Fakultät für Medizin

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Eicosanoid profiles and programs of macrophages in allergic airway inflammation – studies on trained innate immunity and immunomodulation

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Our comforting conviction that the world makes sense rests on a secure foundation: our almost unlimited ability to ignore our ignorance.
Daniel Kahnemann

Summary

Allergic asthma is a chronic inflammatory disease leading to significant health burden. Innate immunity contributes to allergic inflammation and macrophages orchestrate and direct innate immune responses via their plastic production of cytokines, chemokines and eicosanoid lipid mediators. The aim of this dissertation was to characterize eicosanoid profiles in situations of type 2 inflammation, but also to examine persistent alterations of macrophages after allergen exposure, and to determine the immunomodulatory effects of a helminth extract on macrophages in allergic airway inflammation.

We show that house dust mite (HDM) shifts the macrophage eicosanoid profile from 5-lipoxygenase- to cyclooxygenase-derived mediators while strongly inducing proinflammatory and neutrophil-chemoattracting chemokines and cytokines via p38 MAP kinase. Despite the high amounts of chemokines, HDM-exposed macrophages suppressed neutrophil chemotaxis, probably due to the lack of 5-lipoxygenase-derived eicosanoids like LTB_4. During type 2 immune response to house dust mite or nematode parasites in the airways *in vivo*, abundant cysteinyl leukotrienes and 12-/15-lipoxygenase metabolites were detected.

Macrophages derived from the lungs and bone marrow of HDM-sensitized mice produced great amounts of the type 2 mediators cysteinyl leukotrienes (cysLT) and CCL17. This was reflected in monocyte-derived macrophages from HDM-allergic patients, and could be mimicked by repeated HDM exposure *in vitro*, leading to a trained immunity program of abundant CCL17 and cysLT. The trained CCL17 response depended on FPR2 and autocrine TNF signaling and was mediated by 2-hydroxyglutarate and HIF1α, while TLR4 and KDM6B were necessary for the trained cysLT response. Macrophages from HDM-allergic patients exhibited baseline activation and elevated levels of IL17RB, a receptor for the epithelial alarmin IL-25, enabling a proinflammatory crosstalk between trained macrophages and airway epithelial cells.

A helminth extract of *Heligmosomoides polygyrus bakeri* alleviated allergic airway inflammation via an anti-inflammatory switch resulting in high prostaglandin E2 and IL-10 production in macrophages and concomitant suppression of 5-lipoxygenase-derived metabolites like cysLTs. This was dependent on cyclooxygenase, p38 and HIF1α in macrophages, while treatment of granulocytes impaired their chemotaxis due to suppressed chemotactic receptor expression of CCR3 and CRTH2. The active component was identified as a glutamate dehydrogenase, which similarly induced PGE_2 and IL-10 in macrophages and reduced allergic airway inflammation *in vivo*.

Thus, macrophages and their eicosanoid production are implicated in pathomechanisms of allergic asthma, but also a promising target for immunomodulation.

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1 List of publications

1.1. Publications described in this dissertation

This dissertation is based on the original publications listed below, which are referred to in the text by their Roman numbering.

I Fiona D. R. Henkel*, Antonie Friedl*, Mark Haid, Dominique Thomas, Tiffany Bouchery, Pascal Haimerl, Marta de los Reyes Jiménez, Francesca Alessandrini, Carsten B. Schmidt-Weber, Nicola L. Harris, Jerzy Adamski, Julia Esser-von Bieren: House dust mite drives proinflammatory eicosanoid reprogramming and macrophage effector functions. Allergy. 2019 Jun;74(6):1090-1101. https://doi.org/10.1111/all.13700

II Antonie Lechner, Fiona D. R. Henkel, Sina Bohnacker, Carlo Angioni, Yannick Schreiber, Pascal Haimerl, Yan Ge, Dominique Thomas, Agnieszka M. Kabat, Edward J. Pearce, Peter J. Murray, Caspar Ohnmacht, Adam M. Chaker, Carsten B. Schmidt-Weber, Julia Esser-von Bieren: A trained type 2 immunity program drives exaggerated leukotriene and CCL17 responses in allergen-experienced macrophages. *In Submission.*

III Marta De los Reyes Jiménez*, Antonie Lechner*, Francesca Alessandrini, Sina Bohnacker, Sonja Schindela, Aurélien Trompette, Pascal Haimerl, Dominique Thomas, Fiona D.R. Henkel, André Murão, Arie Geerlof, Clarissa Prazeres da Costa, Adam M. Chaker, Bernhard Brüne, Rolf Nüsing, Wolfgang A. Nockher, Matthias J. Feige, Martin Haslbeck, Caspar Ohnmacht, Benjamin J. Marsland, Nicola L. Harris, Carsten B. Schmidt-Weber, Julia Esser-von Bieren: An anti-inflammatory eicosanoid switch mediates the suppression of type-2 inflammation by helminth larval products. Science Translational Medicine. 2020. https://doi.org/10.1126/scitranslmed.aay0605

* equal contribution

1.2. Other publications

During my doctoral work, I contributed to the following publications, which are not discussed in this dissertation but are listed below.

Stephanie I. Mueller, Antonie Friedl, Isabel Aschenbrenner, Julia Esser-von Bieren, Martin Zacharias, Odile Devergne, Matthias J. Feige: A folding switch regulates interleukin 27 biogenesis and secretion of its α-subunit as a cytokine. Proceedings of the National Academy of Science. 2019 XX:X(116), 1585–1590. https://doi.org/10.1073/pnas.1816698116

Pascal Haimerl, Ulrike Bernhardt, Sonja Schindela, Fiona D.R. Henkel, Antonie Lechner, Ulrich M. Zissler, Xavier Pastor, Dominique Thomas, Alexander Cecil, Yan Ge, Mark Haid, Cornelia Prehn, Janina Tokarz, Matthias Heinig, Jerzy Adamski, Carsten B. Schmidt-Weber, Adam M. Chaker, Julia Esser-von Bieren: Inflammatory macrophage memory in NSAID-exacerbated respiratory disease. Journal of Allergy and Clinical Immunology. 2020. https://doi.org/10.1016/j.jaci.2020.04.064

2 List of abbreviations

AAI	Allergic airway inflammation
AIT	Allergen immunotherapy
α-KG	α-ketoglutarate
AlvM	Alveolar macrophage
AM	Airway macrophage
APC	Antigen-presenting cell
ASA	Acetylic salicylic acid
BALF	Bronchoalveolar lavage fluid
BCR	B cell receptor
BMDM	Bone marrow-derived macrophage
CCL	CC-motif ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CRSwNP	Chronic rhinosinusitis with nasal polyposis
COX	Cyclooxygenase
CXCL	CXC-motif ligand
cysLT	Cysteinyl leukotriene
DC	Dendritic cell
DAMP	Danger-associated molecular pattern
FACS	Fluorescence-activated cell sorting
FEV_1	Forced expiratory volume in 1 second
FPR2	Formyl peptide rector 2
GDH	Glutamate dehyrogenase
GM-CSF	Granulocyte monocyte colony stimulating factor
GPCR	G protein coupled receptor
HDM	House dust mite
HETE	Hydroxyeicosatetraenoic acid
2-HG	2-hyroxyglutarate
12-HHT	12-hydroheptadecatrienoic acid
HIF	Hypoxia-inducible factor
Hpb	*Heligmosomoides polygyrus bakeri*
HSCP	Hematopoietic stem cell and progenitor

ICS	Inhalative corticosteroid
IgE	Immunoglobulin E
ILC	Innate lymphoid cell
IL	Interleukin
IM	Interstitial macrophage
IS	Induced sputum
LABA	Long-acting β-agonist
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTRA	Leukotriene receptor antagonist
MDM (aMDM)	Monocyte-derived macrophage (alveolar-like)
MHC	Major histocompatibility complex
N-ERD	NSAID-exacerbated respiratory disease
NSAID	Non-steroidal anti-inflammatory drug
OCS	Oral corticosteroid
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PG	Prostaglandin
PMN	Polymorphnuclear cell
PRR	Pattern recognition receptor
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SABA	Short-acting β-agonist
TCR	T cell receptor
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TX	Thromboxane

3 Introduction

3.1. Allergy and Asthma

Allergy is the misguided type 2 immune response towards innocuous antigens that are recognized as dangerous by the immune system, leading to mucosal reactions like rhinorrhea and conjunctivitis, exanthema and in severe cases, anaphylaxis. Depending on whether the allergen is present continuously during the year or in specific seasons, allergic symptoms occur perennially or seasonally. A typical seasonal allergen is pollen which arises in spring and vanishes throughout the summer and autumn. Sensitization to house dust mite-derived allergen is most frequently the reason for perennial allergy[1]. Allergic symptoms in HDM-allergic patients occur mostly in mucosal barrier membranes such as nasal and pharyngeal mucosa and conjunctiva, resulting in rhinitis, shortness of breath and conjunctivitis.

In addition to symptoms triggered by the allergen directly, allergic patients often suffer from sleep-disordered breathing or obstructive sleep apnea[2]. Also their risk for anxiety is increased[3] and anxiety or depression is associated with poor asthma control[4]. A substantial health burden is related to allergic diseases independently of the trigger of the allergy. Aeroallergens such as pollen, house dust mites or fungal spores[1,5–7] are common causes of sinonasal allergic symptoms while peanuts, cow's milk, eggs and fruits of rosaceae such as apples and strawberries are common triggers of food allergy[8–10] resulting in dermal, oral and gastrointestinal symptoms. Allergic sensitization is a risk factor for asthma[11] and prevention strategies include reduction of exposure to air pollution and improvement of childhood health (maternal heath, vaccinations, nutrition)[12]. Asthma, characterized by bronchial hyperreactivity, cough and airway remodeling, is a chronic inflammatory disease and appears in a multitude of endotypes. Patients experience dyspnea, bronchial hyperreactivity and mucus production, resulting in diminished airway function such as wheezing, cough, chest tightness and reduced physical resilience[13]. Pulmonary remodeling, characterized by airway smooth muscle thickening, excess extracellular matrix deposition, neovascularization, goblet cell metaplasia and epithelial to mesenchymal de-differentiation[14], can already be detectable in preschool-aged asthmatic children[15,16].

Asthma endotypes are defined by their immunopathology (Figure 1). Allergic eosinophilic asthma is characterized by pulmonary eosinophilia, mucus overproduction and remodeling, mainly epithelial damage, thickening of basement membrane and increased airway smooth muscle[13]. Phenotypically, it can hardly be distinguished from non-allergic asthma, only the triggers differ (pollutants, microbes, cold air instead of allergens)[13]. On the other hand, there are non-eosinophilic asthma endotypes, either characterized by lack of immune cell influx

(paucigranulocytic) or type 1 and type 17-biased airway inflammation with neutrophilia, as well as a mixed eosinophilic and neutrophilic phenotype[13].

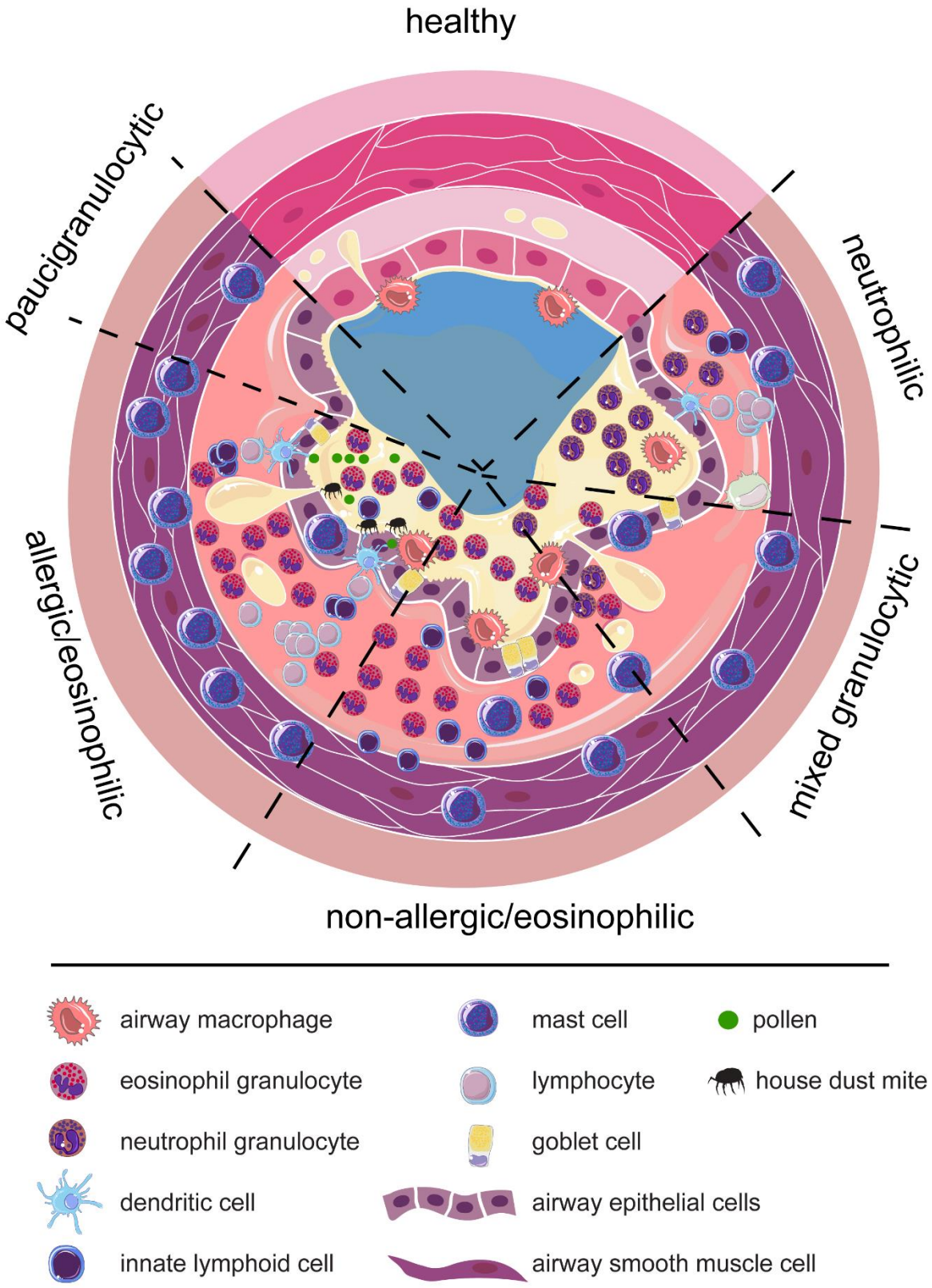

Figure 1 Asthma endotypes defined by the immunopathology. Modified after Papi et al., Lancet 2017

3.1.1. Epidemiology of allergic asthma

Globally, >270 million people suffered from asthma in 2017[17] and >50% of asthma cases are allocated to allergic asthma[18]. In Europe, 150 million people suffer from allergic disease, including 100 million patients with allergic rhinitis and 70 million with asthma[19]. While the overall asthma epidemic seems to have reached a plateau[20], the prevalence of allergic asthma is still increasing[21] and the allergy "tsunami" is still rolling in[22].

House dust mite (HDM) is a frequent sensitizing agent globally[23–26] and HDM sensitization is more prevalent than sensitization to any other allergen according to the European Community Respiratory Health Survey[27]. Estimates suggest that 1 – 2 % of the global population are sensitized to HDM but differences in geographic location, demographic factors and diagnostics make it difficult to provide more exact numbers[1,23,28]. Reported HDM sensitization rates differ considerably, ranging from 21% as a mean of European countries[27], 37% among Latina women in urban USA[29], average >30% in a Korean cohort[30] to ~40% in children and adolescents in Brazil[31]. Early life HDM sensitization is a risk factor for persisting wheeze or allergic asthma[32–34] and HDM sensitization and exposure negatively correlates with lung function in asthmatic children[35]. Thus, HDM sensitization is a frequent and continuous health problem with substantial need for new prevention and treatment strategies.

3.1.2. Asthma etiology

Asthma is a multifactorial disease. Genetic predisposition for asthma has been described in genome-wide association studies (GWAS) for several loci. Obvious genes of interest include type 2 cytokines and alarmins (*IL4*, *IL13*, *IL33* and *TSLP*) as well as HLA-DQB[36,37] but also loci related to immune cell signaling have been reported (*TNF*, *IL18R*, *IL1RL1*, *GATA3*, *STAT6*[36,38]). Some risk loci are common between asthma and atopic diseases[39] but as possibly different genetic variants underlie the multitude of endotypes, and strict clinical stratification is not always performed thoroughly in GWAS, it is difficult to define genetic causes of susceptibility. A large part of asthma etiology is therefore left to "nurture" or environmental factors which is reflected by the high proportion of allergic asthma[18]. Another factor influencing the risk for development of asthma is the exposure to microbes. On one hand, the decreased asthma prevalence in children raised in rural backgrounds due to prenatal, early and continuous contact with diverse microbial agents[40–43] established the "hygiene hypothesis" of inverse correlation of microbial diversity and allergy development in the industrialized world. On the other hand, number and severity of virus infections correlates with childhood wheeze and increases the risk of asthma development[44–46]. Not only exogenous but also commensal bacteria

play a role in asthma development, as delivery of children via Cesarean section results in different microbial colonization[47,48], and may influence asthma risk. Frequent administration of antibiotics to mothers or young children[49,50] is associated with increased allergy risk, possibly due to perturbances of tolerance-inducing microbiota directly affecting the immune system via e.g. short-chain fatty acids (SCFA)[51,52]. Antibiotics can have lasting impact on commensal microbiota[53] but nutrition also influences the composition of the microbiome[54–56]. Besides altered microbial exposure in industrialized countries, intake of strongly processed foods as a part of "Western(ized)" lifestyle perturbates the intestinal microbiome which in turn contributes to the rise of allergic disease[57,58] In addition, exposure to mold in the home highly correlates with asthma and asthma exacerbations as well as allergic disease in children[59,60]. Sensitization to fungal antigens is also linked to severity of asthma[61]. However, not only indoor air quality plays a role but also exposure to outdoor pollution prenatally and in childhood is associated to development of asthma[62,63].

Several allergens exhibit protease activity (e.g. *Der p1*) and cause disruption of the epithelial barrier, which leads to facilitated entry of allergens, immune cell activation as well as alarmin release, which may promote type 2 biasing in the immune response[64,65]. In addition, GWAS revealed filaggrin, an essential structural protein of the epidermis, as a risk factor for atopic disease[66]. Although filaggrin mutations are most strongly associated with atopic dermatitis, they also constitute a risk factor for food allergy[67,68]. Atopic dermatitis can be seen as a starting point of the "atopic march" progressing to allergic rhinitis and finally allergic asthma, and therefore filaggrin might play a role in allergic asthma[69]. Thus, epithelial dysfunction has been suggested as a central pillar of allergy etiology. Besides genetic mutations, epigenetic modifications have been proposed in the development of allergy and asthma as they can transduce environmental exposures into a lasting phenotype, e.g. the protective anti-allergic effects of a rural upbringing[70,71]. Also, epigenome-wide investigation have revealed lower methylation at specific sites in symptomatic asthmatic children which had not been present in their cord blood[72], suggesting they were acquired in response to a stimulus and contribute to disease. Differentially methylated sites were associated to e.g. eosinophil and CD8 T cell activation and immune signaling[72–74].

Altogether, as multiple factors determine the disease outcome in asthma, the profound understanding of asthma etiology is further complicated, and therefore, its prevention.

3.1.3. Pharmacotherapy of asthma and allergy

The Global Initiative of Asthma (GINA) considers asthma as mild if controlled with step 1 or 2 medication (Figure 2) while moderate asthma needs step 3 treatment to be controlled[75]. Asthma is classified as severe if treatment step 4 or 5 are necessary for control or it remains uncontrolled[75]. A considerable number of patients remains uncontrolled due to lack of adherence to treatment[76,77], inadequate therapy of comorbidities, redundancy of pathomechanism[78] or additional risk factors (e.g. obesity, smoking)[75].

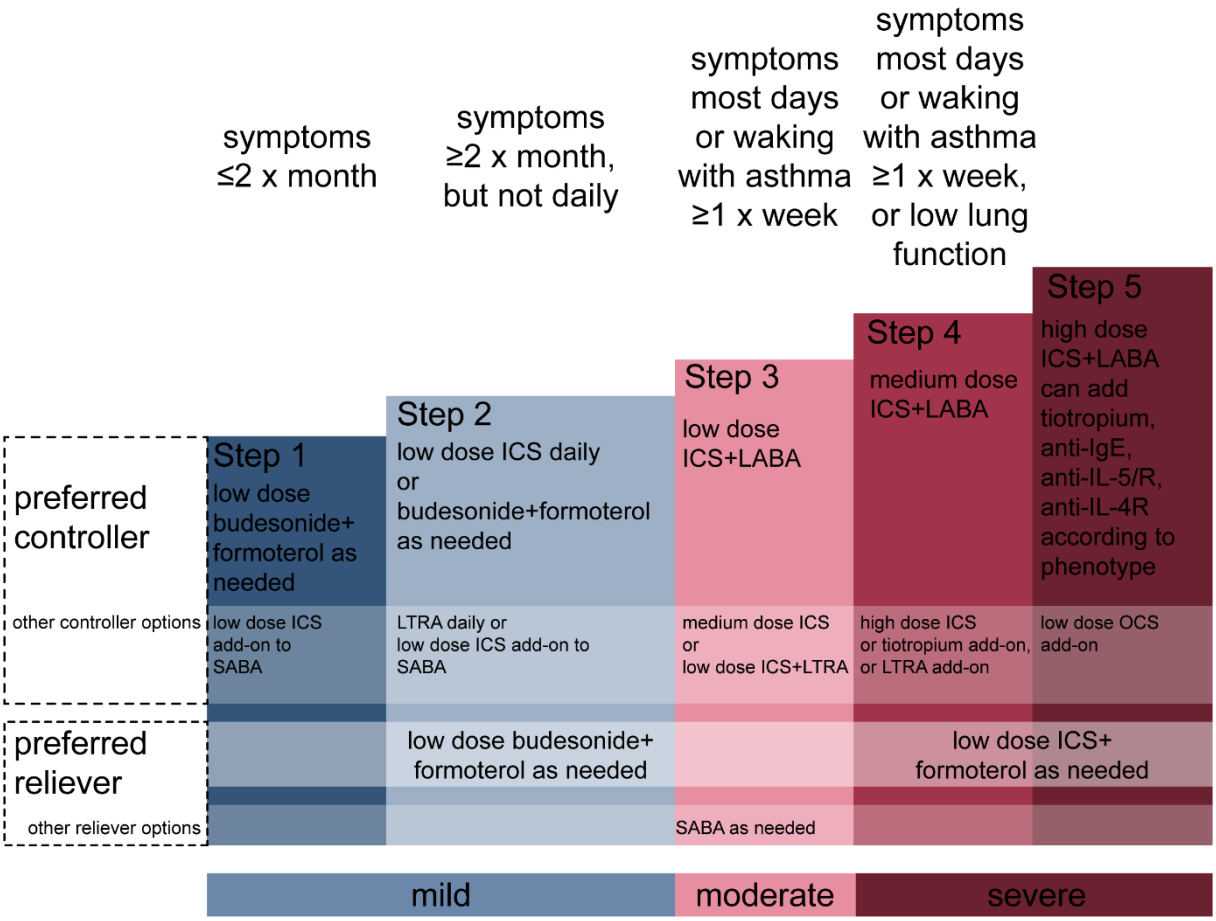

Figure 2 GINA guidelines for treatment of asthma in adults and adolescents. Modified after Global Strategy for Asthma management and Prevention, Update 2020

Asthma pharmacotherapy according to the updated Global Initiative of Asthma (GINA)[75] guidelines begins with inhalative controllers, low dose +LABA, as the early addition of ICS has proven to be superior in the long term[79,80] to initital short-acting β-sympathomimetics (SABA) like salbutamol or fenoterol only for the case of exacerbation. Inhalative corticosteroids (ICS) like budesonide or fluticasone propionate are engineered to act locally in the lung and to get cleared quickly by first-pass if absorbed into the circulation. If the patient reports 3 or more occurrences of 4 scenarios (daytime symptoms or necessity of SABA application more than twice a week, any nightly waking or activity limitation due to asthma) during the last month, symptoms are considered uncontrolled[75]. Severe asthma can even require

systemic corticosteroid therapy to be controlled (Figure 2 step 5). In addition, due to the availability of new "biologics", selected patients with the respective endotype profit from anti-IgE antibody (omalizumab), anti-IL-5 (mepolizumab, reslizumab, benralizumab) or anti-IL-4R (dupilumab) therapy.

Targeting IL-4R signaling is promising not only in allergic asthma, but also in patients suffering from other types of type 2 inflammatory disease, including nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD), previously known as Aspirin-exacerbated respiratory disease (AERD)[81]. This late-onset non-allergic eosinophilic endotype suffers from the trias of asthma, polyposis nasi and intolerance of COX-1 inhibitors (NSAIDs such as ibuprofen, diclofenac or acetylsalicylic acid (ASA)). Age at asthma onset is ~35 years[82,83], asthma is frequently severe[82,83] females are affected twice as frequently as men[84] and nasal polyps are a recurrent problem[85] in N-ERD patients.

81% of N-ERD patients use ICS and 51% require oral corticosteroid treatment[83]. Given the participation of leukotrienes in N-ERD, patients profit from LTRA[82,86]. ASA desensitization has been proposed 40 years ago[87]. A step-wise updosing of ASA to a maintenance dose of 100-1300 mg daily, which is then continued as a daily ASA application[88], can alleviate pulmonary symptoms as indicated by improved FEV_1 and reduced steroid requirement while effects on nasal polyposis are inconsistent[89]. Also, desensitization protocols vary widely and it is unclear which patients will benefit from ASA desensitization[89]. N-ERD patients often require functional endoscopic sinus surgery (FESS) to alleviate sinonasal symptoms such as nasal obstruction and anosmia, but revision FESS is frequently necessary after 2-5 years[90]. In contrast, 3 months of omalizumab treatment can reduce urinary LTE_4 levels after ASA challenge as well as eosinophilic airway inflammation and can lead to ASA tolerance[91]. A retrospective analysis with 22 N-ERD patients which participated in a RCT for mepolizumab in severe asthma, reports improvement of eosinophilia, asthma and sinonasal scores while FEV_1 remained unchanged after 3 months of treatment[92]. As there is significant cross-reaction to NSAIDs depending on the drug's capacity to inhibit COX-1, patients are strongly advised to use only COX-2 selective inhibitors for pain and inflammation (e.g. etoricoxib)[88].

Patients suffering from allergic asthma are advised to practice allergen avoidance, e.g. using very dense, anti-allergic bed casings to minimize exposure to dust mites living in mattresses or using HEPA-filters in vacuum cleaners to reduce the re-emission of fine dust containing allergens[93]. In addition, symptoms can be suppressed by locally administered antihistamines (e.g. azelastine) or glucocorticoids (e.g. betamethasone) as well as peroral antihistamines (e.g. fexofenadine). Although these strategies are effective and safe, the only curative treatment to

date is allergen immunotherapy (AIT). For HDM, subcutaneous as well as sublingual application forms of AIT exist[94]. Sublingual AIT (SLIT) can be performed by educated patients themselves, after a period of assessing safety and risk of anaphylaxis by physicians, but needs strong motivation as sublingual tablets must be administered daily with the possible occurrence of side effects such as oral pruritus[95]. Subcutaneous AIT (SCIT) is mostly administered in the clinic which has the advantage that each dose is applied reliably, and the patients can be monitored for signs of anaphylaxis. However, SCIT requires considerable effort and investment of time as most manufacturers suggest AIT over a course of 3 to 5 years in order to be successful[96]. Hyposensitized allergy patients experience less nasal and ocular systems, need less medication and have an improved quality of life, also after AIT is finished[96]. The mechanism of action of AIT is unclear but seems to include the induction of IgG to compete with IgE, the activation of Treg cells and the attenuation of local mediators of allergy via e.g. IL-10 and TGF-β[96,97].

Allergic asthma and allergic rhinitis are clearly connected as AIT represents the most sustainable treatment option in many patients which experience both[98,99] and airway hyperresponsiveness in allergic patients as well as HDM sensitization in general increase the risk of asthma development[100–102]. Therefore, the concept of “united airways” highlights the interconnection of upper and lower airways linked via epithelium but also blood stream and bone marrow[103].

Taken together, there is still a lot of room to ameliorate the lives of HDM-allergic or asthmatic patients.

3.2. Helminth Infection

Helminths derive their name from Greek ελμινσ, meaning “parasitic worm”. The multicellular macroparasites contain clinically relevant groups like trematodes (flukes, e.g. *Schistosoma mansoni*), cestodes (tapeworms, e.g. *Taenia solium*) and nematodes (roundworms, e.g. *Enterobius vermicularis*)[104] which cause human disease. Nematodes present as a very diverse phylum of 5 clades[105] and can be found free-living in saltwater, freshwater and on, in and under the surface of the earth[106,107], as well as parasitizing plants, invertebrates and vertebrates[106].

3.2.1. Epidemiology

Soil-transmitted helminth infections affect more than 1.5 billion humans world-wide[108,109] causing considerable health burden and cost[109]. This concerns about a quarter of the global population, primarily located in sub-Saharan Africa, North and South America, China and East Asia[109]. Similarly, in South Asia, soil-transmitted helminth infection is present in approximately 25% of the population[110] and prevalence of helminth infection varies between 5-17% in Sub-Saharan African countries[111,112]. Helminth infections are relevant also in Europe[113,114] with a focus on eastern europe[115]. Although helminth adaptation to the host may lead to low symptom burden, serious health problems such as hypovitaminosis, anemia, malnutrition and stunted development in children[116] warrant strategies for deworming, treatment and prevention.

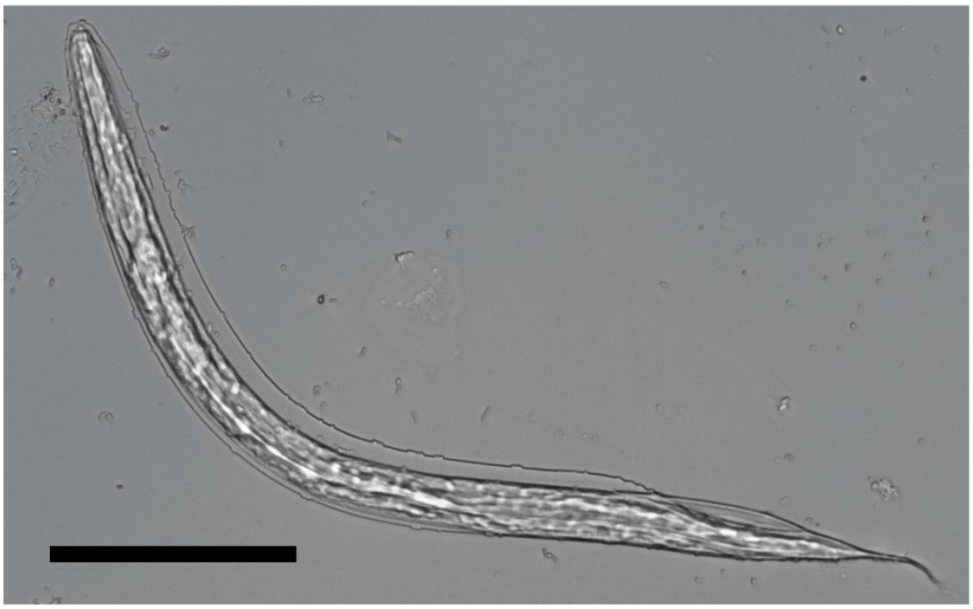

Figure 3 Infective L3 stage larva of Heligmosomoides polygyrus bakeri. Scale bar indicates 50 µm.

3.2.1. *Heligmosomoides polygyrus* life cycle

Heligmosomoides polygyrus bakeri (*Hpb*) is an enteric murine parasite ubiquitous in wild mice[117] and frequently used as mouse model. *Hpb* leads to a chronic infection, which can continue for months in C57BL/6 (BL6) mice if no anthelminthic treatment is applied, while BALB/c mice are resistant and expel the worm within a month[118]. Eggs are released in the feces as soon as 9 days post-infection and L1 larvae hatch from the egg after 36 h if the temperature is >20°C[119]. Larvae feed on bacteria and undergo 2 molts (at 28 h post-hatch and after another 24 h) to transform to the non-feeding infective L3 stage (Figure 3). The infective larvae need to be ingested to infect mice and can remain viable for a substantial amount of time[119] (several months in PBS at 4°C in our laboratory) while they cannot tolerate desiccation[120]. L3 quickly enter the gastric mucosa to appear close the duodenal longitudinal tunica muscularis after 36h, causing eosinophil infiltration but no cyst[121]. 2 days post-infection and 4-6 more days later mark the timing of the third and fourth molt leading to the emergence in the intestinal lumen of adult helminths which use their coiled and ridged body to adhere to intestinal villi in order to remain stationary and feed on host tissue[119]. Male and female adults mate in the intestinal lumen and carry the life cycle forward by releasing eggs to the feces.

3.2.2. Host immune responses

Helminths, as large multicellular parasites which transgress tissues, trigger a type 2 immune response focused on wound healing to limit damage for the host, and mitigating the expulsion of the parasite (see 3.3).

3.2.3. Helminth infections and allergic disease

Given that helminths and their host have shared millions of years of coevolution, it is not surprising that parasites have developed strategies to modulate host immune responses.

The inverse correlation of allergic disease and helminth infection is well described[122–125] and protective effects largely depend on the chronicity of infection[126]. This suggests that extensive host-helminth interaction is necessary to modulate type 2 inflammatory responses. A substantial research effort is focused understanding the mechanisms of helminth-driven immunomodulation with the aim to exploit these as anti-allergic therapeutics. Several helminthic immunomodulatory molecules have been identified and together, they act at every step of the host-helminth interaction. These molecules are either homologues of human signaling molecules or do not exhibit sequential or functional homology to host factors. During tissue migration, epithelial alarmins and their signaling mechanisms are neutralized by helminth

derived products, e.g. excretory-secretory proteins of adult *H. polygyrus* such as "Hp arlarmin release inhibitor" (*HpARI*) and "Hp binds alarmin receptor and inhibits" (*HpBARI*)[127–129]. *HpARI* binds both DNA and reduced IL-33, thus retaining active IL-33 in place in the nucleus of necrotic cells and therefore inhibiting IL-33 signalling[130] while *HpBARI* binds the IL-33 receptor ST2, therefore blocking IL-33 binding[129]. *H.polygyrus* additionally releases extracellular vesicles which contain IL-33 suppressing small RNAs[131,132]. The alarmin suppression strategy is efficacious in suppressing experimental models of allergic airway inflammation[127–129,131]. An alternative strategy to interfere with the induction of an anti-helminth immune response is employed by the trematode *Fasciola hepatica*, which produces a protein (fatty acid binding protein Fh12) able to prevent antigen recognition via TLRs and thus downregulating IL-1β and TNF[133]. The molecule ES-62 from filarial nematode *Acanthocheilonema viteae* also inhibits TLR responses[134] but additionally interferes with BCR activation[135]. Metalloproteinase-like proteins AIP-1 and AIP-2 from human parasitic nematode *Necator americanus* induce accumulation of T regulatory cells in the colon via IL-10 and TGF-β thus activating host anti-inflammatory pathways, and were effective in experimental models of colitis and allergic airway inflammation[136,137]. TGF-β receptor activation and consequently T reg induction is also evident in *H. polygyrus* via the protein TGF-β mimetic (TGM)[138,139]. Furthermore effector cell responses can be modulated via interference with signaling molecules of neutrophils[140] or inducing regulatory macrophages and DCs[141,142]. Finally, helminth immunomodulators can accelerate wound healing and abbreviate immune responses to DAMPs, e.g. by inducing alternative activation in macrophages[143] or promoting epithelial proliferation[144].

Immunomodulation via helminth infection constitutes an experimental therapy option with some positive case studies for inflammatory bowel disease (IBD)[145,146], neurodegenerative disease[147] or type 1 diabetes[148], but randomized controlled clinical trials failed to support these results in IBD[149] as well as asthma, allergic rhinitis and multiple sclerosis[125,150,151]. Also, the risk of potential adverse events of helminth infection[116] must not be ignored. Thus, it is more promising to clarify molecular mechanisms of helminth-driven immunomodulation to harness these as therapeutic options for inflammatory disease.

3.3. Type 2 immunity

Type 2 immunity is the common factor connecting allergy and helminth infection. Type 2 immune responses are classically viewed as the network of innate and adaptive immune cells, prototypic transcription factors (GATA3, STAT6) and signaling molecules (IL-4 and IL-13) that are induced in response to multicellular parasites and toxins - and, in a misaligned manner, to allergens.

3.3.1. Cellular adaptive immunity

Naïve $CD4^+$ T cells are primed when they meet their antigen as presented by antigen-presenting cells (APC). Primed T cells are activated to proliferate and differentiate to effector T_H2 cells if they receive antigen specific TCR ligation and co-stimulation in an environment of type 2 stimulating cytokines. It is still unclear which cell is the initial provider of IL-4 (ILCs, eosinophils, basophils and MCs are discussed as the initial source) and this may indeed depend on the immunological context. T_H2 cells then amplify the type 2 signal by upregulating transcription of IL-4, IL-5 and IL-13 via STAT6 and GATA3[152], but also via STAT5A and NLRP3[153]. T_H2 cells are recruited to tissues via activation of CCR4 by CCL22 and CCL17[154], and their survival depends on IL-2, IL-7 and IL-15[155].

If naïve $CD4^+$ T cells are activated in the presence of TGF-β and retinoic acid without IL-6, they upregulate FOXP3 and differentiate to induced regulatory T (Treg) cells[156]. Tregs suppress immune responses via IL-10 and TGF-β production and are necessary for tolerance at mucosal sites[156]. In lungs of asthmatic children, Treg cells are decreased[157] and Tregs from atopic donors compared to non-atopic donors are impaired in their efficacy to suppress T cell proliferation, particularly during the allergen-season[158]. A proposed mechanism of AIT is the induction of allergen-specific Treg cells[159,160]. T cells can thus be utilized in allergy treatment but the long-lived memory of T cells is also a driving force of allergic inflammation. IL-2, IL-7 and IL-15 drive the survival of effector T cells[161] and their differentiation into effector ($CCR7^-$ but ß-$integrin^+$ and $cytokine\ receptor^+$) or central memory T cells ($CCR7^+$)[162]. Effector memory T cells migrate to tissues where they can be rapidly reactivated while central memory T cells remain in lymphoid tissue[162], both enabling the resurgence of allergic symptoms.

While T_H2 cells represent local effector adaptive immune cells, T follicular helper (T_{FH}) cells remain in lymphoid tissues[163]. Fate decision of naïve $CD4^+$ T cells to T_{FH} cells in mice depends on IL-6 and ICOSL in the absence of IL-2 during TCR ligation, which leads to upregulation of Bcl6 and CXCR5[164]. In contrast, human T_{FH} differentiation depends on IL-12 and TGF-β or IL-12 and activin A but not IL-6[165,166]. T_{FH} cells migrate to the T-B border to interact with B

cells and may play a role in IgE class-switching via BATF-induced IL-4 and possibly IL-21[163]. IL-4 producing GATA3$^-$ T_{FH} cells are elicited by intranasal administration of allergen in combination with low concentrations of IL-33 or IL-1β in mice[163] while IL-13 producing GATA3$^+$ T_{FH} cells are induced by various allergens in human and mice and instruct B cells to produce high-affinity IgE which can cause for anaphylaxis[167].

B cells bind antigen via their BCR and present it via MHC class II and can thus be activated by antigen-specific T cells to form germinal centers, where differentiation into antibody-producing plasma cells along with clonal expansion, somatic hypermutation and affinity-maturation occur. Plasma cells first produce IgM antibodies then undergo class-switching as instructed by cytokines derived from T_{FH} cells: As mentioned above, IL-4 leads to switching to IgG1 and IgE, while TGF-β, IL-5 and IFNγ lead to secretion of IgG2b and IgA, IgA or IgG2a and IgG3, respectively. Some plasma cells migrate to the bone marrow to establish residency as instructed by XBP1, while memory B cells persist in the spleen, lymph nodes and circulation, and upon re-activation, are able to respond to lower doses of antigen, react earlier and faster due to their surface molecule equipment (CD27, high MHC class II and CD80). This results in the rapid secretion of high-affinity antibodies, which confers advantageous – or in the context of allergy, pathological - immunological memory.

3.3.2. Cellular innate immunity

In contrast to the adaptive immune response, which requires 2 – 3 weeks to develop[168], the innate immune system becomes activated within minutes and provides an immediate response to invading pathogens. A diverse set of innate immune cells contributes to this fast immune reaction and, in most cases, stops an infection before the pathogen can proliferate and spread. In the following section, innate immune cells are described in the context of type 2 inflammation.

Dendritic cells (DCs) bridge the innate and the adaptive immune system as they respond to innate signals and sample the environment for antigen, which they present to T cells. It is not entirely clear how dendritic cells (DC) license T cells for T_H2 polarization. Type 2 DCs are characterized by STAT5, IRF3 and -4 as well as KLF4[169–172] and supposed to be dependent on autocrine prostaglandin E2 (PGE_2) signalling[173], Dectin-2-triggered cysLT production[174] or TSLP responsiveness[175] in different models of type 2 inflammation. During microbial challenges, DCs can interact with infected macrophages and pick up antigen from them[176].

Natural Killer (NK) cells are part of the first line of defense and, without the need to be activated[177], kill stressed, infected or neoplastic cells[178–180] using cytotoxic molecules like

perforin and granzymes A and B[181]. Additionally, they support T cell-mediated killing by cytokine secretion[182]. They exhibit self-tolerance by self-MHC class I recognition via inhibitory receptors[183] or hyporesponsiveness[184] to prevent autoimmunity. NK cells have been described to limit type 2 responses by lysing T_H2-polarizing DCs[185] and reducing tissue damage in early helminth infection[186] but can also be regulated by alternatively-activated macrophages[187,188]. In a murine model of house dust mite induced airway inflammation, NK cells were shown to be redundant despite accumulating in the lungs of acutely challenged mice[189] and NK cells sampled from human asthma patients exhibited IL-4 expression, suggestive of "NK2" polarization[190]. While NK cells can be seen as the innate counterpart of cytotoxic $CD8^+$ T cells, innate lymphoid cells (ILCs) reflect the innate counterparts of the different $CD4^+$ T cells flavors (ILC1 and T_H1 cells, ILC2 and T_H2 cells, ILC3 and T_H17 cells)[191]. ILCs were discovered in the context of type 2 responses[192]. They derive from lymphoid progenitors[193,194], depend on IL-2 and are among the first responders reacting to innate and tissue signals[191], including chemoattractants like sphingosine-1 phosphate, CXCL16, prostaglandin D2 (PGD_2) and leukotriene E4 (LTE_4)[195–199]. ILC2 produce IL-5 and IL-13[200], and in some settings also IL-4[201,202], and interact with T_H2 cells via MHC II-antigen presentation, thus shaping and adjusting the type 2 response[203].

Mast cells have for a long time been recognized as immediate mediators of type 1 allergic reactions and amplify or suppress immune responses. Derived from the bone marrow, mast cells reside in most tissues but mainly close to surfaces[204] including the vasculature[205] and become activated by IgE ligation to FcεR1[204]. Due to their location and storage of molecules like histamine, tryptase and proteoglycans[204] ready for release upon degranulation, mast cells are poised for immediate reaction and contribute to anaphylaxis[206]. Also, they can quickly synthesize lipid mediators, mainly leukotrienes and PGD_2[207,208] while PGE_2 can recruit mast cell progenitors but also suppresses mast-cell dependent asthmatic responses[209,210]. Stem cell factor (SCF) ligation to CD117 is the main survival factor but mast cells can react to chemokines and cytokines to shape their cargo, proliferative capacity and receptor repertoire[204]. The mast cell inflammatory output is targeted with antihistaminic drugs (e.g. cetirizine) or directly with mast cell stabilizers (nedocromil and sodium cromoglycate).

Similar to mast cells in regard to bone marrow origin, FcεR1α expression and effector molecules, basophil granulocytes (basophils) exist as a rare population in the circulation and are recruited to tissues by a variety of molecules ranging from chemokines and cytokines like CCL11, CCL5, CXCL8, IL-31, IL-33 to lipid mediators like 5-oxoETE and PAF and pathogen derived products[211–215]. Basophil effector molecules include cysLT and cytokines IL-4, IL-13,

IL-6 and TNF[215], resulting in a protective role during helminth infection as well as pathogenic functions during allergy[216,217]. Basophils were also recently proposed to reside in alveoli and drive alveolar macrophages to an anti-inflammatory phenotype[218].

Neutrophil granulocytes (neutrophils) derive from a common myeloid progenitor and are released from their bone marrow reserve into the circulation in masses[219]. Upon CXCL1, -2, -5 or -8 or LTB_4 signaling neutrophils extravasate and reach inflammatory hotspots to exert their functions as first line of response: phagocytosis and killing of microbes, degranulation resulting in liberation of proteases and formation of reactive oxygen species and release of neutrophil extracellular traps consisting of DNA and proteases, which leads to the death of the neutrophil[219]. The fact that priming of neutrophils with factors like LPS, GM-CSF, IL-18 or TNF results in enhanced responses at the tissue site of inflammation was described already long ago, summarized by Sheppard *et al.*[220]. Although IL-4 impairs neutrophil activation[221], neutrophils can still be useful or detrimental during type 2 immune responses: on one hand, neutrophils contribute to rhinovirus-induced exacerbation of allergic asthma[222] while on the other hand, in a murine model of *Strongyloides* infection, neutrophils supported macrophage-mediated killing of the larvae[223], thereby effectively partaking in type 2 immunity. Severe asthma often has a large neutrophilic component and can be steroid-insensitive[224], and therefore hard to control.

Deriving from the granulocyte macrophage progenitor via an eosinophil lineage-restricted progenitor[225], terminally differentiated eosinophil granulocytes (eosinophils) egress from the bone marrow to circulate for 8 to 12h[226]. Causing the characteristic red eosin stain, eosinophil granules contain cytotoxic cationic proteins like eosinophil peroxidase (EPX), eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and major basic protein (MBP1)[227]. Eosinophils migrate to chemoattractants like CCL5 (RANTES) via CCR5 but also via CCR3 to eosinophil-specific so-called “eotaxins” CCL11, CCL24 and CCL26[228]. Together with IL-4, IL-5 and IL-13, these chemokines play important roles in airway eosinophilia during allergic asthma[228].

For about one week, human eosinophils reside in tissues like the thymus and lungs[229,230] where they are phenotypically different from infiltrating eosinophils and are proposed to contribute to tissue homeostasis. The function of eosinophils was classically assigned to degranulation and immediate host defense, disabling and killing helminths, but especially the finding of tissue-residency has led to the discovery of more varied eosinophil phenotypes and functions, including regenerative functions after muscle injury, homeostasis of visceral adipose tissue or thermoregulation[227].

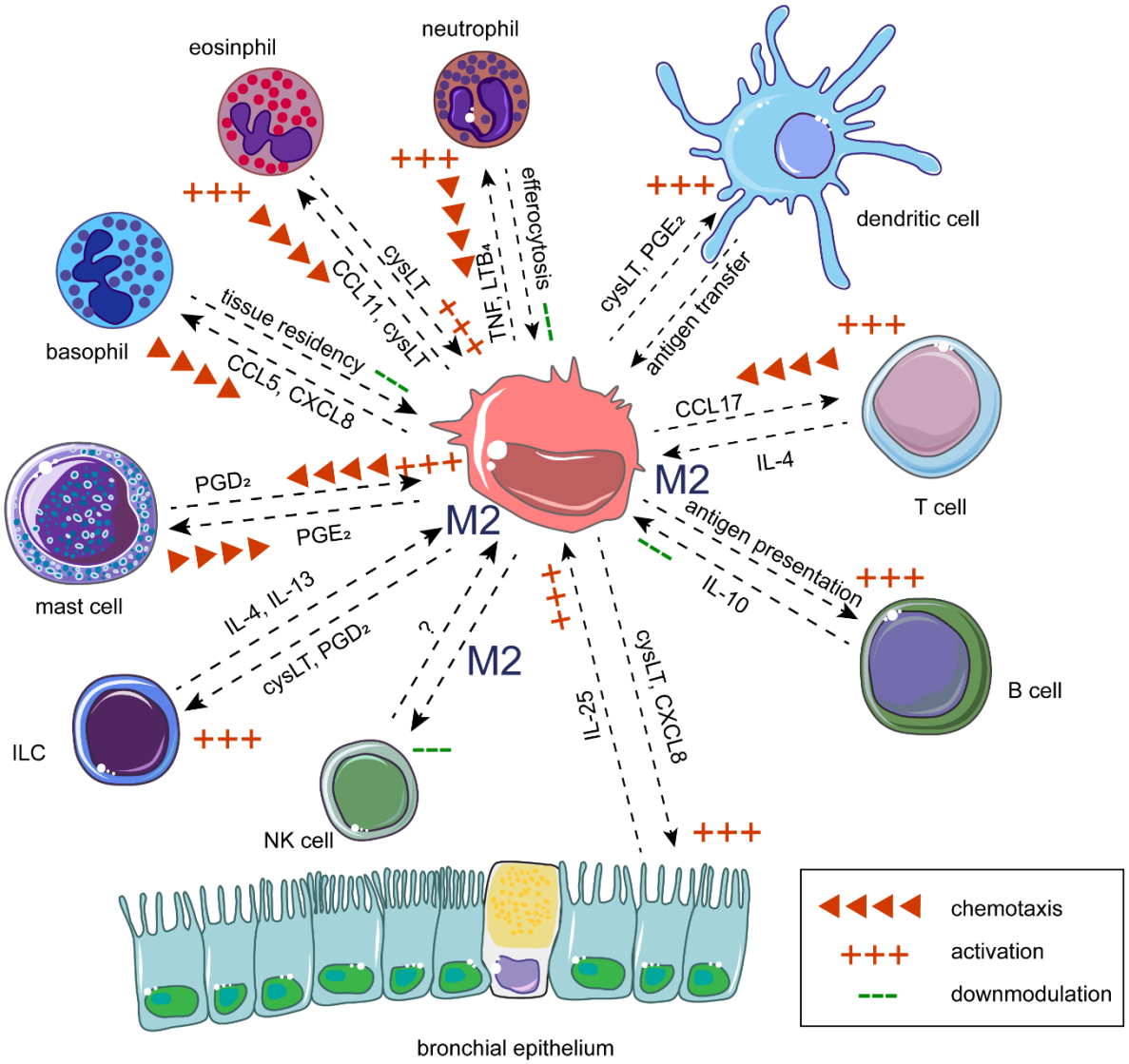

Figure 4 Cytokine-, chemokine and eicosanoid-mediated interactions between macrophages and other immune cells.

Leukotrienes attract and activate eosinophils[231,232] but can also be produced and secreted by eosinophils themselves[233,234], while PGE_2 dampens their activation, e.g. in NSAID-intolerant asthma[235]. Survival of eosinophils depends on IL-5 but also GM-CSF, especially in the lungs[236]. Several therapeutic monoclonal antibodies are directed against signaling involved in eosinophilia, for example reslizumab, mepolizumab (both anti-IL-5) and benralizumab (anti-IL-5 receptor), which are licensed as add-on therapies for adults with uncontrolled eosinophilic asthma.

The innate immune system is thus a versatile and diverse tool defending the organism against pathogens via immediate activation and concerting ongoing immune responses together with cells of the adaptive immune system. Monocytes and macrophages essentially contribute to and orchestrate innate immunity (Figure 4). They are described in detail in the following section.

3.1. Monocytes and macrophages

Macrophages are aptly named "great eaters" from ancient Greek μακροσ (great) and φαγει (eat). Kranid Slavjanskj described in fact alveolar macrophages for the first time in 1863[237] while Eli Metchnikoff recognized their phagocytic capacity and importance for immunity in 1880[238] and also named them in contrast to microphages (later recognized as neutrophils). Monocytes derive their name from the fact that they exhibit a whole, not a polymorphous nucleus like granulocytes[239]. They derive from the bone marrow, circulate for 24 to 72 h[240], and migrate into tissues to establish residency, respond to inflammatory cues or replenish local macrophage populations.

3.1.1. Ontogeny and differentiation

In the bone marrow, the granulocyte-macrophage progenitor commits to monocyte-macrophage/dendritic cell progenitor under the influence of GATA2 and ZEB2. From there, monocytes develop via the common monocyte progenitor by IRF8 and KLF4 signaling[241] although there are hints that oligopotent progenitors can exhibit lineage commitment[242–244] and therefore short-cut the classical tree of hematopoiesis. Nevertheless, PU.1, IRF8, GATA2 and KLF4 are key transcription factors for monocytic development as ablation of these genes results in impaired monocytogenesis[241]. After egress from the bone marrow, which depends on CCR2 in mice but is unclear in humans, monocytes represent ~10% of circulating blood cells and can be described by 2 subsets. Up to 90% of human blood monocytes exhibit the "classical monocyte" surface marker combination HLAII^{+} CD14^{+} CD16^{-}, which correspond to Ly6C^{hi} murine monocytes and were previously considered "proinflammatory monocytes". An intermediate subset (CD14^{+} CD16^{+} in humans or Ly6C^{int} in mice)[245] seems to lead to the "non-classical" CD14^{lo} CD16^{+} monocyte previously called "patrolling monocytes"[241], which is paralleled in the mouse by Ly6C^{lo} monocytes. At least in mice, the transition to Ly6Clo monocytes is driven via Notch2 ligation by Delta-like 1 expressed on vascular endothelial cells[246]. "Classical" monocytes spend 24 to 72h[240] in the circulation before entering the intestine, dermis, heart, pancreas, testes and lung to contribute to the respective tissue-resident macrophage population[241], or undergo changes to become "intermediate" or "non-classical" monocytes which keep circulating for up to 7 days[247].

Using studies in mice, it became clear that Ly6C^{hi} monocytes can be found in most tissues except the epidermis, central nervous system and alveolar lung space[248–251], whose resident macrophage population exhibits a high potential to proliferate although terminally differentiated.

As these tissues are seeded embryonically, macrophages, competent for self-renewal, persist to the adult age. Prominent examples are macrophages in the lung alveolar space and Kupffer cells in the liver[252], with microglia in the brain as an exceptionally long-lived example[253]. In mice, alveolar macrophage development is described in detail by Guilliams *et al.*. On embryonal day E12, yolk sac-derived macrophages seed the embryonal lung while $CD45^+$ cells expand in the lung on E18 but no alveolar macrophage phenotype (defined as $SiglecF^{hi}$ $CD11c^{hi}$) is evident in the mononuclear cell compartment ($CD11b^+$ $F4/80^+$)[250]. Finally, fetal monocytes but not primitive fetal macrophages in the lung were identified to develop into alveolar macrophages via an intermediate state during the first week of life, when the alveolar niche really opens[250]. This depends on a perinatal wave of epithelial-derived GM-CSF[250] as well as PPARγ[254] and TGF-β[255] while maintenance of alveolar macrophages needs persistent TGF-β and GM-CSF signaling[255,256]. In humans though, definitive data are lacking due to ethical restrictions for experiments with early developmental stages.

However, whether macrophage ontogeny plays a major role in terms of phenotype and function can be debated as different monocyte precursors (including those from bone marrow) can develop into functional, self-renewing alveolar macrophages, when transferred into the lung environment[257].

Even though macrophages in different tissues are distinct and show different magnitudes of responsiveness to inflammatory cues, they can polarize in similar ways. Macrophages activated by LPS and interferon γ (IFNγ) produce proinflammatory cytokines like IL-6, IL-12, IL-1β, TNF, CXCL9, CXCL10, CXCL11, reactive oxygen and nitrogen species[258]. This end of the polarization spectrum is called “classical” or “M1” and is geared towards fighting and killing bacteria and viruses[258]. “Alternative activation” or “M2” polarization on the other end is induced by IL-4 and IL-13 stimulation of resting macrophages, resulting in advanced efferocytosis capacity and tissue-reparative functions[259,260]. In between those two imaginary endpoints, there are potentially unlimited grades of polarization, minutely adapted to the current situation in the tissue and fine-tuned by the presence, absence and concentration of signaling molecules. For alternative activation, even subcategories have been proposed, so called M2a, M2b and M2c subsets[261]. IL-4 and IL-13 are supposed to induce M2a characterized by arginase-1, CCL17, CCL22, CCL24 and FcεR2, CD163, CXCR1 and -2 expression, while M2b is induced by immunocomplexes and LPS, exhibiting high IL-10 and low IL-12 and CCL1 expression. Finally, M2c is considered deactivated, elicited by IL-10 exposure in combination with both IL-4 and -13 and expresses CD206, CCL16 and CCL18.

Macrophages are thus potent and plastic mediators of inflammation and determine tissue responses both in a proinflammatory and homeostatic context.

3.1.2. Airway macrophages and their functions

Lung resident macrophage subsets are roughly differentiated due to anatomical location. Macrophages on the surface of the lungs are called alveolar macrophages (AlvM).

In the lung tissue, approximately 2 x 10^9 macrophages are found in the alveolar septa (75%), pulmonary vessel or airway walls of one human right upper lung lobe[262]. They are named interstitial macrophages (IM) and one IM subpopulation shares CD206 (mannose receptor 1) expression[263] with alveolar macrophages as a hallmark of lung macrophages.

IM do not exhibit other bona fide alveolar macrophage markers Marco, SiglecF and Cfs2r in mice[264] but can be grouped into 2 subclusters based on expression levels of MHCII and CD11c or Lyve1[263–265], as well as a tissue-resident monocyte defined as $CD64^+CD16.2^+$ in mice[263]. Recently, IM subsets of the mouse were more closely characterized to reveal differential size, potential to self-maintain, origin and final tissue location[263,265]. Similar to characterization in mice, human IM isolated from digested tissue are smaller than AlvM, not autofluorescent and less phagocytic[266], and the IM subsets were transcriptionally similar[265]. Functions of IM are unclear but it was hypothesized that IM regulate tissue homeostasis in the lung interstitium[267] and protect against fibrosis[265].

Airway macrophages (AM) exert a directing and orchestrating role in the immune system of the airways. In their function as APC, they initiate and direct T cell-mediated immune responses while their phagocytic capacity is part of the immune defense against invading pathogens as well as an essential janitor service to the organism minimizing numbers and impact of mutated and apoptotic cells[268]. The pulmonary mucosa can be seen as an outer surface as inhaled air from outside the organism reaches it (albeit filtered, tempered, and moistened). AlvM are located in the gas-exchange airspaces of the lung, i.e. alveoli, respiratory bronchioles and alveolar ducts, and plentifully so, as one human right upper lung lobe contains in average 1.4 x 10^9 AlvM[262], with 97% of them in the diffusing airspaces rather than the conducting airways. Sex and age do not affect AlvM density and distribution[262]. AlvM location underlines their essential contribution to the main function of the lungs: gas-exchange. Impaired AlvM functionality is the cause of pulmonary alveolar proteinosis with the symptoms of cough, dyspnea, and recurrent lung infections due to the accumulation of debris in the alveolar space[269] and a treatment option is inhalative administration of GM-CSF, as AlvM critically depend on this cytokine for survival and functionality[255,256]. Thus, AlvM are critical for pulmonary

homeostasis as they provide tissue repair functions such as silent efferocytosis, cross-talk to structural cells and immunosuppressive signaling[270,271]. On the other hand, AlvM express high levels of inflammatory cytokines and 5-lipoxygenase at baseline[272,273] and reports of LPS responsivity may be confounded by the high basal inflammatory capacity of AlvM[272,274].
Altogether, macrophages, as the most abundant immune cell in the lungs during homeostasis and potent mediators of inflammation, constitute interesting and understudied targets of research.

3.1.3. Airway macrophages in allergy and asthma

Macrophages in the lungs (Figure 5) have been found to by dysregulated in asthma[275]. Decreased phagocytosis is evident in AM from adults and children with severe asthma[276,277], possibly explaining why asthmatic patients experience increased susceptibility to airway infections[278,279]. Similarly, AM from asthmatic donors were less efficient at efferocytosis[280], the removal of apoptotic cells, which potentially perpetuates inflammation as danger-associated patterns may persist and continuously activate immune cells.
Sampling of AM in the mouse is easily achieved by bronchoalveolar lavage (BAL) but in humans, this invasive technique is hard to justify ethically in healthy individuals. Thus, many studies use induced sputum (IS) to obtain human AM. Considerable diversity though has been described between AM derived from BAL versus IS[271,281]. Still, IS is a valuable technique and studies using sputum-derived AM have contributed important findings about the role of AM in asthma. For example, IS-derived AM from asthmatic subjects exhibit enhanced CCL17 expression which correlated with eosinophilia and was corticosteroid-resistant, while no general alternative activation of asthmatic AM was present[282]. Instead, AM expressing hallmarks of alternative activation (CD206, stabilin-1[283]) are enriched in bronchial biopsies and BAL from asthmatic subjects[284,285] as well as HDM-induced AAI in mice[286]. BAL AM from healthy donors exhibit a mixed phenotype expressing markers of classical as well as alternative activation[287], possibly a reflection of the pronounced plasticity of macrophages. This further emphasizes that macrophage polarization as defined by “M1 versus M2” is hypothetical and must always be regarded in the context of tissue imprinting.
During airway inflammation, monocyte influx further complicates the analysis of macrophage contribution to type 2 inflammation. Several studies suggest, that monocyte-derived macrophages drive pulmonary pathology[288–291]. As the tissue environment dictates macrophage phenotype[268], monocyte-derived macrophages can become very similar to resident AM once tissue-residency has been established[292]. Independently of their origin, it is clear that AM drive

the maintenance and chronification of airway inflammation by directing and promoting T cell-mediated immunity[293,294].

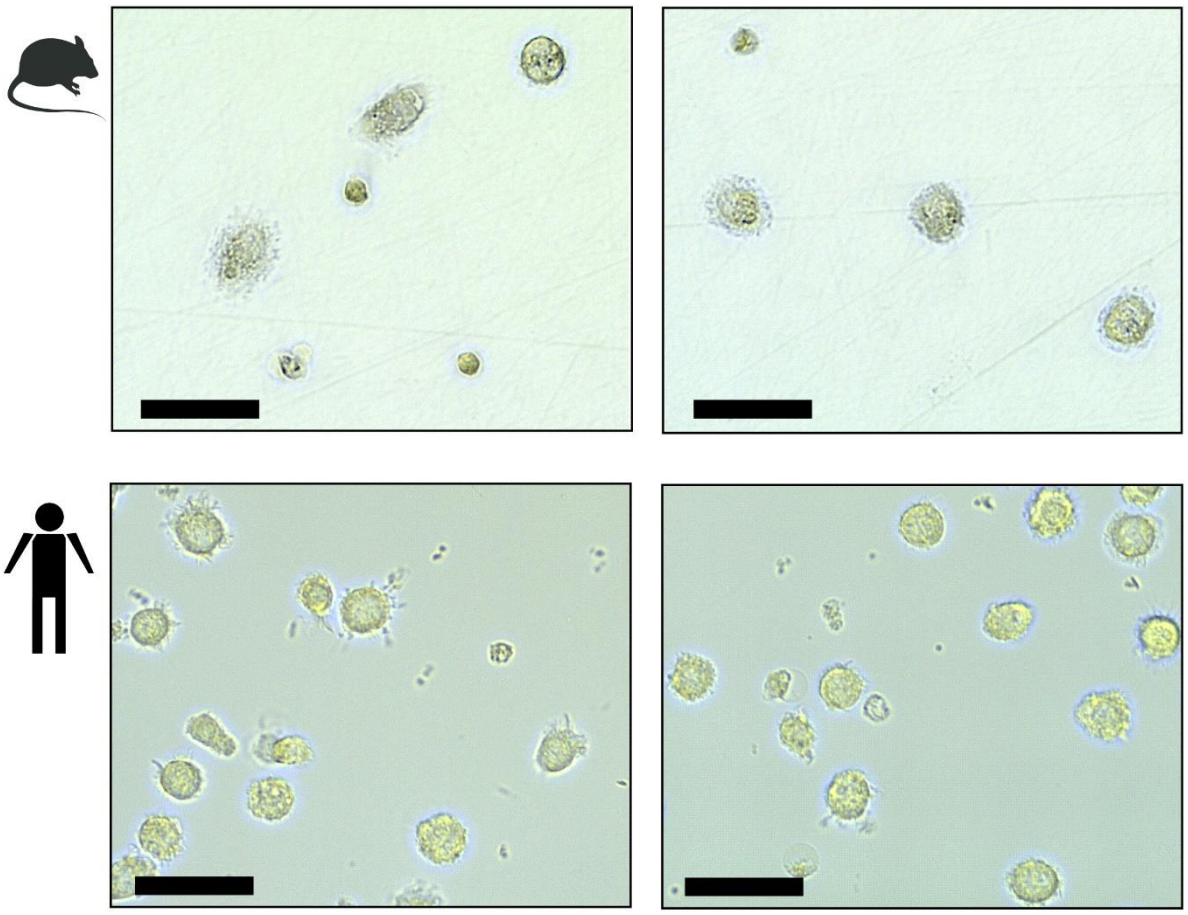

Figure 5 Macrophages derived from bronchoalveolar lavage of mice (upper panels) and human monocyte-derived macrophages differentiated in the presence of TGF-β and GM-CSF (lower panels). Scale bars indicate 20 µm.

3.1.4. Macrophage metabolic and functional reprogramming

To achieve diverse facets of immune activation and regulation, macrophages must be extraordinarily plastic and communicative. Thus, macrophages produce a wide variety of cytokines and chemokines in order to induce the adequate cellular or humoral response to many different immunological challenges. One important means of communication with their direct environment is the eicosanoid pathway as macrophages are a cell type notably well-equipped for eicosanoid biosynthesis. Both LOX and COX are highly abundant or readily inducible in primary human macrophages[295] (I, Figure 4 and Figure 6) as well as terminal synthases like microsomal prostaglandin E synthase (mPGES1) or leukotriene A4 hydrolase (LTA4H). We

recently described reprogramming of lipid mediators towards inflammatory as well as immune-modulatory profiles in macrophages[295] (I, II).

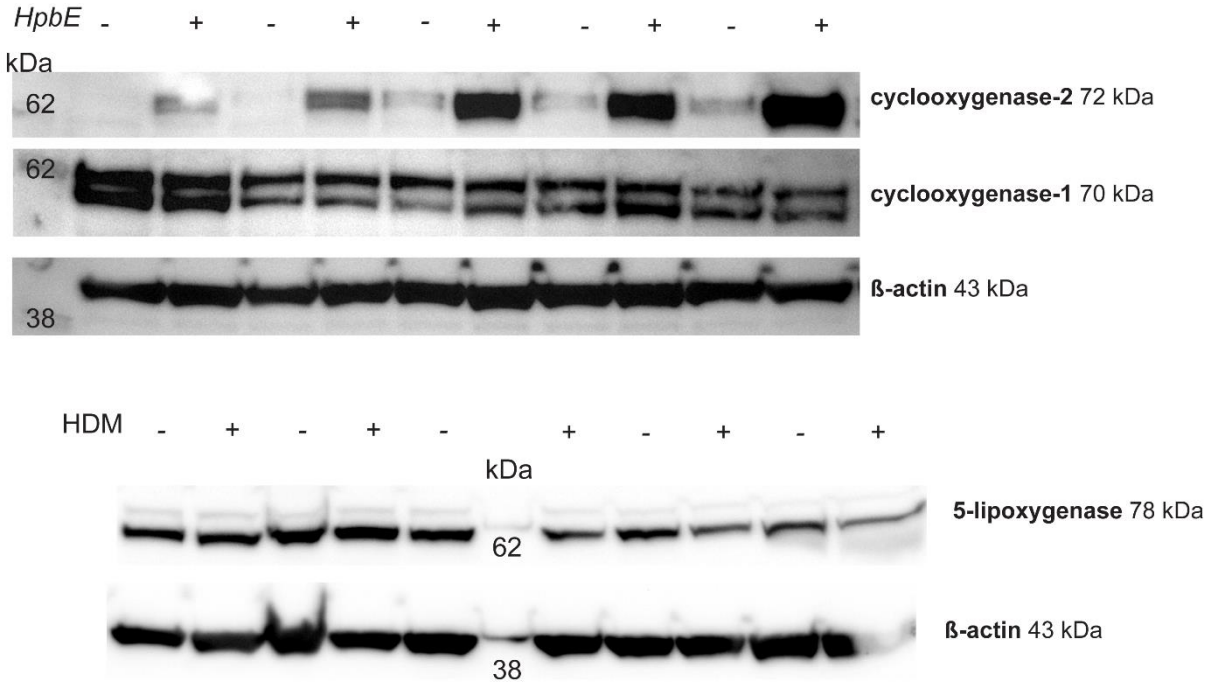

Figure 6 Human macrophages contain abundant eicosanoid biosynthesis enzymes at baseline or after activation with helminth products or house dust mite extract.

Not only lipid mediator metabolism but also energy metabolism is highly plastic in macrophages, which respond to challenges with metabolic adaptations. In case of classical activation, murine macrophages activate glycolysis while mitochondrial respiration is impaired, a phenomenon called "Warburg effect" which was initially described in cancer cells[296]. It is widely agreed that cells use glycolysis if they are in rapid need of energy even if it provides less energy equivalents than mitochondrial respiration[297]. Alternative activation is associated with enhanced used of oxidative phosphorylation[298,299] fueled by lipolysis[300] and mediated by mTOR and IRF4[301]. Re-adaptation of glycolysis is possible in these macrophages in response to subsequent LPS exposure[302]. AlvM especially focus on oxidative phosphorylation and cannot upregulate glycolysis upon activation[303,304], possibly due to the lack of glucose on the surface of the lung.

Metabolites that accumulate during the tricarboxylic (TCA) acid cycle also influence inflammatory output of macrophages. For example, succinate, which accumulates if the TCA cycle is activated in macrophages by LPS, stabilizes HIF1α and in turn increases IL-1β production and therefore inflammatory capacity of macrophages[305]. Another TCA metabolite, citrate, is implicated as a proinflammatory signal resulting in ROS and PGE_2 production[306]. On the other hand, TCA cycle-derived itaconate inhibits succinate accumulation and leads to anti-inflammatory metabolic remodeling[307]. Similarly, an increased α-ketoglutarate (α-

KG)/succinate ratio due to increased glutaminolysis, causes a shift to oxidative phosphorylation and alternative activation of macrophages[308]. There is also an intricate link between metabolic activity and epigenetic remodeling as metabolites constitute important cofactors of histone modifying enzymes[308–310]. For example, α-KG together with O_2 acts as a cosubstrate for dioxygenases like HIF-degrading prolyl hydroxylases or lysine demethylases such as KDM5[311]. In contrast, 2-hydroxyglutarate inhibits histone demethylases and TET 5-methylcytosine hydroxylases[312,313].

Unlike allergen-specific T cells with specific TCR, macrophages rely on cognate pattern recognition receptors (PRR) like TLRs, dectins or Axl to sense conserved patterns of microbes. Components of HDM extract have been shown to activate different PRR. TLR2 and TLR4 mediate the AM response[314] while dectin-2 on DCs is required for HDM-induced AAI in mice[174,315,316]. Dectin signaling has also been suggested to contribute to type 2-licensing in DCs[173]. Formyl peptide receptor bind as diverse ligands as HDM, resolvins and annexin A1[317–319] and FPR2 is associated with classical activation of macrophages[320]. However, recently FPR2 has been reported as a mediator of type 2 immunity as the the soluble PRR serum amyloid A together with *Der p13* activates airway epithelial cells to induce type 2 responses in HDM AAI in mice via FPR2[321]. Axl and MerTK are tyrosine kinase receptors which are important for phagocytosis[322] but MerTK has a role in the resolution of liver inflammation[323] while Axl can also be used to discriminate between IM and AlvM in mice[324].

Thus, macrophages are equipped with a variety of sensing molecules to respond to internal (e.g. metabolites) and external (e.g. tissue-derived) signals, which are then integrated via PRRs, second messengers, and histone modifications and translated into a multi-faceted output of cytokines, chemokines, metabolites and eicosanoids.

3.1.5. Macrophage trained innate immunity

The mammalian immune system consists of innate and adaptive arms intrinsically linked and collaborating to defend the organism and maintain or restore homeostasis. T cell memory is an essential contributor to host defense in mammals, but plants, which lack an adaptive immune system can also become immune to reinfection[325,326], a phenomenon named “systemic acquired resistance”. In mammals in the context of infectious disease, unspecific immune memory is evident in macrophages and other innate immune cells and termed “trained innate immunity”.

Mice that initially experienced a non-lethal infection of *Candida albicans* survived a second, lethal dose of the fungus in the absence of an adaptive immune system[327]. More specifically, macrophages, after an initial stimulation, were rendered hyperresponsive to a second exposure

to the same or another stimulus, with corresponding epigenetic alterations in genes related to the innate immune response[328,329]. A poised state of latent epigenetic enhancers (H3K4me1) was revealed after training of differentiated macrophages which enables a fast switch to active gene expression by acetylation[330]. This clarified that even after terminal differentiation, especially macrophages have a wide range of plasticity to adapt to their ever-changing environment, possibly with long-lasting effects. Also, exogenous manipulations intended to induce training were shown to alter epigenetic landscapes. Differential DNA methylation patterns that correlated with the responsiveness to Bacillus Calmette-Guérin (BCG) vaccination were found in humans[331]. In mice, intravenous BCG treatment led to increased myeloid commitment in hematopoietic stem cells and progenitors (HSCP) resulting in enhanced antimycobacterial activity of bone marrow-derived macrophages (BMDM)[332]. These BMDM exhibited differential histone acetylation and methylation patterns as well. Similarly, trained innate immunity triggered by the tuberculosis vaccine Bacillus Calmette-Guérin (BCG) was shown to occur in the bone marrow niche and results in systemically altered myeloid cells[332], which could potentially lead to altered pulmonary monocyte-derived macrophages. In humans, BCG vaccination persistently alters HSCP epigenome and transcriptome, leading to epigenetic and functional alteration of $CD14^+$ monocytes[333].

Netea and colleagues termed the modulation of progenitors in the bone marrow "central trained immunity" as opposed to "peripheral trained immunity" induced in local cells[334].

Centrally trained progenitors have been shown to provide resistance to chemotherapy-induced myeloablation[335] and contribute to atherosclerosis[336]. Trained immunity of mice in response to β-glucan also depended on increased myelopoiesis[335]. Here, the authors found that HSCPs from trained mice preferentially used glycolysis, but the induction of trained immunity was also dependent on cholesterol synthesis[337]. Similarly, in differentiated cells, the switch to aerobic glycolysis and usage of mevalonate for cholesterol synthesis were shown to be necessary for the on-set of trained immunity[337,338].

Alternatively to enhanced secondary immune responses, trained immunity can also lead to hypo-responsiveness or tolerance, for example by the Toll-like receptor 4 (TLR4) ligand LPS[328]. This phenomenon of persistent immunosuppression was known since long as a complication causing high mortality of sepsis survivors and LPS-induced immunosuppression was attributed to monocytes of probands intravenously challenged with LPS twice within a week[339]. The monocyte inflammatory response remained restricted upon the second LPS challenge[339] or *ex vivo* challenge[340]. LPS tolerance was dependent on COX function after the first challenge as acetylsalicylic acid treatment prevented decreased cytokine response[339].

Instead, trained immunity was induced in murine monocytes resulting in increased cytokine responses 3 months after experimental sepsis[341]. On the other hand, virus infection induced a state of trained immunity in resident murine alveolar macrophages with the help of $CD8^+$ T cells, enhancing antibacterial defense of the lung thereafter[342].

Altogether, macrophages can either be hyper- or hypo-responsive due to central or peripheral trained immunity, which depend on epigenetic modifications, possibly specific to the inducing agent and can be elicited by external (microbial pathogens) or endogenous inflammatory agents (IL-1).

Thus, an enhanced immune response of airway macrophages might be valuable if the organism is repeatedly exposed to pathogens. However, a persistently increased response to harmless components of material deposited in the lungs could unbalance the pulmonary environment and result in chronic inflammation.

During type 2 immune responses in the lung, macrophages orchestrate and perpetuate inflammation and exhibit M2 polarization[274,282,293]. Innate memory of epithelial cells and ILC2s[343,344] has been described to contribute to allergic inflammation in mice while replacement of AM with regulatory monocyte-derived macrophages after viral infection of the lungs protected mice from asthma development[345], suggesting a key role for AM in asthma. Whether innate memory in response to allergens can be triggered in local airway macrophages or progenitors in the periphery, and whether eicosanoid profiles of macrophages are influenced by trained immunity, remained obscure.

3.2. Eicosanoids

Eicosanoids are lipid mediators derived from polyunsaturated fatty acids (PUFA). The name derives from the Greek εικοσι for "twenty" and signifies the 20-carbon atom chain of arachidonic acid metabolites but is also sometimes used for metabolites of C18 PUFAs (linoleic acid, α-linoleic acid) (Figure 7). ω-6 fatty acids are considered proinflammatory while ω-3 fatty acids are widely seen as anti-inflammatory, but the effect of their ratio in human nutrition is still unclear[346]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both ω-3 fatty acids, give rise to resolvins and protectins[347].

Especially in severe asthma endotypes, dysregulation of eicosanoid lipid mediators is prominent[348–350]. These powerful local mediators govern the on-set, amplification and resolution of inflammation and can be produced by myeloid cells, bronchial and nasal epithelial cells, brush and tuft cells and mast cells, and by some cancer cells[351–357].

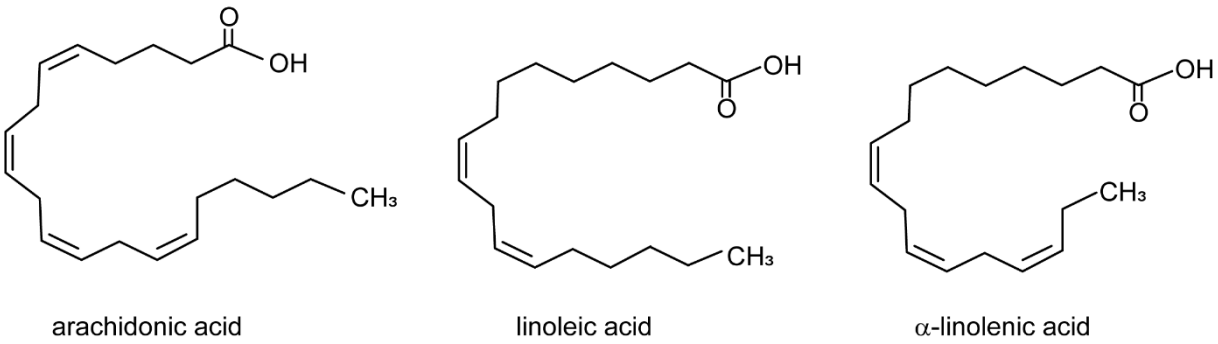

Figure 7 Structural formulas of polyunsaturated fatty acids

3.2.1. Synthesis pathway and functions

To produce eicosanoids, the ω-6 fatty acid arachidonic acid (AA) is cleaved from the membrane by phospholipases A2 (primarily $cPLA_2$, $iPLA_2$ and $sPLA_2$)[358] and utilized in different pathways to give rise to a wide variety of powerful autacoids (Figure 8).

Cyclooxygenases (COX) synthesize in a two-step reaction the instable intermediate peroxide PGH_2 which then is transformed into prostaglandins (PGs) and thromboxane (TX). Cyclooxygenase 1 (COX1) mainly couples to thromboxane synthase (TBXAS1) for the formation of TXA_2, while COX2 feeds PGE_2 synthase (mPGES1) to give rise to PGE_2[359]. PGD_2 biosynthesis is facilitated by coupling of both COX-1 and COX-2 and PGD_2 synthase (PTGDS)[359]. Besides proinflammatory mediators, COX-2 is also involved in the synthesis of prostacyclin (PGI_2)[360]. PGI_2 acts as the antagonist of TXA_2 and is therefore important in the homeostasis of coagulation[361]. This is exemplified in the withdrawal of the selective COX-2 inhibitor rofecoxib (marketed as Vioxx by MSD) in 2004 after the incidence of cardiovascular events was increased in the verum group in a RCT for an additional indication[362]. COX-2 was inhibited while COX-1 was active and feeding TBXAS1, so the ratio between PGI_2 and TXA_2 decreased to a pro-coagulative and cardiotoxic level. On the other hand, there is also evidence for molecule-inherent cardiotoxicity of rofecoxib, which may explain why other "coxibs" lack these severe adverse effects[363].

Lipoxygenases on the other hand introduce a peroxide at either position 5, 12 or 15 of the eicosatetraenoic chain. For of the 5-LOX/FLAP complex, this results in the formation of the instable intermediate leukotriene (LT) A_4, which is either metabolized by the terminal synthase LTA_4 hydrolase (LTA4H) to LTB_4 or by LTC_4 synthase (LTC4S), giving rise to cysteinyl leukotrienes (cysLTs: LTC_4, LTD_4, LTE_4). In non-hematopoietic cells, LTC_4 is produced by MGST2[364]. 12- or 15-hydroxyeicosatetraenoic acids (HETEs) derive from 12/15-lipoxygenase and further peroxidase activity. The specialized pro-resolving mediators (SPM) called lipoxins

can also derive from AA via LOX pathways while further SPM maresins and resolvins derive from ω-3 fatty acids like α-linoleic acid[348]. In addition, the cytochrome P450-system can metabolize PUFAs to biologically active mediators, mainly anti-inflammatory epoxyeicostrienoic acids (EETs) and proinflammatory 20-HETE[365,366].

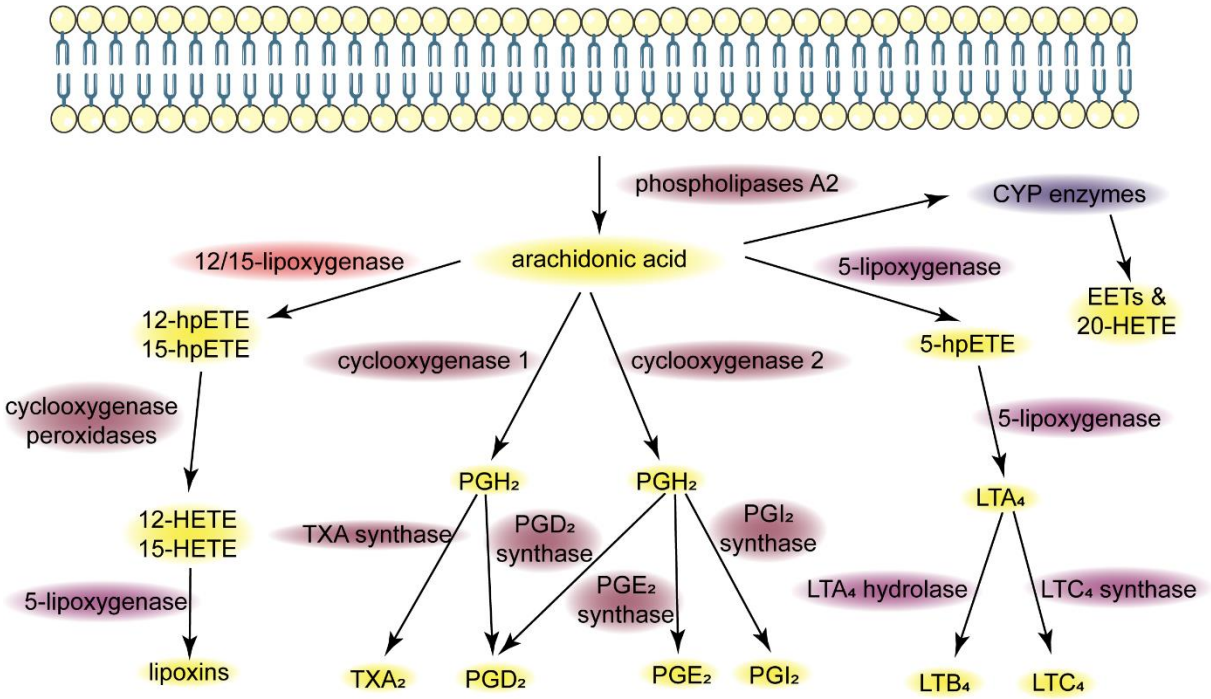

Figure 8 Eicosanoid biosynthesis pathways resulting in the production of lipoxins, prostanoids and leukotrienes

Eicosanoids mediate diverse biological effects dependent on the activation of G-protein coupled receptors. PGE_2 binds 4 dedicated GPCR: EP1, EP2, EP3 and EP4, of which EP2 and EP4 are both Gs-coupled, leading to elevated intracellular cAMP[367] and are expressed in macrophages, enabling autocrine signaling. There is no clear division in anti-inflammatory and pro-inflammatory receptors, but the cellular and tissue context defines the effect of PGE_2. For the pro-inflammatory example, EP2 and EP4 amplify macrophage IL-33 production in response to LPS[368] and regulate T_H17 cell function[369]. On the other hand, EP4 has been reported to mediate anti-inflammatory effects of PGE_2 on airway macrophages[370], to prevent vascular remodeling in allergic lungs[371], to maintain the air-blood barrier[372,373] and to dampen eosinophil functions[374], while EP2 on T cells was necessary to suppress AAI[375] and both EP2 and EP4 suppress ILC2 activation[376]. The specific distribution of EP receptors in pulmonary cells and tissues confers primarily protective and homeostatic effects to PGE_2 in the lungs[377]. PGE_2-mediated anti-inflammatory effects additionally pertain to its inhibitory effect on 5-LOX activity[378].

Designated receptors have also been described for TXA_2 (TP), PGD_2 (DP1 and DP2/CRTH2), $PGF_2\alpha$ (FP) while activation is somewhat promiscuous, e.g. PGD_2 can also bind TP[379,380].

Leukotrienes signal via cysLTR1 and cysLTR2, although there is evidence for further cysLT receptors (e.g. GPR99 or P2Y12)[381,382]. The GPCR cysLTR1 is activated by LTC_4 and LTD_4, and in higher concentrations also by LTE_4[383–385], and couples to $G\alpha_i$ and $G\alpha_q$, therefore lowering intracellular cAMP or activating PLC[385,386]. This results either in reduced PKA activity or mobilization of intracellular calcium via inositolptriphosphate[384,387]. cysLTR2 also signals via $G\alpha_i$ and $G\alpha_q$ and is implicated in NSAID-sensitive asthma[388,389]. For LTB_4, two receptors are described: BLT1 and BLT2 are reported to be high- and low-affinity receptors, respectively[390], of which the first was detected as the most divergent gene in relation to epigenetic modification in comparison to other primates, underlining its importance in immune defense[391]. BLT1 couples to $G\alpha_i$, $G\alpha_q$ or $G\alpha_{16}$ depending on the cell type[390], and can therefore signal via activation of PI3 kinase, inhibition of adenylate cyclase or activation of calcium channels[387].

Altogether, eicosanoid lipid mediators form an immense and intricate signaling network that is far from completely understood.

3.2.2. Roles of eicosanoids in asthma and allergy

Eicosanoids contribute to and regulate the immune response in an anti-or proinflammatory manner. It is unclear whether there is a "type 2" eicosanoid profile although it has been shown that IL-4 suppresses 5-LOX while inducing 15-LOX[392,393] and IL-4 and IL-13 downregulate PGE_2[394].

The expression of CRTH2 on eosinophils, basophils and T_H2[211,395,396] cells suggests that PGD_2 plays a role in type 2 immunity by acting as a chemoattractant for important effector cells. Therefore, PGD_2 leads to airway eosinophilia[397] but also neutrophilia[398] and is considered a fully proinflammatory, type 2-driving prostanoid. Furthermore, PGD_2 causes cough and bronchoconstriction[399,400]. TXA_2, the instable 2,6-dioxabicyloheptan structure of which is hydrated to the inactive metabolite TXB_2 within 30s, was first described as a vasoconstrictor and platelet activator[401]. In the lungs, TXA_2 causes bronchoconstriction[402] but its role in platelet aggregation and blood clotting[403] renders it an important contributor to wound healing as well. If HDM-induced AAI was elicited in *ptges*$^{-/-}$ mice, TXA_2 induced airway eosinophilia, inflammation and hyperresponsiveness, thus demonstrating an important modulating effect of PGE_2 on TP signaling[404]. As described above (3.2.1), PGE_2 downmodulates airway immune responses by dampening effector functions of airway macrophages, ILC2, eosinophils and T_H2 cells[370,374–376,405]. In allergen-challenged, asthmatic human subjects, inhaled PGE_2 diminished

allergen induced bronchoconstriction, sputum eosinophilia and airway hyperresponsiveness in comparison to placebo[406] but PGE_2 so far is only licensed as an abortive and an oxytocic[407,408]. cysLTR1 antagonists in contrast have found their way into asthma pharmacotherapy (see 3.1.2.). The cysLT LTC_4 is the most potent bronchoconstrictor[409] and cysLTs also promote vascular leakage[410,411], enabling immune cell immigration and pulmonary edema, induce airway remodeling by smooth muscle hyperplasia[412,413] and impair ciliar activity[414,415], resulting in decreased mucus clearance. Together with PGD_2, cysLTs enhance T_H2 cell and ILC2 functions and cytokine production[199,416] as well as mast cell activation[417]. Eosinophils are the main producers of cysLTs, which in turn are attracting more eosinophils[418,419]. In addition to hematopoietic cells, tuft cells and brush cells are potent producers of cysLTs, which contribute to host defense against helminth parasites or allergic airway inflammation, respectively[355,420]. Their potent proinflammatory, type 2-inducing actions position cysLTs as central mediators of asthma and allergic airway inflammation. Also allergic nasal symptoms like nasal congestion (vascular leakage), rhinorrhea (mucus overproduction) and local immune cell recruitment (eosinophils) are mediated by cysLTs[421] and nasal allergen challenge can lead to the detection of cysLTs in nasal discharge from allergic subjects[422].

LTB_4 is a neutrophil chemoattractant and activator[423–425] with important roles in host defense against respiratory infections[425–427]. In addition, LTB_4 also promotes the chemotaxis of eosinophils in response to nematodes[232]. 5-HETE mediates vascular permeability[428] and, after oxidation of 5-HETE to 5-oxoETE, it activates a receptor called OXER1, through which it acts as an eosinophil chemoattractant and to a lesser extent an attractant of neutrophils, monocytes and basophils[429]. 15-HETE is abundant in sputum of asthmatic subjects[430] but it exerts weak bronchoconstrictory action in humans[431] and has been shown to inhibit LTB_4-induced neutrophil chemotaxis[432]. A recent Icelandic study revealed that a loss-of-function mutation in ALOX15 is protective for CRSwNP[433], but on the other hand, 15-HETE can be processed to lipoxins, specialized pro-resolving mediators[434], which further complicates a clear functional classification of 15-HETE.

Despite great research effort has been into the synthesis and functions of eicosanoid lipid mediators, which even also brought some benefit to patients in the form of therapy options like LTRA, many questions remain open regarding e.g. eicosanoid receptors, pathological actions and modulatory strategies for lipid mediators - particularly in type 2 immune responses.

4 Aims

1. To characterize the eicosanoid profile of allergen-exposed macrophages.

2. To test the hypothesis that programs of “trained innate immunity” can be induced in macrophages by HDM allergens and thereby contribute to allergic airway inflammation.

3. To analyze the immunoregulatory ability of helminth-derived products on macrophage effector functions and type 2 airway inflammation.

5 Materials and Methods

5.1. Materials

Details about manufacturers of materials used for experimental studies in this dissertation can be found in the "Materials and Methods" sections of the respective papers. All methods, which are described in the publications but are not mentioned here, were performed without my technical contribution.

5.2. Methods

Description of all methods used and in parentheses, in which paper.

5.2.1. Isolation of primary human cells from blood (I, II, III)

Blood was drawn from cubital venipuncture into EDTA-anticoagulated tubes. PBMC and PMN were isolated via density gradient (Lymphoprep for PBMC, Polymorphprep for PBMC and PMN). Cells were sorted using magnetic beads (αCD14 for monocytes from PBMC, αCD16 for neutrophil granulocytes from PMN). An antibody cocktail for negative selection was used to isolate eosinophil granulocytes from PMN. Granulocytes were either used directly for experiments (chemotaxis) or incubated overnight with various stimuli in the presence of 100 ng/mL recombinant human GM-CSF. Monocytes were differentiated in the presence of 10 ng/mL recombinant human GM-CSF and TGF-β1 for 7 days to alveolar-like monocyte derived macrophages (aMDM) or in the presence of 20 ng/mL M-CSF to M-MDM.

5.2.2. Induction of sputum and isolation of primary human cells from induced sputum (II)

Sputum was induced after β-adrenergic relaxation of lungs via salbutamol inhalation (400 µg) by ascending concentrations of sterile NaCl solution (3%, 4%, 5%) for 7 min each. Inhalate was administrated as a Piezo-dispersed aerosol facilitated by a Pari-Boy inhalator device. Coughing was encouraged after each inhalation and expectorated sputum was cooled on ice immediately. Lung function was monitored by measuring FEV_1 before and after salbutamol inhalation and after each NaCl inhalation. A decline of more than 10% from the personal best FEV_1 value was designated as a relative and of more than 20% as an absolute stop criterion. Among all participants, no stop criterion was reached, and no serious adverse events were reported. Sputum was dispersed using Sputolysin and unfractioned cells (~20,000 – 50,000)

were applied to cytospin slides for May-Grünwald staining and differential cell counting. The remainder of the cell suspension was incubated with the RosetteSep monocyte enrichment cocktail and autologous or heterologous erythrocyte concentrate, and furthermore, human primary alveolar macrophages (AM) were purified via centrifugation over a density gradient. AM purity was assessed microscopically, and a fraction (10,000 cells) was subjected to cytospin preparation. The remainder of cells was plated in complete medium, rested for 20 min at 37°C and stimulated for 10 min with 5 mmol/L calcium ionophore A23187. Supernatant was collected and stored in 50% methanol for LC-MS/MS and without for ELISA while AM were lysed in RLT buffer for gene expression analysis.

5.2.3. Culture of normal human bronchial epithelial cells (II)

Normal human bronchial epithelial cells (NHBE) were acquired from Lonza and grown to passage 2 before cryopreservation. For experiments, 200,000 NHBE in passage 4 were plated in 500 µL bronchial epithelium growth medium and grown to 90% confluence in submerged culture. After 6 h starvation in bronchial epithelial basal medium, NHBE were exposed to 10% (vol/vol) cell-free macrophage supernatant overnight. Control treatment with 1 µg/mL HDM was performed to quantify the contribution of background HDM introduced by macrophage supernatant. Control cytokine quantification of 10% macrophage supernatant in bronchial epithelial basal medium without NHBE was performed to measure the cytokine background introduced by macrophage supernatant in parallel with the assay of NHBE supernatants. NHBE were lysed in RLT buffer for gene expression analysis.

5.2.4. Isolation of primary murine cells from bone marrow (II, III)

Murine femores and tibiae were brought under the LAF workbench and residual tissue was removed. Joints were cut and bones opened and flushed with RPMI-1640 medium to obtain bone marrow. To obtain a single cell solution, bone marrow was strained over a 100 µM mesh filter. 1 x 10^6 cells were suspended per mL of complete medium supplemented recombinant murine GM-CSF and recombinant human TGF-β1 and cultured for 7 days with 1 exchange of medium. Differentiated bone marrow-derived macrophages were split as MDM (see above) and plated for further experiments.

5.2.5. Cell culture (I, II, III)

Cells were cultured at 37°C and 5% CO_2 and handled sterilely under a laminar air flow workbench. Myeloid cells and normal human bronchial epithelial cells were cultured in two

different incubators. Description of the specific experimental procedures of *in vitro* experiments are described in detail in the Materials and Methods sections of the respective publications.

5.2.6. Flow cytometry (I, II, III)

Macrophages were detached using 5 mM EDTA in PBS as described above and non-adherent cells (HSCP and granulocytes) were directly stained after harvest. Fc receptor was blocked before extracellular staining using the antibodies indicated in the respective papers. Cells were acquired on a BD FACS Fortessa. Compensation was carried out with antibody-stained beads and FMO stainings of cells. Data was analyzed using FlowJo (BD Life Sciences).

5.2.7. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (I, II, III)

Liquid chromatography-tandem mass spectrometry was performed at the Institute of Clinical Pharmacology, Johann Wolfgang Goethe University in Frankfurt am Main, Germany, by Carlo Angioni and Yannick Schreiber in 2 different panels for prostanoids or LTB_4 and hydroxyeicosatatrenoic acids (HETEs), respectively, as described below. Data was analyzed and released by Dr. Dominique Thomas and PD Dr. Nerea Bouzas Ferreiro.

For the analysis of prostanoids, 200 µL supernatant were spiked with isotopically labeled internal standards (PGE_2-d4, PGD_2-d4, TXB_2-d4, $PGF_2\alpha$-d4, 6-keto $PGF_1\alpha$-d4), 100 µL EDTA solution (0.15 M) and 600 µL ethyl acetate. Samples were vortexed and centrifuged at 20,000 g for 5 min. The organic phase was removed, and the extraction was repeated with 600 µL ethyl acetate. The organic fractions were evaporated at a temperature of 45°C under a gentle stream of nitrogen. The residues were reconstituted with 50 µL of acetonitrile/water/formic acid (20:80:0.0025, v/v/v) and transferred to glass vials.

LC-MS/MS analysis was performed using an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative ESI mode. The chromatographic separation was carried out using a Synergi Hydro-RP column (150 × 2 mm, 4 µm particle size and 80 Å pore size; Phenomenex, Aschaffenburg, Germany). A gradient program was employed at a flow rate of 300 µL/min. Mobile phase A was water/formic acid (100:0.0025, v/v) and mobile phase B was acetonitrile/formic acid (100:0.0025, v/v). The analytes were separated under gradient conditions within 16 min. The injection volume was 10 µL. The gradient program started with 90% A for 1 min, then mobile phase A was decreased to 60% within 1 min, held for 1 min, further decreased to 50% within 1

min and held for 2 min. Within 2 min, mobile phase A was further decreased to 10% and held for 1 min. Within 1 min, the initial conditions were restored, and the column was re-equilibrated for 6 min. Mass spectrometric parameters were set 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.

For analysis and quantification, Analyst Software 1.6 and Multiquant Software 3.0 (both Sciex, Darmstadt, Germany) were used, employing the internal standard method (isotope dilution mass spectrometry). Calibration curves were constructed using linear regression with $1/x^2$ weighting.

Quantification of HETE and LTB_4 was done as described previously[79]. In brief, 150 - 200 µL supernatant were spiked with the corresponding deuterated internal standards and extracted by liquid-liquid-extraction using ethyl acetate. Analytes were separated using a Gemini NX C18 RP-LC-column (150 mm × 2 mm I.D., 5 µm particle size and 110 Å pore size from Phenomenex, Aschaffenburg, Germany) under gradient conditions with water and acetonitrile as mobile phases, both containing 0.01% ammonia solution. The LC system was coupled to a mass spectrometer 5500 QTrap (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative electrospray ionization mode. Data acquisition was done using Analyst Software V 1.6 and quantification was performed with MultiQuant Software V 3.0 (Sciex) employing the internal standard method (isotope dilution mass spectrometry).

For (I), Fiona Henkel partially performed LC-MS/MS analysis in the Genome Analysis Center at the Helmholtz Center Munich in the laboratory of Prof. Dr. Jerzy Adamski.

5.2.8. RNA extraction and quantitative real-time polymerase chain reaction (I, II, III)

Cell lysates in RLT buffer were mixed with equal volumes of 70% [v/v] ethanol then subjected to the spin column protocol of the Quick-RNA MicroPrep kit according to the manufacturer's instructions including a step of DNAse I digestion. RNA was quantified and assessed for purity using a Nanophotometer. RNA quality was accepted if 260/280≥2.0 and 260/230≥2. A maximum of 2 µg RNA per reaction was reverse transcribed using the High Capacity cDNA kit on a Biometra TAdvanced thermocycler using settings as follows: 10 min 25°C, 120 min 37°C, 5 min 85 °C before hold at 12 °C. cDNA was diluted to 2.94 ng/µL and stored at -20°C before use for qPCR. 10 ng cDNA were used as template and mixed with 1.6 µL of 4 µmol/L of the respective forward and reverse primers (primer sequences can be found in the Methods sections of the respective papers) and 5 µL FastStart Universal SYBR Green Master Mix on a

384 well qPCR plate and analyzed using 35 cycles of cycling between 95 °C for 15 s and 60 °C for 1 min on a ViiA7 Real-time PCR system (Applied Biosystems, Thermo Fisher).

5.2.9. Immunofluorescence staining and confocal microscopy (I, II, III)

Macrophages were cultured and stimulated on untreated IBIDI glass slides with 12 well separations before fixation in 4 % paraformaldehyde and permeabilization/antigen retrieval using ice cold acetone before blocking and staining with respective antibodies (see papers) over night. Secondary antibodies labelled with fluorophores (Alexa Fluor488, Alexa Fluor568, Alexa Fluor644) were used at a dilution of 1:500 in 5% BSA for 1 h. Finally, slides were mounted using an aqueous mounting medium containing DAPI for nuclear staining.

Tissues were fixed submerged in 10 % formalin before dehydration and paraffin embedding. Sonja Schindela prepared serial sections. After deparaffination at 60°C, slides were rehydrated using a gradient from lipophilic to hydrophilic solvents. Tissues were permeabilized, antigen-retrieved and blocked before overnight incubation with primary antibodies (specifications can be found in the respective papers). Secondary antibodies were incubated for 1 h and slides mounted as described above.

After 24 h of rest in the dark at 4°C, slides were photographed on a Leica Confocal SP5 microscope using 4 frame averages of each laser (405 nm, 488 nm, 564 nm, 647 nm) and adjusted laser powers for each antigen. Raw images were adjusted for brightness and contrast exactly equal for each series and channels were overlaid (both by using Fiji ImageJ). In some instances of cultured cells, fluorescence intensities were assessed using pipelines in Fiji ImageJ and CellProfiler (open source software from the Broad Insitiute, www.cellprofiler.org).

5.2.10. Hematoxylin/eosin and Periodic acid-Schiff staining

Fixated and dehydrated tissues were sectioned and mounted on slides before subjection to hematoxylin/eosin staining using the staining robot of the Histology Core Laboratory of the Dermatology Clinic (Klinikum rechts der Isar, TU München). PAS staining was performed at the same facility.

5.2.11. Enzyme immunoassays (I, II, III)

Frozen cell culture supernatants, BAL fluids or sera were thawed on ice and diluted in the recommended buffer depending on the range of the respective standard curve. For cytokines, plates were coated using capture antibodies while for eicosanoids, pre-coated plates were used. In general, analyses were performed according to the manufacturer's protocols and read on an

Epoch microplate spectrophotometer. ELISAs were performed for human CCL17, IL-6, IL-10, IL-1β, TNF, and murine CCL17, CCL5, IL-5 and IL-10. EIAs were performed for cysLT, PGE_2 and TXB_2.

5.2.12. Bead-based multiplex cytokine assays (I, II, III)

Frozen cell culture supernatants, BAL fluids or sera were thawed on ice and diluted 1:1 in reagent diluent of the respective kit. Analyses were performed according to the manufacturer's instructions and beads were read on a Bio-Plex 200 system. Standard curve data was reduced using a 5-parameter-logistic fit.

5.2.13. Western Blotting (I, II, III)

Cell lysates were thawed on ice, diluted to result in 1 to 10 µg protein per sample and heated (10 min, 70°C) in the presence of loading buffer and reducing agent. Lysates were separated on a Bis-Tris gradient gel 70 min at 125 V and thereafter blotted to a PVDF membrane. Membranes were blocked and incubated with primary antibodies overnight (specifications can be found in the respective papers). Secondary antibodies coupled to horseradish peroxidase and ECL substrate were used to detect protein bands on an ECL Chemocam System. Densitometry was performed using LabImage 1D software on raw images.

5.2.14. Total RNA Sequencing (II)

Cells harvested from in vitro experiments or patient sampling were lysed in RLT buffer and stored at -70 °C until processing. RNA was extracted as described above (link to 5.2.8), quantified by Qubit fluorescence interference technology and quality assessed using a Nano RNA chip on the Bioanalyzer platform. 10 µL of RNA sample was shipped to the European Molecular Biology Laboratory (GeneCore, EMBL, Heidelberg). Libraries were prepared using the Ultra II NEB stranded kit on a Biomek i7 automation System from 150 ng RNA input. Samples were fragmented for 10 min and multiplied in 13 PCR cycles with an adaptor dilution of 1:25 with the 96 Unique Dual Index Primer Pairs plate (8 bp indices). Libraries were sequenced on a NextSeq500 Illumina sequencer using a 75 cycles high-output kit.

5.2.15. HDM-induced allergic airway inflammation in vivo (II, III)

Female C56J/B6 mice aged 6-8 weeks were purchased from Charles River laboratories, Sulzberg, and housed under specific pathogen-free conditions at the animal facility (E-Streifen) of the Helmholtz Center Munich. Mice were sensitized intranasally to 1 µg HDM extract

(*Dermatophagoides farinae*) under sevoflurane anaesthesia on day 0. On days 8 to 11 of the experiment, mice were challenged with 10 µg intranasally. 72h post-challenge, mice were sacrificed with overdose of ketamin/xylazine. Tracheae were cannulated and lungs were lavaged with 500 µL PBS to assess pulmonary cellularity (differential cell counts) and lung cytokines, chemokines and eicosanoids. Fixated lungs were sectioned and stained for histologic assessment. Bone marrow was harvested as described above (see 5.2.4) for analysis of HSCP and BMDM.

Figure 9 Schematic of the HDM-induced allergic airway inflammation in vivo model

5.2.16. Intranasal instillation of a helminth extract, helminth extract-treated murine macrophages or *HpB* GDH as *in vivo* treatment (III)

Female C56J/B6 mice were sensitized as described above (link to 5.2.15) and on each challenge day, were transferred 3 x 10^5 *HpbE*-pulsed or control-treated alveolar-like BMDM (wildtype, *ptges*$^{-/-}$ or *ptgs2*$^{-/-}$) before HDM challenge. The first repetition of this experiment was performed together with Aurélien Trompette in the Laboratory of Prof. Benjamin Marsland at the the Centre Hospitalier Universitaire Vaudois (CHUV) at the Université de Lausanne (UNIL) in Epalinges, Lausanne, Switzerland, under a TUM GS Internationalization Grant for travel to AL. Similarly, sensitized mice were treated intranasally with *HpbE* (5 µg) or *Hpb* GDH (10 µg) to test treatment efficacy. Analyses were performed as described above (5.2.15).

6 Results

6.1. House dust mite drives proinflammatory eicosanoid reprogramming and macrophage effector functions

Eicosanoids as potent mediators in allergy are notoriously difficult to detect and quantify robustly[435]. The gold standard is liquid chromatography tandem-mass spectrometry[436]. The establishment of the eicosanoid extraction workflow for cell culture supernatants as well as the validation of our LC-MS/MS method for the detection of 52 eicosanoids was performed by Fiona Henkel within the scope of her Master thesis[437]. Using this method, we analyzed the eicosanoid profile of primary human macrophages under conditions which lead to type 2 immune responses in the organism, namely presence of IL-4 and exposure to an extract of HDM. Our cellular model of alveolar-like monocyte-derived macrophages (aMDM, differentiated in the presence of GM-CSF and TGF-β1, which are essential for the differentiation and maintenance of alveolar macrophages[254,255]) hardly responded to the type 2 cytokine IL-4. In contrast, macrophages exposed to the combination of IL-4 and HDM exhibited pronounced changes in their eicosanoid profiles, namely increased output of prostanoids PGE_2, PGD_2, $PGF_2\alpha$ and TXB_2 and reduced levels of leukotrienes as well as 5-HETE. Thus, we stimulated macrophages with HDM only and reproduced the shift from LOX to COX metabolites as observed with the combination of IL-4 and HDM. This was accompanied by a strong induction of proinflammatory chemokines and cytokines. Macrophage eicosanoid biosynthesis pathway were reprogrammed on transcriptional as well as protein level. PRR implicated in the macrophage response to HDM, TLR2, TLR4 and dectin-2, were not required for the eicosanoid switch occurring after 24h HDM stimulation while p38 activation was necessary. Despite detection of neutrophil chemoattractants in the supernatants of HDM exposed macrophages, chemotaxis of human neutrophils was prevented in the presence of HDM-macrophage supernatant, probably due to the downregulation of leukotrienes, which are potent granulocyte chemoattractants. Chemotaxis war partially restored when neutrophils were exposed to secretions of macrophages treated with HDM in the presence of an inhibitor of p38. P38 inhibition restored 5-LOX product levels, probably re-establishing the chemotactic efficacy of macrophages. In the airways of mice during type 2 inflammation induced by HDM-AAI or infection with a lung-passing nematode, *Nippostrongylus brasiliensis*, distinct eicosanoid profiles were apparent, suggesting highly plastic and specific eicosanoid responses towards different type-2 inducing agents.

6.2. A trained type 2 immunity program drives exaggerated leukotriene and CCL17 responses in allergen-experienced macrophages

Macrophages can be trained by PAMPs to exhibit enhanced or subdued responsiveness to a secondary stimulus. While PAMP-induced trained immunity is protective in various infectious diseases[327,332,438], it is unclear if allergens can convey trained immunity. We found increased CCL17 and cysLT production capacity in macrophages isolated from BALF (i.e. local airway macrophages) and from bone marrow (i.e. centrally derived) of HDM-sensitized mice, which for cysLTs, was mirrored in MDM from HDM-allergic donors. Upon *in vitro* HDM exposure, BMDM from HDM-sensitized mice exhibited more pronounced upregulation of prostanoid metabolic genes and higher concentrations of PGE_2, indicative of priming by HDM-AAI *in vivo*. Altered intracellular levels of metabolites in HDM-sensitized BMDM could be related to enhanced cysLT production as GSH and glutamic acid, used in cysLT biosynthesis, were elevated. Still, the overall bioenergetic profile was similar between groups. As progenitors of BMDM are a reservoir of trained immunity[335], we studied HSCP gene expression finding persistently higher *Tgm2* expression in HSCP from HDM-sensitized mice. However, myeloid ablation of *Tgm2* did not alter hallmarks of type 2 inflammation in HDM-AAI (e.g. airway eosinophilia, IL-5). Differentiated human macrophages trained with HDM *in vitro* produced higher levels of CCL17 upon HDM challenge after 5 days of rest as well as increased cysLT output. HDM- but not β-glucan-trained macrophages produced CCL17 HDM-dose-dependently even in the absence of HDM challenge. The trained CCL17 response depended on FPR2 activation and autocrine TNF signaling. 2-HG treatment increased macrophage activation both acutely and after training and HIF1α played a partial role. TLR4 ligation and KDM6B activity during training were both necessary for increased cysLT production upon challenge. Transcriptional differences between MDM from HDM-allergic patients and healthy controls were evident even after 7 days of culture, depicting baseline transcriptional activation as pro-inflammatory genes were more abundant while some modulatory genes were suppressed in macrophages from HDM-allergic donors. *IL17RB*, a gene overlapping with the transcriptional phenotype of *in vitro* HDM-trained and challenged macrophages, was enhanced in MDM from HDM-allergic patients. Supernatants of HDM-trained and challenged macrophages also induced a strong epithelial CXCL8 output. In turn stimulation of HDM-trained macrophages with the epithelial cytokine IL-25 resulted in stronger cysLT and CCL17 production, suggesting a potentially proinflammatory crosstalk between macrophages and epithelium.

6.3. An anti-inflammatory eicosanoid switch mediates the suppression of type-2 inflammation by helminth larval products

Severe type 2 inflammatory conditions in humans require chronic steroid treatment[75] which can have severe adverse effects[439]. Therefore, treatment options with different mechanisms of action are needed. Helminths have co-evolved with their hosts and can dampen type 2 immune responses which are directed against the parasite[130]. *Hpb* is a murine nematode which can regulate type 2 immunity, e.g. by blocking IL-33 or inducing TGF-β, IL-1β and IL-10[128,138,141,440]. Intranasal treatment of mice with an extract (*HpbE*) of stage L3 *Hpb* larvae ameliorated HDM-induced AAI, and the intranasal transfer of previously *HpbE*-pulsed wildtype macrophages also conferred the protective effect. If macrophages differentiated from the bone marrow of *Ptges*$^{-/-}$ mice were treated with *HpbE* and transferred into the airways of HDM-sensitized mice, the modulation of the pulmonary type 2 response was lost. In contrast, the transfer of *Ptgs2*$^{-/-}$ macrophages resulted in a hyperinflammatory response highlighting the role of prostanoids in controlling airway inflammation. Human and murine macrophages were reprogrammed to a potentially immunomodulatory phenotype characterized by high IL-10 and PGE_2 production, as well as TXB_2, 12-HHT and IL-1β, concomitant with suppression of leukotriene and PGD_2 formation. This was partially dependent on ligation of dectin-1, dectin-2 and TLR2, suggesting the presence of molecular patterns in the *HpbE*. HIF1α, p38 and cyclooxygenase activity was necessary to induce PGE_2 and IL-10. *HpbE*-driven eicosanoid reprogramming was similarly evident in granulocytes and PMN chemotactic capacity was diminished upon treatment with *HpbE* due to downregulation of chemotactic surface receptors CCR3 and CRTH2. *HpbE* mainly affected myeloid cells as PBMC depleted of $CD14^+$ cells could not induce PGE_2 and IL-10 while whole PBMC and $CD14^+$ PBMC (mainly monocytes) strongly reacted to *HpbE* treatment with upregulation of PGE_2 and IL-10. Biochemical treatments e.g. with heat and pronase hinted to a protein as the active molecule. Using fractionation and mass-spectrometric analysis, the enzyme *Hpb* glutamate dehydrogenase (*Hpb* GDH) was identified as the most frequent protein in the active fractions. Intranasal treatment with recombinantly expressed *Hpb* GDH during the challenge phase of HDM-induced AAI ameliorated pulmonary symptoms like eosinophilia, IL-5 concentration in BALF and mucus production. Thus, recombinant *Hpb* GDH is an immunomodulator candidate that could be effective when administered locally in type 2-emphasized chronic inflammatory conditions like allergic asthma, N-ERD or CRSwNP.

7 Discussion

7.1. Receptors underlying HDM-driven eicosanoid reprogramming

HDM is a mixture containing diverse components including multiple PAMPs and proteases, and although HDM allergy is evoked by a limited number of allergenic proteins[441], these are not the only immune cell-activating agents. For example, *Der f2* binds LPS[442] and therefore, HDM allergens bring their own adjuvant to induce allergy[443]. LPS contributes to, but was not essential for, HDM-induced macrophage activation, as demonstrated by the failure of TLR4 neutralization or polymyxin B complexation to prevent HDM induced macrophage activation (I, Supplementary Figure 4). TLR4 and TLR2 signaling mediates AM responses to HDM *in vivo*[314] but was not essential for HDM-driven eicosanoid reprogramming (I, II). Dectin-2 is another important HDM receptor[315] but confer its actions rather in the early activation phase instead of mediating reprogramming. Thus, macrophage reprogramming over the prolonged period of 24 h appears to be mediated by other sensors, such as FPR2 (II, Figure 3 h,i).

7.2. Mechanism of HDM-driven leukotriene regulation

Macrophages react to acute HDM exposure by switching their eicosanoid profile from 5-LOX to COX-derived lipid mediators (I, Figure 3 A, B) but the physiological advantage that results from this eicosanoid switch is not completely clear. The most plausible might be a mechanism of immunoregulation, as persisting leukotriene signaling is detrimental as demonstrated by the harmful contribution of leukotrienes and other 5-LOX products to chronic inflammation[357]. PGE_2 suppresses 5-LOX activity by impairing FLAP[378] and by driving cAMP-PKA dependent inhibitory phosphorylation[444], but we have not investigated autocrine contribution of macrophage-intrinsic PGE_2 receptors EP2 and EP4 to 5-LOX suppression, although it could be easily achieved by using EP2- and EP4-specific inhibitors or mPGES1 inhibitors[445]. Also, BMDM from mice unable to respond to PGE_2 (e.g. *Ptger2*$^{-/-}$) or to produce PGE_2 (*Ptges*$^{-/-}$) would provide valuable insights into a potential suppressive role of HDM-induced PGE_2 on LT production. In case of *HpbE*, PGE_2-mediated 5-LOX suppression appears to be a central mechanism (II, Figure 2 F, 5 F-H) and while the switch from 5-LOX to COX metabolites is similar to the one induced by HDM exposure, the massive IL-10 induction concomitant with a lack of PGD_2 production discriminate the anti-inflammatory macrophage phenotype that is induced by *HpbE* from HDM-triggered changes.

Strikingly, HDM-trained macrophages escaped the reprogramming from 5-LOX to COX and instead increased leukotrienes alongside prostanoid production upon HDM challenge (II, Supplementary Figure 6 a). This is reminiscent of PGE_2-resistance implicated in N-ERD[446] and

its molecular mechanism should be pursued further as understanding and possibly, re-establishment of intrinsic PGE_2 effects could ameliorate N-ERD pathology or even chronic type 2 airway inflammation in general. No clear transcriptional hints are evident in regard to eicosanoid production in HDM-trained cells, except that HDM-experienced macrophages could more intensively upregulate *PTGS2* (II, Supplementary Figure 6 b) (Figure 10 A). On the other hand, LT-producing enzymes were not significantly altered transcriptionally (II, Supplementary Figure 6 b) (Figure 10 B). However, there is evidence that 5-LOX and LTC4S can be regulated

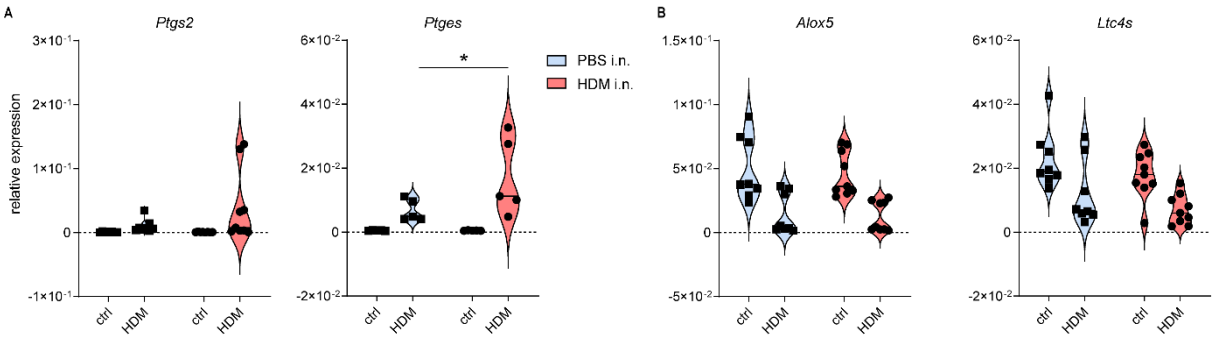

*Figure 10 Gene expression of BMDM from PBS- and HDM-sensitized mice ± in vitro HDM for 24h (normalized to Gapdh, two-way ANOVA, Sidak's multiple comparisons test) i.n.=intranasal, *p≤0.05*

by phosphorylation via PKC and S6 kinase[444,447] without changes in transcription. The lack of commercially available phospho-LTC4S antibodies impedes a simple western blot screen, so activity of the kinases would be interesting to analyze in both *in vitro* trained as well as patient-derived allergen-experienced macrophages. An alternative possibility to explain enhanced eicosanoid synthesis is facilitated liberation of AA from membranes via upregulated phospholipases. Actually, *PLA2G16* was significantly more abundant in MDM from HDM-allergic donors (II, Figure 6 a). In adipose tissue of insulin-resistant individuals, PLA2G16 is activated via HIF, elevates lysophosphatidylcholine levels and thereby mediates inflammasome activation, perpetuating adipocyte and adipose tissue-macrophage activation and chronic activation[448]. Similarly, we recently described elevated lysophosphatidylcholine concentration in nasal lining fluid of N-ERD patients[295], suggesting the existence of a metabolically altered proinflammatory macrophage fingerprint also in chronic type 2 inflammation.

mPGES1 (gene name *PTGES*) responds to HDM stimulation of macrophages (I, Figure 4 A, B) and is coupled to COX-2 to produce PGE_2[359]. Trained macrophages expressed more mPGES1 after 6 days of rest than unstimulated macrophages (II, Figure 7 e), suggesting that mPGES1 remains present and possibly active for prolonged times after initial induction. Alternatively, *PTGES* has been shown to be epigenetically activated via p300-mediated histone acetylation[449], so continuously increased transcription and translation is also possible. *PTGS2*, the gene encoding COX-2, responds rapidly to inflammatory insult, which was confirmed in our

experimental set-up. At 1 h of HDM challenge, *PTGS2* was already upregulated, so together with persisting mPGES1 in trained macrophages, it could contribute to a potentiated PGE_2 response in trained macrophages. Similarly, induction of CCL17 after challenge happened earlier in trained than in control macrophages (II, Supplementary Figure 4 e). Thus, a poised state of the HDM-trained macrophage, which enables quicker response, could be delineated. Therefore, studies of epigenetic modifications which differ between control and HDM-trained macrophages but also between macrophages from healthy and HDM-allergic donors, should be performed to better understand allergen-training.

7.3. Epigenetic regulation of macrophage activation in allergy and asthma

Several studies describe differential epigenetic landscapes between healthy and allergic donor in airway smooth muscle cells, epithelial cells and also myeloid cells[450]. Monocytes from steroid-resistant asthmatics were less responsive to anti-inflammatory actions of dexamethasone and vitamin D due to less successful gene activation via histone 4 acetylation[451]. Exposure to cigarette smoke worsens asthma symptoms in children and BAL AM from passive smoking children exhibited less HDAC2 activity correlating with heightened CXCL8 expression, neutrophilia and steroid resistance[452]. We describe functional alterations in human monocytes and murine bone-marrow derived macrophages from HDM-sensitized patients or mice (II, Figure 1, 2, Supplementary Figure 2), which suggest altered epigenetic states in these cells. However, only epigenomic profiling of patient-derived monocytes compared to healthy/non-allergic monocytes can clarify whether and which alterations are present. In order to study single histone modifications, a screen of the epigenome would be necessary before focusing on particular modifications or sites. ChIP sequencing could provide important information about pathways involved in allergic immunopathology and identify mechanisms of chronification. Epigenetic modifications may even be amenable to therapeutic targeting by chemical compounds inhibiting epigenetic writers or erasers (e.g. particular HMTs, HATs, KDMs or HDACs). Such targeting may however have considerable side effects as epigenetic changes are crucial in many homeostatic processes, e.g. myeloid cell differentiation[453]. Biotechnological epigenome editing e.g. via CRISPR-Cas[454] may provide a possibility to target epigenetic regulators of gene activity in particular cells and might thus “re-set” epigenetic changes with pathologic or disease-accelerating impact. For example, pathological alterations could be particularly targeted in airway macrophages, by therapies that work only locally and on targeted cells. The phagocytotic capacity of macrophages could be exploited by using molecules directed to bind Tyro3, Axl and MerTK (TAM receptors), e.g. Gas 6 or protein S[455].

TAM inhibitors are under investigation to target the tumor-promoting M2 polarization of tumor-associated macrophages[455]. Molecules used to target macrophages do not need to inhibit TAM receptors but rather specifically bind them and activate efferocytosis/phagocytosis in order to bring epigenome-remodeling compounds into the cell. One must keep in mind that *MERTK* was less abundant in MDM from HDM-allergic donors (II, Figure 6 a), so targeting might be less effective in the actual patient population, but consequences of the transcriptional difference for MerTK protein level still need to be verified. Similarly, CD206-directed targeting of macrophages, currently also explored in oncology research[456], possibly via inhalative medications, may be another possibility to administer therapy in a AM-specific manner. However, our results suggest that the proinflammatory activation of macrophages in allergy is imprinted on the level of bone marrow progenitors. On the other hand, adoptive transfer of bone marrow mononuclear cells, similar to the immunomodulatory effects of stem cell transfer but even slightly more effective, can dampen allergic inflammation in mice[457]. For example, a single adoptive transfer of bone marrow-derived mononuclear cells from PBS- or OVA-sensitized and challenged mice into the airways of also OVA-sensitized and challenged mice reduced pulmonary symptoms such as airway resistance and type 2 cytokines[458]. The reduction was less pronounced if bone marrow cells from OVA-sensitized donors were transferred. In a case series, 3 women with severe asthma underwent intravenous autologous transfer of bone marrow mononuclear cells and reported alleviated quality of life scores in the early phase after the transfer[459]. However, efficacy is out of the scope of this study designed to assess safety, and if transfer of bone marrow cells from healthy, non-asthmatic donors could improve asthma symptoms remains unclear. For heterologous transfers though, the problem of histocompatibility arises as for established organ transplantations. A solution to this problem would be to define individual epigenomic modifications in each patient, reverse them *ex vivo* via epigenome editing (similar to the principle of CAR T cell therapy for cancer) and re-infuse them into the patient. Until protocols like this to cure severe inflammatory disease are thinkable, immense fundamental research effort must address metabolic reprogramming, differential activation and epigenetic modifications to clearly define targets for editing.

Thus, systemic or bone marrow-specific targeting may be required to efficiently reverse pathological functions of monocytes and macrophages in asthma patients.

7.4. Future steps and challenges in the development of *Hpb* GDH as a biotherapeutic

The anti-inflammatory and type 2-suppressive activity of *Hpb* GDH was shown in a mouse model of allergic airway inflammation *in vivo* but the enzyme probably needs further refinement before qualifying as a biotherapeutic. Preliminary analyses showed a marked presence of anti-GDH IgG antibodies in serum of GDH-treated HDM-sensitized mice (also in GDH only treated control mice), suggesting immunogenicity even after short and local administration (unpublished). Thus, identification and mutation of the most antigenic regions will be necessary to reduce the immunogenicity of the helminth protein before further preclinical testing. In addition to optimization of the molecule, GMP-compliant production strategies must be explored. *Hpb* GDH can be produced in E.coli (BLI21), which may simplify upscaling, but it is unclear how easily a mutant *Hpb* GDH can be expressed and purified in this system. GDH activity testing can serve as in-process control for biological activity of the enzyme, but it is still unclear whether the therapeutic effect depends on it. The LPS contamination of the E.coli-derived recombinant enzyme must be assessed but in all cases EMA or FDA guidelines on endotoxin testing will apply[460,461]. However, as *Hpb* GDH will potentially be developed as a therapeutic for local administration (e.g. nasal drops or pulmonary aerosol), sterility is required[462]. The contribution of LPS, which is likely to be present in the recombinant preparation we used so far, to the therapeutic effect will have to be elucidated. The mechanism of action as clarified so far suggests topical application as local airway macrophages must be reached to confer the immunomodulatory effect. As the target patient group already suffers from epithelial dysfunction, appropriate caution is mandated for the use of preservatives used for aqueous solutions, like benzalkonium chloride and potassium sorbate, which can irritate the mucosa upon long-term application[463]. Systemic application of a protein is so far only possible by intravenous, intradermal or intramuscular route due to the barrier of the digestive tract. In the stomach, acidic pH and digestive enzymes such as pepsin or chymotrypsin would degrade the protein. In addition, permeation of a large molecule such as an enzyme through the intestinal mucosa could only be facilitated via active transport processes. Despite efforts to develop formulations like nanoparticles, liposomes or polymer coating[464,465] or chemical engineering of peptides[466] to improve oral bioavailability, oral administration of a pure protein is not available in the clinic so far. However, the preferred local administration will possibly avoid these problems. Currently two peptide hormones for nasal application are marketed in Germany[467,468]. Besides advice to store the bottle inside the carton box in an upright position[467,468] and how to clean the spraying opening to maintain accuracy of dosing[467], the administration is rather

straightforward. The simplest therapy regimen is usually the best for adherence, especially if chronic disease warrants long-term medication[469]. Standard toxicity screens[470] will reveal long-term adverse effects that were not evident in the acute treatment of our *in vivo* models. In biologicals, due to their specificity for a target, adverse events are often directly related to the mechanism of action, e.g. re-activation of tuberculosis under anti-TNF therapies[471], or related to immunogenicity and weakening of the therapeutic effect by anti-drug antibodies[472]. Whether these apply to the novel therapeutic enzyme *Hpb* GDH will be studied in the future.

Considering a potential therapeutic development of *HpbE*/*Hpb* GDH, the question arises about simultaneous NSAID administration as this could neutralize a major anti-inflammatory effect of the protein. Asthmatic patients are often advised to use NSAIDs cautiously, and information about the potential interference of NSAIDs with immune regulatory effects of *Hpb* GDH may be conveyed in addition in the case of a successful clinical development as a biotherapeutic.

7.5. Mechanism of action of *Hpb* GDH

In addition to its preclinical characteristics, the mechanisms of action of *Hpb* GDH are largely unclear. One possibility is that GDH activates surface receptors on macrophages which lead to the described anti-inflammatory modulation of the macrophage secretome via intracellular signaling pathways including COX, p38 and HIF1α, as we elucidated for the complete *Hpb* L3 extract. As GDH is an enzyme which catalyzes the conversion of glutamate to α-ketoglutarate as well as the reverse reaction, it is conceivable that macrophage metabolism is altered via skewing of either the immediate surrounding of the cell or – after uptake into the cell via receptor-mediated endocytosis or pinocytosis – the internal metabolite balance of the macrophage itself. Targeted metabolomics of *Hpb* GDH-treated macrophages will provide insights into a potential effect of the enzyme on TCA cycle and amino acid intermediate metabolite profiles. Also, the role of *Hpb* GDH in the living helminth and its predominant expression in the L3 stage is unclear. The environment of the L3 (intestinal tissue) could preferentially induce glutamate metabolism in L3. It is known that availability of amino acids in tissues shapes immune cell functions[473] and especially antigen-stimulated T cells critically depend on glutamine[474]. Thus, glutamine/glutamate depletion via GDH could lead to a more favorable host environment allowing larvae to survive and molt into adult stages. The presence of glutamate in rat duodenum led to activation of defense mechanisms[475] and skewing of amino acid availability might also limit immune cell response in the lungs. However, exhaled breath condensate during acute asthma becomes acidic[476] and glutaminase activity of airway epithelial cells producing ammonia from glutamine contributes to buffering the inflammation-induced

shift in pH[477]. Besides ammonia, glutamate is produced in this reaction but both pathological and protective effects of glutamate signaling in the lungs have been reported[478–480]. In this light, it seems unlikely that *Hpb* GDH operates simply by glutamate availability.

7.6. Glutamate metabolism in the regulation of type 2 immunity by HDM and *Hpb* GDH

Glutamate was more abundant in bone marrow-derived macrophages of HDM-sensitized mice. LTC_4 is converted to LTD_4 in a reaction yielding glutamate from the glutathione residue, but LTC_4 is released from the cell before the reactions to LTD_4 and E_4 take place[386], so increased intracellular levels of glutamate unlikely derive from processing of LTC_4, unless the cell engages in reuptake of glutamate. Oxidized (GSSG) as well as reduced glutathione (GSH) was

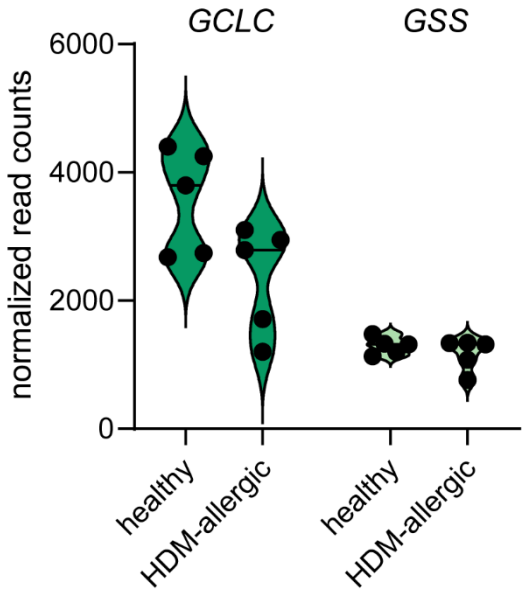

Figure 11 Gene expression of glutamate-cysteine ligase catalytic subunit (GCLC) and glutathione synthase (GSS) in MDM from healthy and HDM-allergic donors (Normalized read counts from transcriptomics dataset)

more abundant in BMDM from HDM-sensitized mice, but it is unclear whether a bigger pool of educts facilitates biosynthesis of cysLT (II, Figure 1), or whether the increased abundance of GSH is due to inherent cellular changes and does not impact cysLT production. Increased levels of educt can also point toward a reduction or break of their metabolism but in our case, the evidence of increased cysLT does not support the hypothesis. However, other metabolic pathways using GSH could be inhibited such as decreased need for neutralization of ROS. In MDM from HDM-allergic donors, the glutamate-cysteine ligase (*GCLC*) tends to be expressed less than in MDM from healthy donors, but there is no difference in glutathione synthethase (*GSS*) (Figure 11), so it is not possible to infer any GSH alterations in human MDM. mPGES1,

the terminal synthase of PGE_2, also depends on GSH[481], so fluctuation of GSH could also affect mPGES1 activity. Studying the redox balance in macrophages from healthy and allergic donors might provide further insight into pathological activation.

7.7. Modulation of BMDM function by HDM and *Hpb* products

Adoptive transfer of *HpbE*-pulsed BMDM into HDM-sensitized mice conveys protection from type 2 airway inflammation (III, Figure 1). The transfer of untreated BMDM slightly increased inflammation, so a part of the *HpbE* effect could be masked by the reaction that is triggered by transfer of BMDM. At baseline, GM-CSF and TGF-β differentiated BMDM contain abundant 5-LOX and being placed in the inflammatory surrounding of HDM-challenged lungs, these BMDM likely become activated and produce proinflammatory 5-LOX derived lipid mediators, which drive HDM-induced airway inflammation[174,315,316].

It is currently unclear if *HpbE* or *Hpb* GDH treatment *in vivo* has a lasting effect on airway macrophages. Gene expression analysis of macrophages isolated from BAL shows induction of *Ccl17* in AM from HDM-challenged mice (part of II, Figure 1) while *Ccl17* was less abundant in AM from GDH-treated HDM-challenged mice 2 days after the last challenge and treatment. In contrast, *Ptges* was slightly enhanced by GDH-treatment (Figure 12), suggesting a local anti-inflammatory mediator switch in airway macrophages. It would thus be interesting to study if

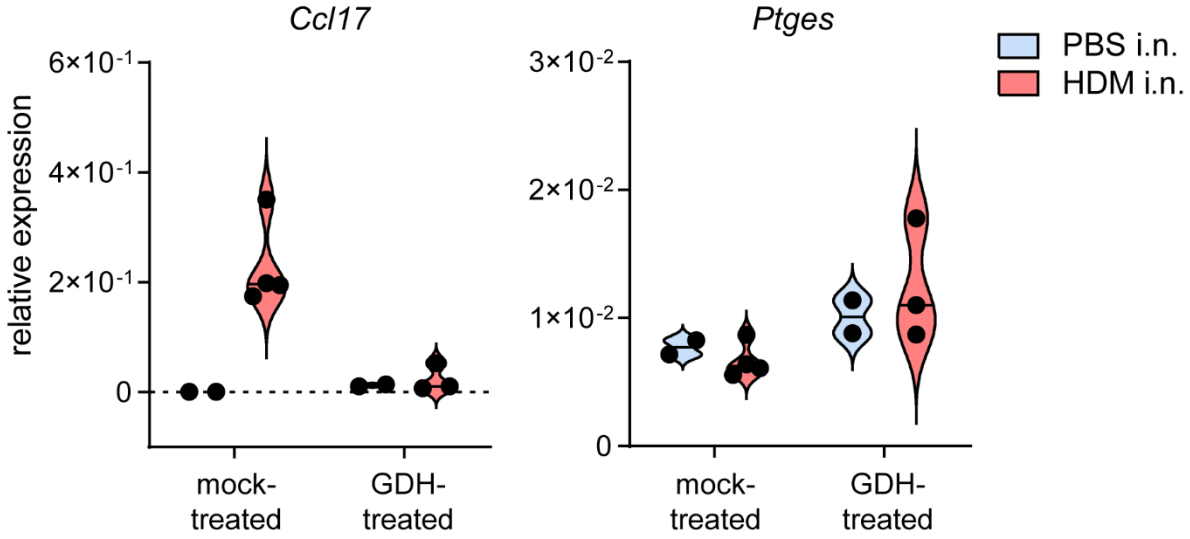

Figure 12 Gene expression in murine AM isolated from BALF of PBS- or HDM-sensitized mice, treated with intranasal Hpb GDH or mock, relative expression normalized to Gapdh.

the anti-inflammatory phenotype of *Hpb* GDH or *HpbE*-treated macrophages is transient or leads to sustained anti-inflammatory activity. The latter could add a favorable kinetic to a possible new medication, as less frequent administration is associated with better adherence[482,483]. On the other hand, sustained anti-inflammatory remodeling of AM could

jeopardize host immunity to infections with bacteria, viruses and fungi e.g. via suppression of LTB_4[484–486]. Further clarification of infection susceptibility under *HpbE* or *Hpb* GDH-treatment is warranted. In this regard, a less persistent AM reprogramming would be beneficial as treatment could be paused in case of infection without lagging of immunosuppression. Indeed, *in vitro* experiments (III, Figures 2, 3) do not show complete suppression of 5-LOX in *HpbE*-treated MDM and cytokine production is generally increased. Thus, a broad immunosuppression with heightened risk for infections as observed with immunosuppressive drugs such as tacrolimus or mycophenolate is unlikely. Experiments with prolonged observation after *HpbE* or *Hpb* GDH-treatment will clarify the duration of the immunomodulatory effect both *in vitro* and *in vivo*.

7.8. Trained type 2 immunity on the level of macrophages

Persistently altered activation and effector mechanisms evoked by allergen exposure have recently been described in ILC2s and respiratory epithelial progenitor cells[343,344]. Airway macrophages as resident regulators of immunity on the surface of the lung decisively modulate allergic and type 2 airway inflammation[258,293]. Trained immunity in macrophages and monocytes has been studied extensively in settings of infectious disease[327–329,332,335]. Murine AM were reported to exhibit a trained phenotype also in response to models of OVA-induced allergic airway inflammation[487], but only their responsiveness to classically activating PAMPs (LPS, Poly(I:C) and imiquimod) was tested while the effect of trained AM responsiveness on type 2 inflammation was neglected.

CysLTs are accepted and well-studied mediators of allergy[488] and AAI[489] and are increased in BALF of HDM-sensitized and -challenged mice (I, Figure 6 B). While important producers of leukotrienes like eosinophils are cleared from the lung after acute inflammation resolves[490,491], alveolar macrophages persist[250]. CCL17 as a T_H2 chemoattractant[154] is overexpressed in alveolar macrophages of allergic patients, but also in asthmatic tissue and DCs[282,492,493] as well as in other type 2 inflammatory diseases like sarcoidosis[494] or atopic dermatitis[495]. We describe a previously unknown overproduction of these mediators in peripherally derived macrophages, while confirming the effect of allergic airway inflammation on locally derived macrophages regarding increased cysLTs and CCL17. This opens two possible routes of reprogramming and implicates central as well as peripheral trained immunity in type 2 inflammation. On one hand, direct allergen exposure can alter macrophages and induce a type 2 trained immunity program locally. This may play a role in AM as they reside at the air interface[496]. Enhanced cysLT production was less prominent in IS-derived human AM compared to BAL AM from HDM-

sensitized mice (II, Figure 1 a, c). Substantial heterogeneity has been reported between BAL and IS derived AM[271,281], so differences in maturity, tissue imprinting and exposure may drive altered trained responses. However, type 2 mediator overproduction at baseline was more prominent in human MDM and murine BMDM as compared to airway macrophages (II, Figure 1 b, d). This suggests that central trained immunity may play a previously unprecedented role in allergic inflammation, as myeloid progenitors in the circulation or the bone marrow affected and reprogrammed by factors which are currently under investigation. Our in vitro data suggest a role for HDM-induced TNF and GM-CSF in trained macrophage responses to HDM. Interfering with these pathways *in vivo* will shed light on their role in reprogramming of myeloid progenitors during type 2 airway inflammation.

"Classical" trained immunity as defined by Netea *et al.* in response to β-glucan[334] is induced at the level of bone marrow progenitors and marked by a shift to increased myelopoiesis[332,335], for which there was a slight trend in our HDM-induced AAI model (Figure 13). *Tgm2*, a marker of alternative activation in macrophages[497] and facilitator of eicosanoid production in allergic

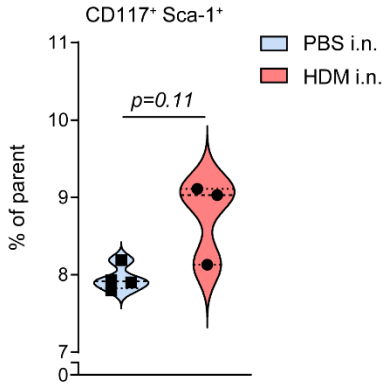

Figure 13 Percentage of myeloid committed progenitor cells of HSCP isolated from PBS- and HDM-sensitized mice (Mann-Whitney test) i.n.=intranasal

asthma[353,498] was consistently expressed at higher levels in HSCP from HDM-sensitized mice (II, Supplementary Figure 3 a) but *Tgm2*$^{flox/flox}$ x LysMcre mice with a myeloid specific knockout of *Tgm2* exhibited unchanged BALF eosinophilia, general cellularity and IL-5 levels suggesting a redundant role for myeloid TGM2 in HDM-induced AAI (II, Supplementary Figure 3 b-d). It is possible that HSCP do not express sufficient LysM to be affected by the myeloid knockout, so progenitor TGM2 could still contribute to central trained immunity while it is redundant in mature myeloid cells. As TGM2 can posttranscriptionally modify proteins by histaminylation and serotonylation[499,500] including histone 3[501], the enzyme can possibly contribute to reprogramming of myeloid cells or myeloid progenitors. It is unlikely that HDM directly affects bone marrow cells, but the inflammatory response it evokes may reach bone marrow compartments and influence myelopoiesis and myeloid progenitors as shown before in

an infection model[335]. On the other hand, HDM contains allergens with protease activity (e.g. *Der f1*) which may disrupt mucosal barriers and facilitate entry of trace amounts of allergen into the circulation or bone marrow. Thus, allergen-training of myeloid cells may share some resemblance with the innate memory of airway epithelial progenitor cells elucidated by Ordovas-Montanes *et al.*[343] as the persistent inflammatory responsiveness prevails at the level of progenitors.

The main read-outs of successful "classical" trained innate immunity in vitro assays are gene expression or concentration of TNF and IL-6[327,329,328,337,336], as most infectious diseases stipulate "classical" activation of macrophages. Also in the cases of oxLDL-induced trained monocytes[502,503], TNF and IL-6 production is the preferred endpoint as they contribute to pathology of atherosclerosis and coronary heart disease[504,505]. HDM exposure also activates IL-6, TNF and other "classically"-associated cytokines and chemokine in human macrophages (I, Figure 5 A), and we found that autocrine TNF effects on macrophages contribute to inducing the type 2 trained immunity program (II, Figure 3 I, j). This is surprising in the perspective of TNF as an inhibitor of type 2 inflammation, as elaborated in the context of cutaneous leishmaniasis, cancer and skin wounds[506–508]. However, reflecting the late appearance of CCL17 in our type 2 trained immunity program, TNF also has been shown to play a role in the induction of late CCL17-mediated inflammation in arthritis[509]. The transcriptomic, epigenetic, and metabolic state of the cell which responds to TNF likely dictates the signal processing and differential outcomes.

Which impact a type 2 trained macrophage progenitor population might have on helminth infections is unclear. Enhanced type 2 immunity mediated by trained macrophages could lead to more successful expulsion of parasites but could also be linked to enhanced type 2 inflammation. A recent study shows that previous HDM-induced AAI and its concomitant pulmonary eosinophilia, alternative macrophage activation and presence of ILC2s resulted in reduced Ascaris burden in the lung and more efficient killing of larvae by eosinophils[510]. Whether protection is conferred also in sites distal to acute type 2 inflammation and persists if the tissue loses its type 2 inflammatory imprint, remains unknown. The fact that protection against allergy in helminth-infected individuals fades after deworming or after change to a Western lifestyle rather suggests that trained type 2 immunity is transient[511,512].

In addition, trained type 2 immune responses may possibly result in potentiated pro-resolving mediators/pathways, similar to the phenomenon of LPS tolerance. We have not addressed whether the pro-resolving activity of macrophages is enhanced by allergen training or by the mechanisms involved in type 2 trained immunity.

In allergic disease, a sustained type 2 immune response is likely to be driving disease. We do not know if seasonal allergens are similarly able to induce trained immunity in innate cells but one could speculate that it is possible, as activators of the innate immune system are present in seasonal allergenic agents as well[513], possibly influencing the immune response as adjuvants.

Epithelial dysfunction, an accepted pathological mechanism in asthma, implicates the potentially vicious crosstalk of trained macrophages and epithelial cells (II, Figure 6 e-g). We do not know whether an epithelial cue on its own can be a training stimulus for AM, but allergen-trained macrophages enhance the production of type 2 inducing mediators (CCL17 and cysLTs) upon activation with the epithelial alarmin IL-25 (II, Figure 6 e, f), possibly due to their enhanced IL17RB expression. This is intriguing, as Ordovas-Montanes *et al.* found a similar IL17RB upregulation in their trained epithelial progenitors[343]. IL-25 and IL17RB signaling may thus play a central role in type 2 trained immunity.

Although we have evidence that AM as well as MDM from allergic individuals are activated (II, Figure 1, 3), we do not know exactly which macrophage in the airway is conveying trained responses. AM are thought to be tightly repressed by the tissue environment[268] although there is evidence that this regulation may be undermined in type 2 airway inflammation[295]. On the other hand, AM are poised for a host defense response, i.e. have high proinflammatory potential even in the absence of chronic inflammation[272]. In inflammatory situations, immigrated monocytes differentiating to AM[292] may mask or dilute evidence of trained immune functions of resident macrophages In addition, the infiltration of inflammatory monocytes may result in inflammatory gene signatures that are falsely attributed to inflammatory reprogramming of resident macrophages. Thus, when performing bulk-sequencing analyses, macrophages have to be subjected to rigorous sorting.

Although centrally trained myeloid cells enter the spotlight in type 2 immunity, a comprehensive analysis of HDM-activated macrophages in the context of their tissue environment remains important. Locally activated macrophages from sensitized or control donors could answer which environmental effects and tissue-derived cues determine the overall phenotype and functions of HDM-trained macrophages. Although this is easily doable in mice, it would be more exciting to assess in the human setting. The effect of real-life HDM exposure on tissue and myeloid progenitor imprinting in humans could e.g. be assessed using an allergen exposition chamber.

7.9. Conclusion

Our work identifies important mechanisms of macrophage reprogramming in type 2 airway inflammation (Figure 14) and raises new questions such as the epigenetic basis of the inflammatory macrophage memory in allergic asthma. As the sample size of participants in (II) was small, more patients need to be recruited to reproduce the current results. Also, *in vivo* proof how allergen-trained macrophages influence HDM-induced airway inflammation and whether the mechanisms identified in vitro play a role *in vivo* as well, is missing. Studies addressing these questions are currently underway.

In addition to elucidating pathomechanisms, a major aim of this thesis was to explore new alleys of immunomodulatory therapy against airway inflammation. *Hpb* GDH could become a first-in-class immunomodulator but further research is necessary to bring this molecule into clinical testing. For example, the protein's stability, toxicity, pharmacokinetics and its effects on the respiratory mucosa need to be clarified. Also, the effect of GDH-mediated immunomodulation on immune responses towards infectious diseases needs to be characterized, especially if prolonged efficacy (potentially by reprogrammed macrophages) is present.

As AM are considered to effectively suppress overshooting inflammation[289] and also the lungs of healthy people are constantly exposed to airborne irritants and allergens, it is unclear why macrophages of allergic patients become reprogrammed while those of healthy persons do not. Genetic susceptibility could play a role, but an initial inflammatory insult along with transient proinflammatory tissue environment could be necessary to start AM reprogramming. A similar model is well studied about the relation of childhood virus infections with asthma onset, where an initial respiratory infection and its concomitant inflammation trigger sustained alterations in the lungs causing wheeze or asthma[514,515]. Thus, targeting macrophages for immunomodulation, especially in the light that allergen-trained macrophages propagate type 2 inflammation, should be pursued also in other respiratory diseases.

Understanding programs of macrophage eicosanoid fingerprints and modulating their eicosanoid production are powerful options to influence type 2 inflammation in the allergic and asthmatic airways. In the duration of this thesis, eicosanoid responses of macrophages in response to acute and repeated allergen stimulation were characterized and reprogramming of macrophages in a trained type 2 immunity program with the potential to drive type 2 inflammation via cysLTs, CCL17 and proinflammatory epithelial cross-talk was described for the first time. In addition, a helminth derived product, which alters the macrophage eicosanoid output to an anti-inflammatory and type 2-suppressing profile, was tested in human cell models

as well as in animal models of AAI and led to the emergence of a candidate biotherapeutic which modulates macrophage reprogramming in allergic airway disease. Therefore, with the focus on macrophages and their eicosanoid production in allergic inflammation, new aspects have been offered for understanding the etiology of allergic asthma. Further characterization of macrophage and progenitor reprogramming can contribute to understanding the chronicity of allergic diseases and thus offer new starting points

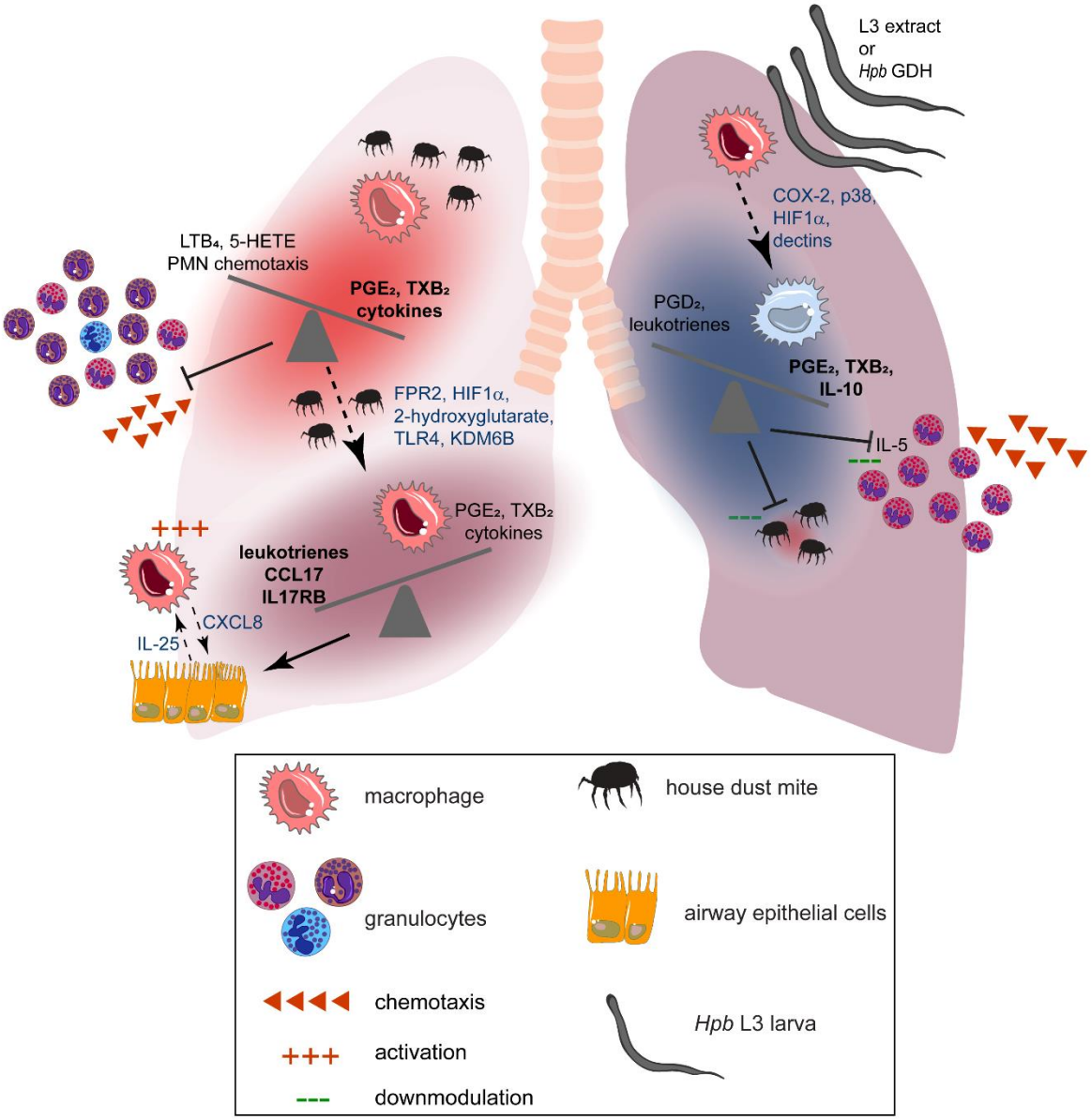

Figure 14 Summary of the findings described in this dissertation

for preventive or therapeutic strategies in the future. Finally, *Hpb* GDH might become the base for a new class of immunomodulators, affecting local cells to adapt an anti-inflammatory profile and modulate type 2 inflammation using their own resources. Thus, this dissertation provides new cues for the deciphering of mechanisms in allergy and allergic asthma, and to progress and evolution of treatment.

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9 Lay summary

9.1. English

Eicosanoids are signaling molecules derived from fatty acids. They for example play a pathological role in allergic asthma. Macrophages, innate immune cells, can produce eicosanoids. As it was unclear, which eicosanoids are produced by macrophages during type 2 immunity and allergic inflammation, we established a mass-spectrometry method to detect and quantify a broad panel of eicosanoids simultaneously. We used it to measure the eicosanoid output of macrophages which were exposed to house dust, a common allergen. After 24 h exposure, macrophages switched their eicosanoid profile from lipoxygenase to cyclooxygenase-derived metabolites. This was due to alterations of gene expression and signaling pathways (p38 MAP kinase, TLR). Macrophages are long-lived cells and remain on the surface of the lung, where they can come in contact with allergens multiple times. In an experimental model of repeated house dust mite exposure, human macrophages upregulated molecules that promote type 2 immune responses and allergic inflammation (CCL17 and cysteinyl leukotrienes). This trained immunity program was mediated via surface receptors (FPR2 and TLR4), self-activation by the cytokine TNF, the metabolite 2-hydroxyglutarate as well as lysine demethylase activity (KDM6B). The exaggerated leukotriene production was reflected in macrophages from allergic donors. To dampen e.g. leukotrienes responsible for symptoms in allergic asthma, we studied a helminth extract which works by suppressing leukotriene production in human macrophages and at the same time enhancing anti-inflammatory molecules (the cytokine IL-10 and the eicosanoid prostaglandin E2). The extract was effective in a mouse model of allergic asthma as well as in granulocytes of human asthmatic patients. We analyzed the components of the extract and identified a protein, Hpb glutamate dehydrogenase, which also suppressed symptoms of allergic asthma in the mouse model. Thus, a deep understanding of eicosanoid production in macrophages can point out new possibilities for their modulation and lead to improved therapeutic opportunities for allergic asthma and related diseases.

9.2. Deutsch

Eikosanoide sind Signalmoleküle, die aus Fettsäuren hergestellt werden, und spielen zum Beispiel bei allergischem Asthma eine krankmachende Rolle. Immunzellen, die Eikosanoide herstellen können, sind sogenannte Makrophagen, Zellen der angeborenen Immunantwort. Da unklar war, welche Eikosanoide von Makrophagen während dem der Allergie zugrunde liegenden Typ-2-Immungeschehen produziert werden, etablierten wir eine Methode, verschiedene Eikosanoide gleichzeitig zu bestimmen und quantifizieren. Damit bestimmten wir das Eicksanoid-Profil von humanen Makrophagen, die mit Hausstaubmilben-Allergenen mittels eines Extrakts stimuliert wurden. Das Eikosanoid-Profil wurde über 24h hinweg von Lipoxygenase-Metaboliten hin zu Zyklooxygenase-Metaboliten umprogrammiert, was durch Veränderung von Genexpression und Signalprozessen (p38 MAK Kinase, TLR) bedingt wurde. Makrophagen sind langlebige Zellen und verbleiben auf der Oberfläche der Lunge, wo sie auch mehrmals mit Allergenen in Kontakt kommen können. In einem experimentellen Modell von mehrmaliger Hausstaub-Stimulation von humanen Makrophagen fanden wir, dass Signalmoleküle, die Typ-2- Immunität und allergische Entzündung fördern (das Protein CCL17 und die Eikosanoid-Signalmoleküle Cysteinyl-Leukotriene) vermehrt produziert wurden. In den Makrophagen geschah diese Art von „Immun-Training“ durch Oberflächenrezeptoren (TLR4 und FPR2), während auch Selbstaktivierung mit dem Zytokin TNF, der Metabolit 2-Hydroxyglutarat und die Aktivität einer Lysin-Demethylase (KDM6B) eine Rolle in der Umprogrammierung spielten. Die überschießende Leukotrien-Produktion war auch in Makrophagen von Hausstaubmilben-Allergikern sichtbar. Um diese überschießende Typ-2-Entzündung durch u.a. Leukotriene während allergischem Asthma und so die Symptome abmildern zu können, untersuchten wir einen Helminthen-Extrakt, der auf menschliche Makrophagen modulatorisch wirkt, indem er die Leukotrien-Produktion unterdrückt und stattdessen zu Produktion von anti-entzündlichen Molekülen (dem Zytokin IL-10 oder dem Eikosanoid Prostaglandin E2) führt. In einem Mausmodell für Hausstaubmilben-allergisches Asthma und bei Granulozyten von Asthma-Patienten war der Extrakt effektiv. Daraufhin untersuchten wir die Zusammensetzung und identifizierten das Protein *Hpb* Glutamat-Dehydrogenase, die ebenfalls Symptome des allergischen Asthmas im Mausmodell dämpfen konnte. Es zeigt sich also, dass sich aus tiefem Verständnis von Eicosanoid-produzierenden Makrophagen Möglichkeiten zur deren Modulation ergeben und so neue Methoden zur verbesserten Therapie von allergischem Asthma und verwandten Erkrankungen entspringen können.

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Annex

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II

A trained type-2 immunity program drives exaggerated leukotriene and CCL17 responses in allergen-experienced macrophages

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Abstract

Infectious or inflammatory insults can “train” innate immune cells to respond more readily to subsequent insults. Macrophages govern type-2 inflammation, e.g. in allergic asthma, but whether these cells develop an inflammatory memory to allergens was not known. We found that macrophages differentiated from bone marrow cells or blood monocytes from house dust mite (HDM)-allergic mice or human patients produced excessive amounts of type-2-inducing mediators (cysteinyl leukotrienes (cysLTs) and CCL17). When trained with HDM *in vitro*, macrophages exhibited a pro-inflammatory M2-like phenotype, producing high amounts of CCL17 and cysLTs upon challenge. Mechanistically, allergen-induced training depended on TLR4 and lysine demethylase KDM6B for cysLTs and formyl-peptide receptor 2- and TNF-signaling for CCL17. HDM-experienced macrophages produced increased amounts of TCA metabolites and 2-hydroxyglutarate, which could replicate HDM-induced training. Thus, a common aeroallergen can trigger an inflammatory macrophage memory that is distinct from trained immunity in infections and specifically drives mediators of type-2 inflammation.

Introduction

Macrophages are central effector cells of type-2 inflammation in allergic diseases or during infection with worm parasites[1]. In response to cues from the tissue micro-environment, macrophages can dynamically adapt their metabolism and effector functions, allowing them to fulfill a broad array of immunological functions[2,3]. External cues such as pathogen molecules or sterile inflammatory challenges can further trigger bioenergetic and epigenetic reprogramming, resulting in persistently altered macrophage effector functions[4–8]. Thus, monocytes and macrophages can be re-reprogrammed or "trained" to exhibit either increased or blunted responsiveness to repeated challenge(s) and to drive both protective and pathological "innate memory" responses[9–12]. Trained immunity is not limited to peripheral tissue macrophages but extends to bone marrow progenitors that provide "central trained immunity" to sustain and augment innate effector responses[12,13].

In addition to bioenergetic reprogramming, macrophages can switch their metabolism of bioactive lipids (eicosanoids), which are key regulators of type-2 inflammation, e.g. in allergic asthma[14,15]. While other eicosanoid-producing myeloid cells (e.g. eosinophils) are cleared from the lung during resolution of acute inflammation[16,17], macrophages persist[18]. Macrophages in the airways of asthmatic patients show an aberrant eicosanoid metabolism and a shift towards proinflammatory mediators[19,20]. Proinflammatory eicosanoids, particularly the leukotrienes (LTs) and recruited (monocyte-derived) macrophages are key drivers of allergic airway inflammation (AAI) triggered by house dust mite (HDM)[21–23]. HDM represents the most prominent aeroallergen and approximately 50% of asthmatics are sensitized to it[24]. The severity of HDM-triggered AAI correlates with numbers of M2 polarized macrophages in the airways of both humans and mice[25,26] and trained monocytes/ macrophages can regulate the development of asthma after viral infection.[27,28] However, if and how

77 macrophages may be “trained” to contribute to chronic type-2 airway inflammation in
78 allergic asthma remained unclear.
79 We found that macrophages exposed to HDM *in vivo* or *in vitro* persistently upregulate
80 effector mechanisms of type-2 inflammation. Allergen-experienced macrophages
81 showed an enhanced inflammatory responsiveness, characterized specifically by an
82 elevated production of mediators of type-2 immunity (cysteinyl leukotrienes (cysLTs)
83 and CCL17). The allergen-induced inflammatory macrophage memory depended on
84 TLR4-, FPR2- and TNF-signaling as well as on KDM6B. Previous exposure to the
85 HDM-induced metabolite 2-hydroxyglutarate induced a trained CCL17 response in
86 macrophages challenged with HDM. Thus, exposure to aeroallergens can persistently
87 alter the metabolism and effector functions of macrophages and their progenitors,
88 providing an innate memory for chronic type-2 airway inflammation.
89
90 **Results**
91 **Macrophages from HDM-allergic patients and HDM-sensitized mice produce**
92 **increased amounts of mediators of type-2 inflammation**
93 Macrophages represent key regulators of lung homeostasis and immunity and they
94 govern type-2 airway inflammation by producing eicosanoids and CCL17[19,21,23,29]. To
95 study a potential inflammatory macrophage memory in the context of type-2
96 inflammation, we assessed eicosanoid and CCL17 responses in macrophages
97 isolated from the airways or differentiated from blood monocytes of HDM-allergic or
98 healthy donors (Supplementary Fig.1 a). Airway macrophages from HDM-allergic
99 patients synthesized high levels of 5-lipoxygenase metabolites (cysLTs, 5-HETE) and
100 CCL17 in response to unspecific activation with a calcium ionophore (Fig.1 a).
101 Peripherally derived macrophages from HDM-allergic patients (aMDM) produced
102 higher concentrations of LTs compared to aMDM from healthy donors (Fig.1 b).

Similarly, murine macrophages isolated from bronchoalveolar lavage fluid (BALF) or differentiated from bone marrow progenitors of PBS- or HDM-sensitized mice (Supplementary Fig.1 b) showed an elevated ionophore-induced production of type-2 inducing mediators (*CCL17* and cysLTs) (Fig.1 c, d). In contrast, differences in the 15-lipoxygenase metabolite 15-HETE, a marker of type-2 airway inflammation, were inconsistent between human and murine macrophages (Supplementary Fig.2 a, b). Differences in eicosanoid responses were not due to altered expression of LT-biosynthetic enzymes (*Alox5* or *Ltc4s)* in murine or human macrophages (Supplementary Fig.2 c-e). Together, these data suggested that allergic airway inflammation is associated with an inflammatory macrophage memory, characterized by persistently increased leukotriene and CCL17 responses in tissue macrophages and their peripheral progenitors.

Peripherally derived macrophages from HDM-allergic mice show an increased metabolite output

As trained immunity is linked to changes in macrophage metabolism[7,30,31], we performed a targeted metabolomic analysis to quantify amino acid- and TCA-cycle metabolites in HDM-experienced macrophages. BMDM from HDM-sensitized mice showed an overall increase in the output of amino acids and TCA-cycle intermediates (Fig.2 a), which included metabolites (glutathione, glutamic acid, adenosine) involved in LT biosynthesis, M2 activation and type-2 immunity (Fig.2 a-c)[32–35]. In addition, 2-hydroxyglutaric acid (2-HG), an inhibitor of α-ketoglutarate-dependent dioxygenases (e.g. lysine demethylases)[36] was increased (Fig.2 d). Despite these metabolite changes, bioenergetic parameters indicative of glycolysis (ECAR) or mitochondrial respiration (OCR) were largely unaltered in HDM-sensitized as compared to mock-sensitized BMDM (Fig.2 e,f). Although bioenergetic reprogramming has been

implicated in macrophage polarization and trained immunity[7,30,31], baseline expression of M2 marker genes in BMDM and genes related to the glycolytic pathway, *Pfkfb3*, *Slc2a1*, *Pdk1* were largely unaltered (Fig.2 g). Thus, despite the lack of prototypic bioenergetic changes involved in trained immunity[8,13], priming of macrophage progenitors in the bone marrow during HDM-induced AAI resulted in the increased production of metabolites involved M2 polarization and type-2 immunity.

Tgm2 is elevated in progenitor cells but myeloid *Tgm2* is dispensable during HDM-induced AAI

Trained immunity can be induced at the level of progenitors and the observed metabolite and mediator changes (Fig. 1, 2) suggested that bone marrow progenitors were affected by HDM-induced airway inflammation[13,10,12]. When comparing transcriptional profiles of isolated hematopoietic stem cells and progenitors (HSCP; lineage-negative, c-kit-positive bone marrow cells) from PBS- and HDM-sensitized mice, we found *Tgm2*, a member of the transglutaminase family and a marker of M2 activation[37], involved in eicosanoid production and allergic airway inflammation[38,39], to be consistently upregulated in HSCP from HDM-sensitized mice (Supplementary Fig.3 a). Thus, we used a genetic approach to test effects of *Tgm2* in HDM-induced AAI. We crossed *Tgm2*$^{flox/flox}$ mice to LysM^{cre} mice to obtain animals with a myeloid specific deletion of *Tgm2* (Supplementary Fig.3 b). However, when sensitized and challenged with HDM, leukocyte and eosinophil counts or IL-5 concentration in BALF were not different between mice with a myeloid *Tgm2* deficiency and littermates (Supplementary Fig.3 c) consistent with similar histological outcomes (Supplementary Fig.3 d). These data suggest that even though *Tgm2* is highly induced during HDM-induced AAI and involved in the regulation of eicosanoid production, other pathways mediate HDM-induced macrophage training.

In vitro HDM training induces a persistent inflammatory macrophage phenotype with enhanced FPR2- and TNF-dependent CCL17 production upon challenge

As myeloid cells can be trained or tolerized by pathogen-associated molecular patterns (e.g. β-glucan or LPS),[40] we studied whether human monocyte-derived macrophages (aMDM)[14,41,42] could be trained with HDM *in vitro*. Thus, aMDM were stimulated with HDM on day 7 of differentiation, challenged after a 5-day wash-out period on day 13 and harvested for RNA sequencing analysis on day 14 (Supplementary Fig.1 c). Trained macrophages with or without HDM challenge exhibited an increased expression of genes involved in M2 polarization (e.g. *IRF4*, *CD163*, *IL4I1*, *VEGFA*) and chemokine/cytokine signaling (CCL17, CCL18, CXCL9) at day 14 (Fig.3 a-d, Supplementary Table 1, 2). In contrast, the HDM-driven induction of interferon-induced genes, implicated in anti-viral immunity, (e.g. OASL, OAS2/3, ISG15/20, USP18, CMPK2) was reduced in HDM-trained aMDM compared to control aMDM (Fig.3 b,d). KEGG pathway analysis showed an enrichment for TNF-signaling in HDM-trained macrophages (Fig.3 e, Supplementary Fig. 4a) as well as cytokine-cytokine receptor interaction, PI3K-Akt-, chemokine-, and JAK/STAT signaling (Supplementary Fig.4 a). After HDM challenge cytokine-cytokine receptor interaction and type-1 immune response-related pathways were enriched (Supplementary Fig.4 b).

In particular, *CCL17* expression was strongly induced in HDM-trained aMDM both without and with challenge (Fig.3 a-d), and significantly elevated CCL17 levels were confirmed in supernatants of trained aMDM (Fig.3 f). CCL17 was initially upregulated after HDM training (day 8), and further increased dose-dependently (Supplementary Fig.4 c) during the 5 days resting phase (until day 13), even in the absence of further stimulation (Fig.3 g). In contrast to HDM, a commonly studied trigger of trained immunity (β-glucan, BGP) did not lead to enhanced CCL17 production (Supplementary Fig.4 d). HDM-trained macrophages also showed a more rapid *CCL17* induction in

response to HDM challenge and no significant changes in cell viability (Supplementary Fig.4 e, f). The trained CCL17 response was dependent on training with the complete extract as training with purified allergens (Der f1 and Der f2) did not result in enhanced CCL17 upon challenge (Supplementary Fig. 4 g). A set of 19 differentially expressed genes (DEG) was shared between trained and trained + challenged macrophages (Supplementary Fig.4 h), including the formyl peptide receptor 2 (FPR2), which mediates HDM-induced cell activation[43,44]. Blockade of FPR2 during HDM-training prevented the massive production of CCL17 after HDM challenge (Fig.3 h) and inhibited the initial induction of TNF after HDM-training (Fig.3 i). As TNF signaling is implicated in the initiation of CCL17-mediated inflammation[45], we neutralized TNF during the training phase, which abrogated the trained CCL17 response (Fig.3 j). The trained CCL17 response was also evident in monocyte derived macrophages differentiated with M-CSF (M-MDM) (Supplementary Fig.4 i) and presence of GM-CSF during the training phase only did not further elevate *CCL17* (Supplementary Fig.4 i). However, intensified *CCL17* expression upon challenge of trained M-MDM was only evident when cells were continuously exposed to GM-CSF during the resting phase, suggesting that the presence of GM-CSF in the tissue microenvironment can greatly enhance the trained CCL17 response (Supplementary Fig.4 i). Thus, HDM training can reprogram human macrophages via FPR2 activation and autocrine TNF signaling to produce high levels of the type-2 chemokine CCL17 upon a secondary challenge.

2-hydroxyglutarate production and HIF1α activation drive allergen-induced macrophage training

HIF1α governs metabolic reprogramming and trained immunity[8]. BMDM from HDM-sensitized mice expressed increased *Hif1a* (Fig.2 g) and several genes related to HIF1α signaling were upregulated in trained macrophages[46–51] (*VEGFA*, *MMP2*,

207 *PLOD2*, *EGR1*, *VLDLR*, *RBP1*, *PPFIA4*) (Fig.3 c,d). In trained human macrophages,
208 the HDM-driven induction of *HIF1a* differed greatly between blood donors (Fig.4 a),
209 and HIF1α inhibition during the training phase only partially abrogated the trained
210 CCL17 response (Fig.4 b). However, HIF1α inhibition reduced the ability of
211 macrophages to activate glycolysis upon HDM training (Fig.4 c), while mitochondrial
212 respiration or cell viability remained unaffected (Supplementary Fig.5 a,b). HIF1α is
213 inactivated by α-ketoglutarate (α-KG)-dependent dioxygenases like prolyl-
214 hydroxylases (PHDs)[52] and 2-hydroxyglutarate (2-HG) competes with α-KG to inhibit
215 PHDs and histone demethylases[36]. 2-HG was elevated in murine BMDM from HDM-
216 sensitized mice as well as in human aMDM after acute HDM exposure (Fig.2 d, Fig. 4
217 d). The expression ratio between lactate dehydrogenase (*LDHA*) and L-2-
218 hydroxyglutarate dehydrogenase (*L2HGDH*), the enzymes responsible for 2-HG
219 production and metabolism in macrophages, gradually increased with HDM-training,
220 acute stimulation and challenge (Fig.4 e). When added during acute activation of
221 macrophages with LPS, 2-HG potentiated the induction of *CCL17*, *IL1B* and *PTGS2*
222 (Fig.4 f). After resting for 5 days and activation with HDM, 2-HG-trained macrophages
223 produced more CCL17 in comparison to acutely HDM exposed macrophages (Fig. 4
224 g), thus providing a potential link to HIF1α-dependent macrophage training. Taken
225 together, 2-HG rewired macrophages towards a type-2 trained phenotype,
226 characterized by exaggerated CCL17 responses.
227
228 **Allergen-trained human macrophages are metabolically activated and exhibit a**
229 **TLR4- and KDM6B-dependent trained leukotriene response**
230 As we observed an exaggerated cysLT production by aMDM from HDM-allergic donors
231 and BMDM from HDM-sensitized mice (Fig.1 b, d), we assessed whether HDM-training

in vitro would affect cysLT production in human macrophages. In contrast to acutely HDM-stimulated aMDM, trained aMDM produced high amounts of cysLTs (Fig.5 a) and further 5-LOX metabolites (5-HETE and LTB_4, Supplementary Fig.6 a) concomitant with high amounts of prostanoids (Supplementary Fig.6 a). The increased eicosanoid output of trained and challenged aMDM was only partially paralleled by changes in LOX and COX pathway gene expression (Supplementary Fig.6 b). To identify potential mechanisms underlying the increased LT response in HDM-trained aMDM, we analyzed our RNAseq data sets for factors involved in M2 activation and type-2 inflammation: *IRF4*, *SOCS1*, *CCL17*, *CCL18*, *CD209*, *IL32* and *CHI3L1*[53] were persistently upregulated in HDM-trained macrophages (Fig.5 b). As an IRF4 – KDM6B - GM-CSF axis had previously been implicated in the epigenetic control of M2 polarization and type-2 immunity[54,55], we studied the expression of the histone demethylase *KDM6B* (JMJD3) in our training setup. Acute stimulation with HDM resulted in the upregulation of *KDM6B* (Fig.5 c) and inhibition of KDM6B during training attenuated the enhanced cysLT response upon HDM challenge (Fig.5 d). In contrast, KDM6B was not involved in the increased CCL17 response in HDM-trained macrophages (Supplementary Fig.6 c).

Alveolar macrophages react to HDM via TLR2 and TLR4 activation, which can result in the activation of KDM6B[54,56]. TLR2-TLR4-double deficient BMDM were unable to respond to HDM (Supplementary Fig.6 d), but TLR2 neutralization during training did not affect the trained CCL17 or cysLT response in human macrophages (Supplementary Fig.6 e). However, TLR4 neutralization during training reduced the exaggerated cysLT response in HDM-trained human aMDM (Fig.5 e). As TLR4-, IRF4- and KDM6B-driven macrophage activation is associated with metabolic reprogramming[7,57,58], we performed metabolic flux analysis in allergen-trained macrophages. Compared to untrained macrophages, HDM-trained macrophages

showed a stronger increase in their oxygen consumption in response to an uncoupling agent (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, FCCP), showing an elevated spare respiratory capacity 6 days after HDM-training (Fig.5 f, g), concomitant with increased glycolysis (Fig.5 h). Despite mediating the trained cysLT response, TLR4 or KDM6B were not required for the metabolic activation of HDM-trained macrophages (Supplementary Fig.6 g,h). After challenge, HDM-trained macrophages exhibited a similar metabolic activation as acutely HDM-exposed macrophages, characterized by increased OCR and ECAR (Supplementary Fig.6 i), suggesting an overall metabolic activation in response to HDM. Taken together, HDM-training induced the metabolic activation of macrophages and the TLR4- and KDM6B-dependent overproduction of cysLTs.

Allergen training differs from trained immunity elicited by microbial products

As previous studies investigating antimicrobial trained immunity focused on IL-6 and TNF responses in macrophages to quantify training effects[4,5,59,10], we analyzed a broad panel of cytokines and chemokines immediately after allergen-training (day 8), after 5 days of rest (day 13) and 24h post-HDM-challenge (day 14). Except for CCL17, HDM-training evoked an increase of cytokines (at day 8) which returned to baseline after the resting phase (Fig. 3f, g, Supplementary Fig.7 a). After 24h HDM challenge, all cytokine and chemokine responses (except for CCL17, Fig. 3f, g) were similarly elevated in HDM-trained compared to acutely stimulated control macrophages (Supplementary Fig.7 b). By contrast, after 8h of HDM challenge, IL-6 was significantly increased in trained relative to control macrophages (Supplementary Fig.7 c), in line with exaggerated HDM-induced *Il6* expression in BMDM from HDM-sensitized mice (Supplementary Fig.7 d). Thus, HDM-trained macrophages show a more rapid IL-6

and CCL17 response during HDM challenge (Supplementary Fig. 7c, 4e), which persist for the type-2 inducing chemokine CCL17.

In addition to their elevated CCL17 and cysLT output, HDM-trained macrophages produced high amounts of prostanoids upon challenge (Supplementary Fig.6 a). Enzymes involved in the production of PGE_2 (*PTGES* (mPGES1), *PTGS2* (COX2), *PTGS1* (COX1)) were strongly induced by HDM challenge of trained aMDM (Supplementary Fig.6 b) and mPGES1, the key terminal enzyme for PGE_2 synthesis, remained upregulated even 6 days after HDM-training (Supplementary Fig.7 e). Cyclooxygenase 2 (*PTGS2*), which produces the substrate for mPGES1, was rapidly induced after HDM stimulation, which may explain the enhanced HDM-triggered PGE_2 production in HDM-experienced human and murine macrophages (Supplementary Fig.6 a, Supplementary Fig.7 g). Thus, HDM training of macrophages results in a persistently enhanced CCL17 response and eicosanoid metabolism, while cytokines related to the classical activation of macrophages and prototypic trained immunity remain largely unaffected.

Macrophages from HDM-allergic donors exhibit a proinflammatory gene expression profile, facilitating a proinflammatory crosstalk with airway epithelial cells

To further study the relevance of HDM-induced macrophage training in human disease, we subjected monocyte-derived macrophages from HDM-allergic individuals (Table 1) and non-allergic volunteers to transcriptional and mediator analysis. RNAseq analysis of aMDM from HDM-allergic compared to non-allergic donors yielded 88 differentially expressed genes (Fig.6 a, b, Supplementary Table 3), indicating a transcriptionally altered macrophage phenotype after 7 days of *ex vivo* differentiation. *IL17RB*, which is induced by TNF signaling and involved in allergic asthma[60], and

CHDH, a regulator of macrophage metabolism and proinflammatory effector functions[61], were upregulated in both *in vitro* HDM-trained and challenged aMDM (Supplementary Table 2) as well as in aMDM from allergic donors compared to non-allergic donors (Fig.6 a, c). Furthermore, *TNFSF10* (TRAIL), *CLEC4D* (dectin-3), LGALS12 (galectin-3) and *IL12RB1*, all implicated in macrophage activation[62–65], were upregulated in aMDM of allergic donors (Fig.6 a). In addition to increased proinflammatory gene expression and LT production (Fig. 1 b), aMDM from HDM-allergic donors exhibited a higher HDM-induced production of IL-1β compared to aMDM from non-allergic donors (Fig.6 d). As the IL-17 receptor B (*IL17RB*) was consistently upregulated in HDM-allergic and trained and challenged macrophages (Fig.6 a, c), we studied whether HDM-trained aMDM showed an altered response to the type-2 inducing epithelial alarmin IL-25, the ligand of IL17RB. The combination of previous HDM training and IL-25 stimulation resulted in a strong CCL17 and cysLTs response (Fig.6 e,f). In turn, when exposed to cell-free supernatant of HDM-trained and challenged macrophages, normal human bronchial epithelial cells (NHBEs) produced significantly higher amounts of CXCL8 compared to cells stimulated with supernatants from acutely HDM-stimulated MDM (Fig.6 g). Together, these data identify a type-2 inducing macrophage reprogramming in human allergic asthma, which may facilitate the proinflammatory crosstalk between allergen-experienced macrophages and the airway epithelium and thus perpetuate type-2 inflammation.

Discussion

Previous studies have shown that innate memory responses on the level of ILC2s and epithelial stem cells can contribute to type-2 inflammation in the context of allergic airway inflammation and nasal polyposis[66,67]. Here, we describe an allergen-driven trained immunity program in macrophages that may contribute to type-2 inflammation

in allergic asthma. Macrophages trained with HDM extract *in vitro* produced high amounts of CCL17 and cysLTs, which are both potent type-2 promoting mediators[68–70]. To our knowledge, previous studies of trained immunity have not described altered eicosanoid profiles. Here, we found that HDM-driven trained type-2 immunity was characterized by exaggerated LT responses in bone marrow- or monocyte-derived macrophages from HDM-sensitized mice or HDM-allergic patients. This heightened LT response could be mimicked by HDM-training and re-exposure of aMDM *in vitro* and was dependent on TLR4 and KDM6B, previously shown to fine-tune macrophage gene expression independently of histone demethylation[71]. Given the potent type-2-inducing functions of LTs, trained LT responses in macrophages may represent an important mechanism of central trained immunity in the context of type-2 airway inflammation.

In addition to altered eicosanoid responses, increased expression of interferon response genes was present in aMDM from HDM-allergic patients, which were also upregulated by acute HDM stimulation. This may contribute to virus-triggered acute exacerbations, which are common in asthmatics[72]. However, HDM-trained macrophages showed a reduced induction of IFN response genes, suggesting that – at least for aMDM from healthy donors – repeated HDM exposure can trigger an immune regulatory state that is partially comparable to the phenomenon of “LPS tolerance”[59] and reminiscent of recently described immunoparalyzed alveolar macrophages after resolution of type-1 inflammation[11]. However, the M2-like gene expression profile and exaggerated CCL17 and LT responses of HDM-trained macrophages is likely to result in a pathologic type-2 immune bias rather than general immunosuppression.

Of note, gene expression profiles of HDM-trained and challenged macrophages from healthy blood donors showed minimal overlap with profiles of macrophages from HDM-allergic patients at baseline. This may be due to relatively high experimental doses of

HDM *in vitro* while *in vivo*, macrophages are likely exposed to lower doses but over a longer period of time. While *in vitro* trained aMDM exhibited an M2-like transcriptional profile, allergic aMDM showed a downregulation of immunoregulatory genes (e.g. *MERTK* and *CD84*), suggesting that tolerogenic pathways may be defective in monocytes/ macrophages from allergic individuals. However, upregulation of *IL17RB* was evident in both allergic aMDM as well as after *in vitro* HDM-training and challenge, suggesting that increased IL-25 responsiveness is a feature of HDM-trained macrophages in allergy patients. While we did not observe heightened baseline *CCL17* expression in aMDM from allergic donors, sputum-derived airway macrophages cultured *ex vivo* for a short time (20 min) released high levels of CCL17 compared to aMDM or compared to airway macrophages from healthy controls. This may suggest that aberrant CCL17 responses depend on tissue priming of monocytes/ macrophages in the lung during type-2 airway inflammation[19].

Importantly, HDM-trained macrophages did not show a general increase in the production of proinflammatory cytokines, but a specific induction of cysLTs and CCL17, which elicit type-2 immune responses. Thus, allergen-induced trained type-2 immunity appears to be distinct from trained immunity programs driven by microbial products[4,12]. Together with a large range of cytokines and eicosanoids, HDM training transiently induced TNF, which is a negative regulator of M2 polarization in cancer or infectious diseases[73–75]. In arthritis, in contrast, TNF signaling was described to be important at early timepoints, while TNF-induced CCL17 appeared as a late mediator[45], mirroring the kinetics of HDM training in macrophages. In the HDM training pathway we uncovered, TNF acted as an early initiator of type-2 inflammatory responses and M2 polarization. These data argue that TNF has a complex effect on M2 myeloid pathways that require further analysis. One prediction emerging from our work is that TNF may have differential inhibitory or enhancing effects depending on acute or chronic

signaling via the two TNF receptors. Understanding these different effects may help understand the spectrum of outcomes observed in TNF neutralization for chronic inflammatory diseases.

Altered expression of TNF-response genes and trained type-2-inducing effector functions persisted during macrophage differentiation from bone marrow- or monocyte progenitors isolated from HDM-sensitized mice or HDM-allergic patients. Thus, HDM exposure does not only trigger local inflammatory responses, but results in a persistent reprogramming of myeloid progenitors and/ or monocytes giving rise to macrophages with elevated type-2 effector responses. The metabolic and epigenetic pathways that drive central type-2 immunity on the level of myeloid progenitors in the bone marrow remain to be determined. Despite the upregulation of *Tgm2* in HSCP from HDM-sensitized mice and its role in allergic airway inflammation[76], myeloid *Tgm2* was dispensable for HDM-triggered AAI. However, as HSCP express low levels of lysozyme, TGM2 deletion may be inefficient in these cells. Thus, we cannot exclude that early expression of TGM2 in HSCP may contribute to the proinflammatory imprinting of monocytes, particularly as TGM2 has been implicated in histone modifications[77].

Indeed, the induction of a trained CCL17 response by 2-HG, an inhibitor of histone demethylases, suggests an involvement of histone modifications in HDM-induced trained immunity. The abrogation of the exaggerated cysLT response in HDM-trained macrophages by an inhibitor of the histone demethylase KDM6B further suggests that trained CCL17 and cysLT responses are uncoupled and that H3K27 demethylation by KDM6B may drive the expression of a positive regulator of LT production. Thus, future studies should assess, which histone modifications are present in HDM-experienced macrophages and how individual modifications regulate CCL17 and cysLT responses, respectively.

Based on our study design, we cannot discern whether HDM itself or the type-2 inflammation triggered by HDM is responsible for macrophage training *in vivo*. The finding that HDM-training of macrophages *in vitro* resulted in exaggerated CCL17 and LT responses upon challenge suggests that resident macrophages in the airways can be trained by HDM itself. In contrast, central trained type-2 immunity on the level of myeloid progenitors in the bone marrow is more likely evoked by the type-2 immune response triggered by HDM. A persistent HDM-driven immune cell reprogramming may also explain why maternal exposure to HDM during pregnancy led to exacerbated type-2 inflammation in offspring in a mouse model of HDM allergy[78]. Our findings suggest that terminally differentiated macrophages can develop an inflammatory, type-2 enhancing memory in response to allergens and that reprogramming of myeloid progenitors further amplifies trained type-2 immunity in allergic airway inflammation. As trained myeloid cells may contribute to the chronicity of type-2 airway inflammation and regulate tissue responses in situations of inflammatory challenge, it will be important to further decipher myeloid trained immunity in the context of type-2 immune responses.

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Methods

Human Study Participants

The institutional ethics committee of the Technical University of Munich (TUM) approved the study. House dust mite allergic patients (IgE concentration and symptom score see Table 1) and healthy control subjects were recruited at the Allergy Section, Department of Otolaryngology, TUM School of Medicine. All participants gave informed written consent in accordance with the Declaration of Helsinki before sampling. The single study visit consisted of completion of questionnaires (SNOT22, MiniRQLQ, PSQ20), blood draw and sputum induction. The clinical diagnostic laboratory of the hospital performed differential blood cell counts, specific mite IgE and total IgE analysis. Patient characteristics are displayed in Table 1.

Sputum Induction and Processing

Sputum was induced as previously described (Haimerl et al. 2020). In brief, participants inhaled 400 µg Salbutamol before inhaling ascending concentrations of sterile NaCl (3%, 4%, 5%, Apotheke des Klinkums Rechts der Isar, Munich) for 7 min each. After every inhalation, coughing was encouraged, and expectorated sputum was collected and cooled on ice. Forced expiratory volume in 1s (FEV_1) was monitored throughout the procedure. Sputum plugs were selected, disintegrated using Sputolysin solution (Merck Millipore, Burlington, MA, USA) and cells were dispersed to a single cell suspension for preparation of cytospins and further cell sorting. Supernatants were stored at -70°C in 50% methanol [v/v] for eicosanoid analysis and undiluted for cytokine assays.

631 **Alveolar macrophage isolation from induced sputum**

632 Sputum cells were suspended in PBS and incubated with erythrocyte concentrate and
633 RosetteSep Monocyte Isolation cocktail (Stemcell Technologies, Vancouver, Canada)
634 for 20 min at room temperature. Macrophages were extracted by density centrifugation
635 using Lymphoprep (Abbott Diagnostics Technologies, Oslo, Norway).

636

637 **Monocyte-derived macrophage isolation and culture**

638 Peripheral blood was drawn from cubital venipuncture of healthy volunteers or HDM-
639 allergic patients. Both groups provided informed written consent in accordance with
640 the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated
641 via density gradient using Lymphoprep (Abbott Diagnostics Technologies) and $CD14^+$
642 monocytes were selected by magnetic bead separation (CD14 Micro Beads, Miltenyi,
643 Bergisch-Gladbach, Germany) and differentiated to alveolar-like monocyte-derived
644 macrophages (aMDM) in RPMI-1640 with 10% fetal bovine serum, 2 mmol/L L-
645 glutamine and 10 ng/mL Gentamicin ("complete medium", all Thermo Fisher, Waltham,
646 MA, USA) supplemented with 10 ng/mL rhGM-CSF (Miltenyi) and 2 ng/mL rhTGF-β1
647 (PeproTech, Hamburg, Germany). Cells were differentiated at 37°C and 5% CO_2 for 6
648 days with a change of 50% medium and replenishment of cytokines on the third day.
649 If indicated, $CD14^+$ cells were differentiated in the presence of 20 ng/mL M-CSF
650 (Miltenyi).

651

652 **Culture and stimulation of normal human bronchial epithelial cells**

653 Commercially available normal human bronchial epithelial cells (NHBEs, Lonza, Basel
654 Switzerland) in passage 4 were grown to 90% confluency in bronchial epithelial growth
655 medium (BEGM, Lonza) and starved overnight in bronchial epithelial basal medium

(BEBM, Lonza). NHBEs were stimulated for 24h with cell-free pooled supernatants from trained human aMDM or complete medium at a final concentration of 10% [v/v].

Experimental Animals

C57BL/6J mice were obtained from Charles River Laboratories (Sulzfeld, Germany). *Tgm2*$^{flox/flox}$ mice (*Tgm2*^{tm1RMgr}, Jax stock number 024694) were bred at the animal facility of the Max-Plack Institute of Biochemistry in Martinsried, Germany before crossing to LysMcre (*Lyz2*$^{tm1(cre)Ifo}$, Jax Stock number 004781) mice at the animal facility of the Helmholtz Center Munich and maintained on a C57BL/6J background to generate LysM^{Cre}Tgm2$^{flox/flox}$ mice with myeloid-specific ablation of Tgm2. All mice were were housed under specific pathogen-free conditions in individually ventilated cages (VentiRack; Biozone, Margate, UK), fed by standard pellet diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) and water ad libitum at the animal facility of the Helmholtz Center Munich. 6-8 weeks old female mice were used for experiments. All animal experiments were approved by the local authorities (Regierung von Oberbayern, Az. 55.2-2532.Vet_02-18-95) and performed at the Helmholtz Center Munich according to institutional guidelines.

House dust mite-induced allergic airway inflammation

Allergic airway inflammation to house dust mite (HDM) was induced as previously described[15]. In short, mice were intranasally sensitized on day 1 to 1 µg HDM extract (*Dermatophagoides farinae,* Stallergènes, Antony, France) and challenged with 10 µg of the same HDM extract on days 8, 9, 10 and 11. Sham-sensitized mice received the same amount of PBS. Three days after the last challenge, mice were sacrificed and bronchoalveolar lavage (BAL) was performed as previously described[15] for differential cell counts and BAL cytokine analysis.

BAL macrophage isolation

Total BAL cells were plated in complete medium and incubated at 37°C and 5% CO_2 for 3 h. Wells were washed twice rigorously with warm PBS and medium was replenished. Phenotype and purity of adherent cells were assessed by microscopy. AM were stimulated for 10 min with calcium ionophore A23187 (5 µmol/L, Merck Chemicals) at 37°C and centrifuged at 4°C. Supernatants were harvested for liquid chromatography tandem mass spectrometry (LC-MS/MS, 50% [v/v] in analysis-grade methanol, Applichem), and for ELISA. Cell lysates were harvested for qPCR analysis (RLT, Quiagen, Hilden, Germany, with 1% β-mercaptoethanol). All samples were stored at -70°C until analysis.

Bone marrow-derived cell isolation and culture

Femurs and tibiae from HDM- or PBS-treated mice were kept in RPMI-1640 medium on ice. Bones were flushed using a 30G needle and cells were forced through a 70 µm cell strainer to prepare a single cell suspension. Cells were resuspended to 1 x 10^6 cells/mL in complete medium with 10 ng/mL rmGM-CSF (Miltenyi) and 2 ng/mL rhTGF-β1 (PeproTech) and differentiated for 7 days with an exchange of 50% medium and replenishment of cytokines on the third day. For isolation of murine hematopoietic stem cells and progenitors (HSCP), total bone marrow cells were incubated with lineage-depletion antibody cocktail and magnetic microbeads (Miltenyi) followed by CD117 positive selection (c-Kit MACS Microbeads, Miltenyi). HSCP were suspended in complete medium (± 10 µg/mL HDM) and left to rest at 37 °C for 20 min before calcium ionophore A23187 stimulation (5 µmol/L) and harvest.

In vitro Training Experiments

For macrophage training (see Supplementary Fig.1 c), D7 aMDM were plated at a density of 10^6 cells/mL and incubated with HDM (10 µg/mL, Stallergenes), β-glucan (10 µg/mL, Invivogen), purified HDM allergens Der f1 and Der f2 (both 10 µg/mL, Citeq) or (2R)-Octyl-α-hydroxyglutarate (10 µmol/L, Cayman Chemical) in the presence (1h pre-incubation before training stimulus) or absence of the following neutralizing antibodies (nAB) or pharmacological inhibitors: TLR2 nAB, TLR4 nAB (both 10 µg/mL, Invivogen), TNF nAB (10 µg/mL, R&D Systems), acriflavine (3 µmol/L, Sigma Aldrich), GSK-J4 (5 µmol/L, Tocris), A485 (100 nmol/L, Tocris) or PBP 10 (10 µg/mL, Tocris). After 24h, supernatant was removed, cells were washed in PBS and fresh medium containing GM-CSF and TGF-β was replenished. On day 11, 50% of medium was exchanged and cells were challenged with 10 µg/mL HDM for 24h in fresh medium containing GM-CSF and TGF-β on day 13 for 24h. Macrophages were harvested as described above. Supernatants were also collected on days 8 and 13.

RNA extraction

Cells lysed in RLT buffer (Quiagen, Hilden, Germany) with 1% ß-mercaptoethanol were mixed with equal volumes of 70% ethanol and RNA was extracted using a spin-column kit according to the supplier's instructions (Zymo Research, Freiburg, Germany) including a DNAse I digestion step. 2 µg RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher) according to the manufacturer's instructions or submitted for total RNA sequencing.

Real-time quantitative polymerase chain reaction (RT qPCR)

10 ng cDNA was used as template. A list of primers (4 µmol/L, Metabion Munich, Germany) can be found in the Supplement (Supplementary Table 4). FastStart

Universal SYBR Green Master Mix (Roche, Mannheim, Germany) was used and the analysis was performed on a ViiA7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The expression levels were normalized to *GAPDH* as house-keeping gene and relative gene expression was represented as $2^{-\Delta CT}$ ($\Delta C_T = C_{T(housekeeper)} - \Delta C_{T(gene)}$).

Metabolic Flux analysis

0.05 x 10^6 MDM or BMDM were plated per well on a Seahorse Miniplate (Agilent) and cultured for training (aMDM) or stimulated overnight (BMDM). On the day of assay, medium was exchanged to the Seahorse XF RPMI medium, pH 7.4 (Agilent) containing 10 mmol/L glucose (Sigma Aldrich), 1 mmol/L pyruvate and 2 mmol/L L-glutamine (both Thermo Fisher). The Mito Stress Test (Agilent) was performed according to the manufacturer's instructions with subsequent injections of oligomycin (1 µmol/L, Agilent), FCCP (aMDM: 1 µmol/L, BMDM: 2 µmol/L, Agilent) and rotenone and antimycin A (both 0.5 µmol/L, Sigma). After the assay, cells were lysed in 40 µL RIPA buffer (Thermo Fisher) and protein concentration was measured for normalization (Pierce BCA protein assay kit, Thermo Fisher).

Metabolomics analysis

0.5 x 10^6 aMDM or BMDM were pelleted for targeted metabolomics. Targeted metabolite quantification by LC-MS was carried out at the Metabolomics Core Facility of the Max Planck Institute for Immunobiology and Epigenetics in Freiburg, Germany. Metabolites were extracted using ice-cold 80:20 methanol:water solution followed by LC separation an Agilent 1290 Infinity II UHPLC inline using a Phenomenex Luna propylamine column (50 x 2 mm, 3 µm particles) with a solvent gradient of 100% buffer B (5 mM ammonium carbonate in 90% acetonitrile) to 90% buffer A (10 mM NH_4 in

760 water) and a flow rate from 1000 to 750 µL/min. Autosampler temperature was 5°C
761 and injection volume was 2 µL. Mass spectrometry was performed using an Agilent
762 6495 QQQ-MS operating in MRM mode and MRM setting were optimized separately
763 for all compounds using pure standards. Data was processed using an in-house R
764 script.
765
766 **Western Blotting**
767 Protein quantification via immunoblotting was performed as described previously. In
768 short, cells were lysed in RIPA buffer (Thermo Fisher) containing protease and
769 phosphatase inhibitor (Roche). 10 µg protein lysate were separated on a Bis-Tris 12-
770 well gel (Thermo Fisher) for 60 min at 125 V. Proteins were blotted to a PVDF
771 membrane (Merck Millipore) for 50 min at 20 V. Membranes were blocked using 5%
772 milk in Tris-buffered saline containing 0.5% Tween (Merck) followed by overnight
773 incubation with primary antibodies (rabbit anti-human mPGES1 1:1000, Cayman
774 Chemical, Ann Arbor, MI, USA; mouse-anti human β-actin, 1:10000 Sigma-Aldrich).
775 Respective HRP-conjugated secondary antibodies were added for 1 hour (goat anti-
776 rabbit IgG or goat anti-mouse IgG, Santa Cruz Biotechnology, Dallas TX, USA) before
777 development using WestFemto ECL substrate (Thermo Fisher) and detection on an
778 ECL ChemoCam imaging system (Intas Science Imaging Instruments). Protein band
779 density was assessed via LabImage 1D software (Kapelan Bio-Imaging).
780
781 **Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)**
782 ***Prostanoids***
783 For the analysis of prostanoids, 200 µL supernatant were spiked with isotopically
784 labeled internal standards (PGE_2-d4, PGD_2-d4, TXB_2-d4, $PGF_2\alpha$-d4, 6-keto $PGF_1\alpha$-
785 d4), 100 µL EDTA solution (0.15 M) and 600 µL ethyl acetate. Samples were vortexed

and centrifuged at 20,000 g for 5 min. The organic phase was removed, and the extraction was repeated with 600 µL ethyl acetate. The organic fractions were evaporated at a temperature of 45°C under a gentle stream of nitrogen. The residues were reconstituted with 50 µL of acetonitrile/water/formic acid (20:80:0.0025, v/v/v) and transferred to glass vials.

LC-MS/MS analysis was performed using an Agilent 1290 Infinity LC system (Agilent, Waldbronn, Germany) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer QTRAP 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative ESI mode. The chromatographic separation was carried out using a Synergi Hydro-RP column (150 × 2 mm, 4 µm particle size and 80 Å pore size; Phenomenex, Aschaffenburg, Germany). A gradient program was employed at a flow rate of 300 µL/min. Mobile phase A was water/formic acid (100:0.0025, v/v) and mobile phase B was acetonitrile/formic acid (100:0.0025, v/v). The analytes were separated under gradient conditions within 16 min. The injection volume was 10 µL. The gradient program started with 90% A for 1 min, then mobile phase A was decreased to 60% within 1 min, held for 1 min, further decreased to 50% within 1 min and held for 2 min. Within 2 min, mobile phase A was further decreased to 10% and held for 1 min. Within 1 min, the initial conditions were restored, and the column was re-equilibrated for 6 min. Mass spectrometric parameters were set 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.

For analysis and quantification, Analyst Software 1.6 and Multiquant Software 3.0 (both Sciex, Darmstadt, Germany) were used, employing the internal standard method (isotope dilution mass spectrometry). Calibration curves were constructed using linear regression with $1/x^2$ weighting.

HETE and LTB_4

Quantification of HETE and LTB_4 was done as described previously[79]. In brief, 150 - 200 µL supernatant were spiked with the corresponding deuterated internal standards and extracted by liquid-liquid-extraction using ethyl acetate. Analytes were separated using a Gemini NX C18 RP-LC-column (150 mm × 2 mm I.D., 5 µm particle size and 110 Å pore size from Phenomenex, Aschaffenburg, Germany) under gradient conditions with water and acetonitrile as mobile phases, both containing 0.01% ammonia solution. The LC system was coupled to a mass spectrometer 5500 QTrap (Sciex, Darmstadt, Germany) equipped with a Turbo-V-source operating in negative electrospray ionization mode. Data acquisition was done using Analyst Software V 1.6 and quantification was performed with MultiQuant Software V 3.0 (Sciex) employing the internal standard method (isotope dilution mass spectrometry).

RNA sequencing and analysis

Library preparation was performed at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany, using the Ultra II NEB stranded kit (New England Biolabs, Ipswich MA, USA) on a Biomek i7 automation system (Beckman Coulter, Krefeld, Germany). Libraries were sequenced on an Illumina NextSeq500 sequencer using a 75 cycles high output kit (Illumina). Reads were aligned to hg18 by GSNAP (version 2018-07-04) using Ensembl 87 for the support of splicesite detection. FeatureCounts (version 1.6.2) was used to assign the reads to genes with the help of Ensembl 87 annotation to return the raw expression matrix. Differential expression analysis of protein coding genes was performed using DESeq2 of the DEBrowser (Bioconductor version 3.10 using R version 3.6.2), assessing genes with a fold change ≥ 2 and an adjusted p value ≤ 0.05. Gene ontology analysis was performed using

DEBrowser's inbuilt KEGG enrichment analysis. Overlap of gene lists was assessed with the R package GeneOverlap version 1.23.0[80].

Data analysis and statistics

Data were analysed using Graphpad Prism 8 (Graphpad, San Diego, CA, USA). T-test or Mann-Whitney test were used to compare two populations depending on normal distribution. For comparison of more groups, Friedmann test or one-way ANOVA was used with correction for multiple comparisons as indicated in the figure legends. See figure legends for respective details of statistical tests and sample size. Heatmaps were generated using Morpheus software, a free online tool provided by the Broad Institute.

Data Availability

The data that support the findings of this study are available upon request from the corresponding author. Analyzed RNAseq data are shown in Supplementary Tables 1, 2 and 3, and complete RNAseq data are deposited at ArrayExpress (identifiers: E-MTAB-9210, E-MTAB-9214, E-MTAB-9215).

References of the Method section

79. Josipovic, I. *et al.* PAFAH1B1 and the lncRNA *NONHSAT073641* maintain an angiogenic phenotype in human endothelial cells. *Acta Physiol.* (2016) doi:10.1111/apha.12700.
80. Li, S. & Mount Sinai. *GeneOverlap: Test and visualize gene overlaps. R package version 1.22.0*. (2019).

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Author contributions

A.L. and J.E.v.B. designed experiments, A.L., F.H., S.B., F.A. and P.H. performed experiments, P.J.M. and F.H. bred *Tgm2*-deficient mice and performed all experiments related to them, A.L., F.H., F.A., D.T., Y.G. and J.E.v.B. analyzed data, F.H., C.A., Y.S. and D.T. performed LC-MS/MS analysis, Y.G. assembled RNA sequencing results, A.M.K. and E.J.P. organized metabolomic analysis, A.L. P.J.M., C.O., C.B.S.W., and J.E.v.B. interpreted data, A.M.C. provided patient samples, A.L. and A.M.C. performed patient sampling, A.L. and J.E.v.B. wrote the manuscript.

Competing interests

CSW received grant support from Allergopharma, PLS Design as well as Zeller AG and received speaker honoraria from Allergopharma. All authors have no conflict of interest in relation to this work.

Figures and Figure Legends

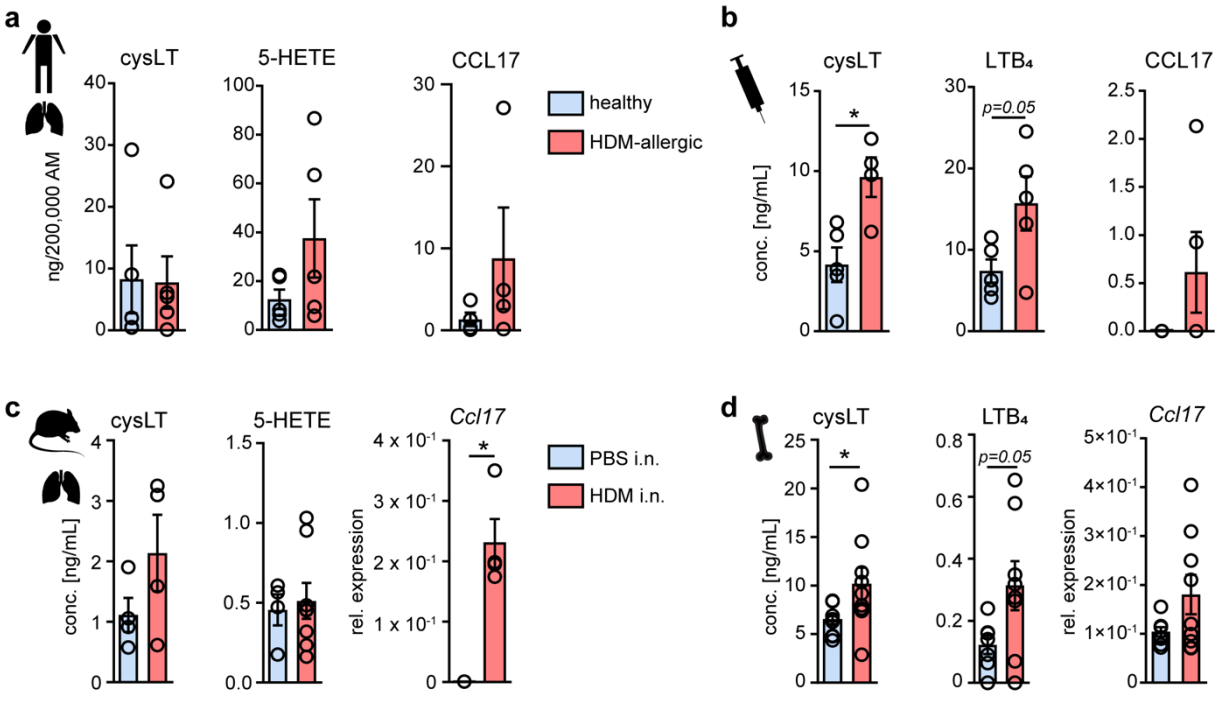


Figure 1: Macrophages from HDM-sensitized humans and mice exhibit trained LT responses

a: Baseline cysLT (EIA), 5-HETE (LC-MS/MS) and CCL17 (ELISA) concentration in supernatants of sputum-derived macrophages from healthy vs. HDM-allergic human donors (after 10 min Ca^{++} ionophore, n=5 per group, Mann-Whitney test test)

b: Baseline cysLT (EIA), LTB_4 (LC-MS/MS) and CCL17 (ELISA) concentration in supernatants of aMDM from healthy vs. HDM-allergic human donors (after 10 min Ca^{++} ionophore, n=5 per group, Mann-Whitney or unpaired t-test)

c: Baseline cysLT concentration (EIA, after 10 min Ca^{++} ionophore, normalized to RNA) in supernatant of, and *Ccl17* gene expression (normalized to *Gapdh*) from BAL AM of PBS- vs. HDM-sensitized mice (n=2-4 per group, unpaired t- test)

d: Baseline total cysteinyl leukotrienes (EIA) and LTB_4 (LCMS) in supernatant of, and *Ccl17* gene expression (normalized to *Gapdh*) in BMDM from PBS- vs HDM-sensitized mice (after 10 min Ca^{++} ionophore, normalized to RNA, Mann-Whitney test, n=5-9 per group)

Data are presented as mean + SEM *p<0.05, **p<0.01. i.n.=intranasal administration, BALF=bronchoalveolar lavage fluid

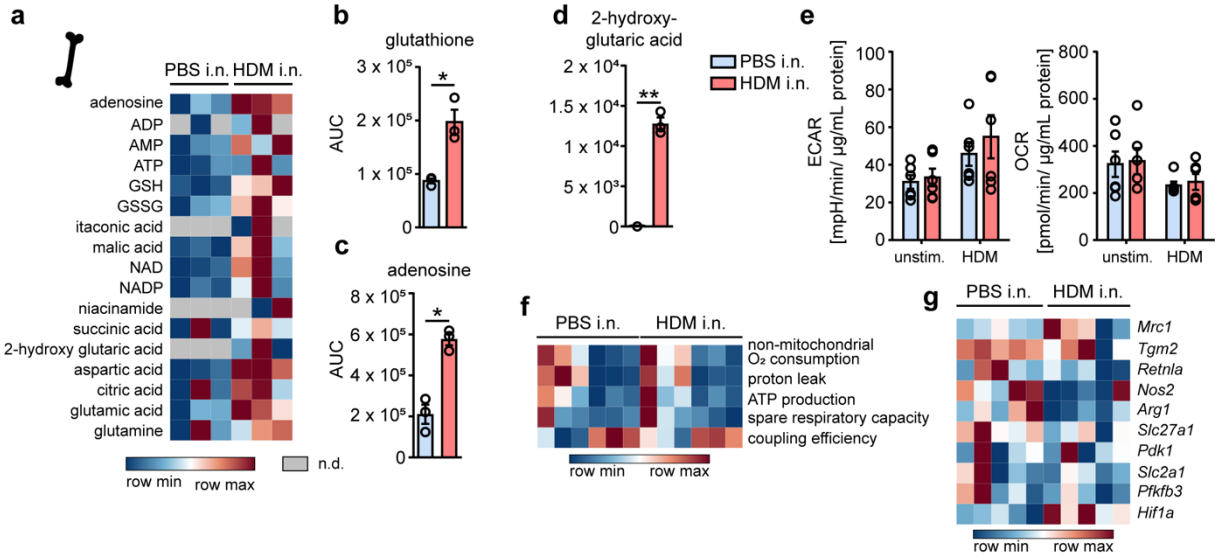


Figure 2: *In vivo* HDM experience alters the metabolism of bone marrow derived murine macrophages

a: Overview of targeted metabolomics, and details for

b: glutathione,

c: adenosine, and

d: 2-hydroxyglutaric acid of BMDM from PBS- vs. HDM-sensitized mice (n=3 per group, paired t-test)

e: Baseline glycolysis and mitochondrial respiration of BMDM from PBS- vs. HDM-sensitized mice, unstimulated or after 24h HDM *in vitro* (n=6 per group)

f: Metabolic parameters of unstimulated BMDM from PBS- vs. HDM-sensitized mice (n=6 per group)

g: Gene expression of macrophage polarization markers and metabogenes in unstimulated BMDM from PBS- vs. HDM-sensitized mice (n=5 per group)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01. AUC=area under curve, ECAR=extracellular acidification rate, OCR=oxygen consumption rate, i.n.=intranasal instillation

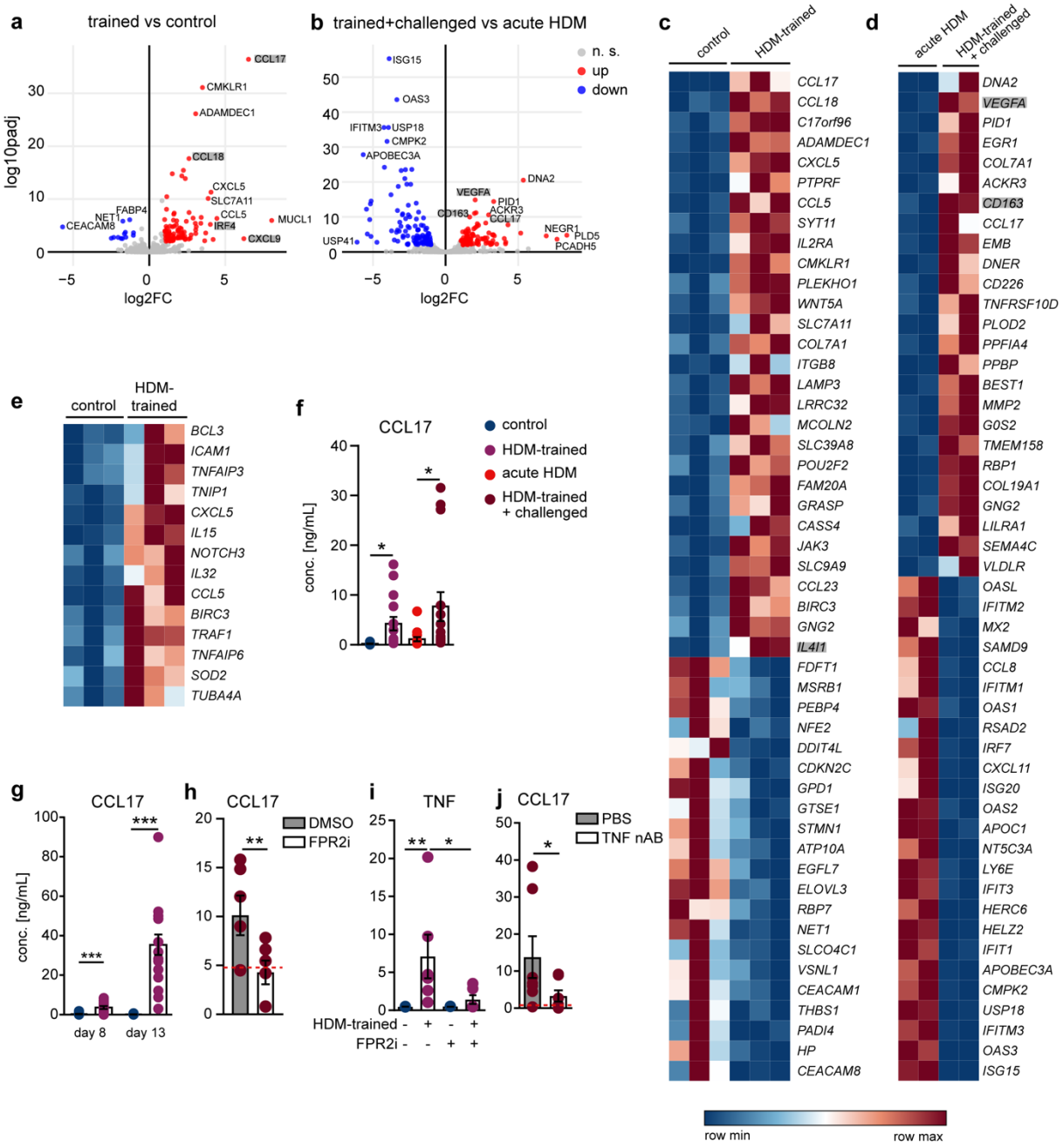


Figure 3: HDM training of differentiated human monocyte-derived macrophages drives a type-2 promoting phenotype via FPR2 and TNF signaling

a: Volcano plot of DEG (fold change>2, p_{adj} <0.05) in HDM-trained versus control macrophages (n=3)

b: Volcano plot of DEG (fold change>2, p_{adj} <0.05) in HDM-trained and challenged versus acutely HDM-exposed macrophages (n=2)

c: Heatmap of 29 most significantly upregulated and 21 most significantly downregulated DEG in HDM trained versus control macrophages (n=3)

d: Heatmap of 25 most significantly upregulated and 25 most significantly downregulated DEG in HDM trained and challenged versus acutely HDM-exposed macrophages (n=2)

e: Genes related to TNF signaling, enriched via KEGG pathway analysis in HDM trained versus control macrophages (n=3)

f: CCL17 concentration in supernatants of control and HDM-trained human macrophages on day 14 (ELISA, n=15, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Holm-Sidak's multiple comparisons test)

g: CCL17 concentration in supernatants of control and HDM-trained human macrophages on days 8 and 13 (ELISA, n=17, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

h: CCL17 concentration in supernatants of challenged HDM-trained human macrophages, ± FPR2 inhibition during training phase, on day 14 (ELISA, after 10 min Ca^{++} ionophore stimulation, n=6, paired t test). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

i: TNF concentration in supernatants of control and HDM-trained human macrophages ± FPR2 inhibition during training phase, on day 8 (ELISA, n=6, Friedmann test, Dunn's multiple comparisons test)

j: CCL17 concentration in supernatants of challenged HDM-trained human macrophages, ± TNF neutralization during training phase, on day 14 (ELISA, n=7, paired t test). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01, ***p<0.001. nAB=neutralizing antibody, FPR2i=Formyl peptide receptor 2 inhibitor, nAB=neutralizing antibody

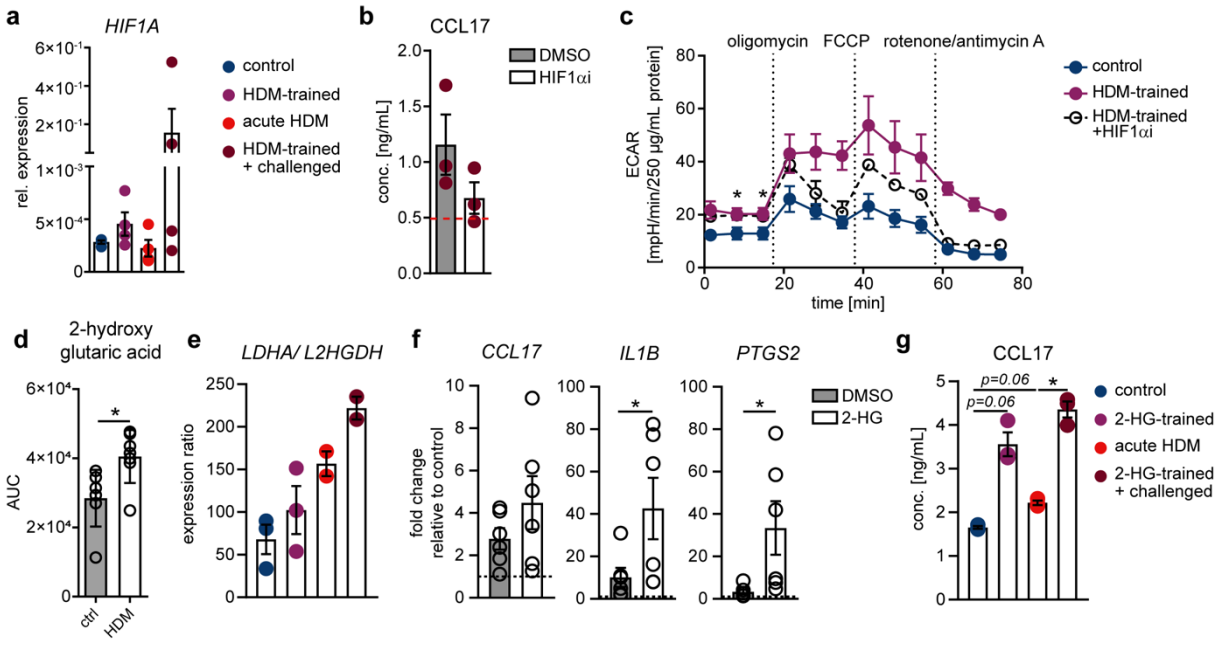


Figure 4: 2-hydroxyglutarate elicits a trained type-2 immunity program in human macrophages

a: *HIF1A* gene expression in control and HDM-trained human macrophages (normalized to *GAPDH*, n=4)

b: CCL17 concentration in supernatants of challenged HDM-trained human macrophages, ± HIF1α inhibition during training phase, on day 14 (ELISA, n=3). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

c: Extracellular acidification rate of control and HDM-trained human macrophages, ± HIF1α inhibition during training phase, on day 14 (n=3)

d: 2-hydroxyglutaric acid in control and HDM-exposed human macrophages on day 7 (n=7, paired t test)

e: Expression ratio of *LDHA* and *L2HGDH* in control and HDM-trained macrophages (normalized read counts from RNAseq dataset, n=2-3)

f: LPS versus control, fold change of *CCL17*, *IL1B* and *PTGS2* ± 2-hydroxyglutarate (normalized to *GAPDH*, n=6, paired t test). Dotted lines indicate fold change=1 (no change)

g: CCL17 concentration in supernatants of control and 2-HG-trained macrophages ± HDM challenge (n=3, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

Data are presented as mean + SEM (bar graphs). *p<0.05. HIF1αi=HIF1α inhibitor, AUC=area under the curve, 2-HG=2-hydroxyglutarate.

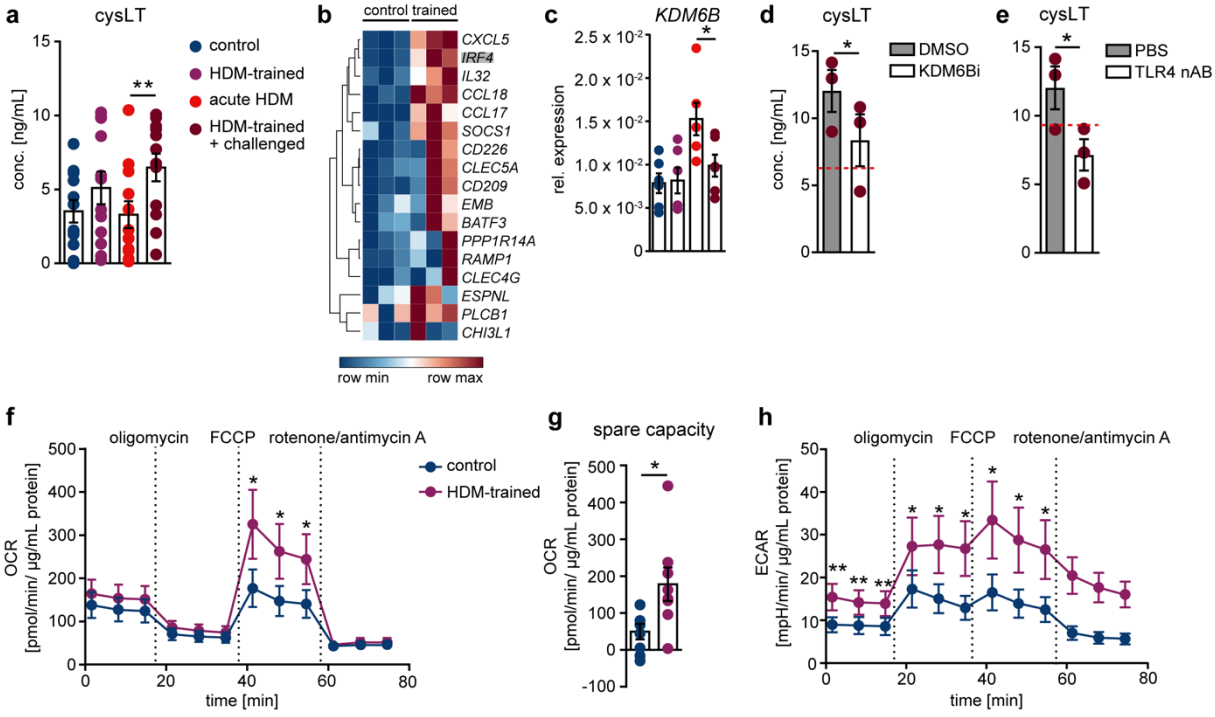


Figure 5: TLR4 and KDM6B activation lead to a trained cysLT response in human macrophages

a: cysLT concentration in supernatants of control and HDM-trained human macrophages on day 14 (EIA, after 10 min Ca^{++} ionophore stimulation, n=12, repeated

measures one-way ANOVA with Geisser-Greenhouse correction, Holm-Sidak's multiple comparisons test)

b: Gene expression of M2-related genes in control and HDM-trained human macrophages on day 14 (normalized read counts from RNA sequencing dataset, n=3)

c: *KDM6B* gene expression in control and HDM-trained human macrophages (normalized to *GAPDH*, n=6, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

d: cysLT concentration in supernatants of challenged HDM-trained human macrophages, ± KDM6B inhibition during training phase, on day 14 (EIA, n=3, paired t test). Red dotted line indicates cysLT concentration of aMDM + acute HDM exposure.

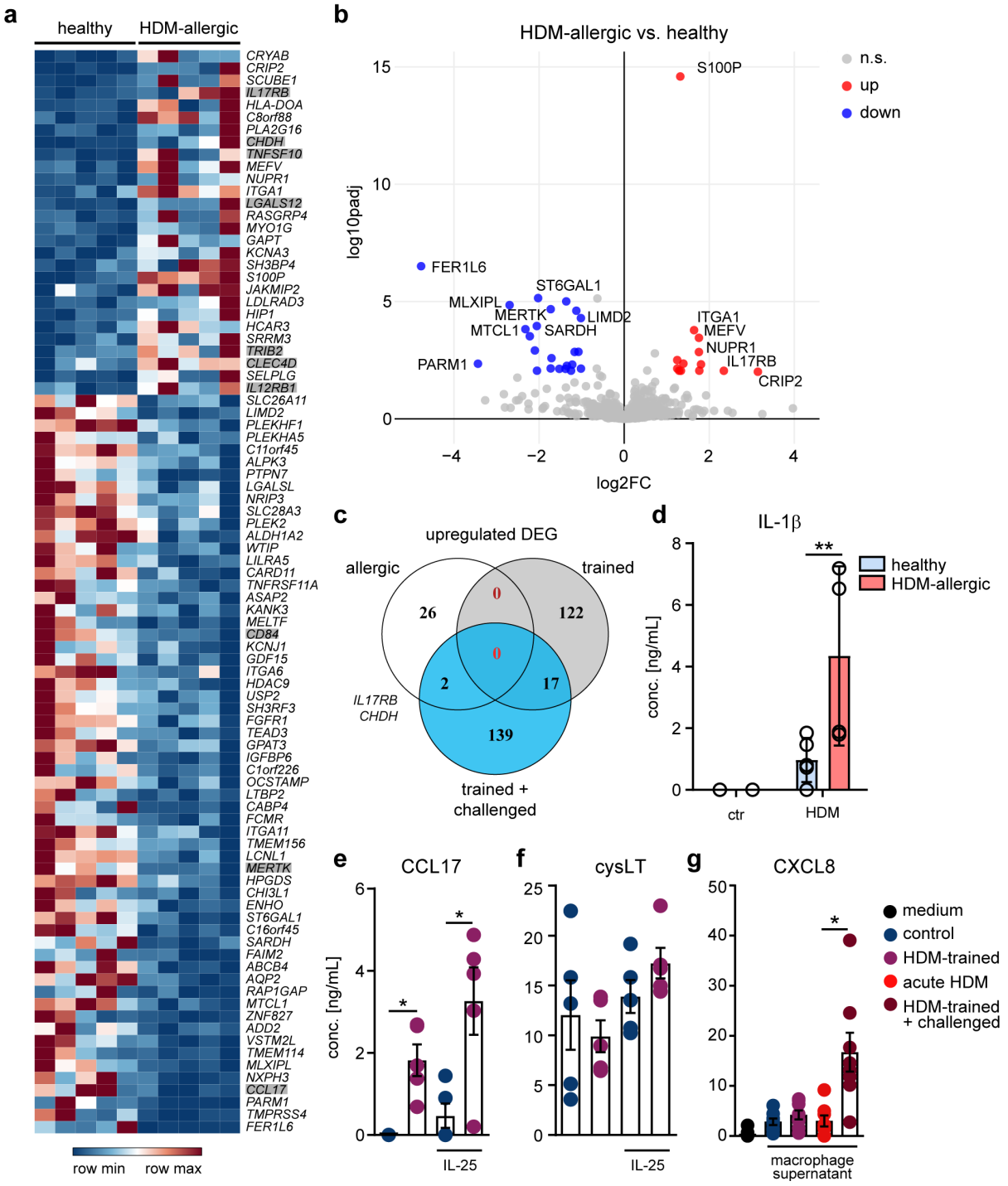
e: cysLT concentration in supernatants of challenged HDM-trained human macrophages, ± TLR4 neutralization during training phase, on day 14 (EIA, after 10 min Ca^{++} ionophore stimulation, n=3, paired t test). Red dotted line indicates cysLT concentration of aMDM + acute HDM exposure.

f: Oxygen consumption rate and

g: spare respiratory capacity, and

h: Extracellular acidification rate of control and HDM-trained macrophages on day 14 (n=7-8, paired t-test)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01, ***p<0.001. KDM6Bi=lysine demethylase 6B inhibitor, nAB=neutralizing antibody, OCR=oxygen consumption rate, ECAR=extracellular acidification rate



1013

1014 **Figure 6: HDM-experienced human monocyte-derived macrophages display**

1015 **suppressed immunoregulatory gene expression and engage in a**

1016 **proinflammatory crosstalk with airway epithelial cells**

1017 a: Heatmap of 28 significantly upregulated and 60 significantly downregulated DEG in

1018 aMDM from HDM-allergic donors versus healthy donors (n=5 per group)

b: Volcano plot of DEG (fold change>2, p_{adj} <0.05) in aMDM from HDM-allergic versus healthy donors (n=5)

c: Venn diagram of upregulated DEG in trained/control, trained+challenged/acute HDM and HDM-allergic/healthy aMDM

d: IL-1β concentration in supernatant of aMDM from HDM-allergic donors versus healthy donors, after 24h HDM exposure *in vitro* (ELISA, n=5 per group, repeated measures two-way ANOVA, Sidak's multiple comparisons test)

e: CCL17 concentration in supernatant of control and HDM-trained human macrophages on day 14, ± IL-25 exposure on day 13 for 8h (ELISA, n=5, repeated measures on-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

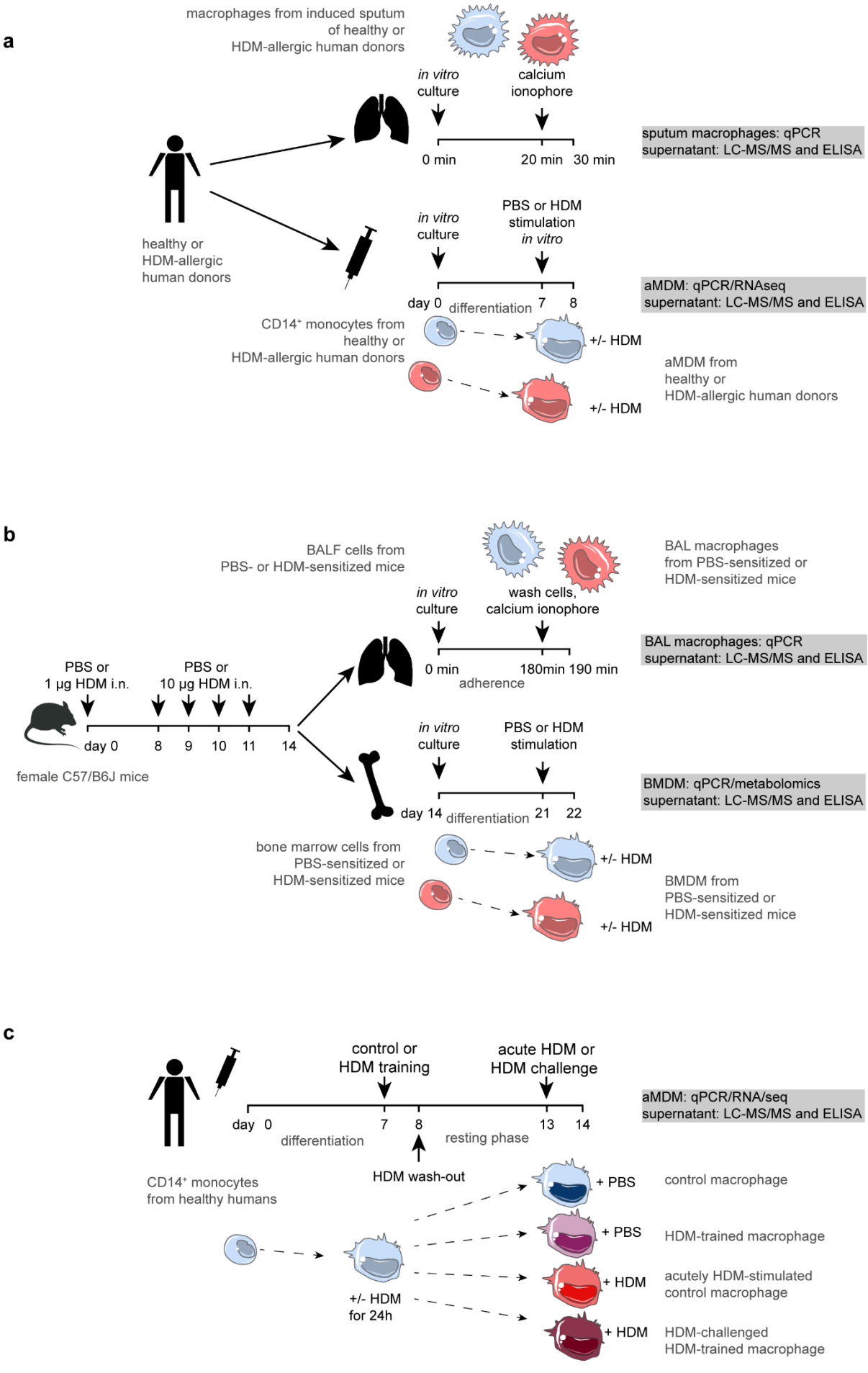
f: cysLT concentration in supernatant of control of HDM-trained human macrophages on day 14, ± IL-25 exposure on day 13 for 8h (EIA, after 10 min Ca^{++} ionophore stimulation)

g: CXCL8 concentration in supernatant of normal human bronchial epithelial cells, exposed to medium or supernatants from control or HDM-trained human macrophages for 24h (ELISA, n=8, Friedmann test, Dunn's multiple comparisons test)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01.

Supplementary Information

Supplementary Figures


a
macrophages from induced sputum of healthy or HDM-allergic human donors
in vitro culture
calcium ionophore
0 min
20 min
30 min
sputum macrophages: qPCR
supernatant: LC-MS/MS and ELISA
healthy or HDM-allergic human donors
in vitro culture
PBS or HDM stimulation in vitro
day 0
differentiation
7
8
aMDM: qPCR/RNAseq
supernatant: LC-MS/MS and ELISA
CD14+ monocytes from healthy or HDM-allergic human donors
+/- HDM
+/- HDM
aMDM from healthy or HDM-allergic human donors
b
BALF cells from PBS- or HDM-sensitized mice
in vitro culture
wash cells, calcium ionophore
BAL macrophages from PBS-sensitized or HDM-sensitized mice
0 min
180min
190 min
adherence
BAL macrophages: qPCR
supernatant: LC-MS/MS and ELISA
PBS or 1 µg HDM i.n.
PBS or 10 µg HDM i.n.
day 0
8
9
10
11
14
female C57/B6J mice
in vitro culture
PBS or HDM stimulation
day 14
differentiation
21
22
BMDM: qPCR/metabolomics
supernatant: LC-MS/MS and ELISA
bone marrow cells from PBS-sensitized or HDM-sensitized mice
+/- HDM
+/- HDM
BMDM from PBS-sensitized or HDM-sensitized mice
c
control or HDM training
acute HDM or HDM challenge
day 0
differentiation
7
8
resting phase
13
14
aMDM: qPCR/RNA/seq
supernatant: LC-MS/MS and ELISA
HDM wash-out
CD14+ monocytes from healthy humans
+/- HDM for 24h
+ PBS
control macrophage
+ PBS
HDM-trained macrophage
+ HDM
acutely HDM-stimulated control macrophage
+ HDM
HDM-challenged HDM-trained macrophage

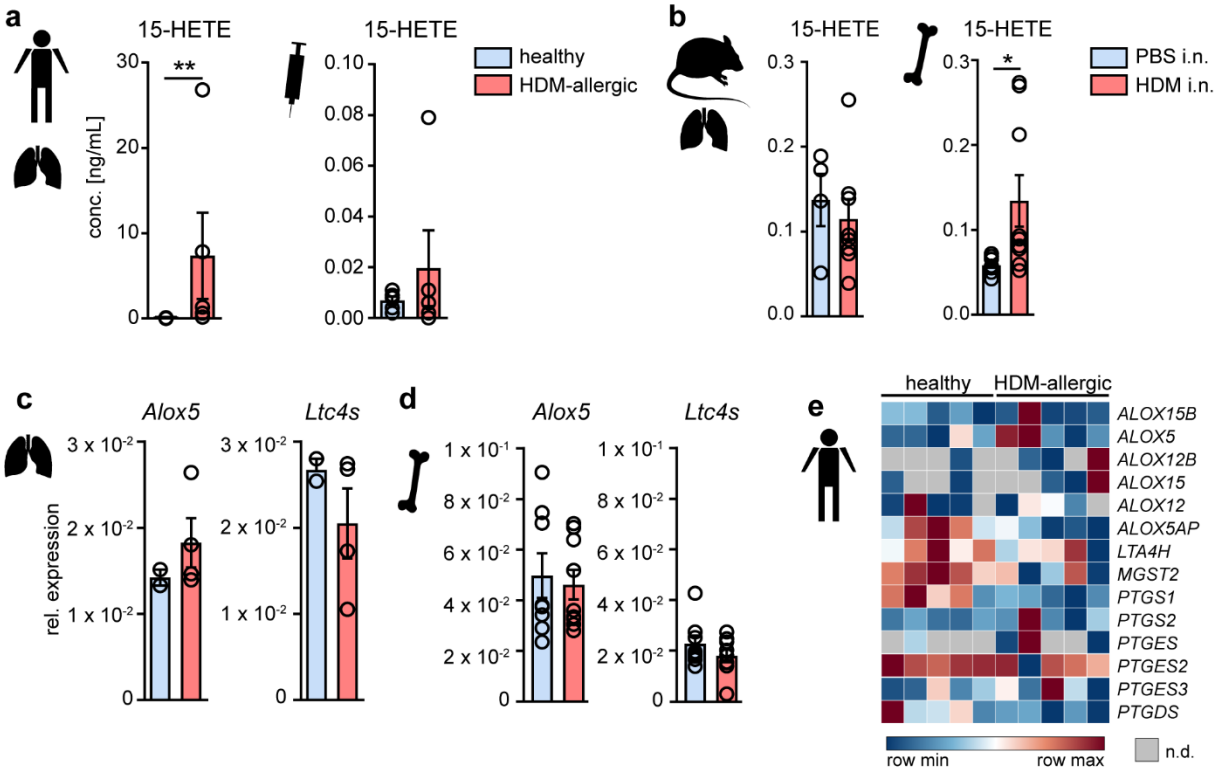
Supplementary Figure 1: Overview of experimental procedures and models

a: Sampling of local lung macrophages from induced sputum and blood-derived monocyte-derived macrophages (aMDM) from healthy and HDM-allergic human donors.

b: Mouse model of HDM-induced allergic airway inflammation in mice and sampling of local lung macrophages from BALF) and bone marrow-derived macrophages (BMDM)

c: In vitro HDM training model using monocyte-derived macrophages (aMDM) from healthy human donors

LC-MS/MS=liquid chromatography-tandem mass spectrometry, i.n.=intranasal, BALF=bronchoalveolar lavage fluid



Supplementary Figure 2: Altered type 2 mediators in *in vivo* HDM-experienced macrophages are not due to transcriptional differences

a: 15-HETE concentration in supernatants of human, and

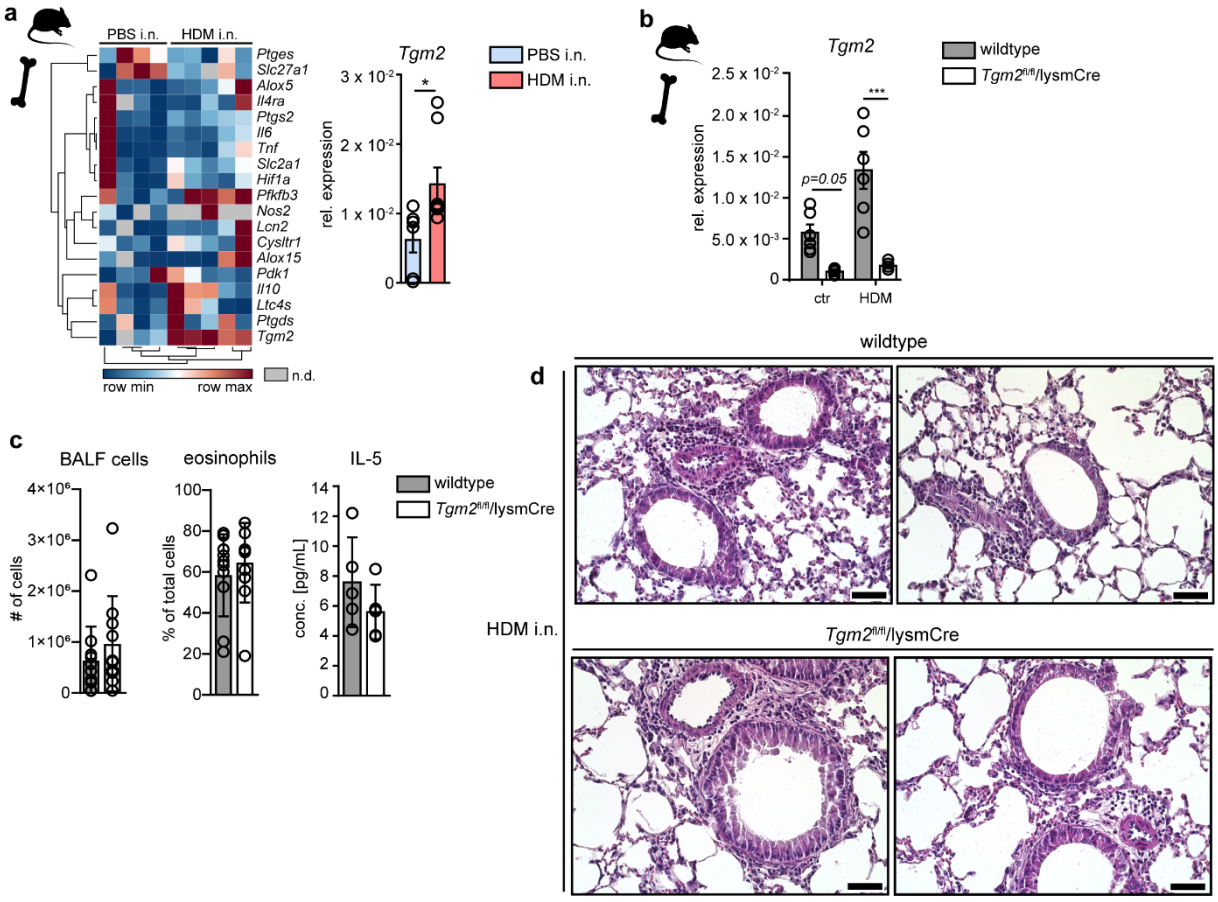
b: murine *in vivo* HDM-experienced locally and peripherally derived macrophages (n=5-9, unpaired t test)

c: *Alox5* and *Ltc4s* gene expression in local airway macrophages (normalized to *Gapdh*, n=4-8), and

d: bone marrow-derived macrophages from PBS- and HDM-sensitized mice (normalized to *Gapdh*, n=8-9, unpaired t test)

e: Eicosanoid pathway gene expression in aMDM from healthy and HDM-allergic human donors (normalized read counts from RNAseq dataset)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01. i.n.=intranasal



Supplementary Figure 3: Tgm2 is upregulated in myeloid progenitors from HDM-sensitized mice, but myeloid *Tgm2* deficiency does not alter HDM-induced AAI *in vivo*

a: Gene expression analysis of HSCP from PBS- and HDM-sensitized mice (normalized to *Gapdh*), detail: *Tgm2* expression level (Mann-Whitney test, n=6 vs n=8)

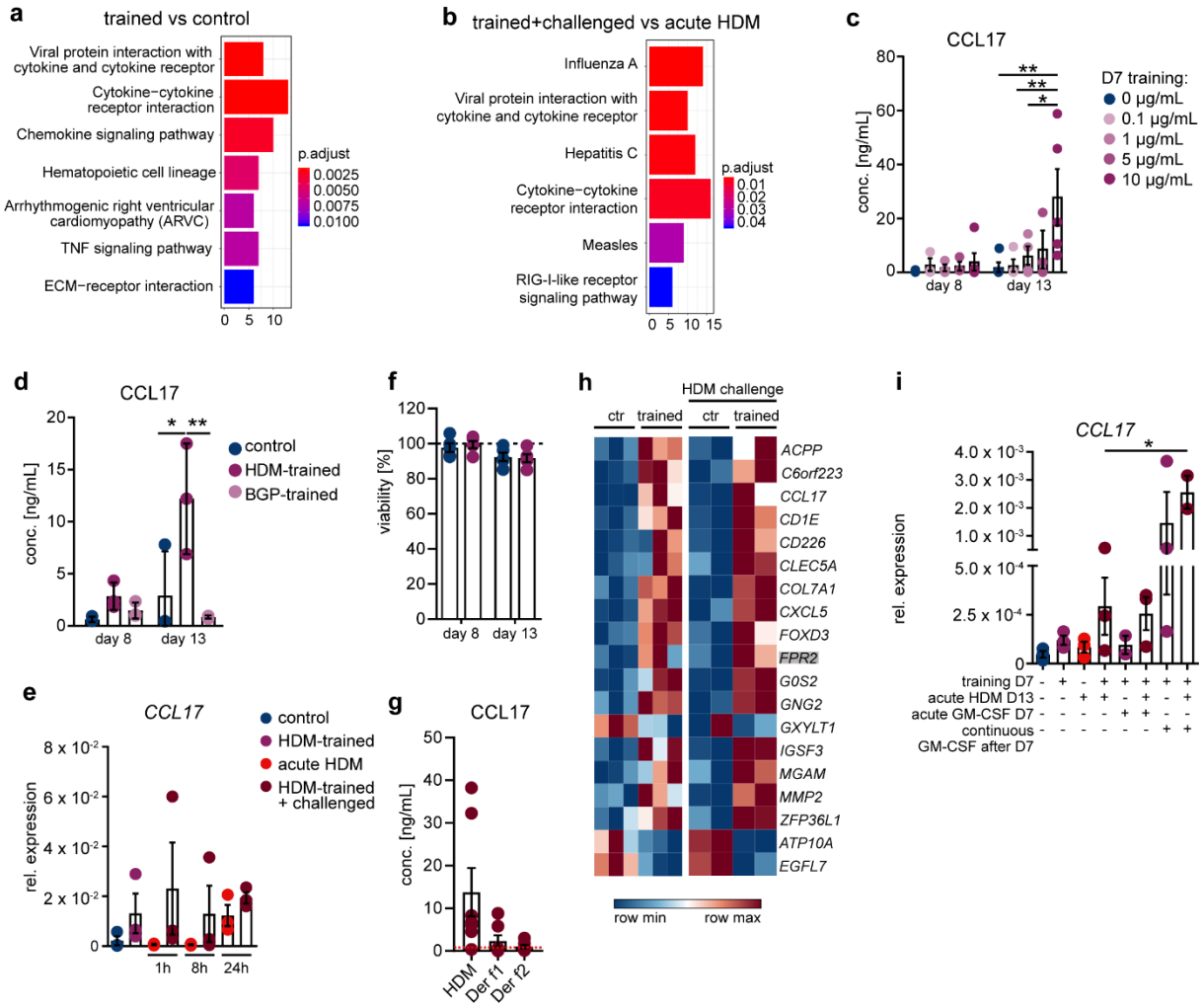
b: Gene expression of *Tgm2* in response to HDM stimulation of BMDM from wildtype versus *Tgm2*$^{fl/fl}$lysmCre (repeated measures two-way ANOVA, Sidak's multiple comparison test, n=6 versus n=5)

c: BALF IL-5 (n=5 per group), total cell count and percentage of eosinophils (n=10 per group) from HDM-sensitized wildtype versus *Tgm2*$^{fl/fl}$lysmCre mice

d: Hematoxylin/eosin staining of lung sections from HDM-sensitized wildtype versus *tgm2*$^{fl/fl}$lysmCre mice, 2 representative images per group, bars indicate 50 µm

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs).

*p<0.05, ***p<0.001. i.n.=intranasal



Supplementary Figure 4: HDM training alters CCL17 production and induces a specific transcriptional signature of upregulated genes in human macrophages

a: KEGG pathway enrichment analysis in HDM trained versus control macrophages (n=3)

b: KEGG pathway enrichment analysis in HDM trained and challenged versus acutely HDM-exposed macrophages (n=2)

c: CCL17 concentration in supernatant of control and HDM-trained human macrophages on day 14, training with different HDM concentrations (ELISA, n=4)

d: CCL17 concentration in supernatant of control, HDM-trained and BGP-trained human macrophages on day 8 and day 13 (ELISA, n=3, repeated measures two-way ANOVA, Sidak's multiple comparisons test)

e: *CCL17* gene expression in control and HDM-trained human macrophages after 1h, 8h and 24h of HDM challenge (normalized to *GAPDH*, n=3)

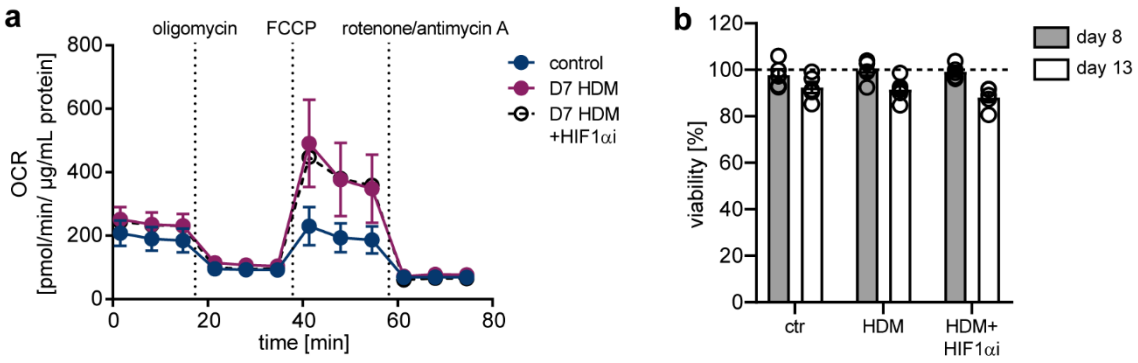
f: Viability of control and HDM-trained human macrophages on day 8 and day 13 (LDH activity assay, n=5, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

g: CCL17 concentration in supernatants of HDM-challenged HDM-, *Der f1*- and *Der f2*-trained human macrophages on day 14 ELISA, n=7)

h: Heatmap of DEG overlapping between HDM-trained/control and trained+challenged/acute HDM macrophages (normalized read counts)

i: *CCL17* gene expression in M-CSF-differentiated control and HDM-trained human macrophages on day 14, treated with GM-CSF during the training or during the training and resting phase (normalized to *GAPDH*, n=3, one-way ANOVA with Holm-Sidak's multiple comparisons test)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01. BGP=β-glucan peptide, Der f1=*Dermatophagoides farinae* purified allergen 1, Der f2= *Dermatophagoides farinae* purified allergen 2

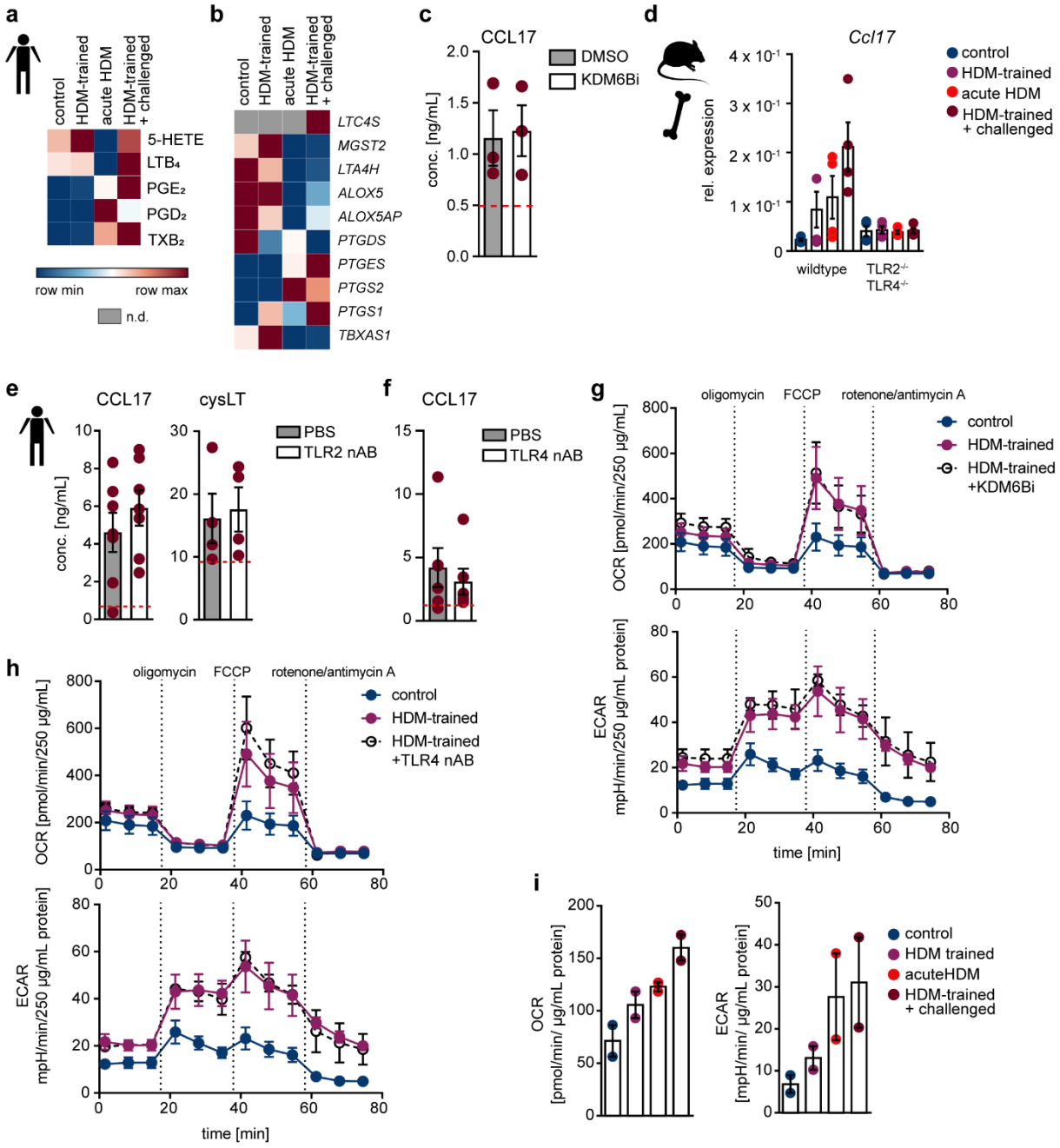


Supplementary Figure 5: HIF1α partially contributes to HDM-training in human macrophages

a: Oxygen consumption rate of control and HDM-trained human macrophages, ± HIF1α inhibition during training phase, on day 14 (n=3)

b: Viability of control and HDM-trained human macrophages, ± HIF1α inhibiton during training phase, on day 8 and day 13 (LDH activity assay, n=5, repeated measures one-way ANOVA with Geisser-Greenhouse correction, Sidak's multiple comparisons test)

Data are presented as mean + SEM (bar graphs). HIF1αi=HIF1α inhibitor



Supplementary Figure 6: HDM-trained macrophages exhibit distinct alterations of eicosanoid and energy metabolism

a: Eicosanoid concentration in supernatants of control and HDM-trained human macrophages on day 14 (LC-MS/MS, after 10 min Ca^{++} ionophore stimulation, averages of n=11)

b: Eicosanoid metabolism genes of control and HDM-trained human macrophages on day 14 (normalized read counts from RNAseq dataset, n=2)

c: CCL17 concentration in supernatants of challenged HDM-trained human macrophages, ± KDM6B inhibition during training phase, on day 14 (ELISA, n=3). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

d: *Ccl17* gene expression in control and HDM-trained BMDM from wildtype or $TLR2^{-/-}$ $TLR4^{-/-}$ mice (n=4, normalized to *Gapdh*)

e: CCL17 and cysLT concentration in supernatants of challenged HDM-trained human macrophages, ± TLR2 inhibition during training phase, on day 14 (ELISA, n=7 and EIA, n=4, paired t test). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

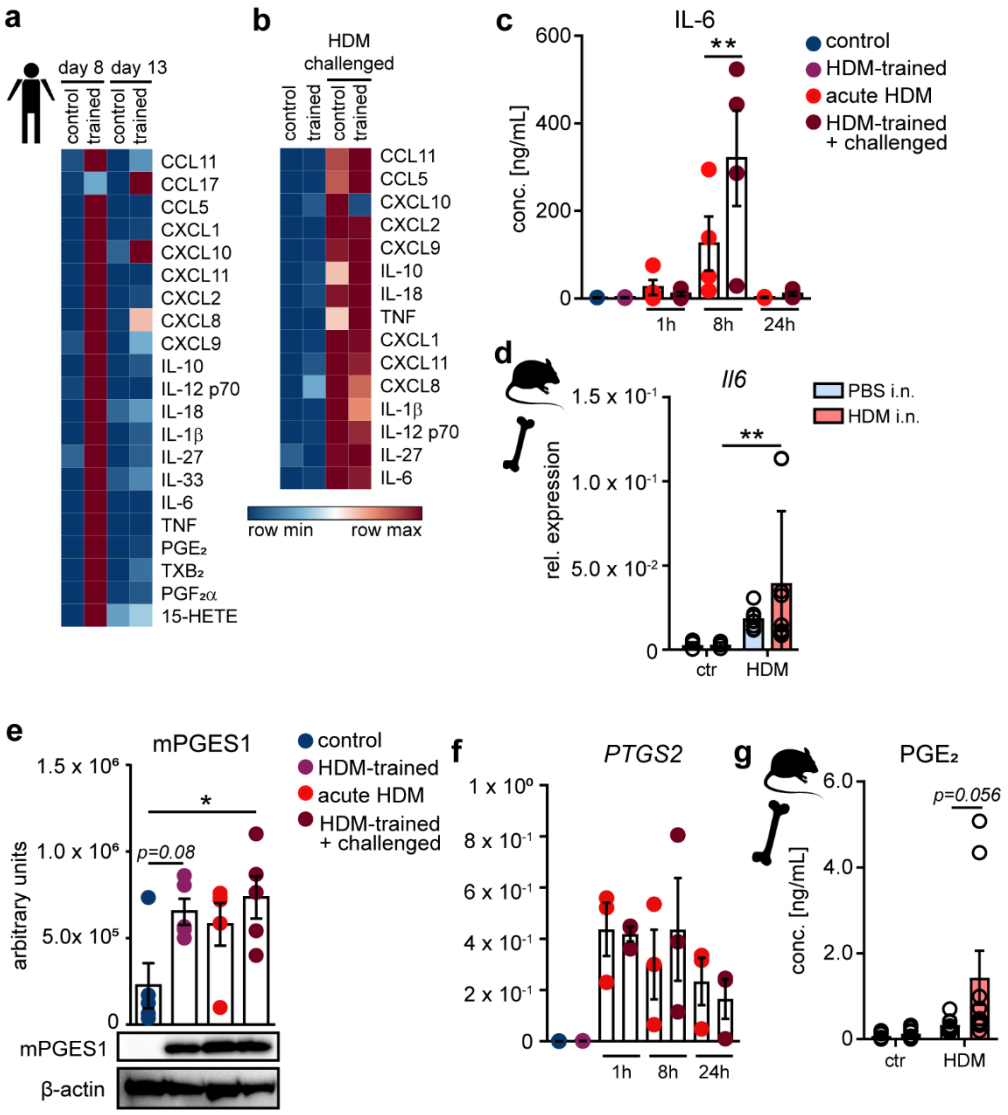
f: CCL17 concentration in supernatants of challenged HDM-trained human macrophages, ± TLR4 inhibition during training phase, on day 14 (ELISA, n=5, paired t test). Red dotted line indicates CCL17 concentration of aMDM + acute HDM exposure.

g: Oxygen consumption rate and extracellular acidification rate of control and HDM-trained human macrophages ± KDM6B inhibition during training phase, on day 14 (n=3)

h: Oxygen consumption rate and extracellular acidification rate of control and HDM-trained human macrophages ± TLR4 inhibition during training phase, on day 14 (n=3)

i: Baseline oxygen consumption rate and extracellular acidification rate of control and HDM-trained human macrophages ± HDM challenge, on day 14 (n=2)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). KDM6Bi=KDM6B inhibitor, nAB=neutralizing antibody, OCR=oxygen consumption rate, ECAR=extracellular acidification rate.



Supplementary Figure 7: HDM-training is characterized less by classical cytokine reprogramming but prostanoid production is altered

a: Cytokine, chemokine, and eicosanoid response over time (on days 8 and 13)

b: Cytokine and chemokine response on day 14 (after HDM challenge)

c: IL-6 concentration of control and HDM-trained human macrophages on day 14, after 1h, 8h or 24h of HDM challenge (ELISA, n=4, repeated measures two-way ANOVA, Sidak's multiple comparisons test)

d: *Il6* gene expression in BMDM from PBS- vs. HDM-sensitized mice, unstimulated or after 24h HDM *in vitro* (n=8-9, repeated measures two-way ANOVA, Sidak's multiple comparisons test)

e: mPGES1 protein concentration of control and HDM-trained human macrophages on day 14 normalized to β-actin (n=5, Friedmann test and Dunn's multiple comparisons test) and representative images of western blot from one donor

f: *PTGS2* gene expression in control and HDM-trained human macrophages after 1h, 8h and 24h of HDM re-exposure (normalized to *GAPDH*, n=3)

g: PGE_2 concentration in supernatant of murine BMDM from PBS- or HDM-sensitized mice ± HDM exposure for 24h *in vitro* (after 10 min Ca^{++} ionophore stimulation, LC-MS/MS, n=8-9, repeated measures two-way ANOVA, Sidak's multiple comparisons test)

Data are presented as z-score transformed (heatmap) or mean + SEM (bar graphs). *p<0.05, **p<0.01. i.n.=intranasal instillation.

Supplementary Table 1: Differentially expressed genes of trained versus control macrophages (separate Excel file)

Supplementary Table 2: Differentially expressed genes of trained versus control macrophages, challenged with HDM (separate Excel file)

Supplementary Table 3: Differentially expressed genes of aMDM from HDM-allergic versus healthy donors (separate Excel file)

Supplementary Table 4: qPCR primer sequences

	Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
Human			
Housekeeper	***GAPDH***	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC
LOX pathway	***ALOX5***	GATTGTCCCCATTGCCATCC	AGAAGGTGGGTGATGGTCTG
	LTC4S	GACGGTACCATGAAGGACGA	GGAGAAGTAGGCTTGCAGCAG
COX pathway	***PTGS2***	GCTGGAACATGGAATTACCCA	CTTTCTGTACTGCGGGTGGAA
	PTGES	TCAAGATGTACGTGGTGGCC	GAAAGGAGTAGACGAAGCCCAG
Inflammatory genes	***CCL17***	AGGGAGCCATTCCCCTTAGA	GCACAGTTACAAAAACGATGGC
	KDM6B	CAGTCCATGAAGCACTGCCA	AAACACCTCCACATCGCACT
	HIF1A	TTCCTTCTCTTCTCCGCGTG	ACTTATCTTTTTCTTGTCGTTCGC
Mouse			
Housekeeper	***Gapdh***	GGGTGTGAACCACGAGAAAT	CCTTCCACAATGCCAAAGTT
LOX pathway	***Alox5***	ATTGCCATCCAGCTCAACCA	ACTGGAACGCACCCAGATTT
	Ltc4s	ATCTTCTTCCACGAAGGAGCC	TCGCGTATAGGGGAGTCAGC
	Alox15	GCGACGCTGCCCAATCCTAATC	CATATGGCCACGCTGTTTTCTACC
	Cysltr1	GCTGAGGTACCAGATAGAGGCT	CTTGGTGCCTTGGAGGTACA
COX pathway	***Ptgs2***	GGGCCATGGAGTGGACTTAAA	TCCATCCTTGAAAAGGCGCA
	Ptges	GAAGAAGGCTTTTGCCAACCC	TCCACATCTGGGTCACTCCT
	Ptgds	GATGGGTTTGGTCCTCCTGG	GCCCCAGGAACTTGTCTTGT
M2 markers and metabolism genes	***Arg1***	GCAACCTGTGTCCTTTCTCC	TCTACGTCTCGCAAGCCAAT
	Retnla	GGGATGACTGCTACTGGGTG	TCAACGAGTAAGCACAGGCA
	Tgm2	TAAGAGTGTGGGCCGTGATG	TTTGTTCAGGTGGTTGGCCT
	Mrc1	TTGCACTTTGAGGGAAGCGA	CCTTGCCTGATGCCAGGTTA
	Il4ra	TGACCTCACAGGAACCCAGGC	GAACAGGCAAAACAACGGGAT
	Nos2	CTGCCTCATGCCATTGAGTT	TGAGCTGGTAGGTTCCTGTTG
	Slc27a1	CTCCAGCACAGGATGCGG	CACGGAAGTCCCAGAAACCA
	Slc2a1	CCCATGTATGTGGGAGAGGTG	GCCAAACACCTGGGCAATAAG
	Pfkfb3	AATGTGGGAGAGTATCGGCG	AAGGCACACTGTTTTCGGAC
	Pdk1	GGCCAGGTGGACTTCTATGC	AGCATTCACTGACCCGAAGT
	Hif1a	CGTTTAGGCCCGAGCGAG	CGACGTTCAGAACTCATCCTATTTT
Inflammatory genes	***Il6***	TGATGCACTTGCAGAAAACA	ACCAGAGGAAATTTTCAATAGGC
	Tnf	TTCTATGGCCCAGACCCTCA	GTGGTTTGCTACGACGTGGG
	Il10	GGCGCTGTCATCGATTTCTC	ATGGCCTTGTAGACACCTTGG
	Ccl17	CTGCTCGAGCCACCAATGTA	ACAGTCAGAAACACGATGGCA

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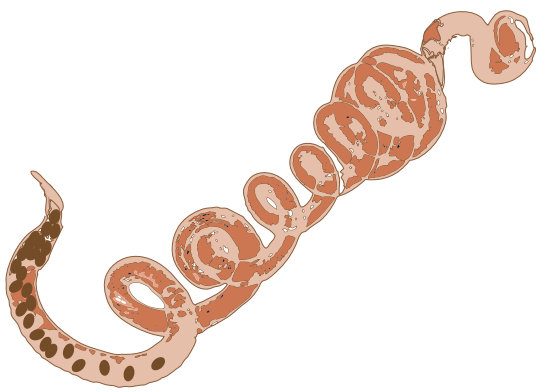

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* equal contribution