# HELMINTH-DERIVED PRODUCTS MODULATE THE MYELOID ARACHIDONIC ACID METABOLISM AND TYPE 2 INFLAMMATION: CHANCES FOR NEW THERAPEUTICS AGAINST AIRWAY INFLAMMATION

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“Obstacles don't have to stop you. If you run into a wall, don't turn around and give up.

Figure out how to climb it, go through it, or work around it.”

**Michael Jordan**

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# 1 ABBREVIATIONS

| | |
|---|---|
| **12-HETE** | 12- hydroxyeicosa-tetraenoic acid |
| **12-HHT** | 12-hydroxy-heptadecatrienoic acid |
| **12-LOX** | 12-lipoxygenase |
| **15-HETE** | 15- hydroxyeicosa-tetraenoic acid |
| **15-LOX** | 15-lipoxygenase |
| **5-HETE** | 5- hydroxyeicosa-tetraenoic acid |
| **5-LOX** | 5-lipoxygenase |
| **AA** | Arachidonic acid |
| **AAM** | Alternative activated macrophages |
| **AHR** | Airway hyperresponsiviness |
| **APC** | Antigen Presenting Cell |
| ***A.s.*** | *Ascaris suum* |
| **BALF** | Bronchoalveolar lavage fluid |
| **BEBM** | Bronchial epithelial basal medium |
| **BEGM** | Bronchial epithelial growth medium |
| **BLT1/2** | Leukotriene B4 receptor 1/2 |
| **BMDM** | Bone-marrow derived macrophage |
| **CCL** | Chemokine ligand |
| **CCR3** | C-C chemokine receptor type 3 |
| **COX** | Cyclooxygenase |
| **cPLA$_2$** | Cytosolic phospholipase A2 |
| **CRSwNP** | Chronic rhinosinusitis with nasal polyposis |
| **CRTH$_2$** | Chemoattractant receptor-like molecule expressed on Th2 cells |
| **CTLA$_4$** | Cytotoxic T-lymphocyte-associated antigen 4 |
| **CysLT** | Cysteinyl leukotriene |
| **CysLT1/2** | Cysteinyl leukotriene receptor 1/2 |
| **DC** | Dendritic cell |
| **DP1/2** | Prostaglandin D2 receptor |
| **EIA** | Enzyme Immune Assay |
| **ELAM** | Endothelial leucocyte adhesion molecule-1 |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **EP1-EP4** | E prostanoid receptors |
| **ERK** | Extracellular signal-regulated kinase |
| **FP** | Fluticasone propionate |
| **GATA-3** | GATA binding protein 3 |
| **GINA** | Global Initiative for Asthma |
| **GM-CSF** | Granulocyte-macrophage colony-stimulating factor |
| **GPCR** | Seven-transmembrane G protein-coupled receptor |
| **HDM** | House dust mite extract |
| **HES** | *Heligmosomoides polygyrus* excretory/secretory products |
| **HIF-1α** | Hypoxia Inducible Factor-1alpha |
| ***Hpb*** | *Heligmosomoides polygyrus bakeri* |
| ***Hpb*E** | *Heligmosomoides polygyrus bakeri* extract |
| **ICAM-1** | Intercellular adhesion molecule-1 |
| **IF** | Immunofluorescence |
| **IFN-γ** | Interferon gamma |

| | |
|---|---|
| **Ig** | Immunoglobulin |
| **IHC** | Immunohistochemistry |
| **IL** | Interleukin |
| **ILC2** | Innate lymphoid type 2 cell |
| **IS** | Internal Standard |
| **KO** | Knockout mice |
| **LC-MS/MS** | Liquid chromatography tandem mass spectrometry |
| **LT** | Leukotriene |
| **$LTA_4$** | Leukotriene $A_4$ |
| **$LTA_4H$** | Leukotriene $A_4$ hydrolase |
| **$LTB_4$** | Leukotriene $B_4$ |
| **$LTC_4$** | Leukotriene $C_4$ |
| **$LTC_4S$** | Leukotriene $C_4$ synthase |
| **LOX** | Lipoxygenase |
| **LPS** | Lipopolysaccharide |
| **MACS** | Magnetic cell sorting |
| **MAPK** | Mitogen-activated protein kinase |
| **MCP-1** | Monocyte chemoattractant protein-1 |
| **MDM** | Monocyte derived macrophages |
| **MGL** | Macrophage galactose type C leptin |
| **MIF** | Migration inhibitory factor |
| **MRC1** | Mannose receptor C type 1 |
| **NFkβ** | Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells |
| **NHBE** | Normal human bronchial epithelial cell |
| **OVA** | Ovalbumin |
| **p** | Passage |
| **PBMC** | Peripheral blood mononuclear cell |
| **PD-1** | Programmed death protein-1 |
| **$PGD_2$** | Prostaglandin D2 |
| **PGDS** | Prostaglandin synthase |
| **$PGE_2$** | Prostaglandin E2 |
| **PGES** | Prostaglandin E synthase |
| **$PGI_2$** | Prostaglandin I2 or prostacyclin |
| **PGIS** | Prostaglandin I2 synthase |
| **PI3K** | Phosphatidylinositol 3-phosphate kinase |
| **PKA** | Protein kinase A |
| **PMN** | Peripheral Mononuclear cells |
| **PRR** | Pattern recognition receptor |
| **PPAR-α** | Peroxisome proliferator-activated receptor alpha |
| **PUFA** | Polyunsaturated fatty acid |
| **qPCR** | Quantitative real-time PCR |
| **RANTES** | Regulated upon activation normal T-cell expressed and secreted |
| **RT** | Room Temperature |
| ***S.m.*** | *Schistosoma mansoni* |
| **SMA** | Smooth muscle actin |
| **SPE** | Solid Phase Extraction |
| **sPLA2** | Secreted phospholipase A2 |
| **SPT** | Skin Prick Test |

| | |
|---|---|
| **Syk** | Spleen tyrosine kinase |
| **TGFβ** | Transforming growth factor beta |
| **TGM2** | Transglutaminase 2 |
| **Th** | T helper cell |
| **TLR** | Toll-like receptor |
| **TNF** | Tumor necrosis factor |
| **TP** | Thromboxane receptor |
| **T regs** | Regulatory T cells |
| **TSLP** | Thymic stromal lymphopoietin |
| **$TXB_2$** | Thromboxane B2 |
| **TXBS** | Thromboxane synthase |
| **VCAM-1** | Vascular cell adhesion molecule-1 |
| **WB** | Western blot |
| **WT** | Wildtype |
| **ZO-1** | Zonula occludens-1 |

# 2 INTRODUCTION

## 2.1 Type 2 immunity

The human immune system is constantly exposed to harmful bacteria, parasites, viruses or allergens, which can trigger the immune system to fight against them and to eliminate the initial cause of damage. The complex biological response of the body tissues is called inflammation, and is a protective response involving immune cells, blood vessels and molecular mediators. The five classical signs of inflammation are heat, pain, redness, swelling, and loss of function. Inflammation can be classified as either acute or chronic, depending on the duration of the inflammatory response. Acute inflammation is the first response of the body against pathogens and is mediated by the infiltration of innate immune cells (especially granulocytes) from the blood into the injured tissue. Prolonged inflammation leads to infiltration of lymphocytes and macrophages, which are features of chronic inflammation and can be associated with destruction or repair of the tissue.

Depending on the nature of the pathogen, the immune system initiates three distinct responses: type 1, type 2 and more recently discovered type 17 that are mainly regulated by subpopulations of $CD4^{+}$ T cells known as T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17), respectively (Mosmann et al., 1986; Harrington et al., 2005). For most infections, type 1 immunity is protective, while type 2 responses aim to regulate the resolution of cell-mediated inflammation (Spellberg & Edwards, 2001). Type 2 immunity is strongly associated with allergy, asthma and helminth infection (Robinson et al., 1992; Hansen et al., 1999; Locksley, 1994), whilst type 17 immune responses contribute to several inflammatory diseases such as psoriasis, rheumatoid arthritris and inflammatory bowel disease (Arican et al., 2005; Irmler et al., 2007; Fujino et al., 2003). Type 17 immunity mediates also protection against fungal infections, especially those caused by Candida albicans (Conti et al., 2009). Since this research project aims to better understand the mechanisms behind the regulation of helminth infections and allergy, the main focus of study were type 2 immune mechanisms, although it is important to understand the balance of type 1 and type 2 immune responses for a comprehensive view of the overall functional outcome.

Type 1 immunity ("cellular immunity") is mostly initiated by bacteria, viruses and some fungi. It is a rapid response and involves antimicrobial effectors and the activation of phagocytic cells such as macrophages and neutrophils (Romagnani, 1999; Kidd, 2003). Type 1 immune responses are directed by Th1 lymphocytes and enhanced through the secretion of cytokines such us interferon-γ (IFN-γ) and to a lesser extend tumour necrosis factor (TNF)-β and interleukin (IL)-2 (Romagnani, 1999; Nagarkatti et al., 1990; Kopf et al., 1993). In contrast, type 2 immunity often results in "humoral immunity" and is initiated by helminths, allergens and venoms (Romagnani, 1999; Kidd, 2003; Palm et al., 2013). This immune response is characterized by the production of high levels of IgE antibodies, eosinophilia and suppression of phagocytosis, involving a wide range of cells such as basophils, eosinophils, mast cells, alternatively activated macrophages (M2) and Th2 lymphocytes, which secrete type 2 cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 (Rogmagnani, 1999; Kopf et al., 1993; Coffman et al., 1989; Anthony et al., 2006).

Of interest, type 2 immune responses are associated with helminth infections and allergy, playing an important role to control parasite infections but causing pathology and chronic inflammation in allergy (Figure 1). Therefore, it is important to better understand the molecular mechanisms behind the regulation of type 2 immune responses. Indeed, such insights may be translated into applications in prevention or treatment of emerging diseases such as allergy and asthma.

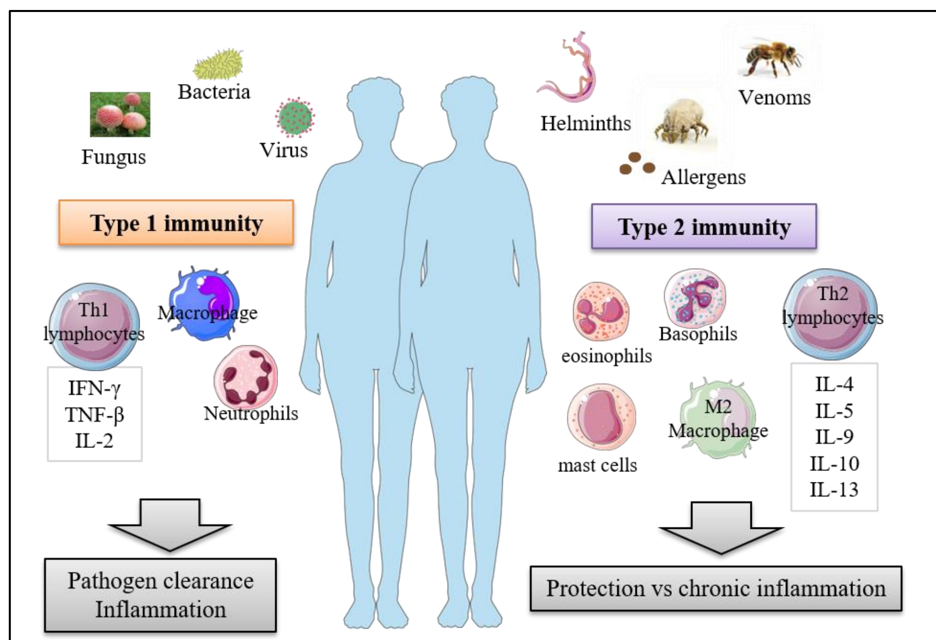


**Figure 1: Type 1 versus type 2 immunity in the fight against pathogens.** The human immune system is constantly expose to pathogens and depending on the nature of the pathogen, the immune system initiates type 1 or type 2 immune responses. Type 1 immunity is initiated by bacteria, viruses and some fungi, while type 2 immunity is initiated by helminths, allergens (e.g. from mites) and venoms. (Abbreviations: IFN- γ, interferon gamma; IL, interleukin; Th, T helper; TNF-β, tumor necrosis factor beta).

## 2.2 Allergic asthma

Asthma is a global health problem that affects 5-20% of the population worldwide, and its prevalence is increasing in many countries (Global Initiative for Asthma, GINA, 2018). Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. The diagnosis of asthma patients is based on their history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough. Since asthma is a complex disease, different phenotypes have been identified depending on demographic, clinical and/or pathophysiological features (Wenzel, 2012):

- Allergic asthma: it usually starts during childhood and is associated with allergic diseases such as eczema, allergic rhinitis and food or drug allergy.
- Non allergic asthma: it is not associated with allergy and usually affects adults with asthma.
- Late-onset asthma: it is present for the first time in adult life, affects adults (particularly women) and these patients tend to be non-allergic.
- Asthma with fixed airflow limitation: some patients with long-standing asthma develop fixed airflow limitation.
- Exercise-induced asthma: these patients show asthma manifestations after a brief period of exercise.
- Asthma with obesity: it refers to obese patients with respiratory symptoms

Over the last 10 years, asthma mortality has dramatically decreased. However, GINA classification has described that only 15% of asthmatic patients using inhaled corticosteroids had

a controlled disease, causing major consequences on morbidity, quality of life, and economic burden (Siroux et al., 2009).

### 2.2.1 Prevalence and disease pattern of allergic asthma

Allergic asthma is the most common type of asthma, which affects 90% of children with childhood asthma, compared with about 50% of asthmatic adults. Epidemiologic studies showed the increase in the prevalence of allergic asthma among adults from 1996 to 2016, rising from 8.4% up to 10.9%, with the highest incidence in women and middle-age population groups (Backman et al., 2017). Allergic asthma is driven by an immune response in the lower airways after chronic exposure to allergens such as plant pollen, house dust mites (HDMs), animal dander or mold. Exposure to allergen in sensitized patients causes irreversible damage in the airways, particularly of the bronchi and respiratory mucosa, leading to chronic inflammation and airway remodelling. Indeed, airway remodelling is described as a thickening of the bronchial wall, alteration of the extracellular matrix deposition, mucus gland hypertrophy, and increased bronchial smooth muscle cell mass, all together, leading to the irreversibility of airway dysfunction and reduced lung function (Kaminska et al., 2009).

### 2.2.2 Underlying immune mechanism of allergic asthma

Airway inflammation plays an important role in the pathogenesis of asthma, characterized by the infiltration of inflammatory cells such as neutrophils, eosinophils and lymphocytes. Indeed, the analysis of bronchial biopsies and bronchoalveolar lavage (BAL) of asthmatic patients reveals the presence of eosinophils, mast cells, and Th2 lymphocytes generating IL-3, IL-4, IL-5, IL-9, IL-13, and granulocyte macrophage colony-stimulating factor (GM-CSF) in the cellular infiltrates (Robinson et al., 1992).

Inhaled allergens trigger a complex immune response characterized by a T helper type 2 (Th2) cell dominated immune response, elevated serum allergen-specific IgE and eosinophilia. Epithelial cells play an important role during asthma, since they are the first barrier in contact with the allergen. Upon allergen recognition, epithelial cells release multiple cytokines including IL-33, IL-25, thymic stromal lymphopoietin (TSLP), cytokines involved in recruiting and activating antigen presenting cells (APC), which modulate innate and adaptive immune responses in the airways (Hammad et al., 2009). For example, IL-33 can activate dendritic cells to induce a Th2 response and promote allergic airway inflammation (Besnard et al., 2011). In addition, IL-33 promoted the polarization of alternatively activated macrophages during airway inflammation and amplified the production of type 2 cytokines, leading to excessive type 2 inflammation and airway hyperreactivity (Kurowska-Stolarska et al., 2009). In a mouse model of allergic asthma, IL-25 was shown to induce mucus production and airway infiltration of macrophages and eosinophils, as well as Th2 cell proliferation and subsequent allergic airway inflammation (Angkasekwinai et al., 2007). TLSP is also involved in the initiation of allergic airway inflammation, triggering the activation of dendritic cells to produce the chemokine CCL17 and therefore promote the proliferation of Th2 cells (Zhou et al., 2005). Therefore, airway epithelial cells play a key role in the recruitment and activation of APC, inducing an allergen specific Th2 response upon antigen presentation.

Recently it was found that innate lymphoid cells 2 (ILC2s) express receptors for epithelial-derived cytokines IL-25, IL-33 and TSLP. IL-33 induced a rapid and potent expansion of ILC2, with the production of IL-13 and type 2 cytokines, contributing to airway contraction and allergic airway inflammation; while IL-25 induced slow and less potent responses (Barlow et al., 2013). In contrast, TSLP seems to be involved in ILC2 survival (Camelo et al., 2017). ILC2 can also respond to lipid mediators, including cysteinyl-leukotrienes (CysLTs) and prostaglandin D2 ($PGD_2$), to produce type 2 and pro-inflammatory cytokines (Salimi et al., 2017).

Interestingly, upon allergen challenge, inhaled allergens can also directly activate APC, such as dendritic cells or macrophages. The APC process the allergen and it is presented to $CD4^+$ T cells, leading to Th2 polarization. Th2 cells play a critical pathogenic role in allergic inflammation as they produce type 2 cytokines such IL-4, IL-5, IL-6, IL-9, and IL-13, which initiated an inflammatory cascade (Robinson et al., 1992; Hansen et al., 1999). IL-4 induces the differentiation and proliferation of Th2 cells, and IL-13 promotes airway hyperresponsiviness (AHR), globet cell hyperplasia and mucus production, characteristic features of asthma (Venkayya et al., 2002). IL-4 and IL-13 support the recruitment and activation of eosinophils, as well as polarization of macrophages to an M2 phenotype (Luttman et al., 1999; Doyle et al., 1994). In addition, IL-4 and IL-13 stimulate IgE production by B cells, which binds to its receptor on mast cells or basophils, leading to cell activation and release of inflammatory cytokines and mediators such as histamine, CysLTs and $PGD_2$, acting as potent bronchoconstrictors of the airways (Hart 2001). Another cytokine important in asthma is IL-5, which induces eosinophil activation and recruitment, contributing to eosinophilic inflammation (Mould et al., 1997). When eosinophils are activated, they also contribute to AHR and remodelling by the release of pro-inflammatory mediators CysLTs, growth factors and metalloproteinases (Hogan et al., 2008). Another type 2 cytokine identified as an important mediator of allergic inflammation is IL-9 because it is involved in the recruitment, proliferation and differentiation of mast cells, promoting the secretion of histamine, $PGD_2$, CysLTs, proteases and pro-inflammatory cytokines which regulate both IgE synthesis and the development of eosinophilic inflammation (Bradding et al., 2006). Therefore, mast cells also contribute to the acute symptoms of asthma, including bronchoconstriction, mucus secretion and mucosal edema (Kearley et al., 2011)

After the establishment of the Th2 immune response, type 2 cytokines in synergy induce cell infiltration, such as basophils, Th2 cells, ILC2s, eosinophils, mast cells and neutrophils. In addition, IL-4 and IL-13 can further drive inflammation in the asthmatic airway by triggering tissue fibroblasts and airway epithelial cells to release GM-CSF, the neutrophil chemoattractant IL-8, TGF-α, the chemokine CCL20 and TSLP (Lordan et al., 2002; Reibman et al., 2003; Kato et al., 2007). In response to IL-13, airway epithelial cells release eotaxin, contributing to the recruitment of eosinophils, basophils, a subset of Th2 cells and mast cells (Matsukura et al., 2001). In addition, allergen exposure and IL-4 can induce the expression of CCL17 in bronchial epithelium that mediates T cell trafficking and Th2 polarization in allergic responses (Heijink et al., 2007). Thus, in allergic asthma, airway epithelial cells do not only present a barrier to inhaled allergens, but also represent an important source of chemokines, which can activate other immune cells, contributing actively to the disease. Interestingly, type 2 cytokines such as IL-4 and IL-13 are major inducers of M2 macrophage polarization, and an increased M2 macrophage population has been described in allergic subjects, suggesting that these cells play a key role in airway inflammation in asthma (Girodet et al., 2016; Melgert et al., 2011). The secretion of IL-33 by the airway epithelium is also involved in the polarization of M2 macrophages (Kurowska-Stolarska et al., 2009). Alternatively activated macrophages secrete high levels of IL-13 and chemokines including CCL-17, CCL-8, CCL-22 and CCL-24, which promote eosinophil infiltration,

leukocyte chemotaxis and activation of Th2 cells, contributing to the pathogenesis of asthma (Siddiqui et al., 2013).

If the inflammatory process is not resolved, infiltrated immune cells will drive further infiltration and activation of inflammatory cells resulting in chronic inflammation and airway remodelling (Figure 2). Thus, a better understanding of the molecular mechanism regulating cell activation and polarization is essential to understand the relationship between allergen exposure and the development of allergic diseases like asthma, helping to improve the discovery of future therapies for asthma treatment.

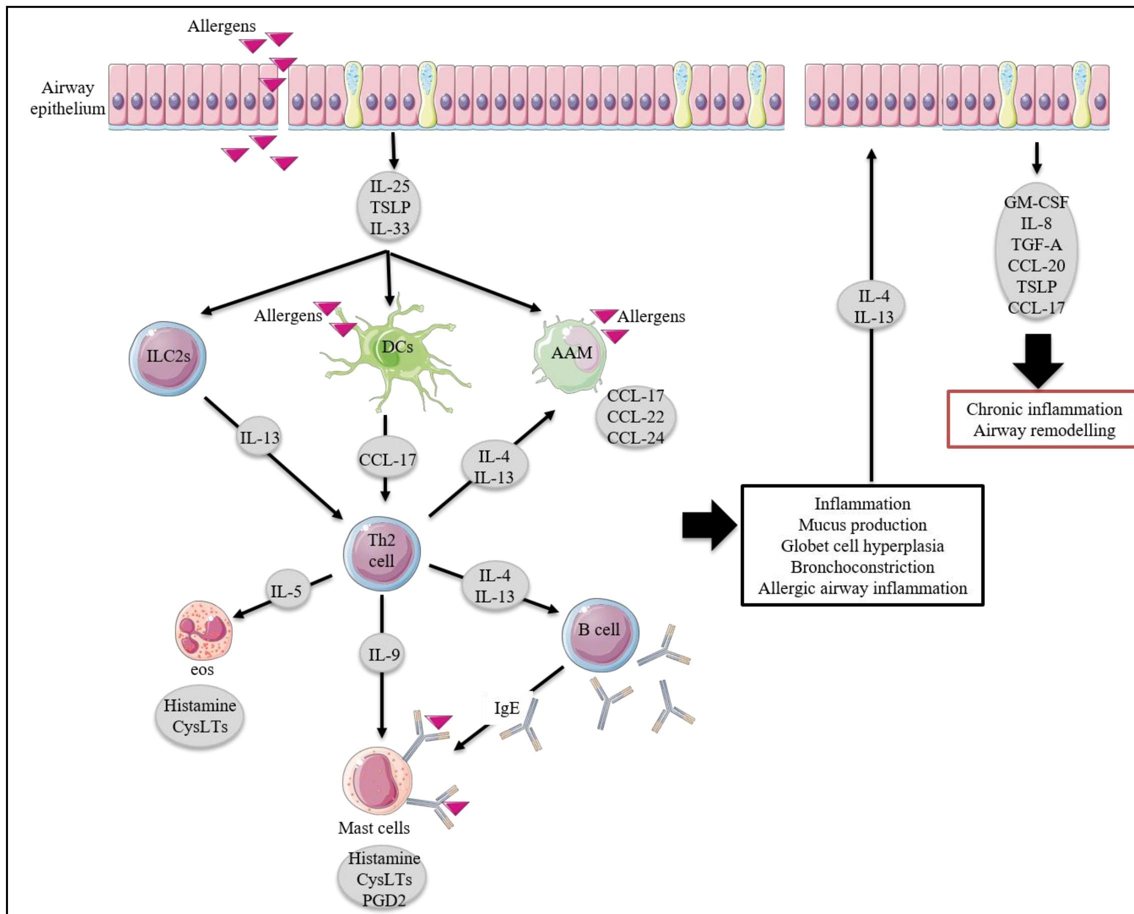


**Figure 2: Airway immune response initiated after allergen recognition.** Upon allergen challenge, lung epithelial cells play an important role in the initiation of the inflammatory response through the release of multiple cytokines, including IL-33, IL-25 and TSLP. Interestingly, inhaled allergens can also directly contact and activate APC, such as dendritic cells or macrophages. Th2 cells orchestrate the inflammatory response through the release of type 2 cytokines IL4 and IL-13 (which stimulate B cells to synthesize IgE, and induces M2 macrophage polarization); IL-5 (which is necessary for eosinophil inflammation), and IL-9 (which stimulates mast cells polarization). After the establishment of Th2 immune response, type 2 cytokines can further drive inflammation and airway remodelling by triggering airway epithelial cells to release chemokines and pro-inflammatory mediators, driving chronic inflammation and airway remodelling. (Abbreviations: AAM, alternatively activated macrophages; CCL, chemokine ligand; CysLTs, Cysteinyl leukotriene; DC, dendritic cell; eos, eosinophil; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; ILC2, innate lymphoid type 2 cell; IgE, immunoglobulin E; PGD2, Prostaglandin D2; TGF-A, transforming growth factor alpha; Th2 cell, T helper type 2 cell; TSLP, thymic stromal lymphopoietin)

## 2.3 Parasite infections

Helminths, also known as parasitic worms, are ¨large, multicellular organisms that are generally visible to the naked eye in their adult stages¨ (source: Centers for Disease control and prevention, CDC). There are three main groups of parasites classified depending on the morphology of the egg, larval, and adult stage: Flukes (trematodes) including a snail intermediate host; tapeworm (cestodes) which larval forms inhabit extraintestinal tissues; and roundworms (nematodes) where adult and larval worms can live in intestinal and extraintestinal sites (Castro, 1996) (Table 1)

| CLASSIFICATION OF HUMAN PARASITIC WORMS | | |
|---|---|---|
| **Flatworm/ Platyhelminth infection** | Fluke/ trematode (Trematode infection) | *Schistosoma mansoni/japonicum/haematobium* (Schistosomiasis)<br>*Clonorchis sinensis* (Clonorchiasis)<br>*Fasciola hepatica/gigantica* (Fasciolosis) |
| | Cestoda (Tapeworm infection) | *Echinococcus granulosus/ multilocularis* (Echinococcosis) |
| **Roundworm/ Nematode infection** | Filarioidea (Filariasis) | *Onchocerca volvulus* (Onchocerciasis)<br>*Wuchereria bancrofti/ Brugia malayi* (Lymphatic filariasis) |
| | Strongylida (hookworm) | *Ancylostoma duodenale/ braziliense* (Ancylostomiasis)<br>*Necator americanus* (Necatoriasis) |
| | Ascaridida | *Ascaris lumbricoides* (Ascariasis)<br>*Anisakis* (Anisakiasis) |
| | Rhabditida | *Strongyloides stercoralis* (Strongyloidiasis) |
| | Oxyurida | *Enterobius vermicularis* (Enterobiasis) |
| | Adenophorea | *Trichuris trichiura* (Trichuriasis-whipworm) |

**Table 1: Human parasitic worm classification.** Helminths can be classified as trematodes, cestodes and nematodes depending on the morphology of the egg, larval, and adult stage. This table includes the parasites mentioned in this thesis as well as the name of the infection caused by each parasite indicated inside the brackets.

Parasitic worms need to infect and live in living hosts. They receive nutrients and protection from the host, and during their life cycle they can migrate through different tissues and organs, causing weakness and disease of the host. In the human host, adult forms of these worms can live in the gastrointestinal tract, blood, lymphatic system or subcutaneous tissues. Helminths have co-evolved with their host for millions of years, and in doing so have developed multiple strategies to manipulate the host immune response in order to longer survive. Better understanding the molecular mechanism developed and the immunomodulatory compounds secreted by parasites is an exciting research field, which offers new possibilities for the modulation of immune responses during human diseases and their potential application in therapeutic treatments.

### 2.3.1 The allergic epidemic and hygiene hypothesis

Allergic diseases are becoming an important public health issue. In the past three decades, the frequency and severity of asthma and allergic diseases have been increasing, particularly in high-income countries where approximately 15-30% of the population is affected (Braman 2006). For

example, the global prevalence of asthma in adults has increased up to 4%, with the highest prevalence in developed countries and lowest in developing countries (Papi et al., 2018). Interestingly, the prevalence of asthma and allergic diseases appear to be low in many rural areas, and a large number of studies have shown that common environmental exposures to bacterial and fungal components from childhood into young adulthood in rural areas confer protection from the development of allergy and asthma (Nicolaou et al., 2005; von Mutius, 2007)

A popular explanation for the increase in the allergic diseases and asthma is the "hygiene hypothesis", introduced for the first time in the 1970s (Strachan, 1989). This hygiene hypothesis links some factors such as improved public health, housing and sanitation to increased allergic diseases, and states that the failure to develop appropriate immune regulation is due to the use of antibiotics or vaccines and the subsequent reduction in the exposure to microbes and their products in childhood (Garn & Renz, 2007).

### 2.3.2 Prevalence of helminth infections

The high prevalence of helminth infections in the global population was estimated for the first time by Stoll in 1947. Stoll introduced the term "this wormy world" to refer to 2.2 billion people worldwide infected with worms (Stoll, 1999). Fifty years later there remained a limited reduction in the overall prevalence of helminth infections, and the most common geohelminth parasites (also known as intestinal and soil-transmitted helminths) include *Ascaris lumbricoides* (roundworm)*, Trichuris trichuria* (whipworm), *Ancylostoma duodenale* and *Necator americanus* (hookworms) (Chan, 1997). These infections are most prevalent in tropical and sub-tropical regions of the developing world where adequate water and sanitation are lacking, combined with lack of access to health care and low levels of education. A more recent study showed that the highest rates of *Ascaris* infection occurs in China and Southeast Asia, in the coastal regions of West Africa, and in Central Africa. Hookworm infections, however, are common throughout much of sub-Saharan Africa, in addition to South China and Southeast Asia (de Silva et al., 2003). The previous study also suggested that despite marked declines in both the Americas and Asia for *Ascaris lumbricoides, Trichuris trichiura* and the hookworms, little recent change had occurred in sub-Saharan Africa (de Silva et al., 2003). Today, more than 1.5 billion people or approximately 24% of the world´s population is infected with various parasitic worms (source: World Health Organization, WHO, 2019). Nevertheless, in industrialized countries there has been a reduction in the prevalence of helminth infection of humans, but as previously mentioned, there has been an increase in the prevalence of allergic diseases. Hence, a picture emerges of helminth infection playing a potential role in suppressing the development of allergic diseases.

### 2.3.3 Association of helminths with allergic diseases

One approach to determine the relationship between helminth infections and allergies is to study people who are naturally infected with helminths and analyse their allergic disease status and immune responses to parasite-specific antigens or allergens. One example is the study of children in Venezuela who were chronically infected with *Ascaris lumbricoides* and *Trichuris trichuria*. After drug treatment to effectively eliminate intestinal helminthic infection, children showed a marked increase in immediate-hypersensitivity skin-test reactivity (from 17% to 68%) and in serum levels of specific IgE against environmental allergens within 2 years of treatment (Lynch et al., 1993). Another study of chronically infected Gabonese children also showed increased skin

sensitivity to house dust mites (HDM) during a 30-month follow up study after anthelminthic treatment (van den Biggelaar et al., 2004). Similarly, in Israel, immigrants from Ethiopia chronically infested with helminths reported a notable increase in skin reactivity to newly acquired allergens and loss of immune suppression after following anthelminthic treatment (Borkow et al., 2000).

A more recent study followed the allergy prevalence of Ethiopian immigrants with high burden of helminths on arrival to Israel and one year later after following a deworming treatment. At time of arrival, immigrants had a low risk of allergy. However, after one year of treatment, all immigrants developed an increase in allergy and skin prick test (SPT) reactivity to common aeroallergens, which was associated with lower helminth burdens (Stein et al., 2016). However, one could argue that the increase in allergy observed in the immigrants might come for the combination of two factors: the loss of immunomodulatory helminth infection and changes in the environmental conditions. In line with the observations described in the Israeli/ Ethiopian study, in Indonesia, 1.000 subjects chronically infected mainly with hookworms underwent a long term deworming treatment, which induced the release of proinflammatory cytokines in response to parasites and unrelated antigens, but attenuated the immunosuppression mechanisms induced by parasite infections (Wammes et al., 2016). These studies showed that anthelminthic treatment of chronically infected individuals resulted in increased allergic responses, further supporting the role of helminth infections in suppressing the development of allergy.

#### 2.3.3.1 Epidemiology of parasite mediated protection against allergies

Several epidemiological studies showed the inverse correlation between the development of allergic disorders and infections with parasitic helminths, suggesting a possible role for worms in suppressing allergies. Allergy suppressing effects of helminth infections are described for individuals infected with the human parasite *Schistosoma,* the human and pig parasite *Ascaris* and *Trichuris,* as well as hookworms. Studies of adults from Gambia described an inverse correlation between intestinal helminth infection and atopy (Nyan et al., 2001). In Ethiopia, infection with intestinal parasites such as *Ascaris lumbricoides (A. lumbricoides)*, *Trichuris suis (T. suis)* or hookworms was linked to a reduction in the risk of asthma (Scrivener et al., 2001). Another study of schoolchildren from Ghana described an inverse association with *Schistosoma mansoni (S. mansoni)* infection and mite atopy, but not wheeze and asthma (Obeng et al., 2014)

Another study in South America demonstrated that subjects living in an *S. mansoni*-endemic area had a milder course of asthma when compared with asthmatic subjects without schistosomiasis (Medeiros et al., 2003). A broader study compared SPT responses to HDM and other allergens in 520 schoolchildren in an area of Gabon where *Schistosoma haematobium (S. haematobium)*, filarial worms and geohelminths were endemic (van den Biggelaar et al., 2004). Infected children had a lower prevalence of positive skin reaction to allergens, but higher concentrations of schistosome-antigen-specific anti-inflammatory cytokine IL-10, compared to responses in uninfected children. The reduction in SPT responses was independent of sex, age and socio-economic factors (van den Biggelaar et al., 2000). In an *S. mansoni*-endemic area of Brazil, infected people also showed a significant negative association between the SPT response to aeroallergens and helminth infection (Araujo et al., 2000).

A reduced risk of atopy was also shown in Ecuadorian school children chronically infected with *A. lumbricoides.* A reduced SPT reactivity was associated with high levels of total IgE and anti-*A. lumbricoides* IgG4 antibodies measured in the serum (Cooper et al., 2003).

A larger study investigated the effect of chronic infection with *A. lumbricoides* and *Trichuris Trichiura (T. Trichiura)* in 1.000 Brazilian children during 5 years. Intestinal helminth infections were associated with the induction of a type 2 immune response, spontaneous production of IL-10, suppression of cytokine response to parasite antigen and elevated levels of total IgE and anti-*Ascaris* IgG4 and IgE, which may contribute to the suppression of SPT reactivity and allergic reactions (Figuedeiro et al., 2010) (Figure 3).

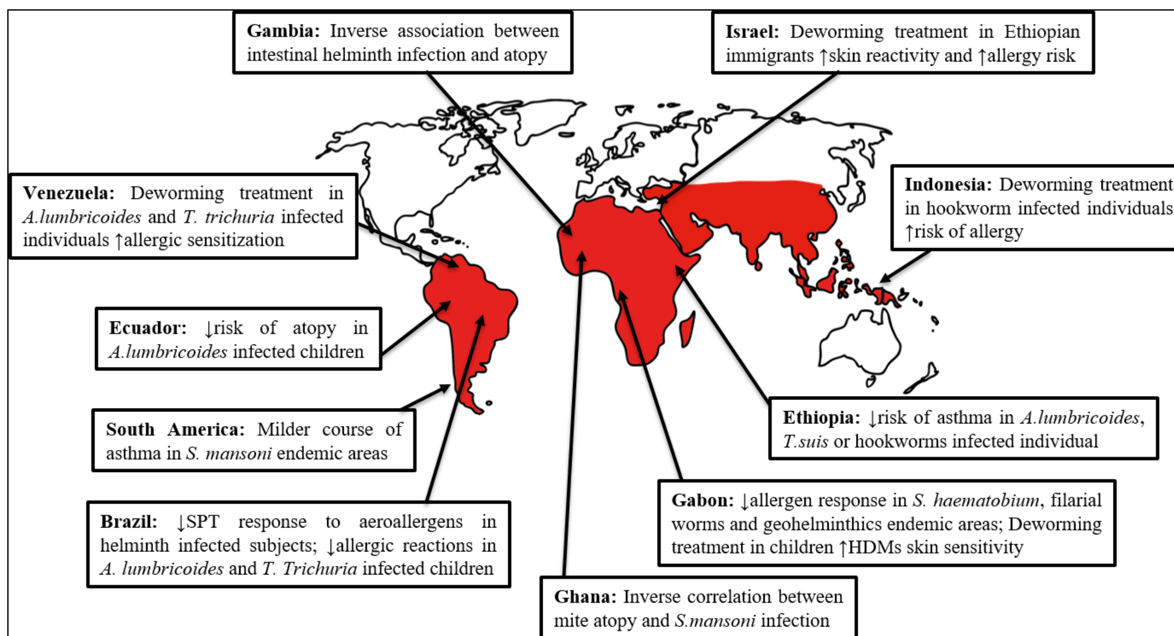


**Figure 3: Epidemiological studies of parasites mediated protection against allergies.** The inverse correlation in the development of allergic diseases and parasitic helminths has been confirmed in several studies performed in countries and endemic areas where helminth infections are highly prevalent and first occur in early life.

### 2.3.3.2 Epidemiology of parasite-induced allergy

The role of helminths in ameliorating allergies is not universally accepted, as several studies do not show a protective role. In a study in Germany, children with *Ascaris* infection had higher levels of total and allergen specific-IgE, with a higher prevalence of allergic rhinitis and asthma (Dolds et al., 1998). Similarly, in Chinese children, infection with *A. lumbricoides* was associated with increased sensitization to common aeroallergens, airway inflammation and asthma risk (Palmer et al., 2002). A more recent study performed in South Africa reported an increased risk of atopic asthma, atopic rhinitis and hypersensitivity to common aeroallergens (mostly HDM) in children with high levels of *Ascaris*-specific IgE antibody (Obihara et al., 2006)

Taken together, the relation between parasite infection and the development of allergic diseases is likely to be species specific. For example, as explained in section 2.3.3.1, infection with helminths like *Schistosoma, Trichuris, Ascaris* and hookworms were associated with a protective effect against allergy. However, some epidemiological studies showed opposite effects for the parasite *Ascaris*, which was associated with increased risk of developing asthma and wheeze. Part

of the inconsistent findings in the epidemiological studies might be explained by variation factors such as age of the population studied, genetic and environmental differences of the human population cohorts, time of first infection and duration of infection, variations between species of helminths that are endemic or the intensity of infection, or differences in study parameters such as clinical symptoms or methods used to measure allergen sensitization (SPT, allergen-specific IgE) (Cooper, 2009).

## 2.4 Parasite mediated protection against allergy

### 2.4.1 Parasite-driven induction of regulatory networks

#### 2.4.1.1 Human studies

Several studies indicate the induction of regulatory networks in individuals infected with parasitic worms. For example, during long-term helminth infections, filarial or schistosomiasis patients have elevated levels of IL-10 compared with non-infected controls, which have been shown to be inversely correlated with allergy (Yazdanbakhsh et al., 2002). In the forest zone of the Republic of Guinea, chronic infection of individuals with the filarial nematode *Onchocerca volvulus* (OV) results in a T cell proliferative hyporesponsiveness to Ov antigen by peripheral blood mononuclear cells (PBMC), with higher levels of TGFβ and IL-10, which are characteristic of the presence of regulatory T cells (Doetze et al., 2000). Patients infected with the filarial parasite *Brugia malayi* showed regulatory-cell networks, characterized by significant increase of $FOXP3^{+}CD4^{+}$T cells and regulatory factors such as TGFβ, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed- death-cell protein 1 (PD-1) and indoleamine 2,3-dioxygenase, but also characterized by a diminished production of IFN-γ, TNFα, IL-4, IL-5 and IL-10, which downregulate both Th1 and Th2 pathways during infection (Babu et al., 2006). Another study compared *Dermatophagoides pteronyssinus*-specific immune responses in patients with asthma and *Schistosoma mansoni*-infected patients with asthma before and after anthelminthic treatment by measuring levels of Th2 cytokines in PBMCs *in vitro* (Araujo et al., 2004). Interestingly, PBMCs from *S. mansoni*-infected patients with asthma produced lower levels of IL-4 and IL-5, but higher levels of IL-10 after allergen exposure than did PBMCs from helminth-free patients with asthma. After anthelminthic treatment, *S.-mansoni* infected patients produced lower levels of allergen-specific IL-10 and appearance of more severe symptoms of asthma, suggesting that IL-10 is the key cytokine that inhibits the inflammatory Th2 immune responses (Araujo et al., 2004).

This propensity of helminths to induce a regulatory environment might significantly affect the allergy profile of infected individuals. In support of this, a study performed in schoolchildren with schistosomiasis showed an inverse correlation between allergic responses to HDM and helminth infections, and suggested that high concentrations of schistosome-antigen-specific IL-10 induced in chronic schistosomiasis is central to suppress atopy in African children (van den Biggelaar et al., 2004). It has been suggested that the production of IL-10 during infection with *S. haematobium* is associated with a high population of regulatory B cells found in peripheral blood of infected children compared to uninfected children. (van der Vlugt et al., 2012). Children infected with *S. haematobium* had a higher frequency of CD4+CD25hiFOXP3+ cells, and that in turn induced regulatory and Th2-polarized cytokines (IL-5, IL-10 and IL-13) but also pro-inflammatory and Th1 cytokines (IFN-γ, IL-17 and TNF) (Schmiedel et al., 2015). Importantly, anti-helminthic treatment decreased the number of IL-10-producing B cells (van der Vlugt et al.,

2012) and decreased the frequency of T regs and their suppressive effect on cell proliferation, but their influence on cytokine production was unaltered (Schmiedel et al., 2015)

Since asthma is a chronic inflammation of the airways associated with airway hyperresponsiviness, an exaggerated Th2 immune response and the release of inflammatory mediators leading to vasodilatation, mucus production and smooth muscle cell contraction (explained in detail in section 2.2), the presence of a regulatory network induced by helminth infection could help to prevent the cascade of events leading to asthma. Indeed, previous studies have shown the protective effect exerted by TGFβ, IL-10 and both $CD4^{+}CD25^{+}$ regulatory T (Treg) cells and inducible regulatory T cells (both expressing the transcription factor FOXP3) in the development of allergy and asthma (Stock et al., 2004 and 2006).

#### 2.4.1.2 Mouse studies

Animal models of parasitic helminth infections in combination with allergic sensitization have made important contributions to the understanding of the helminth-driven regulation of Th2 immune responses. One of the first experimental demonstrations of a role for helminths in the suppression of lung inflammation involved the infection of mice with the gastro-intestinal nematode *Strongyloides stercolaris,* which prevented pulmonary allergic responses to ovalbumin (OVA), and suppressed allergen-specific IgE responses and eotaxin levels in response to allergen exposure in the lungs (Wang et al., 2001). However, no mechanism was described in this study. Similarly, infection with *Nippostrongylus brasiliensis* was shown to reduce OVA-specific IgG1 and IgE levels in the bronchoalveolar alveolar lavage fluid (BALF) and to decrease allergen-induced airway eosinophilia and eotaxin levels, suppressing the development of OVA-induced airway inflammation (Wohlleben et al., 2004). This is the first study to demonstrate a role for IL-10 in the suppression of experimental allergic lung inflammation by a parasitic nematode.

Several epidemiological studies in endemic populations infected with ***Schistosoma mansoni*** have described the protective effect of this worm against allergic diseases (Medeiros et al., 2003; Araujo et al., 2000 and 2004), opening new questions about the mechanisms modulated by this parasite. A lot of studies have investigated its role in allergen-induced airway inflammation in mouse models, although mice are not the natural host of *S. mansoni*. The complexity of helminth modulation depending on the developmental stage of the parasite was addressed using a mouse model of schistosome worm versus worm and egg infection (Mangan et al., 2006). Mice infected with only schistosome male worms (preventing egg production) were protected from OVA-induced airway hyperresponsiviness (AHR), inducing a modified type 2 pulmonary response, with elevated allergen-specific IL-4 and IL-13 but reduced IL-5 in the lung, *via* a mechanism dependent on IL-10 but independent on $CD4^{+}$ or $CD25^{+}$ T-cells. In contrast, infection of mice with *S. mansoni* egg-laying male and female worms exacerbated AHR (Mangan et al., 2006). *S. mansoni* infection can prevent OVA-induced airway inflammation when infection progressed into chronicity but not during acute infection, suggesting an important role of the intensity and duration of the infection in the allergy protective effects (Smits et al., 2007). The protective effect of *S. mansoni* infection during OVA-induced allergic airway inflammation was dependent on the suppression effect of IL-10-producing B cells and partially dependent on the increase of $Foxp3^{+}$regulatory T cells (van der Vlugt et al., 2012, Layland et al., 2013). More recently, infection with *S. japonicum* attenuated airway inflammation in a mouse model of HDM-induced asthma, where induction of T regs was suggested to participate in the helminth-triggered immunoregulation (Qiu et al., 2017)

The hypothesis that helminth infections down-regulate allergic reactions was supported by several studies using infection of mice with ***Heligmosomoides polygyrus***. In contrast to *S. mansoni*, *H. polygyrus* is a murine intestinal nematode, which does not enter the lung in its life cycle, and can establish chronic infection upon primary infection and induces a Th2 cell-dominated immune response. Yet, infection of mice with *H. polygyrus* reduced OVA-induced allergen airway inflammation in part by induction of a $CD4^{+}CD25^{+}Foxp3^{+}$ T cells-mediated mechanism, although protection was not exclusively $CD4^{+}$T-cell-dependent or mediated by parasite-induced IL-10 (Wilson et al., 2005). Another study confirmed and extended the findings that *H. polygyrus* infection protects against atopic responses in a murine model of asthma, showing suppression of allergen-induced airway eosinophilia, bronchial hyperresponsiviness, and *in vitro* allergen-induced Th2 responses in an IL-10-dependent manner and by induction of T regs (Kitagaki et al., 2006). In contrast to the findings of Wilson et al., protective effects of *H. polygyrus* were not seen in the absence of IL-10 in the later study (Kitagaki et al., 2006). This discrepancy could be due to an incomplete ablation of IL-10 responses using an antibody (Wilson et al., 2005), in comparison with the complete abrogation of the cytokine in the knockout mouse (Kitagaki et al., 2006). However, a more recent study suggested that the protection effect of helminth infection against allergy occurred in the lung but not in the skin. Mice infected with *H. polygyrus* were protected in a murine model of OVA-induced airway inflammation but not in a murine model of atopic dermatitis, an effect which was associated with the failure to induce T reg cells in the lymph nodes and an increased mast cell recruitment to skin lesions (Hartmann et al., 2009).

### 2.4.2 Underlying immune mechanism of helminth-mediated protection against allergy

Helminth infections are associated with type 2 immune responses, and several studies have shown the protective effect of helminth infections in mouse models of allergic airway inflammation (as outlined in section 2.4.1.2). However, the initiation of type 2 immune responses on the one hand and the mechanisms behind the protective, immunoregulatory effects of helminths on the other hand are still not completely understood. The mucosal epithelial cell barrier is the first to be exposed to parasites and tissue-migrating parasites cause tissue damage and release of alarmins by epithelial cells. These cytokines can activate innate immune cells such as macrophages, neutrophils, eosinophils or dendritic cells, which provide the critical link between innate and adaptive immunity as they are responsible for Th2 cell polarization (Hammad et al. 2010). Parasites can secrete immunomodulatory compounds and actively manipulate the innate and adaptive immune system to either suppress and/or induce immunomodulatory responses in the host.

#### 2.4.2.1 Modulation of the innate immune system

##### 2.4.2.1.a Macrophages

Macrophages are plastic cells involved in inflammatory and anti-inflammatory functions depending on their state of activation. On response to local microenvironment, macrophages can be polarized into classically activated (M1) or alternatively activated (M2) phenotypes. M1 macrophages are induced by interferon-γ (IFN-γ) and lipopolysaccharide (LPS), and they are generally associated with immunity to bacteria and intracellular pathogens, driving inflammation and clearance of pathogens. M2 macrophages are induced by type 2 cytokines IL-4 and IL-13,

and they are predominant in settings of immunity to helminths, asthma and allergy, involved in wound healing, clearance of apoptotic cell bodies and anti-inflammatory responses (Murray et al., 2017). Recently, it was suggested that macrophage polarization is not fixed, and macrophages can integrate multiple signals and display different activation states within many possibilities, M1 and M2 being just two extreme phenotypes of macrophage polarization (Murray 2017).

Increased M2 polarization and activation are observed in allergic asthma, where macrophages are key players associated with lung injury, fibrosis and globet cell hyperplasia and contribute to airway remodelling by stimulation of smooth muscle cell contraction and extracellular cell matrix degradation (Girodet et al., 2016; Melgert et al., 2011; Wynn et al., 2013). Interestingly, M2 polarization is also associated to helminth infections, but in this case, macrophages are involved in anti-helminth immunity, having effects on worm trapping, immunoregulation and wound healing or repair of tissue injury caused by migrating larvae or eggs (Gause et al., 2013; Esser-von Bieren et al., 2013 and 2015) (Figure 4). However, the different molecular mechanisms involved in the regulation of macrophages during allergic asthma or helminth infections are currently not well understood.

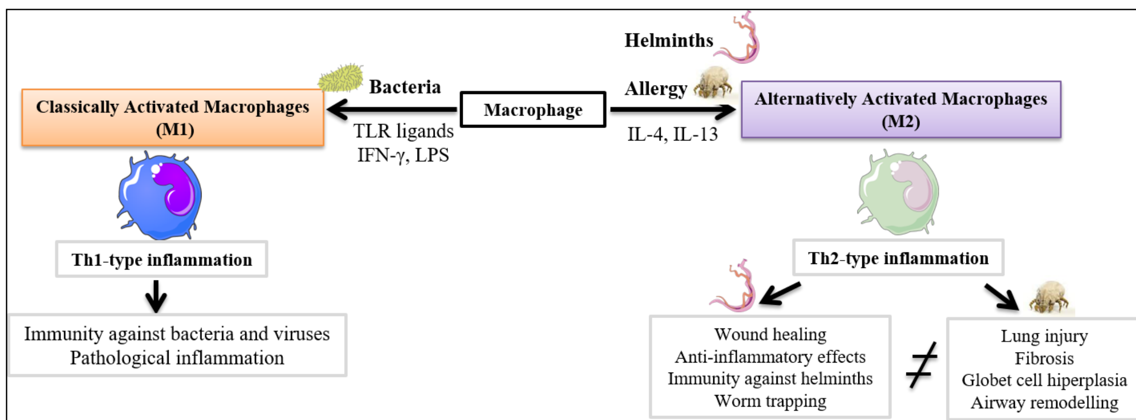


**Figure 4: M1 versus M2 macrophage polarization.** Macrophages can be polarized into classically activated (M1) or alternatively activated (M2) macrophages depending on the local microenvironment. M1 macrophages are associated with immunity to bacteria and intracellular pathogens. In contrast, M2 macrophages are involved in immunity to helminths, asthma and allergy (Abbreviations: IFN-γ, interferon-gamma; IL, interleukin; LPS, lipopolysaccharide; TLR, Toll like Receptor)

Several studies have described the presence of alternative activated macrophages (AAM) during helminth infections with *Schistosoma mansoni*, *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, *Ascaris suis* and filarial parasites (Kreider et al., 2007). As an example, in human filarial infection with ***Brugia malayi***, monocytes from asymptomatic patients showed an alternative activation phenotype characterized by the expression of M2 markers such as resistin, mannose receptor C type 1 (MRC1), macrophage galactose type C leptin (MGL) and chemokine ligand 18 (CCL18). Stimulation of human monocytes with filarial antigen (**BmA**) or live microfilaria *in vitro* recapitulated the expression of typical M2 markers and induced the release of TGF-β and IL-10 (Babu et al., 2009). Furthermore, the regulatory monocyte phenotype induced by this microfilaria can directly inhibit the proliferation of $CD4^{+}$T cells and the production of IFN-γ, IL-13 and IL-10, thus suppressing the adaptive immune system. This effect was dependent on IL-10 producing macrophages (O´Regan et al., 2014). A homolog of macrophage migration inhibitory factor (**MIF**) was identified in *Brugia malayi* as the main responsible for the induction of M2 macrophages in the presence of IL-4 induced during helminth infection (Prieto-Lafuente

et al., 2009). Furthermore, a filarial cystatin identified from *Brugia malayi* (**BmCPI-2**) distinctively contributed to the modulation of human monocytes and macrophages by inducing the synthesis of IL-10 in macrophages, *via* a mechanism dependent on the activation of the mitogen-activated protein kinases (MAPK) p38 and extracellular signal-regulated kinase (ERK) signalling pathways. In contrast, induction of IL-10 in monocytes was independent of the ERK pathway. In addition, BmCPI-2-stimulated macrophages inhibited the proliferation of $CD4^{+}$T cells *in vitro* (Venugopal et al., 2017).

Helminth parasites also secrete immunomodulatory products, which can act directly on macrophages, inducing specific immunomodulatory responses. As an example, the human filarial parasite *Onchocerca volvulus* secretes an immunomodulatory cysteine protease inhibitor (**onchocystatin**), which targets monocytes and upregulates the production of IL-10 (Schönemeyer et al., 2001). An immunomodulatory protease inhibitor (**cystatin, Av17**) is also secreted by the rodent filarial nematode *Acanthocheilonema viteae (A. viteae)*, which suppressed allergic airway inflammation in a murine model of OVA-induced asthma when administered during or after sensitization and before challenge with the allergen. This effect was mediated by macrophages and dependent on IL-10 production, but independent of regulatory T cells (Schnoeller et al., 2008). Macrophages are the primary target of AvCystatin, which is taken up by macrophages and initiates the activation of the MAPK ERK 1/2 and p38, inducing the synthesis of IL-10 (Klotz et al., 2011). A recent study also showed the therapeutic potential of the immunomodulatory AvCystatin in a mouse model of OVA-induced allergic airway inflammation, which was based on the induction of regulatory PD-L1+ and PD-L2+ macrophage populations. The adoptive transfer of AvCystatin-modulated macrophages before allergen challenge was sufficient to suppress the lung infiltration, allergen-specific and total IgE, and type 2 cytokines IL-4, IL-5 and IL-13, both locally and systemically. This effect was partially dependent on the expansion of IL-10 producing $CD4^{+}$ T cells (Ziegler et al., 2015).

The therapeutic potential of the filarial cystatin **AvCystatin** was extended for a clinically relevant allergen and tested in a mouse model of grass pollen-induced allergy. Intraperitoneal application of AvCystatin during sensitisation was enough to prevent airway inflammation and eosinophil recruitment, to reduce the levels of allergen-specific IgE and cytokines measured in the BALF (IL-5 and IL-13) after allergen challenge, suggesting that macrophage IL-10 expression may be a key player in the immunomodulatory capacity of AvCystatin. Furthermore, treatment of human grass pollen allergic-PBMCs with AvCystatin shifted the Th2 response towards a Th1 response, decreasing the levels of IL-13, inducing IL-10 production and IFN-γ in allergic $CD4^{+}$T cells (Danilowicz-Luebert et al., 2013).

Another example of excretory-secretory products, which targets macrophages is the immunomodulatory phosphorylcholine (PC)-containing glycoprotein, excretory-secretory **(ES)-62**, secreted by the filarial nematode *A. viteae.* IFN-γ and LPS triggers the release of type 1 cytokines such as IL-6, IL-12 and TNF-α in macrophages, but treatment with ES-62 abrogated the induction of these proinflammatory cytokines and promoted an anti-inflammatory type 2 phenotype (Goodridge et al., 2001). A different mechanism of action was shown for the immunomodulatory ES protein **Fh12** (and its recombinant form, **Fh15**) from the liver of *Fasciola hepatica (F. hepatica)*. The fatty acid binding protein (FABP) Fh12 induced alternative activation of macrophages and acts as a TLR4 antagonist, suppressing the expression of inflammatory mediators and the TLR4 signalling cascade (p38, ERK and JNK) after LPS stimulation. Thus, Fh12 exerts an anti-inflammatory effect (Ramos-Benitez et al., 2017). In addition, *F. hepatica* can secret a cathelicidin-like peptide (**FhHDM-1**) that acts directly on macrophages, inhibiting

the activation of the NLRP3 inflammasome by lysosomal cathepsine B protease, thus blocking the release of IL-1β (Alvarado et al., 2017)

Thus, macrophages represent an attractive target for helminths and their immunomodulatory products. Indeed, helminths have developed different strategies to modulate macrophage polarization to induce an immune-regulatory network in the host, exerting protective effects during allergic airway inflammation. The characterization of the parasitic compounds and a deep understanding of the mechanisms involved in macrophage modulation will bring new insights for novel therapeutic applications. Interestingly, very few studies have explored therapies based on the transfer of regulatory macrophages modulated by parasitic molecules, which might represent a safer approach in the treatment of allergies and asthma.

#### 2.4.2.1.b Granulocytes

Granulocyte infiltration is a hallmark of allergic asthma. However, neutrophils and eosinophils are also important effector cells during helminth infection. Indeed, eosinophilia increases dramatically during worm infection, although its prevalence in adults is variable (3.1%-50%) and largely depends on the population studied, the areas where infection occurs and the specific helminth infection (Pardo et al., 2006). Eosinophils and neutrophils are able to target the site of parasite infection and kill pathogens. Therefore, parasites and their E/S products have evolved different mechanisms to modulate granulocyte activation and recruitment and escape from the host immune response.

The release of epithelial alarmins after helminth-induced tissue damage is considered to initiate the activation and recruitment of **eosinophils** and it was shown that eosinophils can rapidly migrate towards *Nippostrongylus brasiliensis* and *Caenorhabditis elegans* larvae *in vitro* (Patnode et al., 2014). In addition, eosinophils can secrete the chemoattractant leukotriene $LTB_4$, which activates a paracrine signalling between eosinophils to amplify eosinophil accumulation (Patnode et al., 2014). Eosinophils can play an important role during helminth infections since they are associated with resistance to helminth parasites, killing of nematodes and induction of protective immunity in some, but not all helminth infections (Abraham et al., 2004; Cadman et al., 2014; Ramalingam et al., 2005; Turner et al., 2018).

An alternative approach commonly used to study the relevance of eosinophils during helminth infection is to block the C-C chemokine receptor type 3 (CCR3), the receptor for the potent eosinophil chemoattractant eotaxin. This approach demonstrated that the development of adaptive protective immunity to the filarial worm *Onchocerca volvulus* was dependent on eosinophils and IgE levels, where killing of the larvae was associated to an increase in the number of eosinophils and high levels of eosinophil peroxidase (EPO) (Abraham et al., 2004). Similarly, treatment of *Brugia pahangi*- infected mice with monoclonal antibodies to CCR3 suggested that eosinophils play an important role in the expulsion of the worm and they are essential in host protection (Ramalingam et al., 2005). Upregulation of CCR3 levels seems to be important for the killing of the larvae, as shown in a mouse model of *Brugia malayi*. IL-4-alternatively activated macrophages present in the granuloma formed around the trapped larvae positively regulated eosinophil infiltration during larval infection by the upregulation of CCR3 levels, which contribute to larval killing (Turner et al., 2018). Thus, a cross talk between alternatively activated macrophages and eosinophils present in the granuloma formed around the trapped larvae might be involved in the development of immunity during larval infection.

In contrast, helminth infections can also modulate the expression of CCR3 receptor as a protective mechanism against larval killing. One example is observed for the gastrointestinal parasite *Heligmosomoides polygyrus,* extensively studied for its protective effect against allergen-induced airway inflammation (Wilson et al., 2005; Kitagaki et al., 2006; Hartmann et al., 2009). The suppression of allergen-induced airway eosinophilia during *H. polygyrus* infection was associated with downregulation of CCR3 expression on lung eosinophils, which correlated to lower levels of eotaxin in BALF and impaired eosinophil chemotaxis towards eotaxin. In the same study, lower levels of IFN-γ, IL-4, IL-5, IL-10, IL-6 and TNF-α were measured upon *H. polygyrus* infection and associated with down regulation of allergen-induced immune response (Rzepecka et al., 2007).

Interestingly, other parasites have evolved different strategies to modulate the activation and recruitment of eosinophils by secretion of immunomodulatory products. One example is the metalloproteinases secreted by the hookworm *Necator americanus*, which had a specific proteolytic activity on eotaxin-1 (CCL-1), but not IL-8, eotaxin-2 or LTB4, suppressing the eotaxin-mediated recruitment and activation of eosinophils (Culley et al., 2000). In *Schistosoma mansoni* infection, schistosomal lyso phosphatidyl coline (**lyso-PC**) induced eosinophil activation and recruitment in a TLR2 dependent manner. Lower eosinophilic infiltration in the liver granuloma, lower levels of eotaxin and IL-5 and IL-13 cytokines were measured in *S. mansoni* infected-mice lacking the TLR-2 in comparison to control mice (Magalhães et al., 2010). Thus, helminth parasites have evolved unique strategies to avoid host immune responses aiming to kill and expel the worms by differentially modulating the activation and recruitment of eosinophils.

**Neutrophils** are also recruited to sites of helminth infection and contribute to helminth damage and worm expulsion. One example is the mice infection with *Nippostrongylus brasiliensis*, where neutrophils can accelerate the expulsion of worms by promoting the development of M2 macrophages (Chen et al., 2014). *N. brasiliensis* infection triggers the development of alternatively activated neutrophils (‘N2’) that secrete different factors including IL-13 and prime the polarization of macrophages into a M2 phenotype. Primed macrophages upregulated adhesion molecules and adhered to the larvae to accelerate the mortality of parasites (Chen et al., 2014). Depletion of neutrophils in infected mice abrogated the protective effect, demonstrating the important role of neutrophils in the clearance of helminth infections.

Parasites have also evolved different strategies to modulate neutrophil migration. The filarial parasite responsible for river blindness, *Onchocerca volvuls*, carries the endosymbiotic bacterium *Wolbachia,* which directly activates neutrophil migration. A lipopeptide present in *Wolbachia* (**WoLP**) is a ligand of TLR2 and promoted neutrophil chemotaxis by inducing the expression of integrins on the cell surface and also triggered IL-8 secretion (Tamarozzi et al., 2014). In contrast, a protein secreted by *S. mansoni* eggs, *S.m.* chemokine binding protein (**smCKBP**) has the ability to bind chemokines such as IL-8, CCL2, CCL3, CCL5 and CX3CL1 and therefore blocked their interaction with the receptors, inhibiting neutrophil chemotaxis (Smith et al., 2005).

Another strategy developed by parasites to escape the host immune responses is to inhibit neutrophil activation. The canine hookworm *Ancylostoma caninum* secreted a glycoprotein, neutrophil inhibitory factor (**NIF**), which is a ligand of the integrin CD11b/CD18 and therefore binds this integrin together with fibronectin, blocking the adhesion of neutrophils to the vascular epithelium and their migration (Moyle et al., 1994).

Similar to eosinophils, neutrophils also represent an important cell target for helminth parasites and their E/S immunomodulatory compounds. The modulation of neutrophil activation and

recruitment through different immunoevasion mechanisms evolved by helminth parasites prevents larvae killing and expulsion during infection. Since granulocyte infiltration is a hallmark of allergic asthma, granulocyte modulation by parasites might exert a protective effect during allergy and asthma. However, the effect of granulocyte modulation by helminth products during allergic airway inflammation remains poorly understood, and represents an exciting research field to explore new therapeutic approaches in asthma and allergy treatment.

#### 2.4.2.1.c Innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently discovered cell type of the innate immune system and they play a key role in the initiation of adaptive T cell responses. Upon tissue damage, epithelial cells release alarmins including IL-33 and IL-25 that activate ILC2s, which have been implicated in the induction of Th2 immune responses, causing allergic lung inflammation (Halim et al., 2014).

Of interest, parasites have evolved different strategies to block or neutralize the effect of the alarmins secreted by the epithelium after tissue damage in order to modulate the initiation of type 2 immune responses. For example, excretory secretory products (**HES**) released by the parasite *Heligmosomoides polygyrus* have been studied for their ability to modulate both adaptive and innate immunity. HES administration during OVA sensitization suppressed OVA-induced asthma, reducing pro-inflammatory cytokine production, eosinophil recruitment, IgE production and further type 2 innate response markers such as arginase-1 and RELM-α, and effector T-cell reactivity (McSorley et al., 2012). In an allergy model induced by exposure to the fungus *Alternaria alternata*, co-administration of HES with *Alternaria*/OVA prevented the early innate response to the allergen by blocking the early release of IL-33 induced by *Alternaria*, this inhibiting eosinophilia and the production of type 2 cytokines by ILC2s (McSorley et al., 2014). Interestingly, exogenous administration of IL-33 at sensitization with Alternaria/OVA/HES abrogated HES suppression of allergen-specific response at challenge, suggesting that HES might act on the upstream cytokines which activate ILC2s rather than directly on ILC2s (McSorley et al., 2014). This was the first study describing the suppression of ILC pathways by soluble parasite products. The mechanism of suppression by HES was described in a mouse model of OVA-induced asthma, where co-administration of HES was associated with the suppression of early innate responses, inhibiting IL-5 production and reducing the activation of IL-5 and IL-13-producing ILC2s at the site of administration, thus abrogating the lung pathology (McSorley et al., 2015).

Recently, the IL-33-suppressive protein present in HES was identified as H. polygyrus Alarmin Release Inhibitor (**HpARI**), which can bind directly to the active form of IL-33 and to the nuclear DNA to prevent the release of IL-33 by necrotic cells, suppressing the activation of ILC2 and eosinophilic responses induced by *Alternaria* allergen administration (Osbourn et al., 2017). In addition, the effect of HpARI was tested during infection with *Nippostrongylus brasiliens,* a parasite which migrates through the lung and induce the release of epithelial IL-33, producing ILC2-derived IL-13 and driving a type 2 response and eosinophilia recruitment, two mechanism required for expulsion of the worm (Hung et al., 2013). HpARI administration during *N. brasiliens* infection suppressed the early innate anti-parasite immunity, reducing eosinophilia and abrogating the expulsion of adult parasites from the intestinal lumen (Osbourn et al., 2017).

Thus, modulation of ILC2s by helminth parasites and their immunomodulatory secretory products might represent a promising mechanism to prevent the activation of the innate immune system.

#### 2.4.2.2 Modulation of the adaptive immune system

Induction of regulatory T cells during helminth infection has been suggested to play an important role in the prevention of allergic symptoms in mouse models of allergic airway inflammation. One example is the infection with the filarial parasite ***Litomosoides sigmodontis***. In a mouse model of OVA-induced asthma, infection with *L. sigmodontis* induced regulatory T cells and immunomodulatory cytokines like TGF-β, which in turn ablated the allergic symptoms such as airway eosinophilia, Th2 cytokine production and airway hyperresponsiviness (Dittrich et al., 2008)

During infection with ***H. polygyrus***, induction of Foxp3$^+$ regulatory Tcells was associated with protection to OVA-induced allergen airway inflammation, and adoptive transfer of Treg population from infected mice was able to suppress allergic airway inflammation in sensitized recipient mice (Wilson et al., 2005). A later study demonstrated that excretory secretory products released by *H. polygyrus* (**HES**) directly induced Treg cells by the production of a TGF-β like molecule, which phosphorylated SMAD-2/3 and initiated the TGF signalling pathway, inducing the expression of Foxp3. Adoptive transfer of HES-induced Tregs can suppress allergic airway inflammation to a similar extent as observed by HES (Grainger et al., 2010). Recently, the active molecule present in HES was identified as a TGF-β mimic (Hp-TGM), a protein which has no homology with mammalian TGF-β, but can bind to mammalian TGF-β receptors and induce regulatory T cells (Johnston et al., 2017).

***Schistosoma mansoni*** infection has been inversely correlated with manifestation of allergic diseases and it is characterized by induction of Tregs and IL-10 production in humans and animal models (Araujo et al., 2004; van der Vlugt et al., 2012; Layland et al., 2013). One of the active immunomodulatory compounds is a lysophophatidylserine (**lyso-PS**) present in the tegument of *S. mansoni* adult worm. LysoPS acts as a TLR2 ligand and increases the maturation of DCs, resulting in the activation of regulatory T cells and the production of IL-10 (van der Kleij et al., 2002). Some antigens present in the tegument of *S. mansoni* adult worms (**Sm22.6 and S.m29**) and a fraction of the adult worm extract (**PIII**) strongly induced IL-10 production in PBMCs of asthmatic patients infected with *S. mansoni*, suggesting the potential of these antigens as vaccine candidates (Cardoso et al., 2006). The mechanism of action was described in a mouse model of OVA-induced asthma, where administration of these three antigens before sensitization protected the mice from the development of allergic symptoms, reducing lung inflammation, airway eosinophilia, type 2 cytokines and IgE levels, while inducing Treg expansion (Cardoso et al., 2010). In addition to these immunomodulatory compounds identified from this parasite, a recent study showed that the soluble egg antigen (**SEA**) from *Schistosoma mansoni* is responsible for the production of $PGE_2$ and the induction of Th2 immune responses. The mechanism starts with the binding of SEA to Dectin-1 and Dectin-2 receptors, which activates a downstream signalling cascade involving spleen tyrosine kinase (Syk), ERK, cPLA2, COX-1 and COX-2 activation and secretion of $PGE_2$. In an autocrine manner, $PGE_2$ induced the expression of OX40 ligand (OX40L) in DCs, promoting the polarization of Th2 cells (Kaisar et al., 2018). Interestingly, *S. mansoni* cercaria and the larvae of the whipworm *Trichuris trichuris* can release high amounts of $PGE_2$, helping the infective larvae to migrate through the skin during infection or preventing the release

of pro-inflammatory cytokines in DCs, respectively (Ramaswamy et al., 2000; Laan et al., 2017; section 2.5.3).

Another example of immunomodulatory proteins secreted by helminths is **PAS-1**, a protein isolated from ***Ascaris suum*** worms, which suppressed allergic airway inflammation by reducing lung pathology, eosinophilic airway inflammation, type 2 cytokines and IgE levels in mice immunized with PAS-1 and OVA-challenged. The protection effect of PAS was dependent on IFN-γ and IL-10 production (Araújo et al., 2008). A later study demonstrated that PAS-1 primed CD4+CD25+FoxP3+ and CD8+γδTCR+ T-cell populations, which play an important role in the suppression of allergic airway inflammation (de Araújo et al., 2010). The protein-2 (**AIP-2**) secreted by ***Ancylostoma caninum*** (*A. caninum*) was shown to modulate the activation of DCs and promote the induction of Tregs, resulting in the reduction of allergic symptoms in a mouse model of OVA-induced asthma. The anti-inflammatory properties of AIP-2 are a promising therapeutic alternative as it also modulated *ex vivo* the response of DCs and T cells from HDM-allergic patients (Navarro et al., 2016)

Taken together, helminth parasites and their E/S products are potent immunomodulators of the innate and adaptive immune system (Table 2). They have evolved different strategies to manipulate the host´s immune system, in part by their ability to skew pro-inflammatory responses toward anti-inflammatory or regulatory responses, and therefore survive longer in the host. However, only a few studies have looked at the effect of these parasite products in the treatment of allergic diseases and asthma. A better understanding of the molecular mechanisms behind parasite-mediated immunomodulation during allergic asthma is essential to look for new therapeutic approaches.

| Immunomodulatory compound | Mechanism of action | Reference |
|---|---|---|
| *Acanthocheilonema viteae:* **Av17** (cystatin protease inhibitor) | Induction of IL-10 producing macrophages | Schnoeller et al., 2008; Ziegler et al., 2015; Klotz et al., 2011; Danilowicz-Luebert et al., 2013 |
| *Acanthocheilonema viteae:* **ES-62** (glycoprotein) | Promotes an anti-inflammatory type 2 phenotype in macrophages | Goodridge et al., 2001 |
| *Ancylostoma caninum:* **AIP-2** (anti-inflammatory protein-2) | Activation of DCs and induction of regulatory T cells | Navarro et al., 2016 |
| *Ancylostoma caninum:* **NIF** (neutrophil inhibitory factor) | Blocked neutrophil migration | Moyle et al., 1994 |
| *Ascaris suum:* **PAS-1** (protein of *A. suum*-1) | Induction of IFN-γ and IL-10 production; expansion of regulatory T cells | Araújo et al., 2008; de Araújo et al., 2010 |
| *Brugia malayi:* **BmCPI-2** (cystatin) | Induction of IL-10 producing macrophages and inhibition of CD4+T cell proliferation | Venugopal et al., 2017 |
| *Brugia malayi:* **MIF** (macrophage migration inhibitory factor) | Induction of alternatively activated macrophages in synergy with IL-4 | Prieto-Lafuente et al., 2009 |

| | | |
|---|---|---|
| *Fasciola hepatica:* **Fh12/ Fh15** (fatty acid binding protein) | Induced alternative activation of macrophages; TLR4 antagonist. | Ramos-Benitez et al., 2017 |
| *Fasciola hepatica:* **FhHDM-1** (helminth defence molecule-1) | Inhibit the activation of NLRP3 inflammasome in macrophages | Alvarado et al., 2017 |
| *Heligmosomoides polygyrus*: **HES** (*H.polygyrus* excretory/ secretory products) | Induction of regulatory T cells, blocked the release of IL-33; suppression of ILC-2 activation; TGF-β like activity | McSorley et al., 2012; 2014 Grainger et al., 2010 |
| *Heligmosomoides polygyrus:* **HpARI** (alarmin release inhibitor) | Blocks IL-33; suppression of ILC-2 activation; mimic TGF-β activity; induction of regulatory Tregs | Osbourn et al., 2017; Johnston et al., 2017 |
| *Heligmosomoides polygyrus:* **TGM** (TGF-β mimic) | Binds TGF-β receptors and induce regulatory T cells | Grainger et al., 2010; Johnston et al., 2017 |
| *Necator Americanus:* **Metalloproteinases** | Suppressed eosinophil recruitment and activation by proteolytic activity on eotaxin | Culley et al., 2000 |
| *Onchocerca volvulus:* **Onchocystatin** (cysteine protease inhibitor) | Induction of IL-10 producing macrophages | Schönemeyer et al., 2001 |
| *Onchocerca volvulus*, *Wolbachia*: **WoLP** (lipopeptide) | Promoted neutrophil chemotaxis | Tamarozzi et al., 2014 |
| *Schistosoma mansoni* **Lyso-PC** (lysophosphatidylcoline) | Induced eosinophil activation and recruitment | Magalhães et al., 2010; |
| *Schistosoma mansoni* **Lyso-PS** (lysophosphatidylserine) | Induce DCs maturation and activation of IL-10 producing Treg cells | van der Kleij et al., 2002 |
| *Schistosoma mansoni* **Sm22.6, S.m29, PIII** (*S.mansoni* antigens) | IL-10 production in PBMCs; Induction of regulatory T cells | Cardoso et al., 2006; Cardoso et al., 2010 |
| *Schistosoma mansoni eggs:* **smCKBP** (chemokine binding protein) | Inhibited neutrophil chemotaxis; neutralized chemokine activity | Smith et al., 2005 |
| *Schistosoma mansoni egg antigen:* **SEA** (soluble egg antigen) | Induces $PGE_2$ production; activation of DCs and Th2 polarization | Kaisar et al., 2018 |
| *Trichuris trichuris:* **$PGE_2$** (prostaglandin E2) | Suppression of pro-inflammatory cytokines in DCs | Laan et al., 2017 |

**Table 2: Examples of immunomodulatory molecules secreted by helminths and their cellular mechanisms of action in allergy.**

### 2.4.3 Potential of parasitic helminths or their molecules as future therapies for allergy and asthma

Several studies support the evidence that parasitic helminth infections suppress the development of allergies (as addressed in section 2.4.1), suggesting that helminths can have a therapeutic potential. If one accepts this evidence, it might be worth trying to infect allergic patients with helminths in order to treat allergies. Several clinical trials have tested the therapeutic potential of helminths by life infection of allergic patients with hookworms or whipworms. The first clinical trial of hookworm infection in allergic asthmatic patients was reported in 2010. However, cutaneous infection of asthmatic patients with ten *Necator americanus* larvae did not change clinical measurements such as airway hyperresponsiveness, self-reported asthma symptoms or allergen skin prick tests, but infection was well tolerated in the study group (Feary et al., 2010). Similarly, a clinical trial of *Trichuris suis* infection in allergic rhinitis patients showed no therapeutic effect. Treatment of allergic patients by ingestion of 2500 live worms for 1 month before the peak of pollen season did not change allergic rhinitis symptoms, skin prick test positivity, total histamine levels or grass pollen-specific IgE levels (Bager et al., 2010). The failure of these clinical trials suggested that helminth infection might be more effective in prevention rather than treatment of allergic diseases.

Rather than infecting people directly with worm parasites, an improved approach to modulate the immune system as a therapy is to identify and purify helminth-derived products or synthesize mimics of such products. A large number of helminth derived-immunomodulatory compounds have been already characterized and their mechanism of action described (see table 2). Therefore, current research is now focussing on the characterization of the helminth molecules responsible for the induction of a modified Th2 response to suppress clinical symptoms of allergies and asthma, offering new possibilities for the treatment of these diseases.

An alternative therapeutic approach to treat inflammatory diseases and avoid the administration of live helminths or helminth-derived products is the transfer of cells modulated by parasitic molecules to patients. Parasitic worms and their excretory/secretory products have developed different strategies to escape the host immune response and modulate innate and adaptive immune cells such as macrophages, granulocytes, dendritic cells or Tcells (see section 2.4.2). Thus, therapies based on cells might constitute a safer approach in which cells are differentiated into an immunosuppressive or regulatory phenotype and administered into patients. However, this alternative therapeutic approach still needs to be further investigated.

## 2.5 Eicosanoids as effector mediators in type 2 immunity

### 2.5.1 Eicosanoid biosynthesis and signalling

Eicosanoids were first described by Samuelsson, Bergström and Vane, who jointly won the Nobel price in Physiology or Medicine in 1982 “for their discoveries concerning prostaglandins and related biologically active substances” (source: www.nobelprize.org). Eicosanoids are important lipid mediators involved in many inflammatory diseases but classically known for their effects in asthma and allergy. Upon cell stimulation, the polyunsaturated fatty acid (PUFA) arachidonic acid (AA) is released from membrane phospholipids through the action of cytosolic phospholipase $A_2$ ($cPLA_2$) and/or secreted phospholipase $A_2$ ($sPLA_2$) (Dennis et al., 1997). AA is

the substrate required for the eicosanoid synthesis by two main pathways: lipoxygenase (LOX) pathway to produce leukotrienes and cyclooxygenase (COX) pathway to produce prostanoids (Samuelsson et al. 1982; Vane et al. 1979).

The generation of leukotrienes (LTs) in the LOX pathway is dependent upon the action of 5-LOX, which catalyzes the conversion of AA to the unstable precursor of leukotrienes, $LTA_4$, which is quickly metabolized by the action of $LTA_4$ hydrolase ($LTA_4H$) to leukotriene $B_4$ ($LTB_4$), or by the action of leukotriene $C_4$ synthase ($LTC_4S$) to $LTC_4$ or its metabolites $LTD_4$ and $LTE_4$, commonly known as cysteinyl leukotrienes (cysLTs) (Maycock et al., 1982; Jakschik et al., 1982; Rådmark et al., 2007). The main producers of leukotrienes are myeloid cells (mostly eosinophils, neutrophils and macrophages), and under inflammatory conditions epithelial cells e.g. after stimulation with allergens like house dust mite (Jame et al. 2007, Trian et al., 2015). In addition to 5-LOX, human cells express 12- and 15-LOX enzymes, which participate in the synthesis of hydroxyeicosatetraenoic acids (12-/15-HETE), lipoxins, resolvins or hepoxillins, compounds with anti-inflammatory or proresolving properties (Berger et al., 1998; Serhan, 1994; Schwab et al., 2007; Ackermann et al., 2017).

Regarding the COX pathway, two COX isoenzymes have been identified: a constitute form, COX-1 and an inducible form, COX-2 (Smith & Langenbach, 2001; Naraba et al., 1998). The generation of prostanoids is dependent upon the action of COX, which catalyzes the conversion of AA to the precursor $PGH_2$, which is quickly metabolized by the action of prostaglandin E synthase (PGES) to prostaglandin E2 ($PGE_2$); by the action of thromboxane synthase (TXBS) to the unstable $TXA_2$ or its metabolite $TXB_2$ and 12-hydroxy-heptadecatrienoic acid (12-HHT) produced in equimolar amounts with $TXA_2$; by the action of prostaglandin synthase (PGDS) to prostaglandin D2 ($PGD_2$); or by the action of prostaglandin I2 synthase (PGIS) to prostaglandin I2 ($PGI_2$) also known as prostacyclin (Jakobsson et al., 1999; Hecker et al., 1987; Christ-Hazelhof & Nugteren, 1979; Moncada et al., 1976). The main producers of prostanoids in the lung are myeloid cells (macrophages, mast cells, eosinophils), although airway epithelial cells, airway smooth muscle cells and airway fibroblasts are also involved in the synthesis of prostaglandins and platelets are an important source of thromboxanes.

The regulation and immunological effects of eicosanoids are highly regulated and they depend on the nature of the immune response. In fact, the same lipid mediator might exert different functions depending on the tissue, the signalling pathways activated and the disease type. Function of eicosanoids during allergic asthma and helminth infections will be explained in the next sections.

### 2.5.2 Role of eicosanoids during allergic asthma

#### 2.5.2.1 Lipoxygenase pathway

Leukotrienes play important roles in allergy and asthma as these mediators are potent inducers of several hallmark responses of type 2 inflammation. In asthmatic patients, higher levels of $LTB_4$ and $LTC_4$ were reported in bronchial lavage (BAL) fluid compared to controls subjects without asthma (Wardlaw et al., 1989). Sputum CysLTs concentrations were significantly elevated in asthmatic patients as compared to normal subjects and they were associated with eosinophilic airway inflammation (Aggarwal et al., 2010). LTs exert their biological function by binding to cell surface G protein-coupled receptors (GPCRs) (Samuelsson, 1983). The receptors for $LTB_4$ are BLT1 (high affinity) and BLT2 (low affinity); while the receptors for CysLTs are CysLT1R

(high affinity), CysLT2R (low affinity) and the recently discovered CysLT3R (Yokomizo et al., 1997 & 2000; Lynch et al., 1999; Heise et al., 2000; Bankova et al., 2016).

**$LTB_4$** is a pro-inflammatory mediator and plays a key role as a potent chemoattractant for neutrophils and eosinophils (Ford-Hutchinson et al., 1980). $LTB_4$ promotes vascular leakage (Bray et al., 1981). The $LTB_4$-BLTR axis mediated the recruitment of leukocytes, mostly neutrophils, and the firm leukocyte adhesion within blood vessels during inflammation (Tager et al., 2000). $LTB_4$ binding to BLT1 receptor in dendritic cells promoted the activation and differentiation of T cells in a murine model of OVA-induced asthma, worsening the airway inflammatory and Th2 allergen-induced response in the lung (Miyahara et al., 2008). In contrast to the pro-inflammatory role of BLT1, an anti-inflammatory role of BLT2 was recently published, although the role of BLT2 in asthma is still poorly understood. A recent study showed an inverse correlation between BLT2 expression and airway eosinophilia in a mouse model of OVA-induced asthma (Matsunaga et al., 2013). At high concentrations, $LTB_4$ binds and activates a nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α), which in turn reduced the secretion of $LTB_4$ and promoted its degradation, attenuating the pro-inflammatory effects of this mediator (Narala et al., 2010). Another specific and high affinity ligand described for BLT2 is the COX-derived product 12-hydroxyheptadecatrienoic acid (**12-HHT**). The activation of 12-HHT-BLT2 axis promoted migration of keratinocytes and promoted skin wound healing (Liu et al., 2014). Although the role of 12-HHT in asthma remains poorly understood, this new discovery opened the possibility of new therapeutic strategies to accelerate wound healing.

**CysLTs** are pro-inflammatory mediators acting as potent bronchoconstrictors (they are thousands of times more potent than histamine) and mediators of eosinophilic inflammation by up-regulating the expression of endothelial adhesion molecules, inducing eosinophil chemotaxis and reducing eosinophil apoptosis (Haberal & Corey, 2003). Furthermore, CysLTs increase vascular permeability and edema and increase the mucus production by globet cells (Dahlén et al, 1981; Maron et al., 1982). CysLTs play also an important role in airway remodelling by promoting the proliferation of airway smooth muscle cells and epithelial cells and increasing the collagen deposition (Hay et al., 1995), which is an important characteristic of chronic asthma. Recently, it was shown that CysLTs production after HDM stimulation of bone marrow cells mediated the induction of eosinophil and neutrophil recruitment and the secretion of type 2 cytokines in the lung (Barrett et al., 2011). CysLTs are also involved in the activation of ILC2, inducing migration, promoting cell survival and inducing the production of type 2 cytokines (Salimi et al., 2017). More recently, it was discovered that CysLTs-CysLT3R axis induces globet cell mucin release and promotes IL-25-dependent brush cell expansion in airway epithelial cells, causing type 2 lung inflammation during allergen exposure (Bankova et al., 2016 and 2018).

Taken together, leukotrienes play crucial roles in the pathogenesis of allergic diseases and asthma (Figure 5). Thus, a better understanting of how LT synthesis is regulated and the mechanisms underlying the functions of the receptors that mediate responses to LTs will provide new insights into the development of novel therapeutic agents for the treatment of inflammatory diseases.

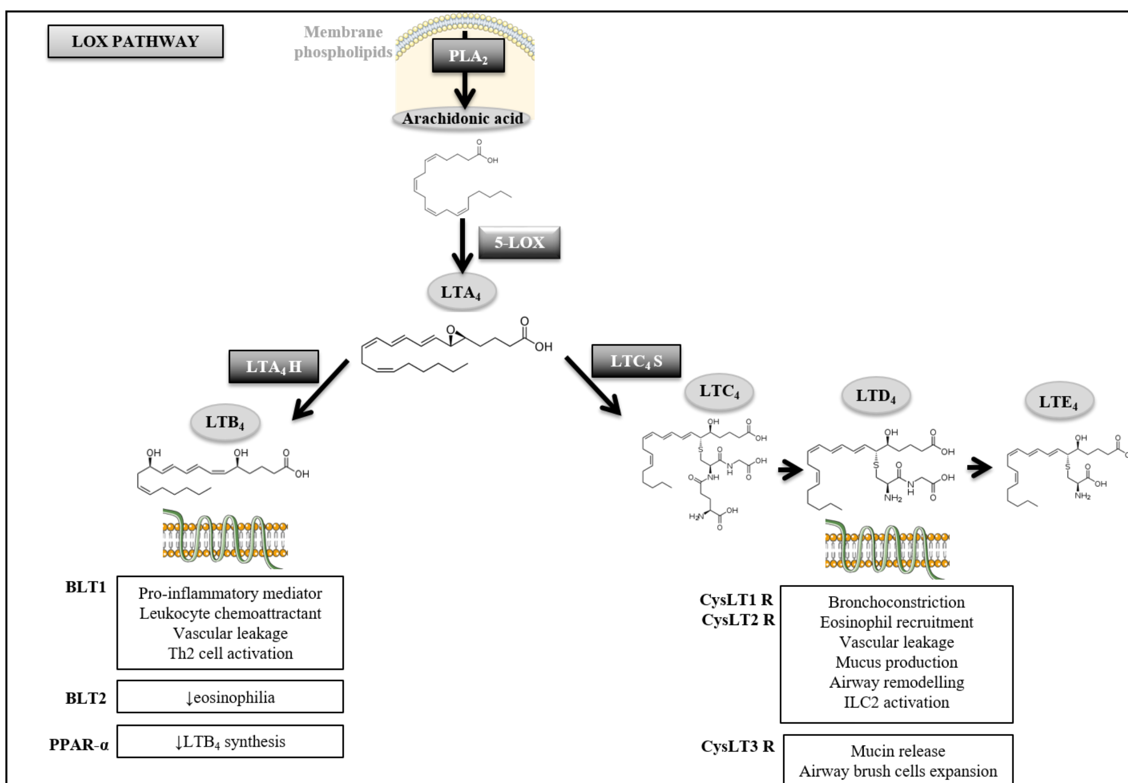


**Figure 5: Lipoxygenase pathway and role of leukotrienes in asthma.** Leukotrienes are synthesized from arachidonic acid by the enzyme 5-LOX. The unstable precursor $LTA_4$ is quickly metabolized by the action of $LTA_4H$ to $LTB_4$, or by the action of $LTC_4S$ to $LTC_4$ or its metabolites $LTD_4$ and $LTE_4$, commonly known as cysLTs. In contrast to the inflammatory role of the $LTB_4$-BLT1 axis, BLT2 has an anti-inflammatory role. CysLTs bind to CysLT1R, CysLT2R and CysLT3R and act as potent pro-inflammatory mediators in asthma. (Abbreviations: 5-LOX, 5-lipoxygenase; BLT1/2, Leukotriene $B_4$ receptor 1/2; CysLT1/2/3R, Cysteinyl leukotriene receptor 1/ 2/ 3; ILC2, innate lymphoid type 2 cell; $LTA_4$, leukotriene $A_4$; $LTA_4H$, $LTA_4$ hydrolase; $LTB_4$, leukotriene B4; $LTC_4$, leukotriene $C_4$; $LTC_4S$, $LTC_4$ synthase; $PLA_2$, phospholipase A2; PPAR-α, peroxisome proliferator-activated receptor alpha; Th, T helper cell).

#### 2.5.2.2 Cyclooxygenase pathway

Prostanoids are key mediators in allergy, as they can regulate the synthesis of leukotrienes and counter-regulate type 2 immune responses. However, prostanoids are tightly regulated and their immunological effect depends on the receptor through which they signal, having even opposing effects depending on the signalling pathways activated. In allergic asthma patients, the analysis of prostanoids in the BAL fluid showed increased levels of $PGD_2$ and PGF2α compared to patients without asthma who had allergic rhinitis (Liu et al., 1990). Another study induced an allergen challenge in asthmatic patients by installation of ragweed antigen via bronchoscopy to a segment of the lung, measuring significantly higher levels of $PGD_2$, $TXB_2$ and 6-keto-PGF1α (metabolite of $PGI_2$, prostacyclin I2) in the BAL fluid in comparison to saline-challenged controls (Liu et al., 1991).

One mediator playing an important role in the pathogenesis of allergic diseases and asthma is **$PGD_2$**. The analysis of BAL fluid of patients with severe asthma showed high levels of $PGD_2$ (Faijt et al., 2013). Furthermore, high levels of $PGD_2$ in the BAL of asthmatic subject were associated with airway bronchoconstriction (Liu et al., 1990). $PGD_2$ signals through three receptors termed as $PGD_2$ receptor 1 ($DP_1$), chemoattractant receptor-like molecule expressed on

Th2 cells ($CRTH_2/DP_2$) or thromboxane receptor (TP). $PGD_2$ plays both, anti- and pro-inflammatory roles depending on the receptor. $PGD_2$ binding to DP1 causes vasodilatation and bronchodilatation in vascular and airway smooth muscle and inhibits platelet aggregation, while it also promotes the polarization and recruitment of Th2 lymphocytes (Giles et al., 1989; Whittle et al., 1985). Similarly, DP2/$CRTH_2$ activation was shown to promote chemotaxis in Th2 cells, eosinophils and basophils (Hirai et al., 2001). Additionally, $CRTH_2$ activation of Th2 cells induces cytokine production, which could promote IgE activation on mast cells, thus further enhancing $PGD_2$ generation (Xue et al., 2009). Thus, treatment of asthmatic patients with antagonists of $CRTH_2$ could be an option as it was shown to efficiently inhibit allergic airway inflammation in asthma patients (Singh et al., 2013). In contrast, $PGD_2$ binding to TP promoted smooth muscle contraction and platelet aggregation, contributing to allergen-induced bronchoconstriction (Johnston et al., 1995). In addition, $PGD_2$ and CysLTs can act synergistically to activate Th2 cells and promote the production of type 2 cytokines and other pro-inflammatory mediators, which will induce neutrophil activation, contributing to the pathology of asthma (Xue et al., 2015). In addition, CysLTs can also potentiate the effect on $PGD_2$ and epithelial cytokines in the activation of ILC2, leading to increased production of type 2 and pro-inflammatory cytokines (Salimi et al., 2017)

**$PGE_2$** is commonly considered a pro-inflammatory mediator, although this mediator can exert a wide array of effects including the suppression of inflammatory responses in the lung. In the sputum of severe asthmatic patients, higher levels of $PGE_2$ were measured in comparison to control subjects, which may represent a bronchoprotective and anti-inflammatory response to counteract the pro-inflammatory effects of CysLTs in the airways. An inverse correlation between sputum $PGE_2$ concentration and eosinophils, as well as a lower ratio $PGE_2$ to CysLTs was reported in eosinophilic asthmatic patients, suggesting a role of $PGE_2$ in the suppression of airway eosinophilia (Aggarwal et al., 2010). This concept is supported by the finding that inhalation of $PGE_2$ in asthmatic patients attenuated the bronchoconstrictor response after allergen challenge, an effect which was explained by inhibitory effects of $PGE_2$ during early allergic responses, blocking the release of $PGD_2$ and CysLTs in mast cells (Hartert et al., 2000). The activity of $PGE_2$ is mediated by four GPCRs, named as E prostanoids receptors (EP1-EP4) (Smith et al., 2011). Each EP receptor signals through a different G protein coupling and activates different downstream signals, some of them even counteracting each other, which can explain the complexity of its physiological role.

$PGE_2$ binding to EP1 and EP4 receptors stimulates early wound healing in the airway epithelium, stimulating spreading and migration of airway epithelial cells after wounding (Savla et al., 2001). Low concentrations of $PGE_2$ showed a protective effect in human small airways acting on the EP2 receptor, which resulted in the inhibition of IgE-dependent contraction and the inhibition of pro-inflammatory histamine and CysLTs release by mast cells (Säfholm et al., 2015). Acting on the EP2 receptor, $PGE_2$ directly inhibited human eosinophil chemotaxis towards chemoattractants such as eotaxin, $PGD_2$ and C5a, and it was involved in the inhibition of eosinophil activation (Sturm et al., 2008). Similarly, LPS-activated macrophages also contributed to the inhibition of eosinophil migration by the release of a COX-derived metabolite, most likely $PGE_2$ (Sturm et al., 2008). EP4 receptor signalling induced relaxation in human airways, opening the possibility of using EP4 receptor antagonism in asthma therapy (Buckley et al., 2011). In contrast, signalling through EP3 receptor is responsible for $PGE_2$-induced airway irritancy and cough (Maher et al., 2009). Interestingly, $PGE_2$ can also countregulate the synthesis of LTs. $PGE_2$ inhibited the action of 5-LOX and thus blocking the production of pro-inflammatory $LTB_4$ in dendritic cells by a mechanism dependent on IL-10 production (Harizi et al., 2003). In addition, $PGE_2$ can activate

the protein kinase A (PKA) via cAMP, leading to the inhibitory phosphorylation of 5-LOX and consequently inhibition of LTs production (Luo et al., 2004). Another difference between the action of $PGD_2$ and $PGE_2$ is their effect in the activation of ILC2: While $PGD_2$ signalling promotes ILC2 function, $PGE_2$ signalling through the EP2 and EP4 receptor inhibited GATA 3 expression in ILC2, thus suppressing the production of type 2 cytokines IL-5 and IL-13 and abrogating the activation and proliferation of ILC2 (Maric et al., 2018).

**$TXA_2$** is a metabolite that regulates the physiology involved in acute asthma. The concentration of $TXA_2$ and its metabolites measured in urine of asthmatic patients were 4-6 fold higher in comparison to control subjects (Taylor et al., 1991). After allergen inhalation, atopic asthmatic patients showed a significant increase of thromboxane-derive products measured in the urine (Sladek et al., 1990). $TXA_2$ is mainly produce by platelets, although another cells such as macrophages, monocytes, neutrophils and lung parenchyma can synthesise this mediator, which play a key role platelet aggregation (Paul et al., 1999). $TXA_2$ binds to TP receptor and promoted vasoconstriction in pulmonary arteries (Cogolludo et al., 2003). This mediator can also regulate adaptive immunity. It was shown that dendritic cells can produce $TXA_2$, negatively regulating its interaction with T cells and thus inhibiting the activation and proliferation of T cells (Kabashima et al., 2003). $TXA_2$ induced the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) or endothelial leucocyte adhesion molecule-1 (ELAM-1) in human vascular epithelial cells, promoting cell recruitment (Ishizuka et al., 1998). Monocyte recruitment is also affected by $TXA_2$ binding to TP receptor in endothelial cells, which induce the expression of monocyte chemoattractant protein-1 (MCP-1), a chemoattractant for monocytes and lymphocytes. (Ishizuka et al., 2000). The use of $TXA_2$ receptor antagonists may prevent exacerbation of inflammation. Of interest, $TXA_2$ is very unstable with a half-life of about 30 seconds, and thus very difficult to measure, and it is usual to measure its stable metabolite $TXB_2$ for analytical and diagnostic purposes.

Thus, prostanoids are key mediators in allergic diseases and asthma, as they are implicated in a wide variety of immune responses, exerting pro- or anti-inflammatory effects depending on the binding receptor and activated pathway (Figure 6). The development of specific enzyme inhibitors and receptor antagonists, but also the beneficial effect of individual prostaglandins offer new possibilities for the treatment of inflammatory diseases.

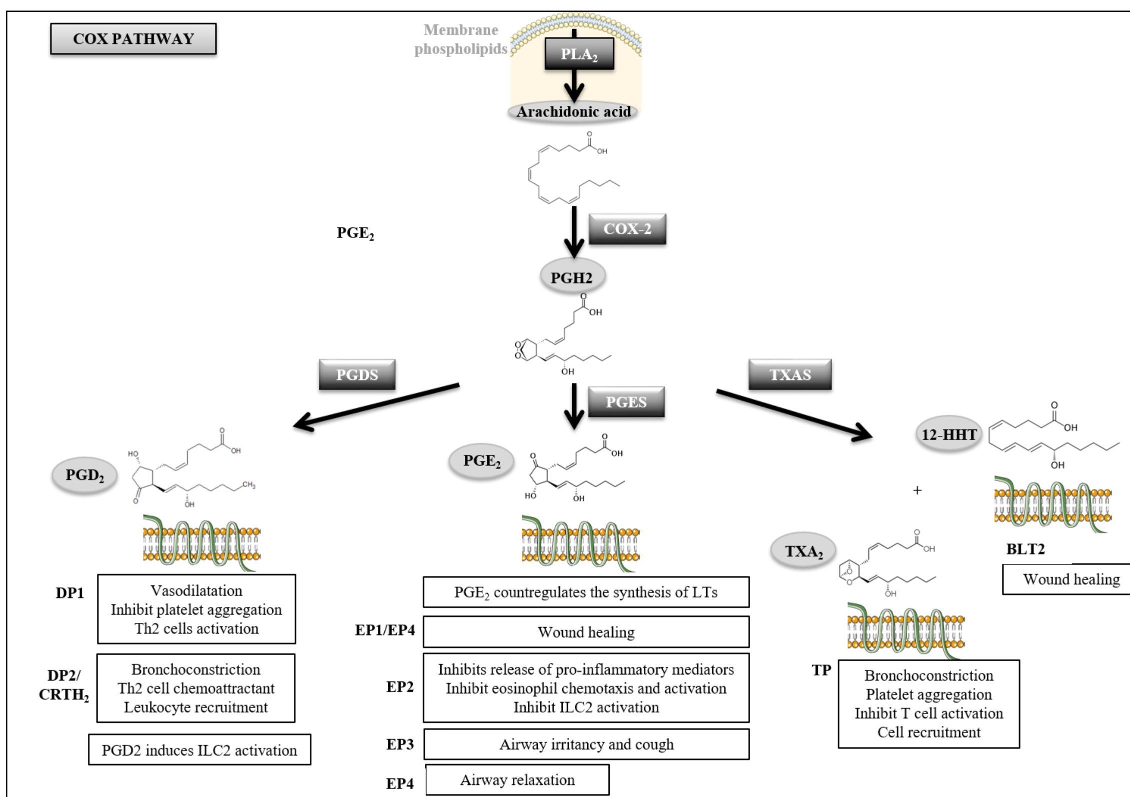


**Figure 6: Cyclooxygenase pathway and role of prostanoids in asthma.** Prostanoids are synthesized from the arachidonic acid by the enzyme COX-2. The unstable precursor $PGH_2$ is quickly metabolized by the action of PGES to $PGE_2$; by the action of TXBS to $TXA_2$ and 12-HHT or by the action of PGDS to $PGD_2$). These mediators can exert pro- or anti-inflammatory effects depending on the receptor and activated pathway, playing different roles in the course of asthma. (Abbreviations: 12-HHT, 12-hydroxyheptadecatrienoic acid; BLT2, leukotriene B4 receptor 2; COX, cyclooxygenase; CRTH2, chemoattractant receptor-like molecule expressed on Th2 cells; DP1-4, $PGD_2$ receptor 1-4; EP1-4, E prostanoids receptor 1-4; ILC2, innate lymphoid type 2 cell; $PGD_2$, prostaglandin D2; PGDS, prostaglandin synthase; $PGE_2$, prostaglandin E2; PGES, prostaglandin E synthase; $PGH_2$, prostaglandin H2; $PLA_2$, phospholipase A2; Th, T helper cell; TP, thromboxane receptor; $TXA_2$, thromboxane A2; TXAS, thromboxane synthase)

Taken together, eicosanoids are key mediators playing an important role in the pathology of allergic diseases such as asthma. Targeting of eicosanoid pathway components (e.g. receptors, enzymes or activating pathways) is an attractive option for therapeutic intervention, especially in asthmatic patients that remain uncontrolled due to resistance to treatment with corticosteroids.

### 2.5.3 Eicosanoid imbalance in aspirin exacerbated respiratory disease patients

Eicosanoids play particularly important roles in the pathogenesis of Aspirin exacerbated respiratory disease (AERD), also known as aspirin-induced asthma, Samter´s triad or Samter´s syndrome. AERD is an asthma phenotype which affects 7% of adult-onset asthmatics and 14% of adult asthmatics with severe asthma (Rajan et al., 2015). AERD, which is slightly more prevalent in women, usually begins in young adulthood, although it has been described in children and presents a diagnostic problem in pediatrics (Szczeklik et al., 2000; Chen et al., 2013). AERD is characterized by three key features: bronchial asthma, chronic rhinosinusitis with nasal polyps, and hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (Samter

& Beers, 1968). Consumption of even small amounts of alcohol is associated with respiratory reactions in the majority of AERD patients (Cardet et al., 2014). These patients often undergo surgery to remove the nasal polyps, although they typically recur within five years after surgery, particularly in patients with asthma and aspirin intolerance (Mendelsohn et al., 2011).

AERD patients show a typical eicosanoid imbalance with over-expression of pro-inflammatory mediators including 5-LOX and $LTC_4S$, leading to over-production of bronchoconstrictory cysLTs, but reduced synthesis and signalling of regulatory $PGE_2$ (Israel et al., 1993; Cowburn et al., 1998; Laidlaw et al., 2014). $PGE_2$ concentrations are reduced in AERD patients, and granulocytes from patients with AERD have been reported to be resistant to $PGE_2$, which might contribute to the severe respiratory tract inflammation and leukotriene overproduction in AERD, as $PGE_2$ can suppress LT production and has protective effects in the lungs (Laidlaw et al., 2014; Luo et al., 2014; Säfholm et al., 2015). The further reduction of $PGE_2$ concentrations by aspirin and other NSAIDs through COX-1 inhibition may accelerate the activation of eosinophils and mast cells in AERD, resulting in the overproduction of pro-inflammatory LTs, which can cause severe exacerbations of asthma in these patients, sometimes in a life-threatening manner (Feng et al., 2006). In contrast, treatment of AERD patients with COX-2 selective inhibitors is considered safe (Morales et al., 2014). This effect could be explained by the relative absence of COX-2 mRNA and protein expression in AERD subjects, whilst COX-1 expression is not significantly changed. Therefore, with this relative absence of COX-2, AERD patients highly depend on COX-1 for the synthesis of $PGE_2$, supporting the importance of COX-1 derived $PGE_2$ in AERD (Pérez-Novo et al., 2005; Gosepath et al., 2005)

Other hallmarks of AERD patients include marked eosinophilic inflammation and a mixed Th-2 and Th-1-like cytokine production characterized by a high expression of IFN-$\gamma$ (Steinke et al., 2013). IFN-$\gamma$ can drive eosinophil maturation and increase the expression of $LTC_4S$, which translates into a high capacity of these eosinophils to secrete cysLTs (Steinke et al., 2013). Increased levels of type 2 cytokine IL-4 are also present in tissue of AERD subjects, and IL-4 can upregulate $LTC_4S$ by mast cells in AERD, thus promoting the synthesis of LTs (Hsieh et al., 2001). Interestingly, a markedly elevated frequency of platelet-adherent eosinophils, neutrophils and monocytes is found in the blood of AERD subjects, and it has been suggested that adherent-platelets contribute up to 50% of the total $LTC_4S$ activity in the blood of AERD, representing an important source of CysLTs in these patients (Laidlaw et al., 2012). Thus, granulocytes (mostly eosinophils) and mast cells are thought to be the major source of pro-inflammatory LTs in AERD and therefore they are two cell types that have important roles in mediating many of the effects observed in this disease. However, macrophages might also play a key role in the pathophysiology of AERD as they express both the LTs and COX biosynthetic pathway enzymes, but their contribution to the development of this disorder remains poorly understood.

Thus, dysregulation of the arachidonic acid metabolism is characteristic in patients with AERD. A better understanding of the mechanisms underlying eicosanoid regulation and the role of macrophages in the synthesis of lipid mediators in AERD patients will provide new insights into the mechanisms involved in this disease and the development of new therapeutic strategies.

### 2.5.4 Role of eicosanoids during helminth infections

During helminth infection, the host immune response activates inflammatory cells but also induces the secretion of lipid mediators to control and favour the expulsion of the parasite.

However, parasites aim to survive longer in the host and have evolved evasion strategies to neutralize or even mimic lipid mediators to benefit from it and to modulate innate and adaptive immune responses, e.g. to induce rapid tissue repair or host immunosuppression.

One parasite already studied for its ability to secrete lipid mediators and modulate the host immune system is ***Schistosoma mansoni***. The life cycle of this parasite starts with the penetration of the infective larvae (cercaria) through the skin. One study has shown that *S. mansoni* cercaria produced high amounts of $PGE_2$ in a COX-2 independent pathway, which in turn helps the infective larvae to migrate through the skin, as $PGE_2$ is a potent vasodilator. The cercarial $PGE_2$ also induced IL-10 production in keratinocytes, contributing to down-regulation of the host immune response and helminth survival in the infective stages of *S. mansoni* infection (Ramaswamy et al., 2000). When the adult male and female worm mate, they produce thousands of schistosome eggs, which enter into the bloodstream and are trapped in the liver microcirculation, resulting in the formation of granulomas and a strong type 2 immune response. The cellular composition of granulomas includes lymphocytes, eosinophils, macrophages, neutrophils, mast cells and fibroblasts (Metwali et al., 1996). The eicosanoid profile measured in macrophages isolated from liver granulomas showed high levels of $TXA_2$ and smaller amounts of $PGE_2$ and $PGI_2$, with the absence of LTs production (Tripp et al., 1988). 5-LOX metabolites contributed to attenuate inflammatory responses and the formation of liver granulomas during *S. mansoni* infection, as the granuloma formation in 5-LOX deficient mice was smaller as compared to WT or 12-LOX deficient mice (Secor et al., 1998). A recent study confirmed that mice lacking the expression of 5-LOX developed smaller lung granulomas with increased signs of tissue regeneration after intravenous infection with purified eggs, which might also affect the helminth type 2-induced immune response, as lower levels of IL-4 and IL-13 and increased TGF-β production were detected in the lung of 5-LOX deficient mice (Toffoli da Silva et al., 2016).

The helminth parasite ***Brugia malayi*** can also stimulate eicosanoid production to modulate the host immune response. It was shown that *B. malayi* microfilaria released high amounts of $PGI_2$ and $PGE_2$, which might contribute to the inhibition of platelet aggregation and vasodilation. In contrast, this parasite did not secrete $TXB_2$, a mediator involved in platelet aggregation and vasoconstriction, contributing to worm migration and survival in the lymphatic vessels (Liu et al., 1990). High concentrations of 12/15 HETE metabolites, $PGD_2$ and $TXB_2$ were measured in the peritoneal lavage after infection with *B. malayi* infected mice, and the eicosanoid profile of alternatively activated macrophages showed an induction of 12/15 HETE and the COX derived 6-keto-PGF1α (auto-oxidation product of $PGI_2$), while no 5-LOX derived metabolites were detectable (Thomas et al., 2012). A previous study found that $PGE_2$ was the main prostaglandin released by *B. malayi* as well as another filarial parasite *Wuchereria bancrofti* (Liu et al., 1992). The whipworm *Trichuris trichuris* also released high amounts of $PGE_2$, which was identified as the main anti-inflammatory compound present in worm secretions, suppressing the induction of pro-inflammatory TNF and IL-12 in LPS-stimulated DCs. Interestingly, the synthesis of $PGE_2$ was COX-independent, but the use of COX-inhibitors reduced the larvae´s motility and metabolic activity (Laan et al., 2017).

Taken together, helminth parasites have evolved different strategies to remodel the AA metabolism and modulate the host immune responses to longer survive in the host. However, it still remains poorly understood how the regulation of lipid mediators might affect the allergy modulation during helminth infection and if parasites and their immunomodulatory excretory/secretory products might have potential therapeutic applications for the treatment of allergic diseases.

## 3 AIM OF STUDY

The overarching objective of this thesis was to contribute to a better understanding of the mechanisms that regulate type 2 inflammation and to explore new immuneregulatory approaches for treating allergic asthma.

Asthma is a global health problem that affects 5-20% of the population worldwide, and its prevalence is increasing in many countries (Global Initiative for Asthma, GINA, 2018). However, several epidemiological studies have shown an inverse correlation between helminth infections and allergy, suggesting a role for the worms in suppressing the development of allergic diseases (Nyan et al., 2001; Scrivener et al., 2001; Obeng et al., 2014; Figuedeiro et al., 2010). Type 2 immune responses are associated with infection with worm parasites or exposure to allergens, playing an important role to control parasitic infections but causing pathology and chronic type 2 inflammation in allergy (Robinson et al., 1992; Hansen et. al., 1999; Locksley, 1994). Helminth parasites and their immunomodulatory excretory/secretory products are potent modulators of the innate and adaptive immune system to either suppress and/or induce immunomodulatory responses in the host. Thus, a better understanding of the type 2 immune mechanisms behind the protective and immunoregulatory effects induced by helminths will bring new insights in the development of therapies against allergic asthma.

Eicosanoids are lipid mediators playing an important role in inflammatory diseases such as asthma and allergy. Leukotrienes (LTs) are potent inducers of several hallmark responses of type 2 inflammation, promoting inflammatory cell recruitment, bronchoconstriction, airway inflammation and remodelling (Barret et al., 2011; Dahlén et al., 1981; Henderson et al., 2002). Although age-dependent clinical manifestations have been previously associated with airway remodelling in asthma (Gollwitzer et al., 2014; Saglani et al., 2013), it remained poorly understood whether the age of sensitization plays a role in the synthesis of LTs and the mechanisms behind its regulation. On the other hand, some prostaglandins (PGs) can countregulate the synthesis of LTs and the release of pro-inflammatory mediators, exerting a protective effect in the lung during airway inflammation (Hartet et al., 2000; Säfholm et al., 2015; Harizi et al., 2013). Thus, the modulation of AA metabolic pathways represents an important drug target in allergy and asthma, and a better understanding of the LTs synthesis depending on the age of sensitization might improve the treatment efficiency.

Interestingly, eicosanoids have also been suggested to participate in the type 2 immune response to helminth parasites. Different parasites can induce the synthesis of PGs which in turns help the infective larvae to migrate through the host and also contribute to suppress the host immune responses (Ramaswamy et al., 2000; Laan et al., 2017). However, it is still not well understood how parasites can modulate the AA metabolism during allergic asthma and whether this modulation might have a protective effect against airway inflammation in settings of type 2 immune responses.

Myeloid cells such as macrophages and granulocytes are main producers of leukotrienes, and thus contribute to the pathogenesis of allergy and asthma. Helminth parasites and their immunomodulatory compounds can directly act on macrophages and modulate type 2 inflammation, exerting anti-inflammatory effects (Goodridge et al., 2001; Ramos-Benitez et al., 2017; Venugopal et al., 2017). Eosinophils and neutrophils also represent important effector cells during helminth infections, and parasites aim to modulate granulocyte activation and recruitment to evade the host immune responses and facilitate the migration and survival of parasitic helminths (Cullet et al., 2000; Magalhaes et al., 2010; Smith et al., 2005). Thus, the identification

of helminth molecules responsible for the induction of a type 2 response to suppress clinical symptoms of allergy and asthma represents a promising alternative for the treatment of these diseases. Furthermore, the transfer of cells modulated by parasitic molecules to patients might represent a safer approach to treat inflammatory diseases and avoid the administration of live helminths or helminth derived products.

Thus, this research project **aimed to explore the modulation of AA metabolic pathways by helminth products as an innate regulatory strategy to modulate type 2 inflammation**. Modulation of the eicosanoid profile was observed *in vivo* during infection with the nematode *Heligmosomoides polygyrus bakeri* (*Hpb*) and *in vitro* by treatment of myeloid cells with an extract of this parasite (*HpbE*) and other helminth parasites. In addition, the mechanism underlying the regulation of lipid mediators by *HpbE* were explored in human and murine macrophages. Potential therapeutic application of this extract or *HpbE*-treated macrophages were tested during house dust mite (HDM)-induced allergic airway inflammation *in vivo*. Additionally,

In addition, this project aims to study the age-dependent activation of the LT pathways and to identify remodelling factors during early- or late-onset allergic airway inflammation in a mice model of HDM-induced allergy. The regulatory mechanism involved in the synthesis of LTs will be further explore in macrophages *in vitro*. The clinical relevance of the identified regulatory mechanism will be addressed by immunohistochemical staining of nasal polyps from Chronic rhinosinusitis with nasal polyposis (CRSwNP) patients *ex vivo.* Additionally, the therapeutic potential of *HpbE* will be compared to another standard drug used for asthma treatment in human macrophages from Aspirin exacerbated respiratory disease (AERD) patients. The characterization of the active immunomodulatory compounds present in *HpbE* will be an important step to translate these findings into a new therapy for type 2 inflammatory diseases. Additionally, the therapeutic potential of *HpbE* was compared to standard drugs used for asthma treatment in human macrophages from Aspirin exacerbated respiratory disease (AERD) patients. The characterization of the active immunomodulatory compounds present in *HpbE* will be an important step to translate these findings into a new therapy for type 2 inflammatory diseases.

In addition, this project **aimed to study the age-dependent activation of the LT pathways and to identify remodelling factors during early- or late-onset allergic airway inflammation in a mouse model of HDM-induced allergy**. The regulatory mechanism involved in the synthesis of LTs were investigated in macrophages *in vitro*. The clinical relevance of the identified regulatory mechanism was assessed by immunohistochemical staining of nasal polyps from Chronic rhinosinusitis with nasal polyposis (CRSwNP) patients *ex vivo.*

# 4 MATERIAL & METHODS

## 4.1 Material

### 4.1.1 Antibodies

| ANTIBODIES | SOURCE | METHOD (concentration/dilution) |
|---|---|---|
| Donkey anti-goat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 568, RRID AB_2534104 | Thermo Fisher Scientific | IF (1:500) |
| Donkey anti-goat IgG (H+L) affinity-purifies polyclonal antibody, HRP-conjugated | Novus Biologicals | IHC (1:500) |
| Donkey anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Biotin, RRID AB_228212. | Thermo Fisher Scientific | IHC (1:500) |
| Donkey anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 647, RRID AB_228212 | Thermo Fisher Scientific | IF (1:500) |
| Donkey anti-rat IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 488, RRID AB_2535794 | Thermo Fisher Scientific | IF (1:500) |
| Goat anti-human COX-2 affinity-purified polyclonal Antibody | Cayman Chemical | IF/IHC (1:100) |
| Goat anti-human $LTA_4$ hydrolase (C-21) affinity-purified polyclonal antibody | Santa Cruz Biotechnology | IF / IHC (1:50) |
| Goat anti-mouse IgG-HRP | Santa Cruz Biotechnology | WB (1:20000) |
| Goat anti-rabbit IgG-HRP | Santa Cruz Biotechnology | WB (1:10000) |
| Mouse anti-human β-actin monoclonal antibody (AC74) | Sigma-Aldrich | WB (1:20000) |
| Mouse anti-human CD193 (CCR3) (5E8) monoclonal antibody, BB700 | BD Biosciences | FACS (1:20) |
| Mouse anti-human Dectin-1 neutralizing monoclonal antibody | Invivogen | 10µg/ml |
| Mouse anti-human Dectin-2 neutralizing monoclonal antibody | Invivogen | 10µg/ml |
| Mouse anti-human IL-1β (8516.311) neutralizing monoclonal antibody | Abcam | 5µg/ml |
| Mouse anti-human TLR2 neutralizing antibody | Invivogen | 10µg/ml |
| Rabbit anti-human 15-LOX polyclonal antibody | Santa Cruz Biotechnology | IHC (1:100) |
| Rabbit anti-human 5-LOX affinity-purified polyclonal antibody | Professor O. Rådmark, Karolinska Institute, Stockholm, Sweden | IHC/ IF (1:50) |
| Rabbit anti-human α–SMA polyclonal antibody | Abcam | IHC (1:100) |
| Rabbit anti-human Collagen-1 polyclonal antibody | BD Bioscience | IHC (1:50) |
| Rabbit anti-human HIF-1α (C-Term) polyclonal antibody | Cayman Chemical | IF (1:100) |

| | | |
|---|---|---|
| Rabbit anti-human $LTC_4$ synthase (S-18)-R affinity-purified polyclonal antibody | Santa Cruz Biotechnology | IF / IHC (1:50) |
| Rabbit anti-human p38 MAPK (D13E1) XP® monoclonal antibody | Cell Signaling Technology | WB (1:1000) |
| Rabbit anti-human phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® monoclonal antibody | Cell Signaling Technology | IF / WB (1:100 / 1:1000) |
| Rabbit anti-human $sPLA_2$-X polyclonal antibody | Santa Cruz Biotechnology | IHC / IF (1:50) |
| Rabbit anti-human TGM2 polyclonal antibody | Cell Signaling Technology | IF/ IHC / WB (1:100 / 1:1000) |
| Rabbit anti-human Wnt5a polyclonal antibody | BD Bioscience | IHC / IF (1:50) |
| Rabbit anti-human ZO-1 polyclonal antibody | Thermo Fisher Scientific | IF (1:50) |
| Rat anti-human CD294 (CRTH2) (BM16) monoclonal antibody, PE-CF594 | BD Biosciences | FACS (1:20) |
| Rat anti-mouse F4/80 monoclonal antibody (BM8), RRID AB_467558 | Thermo Fisher Scientific | IF (1:100) |

**Table 3: List of antibodies used for immunohistochemistry (IHC) or immunofluorescence (IF) stainings, Western blots (WB), Flow cytometry (FACs) or neutralizing experiments in cell culture.**

### 4.1.2 Bacterial Strains

| BACTERIAL STRAINS | SOURCE |
|---|---|
| *Bacillus cereus* | Prof. Clarissa Prazeres da Costa, Technical University of Munich, Germany |
| *Enterococcus faecalis* | |
| *Paenibacillus odorifer* | |
| *Stenotrophomonas maltophilia* | |

**Table 4: List of bacterial strains identified in *Heligmosomoides polygyrus* extract (*HpbE*).**

### 4.1.3 Biological samples

| BIOLOGICAL SAMPLES | SOURCE |
|---|---|
| Human blood cells (healthy and AERD donors) | Center of Allergy & Environment, Technical University of Munich, Germany |
| Patient nasal polyp tissues | Department of Otolaryngology, Klinikum rechts der Isar, Technical University of Munich, Germany |

**Table 5: List of biological samples**

### 4.1.4 Chemicals & inhibitors

| CHEMICAL & INHIBITORS | SOURCE | METHOD (concentration/dilution) |
|---|---|---|
| 3,3′-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride | Sigma-Aldrich | - |
| Acriflavine | Sigma-Aldrich | 3µM |
| Anti-Wnt5a polyclonal antibody | BD Bioscience | 1:400 |
| BAY 11-7085 | Enzo Life Sciences | 5µM |
| Bithionol | Focus Biomolecules | 20µM |

| | | |
|---|---|---|
| CAY10404 | Cayman Chemical | 10µM |
| Cystamine dihydrochloride | Tocris Bioscience | 100µM |
| Fluticasone propionate | Sigma-Aldrich | 1µM |
| H-89 | Cayman Chemical | 10µM |
| HDM extract from *Dermatophagoides farinae* | Stallergenes SA | 5/10µg/ml |
| Human GM-CSF | Miltenyi Biotech | 10ng/ml |
| Human IL-8 | Miltenyi Biotec | 20ng/ml |
| Human RANTES | Miltenyi Biotec | 2ng/ml |
| Human TGF-β1, human | PeproTech | 2ng/ml |
| IL-4 | Promocell | 10ng/ml |
| Indomethacin | Cayman Chemical | 100µM |
| Leukotriene $B_4$ | Cayman Chemical | 2ng/ml |
| LPS | Invivogen | 60ng/ml |
| Monodansylcadaverine | Sigma-Aldrich | 1µM |
| Montelukast | Cayman Chemical | 10µM |
| Mouse GM-CSF | Miltenyi Biotech | 10ng/ml |
| $PGE_2$ | Cayman Chemical | 10µg/ml |
| SF1670 | Cayman Chemical | 250nM |
| Recombinant bovine GDH | Sigma-Aldrich | 10µg/ml |
| Recombinant human GDH | MyBioSource | 10µg/ml |
| Recombinant human liver Ferritin | Sigma-Aldrich | 1µg/ml |
| Recombinant microbial GDH | Swissaustral | 10µg/ml |
| VX-702 | Cayman Chemical | 1µM |
| Wortmannin | Cayman Chemical | 100nM |

**Table 6: List of chemicals & inhibitors used for Western Blot and experiments in cell culture.**

### 4.1.5 Commercial Assays

| COMMERCIAL ASSAYS | SOURCE |
|---|---|
| Bioplex Pro Reagent Kit V | Bio-Rad |
| Cysteinyl Leukotriene ELISA Kit | Cayman Chemical |
| Human IL-10 ELISA Set | BD Biosciences |
| Human IL-1β ELISA Set II | BD Biosciences |
| Leukotriene B4 ELISA Kit | Cayman Chemical |
| Magnetic Luminex Assay | R&D Systems |
| Prostaglandin E2 ELISA Kit | Cayman Chemical |
| Thromboxane B2 ELISA Kit | Cayman Chemical |

**Table 7: List of commercial assays.**

### 4.1.6 Experimental models: Organisms/Stains

| EXPERIMENTAL MODELS | SOURCE |
|---|---|
| Mouse: BALB/c | Charles River Laboratories |
| Mouse: C57BL/6 | École Polytechnique Fédérale de Lausanne (EPFL) |
| Mouse: C57BL/6J | Charles River Laboratories |
| Mouse: HIF-1$\alpha^{floxed/floxed}$ (C57BL/6J background) | Prof. Bernhard Brüne, Goethe-University Frankfurt |
| Mouse*: PTGS2*$^{-/-}$ (C57BL/6J background) | Prof. Rolf Nüsing, Goethe-University Frankfurt |

| | |
|---|---|
| *Heligmosomoides polygyrus bakeri* | Prof. Nicola Harris, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland |
| *Schistosoma mansoni* | Prof. Clarissa Prazeres da Costa, Technical University of Munich, Germany, |
| *Ascaris suum* | Prof. Peter Geldhof, Ghent University, Belgium |

**Table 8: List of mouse strains and helminths used for *in vivo* experiments and cell stimulation *in vitro*.**

### 4.1.7 Primers

Primers for quantitative real-time PCR experiments were purchased from Metabion International AG. Primers were reconstituted in DEPC treated water to a concentration of 100 pmol/µl and stored at -20ºC. The final concentration of a primer pair per qPCR reaction was 0,64 pmol/µl.

| | Gene | Forward primer sequence (5' – 3') | Reverse primer sequence (5' – 3') |
|---|---|---|---|
| **Human** | | | |
| Housekeeper | *GAPDH* | GAAGGTGAAGGTCGGAGT | GAAGATGGTGATGGGATTTC |
| LOX pathway | *ALOX5* | GATTGTCCCCATTGCCATCC | AGAAGGTGGGTGATGGTCTG |
| | *LTA4H* | CAGTGGCTCACTCCTGAACA | TCTGGGTCAGGTGTTTCTCC |
| | *LTC4S* | GACGGTACCATGAAGGACGA | GGAGAAGTAGGCTTGCAGCAG |
| | *CYSLTR1* | CAGTGGACGCTTTGATCTTA | AAGCTTGTCCACGCATATTA |
| COX pathway | *PTGS2* | GCTGGAACATGGAATTACCCA | CTTTCTGTACTGCGGGTGGAA |
| | *PTGES* | TCAAGATGTACGTGGTGGCC | GAAAGGAGTAGACGAAGCCCAG |
| | *PTGDS* | CCCAGGGCTGAGTTAAAGGA | AGAGCAGAGACATCCAGAGC |
| M2 markers | *ALOX15* | GGACACTTGATGGCTGAGGT | GTATCGCAGGTGGGGAATTA |
| | *MRC1* | CGATCCGACCCTTCCTTGAC | TGTCTCCGCTTCATGCCATT |
| | *TGM2* | AGGCCCGTTTTCCACTAAGA | AGCAAAATGAAGTGGCCCAG |
| Type 2 cytokines | *IL4* | GTGTCCTTCTCATGGTGGCT | CAGACATCTTTGCTGCCTCC |
| | *IL5* | TCTCCAGTGTGCCTATTCCC | CGAACTCTGCTGATAGCCAA |
| | *IL13* | GATTCCAGGGCTGCACAGTA | GGTCAACATCACCCAGAACC |
| | *IFNG* | TCAGCCATCACTTGGATGAG | CGAGATGACTTCGAAAAGCTG |
| | *IL10* | CTCATGGCTTTGTAGATGCCT | GCTGTCATCGATTTCTTCCC |
| **Mouse** | | | |
| Housekeeper | *Gapdh* | GGGTGTGAACCACGAGAAAT | CCTTCCACAATGCCAAAGTT |
| LOX pathway | *Alox5* | ATTGCCATCCAGCTCAACCA | ACTGGAACGCACCCAGATTT |
| | *Ltc4s* | ATCTTCTTCCACGAAGGAGCC | TCGCGTATAGGGGAGTCAGC |
| COX pathway | *Ptgs2* | GGGCCATGGAGTGGACTTAAA | TCCATCCTTGAAAAGGCGCA |
| | *Ptges* | GAAGAAGGCTTTTGCCAACCC | TCCACATCTGGGTCACTCCT |
| M2 markers | *Arg1* | GCAACCTGTGTCCTTTCTCC | TCTACGTCTCGCAAGCCAAT |
| | *Tgm2* | TAAGAGTGTGGGCCGTGATG | TTTGTTCAGGTGGTTGGCCT |
| | *Mrc1* | TTGCACTTTGAGGGAAGCGA | CCTTGCCTGATGCCAGGTTA |
| | *Tmed1 / St2l* | AGTAGAGGGTGACCCGGATG | GAACTCGCCGTCCTGGATAG |
| | *Retnla / Fizz1* | GGGATGACTGCTACTGGGTG | TCAACGAGTAAGCACAGGCA |

**Table 9: List of human and mouse primers.**

### 4.1.8 Software

| SOFTWARE | SOURCE | WEBSITE |
|---|---|---|
| Cell profiler | Broad Institute, Cambridge, Mass | cellprofiler.org/ |
| FlowJo v10 software | FlowJo LLC | www.flowjo.com |
| GraphPad Prism version 6 | GraphPad Software | www.graphpad.com |

| ImageJ Fiji | National Institute of Health, Bethesda, Md | imagej.net/Fiji |
|---|---|---|
| LabImage 1D software | Bio-Imaging | |
| MetaboAnalyst version 3.0 | Mc Gill University, Quebec, Canada | www.metaboanalyst.ca |
| Swiss-Prot *Heligmosomoides bakeri* Database | UniProt (24.01.2017 edition) | |
| Proteome Discoverer 1.4 software | ThermoFisher Scientific | |
| TargetLinx application manager | Waters, Mildford | |

**Table 10: List of software used for data analysis.**

### 4.1.9 Instruments

| INSTRUMENTS | SOURCE |
|---|---|
| AKTA pure system with Superdex 75 10/300 GL column | GE Health Care Life Science |
| BD LSRFortessa™ | BD Biosciences |
| ECL ChemoCam Imager | Intas Science Imaging Instruments |
| EVOS™ FL Auto Imaging System | Thermo Fisher Scientific |
| Leica SP5 confocal microscope | Leica Microsystems |
| LC-MS/MS: Agilent 1290 coupled to QTRAP 5500 | Agilent/ AB Sciex |
| LC-MS/MS: UPLC Acquity coupled to Xevo TQ-S | Waters, Milford |
| PCR machine TC-412 | Techne Inc. |
| ViiA 7 Real-Time PCR System Applied Biosystems | Thermo Fisher Scientific |

**Table 11: List of applied instruments.**

## 4.2. Methods

### Mice

C57BL/6J mice were bred and maintained under specific pathogen free conditions at the École Polytechnique Fédérale de Lausanne (EPFL) or at the Centre Hospitalier Universitaire Vaudois (CHUV). Alternatively, BALB/c and C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Unless stated otherwise, 6-12 weeks old mice of both sexes were used. All animal experiments were approved by the local authorities (Swiss Veterinary Office).

### Human blood and tissue samples

Peripheral blood mononuclear cells (PBMCs) or Polymorphonuclear leukocytes (PMN) were isolated from the blood of healthy human donors or patients with Aspirin-exacerbated respiratory disease (AERD). Nasal polyp tissues or inferior turbinates were obtained during polypectomy of patients suffering from chronic rhinosinusitis with nasal polyps (CRSwNP). All patients with CRSwNP received systemic steroids preoperatively. Nasal tissues were washed twice with medium for removal of blood and mucus and cut into several pieces. Some pieces of nasal polyp tissues were fixed in 10% formalin for histology. The rest of nasal polyp pieces were cultured overnight in Airway Epithelial Cell Growth Medium plus antibiotics/antimycotis for 24 hours (5% $CO_2$ at 37°C) under air-liquid interface conditions in order to obtain nasal polyp secretions

for LC-MS/MS analysis or cell culture stimulation. Blood samples and nasal tissues were obtained after informed written consent from the donors. Blood and tissue sampling and experiments including human blood cells were approved by the local ethics committee at the University clinic of the Technical University of Munich (TUM).

### 4.2.1 Parasite Infection and Preparation of Parasite Extracts

#### 4.2.1.1 *Heligmosomoides polygyrus bakeri*

The experiments involving the *in vivo* mice infection with *Heligmosomoides polygyrus bakeri (Hpb)* were performed in collaboration with the laboratory of Prof. Nicola Harris (Lausanne University, Switzerland). Infective stage-three larvae (L3) of *Hpb* were obtained from the eggs of *Hpb*-infected mice (Camberis et al., 2003). Wildtype or HIF-1α$^{floxed/floxed}$ x LysMCre mice were infected with 200 *Hpb* L3 larvae by oral gavage and small intestines were harvested at day 4-7 post-infection. Mice were lavaged two times with 0.5 ml PBS to obtain the peritoneal wash and mixed with MeOH for LC-MS/MS analysis. Intestinal tissues were prepared as "swiss rolls" and fixed in paraffin for preparation of histological section. Small sections of the intestinal tissues were also culture in medium while shaking for 30 minutes and collected with MeOH for eicosanoid analysis.

For preparation of *Hpb* larval extract (*HpbE*), L3 larvae were homogenized in two cycles at 6.000 rpm for 60 seconds in a Precellys homogenizer using Precellys tough micro-organism lysing kits VK05 (Bertin Pharma). Remaining debris were removed by centrifugation (20 min, 14.000 rpm, 4°C). When indicated, heat inactivated-*HpbE* (*HpbE* 90ºC) was prepared by heat treatment at 90ºC overnight.

#### 4.2.1.2 *Schistosoma mansoni*

Mice infection with *Schistosoma mansoni (S.m.)* was performed in collaboration with the laboratory of Prof. Clarissa Prazeres da Costa (TUM, Munich). For the experimental infection, cercaria from a Brazilian strain of *S.m.* were isolated from *Biomphalaria glabrata* snails (Brazilian origin), and mice were infected intraperitoneally with 100–140 cercariae (Ritter et al., 2010). Lung sections and liver sections were obtained 3 or 6 weeks post-infection respectively and fixed in paraffin for preparation of histological section

*Schistosoma mansoni* (*S.m.)* extract (*SmE*) was prepared from newly transformed schistosomula (NTS) (Keiser, 2010). Cercaria from a Brazilian strain of *S.m.* were isolated from *Biomphalaria glabrata* snails using the light induction method as previously described (Ritter et al., 2010). After thorough washing, cercaria were re-suspended in ice-cold HBSS medium, pipetted vigorously and vortexed three minutes at the highest speed to trigger tail loss, which was confirmed by microscopy. NTS were then cultured in hybridoma medium (Biochem) at 37°C, 5% $CO_2$ in ambient air for 7 days. At this time point all NTS had transformed into lung stage larvae. These were harvested and homogenized in culture medium using Precellys tough micro-organism lysing kits (Bertin Pharma) at 6000 rpm for 30 secs in two cycles. Debris were removed by centrifugation at 13.000 rpm for 20 min.

#### 4.2.1.3 *Ascaris suum*

*Ascaris suum* larval extract (*AsE*) was prepared from the infected stage-three larvae (L3) of *A. suum*, a kind gift from Prof. Peter Geldhof (Ghent University, Belgium). *A.s* L3 stage larvae were obtained from eggs (Urban et al., 1981) and L3 larvae were placed in a bath of liquid nitrogen and grinded with a mortar and pestle. The larval homogenate was mixed with PBS and proteinase inhibitor cocktail (Sigma-Aldrich) and inverted at 4°C for 2 hours, followed by centrifugation at 10.000 g for 30 min at 4°C. Supernatants were removed and sterile-filtered (0.22 µm). Filtrates were concentrated at 4°C using a Centripep centrifugal filter with YM-3 membranes (Millipore) (Vlaminck et al., 2016).

The protein concentration in all extracts was determined by the BCA method (Pierce, Thermo Fisher Scientific). Helminth extracts were stored at -80°C until further use.

#### 4.2.1.4 Identification of *Hpb*-associated bacteria

*Hpb* larval extract (*HpbE*) were plated on Columbia and McConkey Agar plates (for aerobic bacteria) and KV (Kanamycin/Vancomycin) and Schaedler plates (anaerobic bacteria) (all plates from Biomerieux) and incubated at 5% $CO_2$ or in anaerobic chambers for 48 hrs at 36°C. No anaerobic growth was observed. Aerobic bacteria (*Bacillus cereus, Enterococcus faecalis, Paenibacillus odorifer, Stenotrophomonas maltophilia)* were counted and identified with Maldi-TOF-MS (Microflex, Bruker). A suspension with a mix of bacteria at identical concentrations as in the original *Hpb* larval extract was prepared in broth prior to use in cell culture.

### 4.2.2 Culture and stimulation of Bone Marrow-derived Macrophages

Bone marrow derived macrophages (BMDM) were obtained from bone marrow of wildtype C57BL/6 or HIF-1$\alpha^{floxed/floxed}$ x LysMCre mice. Skin and muscle were removed from the mouse legs until the bones were clean and bone marrow cells were flushed out onto a cell strainer (70µm), mashed through the filter and washed several times with medium. Cells were counted, resuspended in freezing medium at a density of 10-25x$10^6$ cells/ml and stored at -80ºC until further use. Before every experiment, bone marrow cells were resuspended in medium at a density of 1x$10^6$ cells/ml and culture for 6 days in the presence of 10ng/ml murine recombinant GM-CSF (Miltenyi Biotech) and 2ng/ml human recombinant TGF-β1 (Peprotech). Half medium was exchanged and supplemented with msGM-CSF and hTGF-β1 every other day. On day 6, BMDM were harvested by scraping and only adherent cells were resuspended in medium at a density of 1x$10^6$ cells/ml in the presence of GM-CSF and TGF-β1 (Esser-von Bieren et al., 2013). BMDM were stimulated with 10µg/ml *HpbE* or 10µg/ml HDM (Stallergens) overnight. When indicated, the pharmacological inhibitor Indomethacin (100µM, Cayman Chemical) was added one hour before cell stimulation. After overnight stimulation, BMDM were stimulated with 5µM $Ca^{2+}$-ionophore A23187 (Sigma Aldrich) for 10min and supernatants were stored at -80ºC in 50% MeOH for LC-MS/MS analysis or undiluted for cytokine analysis. Cell pellets were lysated in RLT buffer with 1% β-mercaptoethanol (Merck Millipore) and stored at -80ºC for RNA extraction.

### 4.2.3 Isolation and stimulation of peripheral blood mononuclear cells

Before every experiment, healthy individuals or AERD patients were recruited and blood was freshly drawn in a blood tube pre-coated with EDTA in order to avoid blood coagulation. Blood was carefully overlayed in an equal volume of Polymorphprep (Axis-Shield) and cells were isolated by density gradient. After centrifugation for 35 min at 1600rpm at 20ºC without brake, the upper cell layer corresponding to the Peripheral blood mononuclear cells (PBMCs) (Figure 7) was carefully removed and transferred to a tube with 40ml of PBS. Cells were washed once with PBS, counted and resuspended in medium in the presence of 10ng/ml human GM-CSF (Miltenyi Biotech). PBMC were stimulated with 10 µg/ml *Hpb*E overnight, followed by 5µM $Ca^{2+}$-ionophore A23187 (Sigma Aldrich) stimulation for 10min. Supernatants were stored at -80ºC in 50% MeOH for LC-MS/MS analysis. Cell pellets were lysated in RLT buffer with 1% β-mercaptoethanol (Merck Millipore) and stored at -80ºC for RNA extraction.

### 4.2.4 Isolation and stimulation of Monocyte-Derived Macrophages

Monocyte-derived macrophages (MDMs) were generated from PBMCs by density gradient centrifugation as explained in section 4.2.3. After centrifugation, the PBMCs layer was collected and transferred to a tube with 40ml of PBS. Cells were washed once with MACs buffer and $CD14^+$ monocytes were isolated by magnetic cells sorting (MACS) technology using human CD14 Microbeads, LS columns and the QueadroMACS Separator (all Miltenyi Biotec) according to the manufacturer´s instruction (Figure 7). Monocytes were cultured in medium at a density of $0.5x10^6$ cells/ml and differentiated to macrophages in the presence of 10ng/ml human GM-CSF (Miltenyi Biotech) and 2ng/ml human TGF-β1 (Peprotech) for 6 days. Half medium was exchanged and supplemented with hGM-CSF and hTGF-β1 on day 3. On day 6, MDM were harvested by scraping, counted and resuspended in medium at a density of $1x10^6$ cells/ml for cell culture experiments.

When indicated, MDM were stimulated overnight with 10 µg/ml parasite extracts (*HpbE*, *SmE*), 10 µg/ml heat treated-*HpbE* (*HpbE* 90ºC), 10 µg/ml *H. polygyrus* excretory/secretory products (HES), bacterial homogenate (*HpbE*-bacteria), 60ng/ml LPS (Invivogen), 10ng/ml IL-4 (Promocell), 10µM $PGE_2$ (Cayman Chemical), 1µM Fluticasone Propionate (Sigma-Aldrich), 10µg/ml HDM (Stallergens). For inhibitor studies, the following pharmacological inhibitors were added as indicated one hour before *HpbE* stimulation: 5 µM BAY 11-7085 (Enzo Life Sciences), 10µM CAY10404, 10 µM H-89, 100 µM Indomethacin, 250 nM SF1670, 1 µM VX-702, 100nM Wortmannin (all Cayman Chemical), 3 µM Acriflavine (Sigma-Aldrich), and 20µM Bithionol (Focus Biomolecules). For experiments with neutralizing antibodies, MDM were incubated with 10 µg/ml anti-dectin-1 antibody, 10 µg/ml anti-dectin-2 antibody or 10 µg/ml anti-TLR2 antibody (all Invivogen) or 5 µg/ml anti-IL-1β antibody (Abcam) one hour before *HpbE* stimulation. When mentioned, MDM were overnight stimulated with *HpbE* fractions as indicated. For some experiments, MDM were stimulated with 1 or 10 µg/ml human GDH (MyBioSource), bovine GDH (Sigma-Aldrich) and microbial GDH (Swissaustral) and 1 µg/ml human liver ferritin (Sigma-Aldrich).

In another set of experiments, MDM were overnight stimulated with epithelial derived CM and anti-Wnt5a antibody (R&D Systems) in a 1:400 dilution and with IL-4 supplemented monocyte medium (previously pre-incubated for 24h at 37ºC). MDM were also stimulated with 50ng/ml human recombinant IL-4 (Promokine, Promocells) and treated with TGM2 inhibitors 25 µM

Monodansylcadaverine (Sigma-Aldrich) and 100 µM Cystamine (Tocris Bioscience). After overnight stimulation, MDM were stimulated with 5µM $Ca^{2+}$-ionophore A23187 (Sigma Aldrich) for 10min and supernatants were stored at -80ºC in 50% MeOH for LC-MS/MS analysis or undiluted for cytokine analysis. Cell pellets were lysated in RLT buffer with 1% β-mercaptoethanol (Merck Millipore) and stored at -80ºC for RNA extraction. In some experiments, cell pellets were lysated with RIPA buffer and stored at -80ºC for Western blot or MDM were fixed and stained for Immunofluorescence purposes.

### 4.2.5 Isolation and stimulation of Polymorphonuclear cells

As previously mentioned in section 4.2.3, blood was drawn from healthy individuals or AERD patients and the cells were isolated by density gradient. After centrifugation for 35 min at 1600rpm at 20ºC without brake, the lower cell layer corresponding to Polymorphonuclear cells (PMN) (Figure 7) was carefully removed and transferred to a tube with 40ml of PBS. PMN were washed with PBS, resuspended in 1x lysis buffer and incubated for 10-15min at room temperature. PMN were washed with PBS and resuspended in medium supplemented with 100ng/ml human GM-CSF (Miltenyi Biothec). PMN were stimulated overnight with 10µg/ml parasite extracts (*Hpb*E, *AsE*) followed by stimulation with 5µM $Ca^{2+}$-ionophore A23187 (Sigma Aldrich) for 10min. PMN supernatants were stored at -80ºC in 50% MeOH for LC-MS/MS analysis. Cell pellets were lysated in RLT buffer with 1% β-mercaptoethanol (Merck Millipore) and stored at -80ºC for RNA extraction. In some experiments, cell pellets were fixed and stained for flow cytometry analysis.

### 4.2.6 Isolation and stimulation of Eosinophils

Eosinophils were generated from PMN by density gradient centrifugation as explained in section 4.2.5. After centrifugation and the lysis step, PMN were washed with MACS buffer and CD16- eosinophils were isolated by magnetic cells sorting (MACS) technology using CD16 Microbeads, LS columns and the QueadroMACS Separator (all Miltenyi Biotec) according to the manufacturer´s instruction (Figure 7). Eosinophils were resuspended in medium at a density of $1x10^6$ cells/ml and cultured in the presence of 100ng/ml human GM-CSF and overnight stimulated with 10µg/ml parasite extracts (*Hpb*E, *AsE*), followed by stimulation with 5µM $Ca^{2+}$-ionophore A23187 (Sigma Aldrich) for 10min. Eosinophil supernatants were stored at -80ºC in 50% MeOH for LC-MS/MS analysis.

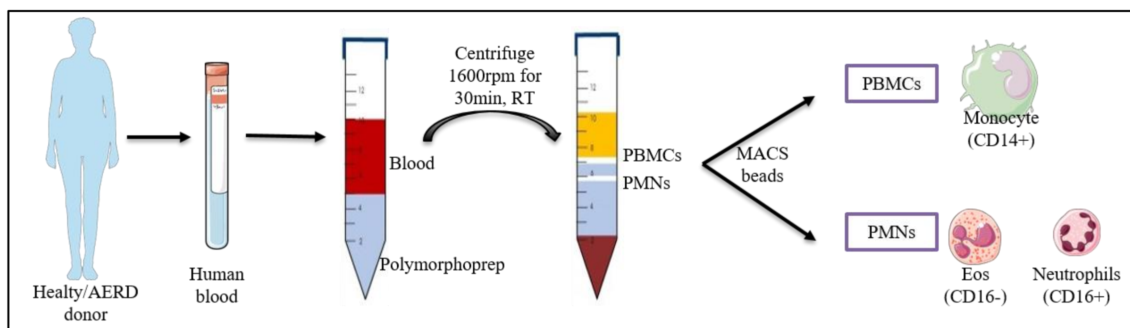


**Figure 7: Isolation of human blood cells from healthy or AERD patients.** Human blood was freshly drawn and blood cells (PBMCs, PMN, monocytes or eosinophils) were isolated by density gradient and isolated by magnetic cell sorting (MACs) beads. (Abbreviations: AERD, Aspirin exacerbated respiratory

disease; CD, cluster of differentiation; Eos, eosinophils; MACs, magnetic cells sorting; PBMCs, peripheral blood mononuclear cells; PMN, Polymorphonuclear cells; RT, room temperature)

### 4.2.7 Culture and stimulation of human bronchial epithelial cells

Normal human bronchial epithelial cells (NHBEs) isolated from non-smoking and non-drinking individuals were purchased from Lonza, expanded in Bronchial Epithelium Growth Medium (BEGM, Lonza) and stored in liquid nitrogen in passage (p) 2 until further use. Before every experiment, NHBEs were further expanded and then seeded in 6 or 12-well plates at a density of 1.5 - 2.5 x $10^5$ cells/well, until they reach 80-85% confluency. Cells were serum-deprived in basal medium (BEBM, Lonza) overnight before stimulation with 10 µg/ml parasite extracts (*HpbE, SmE, AsE, OstE, CoopE*). After 24hours stimulation, NHBEs were ionophore stimulated for 10 min and the cell supernatant were stored at -80ºC in 50% MeOH for LC-MS/MS analysis.

### 4.2.8 Mouse model of House dust mite-induced allergic airway inflammation

#### 4.2.8.1 Treatment with *HpbE* during allergic airway inflammation

The experiments involving the *in vivo* sensitization of mice with House dust mite (HDM) were performed in collaboration with Prof. Francesca Alessandrini (ZAUM, Munich). Eight-weeks old female C57BL/6J mice were sensitized on day 0 by bilateral intranasal (i.n.) instillations of HDM extract from *Dermatophagoides farinae* (1 µg extract in 20 µl PBS; Stallergenes SA) and challenged on days 8-11 with 10 µg HDM dissolved in 20 µl PBS. Control animals received i.n. the same amount of PBS. *HpbE* treatment (5 µg *Hpb* extract in 20 µl PBS) was performed intranasally before sensitization and challenge. In the absence of *HpbE* treatment, the mice received 20 µl PBS (Figure 8A). Three days after the last challenge, the airways of the mice were lavaged five times with 0.8 ml PBS. Aliquots of cell-free BAL fluid were frozen immediately with or without equal volumes of methanol for LC-MS/MS or cytokine analysis respectively. Viability, yield and differential cell count of BAL cells were performed as described before (Alessandrini et al., 2006). Lungs were removed and fixed in 10% buffered formalin. Lung sections were stained with Haematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS).

#### 4.2.8.2 Transfer of Bone Marrow-Derived Macrophages during allergic airway inflammation

The experiments involving the transfer of BMDM during HDM-induced allergy were performed in collaboration with the laboratory of Prof. Nicola Harris (Lausanne University, Switzerland). Eight-weeks old female C57BL/6 mice were sensitized i.n. with HDM extract on d0. For the transfer experiment, BMDM from bone marrow of wildtype C57BL/6 or PTGS2$^{-/-}$ mice were isolated and differentiated for 6 days in the presence of murine recombinant GM-CSF and human recombinant TGF-β1 as previously explained in section 4.2.2. BMDM (wt or PTGS2$^{-/-}$) were pooled from 3 mice and incubated for 24 h in the presence or absence of 10 µg/ml *HpbE*. 45 min before intranasal transfer, BMDM were harvested by scraping, washed and resuspended in PBS. On days 8-11, control mice received 20 µl PBS i.n., and HDM-challenged mice received PBS or 3x$10^5$ BMDM (wt or PTGS2$^{-/-}$) +/- *HpbE* in 20µl PBS i.n. before HDM challenge. Mice were

sacrificed 18 h after the last challenge. BALF was collected using 0.5 ml PBS and stored with or without MeOH for LC-MS/MS or cytokine analysis respectively (Figure 8A). Viability, yield and differential cell count of BAL cells were performed as previously described. Lungs were removed and fixed in 10% buffered formalin. Lung sections were stained with Haematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS).

### 4.2.8.3 Mouse model of allergic airway inflammation at different ages

The experiments involving the *in vivo* mouse sensitization with HDM at different ages was performed in collaboration with Dr. Eva Gollwitzer (Lausanne University, Switzerland). BALB/c mice were i.n. sensitized with 5 µg HDM from day 3, 15 or 60 after birth. Mice were treated with HDM every second day for a total of six exposures (Gollwitzer et al., 2014). Four days after the last challenge, BALF was collected for eicosanoid quantification (Figure 8B). Lungs were fixed in paraffin and lung sections were stained as explained in section 4.2.12 and 4.2.13.

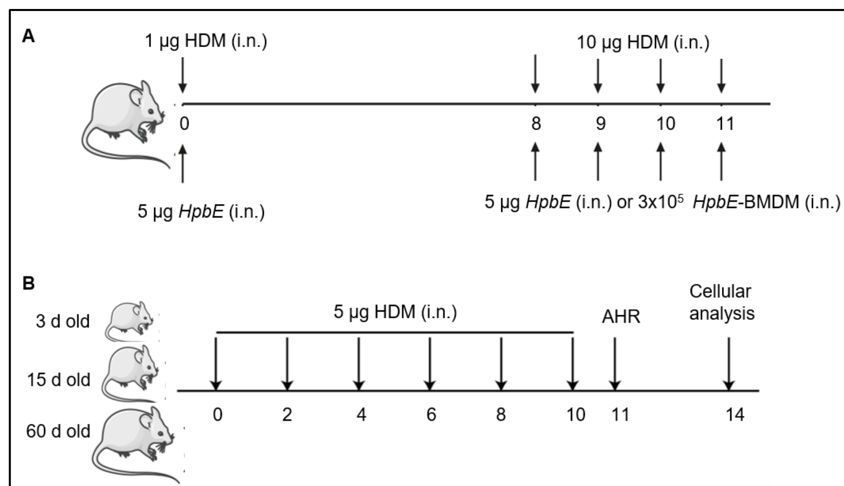


**Figure 8: Experimental model of house dust mite-induced allergic airway inflammation.** (A) Experimental mouse model of HDM-induced allergic airway inflammation and intranasal treatment with *HpbE* (5µg) or transfer of *HpbE*-conditioned BMDM (3x10$^5$ BMDM) (B) Experimental age-dependent mouse model of HDM-induced allergic airway inflammation from day 3, 15 or 60 of life. (Abbreviations: AHR, airway hyperresponsiviness; BMDM, bone marrow derived macrophage; d, days; HDM, house dust mite; *HpbE*, *Heligmosomoides polygyrus bakeri* extract)

### 4.2.9 RNA extraction

Cells pellet were washed with PBS, lysed in 200µl or 350µl RLT buffer with 1% β-mercaptoethanol and stored at -80ºC. RNA was extracted using the Quick-RNA MicroPrep (Zymoresearch) according to the manufacturer´s instructions. DNase digestion was performed using an RNase-free DNase Set (Zymoresearch). The RNA was eluted in 15µl DEPC treated water and the RNA concentration was measured using a NanoDrop 2000 spectrophotometer.

### 4.2.10 cDNA synthesis

Isolated RNA was subjected to reverse transcription by using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) (Table 12). A maximum of 2µg of RNA was

transcribed with a total volume of up to 20µl. The DNA was stored at -20ºC until further analysis using Real-Time Quantitative PCR.

| | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| **Temperature (ºC)** | 25 | 37 | 85 | 4 |
| **Time** | 10 min | 120 min | 5 min | ∞ |

**Table 12: Cycling conditions of cDNA reverse transcription reaction**

### 4.2.11 Real-Time Quantitative PCR

Real-time PCR was performed using a ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). After cDNA synthesis, the samples were diluted in DNase/RNase-Free water to a concentration of 2.941 ng/µl. 3.4µl of cDNA was mixed with 1.6µl of a primer pair solution (4µM each primer) (see table 9 for the list of primers) and 5µl FastStart Universal SYBR Green Master Mix (Roche) in a 384-well PCR Plate. The plate was perfectly sealed and shortly centrifuged before running the method shown in table 13. All samples were run in duplicates. Gene expression levels were determined by the comparative $C_T$ method using the equation 2-$\Delta\Delta C_T$. Transcript levels were normalized to the house-keeping gene *GAPDH*.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| **Initial denaturation** | 98 ºC | 30 s | 1x |
| **Denaturing**<br>**Annealing**<br>**Extension** | 98 ºC<br>59.4/64.3 ºC<br>72 ºC | 10 s<br>30 s<br>30 s/kb | 35x |
| **Final Extension**<br>**Hold stage** | 72 ºC<br>4 ºC | 10 min<br>∞ | 1x |

**Table 13: Cycling conditions for real time qPCR**

### 4.2.12 Immunohistochemistry

Lung-, small intestinal or nasal polyp tissues were fixed in 10% formalin, paraffin embedded and cut in sections of 4µm using a microtome (Leica Microsystems). After dewaxing at 65ºC for 30min, tissues were rehydrated 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 in distilled water and 1x in PBS. Tissues were permeabilized in PBS with 0.2% Triton X-100 for 45 min at RT. Slides were washed 3x with PBS. After blocking unspecific binding with 5% BSA plus 10% donkey serum for 1 hour at RT, sections were incubated with the following primary antibodies: rabbit anti-human 5-LOX antibody (a kind gift of Professor O. Rådmark, Karolinska Institute, Stockholm, Sweden), TGM2 (Cell Signalling Technology), 12/15-LO, α-SMA (both from Abcam), Wnt5a (Lifespan Bioscience), $LTC_4S$, $sPLA_2$-X and $LTA_4H$ (all from Santa Cruz), Collagen-1 A1 (BD Bioscience) (the antibody dilution is included in table 3). After overnight incubation in a moisture chamber at 4ºC, sections were incubated in secondary biotinylated donkey-anti rabbit antibody (see table 3) for 1 hour at RT in a moisture chamber. After a washing step, detection was performed using a horseradish peroxidase-based detection kit (ABC, Thermo Fisher Scientific) by using 3,3´-diaminobenzidene detection reagents (DAB, Sigma-Aldrich). Hämalaum solution (Carl Roth) was used for nuclear counterstaining and tissue were dehydrated in ascending alcohol series. Slides were covered with a coverslip using Histokitt. For Haematoxylin and eosin (H&E)- or Periodic acid-Schiff (PAS) staining, lung tissues were fixed

and stained as described previously (Alessandrini et al., 2006). Images were recorded on an EVOS system (Thermo Fisher Scientific).

### 4.2.13 Immunofluorescence

For immunofluorescent staining, lung tissues were fixed, dewaxed and rehydrated as described in section 4.2.12. For immunofluorescent staining of cells, macrophages were seeded in 12-well chamber slides (Ibidi) and after overnight stimulation, cells were washed 5x with PBS and fixed for 15 minutes with 4% paraformaldehyde at RT, followed by 3x washing with PBS and permeabilization with acetone for 10 minutes at -20°C. Cells were 3x washed with PBS and blocked with 5% BSA and 10% donkey serum for 1 hour. Cells or tissues were incubated with primary antibodies rabbit anti-human 5-LOX, goat anti-human Cyclooxygenase-2 (Cayman Chemical), rabbit anti-human HIF-1α, rat anti-mouse F4/80 (Thermo Fisher), anti-human ZO-1 (Life Technologies), anti-human TGM2 (Cell Signalling Technology) in a moisture chamber overnight at 4ºC (the antibody dilution is included in table 3). After 3 washing steps, cells or tissues were incubated with a fluorescence-conjugated secondary antibodies (see table 3) for 1,5hours at RT in the dark. After 2x washing steps, slides were covered with mounting medium containing DAPI staining (Vectashield, Vector Laboratories) and dry for 1h at 4ºC in the dark. Slides were sealed with nail polish and stored at 4ºC in the dark. Images were recorded on a Leica SP5 confocal microscope (Leica Microsystems) the next day.

### 4.2.14 Western Blotting

Cells were lysed in RIPA buffer (Thermo Fisher Scientific) supplemented with 2% EDTA-free complete protease inhibitor cocktail and 10% phosphatase inhibitor cocktail (both from Roche Applied Science) using a cell scraper. The protein concentration was determined by the BCA method (Thermo Fisher Scientific) and lysates were diluted to equal concentrations. NuPAGE LDS Sample buffer and NuPAGE Sample Reducing Agent (Thermo Fisher Scientific) was added to total lysates, and heated at 70 °C for 10 minutes. Samples were loaded on Bolt 4-12% Bis-Tris Plus gels (Thermo Fisher Scientific) and proteins were separated by electrophoresis at 120V for 90min. Gels were transferred to a PVDF membrane (Merck Chemical) using Mini Blot Module (Thermo Fisher Scientific) at 20 Volt for 90min according to the manufacturer´s instructions. After a washing step in 1x TBS containing 0.02% Tween, the PVDF membrane were blocked in 5% non-fat dry milk in 1x TBS containing 0.02% Tween for 1 hour to prevent unspecific binding. Membranes were incubated overnight with primary antibodies against COX-2 (Cayman Chemical, dilution 1:1000), phospho-p38 and p38 (both Cell Signalling, dilution 1:1000), β-actin (Sigma-Aldrich, dilution 1:10000) or TGM2 (Cell Signalling Technology, dilution 1:1000) at 4ºC in a 50ml Falcon tube on a tube roller. Membranes were washed for 30min at RT and incubated with the corresponding secondary horseradish peroxidase-conjugated antibody (see table 3) for 1hour at RT. Following 30min of washing, detection was performed using enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific, or Amersham ECL Prime, GE Healthcare Life Technologies) according to manufacturer´s instructions and recorded with the ECL ChemoCam Imager (Intas Science Imaging Instruments). LabImage 1D software (Kapelan Bio-Imaging) was used to quantify the protein levels by means of normalization and correction for the amount of β-actin in the samples.

### 4.2.15 Enzyme immunoassays (EIA)

The concentration of $LTB_4$, CysLTs, $PGE_2$ and $TXB_2$ in cell culture supernatants was determined by using commercially available enzyme immunoassay (EIA) kits (Cayman Chemical), according to the manufacturer's instruction.

### 4.2.16 ELISAs

Cell culture supernatants were analysed for IL-10 or IL-1β secretion using the human IL-10 or IL1-β ELISA Set (BD Biosciences), according to the manufacturer's instructions.

### 4.2.17 Bioplex Assay

Mouse cell culture supernatants were analysed by Multiplex cytokine assays (Bioplex Pro Reagent Kit V, Bio-Rad) for the detection of murine IL-1β, IL-6, IL-10, Eotaxin (CCL11), IL-13, RANTES (CCL5) and TNFα.

Human cell culture supernatants were analysed by Magnetic Luminex Assay (R&D Systems) for the detection of human Eotaxin (CCL11), GROα (CXCL1), GROβ (CXCL2), IL-1β, IL-18, IL-6, TARC (CCL17), IP-10 (CXCL10), IL-8 (CXCL8), IL-10, IL-27, TNFα, RANTES (CCL5), ITAC-1 (CXCL11), MIG (CXCL9), IL-12 p70 and IL-33. Both commercially available kits were performed according to the manufacturer's instructions on a Bio-Plex 200 System (Bio-Rad).

### 4.2.18 LC-MS/MS analysis

LC-MS/MS analysis was performed in collaboration with Yvonne Schober and Wolfgang Nockher (Philipps-University Marburg, Germany) and Dominique Thomas (Goethe University Frankfurt, Germany). Intestinal or cell culture supernatants were adjusted to 10% methanol containing deuterated internal standard followed by an extraction using solid reverse phase extraction columns (Bond Elut Plexa 30 mg, Agilent). Samples were eluted into 1.0 ml of methanol, lyophilized and resuspended in 100 µl of water/acetonitrile/formic acid (70:30:0.02, v/v/v; solvent A) and analysed by LC-MS/MS on an Agilent 1290 liquid chromatography separation system. Separation was performed on a Synergi Hydro reverse-phase C18 column (2.1×250 mm; Phenomenex) using a gradient as follows: flow rate =0.3 µl/min, 1 min (acetonitrile/isopropyl alcohol, 50:50, v/v; solvent B), 3 min (25% solvent B), 11 min (45% solvent B), 13 min (60% solvent B), 18 min (75% solvent B), 18.5 min (90% solvent B), 20 min (90% solvent B), 21 min (0% solvent). The separation system was coupled to an electrospray interface of a QTrap 5500 mass spectrometer (AB Sciex). For inhibitor experiments, a smaller LC-MS/MS panel was used. Briefly, analytes were separated under gradient conditions within 16 min: 1 min (90% solvent A), 2 min (60% solvent A), 3 min (50% solvent A), 3 min (10% solvent A). Within 1 min, the initial conditions were restored and the column was re-equilibrated for 6 min. Mass spectrometric parameters were as follows: Ionspray voltage -4500 V, source temperature 500 °C, curtain gas 40 psi, nebulizer gas 40 psi and Turbo heater gas 60 psi. Both quadrupoles were running at unit resolution. Compounds were detected in scheduled multiple reaction monitoring mode. For quantification a 12-point calibration curve for each analyte was used. Data analysis was performed using Analyst (v1.6.1) and MultiQuant (v2.1.1) (AB Sciex).

In some experiments, LC-MS/MS analysis was performed during my EAACI Research Fellowship at Karolinska Institutet, in collaboration with Dr. David Fuchs and Prof. Craig Wheelock (Karolinska Institute, Sweden). Cell supernatants were adjusted to 10% MeOH and 10 µL of the internal standard (IS) working solution was added to each sample in order to correct for variations due to sample preparation or variations in electrospray ionization. Samples were diluted (1:1) with extraction buffer (citric acid: $Na_2HPO_4$, pH=5.6) followed by solid phase extraction (SPE) using an automated liquid handling system Extrahera (Biotage) equipped with SPE cartridges (Evolute Express ABN 60mg, Biotage). After conditioning and equilibration of the cartridges with 2.5 mL of methanol and water, the samples were loaded and washed with 2 mL of water/methanol (90:10, v/v) and eluted with 2.5 mL of methanol. 30 µL glycerol/methanol (30:70, v/v) was added as trap solvent before evaporation using $N_2$ gas (TurboVap LV, Biotage). Samples were reconstituted to a final volume of 70 µL in methanol/water (6:1, v/v) and filtered through a 0.1 µm polyvinylidenfluorid membrane spin filter (Amicron, Merck Millipore) by 5 min centrifugation at 5 °C using 12.000 rcf. LC-MS/MS analysis was performed using an UPLC Acquity coupled to a Xevo TQ-S mass spectrometer system (Waters, Milford). Samples and standard curves were prepared for two different analytical methods (A and B). Method A: For quantification of 109 eicosanoids, measured in negative ionization mode. A BEH C18 column (2.1×150 mm, 1.7 µm, Waters) was used with mobile phase solvents A (water with 0.1% of acetic acid) and B (acetonitrile/isopropanol 90:10, v/v) at a flow rate of 0.5 mL/min. Gradient elution was performed with 80% of A as the starting condition, linearly decreased to 65% at 2.5 min, to 60% at 4.5 min, to 58% at 6 min, to 50% at 8 min, to 35% at 14 min, to 27.5% at 15.5 min and to 0% at 16.6 min. The column was then washed with solvent B for 0.9 min and equilibrated to initial conditions. Method B: cysLTs were measured in positive ionization mode, using the same column but with mobile phase solvents A (water with 0.2% of formic acid) and B (acetonitrile/isopropanol 90:10, v/v + 0.2% formic acid) at a flow rate of 0.45 mL/min. Gradient elution was initiated with 60% of A, linearly decreased to 50% at 4.25 min and to 5% at 4.5 min. The column was then washed with 95% of solvent B for 3 min and equilibrated to initial conditions. For quantification a 12-point calibration curve for each analyte was used. Data analysis was performed using TargetLynx application manager (Waters, Mildford). Relative responses (peak area of analyte/peak area of IS) were used for analyte quantification and a correction-factor was applied to correct for the extracted sample volume.

### 4.2.19 Flow cytometry

After overnight stimulation with 10 µg/ml *HpbE*, human eosinophils were stained with APC-labelled antibodies against $LTC_4S$ or $LTA_4H$ (both Santa Cruz Biotechnologies) (prepared by using an antibody conjugation kit, Innova Biosciences, according to the manufacturer's instructions) as well as with anti-human CCR3 (PE) and anti-CRTH2 (PE-CF594) antibodies (both BD Biosciences). Isotype-matched antibodies were used as controls. All samples were acquired in a BD LSRFortessa™ (BD Biosciences) and analysed by Dr. Julia Esser-von Bieren using FlowJo v10 software (FlowJo LLC)

### 4.2.20 Chemotaxis assays

PMN were resuspended to a concentration of $1x10^6$ cells/ml in the presence of 100 ng/ml human GM-CSF (Miltenyi Biotech) and overnight stimulated with 10 µg/ml *HpbE*. When mentioned,

PMN were pre-treated with 1 µM fluticasone propionate (Sigma-Aldrich), 10 µM Montelukast (Cayman Chemical) or conditioned media from MDM stimulated overnight with 10 µg/ml *Hpb* extract +/- 100 µM Indomethacin for 1 hour. PMN migration in response to nasal polyp secretions or a chemokine cocktail of 2 ng/ml RANTES, 20 ng/ml IL-8 (both Miltenyi Biotech) and 2 ng/ml $LTB_4$ (Cayman Chemical) was tested. Chemoattractants were placed in the lower wells of a chemotaxis plate (3 µm pore size; Corning). After mounting the transwell unit, $2x10^5$ PMN were added to the top of each well and migration was allowed for 3 hours at 37°C, 5% $CO_2$. The number of cells migrating to the lower well was counted microscopically. In some experiments, manual counting was validated by flow cytometry.

### 4.2.21 Fractionation and mass spectrometry analysis of *Hpb* larval extract

Gel extraction chromatography was performed in collaboration with Dr. Maximilian Schiener and Prof. Dr. Simon Blank (ZAUM, Munich). Soluble protein fractions were separated by gel filtration chromatography (SEC) on a Superdex 75 10/300 GL column with the ÄKTA pure system (GE Health Care Life Science). 300 µl of *Hpb* extract was loaded onto the column and eluted isocratically with PBS (pH=8), flow rate 0.8 ml/min. Fractions of 0.5 ml were collected starting when protein presence was detected at λ = 280 nm.

Mass spectrometry analysis was performed by Dr. Martin Haslbeck (TUM, Munich). Selected fractions from the SEC were prepared for liquid chromatography-mass spectrometry analysis, as described previously (Bepperling et al., 2012; Mymrikov et al., 2017). The fractions 10-13 represented positive samples while the flow through and fraction 33 were treated as negative control samples. Proteins in the samples were reduced, alkylated and digested overnight with trypsin. Peptides were extracted in five steps by adding sequentially 200 µl of buffer A (0.1% formic acid in water), acetonitrile (ACN), buffer A, ACN, ACN respectively. After each step samples were treated for 15 min by sonication. After steps 2, 4 and 5, the supernatant was removed from the gel slices and collected for further processing. The collected supernatants were pooled, evaporated to dryness in a speed vac (DNA 120, ThermoFisher Scientific) and stored at -80°C. For the MS measurements the samples were dissolved by adding 24 µl of buffer A and sonicated for 15 min. The samples were then filtered through a 0.22-µm centrifuge filter (Merck Millipore). Peptides were loaded onto an Acclaim PepMap RSLC C18 trap column (Trap Column, NanoViper, 75 µm x 20 mm, C18, 3 µm, 100 Å, ThermoFisher Scientific) with a flow rate of 5 µL/min and separated on a PepMap RSLC C18 column (75 µm x 500 mm, C18, 2 µm, 100 Å, ThermoFisher Scientific) at a flow rate of 0.3 µL/min. A double linear gradient from 5 % (vol/vol) to 28 % (vol/vol) buffer B (acetonitrile with 0.1 % formic acid) in 30 min and from 28% (vol/vol) to 35 % (vol/vol) buffer B in 5 min eluted the peptides to an Orbitrap QExactive plus mass spectrometer (ThermoFisher Scientific). Full scans and five dependent collision-induced dissociation MS2 scans were recorded in each cycle.

The mass spectrometry data derived from the SEC fractions were searched against the Swiss-Prot *Heligmosomoides polygyrus bakeri* Database downloaded from UniProt (24.01.2017 edition) using the Sequest HT Algorithm implemented into the "Proteome Discoverer 1.4" software (ThermoFisher Scientific). The search was limited to tryptic peptides containing a maximum of two missed cleavage sites and a peptide tolerance of 10 ppm for precursors and 0.04 Da for fragment masses. Proteins were identified with two distinct peptides with a target false discovery rate for peptides below 1% according to the decoy search. Proteins detected in the negative control samples were subtracted from the respective hit-lists. For further evaluation two independent

datasets resulting from SEC separations of biological replicates were combined. Only hits that were observed in both datasets were taken into account.

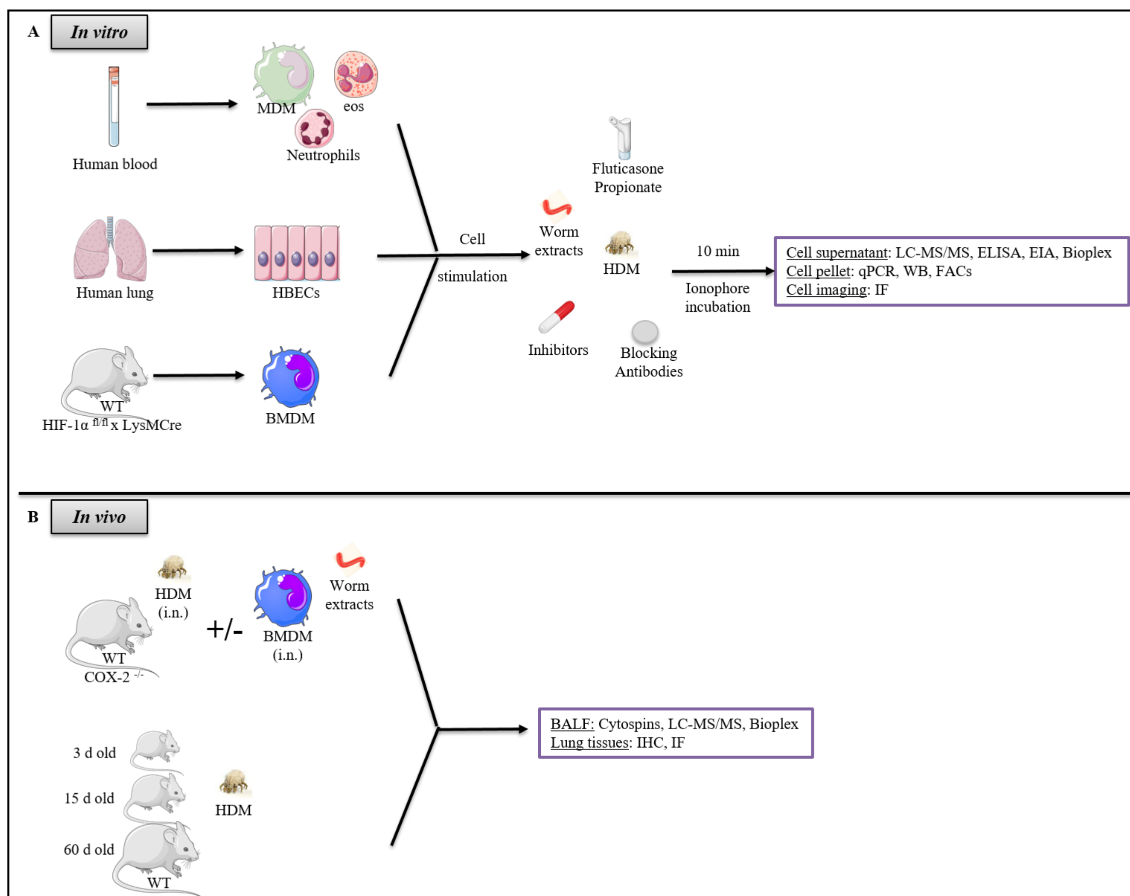


**Figure 9: Schematic representation of the working flow.** (A) Schematic representation of *in vitro* isolation and stimulation of immune cells as well as the different techniques used for sample analysis. (B) Schematic representation of *in vivo* experiments and techniques used for sample analysis. (Abbreviations: BALF, bronchoalveolar lavage fluid; BMDM, Bone Marrow-derived Macrophage; EIA, Enzyme Immunoassay; eos, eosinophils; FACs, Fluorescence Activated Cell Sorting; IF, Immunofluorescence; IHC, Immunohistochemistry; HBECs, Human Bronchial Epithelial Cells; HDM, House Dust Mite; HIF-1α, Hypoxia inducible factor-1 alpha; LC-MS/MS, Liquid Chromatography- Mass Spectrometry; MDM, Monocyte-derived Macrophages; qPCR, Real time quantitative PCR; WB, Western Blot; WT, Wildtype)

### 4.2.22 Quantification and statistical analysis

All statistical analysis for biological data was performed using GraphPad Prism (GraphPad Software Inc). Where two groups were compared, statistical significance was determined by Wilcoxon-Mann-Whitney test. Where more than two groups were compared, Kruskal-Wallis followed by Dunn's multiple comparisons test, Friedman test (paired samples) or 2way ANOVA (unpaired samples) were used. Please see figure legends for statistical tests used and exact value of n. Values are reported as the means +/- SEM. *=p<0.05, **=p<0.01, ***=p<0.001. When mentioned, data analysis was performed using Mann-Whitney test and data were considered statistically significant if P values were less than 0.05

Heat maps were generated using MetaboAnalyst version 3.0 (Mc Gill University, Quebec, Canada), a free online tool for metabolomics data analysis. Image analysis was performed using ImageJ Fiji (National Institutes of Health, Bethesda, Md) and CellProfiler software (Broad Institute, Cambridge, Mass) by using previously published macros (Esser von-Bieren et al., 2013 and 2015)

# 5 RESULTS

## 5.1 Remodelling of myeloid arachidonic acid metabolism and type 2 inflammation by helminth parasites and their products

### 5.1.1 Helminth infection triggers local reprogramming of the arachidonic acid metabolism

Several epidemiological studies have shown an inverse correlation between helminth infection and allergy, suggesting a role for the worms in the suppression of allergic diseases (reviewed in section 2.4). Eicosanoids are important lipid mediators involved in many inflammatory diseases such asthma and allergy, and it has been suggested that parasites have evolved different mechanisms to evade the host immune responses and longer survive in the host (reviewed in section 2.4). However, it is still not well understood how parasites modulate the eicosanoid production *in vivo*. The infection model used for this study is the gastrointestinal parasite *Heligmosomoides polygyrus bakeri* (*Hpb*). *Hpb* has a direct life cycle and mice get orally infected with the infective L3 larvae, migrating larvae, which penetrate the subserosal layer of the duodenum and develop into L4 larvae. At approximately day 8 post infection (p.i.), the larvae return to the intestinal lumen as adult worms (Camberis et al., 2003; Johnston et al., 2015)

To study whether *Hpb* infection could trigger the reprogramming of arachidonic acid (AA) metabolites *in vivo*, the eicosanoid profile in intestinal culture supernatant and peritoneal lavage of naïve and *Hpb*-infected mice was quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS). At day 7 p.i, when no type 2 immune response has been established yet, the formation of AA metabolites was increased during helminth infection. In the cyclooxygenase (COX)-pathway, *Hpb* infection induced the production of prostanoids such as prostaglandin E2 ($PGE_2$), thromboxane B2 ($TXB_2$), 6-keto PGF1α and PGF2α in intestinal culture supernatant and peritoneal lavage, with higher levels measured in intestinal culture supernatants (Figures 10A and 10B). In the lipoxygenase (LOX) pathway, higher production of 12- and 15-hydroxyeicosatetraenoic acid (HETE) metabolites was observed in *Hpb*-infected mice. In contrast, 5-LOX metabolites like 5-HETE, leukotriene B4 ($LTB_4$) or cysteinyl leukotrienes (cysLTs) were close to or below the lower detection limit in intestinal culture supernatants and peritoneal lavage (Figures 10A and 10B).

In line with the abundant production of prostanoids, immunofluorescence stainings of gut sections showed high expression of COX-2 (biosynthetic enzyme involved in the synthesis of prostanoids) and its positive regulator hypoxia inducible factor-1 alpha (HIF-1α) (Scheerer et al., 2013) in the surrounding of the trapped larvae and in cells close to the larvae in *Hpb*-infected mice (Figure 10C). In contrast, 5-LOX (biosynthetic enzyme responsible for the synthesis of leukotrienes) was not present during helminth infection, explaining the absence of leukotrienes measured in the intestinal culture supernatant or peritoneal lavage (Figure 10C). A previous study has shown abundant 12-15 LOX expression in intestinal tissue in *Hpb*-infected mice (Esser-von Bieren et al., 2015), results which are in line with the high production of 12- and 15-HETE measured in intestinal culture supernatant and peritoneal lavage (Figures 10A and 10B).

To further test if reprogramming of the AA metabolism is a general feature during helminth infection, we performed immunohistochemistry stainings of lung and liver sections of mice infected with the human helminth parasite *Schistosoma mansoni* (*Sm*). In this case, *Sm*-infection induced the expression of 5-LOX at sites of larval migration in the lung or egg deposition in the liver (Figure 11A and 11B), suggesting that the suppression of the LOX pathway is not a general

feature of helminths. Thus, infection with *Hpb* triggered local anti-inflammatory reprogramming of the AA metabolism.

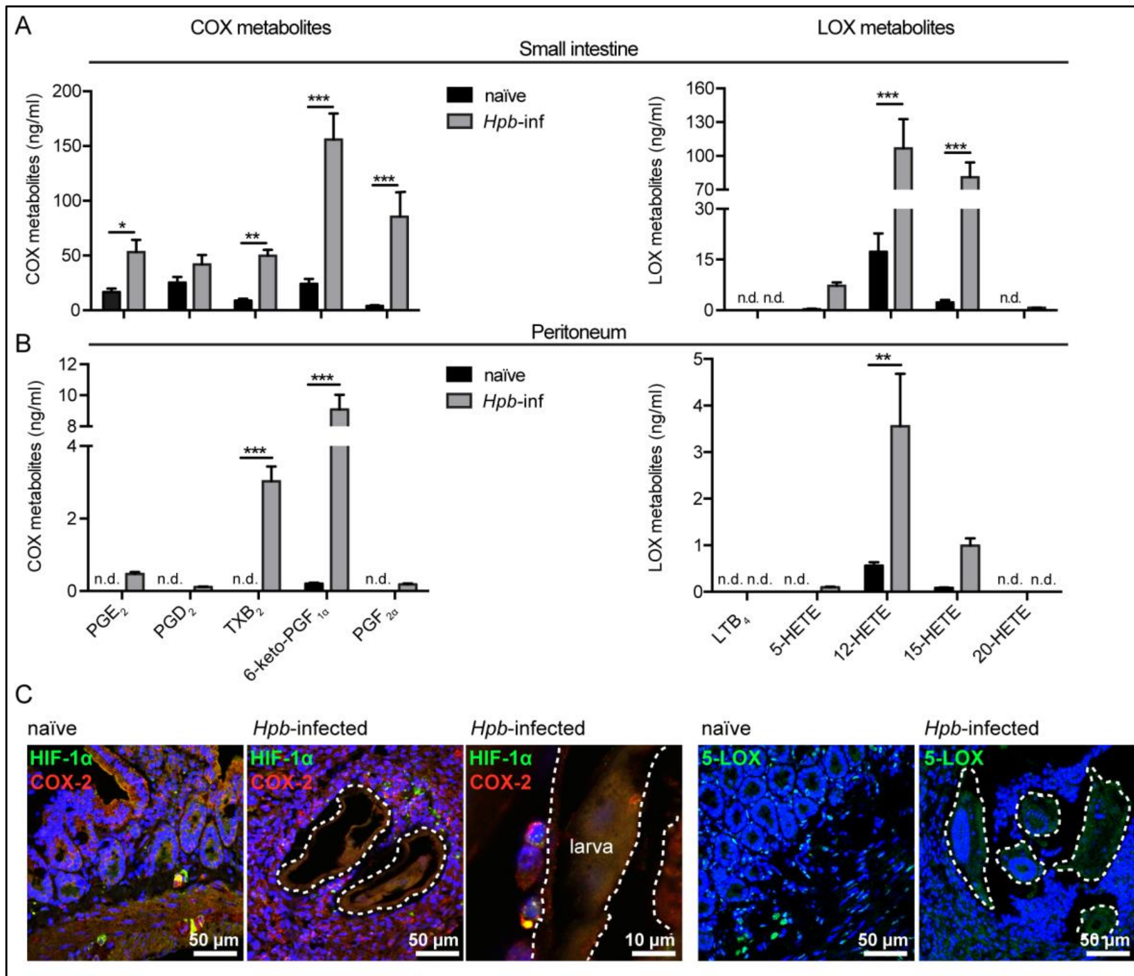


**Figure 10: Infection with *Heligmosomoides polygyrus bakeri* (*Hpb*) remodels the arachidonic acid metabolism at day 7 post-infection (p.i.) *in vivo*.** (A) Levels of COX and LOX metabolites (LC-MS/MS) measured in the intestinal culture supernatant from naïve mice or *Hpb*-infected mice. (B) Levels of COX and LOX metabolites (LC-MS/MS) measured in peritoneal lavage from naïve mice or *Hpb*-infected mice. (C) Representative immunofluorescence stainings of HIF-1α, COX-2 and 5-LOX in section of the small intestinal tissue of naïve and *Hpb*-infected mice. Dashed lines indicate the position of the larvae. Results are pooled from two independent experiments in (A) and (B) or representative of stainings performed for two independent experiments in (C). Results in (A) and (B) are presented as mean ± SEM, n=5-7 per group. Statistical significance in (A) and (B) was determined by 2way ANOVA with Bonferroni correction. *p = 0.05, **p = 0.01, ***p<0.001.

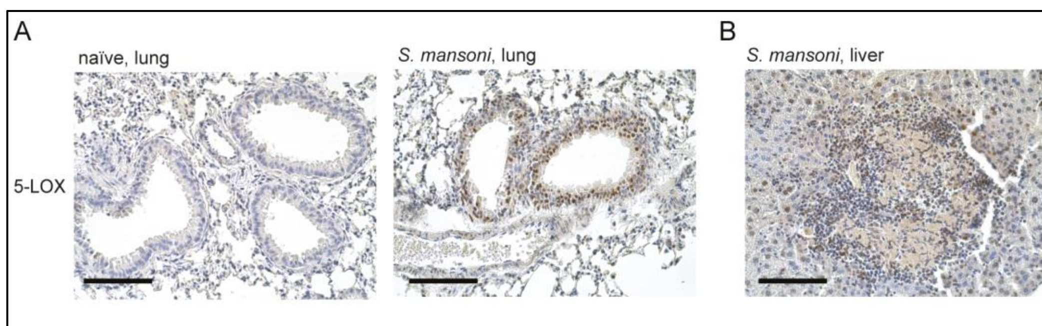


**Figure 11: Infection with *Schistosoma mansoni* induces 5-LOX expression at the site of infection.** (A) Representative immunohistochemical stainings for 5-LOX of lung tissues from naïve (left) or S. mansoni-infected mice (right). (B) Representative immunohistochemical stainings for 5-LOX in the liver of S. mansoni-infected mice. (Scale bar: 100 µm). Stainings are representative for data generated in two independent experiments with n=5 mice per group.

### 5.1.2 An extract of *Hpb* larvae reprograms the arachidonic acid metabolism of murine and human macrophages

During helminth infection, macrophages are plastic cells playing an important role because they are involved in anti-helminth immunity, worm trapping, immunoregulation and repair of any tissue injury caused by migrating larvae or egg deposition (review in 2.4.2.1a.). Since macrophages are also major producers of AA metabolites, we aimed to investigate whether an extract of *Hpb* L3-stage larvae (*HpbE*) could modulate the AA metabolism of murine macrophages. Bone-marrow-derived macrophages (BMDM) were incubated overnight with *HpbE* and the cell supernatant was analysed by LC-MS/MS analysis in order to quantify the eicosanoid profile. Treatment with *HpbE* induced anti-inflammatory $PGE_2$ and prostanoids involved in tissue repair ($TXB_2$), but *Hpb* products inhibited the synthesis of pro-inflammatory and type 2-inducing metabolites ($PGD_2$, $LTB_4$, cysLTs) in murine macrophages (Figure 12A). To test whether this AA reprogramming was a result of transcriptional changes in AA-metabolizing enzymes, gene expression of COX-2 (gene: *Ptgs2*), microsomal prostaglandin E synthase (mPGES-1, gene: *Ptges*), 5-LOX (gene: *Alox5*) and $LTC_4S$ (gene: *Ltc4s*) was measured in control and *HpbE*-stimulated BMDM. In line with the eicosanoid profile, *HpbE* induced the gene expression of *Ptgs2* and *Ptges* (enzymes involved in the synthesis of prostanoids), but suppressed the expression of *Alox5* and *Ltc4s* (enzymes responsible for the synthesis of LTs) (Figure 12A). Thus, the eicosanoid reprogramming in murine macrophages *in vitro* closely resembled local AA metabolism changes during *Hpb* infection *in vivo* (Figure 10)

To investigate a potential clinical relevance of *HpbE* in a human cellular model, human monocyte-derived macrophages (MDM) were isolated and treated with *HpbE* and the eicosanoid profile was measured by LC-MS/MS analysis. For this set of experiments, we used an LC-MS/MS method, which can quantify up to 200 different eicosanoids and PUFAs. In line with the results for *HpbE*-stimulated BMDM, treatment of MDM with *HpbE* modulated the AA metabolism, whilst linoleic acid (LA) metabolites (9-HODE, 13-HODE, 9, (10)-DiHOME) remained largely unaffected as displayed in the heat map (Figure 12C). An induction of COX metabolites such as $PGE_2$, $TXB_2$ and 12-hydroxyheptadecatrenoic acid (12-HHT) but a suppression of LOX metabolites $LTB_4$, $LTC_4$ and 5-HETE was observed in *HpbE*-treated MDM (Figure 12D). *HpbE* also induced a transcriptional regulation of AA biosynthetic enzymes in MDM by inducing the gene expression of enzymes involved in the synthesis of $PGE_2$: *PGTS2* (gene encoding COX-2) and *PTGES* (gene

encoding mPGES-1). In contrast, *HpbE* downregulated the gene expression of *PTGDS* (gene encoding $PGD_2$ synthase), downregulated the gene expression of all LT biosynthetic enzymes: *ALOX-5* (encodes 5-LOX), *LTA4H* (encodes $LTA_4H$) and *LTC4S* (encodes $LTC_4S$) as well as the high affinity receptor for cysLTs (Cysteinyl Leukotriene Receptor-1, *CYSLT1R*) (Figure 12E). Thus, *HpbE* reprogrammed the AA metabolism of human macrophages, inducing a potentially anti-inflammatory eicosanoid profile.

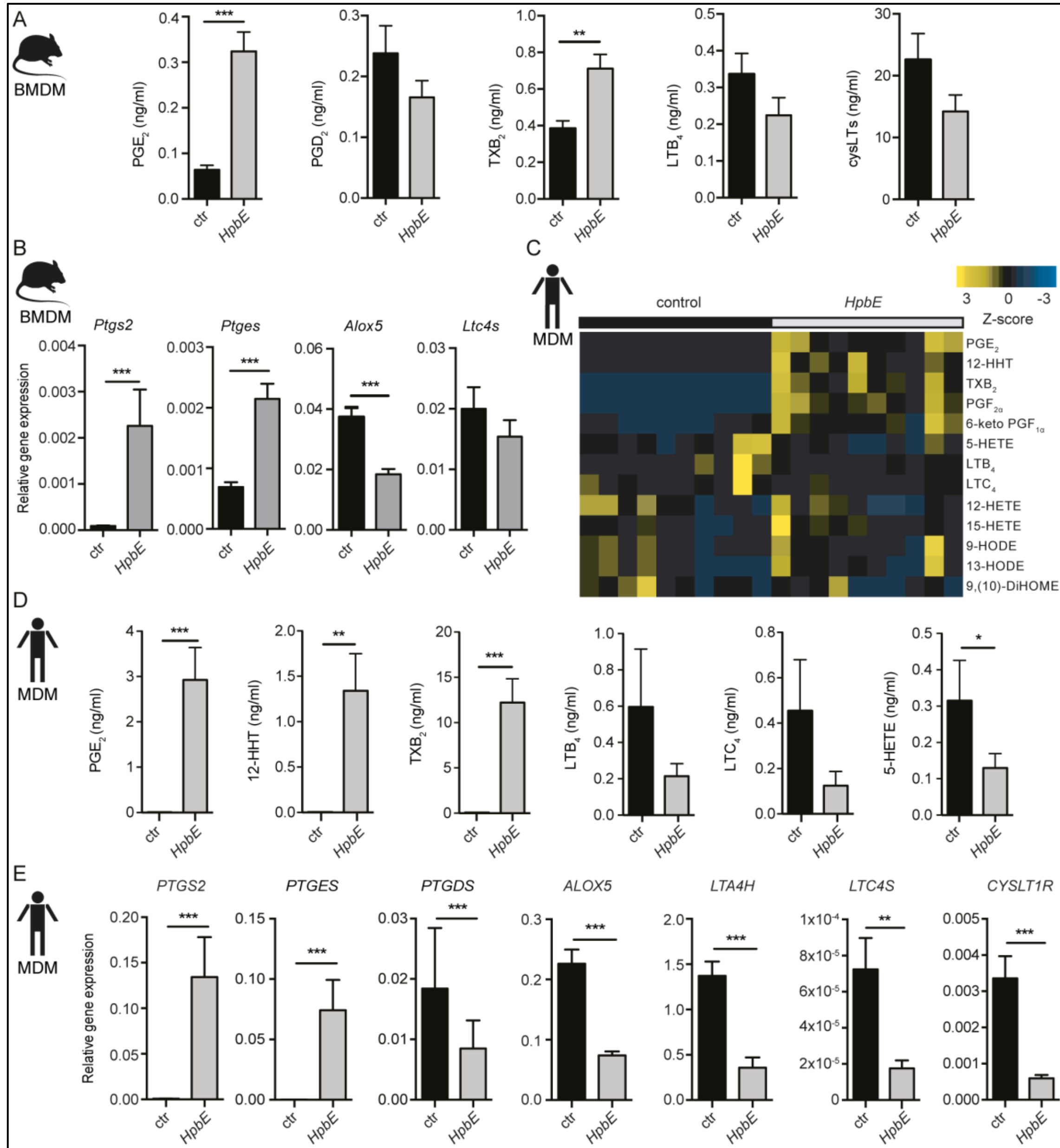


**Figure 12: Hpb larval extract (HpbE) reprograms the arachidonic acid metabolism of murine and human macrophages.** (A) Eicosanoid levels (LC-MS/MS) produced by mouse bone marrow macrophages (BMDM) after treatment with *Hpb* larval extract (*HpbE*) (10µg/ml). (B) Relative gene expression of AA-metabolizing enzymes (qPCR) in mouse BMDM +/- treatment with *HpbE*. (C) Heat map showing PUFA metabolites (LC-MS/MS) detected in human monocyte derived macrophages (MDM) ± treatment with *HpbE*. (D) Levels of major bioactive AA metabolites (LC-MS/MS) produced by human MDM ± treatment with *HpbE*. (E) Relative gene expression of eicosanoid pathway proteins (qPCR) in human MDM ± treatment with *HpbE*. Data are presented as mean ± SEM, n=10-15 MDM from healthy human blood

donors, n=8 BMDM from C57BL/6 mice. Statistical significance was determined by Wilcoxon test. *p<0.05, **p<0.01, ***p<0.001.

Taken together, *HpbE* remodels the AA metabolism of murine and human macrophages by inducing the synthesis of anti-inflammatory mediators and changing the gene expression of major AA-metabolizing enzymes by down-regulation of type 2-inducing pathways. This eicosanoid reprogramming in macrophages *in vitro* closely resembles the local AA metabolism changes during *Hpb* infection *in vivo*.

### 5.1.3 *HpbE* specifically induces type 2-suppressive mediators and prevents M2 polarization in macrophages

To investigate whether treatment with *HpbE* also affects macrophage polarization and type 2 immune responses, quantification of several cytokines was performed by ELISA or Bioplex analysis. Treatment of human and mouse macrophages with *HpbE* resulted in the induction of IL-10 and IL-1β, although the induction of these cytokines was 10-100-fold higher in amplitude in human MDM as compared to murine BMDM (Figure 13A, 13C). IL-10 and IL-1β are known to be involved in the suppression of type 2 immune responses (Schnoeller et al., 2008; Zaiss et al., 2013). *HpbE*-stimulated MDM also secreted high amounts of cytokines involved in the modulation of M2 polarization and type 2 immune responses such as IL-12, IL-18, IL-27 and TNF-α, but interestingly did not affect the production of IL-33 (Figure 13B) (Guo et al., 2009; Kratochvill et al., 2015; Mathie et al., 2015; Qui et al., 2012). In contrast, *HpbE* hardly affected the production of mediators of type 2 inflammation such as IL-33 or CCL17 in MDM (Figure 13B). Together, these results suggest that *HpbE* can remodel macrophage cytokine production to induce a regulatory, type 2-suppressive phenotype.

To further analyse if *HpbE* could induce changes in macrophage polarization, the expression of M2 markers after *HpbE* treatment was assessed in human and murine macrophages. Whilst gene expression of *TGM2* (Transglutaminase 2, TGM2) was not modified, *ALOX-15* (15-Lipoxygenase, 15-LOX) and *MRC1* (Mannose Receptor C-Type 1, MR/CD206) were downregulated, suggesting that *HpbE* counteracted M2 polarization in human MDM (Figure 13D). As several differences in the expression of M2 markers have been shown for mouse and human macrophages (Martinez et al., 2013), macrophage polarization was also studied in murine BMDM using different M2 markers. In contrast to *HpbE*-stimulated MDM, *HpbE* induced gene expression of *Tgm2* and *Arg1*, but down-regulated the gene levels of *Mrc1*, whilst other M2 markers *Tmed1/St2l* and *Rentla/Fizz1* remained unaltered (Figure 13E). Thus, *HpbE* treatment resulted in distinct macrophage polarization, but similar regulatory mediator profiles, most likely inducing an M2-suppresive phenotype in human and murine macrophages.

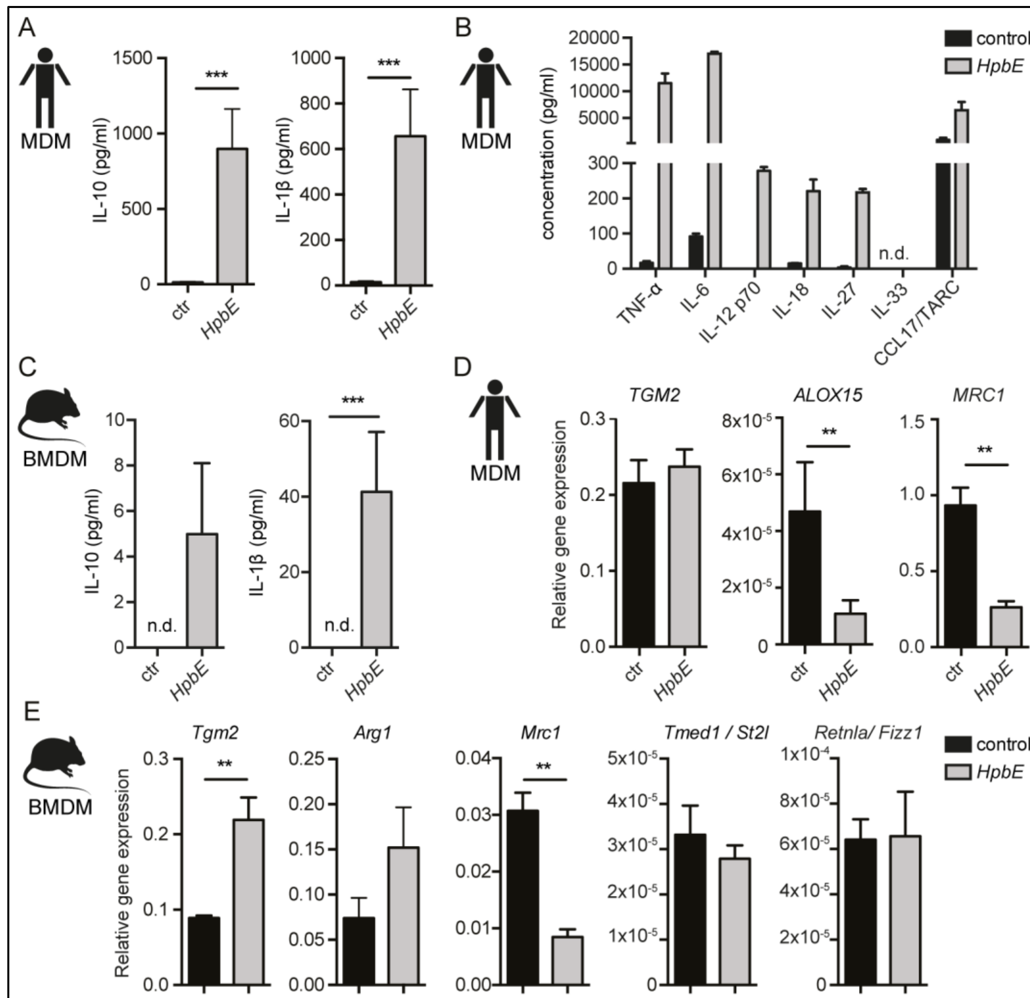


**Figure 13: *HpbE* induces type 2-suppressive cytokines and modulates the polarization of human and murine macrophages.** (A) Levels of IL-10 and IL-1β (ELISA) produced by human MDM ± treatment with *HpbE* (10µg/ml). (B) Levels of TNF-α, IL-6, IL-12p70, IL-18, IL-27, IL-33 and CCL17/TARC (Bioplex) produced by human MDM after treatment with *HpbE*. (C) Levels of IL-10 and IL-1β (Bioplex) produced by mouse BMDM ± treatment with *HpbE*. (D) Gene expression of M2 markers (qPCR) in human MDM ± treatment with *HpbE*. (E) Gene expression of M2 markers (qPCR) in mouse BMDM ± treatment with *HpbE*. Data are presented as mean ± SEM, n=5-8 BMDM from C57BL/6 mice, n=3-15 MDM from healthy human blood donors. Statistical significance was determined by Wilcoxon test. *p<0.05, **p<0.01, ***p<0.001.

### 5.1.4 *HpbE* induces a unique macrophage eicosanoid signature in comparison to other helminth products

To test whether eicosanoid reprogramming of macrophages is a general feature of *Hpb* preparations, excretory secretory products of *Hpb* adult stages (HES) were used to treat human macrophages, since HES are known to modulate type 2 immune responses (McSorley et al., 2012; Osbourn et al., 2017). In contrast to *HpbE*, HES failed to induce IL-10 production or COX metabolites ($TXB_2$ and $PGE_2$) in human MDM (Figure 14A). HES stimulation did not result in transcriptional changes in IL-10 gene expression or AA-metabolizing enzymes (*PTGS2, PTGES*) as observed for *HpbE*-treated MDM (Figure 14B). Interestingly, HES could partially mimic the *HpbE* induced regulation in the LOX pathway. Lower levels of CysLTs were measured after HES

stimulation to a similar extent as observed for *HpbE*, although the gene suppression of ALOX5 was lower than for *HpbE*-stimulated macrophages (Figure 14A, 14B).

During *Hpb* infection *in vivo*, the helminth can induce changes in the intestinal microbiota which have been shown to contribute to *Hpb*-mediated protection against allergic asthma (Zaiss et al., 2015). For the preparation of *HpbE*, L3 larvae were obtained from the eggs in the feces of *Hpb*-infected mice. Although an antibiotic treatment of the larvae was performed before homogenization, it is not clear whether there are still antibiotic resistant bacteria present in *HpbE* due to fecal contamination. Thus, bacterial cultures of *HpbE* were set-up and four different bacterial strains were identified (*Bacillus cereus*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis* and *Paenibacillus odorifer*). A mixture of these bacteria (*HpbE*-bacteria) was used to stimulate human macrophages and the eicosanoid profile and cytokine levels were measured. In contrast to *HpbE*, *HpbE*-bacteria failed to induce IL-10 production or prostanoids ($TXB_2$) at the mediator level (Figure 14C) as well as at the gene expression level, failing to change the gene expression of *IL-10, PTGS2, PTGES* (Figure 14D). Interestingly, *HpbE*-bacteria triggered the inhibition of cysLTs similar as compared to *HpbE*- or HES-stimulated macrophages, but 5-LOX gene expression remained unaltered (Figure 14E and 14D). These results suggested that induction of type 2 suppressive mediators or COX-metabolites was largely due to larval components, while HES and *HpbE*-bacteria might contribute to the regulation of pro-inflammatory mediators such as cysLTs.

To study whether mediator reprogramming in macrophages is a conserved mechanism amongst helminths, larvae of the parasite *S. mansoni* were extracted, homogenized and used as a larval extract (*SmE*) for *in vitro* experiments with human MDM. When analysing the levels of AA-metabolites by LC-MS/MS analysis, *SmE* had only minor effects in the production of COX and LOX metabolites, slightly inducing the production of $PGE_2$ and 5-HETE, whilst other mediators such as 12-HHT, $TXB_2$, $LTB_4$ and $LTC_4$ remained unaltered (Figure 14E). In contrast to *HpbE*, *SmE* did not induce IL-10 production in human macrophages (Figure 14F). Thus, *HpbE* has a unique potential to induce regulatory mediators, which is distinct form HES, *Hpb*-associated bacteria or larval extracts from other helminth parasites.

To further exclude that the *HpbE*-triggered induction of regulatory mediators was mainly due to LPS contamination, the amount of LPS present in *HpbE* was quantified and human macrophages were stimulated with LPS at the concentration present in *HpbE* (60ng/ml). Quantification of lipid mediators by LC-MS/MS analysis showed that LPS alone could not induce COX metabolites ($PGE_2$, 12-HHT and $TXB_2$) at similarly high concentrations as observed for *HpbE*-treated macrophages (Figure 15A). Interestingly, LPS could trigger the inhibition of 5-LOX mediators ($LTB_4$ and $LTC_4$), similar as observed for *HpbE*- stimulated macrophages (Figure 15B). In contrast to the differences observed in the production of prostanoids, LPS induced similar transcriptional changes in the gene expression of $PGE_2$-biosynthetic enzymes (*PTGS2* and *PTGES*) compared to *HpbE*-stimulated MDM; although similar production of LOX metabolites correlated to similar gene expression levels of *ALOX5* in both *HpbE*- and LPS-stimulated macrophages (Figure 15C). Thus, *HpbE* has a unique potential to induce regulatory prostanoids independently of LPS contamination, although LPS might contribute to the suppression of 5-LOX metabolites in *HpbE*-stimulated macrophages.

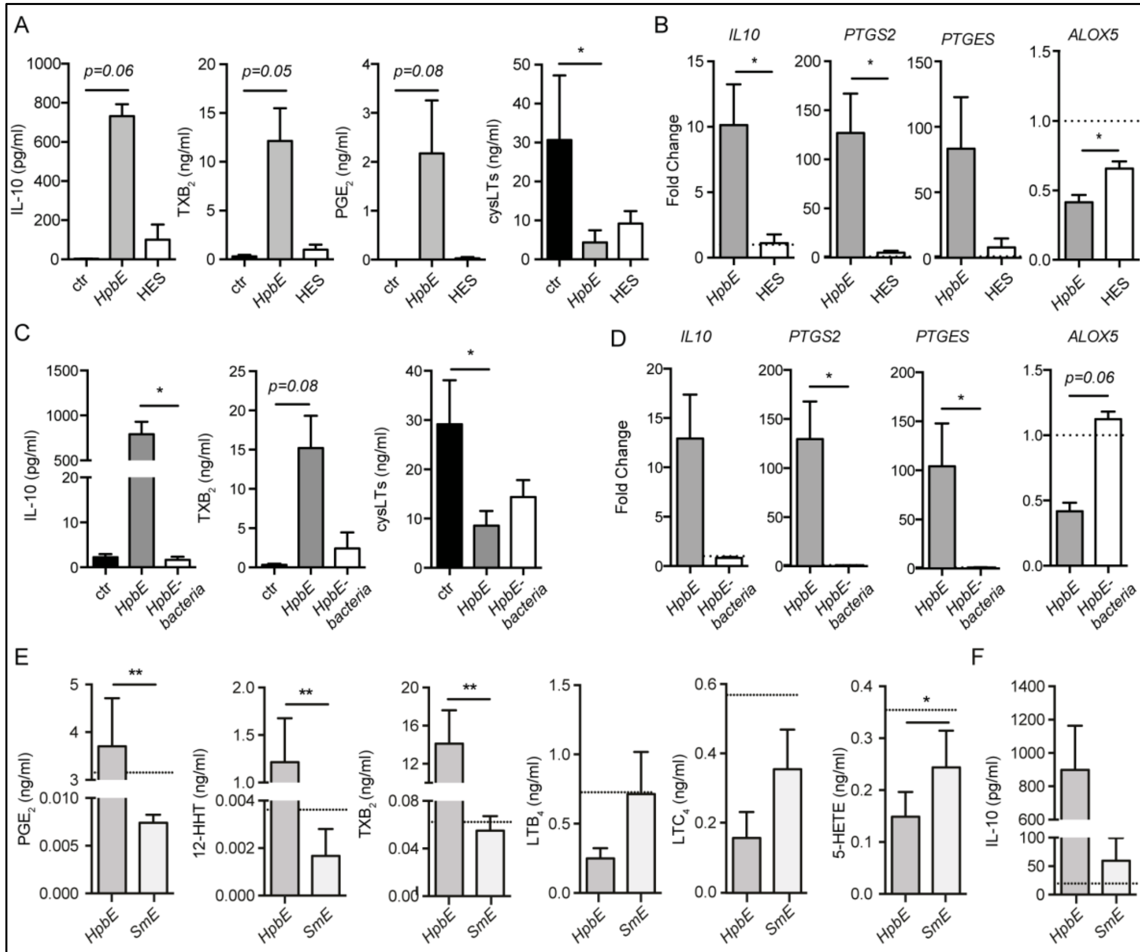


**Figure 14: Secreted products of adult stages of *Hpb* (HES) or a larval extract from a different helminth *S. mansoni* (*SmE*) fail to induce mediator reprogramming in human macrophages.** (A) Levels of IL-10 (ELISA) or prostanoids (LC-MS/MS) in human MDM control or treated with *Hpb* larval extract (*HpbE*) or *Hpb* excretory secretory products "HES" (10 µg/ml). (B) Relative gene expression of IL-10 or AA-metabolizing enzymes (qPCR) in human MDM treated with *HpbE* or HES. (C) Levels of IL-10 (ELISA) or $TXB_2$ and cysLTs (EIA) produced by human MDM after treatment with *HpbE* or a homogenate of major bacterial strains present in *HpbE*. (D) Relative gene expression of eicosanoid pathway proteins or IL10 (qPCR) in human MDM treated with *HpbE* or a homogenate of major bacterial strains present in *HpbE*. (E) Eicosanoid levels (LC-MS/MS) produced by human MDM after treatment with larval extracts from *Hpb* (*HpbE*) or *S. mansoni* (*SmE*) (10 µg/ml). Dashed lines indicate control levels. (F) Levels of IL-10 (ELISA) produced by human MDM ± treatment with *HpbE* or *SmE*. Dashed line indicates control level. Results are expressed as mean ± SEM, n=3-6 per group. Statistical significance was determined by Wilcoxon test (two groups) or Friedman test (more than 2 groups). $*p < 0.05$, $**p < 0.01$.

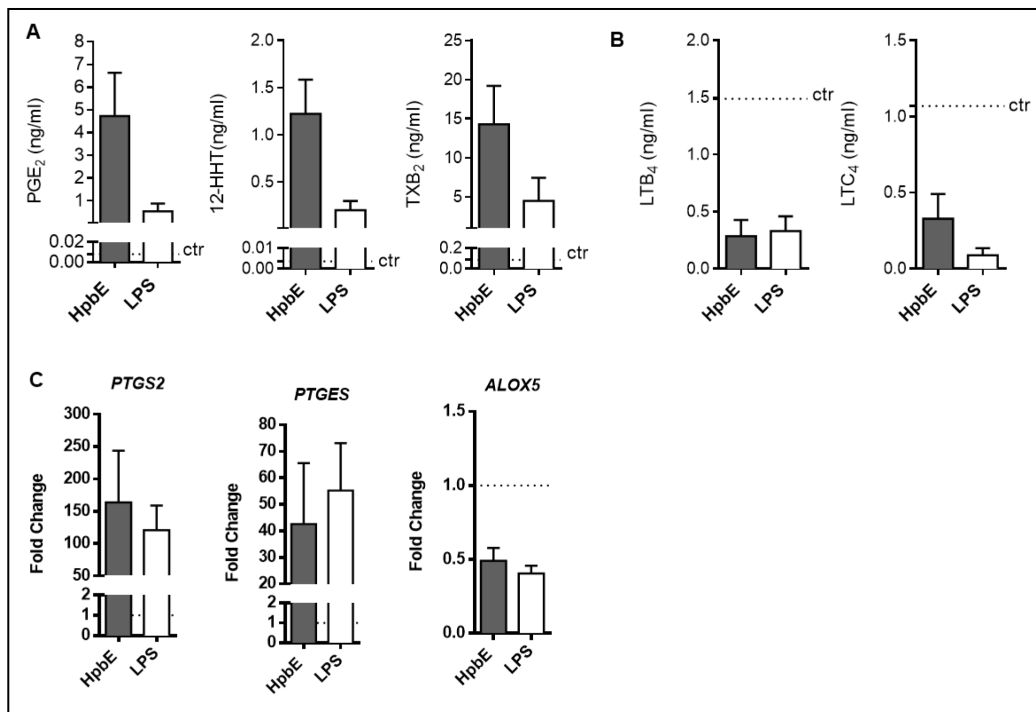


**Figure 15: Effect of LPS on eicosanoid production in human macrophages.** (A) Levels of COX metabolites (LC-MS/MS) produced by MDM treated with *HpbE* (10 µg/ml) or LPS (60ng/ml) (B) Levels of LOX metabolites (LC-MS/MS) produced by MDM treated with *HpbE* or LPS (C) Relative gene expression of AA metabolizing enzymes (qPCR) in MDM treated with *HpbE* or LPS. Dashed line indicates control levels. Results are expressed as mean±SEM, n=3 per group.

### 5.1.5 *HpbE* remodels the AA metabolism of human granulocytes

Granulocyte infiltration represents a hallmark of allergic asthma as neutrophils and eosinophils are a major source of pro-inflammatory eicosanoids during type 2 inflammation. Based on the capacity of *HpbE* to remodel the AA metabolism in macrophages (Figure 12), the next research focus was to test whether treatment with *HpbE* would trigger similar responses in human granulocytes. In line with the results observed for macrophages, the eicosanoid profile of *HpbE*-stimulated granulocytes showed a shift from 5-LOX towards COX metabolism as displayed in the heat map (Figure 16A). *HpbE* treatment induced COX metabolites (particularly 12-HHT and $TXB_2$), whilst inhibiting the production of pro-inflammatory LTs ($LTB_4$ and $LTC_4$) (Figure 16B). Furthermore, treatment of purified human eosinophils with *HpbE* significantly reduced the levels of cysLTs (Figure 16C).

Similar to *HpbE*-driven changes at the transcriptional level of AA metabolizing enzymes in macrophages (Figure 12B, 12E), *HpbE* downregulated the gene expression of enzymes involved in the synthesis of pro-inflammatory mediators (*ALOX5, $LTA_4H$* and *PTGDS*), but induced the expression of $PGE_2$-synthetic enzymes (*PTGS2* and *PTGES*) in human granulocytes (Figure 16D). Thus, *HpbE* remodels the AA metabolism in human granulocytes by inducing the synthesis of anti-inflammatory mediators and changing the gene expression of major AA-producing enzymes, similar to the effects observed in macrophages.

### 5.1.6 *HpbE* inhibits chemotaxis of human granulocytes in settings of type 2 inflammation

Eicosanoid production in myeloid cells play a key role during granulocyte recruitment, but dysregulation in the synthesis of lipids mediators can drive allergic asthma (as reviewed in section 2.5). In order to address whether *HpbE* treatment would affect granulocyte recruitment in a clinical setting of type 2 inflammation, Aspirin exacerbated respiratory disease (AERD) patients were recruited, granulocytes were isolated from the blood and nasal polyp secretions were obtained from nasal polyp tissues that had been cultured overnight after polypectomy. To assess the effect of *HpbE* in granulocyte recruitment, AERD PMN were stimulated with *HpbE* or drugs commonly used for asthma treatment (FP, fluticasone propionate; MK, montelukast) and a chemotaxis assay was performed toward nasal polyp secretions *ex vivo*. Pre-treatment of AERD granulocytes with *HpbE* resulted in a marked reduction in cell recruitment, an effect not achieved by anti-inflammatory drugs (FP, MK) (Figure 16F). Flow cytometry analysis of purified eosinophils showed that levels of LT biosynthetic enzymes ($LTC_4S$, $LTA_4H$) and chemotactic receptors like C-C chemokine receptor type 3 (CCR3) and $PGD_2$ receptor ($CRTH_2$) were down-regulated after *HpbE* treatment of eosinophils (Figure 16E), providing a possible explanation for the suppression of granulocyte recruitment during chemotaxis (Figure 16F). This result was in line with a previous study showing downregulation of CCR3 expression on eosinophils during *Hpb* infection *in vivo,* an effect which contributed to the protection against allergen-induced airway inflammation (Rzepecka et al., 2007).

To investigate the cross-talk between *HpbE*-stimulated macrophages and granulocytes and whether *HpbE* treatment could have an impact on granulocyte recruitment, a chemotaxis assay was now performed in the presence of conditioned medium from MDM previously treated with *HpbE.* Treatment of PMN with conditioned MDM medium did not show any effect on granulocyte chemotaxis, whilst MDM previously stimulated with *HpbE* reduced PMN chemotaxis to levels close to basal migration (Figure 16G), similar to the direct suppression in granulocyte migration of *HpbE* (Figure 16F). Since *HpbE*–stimulated macrophages secrete high levels of COX metabolites, MDM were pre-treated with the unspecific COX inhibitor indomethacin and the conditioned media was used for PMN stimulation, to further test the influence of COX mediators in chemotaxis. Inhibition of PMN migration after treatment with conditioned media of *HpbE*-treated MDM was abrogated when macrophages were previously treated with indomethacin, suggesting that a COX product released by *HpbE*-stimulated MDM was involved in the suppression of granulocyte recruitment (Figure 16G).

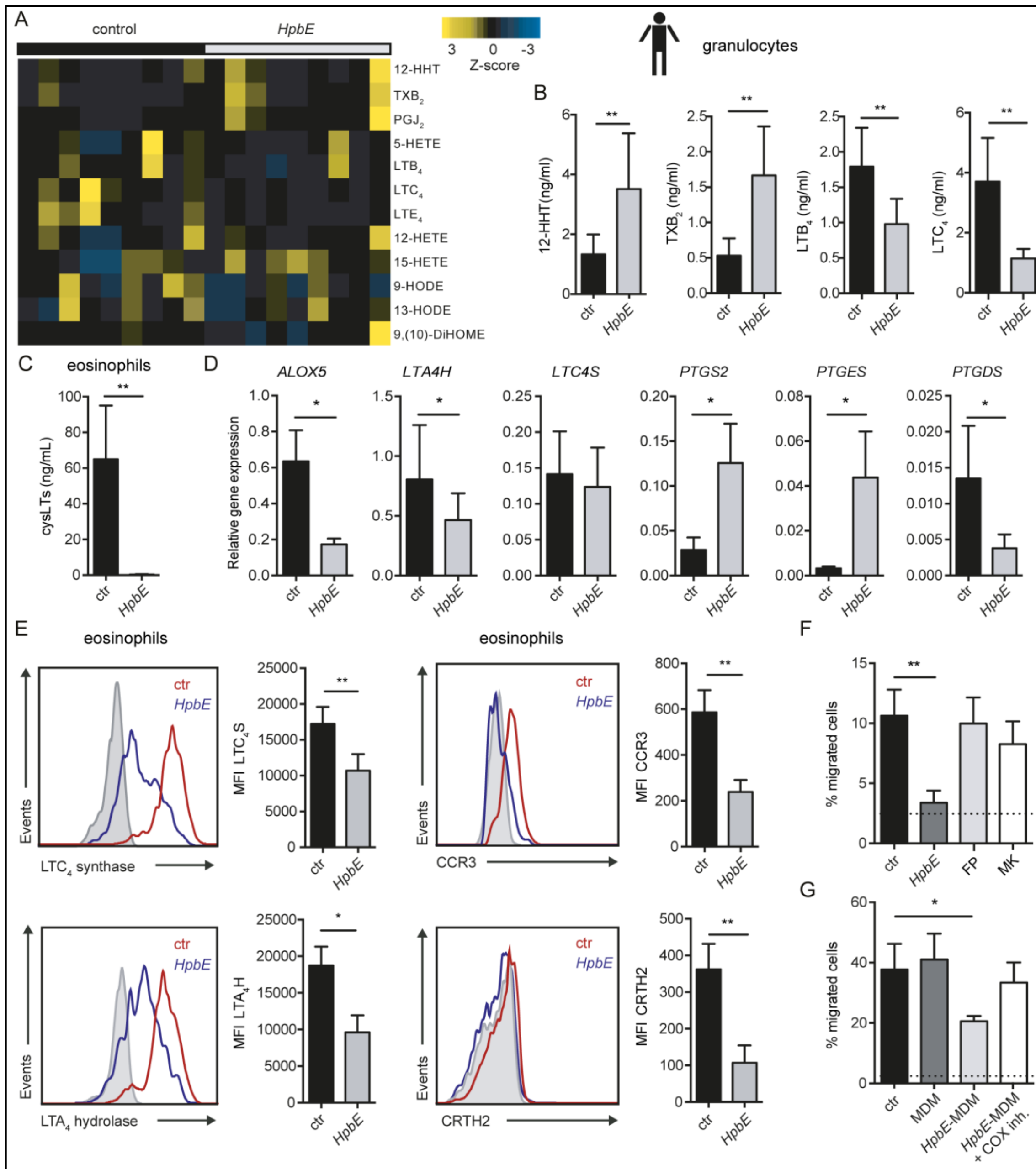


**Figure 16: *HpbE* remodels the AA metabolism and suppresses the activation and chemotaxis of human granulocytes.** (A) Heat map showing PUFA metabolites (LC-MS/MS) detected in mixed human granulocytes ± treatment with *HpbE* (10 µg/ml). (B) Levels of major bioactive AA metabolites (LC-MS/MS) produced by mixed human granulocytes ± treatment with *HpbE*. (C) Levels of cysteinyl leukotrienes (EIA) produced by purified human eosinophils ± treatment with *HpbE*. (D) Relative gene expression of AA-metabolizing enzymes (qPCR) in mixed human granulocytes ± treatment with *HpbE*. (E) Levels of LT-synthetic enzymes ($LTC_4S$ and $LTA_4H$) (flow cytometry) in human eosinophils ± treatment with *HpbE*. (F) Levels of chemotactic receptors (CCR3 and $CRTH_2$) (flow cytometry) in human eosinophils ± treatment with *HpbE*. (G) Chemotaxis of granulocytes from AERD patients (n=6) towards nasal polyp secretions ± treatment with *HpbE* or anti-inflammatory drugs Fluticasone propionate, FP (1µM) or Montelukast, MK (10µM). Dashed line depicts basal migration. (H) Chemotaxis of human granulocytes towards a chemokine cocktail ± pre-treatment with conditioned media from MDM ± *HpbE* (10 µg/ml), ± COX-inhibitor indomethacin (100 µM). Dashed line depicts basal migration. Data are pooled from at least 3 independent experiments and presented as mean ± SEM, n=6-10 mixed granulocytes or purified

eosinophils from human blood donors (healthy or AERD). Statistical significance was determined by Wilcoxon test (two groups) or Friedman test (more than 2 groups). *p<0.05, **p<0.01, ***p<0.001.

### 5.1.7 *HpbE* induces a unique granulocyte eicosanoid signature in comparison to an extract from the parasite *Ascaris suum*

To assess whether granulocyte eicosanoid reprogramming is a common feature of helminth parasites, the effects of the larval extract of the pig parasite *Ascaris suum* (*AsE*) were compared to the effects of *HpbE*. Analysis of the eicosanoid profile by LC-MS/MS showed that *AsE* failed to reduce the production of LOX metabolites ($LTB_4$ and $LTC_4$), but tended to induce the synthesis of COX metabolites ($TXB_2$ and 12-HHT) in human granulocytes, although to a lower extent as compared with *HpbE* treatment (Figure 17A). In line with the eicosanoid profile, flow cytometry analysis of human eosinophils showed that the levels of LT biosynthetic enzymes ($LTA_4H$ and $LTC_4S$) were not altered after *AsE* treatment, whilst *HpbE* treatment downregulated the expression of these enzymes (Figure 17B). Interestingly, *AsE* downregulated the surface levels of the chemotactic receptor $CRTH_2$ to a similar extent as observed during *HpbE* treatment, whilst the levels of CCR3 remained unaltered in *AsE*-treated eosinophils (Figure 17C). Taken together, *AsE* failed to induce eicosanoid reprogramming in human granulocytes and the absence of CCR3 downregulation suggested that *AsE* might not effectively suppress chemotaxis during type 2 inflammation. Thus, *HpbE* has a unique potential to reprogram granulocyte eicosanoid signature and suppress granulocyte recruitment by direct down-regulation of chemotactic receptors.

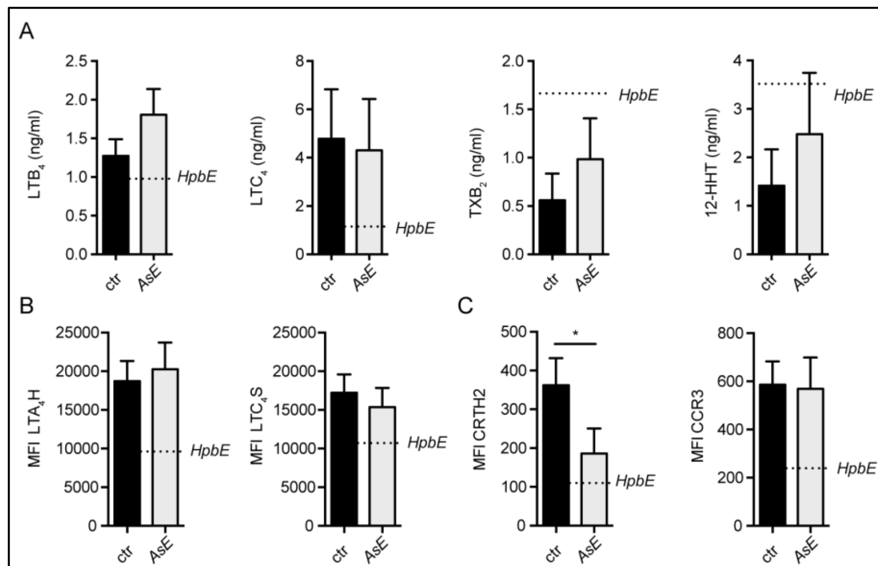


**Figure 17: Larval extract of the nematode parasite *Ascaris suum* fails to modulate eicosanoid pathways in human granulocytes.** (A) Levels of major bioactive AA metabolites (LC-MS/MS) produced by human granulocytes after treatment with *Ascaris suum* larval extract (*AsE*) (10µg/ml). Dashed lines indicate levels for *HpbE*-treated cells (10 µg/ml). (B) Levels of LT-biosynthetic enzymes (flow cytometry) in human eosinophils ± treatment with *AsE*. Dashed lines indicate levels for *HpbE*-treated eosinophils. (C) Levels of chemotactic receptors (flow cytometry) in human eosinophils ± treatment with *AsE*. Dashed lines indicate levels for *HpbE*-treated eosinophils. Data are presented as mean ± SEM, n=5-10 granulocytes from healthy human blood donors. Statistical significance was determined by Wilcoxon test. *p<0.05.

### 5.1.8 *HpbE* remodels the AA metabolism of human bronchial epithelial cells

Human bronchial epithelial cells (HBECs) play an important role in airway inflammation and remodelling during the asthmatic immune response (Hammad et al., 2009) and secretion of cysLTs can be induced under inflammatory conditions like stimulation with the House dust mite extract (HDM) (Jame et al. 2007, Trian et al., 2015). To test whether *HpbE* can reprogram the AA metabolism in HBECs, HBECs were obtained from non-smoking subjects, stimulated with *HpbE* and more than 200 eicosanoids were quantified by LC-MS/MS analysis. In line with *HpbE*-stimulated macrophages, *HpbE* tended to induce the secretion of COX metabolites ($PGE_2$, $PGD_2$ and $TXB_2$), although 12-HETE levels remained hardly unaffected and 5-LOX metabolites ($LTB_4$ and cysLTs) were not detectable in HBECs (Figure 18A). To test whether other parasite extracts can remodel the AA metabolism of HBECs, larval extracts of *Schistosoma mansoni* (*SmE*), *Ascaris suum* (*AsE*), *Ostergaria* (*OstE*) and *Cooperia* (*CoopE*) were obtained to stimulate HBECs. In contrast to *HpbE*, all other parasite extracts failed to induce the secretion of COX metabolites and no changes were observed in the LOX pathway, except for treatment with *AsE* which tended to induce the production of 20-HETE in HBECs (Figure 18B). Thus, *HpbE* induces a unique eicosanoid response in HBECs in comparison to other parasite extracts.

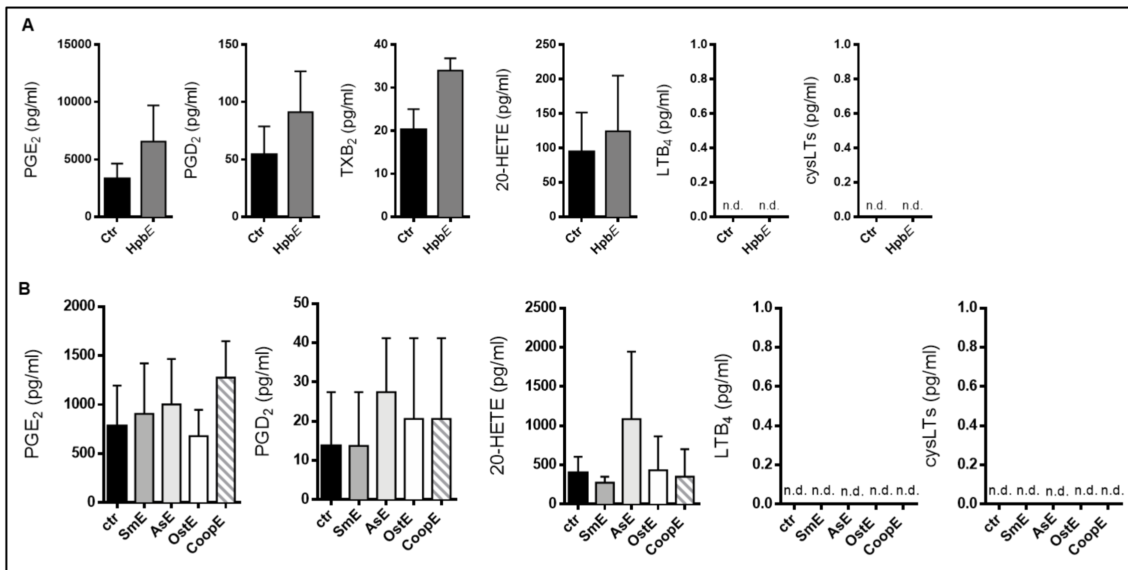


**Figure 18: *HpbE* but not larval extracts from other parasites modulate eicosanoid pathways in human bronchial epithelial cells.** (A) Levels of major bioactive AA metabolites (LC-MS/MS) produced by human bronchial epithelial cells (HBECs) after treatment with *HpbE* (10 µg/ml) (B) Levels of major bioactive AA metabolites (LC-MS/MS) produced by HBECs after treatment with larval extracts of *Schistosoma mansoni* (*SmE*), *Ascaris suum* (*AsE*), *Ostergaria* (*OstE*) and *Cooperia* (*CoopE*) (10µg/ml). Data are presented as mean ± SEM, n=2-4 HBECs.

### 5.1.9 *HpbE* does not impact on type 2 cytokine expression, but induces IFN-γ, IL-10 and eicosanoid reprogramming in human PBMCs

*HpbE* can reprogram the eicosanoid profile and induce the release of anti-inflammatory cytokines in human and mouse macrophages, having similar effects in human granulocytes. The aim of the next experiments was to assess whether *HpbE* could induce type 2 cytokines in human peripheral

blood mononuclear cells (PBMCs). The gene expression of type 2 cytokines was assessed by qPCR analysis in *HpbE*-stimulated PBMCs. However, *HpbE* treatment hardly modified the gene expression of *IL-4, IL-5, IL-13* in PBMCs (Figure 19A). In contrast, there was an induction at the gene level of *IFNG* (Figure 19A) and the induction of anti-inflammatory cytokine IL-10 at both gene and protein level (Figure 19B). Thus, *HpbE* induced a shift towards a type 2 suppressive cytokine profile in different human immune cell populations: macrophages, granulocytes and PBMCs (Figure 13A, 13B, 19A, 19B).

The eicosanoid profile of *HpbE*-treated PBMCs was also analysed by LC-MS/MS. *HpbE* triggered the synthesis of prostanoids ($PGE_2$ and $TXB_2$), whilst it inhibited the production of pro-inflammatory 5-LOX metabolites ($LTB_4$ and 5-HETE) (Figure 19C), similar to the lipid mediator profile measured in *HpbE*-treated macrophages and granulocytes (Figure 12A, 12D, 16B). In contrast to macrophages and granulocytes, *HpbE* strongly induced the production of 12-/15-LOX metabolites (12-HETE and 15-HETE) (Figure 19C), metabolites also measured in the intestinal culture supernatant or peritoneal lavage of *Hpb*-infected mice *in vivo* (Figure 10A, 10B). Thus, in both human and murine leukocytes as well as during helminth infection *in vivo*, immunologically active components of *HpbE* induce an AA-metabolic profile, characterised by induction of regulatory COX metabolites (e.g. $PGE_2$) but suppression of pro-inflammatory LTs ($LTB_4$ and cysLTs).

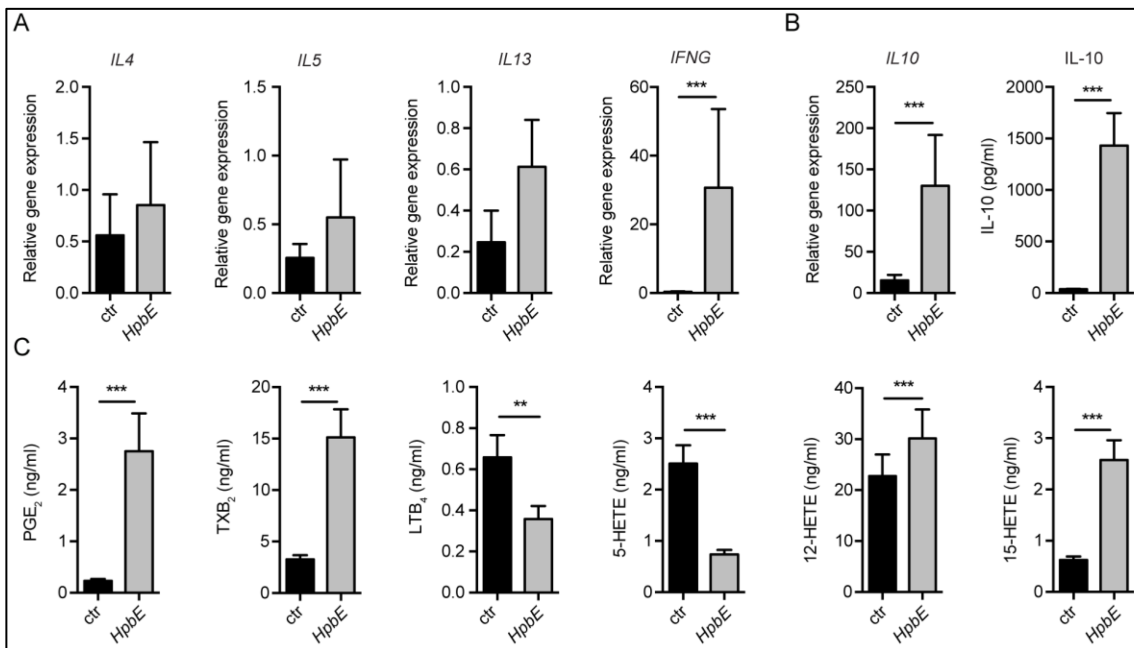


**Figure 19: *HpbE* modulates eicosanoid and cytokine production in human PBMCs.** (A) Gene expression of type 2 cytokines or IFNG (qPCR) in human PBMCs ± treatment with *HpbE* (10 µg/ml). (B) Gene expression (qPCR) and protein levels (ELISA) of IL-10 in human PBMCs ± treatment with *HpbE*. (C) Levels of major bioactive AA metabolites (LC-MS/MS) produced by human PBMCs ± treatment with *HpbE*. Data are presented as mean ± SEM, n=5-6 PBMCs from healthy human blood donors. Statistical significance was determined by Wilcoxon test. **$p<0.01$, ***$p<0.001$.

### 5.1.10 *HpbE*-driven mediator reprogramming is dependent on HIF1α, p38 MAPK, COX and NFκβ in macrophages

During experimental infection of mice with *H. polygyrus*, immunofluorescence stainings of gut tissues showed high expression of HIF-1α in the surrounding of the infective larvae (Figure 10C), suggesting that HIF-1α might be involved in the *Hpb*-driven mediator reprogramming *in vivo*. Thus, to further elucidate a potential involvement of HIF-1α, murine macrophages were stimulated with *HpbE* and the expression of HIF-1α and COX-2 was assessed by immunofluorescence stainings. In line with the *in vivo* data, *HpbE* induced the activation of the transcription factor HIF-1α and its translocation to the nucleus, and further induced high expression of COX-2 and cellular redistribution of the macrophage marker F4/80 in murine macrophages (Figure 20A). To investigate the relevance of HIF-1α in AA-metabolic reprogramming, HIF-1α deficient BMDM (HIF-1α$^{fl/fl}$xLysMCre) were treated with *HpbE* and the eicosanoid profile was measured by LC-MS/MS analysis. In contrast to wildtype (wt) BMDM, HIF-1α deficient BMDM failed to induce the synthesis of prostanoids ($TXB_2$ and $PGE_2$) in response to *HpbE*, whilst inhibition of pro-inflammatory mediators $PGD_2$ or the LOX-metabolite $LTB_4$ remained unaltered after *HpbE* stimulation (Figure 20B). To assess the role of HIF-1α in the synthesis of different cytokines known to be induced by *HpbE* in macrophages (Figure 13), the cytokine profile was assessed by Bioplex analysis after *HpbE* treatment. HIF-1α deficient BMDM showed a reduced *HpbE*-driven induction of IL-6, TNFα and IL-10 (Figure 20C). The expression of M2 markers was also affected by the absence of HIF-1α. Gene expression analysis of M2 makers showed a reduction in the expression of *Tgm2* and *Arg1* in response to *HpbE* in HIF-1α deficient BMDM (Figure 20D). Levels of *Mrc1* were generally higher in BMDM lacking HIF-1α, but *HpbE* down-regulated *Mrc1* gene expression regardless of HIF-1α, while the gene expression of *Rentla/Fizz1* remained intact (Figure 20D). In summary, HIF-1α is largely involved in the regulation of the COX pathway and the induction of type2-suppressive mediators in *HpbE*-treated murine macrophages.

The next research aim was to further decipher the mechanism involved in *HpbE*-driven mediator reprogramming in human macrophages. As HIF-1α is positively regulated by the p38 MAPK (mitogen-activated protein kinase), the expression of phosphorylated p38 (p-p38) MAPK (active form of p38) was confirmed by Western blot in human MDM. Indeed, *HpbE* induced phosphorylation of p38, correlating with higher expression of COX-2 in MDM (Figure 20E). To assess the relevance of HIF-1α at the eicosanoid level, MDM were pre-treated with pharmacological inhibitors of p38 MAPK (VX-702), COX (indomethacin) and HIF-1α (acriflavine). Pre-treatment with VX-702 and indomethacin abrogated the *HpbE*-driven induction of IL-10 and IL-1β (Figure 20F, 21A) and the induction of $PGE_2$-synthetic enzymes at the gene level (*PTGS2* and *PTGES*) in human MDM (Figure 20G). Similar to HIF-1α dependent mediator reprogramming in murine BMDM, pre-treatment with a pharmacological inhibitor of HIF-1α (acriflavine) attenuated the *HpbE*-driven induction of IL-10 and IL-1β (Figure 20F, 21A) and the induction of $PGE_2$-synthetic enzymes (*PTGS2* and *PTGES*) at the gene level in human MDM (Figure 20G). Interestingly, pharmacological inhibition of p38, COX or HIF-1α hardly affected the *HpbE*-driven downregulation of the 5-LOX biosynthetic enzymes (*ALOX5* and *LTA4H*) in MDM (Figure 20G). Thus, *Hpb* products induce the activation of p38 MAPK, leading to the activation of COX and the transcription factor HIF-1α involved in the production of COX mediators and anti-inflammatory cytokines in murine and human macrophages.

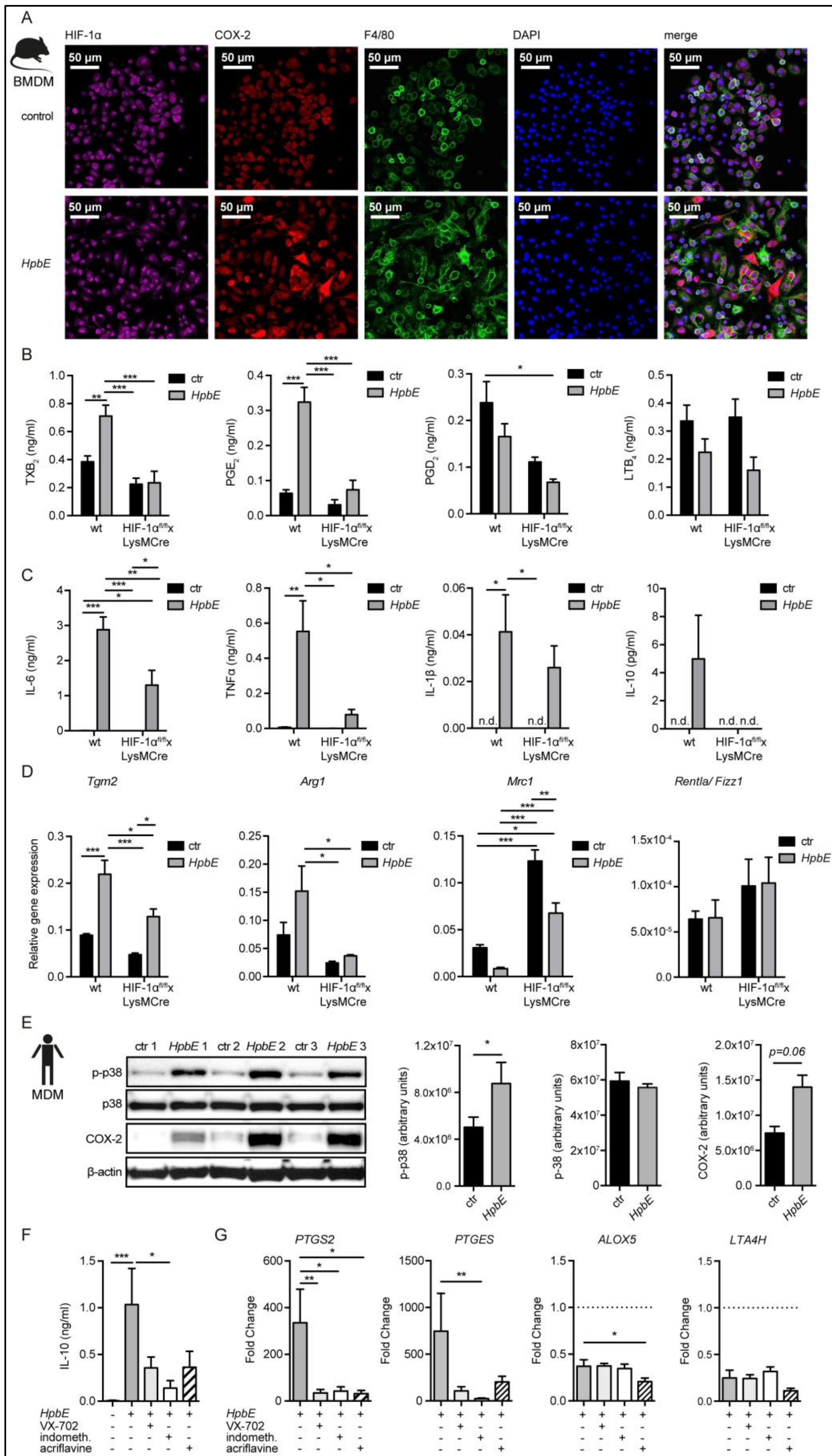

**Figure 20: *HpbE*-driven mediator reprogramming is dependent on HIF-1α and p38 MAPK activity.** (A) Representative immunofluorescence staining of HIF-1α, COX-2, DAPI (cell nuclei) and F4/80 in mouse BMDM ± treatment with *HpbE* (10 µg/ml). (B) Levels of AA metabolites (LC-MS/MS) in mouse

BMDM (wt or HIF-1α $^{floxed/floxed}$ x LysMCre) ± treatment with *HpbE*. (C) Levels of IL-6, TNFα, IL-1β or IL-10 (Bioplex) in mouse BMDM (wt or HIF-1α $^{floxed/floxed}$ x LysMCre) ± treatment with *HpbE*. (D) Gene expression of M2 markers (qPCR) in mouse BMDM (wt or HIF-1α $^{floxed/floxed}$ x LysMCre) ± treatment with *HpbE*. (E) Protein levels of phospho-p38, total p38, COX-2 or β-actin (westernblot) in human MDM ± treatment with *HpbE*. Left: representative blots for human MDM from n=3 blood donors; right: quantification for n=5-8 donors. (F) Levels of IL-10 (ELISA) in human MDM ± treatment with *HpbE* ± inhibitors of p-38 (VX-702, 1 μM), COX (indomethacin, 100 μM) or HIF-1α (acriflavine, 3 μM). (G) Relative gene expression of $PGE_2$- or LT-synthetic enzymes in human MDM treated with *HpbE* ± inhibitors of p-38 (VX-702), COX (indomethacin) or HIF-1α (acriflavine). Dotted lines indicate levels in untreated cells. Data are pooled from at least 2 independent experiments and presented as mean ± SEM, n=5-8 BMDM from wt or HIF-1α $^{floxed/floxed}$ x LysMCre mice or n=5-9 MDM from human blood. Statistical significance was determined by Wilcoxon test for two groups, Friedman test (paired samples) or 2way ANOVA (unpaired samples) for more than 2 groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Since indomethacin is an unspecific inhibitor of COX, a pharmacological inhibitor of COX-2 (CAY 10404) was used to further elucidate the role of COX-2 in *HpbE*-driven reprogramming in human macrophages. Pre-treatment with CAY 10404 did not influence the *HpbE*-driven induction of IL-10 and IL-1β (Figure 21B), although gene expression of IL-10 was upregulated in human MDM (Figure 21C). As expected after using a COX-2 inhibitor, *HpbE*-triggered COX-2 gene expression (*PTGS2*) was reduced by CAY 10404 treatment (Figure 21C). In contrast, the $PGE_2$-biosynthetic enzyme (*PTGES*) was upregulated and the 5-LOX pathway remained largely unaffected by CAY 104 treatment in MDM (Figure 21C). Therefore, a non-selective COX inhibitor (indomethacin), but not a selective COX-2 inhibitor (CAY 10404) reduced the induction of IL-10, IL-1β and *PTGES* (Figure 20F, 20G, 21A, 21B, 21C) suggesting that COX-1 might be involved in *HpbE*-driven reprogramming in human macrophages.

A cross-talk between the transcription factors NFκβ and HIF-1α has been previously demonstrated in innate immune cells (Rius et al., 2008). In order to test whether NFκβ was involved in the pathway activated by *HpbE*, human macrophages were pretreated with an inhibitor of NFκβ (BAY 11-7085) before *HpbE* stimulation. The *Hpb*E-driven production of prostanoids ($PGE_2$) and cytokines (IL-10 and IL-1β) was suppressed by BAY 11-7085 treatment in MDM (Figure 21D). Furthermore, a down-regulation of *HpbE*-induced gene expression of IL-10 and $PGE_2$-biosynthetic enzymes (*PTGES* and *PTGS2*) was shown after inhibition of NFκβ, whilst gene expression of *ALOX5* remained unaltered (Figure 21E). Thus, NFκβ is also involved in the regulation of the COX pathway and cytokine production in *HpbE*-stimulated macrophages.

As the kinases PI3 kinase (PI3K), protein kinase A (PKA) and PTEN can regulate AA-metabolic pathways, the contribution of these kinases in the mechanism of *HpbE*-driven mediator reprogramming was assessed in human macrophages. Pharmacological inhibition of PTEN (SF1670), PI3K (Wortmannin) or PKA (H89) did not interfere with the induction of $PGE_2$, IL-10 and IL-1β in *HpbE*-treated macrophages (Figure 21F). Taken together, *HpbE* activates p38 MAPK and increases the transcription factors NFκβ and HIF1α, leading to the induction of the COX pathway with the synthesis of prostanoids ($PGE_2$ and $TXB_2$) and anti-inflammatory cytokines (IL-10 and IL-1β) in mouse and human macrophages.

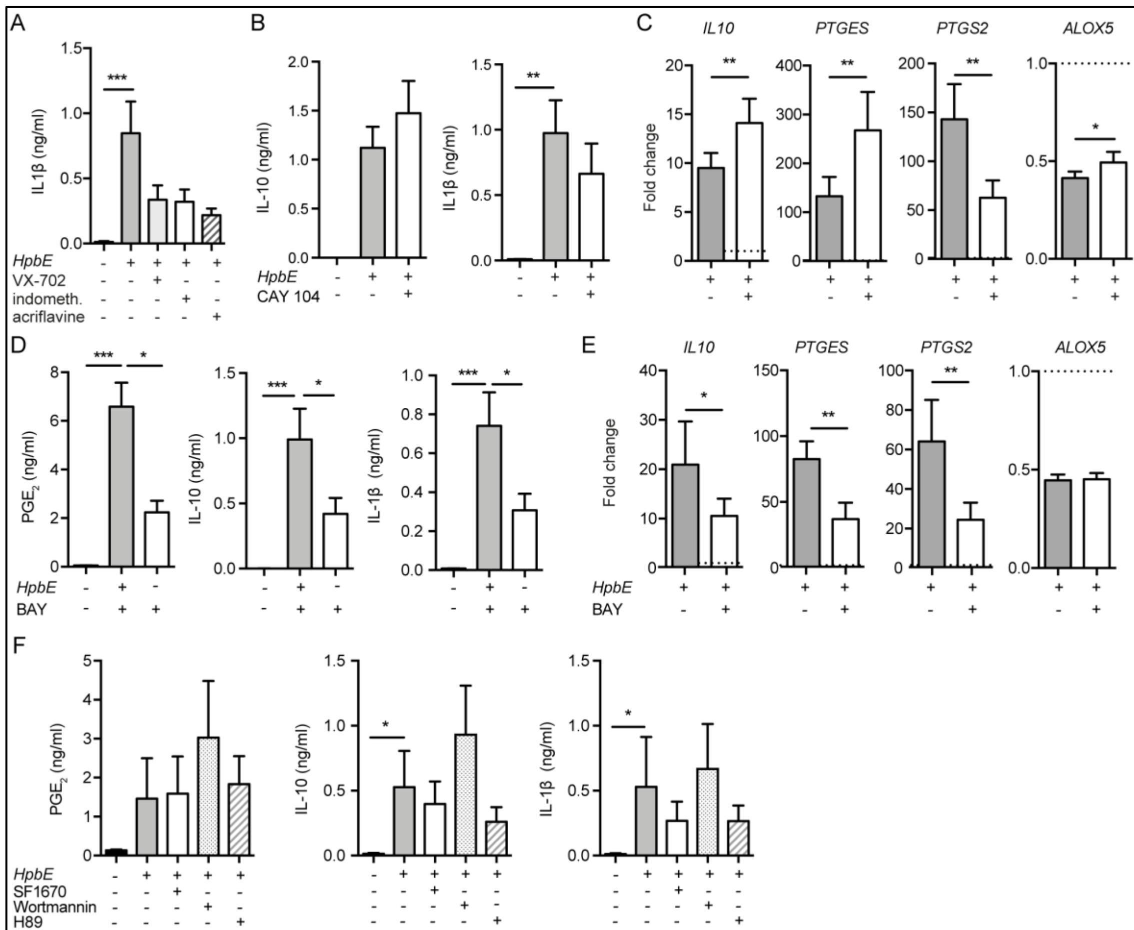


**Figure 21: NFκb, but not PI3K, PKA or PTEN contributes to *HpbE*-driven mediator reprogramming in human macrophages.** (A) Levels of IL-1β (ELISA) produced by human MDM ± treatment with *HpbE* (10 µg/ml) ± inhibitors of p-38 (VX-702, 1 µM), COX (indomethacin, 100 µM) or HIF-1α (acriflavine, 3 µM). (B) Levels of IL-10 or IL-1β (ELISA) produced by human MDM ± treatment with *HpbE* ± selective COX-2 inhibitor (CAY10404, 10 µM). (C) Gene expression of IL10, $PGE_2$-synthetic enzymes or ALOX5 (qPCR) for human MDM treated with *HpbE* ± selective COX-2 inhibitor (CAY10404). (D) Levels of $PGE_2$ (EIA) or IL-10 and IL-1β (ELISA) for human MDM ± treatment with *HpbE* ± NFκb inhibitor (BAY 11-7085, 5 µM). (E) Gene expression of IL10, $PGE_2$-synthetic enzymes or ALOX5 (qPCR) for human MDM treated with *HpbE* ± NFκb inhibitor (BAY 11-7085). (F) Levels of $PGE_2$ (EIA) or IL-10 and IL-1β (ELISA) produced by human MDM after treatment with *HpbE* ± inhibitors of PTEN (SF1670, 250 nM), PI3K (Wortmannin, 100nM) or PKA (H-89, 10 µM). Data are presented as mean ± SEM, MDM from n=5-11 donors. Statistical significance was determined by Wilcoxon test (two groups) or Friedmann test (more than 2 groups). *p<0.05, **p<0.01, ***p<0.001.

### 5.1.11 Upstream mechanisms involved in *HpbE*-driven reprogramming in macrophages

To further elucidate the upstream mechanism underlying prostanoid and cytokine modulation in *HpbE*-stimulated macrophages, neutralizing antibodies were used to block IL-1β or pattern recognition receptors (PRRs) such as TLR2, which can modulate the host immunity and play a role in the modulation of airway inflammation during helminth infection (Cho et al., 2015; Zaiss et al., 2013). Gene expression analysis by qPCR showed that blocking IL-1β before *HpbE* stimulation neither affected the gene expression of *IL-10* or $PGE_2$- biosynthetic enzymes (*PGTS2* and *PTGES*) nor the suppression of the 5-LOX-pathway (e.g. *ALOX5*), suggesting that IL-1β is downstream of the *HpbE*-driven reprogramming in macrophages (Figure 22A). Moreover,

blockade of TLR2 did not influence the gene expression of *IL-10* or *ALOX5*, whilst it abrogated the *HpbE*-driven induction of the COX pathway (*PGTS2* and *PTGES*), suggesting that TLR2 might be involved in the COX regulation and prostanoid synthesis (Figure 22A).

Signalling through other PRRs such as dectin-1 and dectin-2 has been shown to modulate host immunity during helminth infection (Kaisar et al., 2018; Ritter et al., 2010). To further elucidate the role of these PPRs in *HpbE*-stimulated human macrophages, neutralizing antibodies were used to block dectin-1 and dectin-2 signalling pathways. Similar to previous experiments using antibodies against TLR2, blocking dectin-1 and dectin-2 attenuated the induction of $PGE_2$-synthetic enzymes by *HpbE*, whilst the modulation of the gene expression of IL-10 or 5-LOX remained unaltered (Figure 22B). Taken together, *HpbE* activated PRRs (TLR2, dectin-1 and dectin-2) and induced the activation of p38 MAPK and transcription factors NFκβ and HIF1α, which together resulted in the induction of the COX-pathway and increased production of type 2 suppressive mediators in macrophages.

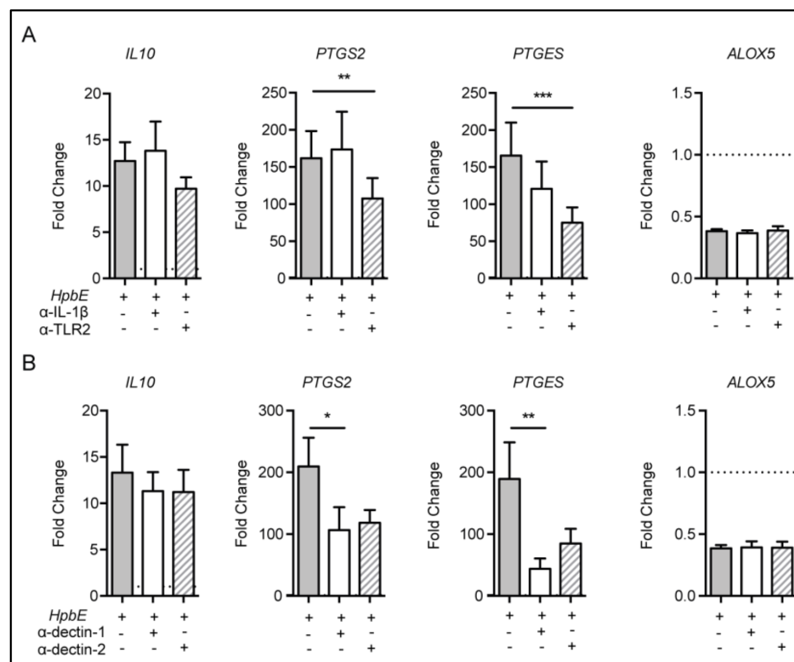


**Figure 22: PRRs (TLR2 and Dectins-1 and -2), but not IL-1β contribute to *HpbE*-driven mediator reprogramming.** (A) Relative gene expression of *IL10*, $PGE_2$-synthetic enzymes or *ALOX5* (qPCR) in human MDM treated with *HpbE* (10 µg/ml) ± blocking antibodies against IL-1β (5 µg/ml) or TLR2 (10 µg/ml). (B) Relative gene expression of *IL10*, $PGE_2$-synthetic enzymes or *ALOX5* (qPCR) in human MDM treated with *HpbE* ± blocking antibodies against dectins-1 or -2 (both 10 µg/ml). Data are pooled from at least 2 independent experiments and presented as mean ± SEM, MDM from n=5-8 donors. Statistical significance was determined by Wilcoxon test (two groups) or Friedman test (more than 2 groups). *p<0.05, **p<0.01, ***p<0.001. Dashed line indicates control level.

### 5.1.12 Role of HIF-1α during *H. polygyrus* infection *in vivo*

HIF-1α is involved in the *HpbE*-driven induction of COX metabolites and type 2 regulatory cytokines in mouse and human macrophages, as demonstrated in HIF1α-deficient murine BMDM as well during HIF1α-pharmacological inhibition in human MDM after *HpbE* stimulation *in vitro* (Figure 20). Furthermore, high expression of HIF-1α was detected by immunofluorescence

staining in the surrounding on the infective larvae in gut sections of *Hpb*-infected mice (Figure 10C). Thus, the next research question was to assess the role of HIF1α during *Hpb*-infection *in vivo*. For this set of experiments, wildtype (wt) and HIF-1α$^{floxed/floxed}$ x LysMCre mice were infected with *Hpb* and at day 7 p.i., worm counts were performed, intestinal culture supernatant and peritoneal lavage were obtained for further eicosanoid analysis by LC-MS/MS. It was unexpected to find no differences in the number of worms counted in the small intestine of WT and HIF-1α$^{floxed/floxed}$ x LysMCre mice, suggesting that depletion of myeloid HIF-1α did not have an effect in the development of *Hpb* infection at early stages of infection (Figure 23A).

As previously shown, *Hpb* infection induced the production of COX metabolites ($PGE_2$, $PGD_2$, $TXB_2$, 6-keto PGF1α and PGF2α) in intestinal culture supernatant and peritoneal lavage, with higher levels measured in intestinal culture supernatants in wildtype infected mice (Figure 10A). However, when comparing the eicosanoid profile of *Hpb*-infected wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice, a similar induction in the COX pathway was observed, with similar levels of COX metabolites measured in both wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice, with the only exception of higher 6-keto PGF1α levels in HIF-1α$^{floxed/floxed}$ x LysMCre (Figure 23B, 23C). In the LOX pathway, high production of 12- and 15- HETE metabolites was observed in *Hpb*-infected mice with similar levels between wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice (Figure 23B, 23C). In contrast, 5-LOX metabolites like 5-HETE, $LTB_4$ or CysLTs were close to or below the lower detection limit in intestinal culture supernatants and peritoneal lavage and no differences were observed in HIF-1α$^{floxed/floxed}$ x LysMCre mice (Figures 23B, 23C). In contrast to the key role of HIF-1α in the induction of prostanoids and production of regulatory cytokines in human and mouse macrophages *in vitro*, depletion of myeloid HIF-1α hardly affects the course of helminth infection or the modulation of the AA metabolism during *Hpb* infection *in vivo*.

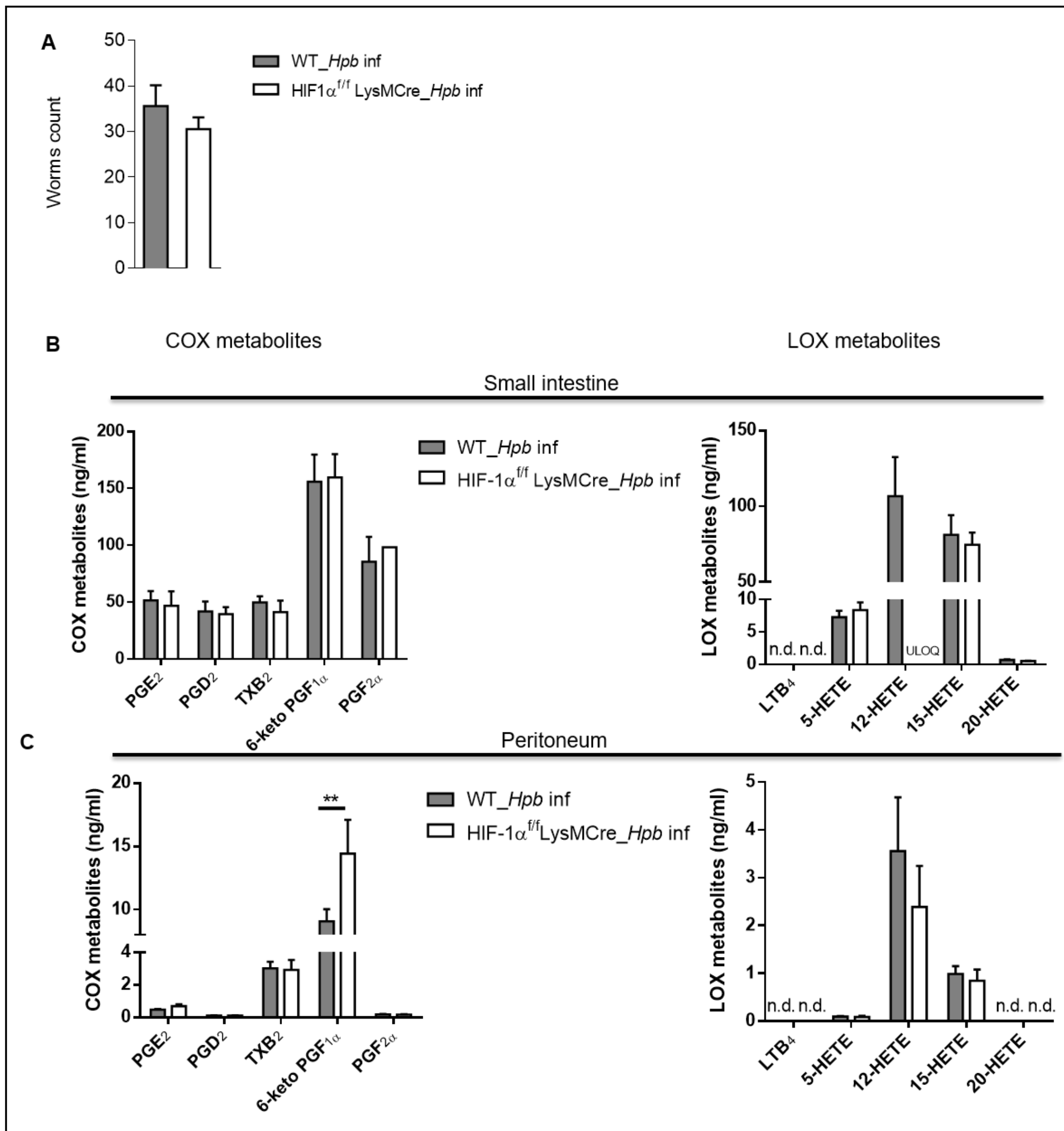


**Figure 23: Myeloid HIF-1α hardly contributes to arachidonic acid reprogramming during infection with *Heligmosomoides polygyrus bakeri* at day 7 post-infection (p.i.) *in vivo*.** (A). Worm count of *Hpb*-infected wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice at day 7 p.i. (B) Levels of COX and LOX metabolites (LC-MS/MS) measured in the intestinal culture supernatant in *Hpb*-infected wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice. (C) Levels of COX and LOX metabolites (LC-MS/MS) measured in peritoneal lavage in *Hpb*-infected wt and HIF-1α$^{floxed/floxed}$ x LysMCre mice. Results in (A), (B) and (C) are presented as mean ± SEM, n=7-8 per group. Statistical significance in (C) was determined by 2way ANOVA with Bonferroni correction. **p = 0.01.

## 5.2. House dust mite (HDM) modulates the myeloid arachidonic acid metabolism and *HpbE* mediates protection during HDM-induced airway inflammation

### 5.2.1 HDM exposure increases COX metabolism in human and mouse macrophages

Eicosanoids are important lipid mediators playing a key role in type 2 immune responses, classically known for their effects in asthma and allergy. During house dust mite (HDM)-inducing

allergic airway inflammation, HDM induces the production of cysLTs in alveolar macrophages and initiates airway inflammation (Clarke et al., 2014). Our group has shown that HDM exposure in the presence of IL-4 reprograms the eicosanoid profile of human macrophages, driving the production of prostanoids ($PGE_2$ and $TXB_2$), but suppressing the synthesis of 5-LOX products ($LTB_4$ and 5-HETE). However, in the absence of IL-4, HDM reprogrammed the eicosanoid profile of human macrophages at a lower extent (Henkel et al., 2018). Here, we aim to study the eicosanoid reprogramming of human and murine macrophages after exposure to HDM. For this set of experiments, human MDM were exposed to HDM and the lipid mediator profile was quantified by LC-MS/MS analysis. HDM triggered the production of COX metabolites ($PGE_2$, $PGD_2$ and $TXB_2$), whilst it suppressed the production of 5-LOX products ($LTB_4$ and 5-HETE) and formation of 15-LOX metabolites remained unaltered (12-HETE and 20-HETE) (Figure 24A). To test whether HDM might have a similar effect on the eicosanoid profile of murine macrophages, BMDM were exposed to HDM and the lipid mediator profile was quantified by LC-MS/MS analysis. Similar to human MDM, HDM triggered the production of COX metabolites ($PGE_2$ and $TXB_2$), although production of $PGD_2$ was slightly suppressed in murine BMDM (Figure 24B). In contrast to human MDM, HDM-stimulated BMDM did not show a suppressed production of pro-inflammatory $LTB_4$ and other metabolites such as 5-HETE or 12-/20-HETE were not detected (Figure 24B). Interestingly, the levels of eicosanoids measured in human MDM were 10-100-fold higher in amplitude as compared to murine BMDM (Figure 24A, 24B).

To test whether HDM treatment also affects macrophage polarization and type 2 immune responses, quantification of several cytokines was performed by Bioplex in murine macrophages. Exposure of murine BMDM to HDM resulted in the induction of immuneregulatory cytokines IL-10 and IL-1β (Figure 24C) (Schoeller et al., 2008; Zaiss et al., 2013). HDM-stimulated BMDM also secreted high amounts of cytokines involved in modulation of M2 macrophages and type 2 immune responses (IL-6, TNF-α) and the chemokine RANTES involved in chemotaxis (Figure 24C) (Guo et al., 2009; Kratochvill et al., 2015).

Taken together, HDM exposure triggered lipid mediator class-switching from 5-LOX to COX metabolism in both human and murine macrophages and could remodel macrophage cytokine production.

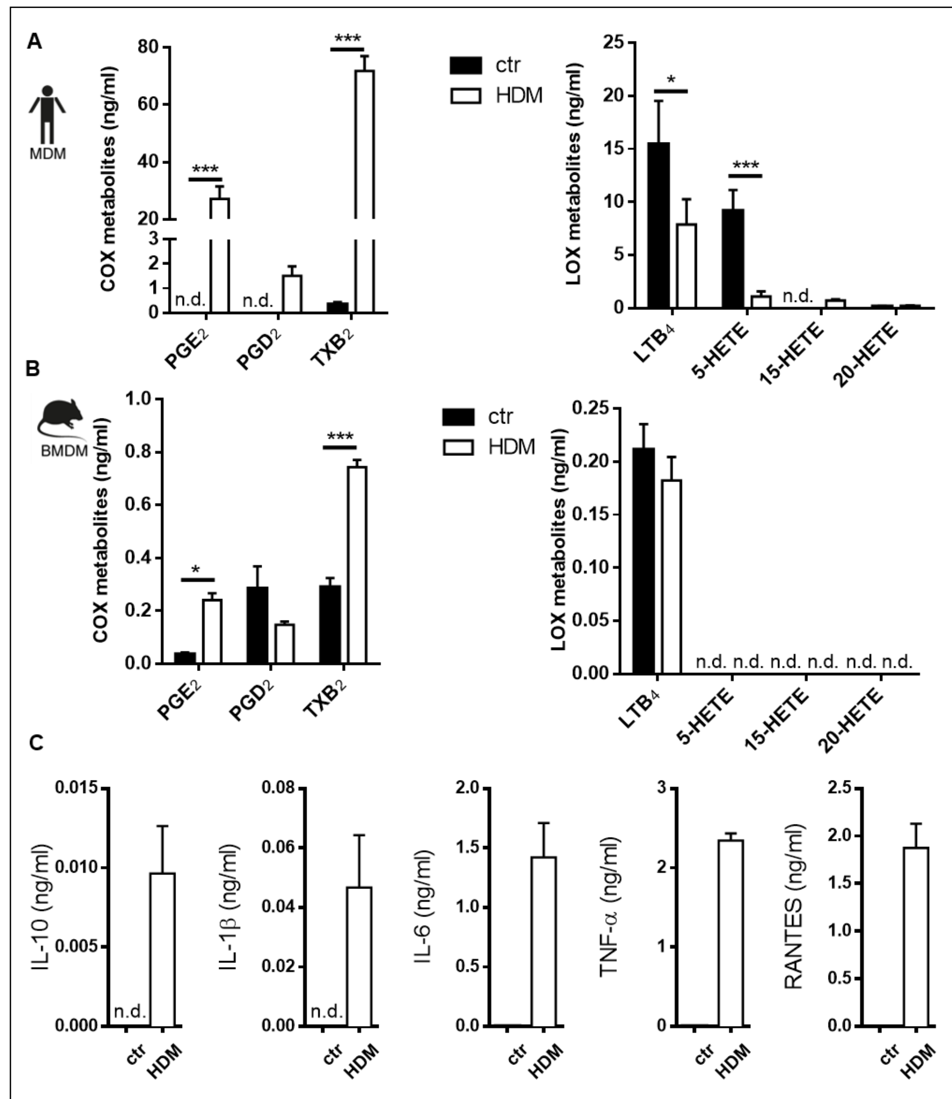


**Figure 24: House dust mite (HDM) triggers COX metabolites and cytokine production in human and mouse macrophages.** (A) Levels of COX and LOX metabolites (LC-MS/MS) produced by human MDM ± treatment with HDM (10µg/ml) (B) Levels of COX and LOX metabolites (LC-MS/MS) produced by murine BMDM ± treatment with HDM (10µg/ml) (C) Levels of IL-10, IL-1β, IL-6, TNF-α and RANTES (Bioplex) in murine BMDM +/- treatment with HDM. Results in (A) are presented as mean ± SEM, n=6 MDM from human blood donors. Results in (B) and (C) are presented as mean ± SEM, n=3 BMDM from C57BL/6 mice. Statistical significance in (A) and (B) was determined by 2-way ANOVA with Bonferroni correction. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

### 5.2.2 HDM and *Hpb* larval extract drive similar eicosanoid reprogramming and cytokine production in a COX-dependent manner in macrophages

As previously shown (see results section 5.1), *Hpb* larval extract (*HpbE*) remodels the AA metabolism of murine and human macrophages by inducing the synthesis of anti-inflammatory prostanoids (Figure 12) and type 2 suppressive cytokines in human and murine macrophages (Figure 13). Similar eicosanoid reprogramming was suggested in HDM-stimulated human and mouse macrophages (Figure 24). Thus, we directly determined similarities and differences in the eicosanoid reprogramming and cytokine production between *HpbE* and HDM-stimulated macrophages form the same donors. Human macrophages were stimulated with *HpbE* and HDM

and the eicosanoid profile was quantified by LC-MS/MS analysis. COX metabolites ($PGE_2$ and $TXB_2$) and 15-LOX product 15-HETE were induced after treatment with *HpbE* and HDM at a similar extent, whilst 5-LOX products ($LTB_4$ and 5-HETE) were suppressed and 20-HETE remained hardly unaltered in human macrophages (Figure 25A). In line with the results observed for human MDM, treatment with *HpbE* or HDM triggered the production of prostanoids ($PGE_2$ and $TXB_2$) in murine macrophages, although lower levels of $LTB_4$ were only observed in *HpbE*-stimulated BMDM but not after HDM stimulation (Figure 25B).

A comparison in the production of cytokines involved in macrophage polarization and type 2 immune responses was also assessed in murine BMDM. Cytokine production such as IL-10, IL-1β, IL-6 and RANTES was similar in *HpbE*- and HDM-stimulated BMDM, except for TNF-α, where HDM tended to induce a higher production in BMDM (Figure 25C). Thus, *HpbE* and HDM trigger a similar eicosanoid reprogramming and cytokine production in human and murine macrophages.

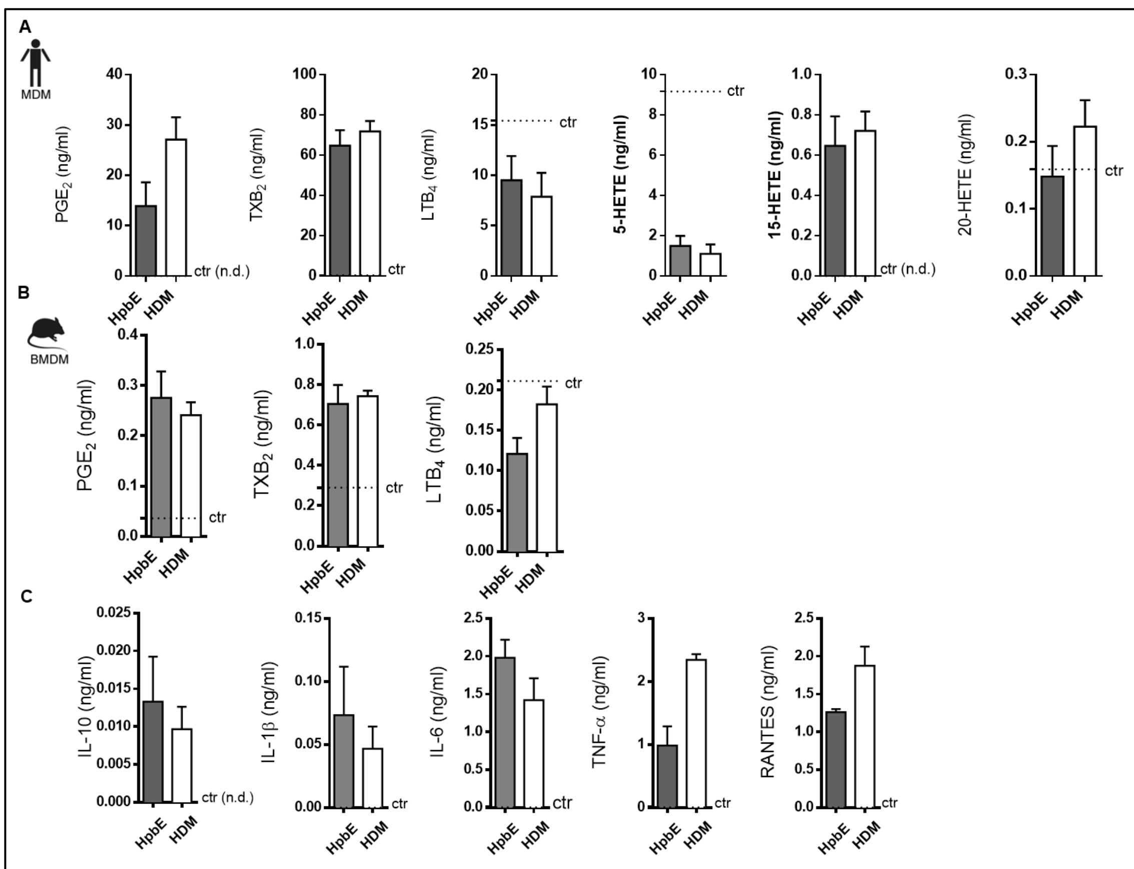


**Figure 25: *HpbE* and HDM drives similar eicosanoid reprogramming in human and mouse macrophages.** (A) Eicosanoid levels (LC-MS/MS) measured in MDM ± treatment with *HpbE* (10µg/ml) and HDM (10µg/ml). (B) Eicosanoid levels (LC-MS/MS) measured in murine BMDM ± treatment with *HpbE* and HDM (C) Levels of IL-10, IL-1β, IL-6, TNF-α and RANTES (Bioplex) in murine BMDM +/- treatment with *HpbE* and HDM. Results in (A) are presented as mean ± SEM, n=6 MDM from human blood donors. Results in (B) and (C) are presented as mean ± SEM, n=3 BMDM from C57BL/6 mice.

To assess whether treatment with *HpbE* would counteract the eicosanoid reprogramming induced by HDM in murine macrophages, BMDM were pre-treated with *HpbE* and exposure to HDM and the eicosanoid profile was quantified by LC-MS/MS analysis. To further elucidate the mechanism involved in eicosanoid modulation, murine macrophages were pre-treated with a pharmacological unspecific inhibitor of COX (indomethacin) before treatment with *HpbE* and HDM. Interestingly, treatment with *HpbE* and HDM together further enhanced prostanoid production ($PGE_2$ and $TXB_2$) triggered by *HpbE* and HDM alone, but hardly affected the $LTB_4$ levels in murine BMDM (Figure 26A). Pre-treatment of BMDM with indomethacin abrogated the production of COX metabolites, but did not alter $LTB_4$ levels in murine macrophages (Figure 26A). To test whether this AA metabolism reprogramming was a result of transcriptional changes in AA-metabolizing enzymes, gene expression of COX-2 (*Ptgs2*), microsomal prostaglandin E synthase (mPGES, *Ptges*) and 5-LOX (*Alox5*) was measured in *HpbE*-/HDM-stimulated BMDM. In line with the eicosanoid profile, *HpbE* and HDM together further induced the gene expression of *Ptgs2* and *Ptges* (enzymes involved in the synthesis of prostanoids), but suppressed the expression of *Alox5* (enzyme responsible for the synthesis of LTs) in murine BMDM (Figure 26B). Pre-treatment with the COX inhibitor prevented the induction of *Ptgs2*, although strongly induced the gene expression of the $PGE_2$-synthetic enzyme (*Ptges*) and hardly affect the gene expression of *Alox5* in murine macrophages (Figure 26B).

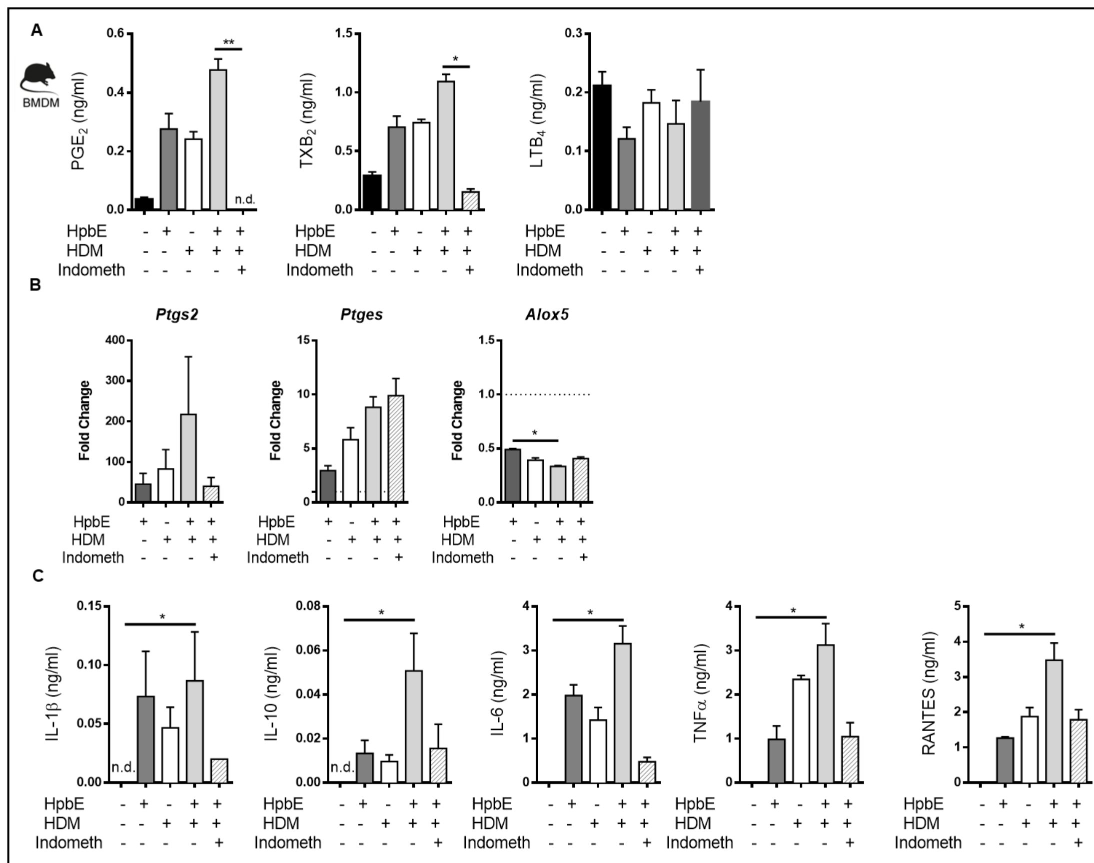


**Figure 26: *HpbE*- and HDM- eicosanoid modulation and cytokine production is COX dependent.** (A) Eicosanoid levels (LC-MS/MS) measured in murine BMDM ± treated with *HpbE* (10µg/ml) and HDM (10µg/ml) +/- inhibitor of COX (indomethacin, 100µM) (B) Fold change gene expression of $PGE_2$- or LT-synthetic enzymes in murine BMDM treated with *HpbE* and HDM +/- COX inhibitor. (C) Levels of IL-10, IL-1β, IL-6, TNF-α and RANTES (Bioplex) in BMDM ± treated with *HpbE* and HDM +/- COX inhibitor. Results are presented as mean ± SEM, n=3 BMDM from C57BL/6 mice. Statistical significance in (A) and (B) was determined by 2-way ANOVA with Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001.

In line with the eicosanoid profile, *HpbE* and HDM together further enhanced the production of cytokines and chemokines such as IL-10, IL-1β, IL-6, TNF-α and RANTES and pre-treatment with indomethacin reduced the production of these cytokines and chemokines in BMDM (Figure 26C). Thus, BMDM stimulation with *HpbE* and HDM together further enhance the reprogramming of the COX pathway and cytokine production in a COX dependent manner in macrophages.

### 5.2.3 Treatment with *HpbE* suppresses allergic airway inflammation *in vivo*

AA metabolites are critical regulators of the type 2 immune responses to house dust mite (HDM) and the immunological modulation of these mediators may represent an alternative strategy to treat allergic airway inflammation (Barret et al., 2011; Draijer et al., 2016). Local AA metabolism changes were shown during *Hpb* infection *in vivo* (Figure 10) and *HpbE* remodels the AA metabolism of myeloid cells *in vitro* (Figure 12, 16). Thus, the next step was to test how treatment with *Hpb* larval extract would affect HDM-induced allergic airway inflammation *in vivo*. Mice were sensitized by intranasal (i.n.) administration of HDM and challenged four times from day 8 to 11. Another mouse group received i.n. administration of *HpbE* before HDM sensitization and challenge (Figure 27A). HDM induced strong type 2 inflammation as shown by the high eosinophil numbers counted in the bronchoalveolar lavage fluid (BALF) of HDM-sensitized mice and treatment with *HpbE* tended to decrease HDM-induced eosinophilia (Figure 27B). Treatment with *HpbE* also reduced airway inflammation and mucus production induced by HDM sensitization (Figure 27C). Eicosanoid analysis of the BALF revealed that the production of 15-HETE in HDM-sensitized mice was abrogated after *HpbE* treatment, results which are consistent with decreased eosinophilia after *HpbE* treatment, as 15-HETE is an AA mediator mainly produced by eosinophils (Figure 27D). Treatment with *HpbE* tended to reduce the production of pro-inflammatory cytokines and chemokines (IL-6, Eotaxin, RANTES) induced by HDM sensitization (Figure 27D). Thus, *HpbE* reprograms the AA metabolism *in vivo* and may thus suppress type 2 inflammation during HDM allergy.

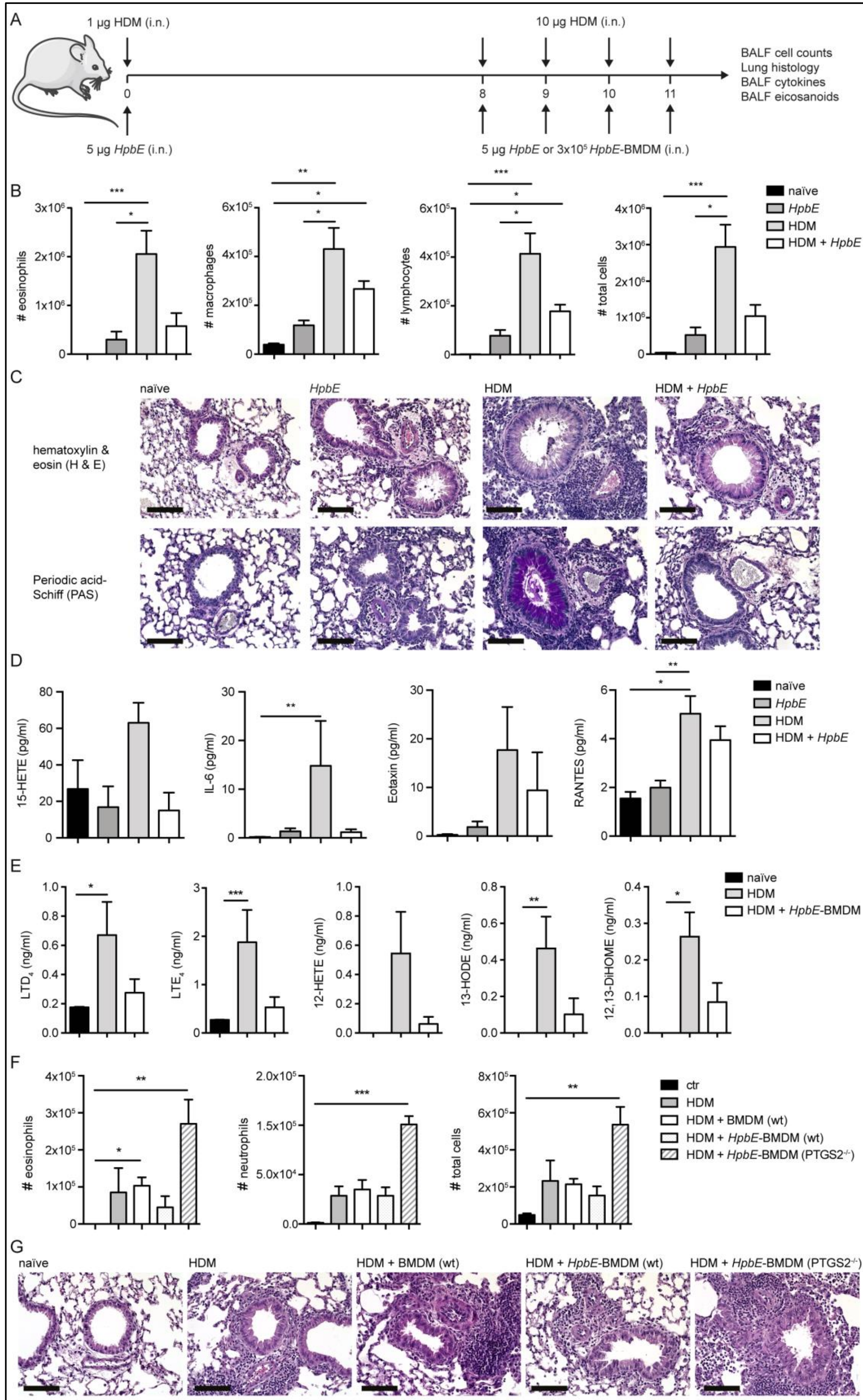


**Figure 27: Treatment with *HpbE* or transfer of *HpbE*-conditioned BMDM modulates AA metabolism and suppresses airway inflammation during HDM allergy *in vivo*.** (A) Experimental model of house

dust mite (HDM)-induced allergic airway inflammation and intranasal (i.n) treatment with *HpbE* (5 µg) or transfer of *HpbE*-conditioned BMDM ($3x10^6$). (B) BALF cell counts in mice sensitized and challenged with HDM (1 µg/ 10 µg) ± intranasal treatment with *HpbE*. (C) Representative haematoxylin and eosin (H&E)- or Periodic Acid-Schiff (PAS) stained lung tissue from mice sensitized to HDM ± treatment with *HpbE*. Scale bar: 100 µm. (D) Levels of eicosanoids (LC-MS/MS) or IL-6, eotaxin and RANTES (Bioplex) in BALF from mice sensitized to HDM ± treatment with *HpbE*. (E) Levels of eicosanoids (LC-MS/MS) in BALF from mice sensitized to HDM ± intranasal transfer of *HpbE*-conditioned BMDM. (F) BALF cell counts in mice sensitized to HDM ± intranasal transfer of *HpbE*-conditioned BMDM (wildtype (wt) or PTGS2-/-). (G) Representative H&E stained lung tissue from mice sensitized to HDM ± intranasal transfer of untreated or *HpbE*-conditioned BMDM (wt or PTGS2-/-). Scale bar: 100 µm. Data are pooled from 2 independent experiments and presented as mean ± SEM, n=3-10 mice per group. Statistical significance was determined by Kruskal-Wallis test followed by Dunn's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001.

### 5.2.4 Modulation of type 2 inflammation by *HpbE*-conditioned macrophages depends on COX-2 metabolites

The data described in previous sections showed that *HpbE* remodels the AA metabolism and induces anti-inflammatory COX metabolites ($PGE_2$) and type 2 suppressive mediators (IL-10, IL-1β) in macrophages (Figure 12, 13). Macrophages are key producers of AA metabolites in the airways and bone marrow- or monocyte-derived macrophages accumulate and drive allergic airway inflammation (Zaslona et al., 2014a). However, transfer of $PGE_2$-stimulated macrophages exerted a protective effect and reduced airway cell infiltration during HDM-induced allergic lung inflammation (Draijer et al., 2016). To further elucidate the mechanisms behind the *HpbE*-driven suppression of type 2 inflammation during HDM-induced airway inflammation (Figure 27A-E) as well as the role of macrophages in this protective effect, intranasal transfer of *HpbE*-treated BMDM was performed before HDM challenges (Figure 27A). Analysis of the eicosanoid profile of BALF samples showed that transfer of *HpbE*-treated BMDM attenuated the production of cysLTs ($LTD_4$ and $LTE_4$), as well as 12-15 LOX metabolites (12-HETE, 13-HODE and 12,13-DiHOME) induced by HDM sensitization (Figure 27E). Since the mechanism involved in *HpbE*-driven mediator reprogramming is dependent on COX activation in macrophages *in vitro* (Figure 20, 26), the contribution of the COX pathway was tested by transfer of *HpbE*-treated BMDM from wildtype (wt) or COX-2 deficient mice ($PTGS2^{-/-}$) *in vivo*. Mice that received untreated BMDM during experimental HDM allergy showed increased airway eosinophilia, overall cell infiltration and airway inflammation as compared to control mice (Figure 27F, 27G). This effect was lost when mice received wildtype BMDM that had been treated with *HpbE* (Figure 27F, 27G). In contrast, transfer of *HpbE*-treated $PTGS2^{-/-}$ BMDM resulted in exaggerated increase in the number of eosinophils and neutrophils counted in the BALF as well as exaggerated airway cell infiltration and inflammation during HDM allergy (Figure 27F, 27G). Taken together, *HpbE* induced COX-2 metabolites in macrophages, which regulated granulocyte recruitment and type 2 inflammation during HDM allergy *in vivo*.

## 5.3 Age dependent synthesis of leukotrienes during allergic asthma and therapeutic application of *Hpb* larval extract

### 5.3.1 Leukotriene production is age dependent during allergic airway inflammation *in vivo*

Leukotrienes (LTs) are critical regulators of the type 2 immune responses to house dust mite (HDM) and play an important role during airway inflammation and remodelling (Figure 27) (Barret et al., 2011; Henderson et al., 2002). However, whether the synthesis of LTs and its regulation depends on the age of sensitization remains poorly understood. Thus, the aim was to test how HDM sensitization at different ages would affect LT production. Mice were sensitized by intranasal (i.n.) administration of HDM immediately after birth (day 3) and compared to mice sensitized in the preweaning (day 15) or adult (day 60) period after birth, and mice were challenged every other day (Figure 28A). Levels of LTs were measured in BALF samples by EIA and expression of LT-biosynthetic enzymes was assessed by immunohistochemistry (IHC) stainings of lung tissues. HDM induced high production of LTs (cysLTs and $LTB_4$) in mice sensitized as neonates and adults, whilst mice sensitized in the preweaning period showed the lowest production of LTs (Figure 28B). In line with LT levels in BALF, IHC stainings of lungs tissues of HDM-sensitized mice as neonates and adults showed high expression of LT enzymes (Figure 28C). 12/15-LO expression was higher in neonatal sensitized mice and 12/15-LO was localized in the cells infiltrating into the lung, most likely eosinophils since 12/15-LO is a marker for eosinophils (Figure 28C, 28D). The high number of eosinophils found in neonates could explain the high levels of cysLTs measured in the BALF, since eosinophils are an important source of LTs in the airways (Hogan et al., 2008). In contrast, 12/15-LO was not detected in preweanling mice but higher expression is observed at day 60 in the perivascular infiltrates (Figure 28C, 28D). Neonatally sensitized mice showed high expression of 5-LO and $LTC_4S$ localized in the airway epithelium, although $LTA_4H$ expression mostly localized in leukocytes and smooth muscle cells (Figure 28C, 28E). Expression of LTs-synthetic enzymes (5-LO, $LTC_4S$, $LTA_4H$) was lower in HDM-sensitized mice after day 15, in contrast to adult sensitized mice which showed higher levels of 5-LO, $LTC_4S$ and $LTA_4H$ localized in the airway epithelium, suggesting a major contribution of the epithelium to LT production during the adult time (Figure 28C, 28E).

In order to exclude that LT production was dependent only on the age and not on HDM sensitization at different ages, LTs levels were measured in the BALF of naïve mice at different ages. LTs levels of naïve mice were 10- to 50- fold lower as measured in HDM-sensitized mice (Figure 28B, 29A). No differences in $LTB_4$ levels were observed between naïve mice at different ages, although there was a slight decrease in cysLTs levels measured in adult compared with younger naïve mice (Figure 29A). In line with the low levels of LTs, expression of 12/15-LO or 5-LO was not detected in the lung tissues of naïve mice (Figure 29B, 29C). These results suggest that LT formation is age dependent. The highest LTs levels were observed in neonatal mice with a major contribution of eosinophils to LTs production. Adult mice showed high production of cysLTs and the immunohistochemistry data suggested that the airway epithelium contributed to this high cysLT production. Preweanling mice seem to be more protected against HDM sensitization as LTs production or expression of LT-biosynthetic enzymes was only observed at low levels in this mouse group.

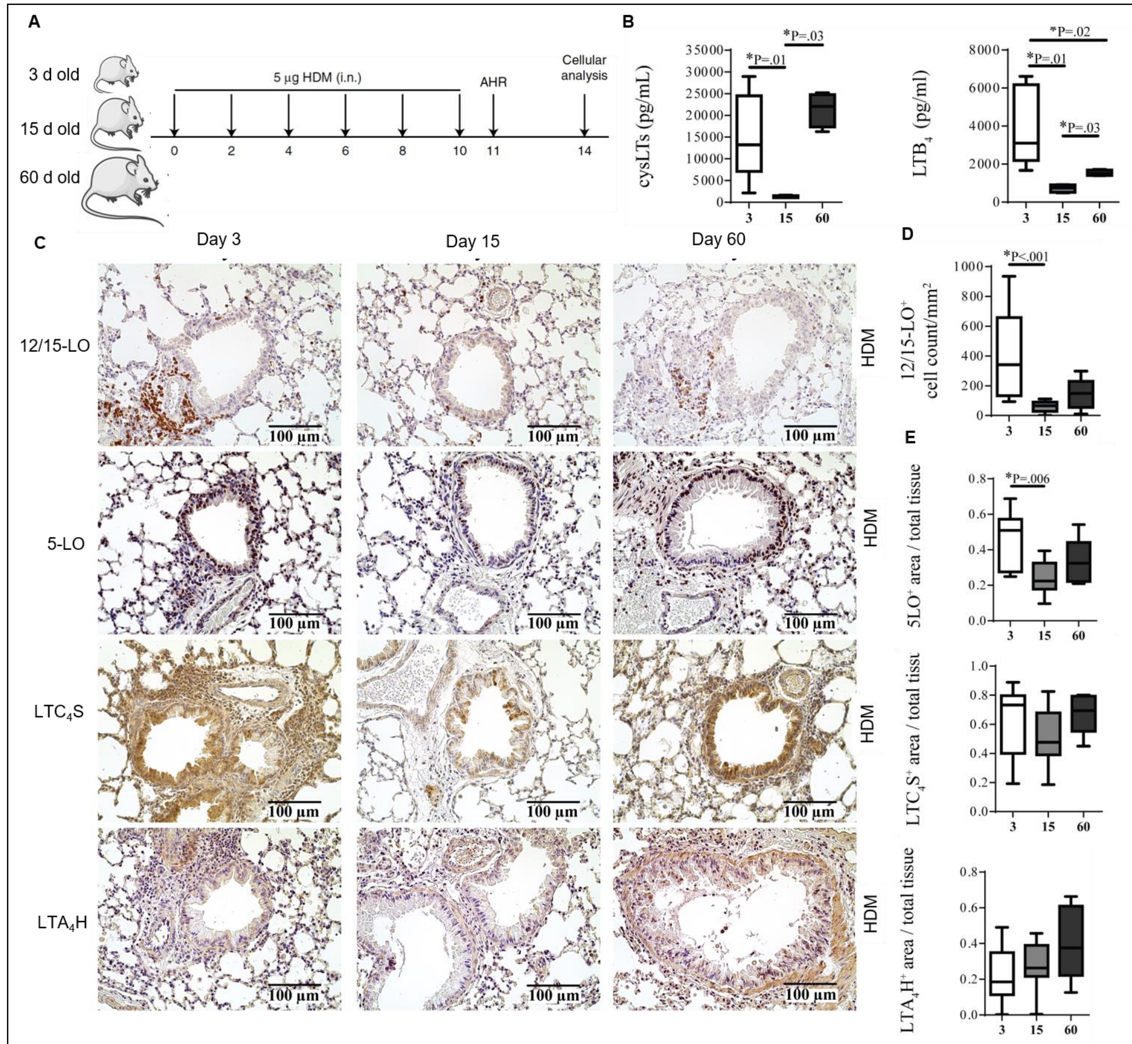


**Figure 28: Leukotrienes production is age dependent in a mice model of HDM-induced allergy *in vivo*.** (A) Experimental model of house-dust mite (HDM)-induced allergic airway inflammation from day 3, 15 or 60 of life. (B) Levels of leukotrienes (cysLTs, $LTB_4$) (EIA) measured in BALF from HDM-sensitized mice. (C) Representative immunohistochemistry (IHC) stained lung tissues from mice sensitized to HDM. Scale bar: 100 µm (D) Counts of $12/15LO^+$ cells per square millimetre lung section (E) Quantification of LT-producing enzymes (5-LO, $LTC_4S$ and $LTA_4H$) expression in lung sections from HDM-sensitized mice. Data is presented in (B), (D) and (E) as mean ± SEM for n=4-6 mice per group. Statistical significance was determined by Mann-Whitney test.

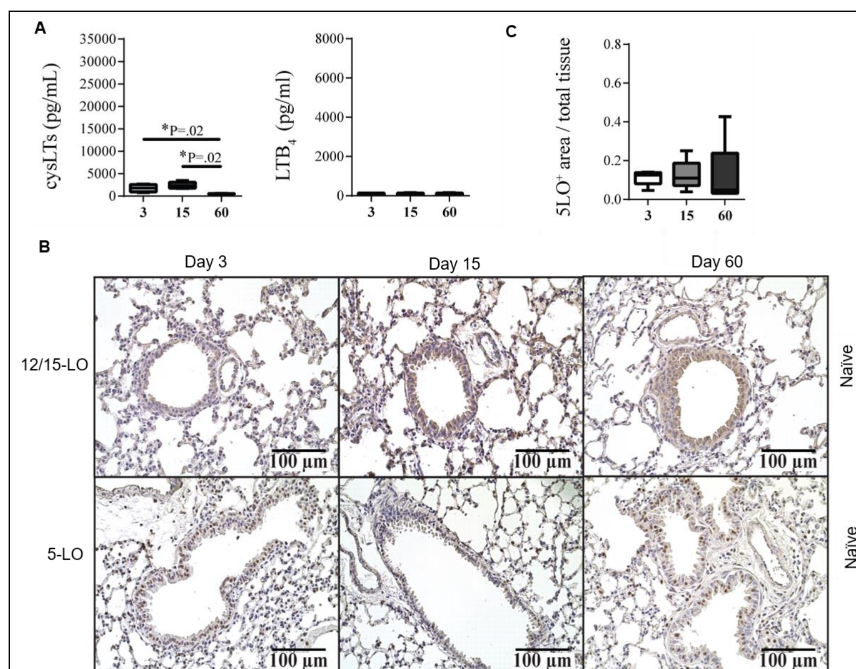


**Figure 29: Absence of LTs formation or expression of LTs-biosynthetic enzymes in naïve mice.** (A) Levels of leukotrienes (cysLTs, $LTB_4$) (EIA) measured in BALF from HDM-sensitized mice. (B) Representative immunohistochemistry (IHC) stained lung tissues from naïve mice. Scale bar: 100 µm (C) Quantification of 5-LO expression in lung sections from naïve mice. Data is presented in (A) and (C) as mean ± SEM for n=4-5 mice per group. Statistical significance was determined by Mann-Whitney test.

### 5.3.2 Signs of HDM-induced airway remodelling are age dependent

Exposure to allergens like HDM can cause irreversible damage in the airways, leading to chronic inflammation and airway remodelling, characterized by thickening of the bronchial wall, alteration of the extracellular matrix deposition, mucus gland hypertrophy and increased bronchial smooth muscle cell (Kaminska et all, 2009). Signs of HDM-induced airway remodelling were studied by IHC staining of α-smooth muscle actine (α-SMA) and the tight junction protein zonula occludens 1 (ZO-1) in lung tissues of HDM-sensitized mice. Higher expression of α-SMA was observed in the lung tissues of HDM-sensitized mice compared with naïve mice, increasing α-SMA expression with age and showing the highest intensity in mice sensitized after 60 days of life with a tendency towards increased thickening of the bronchial wall in adult sensitized mice (Figure 30A, 30B). In contrast, expression of ZO-1 decreased with age. Neonates and younger sensitized mice showed expression of ZO-1 in the surrounding of the airway smooth muscle cells, signs of airway integrity, whilst adult mice showed clear signs of airway remodelling and disruption of the airways as ZO-1 expression is less intense (Figure 30A, 30C). Thus, signs of airway remodelling are age dependent and increased with age in HDM-sensitized mice.

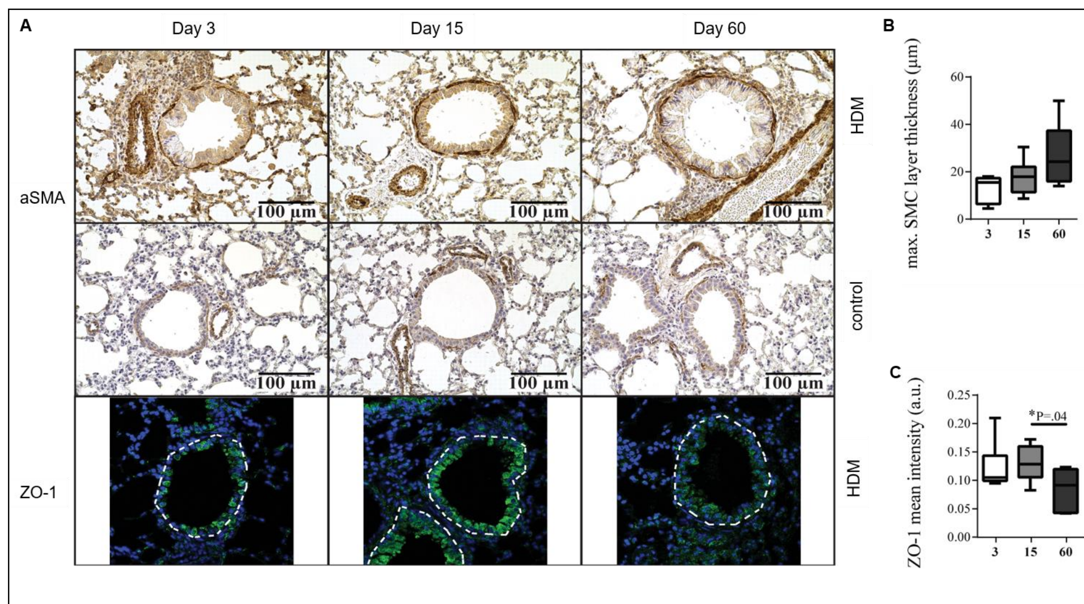


**Figure 30: Age dependent expression of airway remodelling factors in HDM-sensitized mice.** (A) Representative immunohistochemistry (IHC) and immunofluorescence (IF) stained lung tissues from HDM-sensitized and naïve mice. Scale bar: 100 µm (B) Quantification of maximal smooth muscle cell layer thickness in HDM-sensitized mice. (C) Mean fluorescence intensity of ZO-1 in HDM-sensitized mice. Data is presented in (B) and (C) as mean ± SEM for n=4-5 mice per group. Statistical significance was determined by Mann-Whitney test.

### 5.3.3 Expression of TGM2, sPLA2-X, and Wnt5a is age dependent during allergic airway inflammation

Leukotriene production and airway remodelling are age dependent during allergic airway inflammation (Figure 29, 30). However, the regulation of LTs synthesis and the mechanisms depending on the age remained poorly understood. High production of cysLTs was correlated with a high expression of group 10 secreted phospholipase A2 ($sPLA_2$-X) in the airway epithelium of asthmatic patients (Hallstrand et al., 2012 and 2016). sPLA2-X was shown to be regulated by transglutaminase 2 (TGM2), which is also expressed at increased levels in asthma (Hallstrand et al., 2012). Interestingly, a corregulation between TGM2 and Wnt5a was found in airway epithelial cells (Zissler et al., 2016). Thus, we aim to study the expression of $sPLA_2$, TGM2 and Wnt5a at different ages of HDM exposure by IHC stainings of lung tissues of HDM-sensitized mice. Lower expression of $sPLA_2$-X was observed in lung tissues of neonates or younger-sensitized mice, whilst $sPLA_2$ expression increased in adult sensitized mice with localization in smooth muscle cells, infiltrating leukocytes and epithelial cells (Figure 31A, 31B). Interestingly, TGM2 expression was not detected in lung tissues of neonates, young or adult sensitized mice (Figure 31A). Considering the possibility that TGM2 could be produced by the epithelium but rapidly secreted into the BALF, BALF samples were subjected to Western blot analysis. TGM2 was present in BALF samples and increased with age, showing the maximum protein expression in adult sensitized mice (Figure 31D). This result suggested that epithelial cells produced TGM2 which is rapidly secreted, but it is also possible that other cells present in the airways like alveolar macrophages are a source of TGM2. In line with the expression of TGM2 in lung tissues of adult sensitized mice, Wnt5a was only expressed in mice sensitized at 60 days old, mostly localized in

airway smooth muscle cells (Figure 31A, 31C). Taken together, expression of $sPLA_2$, TGM2 and Wnt5a is age dependent and highly abundant in adult sensitized mice.

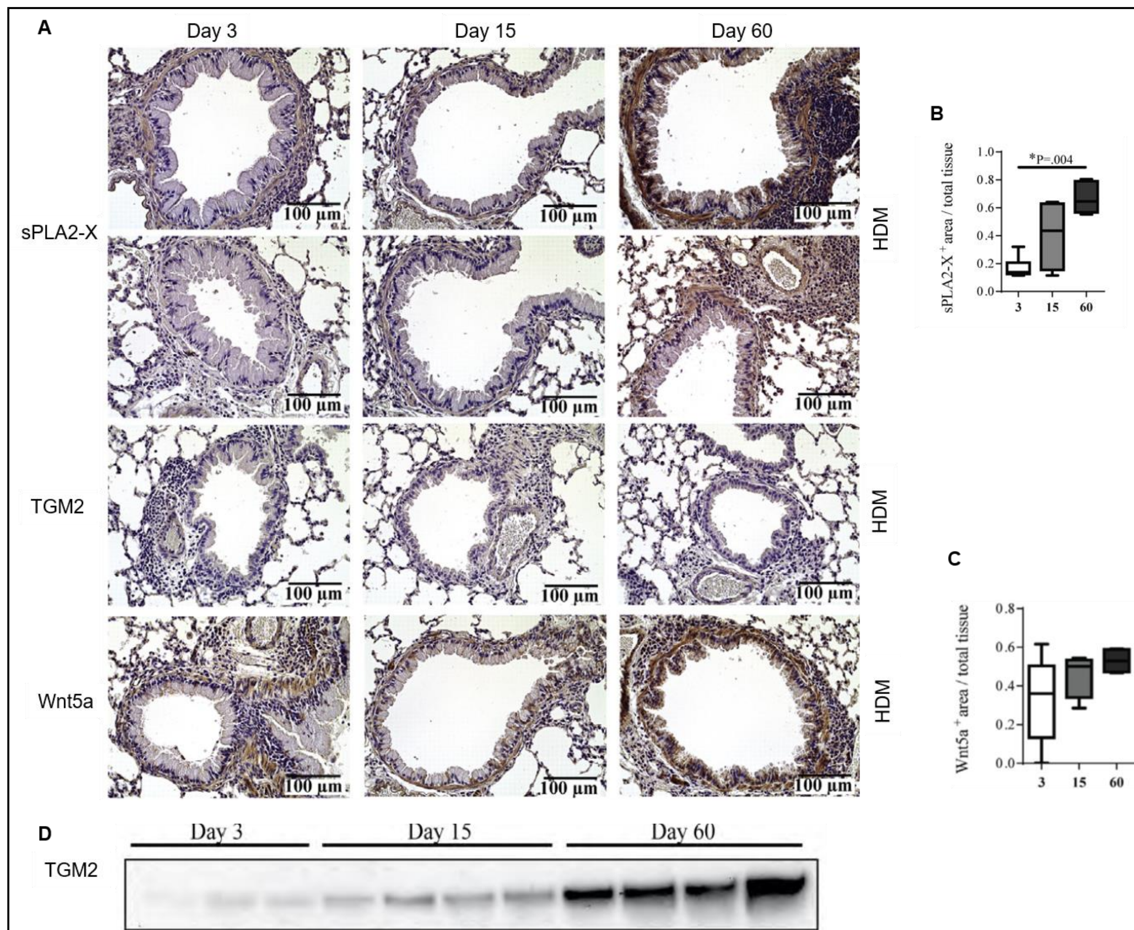


**Figure 31: Expression of $sPLA_2$, TGM2 and Wnt5a are age dependent in HDM-induced asthma.** (A) Representative immunohistochemistry (IHC) stained lung tissues from HDM-sensitized mice. Scale bar: 100 µm. (B), (C) Quantification of $sPLA_2$-X and Wnt5a expression in lung sections from HDM-sensitized mice. (D) Western blot of BALF samples from HDM-sensitized mice. Data is presented in (B) and (C) as mean ± SEM for n=4-5 mice per group. Statistical significance was determined by Mann-Whitney test.

### 5.3.4 Epithelial Wnt5a increases TGM2 expression in alveolar like human macrophages

The airway epithelium plays an important role during allergic asthma and high expression of LT-synthetic enzymes (12/15-LO, 5-LO, $LTC_4S$, $LTA_4H$) and its regulator $sPLA_2$ and Wnt5a was found in adult sensitized mice, but TGM2 expression was not detected in lung tissues (Figure 28 and 31). To further elucidate whether alveolar macrophages present in the airways are a potential source of TGM2, human macrophages were stimulated with IL-4 containing medium (pre-incubated at 4ºC overnight) and conditioned media from IL-4-stimulated human bronchial epithelial cells (HBECs). To mimic the phenotype of alveolar like macrophages, human macrophages were differentiated in the presence of TGF-β (Yu et al., 2017). High expression of TGM2 was observed in IL-4 stimulated MDM and higher expression was induced in MDM pre-treated with HBECs-conditioned medium, suggesting that macrophages are an important source of TGM2 and epithelial secretions can increase TGM2 expression (Figure 32A). To investigate whether Wnt5a can regulate TGM2 expression, MDM were pre-treated with a Wnt5a blocking antibody and stimulated with HBECs-conditioned medium. Pre-treatment with Wnt5a blocking

antibody prevented the expression of TGM2, suggesting that Wnt5a secreted by the airway epithelium can induce TGM2 expression in alveolar macrophages (Figure 32A, 32 B).

Alveolar macrophages are major producers of cysLTs and can initiate airway inflammation during allergic asthma (Clarke et al., 2014). To investigate whether TGM2 can regulate cysLTs production, alveolar macrophages were treated with TGM2 inhibitors (Monodansylcadaverine, MDC or Cystamine, Cys) before IL-4 stimulation. IL-4 stimulated the secretion of cysLTs in macrophages, an effect which was abrogated after treatment with both TGM2 inhibitors (Figure 32C), suggesting that TGM2 triggers the production of cysLTs in macrophages, which are an important source of these mediators in the airways.

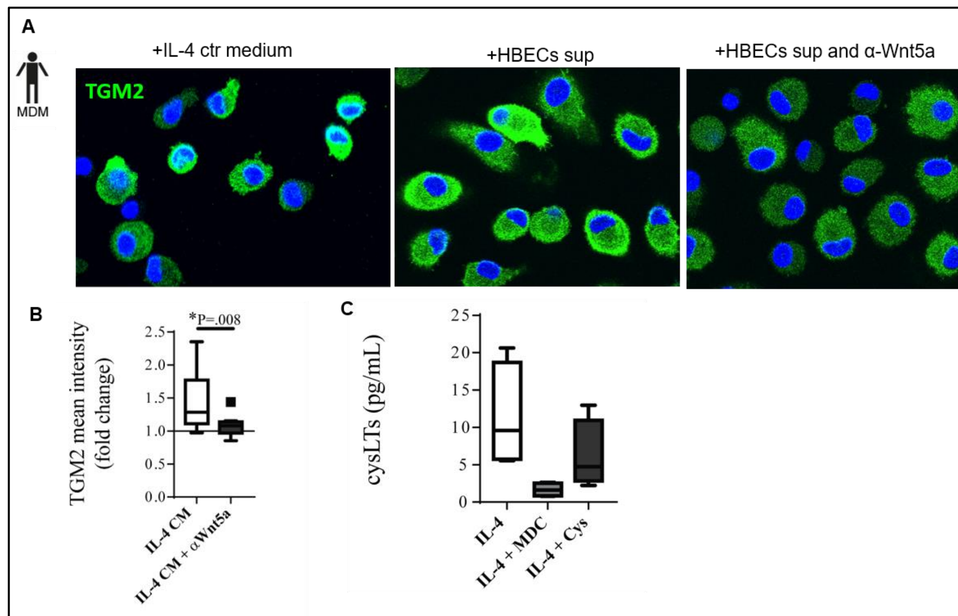


**Figure 32: Epithelial Wnt5a induced TGM2 expression in alveolar like human macrophages.** (A) Representative immunofluorescence (IF) stainings of TGM2 in human macrophages +/- treatment with IL-4 containing medium or HBECs supernatant +/- Wnt5a neutralizing antibody (1:400 dilution) (B) Mean fluorescence intensity of TGM2 in MDM +/- treatment with IL-4 containing medium or HBECs supernatant +/- Wnt5a neutralizing antibody (C) Levels of cysLTs measured in MDM +/- treatment with IL-4 (50ng/ml) and TGM2 inhibitors monodansylcadaverine (MDC, 25µM) and cystamine (Cys, 100µM). Data presented in (B) and (C) as mean ± SEM for n=4-9 MDM from human blood donors. Statistical significance in (B) was determined by Wilcoxon test. *p<0.05.

### 5.3.5 Human nasal polyp tissues express high level of LT enzymes, $sPLA_2$, Wnt5a and TGM2 despite systemic steroid treatment

To address the clinical relevance of the identified remodelling factors, we obtained nasal polyp tissues from patients suffering from chronic rhinosinusitis with nasal polyposis (CRSwNP) after surgery to remove nasal polyps. Expression of LT synthetic enzymes, $sPLA_2$-X, Wnt5a and TGM2 was assessed by IHC staining of nasal polyp tissues and LTs levels were measured in the nasal polyp secretions after overnight tissue culture. Age-dependent expression of the airway remodelling factors could not be assessed because only adult patients (41-68 years old) with nasal polyposis were obtained. Of note, all patients received oral systemic steroid treatment before surgery (table 14).

| CRSwNP patients | |
|---|---|
| **Number of patients (male/female)** | 10 (6/ 4) |
| **Age (years)** | 52.9 +/- 9.2 |
| **Systemic steroid treatment** | 10 / 10 |
| **Asthma** | 6 / 10 |
| **Allergy** | 4 / 10 |
| **AERD** | 4 / 10 |
| **HDM-sensitized** | 2 /10 |

**Table 14: Characterization of patients with chronic rhinosinusitis with nasal polyposis (CRSwNP).** (Abbreviations: Aspirin exacerbated respiratory disease, AERD; house dust mite, HDM)

Despite receiving oral systemic steroid treatment, nasal polyp tissues expressed high levels of 5-LO, $LTC_4S$, $sPLA_2$-X and Wnt5a with predominant localization in the nasal epithelium, whilst only $LTC_4S$ expression was observed in nasal turbinate tissue (Figure 33A). In line with the IHC stainings of nasal polyps, higher levels of cysLTs were measured in nasal polyp tissues of CRSwNP patients when cultured in epithelium cell culture medium overnight, but not in secretions of nasal turbinates (Figure 33B), suggesting that the synthesis of LTs is steroid resistant.

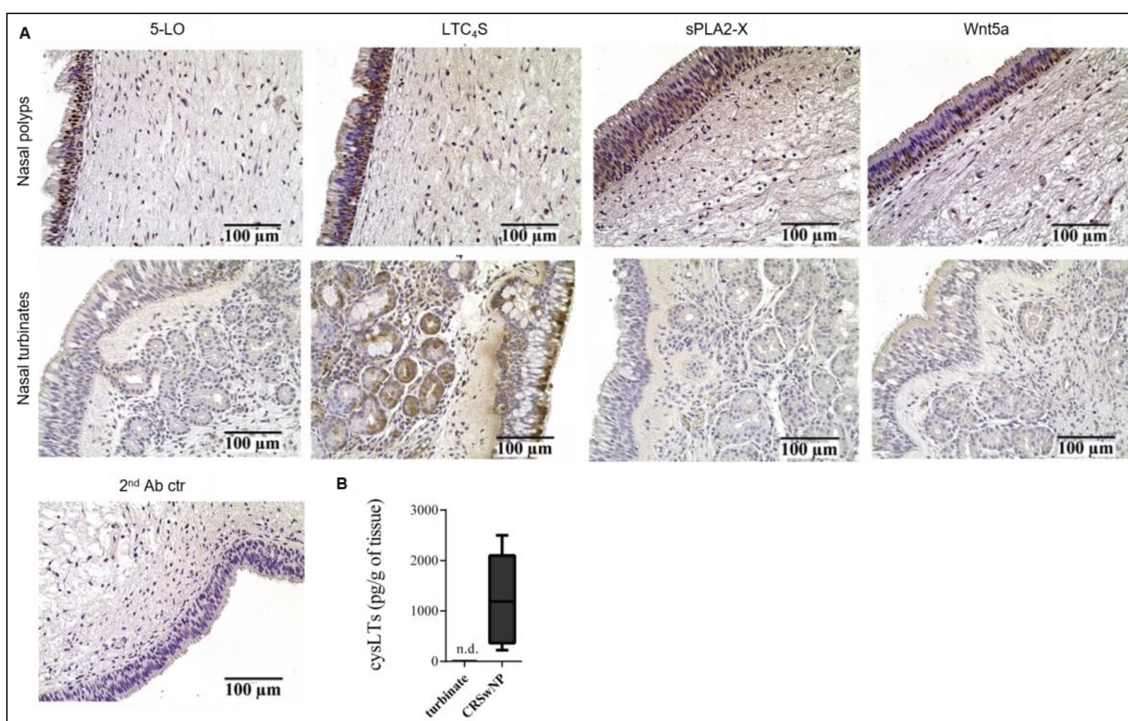


**Figure 33: Expression of remodelling factors in nasal polyp tissues of CRSwNP patients.** (A) Representative immunohistochemistry (IHC) stainings for 5-LO, $LTC_4S$, $sPLA_2$, Wnt5a or control staining in nasal polyp tissues from CRSwNP patients and nasal turbinates from healthy control subjects (B) Levels of cysLTs (EIA) measured in tissue culture supernatant of nasal turbinates (n=2) or nasal polyps (CRSwNP, n=7).

Interestingly, TGM2 expression in the nasal polyp tissues was dependent on the characterization of the CRSwNP patients, being highly abundant in aspirin tolerant (AT) CRSwNP patients and mostly localized in the smooth muscle cells or extracellular space, whilst Aspirin exacerbated

respiratory diseases (AERD) patients did not express TGM2 in the airway epithelium (Figure 34A). Furthermore, expression of collagen (collagen type 1 alpha 1 chain, Col1A1) was found in some patients and co-localized with TGM2 expression in the nasal polyp tissue (Figure 34A). Thus, despite steroid treatment, nasal polyps from adult patients secreted high levels of cysLTs and expressed LT synthetic enzymes, sPLA2-X, Wnt5a and TGM2, the latter being expressed particularly in allergic CRSwNP patients.

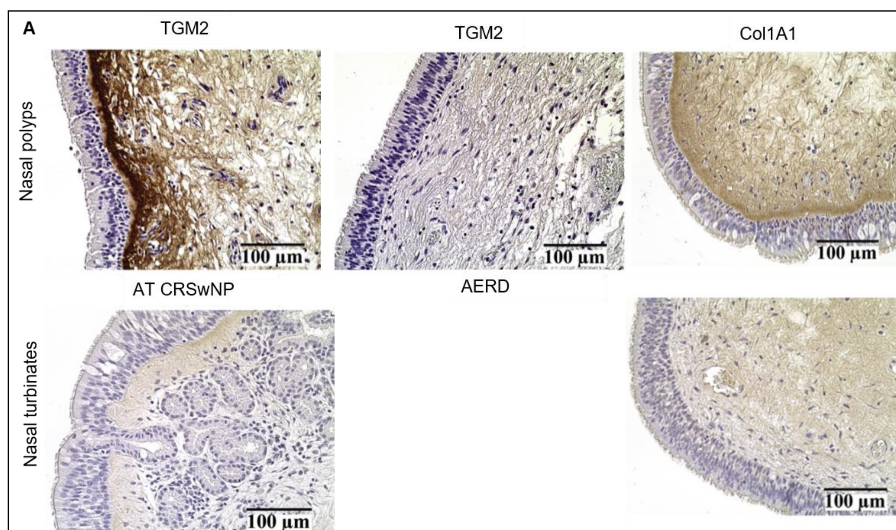


**Figure 34: Expression of TGM2 and collagen in nasal polyp tissues of CRSwNP patients.** (A) Representative immunohistochemistry (IHC) stainings for TGM2 and Col1A1 in nasal polyp tissues of patients with aspirin tolerant (AT) (n=6) or aspirin-exacerbated respiratory diseases (AERD) (n=6), or nasal turbinates (n=2) from healthy control subjects.

### 5.3.6 Therapeutic application of *Hpb* larval extract in comparison to fluticasone propionate for the treatment of AERD patients

Synthesis of pro-inflammatory lipid mediators and expression of LT-biosynthetic enzymes are particularly resistant to steroid treatment in nasal polyps from AERD patients (Figure 33, 34), suggesting that there is a need to look for new therapeutic approaches. Airway remodelling factors secreted by the airway epithelium stimulate the synthesis of cysLTs in alveolar like macrophages, contributing to the development of the airway inflammation (Figure 32). Thus, macrophages play a key role in the pathophysiology and macrophage modulation of the AA metabolism may represent an alternative pathway to treat patients suffering from AERD, disorder characterized by a typical eicosanoid imbalance with over-expression of 5-LOX and $LTC_4S$, leading to over-production of pro-inflammatory mediators cysLTs (Israel et al., 1993; Cowburn et al., 1998; Laidlaw et al., 2014). To further explore the impact of the type 2 cytokine IL-4, which is responsible for the upregulation of $LTC_4S$ by mast cells in AERD patients (Hsieh et al., 2001), macrophages were isolated from heathy and AERD donors, differentiated into alveolar like macrophages and further stimulated with IL-4 in order to analyse the eicosanoid profile by LC-MS/MS analysis. Both healthy and AERD macrophages showed a similar eicosanoid reprogramming in response to IL-4 (Figure 35A). IL-4 stimulation hardly had an effect on the synthesis of COX metabolites, slightly inducing the formation of the prostanoid $PGE_2$ (Figure 35A). In contrast, IL-4 activated the LOX pathway by inducing the release of pro-inflammatory 5-LOX mediators ($LTB_4$ and 5-HETE), whilst 12-/15-LOX mediators were not detected or close

to the lower detection limit (Figure 35A). Thus, IL-4 drives a pro-inflammatory mediator phenotype in human macrophages form healthy and AERD donors.

As described previously in results section 5.1.2 and 5.2.3, the results from our experimental models could potentially be translated into therapeutic applications of *Hpb* larval extract (*HpbE*), which can reprogram the AA metabolism in human and murine macrophages and thereby suppress allergic airway inflammation *in vivo* (Figure 12 and 27). Thus, to test the therapeutic potential of *HpbE* in comparison to standard drugs used for the treatment of asthma, human macrophages from healthy and AERD donors were stimulated with *HpbE* and fluticasone propionate (FP) and the eicosanoid profile was analyzed by LC-MS/MS. Interestingly, both healthy and AERD macrophages showed a similar eicosanoid response to *HpbE* treatment by inducing the synthesis of anti-inflammatory COX metabolites ($PGE_2$ and $TXB_2$) and the 15-LOX metabolite 15-HETE, whilst it suppressed the release of pro-inflammatory LTs ($LTB_4$ and 5-HETE) (Figure 35B). In contrast, treatment with FP failed to induce the synthesis of prostanoids ($PGE_2$ and $TXB_2$) or 15-HETE, and did not contribute to the suppression of 5-HETE drived by *HpbE* in macrophages from healthy or AERD donors (Figure 35B). Thus, *HpbE* and FP distinctly reprogram the AA metabolism of macrophages and only *HpbE* drives an anti-inflammatory eicosanoid mediator response. Since $PGE_2$ concentrations are decreased in AERD patients (Pérez-Novo et al., 2005), induction of $PGE_2$ by *HpbE*-treatment of macrophages from AERD patients offers an alternative therapeutic application in setting of type 2 inflammation.

As described above, *HpbE* can suppress inflammation during type 2 immune responses in a COX dependent manner, most likely by the secretion of the prostanoid $PGE_2$ (Figure 12, 20 and 27). Since granulocytes from AERD patients are known to be resistant to $PGE_2$ and to promote the severe respiratory tract inflammation and LTs overproduction in AERD (Laidlaw et al., 2014), the next research step was to assess the responsiveness to $PGE_2$ in macrophages from healthy and AERD subjects. Similar reprogramming of lipid mediator pathways was observed in $PGE_2$-stimulated macrophages from both healthy and AERD donors. $PGE_2$ hardly affected the regulation of the COX pathway, whilst it effectively suppressed the formation of pro-inflammatory LOX mediators ($LTB_4$ and 5-HETE), suggesting a role of $PGE_2$ in suppressing LTs production in macrophages from healthy and AERD subjects (Figure 35C).

Taken together, in comparison to treatment with FP, *HpbE* reprograms the lipid mediator pathways in human macrophages and might limit type 2 inflammation, suggesting a potential therapeutic application for the treatment of steroid resistant AERD patients with high basal levels of LTs.

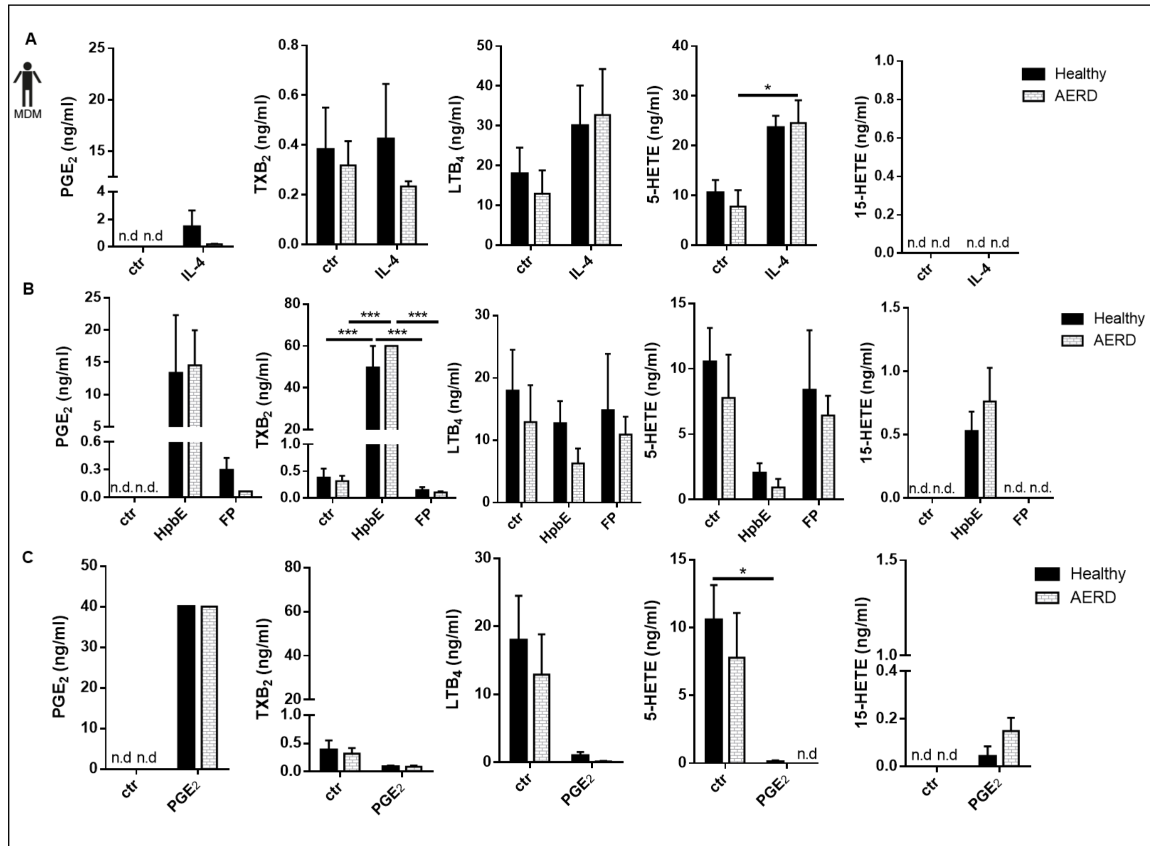


**Figure 35: Treatment with *HpbE* differentially modulates the AA metabolism compared to Fluticasone propionate in macrophages from healthy and AERD subjects.** (A) Levels of COX- and LOX- metabolites (LC-MS/MS) measured in macrophages stimulated with IL-4 (10 ng/ml) from healthy and AERD subjects (B) Eicosanoid levels (LC-MS/MS) produced by macrophages after treatment with *Hpb* larval extract (*HpbE,* 10µg/ml) and Fluticasone propionate (FP, 1µM). (C) Levels of COX- and LOX-metabolites (LC-MS/MS) measured in human macrophages stimulated with $PGE_2$ (10µM). Results are expressed as mean +/- SEM, n=3 healthy and n=3 AERD donors. Statistical significance was determined by 2way ANOVA with Bonferroni correction *$p<0.05$.

### 5.3.7 Characterization of immunoregulatory compounds present in *HpbE*

Treatment with *Hpb* larval extract (*HpbE*) reprogrammed the AA metabolism in myeloid cells and suppressed inflammation in human and murine settings of type 2 immunity. However, the active immune regulatory compounds present in *HpbE* are still not known. In order to identify the nature of the molecules responsible for the immunoregulation by *HpbE*, 90ºC heat treatment was performed in *HpbE* (*HpbE* 90ºC) and its activity was tested by the capacity to induce prostanoids and cytokine production in human MDM. In contrast to the effect induced by *HpbE*, stimulation with *HpbE* 90ºC failed to induce the production of COX metabolites ($PGE_2$ and $TXB_2$) and regulatory cytokines (IL-10 and IL-1β) in human MDM (Figure 36A). As previously shown, *HpbE* can directly suppress granulocyte recruitment in setting of type 2 inflammation (Figure 16F). To study the effect of heat treatment, a chemotaxis assay was performed with *HpbE* 90ºC-stimulated PMN. *HpbE*-driven suppression of granulocyte recruitment was prevented after heat treatment of *HpbE* (Figure 36B). This suggested that mediator reprogramming in macrophages and suppression of granulocyte recruitment was largely dependent on heat-labile molecules, most likely proteins.

In order to identify immunoregulatory proteins present in *HpbE*, the different compounds present in the extract were separated by size exclusion chromatography collecting a total of 54 fractions (Figure 36C). To identify the active fractions, only the fractions with protein content (detected absorbance at 280 nm) were used to stimulate human macrophages and the eicosanoid profile and production of cytokines were measured by EIA or ELISA. Fractions number 8-11 were identified as positive fractions for their capacity to induce the COX metabolite $TXB_2$, as well as IL-10 to a similar extent as observed for *HpbE*-treated MDM (Figure 36D). Further characterization of the protein compounds in active fractions 8-11 was performed by mass spectrometry analysis, thus highlighting *Hpb* glutamate dehydrogenase (GDH) and *Hpb* ferritin as candidate immunoregulatory proteins present in *HpbE* (Table 15).

| Protein | Score | Coverage | # Unique Peptides | MW (kDa) | uniprot ID |
|---|---|---|---|---|---|
| **Glutamate dehydrogenase** | 67.93 | 70.02 | 33 | 59.1 | A0A183FP08 |
| **Ferritin** | 18.95 | 44.74 | 5 | 17,2 | A0A183FLG6 |
| **Proteasome subunit alpha type** | 12.27 | 21.77 | 4 | 27.2 | A0A183GNP5 |
| **Proteasome subunit alpha type** | 4.12 | 21.95 | 2 | 23.1 | A0A183FS25 |

**Table 15: Proteins uniquely present in active fractions of *Hpb* larval extract identified by mass spectrometry.** Four proteins present only in active fractions were identified among the top 50 proteins identified in active and non-active fractions (ordered by score, coverage and number of unique peptides).

The best approach to determine the contribution of *Hpb* GDH in *HpbE*-driven reprograming in human macrophages is to recombinantly produce *Hpb* GDH. However, recombinant production of soluble GDH in *Escherichia Coli (E. Coli)* was unsuccessful, and further optimization is required. This is the reason why commercially available GDH was purchased to test its capacity to reprogram eicosanoid signalling pathways and cytokine production. As mammalian and microbial GDHs are structurally distinct (Li et al., 2009), human MDM were treated with commercially available GDH from mammalian species (human, bovine) or a thermophilic bacterium. Whilst mammalian GDH failed to induce the COX metabolite $PGE_2$ and the cytokine IL-10, the production of both mediators was dose-dependently increased by microbial GDH (Figure 36E). In addition, an inhibitor of GHD (Bithionol), which is also used as anti-helminthic, reduced the *HpbE*-triggered induction of $PGE_2$ and IL-10 (Figure 36F).

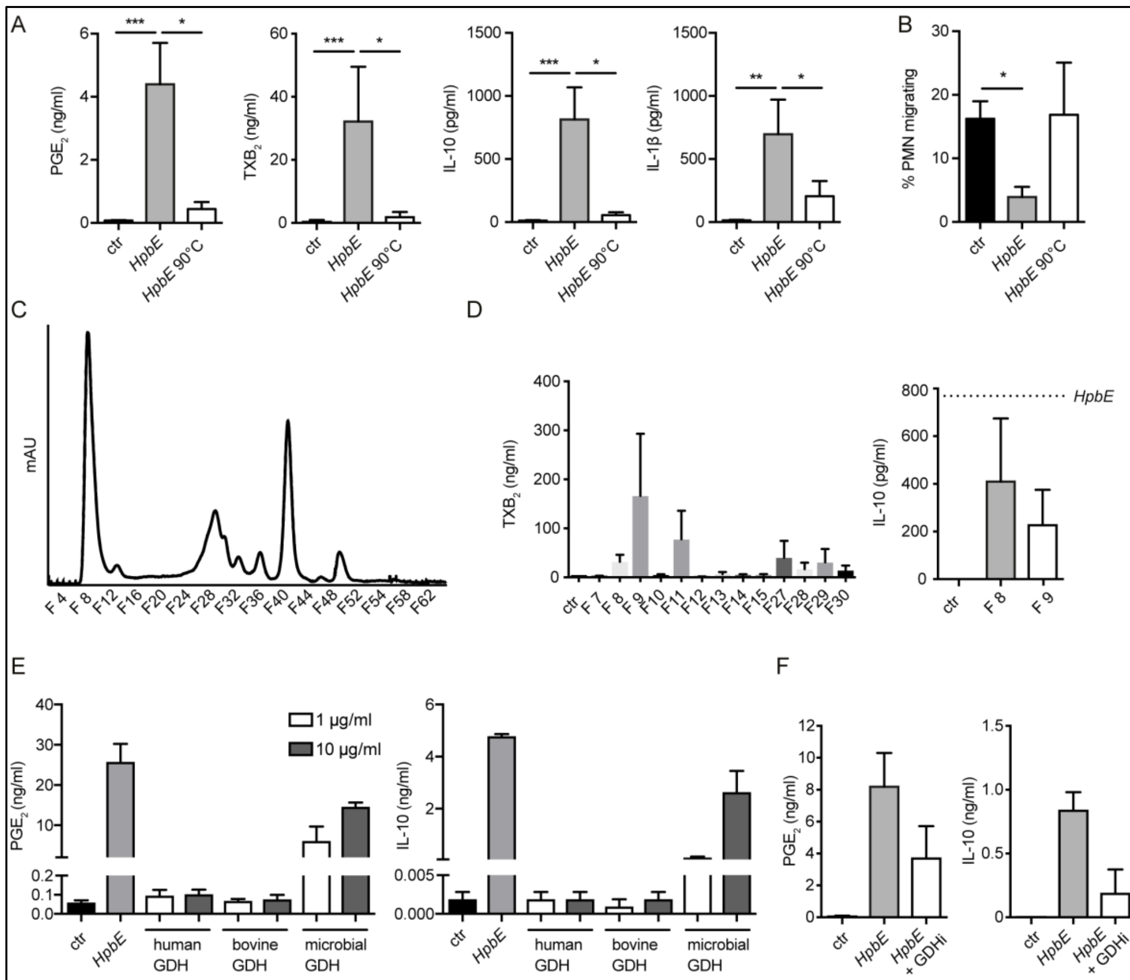


**Figure 36: Glutamate dehydrogenase is a major immunoregulatory protein in Hpb larval extract.** (A) Levels of prostanoids (EIA) or IL-10 and IL-1β (ELISA) in human MDM ± treatment with *HpbE* (10µg/ml) or heat-inactivated *HpbE* (*HpbE* 90°C, 10µg/ml). (B) Chemotaxis of human PMN ± treatment with *HpbE* or heat-inactivated *HpbE* (*HpbE* 90°C). (C) Size exclusion chromatogram for fractionation of *HpbE*. (D) Levels of $TXB_2$ (EIA) or IL-10 (ELISA) in human MDM ± treatment with H*pbE* fractions. (E) Levels of $PGE_2$ (EIA) or IL-10 (ELISA) in human MDM ± treatment with *HpbE* or recombinant human, bovine or microbial glutamate dehydrogenase (GDH, 1 or 10 µg/ml). (F) Levels of $PGE_2$ (EIA) or IL-10 (ELISA) in human MDM ± treatment with *HpbE* ± inhibitor of GDH (GDHi, Bithionol, 20µM). Data are pooled from at least 2 independent experiments and presented as mean ± SEM for MDM from n=3-10 healthy human blood donors. Statistical significance was determined by Friedman test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Another potential candidate identified by mass spectrometry analysis was *Hpb* ferritin. To further assess the effect of *Hpb* ferritin in human MDM, recombinant production of the heavy and light chain of *Hpb* Ferritin was attempted, although the recombinant production in *E. coli* was unsuccessful. Similar to the approach used for GDH, Ferritin is also commercially available. Liver mammalian ferritin was purchased and used for *in vitro* stimulation of human macrophages. In contrast to microbial GDH, ferritin does not appear to be responsible for the regulation of the COX pathway as production of prostanoids was not induced in human macrophages (Figure 37A). Treatment with ferritin slightly upregulated the gene expression of $PGE_2$-biosynthetic enzymes (*PTGS2, PTGES*), but at a lower extent compared to *HpbE*-stimulated macrophages (Figure 37B). Gene expression of the regulatory cytokine IL-10 remained unaltered, in contrast to the

downregulation of the 5-LOX biosynthetic enzyme (*ALOX5*) induced after treatment with ferritin (Figure 37B). The effect of 5-LOX downregulation was not further explored by LC-MS/MS or EIA and whether ferritin could induce changes in the levels of $LTB_4$ or cysLTs mediators remains to be investigated.

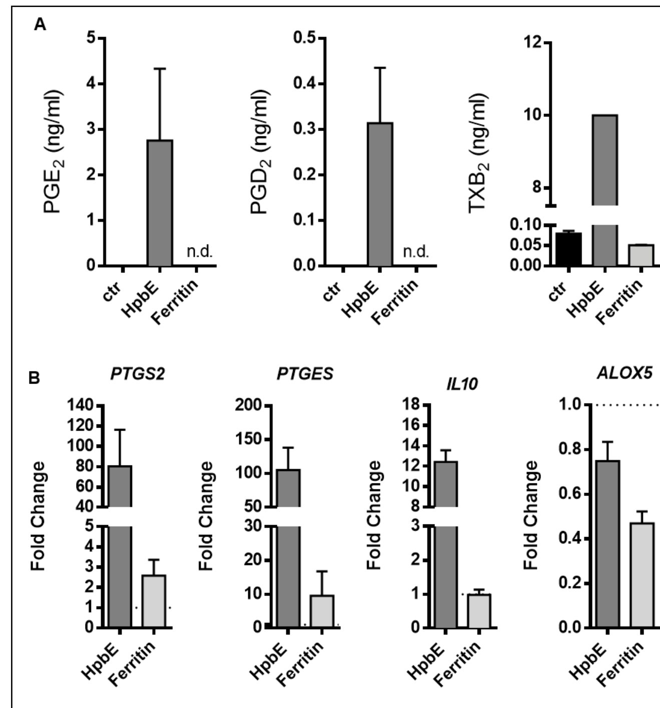


**Figure 37: Ferritin cannot mimic the modulation of COX metabolites in *HpbE*-treated macrophages.** (A) Levels of prostanoids (LC-MS/MS) in human MDM ± treatment with *HpbE* (10µg/ml) or liver mammalian ferritin (1µg/ml) (B) Relative gene expression of $PGE_2$-synthetic enzymes, IL-10 or 5-LOX (qPCR) in human MDM ± treatment with *HpbE* or ferritin. Data are presented as mean ± SEM for MDM from n=2-3 healthy human blood donors.

Taken together, glutamate dehydrogenase is a major immunoregulatory protein present in *HpbE*. Microbial GDH acts as a modulator of the host AA metabolism, able to modulate the COX pathway and induce regulatory mediators, thus potentially modulating type 2 immune responses. Ferritin represents a potential candidate for the immunoregulation of the 5-LOX pathway. Indeed, a mammalian ferritin (from liver) was able to downregulate the gene expression of *ALOX5*, although its role in the regulation of the 5-LOX pathway remains to be studied in future experiments. The potential roles of *Hpb* GDH and *Hpb* ferritin in the regulation of eicosanoid pathways and type 2 inflammation need to be confirmed by performing further *in vivo* and *in vitro* experiments with the recombinant proteins.

# 6 DISCUSSION

## 6.1 Helminth parasites differentially remodel the AA metabolism *in vivo*

Eicosanoids are bioactive lipid mediators derived from arachidonic acid (AA) playing key roles in the induction of type 2 immune responses (Barret et al., 2011; Miyahara et al., 2008). However, helminth parasites have evolved different mechanisms to modulate type 2 inflammation by targeting innate immune effector mechanisms, including the blockade of IL-33 release, suppression of ILC2 activation and the induction of alternative activation of macrophages (Klotz et al., 2011; McSorley et al., 2014; Osbourn et al., 2017; Schnoeller et al., 2008). Given the important role of eicosanoids in type 2 inflammation, AA metabolic pathways are important targets both in allergy and during helminth infection. The research conducted within the frame of this thesis showed that one of the evasion strategies used by the parasitic nematode *Heligmosomoides polygyrus bakeri* (*Hpb*) is to directly reprogram the AA metabolism and suppress type 2 immune responses. At day 7 post-infection, *Hpb* drives the production of regulatory prostanoids ($PGE_2$ and 6-keto PGF1-α, a degradation metabolite of $PGI_2$), which can modulate type 2 immune responses by inducing regulatory macrophage polarization and limit type 2 cytokine production by ILC2s (Draijer et al., 2016; Maric et al., 2018). *Hpb* also triggers the production of COX metabolites 12-HHT and $TXB_2$, involved in tissue repair and blood clotting (Liu et al., 2014; Paul et al., 1999). In contrast, *Hpb* suppressed the secretion of the pro-inflammatory prostanoid $PGD_2$, known to drive activation of ILC2 and production of type 2 and pro-inflammatory cytokines (Salimi et al., 2017; Xue et al., 2015). *Hp*b also prevented the release of pro-inflammatory mediators including $LTB_4$ and cysLTs, which induce Th2 cell activation, vascular leakage, eosinophil recruitment and mucus production by globet cells (Barret et. al., 2011; Haberal & Corey et al., 2003; Miyahara et al., 2008). In contrast, a different eicosanoid profile was shown during helminth infection with *Brugia malayi*, which induced $PGD_2$ and $TXB_2$ production (Thomas et al., 2012), suggesting that different helminth species may differentially affect the AA metabolism.

The *Hpb*-driven modulation of AA metabolic pathways might represent an immune evasion strategy of this parasite to escape from the type 2 immune response initiated by the host immune system. The induction of COX metabolites involved in tissue repair and blood clotting may be important after helminth infection to repair the tissue disruption caused by the tissue migrating larvae. In keeping with the observed induction of prostanoids by *Hpb*, the infective larvae of *S. mansoni* were shown to produce high amounts of $PGE_2$, which is also a potent vasodilator mediator and helped the infective larvae to migrate through the skin (Ramaswamy et al., 2000). Furthermore, *Hpb*-driven suppression of pro-inflammatory AA mediators might limit granulocyte infiltration and recruitment and thus larval killing as well as prevent mucus production and larval expulsion. In line with the LTs profile, low levels of granulocyte infiltration and 5-LOX expression were observed in the surroundings of the trapped *Hpb* larvae in gut tissues at day 7 p.i. The absence of LTs from tissues of *Hpb*-infected mice was unexpected as helminth infection with other nematodes (*C. elegans* and *N. brasiliens*) has been reported to induce LT production and eosinophil recruitment (Patnode et al., 2014). As compared to *Hpb*, a different modulation of the 5-LOX pathway was described during infection with *Schistosoma mansoni*, with high expression of 5-LOX at the sites of larval migration in the lung or egg deposition in the liver. Thus, our results are in line with previous studies showing 5-LOX expression in lung granuloma of *S. mansoni* infected mice (Toffoli da Silva et al., 2016)

Taken together, these findings suggest that different helminth species may differentially affect the AA metabolism and thus either promote or suppress type 2 immune responses.

### 6.2 *H. polygyrus* larval extract remodels the AA metabolism of myeloid cells *in vitro*

Macrophages are plastic cells involved in anti-helminth immunity, having effects on worm trapping, immunoregulation and wound healing or repair of tissue injury caused by migrating larvae (Gause et al., 2013; Esser von-Bieren et al., 2013 and 2015). It has been previously described that helminth parasites secrete immunomodulatory products which can directly target macrophages, inducing immunomodulatory responses (Klotz et al., 2011; Schnoeller et. al., 2008; Schönemeyer et al., 2001). Our studies showed that a larval extract of *Hpb* (*HpbE*) can directly reprogram the AA-metabolism of **macrophages** *in vitro*, shifting the synthesis of 5-LOX towards COX metabolites. *HpbE* treatment of murine and human macrophages induced the synthesis of regulatory prostanoids ($PGE_2$) and prostanoids involved in tissue repair and blood clotting (12-HHT and $TXB_2$), whereas it suppressed the production of pro-inflammatory lipid mediators (LTs and $PGD_2$). A similar suppression of 5-LOX derived metabolites was observed in M2 macrophages during helminth infection with *Brugia malayi*, although this parasite distinctly induced the release of 12/15-HETE metabolites and COX-derived 6-keto PGF1α (Thomas et al., 2012), suggesting that different parasites may differentially affect the reprogramming of the AA metabolism in macrophages. In line with the eicosanoid profile, *HpbE*-induced changes in the AA metabolism were a result of transcriptional changes, inducing the gene expression of AA-metabolic enzymes involved in the synthesis of prostanoids (*Ptgs2* and *Ptges*), whilst suppressing the expression of enzymes responsible for the synthesis of LTs (*Alox5* and *Ltc4s*). Thus, *HpbE*-triggered eicosanoid reprogramming in macrophages *in vitro* closely resembled the AA metabolism changes during helminth infection *in vivo*.

Furthermore, *HpbE* distinctly induced the synthesis of type 2 suppressive mediators IL-10 and IL-1β (Schnoeller et al., 2008; Zaiss et al., 2013) in both human and murine macrophages and in addition modulated the production of cytokines involved in modulation of M2 polarization and type 2 inflammation (IL-12, IL-18, IL-27 and TNF-α) in human macrophages (Guo et al., 2009; Kratochvill et al., 2015; Mathie et al., 2015; Qui et al., 2012). Of interest, *HpbE* did not affect the production of IL-33 in MDM. This observation is in line with a recently discovered protein present in excretory/secretory products of *H. polygyrus* (HES) called H. polygyrus Alarmin Release Inhibitor (HpARI), which can block the release of IL-33 (Osbourn et al., 2017). However, it is also controversial if macrophages represent a relevant source of IL-33 as this cytokine can only be detected in large amounts in secretions of tissue cells (e.g. epithelial cells) rather than in leukocytes. Thus, future studies should address the effects of *HpbE* on epithelial cells of the lung and intestine.

In terms of macrophage polarization, *HpbE* prevented M2 polarization by downregulation of the gene expression of M2 markers such as *ALOX15* and *MRC1*, whilst expression of *TGM2* was not affected in human macrophages. However, a different macrophage polarization profile was observed in murine macrophages, where gene expression of *Mrc1* was downregulated but expression of *Arg1* and *Tgm2* was upregulated after *HpbE* stimulation. Interestingly, macrophages expressing Arginase-1 have been shown to suppress the production of type 2 cytokines and prevent the inflammation and fibrosis during parasite infection with *S. mansoni* (Pesce et al., 2009). Since TGM2 and MRC1 are the only conserved M2 makers in human and murine macrophages (Martinez et al., 2013), these results suggest that *HpbE* might drive a

partially distinct polarization profile in human and murine macrophages, but a similar regulatory mediator profile, most likely inducing an M2-suppressive phenotype. The absence of a clear pattern of M2 polarization was unexpected as immunomodulatory compounds secreted by other parasites (*Brugia malayi* and *Fasciola hepatica*) had been reported to induce macrophage M2 polarization (Prieto-Lafuente et al., 2009; Ramos-Benitez et al., 2009). Furthermore, it has been described that monocytes from patients infected with *Brugia malayi* had an M2 phenotype characterized by expression of M2 markers like MRC1 (Babu et al, 2009), implying that different helminth parasites might induce a distinct pattern of macrophage polarization. As M2 macrophages have been associated with asthma and other chronic airway diseases (Girodet et al., 2016; Melgert et al., 2011), the suppression of M2 polarization by *HpbE* might be harnessed for future therapies against chronic airway inflammation.

Granulocytes (neutrophils and eosinophils) are important effector cells during helminth infection, able to rapidly infiltrate the site of infection and to kill the larvae and promote worm expulsion (Abraham et al., 2004; Chen et al., 2014; Patnode et al., 2014) Thus, granulocytes represent an attractive cellular target for helminth-driven immune evasion, aiming to modulate the activation and recruitment of granulocytes. Similar to *HpbE*-driven eicosanoid reprogramming in macrophages, the present study has demonstrated that *HpbE* remodels the AA- metabolism of **human granulocytes**, showing a shift of LOX towards COX metabolism. *HpbE* triggered the induction of COX metabolites (12-HHT and $TXB_2$), whilst it inhibited the production of pro-inflammatory LTs (cysLTs and $LTB_4$) in granulocytes. The rapid induction of COX metabolites involved in tissue repair and blood clotting may be important after helminth infection to quickly repair the tissue disruption caused by the tissue migrating larvae. The suppression of LTs is likely important as it avoids the recruitment of eosinophils. Indeed, a previous publication suggested that eosinophils can rapidly migrate towards *N. brasiliensis* and *C. elegans* larvae and induce the secretion of $LTB_4$, thus amplifying eosinophil accumulation (Patnode et al., 2014).

We further show that *HpbE* can act directly on human granulocytes and inhibit granulocyte chemotaxis more efficiently than standard drugs used for the treatment of asthma (Fluticasone propionate and Montelukast) in *ex vivo* settings of type 2 inflammation. The suppression of granulocyte recruitment might be a result of the reduced expression of LT-biosynthetic enzymes ($LTA_4H$ and $LTC_4S$), which may limit the autocrine production of chemoattractive LTs as well as reduced expression of chemotactic receptors (CCR3 and $CRTH_2$). Indeed, modulation of CCR3 might represent an immunoevasion strategy of *Hpb* to longer survive in the host, since upregulation of CCR3 levels are important for the killing and expulsion of the larvae and to develop adaptive protective immunity during helminth infection (Abraham et al., 2004; Ramalingam et al., 2018; Turner et al., 2018). *HpbE*-induced downregulation of $CRTH_2$ might also prevent granulocyte recruitment, as $PGD_2$ binding to its receptor $CRTH_2$ has been shown to promote chemotaxis in Th2 cells, eosinophils and basophils (Hirai et al., 2001). In line with this *HpbE*-driven suppression of CCR3 in granulocytes, downregulation of CCR3 levels in eosinophils was previously described during *in vivo* infection with *Hpb* and linked to the suppression of allergen-induced airway eosinophilia (Rzepecka et al., 2007). In addition, our study suggested a cross-talk between *HpbE*- stimulated macrophages and granulocytes, where *HpbE*-treated macrophages attenuated chemotaxis of human granulocytes in a COX dependent manner, as pre-treatment with a COX inhibitor prevented the suppression of granulocyte recruitment. A COX-derived mediator, most likely $PGE_2$, might be responsible for the inhibition of granulocyte trafficking since $PGE_2$ at high concentrations is known to suppress eosinophil and macrophage migration (Sturm et al., 2008; Osma-Garcia et al., 2016).

## 6.3 *H. polygyrus* larval extract remodels the AA metabolism of HBECs and PBMCs, but does not have an impact on type 2 cytokine production *in vitro*

Epithelial cells play an important role during asthma, since they are the first barrier in contact with the allergen. The present study demonstrated that *HpbE* can remodel the AA-metabolism of **human bronchial epithelial cells** (HBECs) by modulation of the COX metabolism and induction of prostanoid synthesis ($PGE_2$ and $TXB_2$), similarly as observed in *HpbE*-stimulated myeloid cells. Although synthesis of LTs can be induced in HBECs under inflammatory conditions (Jame et al., 2007; Trian et al., 2015), LTs secretion was not detected in *HpbE*-stimulated HBECs. HBECs treatment with other parasite extracts (*S. mansoni*, *Ostergaria*, *Ascaris* or *Cooperia*) hardly affected the AA metabolism, suggesting that *HpbE* might induce a unique eicosanoid profile in the airway epithelium. Interestingly, a recent study reported that *H. polygyrus* infection *in vivo* induced the expression of $sPLA_2$ in epithelial cells, playing an important role as antihelminthic to confer resistance to helminth infection (Entwistle et al., 2017). Thus, epithelial lipid mediator pathways may control immune evasion *vs.* protective immunity during *Hpb* infection.

With regards to the broader propensity of *HpbE* to induce a regulatory network in human subjects, this research project has shown that *HpbE* does not have a direct impact on type 2 cytokines (IL-4, IL-5 and IL-13), although it induced the production of IFN-γ, IL-10 and eicosanoid reprogramming in human **peripheral blood mononuclear cells** (PBMCs). Interestingly, *HpbE* shifted the production of 5-LOX towards COX metabolites, but triggered the synthesis of 12/15-HETE metabolites, resembling the eicosanoid profile measured during *Hpb*-infection *in vivo*. Induction of IL-10 producing PBMCs was previously described in *S. mansoni* infected patients with asthma, where lower levels of IL-4 and IL-5 were measured after allergen exposure compared to helminth-free patients with asthma, demonstrating that administration of antigens present in *S. mansoni* protected against allergen-induced airway eosinophilia and inflammation (Araujo et al., 2004; Cardoso et al., 2006 and 2010).

Taken together, the parasitic nematode *H. polygyrus* can directly reprogram the AA metabolism *in vivo* and treatment of murine and human leukocytes with the parasitic L3 larval extract (*HpbE*) similarly remodels the AA-metabolism by inducing regulatory COX metabolites but suppressing pro-inflammatory LTs, thereby suppressing type 2 inflammation and inducing a regulatory network, which might have protective effects during allergic asthma.

## 6.4 Other parasite extracts or excretory/secretory products failed to induce AA-metabolic reprogramming in human myeloid cells

*HpbE* induces a unique eicosanoid signature in myeloid cells in comparison to other helminth products. This study showed that human **macrophages** stimulated with excretory/secretory (ES) products of *Hpb* (HES) failed to induce IL-10 production or COX metabolites ($PGE_2$ and $TXB_2$), although HES could also trigger 5-LOX downregulation. The absence of the modulation of COX metabolites by HES was unexpected given that HES are known to modulate type 2 immune responses (Mc Sorley et al., 2012; Osbourn et al., 2017). An extract of *S. mansoni* (*SmE*) had only minor effects in the production of COX and LOX metabolites when compared to *HpbE* or eggs from *S. mansoni*, where the identified soluble egg antigen (SEA) induced $PGE_2$ production in dendritic cells (Kaiser et al., 2018). These results suggest that different stages during the life cycle

of the same parasite might differentially modulate the host immune system. Given the different sizes, habitats and nutritional requirements of the different stages this likely reflects an evolutionary adaptation, which allows the parasite to optimally survive in the host.

In contrast to *HpbE*, *SmE*-stimulated macrophages failed to induce the release of the anti-inflammatory cytokine IL-10 in human macrophages. However, other immunomodulatory compounds identified in different helminth parasites such as BmCPI-2 (*Brugia malayi*), onchocystatin (*Onchocerca volvus*) and AvCystatin (*Acanthocheilonema vitaea*) can induce IL-10 producing macrophages which contribute to attenuate allergen-induced airway inflammation (Venugopal et al., 2017; Schönemeyer et al., 2001; Klotz et al., 2011; Ziegler et al., 2015). These results suggest that different parasites and even different ES products released by the same parasite can differentially reprogram the AA metabolism and modulate type 2 inflammation.

When comparing the eicosanoid reprograming induced by *HpE* in human granulocytes, our research demonstrated that the larval extract of a different parasite *Ascaris* (*AsE*) did not induce a shift from 5-LOX to COX metabolism. In contrast to *HpbE*, *AsE* did not modulate the expression of the chemotaxis receptor CCR3, suggesting that *AsE* might not effectively suppress granulocyte chemotaxis during type 2 inflammation. Indeed, parasite-induced eosinophil activation and recruitment have been shown during helminth infection with *S. mansoni* (Magalhães et al., 2010). Thus, different parasites may differentially promote or suppress granulocyte activation and recruitment.

### 6.5 *HpbE*-driven AA reprogramming is dependent of HIF1α, p38 MAPK, COX and NFκβ in macrophages

Based on the capacity of *HpbE* to induce a unique eicosanoid feature and modulation of type 2 immune responses in human and murine macrophages, this research project has identified the mechanistic pathways activated by this larval extract *in vitro*. *HpbE*-driven reprogramming of COX metabolites and type 2 immunosuppressive cytokines is dependent on p38 MAPK, COX and the transcriptional factors HIF-1α and NFκβ. *Hpb* products induce the activation of p38 MAPK, which activates COX and promote the production of prostanoids ($PGE_2$ and $TXB_2$) and anti-inflammatory cytokines (IL-10 and IL-1β). Of interest, a similar mechanism of action was described for a protease inhibitor (AvCystatin) from a filarial nematode, which induced the synthesis of IL-10 through the activation of p38 MAPK and therefore modulated the macrophage activation (Klotz et al., 2011). The finding that indomethacin (unspecific COX inhibitor) efficiently suppressed the *HpbE*-driven induction of COX-2, mPGES-1 and IL-10, whilst CAY 104 (specific COX-2 inhibitor) did not interfere with the induction of mPGES-1 or IL-10, suggested that COX-1 might be involved in *HpbE*-driven remodelling of the AA metabolism. However, it should be taken into account that indomethacin inhibits COX but also acts as a mitochondrial uncoupler (Moreno-Sánchez et al., 1999), implying that metabolic pathways such as glycolysis can be also activated by *HpbE* in macrophages, although further research in this direction needs to be done in order to confirm this hypothesis.

Downstream signalling pathways activated by *HpbE* included the transcriptional factors HIF-1α and NFκβ, which contributed to the *HpbE*-triggered induction of type 2 suppressive mediators ($PGE_2$, IL-10 and IL-1β). The expression of both transcription factors in myeloid cells have been suggested to play a protective and anti-inflammatory role during allergic airway inflammation (Toussaint et al., 2013; Han et al., 2009). To our surprise, depletion of myeloid HIF-1α hardly

affected the course of helminth infection or the modulation of the AA metabolism during *Hpb* infection, although it is possible that other HIF-1α-expressing cells contributed to the *Hpb*-driven immuneregulation *in vivo*. In addition, LysMCre-driven deletion of HIF-1α may also not be sufficient, as this strategy may not work properly for all macrophage populations, especially under inflammatory conditions (Vannella et al. 2014).

Furthermore, upstream mechanisms underlying the induction of the COX pathway included several PRRs (TLR2, Dectin-1/2), whilst they were not involved in the *HpbE*-driven induction of IL-10. These results suggested that there might be several immunomodulatory active compounds present in *HpbE*, some of them are most likely carbohydrate structures which contribute to the induction of prostanoids by *HpbE*. This is in line with soluble egg antigen (SEA) from *S. mansoni*, which binds to dectin-1 and dectin-2 receptors and activates the secretion of $PGE_2$ in dendritic cells (Kaisar et al., 2018).

Regarding the modulation of the 5-LOX pathway by *HpbE*, kinases such as PI3K, PKA and PTEN were not involved in 5-LOX regulation, which thus remains poorly understood. Nevertheless, *Hpb*-associated bacteria and HES showed similar effects on the inhibition of cysLTs as observed in *HpbE*-stimulated macrophages. This may suggest that bacterial products or an immunomodulatory compound present in both *HpbE* and HES might contribute to the suppression of pro-inflammatory LTs by *HpbE*. In contrast, an immunomodulatory compound present in *HpbE* but not in HES might contribute to the modulation of the COX pathway.

Taken together, several active compounds are present in *HpbE* and are responsible for activation of PPRs (TLR2, dectin 1-/2), p38 MAPK, COX and activation of the transcription factors HIF-1α and NFκβ, leading to an anti-inflammatory remodelling of the AA metabolism and induction of type 2 suppressive cytokines, leading to suppression of type 2 immune responses (Figure 38).

### 6.6 Treatment with *HpbE* suppressed type 2 inflammation during allergic airway inflammation *in vivo*

The present study has shown that the parasitic nematode *H. polygyrus* can directly reprogram the AA metabolism *in vivo* and treatment of murine and human leukocytes with the parasitic larval extract (*HpbE*) similarly remodels the AA-metabolism by inducing regulatory COX metabolites but suppressing pro-inflammatory LTs, thereby suppressing type 2 inflammation and inducing a regulatory network which might have protective effects during allergic asthma. The therapeutic potential of *HpbE* was tested in a mouse model of house dust mite (HDM)-induced allergy, where the central role of prostanoids and LT was previously reported (Barret et al, 2011; Draijer et al., 2016). Intranasal treatment with *HpbE* reprogrammed the AA metabolism *in vivo* and prevented the HDM-induced eosinophilia, cell infiltration, airway inflammation and mucus production during HDM-induced airway inflammation. Although previous studies have shown that *Hpb* infection *in vivo* or treatment with *Hpb* excretory/ secretory products (HES) can suppress allergic airway inflammation by modulation of type 2 cytokine responses (McSorley et al., 2012; Osbourn et al., 2017; Wilson et al., 2005), here we demonstrated that an extract of the migratory larval stages of *Hpb* has a potential therapeutic application during allergic asthma.

During HDM-induced allergic airway inflammation, macrophages play a key role as initiators of the airway inflammation by the production of cysLTs (Clarke et al., 2014). However, in our hands, *in vitro* exposure to HDM modulated the AA metabolism in human and mouse macrophages by

inhibiting the synthesis of pro-inflammatory LOX metabolites ($LTB_4$ and 5-HETE), whilst inducing the production of COX metabolites ($PGE_2$ and $TXB_2$). HDM exposure also triggered the production of type 2 suppressive cytokines (IL-10 and IL-1β) and cytokines involved in modulation of M2 macrophages and type 2 immune responses (IL-6 and TNF-α). HDM-stimulated macrophages also produced RANTES (chemokine involved in chemotaxis), although it has been shown that HDM-exposed MDM prevented the chemotaxis of human granulocytes (Henkel et al., 2018). It was unexpected to find that the eicosanoid profile of *HpbE*-stimulated macrophages was similar as compared to HDM-exposed macrophages *in vitro*, since HDM is known to promote allergic airway inflammation *in vivo* (Barret et al., 2011; Clarke et al., 2014), whereas intranasal administration of *HpbE* had a protective effect during HDM-induced allergic airway inflammation. Indeed, pretreatment with *HpbE* before HDM stimulation *in vitro* further enhanced the production of COX metabolites ($PGE_2$ and $TXB_2$) and the anti-inflammatory cytokine IL-10 in murine macrophages in a COX-dependent manner. Similar to the signalling pathway activated by *HpbE*, HDM activates p38 MAPK and COX-2 expression in human macrophages (Henkel et al., 2018), an effect, which could explain the enhanced production of COX metabolites when macrophages were stimulated with both *HpbE* and HDM. A better understanding of the regulatory network activated by *HpbE* during allergic airway inflammation might be useful to understand the different modulation of type 2 immune responses during HDM-induced allergy and the protective effect of *HpbE* during allergic asthma *in vivo*. One difference between the effects of HDM and *HpbE* on murine macrophages is the $PGE_2$/ $PGD_2$ ratio and the suppression of $LTB_4$, which was higher for *HpbE* and might thus explain the type 2-suppressive effect of *HpbE*-treated BMDM during experimental HDM allergy.

Modulation of type 2 inflammation by *HpbE* during HDM-induced allergic asthma was mainly mediated by a COX-2 metabolite released by *HpbE*-stimulated macrophages. Indeed, adoptive transfer of *HpbE*-stimulated wild type but not COX-2 deficient macrophages attenuated granulocyte recruitment and type 2 inflammation during HDM allergy *in vivo*. According to a previous study, adoptive transfer of $PGE_2$-treated macrophages suppressed allergic lung inflammation (Draijer et al., 2016), making $PGE_2$ a potential mediator secreted by *HpbE*-macrophages responsible for the modulation of type 2 immune responses. This beneficial effect of *HpbE*-stimulated macrophages is a novel avenue to be explored for therapeutic applications.

Taken together, infection with the parasitic nematode *Hpb* or treatment with *Hpb* larval extract can reprogram the AA metabolism. In both human and murine settings of type 2 inflammation, *HpbE* treatment suppressed the pro-inflammatory LOX pathway but induced the COX pathway by the activation of p38 MAPK, COX-2, HIF-1α and NFκβ. This resulted in reduced chemotaxis of human granulocytes and attenuated allergic airway inflammation during HDM-allergy in mice (Figure 38).

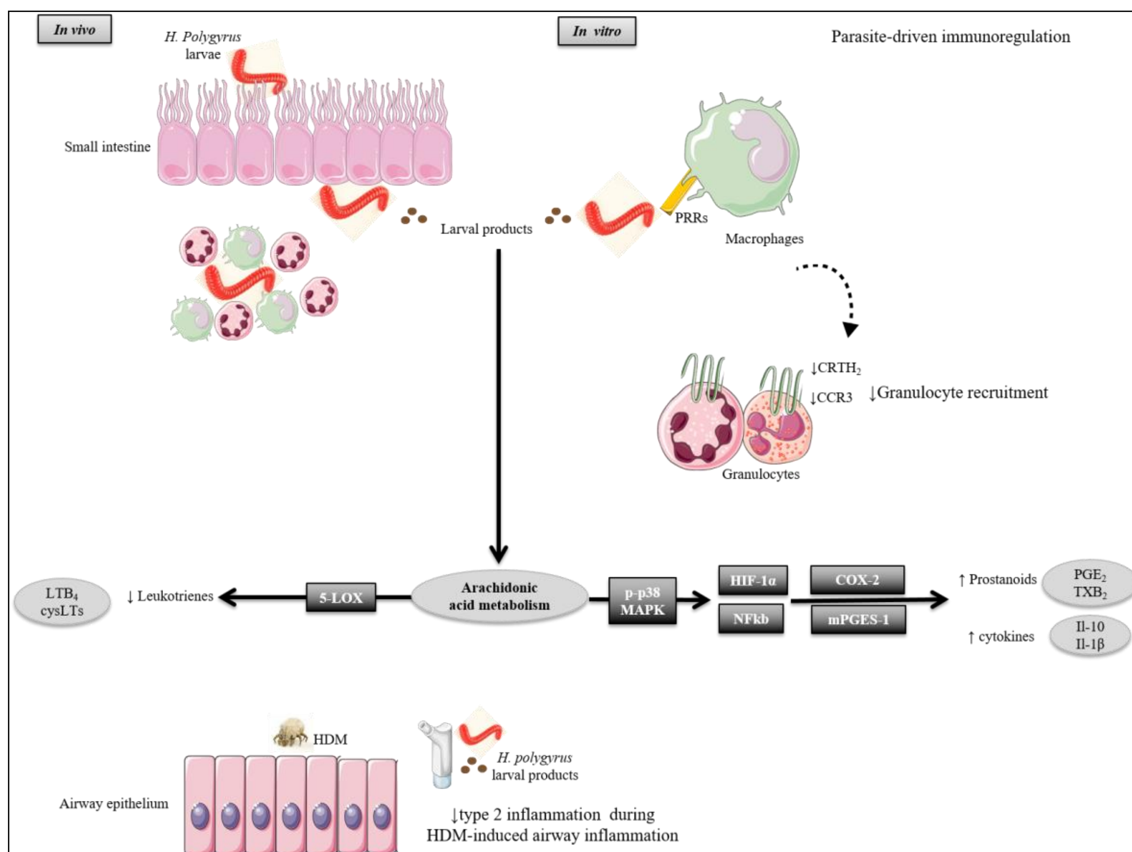


**Figure 38: Infection with *Hpb* or treatment with *HpbE* reprograms the AA metabolism in settings of type 2 inflammation.** Infection with *H. polygyrus in vivo* or treatment with *Hpb* larval extract *in vitro* remodels the AA metabolism by suppressing the pro-inflammatory 5-LOX pathway whereas inducing the COX pathway. *HpbE* signalling pathway involved the activation of PPRs, p38 MAPK, HIF-1α, NFκβ and COX-2. This resulted in reduced chemotaxis of human granulocytes and attenuated allergic airway inflammation during HDM-allergy in mice. (Abbreviations: 5-LOX, 5-lipoxygenase; COX-2, cyclooxygenase-2; $CRTH_2$, chemoattractant receptor-like molecule expressed on Th2 cells; CCR3, C-C chemokine receptor type 3; cysLTs, cysteinyl leukotriene; HIF-1α, hypoxia inducible factor 1-alpha; HDM, House Dust Mite; IL, interleukin; $LTB_4$, leukotriene B4; mPGES, membrane prostaglandin E synthase; NFκβ; nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; $PGE_2$, prostaglandin E2; mPGES, membrane prostaglandin E synthase; p-p38 MAPK, phosphorylated p-38 Mitogen-activated protein kinase; PRRs, patter recognition receptors; $TXB_2$, thromboxane B2)

### 6.7 LTs production and signs of airway remodelling during allergy are age dependent

Chronic exposure to allergens such as house dust mite (HDM) in sensitized patients causes irreversible damage in the airways, leading to chronic inflammation and airway remodelling (Kaminska et al., 2009). Leukotrienes play important roles in allergy and asthma as these mediators are potent inducers of several hallmark responses of type 2 inflammation, inducing cell recruitment and airway remodelling (Figure 38, Barret et al, 2011; Henderson et al., 2002 Ochkur et al., 2013). Using a mouse model of HDM-induced allergic airway inflammation at different ages, our study has shown that the production of LTs and expression of LT-biosynthetic enzymes and airway remodelling factors were age dependent. Mice sensitized as neonates showed high levels of LTs (cysLTs and $LTB_4$) with a major contribution of eosinophils to LT production. As previously reported, HDM-induced eosinophilia in the airways of neonates was higher than in preweanling or adult mice, and eosinophils are mayor producers of LTs in the airways (Gollwitzer

et. al., 2014; Hogan et al., 2008). Preweanling mice were more protected from HDM sensitization, which correlated with the finding that LTs levels measured in the airways were close to the lower detection limit and expression of LT-biosynthetic enzymes was mostly absent in contrast to neonates or adult mice. Adult sensitized mice showed high production of LTs despite lower number of infiltrating granulocytes as compared to neonates. This might be explained by high expression of LT-biosynthetic enzymes (5-LO, $LTC_4S$ and $LTA_4H$) in the airway epithelium, suggesting that airway epithelial cells significantly contribute to the LT production in adult airways. Indeed, HDM exposure can induce the synthesis of LTs biosynthetic enzymes (5-LO and $LTC_4S$) in airway epithelial cells, contributing to the synthesis of LTs (Jame et al., 2007; Trian et al., 2015).

Lung tissues of adult sensitized mice showed more pronounced signs of airway remodelling such as increased thickening of the bronchial wall (high expression of α-SMA) and disruption of the airways (loss of tight junction protein ZO-1) as compared to neonates and preweanling mice. Signs of airway remodelling were not observed in neonatal mice, despite the high levels of cysLTs measured in the BALF. Interestingly, previous publications have shown age differences in the production of type 2 cytokines and IL-33 levels. Neonatally sensitized mice showed exacerbated airway inflammation due to high production of type 2 cytokines (IL-4, IL-5 and IL-13) as compared to both younger and adult mice; whilst IL-33 levels are lower in neonates (Gollwitzer et al., 2014; Saglani et al., 2013). However, type 2 cytokine production decreases with age while IL-33 levels were shown to increase with age and play a key role in the induction of airway remodelling and collagen deposition (Gollwitzer et al., 2014; Saglani et al., 2013), suggesting that type 2 cytokines can induce exacerbated airway inflammation in sensitized neonates, followed by increased production of epithelial remodelling factors and LT-biosynthetic enzymes after longer allergen exposure during adult stage. The type 2 cytokine IL-4 can induce the synthesis of LTs in myeloid cells and IL-5 promotes eosinophil survival (Henkel et al., 2018; Mould et al., 1997), which could partially explain the HDM-induced eosinophilia and high cysLTs production in neonatally sensitized mice. The increased production of IL-33 and expression of epithelial airway remodelling factors and LT-biosynthetic enzymes suggest an epithelial contribution to the production of cysLTs during chronic inflammation.

Taken together, LTs production and signs of airway remodelling during allergy are age dependent. Allergen exposure during childhood induces a strong type 2 inflammatory response, where eosinophils play a key role as the major producers of cysLTs. There is a period of reduced susceptibility at young life stages, with lower levels of LTs production. However, strong inflammation and airway remodelling are observed during adult age, the airway epithelium being the main source of airway remodelling factors (sPLA2-X, TGM2, Wnt5a) and LT-biosynthetic enzymes, explaining the high levels of cysLTs detected in adult airways (Figure 39).

## 6.8 LTs production and expression of airway remodelling factors are steroid resistant in patients with CRSwNP

The mechanism behind LT production and airway remodelling in the epithelium of adult sensitized mice involved the activation of remodelling factors such as $sPLA_2$-X, TGM2 and Wnt5a. As compared to neonates or younger sensitized mice, high expression of $sPLA_2$-X was detected in lung tissues of adult sensitized mice, mostly localized in smooth muscle cells, infiltrating leukocytes and epithelial cells. Interestingly, $sPLA_2$-X promotes the synthesis of

cysLTs in eosinophils during inflammation (Hallstrand et al., 2016), suggesting that epithelial expression of $sPLA_2$-X might promote LTs synthesis in adult sensitized mice. Since $sPLA_2$-X is regulated by TGM2 (Hallstrand et al., 2010), it was unexpected not to detect TGM2 expression in the airway epithelium, although TGM2 levels were found in the BALF and increased with age, showing high levels of secreted TGM2 in adult-sensitized mice. It is possible that epithelial cells produce and rapidly secrete TGM2, although our study suggests that other cells present in the airways like alveolar like macrophages can express high levels of TGM2. Another remodelling factor produced by the airway epithelium was Wnt5a, showing higher expression in the airway epithelium and BALF of adult sensitized mice (Dietz et al., 2017) Wnt5a can promote airway remodelling in asthmatic patients by inducing deposition of extracellular matrix and expression of collagen and fibronectin (Kumawat et al., 2013). In agreement with these results, Wnt5a and another member of the $sPLA_2$ family ($sPLA_2$ group IID), were previously reported to increase with age (Florian et al., 2013; Vijay et al., 2015). Thus, a cascade of airway remodelling factors including $sPLA_2$-X, TGM2 and Wnt5a is age dependent.

Furthermore, our study showed a cross-talk between inflamed airway epithelium and alveolar like macrophages, leading to macrophage activation and synthesis of LTs. Wnt5a secreted by the airway epithelium activates the expression of TGM2 in alveolar like macrophages and induced the production of pro-inflammatory cysLTs, further supporting the role for macrophages in adult inflamed airways. As TGM2 is an important marker of M2 macrophage polarization (Martinez et al., 2013), these results suggest that Wnt5a may promote macrophage polarization towards an M2 phenotype. Since TGM2 inhibition prevented the production of cysLTs, it was suggested that TGM2 can regulate the synthesis of cysLTs in macrophages. Since $sPLA_2$-X is regulated by TGM2 (Hallstrand et al., 2010), most likely TGM2 activates $sPLA_2$-X which enhances the arachidonic acid release and the conversion into cysLTs by the action of LT-biosynthetic enzymes (5-LOX and $LTC_4S$), highly expressed in the airway epithelium of adult sensitized mice. Thus, this study suggests a cross talk between airway epithelial cells and alveolar-like macrophages through Wnt5a, TGM2, and $sPLA_2$ to further promote LTs production and remodelling during allergic asthma.

The relevance of the identified airway remodelling cascade was assessed in patients with chronic rhinosinusitis and nasal polyposis (CRSwNP). Human nasal polyp tissues from CRSwNP patients expressed high levels of LT enzymes (5-LO, $LTC_4S$, $LTA_4H$), $sPLA_2$-X, TGM2 and Wnt5a despite receiving systemic steroid treatment. In fact, expression of TGM2 and $sPLA_2$-X in HDM-stimulated NHBEC was observed despite treatment with Fluticasone Propionate, a glucocorticosteroid commonly used for asthma treatment (Dietz et al., 2017). In addition, high levels of cysLTs were found in nasal polyp secretions of CRSwNP patients despite systemic steroid treatment, suggesting that expression of airway remodelling factors, LT-biosynthetic enzymes and synthesis of LTs are steroid resistant. Thus, this cascade of remodelling factors might represent an alternative therapeutic target for the treatment of asthma, particularly in patients with steroid-resistant forms of airway inflammation (Figure 39).

### 6.9 Potential therapeutic application of *HpbE* in the treatment of AERD patients

Our research has shown that treatment with *Hpb* larval extract can reprogram the AA metabolism in both human and murine settings of type 2 inflammation. Treatment with *HpbE*- or adoptive transfer of *HpbE*-stimulated macrophages prevented allergen-induced allergic airway inflammation, implying the beneficial effect of *HpbE*-stimulated macrophages in the treatment of

allergic asthma (Figure 38). The clinical relevance of this finding was assessed by the treatment of macrophages from AERD patients with *HpbE*. Similar to the effect observed in healthy macrophages, *HpbE* also modulated the AA metabolism in macrophages of AERD patients inducing the synthesis of COX metabolites like $PGE_2$, which can suppress inflammatory responses in the lung, contribute to the inhibition of eosinophils chemotaxis and induce wound healing in the airways (Säfholm et al., 2015; Savla et al., 2001; Sturm et al., 2008). *HpbE* also induced the synthesis of the prostanoid $TXB_2$, which can inhibit the activation and proliferation of T-cells (Kabashima et al., 2003). In contrast, *HpbE* suppressed the release of pro-inflammatory 5-LOX mediators ($LTB_4$ and 5-HETE) known to act as potent chemoattractants, bronchochonstrictors and promote airway inflammation in the lung during allergic asthma (Tager et al., 2000; Miyahara et al., 2008).

The therapeutic potential of *HpbE* was compared to a standard drug used for the treatment of asthma, Fluticasone propionate (FP). Treatment with FP hardly modified the synthesis of lipid mediators when compared to *HpbE*-stimulated macrophages. FP failed to induce prostanoids ($PGE_2$ and $TXB_2$) and hardly suppressed the release of LTs, suggesting that *HpbE* uniquely modulates the AA metabolism and induces an immunosuppressive eicosanoid profile in macrophages. In line with these results, treatment with FP failed to suppress the chemotaxis of AERD granulocytes toward nasal polyp secretions, whilst *HpbE* efficiently suppressed granulocyte recruitment, potentially by downregulation of chemotactic receptors CCR3 and $CRTH_2$. Decreasing the number of eosinophils through the blockade of eotaxin-CCR3 axis might have potential therapeutic applications in asthma. Taken together, *HpbE* might represent a therapeutic alternative in steroid resistant AERD patients where high expression of LTs-biosynthetic enzymes and high levels of LTs are found despite receiving systemic steroid treatment.

Indeed, treatment with *HpbE* might counterregulate the effect of IL-4, the type 2 cytokine responsible for the upregulation of $LTC_4S$ in AERD patients (Hsieh et al., 2001). Here we found that IL-4 stimulation induced the release of pro-inflammatory 5-LOX mediators ($LTB_4$ and 5-HETE) but hardly modulated the COX pathway and did not induce the synthesis of the 15-LOX mediator 15-HETE. The observed IL-4 modulation of the AA metabolism was quite unexpected in comparison to previous studies showing the opposite effect, down-regulation of 5-LOX but up-regulation of 15-LOX expression (Spanbroek et al., 2001). However, in the present study, macrophages are differentiated in the presence of GM-CSF and TGF-$\beta$1 to mimic an alveolar-like macrophage phenotype (Yu et al., 2017), and it has been suggested that TGF-$\beta$1 stimulation prevents the IL-4-driven regulation of lipid mediators (Henkel et al., 2018). Thus, *HpbE*-driven induction of prostanoid formation and suppression of 5-LOX mediators might counteract the effect of IL-4 in the modulation of the AA metabolism.

One of the main immunomodulatory eicosanoids secreted by *HpbE*-treated macrophages is $PGE_2$, a mediator which attenuated bronchoconstrictor responses after allergen challenge in asthmatic patients (Hartert et al., 2000). However, granulocytes of AERD patients are known to be resistant to $PGE_2$ and to promote overproduction of LTs and inflammation in AERD (Laidlaw et al., 2014). The present study has demonstrated that macrophages of AERD patients respond to $PGE_2$ stimulation by suppressing the production of 5-LOX metabolites ($LTB_4$ and 5-HETE). Taking into account that $PGE_2$ concentrations are decreased in AERD patients (Pérez-Novo et al.,2005) and that $PGE_2$ can counteract the pro-inflammatory effects of cysLTs and $PGD_2$ in the airways (Aggarwal et al., 2010; Hartert et al., 2000; Säfholm et al., 2015), treatment of macrophages with

*HpbE* may represent a promising future therapeutic alternative for the treatment of AERD patients.

### 6.10 GDH and ferritin as potential immunomodulatory compounds present in *HpbE*

The characterization of the active immunomodulatory compounds present in *HpbE* will be an important step to elucidate the eicosanoid modulatory effects of *HpbE* and translate these into future applications in the therapy of allergic asthma or AERD. **Glutamate dehydrogenase** (GDH) was identified as a major immunoregulatory component of *HpbE*, able to modulate the AA metabolism and induce the release of the regulatory prostanoid $PGE_2$ and the anti-inflammatory cytokine IL-10. When comparing the proteomic analysis of excretory/secretory products (HES) of adult stages of *Hpb* larvae (Hewitson et al., 2011), it was interesting to observe that GDH was not found in HES and is only present in *HpbE,* suggesting a different protein profile depending on the stage of the same parasite. Differences in the protein composition might explain the different reprograming of the AA metabolism observed for *HpbE*, GDH or HES, as only HES failed to induce the release of prostanoids in macrophages. Interestingly, GDH was identified from the parasite *Trypanosoma cruzi* as the major immunomodulatory compound responsible for the production of IL-10 and IL-6 (Montes et al., 2006). *HpbE*-treated macrophages also release high amount of IL-10 and IL-6, making GDH a potential active compound present in *HpbE*. As GDH of different organisms and even different stages of the same parasite can differ considerably in their structure and function (Li et al., 2009), further work is required for the detailed characterization of *Hpb* GDH.

Another potential candidate identified in *HpbE* but not in HES is **Ferritin**, which failed to modulate the COX pathway in human macrophages, but reprogrammed the transcriptional gene expression of 5-LOX, suggesting a role of ferritin in the suppression of LTs. According to a previous publication, ferritin induced HIF-1α stabilization by decreasing the intracellular available iron which is essential for PHD (prolyl hydroxylase domain enzyme) as a cofactor and thus for subsequent inhibition of HIF-1α degradation (Siegert et al., 2015). Although HIF-1α activation is involved in the signalling pathway induced by *HpbE*, this role of ferritin in the modulation of the HIF-1α is quite unlikely because ferritin did not modulate the COX pathway in human macrophages. In addition, placenta ferritin has been shown to promote IL-10 production in macrophages via the activation of p38 MAPK (Zahalka et al., 2003). Nevertheless, in our hands, liver ferritin did not modulate the IL-10 expression in macrophages. However, ferritin can act as an iron-storage protein and inactivate the 5-LOX, enzyme which requires the presence of iron as a cofactor to be active (Rådmark et al., 2007), suggesting a direct inhibitory effect of ferritin on 5-LOX activity. However, thus far, we only observed an effect of human liver ferritin on 5-LOX gene expression. Thus, further work is required to confirm this hypothesis and to further elucidate the mechanism of action and characterization of *Hpb* Ferritin.

In conclusion, GDH and ferritin are main immuneregulatory candidate proteins characterized in the *Hpb* larval extract, which induce remodelling of the AA metabolism. As AA metabolites represent important mediators of severe and therapy-resistant type 2 inflammation, their modulation by active components of *HpbE* may represent a promising future therapeutic approach (Figure 39). Thus, the recombinant production and detailed characterization of immuneregulatory

*HpbE* proteins is the next key step, which should allow the translation of the main results from this thesis into a new therapy for type 2 inflammatory diseases.

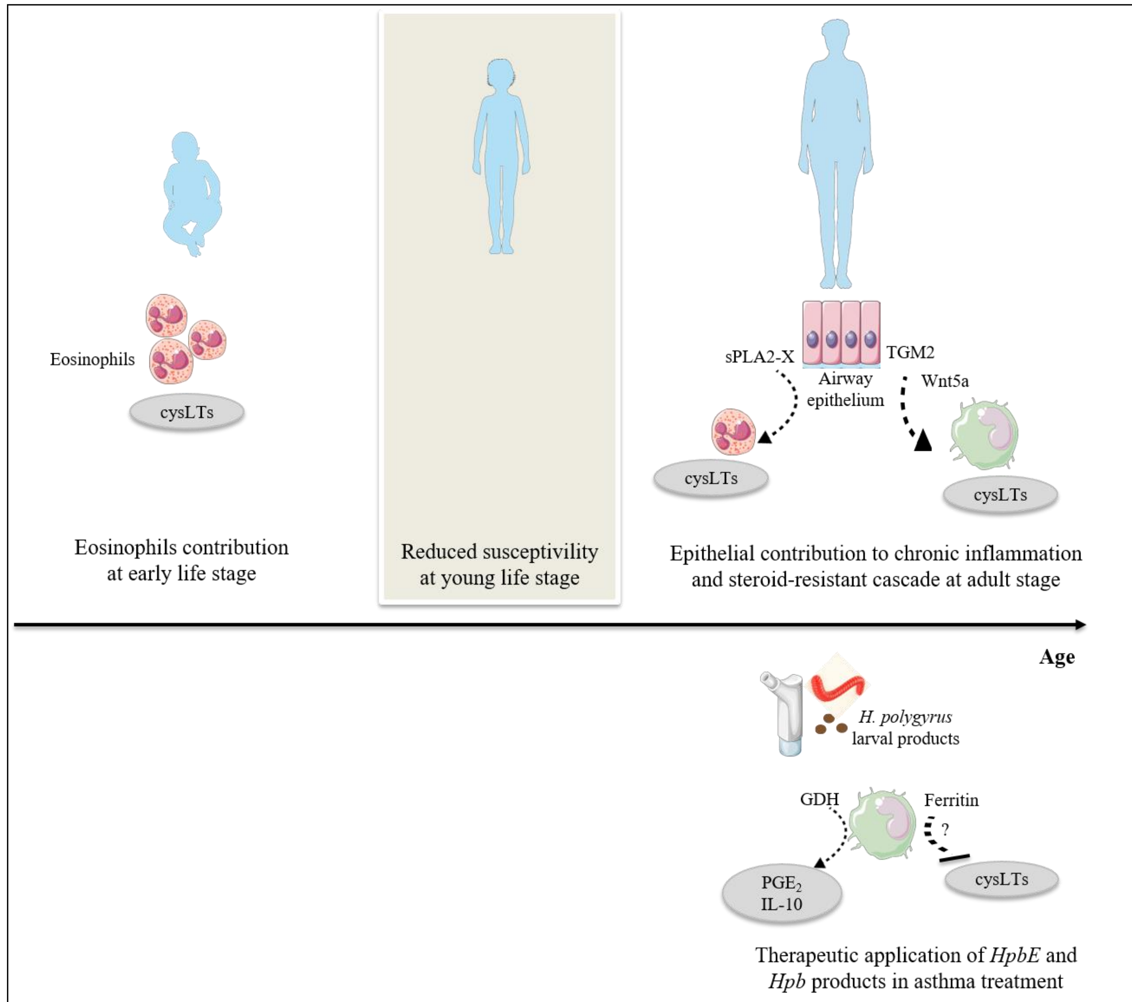


**Figure 39: Age dependent regulation of LTs and airway remodelling factors and potential application of parasite products in asthma treatment.** Allergen exposure during childhood induce a strong type 2 inflammatory response, where eosinophils play a key role as the major producers of cysLTs. There is a period of reduced susceptibility at young life stages, with lower levels of LTs production. However, strong inflammation is observed during adult stage and the airway epithelium is the main source of airway remodelling factors ($sPLA_2$-X, TGM2, Wnt5a) and LT-biosynthetic enzymes at adult stage, interacting with myeloid cells to promote LT production. Treatment with *Hpb* larval extract (*HpbE*) or *Hpb* products (glutamate dehydrogenase, GDH and ferritin) modulates the arachidonic acid metabolism and induce the production of type 2 suppressive cytokines. Therefore, parasite products may represent a promising future therapeutic alternative for the treatment of asthmatic patients. (Abbreviations: cysLTs, cysteinyl leukotrienes; GDH, Glutamate Dehydrogenase; *Hpb*, *Heligmosomoides polygyrus bakeri*; *HpbE*, *Hpb* extract; $PGE_2$, prostaglandin E2; sPLA2, secreted phospholipase A2; TGM2, transglutaminase 2)

# 7 SUMMARY

In this study we have explored the modulation of AA metabolic pathways during helminth infection with the nematode *Heligmosomoides polygyrus bakeri* (*Hpb*) as an immune regulatory approach to modulate type 2 inflammation. We have shown that at early stages of infection (day 7), *Hpb* drives the production of regulatory prostanoids ($PGE_2$ and 6-keto PGF1-α, a degradation metabolite of $PGI_2$) and COX metabolites 12-HHT and $TXB_2$, but suppressed the secretion of the pro-inflammatory mediators $PGD_2$ and LTs ($LTB_4$, cysLTs). In line with the abundant production of prostanoids, immunofluorescence stainings of gut sections showed high expression of COX-2 (biosynthetic enzyme involved in the synthesis of prostanoids) and its positive regulator hypoxia inducible factor-1 alpha (HIF-1α) in the surrounding of the trapped larvae and in cells close to the larvae in *Hpb*-infected mice. In contrast, 5-LOX (biosynthetic enzyme responsible for the synthesis of leukotrienes) was not present during helminth infection, explaining the absence of leukotrienes measured in the intestinal culture supernatant or peritoneal lavage. The *Hpb*-driven modulation of AA metabolic pathways might represent an immune evasion strategy of this parasite, aiming to escape from the type 2 immune responses initiated by the host, intending to limit infiltration and recruitment of granulocytes and thus larval killing, as well as to prevent mucus production and larval expulsion. The *Hpb*-induced production of COX metabolites involved in tissue repair and blood clotting may be important to rapidly repair the tissue disruption caused by the tissue migrating larvae. However, the exact contribution of eicosanoids to the protective immune response against *Hpb* remains to be explored in follow-up studies.

As the main focus of this thesis, we have shown that a larval extract of *Heligmosomoides polygyrus bakeri* (*HpbE*) modulates AA metabolic pathways in myeloid cells such as macrophages and granulocytes, by shifting the synthesis of 5-LOX towards COX metabolites. *HpbE* treatment of murine and human macrophages induced the synthesis of regulatory prostanoids ($PGE_2$) and prostanoids involved in tissue repair and blood clotting (12-HHT, $TXB_2$), whereas it suppressed the production of pro-inflammatory lipid mediators (LTs, $PGD_2$). *HpbE* also induced a transcriptional regulation of AA biosynthetic enzymes in human macrophages by inducing the gene expression of enzymes involved in the synthesis of $PGE_2$: *PGTS2* (gene encoding COX-2) and *PTGES* (gene encoding mPGES-1). In contrast, *HpbE* downregulated the gene expression of *PTGDS* (gene encoding $PGD_2$ synthase), downregulated the gene expression of all LT biosynthetic enzymes: *ALOX-5* (encodes 5-LOX), *LTA4H* (encodes $LTA_4H$) and *LTC4S* (encodes $LTC_4S$) as well as the high affinity receptor for cysLTs (Cysteinyl Leukotriene Receptor-1, *CYSLT1R*). In murine macrophages, *HpbE* induced the gene expression of *Ptgs2* and *Ptges*, but suppressed the expression of *Alox5* and *Ltc4s*. Thus, *HpbE* reprogrammed the AA metabolism of human and murine macrophages, inducing a potentially anti-inflammatory eicosanoid profile.

Furthermore, *HpbE* induced the synthesis of type 2 suppressive mediators IL-10 and IL-1β in both human and murine macrophages, and modulated the production of cytokines involved in the modulation of M2 polarization and type 2 inflammation (IL-12, IL-28, IL-27 and TNF-α) in human macrophages. In terms of macrophages polarization, *HpbE* did not modified the gene expression of M2 markers like *TGM2* (Transglutaminase 2, TGM2), but *ALOX-15* (15-Lipoxygenase, 15-LOX) and *MRC1* (Mannose Receptor C-Type 1, MR/CD206) were downregulated, suggesting that *HpbE* counteracted M2 polarization in human MDM. In contrast to *HpbE*-stimulated MDM, *HpbE* induced gene expression of *Tgm2* and *Arg1*, but down-regulated the gene levels of *Mrc1*, whilst other M2 markers *Tmed1/St2l* and *Rentla/Fizz1* remained unaltered in murine macrophages. Thus, *HpbE* might drive a partially distinct polarization profile

in human and murine macrophages, but a similar regulatory mediator profile, most likely inducing an M2-suppressive phenotype.

Similar to *HpbE*-driven eicosanoid reprogramming in macrophages, we found that *HpbE* remodels the AA-metabolism of human granulocytes, showing a shift from LOX towards COX metabolism. *HpbE* triggered the induction of COX metabolites (12-HHT, $TXB_2$), whilst it inhibited the productions of pro-inflammatory LTs (cysLTs, $LTB_4$) in human granulocytes. Similar to *HpbE*-driven changes at the transcriptional level of AA metabolizing enzymes in macrophages, *HpbE* downregulated the gene expression of enzymes involved in the synthesis of pro-inflammatory mediators (*ALOX5, $LTA_4H$* and *PTGDS*), but induced the expression of $PGE_2$-synthetic enzymes (*PTGS2* and *PTGES*) in human granulocytes. In addition, *HpbE* can act directly on human granulocytes and inhibit chemotaxis more efficiently than standard drugs used for asthma treatment by reducing the expression of LT-biosynthetic enzymes ($LTA_4H$ and $LTC_4S$) as well as down-regulating the expression of chemotactic receptors CCR3 (C-C chemokine receptor type 3) and $CRTH_2$ (Prostaglandin D2 receptor 2). Our study has also suggested a cross-talk between macrophages and granulocytes, where *HpbE*-treated macrophages attenuated chemotaxis of human granulocytes in a COX dependent fashion.

In comparison to other helminth parasite extracts or excretory/ secretory (E/S) products, we have demonstrated that *HpbE* induces a unique eicosanoid signature in myeloid cells. In contrast to *HpbE*, E/S products of *Hpb* (HES) failed to induce IL-10 production or prostanoids ($PGE_2$ and $TXB_2$) in human macrophages, suggesting that different stages during the life cycle of the same parasite might differentially modulate the host immune system. Other parasite extracts of *Schistosoma mansoni* (*SmE*) or *Ascaris suum* (*AsE*) had minor effects in the production of COX and LOX metabolites when compared to *HpbE* in human macrophages and granulocytes, respectively. Regarding the modulation of the 5-LOX pathway, macrophage stimulation with HES or *Hpb*-associated bacteria showed a similar reduction compared to *HpbE* in the cysLTs production, suggesting that bacterial products might contribute to the suppression of pro-inflammatory LTs by *HpbE*.

With regards to the broader propensity of *HpbE* to induce a regulatory network in human subjects, this study has shown that *HpbE* does not have a direct impact on type 2 cytokines (IL-4, IL-5 and IL-13), although it induced the production of IFN-γ, IL-10 and eicosanoid reprogramming in human peripheral blood mononuclear cells (PBMCs). Interestingly, *HpbE* shifted the production of 5-LOX towards COX metabolites, but triggered the synthesis of 12/15-HETE metabolites, resembling the eicosanoid profile measured during *Hpb*-infection *in vivo*.

We have identified the mechanistic pathways underlying the *HpbE*-driven eicosanoid reprogramming in macrophages. *Hpb* products induced the activation of p38 MAPK, which activates COX and the transcriptional factors HIF-1α and NFκβ, promoting the production of prostanoids ($PGE_2$ and $TXB_2$) and the type 2-suppressive cytokines IL-10 and IL-1β. The upstream mechanism underlying the induction of COX pathway included several PPRs (TLR2, Dectin-1/2), whilst they were not involved in the *HpbE*-driven induction of IL-10. Regarding the modulation of the 5-LOX pathway by *HpbE*, kinases such as PI3K, PKA and PTEN were not involved in the down-regulation of 5-LOX, a mechanism, which still needs to be further explore.

The therapeutic potential of *HpbE* or *HpbE*-treated macrophages was shown in a mouse model of HDM-induced allergic airway inflammation *in vivo*. Intranasal treatment with *HpbE* before HDM sensitization and challenge reprogrammed the AA metabolism and prevented the allergy-induced eosinophilia, cell infiltration, airway inflammation and mucus production. In addition, we have

shown that adoptive transfer of *HpbE*-stimulated wild type macrophages before HDM challenge attenuated granulocyte recruitment and type 2 inflammation. Interestingly, this effect was not observed after adoptive transfer of COX-2 deficient, suggesting that modulation of type 2 inflammation by *HpbE* was mainly mediated by a COX-2 metabolite released by *HpbE*-stimulated macrophages.

Chronic exposure to allergens such as HDM in sensitized patients causes irreversible damage in the airways, leading to chronic inflammation and airway remodelling (Kaminska et al., 2009). By investigating HDM-induced allergic airway inflammation at different ages (neonate, preweanling and adult), we could show that the age of sensitization plays a key role in the production of LTs and expression of LT-biosynthetic enzymes and airway remodelling factors. Mice sensitized as neonates showed high levels of LTs (cysLTs and $LTB_4$) with a major contribution of eosinophils to LTs production. Adult sensitized mice showed high production of LTs and high expression of LT-biosynthetic enzymes (5-LO, $LTC_4S$ and $LTA_4H$) in the airway epithelium, suggesting that airway epithelial cells significantly contribute to the LT production. In contrast, preweanling mice were more protected from HDM sensitization, showing lower LTs levels measured in the airways. Signs of airway remodelling were only observed in lung tissues of adult sensitized mice. We also identified the mechanism behind the LT production and airway remodelling in the epithelium of adult sensitized mice, which involved the activation of remodelling factors such as $sPLA_2X$, TGM2 and Wnt5a.

Furthermore, our study showed that a cross-talk between inflamed airway epithelium and alveolar like macrophages can promote the synthesis of LTs. The mechanism proposed starts with the secretion of Wnt5a by the inflamed airway epithelium, which activates the expression of TGM2 in alveolar like macrophages and induced the production of pro-inflammatory LTs, further supporting the role for macrophages in adult inflamed airways. The relevance of the identified remodelling cascade was assessed in patients with chronic rhinosinusitis and nasal polyposis (CRSwNP). High levels of LT enzymes (5-LO, $LTC_4S$ $LTA_4H$), $sPLA_2$-X, TGM2 and Wnt5a were observed in human nasal polyp tissues, and high levels of cysLTs were measured in nasal polyp secretions of these patients despite undergoing glucocorticosteroids treatment, suggesting that the expression of airway remodelling factors, LT-biosynthetic enzymes and LT synthesis are steroid resistant. Thus, this cascade of remodelling factors might represent an alternative therapeutic target for the treatment of asthma, particularly in patients with steroid-resistant forms of airway inflammation.

Based on the potential of *Hpb* larval extract to reprogram the AA metabolism in human settings of type 2 inflammation, we further assessed the clinical relevance of these findings in macrophages from patients with AERD (aspirin exacerbated respiratory disease), a disorder characterized by dysregulation in eicosanoid pathways. AERD is characterized by three key features: bronchial asthma, chronic rhinosinusitis with nasal polyps, and hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (Samter & Beers, 1968). AERD patients show a typical eicosanoid imbalance with over-expression of pro-inflammatory mediators including 5-LOX and $LTC_4S$, leading to over-production of bronchoconstrictory cysLTs, but reduced synthesis and signalling of regulatory $PGE_2$ (Israel et al., 1993; Cowburn et al., 1998; Laidlaw et al., 2014). Similar to the effect observed in healthy macrophages, *HpbE* also modulated the AA metabolism of AERD macrophages by inducing the synthesis of COX metabolites ($PGE_2$ and $TXB_2$), whilst suppressing the release of LTs ($LTB_4$ and 5-HETE). Fluticasone propionate (FP, standard drug used for asthma treatment) failed to induce prostanoids and hardly suppressed the release of LTs, suggesting that *HpbE* uniquely modulates the AA

metabolism and induces an immunosuppressive eicosanoid profile in macrophages from AERD patients.

Based on the potential therapeutic applications of *HpbE*, we have started to work on the characterization of the active immunomodulatory compounds present in *HpbE*. Glutamate dehydrogenase (GDH) was identified as a protein able to modulate the AA metabolism and induced the release of the regulatory prostanoid $PGE_2$ and the anti-inflammatory cytokine IL-10. Another potential candidate is Ferritin, which failed to modulate the COX pathway but down-regulated the gene expression of 5-LOX, suggesting a role in the suppression of LTs synthesis. However, further work is required to recombinantly produce these proteins present in *HpbE* and to further elucidate the mechanism of action in setting of type 2 inflammation.

Taken together, we have shown that *Hpb* larval extract can reprogram the AA metabolism in human setting of type 2 inflammation. Treatment with *HpbE* or adoptive transfer of *HpbE*-stimulated macrophages prevented HDM-induced airway inflammation, implying the potential of *HpbE* in allergic asthma treatment as well as exploring the beneficial effect of *HpbE*-stimulated macrophages as a cell-based therapy. Furthermore, we identified that a cascade of airway remodelling factors and LT synthesis are age dependent and steroid resistant in CRSwNP patients. GDH and ferritin were identified as potential immunomodulatory compounds present in *HpbE*, representing a first step into the development of a novel therapy for type 2 inflammatory disease, particularly in patients with steroid-resistant forms of airway inflammation.

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# 8 ZUSAMMENFASSUNG

Asthma ist eine chronische Erkrankung, von der weltweit 5 bis 20 % der Bevölkerung mit zunehmender Prävalenz betroffen sind. Bei sensibilisierten Patienten führt die chronische Exposition gegenüber Allergenen wie Hausstaubmilben (HDM) zu irreversiblen Schäden in den Atemwegen, die zu chronischen Entzündungen und strukturellen Umbauprozessen in den Atemwegen (Remodeling) führen (Kaminska et al., 2009). Mehrere epidemiologische Studien haben jedoch eine inverse Korrelation zwischen Wurminfektionen und Allergien gezeigt, was darauf hindeutet, dass Würmer die Entwicklung einer allergischen Erkrankung unterdrücken (Nyan et al., 2001; Scrivener et al., 2001; Obeng et al., 2014; Figuedeiro et al., 2010).

Eicosanoide sind bioaktive Lipidmediatoren, die aus der Arachidonsäure (AA) gebildet werden und eine Schlüsselrolle in der Induktion der Typ-2-Immunantwort bei Allergie und Asthma spielen (Barret et al., 2011; Miyahara et al., 2008). Leukotriene (LTs) sind entscheidende Botenstoffe verschiedener charakteristischer Reaktionen der Typ-2-Entzündung, wie Bronchokonstriktion, Zellrekrutierung, Atemwegsentzündung und -umbau (Barret et al., 2011; Henderson et al., 2002). Würmer können jedoch auch eine Typ-2-Immunantwort regulieren, indem sie die Produktion von Typ-2 Zytokinen unterdrücken oder durch gezieltes Ansprechen angeborener Effektor Zellen wie Makrophagen oder Granulozyten (Navarro et al., 2016; Wilson et al., 2005; Klotz et al., 2011; Magalháes et al., 2010). Ob und wie Würmer Lipidmediatoren regulieren können, ist jedoch nicht bekannt.

In dieser Studie haben wir die Modulation von AA-Stoffwechselwegen während einer Wurminfektionen mit dem Fadenwurm *Heligmosomoides polygyrus bakeri* (*Hpb*) als angeborene regulatorische Strategie zur Modulation der Typ-2-Entzündung untersucht. Wir zeigten, dass *Hpb* in frühen Stadien der Infektion (Tag 7) die Produktion von regulatorischen Prostaglandinen ($PGE_2$ und 6-keto PGF1-α, ein Abbauprodukt von $PGI_2$) und Cyclooxygenase (COX)-Metaboliten (12-HHT und $TXB_2$) fördert, jedoch die Sekretion von entzündungsfördernden Mediatoren $PGD_2$ und LTs ($LTB_4$, cysLTs) unterdrückt. Entsprechend der hohen Produktion von Prostanoiden zeigten Immunfluoreszenzfärbungen von Darmschnitten eine hohe Expression von COX-2 (Biosyntheseenzym, das an der Synthese von Prostanoiden beteiligt ist) und seines positiven Regulators Hypoxie-induzierbaren Faktors-1 alpha (HIF-1α) in Zellen in der direkten Umgebung von *Hpb*Larven im Darmgewebe. Im Gegensatz dazu war die 5-Lipoxygenase (5-LOX, Biosyntheseenzym, das für die Synthese von Leukotrienen verantwortlich ist) während der Helmintheninfektion nicht vorhanden, was die Abwesenheit von Leukotrienen im Darmkulturüberstand oder in der Peritonealwaschflüssigkeit erklärt. Die *Hpb*-gesteuerte Regulierung des AA-Stoffwechsels könnte eine Strategie zur Immunevasion dieses Parasiten darstellen, die darauf abzielt, die vom Wirt ausgelösten Immunantworten des Typs-2 zu unterbinden und sowohl die Infiltration und Rekrutierung von Granulozyten als auch die Schleimproduktion zu begrenzen und auf diese Weise das Abtöten bzw. Ausscheiden der Larven zu verhindern. Die *Hpb* vermittelte Produktion von COX-Metaboliten, die an der Wundheilung und Blutgerinnung beteiligt sind, kann wichtig sein, um die durch die migrierenden Gewebslarven verursachte Gewebezerstörung schnell zu reparieren. Der genaue Beitrag von Eicosanoiden zur schützenden Immunantwort gegen Hpb muss jedoch noch in Folgestudien untersucht werden.

Wir konnten ebenso zeigen, dass ein Larvenextrakt aus *Heligmosomoides polygyrus bakeri* (*HpbE*) den AA Stoffwechsel in myeloiden Zellen wie Makrophagen und Granulozyten moduliert, indem die Synthese von 5-LOX in Richtung COX-Metaboliten verschoben wird. Die Behandlung von murinen und humanen Makrophagen mit *HpbE* induzierte die Synthese von

regulatorischen Prostaglandinen ($PGE_2$) und Prostaglandinen, die an der Wundheilung und Blutgerinnung beteiligt sind (12-HHT, $TXB_2$), wohingegen die Produktion von entzündungsfördernden Lipidmediatoren (LTs, $PGD_2$) unterdrückt wurde. *HpbE* induzierte auch eine Regulation der Transkription von AA-Biosyntheseenzymen in menschlichen Makrophagen: So kam es zu einer Induktion der Genexpression von Enzymen, die an der Synthese von $PGE_2$: *PGTS2* (für COX-2 kodierendes Gen) und *PTGES* (für mPGES-1 kodierendes Gen) beteiligt sind. Im Gegensatz dazu regulierte *HpbE* die Genexpression von *PTGDS* (für $PGD_2$-Synthase kodierendes Gen) und die Genexpression aller LT-Biosyntheseenzyme herunter: *ALOX-5* (für 5-LOX kodierend), *LTA4H* (für $LTA_4H$ kodierend) und *LTC4S* (für $LTC_4S$ kodierend), sowie der Hochaffinitätsrezeptor für cysLTs (Cysteinyl-Leukotrien-Rezeptor-1, *CYSLT1R*). Auch in murinen Makrophagen induzierte *HpbE* die Genexpression von *Ptgs2* und *Ptges*, unterdrückte jedoch die Expression von *Alox5* und *Ltc4*s. So programmierte *HpbE* den AA-Metabolismus von menschlichen und murinen Makrophagen um und induzierte ein potentiell entzündungshemmendes Eicosanoid-Profil.

Weiterhin induzierte *HpbE* in humanen und murinen Makrophagen die Synthese der Typ-2 hemmenden Mediatoren IL-10 und IL-1β und modulierte die Produktion von Zytokinen, die an der Regulierung von M2-Polarisierung und der Typ-2-Entzündung (IL-12, IL-28, IL-27 und TNF-α) in humanen Makrophagen beteiligt sind. In Bezug auf die Polarisation von humanen Makrophagen veränderte *HpbE* nicht die Genexpression des M2-Markers *TGM2* (Transglutaminase 2, TGM2), führte aber zu einer verminderten Expression von *ALOX-15* (15-Lipoxygenase, 15-LOX) und *MRC1* (Mannose-Rezeptor C-Typ 1, MR / CD206), was darauf hindeutet dass *HpbE* der M2-Polarisation in humanem MDM entgegenwirkt. Im Gegensatz zu *HpbE*-stimuliertem humanen MDM induzierte *HpbE* in murinen Makrophagen die Genexpression von *Tgm2* und *Arg1*, regulierte jedoch *Mrc1* herunter. Hingegen blieben andere M2-Marker *Tmed1/St2l* und *Rentla/Fizz1* unverändert. Deshalb konnte *HpbE* zwar ein teilweise unterschiedliches Polarisationsprofil in menschlichen und murinen Makrophagen hervorrufen, induzierte jedoch ein ähnliches regulatorisches Mediatorprofil. Da M2-Makrophagen mit Asthma und chronischen Atemwegserkrankungen assoziiert sind, könnte die Unterdrückung der M2 Polarisation durch *HpbE* eine Alternative für zukünftige therapeutische Anwendungen gegen chronische Atemwegserkrankungen bedeuten.

Ähnlich zu der *HpbE*-vermittelten Eicosanoid-Umprogrammierung in Makrophagen, modelliert *HpbE* auch den AA-Stoffwechsel humaner Granulozyten und zeigt eine Verschiebung von LOX- in Richtung COX-Metabolismus. In humanen Granulozyten löste *HpbE* die Induktion der COX-Metaboliten (12-HHT, $TXB_2$) aus und hemmte die Produktion von entzündungsfördernden LTs (cysLTs, $LTB_4$). Ähnlich wie *HpbE*-getriebene Veränderungen auf der Transkriptionsebene von AA-metabolisierenden Enzymen in Makrophagen, unterdrückte *HpbE* die Genexpression von Enzymen (*ALOX5, $LTA_4H$* und *PTGDS*), die an der Synthese von proinflammatorischer Mediatoren beteiligt waren, induzierte jedoch die Expression von $PGE_2$-synthetischen Enzymen (*PTGS2* und *PTGES*) in menschlichen Granulozyten. Außerdem kann *HpbE* direkt auf humane Granulozyten wirken und deren Chemotaxis effizienter hemmen als zur Asthmabehandlung verwendete Standardarzneimittel, indem es die Expression von LT synthetisierenden Enzymen ($LTA_4H$ und $LTC_4S$) verringert und die Expression von chemotaktischen Rezeptoren CCR3 (C-C-Chemokinrezeptor Typ 3) und $CRTH_2$ (Prostaglandin D2 Rezeptor 2) herunterreguliert. Darüber hinaus lässt unsere Studie auch darauf schließen, dass sich Makrophagen und Granulozyten gegenseitig beeinflussen, da *HpbE* behandelte Makrophagen die Chemotaxis von humanen Granulozyten mittels eines COX-abhängigen Mechanismus abschwächen.

Im Vergleich zu anderen Extrakten oder Ausscheidungs- bzw. Sekretionsprodukten (E/S) von Helminthen, haben wir gezeigt, dass *HpbE* in myeloiden Zellen eine einzigartige Eicosanoid-Signatur hervorruft. Im Gegensatz zu *HpbE* können E/S Produkte von *Hpb* (HES) in humanen Makrophagen keine IL-10 oder Prostaglandin- Produktion ($PGE_2$ und $TXB_2$) induzieren. Das lässt vermuten, dass verschiedene Stadien während des Lebenszyklus desselben Parasiten das Immunsystem des Wirtes unterschiedlich modulieren können. Im Vergleich zu *HpbE* hatten andere Parasiten-Extrakte aus *Schistosoma mansoni* (*SmE*) oder *Ascaris suum* (*AsE*) weniger Einfluss auf die Produktion von COX- und LOX-Metaboliten in humanen Makrophagen und Granulozyten. Im Hinblick auf die Modulation des 5-LOX Signalwegs zeigten Makrophagen, die mit HES oder *Hpb*-assoziierten Bakterien stimuliert wurden, verglichen mit *HpbE* eine ähnliche Reduzierung der cysLT-Produkion. Dies lässt vermuten, dass bakterielle Produkte bei der Unterdrückung der entzündungsfördernden LTs durch *HpbE* mitwirken könnten.

Weiterhin hat diese Studie gezeigt, dass *HpbE* keinen direkten Einfluss auf Typ-2-Zytokine (IL-4, IL-5 und IL-13) hat, obwohl es die Produktion von IFN-γ, IL-10 und die Reprogrammierung der Lipidmediatoren in humane mononukleären Zellen aus peripherem Blut (PBMCs) induziert. Dabei wird ebenfalls die Synthese von 5-LOX in Richtung COX-Metaboliten verschoben. *HpbE* induzierte in PBMCs auch insbesondere die Produktion von 12/-15-HETE-Metaboliten, ähnlich zu dem Eicosanoid-Profil, das während der *Hpb*-Infektion *in vivo* gemessen wurde.

Zusätzlich haben wir die Mechanismen identifiziert, die der *HpbE*-gesteuerten Eicosanoid-Umprogrammierung in Makrophagen zugrunde liegen. *Hpb* Produkte induzierten die Aktivierung von p38 MAPK, welche COX und die Transkriptionsfaktoren HIF-1α und NFκβ (Nuklear Faktor-κβ) aktiviert und die Produktion von Prostaglandinen ($PGE_2$ and $TXB_2$) sowie der Typ-2 unterdrückenden Zytokine IL-10 and IL-1β fördert. Der der Induktion des COX-Signalwegs zugrunde liegende Upstream-Mechanismus umfasste mehrere Pattern-Recognition-Rezeptoren (PPRs) (TLR2, Dectin-1/2), während sie nicht an der *HpbE*-gesteuerten Induktion von IL-10 beteiligt waren. Allerdings waren Kinasen wie PI3K (Phosphoinositid-3-Kinasen), PKA (Proteinkinase A) und PTEN (Phosphatase and Tensin homolog) nicht an der Herunterregulierung des 5-LOX-Synthesewegs durch *HpbE* beteiligt, sodass der zugrunde liegende Mechanismus noch weiter erforscht werden muss.

Das therapeutische Potential von *HpbE* oder *HpbE*-behandelten Makrophagen wurde weiterhin in einem Maus Model der HDM-induzierten allergischen Atemwegsentzündung *in vivo* gezeigt. Eine intranasale Behandlung mit *HpbE* vor HDM-Sensibilisierung und -Provokation führte zu einer Umprogrammierung des AA-Stoffwechsels und verhinderte die Allergie-induzierte Eosinophilie, Zellinfiltration, Atemwegsentzündung und Schleimproduktion. Die Modulation der Typ-2-Entzündung durch *HpbE* wurde vor allem durch COX-2-Metabolite vermittelt, die von *HpbE*-stimulierten Makrophagen freigesetzt wurden. Dies zeigte sich darin, dass der Transfer von *HpbE*-stimulierten Wildtyp- aber nicht COX-2-defizienten Makrophagen vor Provokation die Granulozyten Rekrutierung und Typ-2-Entzündung während der HDM-induzierten Allergie *in vivo* abschwächte.

Eine chronische Exposition gegenüber Allergenen wie HDM bei sensibilisierten Patienten führt zu irreversiblen Schäden in den Atemwegen, die zu chronischen Entzündungen und strukturellen Veränderungen (Remodeling) in den Atemwegen führen (Kaminska et al., 2009). Mittels einem Maus Model für die HDM-induzierte allergische Atemwegsentzündung in unterschiedlichen Altersstufen (Neugeborenes, Jungtier und ausgewachsenes) konnte dieses Forschungsprojekt zeigen, dass das Alter der Sensibilisierung eine Schlüsselrolle bei der Produktion von LTs, der Expression von LT-Synthese Enzymen sowie von Remodelingfaktoren spielt. Mäuse, die als

Neugeborene sensibilisiert wurden, zeigten hohe LT-Werte (cysLTs und $LTB_4$) mit einer starken Beteiligung von Eosinophilen bei der LT Produktion. Erwachsene sensibilisierte Mäuse zeigten eine hohe Produktion von LTs und eine hohe Expression von LT-Synthese Enzymen (5-LO, $LTC_4S$ und $LTA_4H$) im Atemwegsepithel, was vermuten lässt, dass Atemwegsepithelzellen signifikant an der LT Produktion beteiligt sind. Im Gegensatz dazu waren Jungtiere vor einer HDM Sensibilisierung stärker geschützt und zeigten in den Atemwegen niedrigere LT-Werte. Anzeichen von Atemwegs-Remodeling wie eine gesteigerte Verdickung der Bronchialwand (hohe Expression von α-smooth muscle actin, α-SMA) und eine Gewebeschädigung in den Atemwegen (Verlust von Tight-junction Protein Zonula Occludens-1, ZO-1) wurden nur in Lungengeweben von ausgewachsenen sensibilisierten Mäusen beobachtet. Hier haben wir auch festgestellt, dass der Mechanismus hinter der LT-Produktion und dem Atemwegs-Remodeling im Epithel von ausgewachsenen sensibilisierten Mäusen die Aktivierung von Remodeling-Faktoren wie $sPLA_2X$ (sekretorische Phospholipasen A2), TGM2 und Wnt5a beinhaltet.

Des Weiteren zeigte unsere Studie, dass eine Wechselwirkung zwischen entzündetem Atemwegsepithel und Makrophagen die Synthese von LTs fördern kann. Der vorgeschlagene Mechanismus startet mit der Sekretion von Wnt5a durch das entzündete Atemwegsepithel, welches die Expression von TGM2 in Makrophagen aktiviert und die Produktion von entzündungsfördernden LTs induziert, wodurch die Rolle der Makrophagen in entzündeten Atemwegen bei Erwachsenen weiter unterstützt wird. Die Relevanz der entdeckten Kaskade wurde auch in Atemwegsgewebe von Patienten mit chronischer Rhinosinusitis und Nasenpolypen (CRSwNP) validiert. Große Mengen von LT Enzymen (5-LO, $LTC_4S$ $LTA_4H$), $sPLA_2$-X, TGM2 und Wnt5a wurden in humanen Nasenpolypen Geweben beobachtet, und hohe Konzentrationen von CysLTs wurden in Nasenpolyp Sekreten dieser Patienten gemessen. Dies lässt vermuten, dass die Expression von Atemwegs Remodeling-Faktoren, LT-Synthese Enzymen und die LT Synthese steroidresistent sind. Daher könnte diese entzündliche Kaskade ein alternatives therapeutisches Ziel für die Behandlung von Asthma und Nasenpolypen darstellen, speziell bei Patienten mit steroidresistenten Formen von Atemwegsentzündung.

Unter Berücksichtigung des Potenzials von *Hpb*-Larvenextrakt zur Umprogrammierung des AA-Metabolismus untersuchten wir ferner die klinische Relevanz dieser Ergebnisse bei Makrophagen von Patienten mit AERD (aspirin exacerbated respiratory disease), einer Störung, die durch eine Dysregulation der Eicosanoid-Regulation und einer Überproduktion von CysLTs gekennzeichnet ist. AERD ist durch drei Hauptmerkmale gekennzeichnet: Asthma bronchiale, chronische Rhinosinusitis mit Nasenpolypen und Überempfindlichkeit gegen Aspirin und andere nicht steroidale entzündungshemmende Arzneimittel (NSAIDs) (Samter & Beers, 1968). AERD-Patienten zeigen ein typisches Eicosanoid-Ungleichgewicht mit Überexpression von proinflammatorischen Mediatoren, einschließlich 5-LOX und $LTC_4S$, was zu einer Überproduktion von bronchokonstriktiven CysLTs führt, aber die Synthese und Signalgebung von regulatorischem $PGE_2$ reduziert (Israel et al., 1993; Cowburn et al., 1998; Laidlaw et al., 2014). Wir könnten zeigen, dass ähnlich wie bei gesunden Makrophagen modulierte *HpbE* auch den AA-Metabolismus, indem es die Synthese von COX-Metaboliten ($PGE_2$ und $TXB_2$) induzierte und gleichzeitig die Freisetzung von proinflammatorischen 5-LOX-Mediatoren ($LTB_4$ und 5-HETE) unterdrückte.

Beim Vergleich des Eicosanoidprofils von Makrophagen aus AERD Patienten, die mit *HpbE* behandelt wurden, induzierte Fluticasonpropionat (FP, Standardmedikament zur Behandlung von Asthma) keine Prostaglandine und unterdrückte die Freisetzung von LT kaum, was vermuten lässt, dass *HpbE* den AA-Metabolismus auf einzigartige Weise moduliert und ein

immunsuppressives Eicosanoid-Profil in Makrophagen von AERD-Patienten induziert. Die Behandlung mit FP unterdrückte die Chemotaxis von AERD-Granulozyten gegenüber nasalen Polypensekreten nicht, während *HpbE* die Granulozytenrekrutierung wirksam unterdrückte, möglicherweise durch Herunterregulierung der chemotaktischen Rezeptoren CCR3 und $CRTH_2$. Die Verringerung der Anzahl von Eosinophilen durch die Blockade der Eotaxin-CCR3-Achse könnte potenzielle therapeutische Anwendungen bei Asthma haben. Zusammengenommen könnte *HpbE* eine therapeutische Alternative bei steroidresistenten AERD-Patienten darstellen, bei denen trotz systemischer Steroidbehandlung eine hohe Expression von LT-Biosyntheseenzymen und hohe LT-Spiegel festgestellt werden.

Basierend auf den möglichen therapeutischen Anwendungen von *HpbE* haben wir begonnen, an der Charakterisierung der im *HpbE* vorhandenen immunmodulatorischen Wirkstoffe zu arbeiten. Glutamatdehydrogenase (GDH) wurde als ein Protein identifiziert, das den AA-Metabolismus moduliert und die Freisetzung des regulatorischen Prostaglandins $PGE_2$ und des entzündungshemmenden Zytokins IL-10 induzieren kann. Beim Vergleich der proteomischen Analyse von Ausscheidungs- / Sekretionsprodukten (HES) adulter Stadien von Hpb-Larven (Hewitson et al., 2011) wurde GDH nicht in HES gefunden und ist somit vermutlich nur in *HpbE* vorhanden, was auf ein je nach Stadium unterschiedliches Proteinprofil hindeutet. Unterschiede in der Proteinzusammensetzung könnten die unterschiedliche Umprogrammierung des AA-Metabolismus erklären, die für *HpbE*, GDH oder HES beobachtet wurde, da nur HES die Freisetzung von Prostanoiden in Makrophagen nicht induzieren konnte. Ein weiterer möglicher Kandidat ist Ferritin, das den COX-Weg zwar nicht modulieren konnte, aber die Genexpression der 5-LOX herunterregulierte, was auf eine Rolle bei der Unterdrückung der LT-Synthese schließen lässt. Es sind jedoch weitere Forschungsarbeiten erforderlich, um diese in *HpbE* vorhandenen Proteine rekombinant herzustellen und den Wirkungsmechanismus in der Typ-2-Entzündung weiter aufzuklären.

Zusammengefasst haben wir gezeigt, dass *Hpb*-Larvenextrakt den AA-Metabolismus von menschlichen Immunzellen im Rahmen von Typ-2-Entzündungen umprogrammieren kann. Die Behandlung mit *HpbE* oder der adoptive Transfer von *HpbE*-stimulierten Makrophagen verhinderte eine HDM-induzierte Atemwegsentzündung, was auf ein potentielles therapeutisches Potenzial von *HpbE* bei der Behandlung von allergischem Asthma und anderen Typ-2 Entzündungen schließen lässt. Darüber hinaus konnten wir zeigen, dass eine Kaskade von Faktoren, die am Atemwegsremodelling beteiligt sind, sowie die Synthese von LTs altersabhängig und steroidresistent bei CRSwNP-Patienten sind. GDH und Ferritin wurden als potenzielle immunmodulatorische Komponenten in *HpbE* identifiziert. Dies ist ein erster Schritt in der Entwicklung einer neuartigen Therapie zur Behandlung der Typ-2-Entzündung, insbesondere bei Patienten mit steroidresistenten Formen der Atemwegsentzündung.

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# 10 REGISTERS

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## 11 CURRICULUM VITAE

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# 12 LIST OF PUBLICATIONS

PROJECT-RELATED PUBLICATIONS

- **de los Reyes Jiménez M***, Friedl A*, Alessandrini F, Bohnacker S, Schindela S, Trompette A, Haimerl P, Thomas D, Henkel FDR, Mourao A, Geerlof A, Prazeres da Costa C, Chaker A, Brüne B, Nüsing R, Korotkova M, Nockher WA, Feige M, Haslbeck M, Ohnmacht C, Marsland BJ, Voehringer D, Harris NL, Schmidt-Weber CB, Esser-von Bieren J (2020). An anti-inflammatory eicosanoid switch mediates the suppression of type 2 inflammation by helminth larval products. *Science Translational Medicine*. doi: 10.1126/scitranslmed.aay0605

- Henkel FDR*, Friedl A*, Haid M, Thomas D, Bouchery T, Haimerl P, **de los Reyes Jiménez M**, Alessandrini F, Schmidt-Weber CB, Harris NL, Adamski J, Esser-von Bieren J (2018). House dust mite drives proinflammatory eicosanoid reprogramming and macrophage effector functions. *Allergy*. doi: 10.1111/all.13700

- Dietz K, **de Los Reyes Jiménez M**, Gollwitzer ES, Chaker AM, Zissler UM, Rådmark OP, Baarsma HA, Königshoff M, Schmidt-Weber CB, Marsland BJ, Esser-von Bieren J (2017) Age dictates a steroid-resistant cascade of Wnt5a, transglutaminase 2, and leukotrienes in inflamed airways. *J Allergy Clin Immunol*.;139(4):1343-1354.e6. doi: 10.1016/j.jaci.2016.07.014

OTHER PUBLICATIONS

- Martin AH, **de los Reyes Jiménez M**, Pouvreau L (2016) Modulating the aggregation behaviour to restore the mechanical response of acid induced mixed gels of sodium caseinate and soy proteins. *Food Hydrocolloids*; volume 58: 215-223; doi.org/10.1016/j.foodhyd.2016.02.029

- de Jongh HH, **de los Reyes Jiménez M**, Baumert JL, Taylor SL, Koppelman SJ (2015) Electrophoretic Behavior in Relation to the Structural Integrity of Codfish Parvalbumin upon Heat Treatment. *J Agric Food Chem.*; 63(18):4683-9. doi: 10.1021/jf505990h

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