Glucocorticoiden und es ist unwahrscheinlich, dass neue Therapien, die auf Typ 2 Zytokine ausgerichtet sind, die Kaskade beeinflussen. Wnt5a, TGM2 und sPLA2-X repräsentieren daher interessante Ziele für neue Medikamente. Therapeutisch auf die neue Kaskade abzuzielen, könnte ein erster Schritt in die Richtung einer Anti-Remodellierungs-Therapie darstellen, welche besonders für schwere Asthmatiker interessant sein könnte.

7 Literature

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Pearl S. Buck

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