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Regulation of macrophage derived eicosanoids in allergy and infection

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

Marie Curie

Abstract

Macrophages are plastic cells that can sense and respond to external stimuli via secretion of a wide range of chemokines, cytokines and lipid mediators. These innate immune cells thereby contribute to host defence and induction of inflammatory responses. The aim of this dissertation was to investigate how macrophage derived lipid mediators are regulated in viral type 1 and allergic/ parasitic type 2 immune responses, which was analysed in three different immunological settings.

First, we showed that priming of monocyte-derived macrophages (MDM) with house dust mite (HDM) shifted their eicosanoid metabolism from a 5-lipoxygenase (5-LOX) dominated profile towards cyclooxygenase (COX) derived lipid mediators. This shift in lipid mediators was based on altered gene expression and protein levels induced via p38 MAPK signalling. Also pro-inflammatory chemokine and cytokine levels were enhanced in HDM stimulated MDM, while their capacity to induce granulocyte chemotaxis was reduced. In contrast to the *in vitro* effect, *in vivo* HDM induced airway inflammation led to increased cysLT production in the lung, while another type 2 setting, infection with the parasite *N. brasiliensis*, resulted in elevated pulmonary prostanoid levels.

In the second study, we investigated transcriptional and eicosanoid reprogramming in MDM of individuals that had been infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). We showed that individuals with a mild COVID-19 disease course have a persistent transcriptional and metabolic imprint 3-5 months after infection, evident by increased pro-inflammatory 5-LOX metabolite production that returned to baseline 12 months after infection. SARS-CoV-2 spike protein or LPS stimulation exaggerated prostanoid, type I IFN and chemokine responses in MDM from convalescent SARS-CoV-2 infected individuals. Pro-inflammatory lipid mediators were enhanced in post COVID-19 MDM and stimulation further augmented the disparity to healthy MDM which is indicative of an epigenetic reprogramming resulting in long-term modification of the innate immune compartment after COVID-19 infections.

Third, we assessed the role of transglutaminase 2 (TG2), a marker of alternatively activated macrophages involved in allergic inflammation and tissue remodelling, in type 2 immune responses. We found that TG2 is induced in macrophages in the context of type 2 settings. Using conditional TG2 knockout mice we showed that myeloid TG2 is not involved in allergic airway inflammation, while it plays a role in expulsion of the parasite *H. polygyrus*. Myeloid TG2 deficiency did not impact on *N. brasiliensis* host defence, although it might regulate lung remodelling. Mechanisms of action of myeloid TG2 in type 2 immunity have to be further explored.

Thus, macrophages and their lipid mediator profiles provide immune-regulatory targets in diverse inflammatory and host defence settings.

Deutsche Zusammenfassung

Makrophagen sind plastische Zellen, die externe Reize wahrnehmen und über die Sekretion einer Vielzahl von Chemokinen, Zytokinen und Lipidmediatoren darauf reagieren können. Diese innaten Immunzellen tragen dadurch zur Verteidigung des Wirts und zur Induktion von Entzündungsreaktionen bei. Das Ziel dieser Dissertation war es zu untersuchen, wie von Makrophagen gebildete Lipidmediatoren in viralen Typ-1- und allergischen/ parasitären Typ-2-Immunantworten reguliert werden. Dies wurde in drei verschiedenen immunologischen Situationen analysiert. Zuerst zeigten wir, dass die Stimulation der von Monozyten abstammende Makrophagen (MDM) mit Hausstaubmilben (HDM) deren Eicosanoid Metabolismus von einem 5-Lipoxygenase (5-LOX) dominierten Profil hin zu Cyclooxygenase (COX) abgeleiteten Lipidmediatoren verschob. Diese Veränderung der Lipidmediatorproduktion basierte auf einer modifizierten Genexpression sowie Änderungen auf Proteinebene, die über die p38-MAPK Signalkaskade vermittelt wurden. Auch die entzündungsfördernden Chemokin- und Zytokinspiegel waren bei HDM-stimulierten MDM erhöht, während ihre Fähigkeit Granulozyten-Chemotaxis zu induzieren reduziert war. Im Gegensatz zum *in vitro* Effekt führte eine *in vivo* HDM-induzierte Atemwegsentzündung zu einer verstärkten cysLT-Produktion in der Lunge, während eine andere Typ-2-Entzündung, eine Infektion mit dem Parasiten *N. brasiliensis*, zu erhöhten pulmonaren Prostanoidspiegeln führte. In der zweiten Studie untersuchten wir die Transkriptions- und Eicosanoid-Reprogrammierung in MDM von Personen, die mit dem schweren akuten Atemwegssyndrom Coronavirus 2 (SARS-CoV-2) infiziert worden waren. Wir zeigten, dass Individuen mit einem milden COVID-19 Krankheitsverlauf 3-5 Monate nach der Infektion eine anhaltende transkriptionelle und metabolische Prägung aufwiesen, die sich in einer erhöhten Produktion von entzündungsfördernden 5-LOX Metaboliten zeigte, die 12 Monate nach der Infektion auf den Ausgangswert zurückkehrten. Stimulation mit SARS-CoV-2 Spike-Protein oder LPS erhöhte die Prostanoid-, Typ-I-IFN- und Chemokin-Bildung bei MDM von genesenen SARS-CoV-2 infizierten Personen weiter. Entzündungsfördernde Lipidmediatoren wurden von post COVID-19 MDM vermehrt gebildet, und eine Stimulation verstärkte den Unterschied zu gesunden MDM, was auf eine epigenetische Reprogrammierung hinweist, die zu einer langfristigen Modifikation des angeborenen Immunkompartiments nach COVID-19 Infektionen führt.
Zudem untersuchten wir die Rolle von Transglutaminase 2 (TG2), einem Marker für alternativ aktivierte Makrophagen, die an allergischen Entzündungen und Gewebeumbau beteiligt sind, bei Typ-2-Immunantworten. Wir zeigten, dass TG2 in Makrophagen während Typ-2-Entzündungen induziert wird. Unter Verwendung konditioneller TG2 Knockout Mäuse zeigten wir, dass myeloide TG2 nicht an allergischen Atemwegsentzündungen beteiligt ist, obwohl es eine Rolle bei der Ausscheidung des Parasiten *H. polygyrus* spielt. Das Fehlen myeloider TG2 hatte keine Auswirkungen auf die *N. brasiliensis* Abwehr, während es den Lungenumbau regulieren könnte. Die Wirkungsmechanismen von myeloider TG2 bei der Typ-2-Immunität sollten weiter erforscht werden. Insgesamt stellen Makrophagen und deren Lipidmediatorprofile immunregulatorische Ziele in verschiedenen Entzündungs- und Abwehrsituationen des Wirts dar.

Contents

Nomenclature

15-LOX	15-Lipoxygenase
5-LOX	5-Lipoxygenase
AA	Arachidonic acid
AM	Alveolar macrophage
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
Arg1	Arginase 1
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guérin
BMDM	Bone marrow derived macrophages
CD206	Mannose receptor
COVID-19	Coronavirus disease 2019
COX	Cyclooxygenase
CYP	Cytochrome P450
CoA	Coenzyme A
DAMP	Damage-associated molecular patterns
DC	Dentritic cell
DHA	Docosahexaenoic acid
EP	E-type prostanoid receptor
EPA	Eicosapentaenoic acid
$FAD/FADH_2$	Flavin adenine dinucleotide - oxidised and reduced form
FLAP	5-Lipoxygenase activating protein
G-6-P	Glucose-6-phosphate

GM-CSF	Granulocyte macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDM	House dust mite
HSC	Hematopoietic stem cell
IAV	Influenza A virus
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
IM	Interstitial macrophage
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
KDM	Lysine demethylase
KMT	Lysine methyltransferase
LPS	Lipopolysaccharide
LT	Leukotriene
MΦ	Macrophage
MAPK	Mitogen-activated protein kinase
MDM	Monocyte-derived macrophages
MHC	Major histocompatibility complex
N-ERD	NSAID exacerbated respiratory disease
NAD^+/NADH	Nicotinamide adenine dinucleotide - oxidised and reduced form
NSAID	Nonsteroidal anti-inflammatory drug
OVA	Ovalbumin
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PG	Prostaglandin
PGES	PGE synthase

PGI_2	Prostaglandin I_2 / Prostacyclin
PLA_2	Phospholipase A_2
PMN	Polymorphonuclear leukocyte
PPARγ	Peroxisome proliferator-activated receptor γ
PPP	Pentose phosphate pathway
PRMT	Protein arginine methyltransferase
PRR	Pattern recognition receptor
PTM	Posttranslational modification
PUFA	Polyunsaturated fatty acid
RALDH	Retinal dehydrogenases
RDM	Arginine demethylase
ROS	Reactive oxygen species
S-protein	SARS-CoV-2 spike protein
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SPM	Specialized pro-resolving lipid mediators
STAT	Signal transducer and activator of transcription
TCA cycle	Tricarboxylic acid cycle
TG2	Transglutaminase 2
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TX	Thromboxane
Th	T helper cell
Hpb	*Heligmosomoides polygyrus bakeri* (*H. polygyrus*)
Nb	*Nippostrongylus brasiliensis* (*N. brasiliensis*)
cysLT	cysteinyl LT, including LTC_4, LTD_4, LTE_4
iNos	Inducible nitric oxide synthase

List of Publications

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1 Introduction

1.1 Macrophages as players in immune responses

1.1.1 Macrophages recognize antigens via a variety of receptors

The immune system protects the body from foreign organisms and diseases. Physical, chemical and mechanical barriers form a first line of defence. The cellular immune system consists of an innate branch involved in the non-specific, but fast immune response and the adaptive immune system conferring slower, antigen-specific immunity. The innate immune system is formed by myeloid cells (monocytes, macrophages, DCs and granulocytes), and innate lymphoid cells (ILCs) including natural killer (NK) cells. They are the first to act in the recognition of micro-organisms through pattern recognition receptors (PRR) detecting either conserved surface molecules on pathogens, the pathogen-associated molecular patterns (PAMPs), or molecules released from damaged or dead host cells, the damage-associated molecular patterns (DAMPs). PRR can be intracellularly or extracellularly located and are divided into five subfamilies, namely toll-like receptors (TLRs), C-type lectin receptors (CLRs), as well as, nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and absent in melanoma-2 (AIM2)-like receptors (ALRs). PAMPs and DAMPs recognized by various PRRs are summarized in Table 1.1.

1.1.2 Immune response induction

PRR are expressed on cells of the innate immune system, but also by non-immune cells like epithelial cells and fibroblasts [15, 16]. Signalling through these receptors activates different signalling cascades resulting in transcriptional regulation of inflammatory response genes like pro-inflammatory cytokines, chemokines and interferons (IFN). Also the release of lipid mediators, phagocytosis and cell death are regulated (in part) through PRR signalling [17–20]. Inflammatory mediator release will also induce chemotaxis of granulocytes, for example interleukin (IL)-1β and LTB_4 induce neutrophil recruitment [21, 22], while monocyte chemotaxis involves MCP-1 and CC chemokine receptor 2 (CCR2) signalling [23, 24]. After recognition of PAMPs or DAMPs, antigen-presenting cells (APCs) phagocytose, process and present antigens within MHC II molecules for T cell activation [25] which may involve migration to the draining lymph nodes [26].
T helper (Th) cells are activated through T cell receptor (TCR) and CD4 coreceptor binding to peptide-loaded MHC II molecules on APCs [27, 28].
Th cells are categorized based on their cytokine production and effector function into Th1, Th2, and Th17 cells [29, 30]. Induction of signal transducer and activator of transcription (STAT) 4 following IL-12 signalling activates T-bet and results in Th1 cell differentiation, characterized by IFN-γ secretion [31, 32]. Commitment to Th2 cells is regulated by STAT6 and GATA3 and

Table 1.1: Example of different pattern recognition receptors, their ligands and physiological response

Family	Receptor	Ligand	Location	Physiological response	Ref.
TLR	TLR4	e.g. LPS	Plasma membrane	Type I IFN induction, pro-inflammatory cytokine production	[1–3]
	TLR9	CpG-DNA	Endosome, lysosome	Type I IFN induction, pro-inflammatory cytokine production	[3–5]
CLR	Dectin-1	β-glucans, zymosan	Plasma membrane	PGE_2, production of chemo-, cytokines, induction of phagocytosis	[6, 7]
NLR	NOD2	Peptidoglycans	Cytosol	NFκB / MAPK activation, pro-inflammatory cytokine production	[8–10]
RLR	RIG-I	viral dsRNA	Cytosol	Type I IFN induction, pro-inflammatory cytokine production	[11, 12]
ALR	AIM2	dsDNA	Cytosol	Inflammasome activation	[13, 14]

controls the production of IL-4, IL-5, and IL-13 [33, 34]. Th17 cells are identified by RORγt expression and secretion of IL-17A, IL-17F and IL-22 [35, 36].
Different Th cell subsets have different functions, whereas Th1 cells mediate protection against viral and bacterial infections [37, 38], Th2 cells are important for defence against extracellular parasites [39, 40], while also inducing pathogenesis of allergic diseases and asthma [41]. Th17 cells organize immunity to extracellular bacteria and fungi [42], just as being responsible for severe asthma and autoimmune responses [43, 44].
T cells have innate counterparts, the innate lymphoid cells (ILCs), divided into ILC1s, ILC2s, ILC3s, lymphoid tissue inducer (LTi) cells and NK cells, which are mainly tissue resident cells [45, 46]. ILCs mirror different Th subsets, ILC1s reflect Th1 cells, ILC2s are counterparts for Th2 cells, as are ILC3s for Th17 cells, expressing the same master regulatory genes and cytokines [47–49]. LTi cells initiate development of secondary lymph nodes and Peyer's patches during embryogenesis [50, 51]. NK cells are involved in cytolysis of infected or tumour cells [52], and also play a role in immune tolerance and memory [53].
Specific recognition and response to foreign antigens is established through the adaptive immune system, including B- and T-cells. Antigen recognition by T cells leads to their clonal expansion [54]. After antigen encounter a long-term memory can be formed involving memory B cells and plasma cells [55, 56], enabling rapid antibody production in future encounters [57, 58].

1.1.3 Impact of milieu on macrophage response

The cytokine milieu impacts on the macrophage (MΦ) effector profile. Macrophages are plastic cells that can respond via secretion of a wide range of chemokines, cytokines and lipid mediators. Viruses, bacteria and protozoa induce a type 1 immune response leading to M1 MΦ activation

through TLR or IFN signalling [59]. Inflammatory M1 MΦ are characterized by expression of pro-inflammatory genes, like IL-6, tumor necrosis factor (TNF), IL-1β, and iNos (inducible nitric oxide synthase) [60, 61].
Contrarily, allergens or parasitic infections induce a type 2 immune response characterized by production of IL-4, IL-5 and IL-13 by Th2 cells or ILC2s and M2/ alternative activation of MΦs [62]. M2 MΦ produce IL-10, upregulate mannose receptor (MRC1/CD206), transglutaminase 2 (TG2) and murine MΦs augment arginase 1 (Arg1) expression [62, 63]. M2 MΦ contribute to resolution, wound healing and tissue repair [64–66]. Type 2 immune responses can also lead to pathological fibrosis. The enzyme TG2 is involved in tissue remodelling [67, 68] through its transamidation and crosslinking activity [69] and can thereby contribute to the development of fibrosis [70, 71]. Additionally, M2 MΦ are responsible for trapping and killing of helminth larvae [72–74].
Also, the secreted lipid mediator profile depends on the macrophage activation status, M2 MΦ are characterized by increased 15-lipoxygenase (15-LOX) expression, while M1 MΦ induce cyclooxygenase-2 (COX-2) [62, 75, 76], which will be further elaborated on in Section 1.3.2.

1.2 Epigenetics and metabolism in innate immunity

1.2.1 Trained immunity

In recent years the compartmentalization between the innate and adaptive branch of the immune system has started to blur, as innate immune cells like monocytes and macrophages are also able to show adaptive properties. In 1978, Di Luzio and Williams showed that previous β-glucan administration reduced mortality of mice infected with *Staphylococcus aureus* (*S. aureus*) [77]. Other fungal or bacterial products, e.g. *Candida albicans* (*C. albicans*) infection or muramyl dipeptide exposure, could protect against following lethal bacterial infections or sepsis in mice [78, 79]. This effect was also present in Rag1 KO mice, forming only immature B- and T- cells, and thereby independent of antibody formation [78, 79].
Another example of adaptive features in innate immunity is the vaccination with Bacillus Calmette-Guérin (BCG), the vaccine developed against tuberculosis. BCG vaccination has beneficial side effects, decreasing overall child mortality and providing non-specific protection against other infections [80, 81]. The protective effects are conferred independent of T-cells [82, 83]. Therefore it was hypothesized that also innate immune cells can form a memory, the innate immune memory [79, 84]. Innate memory responses can either manifest trough a reduced response on second exposure to the same or a different effector molecule, termed induction of tolerance. Alternatively, the second encounter leads to an increased immune response called trained immunity. Innate memory is rendered through epigenetic modifications and altered metabolism [83–85] which will be explained in the further sections.

1.2.2 Epigenetic modification

Gene transcription depends on the availability of transcription factors, RNA polymerase, activator proteins but also on the chromatin structure and accessibility. DNA in the eukaryotic cell is organized in nucleosomes, two groups of four core histones, H2A, H2B, H3 and H4, forming an octamer wrapping around 147 bp of DNA that is stabilized by a histone H1 linker [86]. Posttranslational modifications (PTM) of histone N-terminal 'tails' are catalyzed by enzymes referred to as *writers* or *erasers*. PTMs alter the chromatin structure and thereby support or

decrease transcription. *Writers* encompass enzymes that add PTMs to histones, while *eraser* remove them. The *readers* recognize modifications and exert biological effects. Mechanisms will be explained in the further section, an overview is shown if Figure 1.1. Apart from histones, DNA can be directly modified, via methylation of cytosine by DNA methyltransferases (DNMTs) [87].

Histone acetylation
Histone acetylation is catalyzed by histone acetyl transferases (HATs), that add an acetyl group from acetyl-coenzyme A (CoA) to an ε-amino lysine residue. This modification alters the binding capacity of histones to DNA, as the positively charged lysine is exchanged by a neutral acetyl residue, making the negatively charged DNA in most cases more accessible for transcription [88]. HATs are categorized into two classes, the nuclear localized type A and the cytoplasmic type B. Type B HATs acetylate histones in the cytoplasma after their synthesis [89]. The larger class of type A HATs can be sub-divided based on their structure and function into the GNAT (Gcn5-related N-acetyltransferases) family, the MYST (MOZ, Ybf2, Sas2, Tip60) family, the p300/CBP family and steroid receptor co-activators, as well as, transcription factor related HATs [90]. Histone deacetylases (HDACs), subdivided into class I to IV, exert the opposing effect by transferring acetyl-residues to CoA. The network of HATs and HDACs regulates gene transcription and cell activation status [91]. Histone acetylation is recognized by a diverse class of proteins, most of them containing bromodomains [92]. Histone acetylation and recruitment of bromodomain proteins is involved in cell differentiation and cell division trough activation of gene transcription [93, 94].

Histone methylation
Lysine residues are also targets for methylation, potentially adding up to three methyl-residues to the ε-amino side group, forming mono-, di- or trimethylated lysine. Histone lysine methylation has no impact on charge and is added by different histone lysine methyltransferases (KMTs) or removed by lysine demethylases (KDMs). The location of the lysine methylation defines the effect, for example H3K4 methylation is associated with active transcription, while H3K27me3 leads to gene silencing [85, 95]. There are two enzyme domains functioning as KMTs: the SET (Su(var), Enhancer of zeste, Trithorax) domain, first described as a conserved sequence in *Drosophila melanogaster* proteins and the seven β-strand (7BS) domain, sometimes referred to as non-SET histone KMTs. SET domains include a pre-SET and post-SET domain, catalyzing the methyl transfer from S-adenosylmethionine to ε-amine lysine. Different KMTs recognize specific histone residues, for example H3K4 is methylated in humans by the SET1 family, e.g. MLL1 [96]. The non-SET histone methyltransferase DOT1, or DOT1L in mammals, is responsible for mono-, di- or tri-methylation of H3K79 [97]. H3K79me2 and H3K79me3 at enhancer sites leads to an open chromatin structure and gene transcription [98].

Also arginine can be mono- or di-methylated at its guanidino group. Protein arginine methyltransferases (PRMTs) are separated into type I, II and III PRMTs. All three types catalyse the formation of monomethyl-arginine, type I PMRTs further create the asymmetric N,N-dimethylarginine or ADMA, while type II PRMTs form the symmetric form SDMA (N,N′-dimethylarginine). The effect of histone arginine methylation depends on the residue, H4R3 methylation facilitates p300 mediated histone acetylation and thereby transcription [99, 100], while H3R2me2a is associated with inactive promoters (in yeast) and inhibits H3K4 methylation [101]. Arginine demethylation is less well studied, there have been no unique arginine demethylases (RDMs) identified, but lysine demethylase Jumonji domain containing 6 (JMJD6) has been suggested to catalyze arginine demethylation as well [102]. Walport *et al.* [103] identified

several Jumonji C domain-containing KDMs that can serve as RDMs *in vitro*. Methyl-arginine or arginine can also be deiminated by peptidyl arginine deiminases (PADs), converting them to citrulline and thereby changing the protein's chemical properties [104]. PAD4 activity is also necessary for neutrophil extracellular trap (NET) formation [105, 106], extracellular fibers containing histones, DNA and granule-derived enzymes [107]. During NETosis PAD4 translocates to the nucleus and promotes chromatin decondensation through histone citrullination [108].

Figure 1.1: (On next page) Schematic overview of different histone modifications. A) Histone lysine methylation and demethylation are catalysed by lysine methyltransferases (KMTs) and lysine demethylases (KDMs), respectively. B) Histone arginine methylation and demethylation are regulated by arginine demethylases (RDMs) and protein arginine methyltransferases (PRMTs). Methyl-arginine or arginine are also targets for citrullination (Cit), catalyzed by peptidyl-arginine deiminases (PADs). C) Histone lysine acetylation via histone acetyl transferases (HATs) concomitantly leads to a loss of charge and chromatin opening, which is reversed by histone deacetylases (HDACs). D) TG2 catalyses serotonylation (5-HT/ serotonin) of glutamine, while its reversibility is to date unknown.
Abbreviations: SAM: S-adenosylmethionine, SAH: S-adenosylhomocysteine

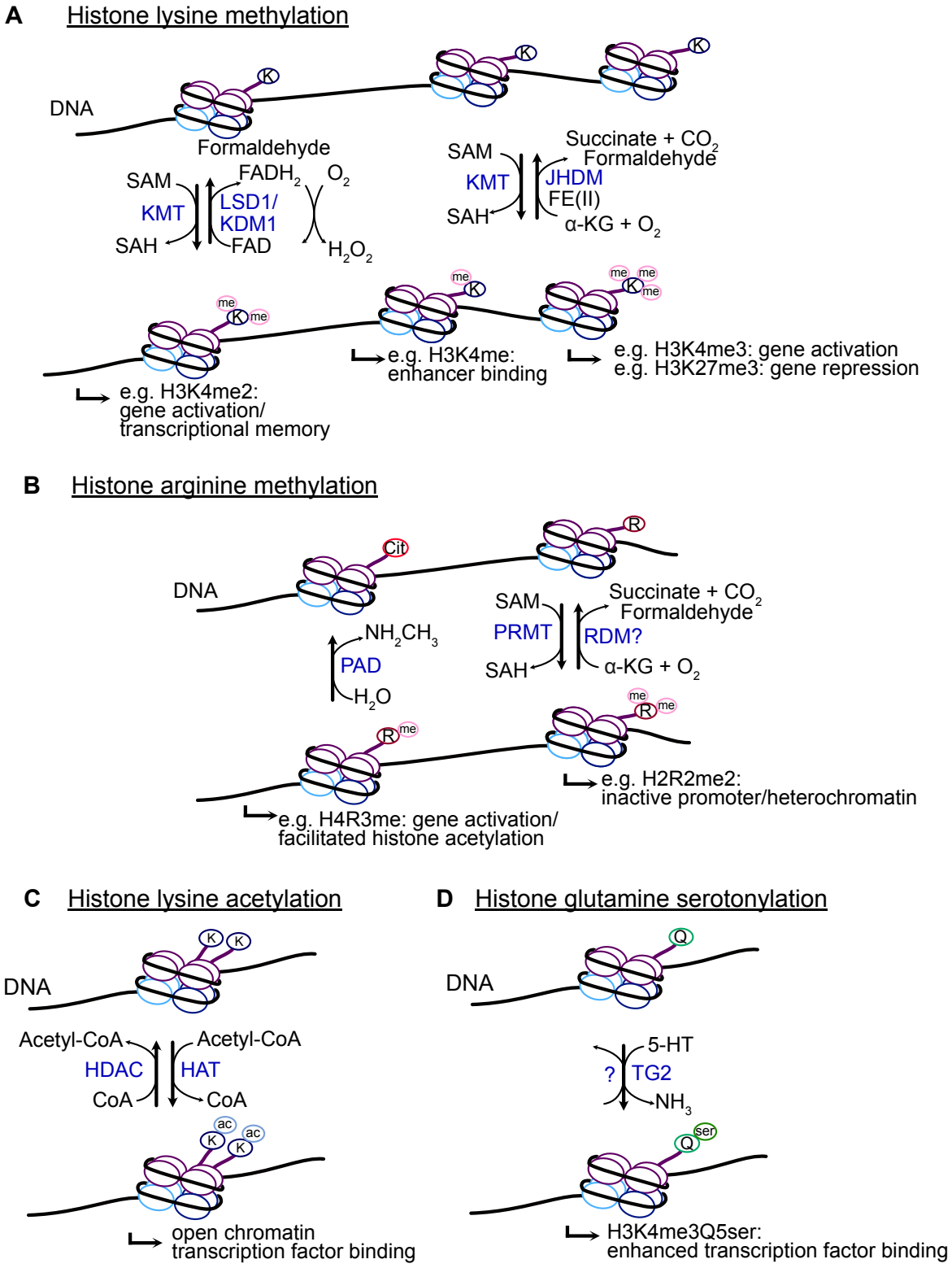

Figure 1.1: Caption on previous page.

Other histone modifications

Apart from acetylation and methylation also more complex modifications have been discovered, for example transglutaminase 2 can serotonylate H3Q5 or H3K4me3Q5, leading to enhanced transcription factor binding and thereby gene expression [109]. Histone lysine lactylation, addition of lactate derived from glycolysis can promote transcription. Histone lactylation is elevated in situations of increased glycolysis and lactate production like hypoxia or M1 macrophage differentiation [110], as well as, under inflammatory stress conditions [111].

1.2.3 Cell metabolism

To compensate or to react to extrinsic stimuli, cells need to be able to adjust their metabolic demand. For production of metabolites, proliferation, repair or growth, energy is needed. This can be supplied through several pathways. An overview of metabolic pathways is given in Figure 1.2.

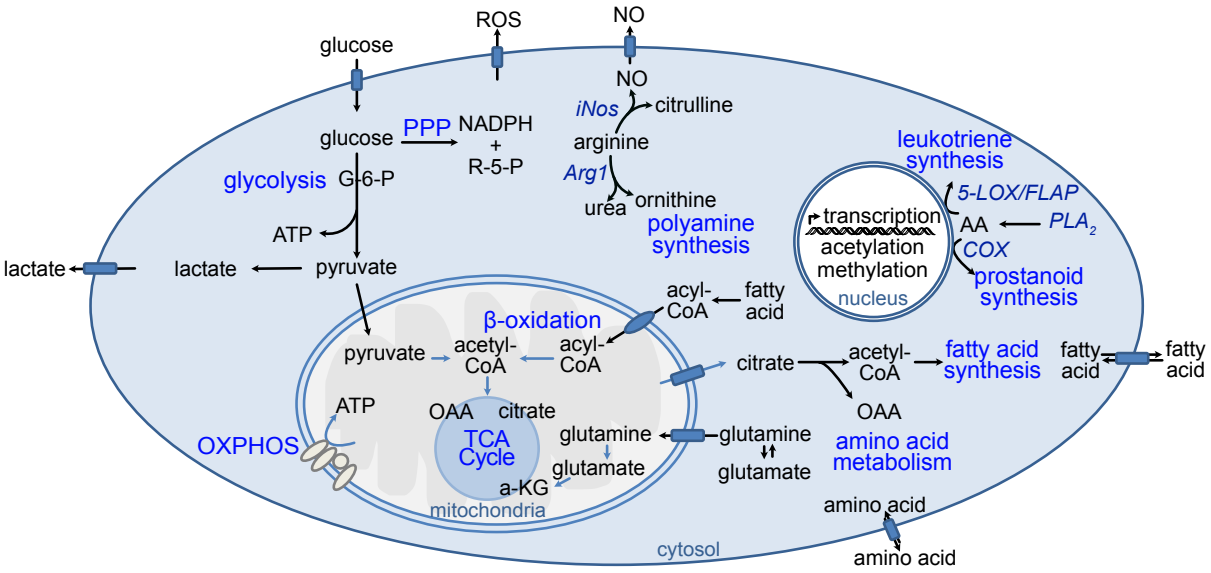

Figure 1.2: Overview of cellular metabolism. Glycolysis, the pentose phosphate pathway (PPP) and polyamine synthesis take place in the cytoplasm, while fatty acid β-oxidation, the TCA cycle and OXPHOS occur in the mitochondria. Amino acids can replenish the TCA cycle. Acetyl- and methyl- status also affect transcription via epigenetic modification. Polyunsaturated fatty acids like arachidonic acid (AA) serve as precursors for leukotriene and prostanoid synthesis. Abbreviations: a-KG: α-ketoglutarate, G-6-P: glucose-6-phosphate, NO: nitric oxide, OAA: oxalacetate, R-5-P: ribose-5-phosphate

Glycolysis

Glucose is taken up from the environment via glucose transporters in the extracellular membrane. Per unit glucose two ATP (adenosine triphosphate) and two pyruvate molecules are formed through glycolysis, making this pathway relatively inefficient. However, NAD^+ (nicotinamide adenine dinucleotide) is reduced to NADH and several metabolic intermediates for other pathways are provided, e.g. glucose-6-phosphate (G-6-P) for the pentose phosphate pathway, pyruvate for fatty acid synthesis and fuelling of the tricarboxylic acid cycle (TCA cycle). Pyruvate can further be converted to lactate. Glycolysis is the way of choice for fast energy generation and seen in rapidly proliferation cells or under inflammatory conditions [112, 113].

TCA cycle
The TCA cycle in the mitochondrial matrix is fuelled with acetyl-CoA derived either from glycolysis or fatty acid oxidation. Transfer of the acetyl group to oxaloacetate leads to the formation of citrate. Also amino acids can replenish the TCA cycle, glutamate can be converted by glutamate dehydrogenase to α-ketoglutarate (α-KG) and aspartate via aspartate transaminase to oxaloacetate. Net energy yield of one cycle are three molecules NADH, one $FADH_2$ (flavin adenine dinucleotide) and one GTP (guanosine-5'-triphosphate) which can be used to transport electrons across the electron transport chain and generate ATP via the oxidative phosphorylation (OXPHOS). The TCA cycle is not only used for energy generation but also a metabolic hub for substrate conversion and biosynthesis. Different TCA metabolites are involved in cell signalling. Acetyl-CoA is used for histone acetylation, succinate accumulation stabilizes HIF-1α and IL-1β production [114].

Pentose phosphate pathway
Also taking place in the cytoplasma, the pentose phosphate pathway (PPP) uses G-6-P, derived from glycolysis, for the generation of NADPH and ribose 5-phosphate. Followed by this oxidative PPP, ribose 5-phosphate can be utilized for nucleotide synthesis in the non-oxidative PPP branch. The reduced form, NADPH, is a cofactor of different pathways like fatty acid and cholesterol synthesis, as well as, protection against oxidative stress through reduction of glutathione disulfide (GSSG) to glutathione (GSH) [115]. NADPH can contrarily lead to the formation of reactive oxygen species (ROS) via NADPH oxidases [116]. NAD metabolism plays an important role in inflammatory macrophages [117].

1.2.4 Epigenetic and metabolic reprogramming in macrophages

Macrophage differentiation and polarization concomitantly shift metabolism. Monocyte to macrophage differentiation is accompanied by major changes in histone accessibility, including H3K27ac and thereby altered gene expression [85].
M1 MΦ activation increases glycolysis via upregulation of glycolytic enzymes, as well as, augmented glucose uptake [112] and reduced oxidative phsophorylation. The TCA cycle in M1 MΦ is disrupted at isocitrate dehydrogenase, converting isocitrate to α-KG, thereby accumulating citrate [113]. Citrate can be converted to acetyl-CoA via ATP-citrate lyase and thus lead to histone acetylation and facilitated RNA polymerase II binding and gene transcription [118, 119]. Alternatively, citrate is metabolised via immune-responsive gene 1 (IRG1) to itaconate or exported via the citrate transporter and contributes to fatty acid synthesis promoting further membrane biogenesis or eicosanoid synthesis. Itaconate has been shown to inhibit succinate dehydrogenase (SDH) and thus contributing to succinate accumulation, while also limiting inflammatory macrophage responses [120]. Succinate is exported from the mitochondria, also contributing to HIF-1α stabilization and IL-1β induction [114]. Metabolic pathways are shifted to increased ROS production via NADPH from the PPP or mitochondrial ROS production necessary for bacterial killing and phagocytosis [117, 121]. ROS production also induces IL-1β production via HIF-1α stabilization [114].
M2 MΦ have an intact TCA cycle and mainly meet their energy demand via fatty acid oxidation and oxidative phosphorylation. Part of the alternative macrophage activation via IL-4 signalling is regulated by histone modifications as STAT6 activation leads to repression of selective enhancers, diminished p300 and increased HDAC binding, and further to a reduced inflammatory

response [122].
IL-4 MΦ polarization is mediated via STAT6 and partly through peroxisome proliferator-activated receptor γ (PPARγ) [123, 124]. Satoh *et al.* [95] showed that Jmjd3, a H3K27 demethylase, and interferon regulatory factor (IRF) 4 signalling is necessary for M2 macrophage polarization in helminth infections, while it is redundant for M1 polarization. M2 macrophage activation via IL-4 also induces glucose metabolism to promote fatty acid synthesis and oxidative phosphorylation, which depends on mTORC2 and IRF4 signalling [125]. The dependency of M2 MΦ on glycolysis has been challenged by Wang *et al.* [126], stating that it is not required for M2 differentiation, as long as oxidative phosphorylation is functional.

As mentioned in Section 1.2.1, previous encounter to a stimulus can lead to a non-specific protective effect. BCG vaccination or β-glucan can induce trained innate immunity. Quintin *et al.* [79] stated that β-glucan training induced epigenetic modifications that resulted in increased pro-inflammatory cytokine production and thereby provided protection against *C. albicans* infections. Monocytes that had been trained with β-glucan showed increased H3K4me3 and H3K27ac at promoter regions of glycolytic genes and thereby induced glycolysis [127]. This effect was dependent on mammalian target of rapamycin (mTOR) activation. Another metabolic pathway implicated with trained immunity is the TCA cycle. Domínguez-Andrés *et al.* [128] demonstrated that lipopolysaccharide (LPS) induced tolerance through increased itaconate synthesis which was based on histone acetylation and increased expression of IRG1. In contrast β-glucan counteracted LPS-induced immune tolerance by inhibiting IRG1 induction.
Part of BCG's protective effect to following infections is provided through increased H3K4me3 and enhanced innate immune responsiveness of monocytes [129].
To grant long-time memory in rather short-lived cells like monocytes (up to 7 days in circulation [130]) or macrophages, metabolic or epigenetic reprogramming *in vivo* has to take place at the progenitor level. It has been confirmed by Kaufmann *et al.* [131] that BCG can enter the bone marrow and impact on hematopoietic stem cells (HSCs) when administered intravenously. Bone marrow derived macrophages (BMDM) derived from those HSCs were epigenetically modified and bestowed better protection against *Mycobacterium tuberculosis* (*M. tuberculosis*) infection.

1.3 Lipid mediators synthesis

Eicosanoids, bioactive lipid mediators derived from 20 carbon polyunsaturated fatty acids (PUFAs), can function as signalling molecules. The precursor PUFAs, e.g. arachidonic acid (AA), are cleaved off the phospholipid bilayer by phospholipase A_2 (PLA_2) [132], and can be converted through the cyclooxygenase (COX) pathway to prostanoids, including thromboxane (TX) and prostaglandins (PG). COX is present in two forms, the constitutively active COX-1 and the inducible COX-2 which expression is elevated under inflammatory conditions [133]. Terminal synthases lead to formation of different metabolites that confer their effect through G-protein coupled receptors (GPCR). TXA_2, converted from PGH_2 by TXA synthase, contracts smooth muscle cells and leads to platelet aggregation, however it is unstable and spontaneously hydrolyzed to TXB_2 [134, 135]. The counterpart prostacyclin (PGI_2) prevents the formation of blood clots and leads to vasodilation [136–138].
PGE_2 synthesized by PGE synthases (PGES), binds to four GPCR, the E-prostanoid receptors EP1-4. Intracellular signalling and downstream effects depend on the activated receptor. In type 2 immune responses PGE_2 has anti-inflammatory properties, for example through reduction of eosinophil migration [139]. PGE_2 induces an anti-inflammatory M2 MΦ phenotype and DC polarization [140, 141].

AA can also be converted by 5-lipoxygenase (5-LOX) and 5-LOX activating protein (FLAP) to 5-HETE and the leukotrienes (LTs). LTB_4 is formed via LTA_4 hydrolase (LTA4H). LTA_4 is also metabolized to LTC_4 via LTC_4 synthase and further to LTD_4 by γ-glutamyl transferase and LTE_4 by dipeptidase. LTC_4, LTD_4 and LTE_4 form the cysteinyl-LTs (cysLTs) [142]. LTs have chemoattractant properties and cysLTs can lead to vascular leakage and constriction [21, 22, 143].
Additionally, 12-/15-LOX can transform AA to 15-HETE or 12-HETE, the product depends on the host species and enzyme isoform, and further to lipoxins (LXs). Lipoxins have anti-inflammatory properties, apparent as inhibition of neutrophil recruitment, increased macrophage phagocytosis and reduced ROS production [144, 145]. They are part of the so-called specialized pro-resolving lipid mediators (SPM) that also include resolvins and protectins. SPMs, as the name suggests, mediate inflammatory resolution and tissue regeneration. Figure 1.3 depicts eicosanoid metabolic pathways.

Apart from the named pathways, PUFAs can undergo auto-oxidation or be metabolized by

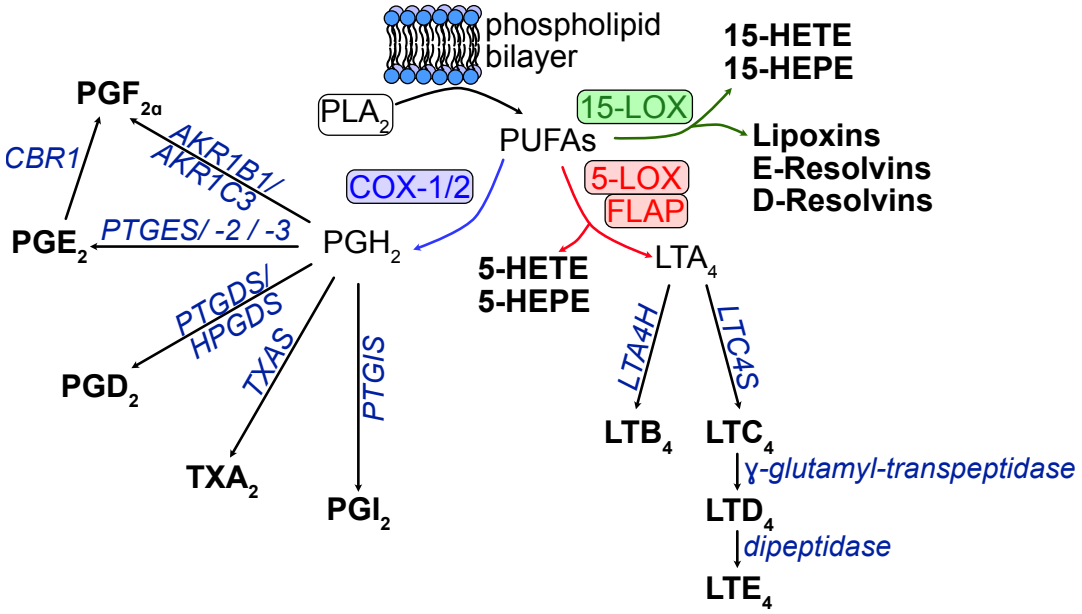

Figure 1.3: Overview of eicosanoid metabolism. Polyunsaturated fatty acids (PUFAs) are cleaved off the phospholipid bilayer by PLA_2 and can be converted via either COX-1 or COX-2 to prostanoids, via 5-LOX/FLAP to 5-HETE or 5-HEPE and leukotrienes (LTs), as well as, via 15-LOX to 15-HETE/15-HEPE, and SPMs, including resolvins and lipoxins.

cytochrome P450 (CYP) enzymes [165]. An overview of some eicosanoids and their biological effects are shown in Table 1.2.
Also other PUFAs can give rise to lipid mediator series, for example, docosahexaenoic acid (DHA) to the D-series resolvins, protectins and maresins and eicosapentaenoic acid (EPA) to E-series resolvins [166, 167].
Different immune cells are capable of forming diverse lipid metabolites depending on their expressed enzymes, for example (murine) neutrophils mainly generate LTB_4, while eosinophils produce higher levels of cysLTs [168]. The species impacts mediator profile of different cell types, main leukotriene metabolite of murine eosinophils is LTC_4, while human eosinophils produce primarily LTB_4 [169]. Also trans-cellular eicosanoid synthesis occurs, for example platelets

Table 1.2: Eicosanoids and their physiological role

Pathway	Mediator	Receptor	Physiological response	Ref.
COX	PGE_2	EP1	activation of phospholipase C and protein kinase C, intracellular Ca^{2+} ↑, nociception	[146, 147]
		EP2/ EP4	cAMP ↑, activation of protein kinase A, vasodilation, promotes Th1 cell differentiation, anti-inflammatory	[139, 148, 149]
		EP3	cAMP ↓, PI3K activation, mast cell activation, fever	[150, 151]
	PGD_2	DP1	cAMP ↑, inhibits allergic inflammation	[152, 153]
		DP2	chemoattraction, induction of Th2 cytokines	[154–156]
	PGI_2	IP	cAMP ↑, platelet aggregation ↓, suppressor of allergic inflammation	[136–138]
	TXA_2	TP	intracellular Ca^{2+} ↑, vasoconstriction, platelet aggregation ↑	[135, 157]
5-LOX	LTB_4	BLT1/ BLT2	chemotaxis, neutrophil activation and degranulation	[21, 22, 143]
	cysLTs	cysLTR1 & 2	bronchoconstriction, vascular leakage, type 2 inflammation	[158–161]
15-LOX	LXs	FPR2	inhibits chemotaxis, phagocytosis ↑	[162–164]

do not express 5-LOX but can generate cysLTs through uptake of neutrophil secreted LTA_4 [170]. Recently, tuft cells have been identified as another source of cysLTs [171–173].

1.3.1 Regulation of eicosanoid metabolism

Eicosanoid metabolism can be regulated through enzyme expression, epigenetic or posttranslational modifications. Release of fatty acids by PLA_2 is the first step of eicosanoid synthesis. Activity of PLA_2 is increased by TG2-catalyzed post-translational modifications [174, 175]. 5-LOX translocates from the cytosol to the nucleus upon Ca^{2+} binding where it colocalizes with PLA_2 and FLAP. 5-LOX activity depends highly on PTMs. Phosphorylation at Ser271 and Ser663 by extracellular signal-regulated protein kinase (ERK) 1/2 or mitogen-activated protein kinase (MAPK) activated protein kinase (MK) -2/3 lead to enhanced LTA_4 formation, while phosphorylation at Ser523 by protein kinase A (PKA) inhibits 5-LOX nuclear import [176, 177]. More recent studies showed a variability in phopshorylation status depending on the cell type and stimulation, as well as additional tyrosine phosphorylation sites [178, 179]. 5-LOX expression has been shown to be epigenetically regulated through H3K4 methylation, as well as, H3 and H4 acetylation [180].
Also the prostanoid pathway is epigenetically regulated. p300 recruitment and H3K27ac were shown to be necessary for PTGES1 expression, which was regulated by HDACs [181]. In patients

suffering from N-ERD (Nonsteroidal anti-inflammatory drug (NSAID) – exacerbated respiratory disease), a chronic type 2 inflammatory disease characterized by asthma, intolerance to NSAIDs and nasal polyposis, EP2 receptor expression was epigenetically regulated, suggesting part of the disease pathology is mediated by reduced PGE_2 response in fibroblasts [182].

1.3.2 Macrophages as eicosanoid producers

Monocytes and macrophages are able to produce a wide range of lipid mediators. As mentioned in Section 1.2.4, M1 or M2 MΦ activation alters their metabolism. Inflammatory macrophage activation results in a COX dominated mediator profile, while IL-4 activated MΦ mainly produce 15-HETE and downstream SPMs, mediated through a differential enzyme expression profile [183]. Formation of SPMs under those conditions was restricted to M2 MΦ. Macrophage efferocytosis is important for apoptotic cell clearance and tissue homoeostasis. Dalli & Serhan [184] showed that uptake of apoptotic PMN by MΦ results in SPM production that probably aids in curtailing inflammation. Macrophages also express diverse eicosanoid receptors, permitting sensing of the local tissue-dependent lipid mediator profile and enabling autocrine eicosanoid signalling.

1.4 Role of macrophages in immune homoeostasis

Macrophages, together with DCs and monocytes, belong to the mononuclear phagocyte system. In different tissues resident macrophages are responsible for homeostasis under steady-state conditions [185]. Macrophage phagocytosis is important for cell debris or pathogen clearance and tissue maintenance. Tissue macrophages are mainly constituted before birth from fetal monocytes and can locally self-maintain [186], which is also true for alveolar macrophages [187, 188]. Upon depletion, tissue-resident macrophages, as well as, monocytes are able to replenish the population in steady-state [189, 190]. The environmental milieu defines the transcriptional and metabolic macrophage profile [191, 192]. Lavin *et al.* [193] showed that different tissue resident macrophage have a defined gene expression and enhancer landscape that is dictated, at least partly, by the microenvironment. They demonstrated that differentiated tissue-resident macrophages transferred to a new microenvironment adjust their gene expression profile. Plasticity to adapt and colonize a new tissue environment is higher for precursor cells compared to differentiated tissue macrophages [194].

1.4.1 Lung tissue macrophages in steady-state conditions

Because of its role in gas exchange the lung is always in contact with the environment. It needs to differentiate between self and foreign antigens, promote tolerance to innocuous particles or aeroallergens or in case of a pathogen infiltration mount an immune response. Therefore maintenance of immune homeostasis in the lung is very important. The lung environment promotes tolerance and controls inflammation. In the airways, macrophages form part of the barrier defence, phagocytosing and presenting infiltrating microbes. They also clear surfactant [195, 196], as well as, apoptotic cells and debris, which is regulated through production of anti-inflammatory mediators like transforming growth factor-β (TGF-β) and PGE_2 [197].
There are at least two populations of macrophages in the lung, alveolar macrophages (AMs), residing in the alveolar space, and several subsets of interstitial macrophages (IMs) located in the alveolar interstitium. They are phenotypically and functionally distinct. Murine AMs and IMs can be distinguished based on their surface marker expression, as specified in Figure 1.4 [198]. AMs depend on granulocyte macrophage colony-stimulating factor (GM-CSF), and TGF-β for

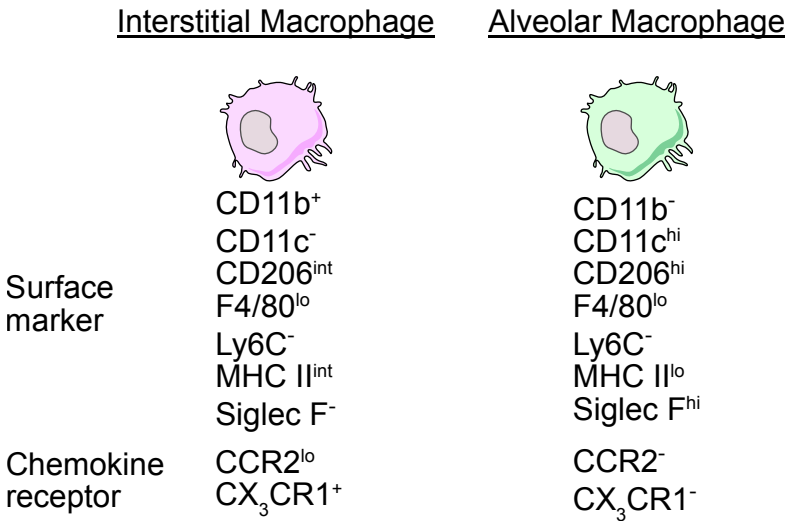

Figure 1.4: Phenotypic differences of murine interstitial and alveolar macrophages.

their maturation and differentiation [199, 200]. GM-CSF induces PPARγ expression [195]. Loss of either results in defective AM differentiation, accumulation of surfactant and lipids besides impaired pathogen clearance [195, 199, 201].
TGF-β can be secreted by MΦ themselves and work in an autocrine manner, or by lung epithelial cells [202]. Soroosh *et al.* [203] have shown that TGF-β together with retinoic acid/retinal dehydrogenases (RALDH) promotes lung homeostasis through induction of Foxp3$^+$ Tregs. Additionally, DC-*Tgfbr2* KO or hematopoietic *Tgfbr2* KO mice showed aberrant inflammation, decreased peripheral Foxp3$^+$ Tregs and pro-inflammatory DCs [204, 205]. IMs contribute to lung homeostasis by secretion of IL-10. IM secreted IL-10 can hinder DC migration to lymph nodes after LPS stimulation and thereby prevent inflammatory immune responses [206, 207]. The lipid mediator PGE_2 also exerts anti-inflammatory effects in the lung and controls tissue damage upon efferocytosis [208, 209]. PGE_2 induces IL-10 expression and can inhibit lung inflammation through initiation of an anti-inflammatory macrophage phenotype [140]. As shown by Svedberg *et al.* [192], the hypo-responsiveness of AMs, and to a lesser extend also IMs, to type 2 activation is determined by the lung environment.

1.4.2 Monocyte recruitment and replenishment during infections

Lung homeostasis is disturbed upon inflammatory induction by a viral type 1 or a parasitic/ allergic type 2 immune response. Circulating monocytes (derived from the bone marrow) can infiltrate mucosal tissues from the blood stream. Murine monocytes can be categorized into inflammatory and patrolling ones [210], while human monocytes are divided into classical, intermediate and non-classical monocytes based on their receptor expression [211], as shown in Figure 1.5.

Recruitment of monocytes from the bone marrow depends on chemokine signalling through CCL2/CCR2 for Ly6C^{hi} monocytes [23]. Chemoattractant release, during inflammation results in monocyte (and other lymphocytes) migration. Monocytes take part in apoptotic cell clearance and can themselves undergo apoptosis [213]. Depending on the environmental milieu, monocytes will become inflammatory or resolving monocyte-derived macrophages [214]. During the course of inflammation, monocyte/ macrophage phenotype can switch from inflammatory to resolving cells that promote tissue regeneration [215].

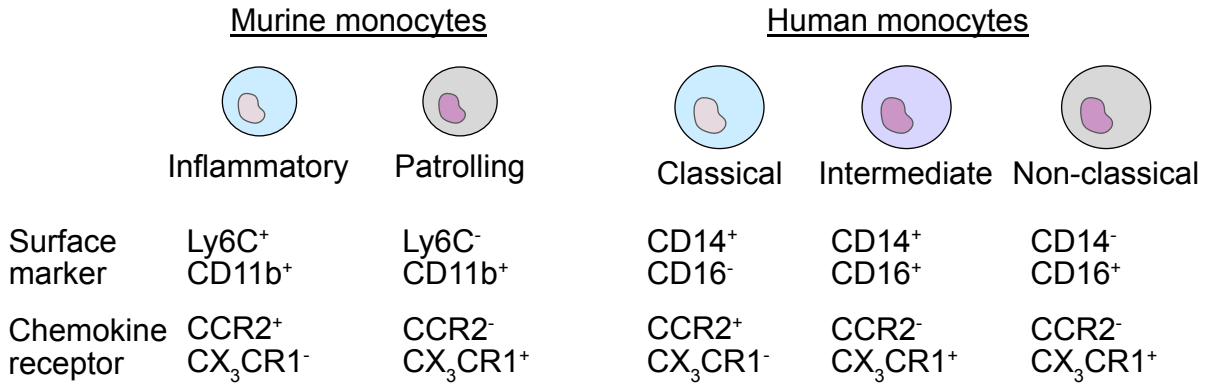

Figure 1.5: Monocyte subsets in human and mice, adapted from Murray [212].

1.5 Macrophages in type 2 immune responses

Allergic disease are initiated by release of proteases and alarmins like IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) from the epithelium upon damage. Some allergens are also proteases themselves, like Der p1, a house dust mite (HDM) allergen [216]. IL-25 and IL-33 release from epithelial cells induces eosinophilia, Th2 cell differentiation and ILC2 activation [217, 218]. Tuft cells also contribute to IL-25 release and subsequent ILC2 activation [219, 220]. IL-5 is essential for eosinophil survival, their recruitment under type 2 conditions is mediated by ILC2s [221]. Recruited eosinophils contribute to type 2 immunity through production of cysLTs and inflammatory cytokines [222, 223]. IL-33 also acts on MΦ and promotes their M2 polarisation [224, 225]. TSLP induces DCs to promote Th2 priming [226] or takes effect on $CD4^+$ T cells directly and drives Th2 polarisation [227]. Th2 cells and ILC2s produce type 2 cytokines, including IL-4, IL-5 and IL-13, which amplify the type 2 immune response. IL-4 and IL-13 share part of their signalling cascade through the IL-4Rα and IL-13Rα1 receptor, but also have unique functions. IL-13 leads to goblet cell and tuft cell hyperplasia and mucus production [219, 228], while IL-4 is important for IgE isotype switching in B cells [229]. IL-4 and IL-13 also lead to M2 polarization of macrophages. An overview of these pathways is given in Figure 1.6.
Inflammation and tissue damage lead to recruitment of monocytes, however, macrophage accumulation in inflamed lungs can be maintained through local proliferation [230]. This is also the case in another type 2 setting, namely in nematode infections, where IL-4 triggers proliferation of tissue resident macrophages [231]. Thus, *in vivo* recruited monocyte derived macrophages and tissue resident macrophages polarise to M2 macrophages and are involved in type 2 immunity. Zasłona *et al.* [230] found that not tissue resident but recruited monocyte-derived macrophages are responsible for allergic lung inflammation, as depletion of resident AMs augmented, while loss of circulating monocytes attenuated lung inflammation in an HDM allergic-airway inflammation model. This effect is supported by Lee *et al.* [232] who state that epithelial cell secreted CCL2 leads to monocyte recruitment and airway inflammation.
Macrophage reprogramming during type 2 immune responses results in an anti-inflammatory phenotype that may promote wound healing [65] and resolution of inflammation [184]. Nevertheless, M2 polarisation is also involved in type 2 pathologies. The M2 MΦ marker TG2 is increased in asthmatic patients [233, 234] and associated with airway hyper-responsiveness [235, 236]. Increased numbers or M2 MΦ were linked to disease severity in human asthmatic patients [237], while number of IL-10 producing MΦ were reduced [238]. Macrophages from severe asthmatics also take up less apoptotic cells while concomitantly producing less 15-HETE and

PGE_2 [239]. Reduction of IL-10 producing macrophages in allergic asthmatics could be inhibited by PGE_2 administration [140], speaking for a dysregulated IL-10 / PGE_2 axis in macrophages from asthmatic patients. During lung fibrosis increased macrophage numbers can be ascribed to monocytic infiltration and differentiation that persist after inflammation has resolved [240].

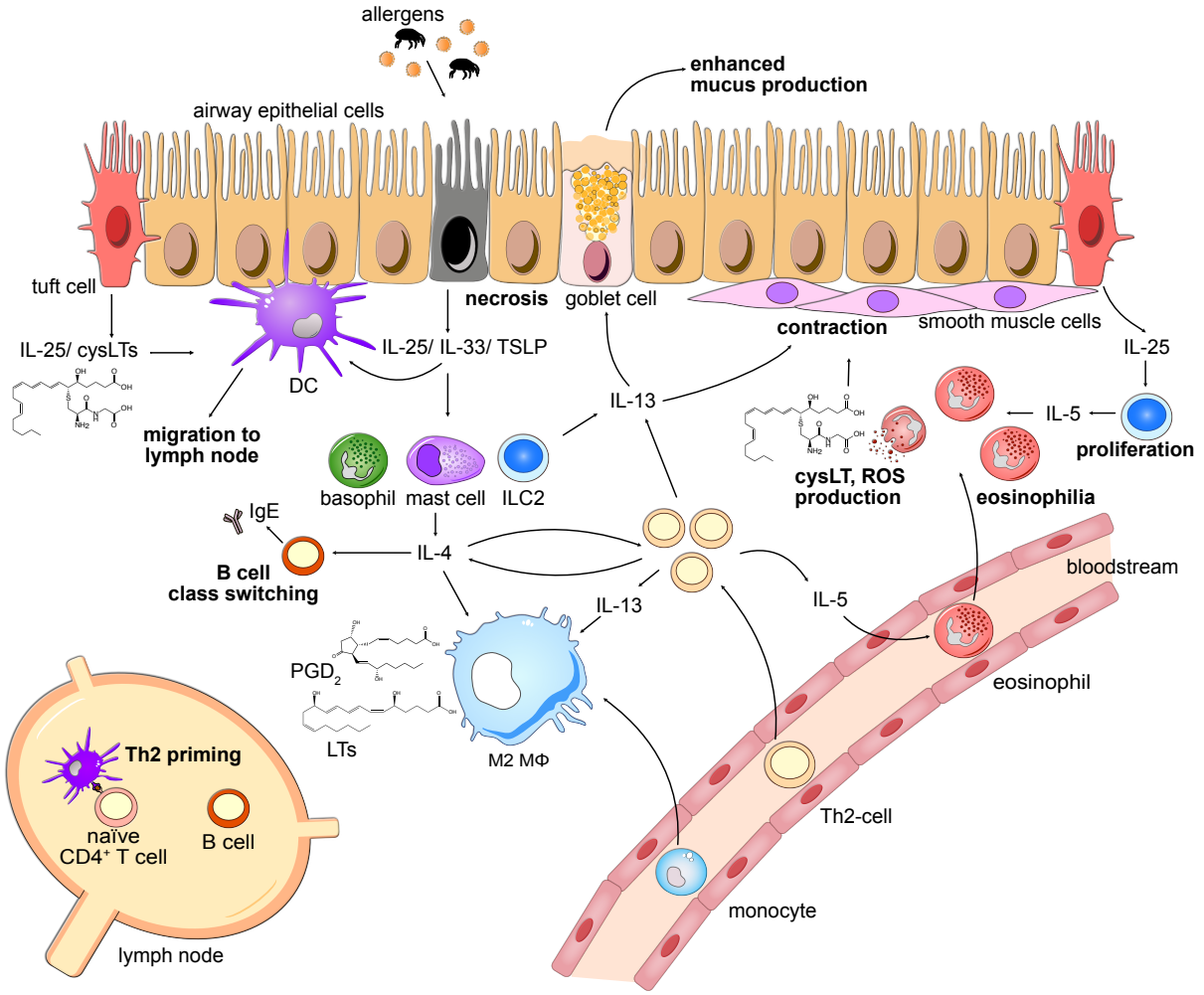

Figure 1.6: Mechanisms of allergic type 2 immune responses initiation. Allergen exposure leads to epithelial barrier injury and release of thymic stromal lymphopoietin (TSLP), IL-25 and IL-33. Those alarmins further activate basophils, mast cells and ILC2s to release the type 2 cytokines IL-4, IL-5 and IL-13, as well as, lipid mediators like PGD_2. Tuft cells contribute to IL-25 and cysLTs production, thereby activating different cell types including Th2 cells and ILC2s. IL-25 and IL-33 also induce DC migration to lymph nodes where they present antigen and promote naïve $CD4^+$ T cells to Th2 differentiation. Th2 cells produce IL-4, IL-5 and IL-13 leading to goblet cell hyperplasia, enhanced mucus production, smooth muscle cell contraction, eosinophil recruitment and M2 macrophage polarization. Mast cells release eosinophil chemotactic factors and IL-5 leading to eosinophil accumulation. Eosinophils contribute to type 2 inflammation through production of cysLTs and ROS.

1.6 Macrophages in respiratory viral infections

Entry of a virus into the host leads to the activation of innate immune cells via PRR sensing of viral nucleic acids, membrane particles or damaged and infiltrated host cells which induces the secretion of inflammatory cytokines and IFNs. IFNs are divided in type I, II and III IFNs, with type I encompassing several IFN-α, IFN-β, IFN-ε, IFN-ϰ and IFN-ω, while IFN II only includes IFN-γ, and IFN III encompassing four IFN-λ molecules [241]. IFNs bind to receptors containing two different subunits, type I IFNs bind to the heterodimeric IFNAR1/ IFNAR2 receptor, expressed on all cells. Type II IFNs to IFNGR1/IFNGR2 and type III IFNs bind to IL-10R2/ IFNLR1 heterodimers [242]. After receptor binding, canonical IFN-I and IFN-III signalling via Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) phosphorylates STAT1/2, recruits IRF9, and leads to formation of the complex ISGF3 (IFN-stimulated gene factor 3) [242, 243]. Translocation of the ISGF3 complex to the nucleus results in the expression of ISGs (IFN-stimulated genes). ISGs encompass many different molecules responsible for anti-viral immunity, via inhibition of viral entry or viral replication, induction of apoptosis, or recruitment of immune cells. For example ISG20 is involved in RNA degradation [244], while IRF7 promotes transcription of type I IFNs [245]. IFNs also induce maturation of monocytes to DCs, via upregulation of MHC and co-stimulatory molecules, and their migration to lymph nodes [26, 246]. IFN activated MΦ and DCs secrete cytokines promoting Th1 polarisation [247]. Type I IFNs also act directly on $CD4^+$ and $CD8^+$ T cells, inducing T cell polarisation to Th1 or cytotoxic T cells [247].
In influenza A viral infections, affecting the respiratory tract, depletion of AMs results in increased susceptibility to some viral strains [248], demonstrating the important role of macrophages in early anti-viral responses. On the other side macrophages participate in viral spreading or dysregulated immune responses. As is the case in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections.

1.6.1 The COVID-19 pandemic

SARS-CoV-2 is the virus responsible for the coronavirus disease 2019 (COVID-19) [249]. COVID-19 turned into a pandemic, worldwide infecting over 390 million and killing more than 5.7 million people worldwide ([250], data Feb. 2022). SARS-CoV-2 belongs to the class of betacoronaviruses containing single-stranded positive-sense RNA [251]. Two other severe diseases originated from coronaviruses, SARS-CoV leading to SARS and Middle East respiratory syndrome–related coronavirus (MERS-CoV) to MERS. SARS-CoV-2 infection takes place via contact with a positive persons or aerosol and droplet inhalation, while transmission through infected surfaces is less common [252, 253].
The main entry route for SARS-CoV-2 occurs via angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) [254]. RNAseq analysis identified substantial ACE2 expression in epithelial cells of diverse organs like the respiratory or the gastrointestinal tract [255]. SARS CoV-2 spike protein is highly glycosylated and can also be recognized by C-type lectin receptors like L-SIGN and DC-SIGN [256, 257], which are also involved in human immunodeficiency virus or *M. tuberculosis* infections [258, 259]. L-SIGN (CD209L) is mainly expressed on type II alveolar cells, as well as, lung, liver and lymph node endothelial cells [260], while DC-SIGN (CD209) is expressed on DCs and MΦs [261, 262].
The majority (ca. 80% to 90%) of COVID-19 infected patients display mild symptoms like headache, cold or anosmia [263, 264], while severe disease can result in dyspnoe, hypoxia, multiorgan failure and acute respiratory distress syndrome (ARDS) [265]. Dysregulated activation of myeloid cells contributes to the cytokine storm, that is linked to disease severity and death [266, 267], as shown in Figure 1.7. Overshooting inflammatory responses are coupled to an

adverse disease outcome, observable by high plasma levels of pro-inflammatory cytokines like IL-1β, IL-6 and CCL2 [256, 266, 268]. Myeloid cell frequencies in the blood are altered between healthy, mild and severe COVID-19 patients, represented by increased CD163^{hi}HLA-DR^{low} and S100A^{hi}HLA-DR^{low} monocytes [265, 267, 269]. The alarmins S100A8 and S100A9 are involved in Ca^{2+} signalling, arachidonic acid metabolism and cytoskeletal rearrangement needed for cell migration and phagocytosis [270]. Also increased numbers of neutrophils and immature neutrophils are found in the circulation during severe disease speaking for emergency myelopoiesis [266, 269]. While severe COVID-19 patients had high levels of inflammatory chemokines and cytokines in plasma, IFN levels were not higher in severe compared to mild disease. Indeed, SARS-CoV-2 defers and antagonizes the early induction of IFN responses [271], which, especially in severely ill, can lead to untimely induction of antiviral responses [266]. Also the serum lipidome of COVID-19 patients was altered. Schwarz *et al.* [272] showed a shift from PUFA-containing phosphatidylcholine, and phosphatidylserine towards PUFA free fatty acids and PUFA-containing triacylglycerols in serum of COVID-19 patients compared to healthy controls, which was exacerbated in severely ill. Especially 5-LOX and CYP derived lipid mediators were elevated in severe COVID-19 patients, with concomitant increase of 5-LOX expression in monocytes, macrophages, as well as, neutrophils [272]. Another study found increased prostanoid and leukotriene levels, but also SPMs, in the bronchoalveolar lavage (BAL) fluid of COVID-19 patients compared to healthy controls [273], showing that the increased number of immune cells in the BAL also influences disease through secretion of inflammatory lipid mediators. As mentioned above, TXA_2 promotes vasoconstriction and platelet aggregation. Microthrombosis events have been reported in COVID-19 pathology. SARS-CoV-2 infection results in increased neutrophil extracellular trap (NET) formation [274]. Also hyper-reactive platelets and increased aggregate appearance are involved in the thrombotic pathophysiology [275] displayed in Figure 1.8.

In 2020, when the COVID-19 pandemic commenced, BCG vaccination was advocated to reduced SARS-CoV-2 mortality, however, current results state no protective effect of BCG vaccination on infection [278, 279], while other studies suggested a boosting effect of BCG vaccination on subsequent COVID-19 vaccination [280, 281]. Apart from the acute disease, SARS-CoV-2 infections can also lead to long-term sequelae after virus clearance, reaching from fatigue and headache, to airway dysfunction, including impaired pulmonary diffusion [282]. This pathological state is termed 'long-COVID', with the reported persistence of more than 1 symptom ranged from 30% 3-9 months [283], 76% 6-months after COVID-19 infection [282] or up to 87%, 6-weeks after infection [284]. Variety between prevalence depends on the selected study population and time after infection [285].

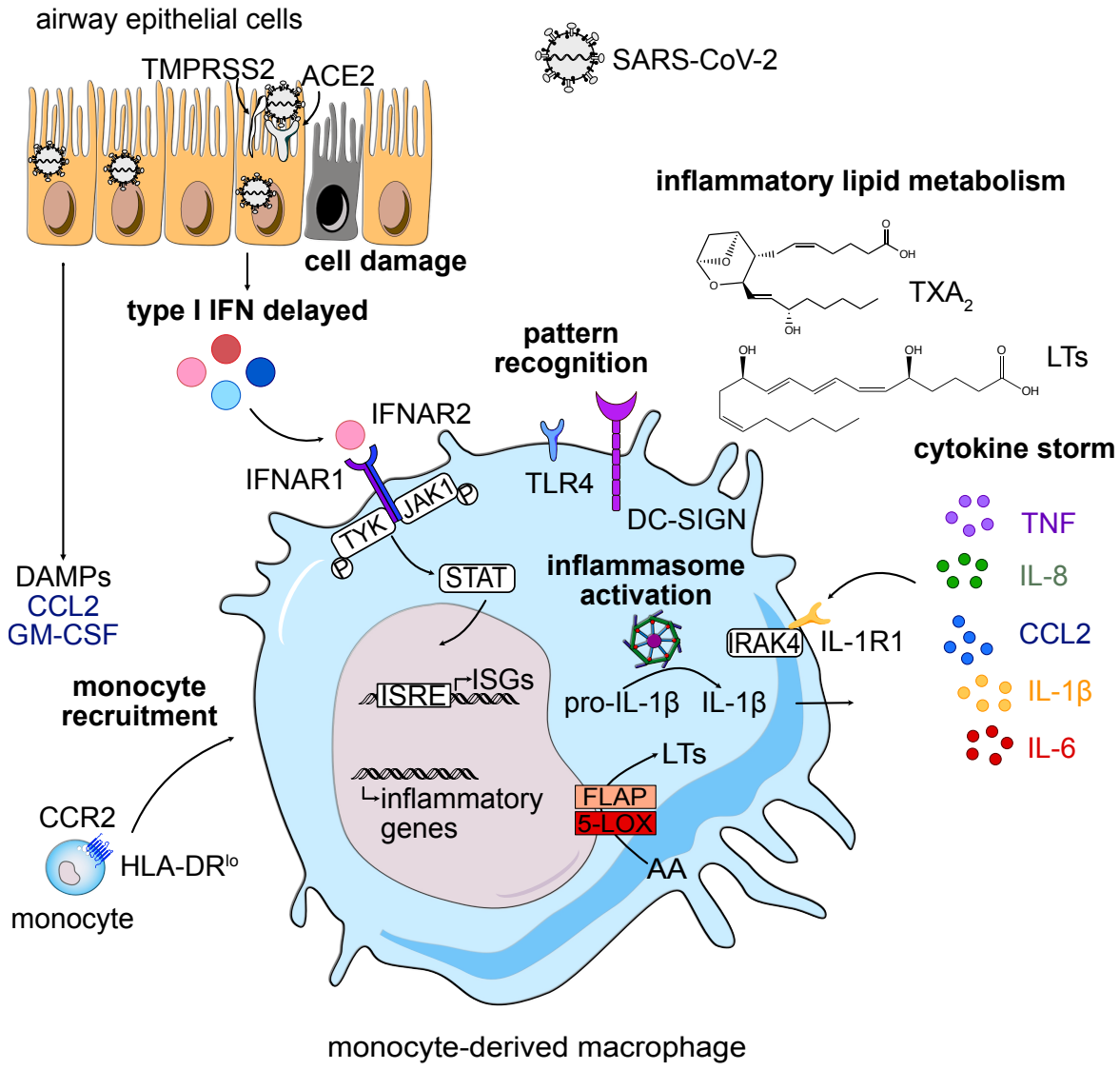

Figure 1.7: Recruitment of monocytes and their contribution to COVID-19 cytokine storm. Monocytes are recruited though chemoattractant release and differentiate into inflammatory macrophages through JAK/STAT signalling. Activated monocyte-derived macrophages contribute to the inflammation by release of pro-inflammatory chemo- and cytokines. NLRP3 inflammasome activation leads to IL1β maturation and release. Released cytokines can also function in an autocrine manner, sustaining the inflammatory response. Apart from chemo- and cytokines also inflammatory eicosanoids like LTB_4 and thrombosis promoting TXA_2 are released. Figure adapted from Merad & Martin [276].
Abbreviations: ISRE: IFN-stimulated response element, ISG: interferon-stimulated gene

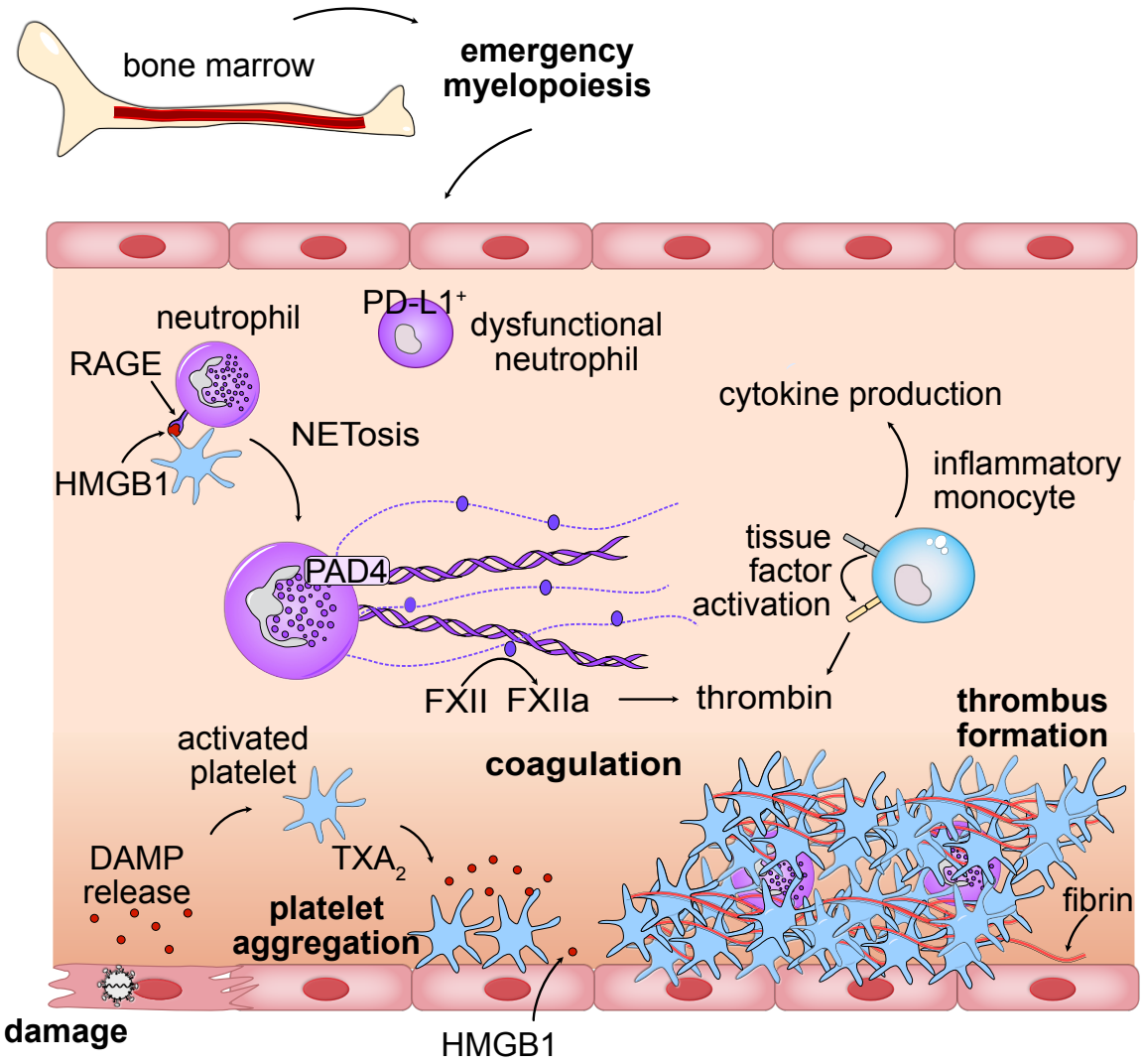

Figure 1.8: Involvement of monocytes, neutrophils and platelets in immunothrombosis, a pathology negatively associated with COVID-19 survival. Infected cells release DAMPs including HMGB1 (high mobility group box 1 protein), leading to platelet, neutrophil and monocyte recruitment. TXA_2, produced by activated platelets, promotes platelet aggregation and HMGB1 release. Platelet-derived HMGB1 can evoke NETosis through receptor for advanced glycation end products (RAGE) or TLR4 signalling. Tissue factor on monocytes is activated initiating the extrinsic coagulation cascade and thrombin formation. Also NETs promote coagulation via activation of Factor XII (FXII) to FXIIa, merging on thrombin and further fibrin generation. Accumulation of activated platelets and coagulation can lead to thrombus formation. Figure adapted from Stark & Massberg [277].

2 | Aim

Lipid mediators, or eicosanoids, are involved in inflammatory and resolving immune responses. As macrophages are capable of forming and secreting a wide range of these mediators, we wanted to contribute to the understanding of macrophage derived lipid mediators in type 1 and type 2 immune responses.

The aim of the first publication was to analyse eicosanoid production of human or murine macrophages upon allergen exposure. The intention was to establish and employ an LC-MS/MS based approach for a more reliable quantification of lipid mediators.

As monocytes and macrophages are involved in the SARS-CoV-2 immune response and its severity, we wanted to investigate which persistent alterations in the myeloid compartment contribute to long term complications (post COVID). We were particularly interested if previous SARS-CoV 2 infection can persistently alter eicosanoid formation of macrophages, which we investigated in the second publication.

The purpose of the third manuscript (in preparation) is to investigate the role of transglutaminase 2 (TG2) in type 2 immune responses. Transglutaminase 2 has been shown to be increased in asthmatic patients and involved in allergic airway inflammation in mice. We therefore hypothesized that myeloid derived TG2 is responsible for the induction of allergic inflammation and involved in anti-helminth immunity.

3 | Material and Methods

3.1 Material

A list of the material is provided in the corresponding publication.

3.2 Methods

A brief method description is appended below, while a more detailed account of all methods can be found in the enclosed manuscripts.

3.2.1 Isolation and culture of PMN, PBMCs and monocyte derived macrophages

All human experiments were approved by the local ethics committee at the university clinic of the Technical University of Munich. After written informed consent, blood from healthy human donors or SARS-CoV-2 seropositive patients was used for the isolation of peripheral blood mononuclear cells (PBMCs) or polymorphonuclear leukocytes (PMNs) via density gradient centrifugation. Monocytes from the PBMC section were enriched via CD14 positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) and differentiated to monocyte derived macrophages (MDM) for 6 days with human GM-CSF (Miltenyi Biotec) and TGF-β (PeproTech, London, UK) to induce an alveolar-like phenotype. The PMN section was subjected to red blood cell lysis and cultured over night or the specified time with GM-CSF. PMN, PBMCs and MDM were subjected to further analysis as specified.

3.2.2 Cytokine analysis via ELISA or EIA

Chemo- and cytokines in cell culture supernatant were quantified using commercially available multiplex or enzyme-linked immunosorbent assay (ELISA) kits (BD or R&D systems, Minneapolis, USA). For eicosanoid detection, enzyme immune assays (EIA) were used (Cayman Chemicals, Ann Arbor, USA).

3.2.3 Chemotaxis assay

PMN were isolated as stated above. After isolation cells were primed for 30 min with conditioned media of MDM. PMN were further subjected to chemotactic assays through transwells (3 μm pore size, Corning, USA) for 3h at 37°C towards medium containing LTB_4 (Cayman Chemicals), IL-8 and CCL5 (both Miltenyi Biotec). Migration of PMN was evaluated microscopically.

3.2.4 RNA extraction and cDNA synthesis

Cells were lysed in RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol (β-ME). RNA was isolated via commercially available spin-columns (Zymo research, Irvine, USA) and further reverse transcribed to cDNA (Thermo Fisher Scientific, Carlsbad, USA) or used for bulk RNA sequencing.

3.2.5 RNA sequencing

RNA sequencing was performed at the Helmholtz Genomics Core Facility. Library preparation was performed using the TruSeq Stranded mRNA Library Prep Kit. Total RNA quantity was assessed by a Qubit 4 Fluorometer (Thermo Fisher Scientific) and RNA integrity number was determined with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, USA). For library preparation, 1 µg of RNA was poly(A) selected, fragmented, and reverse transcribed with the Elute, Prime, Fragment Mix (Illumina, San Diego, USA). A-tailing, adaptor ligation, and library enrichment were performed as described in the TruSeq Stranded mRNA Sample Prep Guide (Illumina). RNA libraries were assessed for quality and quantity with the Agilent 2100 BioAnalyzer and the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Thermo Fisher Scientific). RNA libraries were sequenced as 150 bp paired-end runs on an Illumina NovaSeq 6000 platform.

3.2.6 Real-time quantitative PCR

Primers for real-time quantitative PCR analysis can be found in the manuscript sections. The PCR was conducted using 10 ng cDNA as template for FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) on a ViiA7TM Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific).

3.2.7 Lipid mediator quantification via LC-MS/MS

Cell culture supernatant, BALF or intestinal culture supernatant was stored in equivalent volume of MeOH at $-80°C$ until sample extraction. Three different extraction and quantification methods were used. The method described here is the one that was established and described in publication I.
Automated solid phase extractions were performed with a Microlab STAR robot (Hamilton, Bonaduz, Switzerland). Prior to extraction all samples were diluted with H_2O to a MeOH content of 15% and 10 µl of internal standard solution was added. Samples were extracted using Strata-X 96-well plates (30 mg, Phenomenex, Aschaffenburg, Germany) and eluted with MeOH. Samples were evaporated to dryness under N_2 stream and redissolved in MeOH/H_2O (1:1). Chromatographic separation of eicosanoids was achieved with a 1260 Series HPLC (Agilent) using a Kinetex C18 reversed phase column (2.6 µm, 100 x 2.1 mm, Phenomenex) with a SecurityGuard Ultra Cartridge C18 (Phenomenex) precolumn. The QTRAP 5500 mass spectrometer (Sciex, Darmstadt, Germany), equipped with a Turbo-VTM ion source, was operated in negative ionization mode. Samples were injected via an HTC PAL auto-sampler (CTC Analytics, Zwingen, Switzerland), set to 7.5°C. Identification of metabolites was achieved via retention time and scheduled multiple reaction monitoring (sMRM) as specified. Acquisition of LC-MS/MS data was performed using Analyst Software 1.6.3 followed by quantification with MultiQuant Software 3.0.2 (both Sciex).

3.2.8 Fluorescence activated cell sorting (FACS) analysis

MDM were stained with antibodies against EP2 (PE) (Columbia Biosciences Corp, Maryland, USA) , HLA-DR (BV650), CD206 (BB515) and CD11b (BV421), all BD. All samples were acquired on a BD LSRFortessa and analysed using FlowJo v10 (FlowJo, BD) software.

3.2.9 Western blot analysis

Western blotting was performed as previously published [286].

3.2.10 In vivo HDM-induced allergic airway inflammation

Mice were sensitized with 1 µg *Dermatophagoides farinae* (HDM) extract, followed by HDM challenges on day 8-11 with 10 µg extract. Animals were sacrificed on day 14. BALF was collected and differential cell count, lipid mediators and gene expression analysed.

3.2.11 In vivo *Nippostrongylus brasiliensis* infection

Mice were infected with 200 or 500 *N. brasiliensis* larvae and subjected to BALF after 5 days of infection as previously published [40], or on day 3 and 6 for the transglutaminase 2 experiments. Alveolar macrophages in the BALF were left to adhere for 4h at 37°C, while other cells were washed away, followed by lysis in RLT Buffer (Qiagen) containing β-ME for RNA isolation.

3.2.12 In vivo *Heligmosomoides polygyrus* infection

Mice were infected with 200 *H. polygyrus* larvae and sacrificed on day 14 or day 24 after infection. The small intestine was removed, opened and parasites were counted. For intestinal supernatant analysis, duodenum was washed thoroughly with PBS containing Penicillin-Streptomycin and incubated for 6h at 37°C. Supernatant was removed and stored in equivalent volume of MeOH at −80°C for lipid mediator analysis. Peritoneal macrophages were obtained by adhering peritoneal wash for 4h at 37°C, washing away non-adherent cells and lysis of adherent cells in RLT Buffer (Qiagen) containing β-ME for RNA isolation.

3.2.13 Mice

C57BL/6J Tgm2$^{fl/fl}$ mice were purchased from Jackson Laboratories and crossed to either LysM^{Cre} or Vav^{iCre}. Mice were bred and housed at the Helmholtz Zentrum München, Germany under specific pathogen-free conditions with *ad libitum* access to food and water. Experiments were performed with either male or female mice in accordance with approved guidelines by the Bavarian government.

3.2.14 Data analysis and statistics

Data was analysed either via GraphPad Prism (GraphPad Software, San Diego, USA) or R (open source, [287]), versions as specified in the publication. The statistical test and data exclusion or replacement criteria used in the individual studies is stated in detail in the method sections of the publications. Intestinal lipid mediator levels were normalized to duodenal weight. Chemical structures and figures were generated using ChemDraw v20.1 and Affinity Designer.

4 Results

4.1 House dust mite drives proinflammatory eicosanoid reprogramming and macrophage effector functions

Lipid mediators play an important role in type 2 immune responses like allergy and asthma. In the frame of this thesis we have established an LC-MS/MS method for the quantification of 52 oxylipins.
To validate the method in a well-established cellular model, we analyzed supernatant from human PMN stimulated with zymosan, a fungal cell wall component, or left them untreated. Zymosan stimulation reduced production of leukotrienes compared to unstimulates PMN levels. The LC-MS/MS measurement was reproducible over technical replicates.
To evaluate the impact of allergen exposure on macrophages and their effector functions, we stimulated human alveolar-like monocyte-derived macrophages (MDM) with house dust mite (HDM) extract in combination or not with IL-4. Lipid mediator profiles indicated macrophage reprogramming by HDM to a COX dominated profile with decreased 5-LOX metabolism, while additional IL-4 had minimal impact on the mediator levels. Macrophage reprogramming was also visible on gene expression level. HDM exposure led to induction of COX-2 (*PTGS2*) and prostaglandin E synthase (*PTGES*) expression and protein abundance, while 5- and 15-LOX were down-regulated. Additionally, HDM exposed MDM increased their production of proinflammatory cytokines and chemokines like IL-6 and TNF.
We evaluated the impact of inhibiting either dectin-2, shown to recognize HDM [18], or p38 MAPK, implicated in eicosanoid regulation [288, 289], on macrophage eicosanoid reprogramming. Phosphorylation of p38 was involved in HDM signal transduction and effector mechanism in MDM, as p38 inhibition before HDM stimulation diminished the increase of PGE_2 and PGD_2, while also preventing 5-HETE reduction. Dectin-2 inhibition had no impact on HDM-triggered prostanoid and cytokine induction. Figure 4.1 depicts effects of HDM stimulation on macrophages.
To investigate if the HDM-induced macrophage reprogramming altered their effector function, we evaluated the capacity of MDM to recruit granulocytes. Indeed, PMN chemotaxis to an MDM medium pool was reduced if the MDM had been stimulated with HDM. This effect was also present, if MDM had been treated with indomethacin, a COX inhibitor, before addition of HDM. However, inhibition of p38 could restore macrophage chemotactic potential. This suggested that prostanoids were not responsible, while reduced LTB_4 levels might explain the impaired chemotactic capacity of MDM after HDM stimulation.
To assess if eicosanoid reprogramming takes place in an *in vivo* type 2 setting, we quantified lipid mediators in the BAL fluid of mice that had been sensitized and challenged with HDM or infected with the nematode *Nippostrongylus brasiliensis* (*Nb*). *Nb* infected mice showed high levels of prostanoids in the BAL fluid, while those were not detected in HDM sensitized or naïve

mice. HDM and *Nb* infection both reduced LTB_4 levels and increased cysLTs compared to naïve BAL fluid levels. We could also detect 17-HDHA, a precursor for resolvin production, in the BAL of *Nb* infected, as well as, HDM sensitized animals.
Thus, HDM and *Nb* both induced a type 2 inflammation and concomitant lipid mediator reprogramming, while differing in their mediator profile in the BAL fluid. The discrepancy in the lipid mediator profile between *in vitro* HDM stimulation and *in vivo* HDM sensitization could be a matter of timing or contributions of additional cells (granulocytes, tuft cells) to eicosanoid production *in vivo*. An induction of the COX and repression of the 5-LOX pathway in macrophages may represent an early reaction that is followed by a type 2 immune response to allergens or helminths.

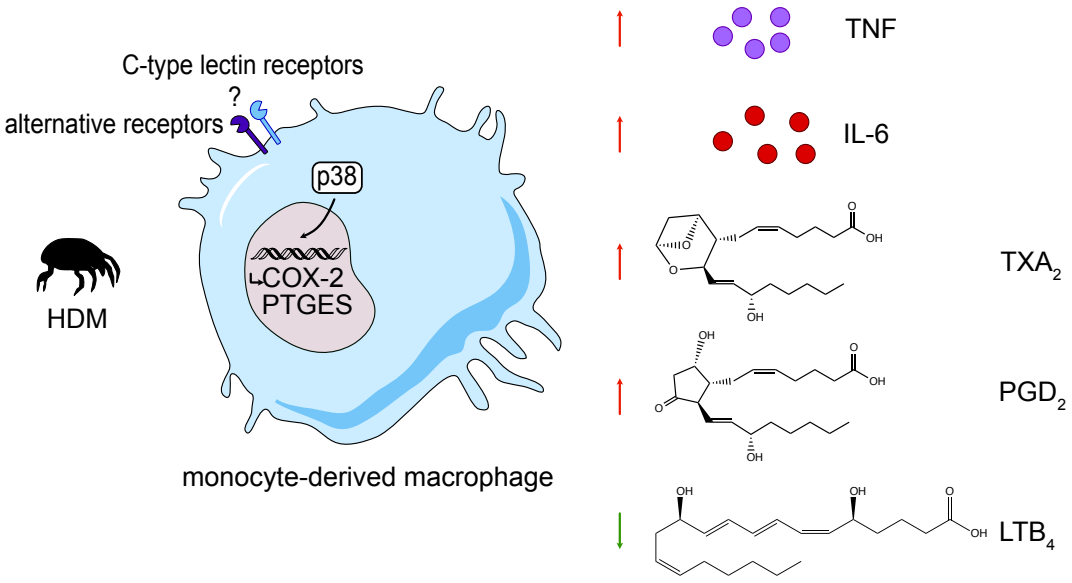

Figure 4.1: House dust mite (HDM) stimulation resulted in macrophage eicosanoid reprogramming via p38 MAPK. The involvement of C-type lectins or alternative receptors in HDM recognition in our study was unclear. Monocyte-derived macrophages produced more prostanoids and less 5-LOX metabolites like LTB_4 after 24h *in vitro* HDM stimulation which was transcriptionally regulated.

4.2 Mild COVID-19 imprints a long-term inflammatory eicosanoid- and chemokine memory in monocyte-derived macrophages

Monocyte-derived macrophages are responsible for the cytokine storm in severe COVID-19 [266, 267]. Therefore, we investigated if a long-term reprogramming of the monocyte compartment is present upon SARS-CoV-2 infection.

Health care workers from the TUM university hospital were retrospectively assigned into SARS-CoV-2 seronegative or seropositive (post CoV) patients based on their SARS-CoV-2 IgG titer. The final cohort included 36 seronegative and 68 previously COVID-19 infected individual. At the time of infection (Spring 2020), 74% of patients reported symptoms with the most frequent being fatigue, while only 16% described persistent symptoms 3-5 months after infection. Also 31% of the seronegative control group reported symptoms in Spring 2020, the main symptom being fatigue as well.

Monocytes from patients 3-5 months after COVID-19 infection or seronegative controls were differentiated into alveolar-like MDM to simulate the lung environment. RNAseq analysis identified 163 differentially expressed genes between seronegative and seropositve MDM. Especially inflammatory chemokines like CCL2 and CCL8 were expressed at higher levels in MDM of patients that had been infected with SARS-CoV-2. This effect was exaggerated upon 24h SARS-CoV-2 spike protein (s-protein) or LPS stimulation. S-protein stimulation resulted in induction of ISGs (e.g. ISG20, OAS1) and chemokines (e.g. CCL8, CXCL10). Our data indicated an inflammatory imprint and increased reactivity of MDM 3-5 months after SARS-CoV-2 infection. Gene expression differences between seronegative and convalescent COVID-19 MDM were greater after restimulation, indicating a "training" effect of previous SARS-CoV-2 infection.

Eicosanoids have been implicated in severe, acute COVID-19 [272, 273], and we measured increased expression of genes involved in fatty acid synthesis (e.g. FASN, CYB5R2) after COVID-19 infection. Thus, we analysed eicosanoid metabolites from seropositive or seronegative MDM. MDM from previously infected patients secreted significantly more pro-inflammatory 5-LOX metabolites including LTs and 5-HEPE, as well as, prostanoids like $PGF_{2\alpha}$ and 12-HHTrE. Addition of s-protein further increased production of prostanoids, both in seropositive and seronegative MDM, with a higher amplitude in previously infected, as depicted in Figure 4.2. We also showed that MDM could be categorized into 5-LOX high and low producers 3-5 months after infection. 5-LOX low producers had lower symptom levels at time of infection, while also exhibiting a more rapid decline in SARS-CoV-2 serum IgG titers. Analysis of MDM eicosanoid levels 12 months after infection indicated a return to seronegative levels, implying a temporary eicosanoid reprogramming.

Contrary to the eicosanoid effect, cytokine and chemokine production was not significantly different between MDM from seronegative or seropositive subjects 3-5 months after infection, independent of the stimulation.

Glucocorticoids are commonly used for the treatment of COVID-19. We investigated if treatment with fluticasone propionate, an inhaled glucocorticoid, could suppress the inflammatory MDM phenotype. Inflammatory cytokines like IL-6 and prostanoids were reduced by fluticasone propionate application, however, 5-LOX metabolites like LTB_4 and LTC_4 were significantly increased. On the one side, glucocorticoid administration could decrease cardiovascular events by reducing pro-thrombotic metabolites, while on the other, it could contribute to inflammation through enhanced leukotriene formation.

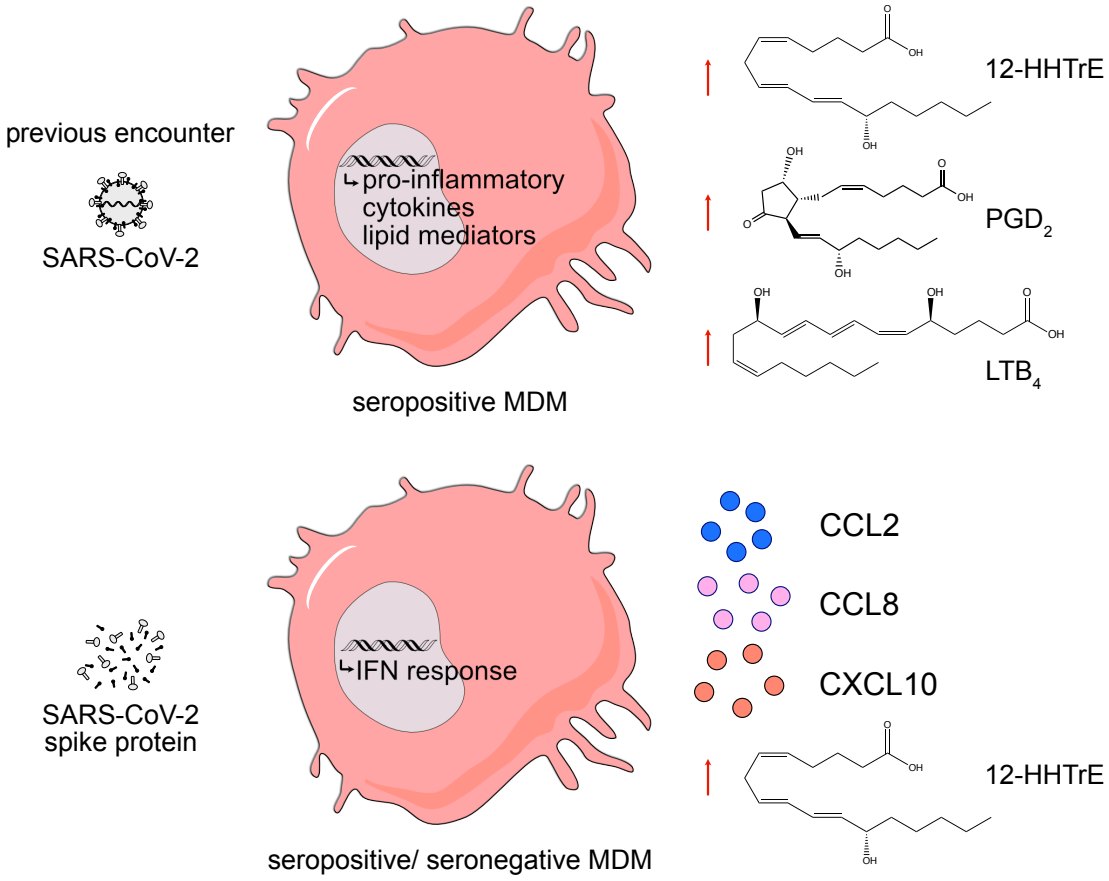

Figure 4.2: Previous SARS-CoV-2 infection resulted in inflammatory MDM reprogramming defined by increased prostanoid and 5-LOX mediator production. SARS-CoV-2 spike protein stimulation induced an IFN response in MDM and resulted in production of inflammatory cytokines and chemokines.

4.3 Role of transglutaminase 2 in type 2 immune responses

TG2 is an abundantly expressed enzyme and considered a marker of alternatively activated MΦ [290] that has been implicated in allergic airway inflammation [235]. To investigate if macrophage TG2 is involved in type 2 immune responses we generated conditional TG2-deficient mice. To induce a myeloid deficiency, $Tgm2^{fl/fl}$ mice were crossed to $LysM^{Cre}$ mice (mTG2 KO), or to induce a hematopoietic knock-out, to Vav^{iCre} mice (hTG2 KO). Both KO displayed only minor Tgm2 expression in different MΦ species, including bone marrow derived macrophages (BMDM), as well as, alveolar MΦ (AM) or peritoneal MΦ (PM) (Figure 4.3 A, B).
To investigate if type 2 immune responses impact on Tgm2 expression levels in MΦ, we infected mice with either the intestinal parasite *Heligmosomoides polygyrus bakeri* (*Hpb*) or *Nb* (as depicted in Figure 4.3 C, D) and evaluated Tgm2 expression during infection. We detected an increase in MΦ transglutaminase 2 upon infection in wild type animals, with only a slight increase in myeloid or hematopoietic TG2 KO mice (Figure 4.3 E-G). Expression of Tgm2 in WT naïve peritoneal macrophages differed between different experiments.
As TG2 had been shown to activate PLA_2 [233], we investigated the eicosanoid profile of BMDM from either wild type (WT) or mTG2 deficient mice. To induce an inflammatory type 2 setting, we added HDM extract for 24h (Figure 4.3 H). HDM stimulation increased prostanoid formation, while reducing leukotriene generation (as shown in publication I for human MDM). TG2 deficieny of BMDM did not alter eicosanoid output.
Lipid mediators are also involved in the host defence and immune modulation during parasite infections, for example PGE_2 secretion by parasites or induction of its formation by the host are engaged in immune evasion [291, 292]. Therefore, we quantified prostanoid formation in intestinal supernatant of mice that had been infected with *Hpb* for 14 or 24 days (Figure 4.3 I-L). mTG2 deficient mice tended towards lower production of PGE_2 at d14 compared to WT mice, while its production at d24 was not different (Figure 4.3 J). Prostanoid production peaked on day 14, while hTG2 KO mice produced significantly lower amounts compared to WT animals (Figure 4.3 K, L). To see if this coincided with reduction in parasite burden, we counted adult worms (L5 stage) in the intestine. Worm counts on day 14 were comparable between WT and mTG2 KO or hTG2 KO mice, while on day 24 mTG2 KO mice had significantly less parasites in the intestine (Figure 4.3 M), with a trend also visible for hTG2 KO mice (Figure 4.3 N).
While we detected reduced numbers of *Hpb* larvae in the intestine of myeloid TG2 deficient mice, we did not perceive different numbers of parasites in the intestine after *Nb* infection.
Oh *et al.* [235] showed reduced inflammation and eosinophilic infiltration in TG2-deficient mice in an ovalbumin (OVA) allergic airway inflammation model. To evaluate if myeloid or hematopoietic TG2 is necessary for the induction of airway hyper-responsiveness we sensitized and challenged mice with HDM and assessed airway inflammation in the lung. Neither mTG2 nor hTG2 KO mice showed lower eosinophilic infiltration into the lung compared to WT mice.
Therefore, myeloid or hematopoietic TG2 seems to play a role in *Hpb* expulsion, while it is redundant during *Nb* infection or HDM sensitization.

Figure 4.3: (On next page) (A) Macrophage subsets from either myeloid deficient TG2 KO mice (LysM^{Cre}Tgm2$^{fl/fl}$, mTG2KO) or (B) hematopoietic TG KO mice (Vav^{iCre} Tgm2$^{fl/fl}$, hTG2 KO) express less Tgm2. (C) *Hpb* infection model: mice were infected on day 0 with 200 L3 *Hpb* larvae and analyzed on day 14 or 24. (D) *Nb* infection model: mice were infected on day 0 with 500 L3 *Nb* larvae and analyzed on day 3 or 6. (E-G) Expression of Tgm2 in either AM or PM at specified time points after *Hpb* or *Nb* infection. (H) Eicosanoid production quantified via LC-MS/MS in WT or mTG2 KO BMDM stimulated for 24h with HDM or not. (I,J,K,L) LC-MS/MS measurement of intestinal culture supernatant after indicated day of *Hpb* infection in WT and mTG2 KO mice or hTG2 KO mice. (M,N) Intestinal worm count of mTG2 KO or hTG2 KO mice on day 14 or 24 after *Hpb* infection. Statistical significance was determined using Mann-Whitney test, $*$: $p \leq 0.05$, $**$: $p \leq 0.01$, $***$ $p \leq 0.001$.

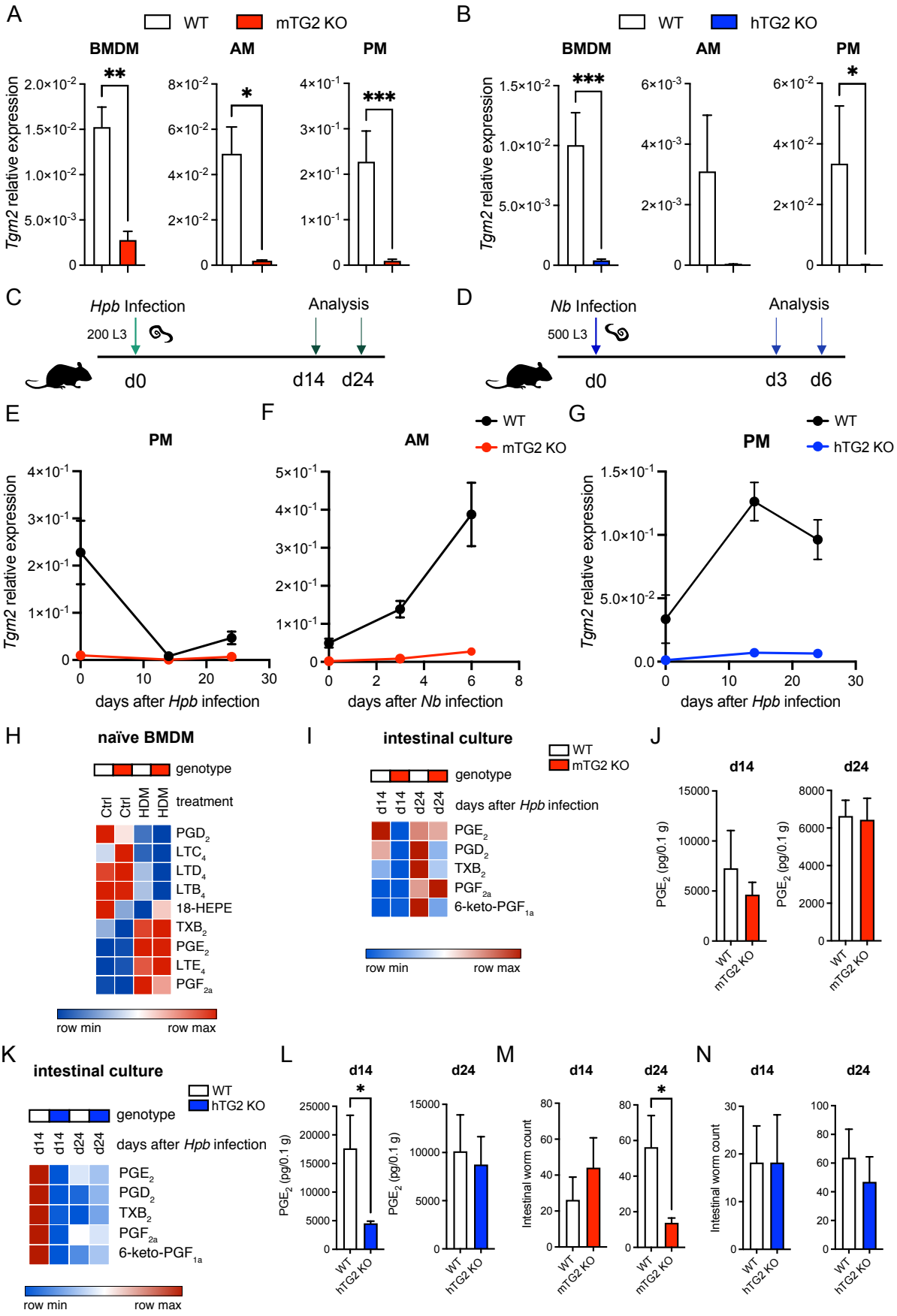

Figure 4.3: Caption on previous page.

5 Discussion

5.1 Necessity of reproducible lipid mediator quantification

Lipid mediators play vital roles in the regulation of inflammation and resolution. We showed that their quantification via ELISA resulted in an overestimation compared to quantification via our developed LC-MS/MS method. Using LC-MS/MS allows a more specific quantification, (e.g. LTC_4, LTD_4, LTE_4 versus all cysLTs) and enables detection of metabolites at lower levels of detection, as well as, a broader ranged profiling of lipid mediators [293]. This is especially important for a class of structurally similar molecules possessing different functions, where stereochemistry can influence physiological response [294]. Thus, quantification via LC-MS/MS enables an extended targeted analysis of lipid mediators important for understanding physiological changes in health and disease.

5.2 Monocyte-derived macrophages as an *in vitro* model for allergic lung inflammation

In allergy and asthma macrophages are important mediators of disease and resolution. Recruited monocyte-derived macrophages, in contrast to tissue resident macrophages, were main contributors to the lung inflammation in allergy [230].
Therefore, we chose to differentiate monocytes into alveolar-like macrophages in the presence of GM-CSF and TGF-β, two cytokines determining alveolar phenotype in the lung [199, 200]. We could recapitulate some characteristics of alveolar macrophages, like the high capacity for LT formation [295], production of pro-inflammatory cytokines and chemokines [296], as well as, a diminished responsiveness to IL-4 [192]. Even though those MDM might not fully reflect alveolar macrophages, they represent a useful model.
Single cell models can not substitute the lung where multiple cell types interact and influence each other. We therefore compared the *in vitro* macrophage eicosanoid metabolome to BAL fluid of HDM sensitized mice. While HDM stimulation of the MDM culture resulted in short-term induction of prostanoids, and reduction of 5-LOX metabolites that returned to baseline after 96h of HDM exposure, the two week *in vivo* HDM sensitization and challenge resulted in increased cysLT production in the BAL. In general, cysLTs are considered to be rapidly released upon allergen exposure [159], while macrophages in type 2 immune setting (during IL-4 or allergen exposure) seem to induce prostanoid or 15-LOX metabolite formation, while suppressing 5-LOX metabolites [297, 298]. Hence, timing and environmental milieu play a role in lipid mediator production.
In the BAL fluid also other immune cells like eosinophils, recruited during allergic airway inflammation, and epithelial cells (particularly tuft cells) could add to the production of LTs [286, 299, 300]. However, macrophages are the most abundant immune cell in the naïve lung [301]

and likely contribute to the initiation of the immune response and lipid mediator production upon HDM exposure.
Nevertheless, more and more publications stress the importance of epithelial cells in type 2 immune responses. Specialized epithelial cells like tuft or brush cells can produce leukotrienes, as well as, PGD_2 and thereby participate in type 2 inflammation [171, 302]. Recently, Ualiyeva *et al.* [300] showed that tuft cells can initiate allergic airway inflammation. Further investigations should clarify interactions of different cells types *in vivo* in the induction and perpetuation of allergic airway inflammation and if this process can be translated from mice to humans.

5.2.1 House dust mite recognition

Previous published data suggested dectin-2 as a receptor for HDM recognition [18, 303] and inflammatory response [304]. Neutralization of dectin-2 during HDM exposure, as performed in study I, had no impact on eicosanoid metabolism. While metabolites were determined after 24h of HDM exposure, other studies measured effector response after shorter incubation time (up to 1h) [18] or during *in vivo* HDM sensitization [303, 304]. Therefore, dectin-2 inhibition might impact on early metabolite levels during HDM stimulation, while other signalling pathways could impact on metabolite levels at later time points. We showed that phosphorylation of p38 was necessary for HDM eicosanoid and cytokine reprogramming. Activation of p38 had already been implicated in the regulation of eicosanoid metabolism [288, 289]. We did not investigate upstream p38 activation, but HDM mediated MDM reprogramming was independent of TLR-2/4 signalling. In a later study our group identified formyl peptide receptor 2 (FPR2) as an important receptor for HDM involved in macrophage reprogramming [305], which is also in line with another more recent publication [306].

5.2.2 Macrophages in allergy

Macrophages can secrete a wide range of cytokines, chemokines and lipid mediators and thereby play a role in type 2 immune responses. They are reprogrammed by the type 2 cytokines IL-4 and IL-13 to an M2 phenotype. M2 macrophages are increased in asthmatic patients [307]. However, alternative activation of macrophages was not necessary for allergic airway inflammation and eosinophil recruitment in mice [308], while recruited monocytes contributed to allergic lung inflammation [230]. Reduced monocyte recruitment through CCL2 receptor blockage could diminish airway inflammation during OVA-induced allergic airway inflammation [309, 310]. Another study affirmed that allergen sensitization altered macrophage phenotype, as only non-sensitized macrophages could inhibit development of allergic airway inflammation, while sensitized ones lost this ability [311]. Draijer *et al.* [140] showed that PGE_2 might reverse allergic inflammation, as PGE_2 treatment of macrophages lowered lung cell infiltration during HDM-induced airway inflammation. Severe asthma and allergen sensitization reduced phagocytic capacity of alveolar macrophages and could contribute to disease exacerbation through increased bacterial burden in the lung [312, 313]. In line with this, we demonstrated that monocyte-derived macrophages from N-ERD (Nonsteroidal anti-inflammatory drug–exacerbated respiratory disease) patients exhibited a pro-inflammatory phenotype indicative of an inflammatory memory [314]. As displayed in publication I, allergy or asthma leads to macrophage reprogramming apparent by altered production of pro-inflammatory cytokines and lipid mediators, which present targets for novel therapies. Future studies should identify the exact receptors and signalling cascades or epigenetic mechanisms necessary for macrophage reprogramming.

5.3 Implications of eicosanoid reprogramming in COVID-19

During SARS-CoV-2 infections mainly monocyte-derived cells are responsible for the overshooting inflammatory response in the lung [265]. Besides a hyper-inflammation characterized by increased chemo- and cytokine production (the "cytokine storm") [268], also a dysregulated eicosanoid metabolism has been described during COVID-19 infection [272]. We did not observe differences in cytokine production between seronegative or seropositive MDM 3-5 or 12 months after infection, which might be explained by the *in vitro* differentiation of monocytes to macrophages or the interval between infection and our investigations. However, we showed that patients suffering from mild COVID-19 exhibited a distinct eicosanoid formation in MDM 3-5 months after SARS-CoV-2 infection. This was characterized by increased inflammatory LTs and prostanoid mediators including 12-HHTrE.
We could stratify seropositive patients based on the amount of MDM derived 5-LOX metabolites. 5-LOX high producers had lower symptoms at the time of infection, but also a more rapid decline in SARS-CoV-2 serum IgG titer. As LT production in other diseases like asthma is associated with a more severe phenotype [315, 316], we would have expected a higher symptom burden in the 5-LOX high producers. What and if there is a causal connection between LT production and symptoms or IgG titers was not investigated the frame of this study, but would further contribute to the understanding of SARS-CoV-2 pathology.
Following stimulation with LPS or s-protein a higher increase in lipid mediator formation in post CoV MDM compared to seronegative controls was measured, which indicates that SARS-CoV-2 infection could lead to a "training" of the monocyte compartment. Eicosanoids produced by MDM returned to baseline levels 12 months after SARS-CoV-2 infection.
Previous encounter can lead to trained innate immunity that is characterized by either an enhanced or a tolerized response [84]. Several other viral infections like herpes virus [317] or influenza [318] can evoke macrophage training. While herpes virus infection could inhibit development of allergic asthma through replacement of AMs by monocyte-derived MΦ in the lung [317], the effect on influenza A virus (IAV) infection is more complex. On the one side, Aegerter *et al.* [318] found that IAV infection resulted in increased numbers of AM that induced protection to subsequent *Streptococcus pneumoniae* infection. On the other side, Shirey *et al.* [319] showed that previous influenza infection was detrimental for following bacterial infections. The high death toll in the 1918-1919 influenza pandemic can presumably be ascribed to viral and bacterial co-infections [320]. Timing between viral and bacterial infections dictates severity, while Aegerter *et al.* [318] investigated infections that took place after complete resolution of infection, Shirey *et al.* [319] analysed bacterial infections during acute viral infection.
Additionally, increased macrophage leukotriene production after COVID-19 infection (shown in publication II) could lead to decreased susceptibility to following IAV infections, as LTB_4 is crucial for resistance against IAV [321]. However, previous viral infection could also lead to immune-paralysis including reduced macrophage phagocytic capacity [319, 322]. Theobald *et al.* [323] showed that only monocytes from previously SARS-CoV-2 infected patients released IL-1β after s-protein stimulation due to a long-lasting inflammasome activation, which might be protective in following viral infections [324]. Also, in our study IL-1β production after s-protein stimulation was only significantly increased in MDM from post CoV patients. If SARS-CoV-2 infection will therefore increase or decrease susceptibility to further infections remains open for future investigations.
Another impact is exerted by the virus variant. We conducted the study in Spring 2020, when people were infected with wild-type SARS-CoV-2. If other SARS-CoV-2 variants induce a different metabolite profile and training in the monocyte compartment is unknown so far.

5.3.1 Limitations and hints for therapy

While several vaccines against SARS-CoV-2 have been approved, to date no specific treatment is available. Common therapy options include remdesivir, a viral RNA polymerase inhibitor, with conflicting data concerning efficacy. While some studies reported beneficial outcome of remdesivir medication [325], others indicated no effect concerning mortality or duration of hospital stay [326–328].
Altered eicosanoid metabolism has been detected in BAL and plasma of severely ill patients characterized by increased pro-inflammatory mediators like LTs, pro-thrombotic metabolites (e.g. TXB_2), as well as, SPMs [272, 273, 329]. Anti-inflammatory drugs like corticosteroids, including dexamethasone, are another therapy option. Dexamethasone administration lowered mortality compared to standard care in one study [330], while being ineffective in another [326]. As shown in publication II, treatment of MDM with the glucocorticoid fluticasone propionate reduced pro-thrombotic metabolites like TXB_2 and cytokines (e.g. IL-6), while increasing pro-inflammatory leukotrienes, and could thus aggravate airway inflammation. It also reduced 12-HHTrE formation, implicated in wound healing [331]. While Koenis *et al.* [329] reported *in vivo* dexamethasone administration to lower inflammatory mediators (not LTB_4 and LTD_4) and increase SPM formation in plasma of COVID-19 patients it did not impact on mortality or duration of hospitalisation.
In general, corticosteroid treatment has severe side effects and consequences of its immunosuppressive action could also be detrimental in COVID-19 [332]. Therefore, individual pros and cons have to be weighed before medication with steroids. Co-administration of montelukast, a cysLT receptor antagonist, might be recommended and improve disease severity [333, 334].
SPM formation was increased in COVID-19 patients [329, 335] and resolvin administration could improve thrombosis, a co-pathology of SARS-CoV-2 infections [336]. How SPMs interact with pro-inflammatory lipid mediators and if their levels correlate to disease severity remains to be investigated. However, obesity, a risk-factor for severe COVID-19, is associated with reduced SPM formation that could explain exacerbated vulnerability to COVID-19 [337]. Therefore, dietary intervention leading to increased plasma SPM concentration, as shown for fish oil supplementation [338], has been proposed as a potential therapy. Studies supplementing omega-3 fatty acids or fish oil are under way and preliminary data hint to a lower death rate [339]. Generally, eicosanoid metabolism is an important player in COVID-19 pathology and should be evaluated in patient treatment.
A major limitation in our study is the cohort, as we only investigated mild COVID-19 cases that had mostly resolved 12 months after infection, our findings might not be transferable to severe cases. Also, patients had been retrospectively categorized 3-5 months after COVID-19 infection, which impeded investigation of trajectories from active infection to convalescence.
While we found a training effect on monocytes by SARS-CoV-2 we did not address the upstream mechanisms leading to them. Therefore, further investigations might lead to a better understanding of the eicosanoid aberrations and their involvement in disease and provide additional therapeutic options for the prevention of long-COVID.

5.4 Myeloid TG2 is not involved in HDM induced allergic inflammation

We had hypothesized that myeloid TG2 is important for the induction of allergic airway inflammation based on the observation by Oh *et al.* [235] that TG2 deficient mice had lower airway hyper-responsiveness in an OVA induced allergic airway inflammation model. The authors did

not delineate which cell type was responsible for the described response. In either a myeloid or hematopoietic conditional TG2 KO we could not reproduce the effect in an HDM allergic airway model. Neither mTG2 KO nor hTG2 KO mice had lower eosinophilic infiltration into the lungs compared to WT mice. As TG2 is ubiquitously expressed [340], and can be secreted [341] or released upon oxidative stress [342], we propose that TG2 formation and release by non-myeloid cells is sufficient to induce allergic inflammation.
Additionally, conditional knock-out of TG2 did not result in complete elimination of the protein, as we could detect residual enzyme expression. The remaining TG2 might contribute to inflammatory responses.
If for example epithelial derived TG2 is important for allergic airway inflammation and eosinophil recruitment has to be evaluated in another conditional knock-out experiment.

5.5 Myeloid TG2 engages in *Hpb* expulsion

TG2 was induced in peritoneal (and alveolar) macrophages during type 2 immune responses, which was not the case in mTG2 KO and hTG2 KO mice. TG2 can post-transcriptionally modulate activity and expression of different enzymes [109, 343] and thereby contribute to tissue remodelling and inflammation [70, 344, 345]. We could show that myeloid TG2 is involved in the expulsion of *Hpb* parasites, as mTG2 mice had reduced numbers of intestinal parasites on day 24 after infection compared to WT mice, while worm counts were similar on day 14. This effect was mirrored to a lesser effect in hTG2 KO mice, also showing reduced numbers of intestinal *Hpb* parasites on day 24, but not on day 14.
Different factors contribute to anti-helminth immunity. We had speculated that lipid mediator production might be different if TG2 was missing in macrophages, supported by the role of TG2 on PLA_2 activation [233]. While mTG2 KO mice displayed a similar lipid mediator profile in intestinal supernatant between WT and mTG2 KO mice, the intestinal culture of hTG2 KO mice contained significantly lower amounts of prostanoids, including PGE_2 and PGD_2. PGE_2 had been shown to have an immuno-regulatory effect and induce Th2 priming by DCs [20, 292]. It is also highly increased in MΦ stimulated with *Hpb* extract [346]. PGD_2 signalling via CRTH2 induces chemotaxis [155] and type 2 cytokine production, as well as, ILC2 accumulation and Th2 differentiation [347, 348], which could impact on worm clearance though increased mucus production [349]. However, Oyesola *et al.* [350] showed, that PGD_2 in the intestine counteracts IL-13 effects and suppresses goblet cell hyperplasia. CRTH2 deficient mice had lower parasite burden and PGD_2 production in the intestine originated from tuft cells [350]. Vav1 is also expressed in tuft cells [300], hence tuft cells in hTG2 KO mice are also TG2 deficient. If and how tuft-cell derived TG2 regulates immune responses was not investigated, but could explain the reduction of prostanoids in the intestinal culture in hTG2 KO mice compared to WT ones. Thus, lower prostanoid levels might reduce intestinal worm burden, which we could not confirm in our experiments. However, epithelial or tuft cell and macrophage interplay could also be regulated by TG2.
More recent studies report tuft-cell derived IL-25 to mediate anti-helminth immunity through induction of type 2 immune responses [172, 219, 351]. While tuft-cells might induce type-2 immunity, several studies indicate that macrophages are responsible for parasite expulsion. Smith *et al.* [352] stated that IL-25 signalling and alternative MΦ activation are necessary for *Hpb* expulsion, which is in line with Chen *et al.* [353] showing that M2 polarized macrophages mediate expulsion of *Nb* parasites. Nematode clearance is mediated through IL-13 signalling, which apart from M2 MΦ polarization also induces smooth muscle contraction and enhances goblet cell mucus production [353–356]. Which of this signalling mechanism is differently regulated in

TG2 deficient macrophages remains open for investigation, but as TG2 is considered a marker of alternative activation and macrophages are important mediators in parasite host defence, differences in their expression profile could impact on effector function.
Arginine metabolism is also involved in parasite resistance. Macrophages express Arg1 or Nos2 depending on their polarisation status, both enzymes competing for the substrate arginine. [63]. The Arg1 products ornithine and polyamines can directly hinder *Hpb* motility [72]. Increased Arg1 expression by M2 MΦ can lead to Th2 cell starvation, suppress chronic Th2 responses [357], and reduce T cell proliferation [358]. Acute Th2 responses are beneficial in providing resistance to parasites [354], while chronic states can lead to fibrosis [359]. Therefore, arginine metabolism might be another pathway differentially regulated by myeloid TG2 deficient mice.
The effect of macrophage transglutaminase 2 deficiency on their activation and effector function has to be investigated in more detail. RNAseq and ChIPseq approaches might shed light on these open questions.

5.6 Differential impact of TG2 on different parasites

While TG2 did not play a role in parasite resistance during *Nb* infection, we observed signs for differences in tissue damage in the lung. In line with this, TG2 has been shown to be involved in fibrosis and tissue remodelling [67, 70], therefore even though TG2 might not play a role during acute *Nb* infection, it could drive lung fibrosis after parasite clearance. To state if the role of myeloid TG2 in tissue remodelling or repair after *Nb* elimination is beneficial its role has to be analyzed in more detail, for example by histological scoring of lung damage.
The finding that TG2 might play a role in *Hpb*, but not during acute *Nb* infections, could be explained by their varying life cycle. The native murine *Hpb* larvae solely remain in the intestine, where they induce a chronic infection [360]. In contrast *Nb* parasites migrate through the skin to the lung and further on to the intestine, where they will be expelled after around 2 weeks [361]. Host defence mechanisms overlap between *Hpb* and *Nb* infections, for example, induction of a type 2 immune response and alternative activation of macrophages occurs in both [40, 362]. However, the response on tuft cell and succinate signalling differs between *Hpb* and *Nb* infection [363]. Succinate administration leads to tuft cell hyperplasia and induces a type 2 immune response via SUCNR1 signalling [364, 365]. While previous succinate administration could reduce *Nb* worm burden [364] and increase tuft cell frequency in the intestine [172], *Hpb* products could inhibit tuft cell expansion [363]. Thus, ILC2 activation by tuft-cell derived IL-25 and cysLTs as well as type 2 immune responses [220] are probably reduced in *Hpb* compared to *Nb* infections [173]. To answer why we see a difference in the one, but not in the other parasite infection, the effect and downstream mechanisms of myeloid TG2 in parasitic infections have to be investigated in more detail.

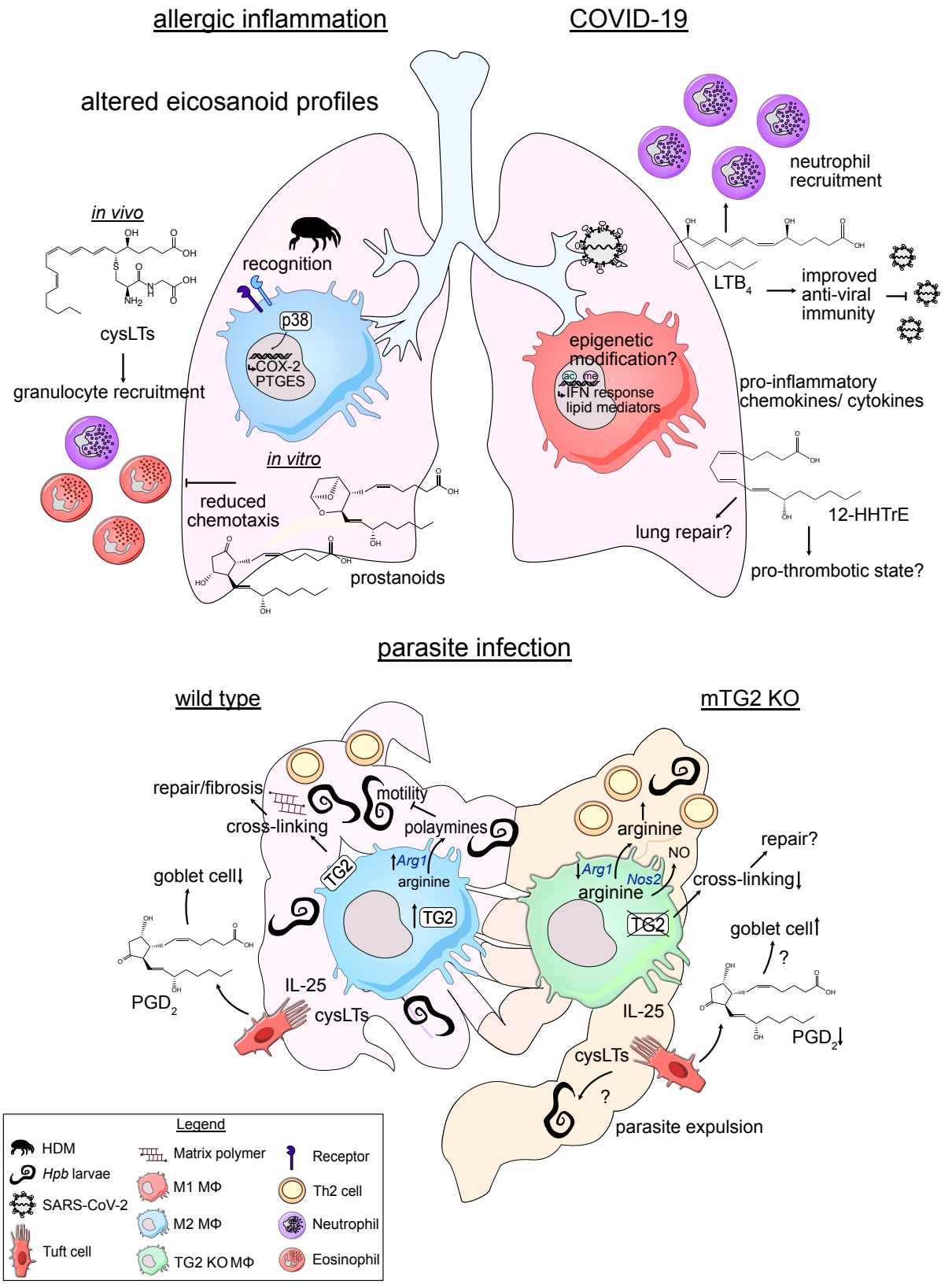

Figure 5.1: Summary of results from the three different projects. Upper Figure left: HDM induces macrophage lipid mediator reprogramming. Upper Figure right: COVID-19 infection primes macrophages and alters their lipid mediator output. Lower Figure left: *Hpb* infection results in chronic type 2 inflammation in the intestine of wild type mice. Lower Figure right: Hypothesized immune-regulatory mechanisms in mTG2 KO mice resulting in increased parasite expulsion.

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ORIGINAL ARTICLE

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House dust mite drives proinflammatory eicosanoid reprogramming and macrophage effector functions

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Abstract

Background: Eicosanoid lipid mediators play key roles in type 2 immune responses, for example in allergy and asthma. Macrophages represent major producers of eicosanoids and they are key effector cells of type 2 immunity. We aimed to comprehensively track eicosanoid profiles during type 2 immune responses to house dust mite (HDM) or helminth infection and to identify mechanisms and functions of eicosanoid reprogramming in human macrophages.

Methods: We established an LC-MS/MS workflow for the quantification of 52 oxylipins to analyze mediator profiles in human monocyte-derived macrophages (MDM) stimulated with HDM and during allergic airway inflammation (AAI) or nematode infection in mice. Expression of eicosanoid enzymes was studied by qPCR and western blot and cytokine production was assessed by multiplex assays.

Results: Short (24 h) exposure of alveolar-like MDM (aMDM) to HDM suppressed 5-LOX expression and product formation, while triggering prostanoid (thromboxane and prostaglandin D_2 and E_2) production. This eicosanoid reprogramming was p38-dependent, but dectin-2-independent. HDM also induced proinflammatory cytokine production, but reduced granulocyte recruitment by aMDM. In contrast, high levels of cysteinyl leukotrienes (cysLTs) and 12-/15-LOX metabolites were produced in the airways during AAI or nematode infection in mice.

Abbreviations: 5-LOX, 5-lipoxygenase; COX, cyclooxygenase; cysLTs, cysteinyl leukotrienes; IS, internal standard; LC-MS/MS, liquid chromatography tandem mass spectrometry; LTA4H, leukotriene A4 hydrolase; LTB4, leukotriene B4; LTC4S, leukotriene C4 synthase; MDM, monocyte-derived macrophages; PGs, prostaglandins; PMN, polymorphonuclear leukocytes; SPM, specialized pro-resolving mediator.

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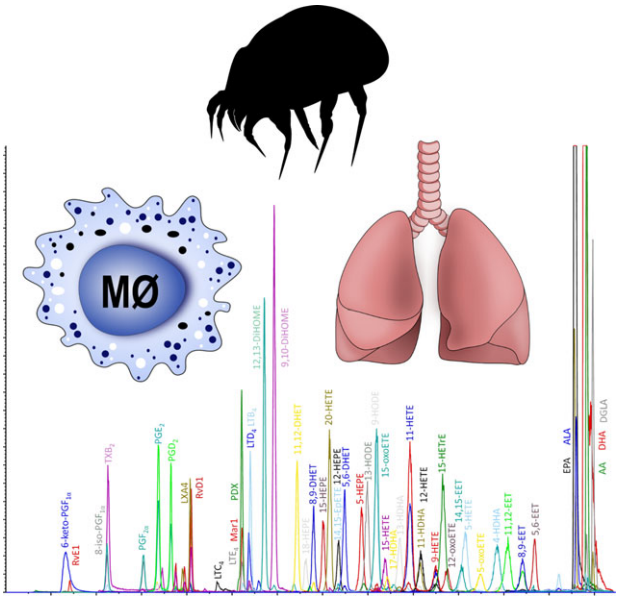
Conclusion: Our findings show that a short exposure to allergens as well as ongoing type 2 immune responses are characterized by a fundamental reprogramming of the lipid mediator metabolism with macrophages representing particularly plastic responder cells. Targeting mediator reprogramming in airway macrophages may represent a viable approach to prevent pathogenic lipid mediator profiles in allergy or asthma.

KEYWORDS
eicosanoids, house dust mite, LC-MS/MS, macrophages, type 2 inflammation



GRAPHICAL ABSTRACT

Macrophages respond to house dust mite (HDM) by increased cyclooxygenase (COX) metabolism, while the type 2 immune response to HDM or parasitic worms in the airways is characterized by increased lipoxygenase (LOX) metabolism

1 | INTRODUCTION

Lipid mediators govern immune responses in a multitude of infectious or chronic inflammatory settings.[1] In allergy and asthma, prostanoids and leukotrienes (LTs) derived from the polyunsaturated fatty acid (PUFA), arachidonic acid (AA), drive hallmark type 2 immune responses such as eosinophil accumulation.[2,3] AA metabolites (eicosanoids) have also been suggested to contribute to type 2 immunity during nematode infection.[4-6] Despite these important immunological functions, few studies have comprehensively assessed lipid mediator profiles during type 2 immune responses. This is possibly due to the limited availability of adequate LC-MS/MS workflows, which are required for the simultaneous quantification of a multitude of structurally similar but functionally distinct mediators. Indeed, most immunological studies in allergy or nematode infection have used immunoassays to quantify less than a handful of mediators.[5,7,8] However, LC-MS/MS analysis of 18 eicosanoids in macrophages from nematode-infected mice suggested abundant and plastic eicosanoid production during type 2 immune responses.[6] In addition, a number of studies have applied LC-MS/MS approaches to quantify up to 88 lipid mediators in *ex vivo* samples from allergy and asthma patients.[9-12] Moreover, using macrophages as a model system, targeted lipidomics approaches were applied to quantify more than 100 eicosanoid metabolites.[13] Due to their plasticity and abundant expression of eicosanoid biosynthetic pathways, macrophages present an attractive cellular model to study lipid mediator production in immunological settings.[14] In the context of inflammasome activation, targeted lipidomics workflows allowed for the characterization of an "eicosanoid storm" during macrophage activation.[15,16] However, despite these recent advances in lipidomics technologies, information about the lipid mediator profiles in type 2 immune responses remains scarce.

Here, we have established a targeted lipidomics workflow for the simultaneous quantification of 52 oxylipins from several PUFAs (AA, LA, and DHA). We applied this workflow to demonstrate that HDM exposure of human macrophages results in a pronounced eicosanoid reprogramming, characterized by high levels of prostanoids (particularly thromboxane), but low levels of 5-LOX products. This

eicosanoid reprogramming was dependent on p38 MAPK activation, but independent of dectin-2. We further show that HDM-driven eicosanoid reprogramming occurs on the mRNA and protein level and is associated with the production of proinflammatory cytokines and chemokines. However, HDM-exposed macrophages showed a reduced chemotactic potential toward granulocytes, correlating with suppressed LTB_4 production. Together, these findings suggest that HDM induces a proinflammatory macrophage phenotype with impaired effector function. Finally, we quantified mediator profiles in bronchoalveolar lavage fluid (BALF) from HDM-sensitized and nematode-infected mice, thus revealing profound changes in COX and LOX metabolites during type 2 immune responses *in vivo*. In summary, these data show that the AA metabolism is fundamentally reprogrammed during type 2 immune responses and suggest macrophage reprogramming as an attractive target in type 2 inflammation.

Highlights

- House dust mite triggers the production of cyclooxygenase metabolites (prostaglandin D_2, prostaglandin E_2, and thromboxane) as well as of proinflammatory cytokines in human macrophages.
- House dust mite-exposed macrophages produce low levels of 5-lipoxygenase metabolites (eg, leukotriene B_4) and suppress neutrophil recruitment.
- High levels of cysteinyl leukotrienes and 12-/15-lipoxygenase metabolites are produced during the type 2 immune response to house dust mite or nematode parasites in the airways.

2 | MATERIALS AND METHODS

Animal experiments were performed according to institutional guidelines and to Swiss federal and cantonal laws on animal protection.

2.1 | Material

Eicosanoids, PUFAs, and deuterated internal standards (ISs) were purchased from Cayman Chemical (Ann Arbour, MI, USA). An analyte and IS working solution were prepared as shown in Tables S1 and S2. LC-grade solvents [2-propanol, Carl Roth (Karlsruhe, Germany), acetonitrile, Thermo Fisher Scientific (Waltham, MA, USA), methanol, Applichem (Darmstadt, Germany)) and ultrapure H_2O (supplied through a MilliQ system (Merck Millipore, Darmstadt, Germany)] were used for mobile phase preparation.

2.2 | Isolation and culture of polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC)

Written informed consent in accordance with the Declaration of Helsinki was obtained from healthy volunteers before blood collection, which had been approved by the local ethics committee at the Technical University of Munich. PMN and PBMC were isolated and cultured in medium containing 10% heat-inactivated FBS and monocytes were differentiated to aMDM as described previously.[17,18] Supernatants were stored at −80°C in 50% MeOH for LC-MS/MS or undiluted for cytokine analysis.

2.3 | Chemotaxis assay

PMN were incubated for 30 min at 37°C with pooled conditioned medium of aMDM ± HDM ± indomethacin (100 μM, Cayman Chemical) ± DBM-1285. 2×10^5 PMN were transferred to transwells (3 μm pore size, Corning, NY, USA) and allowed to migrate for 3 h at 37°C toward conditioned medium containing chemoattractants: 2 ng/mL LTB_4, Cayman Chemical; 20 ng/mL IL-8; 2 ng/mL CCL5, both Miltenyi Biotec. Migrated PMN were counted microscopically.

2.4 | In vivo model of *Nippostrongylus brasiliensis* infection

Mice were infected subcutaneously with 200 larvae of *N. brasiliensis* (*Nb*), and BALF was collected on day 5 postinfection as previously described.[19,20]

2.5 | In vivo model of HDM-induced allergic airway inflammation

C57BL/6J mice were sensitized by bilateral intranasal (i.n.) instillations of extract from *Dermatophagoides farinae* ("HDM") (1 μg in 20 μL PBS; Stallergenes). Challenges were performed on days 8-11 with 10 μg HDM extract. Three days after the final challenge, BALF (600 μL) was collected, equal volumes of methanol were added, and samples were frozen immediately at −80°C until further processing.

2.6 | Real-time PCR

aMDM were lysed in RLT Buffer (Qiagen, Hilden, Germany) with 1% β-mercaptoethanol (Merck Millipore), followed by RNA extraction (Zymo Research, Irvine, CA, USA) and reverse transcription according to the manufacturer's instructions (Thermo Fisher Scientific). qPCR analysis was performed as described previously (primers shown in Table S3).[18]

2.7 | Western blotting

Western blotting was performed similarly to previously published procedures.[18] A detailed procedure can be found in the Appendix S1.

2.8 | Multiplex cytokine assay and ELISA

Multiplex cytokine assays were performed as detailed in the Appendix S1.

2.9 | Sample preparation for LC-MS/MS

Samples for method validation were prepared as triplicates in medium/MeOH (1:1) or PBS/MeOH (1:1) with an analyte concentration of 0.1, 1, or 10 ng/mL [10× higher concentrations for PUFAs (Tables S1 and S2)]. Automated solid phase extractions were performed with a Microlab STAR robot (Hamilton, Bonaduz, Switzerland). Prior to extraction, all samples were diluted with H_2O to a MeOH content of 15% and 10 μL of IS stock solution was added. Samples were extracted using Strata-X 96-well plates (30 mg, Phenomenex, Aschaffenburg, Germany) and eluted with MeOH. Samples were evaporated to dryness under N_2 stream and redissolved in 100 μL MeOH/H_2O (1:1).

2.10 | LC-MS/MS lipid mediator analysis

Chromatographic separation of eicosanoids was achieved with a 1260 Series HPLC (Agilent, Waldbronn, Germany) using a Kinetex C18 reversed phase column (2.6 μm, 100 × 2.1 mm, Phenomenex) with a SecurityGuard Ultra Cartridge C18 (Phenomenex) precolumn. The Sciex QTRAP 5500 mass spectrometer (Sciex, Darmstadt, Germany), equipped with a Turbo-V™ ion source, was operated in negative ionization mode. Identification of metabolites was achieved via retention time and scheduled multiple reaction monitoring (sMRM). Unique Q1/Q3 transitions were selected for each analyte by using single analyte injections and comparison with the literature.[14] Analytes with identical MRM transitions were differentiated by retention time (Figure S1). A more detailed method description can be found in the Appendix S1.

2.11 | Data analysis

All data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) or R 3.4.3.[21] For LC-MS/MS analysis, all samples were normalized to their RNA content. Data were analyzed using Wilcoxon, Friedman, or Kruskal-Wallis test with respective post hoc test as specified in the Figure legends and considered statistically significant if $P < 0.05$.

3 | RESULTS

3.1 | Lipid mediators involved in type 2 immune responses can be detected with high accuracy, precision, and recovery by LC-MS/MS

Quantification of lipid mediators in type 2 immune settings has resulted in discrepancies, depending on the analytical method (EIA or LC-MS/MS).[5,22] Thus, we compared these methods for leukotrienes, PGE_2, and TXB_2 in supernatants of human PMN and aMDM. Quantification by EIA showed higher variability, particularly for LTs: SD = 8.48 (EIA) vs SD = 0.72 (LC-MS/MS) for LTB_4; SD = 6.44 (EIA) vs SD = 0.23 (LC-MS/MS) for cysLTs. Levels obtained by EIA were also significantly higher as compared to LC-MS/MS and did not correspond well to AA-metabolizing enzymes (Figure 1A).[17]

Thus, we established an LC-MS/MS workflow for the comprehensive and simultaneous quantification of PUFA metabolites involved in type 2 inflammation (Figure 1B, Figure S1, Tables S4-S9). At 1 ng/mL, we could detect 36 metabolites according to FDA guidelines (accuracy: ±15%, RSD <20%) (Table S4). This included eicosanoids (LTs, TXB_2, and PGD_2) as well as specialized pro-resolving mediators (SPMs) [resolvin E1/D1 (RvE1/RvD1) and protectin D1 (PDX)] (Figure 1C,D, Table S4). The recovery ranged from 69% to 127% for key lipid mediators of type 2 inflammation with a matrix effect in a similar range (Figure 1E, Table S5). Thus, at concentrations ≥ 1 ng/mL eicosanoid mediators of type 2 immunity (LTs, TXB_2, and PGD_2) and several SPMs could be quantified with good accuracy, precision, and recovery.

3.2 | Zymosan exposure reprograms the eicosanoid metabolism of myeloid cells

In order to validate our LC-MS/MS workflow in a well-characterized cellular model, we processed and analyzed culture supernatants from human PMN that were either left untreated or exposed for 24 h to zymosan prepared from fungal cell walls. First, a pool of PMN supernatants was measured in three technical replicates (Figure 2A-E), and second, levels of eicosanoids produced by PMN from different individuals (n = 5) were analyzed separately (Figure 2F). Untreated PMN produced mainly 5-LOX metabolites (5-HETE and LTB_4) at a concentration of around 1.4 ng/mL and low levels of cysLTs (Figure 2A). PMN preparations contained neutrophils and eosinophils and thus had the capacity to generate LTs and 15-LOX metabolites (Figure 2A,B). Treatment with zymosan resulted in reprogramming of the eicosanoid metabolism, characterized by reduced production of LTB_4, cysLTs, and 5-HETE ($P = 0.06$) (Figure 2A,D-F). In contrast, zymosan exposure triggered the formation of COX metabolites with a fivefold increase in TXB_2 levels. Additionally, zymosan-exposed PMN released PGE_2 and $PGF_2\alpha$ that were undetectable in unstimulated PMN (Figure 2C). Taken together, lipid mediator class switching could be tracked by the developed LC-MS/MS workflow, allowing us to reveal previously reported as well as unprecedented zymosan-induced changes in the eicosanoid profile.[23,24]

3.3 | TGFβ1 induces a macrophage phenotype that resembles alveolar macrophages and resists IL-4-mediated regulation of eicosanoid pathways

Based on recent studies showing key roles for GM-CSF and TGFβ1 in alveolar macrophage (AM) differentiation,[25,26] we differentiated human monocytes into alveolar-like macrophages (aMDM) and characterized their eicosanoid profile. At baseline, aMDM expressed high levels of 5-LOX and its respective oxylipin products (Figure 3B,E). In addition, aMDM expressed higher levels of 5-LOX and IL-1β as compared to MDM, suggesting that they adapted features of AM (Figure S2A,B).[27,28] IL-4 is known to reprogram the AA metabolism of macrophages by inducing 15-LOX, but suppressing 5-LOX and COX. We confirmed the IL-4-triggered induction of *ALOX15* in MDM from most donors during differentiation in the absence of TGFβ1 (Figure S2C). However, IL-4 had no significant impact on the eicosanoid profile of aMDM (Figure 3A-C), suggesting that aMDM

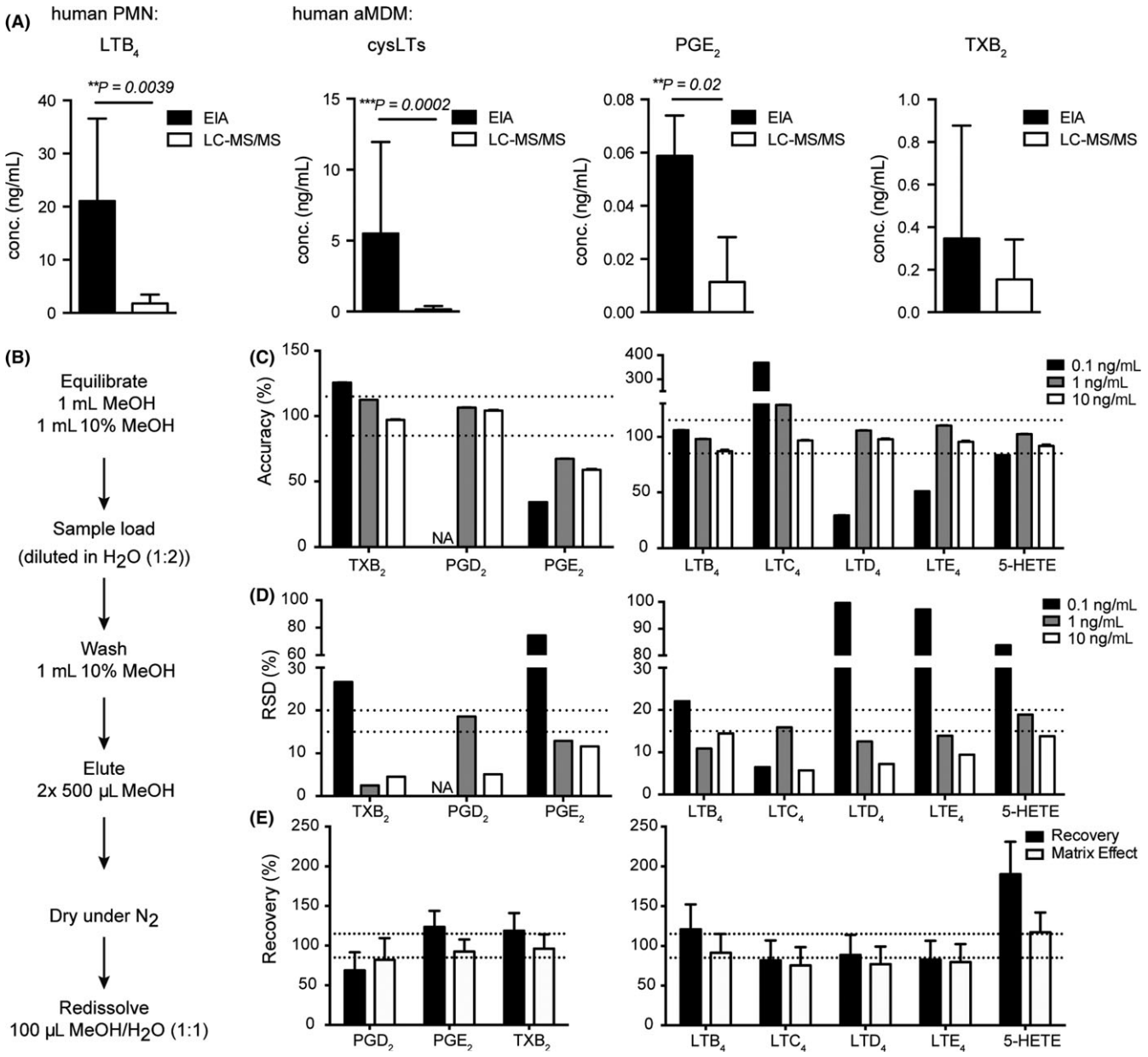


FIGURE 1 Lipid mediators involved in type 2 immune responses can be detected with high accuracy, precision, and recovery by LC-MS/MS. (A) Levels of major bioactive eicosanoids (mean + SD) in supernatants from PMN (n = 5) or MDM (n = 11-30) quantified by EIA or LC-MS/MS; (B) Sample preparation workflow; (C) Accuracy (%) at three different concentrations for key eicosanoids, shown as mean + SD. Dotted lines: ± 15% range; (D) Precision calculated as relative standard deviation (RSD) (%), shown as mean. Dotted lines: 15% and 20% RSD; (E) Recovery at 1 ng/mL. Dotted lines: ± 15% range. Samples in (C-E) were extracted and measured in triplicates on the same day. Statistical significance was determined using Wilcoxon test

resist IL-4-driven induction of 15-LOX as well as suppression of COX and 5-LOX. Indeed, *PTGS2*, *PTGES*, *ALOX5*, and *ALOX15* mRNA levels remained unaffected by IL-4 (Figure 4A).

3.4 | HDM exposure decreases 5-LOX metabolism but increases COX metabolism in human alveolar-like macrophages via p38 MAPK

Next, we assessed the eicosanoid profile of aMDM during 24-96 h exposure to IL-4 and HDM. After 24 h of HDM+IL-4 exposure, formation of 5-LOX products (LTB_4 and 5-HETE) was reduced (Figure 3B). Contrary to the effect on 5-LOX, HDM+IL-4 stimulation resulted in an increase of prostanoids (Figure 3A, Figure S3A). In line with the LC-MS/MS data, 5-LOX mRNA levels were reduced by IL-4 + HDM, while COX-2 was induced (Figure 4A). At later time points, we observed only minor changes in eicosanoid concentrations with the exception of PGD_2, which decreased after prolonged HDM exposure (Figure S3A), and 5-HETE and LTB_4, which initially decreased but increased back to control levels at 96 h (Figure S3B,C).

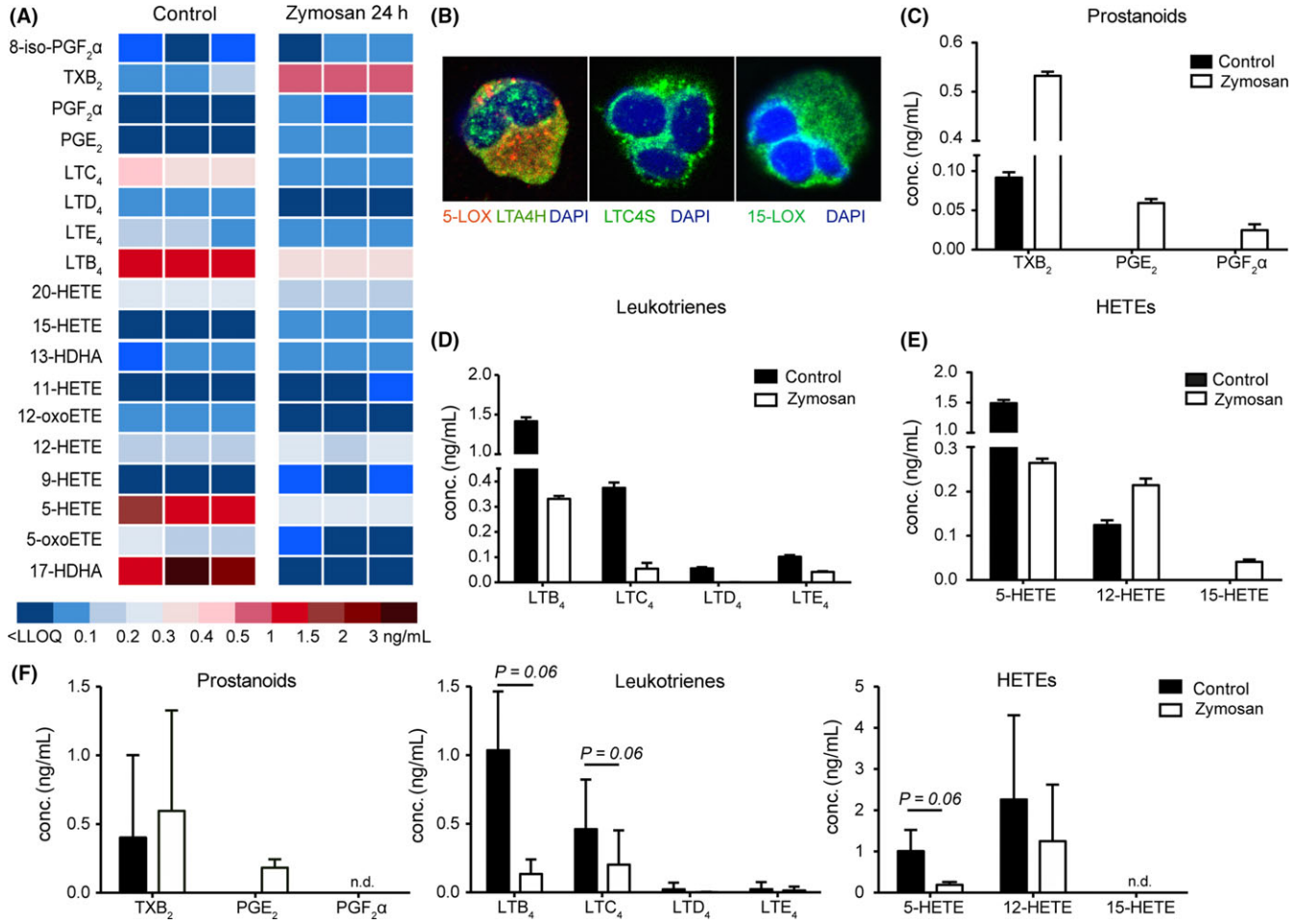


FIGURE 2 Zymosan triggers eicosanoid reprogramming in human granulocytes. (A) Heatmap of LC-MS/MS data for human PMN (pool of n = 6 donors) ± zymosan, analyzed as three technical replicates. (B) Neutrophil (left) or eosinophils (right) stained for 5-LOX and LTA4H or LTC4S and 15-LOX, respectively. Blue: DAPI (nuclei). (C-E) Levels of COX metabolites (C), leukotrienes (D), and HETEs (E) produced by PMN, presented as mean + SD (pool of n = 6, measured in triplicates). (F) Levels of prostaglandins, leukotrienes, and HETEs produced after 24 h ± zymosan (n = 5). Statistical significance was determined using Wilcoxon test

In the absence of IL-4, HDM also triggered prostanoid production, while 5-LOX products were reduced in aMDM from five out of seven donors (Figure 3D,E).

To identify the mechanisms underlying this eicosanoid reprogramming, we first neutralized dectin-2, which has been described as the major C-type lectin receptor recognizing HDM.[7] However, blocking dectin-2 did not interfere with HDM-triggered changes in either COX or LOX metabolites (Figure 3F). Similarly, blockade of TLR-2 or TLR-4 or addition of polymyxin B to inactivate LPS did not affect mediator reprogramming by HDM (Figure S4). As the MAP kinase p38 has been implicated in the regulation of eicosanoid pathways,[29] we assessed p38 phosphorylation in response to HDM. Levels of phosphorylated p38 were increased in HDM-exposed as compared to unstimulated aMDM and prostanoid formation was significantly reduced when macrophages were co-incubated with HDM and a p38 inhibitor (Figure 3F,G). In addition, p38 inhibition during HDM exposure restored 5-LOX product formation (Figure 3F, Figure S5A).

We further examined the effect of HDM on mRNA and protein levels of COX and LOX pathway enzymes. *PTGES* (mPGES1) and *PTGS2* (COX-2) were increased, while *ALOX5* (5-LOX) expression was down-regulated by HDM on both transcript and protein level (Figure 4B,C). M2 polarization markers were either significantly reduced (*ALOX15*) or unaffected by HDM exposure (*TGM2*) (Figure 4A-C). Altogether, HDM-induced eicosanoid reprogramming likely occurred as a result of profound changes in the expression of eicosanoid pathway genes.

3.5 | HDM exposure triggers the production of proinflammatory cytokines and chemokines

To study whether allergen-driven eicosanoid reprogramming was associated with an altered cytokine profile, we performed multiplex bead assays of supernatants from aMDM. We observed a significant increase in proinflammatory cytokines (IL-6, IL-1β, TNF-α, and IL-12 p70) after 24 h HDM exposure (Figure 5A,B). HDM also triggered the release of chemokines (CXCL9/10, IL-8) involved in granulocyte recruitment (Figure 5A). Similar to the effects on eicosanoid reprogramming, p38 inhibition reduced the HDM-induced production of

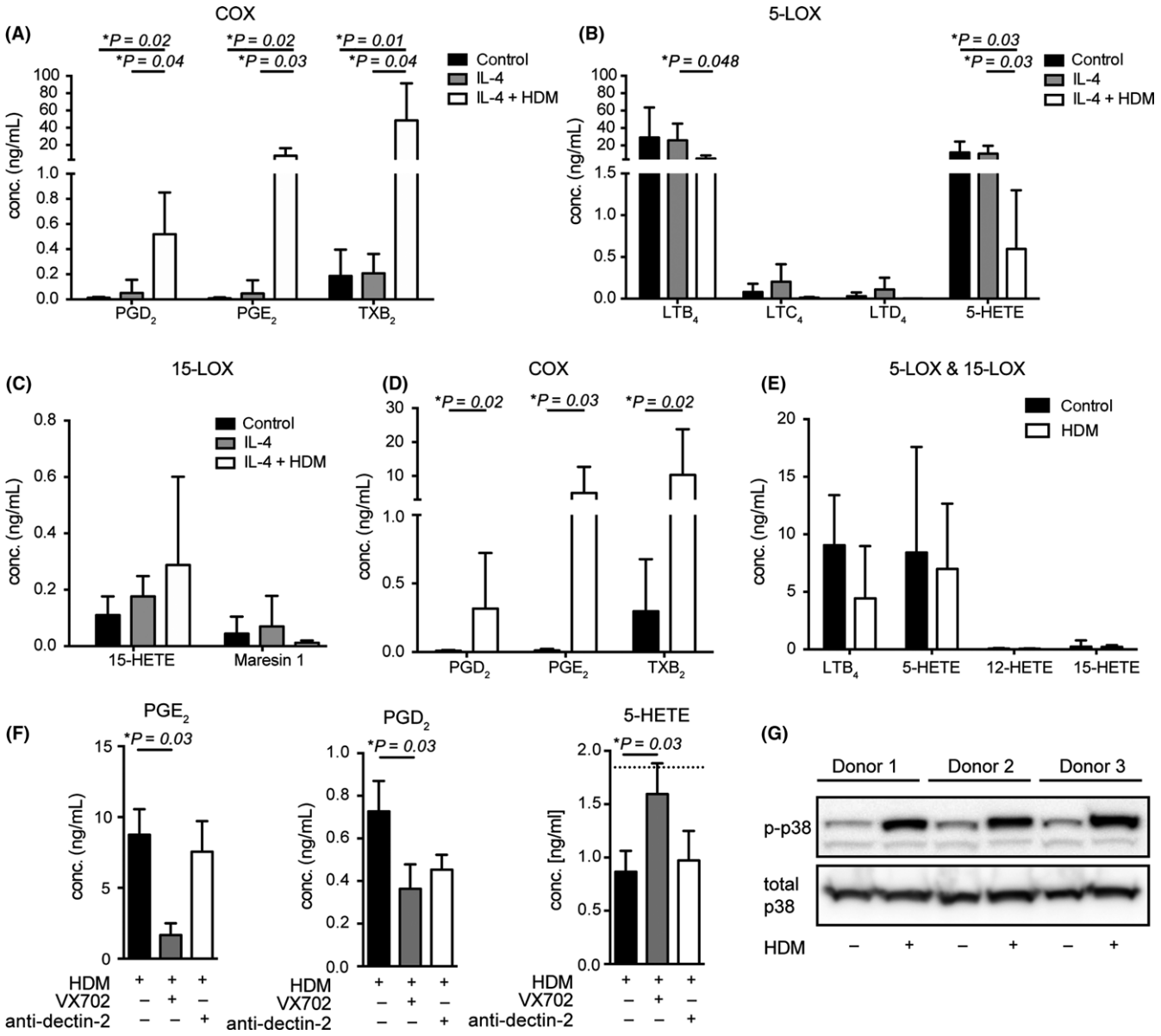


FIGURE 3 House dust mite extract triggers COX metabolism but suppresses 5-LOX metabolism in human alveolar-like macrophages via p38 MAPK. (A-C) LC-MS/MS data for 5-LOX (A), COX (B), or 15-LOX (C) metabolites of aMDM, stimulated or not with 10 ng/mL IL-4 ± 10 μg/mL HDM (n = 4); (D-F) LC-MS/MS data for COX (D) or 5/15-LOX metabolites (E), (n = 7) and of aMDM pre-incubated with p38 inhibitor or dectin-2 neutralizing antibody (F) before HDM exposure (n = 5). (G) Representative WB for total and phosphorylated p38 in aMDM (n = 3). Data are shown as mean + SD; statistical significance was determined using Kruskal-Wallis test with Dunn's correction (A-C, F) or Wilcoxon test (D)

IL-6 and TNF-α, while dectin-2 neutralization did not affect the expression of these cytokines (Figure 5C,D, Figure S5B).

3.6 | HDM-exposed macrophages have a reduced capacity to recruit granulocytes

The recruitment of inflammatory neutrophils and eosinophils is a hallmark response of asthma. Thus, we addressed the functional consequence of HDM-driven mediator reprogramming by performing chemotaxis assays with human PMN. Migration of PMN toward a chemoattractant mixture was diminished if PMN were exposed to supernatant from aMDM stimulated with HDM as compared to supernatant from unstimulated aMDM (Figure 5E). Addition of the COX inhibitor indomethacin during HDM stimulation did not affect the HDM-triggered reduction in chemotaxis, suggesting that prostanoids were not responsible for this effect. In contrast, p38 inhibition could restore PMN chemotaxis, correlating with increased LTB_4 levels (Figure S5A,C) and reduced IL-6 and TNF-α concentrations (Figure S5B).

Taken together, HDM exposure induced a proinflammatory macrophage phenotype characterized by abundant production of bronchoconstrictive thromboxane and TNF-α, but low production of LTB_4 and impaired chemotactic potential.

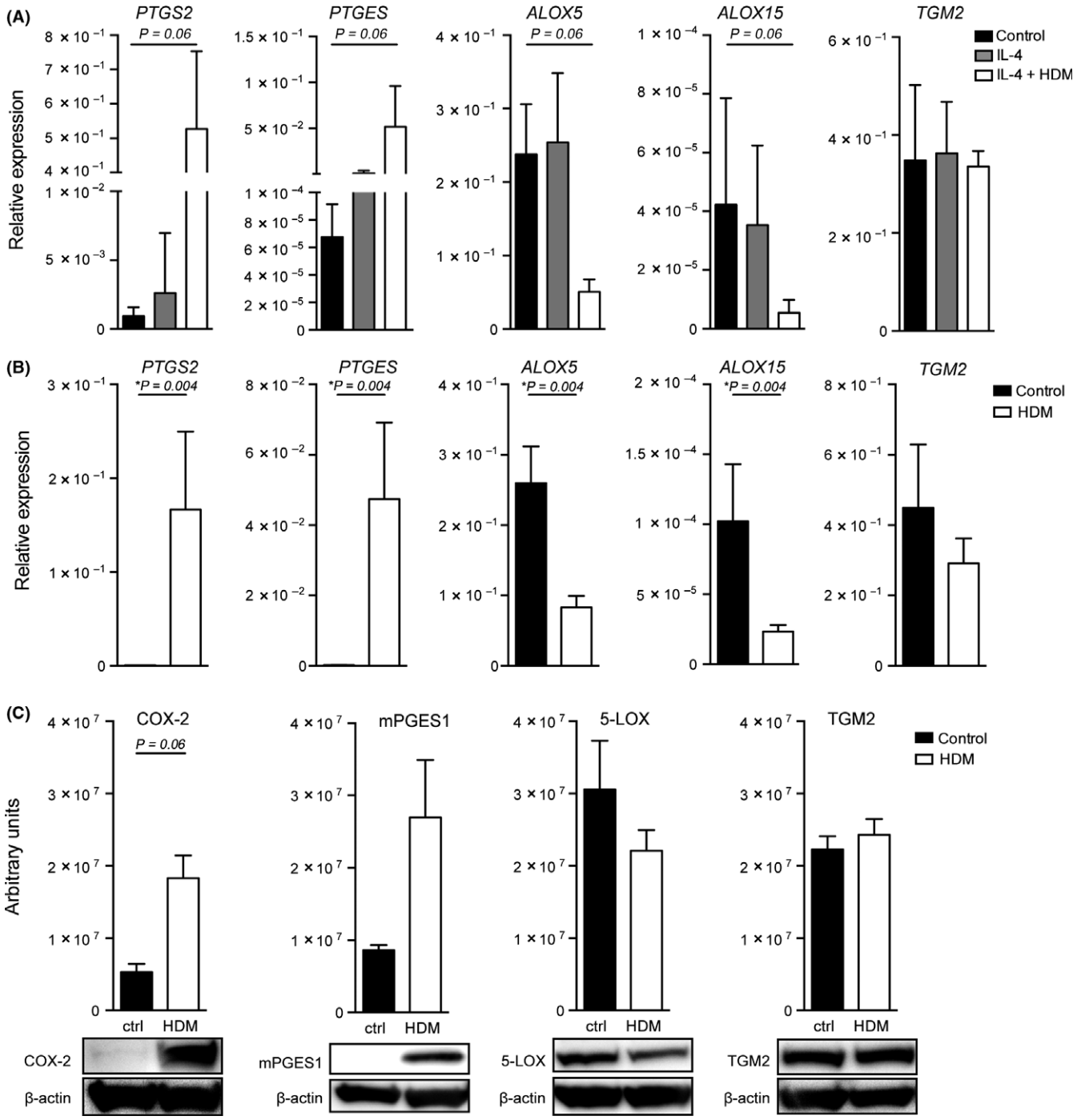


FIGURE 4 HDM-driven eicosanoid reprogramming occurs on the mRNA and protein level. (A and B) relative gene expression of aMDM stimulated or not with 10 ng/mL IL-4 ± 10 μg/mL HDM, (n = 7) (A) or with 10 μg/mL HDM (n = 9) (B) for 24 h; (C) protein levels normalized to β-actin (upper panels) and representative WB images (lower panels) of aMDM ± 10 μg/mL HDM for 24 h (n = 5-7). Data are shown as mean + SD; statistical significance was determined using Wilcoxon test

3.7 | Distinct eicosanoid profiles are induced during the type 2 immune response to HDM or nematode infection in the airways

To assess whether eicosanoid reprogramming is a general feature of type 2 immune responses, we characterized lipid mediator profiles in the airways of HDM-sensitized or nematode-infected mice. When comparing eicosanoid profiles after sensitization and challenge with HDM or infection with the lung-migrating nematode *Nippostrongylus brasiliensis* (*Nb*), we observed an abundant formation of prostanoids in BALF from *Nb*-infected, but not from HDM-sensitized mice ($P < 0.05$ for all COX metabolites) (Figure 6A). In addition, no prostanoids could be detected in the BALF of naïve mice (Figure 6A). In contrast, cysLTs were detectable in the airways of *Nb*-infected as well as of HDM-sensitized mice (Figure 6B). Moreover, high levels of 12-/15-LOX metabolites (particularly 12-HETE and 13-HODE) were

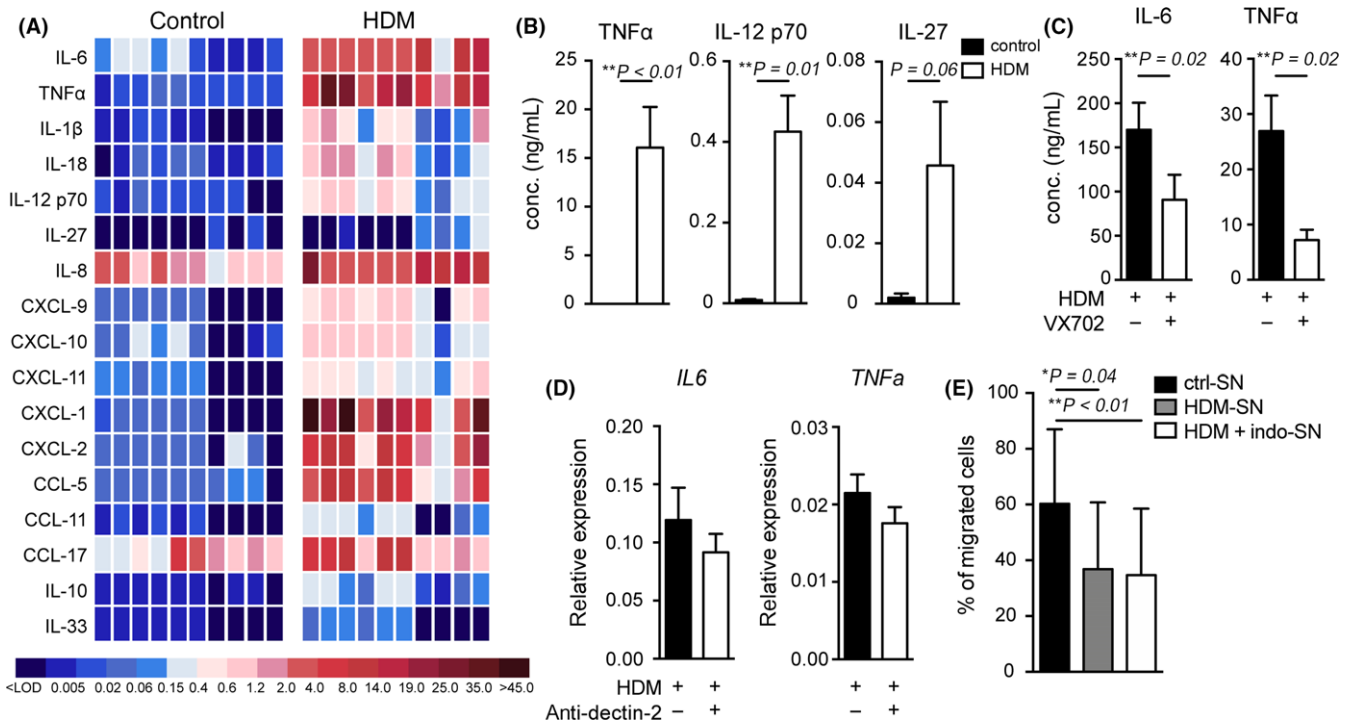


FIGURE 5 HDM exposure triggers the production of proinflammatory cytokines and chemokines, but reduces the granulocyte-chemotactic potential of human macrophages. (A) Overview of cytokine levels [ng/mL], (B) TNF-α, IL-12 p70, and IL-27 (mean + SD) for aMDM from 10 different blood donors ± 10 μg/mL HDM for 24 h; (C) concentration (n = 10) and (D) gene expression (n = 6) of IL-6 and TNF-α in aMDM pre-incubated with p38 inhibitor VX702 or dectin-2 neutralizing antibody before HDM exposure; (E) Percentage of granulocytes migrating toward supernatants (SN) of aMDM ± 10 μg/mL HDM ± 100 μM indomethacin (n = 7). Statistical significance was determined using Wilcoxon test (B-D) or Friedman test with Dunn's correction (E)

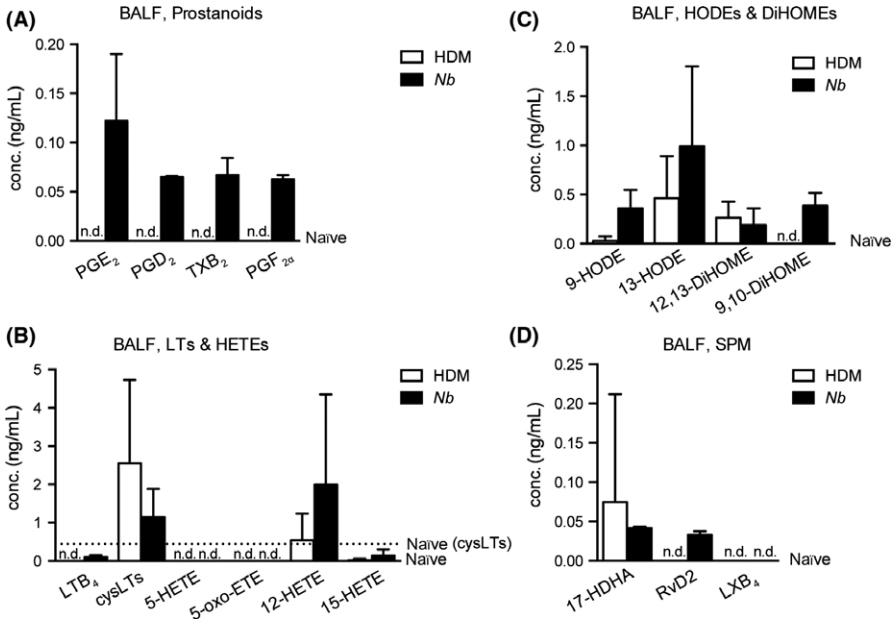


FIGURE 6 Distinct eicosanoid profiles are induced during the type 2 immune response to HDM or nematode infection in the airways. (A-D) LC-MS/MS analysis of prostanoids (A), LOX metabolites of AA (B), LA metabolites (C), and SPMs (D) in BALF from HDM-sensitized or *Nb*-infected mice (n = 3-6), representative data from two independent experiments are presented as mean + SD. Dotted lines represent levels for naïve mice. Statistical significance was determined using Kruskal-Wallis test with Dunn's correction

produced in the airways of *Nb*-infected and HDM-sensitized mice (Figure 6B,C). LA-derived metabolites (9-/13-HODE, 9,10-/11,13-DiHOME) were synthesized in similar quantities as compared to AA metabolites in the airways after challenge with HDM or infection with *Nb* with a tendency for higher levels in *Nb*-infected mice ($P = 0.025$ for 9,10 DiHOME, $P = 0.124$ for 9-HODE). Finally, BALF from *Nb*-infected mice also contained detectable levels of SPMs (17-HDHA and RvD2) (Figure 6D). Thus, lipid mediator reprogramming occurs during the type 2 immune response to HDM or nematode parasites in the airways in vivo with partially distinct profiles.

The induction of the COX pathway and simultaneous suppression of the 5-LOX pathway may represent an early response of macrophages in type 2 immune settings, which then governs the ensuing type 2 immune response to allergens or helminth infection.

4 | DISCUSSION

Eicosanoid lipid mediators play central roles in type 2 immune responses, particularly in allergic inflammation. Thus, the comprehensive assessment of eicosanoid profiles in settings of type 2 inflammation can provide important information about the ensuing immune response and the functional plasticity of the cell types involved. Here, we describe an LC-MS/MS workflow, which allowed us to characterize eicosanoid reprogramming in two distinct settings of type 2 inflammation. First, we show that the lipid mediator metabolism of human alveolar-like macrophages (aMDM) is highly responsive to allergen-driven reprogramming. Second, we describe profound changes in lipid mediator profiles during the type 2 immune response to HDM or nematode infection in vivo.

Using a newly developed LC-MS/MS workflow, up to 52 oxylipins could be quantified in cell culture supernatants and biological samples from the airways. To our knowledge, this represents one of the largest oxylipin panels that has been validated and applied in the context of type 2 immune responses. This workflow allowed for the sensitive and reliable quantification of central eicosanoid mediators of type 2 inflammation (eg, LTs, TXB_2, PGD_2), while the accuracy should be improved for other mediators (eg, PGE_2). To initially validate the LC-MS/MS workflow, we studied zymosan-triggered eicosanoid reprogramming in human PMN. At baseline, stimulation with Ca^{2+} ionophore resulted in the release of 5-HETE and LTs, which is consistent with previous studies.[30,31] In keeping with the literature, zymosan induced a shift in the eicosanoid metabolism, characterized by higher amounts of prostanoids.[32] Previous studies largely focused on the acute effects of zymosan or HDM and showed that both stimuli could trigger LT production by myeloid cells, when applied for short times (2-60 min).[7,33] Here, we focused on the prolonged exposure to TLR2/dectin ligands (zymosan and HDM) as they are involved in the initiation of type 2 inflammation.[2,8,34] Lipid mediator class switching from 5-LOX to COX metabolites occurred for both stimuli, thus suggesting that lipid mediator reprogramming during type 2 inflammation happens analogous to settings of type 1 inflammation.[16]

The induction of prostanoids and suppression of 5-LOX metabolites appears to be a common feature of macrophages in type 2 immune settings in response to allergens, IL-4 or nematode infection. Indeed, the reduced production of 5-LOX metabolites could be a result of high levels of IL-4 produced by T_H2 cells, ILC2s and/ or basophils [35] as IL-4 is known to suppress 5-LOX expression in various cell types, including macrophages.[17,36] In a model of filarial nematode infection, eicosanoid reprogramming in nematode-elicited macrophages was shown to depend on IL-4 receptor signaling.[6] In line with this study, we confirmed the induction of prostanoids for a different nematode parasite, thus suggesting that activation of the COX pathway is a general feature of the immune response to nematodes. Recently, soluble egg antigen of a distinct helminth species (the trematode *Schistosoma mansoni*) was reported to induce PGE_2, which contributed to T_H2 polarization.[37] This suggests an important functional role of prostanoids during the type 2 immune response to helminth infection.

The plasticity of macrophages and their extraordinary capacity to produce lipid mediators suggests that these cells are key drivers of eicosanoid reprogramming in type 2 immunity. During allergen-triggered type 2 immune responses in the airways, the macrophage pool consists of resident alveolar macrophages (AMs) and macrophages derived from recruited monocytes.[38] We used aMDM (differentiated in the presence of GM-CSF and TGFβ1) as a cellular model to mimic this mixed macrophage population. Although aMDM may not fully recapitulate macrophages in the lung, these cells showed several typical features of AMs, including high baseline expression of LT-biosynthetic enzymes and of the proinflammatory cytokine IL-1β.[27,28]

We particularly focused on HDM extract as a trigger of type 2 inflammation with well-established functional roles for lipid mediators.[2,8,39] Exposure of aMDM to HDM for 24-96 h resulted in a dynamic mediator class switching of LOX and COX metabolites. While the production of regulatory mediators (eg, PGE_2) peaked after 48 h of HDM exposure, proinflammatory 5-LOX metabolites were initially suppressed, but increased back to baseline over time. This may explain why cysLTs were formed in the airways of HDM-sensitized mice during a two-week model of allergic airway inflammation. However, in addition to macrophages, other cell types including eosinophils and airway epithelial cells can contribute to the formation of LOX metabolites (including 5-LOX-derived cysLTs and 12/15-LOX-derived HETEs and HODEs) during HDM-triggered airway inflammation in vivo.[18] Nevertheless, macrophages likely represent a major source of lipid mediators during the initial exposure to HDM as they are abundant in the airways and highly express or readily upregulate LOX and COX enzymes.

Given the production of several neutrophil-chemotactic factors by HDM-exposed aMDM, we hypothesized that aMDM would show an increased potential to trigger granulocyte chemotaxis after HDM exposure. However, in line with previous in vivo studies, secretions from HDM-exposed aMDM rather tended to decrease granulocyte chemotaxis.[40] This may result in impaired host defense and thus increased susceptibility to infections, which is a common complication in asthmatic patients.[41] To address the functional contribution of COX metabolites, we studied granulocyte chemotaxis in the presence of secretions from HDM-exposed aMDM, which had been treated with the COX inhibitor indomethacin. However, COX inhibition did not affect the chemotactic potential of HDM-exposed aMDM. Instead, a p38 inhibitor restored LTB_4 production and neutrophil chemotaxis, suggesting that the reduced production of the neutrophil chemoattractant LTB_4 by HDM-exposed aMDM may contribute to the impaired chemotactic potential.

Contrary to previous reports of dectin-2 as an essential HDM receptor,[7,8] we did not observe a reduction of HDM-triggered prostanoid or cytokine production when neutralizing dectin-2. However, this may be due to the timing of dectin-2 ligation as previous studies

were focused on acute responses (20-60 min) after HDM exposure. Indeed, while the initial response might depend on dectin-2, other mechanisms likely drive mediator reprogramming during longer exposure. Our results suggest that p38 activation by a dectin-2 and TLR-2/-4-independent mechanism contributed to eicosanoid and cytokine reprogramming in macrophages.

Taken together, HDM exposure induced a potentially pathogenic macrophage phenotype, characterized by abundant production of prostanoids (particularly TXB_2) and proinflammatory cytokines (particularly TNF-α). Given that several of the HDM-triggered macrophage mediators are implicated in severe, steroid-resistant airway inflammation, mediator reprogramming in macrophages should be explored as a therapeutic target in therapy-resistant allergy and asthma.

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CONFLICTS OF INTEREST

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.

AUTHOR CONTRIBUTIONS

FDRH, AF, MH, DT, TB, PH, MRJ, and FA performed experiments. FDRH, AF, MH, DT, and JEvB analyzed data. JEvB, JA, NLH, and CBSW designed the study; JEvB, FDRH, and AF wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Publication II

BRIEF COMMUNICATION OPEN

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Mild COVID-19 imprints a long-term inflammatory eicosanoid- and chemokine memory in monocyte-derived macrophages

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Monocyte-derived macrophages (MDM) drive the inflammatory response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and they are a major source of eicosanoids in airway inflammation. Here we report that MDM from SARS-CoV-2-infected individuals with mild disease show an inflammatory transcriptional and metabolic imprint that lasts for at least 5 months after SARS-CoV-2 infection. MDM from convalescent SARS-CoV-2-infected individuals showed a downregulation of pro-resolving factors and an increased production of pro-inflammatory eicosanoids, particularly 5-lipoxygenase-derived leukotrienes. Leukotriene synthesis was further enhanced by glucocorticoids and remained elevated at 3–5 months, but had returned to baseline at 12 months post SARS-CoV-2 infection. Stimulation with SARS-CoV-2 spike protein or LPS triggered exaggerated prostanoid-, type I IFN-, and chemokine responses in post COVID-19 MDM. Thus, SARS-CoV-2 infection leaves an inflammatory imprint in the monocyte/ macrophage compartment that drives aberrant macrophage effector functions and eicosanoid metabolism, resulting in long-term immune aberrations in patients recovering from mild COVID-19.

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INTRODUCTION

The Coronavirus disease 2019 (COVID-19) has emerged as a global pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections[1]. Long-term symptoms of COVID-19 are common after severe disease[2], but may also affect 15–20% of individuals with previous mild disease[3]. Monocyte-derived macrophages (MDM) drive the inflammatory response to SARS-CoV-2 and contribute to cytokine storms in severe COVID-19[4,5]. Severe COVID-19 is associated with profound changes in the myeloid compartment, including expansion of dysfunctional, pro-inflammatory monocytes during the first weeks after SARS-CoV-2 infection[6,7].

Eicosanoids are bioactive metabolites of polyunsaturated fatty acids (PUFAs) with key roles in infection and inflammation[8]. Eicosanoids are formed from arachidonic acid (AA) through different enzymatic pathways, including the cyclooxygenase (COX) pathway, synthesizing prostanoids and the 5-lipoxygenase (5-LOX) pathway, generating leukotrienes (LTs)[8]. LTs are potent granulocyte-chemotactic metabolites which cause bronchoconstriction, vascular leakage, and airway remodeling[9]. Resident and recruited macrophages in the lung produce high levels of cysteinyl LTs (cysLTs) and leukotriene B_4 (LTB_4), thereby promoting granulocyte infiltration, airway inflammation and tissue remodeling[8]. Serum and airway prostanoid- and LT levels are increased in severe COVID-19[10,11], suggesting a role for eicosanoids in the immune response to SARS-CoV-2 infection.

By studying transcriptome- and lipid mediator profiles in MDM of convalescent SARS-CoV-2-infected individuals with previous mild disease, we show that inflammatory gene expression and eicosanoid profiles as well as altered responsiveness to inflammatory cues are maintained at 3–5 months post infection as well as throughout macrophage differentiation. Pro-inflammatory 5-LOX metabolites were selectively increased in post COVID-19 MDM, suggesting that SARS-CoV-2 infection drives a pro-inflammatory eicosanoid reprogramming that contributes to long-term alterations in innate immune cell function.

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RESULTS AND DISCUSSION

Recent studies have identified immunological changes in individuals recovering from severe or moderate acute COVID-19 for up to 12 weeks post infection[6,7,12,13]; however potential immune aberrations in the majority of SARS-CoV-2-infected patients, affected by mild disease, have remained obscure.

Monocyte-derived macrophages of convalescent COVID-19 patients show pro-inflammatory transcriptional reprogramming and enhanced LPS responses

Our recent work had shown that patients suffering from chronic airway inflammation exhibit transcriptional reprogramming of MDM[14], a cell type implicated in COVID-19 pathogenesis[7]. To investigate whether SARS-CoV-2 infection induces persistent changes in MDM gene expression, we studied a sub-cohort from a large SARS-CoV-2 seroprevalence study in healthcare workers[15] (Table S1, Figs. 1a, S1a). To mimic the pulmonary cytokine milieu, in which infiltrating monocytes differentiate into macrophages, MDM were differentiated in the presence of GM-CSF and TGF-β1[16,17], which resulted in a similar MDM population in seronegative and seropositive subjects (Fig. S1b).

At 3–5 months after SARS-CoV-2 infection, antibody levels in the seropositive group had dropped by ~30% and 16.2% (vs. 2.8% in the seronegative group) reported persistent symptoms (Figs. 1a, S1c, Table S1). Differential blood cell counts were similar between seronegative and seropositive individuals (Table S1).

CCL2, which is increased in monocytes during severe, acute disease[6], was upregulated in post COVID-19 monocytes, suggesting a persistent inflammatory imprint despite mild disease in the investigated cohort (Fig. 1b).

RNA-sequencing (RNAseq) analysis identified 163 differentially expressed genes (DEGs) in MDM differentiated from monocytes of seropositive individuals 3–5 months post infection compared to MDM from seronegative subjects (Fig. 1c, d, Table S1). Post COVID-19 MDM showed higher expression of pro-inflammatory chemokines (*CCL2*, *CCL8*, *CCL7*), driving neutrophil recruitment, including in COVID-19[18,19] (Fig. 1c, d, Table S1).

FCGBP and endothelin-1 (*EDN1*), implicated in anti-viral defense and pro-fibrotic macrophage activation[20,21] were also upregulated in post COVID-19 MDM, together with cytochrome B5 reductase 2 (*CYB5R2*), involved in respiratory burst and fatty acid metabolism[22] (Fig. 1d). In contrast, Semaphorin-7A (*SEMA7A*), implicated in the synthesis of pro-resolving lipid mediators[23], was downregulated in post COVID-19 MDM (Fig. 1d). Post COVID-19 MDM further showed enhanced inflammatory responses to lipopolysaccharide (LPS), characterized by an exaggerated induction of chemokines involved in neutrophil recruitment[24,25] (Table S1, Fig. 1e–g). Increased expression of perforin-2 (*MPEG1*) in post COVID-19 MDM at baseline or upon LPS stimulation (Fig. 1d–g) further suggested persistently enhanced interferon (IFN) signaling following SARS-CoV-2 infection[26]. In contrast, expression of nerve growth factor receptor (*NGFR*), X inactive specific transcript (*XIST*) and *SEMA7A*, mediating anti-inflammatory or pro-resolving effects on macrophages[23,27,28], was reduced in LPS-stimulated post COVID-19 MDM (Figs. 1f, g, S1h, Table S1). Thus, despite mild acute disease in the investigated cohort, MDM exhibited a persistent inflammatory imprint, which was associated with increased symptom burdens and aberrant LPS responses at 3-5 months post infection (Figs. S1c, 1e–g).

SARS-CoV-2 S-protein-triggered IFN response is exaggerated in post COVID-19 MDM

To define consequences of SARS-CoV-2-induced macrophage reprogramming for re-infection or vaccination, we investigated the response of post COVID-19 MDM to SARS-CoV-2 spike (S)-protein. Entry of SARS-CoV-2 is mainly mediated via recognition of its transmembrane S-glycoprotein by angiotensin-converting enzyme 2 (ACE2) and processing by TMPRSS2[29]. However, *ACE2* and *TMPRSS2* expression in MDM was 100 times lower compared to airway epithelial cells, the major cellular targets of SARS-CoV-2, regardless of inflammatory stimulation or glucocorticoid treatment (Fig. S1d–f). Yet, macrophages can respond to S-proteins of SARS-CoV-1 or SARS-CoV-2 via innate sensing mechanisms including C-type lectins[30,31], which were upregulated in post COVID-19 MDM (Fig. S1g).

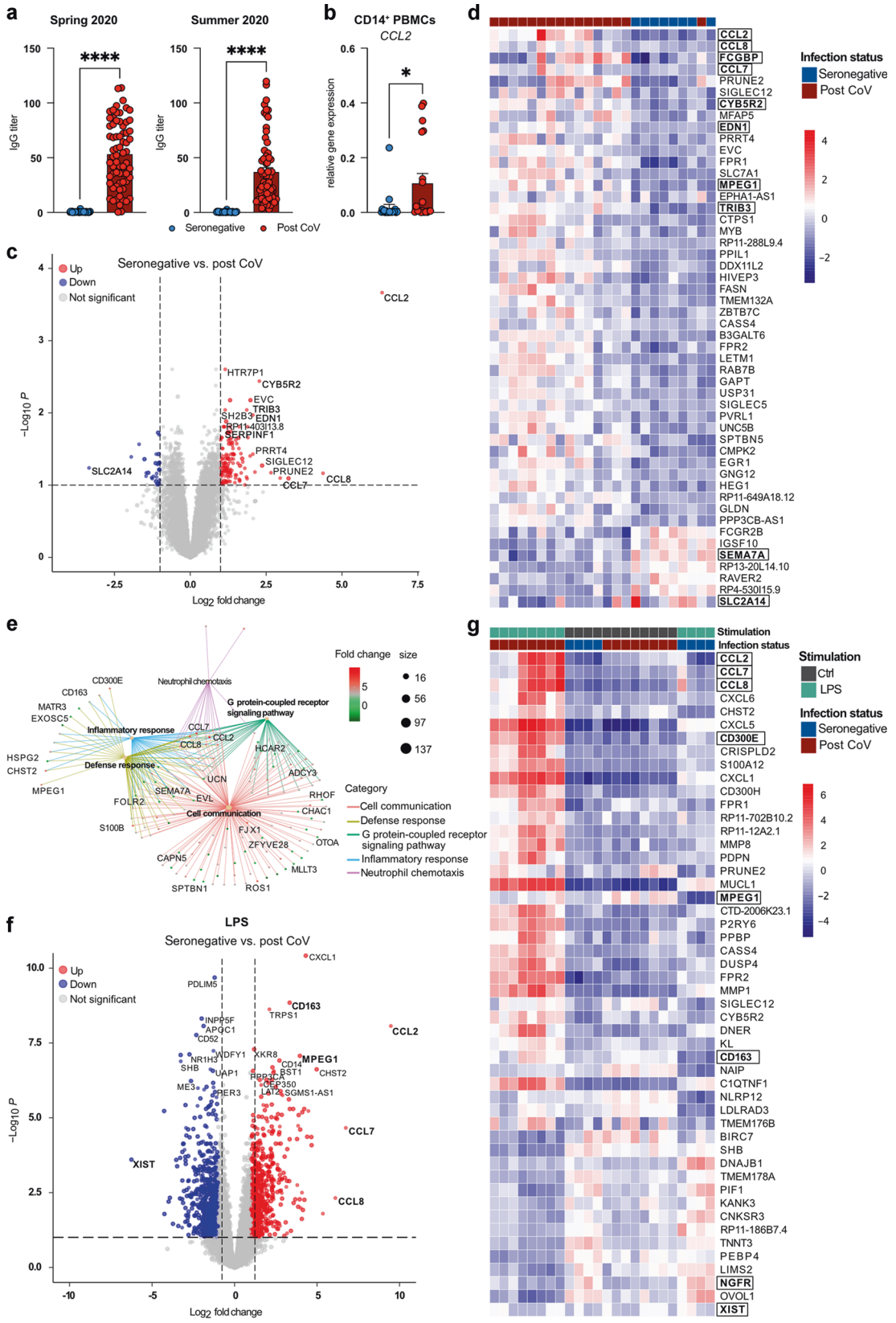
Indeed, MDM readily responded to S-protein and transcriptional differences between seronegative and post COVID-19 MDM were exacerbated by both S-protein and LPS (Fig. 2a). S-protein induced multiple interferon-stimulated genes (ISGs) (e.g. *IFI27*, *IFITIM1/3*, *APOBEC3A, ISG20, MX1/2, OAS1/3*) (Fig. 2b, c, Table S1), demonstrating that it induces an antiviral state in MDM. S-protein stimulation of post COVID-19 MDM resulted in a higher number of DEGs compared to seronegative MDM (858 vs. 220), indicative of a persistently enhanced responsiveness to SARS-CoV-2 several months post infection (Table S1).

The induction of IFN-induced genes (e.g. *CXCL10*, *CXCL11*, *MPEG1*) was increased in S-protein-stimulated post COVID-19 MDM (Fig. 2d, e, Table S1), supporting a role for type I IFN signaling in macrophage reprogramming by SARS-CoV-2 infection. MDM from convalescent SARS-CoV-2-infected subjects showed an enhanced LPS- and S-protein-triggered induction of chemokines (*CCL2*, *CCL8*, *CXCL10*, *CXCL11*) and M2-associated genes (*CD226*, *CD163, CD209*, *TIMP3*, *MERTK*, *TNIP3*), suggesting a pro-inflammatory, T-cell suppressive[32,33] MDM phenotype (Figs. 1e–g, 2d, e, Table S1). This was in agreement with the exaggerated S-protein- or LPS-mediated induction of immune regulatory enzymes and receptors, including *ACOD1/ IRG1*, *PTGES* and *CD300E* in post COVID-19 MDM (Figs. 1e–g, 2d, e, Table S1)[32,34,35].

Thus, previous SARS-CoV-2 infection imprints a pro-inflammatory macrophage phenotype, that mounts exaggerated chemokine- and IFN responses, but likely exhibits impaired T-cell stimulatory and pro-resolving capacities. This was in line with previous studies identifying a dysfunctional, pro-inflammatory monocyte activation for up to 12 weeks after SARS-CoV-2 infection[7,13] and additionally suggested the long-term persistence of a pro-inflammatory macrophage state following mild disease. Changes in gene expression of post COVID-19 MDM were amplified by inflammatory stimuli, suggesting a "trained" state that lasted for at least 5 months post infection. Mechanistically, this may be driven by IFN-mediated reprogramming as post COVID-19 MDM exhibited an exaggerated upregulation of multiple ISGs, including perforin-2 (*MPEG1*), a driver of type I IFN signaling[26].

Post COVID-19 MDM produce increased amounts of inflammatory 5-lipoxygenase metabolites at 3–5 months post SARS-CoV-2 infection

Previous studies had suggested an involvement of pro-inflammatory eicosanoids in severe, acute COVID-19[10,11,36] and our RNAseq data indicated aberrant expression of genes involved in fatty acid and- eicosanoid synthesis in MDM and monocytes of convalescent, SARS-CoV-2 infected individuals (Figs. 1, 2, 3a, b, Table S1). Thus, we performed LC-MS/MS quantification of lipid mediators following stimulation with calcium ionophore to trigger PUFA mobilization and eicosanoid production. Compared to MDM from seronegative individuals, exhibiting considerable production of soluble epoxide hydrolase (sEH) metabolites (11,12-DiHETrE, 19,20-DiHDPA, 17,18-DiHETE), post COVID-19 MDM displayed broadly altered eicosanoid profiles that were dominated by pro-inflammatory 5-lipoxygenase (5-LOX) metabolites (Fig. 3c–e). Post COVID-19 MDM synthesized increased amounts of pro-inflammatory 5-LOX metabolites (LTB_4, 5-KETE, 5-HEPE and LTD_4), implicated in granulocyte chemotaxis and airway remodeling (Fig. 3d, e). In addition, the production of pro-inflammatory COX metabolites $PGF_{2\alpha}$ and 12-HHTrE was increased in post COVID-19 MDM (Fig. 3d, f).



This suggested that the prominent synthesis of inflammatory eicosanoids is not limited to acute and severe COVID-19[10,11] and that reprogramming of innate immune cells may result in persistently enhanced LT production even following mild disease. Of note, we did not analyze spontaneous eicosanoid production, but used Ca^{2+} ionophore to elicit maximal eicosanoid responses, which allowed us to quantify lipid mediators in limited numbers of patient cells. Thus, eicosanoid profiles identified in the current

Fig. 1 Pro-inflammatory transcriptional reprogramming and heightened LPS response in post COVID-19 MDM. a Serum IgG titers of seronegative ($n = 36$) or SARS-CoV-2 seropositive (post CoV) ($n = 68$) individuals in Spring 2020 or at 3–5 months post infection (p.i.) (Summer 2020). Data are shown as mean + SEM. **b** Expression of *CCL2* in $CD14^+$ PBMCs of seronegative ($n = 20$) vs. post CoV ($n = 19$) subjects at 3–5 months p.i. **c** Volcano plot showing DEGs between seronegative ($n = 8$) and post CoV MDM ($n = 16$). Top 10 DEGs (base mean > 50), log2 FC > 2 or adjusted p value (padj < 0.016 labeled), DEGs with log2 FC > 1 and padj < 0.1 marked. **d** Heatmap of top 50 DEGs between seronegative ($n = 8$) and post CoV ($n = 16$) MDM, padj < 0.1, log2 FC > 1; base mean > 50. **e** GSEA between post CoV ($n = 8$) and seronegative ($n = 4$) MDM + LPS, log2 FC > 2, p value < 0.01. **f** Volcano plot showing DEGs between seronegative ($n = 4$) and post CoV ($n = 8$) MDM + LPS. DEGs with log2 FC > 3 or padj < 1×10^{-6} labeled, DEGs with log2 FC > 1 and padj < 0.1 marked. **g** Heatmap of top 50 DEGs between seronegative ($n = 4$) and post CoV ($n = 8$) MDM ± LPS, padj < 0.1, log2 FC > 1, base mean > 50. Statistical significance was determined by Mann–Whitney test (**a**, **b**) or DESeq2 (**c**–**f**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

study reflect a setting of acute inflammatory challenge. MDM of convalescent subjects also revealed a marked lower inferred soluble epoxide hydrolase activity. The epoxides of arachidonic acid have been reported to promote the resolution of inflammation, including mitigation of cytokine storms[37]. Accordingly, inhibition of the sEH has been proposed as a potential therapeutic target for COVID-19[38]. Our findings suggest that subsequent to mild COVID-19, MDM may exhibit a compensatory sEH activity that is shifted towards a pro-resolution state. In contrast to acute infection, which resulted in increased *ALOX5* expression in neutrophils and monocytes[10], we did not find evidence of increased 5-LOX pathway gene expression in post COVID-19 MDM (Fig. 3g). Instead, genes involved in upstream events of fatty acid and lipid mediator biosynthesis (e.g., *FASN*, *DGAT2*, *PLA2G4C*) were upregulated in post COVID-19 MDM compared to MDM from seronegative subjects, suggesting an MDM phenotype in position for rapid activation of lipid metabolic pathways.

Analysis of MDM eicosanoid profiles from donors of the same cohort at 12 months post infection indicated that LT and prostanoid synthesis of post COVID-19 MDM had largely returned to baseline levels at this time point (Fig. 3h, i). This suggested that pro-inflammatory eicosanoid reprogramming in mild COVID-19 is transient, but that it may contribute to an enhanced inflammatory propensity during the first months post SARS-CoV-2 infection.

When stratified into 5-LOX low- or high producers, post COVID-19 subjects with high MDM LT production exhibited less acute symptoms but a faster decline in SARS-CoV-2 specific IgG titers (Fig. 3j), indicative of an efficient acute anti-viral response[39]. However, the lack of a defined clinical diagnosis of long COVID and poor reporting of long-term symptoms in the studied post COVID-19 cohort, prevented us from establishing a clear link between high MDM LT production and long-term symptoms of SARS-CoV-2 infection. Thus, future studies should investigate eicosanoid reprogramming in a cohort with clinically defined long COVID. Such studies would be imperative to define a potential pathological relevance of the inflammatory macrophage memory observed in the current study.

As patients in our study were enrolled following seroconversion, we were not able to compare monocyte and macrophage profiles at 3–5 months post infection to those during acute disease. However, we observed a considerable overlap between transcriptional profiles of post COVID-19 MDM and published transcriptomes of macrophages from SARS-CoV-2-infected individuals with mild acute disease[40]. Thus, several of the DEGs identified in our analysis (*MPEG1*, *CD163*, *CXCL9*, *MERTK*, and *MRC1*) were increased and correlated with higher expression of 5-LOX pathway genes in mild vs. severe acute disease[40]. It will be important to compare macrophage reprogramming between convalescent COVID-19 patients with different disease severities as well as following infection with other respiratory viruses (e.g., influenza). While previous studies have suggested an acute and transient increase in eicosanoids during respiratory syncytial virus (RSV) or influenza A virus (IAV) infection[41–43], a comprehensive assessment of macrophage eicosanoid profiles in these diseases is currently lacking. PGE_2 production was increased following IAV infection, however we did not observe increased PGE_2 production in post COVID-19 MDM. Similarly, transcriptional profiles of post COVID-19 MDM showed minimal overlap with post influenza macrophage gene expression profiles[44,45], suggesting that infection with different respiratory viruses results in distinct macrophage reprogramming. Increased macrophage LTB_4 production may however contribute to protective immunity during acute infection with multiple respiratory viruses[41,43]. It will be important to determine, whether the persistent increase of LTB_4 may contribute to a decreased susceptibility to respiratory viral infection during the first months following SARS-CoV-2 infection.

As airway inflammation, including in COVID-19, is commonly treated by glucocorticoids, we investigated potential effects of glucocorticoids on LT synthesis by post COVID-19 MDM. Fluticasone propionate, a commonly used inhaled glucocorticoid, further increased LT synthesis by post COVID-19 at baseline or after stimulation with house dust mite (HDM), used as a ubiquitous trigger of airway inflammation (Fig. S2a–d). This suggested that glucocorticoid treatment may further aggravate the pro-inflammatory eicosanoid reprogramming in post COVID-19 subjects. Given the therapeutic efficacy of glucocorticoids in airway inflammation, the finding that glucocorticoids enhanced LT synthesis may be surprising. However, it is in keeping with studies showing no reduction in LTs following glucocorticoid treatment in humans or enhanced LT production following in vitro treatment with glucocorticoids[46–48].

S-protein-triggered prostanoid response is enhanced in post COVID-19 MDM

To assess potential differences in eicosanoid production capacities under inflammatory conditions, we compared Ca^{2+} ionophore-elicited eicosanoid production in post COVID-19 and seronegative MDM stimulated for 24 h with S-protein or LPS. S-protein stimulation profoundly altered eicosanoid profiles (Fig. 4a, b), provoking a prominent induction of prostanoids from the thromboxane pathway (TXB_2 and 12-HHTrE), while 5-LOX metabolites were reduced (Fig. 4b).

Compared to seronegative MDM, post COVID-19 MDM exhibited enhanced S-protein-induced prostanoid production, which was particularly evident for the thromboxane synthesis metabolite 12-HHTrE (Fig. 4b, c). Similarly, the cytochrome P450 metabolite 19-HETE was significantly increased in S-protein-stimulated post COVID-19, indicative of increased S-protein-mediated induction of vasoactive eicosanoids at 3-5 months post infection. In contrast at 12 months post infection, S-protein-triggered eicosanoid responses did not differ between SARS-CoV-2 seronegative and seropositive subjects (Fig. 4d). Compared to S-protein, LPS induced a stronger eicosanoid shift, thus overriding aberrant lipid mediator synthesis of post COVID-19 MDM (Fig. S3a, b). While upregulating prostanoids, LPS reduced the heighted production of LTD_4 in post COVID-19 MDM (Fig. S3c), in line with suppressive effects of 24 h LPS stimulation on LT production by alveolar macrophages[49]. Together, this suggested that eicosanoid responses remain increased for several months following SARS-CoV-2 infection. In addition, during challenge with LPS or S-protein, eicosanoid profiles switch towards prostanoids with tissue reparative, vasoconstrictor and immune regulatory functions,

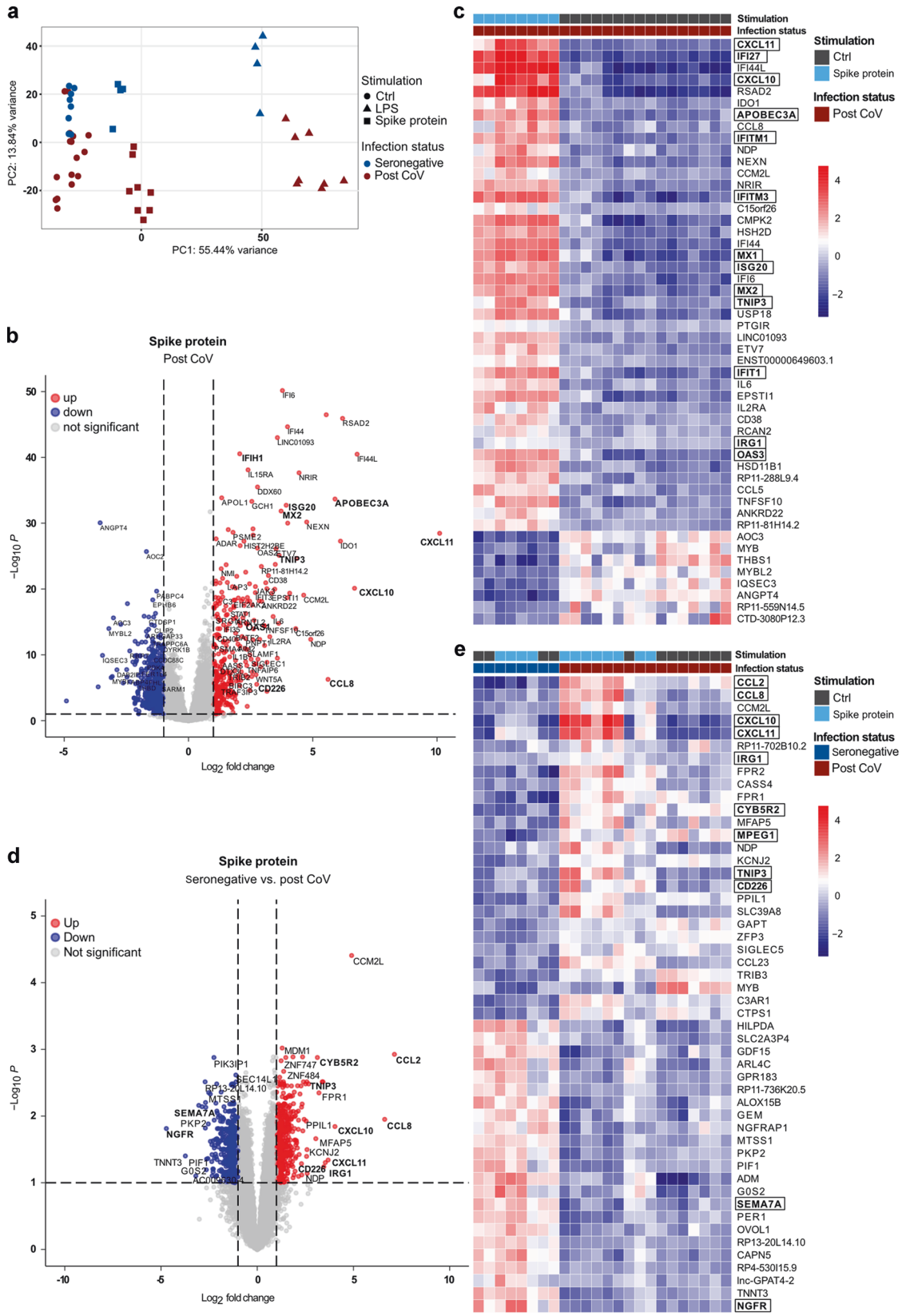
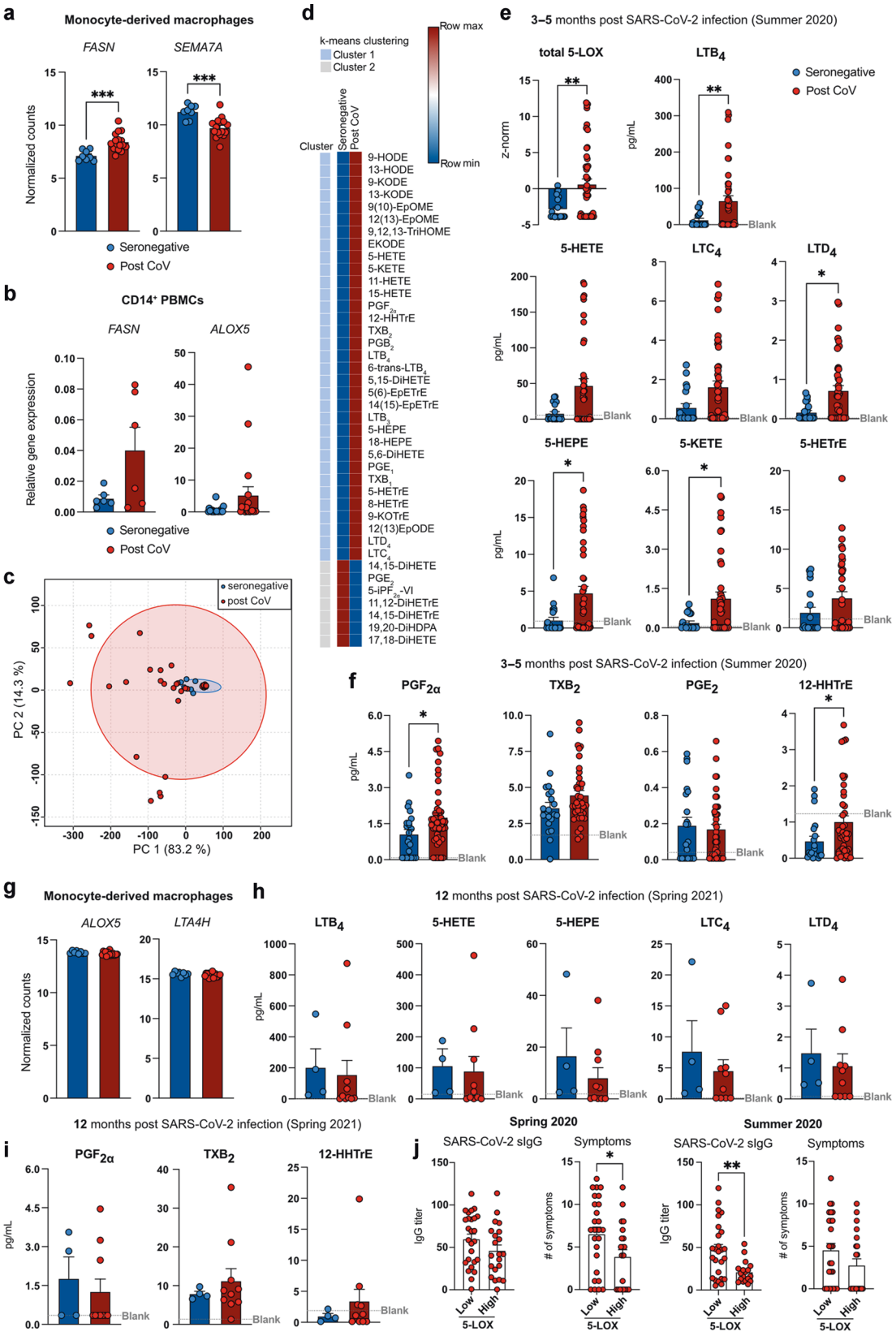


Fig. 2 S-protein-induced type I IFN and chemokine responses are exaggerated in post COVID-19 MDM. a PCA of RNAseq datasets (baseline, S-protein, LPS) for seronegative ($n = 4$–8) or post CoV ($n = 8$–16) MDM. **b** Volcano plot of DEGs for post CoV MDM ($n = 8$) ± S-protein. DEGs with log2 FC > 5 or padj < 0.00001 (DESeq2) are labeled, DEGs with log2 FC > 1 and padj < 0.1 are colored. **c** Heatmap of top 50 DEGs in post CoV MDM ($n = 8$–16) ± S-protein, padj < 0.1, log2 FC > 1, base mean > 50. **d** Volcano plot of DEGs of S-protein-stimulated MDM from seronegative ($n = 4$) vs. post-CoV ($n = 8$) donors. DEGs with log2 FC > 2.5 or padj < 0.003 are labeled, DEGs with log2 FC > 1 and padj < 0.1 are colored. **e** Heatmap of top 50 DEGs of MDM ± S-protein from seronegative ($n = 4$) or seropositive ($n = 8$) donors, padj<0.1, log2 FC > 1, base mean > 50.



potentially promoting repair of inflammation-induced tissue damage.

In contrast to eicosanoid profiles, cytokine production at baseline or following stimulation was not significantly different between post COVID-19 and seronegative MDM (Fig. S2e–h), suggesting that cytokine aberrations may not persist for >12 weeks or during monocyte-macrophage differentiation. However, in contrast to LTs, cytokine and prostanoid production by MDM

Fig. 3 Post COVID-19 MDM produce increased amounts of inflammatory 5-lipoxygenase metabolites. a *FASN* and *SEMA7A* expression in seronegative ($n = 8$) and post CoV MDM ($n = 16$). **b** Gene expression of *FASN* and *ALOX5* in seronegative ($n = 6/20$) or post CoV ($n = 6/19$) $CD14^+$ PBMCs. **c** PCA of lipid mediator profiles of MDM from seronegative (n = 22) or seropositive (n = 47) individuals. Red and blue circles: 95% CI. **d** Heatmap of lipid mediators produced by seronegative ($n = 22$) or post CoV ($n = 47$) MDM (LC-MS/MS). Clustering: with k-means using Pearson correlation. Data is shown as mean. **e** Sum of z-scored arachidonic acid derived 5-LOX metabolite concentrations for each donor. Levels of major 5-LOX (**e**, **h**) and COX (**f**, **i**) metabolites produced by MDM at 3-5 (**e**, **f**) or 12 (**h**, **i**) months p.i. (LC-MS/MS) shown as mean + SEM of $n = 22/ n = 4$ seronegative or $n = 47/n = 10$ seropositive individuals. **g** Expression of *ALOX5* and *LTA4H* (RNAseq) in MDM from seronegative ($n = 8$) or post CoV ($n = 16$) individuals. **j** IgG titers in serum or number of symptoms in MDM from post CoV donors stratified into 5-LOX low (z-score < 1) and high producers (z-score > 1). Bar graphs are depicted as mean + SEM. Statistical significance was determined by Mann–Whitney test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

was efficiently suppressed by fluticasone propionate (Fig. S2a–d, i). This suggested that cytokines and prostanoids are efficiently targeted, while exaggerated LT responses of post COVID-19 MDM are further exacerbated by glucocorticoids. Indeed, thromboxane is a major eicosanoid produced by inflammatory macrophages and involved in vascular and airway remodeling, thus its inhibition by glucocorticoids may provide a therapeutic benefit. However, glucocorticoids may in turn further enhance the heightened production of pro-inflammatory LTs by post COVID-19 MDM, thus promoting LT-driven airway inflammation and remodeling. Based on the enhanced production of 5-LOX-derived lipid mediators both in acute[10,11] and post-acute COVID-19 (this study), approved LT pathway inhibitors should be considered as regimens to treat and/ or prevent airway inflammation and remodeling during the first 6 months following SARS-CoV-2 infection.

Future studies should further decipher upstream receptors and epigenetic pathways that drive the persistent pro-inflammatory macrophage and eicosanoid reprogramming during SARS-CoV-2 infection. In addition, a potential heterogeneity in GM-CSF and TGF-β1-differentiated MDM from seronegative and seropositive individuals should be addressed in single cell analyses. LTs have been reported to induce CCL2 in monocytes[50,51], suggesting that enhanced LT synthesis may drive exaggerated pro-inflammatory chemokine responses in post COVID-19 MDM. In turn, increased CCL2 production by post COVID-19 MDM or monocytes may promote LTB_4 production[52]. Thus, our combined RNAseq and LC-MS/MS data suggest a crosstalk between CCL2 and LTs, which perpetuates the persistent pro-inflammatory activation of monocytes and macrophages following SARS-CoV-2 infection. Due to limitations in patient material, we could not perform a comprehensive comparison of MDM and monocytes, however our data suggest that differences in CCL2 and fatty acid synthesis are at least partially present in undifferentiated post COVID-19 monocytes, which differentiate into inflammatory monocyte-derived macrophages when entering the lung[5]. The persistent upregulation of pro-inflammatory eicosanoids in post COVID-19 macrophages may have multiple consequences for subsequent immune responses, e.g. during bacterial or viral infection or in patients suffering from chronic inflammatory diseases such as asthma, thus requiring future investigation.

METHODS

Study design

Symptoms of seronegative (SARS-CoV-2 seronegative) and post COVID-19 (SARS-CoV-2 seropositive) individuals were determined through a questionnaire in Spring 2020 and 3–5 months later, in Summer 2020. Percentage of each symptom was calculated separately for seropositive and -negative individuals (table S1). Sample sizes for each experiment are specified in the corresponding figure legends; an overview is depicted in Fig. S1a. All blood donors participated in the study after informed written consent. All procedures were approved by the local ethics committee at the University clinic of the Technical University of Munich (internal references: 216/20S, 263/21S) and in accordance with the declaration of Helsinki.

Monocyte-derived macrophage culture

Isolated peripheral blood mononuclear cells (PBMCs) of post COVID-19 or seronegative individuals were used to generate monocyte-derived macrophages (MDM), as previously reported[53,54]. MDM were cultured in the presence of 10 ng/mL human GM-CSF (Miltenyi Biotec, Bergisch-Gladbach, Germany) and 2 ng/mL human TGF-β (Peprotech, Hamburg, Germany). After 7 days incubation, cells were harvested and stimulated for 24 h with 100 ng/mL LPS (Invivogen, San Diego, CA, USA), 20 nM spike protein (antibodies-online GmbH), 10 µg/mL house dust mite extract (HDM) (Citeq Biologics, Groningen, The Netherlands), 1 µM fluticasone propionate (FP) (Sigma-Aldrich, St. Louis, MO, USA), 5 µM or 100 nM dexamethasone (DXM) (Sigma-Aldrich, Merck). After 24 h of stimulation cells were harvested in presence of Ca^{2+}-ionophore A23187 (Sigma-Aldrich, Merck).

NHBE and ALI culture

Primary normal human bronchial epithelial cells (NHBEs) (Lonza, Basel, Switzerland) from non-smokers in passage 3 were grown to 80–90% confluency in Bronchial Epithelial Cell Growth Medium (BEGM) (Lonza). Following starvation overnight in bronchial epithelial basal medium (BEBM) (Lonza), NHBEs were stimulated for 24 h with 1 µg/mL HDM (Citeq) or 1 µM FP (Sigma-Aldrich, Merck). For air-liquid interface (ALI) cultures, NHBEs were split at 60–80% confluency and 1×10^5 cells were seeded on 12 mm transwells (0.4 µm pores, Stemcell Technologies, Vancouver, Canada). Cultures were maintained in BEGM (500 µL apical and 1000 µL basal) until cells reached full confluency. Subsequently, cells were "airlifted" by removing the apical medium, and basal medium was replaced with PneumaCult-ALI Maintenance Medium (Stemcell Technologies). Medium was replaced every 2 days and excessive mucus washed away with DPBS (Gibco). Cells were cultured at air liquid interface for 3–4 weeks. Before stimulation, cells were starved overnight in PneumaCult-ALI Basal Medium (Stemcell Technologies). ALI cells were stimulated on the apical side with 1 µg/mL HDM (Citeq), 1 µM FP (Sigma-Aldrich) or corresponding control for 24 h.

Histology

For histology ALI cells were fixed in 4% formaldehyde and embedded in paraffin. Sections were cut and hematoxylin & eosin (H&E) stained at the Klinikum rechts der Isar, Dermatology Department.

RNA isolation

Cells were lysed in RLT buffer (Qiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol. RNA was extracted using a spin-column kit according to the manufacturer's instructions (Zymo Research, Freiburg, Germany) and transcribed into DNA using the HighCapacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems) or submitted for total RNA sequencing.

RNA sequencing

Library preparation was performed using the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA). Briefly, RNA was isolated from MDM cell lysates according to the

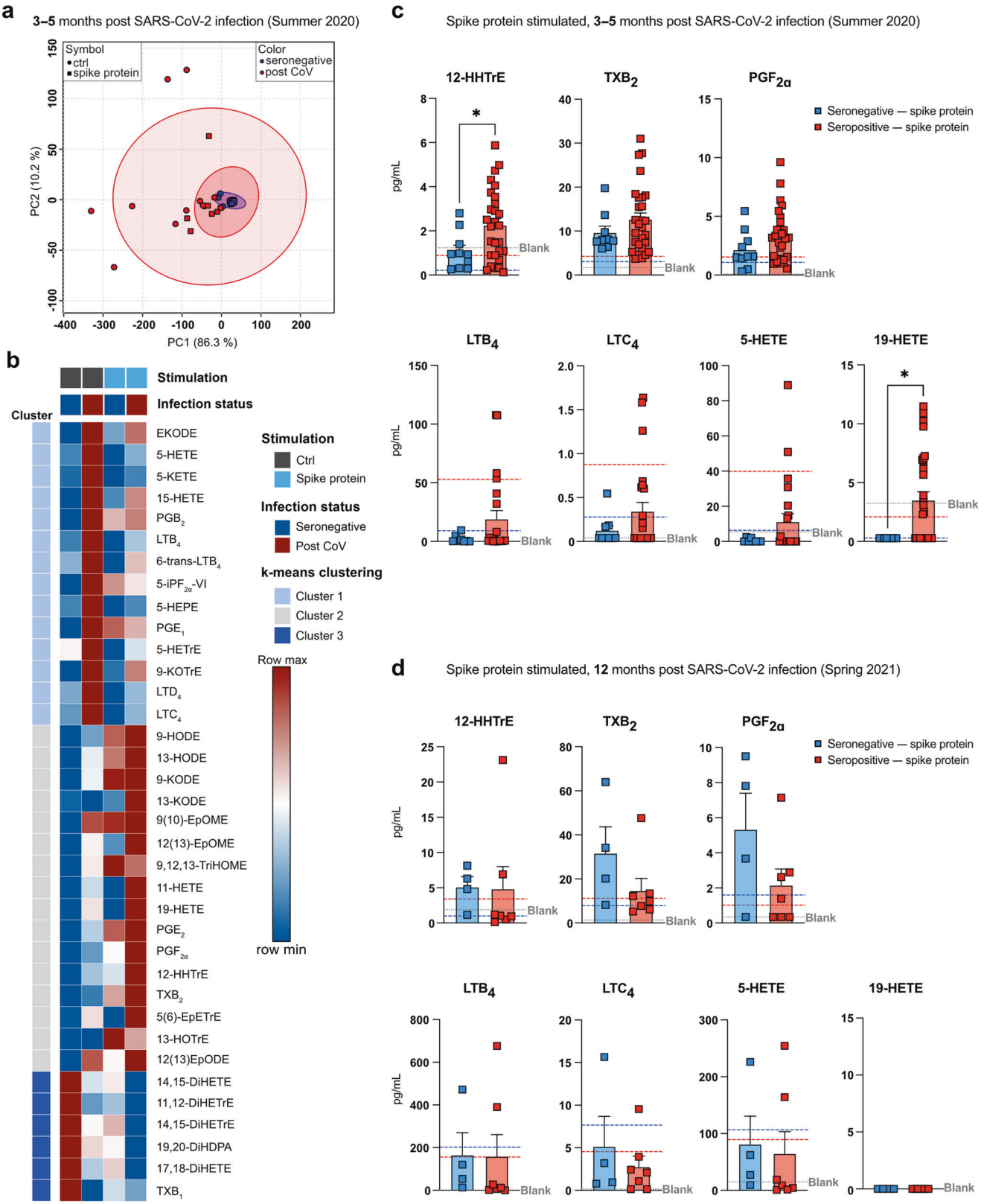


Fig. 4 Increased S-protein-triggered prostanoid response in post COVID-19 MDM. a PCA of lipid mediators quantified in seronegative ($n = 10$) or post CoV ($n = 29$) MDM ± S-protein. Red and blue circles: 95% CI (LC-MS/MS at 3-5 months p.i). **b** Heatmap of lipid mediators produced by MDM (seronegative/ post CoV) ± S-protein; clustered with k-means using Pearson correlation. Data are shown as mean of seronegative ($n = 10$) or post CoV ($n = 29$) MDM. Concentrations of 12-HHTrE, TXB_2, $PGF_{2\alpha}$ and 19-HETE/ 5-HETE produced by MDM + S-protein, at 3-5 months (**c**) or 12 months (**d**) p.i.; **d** $n = 4$ (seronegative); $n = 7$ (post CoV). Dashed lines indicate average ctrl level of either seronegative (blue) or seropositive (red) MDM. Bar graphs are depicted as mean + SEM. Statistical significance was determined by Mann–Whitney test. *$p < 0.05$.

manufacturer's instructions (Zymo Research). Total RNA quality and quantity was assessed by Qubit 4 Fluorometer (Invitrogen) and RNA integrity number (RIN) was determined with the Agilent 2100 BioAnalyzer (RNA 6000 Nano Kit, Agilent).

For library preparation, 1 µg of RNA was poly(A) selected, fragmented, and reverse transcribed with the Elute, Prime, Fragment Mix (Illumina). A-tailing, adaptor ligation, and library enrichment were performed as described in the TruSeq Stranded mRNA Sample Prep Guide (Illumina). RNA libraries were assessed for quality and quantity with the Agilent 2100 BioAnalyzer and the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Thermo Fisher Scientific). RNA libraries were sequenced as 150 bp paired-end runs on an Illumina NovaSeq 6000 platform. Sequencing was performed at the Helmholtz Zentrum München (HMGU) by the Genomics Core Facility.

Cytokine analysis (ELISA)

Cell culture supernatants were analyzed for IL-6, IL-1β and IL-8 secretion using the human ELISA Sets (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Lipid mediator quantification

Briefly, cell supernatants from 200,000 cells, stored in equal volume of methanol, were extracted using solid phase extraction (Evolute Express ABN, Biotage, Uppsala, Sweden) and lipid mediators (see Table S1) were quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)[55]. Given that cell culture media has significant background levels of many lipid mediators, compounds whose concentration was below the media level were excluded from data analysis.

Real-time quantitative PCR

10 ng cDNA was used as a template. The list of applied primers (4 µmol/L, Metabion international AG, Planegg, Germany) can be found in the Supplement. FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) was used and fluorescence was measured on a ViiA7™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The expression levels were normalized to the house-keeping genes GAPDH (for MDM), ACTB, HPRT1 and TFRC *(average for NHBEs and ALI cultured cells)*. Relative gene expression was calculated as $2^{\Delta CT}$ ($\Delta C_T = C_{T\ (Housekeeper)} - C_{T(Gene)}$). For genes where expression could not be quantified, CT values were set to 40.

Data analysis and statistics

LC-MS/MS and RNAseq data were analyzed using previously published procedures[14,48,55,56]. Details can be found in the Supplement.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.E.v.B., C.E.W., C.B.S.W., A.M.C., P.K., P.L., U.P. Methodology: A.Q., J.K., C. E.W., M.U.D. Investigation: S.B., F.D.H., F.H., A.Q., J.H., A.L., A.P., S.Y, J.E. Visualization: S.B., F. D.H., F.H., M.U.D. Funding acquisition: J.E.v.B., C.E.W. Project administration: J.E.v.B., A.M.C., P.K., U.P., P.L. Supervision: J.E.v.B., A.M.C., P.K. Writing—original draft: J.E.v.B., S.B., F.D.H., F. H. Writing—review & editing: C.E.W., C.B.S.W., A.L., A.M.C, A.P., P.K., P.L.

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COMPETING INTERESTS

C. B. S.-W. received grant support from Allergopharma, PLS Design, as well as Zeller AG; and received speaker honoraria from Allergopharma. U. Protzer is co-funder, board member and share holder of SCG Cell Therapy Inc. and serves as ad hoc advisor for Sanofi-Pasteur, BioNTech, Janssen and Swedish Orphan Biovitrum concerning COVID-19. The rest of the authors declare that they have no relevant conflicts of interest related to this work.

ADDITIONAL INFORMATION

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