Aus dem Institut für Laboratoriumsmedizin und Pathobiochemie,
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des Fachbereichs Medizin der Philipps-Universität Marburg

# Resolution of airway remodelling in a mouse model of chronic allergic asthma

Inaugural-Dissertation zur Erlangung

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# 1 Introduction

## 1.1 Bronchial asthma

Bronchial asthma is a chronic disease characterized bychronic airway inflammation, airway hyperresponsiveness and airway remodelling (Murdoch und Lloyd 2010). It is known as a costly chronic disorder (Böcking et al. 2012b), this enhances the need for further research to better understand the mechanisms of this disease and finally to develop curative medication (Akinbami et al. 2011).

### 1.1.1 Epidemiology

Asthma is a multifactor disease influenced by genetic and environmental components. Remarkable development in studying asthma genetics has led to identification of several candidate genes that are associated with asthma-related traits (Vercelli 2008). Furthermore, immune responses in asthmatic patients are also regulated by epigenetic mechanisms (Yang und Schwartz 2012). Asthma is one of the most common diseases worldwide, it`s global prevalence is ranging from 1% to 18% of the populations, with high prevalence (>10%) in developed countries and increasing rates in developing regions as they become more westernized (Braman 2006). Within one decade the average prevalence of asthma in Western Europe has nearly doubled to now 5.9%, the highest rate found in Scotland (18.4%), whereas prevalence in the German population is 6.9% (Masoli et al. 2004). The increased prevalence of allergic diseases implied an important aspect in terms of health costs as well as life quality (Böcking et al. 2012a). Only a few data are available about the prevalence of allergic diseases in Arabic countries and Middle East (Al et al., 2010).

### 1.1.2 Pathophysiology

Human bronchial asthma is a chronic airway inflammatory disease, which affects the bronchial airways. Airway inflammation is recognized as the key component of the disease. Different cell types are supposed to be responsible of regulating this airway inflammation such as mast cells, eosinophils, airway epithelial cells and CD4 positive lymphocytes (Van Hove, C L et al. 2008).

Eosinophilic and noneosinophilic asthma is clinically an important classification because it identifies groups with markedly different responses to corticosteroids and other drugs (Furukawa et al. 2014).

The detailed phenotyping of asthma can allow successful targeting of existing and novel therapies of this disease (Olin und Wechsler 2014).

#### 1.1.2.1 Immune response

The immunological response in allergic asthma can be divided into two phases:
Phase I: The awareness or sensitization phase of the allergen which begins with an inclusion of an antigen on the mucosa of the airways. There specialized antigen presenting cells (APC) such as dendritic cells (DC) uptake the allergen and present it via the major histocompatibility complex (MHC) II (Hsieh et al. 1993). Activated APCs migrate to the draining lymph nodes (Paul und Seder 1994) where naive T cells detect this antigen via the antigen-specific T-cell receptor (TCR) (Ting et al. 1996). The differentiation of the naive CD4+ T cells to Th1, Th2, Th17 or Treg cells depends on both the binding of antigen to the TCR and the effect of local cytokines (Vernal und Garcia-Sanz 2008). E.g. the differentiation of naïve Th0 cells into Th1 cells occurs under the influence of the cytokines IL-12 and IL-18 which are usually secreted by APCs when high doses of allergen are present. On the other hand, presence of the cytokine IL-4 drives the differentiation toward Th2 cells (Kinet 1999) (Figure 1). Transcription factor GATA-3 - expressing Th2 cells produce primarily IL-4, IL-5 and IL-13, whereas Th1 cells, which express the transcription factor Tbet, produce mainly the cytokines IL-2 and IFN-γ (Bousquet et al. 2000). Although Th2 cells have been considered as main orchestrators of allergic airway inflammation, recent studies have shown a potential interaction of other helper T cells as Th17 cells, an IL-9-producing subset called Th9 cells, Th22 cells which primarily secrete IL-22, IL-13 and tumor necrosis factor-α TNF-α and Th25 cells producing IL-25 (Pawankar et al. 2015). Th2 cells can also induce B cell differentiation as well as activation through producing IL-4 which instructs B cells to switch from IgM to allergen specific IgE antibody production (Heusser und Brinkmann 1994).
Phase 2: Repeated allergen contact leads to formation of allergic airway inflammation. The allergen is absorbed by the airway and leads to cross linking of IgE molecules on mast cells which induces an immediate response by a quick release of mediators such as histamine, prostaglandins and leukotrienes (Jeffery 1992). These mediators cause contraction of the airway smooth muscles, mucus production and vasodilation (Homer und Elias 2000) (Figure 2). Meanwhile, the DCs take the allergen to the local lymph nodes, where they present the antigen again to both the memory and naive T cells, leading to reactivation of Th2 memory cells and further differentiation of naive T cells (Chetta et al. 1996). In the late phase of the inflammatory response, there is an infiltration of activated

CD4+ T cells, eosinophils, basophils, neutrophils and macrophages under the influence of IL-5 and GM-CSF (Douwes et al. 2002).

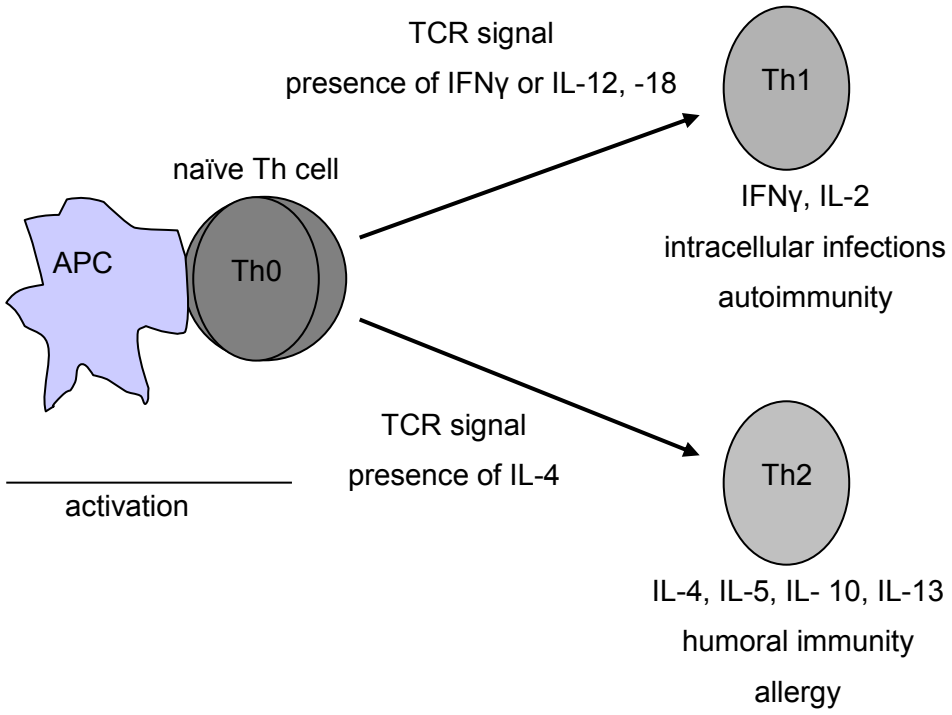


**Figure 1:** Differentiation of Th0 to Th1 and Th2 in the presence of different cytokines (Biederman et al., 2002, modified)

#### 1.1.2.2 Airway inflammation

Airway inflammation processes are early present in the clinical picture of the disease. This acute allergic airway inflammation is characterized by the infiltration of eosinophils and Th2 cells (Lederlin et al. 2010). Many cell types are involved in the chronic allergic airway inflammation such as activated macrophages, lymphocytes, eosinophils and neutrophils (Wegmann et al. 2005). The interaction between the different inflammatory as well as epithelial cells, inflammatory mediators and matrix proteins induce the airway remodelling namely collagen deposition and smooth muscle thickening (Fang et al. 2008).

The persistent inflammation lead to an increased airway hypersensitivity, which is defined as an increased bronchi obstructive reaction to the allergen or a non-specific stimulus (e.g., methacholine) (Zosky und Sly 2007).

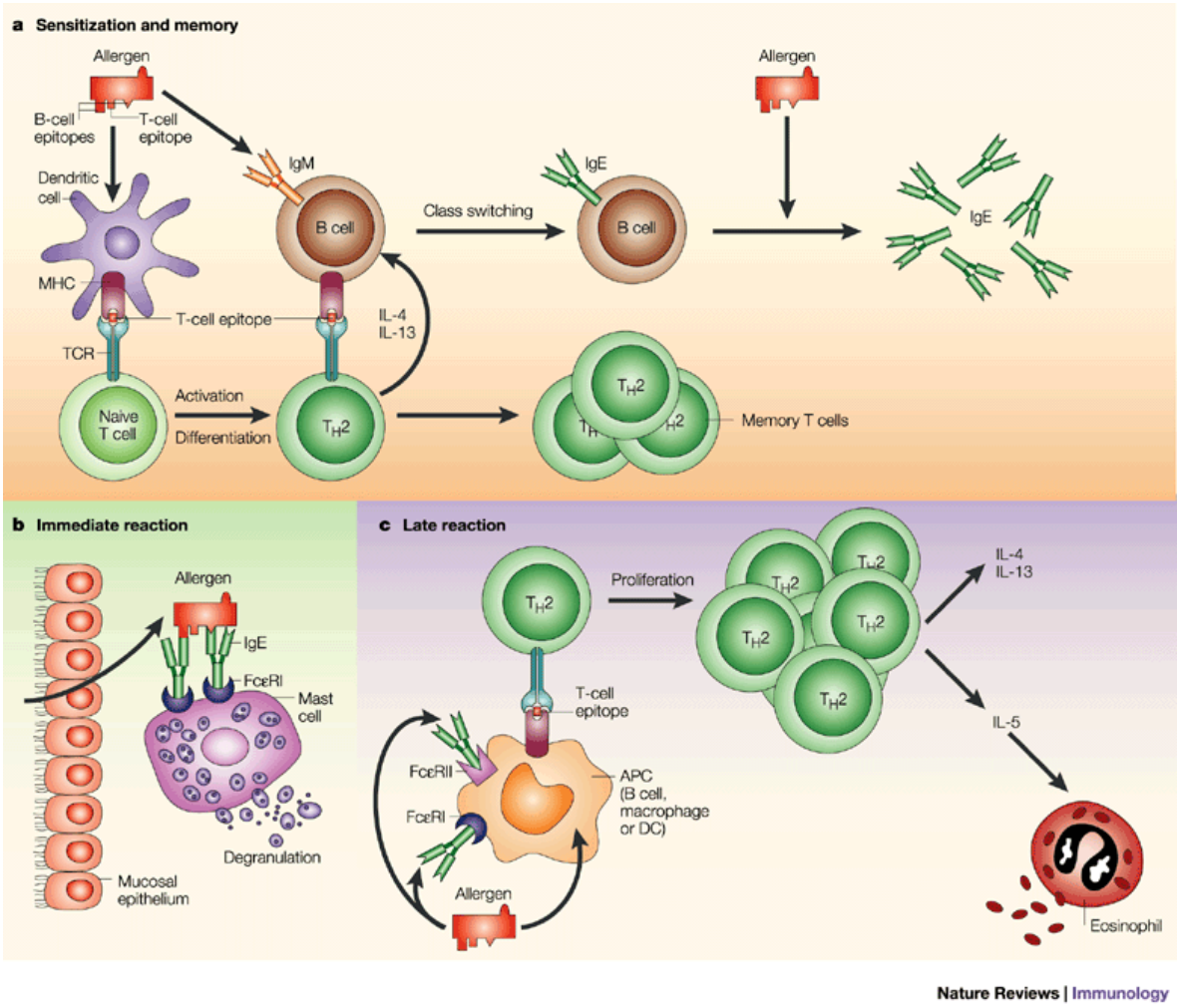


**Figure 2:** Immune mechanisms of asthma (Valenta R., 2002)

#### 1.1.2.2.1 Dendritic cells

Dendritic cells (DCs) play an important role in the development of allergies by capturing antigens, transporting them from the airway surface to regional lymph nodes and presenting them to T cells. In the lymph nodes, DCs present processed antigens to T cells and stimulate the differentiation of naïve T cells into different T cell subtypes. Airway DCs also play a crucial role in the local restimulation of circulating effector T cells upon allergen challenge (Pouliot et al. 2010).

#### 1.1.2.2.2 Lymphocytes

Lymphocytes play a major role in allergic asthma. There are at least six main T cell subpopulations known so far (Th1, Th2, Th17, Th9, Th22, Th25, Treg). Th1 cells provide protection against intracellular bacteria and work mainly via the secretion of cytokines such as IFNγ. In addition, they maximize the killing efficiency of macrophages and are the

main contributors to the proliferation of cytotoxic CD8+ T cells (Schmid et al. 2010). The central function of Th2 cells is fighting parasites via the secretion of cytokines IL-4, IL-5 and IL-13, as well as stimulation of B cell proliferation and induction of antibody class switching to IgE. Th2 cells are a key factor in the initiation and exacerbation of allergy and asthma (Robinson et al. 1993).

Th17 cells provide an anti-microbial immunity at epithelial barriers via the secretion of cytokines such as IL-17 and IL-22 (Liang et al. 2006). When the immune response is dysregulated, Th17 cells are thought to play a key role in autoimmune diseases such as multiple sclerosis and SLE (system lupus erythematosus) (Selmi 2010).

The fourth major T cell subsets are the T regulatory cells (Treg). They are specialized to suppress activation of the immune system and thereby maintain an immune system homeostasis and tolerance to self-antigens (Harrington et al. 2005; Stockinger und Veldhoen 2007).

In human allergic asthma, the development of a Th2 cytokine profile results in airway inflammation, development of allergen specific IgE, presence of eosinophils in the lung and hyperplasia of goblet cells resulting in mucus production (Cohn et al. 2004). Alternatively, asthma may also occur due to a reduction of Treg cells (Karagiannidis et al. 2004). Lack of immune suppression from Treg cells results in production of IL-4 and IL-13 which leads to a stronger Th2 phenotype and more severe asthma (Ray et al. 2010).

The principal functions of B cells are to produce specific antibodies against different antigens. Upon secondary contact with an antigen, memory B cells proliferate and differentiate into plasma cells, which produce antibodies to resolve an infection (Townsend et al. 2010). In case of immune system dysregulation, B cells produce antibodies against harmless molecules such as birch pollen or house dust mite. In allergies, IgE produced by B cells causes mast cell activation and further enhancement of Th2 immune response (Vicario et al. 2010).

#### 1.1.2.2.3 Mast cells

Mast cells are resident cells in mucosa / tissues and play a major role in allergic asthma. Activation of these cells through cross-linking of high-affinity receptors loaded with IgE causes the release of preformed mediators stored in granules, which significantly contribute to the asthma phenotype (Wasserman 1984). Increased numbers of mast cells in airways may be linked to airway hyperresponsiveness due to the release of bronchoconstriction mediators (histamine, cysteinyl-leukotrienes and prostaglandin D2) (Galli et al. 2005; Schroeder 2009; Bradding und Brightling 2007). In addition, release of

histamine increases mucus production, which worsens the asthma phenotype and can partially contribute to remodelling during chronification (Prussin und Metcalfe 2003).

#### 1.1.2.2.4 Eosinophils

Eosinophils are by nature responsible for combating multicellular parasites, but in a dysregulated immune response they play a central role in different types of allergic diseases as in asthma (Chu und Martin 2001; Sampson 2000). These cells produce growth factors such as TGF-β1, VEGF, and PDGF, having important functions in collagen deposition and airway remodelling, TGF-β1 is believed to be a key regulator of the immune system by driving the development of CD25+ regulatory T cells (Romagnani 2006). Eosinophils generate also leukotrienes which increase mucus production in bronchi as well as vascular permeability leading to infiltration of inflammatory cells in the airway wall (Rothenberg und Hogan 2006). Most of asthma phenotypes are associated with an increase of eosinophils either in lung tissue, blood or in bone marrow. IL-5, which is produced mainly by Th2 cells, is the key cytokine of eosinophils differentiation, maturation and recruitment (Bates et al. 2009).

#### 1.1.2.2.5 Neutrophils

Neutrophils are the most abundant immune cells in the body, they are essential in innate immunity and are usually the first cells migrating to an inflammation site (Witko-Sarsat et al. 2000). Similar to eosinophils, neutrophils are present in the airways of asthmatic patients and they are the most abundant cell type in cases of corticosteroid resistant asthma, (Macdowell und Peters 2007). Neutrophils contribute to the inflammatory process by secreting both lactoferrin and cathelicidin, which work as antimicrobial compounds and act to attract other immune cell types such as macrophages and lymphocytes to the inflammation site. Presence of neutrophils has been linked to severe asthma attacks and the development of a more chronic state of the disease (Monteseirín 2009).

### 1.1.2.3 Airway remodelling

Airway remodelling in asthma is characterized by structural abnormalities like hypertrophy of airway smooth muscle, subepithelial fibrosis, goblet cell hyperplasia, and proliferation of airway blood vessels and nerves (Lederlin et al. 2010; Leung et al. 2004) (Figure 3). Airway remodelling is thought to arise either via an excessive repair process of the

airways (Nials und Uddin 2008), or via an unresolved allergen driven inflammation that leads to irreversible airway damage (Blacquière et al. 2010).

#### 1.1.2.3.1 Goblet cells hyperplasia

Goblet cells are located in the epithelium of the conducting airways where they produce mucus in response to inhaled airway insults (Caramori et al. 2008). The proliferation and hyperplasia of this cell type as well as overproduction of mucus are important findings characterizing asthma (Zuhdi Alimam et al. 2000). It has been shown that the Th2 cytokine IL-13 is the main driver of goblet cells hyperplasia and mucus production, it induces the differentiation of airway epithelial cells into goblet cells resulting in overproduction of mucus in the airways (Shim et al. 2001). *MUC5AC* is one of different genes identified to be overexpressed in human airways of asthma patients (Fahy 2001), This gene is overexpressed by goblet cells hyperplasia both in vitro and in vivo (Rose et al. 2000). Goblet cells hyperplasia has recently been also described as a suitable environment of infections with rhinovirus in asthma patients (Lachowicz-Scroggins et al. 2010).

#### 1.1.2.3.2 Smooth muscle thickening

The smooth muscle thickening is the result of the hypertrophy of airway smooth muscle cells and as was recently shown, the migration of such cells from other regions to the subepithelial area in asthma patients (Bergeron et al. 2009).

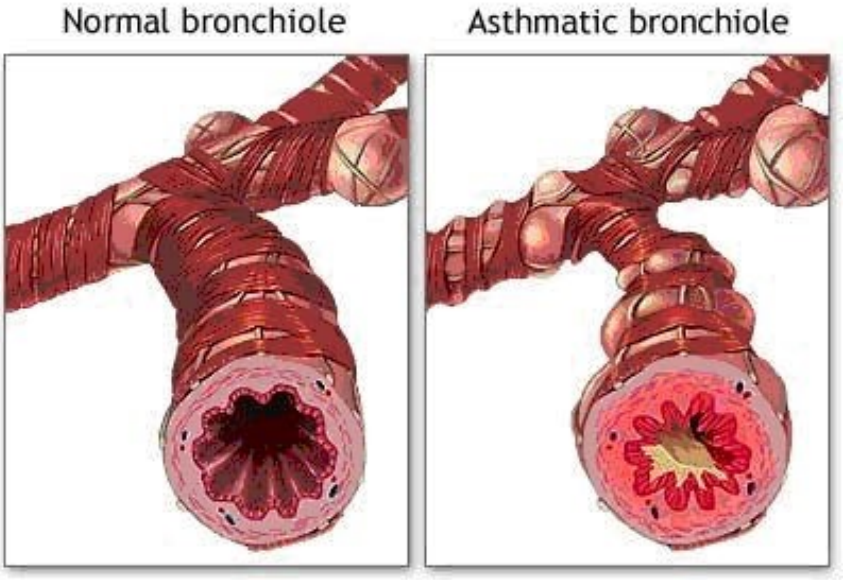


**Figure 3:** Airway remodelling in asthma. (Francis et al., Pulmonary internal medicine 2008)

The role of the bronchial smooth muscle remains controversial in healthy subjects. It assists likely in gas exchange regulation, mucus clearance, defence mechanisms and coughing (Solway und Irvin 2007), whereas its role in asthmatics is well established: smooth muscle support the bronchial inflammation by producing different inflammatory mediators necessary for the recruitment and activation of different inflammatory cells such as mast cells and T lymphocytes (Bara et al. 2010; Damera und Panettieri 2011). In addition, the thickening of airway smooth muscle plays an important role in increasing the bronchomotor responsiveness which characterizes asthma. Exaggerated broncho-constriction and airflow obstruction is caused by the excessive contraction of airway smooth muscle during the asthma attack (Stewart et al. 1994). Despite smooth muscle cells are the final target of different cytokines and chemokines, they are also secreting different mediators in response to different stimulants (Tliba und Panettieri 2009)(Figure.4). Through the allergic disease, smooth muscle cells proliferation increases 3-5 fold more than in healthy people (Hassan et al. 2010). In the past, smooth muscle thickening was a therapeutic target of different studies, many different medications have been used to minimize either the proliferation or the exaggerated contractibility of these cells (Delmotte et al. 2010).

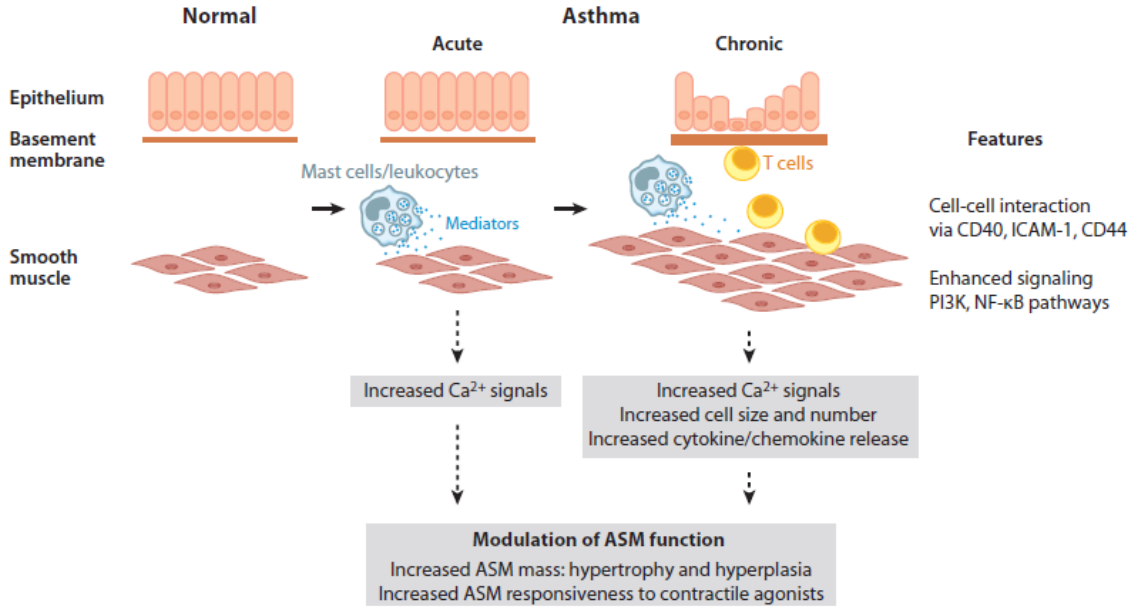


**Figure 4:** Interaction between SMA cells and other inflammatory cells during asthma development. [Annu. Rev. Physiol. 2009.71:509-535].

#### 1.1.2.3.3 Collagen deposition

The thickening of the basement membrane is a marked manifestation of airway remodelling in asthma patients. It ranges between 7 - 23 µm in asthma patients compared to 4 - 5 µm in normal subjects.

Collagen deposition originates from the recruitment, proliferation and activation of local connective tissue cells in response to different inflammatory factors (Kim et al. 2009), as well as from the migration of precursor cells such as fibroblasts from the bone marrow and circulation to the lung tissue (Pereira et al. 1995). A marked increase of fibrocytes which are identified by CD34/procollagen-1a expression have been also detected in lungs and blood of patients with severe chronic asthma (Schmidt et al. 2003). TGF-β1 plays an important role in mediating remodelling by inducing the production of extracellular matrix proteins and cell proliferation. Additionally, increased TGF-β1 expression, which has been observed in asthmatic patients, correlates with subepithelial fibrosis (Ohno et al. 1996) (Vignola et al. 1997).

Matrix metalloproteinases (MMPs) are also involved in inducing airway inflammation and remodelling. An increase of MMP-9 during acute asthma facilitates the migration of inflammatory cells to the tissue, which may also participate in the airway remodelling by releasing or activating pro fibrotic factors (Kelly und Jarjour 2003).

### 1.1.3 Asthma treatment and therapy

#### 1.1.3.1 Therapy aspects in humans

Anti-inflammatory therapy with inhaled / oral corticosteroids, beta 2-agonists and leukotriene receptor antagonists are the most used medication in patients with asthma (global initiative for asthma GINA2017). The current anti-inflammatory treatment of asthma is predominately based on the use of inhaled glucocorticoids (ICS). Although these drugs are highly effective in preventing life threatening consequences of asthma (Suissa et al. 2002), their effect is limited in modulating airway remodelling (Caramori et al. 2008). The synthetic glucocorticoid “budesonide” is a well-established compound used locally to treat allergic diseases and asthma (Chian et al. 2011). The therapeutic potential of budesonide has been extensively studied in acute allergic inflammation models but only few studies have investigated efficacy on established airway remodelling and chronic asthma (Bos et al. 2007; Kelly et al. 2010). There are new therapeutic options which target immunoglobulin E and cytokines such as (anti Ig E (omalizumab), antiIL-13 (lebrikizumab), anti IL4Rα (dupilumab) and anti-IL-5 (mepolizumab, reslizumab) (Roth and Tamm 2010; Pepper et al. 2017)

#### 1.1.3.2 Therapy aspects in animals

The majority of asthma studies which utilized animal models were based on the acute allergic airway inflammation model (Zosky und Sly 2007). Although this model induces features of a strong acute allergic inflammation, it does not develop major characteristics of chronic airway remodelling such as collagen deposition and smooth muscle thickening (Bates et al. 2009). Alternative models which reflect the pathological changes observed in patients are chronic asthma models, in which a variety of parameters with regard to airway remodelling have been developed (Wegmann et al. 2005; McMillan und Lloyd 2004; Temelkovski et al. 1998). Such models are required to study novel intervention methods in a therapeutic rather than a prophylactic setting as investigated in acute asthma models (Nials und Uddin 2008).

# 2 Aim of the study

Although the development of allergen-induced airway inflammation and remodelling has been extensively examined, few studies have addressed the resolution of allergic inflammation (Kearley et al. 2009). We still need further information about resolution of airway remodelling in case of an effective therapy by asthma patients. Moreover, we need a therapeutic model of airway resolution, in order to evaluate the efficacy of different novel asthma therapies.

We are aiming in our study to characterize the inflammatory and remodelling events that contribute to the transition from acute to chronic experimental asthma. Furthermore we have studied the impact of ICS treatment during this transition phase, to specifically identify steroid-sensitive and resistant pathways. The reversibility of remodelling has been also examined following a period of ICS therapy and to as well as in the optimal situation of allergen avoidance.

# 3 Material and Methods

## 3.1 Animals

Female BALB/c mice aged 6–8 weeks were purchased from Harlan Winkelmann (Borchen, Germany) and were maintained under pathogen-free conditions in isolated ventilated cages with 12 hour light/dark cycles. Water and ovalbumin (OVA)-free diet were supplied ad libitum. All mouse experiments met German and international guidelines and were approved by the Regierungspraesidium Giessen, and all measures were taken to keep animal suffering to a minimum.

## 3.2 Material and equipment

| | |
|---|---|
| 20G Catheter | BD, Drogheda, Ireland |
| 96-well Microtiterplates Maxisorp Flachboden | Nunc, Wiesbaden, D |
| Absorptionsphotometer Magellan | Tecan, Männedorf, Switzerland |
| Analysis software Magellan | Tecan, Männedorf, Switzerland |
| BALB/c mice | Harlan Winkelmann, Borchen, Germany) |
| BD OptEIA Set Mouse IL-4 (ELISA kit) | BD Pharmingen, San Diego, USA |
| BD OptEIA Set Mouse IL-5(ELISA kit) | BD Pharmingen, San Diego, USA |
| BD OptEIA Set Mouse IL-13(ELISA kit) | BD Pharmingen, San Diego, USA |
| BD OptEIA TM Set Mouse IFN-γ(ELISA kit) | BD Pharmingen, San Diego, USA |
| Biotinylated goat anti-rabbit IgG antibody | Vector, Brockville, Canada |
| Biotinylated rabbit anti-goat IgG antibody | Vector, Brockville, Canada |
| Budesonide | Astra Zeneca, Lund, Sweden |
| CAST-Grid System | Visiopharm, Hoersholm, Denmark |
| Casy Cell Counter System | Schaerfe Systems, Reutlingen, D |
| Cell-F System | Olympus, Hamburg, D |
| Centrifuge Megafuge 1.0R | Heraeus, Osterode, D |
| Cytocentrifuge Cytospin 3 | Thermo Electron Corporation, US |
| Complete Protease Inhibitor tablets | Roche, Mannheim, D |
| DAB Substrate | Vector, Brockville, Canada |
| Diff-Quick solution | Dade-Behring, Marburg, D |

| | |
|---|---|
| Dulbecco's Phosphate Buffered Saline (PBS) | PAA Laboratories GmbH, Cölbe, D |
| Eosin 1% | Merck, Darmstadt, D |
| Ethanol | Roth, Karlsruhe, D |
| Ethanol absolute | Roth, Karlsruhe, D |
| FCS Gold | PAA Laboratories GmbH, Cölbe, D |
| Goat anti-CD3 antibody | Santa Cruz Biotechnology, UK |
| Hämatoxylin nach Gill II | Merck, Darmstadt, D |
| Hydrogen peroxide 35% solution | Acros, New jersey. USA |
| Immuno$^{TM}$wash 12 Microtiterplates -Washer | Nunc, Wiesbaden, D |
| Inject® Alum ($Al(OH)_3$) | Pierce, Rockford, USA |
| Ketamin (10mg/ml) | Inresa, Freiburg, D |
| L-Glutamine | PAA Laboratories GmbH, Cölbe, D |
| Mikrobiologie Agar | Merck, Darmstadt, D |
| Microtiterplate Reader Sunrise | Tecan, Crailsheim, D |
| OVA Grade V | Sigma, Hamburg, D |
| OVA Grade VI | Sigma, Hamburg, D |
| Periodic acid | Merck, Darmstadt, D |
| PC-based Olympus light microscope BX51 | Olympus, Hamburg, D |
| Phosphat-Puffer (PBS) | Biochrom, Berlin, D |
| Prism 5 Graph Pad Software | GraphPad Software, Inc.,San Diego, USA |
| Rabbit anti-SMA antibody | Abcam, Cambridge, UK |
| Roticlear® | Roth, Karlsruhe, D |
| RPMI (1x) without L-Glutamine | PAA Laboratories GmbH, Cölbe, D |
| RTU Horseradish peroxidase strepavidin | Vector, Brockville, Canada |
| SCHIFF reagent | Merck, Darmstadt, D |
| Set Mouse TGF-β (ELISA kit) | R&D Systems, Minneapolis, MN, USA |
| Set Mouse TNFα (ELISA kit) | R&D Systems, Minneapolis, MN, USA |
| Sodium carbonate ($NaHCO_3$) | Merck, Darmstadt, D |
| Sodium Phosphate ($Na_2HPO_4$) | Merck, Darmstadt, D |
| Streptavidin / Phosphatase | Sigma, Taufkirchen, D |

| | |
|---|---|
| substrate diaminobenzidine (DAB, SK-4100,) | Vector, Brockville, Canada |
| Sulfuric acid ($H_2SO_4$) | Merck, Darmstadt, D |
| Tween 20 | Roth, Karlsruhe, D |

### 3.3 Experimental animal model

Mice were sensitized to OVA by three intraperitoneal (i.p.) injections 10 µg OVA grade VI adsorbed to 1.5 mg $Al(OH)_3$ diluted in 200 µl phosphate-buffered saline (PBS). Mice were challenged with OVA (grade V) aerosol (1% wt/ vol in PBS) twice a week on 2 consecutive days over a period of 18 weeks (Figure 5, protocol A). Control groups were sensitized and challenged with PBS. To investigate the resolution of airway inflammation and remodelling, mice were challenged with OVA for 12 weeks, then OVA aerosol was replaced by PBS during the resolution phase. Animals were analysed after 4, 8 weeks respectively (Figure 5, protocol B). Budesonide solution was diluted in PBS to 200 µg/ml and 50 µl were given intranasal (ICS) four hours before OVA challenge. Budesonide treatment in the chronic asthma model was performed concurrently with allergen exposure beginning at week 7 until week 15 and then discontinued; analyses were performed at weeks 6, 14 and 18 (Figure 5, protocol C ). As no differences were detected between control mice at different time points, data is shown only for one PBS group (12 weeks). All experiments were performed once with a group size of 6–8 mice treated in parallel in accordance to German animal ethic regulations. Each group was coded and analysed by an investigator blinded to the experimental conditions.

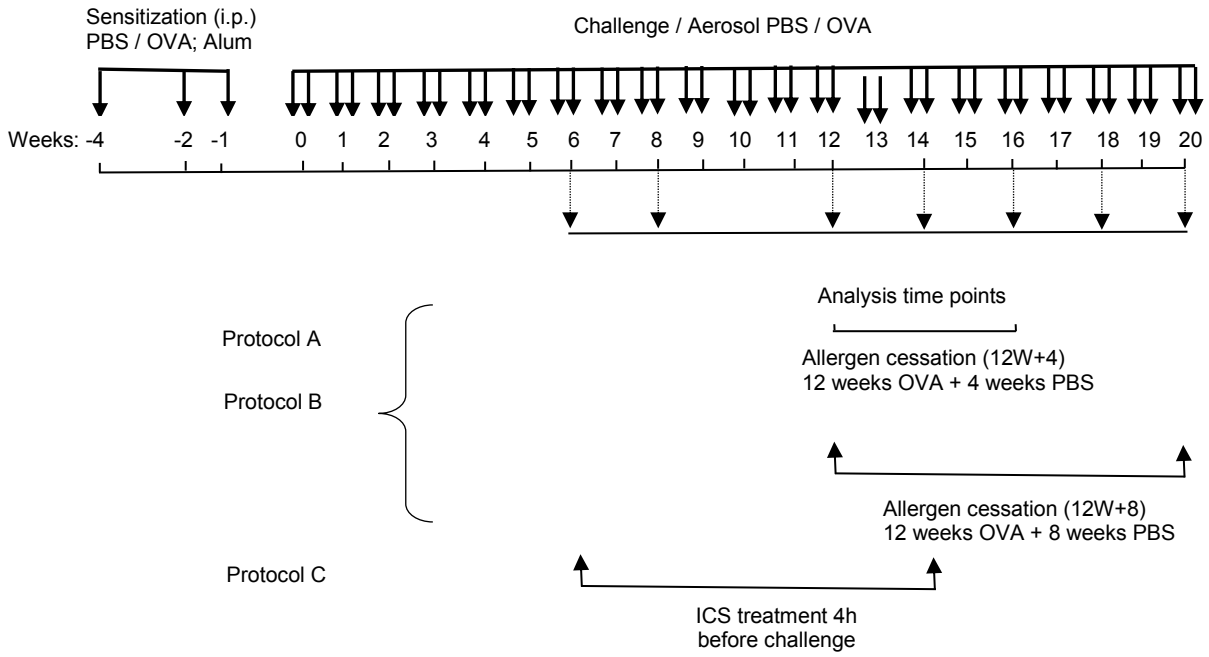


**Figure 5: Schematic representation of treatment protocol**: Mice were sensitized to OVA by three intraperitoneal (i.p.) injections on days 0, 14 and 21 with OVA absorbed to alum. Mice were then challenged with aerosolized OVA twice weekly for up to 18 weeks as indicated. Control mice were sensitized and challenged with PBS. Analyses were performed after 6, 8, 12, 14, 16 and 18 weeks of aerosol challenge (Protocol A). To investigate the resolution of airway inflammation and remodelling, OVA aerosol was replaced with PBS after 12 weeks for either 4 or 8 weeks (Protocol B). In a third study, corticosteroids were given intranasal (ICS) starting after 6 weeks of OVA challenge for 8 weeks (Protocol C). doi:10.1371/journal.pone.0085839.g001

### 3.4 Broncho Alveolar Lavage Fluid (BALF)

Bronchoalveolar lavage (BAL) is the suitable method to obtain the inflammatory cells from the lung airways. 48 hours after the last aerosol challenge, animals were anesthetized with 200 µl Ketamin, then chest was opened and the trachea was cannulated through a small incision using a 20G catheter, 1 ml PBS containing Complete® protease inhibitor (1 tablet Complete®/50ml PBS) was slowly injected through the catheter and then removed and kept in ice.

#### 3.4.1 BALF inflammatory cell counts

The BALF was then centrifuged at 350 g for 10 minutes, and the supernatant was taken in small tubes and kept at -20 °C fridges. The cells sediment was again resuspended in 1 ml PBS/1%BSA and the cells were counted using the cell counters system (Casy).

### 3.4.2 BALF differential cell counts

For the differentiation of cells in the BALF, cytospins were prepared as follows:
50 µl of BALF in PBS / 1% BSA were already used for cell counting, then these cells were diluted with 150 µl PBS / 1% BSA, and then pipetted and centrifuged for 5 min at 700 rpm, the cells were distributed on a slide and the liquid was absorbed by a filter paper. The slides were dried for 1 h at room temperature and then stained using Diff-Quick Solution. The differentiation of the individual cell types was based on staining and morphology using light microscopy at 400-fold Magnification.

### 3.4.3 Determination of BALF cytokines using Enzyme-linked immunosorbent assay (ELISA)

96 well plates were coated with the primary antibody (IL-5, IL-13, TGFβ-1, IFNγ, and TNFα). The antibodies were diluted with a coating buffer either sodium carbonate for (IL-4, IL-5, IL-13, IFNγ, TNFα) or PBS for TGFβ-1 according to manufactures recommendations. The plates were left overnight in 4°C. On the second day the plates were washed 4 times with a wash buffer (PBS 0.1% tween) except TGFβ-1 with (PBS 0.5% tween), then blocked for 2 hours with the appropriate blocking buffer (PBS 1% BSA) and (200 µl PBS 5 % Tween) for TGFβ-1. During incubation time, serial dilutions of the standards were performed in order to establish a standard curve for each of the measured cytokines. Once the blocking period was finished, plates were washed 4 times with wash buffer and then samples and standards were added. The plates were next incubated overnight in 4°C. Next day the plates were washed 4 times and then detecting antibodies were added according to the manufactures recommendation. The plates were incubated at room temperature for another 2 hours, then washed 4 times, streptavidin-peroxidase was added at a dilution of 1:1000 and incubated in the dark for 30 minutes in room temperature. After a final eight-time washing, a peroxidase substrate solution was added and incubated in the dark until colour reaction was completed, in another step the whole reaction was stopped using sulfuric acid. The reaction stops once the colour changes from blue to yellow. This was followed by photometric measurement of the plate at 450 nm and evaluation the measurement data using the Magellan software.
Total TGF-*β*1 levels were first determined after acid activation (100 µl sample + 20 µl 1M HCl mixing and incubation for 10 minutes in Room Temperature (RT), then neutralizing the sample with 20 µl 1,2 M NaOH/ 0,5M HEPES (Hydroxyethylpiperazin-Ethansulfonacide-Puffer) and after good mixing the sample was ready to testing).

## 3.5 Lung Histology

Lungs were fixed in situ with 6% (wt/vol) paraformaldehyde via the trachea, removed and stored in 6% par formaldehyde. From the fixed lungs the remaining trachea and heart were removed. To include randomness in the selection, the lungs were embedded in a liquid solution containing 2% agar in distilled water, refrigerated for 1 hour at 4 ° C. until the agar was solid, the lungs were cut in 2 mm thick sections in a plane rectangular shape according to the "Systematic Randomized Uniform Sampling" (SURS) and covered again with a 2% agar. This block was put then in an Embedding cassette in formalin, transferred and waxed in paraffin. Thin sections of 3 µm thickness were made by microtome, and placed on slides and left for 48 hours in room temperature to dry.

### 3.5.1 Hematoxylin-Eosin (HE)-Staining

Slides were deparaffinised and rehydrated as follows: first the slides were dipped in Xylol for 15 min, then they were dipped in Ethanol (100%) two times each for one minute, then in ethanol (90%, 80%, 70%) each for one minute respectively. After that, slides were washed in distilled water then dipped in haematoxylin solution for 3 min. Then slides were dipped for 10 min in running warm tap water, then washed for short time in distilled water. The slides were dipped in eosin solution for 2 min and then washed in distilled water. At the end the slides were dipped in the raw of Ethanol (70%, 80%, 90%, 100%) each for one min respectively, and in Xylol for 15 min. At the end the slides were covered with Neo Mount.

### 3.5.2 Periodic Acid-Schiff Staining

To stain the sections, the slides were deparaffinised and rehydrated as mentioned before (2.5.1), then dipped in periodic acid 0.5 % in distilled water for 10 min. Slides were then dipped for 3 min in running tap water, then washed for short time in distilled water. The slides were dipped in Schiff reagent (always fresh prepared) for 15 min, then slides were dipped for 15 min in running tap water and washed for short time in distilled water. For contrastivity the slides were dipped in Haematoxylin solution for 1 min, then in running tap water for another 3 min. At the end the slides were dipped again in alcohol and Xylene as described in 2.5.1.

### 3.5.3 Sirius Red/Fast Green Staining

A Sirius Red/Fast Green staining was used as a specific fibrous collagen staining in the lung tissue. The Sirius Red dye accumulates in the triple helix structure of collagen molecules. The Fast Green staining serves as a Counter stain. After deparaffinisation and rehydration with xylene and alcohol as described in 2.6.1, sections were dipped for 48 hours in Sirius Red/Fast Green staining in dark, then were rinsed with water for a short time and at the end slides were dipped again in alcohol series and Xylene as described in 2.5.1.

### 3.5.4 Immunohistochemistry

Tissue sections were first deparaffinised and then rehydrated. Endogenous tissue peroxidase activity was inactivated with 1% $H_2O_2$ (vol/vol in methanol) for 30 min. Antigen retrieval was performed through immersing slides in plastic staining holders containing 10 mM citrate buffer in Aqua dest., then putting the holders inside the microwave oven (450W) for 3 – 5 minutes or until the solution started to boil. Sections were blocked for 60 mins with 3% milk powder in PBS. Smooth muscle actin (SMA) expression and T lymphocytes were detected by incubation with rabbit anti-SMA antibody and goat anti-CD3 antibody, respectively, in a humid chamber overnight at 4°C. Sections were then washed with PBS, incubated for 60 min with biotinylated goat anti-rabbit antibody or biotinylated rabbit anti-goat antibody (1:100 in PBS / 3% milk powder). Slides were washed with PBS, then ABC complex (RTU Horseradish peroxidase Streptavidin) was added and incubated for 30 min in dark, washed and then incubated with the substrate diaminobenzidine (DAB, SK-4100) for 10 min in the dark. The sections were then rinsed briefly with distilled water and then counterstained for 1 min in haematoxylin. At the end, slides were dipped again in alcohol series and xylene as described in 2.5.1.

## 3.6 Quantitative morphology

H&E-stained tissue sections were microscopically viewed and random images were collected under 20 x objective. Degree of inflammation was expressed as a peribronchial airway inflammation score (0, normal; 1, few inflammatory cells; 2, one to two cell layers ring of inflammatory cells; 3, three to four cell layers ring of inflammatory cells and 4, more than four layers ring of inflammatory cells) (Conrad et al. 2009). H&E and CD3 immunohistochemical stained lung sections were selected by random sampling (40–50 images) using the 40 x objective. The number of eosinophils and CD3-positive cells were quantified and expressed as cell numbers per field. PAS-stained sections were viewed

and random images collected under 20 x objective. The fraction of the analysed basal membrane covered by goblet cells was then evaluated through calculating the goblet cells per mm basement membrane. Inflammation and goblet cells were quantified using a PC-based Olympus light microscope BX51 equipped with a Cell-F System. Paraffin sections stained with Sirius Red, anti-SMA, were used to quantify changes in airway collagen deposition, smooth muscle cell layer thickening, respectively, using the BX51 microscope equipped with a CAST-Grid System [3]. All sections were delineated and the fields of view analysed (at 400x) were automatically defined according to systematic uniform random sampling, 150 random samples (30% of total lung tissue area and thus representing all parts of the airway tree) were taken of each section. The arithmetic mean thickness ($T_{comp}$) was determined as the volume of the respective component, determined by counting all points intercepting the airway epithelium and Sirius Red- and a-SMA-positive components, respectively (Cruz-Orive und Weibel 1990; Hsia et al. 2010). Results were referred to the reference surface determined by counting all intersections with the airway epithelial basal membrane. The arithmetic mean thickness was calculated according to the formula: $T_{comp} = L(P) \times \Sigma P_{comp}/(2 \times \Sigma I_{bi})$ (Weibel ER, Cruz-Orive LM 1997). L(P) is the line length per test point, $P_{comp}$, the number of points hitting the respective component and $I_{bl}$ the number of intersections between the test line and the epithelial basal membrane.

### 3.7 Statistical analysis

Graphing and statistical analysis of normally distributed data was performed using Prism 5 (Graph Pad Software, San Diego, CA, USA). Data are expressed as mean ± SEM and are analysed for significance using one-way ANOVA with Tukey's Multiple Comparison Test (for multiple group comparisons) or the Student’s unpaired t-test for two groups comparison. Statistical significance was referred to as follows

*p⩽0.05, **p⩽0.01, ***p⩽0.001

# 4 Results

## 4.1 Chronic exposure to OVA results in prolonged airway inflammation and remodelling

### 4.1.1 Airway inflammation

The kinetic of airway inflammation was investigated using a chronic mouse model of experimental asthma by challenging mice with aerosolised OVA over an 18 week period (Figure 5, protocol A). In the bronchoalveolar lavage fluid (BALF), peak cell infiltration was observed at 6 weeks of OVA challenge, which steadily decreased until 12 weeks and then remained at almost baseline levels. In contrast, the high level of peribronchial tissue inflammation observed at 6 weeks, persisted throughout all analysed time points (Figure 6).

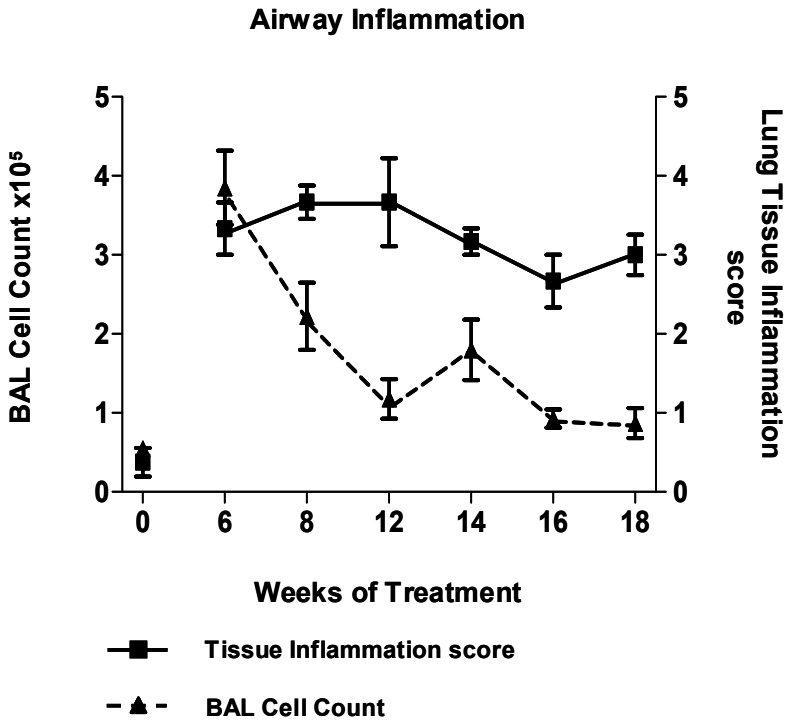


**Figure 6:** Chronic allergen exposure induces airway inflammation: Mice were challenged with OVA for up to 18 weeks (Figure 5, Protocol A) and analysed for airway inflammation as determined by BALF cell counts and morphometric quantification of peribronchial inflammation.

The maintenance of allergen challenge was accompanied by decreased bronchoalveolar inflammation. A marked proportional decrease of eosinophils after 12 weeks without dominance as seen before 12 weeks (Figure 7A-D)

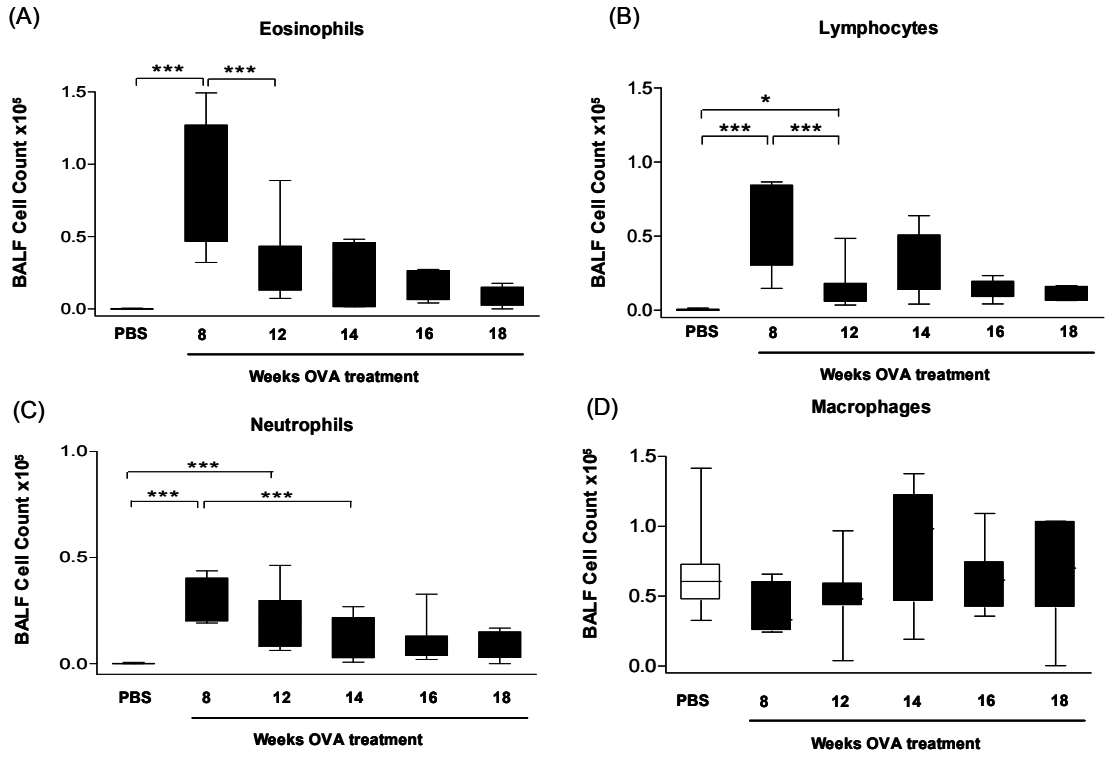


**Figure 7:** Prolonged allergen challenge results in decreased BALF inflammatory cell recruitment: Differential cell counts from chronic allergic inflammation model following allergen challenge for up to 18 weeks, 6-8 animals per group, *p≤0.05, ***p≤0.001.

### 4.1.2 Airway remodelling

Hallmarks of airway remodelling are goblet cell hyperplasia, thickening of the smooth muscle cell layer and extracellular matrix deposition. To quantify changes in airway remodelling immunohistochemical analysis in combination with stereological quantification was performed. Comparison of PAS-stained lung sections from chronically OVA treated mice revealed that ~45% of all cells lining the bronchial airways throughout the whole observation period were Goblet cells (Figure 8). Advanced structural changes, such as collagen deposition and smooth muscle thickening, in the airways were not visible until eight weeks of OVA exposure as determined by collagen and a-SMA staining, respectively. Between 8 and 12 weeks the amount of airway collagen rapidly increased by approximately 3 fold and then remained constant. Thickening of the smooth muscle layer was less pronounced than collagen deposition, but increased approximately two fold between 6 and 12 weeks (Figure 8). Together these data show that in this mouse model of chronic experimental asthma, continued allergen exposure is associated with prominent

and persistent airway inflammation and structural alterations (Alrifai et al. 2014) publication attached.

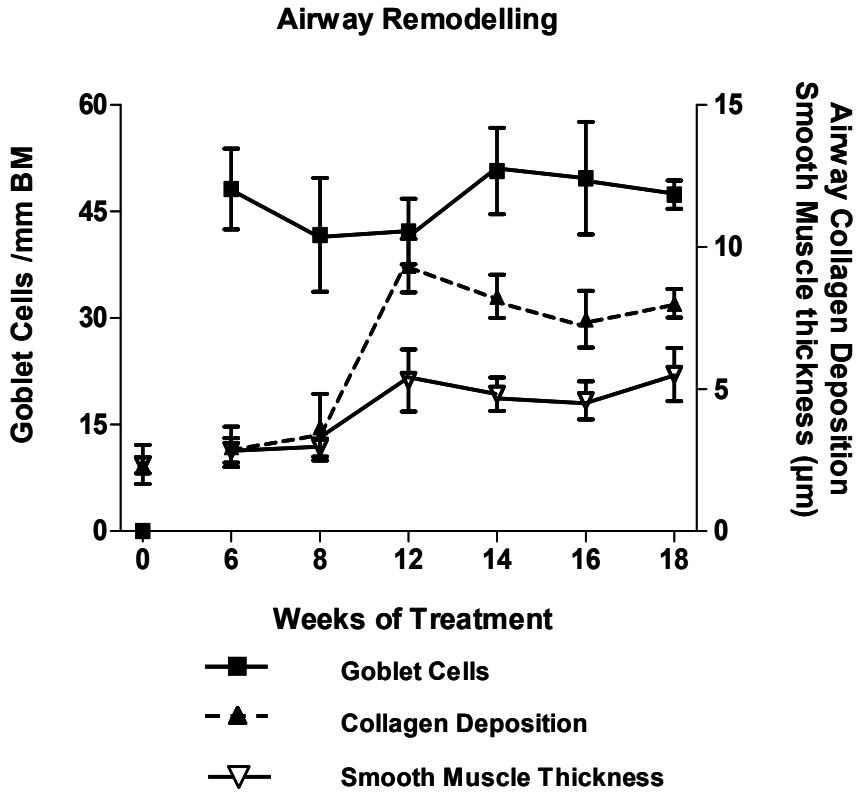


**Figure 8:** Chronic allergen exposure induces airway remodelling: Mice were challenged with OVA for up to 18 weeks (Figure 5, Protocol A) Airway remodelling was determined by quantification of Goblet cell hyperplasia, subepithelial collagen deposition and smooth muscle thickening. Data points represent means 6 ± ∓ SEM of n=6-8 animals per group.

## 4.2 Cessation of allergen exposure reverses airway inflammation and remodelling

To mimic the situation of effective allergen avoidance, it was further investigated how allergen cessation can affect established airway remodelling and inflammation.

### 4.2.1 Airway inflammation

#### 4.2.1.1 BAL inflammation

Following 12 weeks of OVA challenge mice exhibited robust airway inflammation and fully established remodelling, including thickening of the smooth muscle layers and increased deposition of collagen. Therefore, this time point was chosen as the reference point for the

chronic situation and used to investigate the effects of allergen cessation over the following 8 week period. After 4 weeks of resolution, total BALF cell numbers remained constant while the numbers of eosinophils and neutrophils decreased to baseline levels during this period (Figure 9B, D). The number of lymphocytes in the BALF resolved much slower, returning to control levels after 8 weeks together with total BALF cell counts (Figure 9A, C). Interestingly, the number of alveolar macrophages initially increased significantly then decreased almost to control levels after 8 weeks of resolution (Figure 9E).

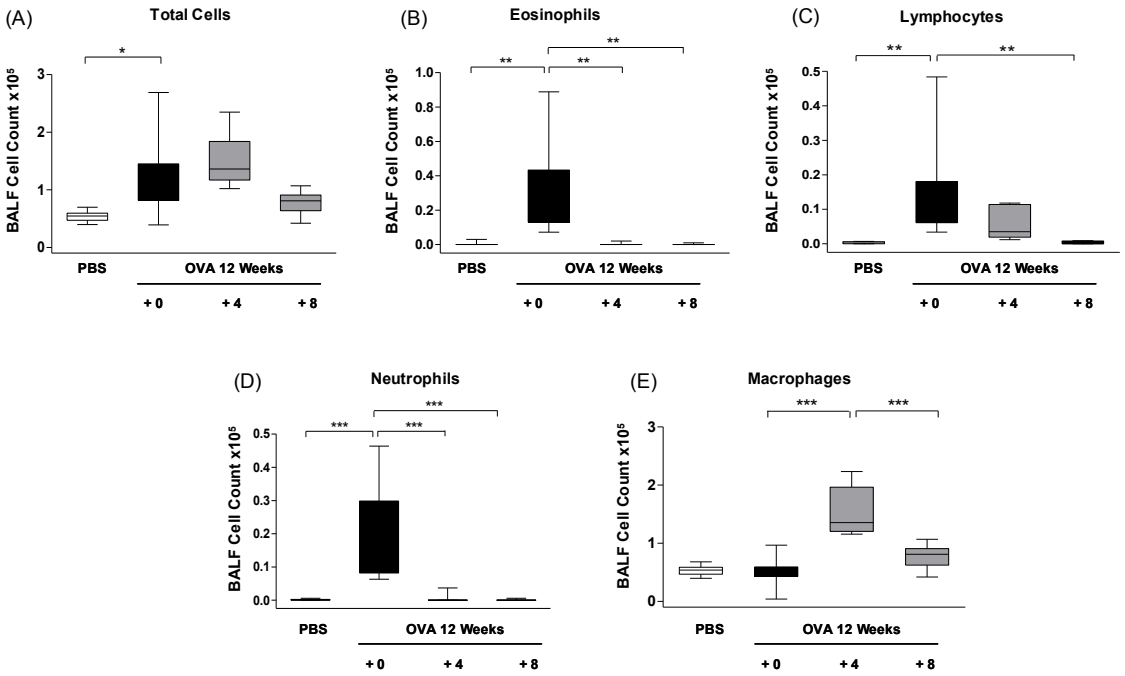


**Figure 9:** Resolution of BALF inflammation following allergen cessation: Mice were challenged with OVA twice weekly for 12 weeks to establish features of chronic asthma; OVA challenge was then discontinued and replaced with PBS for subsequent 4 or 8 weeks (Figure 5, Protocol B). Bronchoalveolar (BAL) inflammation was analysed after 12 (+ 0), 16 (+ 4) or 20 (+ 8) weeks of challenge. The results represent data from n=6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

### 4.2.1.2 Lung tissue inflammation

Tissue inflammation was assessed by simple H&E and immunohistochemical staining in combination with stereological quantification (Figure 10). The high level of lung tissue inflammation observed before allergen cessation slowly decreased and returned to baseline levels after the 8 week resolution period (Figure 10A, C). While lung tissue eosinophils rapidly disappeared (Figure 10D), many $CD3^+$ T lymphocytes were still

present after 4 weeks and only reduced after 8 weeks of allergen cessation (Figure 10B, E).

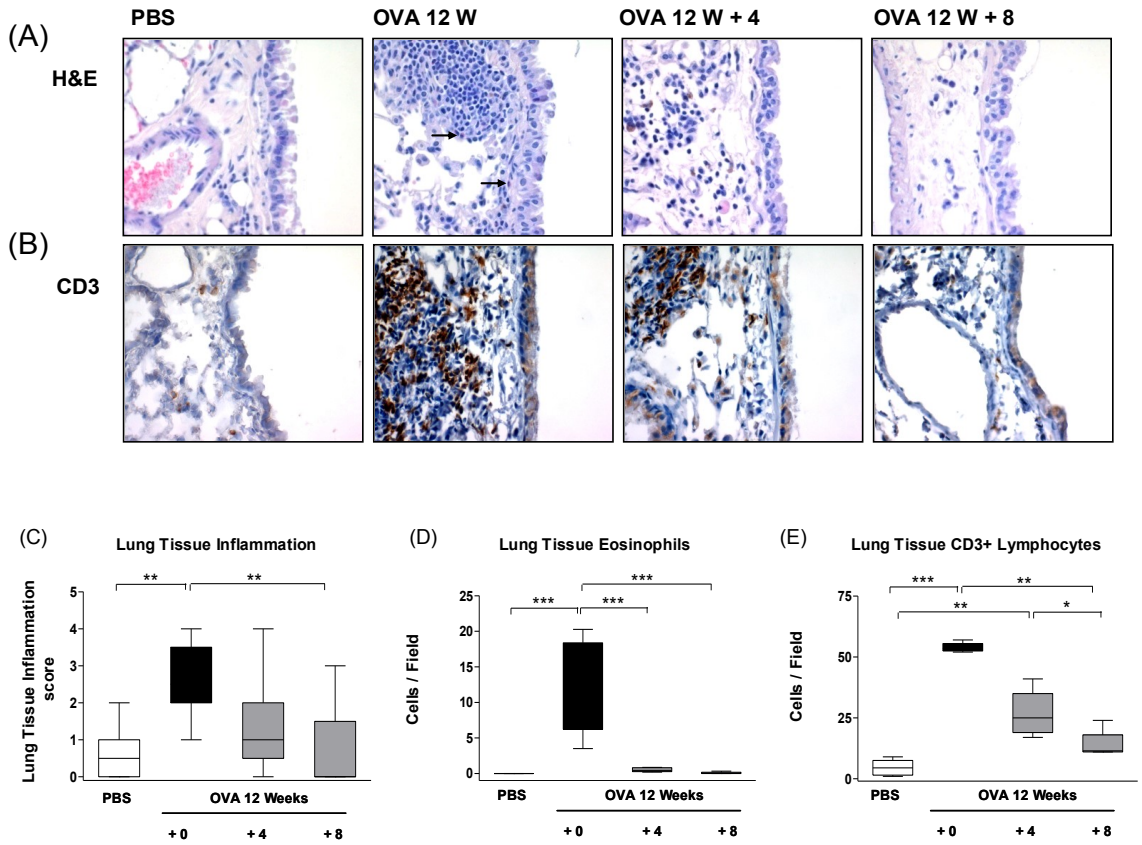


**Figure 10:** Resolution of airway tissue inflammation and remodelling following chronic allergen exposure: Chronic asthma was established by challenging OVA sensitized mice for 12 weeks twice weekly. OVA challenge was then discontinued and replaced with PBS for subsequent 4 or 8 weeks. Outcome measurements were made at either 12 (+ 0), 16 (+ 4) or 20 (+ 8) weeks of challenge (Figure 5, Protocol B). (Figure 10A) Representative photomicrographs of haematoxylin & eosin (H&E) stained sections (arrows indicate eosinophils), (Figure 10B) immunohistochemical staining of CD3. (Figure 10C) Histological quantification of lung inflammation, (Figure 10D) eosinophil numbers as determined by morphological criteria in H&E stained lung sections, (Figure 10E) CD3+ lymphocytes per field of observation. Box and whisker plots show mean and percentiles with n=6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

### 4.2.2 BAL cytokines profile

Before allergen cessation and during the 8 week resolution phase, the levels of IL-5 in the BALF were indistinguishable from the PBS group (Figure 11A). The levels of IL-13 were increased at the chronic reference point (12 weeks of OVA challenge) then decreased during resolution period, however, these changes did not reach statistical significance

(Figure 11B). In contrast, the levels of IFN-Ɣ were increased during the resolution phase (Figure 11C). High levels of the pro-remodelling cytokine TGF-β were detected in the BALF at 12 weeks of OVA challenge, which then returned to baseline levels after 8 weeks of allergen cessation (Figure 11D).

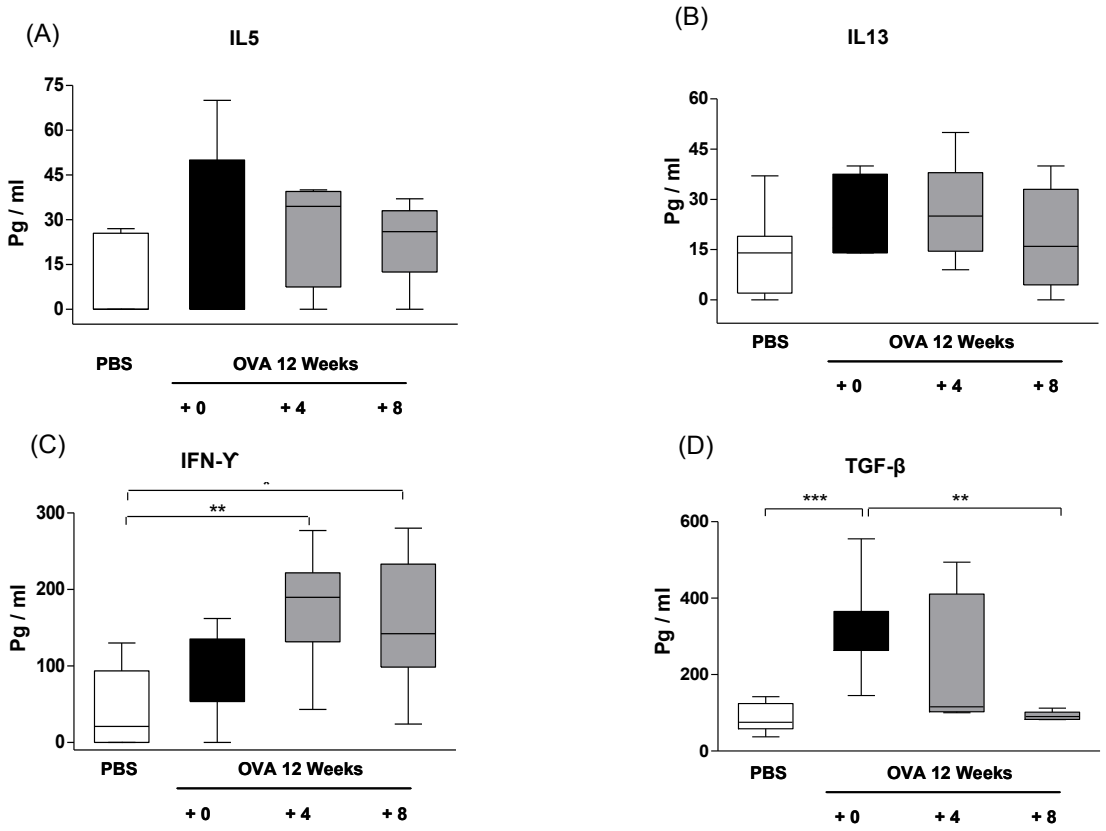


**Figure 11:** Cytokine profiles following allergen cessation: Mice were challenged with OVA twice weekly for 12 weeks to establish features of chronic asthma; OVA challenge was then discontinued and replaced with PBS for subsequent 4 or 8 weeks (Figure 5, Protocol B). Bronchoalveolar (BAL) cytokines were analysed after 12 (+ 0), 16 (+ 4) or 20 (+ 8) weeks of challenge. The results represent data from n=6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

### 4.2.3 Airway remodelling

Before resolution ~40% of the lining airway epithelium consisted of goblet cells, which decreased to ~8% after 4 weeks of allergen cessation then further decreased to complete absence after 8 weeks of resolution (Figure 12A, D). Airway collagen deposition exhibited a similar trend, rapidly decreasing in thickness during the initial four weeks of allergen avoidance and finally resolving at eight weeks (Figure 12B, E). On the other hand, smooth muscle thickening was much slower to resolve, requiring the full eight weeks to return to

control levels (Figure 12C, F). Together these data show that the resolution of inflammation and remodelling are highly dynamic processes that occur with different kinetics for individual parameters.

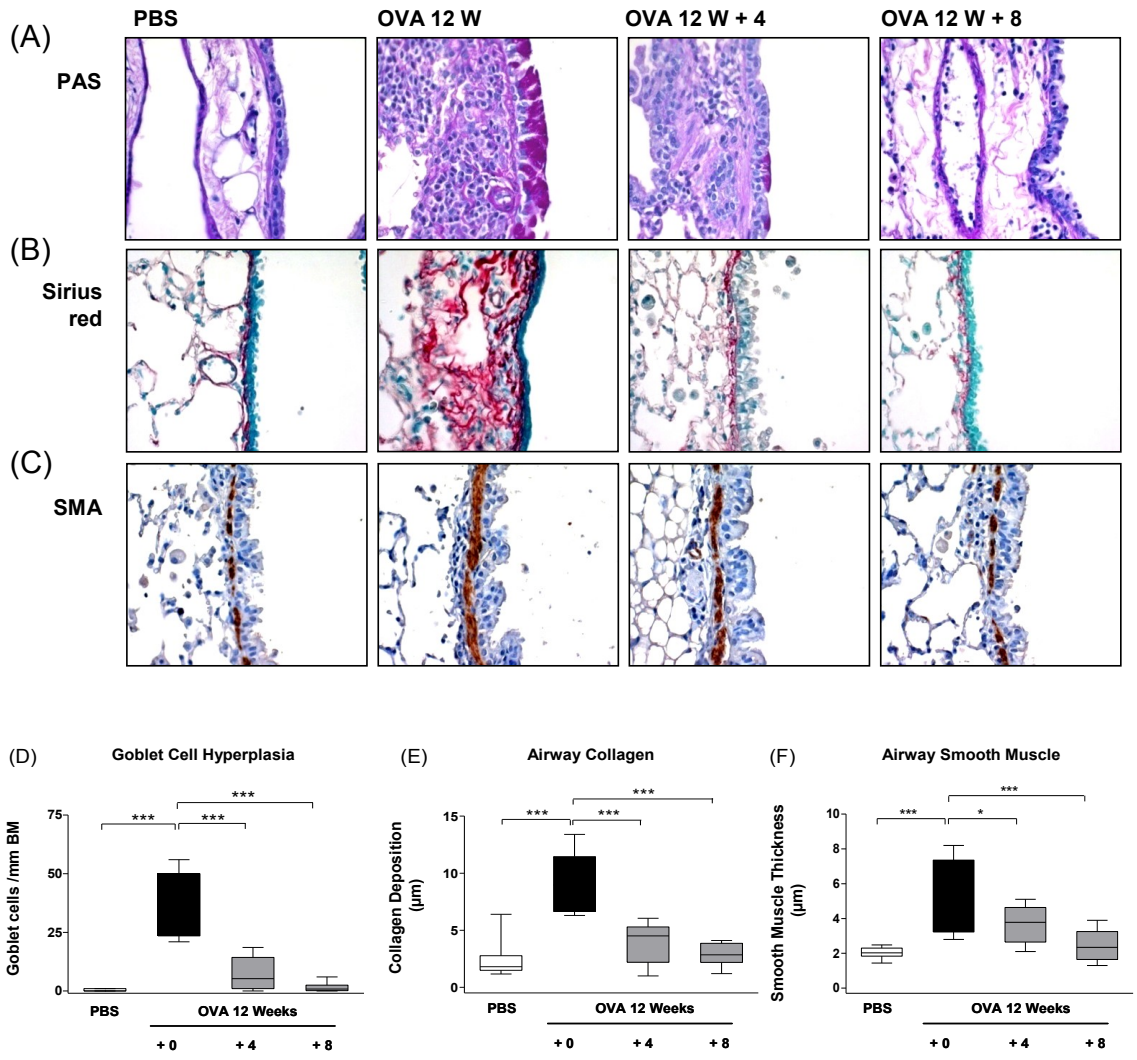


**Figure 12:** Resolution of airway tissue inflammation and remodelling following chronic allergen exposure: Chronic asthma was established by challenging OVA sensitized mice for 12 weeks twice weekly. OVA challenge was then discontinued and replaced with PBS for four or eight weeks. Outcome measurements were made at either 12 (+ 0), 16 (+ 4) or 20 (+ 8) weeks of challenge (Figure 5, Protocol B). (Figure 12A) Representative photomicrographs of periodic acid-Schiff (PAS), (Figure 12B) Sirius Red staining and (Figure 12C) immunohistochemical staining of smooth muscle actin (SMA). (Figure 12D) Histological quantification of PAS positive Goblet cells, (Figure 12E) collagen deposition and (F) SMA thickness. Box and whisker plots show mean and percentiles from n=6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

## 4.3 Corticosteroids protect against the full establishment of airway remodelling during development of chronic asthma

It was finally investigated whether therapeutic intervention could interfere with the development of advanced airway remodelling. The efficacy of Steroid treatment at the given dose was first determined using an acute model of experimental asthma (Figure 13A). As expected, acute OVA challenge induced a strong recruitment of inflammatory cells into the BALF, predominately consisting of eosinophils as well as high levels of Goblet cell hyperplasia. ICS administration significantly attenuated the OVA-induced asthma phenotype. However, treatment did not completely attenuate experimental asthma manifestation, as the numbers of eosinophils and Goblet cells remained significantly higher than in control mice (Figure 13B-G).

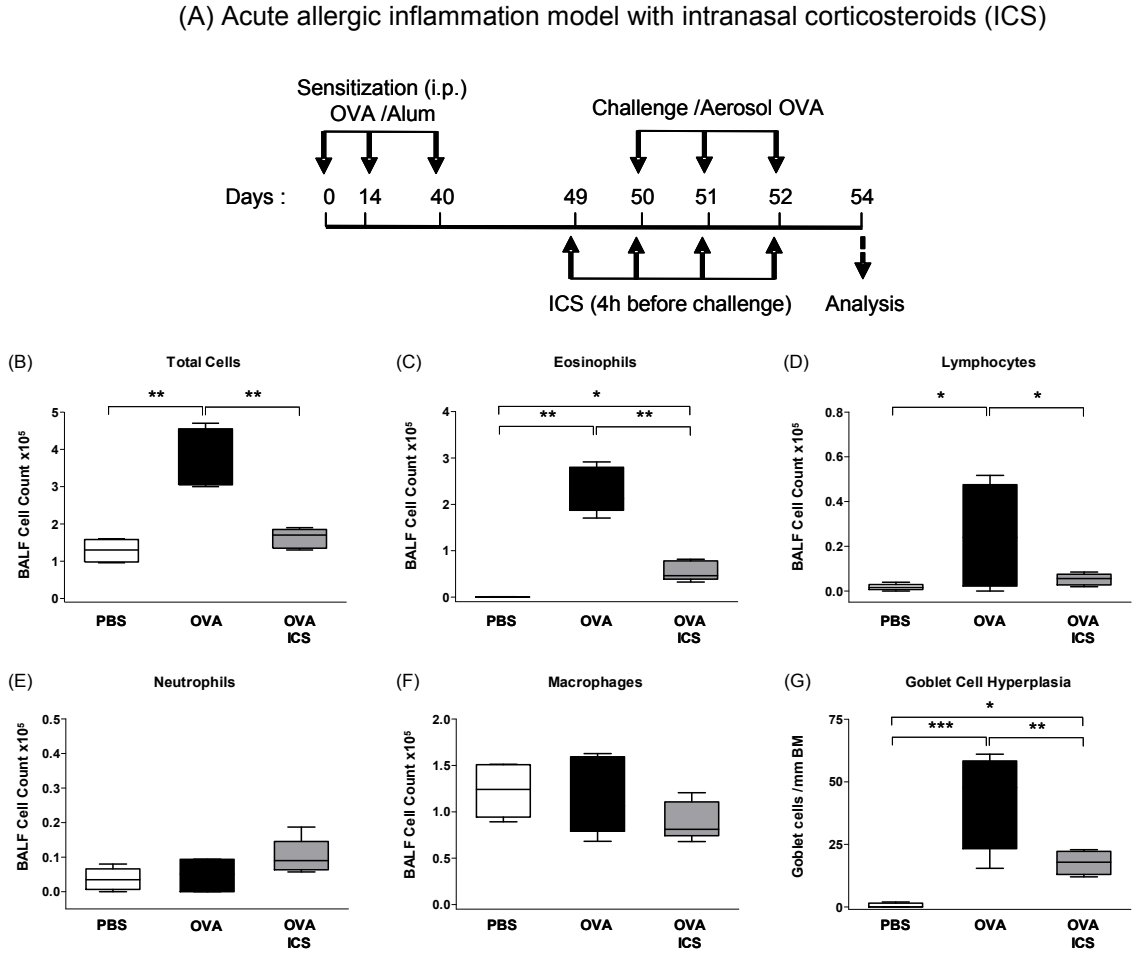


**Figure 13:** corticosteroids attenuates features of acute airway inflammation: (A) Acute airway inflammation was generated in mice via intraperitoneal (i.p.) injection of OVA conjugated to Alum and subsequent challenge with OVA for three days with or without the prior treatment with intranasal corticosteroids (ICS). Mice were analysed 48 hrs after last challenge for; (B) total BALF cells, (C) eosinophils, (D) lymphocytes, (E) neutrophils, (F) macrophages and (G) quantified for the number of Goblet cells. Basement membrane (BM). The results are shown as box and whiskers-plots showing mean and percentiles with 6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

The effect of ICS on the development and progression of airway remodelling was then examined in the chronic model; Corticosteroid treatment started at a time when initial remodelling processes are observed (week 6) and then continued during the period of reinforcement and full establishment of remodelling until week 14 (Figure 5).

Prior to ICS therapy (week 6), OVA challenge induced a strong bronchoalveolar and tissue inflammation, recruiting eosinophils, neutrophils and lymphocytes to the BALF (Figure 14, black bars). Treatment of mice with ICS for 8 weeks did not alter the level of bronchoalveolar inflammation (Figure 14A-E, light grey bars), however, significantly reduced tissue inflammation (Figure 14F, light grey bars) compared to mice challenged with OVA alone. ICS therapy also diminished Goblet cell numbers and collagen deposition but had no effect on smooth muscle thickening (Figure 14G-I, light grey bars).

To investigate whether this protection persisted following discontinuation of ICS, mice were further exposed to OVA for another four weeks in the absence of corticosteroids. Mice previously treated with ICS exhibited increased eosinophil numbers in the BALF as compared to mice that never received ICS (Figure 14B, dark grey bars). However, mice that were treated with ICS maintained lower Goblet cell numbers and reduced collagen deposition compared to mice that did not receive ICS (Figure 14G H, dark grey bars). Together, these data show that ICS confers some protection from advance remodelling during the transition from the acute to the chronic phase. However, some beneficial effects of ICS are lost in the case of subsequent allergen exposure.

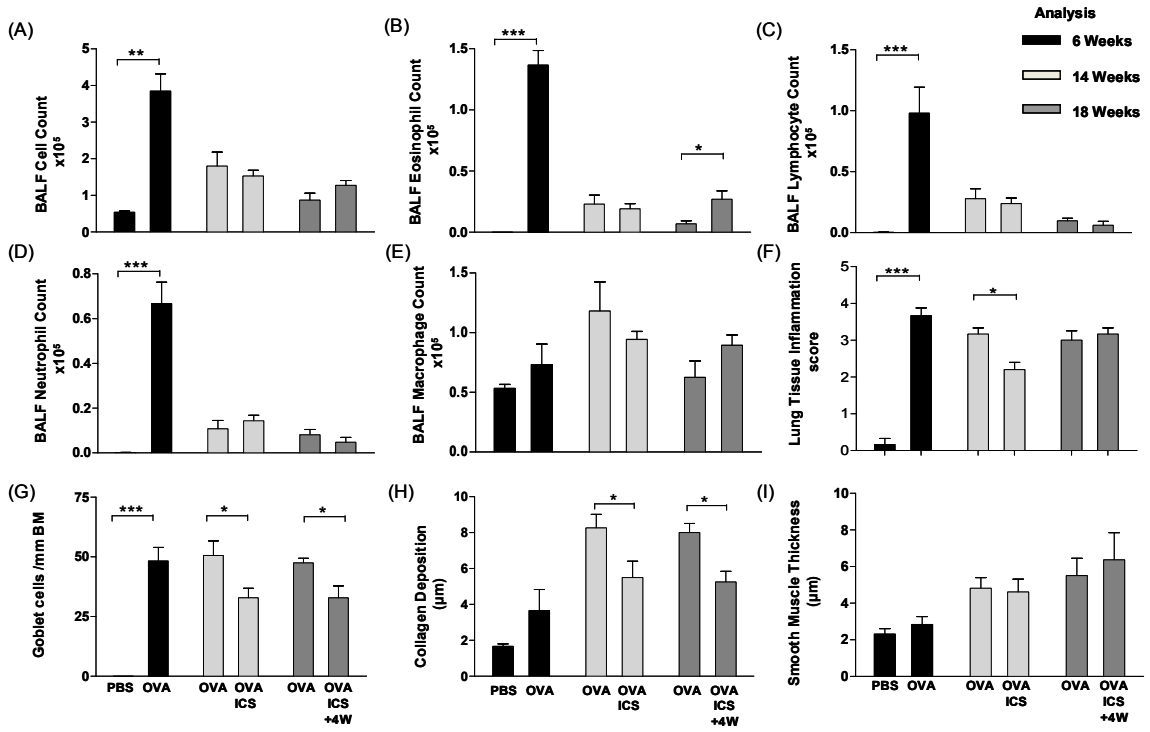


**Figure 8:** intranasal corticosteroids attenuate some but not all characteristics of chronic asthma: Mice were sensitized and challenged for 6 weeks with PBS as control and OVA (black bars). OVA treatment was continued for another 8 weeks (light grey bars), with or without parallel treatment with intranasal corticosteroids (ICS). OVA treatment was then continued for another 4 weeks without ICS application (dark grey bars) (Protocol C). Data are presented as mean +/- SEM, n=6-8 animals per group, *p≤0.05, **p≤0.01, ***p≤0.001.

# 5 Discussion

Pronounced airway remodelling is a hallmark of chronic asthma and is characterised by Goblet cell hyperplasia, deposition of extracellular matrix components and thickening of the smooth muscle layer. The presence of advanced airway remodelling is associated with a poorer clinical prognosis and is therefore, considered an important therapeutic target (Ge et al. 2010; Murdoch und Lloyd 2010). Unfortunately, current anti-inflammatory therapeutic strategies including corticosteroids, while effective for reducing inflammation are less successful in treating structural alterations in airway remodelling (Kelly et al. 2010). Furthermore, the reversibility of airway remodelling is still unclear; it is not fully understood whether cessation of allergen exposure can lead to the full resolution of established remodelling (McMillan und Lloyd 2004).

To address these open questions, we have utilised a mouse model of chronic asthma which exhibits pronounced airway remodelling at 12 weeks of aerosol allergen exposure and is maintained throughout the entire challenge period of 18 weeks. The maintenance of chronic asthma and tissue inflammation was accompanied by decreased bronchoalveolar inflammation but persistence of tissue inflammation. The low levels of eosinophils and lymphocytes present within the BALF at the later time points during allergen challenge is consistent with previous studies (Wegmann et al. 2005; Sakai et al. 2001). This data also supports clinical observations by Persson et al. who described that a decrease in inflammatory BALF cells but the persistence of lung tissue inflammation is an index of worse outcome in asthma (Persson und Uller 2010). These data demonstrate that decreased inflammatory cell numbers in the BALF but maintenance of tissue inflammation correlates with the progression of chronic allergic asthma and is independent from resolution. Although compartmentalization of airway-inflammation seems to be a critical step during the transition from an acute to a chronic phenotype, the underlying molecular mechanisms which regulate compartmentalization of inflammatory cells are still not known. It is likely that selective and spatial recruitment processes direct this phenotype, which includes the expression of adhesion molecules, chemokines and/or chemokine receptors. It has been reported that prolonged allergen challenge can lead to immune tolerance and loss of inflammation (van Hove et al. 2007). However, in this and other studies it has been demonstrated that prolonged allergen exposure results in persistent Goblet cell hyperplasia and chronic tissue inflammation (Sakai et al. 2001). The discrepancy between these reports is most likely due to the use of different mice strains; C57BL/6 versus BALB/c as used in our study (Chu et al. 2006; Hogan et al. 2008; Nygaard et al. 2005), which indicates an underlying genetic component in asthma susceptibility and recovery.

In mice with fully established airway inflammation and remodelling, allergen cessation (four weeks) resulted in a rapid decrease in inflammatory cells such as eosinophils and neutrophils from the BALF. In contrast, macrophage numbers revealed a different kinetics initially increasing in numbers before returning to baseline levels. This temporary increase in macrophage numbers adds further support to the important role of this cell type in the resolution of inflammation (Leung et al. 2004, Porcheray et al. 2005, 2005).

Allergen cessation resulted in a rapid decrease in Goblet cell numbers, which is in line with observations made by other investigators (Blyth et al. 2000; Southam et al. 2008b; Kumar et al. 2004). Our study expands on these investigations by performing comprehensive analysis of both inflammatory and remodelling parameters. It has been proposed that the cessation of allergen exposure does not completely attenuate airway remodelling (Henderson, JR et al. 2006; McMillan und Lloyd 2004; Kumar et al. 2004; Leigh et al. 2002). In the studies by McMillan et al. and by Kumar et al. four weeks of allergen cessation was not sufficient to fully resolve airway remodelling (Kumar et al. 2004; McMillan und Lloyd 2004). This observation was confirmed by the results of our study, however, prolongation of the resolution period to eight weeks completely attenuated lung tissue inflammation and fully reversed airway remodelling (Alrifai et al. 2014). Together this supports the notion that continued allergen exposure is required for the persistence of allergic airway inflammation and remodelling, and that avoidance of allergen exposure could ameliorate airway inflammation and remodelling at least in mice.

The extensive airway remodelling at twelve weeks of OVA challenge correlated with high levels of TGF- β in the BALF. TGF- β has important roles in mediating remodelling by inducing the production of extracellular matrix proteins and cell proliferation. It has been shown that TGF- β has a significant role in pulmonary fibrosis (Khalil et al. 1991). Additionally increased TGF- β expression has been observed in asthmatic patients, which correlated with subepithelial fibrosis (Redington et al. 1997; Halwani et al. 2011; Vignola et al. 1997). Furthermore, in our study the decreasing level of TGF- β in BALF following allergen cessation also correlated with the resolution of airway remodelling, which further indicates the important role of this cytokine in remodelling and resolution. The increased IFN-Ɣ levels observed during resolution phases may also serve to further antagonise the profibrotic effects of TGF- β (Eickelberg et al. 2001).

ICS are the mainstay of asthma therapy in humans (Baran 1987). Studies in mice have predominately focused on the effects of ICS in acute asthma models (Chian et al. 2011; Schmidt et al. 1994; Shen et al. 2002). We have here investigated the effects of ICS during the establishment of airway remodelling. The experimental protocol closely mimics the clinical situation, in which patients suffer from acute allergic asthma symptoms at the starting point of treatment. The data from the acute model confirmed the efficacy of the

ICS treatment and is consistent with other studies (Chian et al. 2011; Schmidt et al. 1994; Shen et al. 2002). Applying ICS during the transition from acute to chronic asthma, resulted in lower lung tissue inflammation, Goblet cell hyperplasia and collagen deposition. ICS however did not alter allergen induced smooth muscle thickening. Together these results indicate that despite ICS OVA sensitization is retained and that ICS delay some but not all characteristics of chronic remodelling. Similar observations were reported in a chronic OVA-induced asthma model when budesonide was given for four weeks following allergen cessation, however in this case no differences in collagen deposition and smooth muscle mass were observed (Southam et al. 2008a).

Similar results have been obtained following the co-application of OVA and dexamethasone, which reduced Goblet cell hyperplasia but did not affect smooth muscle thickness (Karras et al. 2007; Miller et al. 2006). These observations again demonstrate that slowly progressing remodelling features are more resistant to therapeutic interventions. Our study also expands on works of Kumar and Herbert in which the authors showed that dexamethasone treatment resulted in reduced lung inflammation and collagen deposition (Herbert et al. 2008; Kumar et al. 2003), by investigating airway inflammation and remodelling over a longer treatment period and by maintaining allergen challenge after the cessation of ICS.

In a study by Southam et al. the simultaneous removal of both, the allergen and ICS, resulted in a marked rebound of Goblet cell hyperplasia, which was most apparent after prolonged co-application of budesonide and allergen (Southam et al. 2008b). Interestingly a minimum of six weeks of concurrent budesonide/ICS administration was required to confer this rebound effect. In our study the continuation of allergen challenge after the discontinuation of ICS resulted in slightly increased eosinophil counts but did not affect Goblet cell numbers or other remodelling characteristics. An important difference between these studies was that we maintained allergen challenge after cessation of ICS, a situation which reflects a non-compliant patient. The disparity between ICS effects in acute and chronic asthma supports the concept that there is a shift in immune responses throughout disease progression in allergic asthma (Wegmann et al. 2005). Therefore, the same therapy could confer different efficacy because of variability in the immune response pattern of different asthma patients. ICS are highly effective in reducing allergen induced eosinophilia and consequently in treating acute experimental asthma in which the eosinophils are the dominant cell type (Gauvreau et al. 2000). However, in chronic asthma phenotypes, which exhibit decreased eosinophils counts, other inflammatory cells have a more predominate role and are less responsive to corticosteroid therapy.

In conclusion, using a chronic model of experimental asthma we have shown that continuous allergen exposure in mice induces reversible airway remodelling. Treatment of

established inflammation and remodeling can be partially accomplished with corticosteroids, however, most prominent beneficial effects are observed by allergen avoidance. This model offers new opportunities to further delineate the cellular and molecular signaling pathways that contribute to the transition from the acute to the chronic phenotype, and to elaborate the pathways of normal repair and structural reorganisation.

## Summary

Asthma is associated with chronic airway inflammation and progressive airway remodelling. However, the dynamics of the development of these features and their spontaneous and pharmacological reversibility are still poorly understood. We have therefore investigated the dynamics of airway remodelling and repair in an experimental asthma model and studied how pharmacological intervention affects these processes.

Using BALB/c mice, the kinetics of chronic asthma progression and resolution were characterised in absence and presence of inhaled corticosteroid (ICS) treatment. Airway inflammation and remodelling was assessed by the analysis of bronchoalveolar and peribronichal inflammatory cell infiltrate, Goblet cell hyperplasia, collagen deposition and smooth muscle thickening. Chronic allergen exposure resulted in early (goblet cell hyperplasia) and late remodelling (collagen deposition and smooth muscle thickening). After four weeks of allergen cessation eosinophilic inflammation, goblet cell hyperplasia and collagen deposition were resolved, full resolution of lymphocyte inflammation and smooth muscle thickening was only observed after eight weeks. ICS therapy when started before the full establishment of chronic asthma reduced the development of lung Inflammation, decreased goblet cell hyperplasia and collagen deposition, but did not affect smooth muscle thickening. These effects of ICS on airway remodelling were maintained for a further four weeks even when therapy was discontinued.

Utilising a model of experimental chronic asthma we have shown that repeated allergen exposure induces reversible airway remodelling and inflammation in mice. Therapeutic intervention with ICS was partially effective in inhibiting the transition from acute to chronic asthma by reducing airway inflammation and remodelling but was ineffective in preventing smooth muscle hypertrophy.

## Zusammenfassung

Asthma bronchiale ist eine chronisch-entzündliche Erkrankung der Atemwege mit progressiv ausgeprägten pathologischen Umbauprozessen in den Atemwegen (Remodelling). Die Dynamik der Entwicklung dieser Merkmale und ihre spontane und pharmakologische Reversibilität sind bisher nicht ganz verstanden. Wir haben daher in einem experimentellen Asthmamodell die Dynamik des Remodelling und der Reparatur der Atemwege sowie die Auswirkung pharmakologischer Interventionen auf diese Prozesse untersucht.

Unter Verwendung von BALB/c-Mäusen wurde die Kinetik der Entwicklung und des Rückgangs des chronischen Asthmas in Abwesenheit bzw. Anwesenheit einer inhalativen Corticosteroid (ICS)-Behandlung charakterisiert. Die Entzündung und das Remodelling der Atemwege wurden mittels der Analyse des entzündlichen Zellinfiltrats der Bronchoalveolar- und Peribronchialzellen, der Becherzell-Hyperplasie, der Kollagenablagerung und der Verdickung der glatten Muskulatur beurteilt. Chronische Allergenexposition führte zu frühem (Becherzell-Hyperplasie) bzw. zu spätem Remodelling (Kollagenablagerung und Verdickung der glatten Muskulatur). Vier Wochen nach dem Absetzen des Allergens waren eosinophile Infiltration, Becherzell-Hyperplasie und Kollagenablagerung wieder vollständig zurückgegangen; bis zum kompletten Rückgang der lymphozytären Infiltration und der Verdickung der glatten Muskulatur dauerte es 8 Wochen. Wurde die ICS-Therapie vor der vollständigen Etablierung eines chronischen Asthmas begonnen, reduzierte sie die Entwicklung von Lungenentzündung und verringerte Becherzell-Hyperplasie sowie Kollagenablagerung, beeinflusste jedoch nicht die Verdickung der glatten Muskulatur. Diese Effekte von ICS auf das Remodelling der Atemwege wurden für weitere vier Wochen aufrechterhalten, selbst wenn die Therapie unterbrochen wurde.

Unter Verwendung eines Modells von experimentellem chronischem Asthma haben wir gezeigt, dass wiederholte Allergenexposition bei Mäusen reversibel Remodelling und Entzündung der Atemwege induziert. Therapeutische Intervention mit ICS war teilweise wirksam bei der Hemmung des Übergangs von akutem zu chronischem Asthma durch die Verringerung der Entzündung der Atemwege und Remodelling, war aber unwirksam bei der Verhinderung der Hypertrophie der glatten Muskulatur.

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# LIST OF ACADEMIC TEACHERS

My academic teachers include the Professors and Privatdozents from:

Philipps University Marburg: Garn, Renz

Damascus University: Abu-Asali, abu-Samra, Al-Haffar, Ali, Al-Jokhadar, Abbas, Bukdash, Hajar, Kabani, Katan, Jairoudi, Makhlof, Mansour, Mourad, Nahas, Othman, Reehawi, Sabah, Shaikha

# Curriculum Vitae

***Mohammed Alrifai M.D.***

| | |
|---|---|
| **1993-1999** | Study of human medicine, Damascus University, Syria. |
| **1999** | Sate examination and licence to practise medicine, |
| **1999** | Master degree on medicine. Title (Role of old and New laboratory diagnostics to investigate causes of liver Cirrhosis in Syria) . |
| **Professional Career** | |
| **1999-2003** | Resident physician at the department of laboratory medicine, Al Mouassat university hospital, Damascus University, Syria. |
| **2003** | Specialist in the field of laboratory medicine |
| **11/2003 – 06/2006** | Head of private Laboratory, Damascus, Syria. |
| **06/2006 – 02/2008** | Specialist in the central laboratory, Al Mouassat university hospital, Damascus University, Syria. |
| **02/2008 – 10/2011** | Doktorand in the field of immunology and haematology, biomedical research centre, Philipps University, Marburg, Germany (Head: Prof. Dr. H. Renz) |
| **02/2008 – 10/2011** | Resident in the institute of laboratory medicine and pathochemistry and molecular diagnostics, University hospital Giessen and Marburg GmbH, Marburg, Germany (Head: Prof. Dr. H. Renz) |
| **Since 10/2011** | Physician in the Department of Hemostaseology (Head: Prof. Dr. B. Kemkes-Matthes), University Hospital Giessen and Marburg GmbH, Giessen, since 01.08.2018 (partial time 51%) |
| **28.09.2016** | Recognition of the specialist title for Laboratory Medicine |
| **01.08.2017** | Specialist (partial time 49%) in the institute of laboratory medicine and pathochemistry and molecular diagnostics, University hospital Giessen and Marburg GmbH, Giessen, Germany (Head: Prof. Dr. H. Renz) |
| **20.10.2017** | Specialist in the field of Hemostaseology |
| **Languages** | Arabic, German and English |

## Publications:

- Compartmental and temporal dynamics of chronic inflammation and airway remodelling in a chronic asthma mouse model. **Mohammed Alrifai**, Leigh M. Marsh, Tanja Dicke, Ayse Kılıç, Melanie L. Conrad, Harald Renz, and Holger Garn. PLoS One. 2014 Jan 21;9(1):e85839. doi: 0.1371/journal.pone. 0085839. eCollection 2014.

- Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma. Hagner S, Welz H, Kicic A, **Alrifai M**, Marsh LM, Sutanto EN, Ling KM, Stick SM, Müller B, Weissmann N, Renz H. Allergy. 2012 Aug;67(8):998-1006. doi: 10.1111/j.1398-9995.2012.02851.x. Epub 2012 Jun 12.

- Neonatal supplementation of processed supernatant from Lactobacillus rhamnosus GG improves allergic airway inflammation in mice later in life. Harb H, van Tol EA, Heine H, Braaksma M, Gross G, Overkamp K, Hennen M, **Alrifai M**, Conrad ML, Renz H, Garn H. Clin Exp Allergy. 2013 Mar;43(3):353-64. doi: 10.1111/cea.12047.

- A highly specialised self-made computer program enhances efficiency and safety of immunohaematology reports. Slonka J, **Alrifai M**, Bein G, Sachs UJ. Transfus Med. 2013 Jun;23(3):207-14. doi: 10.1111/tme.12024. Epub 2013 Mar 21.

## Conferences participations:

- 62th Annual Meeting of the Society of Thrombosis and Haemostasis Research,
  **GTH 2018 in Vienna, poster presentation:**
  Prevalence of common thrombophilic disorders in patients with ocular thrombosis
  **GTH 2018 in Vienna, oral presentation:**
  Hämophilie - Behandlung in der 3-Welt

- 61th Annual Meeting of the Society of Thrombosis and Haemostasis Research,
  **GTH 2017 in Basel, poster presentations:**
  Management of epidural bleeding in a patient with acquired hemophilia A masked by phenprocoumon therapy

- 60th Annual Meeting of the Society of Thrombosis and Haemostasis Research,
  **GTH 2016 in Muenster, poster presentations:**
  1- Impact of Thrombophilia screening and Anticoagulation on clinical course of patients with ocular thrombosis
  2 - Clinical course of pregnancy and delivery in a patient with Factor XI deficiency

- 59th Annual Meeting of the Society of Thrombosis and Haemostasis Research,
  **GTH 2015 in Dusseldorf, poster presentations:**
  **"**- Clinical course of pregnancy and delivery in a woman with von Willebrand syndrome Type 2 N"
  **"**Impact of Thrombophilia Screening and Anticoagulation in patients with Ocular Thrombosis"

- 58th Annual Meeting of the Society of Thrombosis and Haemostasis Research,
  **GTH 2014 in Vienna, poster presentations:**
  **"**Importance of Thrombophilia screening and Anticoagulation in patients with ocular venous thrombosis"
  **"**Effect of ABO Blood group on the Ristocetin induced platelet function in patients with von Willebrand`s disease"

- XXIV Congress of the international Society on Thrombosis and Haemostasis,
  **ISTH 2013 in Amsterdam, poster presentation:**
  **"**Role of thrombophilia in patients with ocular thrombosis"

Conferences participations:

- 57th Annual Meeting of the Society of Thrombosis and Haemostasis Research, **GTH 2013 in Munich, poster presentations:**
  "Immune tolerance induction in a patient with acquired haemophilia"
  "Role of thrombophilia in patients with ocular thrombosis"

- The 13th Arab congress of clinical biology 2012, Marrakech, oral presentation: "Basic concepts of haemophilia"

## Ehrenwörtliche Erklärung:

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel:

"Resolution of airway remodelling in a mouse model of chronic allergic asthma"

im Institut für Laboratoriumsmedizin und Pathobichemie, Molekular Diagnistik unter Leitung von Prof. Dr. med. Harald Renz ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Teile dieser Arbeit wurden in bei dem Journal **PLoS One** publiziert.

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| **.....** | **.....** |
| **(Ort, Datum)** | **(Mohammed AlrifAl)** |

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