Defining local and systemic pathways that influence allergic lung inflammation

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ABBREVIATIONS

(i)EOS	Inflammatory eosinophils
(r)EOS	Resident eosinophils
5-HT	5-hydroxytryptamine
ACK	Ammonium-chloride-potassium
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADRB2	β2 adrenergic receptor
AGM	Aorta gonads mesonephros
AHR	Airway hyperresponsiveness
AM	Alveolar macrophage
ANOVA	Analysis of variance
APC	Antigen presenting cell
APRIL	A proliferation-inducing ligand
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BCH	2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid
BCR	B cell receptor
BLD	Blood
BM	Bone marrow
C/EBPα	CCAAT-enhancer-binding proteins alpha
cAMP	Cyclic adenosine monophosphate
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Chron's disease
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COPD	Chronic obstructive pulmonary disorder
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
CysLT	Cysteinyl leukotriene
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid

EET	Eosinophil-derived extracellular trap
eIF3	Eukaryotic initiation factor 3
ELISA	Enzyme linked immunosorbent assay
EoP	Eosinophil progenitor
EoPre	Eosinophil precursor
EPX	Eosinophil peroxidase
ERK	Extracellular signal-regulated kinases
ES	Excreted/secreted products
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FFAR	Free fatty acid receptor
FFPE	Formalin fixed and paraffin embedded
ES	Excreted/secreted products
FACS	Fluorescence-activated cell sorting
FL	Foetal liver
Foxp3	Forkhead box P3
Fuc	Fucose
Gal	Galactose
GalNAc	Galactosamine
GATA1	GATA-binding factor 1
GF	Germ-free
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte macrophage progenitor
GPR	G protein-coupled receptor
H&E	Haematoxylin and Eosin
HDAC	Histone deacetylase
HDM	House dust mite
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia inducible factor 1 alpha
HSC	Haematopoietic stem cell
i.n.	Intranasal
i.t.	Intratracheal
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IE	Intraepithelial
IF	Immunofluorescence

IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-1RacP	IL-1 receptor accessory protein
ILC	Innate lymphoid cell
IL-Rα	Interleukin receptor alpha
IM	Interstitial macrophage
LAT1	Large amino acid transporter 1
LI	Large intestine
LP	Lamina propria
LPS	Lipopolysaccharide
LT	Leukotriene
MAMP	Microbial-associated molecular pattern
MBP	Major basic protein
MCh	Methacholine
MDP	Macrophage and dendritic cell precursor
MED LN	Mediastinal lymph node
MEP	Megakaryocyte erythrocyte progenitor
MES LN	Mesenteric lymph node
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
Muc2	Mucin 2
NK	Natural killer
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
o.g.	Oral gavage
ORF	Open reading frame
OVA	Ovalbumine
PAF	Platelet activating factor
PAMP	Pattern associated molecular pattern
PAS	Periodic acid schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-L1	Programmed death-ligand-1
PFA	Paraformaldehyde
PG	Prostaglandin
PKC	Protein kinase C

PL	Peritoneal lavage
PNA	Peanut agglutinin
PP	Payer's patch
qPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
Relm-α	Resistin-like molecule alpha
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SBA	Soybean agglutinin
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SFB	Segmented filamentous bacterium
SI	Small intestine
Siglec-F	Sialic acid-binding immunoglobulin-like lectin F
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
SPINK5	Serine peptidase inhibitor Kazal type 5
ST2	Suppression of tumorigenicity 2
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll like receptor
TNF-α	tumour necrosis alpha
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
VCAM-1	Vascular adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA–4	Very late antigen 4
XBP-1	X-box binding protein 1

ABSTRACT

Asthma is one of the most common chronic inflammatory disorders in the world, resulting in significant disability, poor quality of life and healthcare costs. The inflammatory response in asthma primarily involves aberrant Th2 responses and eosinophil infiltration in the airways. However, patients also show evidence of non-Th2-mediated asthma, characterised by airway neutrophilia and Th1/Th17 responses. Current treatment for the disease can induce long-term side effects and patients lack more tailored and effective therapies. Moreover, there is increasing evidence for asthmatic patients developing concomitant chronic inflammation of the gut. There is therefore the necessity to further investigate the mechanisms underlying the development of asthma and whether inter-organ communication may be relevant in its etiology with the aim of finding alternative therapeutic targets.

Using a murine model of acute allergic asthma, the results presented in this thesis describe a system in which different immune compartments can be interconnected with each other. Focussing on eosinophils, we show that these cells can adapt to the characteristics of the tissue microenvironment where they reside by acquiring features that may be correlated with homeostatic or pro-inflammatory properties. Pro-inflammatory features of eosinophils were associated with a metabolic reprogramming of these cells, which was supported by factors enriched in the lung environment, such as IL-33, GM-CSF and IL-5. We found that lung pro-inflammatory eosinophils and neutrophils expressed GPR109a and that agonists of this receptor, including the metabolite butyrate and the vitamin niacin, had anti-inflammatory effects in both eosinophilic and neutrophilic asthma. Eosinophil and lymphocyte populations in the gut were also affected by the ongoing lung inflammatory responses. Eosinophil heterogeneity and acquisition of pro-inflammatory features was present in the small intestine, whereas T cell- and type-2 innate lymphoid cell-mediated Th2 responses dominated the large intestine. We provide evidence for T cell recirculation as well as structural, biochemical and microbial changes in the large intestine, that may rewire the gut metabolic machinery to restore body homeostasis.

In summary, this work highlights GPR109a as a potentially exciting target for the treatment of Th2 and non-Th2 asthma and provides additional mechanistic insights into inter-organ communication. These findings may inform unappreciated therapeutic interventions that could impact treatment of asthma.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Rossana Azzoni

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EXPERIMENTAL CONTRIBUTIONS

All experiments were designed, performed and analysed by myself with the input of my supervisors, Dr. John Grainger[1], Dr. Joanne Konkel[1], Dr. Tara Sutherland[1], Prof. Tracy Hussell[1], Dr. Yashaswini Kannan[2] and Dr. Edith Hessel[3]. Hayley Bridgeman, Dr. Kara Filbey, Dr. Tovah Shaw and Dr. Kelly Wemyss assisted with sample processing. Dr. Ian Prise analysed the microarray gene sequencing from Mesnil et al. (2016). Seahorse extracellular flux analysis was performed by Dr. Maria Zancanaro-Krauss (Hepworth lab). Cell sorting was carried out by Michael Jackson within the Flow Cytometry Core Facility (University of Manchester).

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 The immune system

The immune system is composed of a complex network of cellular and soluble elements that interact to respond to both infectious and non-infectious agents affecting the body. Specialised lymphoid organs, such as the spleen and lymph nodes, are the sites where specific immune responses are mounted and are distributed throughout the body to provide ample protection (Parkin and Cohen, 2001).

1.1.1 Haematopoiesis

Blood cells are generally distinguished into two separate lineages: lymphoid and myeloid. As shown in Fig. 1.1, the lymphoid lineage consists of T, B, natural killer (NK) cells and innate lymphoid cells (ILCs) (Serafini, et al., 2014). The myeloid lineage includes granulocytes (neutrophils, eosinophils, basophils and mast cells) in addition to monocytes, macrophages, erythrocytes and megakaryocytes (Iwasaki and Akashi, 2007). Dendritic cells (DCs) are unique as they can derive both from the myeloid or the lymphoid lineage (Manz et al., 2001). Lymphoid and myeloid cells are generated through the process of haematopoiesis. Haematopoiesis consists of a cascade of finely regulated events leading to differentiation of totipotent stem cells into all cells present in the blood. Firstly, stem cells lose their self-renewing capability and differentiate into cells with limited differentiation potential. Secondly, those cells committed to a specific lineage undergo terminal differentiation (i.e. maturation). Haematopoiesis can be divided in two phases: a primitive phase, which takes place in the yolk sac (YS) and is restricted to myeloid development, and a definitive phase, which takes place in the aorta–gonads–mesonephros (AGM).
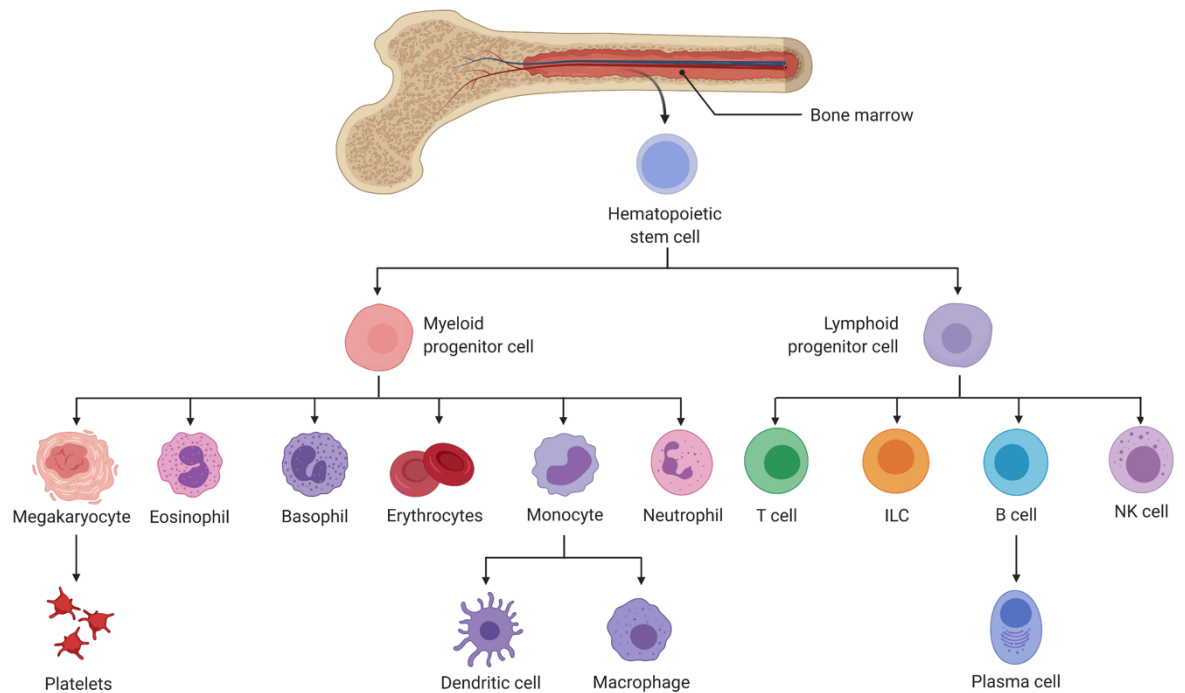
Figure 1.1: Current model for haematopoiesis (created with BioRender.com)

YS-derived progenitors spread following development of the circulation and colonize the whole embryo to give rise to foetal primitive macrophages. Concomitantly, definitive haematopoiesis results in the generation of haematopoietic stem cells (HSCs) with multi-lineage haematopoietic potential. These give rise to progenitors that colonize the foetal liver (FL) (Wynn et al., 2013). Other haematopoietic organs, such as the spleen and the bone-marrow (BM), are then colonized via the circulatory system by haematopoietic progenitors that will ultimately undergo differentiation. Coincident with the formation of bone, FL haematopoiesis declines and is replaced by BM haematopoiesis (Wynn et al., 2013).

In the BM, multipotent activity is retained by a small fraction of cells that lack expression of lineage-associated surface markers (Lin) but expresses high Sca-1 and c-Kit, hence defined LSKs (Spangrude et al., 1988). Based on their reconstituting activity, LSKs have been further subdivided into long-term haematopoietic stem cells (LT-HSCs) that are $CD34^-CD38^+$ or $Thy1.1^{lo}$, and short-term (ST)-HCSs, also known as or multipotent progenitors (MPPs), that are $CD34^+CD38^-$ or $Thy1.1^-$ (Randall et al., 1996). MPPs can give rise to common lymphoid progenitors (CLPs), which express the receptor for IL-7 (IL-7R), a cytokine essential for lymphocyte development. Indeed, CLPs generate all lymphocytes but not myeloid cells (Kondo et al., 1997). MPPs can also give rise to common myeloid progenitors (CMPs), which do not express IL-7R and generate all myeloid cell types. CMPs can be further subsetted based on expression of CD34, FcγRII and FcγRIII into megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) (Akashi et al., 2000) (Fig. 1.1).

1.1.2 Innate and adaptive immunity

Immune cells are traditionally subdivided into two arms depending on the speed and specific nature of the resulting immune response. These systems, defined as innate and adaptive, are separate but interaction between them is essential to allow body homeostasis (Janeway Jr et al., 2001).

Innate immunity includes physical, chemical, and microbiological barriers. Further action of immune system elements occurs when these barriers are not sufficient to counteract pathogenic agents or tissue injury (Chaplin, 2010). Innate immune cells include monocytes, macrophages, DCs, neutrophils, NK cells, ILCs, mast cells, basophils and eosinophils, and provide immediate host defence (Fig. 1.2). Innate immune responses involve both soluble and cell surface molecules, such as complements, cytokines and pattern recognition receptors (PRRs) that recognize a fixed set of molecular patterns associated with certain pathogens and tissue damage.
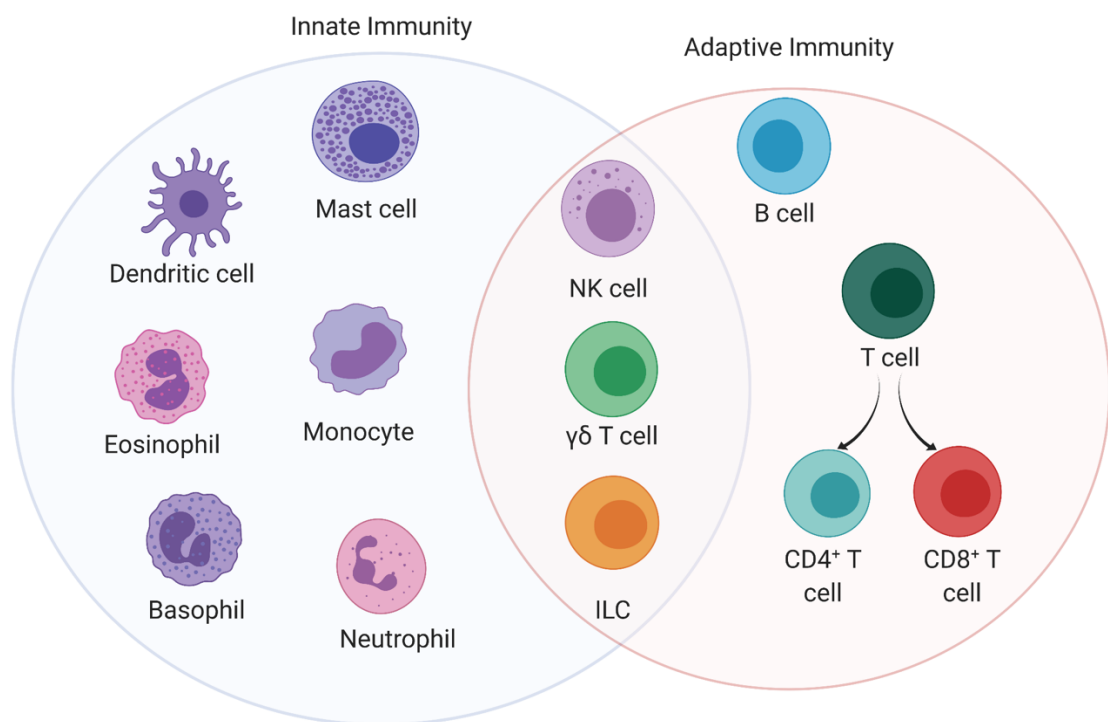
Figure 1.2: Cells of the innate and adaptive immune system (created with BioRender.com).

These are also known as pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). Since innate immune responses are not antigen specific they lack 'immunological memory', thus innate responses are not enhanced upon repeated exposure to the same antigen. Innate immunity is present even in the simplest animals, confirming its conserved nature and importance in survival (Parkin and Cohen, 2001).

Adaptive immunity is what distinguishes more advanced animals, as it involves antigen-specific reactions mediated by T and B lymphocytes (Marshall et al., 2018) (Fig. 1.2). These cells can somatically rearrange their receptor genes to confer specific recognition of antigens or epitopes. Moreover, they can also generate amplified and more rapid responses upon secondary antigen exposure, resulting in the ability to build immunological memory to specific challenges. T lymphocytes are distinguished into two main subtypes by the presence of cell surface molecules known as cluster of differentiation (CD)8^{+} and CD4^{+} and by contact with innate antigen presenting cells (APCs) through binding of T-cell receptors (TCRs) to peptide antigens loaded on molecular histocompatibility complex class I or II (MHC-I or -II) respectively (Nicholson, 2016).

CD8^{+} T-cells are very important for immune defence against intracellular pathogens, including viruses and bacteria, and for tumour surveillance (Nicholson, 2016). They induce direct cell lysis through secretion of cytotoxic granules clustered at the point of cell contact, including perforin, granzymes and granulysin and secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α), which have anti-tumour and anti-viral microbial effects (Berger, 2000).

CD4^{+} T cells further differentiate into helper T-cells (Th), with these regarded as being the most prolific cytokine producers (Romagnani, 2000). This subset can be further subdivided into Th1, Th2, Th17 and T regulatory (Treg) cells, depending on the cytokine stimulation they receive (Broere and van Eden, 2019). Th1 cells differentiate in the presence of the pro-inflammatory cytokines interleukin-12 (IL-12) and IFN-γ, upregulate expression of the transcription factor T-bet and produce pro-inflammatory factors responsible for killing intracellular parasites through further production of pro-inflammatory cytokines and consequent increased MHC-I expression and CD8^{+} T-cell activity (Zhang and Bevan, 2011). Conversely, Th2 cells differentiate in the presence of IL-4, upregulate the transcription factor GATA3 and further produce IL-4, IL-5 and IL-13, which are associated with the promotion of B-cell proliferation, antibody class switching to IgE, eosinophilic responses to extracellular pathogens and suppression of Th1 differentiation, which is mediated by IL-4 antagonistic activity on IFN-γ (Luckheeram et al., 2012). Th17 cells have been discovered more recently when IL-23 was found to induce the generation of IL-17A-producing CD4^{+} T cells (Murphy et al., 2003). These T cells have been implicated in protection from extracellular bacteria and fungal pathogens, but also in the development of autoimmune diseases in animals and humans (Wei et al., 2007). More recently, Th17-derived IL-17A has also been found to regulate Th2 immunity in the lung, by balancing protective versus excessive Th2 responses to nematode infection (Ajendra et al., 2020). Tregs are a subset of CD4^{+} T cells with potent immunosuppressive features that are crucial for maintenance of immune homeostasis. High amounts of TGF-β and the vitamin A metabolite retinoic acid (RA) favours

differentiation of these cells, which are characterised by expression of the transcription factor Forkhead box P3 (Foxp3) and the IL-2 receptor α-chain (CD25) (Plitas and Rudensky, 2016).

B cells express B-cell receptors (BCRs), which are cell surface immunoglobulins that recognise whole antigens without the need to be trimmed into peptides and presented on MHCs, through T-cell-dependent or T-cell-independent interactions (LeBien and Tedder, 2008). Following activation, B-cells differentiate into plasma cells producing antibodies, which can mediate a variety of responses, such as neutralisation of toxins and viruses, opsonisation of pathogens to promote phagocytosis, activation of the complement cascade which helps to fight pathogens, and antibody-dependent cell cytotoxicity, mediated via NK cell activation (Hoffman et al., 2016).

Although it is clear that generation of tailored adaptive immune responses is essential to protect the host, cells of the innate system are vital mediators for the initiation of such responses. Particularly granulocytes, which are the most abundant leukocytes, are the very first responders during inflammatory responses and play an essential role in host protection from infections and in wound healing (Wang, 2018).

1.2 Eosinophils

Eosinophils are primitive myeloid cells found in all vertebrate species (Klion, 2017). They typically measure 10–16 μm in diameter, show characteristic bi-lobed nuclei and can be easily recognised by staining with acidophilic dyes (McBrien and Menzies-Gow, 2017).

Eosinophils represent a minor component of circulating leukocytes, but higher proportions can be found in tissues outside the circulatory system, such as the small intestine (Weller and Spencer, 2017). Traditionally, eosinophils have been considered as effector cells mainly involved in fighting parasite infections and in allergic responses. Their defensive response has largely been associated with the secretion of potent cytotoxic granule proteins, which can be detrimental to the host by contributing to the ongoing inflammatory response and tissue damage (Ramirez et al., 2018). Despite this, increasing evidence suggests that eosinophils contribute to vital body processes, including antibody production, metabolic homeostasis, and tissue regeneration (Klion, 2017; Ramirez et al., 2018). It is now clear that eosinophils have a broader tissue distribution than previously appreciated and are more than just terminally differentiated effector cells (Weller and Spencer, 2017). As such, there is significant interest in the manipulation of eosinophils for therapeutic benefit by identification of novel biomarkers and characterisation of various phenotypes.

1.2.1 Eosinophil development

Eosinophils are terminally differentiated granulocytes that develop in the BM under the control of the key transcription factors GATA-binding factor 1 (GATA1), CCAAT-enhancer-binding proteins (C/EBP) α, PU.1, and X-box binding protein (XBP) 1 (Bochner, 2018). They derive from CD34$^+$ GMPs through intermediate eosinophil lineage-committed progenitors (EoPs) in mice, whereas human EoPs are derived from CMPs or their upstream multipotent progenitors (Fig. 1.3). EoPs differentiation depends on the appearance of the IL-5 receptor (IL-5Rα) on their surface. It consists of a specific α-chain (CD125), which is also found on basophils, and a β-chain common to the IL-5 receptor (CD131), with the latter also part of the receptors for IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Bochner, 2018; Kouro et al., 2009). EoPs are therefore identified as CD34$^+$IL-5Rα$^+$ cells also expressing low levels of c-Kit (CD117), which is a tyrosine kinase receptor for stem cell factor (SCF). c-Kit is crucial to support haematopoietis as well as cell survival and expansion (Lennartsson and Rönnstrand, 2012). Although mature mast cells and melanocytes express c-Kit, its expression is normally lost during cellular maturation. In fact, dysregulated c-Kit expression and function in mature cells leads to development of diseases such as cancer (Frumento et al., 2019). EoPs reside in small numbers primarily in the BM, and lower levels can be found in peripheral blood and in human umbilical cord blood (Fulkerson et al., 2014).
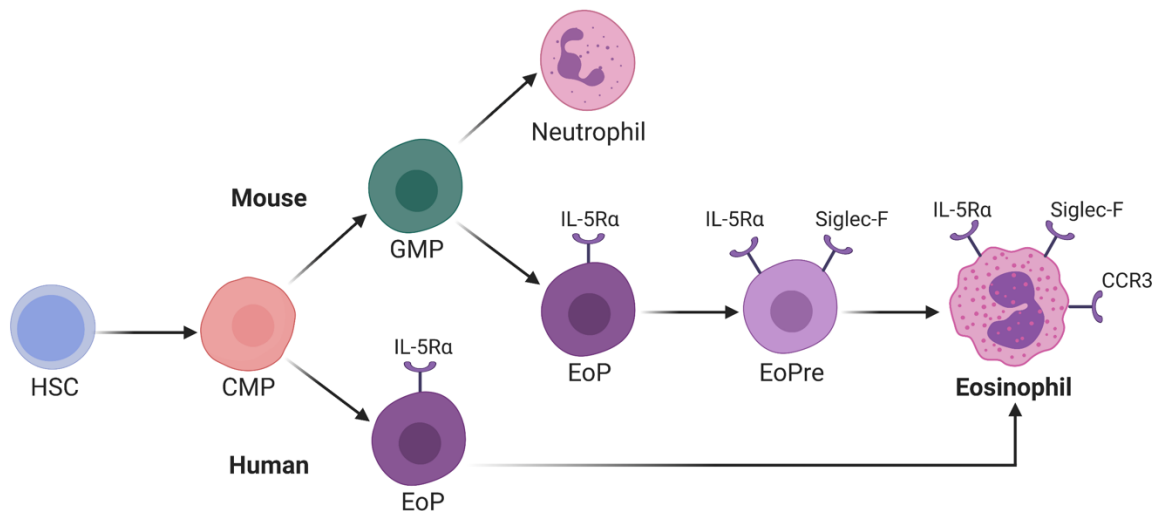
Figure 1.3: Eosinophil development in the BM in humans and mice (created with BioRender.com).

Recently, Doyle et al., 2013, have also shown that expression of the genes encoding for the granule proteins major basic protein-1 (MBP-1) and eosinophil peroxidase (EPX) is not a consequence of commitment to the eosinophil lineage but is essential for normal eosinophilopoiesis. This was demonstrated by complete loss of peripheral eosinophils and EoPs in the BM of mice deficient in both MBP-1 and EPX.

EoPs differentiate into eosinophil precursors (EoPre), which express IL-5Rα and sialic acid-binding immunoglobulin-like lectin F (Siglec-F) on their surface. EoPre further differentiate into mature eosinophils identified by surface expression of IL-5Rα, Siglec-F, and C-C chemokine receptor type 3 (CCR3) (Fulkerson et al., 2014). Although IL-3, IL-5, and GM-CSF have been shown to induce eosinophil-containing colonies from BM progenitors, only IL-5 selectively produces eosinophils (Fulkerson et al., 2014).

These findings have enabled the generation of genetically modified mice to improve the study of eosinophil biology. Transgenic (Tg) mice overexpressing IL-5 have been created to induce systemic eosinophilia (Kouro et al., 2009), whereas eosinophil deficiency has been achieved in different ways. IL-5 deficient mice have been generated by gene targeting in C57BL/6 embryonic stem cells by homologous recombination (Kopf et al., 1996). These mice do not develop blood and tissue eosinophilia following helminth infection or allergen challenge but still show basal eosinophil production in the BM. This suggested that IL-5 is essential for parasite- and allergen-induced eosinophilia but not for homeostatic generation of all eosinophils, implying that eosinophils may rely on factors other than IL-5 for their development, as explained later on in this chapter (Kopf et al., 1996; Matthaei et al., 1997). Although the transcription factor GATA1 is necessary for generation of eosinophils, as well as megakaryocytes and mast cells from immature myeloid cells, a high-affinity GATA-binding site, named double GATA-site (ΔdblGATA), within the GATA1 gene was found to be required for eosinophil development only. This discovery led to generation of eosinophil-deficient mice by deletion of the ΔdblGATA from the promoter region of the transcription factor GATA1 (Yu et al., 2002). In contrast to IL-5 deficient mice, ΔdblGATA mice show complete eosinophil ablation from the BM at steady-state and following allergen challenge, demonstrating the requirement of this site for eosinophil lineage specification (Yu et al., 2002; Humbles et al., 2004). Eosinophil-deficient mice named PHIL were also created by targeting the eosinophil-specific EPX promoter, which was used to drive expression of the cytocidal protein diphtheria toxin A chain (Lee et al., 2004). PHIL mice are specifically devoid of eosinophils and show reduced inflammatory responses in the lung following allergen exposure (Lee et al., 2004). Similar to PHIL mice, inducible PHIL (iPHIL) mice have been created by insertion of the diphtheria toxin receptor into the EPX gene locus (Jacobsen et al., 2014). Importantly, compared to PHIL mice, iPHIL mice allow 'on demand' eosinophil elimination by addition of exogenous diphtheria toxin, which has enabled assessment of the specific role of eosinophils in allergic lung inflammation without the issues associated with congenital eosinophil deficiency. Notably, eosinophil ablation during each stage of the immune response to allergen exposure showed that eosinophils are not essential contributors to the inflammatory response during sensitisation, but that eosinophil ablation during challenge led to neutrophil-mediated and steroid-resistant airway inflammation (Jacobsen et al., 2014). The EPX promoter was also targeted to generate knock-in eosinophil-targeting Cre recombinase-

expressing (EoCre) mice, by inserting an open reading frame (ORF) encoding a mammalianised Cre recombinase at the endogenous AUG start codon of the EPX gene (Doyle et al., 2013). In these mice, Cre expression was shown to be limited to eosinophil-lineage committed cells with no evidence of Cre-mediated toxicity. Importantly, this novel approach enables eosinophil-specific gene deletion or overexpression, allowing to further explore eosinophil characteristics and functions in a gene-specific manner (Doyle et al., 2013).

As mentioned previously, overexpression of IL-5 in mice results in profound eosinophilia, suggesting that IL-5 not only regulates differentiation, but also enhances EoP proliferation (Fulkerson et al., 2014). However, overexpression of IL-5Rα in GMPs does not increase the frequency of EoPs, suggesting that IL-5 does not promote eosinophil lineage commitment (Kouro et al., 2009). The discovery that IL-5 deficient mice do not show complete eosinophil depletion in the BM suggested that other mediators can promote eosinophil development in the absence of IL-5 (Fulkerson et al., 2014). In fact, the cytokine IL-33 was then reported to have important roles in eosinophil development upstream of IL-5 (Johnston et al., 2016 and 2017). IL-33 was shown to specifically expand the EoPre pool and to upregulate IL-5Rα to allow responsiveness of these precursors to IL-5, which is released by type-2 innate lymphoid cells (ILC2s) in the BM (Tsuzuki et al., 2017). Mice deficient in IL-33 or its receptor are almost completely devoid of eosinophil populations, suggesting that IL-33 is not only involved in disease pathogenesis but may promote homeostasis (Johnston et al., 2016 and 2017). Importantly, many of the transcription factors and cytokines involved in eosinophilopoiesis are also necessary for the development of basophils and mast cells. This is important when considering the development of eosinophil-targeted therapies as they may have non-selective effects (Klion, 2017).

1.2.2 Eosinophil trafficking

Under steady-state, eosinophils survive for approximately 24 hours in the circulation. Some eosinophils migrate to the liver and spleen, but they predominantly reach the gastrointestinal (GI) tract. Normally there are very few eosinophils in the upper and lower airways or in the oesophagus (Klion, 2017). Eosinophil migration is achieved by two processes: cell adhesion molecules present on the endothelium and epithelium, and chemotactic signals triggered by chemokines and chemo-attractant receptors (Simson and Foster, 2000).

Intravascular eosinophils do not adhere to the non-inflamed endothelium as it does not normally express high levels of adhesion molecules. Inflammatory stimuli, such as IL-1, TNF-α, IL-13 and IL-4 induce the expression of adhesion molecules, such as vascular adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, P- and E-selectin (Broide and Sriramarao, 2001). Eosinophils interact with adhesion molecules through their expression of L-selectin

(CD62L), P-selectin glycoprotein ligand (PSGL)-1, as well as integrins, particularly the β1 integrin very late antigen (VLA)–4 (Broide and Sriramarao, 2001).

Expression of CCR3 on mature eosinophils allows early eosinophil-specific recruitment from the BM through binding of the chemokines C-C motif chemokine 11 (CCL11), CCL24 and CCL26, also known as eotaxin 1, 2 and 3 respectively (Klion, 2017). Eotaxins are secreted in the lung by several cell types, including epithelial cells, smooth muscle cells, vascular endothelial cells, macrophages, as well as eosinophils themselves, in response to the Th2 cytokines IL-4 and IL-13 (Conroy and Williams, 2001). CCL11 is secreted at early time points and is followed by CCL24 and CCL26. CCL11 and CCL24 have been shown to promote eosinophil accumulation into tissues together with IL-5, whereas CCL26 is involved in maintaining prolonged eosinophil recruitment (Rosenberg et al., 2007). IL-5, together with IL-3 and GM-CSF, act as a chemokinetic agent that prime eosinophils to amplify their migratory responses to chemoattractants (Simson and Foster, 2000). The importance of eotaxins in eosinophil recruitment has been demonstrated by using mice deficient in CCR3 or eotaxin-1 or treated with anti-eotaxin-1 antibodies, that show reduced eosinophilia and airway hyperresponsiveness (AHR) in the lung (Humbles et al., 1997; Gonzalo et al., 1998; Campbell et al., 1998). However other studies have shown no differences in eosinophil recruitment to the lung between WT and eotaxin-1 deficient mice following allergen exposure (Yang et al., 1998; Tomkinson et al., 2001). The role of eotaxin-1 in eosinophil recruitment has been found to depend on the animal strain and species, the challenge and sensitization protocol and the phase of the response (Conroy and Williams, 2001). Moreover, eosinophil recruitment can also be eotaxin-independent as other chemokines, such as CCL5, CCL12 and CCL3, contribute to the process (Broide and Sriramarao, 2001), together with other non-chemokine mediators, as explained below.

The complement anaphylatoxins C3a and C5a can also enhance eosinophil chemotaxis. These are small cationic activation peptides released from components C3 and C5 by the action of C3/5 convertases. C5a can induce chemotaxis of both eosinophils and neutrophils, whereas C3a is eosinophil specific. Engagement of several eosinophil-expressed integrins is shown to be required for complement-induced eosinophil adhesion on the endothelium (DiScipio et al., 1999).

Chemotaxis of eosinophils, as well as neutrophils, macrophages and mast cells, can be promoted by other mediators abundantly released during inflammatory responses, such as the lipids platelet activating factor (PAF), leukotriene B_4 (LTB_4), prostaglandin-D_2 (PGD_2) (Gomes et al., 2013) and the neurotransmitter 5-hydroxytryptamine (5-HT) also known as serotonin. Although 5-HT is mostly present in the central nervous system, it is known to have multiple functions in the periphery. One of these includes eosinophil chemotaxis, which is dependent on

the 5-HT$_{2A}$ receptor by increasing intracellular Ca^{2+} levels, thereby promoting actin polymerization and cytoskeletal changes (Kang et al., 2013).

1.2.3 Eosinophil functions

In 1879, the name eosinophil was firstly coined by Paul Ehrlich, who discovered that these cells contained granules that heavily stained with the acidic dye eosin (Gleich and Adolphson, 1986). Further examination of eosinophils revealed that these cells contained four different types of crystalloid granules: major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) (Hogan et al., 2008). These proteins were then purified and characterised to investigate eosinophil associated diseases (Slifman et al., 1986; Peterson et al., 1988).

Peripheral blood eosinophilia with concomitant release of cytotoxic granule proteins was firstly identified in helminth infections. These parasites are large multicellular organisms that require several phases to fully develop during infection (Klion and Nutman, 2004). Helminth infection is typically associated with the induction of Th2 responses, characterised by increased levels of IL-13, IL-4 and immunoglobulin (Ig)E (Allen and Sutherland, 2014). However, each developmental stage of helminths can be antigenically diverse, thereby inducing distinct immune responses that occur at different times and vary in a tissue-dependent manner (Maizels and Yazdanbakhsh, 2003).

In vitro studies firstly revealed that eosinophils provided protection during such infections by killing parasitic larvae through release of EPX and reactive oxygen species (ROS) (Buys et al., 1981; Capron et al., 1979). The identification of IL-5 as an essential factor for eosinophil development further enabled the evaluation of eosinophil functions *in vivo.* Eosinophilia and high levels of circulating IgE were found to depend on IL-4 and IL-5 produced by Th2 cells. IL-5 especially was determined to be sufficient for the development of eosinophilia and eosinophil activation, as shown by injection of neutralizing anti-IL-5 monoclonal antibodies (Coffman et al., 1989), whereas IL-4 and IL-13 induced expression and release of eotaxins by fibroblasts, resulting in tissue recruitment of eosinophils (Mochizuki et al., 1998; Gurish et al., 2002). Activated eosinophils from the blood of helminth-infected patients and mice showed upregulation of surface receptors, decreased density, increased survival and release of cytotoxic mediators (Mawhorter et al., 1996; Sugaya et al., 2001). The transcription factor GATA3, which regulates expression of IL-5, was also found to directly control helminth-induced eosinophilia and IgE levels, as demonstrated by GATA3 transgenic mice (Watanabe et al., 2003).

The availability of transgenic and eosinophil-deficient mice led to further understanding of the role of eosinophils in helminth infection. Despite their role in protection, eosinophils can also have detrimental effects on the host during helminth infection by contributing to tissue damage or by promoting parasite survival (Cadman et al., 2014). For example, mice infected with *Brugia malayi, Toxocara canis* or *Ascaris suum* develop AHR in an IL-5 dependent manner along with increased levels of eosinophils and IgE (Hall et al., 1998; Pinelli et al., 2005; Enobe et al., 2006). Although the release of eosinophil granule proteins has been correlated with parasite killing, these proteins are also toxic to tissues and cells both *in vitro* and *in vivo* (Gleich et al., 1979; Ackerman et al., 1990; Shin et al., 2009). Other mediators released by activated eosinophils, such as leukotrienes, PAF and ROS may also contribute to the ongoing inflammatory response and tissue damage (Klion and Nutman, 2004).

It is now known that eosinophil-mediated host protection depends upon the species of infecting helminth. For example, systemic eosinophilia present in IL-5 Tg mice enhanced resistance during the early stages of primary infection with *Nippostrongylus brasiliensis,* but did not promote clearance of *T. canis* larvae (Dent et al., 1999). Eosinophils mediated their protective effect by trapping *N. brasiliensis* larvae in the skin during the first few hours of primary infection. In contrast, *T. canis*, which remains in the host for longer periods of time, is thought to have evolved strategies to eliminate susceptibility to eosinophils (Dent et al., 1999). The results obtained with IL-5 Tg mice were confirmed with the use of eosinophil deficient mice, showing that resistance to *N. brasiliensis* was impaired in either IL-5 deficient or ΔdblGATA mice in the intestinal phase of primary infection and in the early pre-lung phase of secondary infection (Knott et al., 2007). Other studies demonstrated that infection with *Schistosoma mansoni* or *Trichinella spiralis* increased worm burdens in IL-5 Tg mice compared to WT controls (Dent et al., 1997; Behm and Ovington, 2000). Gebreselassie et al. (2012) showed that, in mice infected with *T. spiralis*, eosinophils can dampen macrophage-induced pro-inflammatory responses whilst promoting Th2 polarisation, which resulted in reduced parasite death. In fact, low levels of Th2 responses, that are not sufficient to trigger effector mechanisms but downregulate Th1 inflammation, can enhance parasite survival during long-term infections (Maizels et al., 2004). Enhanced protection against the parasite *Entamoeba histolytica,* which causes diarrhoea in poor countries with low hygiene standards, was found to depend on IL-4 produced by eosinophils recruited to the intestine in an IL-25-dependent manner (Noor et al., 2017). Eosinophils can also be exploited by filarial parasites as a cue to alter their reproduction in response to IL-5 and IL-4, for it to be maximised before Th2 responses can clear the infection (Babayan et al., 2010). These studies importantly demonstrated that eosinophils can either mediate host protection or aid parasite survival in a parasite-dependent manner.

Several helminth and hookworm parasites migrate to the lung during their life-cycle and this is associated with significant airway eosinophilia and generation of Th2 responses (Culley et al., 2002; Cheepsattayakorn, 2014). Eosinophil recruitment to the airways of mice infected with *Necator americanus* was shown to occur as a result of parasitic larvae remaining trapped in the pulmonary vasculature after entering the circulatory system, which induced the release of eosinophil chemoattractants and IL-5 (Culley et al., 2002). In the 1980's, Th2 allergic inflammation of the airways was also found to involve blood and lung eosinophilia (De Monchy et al., 1985; Durham et al., 1989) and eosinophils have since been implicated in the pathogenesis of allergic disease (Kay et al., 1997). Considering the similarity in the nature of the inflammatory response triggered by parasites and allergens in the airways, several studies aimed to investigate the relationship between helminth infections and allergies (Cooper, 2009). This led to the discovery that some but not all parasites may enhance Th2 inflammation during allergic responses (Fallon and Mangan, 2007 and references therein). For instance, infection with *Ascaris lumbricoides*, has been associated with increased incidence of allergic reactions in humans and primates (Sugane, 1988; Dold et al., 1998; Richards et al., 1983). Mouse models also showed that infection with *Ascaris* triggers airway eosinophilia, IgE production and Th2 lung pathology (Enobe et al., 2006; Weatherhead et al., 2018), and that pre-sensitization with *Ascaris* antigens accelerates IgE release upon HDM inhalation (Suzuki et al., 2016). Conversely, other studies showed that helminths may dampen allergic responses by promoting a regulatory environment, characterised by high levels of IL-10, TGF-β and IL-10 producing Tregs or B cells that counteract Th2 responses, therefore protecting against asthma (Stock et al., 2006; Doetze et al., 2000; van den Biggelaar et al., 2000; Wilson et al., 2005; Mangan et al., 2006). Despite the regulatory responses that helminths can induce in the host, these may not be sufficient to counteract allergic Th2 responses, suggesting that a tight balance between pro- and anti-allergenic responses is required to maintain homeostasis (Santiago and Nutman, 2016). In fact, a study analysing the effect of pre-existing asthma on helminth infection showed that HDM-induced inflammation induced a strong Th2 response in the lung involving eosinophilia, M2 macrophage polarisation and mucus hyperproduction, which impaired *Ascaris* migration and development, resulting in reduced parasite burden. Importantly, this effect was dependent on eosinophils, since parasite development was not altered in eosinophil deficient mice (Gazzinelli-Guimaraes et al., 2019).

1.3 Immunity in the lung

The lungs are the site of gas exchange in the body, which is essential for survival and health, but also results in the constant exposure to inhaled particulates, including pathogens. Moreover, only recently the lungs have been found to contain their own microbiota (Moffatt and Cookson, 2017). Rapid and effective immune responses are therefore required to protect the host without initiating aberrant inflammatory responses against harmless components (Hasenberg et al.,

2013). Innate immune cells are the main providers of local protection within the lung, but if dysregulated, their responses can be detrimental to the host and cause disease, such as asthma (Hartl et al., 2018).

Macrophages predominantly provide protection under steady state conditions in the lung, but other cell types including DCs, NK cells and ILCs may also initiate defence mechanisms. Macrophages in the lung can be broadly divided into alveolar macrophages (AMs) and interstitial macrophages (IMs) (Ardain et al., 2019). AMs are the main phagocytic and antigen-presenting cell in the respiratory tract and are also the most abundant cellular population within bronchoalveolar lavage (BAL) fluids (Hartl et al., 2018). They are located closely to alveolar epithelial cells and can be easily distinguished from IMs due to their unusual expression of specific markers: they express low levels of CD11b but elevated CD11c, normally expressed by DCs, as well as Siglec-F, usually expressed by eosinophils (Hussel and Bell, 2014). Compared to IMs, AMs are self-renewing long-lived resident cells that do not require replenishment from the BM (Guilliams et al., 2013). Phagocytosis of dead cells and foreign particles by AMs plays an essential role in the defence against invading pathogens and in the resolution of inflammation (Nagre et al., 2019). AMs express a variety of PAMP receptors that allow them to detect microbial- and host-derived signals leading to the release of pro- or anti-inflammatory cytokines, including IL-1-β, IL-6, IL-10, or TNF-α. They can also produce TGF-β, which induces differentiation of CD4$^+$ T cells into Tregs with associated anti-inflammatory responses (Coleman et al., 2013). However, AMs may lose the ability to induce immune tolerance when they encounter a mixture of harmless antigens and allergens able to trigger toll-like receptor 4 (TLR4)-mediated responses (Mathie et al., 2015).

Whilst there are low levels of leukocytes present at steady state, their levels rise dramatically under acute or chronic inflammatory conditions. Eosinophils, and to a lesser extent neutrophils, accumulate in the airways and play a major role in contributing to inflammatory responses (Goyal et al., 2019).

1.3.1 The immune response in allergic asthma

Allergic asthma is one of the most common chronic inflammatory diseases in the world. Although asthma-related mortality has declined by ~18% over the past 20 years, the number of diagnoses has increased by 20-30% (Mattiuzzi and Lippi, 2020).

Allergic asthma affects the airways and involves aberrant Th2 inflammatory responses and AHR to innocuous inhaled antigens. Clinically, asthma presents itself in attacks or episodes, although the inflammation underlying asthma is chronic in most cases. These episodes are the result of airway narrowing, which is caused either by swelling, mucus plugging or bronchoconstriction.

This leads to development of symptoms including wheezing, breathlessness, chest tightness and cough (Finn and Bigby, 2009). Many asthma episodes resolve following minor treatment, whereas others require hospitalisation and may lead to death (Mims, 2015).

Exposure to inhaled allergens is not the only factor responsible for the development of allergic asthma; a combination of genetic predisposition and environmental factors, including birth mode, birth order, antibiotic intake and diet, may promote susceptibility to the disease (van Tilburg Bernardes and Arrieta, 2017).

It has long been recognised that asthma-associated traits can be inherited and several regions of the chromosome with genes that are highly replicated during asthma have been established (Subbarao et al., 2009). Most of these genes have been identified through analysis of gene variants, such as single nucleotide polymorphisms (SNPs), in the main signalling pathways that influence allergic inflammation and asthma (Vercelli, 2008). For example, the genes encoding for IL-4, IL-13, CD14, the β2 adrenergic receptor (ADRB2) and serine peptidase inhibitor Kazal type 5 (SPINK5) are some of the most replicated in allergic inflammation (Ober and Hoffjan, 2006). SNPs of genes associated with eosinophils, such as IL-5, IL-5R and GM-CSF have also been shown, although not as prominent as in other Th2 genes (Kabesch et al., 2007). Although the role of epigenetics in the pathogenesis of asthma is still not fully understood, there is evidence for changes in methylation to be associated with eosinophilia and asthma susceptibility genes, such as IL-5, EPX, IL-13 and IL-4 (Boorgula et al., 2019).

The last few decades have seen a dramatic increase in the global prevalence of asthma and allergies, especially in industrialised countries (Brooks et al., 2013). This is correlated with improved hygiene, medical treatments and vaccinations, resulting in the decreased prevalence of infectious diseases. In contrast, fewer cases of asthma or other atopic diseases are encountered in poorer countries where unhygienic conditions and overcrowding prevail (Brooks et al., 2013). The concept by which increased cleanliness is one of the factors that has caused a rapid rise in allergic disorders has been defined as the "hygiene hypothesis" (Weber et al., 2015). This has also been correlated with alterations of the infant gut microbiome, now considered as one of the main factors driving asthma pathogenesis (Liu, 2015). The fact that the environment has powerful effects on the onset and prevalence of asthma has also been evidenced by migration studies. People migrating from countries with low incidence of asthma to countries with high incidence were shown to acquire the higher rates of the disease in a time and age-dependent manner (Cabieses et al, 2014). Although the incidence and prevalence of asthma are indeed higher in children, hospitalisation and mortality are higher in adults. Moreover, incidence and prevalence of asthma differs by sex across the lifespan. Before puberty, male individuals are more affected by asthma, whereas after puberty females show a

higher burden of the disease, suggesting that hormonal changes play a role in the pathogenesis of asthma (Dharmage et al., 2019).

The main features of the inflammatory response include significant infiltration of the bronchial mucosa by lymphocytes and granulocytes, goblet cell hyperplasia and increased serum IgE levels (Nathan et al., 2009).

Although Th2 cells are recognised to have key roles in the initiation of the inflammatory response, it has become evident that a crosstalk between the innate and adaptive immune system plays a critical role in the initiation and development of Th2-biased responses (Nathan et al., 2009). In fact, allergic asthma has been determined to be a heterogeneous disease with several distinct pathologic phenotypes. Clinically, these are distinguished according to symptoms, concomitant comorbidities and identifiable triggers, as well as identification of whether the inflammatory infiltrate is eosinophilic or neutrophilic (Borish, 2016; Brussino et al., 2018). More recently asthma has also been classified into high or low Th2 endotypes with the aim of defining the specific biological mechanism underlying a given phenotype. The identification of different asthma endotypes has been useful for the development of more tailored therapies aimed at targeting specific inflammatory mediators (Brussino et al., 2018).

Although eosinophilic asthma is more prevalent, there is evidence for neutrophils being the predominant cellular infiltrate in cases of severe and persistent asthma, asthma exacerbations and corticosteroid-resistant asthma (Wang et al., 2016). The mechanisms underlying neutrophilic asthma are still unclear, but there is evidence for increased levels of IL-8 in the airways, which is a chemotactic factor for neutrophils, and that IL-17A produced by Th17 cells may be responsible for upregulation of IL-8 (Nakagome et al., 2012). Increased levels of other pro-inflammatory cytokines, including IL-1β, IL-6, IL-12 and TNF-α, in addition to changes in the lung microbiota, have been associated with neutrophilic asthma compared to non-neutrophilic asthma (Yang et al., 2018). Neutrophilic asthma has also been correlated with 'occupational' asthma, since exposure to bacterial endotoxins, particulate air pollution and ozone have been shown to induce non-allergic neutrophilic airway inflammation. This suggests a common mechanism in the generation of chemokine release and neutrophil influx both in occupational and non-occupational asthma, involving activation of innate immune responses in the general population (Douwes et al., 2002).
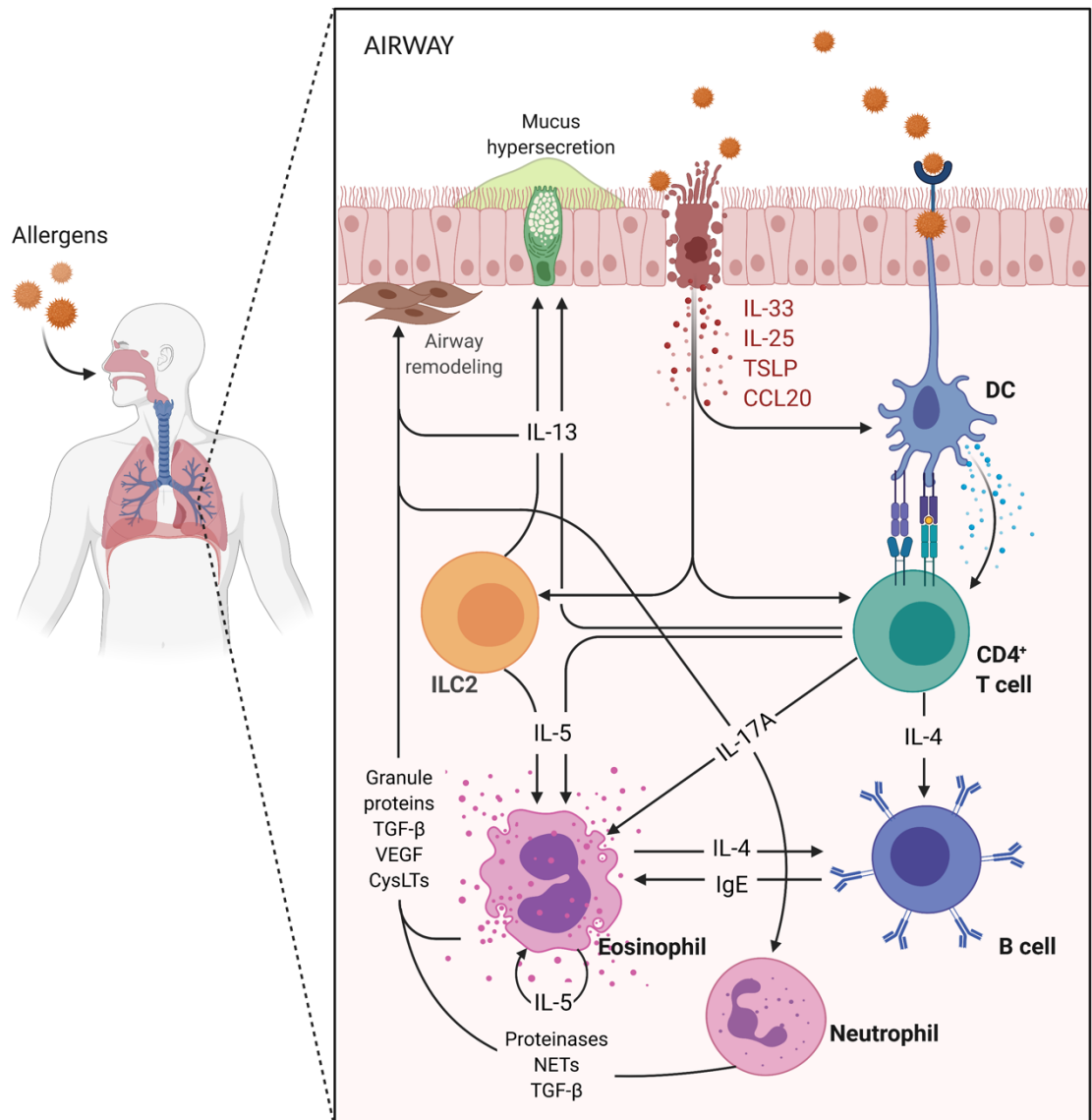
Figure 1.4: The immune response in allergic asthma (created with Biorender.com).

As shown in Fig. 1.4, the first stages of the allergic response typically involve allergens directly stimulating airway epithelial cells to secrete cytokines and chemokines, such as IL-33, IL-25, thymic stromal lymphopoietin (TSLP) and CCL20. IL-33 is constitutively expressed by endothelial cells and epithelial cells of barrier sites, such as the lung, intestine and skin. IL-33 is a pro-inflammatory cytokine released upon cell death and its expression is tightly regulated at the level of transcription, translation, and receptor signalling in order to avoid detrimental effects. The IL-33 receptor consists of the suppression of tumorigenicity 2 (ST2) protein and the IL-1 receptor accessory protein (IL-1RAcP), which is expressed on several adaptive and innate cells in the lung, such as eosinophils, basophils, mast cells, macrophages, ILC2s, Th2 cells and Tregs (Johansson and McSorley, 2019). IL-33 expression and signalling is highly upregulated during allergic responses in the airways and induces ILC2 proliferation and production of IL-4, IL-5 and IL-13, M2 macrophage polarisation, and mast cell, eosinophil and basophil activation

(Amin et al., 2016). Together with IL-33, TSLP and CCL20 bind to TLSPR and CCR6 respectively expressed on immature DCs, which undergo maturation and activation (Liu et al., 2018). Release of the chemokines CCL17 and CCL22 by DCs promotes ILC2 recruitment to the airways and coordinates their co-localisation with naïve CD4$^+$ T cells in the local lymph nodes where DCs act as APCs. TSLP promotes expression of the co-stimulatory molecules CD40, CD80 and CD86 on DCs, which induce CD4$^+$ T cell differentiation and activation into antigen-specific Th2 cells. These cells migrate back to the lung mucosal tissue, where they release IL-4, IL-5 and IL-13 (Grinnan et al., 2006). Production of IL-13 and IL-5 can also be triggered by IL-33 binding to ST2 expressed on differentiated Th2 cells (Chen et al., 2017). These cytokines play key roles in the onset and amplification of allergic inflammation in asthma: IL-4 promotes the production of IgE by B cells, which in turn further promote degranulation of eosinophils, mast cells and basophils; IL-5 triggers eosinophil differentiation, recruitment and activation; IL-13 acts on many cell types and promotes mucus hyper-production and tissue remodelling (Elias et al., 2005).

In addition to Th2 effector cells, Th17 and Tregs are involved in the pathogenesis of asthma (Song et al., 2008). Th17 cells mainly produce IL-17A, which induces the recruitment of both neutrophils and eosinophils into the airways (Zhang et al., 2017), as well as tissue remodelling by promoting fibroblast proliferation (Bellini et al., 2012). On the other hand, Tregs have an essential role in promoting tolerance to innocuous environmental antigens, such as allergens, by controlling Th2 immune responses to prevent the development of allergic disorders (Rivas and Chatila, 2016). However, there is evidence for disrupted Treg activity in asthma (Lambrecht and Hammad, 2015). For example, ST2-expressing Tregs may lose their suppressive properties and upregulate expression of Th2 cytokines and GATA3 (Chen et al., 2017).

1.3.2 Animal models of allergic asthma

Animal models have been widely used to study the pathophysiology of allergic airway inflammation seen in humans. Mice, like the majority animals, do not naturally develop periodic lung inflammatory responses similar to asthma, therefore murine models need to be firstly exposed or 'sensitised' to an allergen and subsequently re-exposed or 'challenged' to the same allergen to trigger an allergic response (Chapman et al., 2014).

Ovalbumin (OVA) administration has long represented the standard protocol to induce murine models of allergic airway inflammation. Although this model reproduces many characteristics of the inflammatory responses seen in asthma, it does not appropriately mimic the human condition. Firstly, humans are generally not allergic to chicken egg protein (OVA) unless caused by a food allergy. Secondly, OVA is systemically administered to animals (intraperitoneally)

together with the Th2 skewing adjuvant aluminium hydroxide, whereas sensitisation to allergens in humans occurs via the airways. Finally, prolonged exposure to OVA may lead to tolerance in mice, which does not allow reproducibility of chronic disease (Chapman et al., 2014).

For these reasons, murine models of allergic airways disease have more recently switched from the use of OVA to inhaled house dust mite (HDM) extract (Gregory and Lloyd, 2011; Buday and Plevkova, 2014). HDM is one of the most prevalent aeroallergens worldwide, with up to 85% of asthmatics showing allergic reactions to HDM and affecting people of all ages (Buday and Plevkova, 2014). HDM extract contains many allergenic components, including faecal matter, lipopolysaccharide (LPS), chitin and proteolytic enzymes, all of which are directly relevant to human allergic responses (Nathan et al., 2009). A major advantage of the HDM model is its topical administration (intranasal), which allows allergic disease to rely on local mucosal sensitization within the lungs, as it occurs in humans. Moreover, HDM-challenged mice do not normally show induction of tolerance even over prolonged exposures (Buday and Plevkova, 2014).

For decades, fungi have been associated with the pathogenesis of asthma. Among the fungi, *Aspergillus fumigatus* is the most prevalent cause for allergic asthma in atopic people and has been used to generate mouse models of allergic lung inflammation. *A. fumigatus* is a source of 23 listed allergens that have been shown to promote a Th2 response in the murine lung associated with recruitment of both eosinophils and neutrophils, tissue damage and mucus hyperproduction, without the need of sensitisation or adjuvants (Pandey et al., 2013; Chaudhary and Marr, 2011).

To assess airway function in mice models of allergic lung inflammation both non-invasive and invasive analysis methods have been used. Whole-body plethysmography is a non-invasive method that measures airway function with pause (Penh), with enhanced pause being correlated to increased bronchoconstriction. Although this method is simple and does not require anaesthesia or surgery, pause is argued to be a measure of breathing rather than an indicator of mechanical lung function. Moreover, with this method animals experience stress, which may affect physiological measurements (Verheijden et al., 2014). Invasive lung resistance measurement (R_L) is a more reliable method to measure lung function. Administration of bronchoconstrictor agents through a cannula inserted in the trachea, such as methacholine (MCh), is often performed with this method. Increased sensitivity to MCh reflects the presence of AHR in the airways, the degree of which was found to depend on the number of allergen challenges mice are exposed to (Zosky et al., 2004). Nevertheless, this method requires anaesthesia, surgery and artificial ventilation, procedures that can all affect physiological parameters (Verheijden et al., 2014).

Histopathological analysis of paraffin-embedded lung sections is also routinely used to assess the level of airway inflammation. The histopathology of asthma is characterized by several structural changes, including inflammatory cell infiltrate, epithelial detachment, goblet cell hyperplasia, fibrosis, bronchial smooth muscle hypertrophy and thickening of the airway wall. These changes are observed in both central and peripheral airways (Hamid, 2003; Saetta and Turato, 2001). The most common staining techniques used to identify such changes are Haematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) together with Alcian-Blue. In H&E, Hematoxylin is used to stain cellular nuclei in blue, whereas Eosin is a pink dye used as a counterstain to distinguish between the cytoplasm and nuclei of cells (Zhou and Moore, 2017). PAS and Alcian-Blue identify goblet cells by staining mucins containing neutral and acidic glycoproteins respectively (McManus, 1948; Yamabayashi, 1987).

1.3.3 Eosinophils in allergic asthma

It is accepted that high levels of eosinophils in the airways, blood and BM are a characteristic feature of allergic asthma and that this correlates with severity of disease (Fahy, 2009).
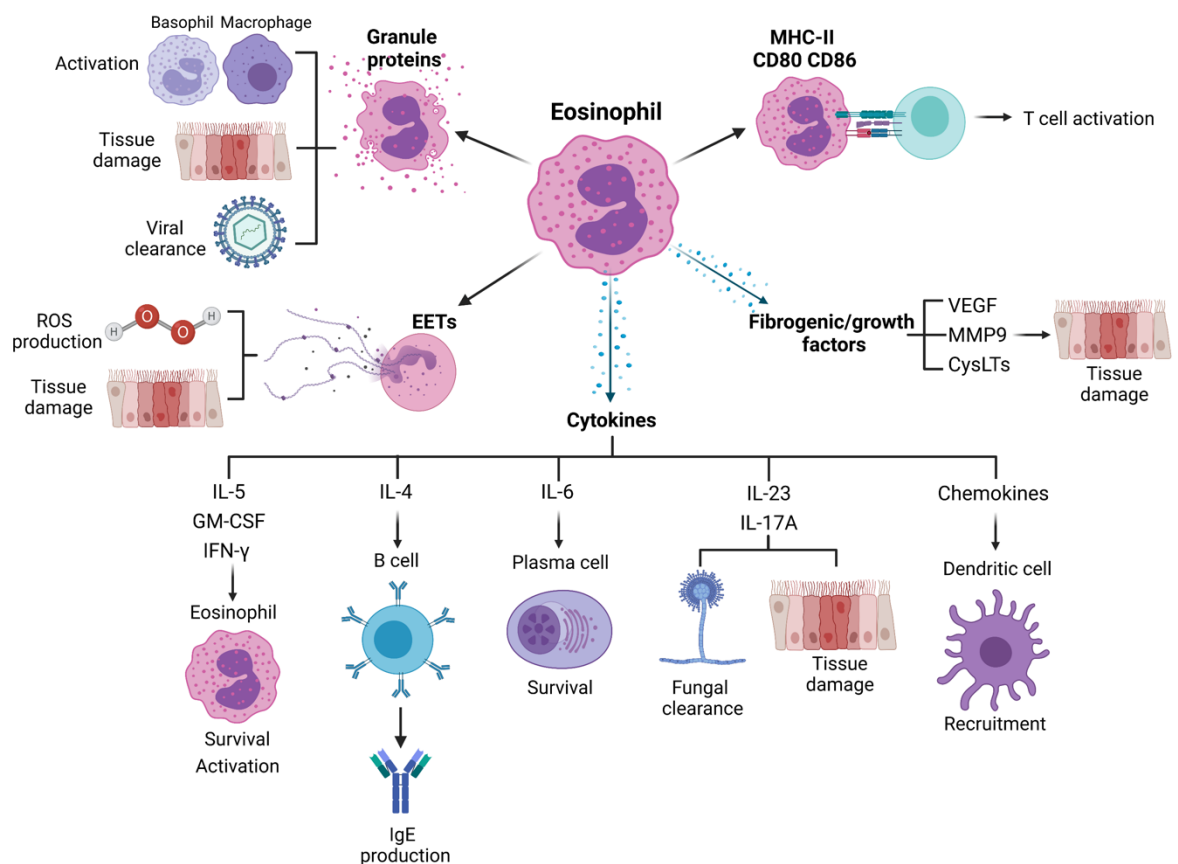
Figure 1.5: Eosinophils in allergic asthma (created with BioRender.com)

Degranulation of eosinophils is one of the most pathogenic functions of these cells in allergic asthma (Fig. 1.5). As mentioned previously (section 1.2.3), eosinophils contain four types of crystalloid granule proteins, MBP, EPX, ECP and EDN. MBP is toxic to the airway epithelium, induces airway smooth muscle contraction, and stimulates mast cell and basophil degranulation (Frigas et al., 1980), as well as IL-8 secretion by neutrophils (Page et al., 1999), which induces recruitment and activation of both neutrophils and eosinophils (Ulfman et al., 2001; Norzila et al., 2000). EPX promotes ROS production and enhances macrophage phagocytosis (Lefkowitz et al., 1997), whereas ECP is bactericidal and induces fibrosis by acting on fibroblasts (Amin et al., 2016). EDN, whose expression is also detected on mononuclear cells, promotes DC activation (McBrien and Menzies-Gow, 2017) but also has antiviral activity (Rosenberg and Domachowske, 2001). In addition to crystalloid granules, eosinophils have been found to contain other granules, named primary, small and secretory vesicles (Hogan et al., 2008). The primary granules appear during the promyelocytic stage of eosinophil development and are enriched in Charcot-Leyden crystal (CLC) protein. Small secretory vesicles and small granules contain pre-formed cytokines, chemokine and growth factors in humans, however it is still not fully clear whether all cytokines are pre-formed in mice (Weller and Spencer, 2017).

Granule proteins are mainly released via piecemeal degranulation or cytolysis. Piecemeal degranulation involves the release of vesicles from granules within the cytoplasm, which reach the plasma membrane where they release their content into the extracellular space. Cytolysis, or cell lysis, occurs when the plasma membrane is ruptured and eosinophils release all of their contents, including intact granules, into the extracellular space (McBrien and Menzies-Gow, 2017).

Similarly to neutrophils, eosinophils have also been found to secrete extracellular traps, named eosinophil-derived extracellular traps (EETs) (Yousefi et al., 2008) (Fig. 1.5). These consist of mitochondrial DNA released in less than one second, which has been associated with protection against bacteria but also with the development of pathologies, including allergic diseases (Hwang et al., 2019). Eosinophil influx in the airway has been correlated with production of EETs that stain positive for DNA and MBP, and EET[+] eosinophils have been negatively correlated with lung function (Mukherjee et al., 2018). EETs contribute to eosinophil degranulation and ROS production in an autocrine fashion and have been associated with epithelial detachment, epithelial cell activation and pro-inflammatory cytokine secretion (Choi et al., 2018).

Eosinophils have been shown to secrete both Th1 and Th2 cytokines (Fig. 1.5). Although Th2 cells and ILC2s are the main IL-5-producing cells, eosinophils can also release IL-5 and GM-CSF, which promote their own survival in an autocrine fashion (Wen and Rothenberg, 2017). Eosinophil activation is also correlated with enhanced glycolysis as well as mitochondrial

respiration, both triggered by IL-5 and GM-CSF (Jones et al., 2019). Eosinophils can also release IL-4 (Chen et al., 2004), which simulates the proliferation of B cells and IgE production, and IL-6, which enhances survival of plasma cells (McBrien and Menzies-Gow, 2017).

Eosinophils also enhance airway remodelling and fibrosis (Wen and Rothenberg, 2017). Eosinophils are indeed a source of several fibrogenic and growth factors, including TGF-β, vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-9 (Hogan et al., 2008) and cysteinyl leukotrienes (cysLTs) (Thompson-Souza et al., 2017) (Fig. 1.5). In fact, subjects with eosinophilic asthma show increased thickness of the subepithelial basement membrane, not detected in patients with non-eosinophilic asthma (Fahy, 2009). Eosinophils also produce several chemokines, in particular the CCR1-binding C10, MIP-1γ and MIP-1α, as well as CCL11, that may altogether contribute to the recruitment of DCs and other inflammatory cells (Rose et al., 2010). The Th1 cytokine IFN-γ can also be produced by eosinophils and may contribute to lung inflammation by enhancing eosinophil-effector functions via IFN-γR1 in an autocrine manner (Kanda et al., 2009) (Fig. 1.5).

It is clear that eosinophils are key contributors to the inflammatory response in allergic asthma. However, recent studies suggest that the role of eosinophils may be more complex than previously realised (Weller and Spencer, 2017). Mesnil et al. (2016) identified two populations of eosinophils in the airways and blood of allergic mice, defined as resident (r) and inflammatory (i)Eos. (r)Eos were identified both at steady-state and following administration of HDM, whereas (i)Eos only in allergic mice. These two populations showed different phenotypes and morphology, as (r)Eos were Siglec-F^{int}IL-5R^{int}CD62L^{+} cells with a ring-shaped nucleus, whereas (i)Eos were Siglec-F^{hi}IL-5R^{int}CD101^{+} cells with a segmented nucleus. (i)Eos were also found to be localised in the peribronchial areas of the lung, whereas (r)Eos in the parenchyma. In this study, the presence of (i)Eos was dependent on IL-5 since treatment with anti-IL-5 antibodies did not affect numbers of (r)Eos but reduced (i)Eos. Microarray analysis showed that (r)Eos expressed genes associated with homeostatic responses in the lung, including *Anxa1*, *Runx3, Serpinb1a,* and *Ldlr*, that have been implicated in the negative regulation of lung Th2 responses, whereas (i)Eos expressed pro-inflammatory genes, such as *Slc3a2, Tlr4, C3ar1, Il13ra1,* and *Il6*. Functionally, (r)Eos were shown to inhibit the development of Th2 responses in the lung by suppressing expression of costimulatory molecules on allergen-loaded BM-derived DCs, therefore also T cell activation.

Eosinophils are also reported to have immunomodulatory functions in allergic lung inflammation. They have been shown to express MHC Class II and costimulatory molecules, such as CD80 and CD86, that are required to present exogenous antigens to naïve T cells and trigger their activation (Akuthota et al., 2010; Farhan et al., 2016) (Fig 1.5). Eosinophils may act as

professional APCs, as they can traffic to lymph nodes where they physically interact with T cells and bias their differentiation into Th2 cells. Eosinophils could therefore help traditional APCs in amplifying the adaptive immune response to inhaled allergens (Shi et al., 2000).

Respiratory viruses are considered as an additional causative factor in asthma exacerbations. Despite the reported increases in eosinophil levels following virus exposure, eosinophils can be directly activated through TLR-7 binding and provide antiviral activity toward single-stranded RNA viruses via release of cytotoxic granule proteins (Phipps et al., 2007) (Fig. 1.5).

Mice exposed to *A. fumigatus* also showed that eosinophils can eliminate the spores of the fungus via secretion of IL-23 and IL-17, whereas absence of eosinophils led to enhanced susceptibility to fungal infection. Eosinophils may therefore contribute to fungal clearance but at the same time their release of IL-17 and IL-23 may contribute to lung damage (Guerra et al., 2017) (Fig. 1.5).

1.3.4 Eosinophil-targeted therapies

Glucocorticoids are the most frequently used therapy for allergic asthma. They decrease eosinophilia by suppressing the transcription of the genes for IL-3, IL-4, IL-5, GM-CSF, and various chemokines, including eotaxins (Hogan et al., 2008). Although short-term treatment with glucocorticoids is safe and effective, long-term therapy causes significant side effects (Dunlap and Fulmer, 1984) such as impaired growth and suppression of the hypothalamic-pituitary-adrenal-axis (Dahl, 2006). Despite administration of high doses of glucocorticoids, some patients are also resistant to the drugs and airway eosinophilia cannot be reduced (Barnes and Adcock,1995). Glucocorticoid-resistant patients may be treated with myelosuppressive drugs (e.g. hydroxyurea or vincristine) or IFN-α, which inhibits eosinophil degranulation and effector functions (Aldebert et al.,1996). Treatment with lidocaine was also shown to be effective in reducing eosinophil survival similarly to glucocorticoids but without their cytotoxicity (Hunt et al., 2004). Drugs that inhibit eosinophil cheomotaxis and activation are in use and include inhibitors of the synthesis and receptor binding of cysLTs (Ogawa and Calhoun, 2006), and nedocromil sodium or sodium cromoglicate that inhibit eosinophil degranulation (Gonzalez and Brogden, 1987).

The identification of different asthma phenotypes has led to development of more tailored treatments, such as biologic therapies to target specific inflammatory mediators (Bussino et al., 2018). Antibodies blocking IL-13, such as Lebrikizumab and Tralokinumab, have been developed since the cytokine is a key mediator in the pathogenesis of asthma. Despite showing promise in pre-clinical trials, they were found to provide little clinical benefit in patients with refractory asthma (Blanchard et al., 2005). Alternatively, Dupilumab has been developed to

block IL-4Ra, the common receptor for IL-4 and IL-13, and has shown more efficacy than IL-13 blockade in improving symptoms. However, it is not clear whether the benefits are correlated with decreased eosinophil levels or activation (Barnes, 2017).

Omalizumab, which binds IgE, has shown more efficacy in reducing symptoms and has been approved for the treatment of severe allergic asthma. IgE binding prevents activation of FcεRI, predominantly leading to inhibition of mast cell, basophil and DC activation. Although the function of FcεRI on eosinophils is not fully understood, treatment with anti-IgE also decreases eosinophilia, eosinophil activation and promotes apoptosis without inducing cell lysis (Matucci et al., 2018).
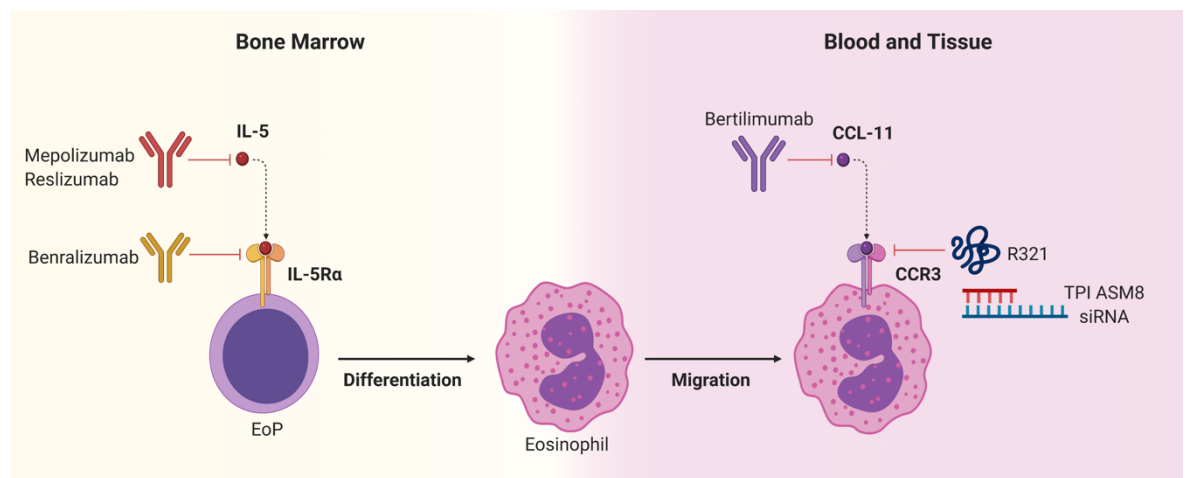
Figure 1.6: Eosinophil targeted therapies (created with BioRender.com).

The above-mentioned treatments have beneficial effects in reducing eosinophilia but are not eosinophil-specific. Considering the fundamental role of IL-5 in eosinophil differentiation and survival, two different humanised anti-IL-5 antibodies, Mepolizumab and Reslizumab, have been developed. Anti-IL-5 antibodies target eosinophils by binding to IL-5 and preventing its binding to IL-5Rα (Wechsler et al., 2012) (Fig. 1.6). However, although treatment with anti-IL-5 antibodies eliminates eosinophils, it did not lead to a significant clinical improvement in asthma patients (Borish, 2016). Moreover, treatment with anti-IL-5 antibodies increases circulating levels of IL-5, with the majority of it bound in IL-5-anti-IL-5 antibody complexes (Matucci et al., 2018). These immune complexes can act as a storage for IL-5 potentially increasing the half-life of the cytokine, which may potentiate and not neutralise its target activity (Roufosse et al., 2018).

To alternatively compromise IL-5 signalling, the humanised antibody Benralizumab has been developed to target IL-5Rα, resulting in inhibition of IL-5-mediated receptor activation (Fig. 1.6). Benralizumab is produced in Chinese hamster ovary cells deficient in the enzyme α 1,6 fucosyltransferase (FUT8), resulting in no fucosylation of the antibody. This enhances the

binding of Benralizumab to human FcγRIIIa, leading to enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), therefore eosinophil apoptosis. Targeting IL-5Rα over IL-5 also enables depletion of eosinophils with lower expression of the receptor, therefore eosinophils that no longer rely on IL-5 as a survival factor (Wechsler et al., 2012). Despite reductions in blood and BM eosinophils, treatment with Benralizumab was not found to affect bronchial eosinophilia or ECP levels in the sputum of asthmatic patients (Brussino et al., 2018). These data suggest that targeting one single cytokine may limit therapy efficacy due to redundant pathways involved in supporting eosinophil survival and activation (Matucci et al., 2018). At present, the candidates receiving the most benefit from anti-IL-5 therapy are subjects with severe eosinophilic asthma and a history of frequent exacerbations, but more studies are necessary to fully confirm this profile (Garcia et al., 2013).

Strategies aimed at blocking eosinophil chemotaxis by targeting the CCR3/eotaxin axis are also being investigated (Fig. 1.6). Numerous inhibitors of the eotaxin/CCR3 pathway, including small-molecule inhibitors of CCR3 and a human anti-eotaxin-1 antibody (Bertilimumab), are being developed and have showed some promising effects (Rosenwasser et al., 2003). CCR3 is also targeted by TPI ASM8, an antisense oligonucleotide designed to downregulate mRNA expression and gene transcription of CCR3, which has been shown to reduce airway eosinophilia and symptoms in mild asthmatics (Wechsler et al., 2012). Similarly, small interfering (si)RNA molecules that prevent gene transcription of IL-5 or eotaxin are also under investigation (Garcia et al., 2013). Recently, the peptide-based CCR3 antagonist R321 has shown more promising results. The compound does not inhibit receptor endocytosis suggesting that eosinophils do not become desensitised to R321 as it does not remain bound on the cell membrane (Pease and Williams, 2019).

1.4 Homeostatic functions of eosinophils

Recent studies investigating the role of eosinophils have demonstrated that the activities of these granulocytes are not just destructive and correlated with disease progression, but they can also be beneficial and promote maintenance of tissue homeostasis (Jacobsen et al., 2012). This has led to the hypothesis that eosinophils do not accumulate in tissues simply as a result of disrupted immunity, but they may be attracted to any tissue with high cell turnover and the necessary factors for their survival, in either health (e.g. GI mucosa and BM) or disease (e.g. tumours and inflamed lung). The functions of recruited eosinophils are partly determined by the tissue where they accumulate. However, independently of this, eosinophil-released bioproducts generally promote tissue remodelling and repair. The latter processes are suggested not to change in nature between health and disease, but in magnitude and extent. As a result, eosinophils may contribute to maintaining tissue homeostasis, but in certain circumstances their activities may become predominant to local immune responses. These observations suggest

that eosinophils may contribute to homeostasis with variety of functions that still need to be fully explored (Lee et al., 2010).

Bone Marrow	Spleen	Adipose tissue	Liver	Gut	Thymus
APRIL IL-6 → Plasma cell → Antibody production	IL-4 → B cell → IgM production	IL-4 → M2 macrophage → Beige fat	IL-4 → Fibro/adipocyte progenitor → Fibroblast → Tissue regeneration	TGF-β IL-1β → Plasma cell → IgA production	Apoptotic T-cell phagocytosis
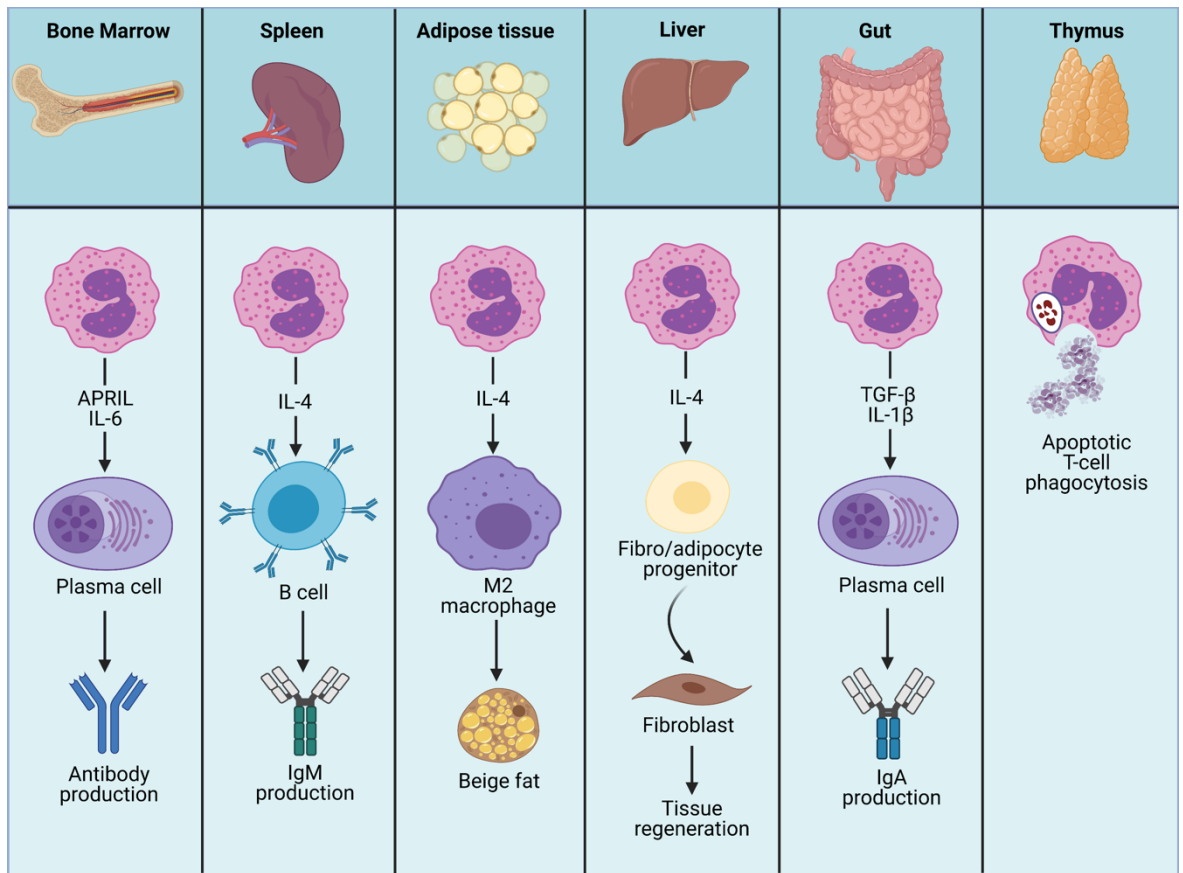
Figure 1.7: Homeostatic functions of eosinophils (created with BioRender.com).

Eosinophils have been correlated with maintenance of B cell homeostasis. Eosinophils have been demonstrated to localise close to B cell-derived plasma cells in the BM and to promote their survival by producing IL-6 and A proliferation-inducing ligand (APRIL). Plasma cell antibody production was also enhanced following secondary immunisation, suggesting that during immune responses eosinophils can be primed to prevent plasma cells from undergoing apoptosis (Chu and Berek, 2012) (Fig. 1.7).

B cell priming for the production of antigen-specific IgM was also shown to be dependent on IL-4 released by eosinophils in the spleen (Wang and Weller, 2008). Modulation of B cells was found to occur irrespective of eosinophil activation state, T-independent and T-dependent B cell activation (Wong et al., 2014) (Fig. 1.7).

Eosinophils are known to reside in adipose tissue but until recently their function there was unclear. Eosinophils have been shown to maintain appropriate glucose metabolism and insulin levels in adipose tissue by producing IL-4 and subsequent M2 macrophage polarisation, which

is necessary to maintain metabolic homeostasis (Wu et al., 2011). Conversion of subcutaneous white adipose tissue into beige fat produces heat, which is essential to allow regulation of body temperature. This process was also found to depend on eosinophil-derived IL-4 and M2 macrophage polarisation, which enhances catecholamine production and consequent biogenesis of beige fat (Qiu et al., 2014) (Fig. 1.7). Eosinophil-derived catecholamines have also been linked to maintaining the capacity of perivascular adipose tissue to mediate an anti-contractile effect on the vasculature, necessary to prevent the pathogenesis of diseases such as hypertension, diabetes or obesity. This process is mediated by binding of catecholamines to β3 adrenoreceptors on adipocytes with subsequent release of adiponectin and nitric oxide that induce smooth muscle relaxation (Withers et al., 2017).

Eosinophil release of IL-4 can also mediate regenerative responses to tissue injury. Muscle damage results in eosinophil recruitment, which through IL-4 production controls the proliferation and differentiation of resident fibro/adipocyte progenitors into fibroblasts, and not adipocytes, supportive of myogenesis and necrotic fibre clearance (Heredia et al., 2013). Liver injury also requires IL-4 signalling to induce hepatocyte proliferation and the eosinophils were found to be the major producers of the cytokine (Goh et al., 2013) (Fig 1.7).

Together with the adipose tissue, high levels of eosinophils can be found in the female reproductive tract, especially in the ovaries, preovulatory follicles, placenta and uterus. These tissues are tightly regulated by remodelling processes initiated by infiltrated leukocytes to facilitate the reproductive process. Eosinophils do so by promoting cervix dilation at birth and postpartum remodelling via release of cytotoxic granule proteins (Timmons et al., 2009). The importance of eosinophils in the reproductive tract is also evidenced by eosinophil- or eotaxin-deficient mice that show alterations in the development and function of the reproductive tract, such as delayed puberty and placental growth (Sferruzzi-Perri et al., 2003), as well as mammary gland development (Gouon-Evans et al., 2000).

As previously mentioned (section 1.1.2), eosinophils predominantly migrate to the GI tract and can be found in all regions, apart from the oesophagus. Eosinophils promote intestinal homeostasis by supporting production of plasma cell-derived IgA via the release of TGF-β and IL-1β (Beller et al., 2014; Jung et al., 2015) (Fig. 1.7). Absence of eosinophils is indeed associated with microbial dysbiosis, altered integrity of the mucosal barrier and development of Peyer's patches, as well as decreased numbers of CD103^{+} T cells and DCs in the lamina propria (Beller et al., 2014; Jung et al., 2015).

The number of intestinal eosinophils has also been inversely correlated to the number of Th17 cells in the intestine. In fact, regulation of Th17 cell development has been attributed to

eosinophil-derived IL-1Ra. IL-1β production by intestinal macrophages is required for the development of Th17 cells, therefore the balance between IL-1β and IL-1Ra is important in regulating the number of Th17 cells in the intestine to prevent the pathogenesis of diseases such as inflammatory bowel disease (IBD) (Sugawara et al., 2016).

Eotaxin-recruited eosinophils can be found in the embryonic thymus located within the cortico-medullary region. The thymus is an essential organ as it is the site of T cell selection, a process by which T cells that are reactive to self-antigens are eliminated by apoptosis (Weller and Spencer, 2017). Although thymic macrophages are mainly responsible for phagocytosing apoptotic T cells, eosinophil or neutrophil depletion reduces efficiency of the clearance process, suggesting their involvement (Kim et al., 2010) (Fig. 1.7). Eosinophils express Indoleamine 2,3-dioxygenase (IDO) which catalyses the conversion of tryptophan to kynurenine. Tryptophan is essential for T cell survival and proliferation, whereas increased kynurenine levels promote T cell apoptosis. Eosinophil expression and activation of IDO may therefore be one of the mechanisms by which these cells contribute to T cell immunomodulation in the thymus as well as immune responses to apoptosis (Odemuyiwa et al., 2004).

1.5 The gut-lung axis

As previously mentioned (section 1.2), the immune system of the lung has developed multiple mechanisms of tolerance toward the constant exposure to microbiota as well as prompt defence from foreign agents. The gut shares similarities with the lung, as they have the same embryonic origin, structure, as well as exposure to commensals and pathogens (Tulic et al., 2016). The existing complex and mutual relationship with the microbiota have raised great interest in elucidating how it may alter immune function in a tissue- and context-specific manner. Further research in this area may uncover further physiological mechanisms involved in maintenance of homeostasis as well as in the generation of inflammatory diseases. Drugs targeting the microbiome, or the microbiome itself, may represent additional innovative treatments for diseases linked to inflammation (Shreiner et al., 2015).

1.5.1 The gut-lung axis in allergic asthma

The "hygiene hypothesis", as previously discussed (section 1.2.1), suggests that environmental changes have impaired the mechanisms involved in mucosal immune tolerance and have created a predisposition for allergic disease. This suggests that there is an existing relationship between the gut and the lung. Several studies over the last 50 years have indeed demonstrated cross-communication between these distant mucosal sites (Tulic et al., 2016 and references therein).

Altered gut microbiota during the first three months of life due to pre- and peri-natal antibiotics, delivery by caesarean section, urban living or formula feeding, has been found to predispose infants to a relatively high risk of asthma (Fig. 1.8). This is associated with significant decreases in the bacterial genera of *Faecalibacterium, Lachnospira, Veillonella, Rothia* and *Akkermansia muciniphila*, as well as reduced faecal levels of short-chain fatty acids (SCFAs) (Arrieta et al., 2015; Fujimura et al., 2016; Wesemann and Nagler, 2016; Zhang et al., 2020). In support of this, antibiotic intake during pregnancy and in infants is associated with an increased incidence of childhood asthma (Mulder et al., 2016). Furthermore, inoculation of the bacteria absent in the microbiota of asthmatic patients decreases airways inflammation. For example, *Bacteroides fragilis* is reported to balance Th1 and Th2 responses systemically, thereby reducing susceptibility to allergic asthma (Enaud et al., 2020). Increased incidence of GI symptoms has also been described in children with asthma or atopic dermatitis (Powell et al., 2010). Some asthmatic patients commonly suffer from GI disorders such as primary eosinophilic GI disease, gastro-oesophageal reflux disease or the more severe eosinophilic esophagitis (Tulic et al., 2016). Moreover, increased levels of eosinophils, mast cells, lymphocytes and Th2 cytokines have not only been detected in the airways but also in the duodenal mucosa of asthmatic patients compared with healthy individuals (Powell et al., 2010). Asthmatic patients also show decreased circulating levels of the hormone motilin, which regulates interdigestive migrating contractions in the GI tract, whereas patients affected by heart failure and non-GI disease displayed unchanged levels of the protein (Ni and Gao, 2012). Communication between the lungs and the gut has also been evidenced in mice following intranasal administration of antigens, which triggered specific CD4^{+} T-cell proliferation not only in the lymph nodes draining the lungs but also in the gut-draining mesenteric lymph nodes, and not in peripheral non-draining nodes (Lambrecht et al., 2000). Another study showed that mice treated with broad-spectrum antibiotics in conjunction with a low intestinal colonisation with *Candida albicans,* used to induce microbial dysbiosis, developed airway allergic responses to intranasally administered OVA, demonstrated by increased eosinophil number, IgE levels, IL-5 and IL-13 production as well as goblet cell hyperplasia. Conversely, absence of microbial dysbiosis led to minor eosinophil recruitment and cytokine release (Beck et al., 2012; Noverr et al., 2005). Age-related alterations of the gut microbiota in old mice were also found to mimic the phenotype of HDM-exposed mice and were associated with increased Th17 cells in the spleen and IL-17A levels in the BAL (Shukla et al., 2017).

Inflammation in the gut may also affect the lung, as patients with IBD display airway inflammation even in the absence of any bronchopulmonary symptoms and with normal pulmonary functions (Wang et al., 2013; Luo et al., 2008). Changes were detected in both large and small airways, including thickening of the epithelium, fibrosis and inflammation of the pulmonary parenchyma;

these symptoms were more pronounced in in patients with active disease than those in remission (Tulic et al., 2016).

Recent studies have provided evidence for the lung microbiome to also be involved in the gut-lung axis (Fig. 1.8). For example, pneumonia caused by multi drug resistant *Staphylococcus aureus* or *Pseudomonas aeruginosa*, which originate in the lung, has been shown to induce intestinal injury (Lobo et al., 2005; Perrone et al., 2012) and intranasal administration of LPS has been associated with microbial dysbiosis in the lung and caecum (Sze et al., 2014).
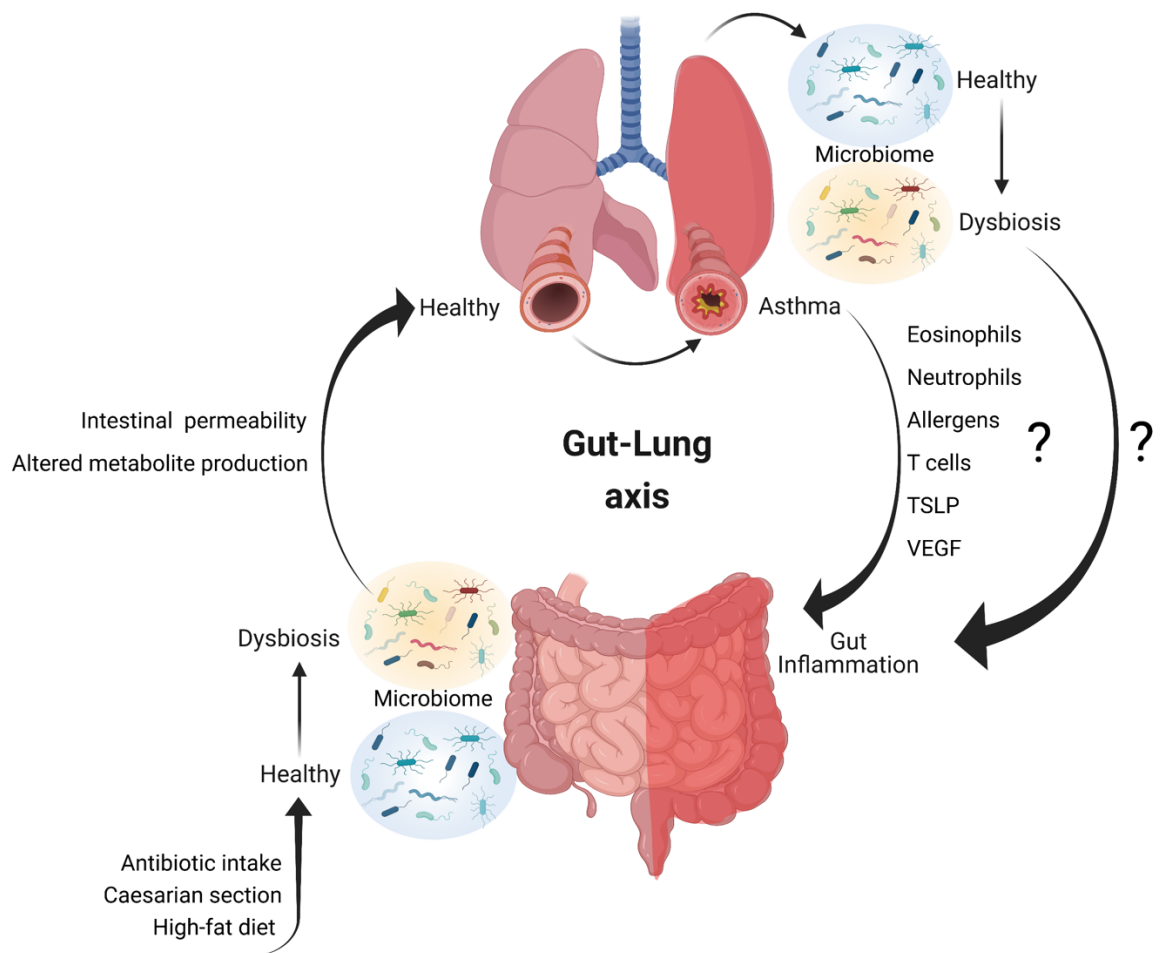
Figure 1.8: The gut-lung axis in allergic asthma (created with BioRender.com)

These results suggest that the relationship between the lung and the gut is bi-directional. They support the hypothesis that microbial colonisation during a developmental window early in life protects against childhood asthma but also that Th2 responses at one mucosal site can prime for similar responses in distant mucosal sites. Particularly communication between the gut and lung seems to be specific, as evidenced by a rat model of ulcerative colitis (UC) (Liu et al., 2013), and by intranasal infection with Influenza virus (Wang et al., 2014)

Although there is now robust evidence for the presence of concomitant inflammation in the lung and gut, the mechanisms underlying the lung-gut crosstalk are still poorly understood (Tulic et al., 2016) (Fig. 1.8).

Elevated numbers of eosinophils are known to be present in the airways during allergic lung inflammation, but increased levels have also been associated with the development and severity of IBD. Fort these reasons, eosinophils have been investigated as possible mediators of the lung-gut crosstalk. In support of this, increased expression of CCL11 mRNA is detected in the sputum and intestinal biopsy samples from both asthmatics and individuals with GI pathologies (Waddell et al., 2011). Together with eosinophils, neutrophils may also be elevated in both asthma and IBD, therefore they may be implicated in the establishment of the lung-gut crosstalk (Zhou and Liu, 2017). Damage to the epithelial barrier is another feature common between lung and gut inflammatory diseases. *Dermatophaghoides Pteronyssinus* (Derp1), which is a component of HDM with proteolytic activity in the lung, was found to reach the GI tract and to equally affect the gut epithelium, suggesting that it could link the similar pathology in the two organs (Tulic et al., 2016). Another possibility is that lung-resident DCs promote antigen-specific T cells to migrate back to the gut by inducing upregulation of gut-specific homing molecules such as integrin α4β7 and CCR9 (Ruane et al., 2013). The overlapping inflammation between the lung and the gut may also be caused by shared expression of inflammatory mediators. TSLP and VEGF have been suggested as potential candidates. TSLP is produced by pulmonary epithelial cells and mediates Th2 allergic responses in the lung. The same responses were also found to be mediated by TSLP in patients with Crohn's Disease (CD), suggesting that this molecule may be involved in the cross-talk (Takai, 2012). VEGF is involved in the pathogenesis of IBD (Scaldaferri et al., 2009) but high levels are also detected in asthmatic patients (Lee at al., 2017), suggesting that it may also represent a mediator of inflammatory responses between the lung and the gut (Liu et al., 2013).

1.5.2 Manipulation of the gut microbiota for the treatment of allergic asthma

The human gut microbiota is complex and is composed of at least a thousand distinct bacterial species. They are typically composed of five phyla: *Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria,* and *Tenericutes*, with *Bacteroides, Faecalibacterium,* and *Bifidobacterium* being the most prevalent genera in healthy adults (Barcik et al., 2020). These commensal species are not yet present in the foetal GI tract, which becomes colonised only at birth by the mother's urogenital tract (Belkaid and Hand, 2014).

The neonatal immune system adapts to the presence of bacteria with the aid of breast milk-derived IgA, cytokines and immune cells that promote induction of tolerogenic responses over inflammatory ones to allow establishment of the microbiota. The gut microbiota then contributes to the post-natal development of the immune system, including lymphoid structures, cellular

development and function by providing a vast array of antigenic stimuli (Belkaid and Hand, 2014). This is achieved through sensing of microbial-associated molecular patterns (MAMPs) by PRRs, mainly TLRs, expressed on intestinal epithelial and immune cells. For example, TLR-2 recognises lipoteichoic acid, whereas TLR-4 recognises LPS, that are MAMPs present on Gram-negative and Gram-positive bacteria respectively. These signals determine the nature of the immune response leading to establishment of a symbiotic relationship with the host (Toh et al., 2012). In fact, impaired immune responses in germ-free (GF) mice are restored by simply reintroducing the microbiome via faecal transplants (Dang and Marsland, 2019). Induction of BM myelopoiesis by the microbiota is also suggested to be mediated via sensing of MAMPs by TLRs expressed on HSCs and subsequent activation of MyD88 signalling (Nagai et al., 2006).

The respiratory microbiota develops together with the gut microbiome and shares a similar phylum structure but *Prevotella, Veillonnella* and *Streptococcus spp.* are more prevalent as genera (Moffatt and Cookson, 2017). In healthy lungs, there is not a high abundance of bacteria due to inhospitable conditions. However, during inflammatory responses, specific bacterial species can adapt to changes occurring in the lung environment allowing their growth and survival (Sommariva et al., 2020). Although studies analysing the airway microbiome are scarcer, there is evidence for it being essential in maintaining immune tolerance in the lungs (Enaud et al., 2020). In humans high bacterial load in the airways is associated with reduced pro-inflammatory responses to bacterial products such as LPS or to chronic viral infections (Sommariva et al., 2020).

It is therefore clear how the very first interaction between commensals and the foetal immune system may determine further development of the host, as well as protection or propensity to diseases at mucosal sites, such as allergic asthma (Belkaid and Hand, 2014). One of the main mechanisms by which the microbiome mediates induction of gut tolerance is via generation of Tregs. These cells produce anti-inflammatory cytokines such as IL-10 and promote IgA production in an antigen-specific manner, thereby limiting mucosal pro-inflammatory responses. Differentiation of CD4^{+} T cells into Tregs is normally induced by TGF-β and RA, secreted by specialised CD103^{+}CD11b^{+} DCs in the gut. Commensals may directly enhance Treg induction by several mechanisms, such as creating a TGF-β rich environment or stimulating TLR-2 on T cells (Belkaid and Hand, 2014). Other studies showed that intestinal *Clostridia spp.* and *Escherichia* promote the development of Tregs through the generation of SCFAs or IL-10 production by monocytes respectively (Furusawaet al., 2013; Orivuor et al., 2015). The induction of Tregs may be a potential mechanism by which *Clostridia spp*. mediates protection against wheezing and asthma (McAleer and Kolls, 2018).

Induction of tolerance by the lung microbiome has also been associated with Treg activity. For instance, Gollwitzer et al. (2014) has shown that juvenile mice (15-60 days old) could mount tolerogenic responses to HDM. Conversely, a short time after birth (3 days), mice were more susceptible to development of aberrant Th2 inflammatory responses in the lungs despite high levels of Tregs present. The ability of the immune system to suppress inflammatory responses was correlated to increased bacterial load and a shift from a prevalence of *Gammaproteobacteria* and *Firmicutes* toward *Bacteroidetes* as mice aged. This resulted in increased expression of programmed death-ligand-1 (PD-L1) on DCs, which was required for the development of immunosuppressive Helios⁻ Tregs.
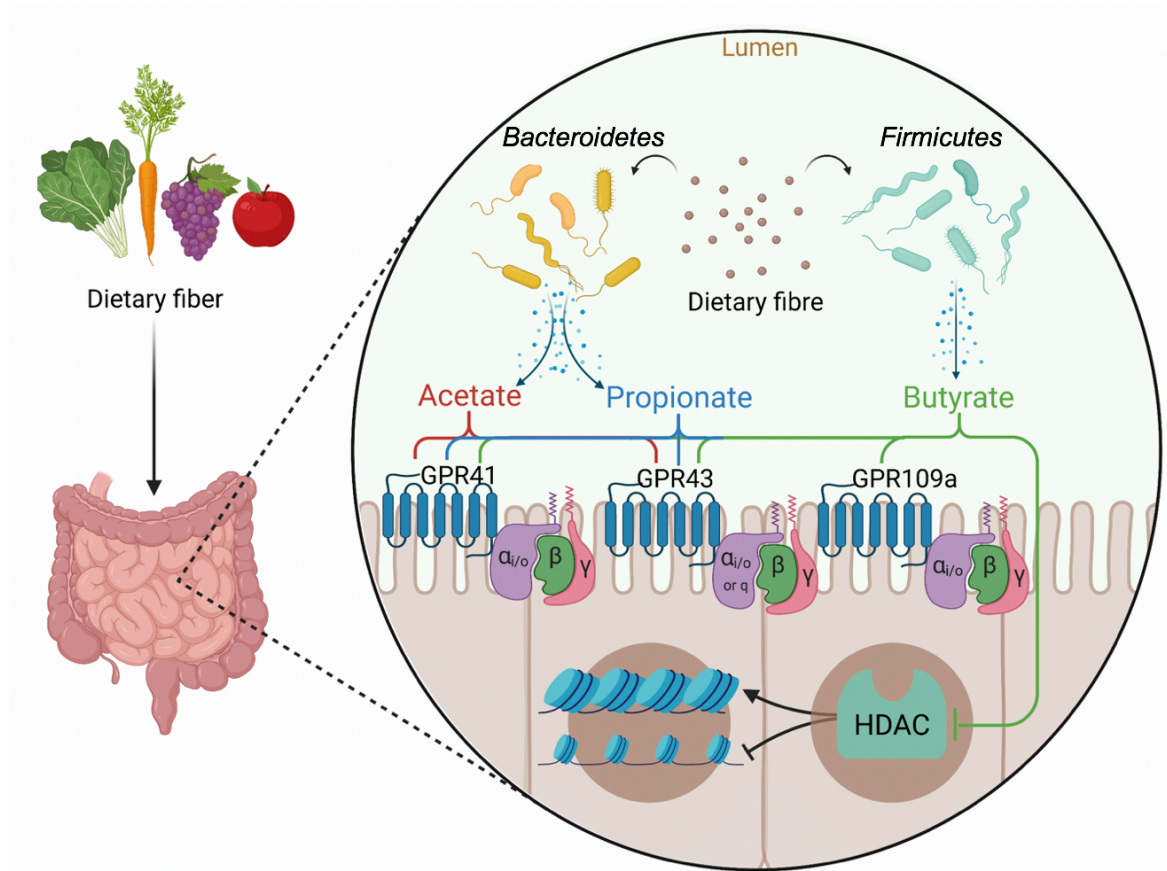
Figure 1.9: SCFAs mechanisms of action (created with BioRender.com)

Dietary fibers, but also proteins and peptides, which escape digestion in the upper gut, are metabolized by the microbiota leading to production of SCFAs that fortify barriers of defense and provide anti-inflammatory effects to maintain immune homeostasis in the gut. A diet rich in fibre is therefore important to maintain a healthy gut microbiome compared to high fat and sugar intake, which leads to reduced production of SCFAs and microbial diversity (Koh et al., 2016). As shown in Fig. 1.9, the most abundant SCFAs in the gut are butyrate, acetate and propionate, with their collective concentrations in the human gut ranging from 50 to 150 mM (Wu et al., 2017). SCFA-mediated effects occur mainly via binding to three G-protein coupled receptors

(GPCRs): GPR43 (also known as free fatty acid receptor 2, FFAR2), GPR41 (FFAR3) and GPR109a (NIACR1). GPR41 and GPR109a are coupled to $G_{i/o}$ which inhibits cyclic adenosine monophosphate (cAMP) accumulation, whereas GPR43 is coupled to both $G_{i/o}$ and G_q, which stimulates activation of protein kinase C (PKC) and intracellular Ca^{2+} release (Mizuno and Itoh, 2009). Additionally, SCFAs, primarily butyrate, can act as inhibitors of histone deacetylases (HDACs), thereby directly modulating gene transcription. They may reach the cell nucleus by passive diffusion or absorption via the high-affinity Na^+-coupled monocarboxylate transporter SLC5A8 or the low-affinity H^+-coupled carrier SLC16A1 (Dang and Marsland, 2019).

In the gut, SCFAs may mediate anti-inflammatory effects via induction of Tregs. SCFAs may signal through GPR109a on macrophages and DCs to induce IL-10 production, which in turn triggers the differentiation of Tregs (Singh et al., 2014). Another study showed that binding of SCFAs to GPR43 and HDAC inhibition can also generate Tregs in the gut by increasing transcription of the Foxp3 gene (Smith et al., 2013). SCFAs, acetate in particular, can enhance intestinal protection by signalling through GPR43 and enhance B-cell production of IgA, one of the main mechanisms of defence at the intestinal mucosal barrier (Wu et al., 2017). SCFAs were also found to increase prostaglandin E_1 (PGE_1) release by subepithelial myofibroblast and epithelial cells that in turn increases expression of mucin-2 (MUC-2), therefore mucus production (Willemsen et al., 2003). A recent study further demonstrated that SCFAs signal through GPR43 and GPR109a on epithelial cells to activate the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and IL-18 production, which is necessary for maintenance of epithelial integrity, repair and intestinal homeostasis (Macia et al., 2015).

Despite SCFAs being largely concentrated in the gut, there is evidence for SCFA-mediated anti-inflammatory effects in the periphery and especially along the gut-lung axis. In support of this, several studies report a direct relationship between obesity and a Westernised diet with asthma incidence and worsening, whereas a Mediterranean diet was associated with protection against the onset of asthma in children. The diet regimen adopted by pregnant women was also found to significantly affect the developing foetus and further susceptibility to allergic lung inflammation (Thorburn et al., 2015). The mesenteric lymphatic system is an essential pathway between the lungs and the intestine, through which SCFAs may translocate across the intestinal barrier, reach the systemic circulation, and modulate the lung immune response (Dang and Marsland, 2019). For example, during allergic lung inflammation, propionate was found to promote generation of macrophage and dendritic cell precursors (MDPs) in the BM and this effect was dependent on GPR41. Subsequent migration of the precursors to the lungs and differentiation in mature DCs showed that these cells had high phagocytic activity but were impaired at acting as APCs. Therefore, Th2 cells could not be activated and this prevented the development of airway inflammation (Trompette et al., 2014). Similarly, during influenza infection, propionate

promoted generation of MDPs via GPR41. In this scenario, MDPs committed to Ly6C⁻ monocytes without affecting the Ly6C⁺ subset, and when in the lung, they differentiated into M2 macrophages with inefficient release of the neutrophil chemokine CXCL1. As a result, decreased neutrophil recruitment limited virus-induced inflammatory responses in the lung. In the same study, SCFAs also increased the metabolic activity of CD8⁺ T cells which was correlated with an activated phenotype indicative of enhanced antiviral activity (Trompette et al., 2018). Increased levels of SCFAs and GPR41-dependent activation of Tregs was also found to mediate the beneficial effects of exposure to the parasite *Heligmosomoides polygyrus bakeri* in allergic lung inflammation (Zaiss et al., 2015).

Considering the homeostatic role that commensals and their metabolites provide, manipulation of the gut microbiome may be a promising therapeutic strategy to prevent or ameliorate allergic asthma (Anand et al., 2018). For example, a combination of five traditional chinese herbal medicines was shown to decrease Th2 cytokine release, cellular infiltration, tissue remodelling in the lung of OVA-induced asthmatic mice. The anti-inflammatory effects were achieved via alterations in the diversity of the gut microbiome with increased abundance of *Firmicutes,* therefore increased production of butyrate (Tsang et al., 2018). Commensals themselves given orally as probiotics, such as *Lactobacillus rhamnosus, Bifidobacterium lactis*, and *Bifidobacterium breve* have been found to suppress asthma symptoms by increasing TGF-β and levels of Tregs in the airways of mice (Feleszko et al., 2007; Jang et al., 2012; Hougee et al., 2010). Other studies have shown that treatment with several lactic acid bacteria attenuates asthma symptoms in allergic mice (Lee et al., 2007) as well as Th2 cytokine release by human peripheral blood mononuclear cells (PBMCs) from allergic patients (Pochard et al., 2002). More importantly, probiotic treatment was effective in ameliorating lung inflammation in infants but also asthma exacerbations in adults (Zhang et al., 2020). For instance, a single dose of inulin with probiotics significantly decreased airway inflammation and increased expression of GPR41 and GPR43 in the sputum of asthmatic patients (Halnes et al., 2017). Another study showed that ingestion of *Bifidobacterium infantis* increases expression of IL-10 and Foxp3 in the blood of human healthy subjects, which was dependent on TLR-2 and IDO expression by DCs (Konieczna et al., 2012). Treating asthmatic children with *Lactobacillus reuteri* for 60 days was also found to reduce lung inflammation (Del Giudice et al., 2012). Despite evidence for probiotics being promising in suppressing allergic lung inflammation, some studies report that they are not effective in improving all symptoms of the pathology of asthma. The efficacy of probiotic is suggested to be age-dependent, transient, as well as sufficient to suppress lung inflammation only when other immunotherapies are given together (McAleer and Kolls, 2018). Also, database reviews report that there is more evidence for probiotics being beneficial in eczema and allergic rhinitis rather than in asthma (Hufnagl et al., 2020).

Given the variety of effects that commensals and their metabolites exert, further studies are needed to better define the molecular and immunological mechanisms underpinning gut-lung cross-communication. This would allow subsequent refining of treatment regimens or possibly lead to discovering novel therapies that can prevent lung allergic inflammation.

1.6 Aims and objectives

In order to understand whether microbial metabolites can be used as eosinophil-targeted therapies in allergic asthma it is firstly necessary to better elucidate the precise effects of Th2 inflammation on eosinophils and secondly whether these responses extend to peripheral organs. This project therefore aims to:

1. Utilising a murine model of allergic lung inflammation clearly define how Th2 responses affect eosinophil phenotype and function in the airways
2. Assess whether lung eosinophils can be targeted from a therapeutic standpoint
3. Describe the systemic effects of allergic lung inflammation on immune cells in distal tissues with particular focus on the gut
4. Investigate how inter-organ communication may occur during allergic lung inflammation

1.7 Expected outcomes of research

Asthma is one of the most common chronic inflammatory disease worldwide and due to the inability of current therapies to fully restore airway homeostasis, the incidence of the disease and related complications is still rising. By addressing the objectives detailed above, this work aims to identify novel therapeutic options for the treatment of allergic asthma. Using flow cytometry, histology and transcriptomic analysis, this project aims to enhance our understanding of lung Th2 inflammation and the mechanisms through which they affect cell phenotype and function in the airways but also at distal sites, with a focus on eosinophil populations. Characterisation of these responses will aid in determining whether manipulating the gut microbiome and associated metabolites may be an effective strategy to ameliorate the pathology of allergic asthma.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Mice

Wild-type (WT) BALB/c and C57BL/6J (CD45.2) female mice were purchased from Envigo and housed in individually ventilated cages under specific pathogen free (SPF) conditions. Congenic CD45.1$^+$ mice (serially backcrossed from SJL/J onto C57BL/6) were all bred in-house and were backcrossed to a C57BL/6 background for at least 10 generations. Germ-free (GF) and SPF controls were bred in-house and were on a C57BL/6 background. Il-33$^{Gt/Gt}$ and Il-33$^{+/Gt}$ mice (Pichery et al., 2012) were on a C57BL/6 background and were bred in-house and kindly shared by Prof. Richard Grencis. All experiments were approved by The University of Manchester Local Ethical Review Committee and were performed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

2.2 *In vivo* treatments and infections

2.2.1 House dust mite (HDM)

Mice were sensitised with 100 µg HDM extract (Greer Laboratories) in 40 µl Phosphate Buffer Saline (PBS; Sigma Aldrich) or with PBS alone given intranasally (i.n.) on day 0. Mice were challenged with 10 µg HDM extract in PBS or with PBS alone given i.n. on days 7-11. Animals were sacrificed 24 hours after the last HDM challenge (day 12).

For some experiments, HDM was labelled with AF647 Antibody Labelling Kit (Invitrogen) according to manufacturer instructions and delivered i.n. at 100 µg in 40 µl PBS.

2.2.2 Sodium butyrate and niacin

Sodium Butyrate (Sigma Aldrich) was administered in the drinking water at 300 mM for two weeks prior and during HDM treatment. In some experiments, sodium butyrate and niacin (Sigma Aldrich) were delivered i.n. in 40 µl of PBS at 30 mM twice a week for two weeks prior and during HDM treatment or during the challenge of HDM treatment alone.

2.2.3 IL-33

Mice were i.n. administered 4 µg murine recombinant IL-33 (Peprotech) in 40 µl PBS or PBS alone daily for four consecutive days and euthanised 24 hours after the last instillation.

2.2.4 *Heligmosoides polygyrus bakeri*

For infection with *H. polygyrus*, mice were given 200 L3 larvae by oral gavage on day 0 and tissues were harvested on day 7.

2.2.5 *Nippostrongylus brasiliensis*

For infection with *N. brasiliensis*, 250 L3 larvae were injected subcutaneously (s.c.) on day 0 and tissues were harvested on day 6.

2.2.6 *Schistosoma mansoni*

For infection with *S. mansoni*, 20 cercariae were injected s.c. on day 0 and 7 and subsequently 80 cercariae were injected on day 14. Tissues were harvested on day 28.

Biomphalaria glabrata snails exposed to *S. mansoni* miracidia (NMRI strain) were acquired from Biodefense and Emerging Infections research repository or from Karl Hoffmann (Aberystwyth University). Snails were maintained in aquaria until patency, 4-5 weeks after exposure to *S. mansoni* miracidia, with a regular 12 hour light 12 hour dark cycle. Approximately 24 hours before infection, lights were turned off in the room containing the snails. The following day, patent snails were moved to a beaker containing 5-10 ml of aquarium water and exposed to a warm light source for 50 minutes to induce shedding of cercariae. The water containing cercariae was then poured into a 50 ml falcon for counting using a 1:1 dilution Lugol's iodine solution (Sigma Aldrich).

2.2.7 Influenza

For infection with Influenza virus (PR8 strain), mice were administered 30 µl of virus suspension (5 pfu) on day 0 and tissues were harvested on day 3 or day 7.

2.2.8 Generation of shield chimeras

WT CD45.2$^+$ host mice aged 6-8 weeks were anaesthetized by intraperitoneal (i.p.) administration of ketamine (80 mg/kg; Vetoquinol) and xylazine (8 mg/kg; Bayer). Anaesthetized mice were positioned beneath a lead sheet shielding the thorax from a split dose of irradiation (2 × 5.5 Gy). Mice therefore received partial body irradiation with only the head, the lower two thirds of the body and forelimbs left exposed. After recovery from anaesthesia, mice were reconstituted by intravenous injection with 2 × 10^6 CD90.2$^+$ T cell–depleted donor BM cells from congenic CD45.1$^+$ WT donor animals. T cells were depleted using CD90.2 microbeads (Miltenyi Biotec). Mice were maintained on 0.03% enrofloxacin in drinking water for up to 1 week before and for 2 weeks after irradiation and then were housed in autoclaved cages with sterile water, diet and bedding. Reconstitution was allowed to occur for a minimum of 7 weeks before analysis.

2.2.9 Intravascular staining

Mice were administered AF700-conjugated CD45 antibody (Biolegend) intravenously (i.v.) in 200 µl PBS 5 minutes before euthanasia.

2.2.10 Euthanasia and tissue harvesting

In all experiments, mice were euthanized by exposure to a rising concentration of CO_2 and tissues harvested at the indicated times post-treatment.

2.3 Murine tissue preparation and cell isolation

2.3.1 Lung, spleen, eyes and lymph nodes

Tissues were chopped finely in 100 µg/ml liberase TL (Roche) and 250 µg/ml DNaseI (Sigma Aldrich) and digested for 20 minutes at 37°C in a shaking incubator. 5 mM EDTA (Sigma Aldrich) was added for 5 minutes, after which the resulting suspension then passed through a Corning® 70 µm cell strainer (Sigma Aldrich). After pelleting by centrifugation (500 x g, 5 minutes, 4°C), it was resuspended in ACK lysing buffer (Lonza; it contains ammonium chloride, potassium bicarbonate and EDTA sodium salt dihydrate) for 3 minutes on ice. Suspensions were then washed with PBS and resuspended in complete RPMI 1640 (Sigma Aldrich) (supplemented with 10 mM HEPES (Sigma Aldrich), 5 µg/ml penicillin-streptomycin (Sigma Aldrich), 2 mM L-glutamine (Sigma Aldrich), Minimal non-essential amino acids 100x (Sigma Aldrich)) containing 10% foetal calf serum (FCS; Sigma Aldrich) until staining.

2.3.2 Bronchoalveolar lavage (BAL)

BAL was performed three times with 400 µl PBS containing 5mM EDTA and 2% FCS. Fluid was centrifuged and supernatant was collected and stored at -80°C for cytokine detection. Pellets were resuspended in resuspended in complete RPMI containing 10% FCS until staining.

2.3.3 Blood

Blood was collected into EDTA-coated syringes by cardiac puncture. Suspensions were washed and resuspended in ACK lysing buffer for 3 minutes on ice twice. Suspensions were then washed with PBS and resuspended in complete RPMI containing 10% FCS until staining.

2.3.4 Bone-marrow

Femurs were collected and removed of their ends. Each bone was placed in 0.5 ml Eppendorf tubes pierced with a 21G syringe. These in turn were inserted into 1.5 ml Eppendorf tubes and pulsed to maximum speed in a microcentrifuge. Cell pellets were resuspended in ACK lysing buffer for 3 minutes on ice. Suspensions were then washed with PBS and resuspended in complete RPMI containing 10% FCS until staining.

2.3.5 Small intestine and colon lamina propria and muscularis

Cells were isolated as previously described with some modifications (Sun et al., 2007). Small intestine and colon were dissected and cut longitudinally, Peyer's patches were removed from

the length of the small intestine and both tissues were washed with PBS on ice. To remove intestinal epithelial cells and leukocytes, small intestines and colons were cut into segments (2-3 cm) and incubated in prewarmed RPMI supplemented with 20 mM HEPES, 5 mM EDTA and 1 mM freshly thawed dithiothreitol (DTT; Sigma Aldrich) containing 3% FCS for 20 minutes at 37°C in a shaking incubator. Gut segments were then repeatedly shaken in RPMI supplemented with 2 mM EDTA and 20 mM HEPES. Remaining tissue (lamina propria and muscularis) was chopped finely and digested at 37°C for 30 minutes with continuous stirring in complete RPMI supplemented with 20 mM HEPES, 0.1 mg/ml Liberase TL, and 0.5 mg/ml DNaseI. Digested tissue was passed through a 70 µm filter followed by a 40 µm cell strainer, and after pelleting, it was resuspended in complete RPMI containing 10% FCS until staining.

2.4 Flow cytometry

2.4.1 Surface marker staining

Single-cell suspensions (5×10^5–2×10^6 total cells) were washed with PBS and stained with the Live/Dead Fixable UV dead cell stain kit (Biolegend) to exclude dead cells. Subsequently, cells were stained in the dark for 20 min at 4°C with fluorochrome- or biotin-conjugated antibodies in PBS containing anti-CD16/CD32 (2.4G2; BioXcell) in the dark. Cells were washed and, where necessary, incubated for a further 15 min with fluorochrome-conjugated streptavidin and then washed. In some cases, cells were immediately acquired live, or alternatively, after further washing, cells were fixed in 2% paraformaldehyde (PFA; Sigma Aldrich) for 10 minutes at room temperature and ultimately resuspended in PBS before acquisition.

Cells were stained with: CD11b (M1/70), CD11c (N418), CD45 (30F11), CD45.1 (A20), CD45.2 (104), CD98 (RL388), CD34 (RAM34), CD16/32 (93), CD125 (DIH37), CD117 (2B8), CD69 (H1.2F3), IgE (RME-1), CD300c/d (TX52), CXCR4 (L276F12), TLR-4 (SA15-21), CD29 (HMβ1-1), CD86 (GL-1), IDO (2E2/IDO1), CD5 (53-7.3), CD127 (A7R34) and CD4 (RM4-5) from BioLegend, KLRG-1 (2F1), CD90.1 (HIS51) from eBioscience, Siglec-F (E50-2440) from BD, CD101 (REA301) from Miltenyi, rabbit anti-mouse GPR55 from Abcam and rabbit anti-mouse GPR41 from Novus Biologicals. The lineage antibody cocktail for excluding lymphocytes, other granulocytes and monocytes/macrophages for eosinophil analysis included TCRβ (H57-597), CD3 (17A2), B220 (RA3-6B2), Ly6G (1A8), Ly6C (HK1.4), CD19 (1D3/CD19), NK1.1 (PK136) and Ter119 (TER-119) from BioLegend.

2.4.2 Intracellular marker staining

Where intracellular staining was required cells were pelleted by centrifugation, after surface marker staining, and resuspended in Foxp3/Transcription Factor Fixation/Permeabilisation concentrate and diluent buffer (eBioscience) diluted 1:4 overnight. Cells were then washed and

stained with cocktail of antibodies made in Intracellular Staining Permeabilization Wash Buffer 10X (eBioscience) diluted 1:10 in PBS, and incubated for 45 minutes at 4°C in the dark. Following staining, cells were washed and resuspended in PBS before acquisition. Cells were stained with mouse anti-rabbit Relm-α (500-P214) from Peprotech, Ki67 (16A8), IL-4 (11B11), IL-5 (TRFK5) and T-bet (4B10) from Biolegend, Foxp3 (FJK-16s) and IL-33 (396118) from Thermofisher, IL-13 (eBio13A), IFN-γ (XMG1.2) and GATA3 (TWAJ) from eBioscience.

To assess protein phosphorylation, cells were fixed and permeabilised using BD Phosphoflow kit according to manufacturer instructions. Cells were stained with pSTAT1 (58D6), pSTAT5 (C71E5) and pERK1/2 from Cell Signaling, and pmTOR (MRRBY) and pS6 (cupk43k) from Thermofisher.

2.4.3 Sample acquisition

Appropriate compensation was performed using UltraComp eBeads™ (Invitrogen) and samples were acquired on a BD LSRFortessa™ running BD FACSDiva 8 software (BD) and saved as FCS files.

2.4.4 Fluorescence activated cell sorting (FACS)

To sort eosinophil populations from the lung (L/D⁻Lin⁻CD11b$^{int/hi}$Siglec-F$^{int/hi}$) single-cell suspensions were prepared (Section 2.3.1) and resuspended in PBS with 2% FCS and 2 mM EDTA. Sorting was performed using a FACSAria Fusion (BD) and sorted cells were collected in complete RPMI with 20% FCS and stored on ice for use in cytospins and *in vitro* cell culture assays.

2.5 Histology

2.5.1 Cytospin preparation and staining

Sorted lung eosinophils, monocytes or neutrophils were mounted on SuperFrost Plus™ Adhesion Slides (Thermofisher) using a Cytospin centrifuge (Cytospin 4; Thermo Fisher Scientific) operating for 5 minutes at 500 rpm. Cells were fixed with ice-cold methanol and stored at room temperature. Unspecific antibody binding on sorted cells was blocked with TSA blocking reagent (PerkinElmer) for 30 minutes. Sections were then incubated with either mouse anti-rabbit GPR109a (1:100; Abcam), mouse anti-rabbit GPR41 (1:50; Invitrogen) or Cy3-cojugated mouse anti-rabbit GPR43 (1:50; Bioss) for 1 hour at room temperature. Primary antibody staining was followed by secondary antibody staining with FIT-C goat anti-rabbit (1:200; Invitrogen) for 1 hour at room temperature. Cells were mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermofisher).

Sorted lung eosinophils were also stained with Diff-Quik (Thermofisher) according to manufacturer's instructions and mounted with DPX Mounting Medium (Fisher Scientific).

2.5.2 Tissue processing and sectioning

Tissues were collected into 4% PFA and stored for 24 hours before being transferred to 70% ethanol until processing. Fixed tissues were dehydrated through increasing series of ethanol (50%-100%), cleared in Xylene (Fisher Scientific) and infiltrated with paraffin in a dehydration automat (Leica ASP300 S) using a standard protocol. Specimens were embedded in paraffin (Histocentre2, Shandon). Tissues were sectioned on a microtome (5 µm thick) with a S35 Blade and dried overnight at 37°C before staining.

2.5.3 Haematoxylin and Eosin (H&E)

Formalin-fixed paraffin-embedded (FFPE) sections were dewaxed with xylene, rehydrated with decreasing series of ethanol (100%-50%) and rinsed with distilled water. Sections were then stained with Harris modified haematoxylin solution (Sigma Aldrich) for 4 minutes and washed in tap water for another 4 minutes. Sections were differentiated with one dip in acid alcohol (70% ethanol with 1% HCl), washed and blued in running tap water for 5 minutes. Sections were treated with Alcoholic Eosin Y solution (Sigma Aldrich) for 30 seconds and washed in tap water for 5 minutes. Sections were finally dehydrated with rising ethanol gradients (50%-100%), cleared with xylene and cover slipped using DPX mounting medium (Fisher Scientific).

2.5.4 Periodic acid Schiff (PAS) and Alcian-blue

FFPE sections were dewaxed, rehydrated and rinsed in distilled water as before. Sections were then stained with Alcian blue solution (Sigma Aldrich) for 15 minutes and washed in tap water for 4 minutes. Sections were then treated with periodic acid (Sigma Aldrich) for 5 minutes, washed for 5 minutes, and stained with Schiff's reagent (Sigma Aldrich) for 10 minutes. After washing for 5 minutes, nuclei were stained with Harris haematoxylin for 1 minute and immediately washed for 2 minutes. Sections were differentiated through one dip in acid alcohol, washed and blued in running tap water for 5 minutes. Sections were finally dehydrated, cleared and cover slipped using DPX mounting medium.

2.5.5 Immunofluorescence

FFPE sections were deparaffinized, rehydrated and rinsed in distilled water as before. To stain for the eosinophil-specific marker major basic protein (MBP), sections were subjected to antigen retrieval using Pepsin (Thermofisher) followed by quenching of endogenous peroxidase activity with 0.3% hydrogen peroxide (Sigma Aldrich) before blocking with TSA for 30 minutes. Sections were incubated with rat anti-mouse MBP (1:200; provided by Dr. E. Jacobsen, Mayo Clinic Arizona, Scottsdale, AZ) overnight at 4°C. Sections were subsequently washed and incubated

with secondary antibody goat anti-rat AF-647 (1:200; Invitrogen) for 1 hour at room temperature and finally mounted with ProLong™ Gold Antifade Mountant with DAPI. All dilutions and washes were performed with 1X Tris-buffered saline (TBS).

To stain for Relm-α, Ym-1, EPX, CD4, ALOX15, HIF-1α, Muc2 and lectins, sections were subjected to heat-induced antigen retrieval (HIER) using citrate buffer (pH 6), microwaving for 10 minutes on full power, followed by another 10 minutes at 50% power. Sections were permeabilised through incubation in PBS containing 0.1% Triton™ X-100 (Sigma Aldrich) for 15 minutes before blocking with TSA for 30 minutes. For Ym-1 staining, Avidin-biotin blocking was subsequently carried out as per the manufacturer's instructions (BioLegend). Sections were incubated with either rabbit anti-mouse Relm-α (1:100; Peprotech) and biotinylated anti-mouse Ym-1 (1:50; R&D systems); rabbit anti-mouse CD4 (1:100; Abcam); mouse anti-mouse EPX (1:100; provided by Dr. E. Jacobsen, Mayo Clinic Arizona, Scottsdale, AZ) and rabbit anti-mouse 15 Lipoxygenase 1 (1:100; Abcam) or rabbit anti-mouse HIF1-α (1:100; Abcam); rabbit anti-mouse Muc2 (1:400; Novus Biologicals) and biotinylated lectin kit (Vector laboratories) overnight at 4°C. Sections were subsequently washed and incubated with the secondary antibodies goat anti-rabbit FIT-C (1:200; Invitrogen) and Streptavidin Cy3 (1:300; Biolegend) for 1 hour at room temperature and finally mounted with ProLong™ Gold Antifade Mountant with DAPI. All dilutions and washes were performed with TBS.

2.5.6 Image acquisition

Brightfield images were collected on an Olympus BX63 upright microscope using either 10x, 20x or 40x objectives and captured and white-balanced using an Olympus DP80 camera in colour mode through Olympus CellSens Dimension v. 1.16.

Fluorescent images were collected on a Zeiss Axioimager.D2 upright microscope using either a 10x, 20x or 40x objectives and captured using a Photometrics Coolsnap HQ2 camera through Micromanager software v. 1.4.23.

2.6 Quantitative polymerase chain reaction (PCR)

2.6.1 RNA and DNA extraction

Tissues were collected into RNAlater™ stabilisation solution (Invitrogen) and stored at -80°C. To isolate RNA, 1 ml TRIzol™ reagent (Invitrogen) was added to tissues transferred into Lysing Matrix D tube (MP Biomedicals). Tissues were homogenised using a MP Biomedicals FastPrep-24™ Classic Grinder (4.0 m/s, 40 seconds) and rested on ice. Tissue suspensions were transferred to a clean Eppendorf tube and 200 μl chloroform (Sigma Aldrich) was added. Tubes were manually shaken for 15 seconds and rested at room temperature for 5 minutes. Tubes

were centrifuged (12,000 x g, 15 minutes, 4°C) and the upper aqueous layer was transferred into a clean Eppendorf tube. To precipitate RNA, 500 µl propan-2-ol (Sigma Aldrich) was added and samples were incubated at room temperature for 10 minutes. Tubes were centrifuged (12,000 x g, 10 min, 4°C) to pellet the RNA. The supernatant was removed by tipping, and the RNA pellet was washed by vortexing with 1ml 75% ethanol. Samples were centrifuged once more and RNA pellets were air-dried for 30-60 minutes at room temperature before resuspending in 30 µl RNase-free water (Qiagen). The quality and quantity of RNA was measured using a NanoDrop Microvolume spectrophotometer. Samples with 260/280 values of 1.60-2.00 and 260/230 values of 1.50-2.20 were considered of good quality. Samples were stored at -20°C until cDNA synthesis.

DNA was extracted from faecal samples using the DNeasy® PowerLyzer® PowerSoil® Kit (Qiagen) according to manufacturer instructions. The quality and quantity of DNA was also measured using a NanoDrop Microvolume spectrophotometer and 260/280 and 260/230 values were checked. DNA samples were stored at -20°C until qPCR analysis.

2.6.2 cDNA synthesis

RNA concentrations were normalised across samples through dilution with RNase-free water. cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions and a Veriti 96-well thermal cycler.

2.6.3 Quantitative PCR (qPCR) and analysis

cDNA samples were diluted 1:8 in nuclease free water. DNA samples from faeces were not diluted. Each sample was added in triplicates per RNA transcript on a 384 well qPCR plate (Applied Biosystems). Each well contained 4.5 µl cDNA, 5 µl Fast SYBR™ Green Mastermix (Applied Biosystems) and 0.5 µl 10 µM forward and reverse primers (Table 2.1). Samples were amplified using a QuantStudio 12 K Flex system.

Table 2.1: PCR primer sequences.

Target Gene		Primer Sequence
IFN-γ	F	ATGAACGCTACACACTGCATC
	R	CCATCCTTTTGCCAGTTCCTC
CCL11	F	GAATCACCAACAACAGATGCAC
	R	ATCCTGGACCCACTTCTTCTT
IL-13	F	CCTCTGACCCTTAAGGAGCTTAT
	R	CGTTGCACAGGGGAGTCT
IL-4	F	GAGAGATCATCGGCATTTTTGA
	R	TCTGTGGTGTTCTTCGTTGC
Relm-α	F	TATGAACAGATGGGCCTCCT
	R	GGCAGTTGCAAGTATCTCCAC
Muc5ac	F	GTGATGCACCCATGATCTATTTTG
	R	ACTCGGAGCTATAACAGGTCATGTC
IL-33	F	GGTGAACATGAGTCCCATCA
	R	CGTCACCCCTTTGAAGCTC
GAPDH	F	AGGTCGGTGTGAACGGATTTG
	R	TGTAGACCATGTAGTTGAGGTCA
TNF-α	F	CCCTCACACTCAGATCATCTTCT
	R	GCTACGACGTGGGCTACAG
IL-17A	F	TTTAACTCCCTTGGCGCAAAA
	R	CTTTCCCTCCGCATTGACAC
Firmicutes	F	GGAGYATGTGGTTTAATTCGAAGCA
	R	AGCTGACGACAACCATGCAC
Bacteroidetes	F	GTTTAATTCGATGATACGCGAG
	R	TTAASCCGACACCTCACGG
Verrucomicrobia	F	TCAKGTCAGTATGGCCCTTAT
	R	CAGTTTTYAGGATTTCCTCCGCC
Actinobacteria	F	TGTAGCGGTGGAATGCGC
	R	AATTAAGCCACATGCTCCGCT
Prevotella	F	CACRGTAAACGATGGATGCC
	R	GGTCGGGTTGCAGACC
Veillonella	F	AYCAACCTGCCCTTCAGA
	R	CGTCCCGATTAACAGAGCTT
Lactobacillae	F	AGCAGTAGGGAATCTTCCA
	R	CACCGCTACACATGGAG
F. prausnitzii	F	GGAGGAAGAAGGTCTTCGG
	R	AATTCCGCCTACCTCTGCACT
β-Proteobacteria	F	AACGCGAAAAACCTTACCTACC
	R	TGCCCTTTCGTAGCAACTAGTG
γδ-Proteobacteria	F	GCTAACGCATTAAGTRYCCCG
	R	GCCATGCRGCACCTGTCT
SFB	F	AGGAGGAGTCTGCGGCACATTAGC
	R	TCCCCACTGCTGCCTCCCGTAG

2.7 Supernatant analysis

2.7.1 Preparation of supernatant from tissues

Lysis buffer was prepared by adding 1 M tris-hydrochloric acid (pH = 8.0), 5 M NaCl and 0.1% Triton^{TM} X-100 to distilled water supplemented with Protease Inhibitor Cocktail (Sigma Aldrich) diluted 1:200. Fresh tissues were cut in 1 cm length pieces, weighed and stored in 1.5 ml Epperdorf tubes on dry ice until processing. Tissues were then transferred to Lysing Matrix D tubes containing 1ml lysis buffer and they were homogenised using a MP Biomedicals FastPrep-24^{TM} Classic Grinder (4.0 m/s, 40 seconds). Supernatants were transferred into clean 1.5 ml Epperdorf tubes and centrifuged (3300 x g, 5 minutes, 4°C). Supernatants were collected discarding the resulting pellet and stored at -80°C until use.

2.7.2 Enzyme-linked immunosorbent assay (ELISA)

Supernatants derived from homogenised tissues were screened for IL-33, Relm-α, Ym-1, IL-4, IL-13, whereas IL-6, IL-4 and EPX were examined from supernatants of cultured eosinophils (Table 2.2). Appropriate primary antibodies were diluted in ELISA coating buffer (Biolegend) and added to 96 well high binding ELISA plates (Greiner Bio-One) overnight at 4°C. The coating solution was then flicked off and the plate was washed three times with PBS containing 0.1% Tween® 20 (Sigma Aldrich). Plates were then blocked with PBS containing 10% FCS for 1 hour at room temperature. The blocking solution was flicked off and samples and standards were added and incubated overnight at 4°C. Plates were washed three times and secondary antibodies were then added and incubated for 2 hours at room temperature. Following washing for three times, streptavidin-peroxidase was added and incubated for 30 minutes at 37°C. The plate was finally washed eight times and was developed with the TMB substrate kit (Biolegend) according the manufacturer's instructions. The reaction was stopped by adding Stop Solution for TMB substrate (Biolegend). The plates were read using a Tecan Infinite M200 PRO microplate reader running I-control 1.9 software at 450 nm, with reference of 570 nm subtracted.

Table 2.2: Antibodies and standard concentrations used for ELISA.

Coating antibody	Coating antibody concentration	Top Standard concentration	Detection antibody	Detection antibody concentration
LEAF™ purified anti-mouse IL-6 (BioLegend)	1 µg/ml	50 ng/ml recombinant mouse IL-6 (BioLegend)	Biotin anti-mouse IL-6 (BioLegend)	0.5 µg/ml
IL-4 capture antibody (R&D systems)	4 µg/ml	1 ng/ml recombinant standard (R&D systems)	IL-4 detection antibody (R&D systems)	0.15 µg/ml
IL-13 capture antibody (R&D systems)	4 µg/ml	4 ng/ml recombinant standard (R&D systems)	IL-13 detection antibody (R&D systems)	0.2 µg/ml
IL-33 capture antibody (R&D systems)	0.8 ug/ml	1 ng/ml recombinant standard (R&D systems)	IL-33 detection antibody (R&D systems)	0.075 ug/ml
EPX capture antibody (Di Develop)	-	2 ng/ml EPX recombinant standard (Di Develop)	EPX detection antibody (Di Develop)	-
Anti-murine Relm-α (Peprotech)	1 µg/ml	100 ng/ml recombinant Murine Relm-α (Peprotech)	Biotinylated anti-murine Relm-α (Peprotech)	0.25µg/ml
Ym-1 capture Antibody (R&D systems)	1 µg/ml	50 ng/ml recombinant standard (R&D systems	Ym-1 detection Antibody (R&D systems)	0.5 µg/ml

2.8 *In vitro* assays

2.8.1 Seahorse extracellular flux analysis

Sorted eosinophils were plated at 150,000 cells/well and allowed to adhere for at least 1 hour. ECAR and OCR were measured in XF media (modified DMEM containing 2 mM L-glutamine) under basal conditions and in response to 10 mM glucose, 2.5 µM oligomycin, 100 mM 2-DG using a 96-well extracellular flux analyzer XFe-96 (Seahorse Bioscience).

2.8.2 L-amino acid transport

The assay was performed as previously described (Sinclair et al, 2018). Surface cell antibody staining (Section 2.5) was performed prior to uptake assay protocol. Kynurenine (Sigma Aldrich) was diluted in Hank's balanced salt solution (HBSS; Sigma Aldrich) at 800 µM, Lysine (Sigma Aldrich) at 20 mM and 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH; Sigma Aldrich) at 40 mM, and they were all pre-warmed at 37°C before use. Samples were resuspended in HBSS and lysine, BCH and kynurenine were added in the order described to achieve final concentrations of 5 mM, 10 mM and 200 µM respectively. Samples were incubated for 5 minutes at 37°C and the reaction was stopped by addition of 2% PFA for 15 minutes at room temperature. Cells were washed and resuspended in HBSS before acquisition by flow cytometry. The 405 nm laser and 450/50 BP filter were used for kynurenine fluorescence detection.

2.8.3 Glucose transport

To assess glucose transport, cell suspensions were first resuspended in XF media for 20 minutes at 37°C. The glucose analogue 2-NBDG (Thermofisher) was then added at 50 µg/ml and samples were further incubated for 20 minutes at 37°C. Cells were washed and surface stained (Section 2.5). Samples were resuspended in PBS before acquisition by flow cytometry. The 488/530 nm laser was used for 2-NBDG fluorescence detection.

2.8.4 Stimulation of cell suspensions

Single cell suspensions were obtained from the gut (Section 2.4.5) and stimulated in a 96-well plate with Cell Stimulation Cocktail (eBioscience) for 4 hours at 37°C. Cells were washed and stained for flow cytometric analysis (Section 2.5).

2.8.5 Eosinophil stimulation

Sorted lung eosinophils (Section 2.5.4) were resuspended in complete RPMI with 10% FCS and added to a 96 well plate at a density of 1×10^6/ml. Murine recombinant IL-33, GM-CSF and IL-5 (Peprotech), all at 100 ng/ml in combination, or A23187 (Sigma Aldrich) at 5 µM were added and samples were incubated for 24 hours at 37°C. Cell supernatants were collected and analysed by ELISA (Section 2.8.2).

Bone-marrow-derived cell suspensions (Section 2.4.4) were resuspended in complete RPMI with 10% FCS and added to a 48 well plate at a density of 1×10^6/ml. Murine recombinant IL-33, GM-CSF and IL-5 (Peprotech), all at 100 ng/ml alone or in combination, or platelet activating factor (200 ng/ml; Sigma-Aldrich), IL-33 (100 ng/ml) and Ionomycin (50 ng/ml) alone or in combination were added with or without butyrate or niacin (300 µM) and samples were

incubated for 24 hours or 3 hours at 37°C. Cells were washed and stained for flow cytometric analysis (Section 2.5).

2.9 Human tissue preparation and cell isolation

2.9.1 Human samples

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

2.9.2 Lung

Lung tissue was weighed to be digested in 5-8 g/tube. Tissue was then chopped in a petri dish using a razor blade, transferred to a 50 ml tube containing HBSS supplemented with 125 µg/ml Liberase TL and 150 U/ml DNaseI, and digested for 40 minutes at 37º C in a shaking incubator. The tube with tissue was then topped up with complete RPMI supplemented with 10% FCS and 0.5 mM EDTA and passed through a 100 µm filter removing any undigested connective tissue to allow passage of cells through the filter. The tube was again topped up with complete RPMI and passed through a 70 µm filter. The cell suspension was centrifuged and resuspended in distilled water for 30 seconds to lyse red blood cells. The reaction was stopped by addition of complete RPMI and, following pelleting, cells were resuspended in complete RPMI with 10% FCS until staining.

2.9.3 Blood

Blood was centrifuged and resuspended in distilled water for 30 seconds to lyse red blood cells, topped up with complete RPMI and centrifuged, twice. Cells were then resuspended in complete RPMI with 10% FCS until staining.

2.9.4 Cell freezing

If freezing, 10-20x10^6 cells per cryovial were resuspended in 1 ml Freezing media – Recovery^{TM} (Gibco/Fisher) and stored at -80º C.

2.9.5 Flow cytometry

Single-cell suspensions (5 × 10^5–2 × 10^6 total cells) were washed with PBS and stained with the Live/Dead Fixable UV dead cell stain kit (Biolegend) to exclude dead cells. Subsequently, cells were stained in the dark for 20 minutes at 4°C with fluorochrome-conjugated antibodies in PBS containing Human TruStain FcX (Biolegend). Cells were washed then fixed in 2% PFA for 10 minutes at room temperature and ultimately resuspended in PBS before acquisition.

Cells were stained with: CD101 (BB27), CD45 (HI30), CCR3 (5E8) and Siglec-8 (7C9) from Biolegend, CD125 (A14) and CD24 (ML5) from BD, CD98 (MEM-180) from Invitrogen, CD11b (ICRF44) and GPR109a (4NZBRGO) from eBioscience. The lineage antibody cocktail for excluding lymphocytes, other granulocytes and monocytes/macrophages for eosinophil analysis included TCRβ (IP26), CD3 (OKT3), CD20 (2H7), CD14 (M5E2), CD16 (3G8), CD123 (6H6), CD117 (104D2) and CD56 (5.1H11) from Biolegend.

2.9.6 Eosinophil isolation and stimulation

Blood eosinophils were isolated using EasySep™ Direct Human Eosinophil Isolation Kit (Stem Cell Technologies) according to manufacturer instructions. Cells were resuspended in complete RPMI with 10% FCS and added to a 96 well plate at a density of $1x10^6$/ml. Human recombinant IL-33, GM-CSF and IL-5 (Peprotech), all at 100 ng/ml in combination with or without butyrate or niacin (300 μM) were added and samples were incubated for 24h at 37°C. Cells were washed and stained for flow cytometric analysis (Section 2.11).

2.9.7 Cytospin preparation and staining

Isolated blood eosinophils were mounted on SuperFrost Plus™ Adhesion Slides (Thermofisher) using a Cytospin centrifuge (Cytospin 4; Thermo Fisher Scientific) operating for 5 minutes at 500 rpm. Cells were fixed with ice-cold methanol and stored at room temperature. Isolated blood eosinophils were permeabilised through incubation in PBS containing 0.1% Triton™ X-100 (Sigma Aldrich) for 5 minutes before blocking with TSA for 30 minutes. sections were then incubated with mouse anti-human EPX (1:100; Abcam) for 1 hour at room temperature. Primary antibody staining was followed by secondary antibody staining with goat anti-mouse AF-647 (1:200; Invitrogen) for 1 hour at room temperature. Cells were mounted with ProLong™ Gold Antifade Mountant with DAPI (Thermofisher).

Isolated blood eosinophils were also stained with Diff-Quik (Thermofisher) according to manufacturer's instructions and mounted with DPX Mounting Medium (Fisher Scientific).

2.10 Analysis

2.10.1 Images
All images were processed and analysed using Fiji Image J (http://imagej.net/Fiji/Downloads).

2.10.2 Flow cytometry
Data was analysed using FlowJo software (TreeStar) was used for analysis and gates were set using appropriate fluorescence-minus-one controls.

2.10.3 qPCR

Relative expression of target genes within experimental treatment groups was determined through comparison to an internal control gene (*Gapdh*) and the experimental control group.

2.11 Statistics

Statistical analyses were performed using Prism (8.0; GraphPad software) and data were presented as mean ± SEM in all cases. Two experimental groups were compared using a Student's t test for unpaired data. Where more than two groups were compared, a one-way ANOVA or two-way ANOVA with Bonferroni's correction was used. Significance was set at P ≤ 0.05.

CHAPTER 3 – PAPER 1

Agonism of GPR109a is associated with reduced activation of inflammatory eosinophils in allergic asthma

3.1 ABSTRACT

Eosinophils are known to be key contributors to inflammatory responses in allergic lung disease. Nevertheless, eosinophil-targeted therapies are still not curative. Alongside their inflammatory effector functions, eosinophils have been shown to promote homeostatic responses at steady-state and in disease. Better understanding of eosinophil recruitment, signalling and phenotype is therefore necessary to provide mechanistic insight and offer novel therapeutic strategies for allergic disease. In the lung, we found that Siglec-F^{hi}CD11b^{hi} eosinophils were more inflammatory than Siglec-F^{int}CD11b^{int} eosinophils and showed enhanced CD98 expression as well as altered metabolic activity that represented common features of lung inflammatory responses. We also established that the SCFA butyrate could specifically decrease levels of (i)EOS as well as reduce their metabolic activity in the lung of allergic mice and that this mechanism may be mediated by GPR109a. Thus, selective targeting of Siglec-F^{hi}CD11b^{hi} eosinophils may be beneficial in ameliorating allergic lung inflammation.

3.2 INTRODUCTION

Allergic asthma is a chronic inflammatory disorder affecting the upper airways. It is characterised by breathlessness, wheezing, bronchoconstriction and mucus production in response to inhaled environmental allergens. The inflammatory response in the airways is characterised by cellular infiltration and aberrant Th2 cytokine responses (i.e. IL-4$^+$, IL-5$^+$, IL-9$^+$ and IL-13$^+$ mucosal immune cells) resulting in elevated production of IgE and, over the long term, airway remodelling and fibrosis (Kay, 2005).

The World Health Organization estimates that 300 million people worldwide currently suffer from asthma (Masoli et al., 2004). Moreover, asthma is the most common chronic disease among children, causing approximately 250,000 deaths per year. Although the prevalence of asthma is 7-10% worldwide, higher asthma rates tend to exist in more developed and westernized countries than developing countries, with as much as 20- to 60-fold differences (Kim et al., 2013).

It is known that during allergic responses eosinophil numbers rise dramatically in the airways, blood and BM and that increased levels correlate with disease severity (Fahy, 2009). These cells are thought to be recruited from the BM to the lung by T-cell- and ILC2- derived IL-5 and eotaxins which are mainly secreted by airway epithelial cells under the influence of Th2

cytokines (Uhm, et al., 2012; Walsh et al., 2010). After tissue ingress eosinophils can release cytotoxic granule proteins as well as cytokines and lipid mediators, contributing to the ongoing inflammatory response and tissue damage (Liu et al., 2006; Moqbel and Odemuyiwa, 2008).

Despite these effector functions, eosinophils have also been shown to promote homeostatic responses at steady-state and in a variety of disease settings (Marichal et al., 2017; Carretero et al., 2015). Recently, two populations of eosinophils have been identified in the airways of mice during allergen-induced allergic inflammation (Mesnil et al., 2016; Abdala-Valencia et al., 2016). They have been described as resident (r)EOS and inflammatory (i)EOS and defined as two terminally differentiated eosinophil populations. (r)EOS are proposed to be IL-5 dependent, to promote lung homeostasis and to be located in the parenchyma. (i)EOS were identified as IL-5 independent cells recruited from the BM, showing expression of CD101 and upregulated Siglec-F. (i)EOS were localised in the peribronchial area of the lungs and were reported to enhance the inflammatory response in the airways (Mesnil et al., 2016). Nevertheless, it remains unclear how and where these two populations of eosinophils originate and how their phenotype correlates with their function. The factors that may dictate properties of (r)EOS and (i)EOS have also not been defined. Moreover, whether it is possible to identify (r)EOS and (i)EOS in the human lung and target one of the two populations from a therapeutic stand point has not been addressed.

In this study we found that even though lung eosinophils are defined as tissue-resident cells, they do not represent long-lived cells and that, contrary to current assumptions, (i)EOS may be generated locally from lung (r)EOS. Similar to the findings of Mesnil et al. (2016), we also detected (i)EOS in the circulation but we established unique phenotypes belonging to (i)EOS in each tissue compartment. This led us to identify IL-5, IL-33 and GM-CSF as lung-specific factors that may be able to locally modulate eosinophil phenotype and activation. Lung (i)EOS, but not (r)EOS, expressed high levels of CD98, which correlated with greater activation of the mTOR signalling pathway and, in turn, with amino acid and glucose uptake, as well as with enhanced metabolic activity. Functionally, (i)EOS were pro-inflammatory and more activated than (r)EOS. Importantly, we also determined that (i)EOS-expressing CD98 are not only present in allergic mice but represent a common feature of lung inflammatory responses. Therapeutically, we found that oral or intranasal administration of the SCFA butyrate could ameliorate HDM-induced lung eosinophilia and that this process may be mediated by GPR109a. Finally, analysis of lung and blood from asthmatic patients showed the presence of Siglec-8[int/hi] cells with upregulated expression of CD98 and GPR109a, which may represent an analogous population to the murine (i)EOS we characterised in the lung after allergen challenge. These data better define our understanding of eosinophil biology and development and provide potential druggable targets to ameliorate allergic lung disease.

3.3 RESULTS

3.3.1 Levels of Siglec-F^{hi}CD11b^{hi} eosinophils are increased following HDM administration and may be generated locally

Eosinophils have long been considered to exclusively function as pathogenic cells in the development of allergic asthma. However, recent evidence suggests that the relationship between eosinophils and lung pathology is more complex than previously appreciated (Jacobsen et al., 2012; Lee at al., 2010). To better understand how allergic lung inflammation affects eosinophil phenotype and function we firstly established a mouse model of allergic asthma, similar to the one used by Hammad et al. (2010). We exposed wild-type (WT) BALB/c mice to a 11-day sensitisation and challenge protocol using 100 µg and 10 µg of HDM respectively and, 24 hours after the last challenge, we digested tissues to obtain single cell suspensions (Fig. 3.1A). Eosinophils were identified in the bronchoalveolar lavage (BAL) and lung tissue by expression of their characteristic markers Siglec-F and CD11b. Alveolar macrophages (AMs) also express Siglec-F but they are negative for CD11b, and therefore could be excluded from the analysis (Supplementary Fig. 1A). This approach identified two populations of Siglec-F$^+$CD11b$^+$ eosinophils in the airways of HDM treated mice: Siglec-F^{int}CD11b^{int} and Siglec-F^{hi}CD11b^{hi} cells (Fig. 3.1B). Similar to our findings, two populations of eosinophils have previously been characterised in the allergic lung based on their expression of Siglec-F, and were defined as resident (r)EOS (Siglec-F^{int}) and inflammatory (i)EOS (Siglec-F^{high}) (Mesnil et al., 2016). Therefore, we adopted this nomenclature in our study. Allergen exposure resulted in increased total numbers of eosinophils in the airways, but specifically increased and decreased levels of (i)EOS and (r)EOS respectively compared to naïve controls (Fig. 3.1B). Temporal dynamics of eosinophils in the lung showed that proportions of (i)EOS markedly increased 24 hours after the last challenge with HDM (~10% of CD45$^+$ cells) and their levels peaked at 72 hours (~17% of CD45$^+$ cells), almost returning to baseline at 120 hours (~3% of CD45$^+$ cells) (Fig. 3.1C).

Allergic asthma is known to trigger BM and blood eosinophilia (Fahy, 2009; Inman 2000), therefore we analysed the eosinophil composition in those tissue compartments as well. As expected, HDM induced BM and blood eosinophilia, but interestingly, (i)EOS were only present in the circulation (Fig. 3.1D). Flow cytometry analysis of other mucosal tissues highlighted that allergen inhalation induced eosinophilia in the spleen but not in the intestines or the eyes (Supplementary Fig. 1B).

At steady-state, eosinophils are considered to be tissue-resident cells (Weller and Spencer, 2017; Xenakis et al, 2017). For this reason, we aimed to verify whether tissue residency actually varies between (r)EOS and (i)EOS in the lung. To this end, we used lung-shielded BM chimeras,

which were generated by a process in which WT CD45.2$^+$ mice were irradiated with their thorax shielded from irradiation. Animals were then reconstituted with congenic (CD45.1$^+$) WT BM. Shield irradiation aimed to limit confounding effects of irradiating eosinophil populations in the lung and prevent development of a local inflammatory response. At 9 weeks after irradiation, chimerism of lung eosinophils was similar to blood eosinophils both at steady-state and after HDM treatment (Fig. 3.1E). Compared to alveolar macrophages (AMs), which are known to be long-lived self-renewing resident cells (Guilliams et al., 2013) and therefore show very little contribution from donor CD45.1$^+$ cells (Supplementary Fig. 1C), lung eosinophils displayed a high degree of chimerism (~50%), implying that all eosinophils were rapidly replenished by circulating BM-derived eosinophils both at steady-state and after HDM treatment (Fig. 3.1E). The ratio of host:donor eosinophils was higher in the lung, blood and peritoneal lavage (PL) following HDM exposure compared to naïve mice, suggesting that allergen exposure may trigger local proliferation or higher turnover of eosinophils (Fig. 3.1E).

We observed that leukocytes in the lung vasculature fail to be completely removed by perfusion (data not shown), which has been reported previously (Anderson et al., 2012). Several studies suggest that intravascular staining may discriminate between tissue-localised and blood-borne cells in the mouse lung (Anderson et al., 2014; Bittner-Eddy et al., 2017; Patel et al., 2015). Therefore, to further verify whether there were differences in the origin of (r)EOS and (i)EOS, we administered AF700-conjugated CD45 antibodies (CD45 is expressed on the surface of all leukocytes) intravenously in naïve and HDM-treated mice (WT CD45.2$^+$) 5 minutes before they were euthanised. This method allowed us to label all blood-borne leukocytes and discriminate between tissue-resident cells, which stained for CD45.2. Interestingly, the results showed that despite the fact that (r)EOS in the lung have previously been defined as tissue resident cells, about 80% of them were labelled with the antibody in the lung and in the blood of both naïve and HDM-treated mice (Fig. 3.1F). This suggests that lung eosinophils are constantly being replenished by BM-derived eosinophils and cannot be considered as tissue-resident cells. Unfortunately, this method did not allow us to confirm whether (i)EOS are blood-borne or tissue-resident cells as they were not labelled by the antibody in the blood or in the lung (Supplementary Fig. 1D), suggesting that these cells are able to cleave the antibody prior to or after tissue digestion.

We also noted that exposure to lower doses of HDM (0.4 µg and 4 µg) did not trigger an increase in total eosinophils in the BM, blood or lung in contrast to the higher dose used in our initial experiments (Supplementary Fig. 1E). However, when (i)EOS were assessed specifically, we found that even a low dose of HDM could trigger a proportional increase of this eosinophil subset in the lung (Supplementary Fig. 1E), suggesting the possibility that (i)EOS may originate in the pulmonary tissue.

Eosinophil progenitors (EoPs) have been reported to extravasate into the circulation from the BM and reach the lungs where they may give rise to mature eosinophils (Johnston and Bryce, 2017). We therefore hypothesised that changes in the proportions of EoPs would be detectable if lung eosinophils derived from them. We identified EoPs as lin$^-$c-kit$^+$CD34$^+$IL-5Rα^+Siglec-F$^-$ cells (Zhu et al., 2018) (Supplementary Fig. 1F) not only in the BM but also in the circulation and in the lung, with their levels being unchanged at all three sites following HDM treatment (Supplementary Fig. 1G). These results also open up the possibility that (i)EOS may originate in the pulmonary tissue from BM-derived (r)EOS and not progenitor cells. Nevertheless, additional experiments would be needed to assess EoPs at additional timepoints in the course of allergic inflammation.

Together these data indicate that two populations of eosinophils are present in the airways following HDM exposure and that, contrary to current assumptions, they are not tissue-resident cells but constantly require replenishment from the BM.
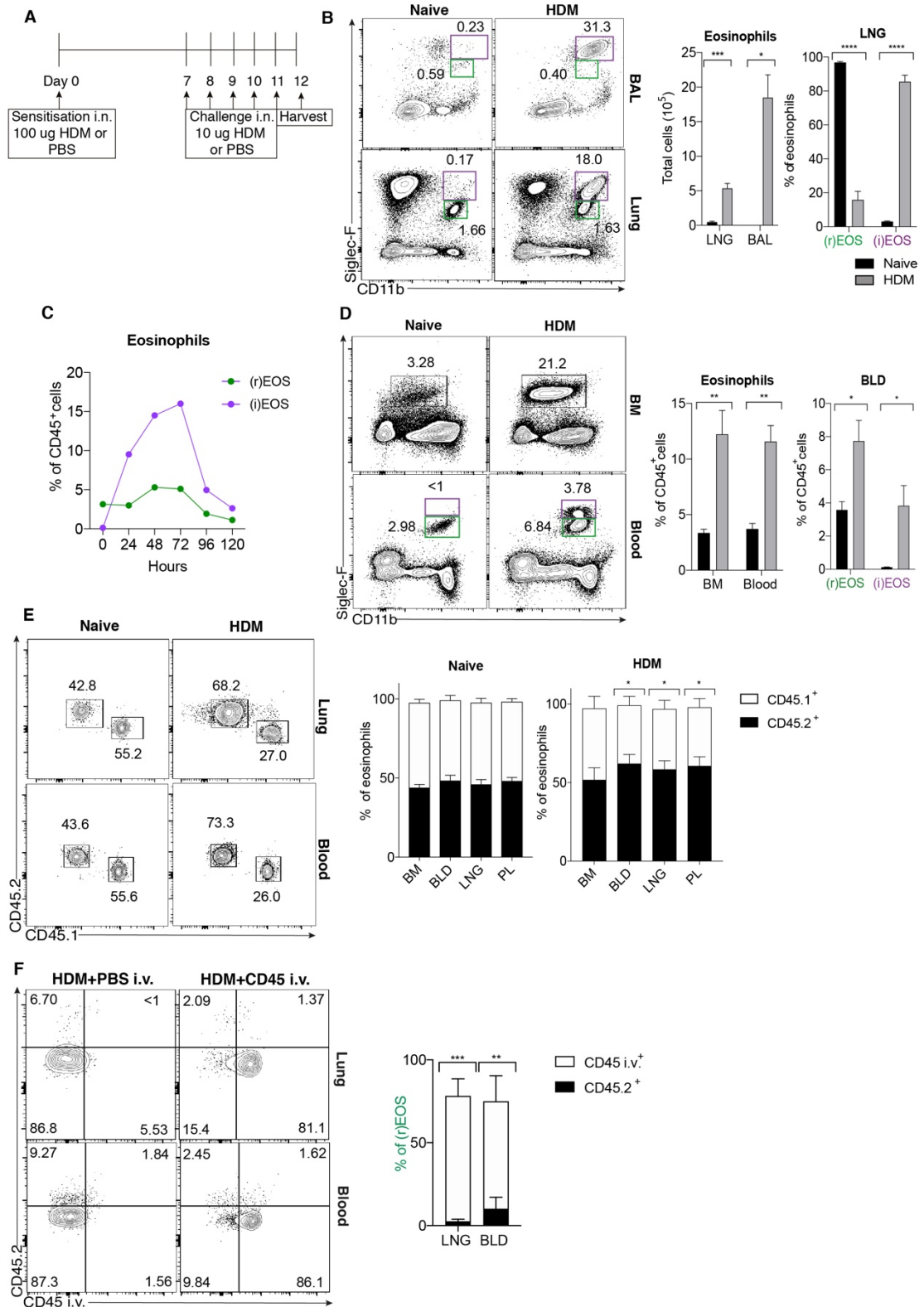
Figure 3.1: Levels of Siglec-F^{hi}CD11b^{hi} eosinophils are increased following HDM administration and may be generated locally

A) Experimental timeline. 8-12 week old female BALB/c WT mice were sensitised on day 0 with 100 µg HDM and challenged on day 7-11 with 10 µg HDM. Control mice sensitised and challenged with PBS. Mice were euthanised 24 hours after the last challenge (on day 12). B) Eosinophils were quantified by flow cytometry in the lung of naïve and HDM-treated mice and expressed as absolute cell numbers and quantification of (r)EOS

and (i)EOS was expressed as percentage of total eosinophils. C) (i)EOS and (r)EOS were quantified by flow cytometry in the lung of HDM-treated mice 24 to 120 hours after the last challenge and expressed as a percentage of CD45+ cells. D) Eosinophils were quantified by flow cytometry in the BM and blood of naïve and HDM-treated mice and expressed as a percentage of CD45+ cells. E) The percentage of eosinophils (as gated in Sup. Fig. 1A) expressing CD45.1 and CD45.2 was quantified by flow cytometry in the lung of naïve and HDM-treated lung-shielded chimeric mice. F) The percentage of (r)EOS expressing AF700-conjugated CD45 administered i.v and CD45.2 was quantified by flow cytometry in the lung and blood of HDM-treated mice. n = 3-6 per experiment. n = 1 for E. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001.

3.3.2 (i)EOS express high levels of CD98 and are more activated than (r)EOS

Although (r)EOS and (i)EOS have been characterised to some extent during allergic lung inflammation, their phenotype and functions still need to be fully determined. Mesnil et al. (2016) found that the immunoglobulin CD101 was expressed on lung (i)EOS but not (r)EOS after allergen exposure (Supplementary Fig. 2A). Similarly, our results showed that (i)EOS expressed elevated CD101 compared to (r)EOS (Fig. 3.2A).

CD98 is a transmembrane glycoprotein expressed across a broad range of cells that regulates multiple cellular processes, including amino acid transport and integrin signalling. However, aberrant expression of CD98 and associated signalling have been associated with inflammation and cancer (Kucharzik et al., 2005; Nguyen et al., 2011). Particularly eosinophils from IBD patients display increased CD98 expression and are thought to contribute to the initiation of intestinal inflammation (Xue et al., 2012). Moreover, we observed that in addition to *cd101*, the gene *Slc3a2*, which encodes for CD98 heavy chain, was also upregulated on (i)EOS compared to (r)EOS from allergic mice in the study by Mesnil et al. (2016) (Supplementary Fig. 2A).

Our study highlighted that a high proportion of lung (i)EOS expressed CD98, which like CD101, was not highly expressed on (r)EOS (Fig. 3.2A). This was also shown with t-distributed stochastic neighbor embedding (t-SNE) plots, in which each dot represents individual cells and clusters identify groups of cells with common marker expression. In fact, this method allowed us to identify two neighbouring but separate clusters in the lung corresponding to Siglec-F^{int}CD11b^{int} and Siglec-F^{hi}CD11b^{hi} eosinophils, with the latter expressing CD101 and CD98 (Supplementary Fig. 2B).

We noticed that CD101 and CD98 were largely not co-expressed by (i)EOS, with the majority of cells being single positive for each marker and a small population of double positive cells (Fig. 3.2B). Moreover, expression of these molecules did not follow the same pattern over a period of 5 days after the last HDM challenge. Expression of CD101 on (i)EOS peaked 72 hours after the last HDM challenge and progressively decreased. Conversely, CD98 expression peaked 24 hours after the last HDM challenge, subsequently decreased at 48 hours and remained unaltered at 72 hours, before increasing again at 96 hours and finally dropping at 120 hours

(Fig. 3.2C). These observations suggest that (i)EOS may be subdivided into four further populations with potentially distinct characteristics.

To further explore the phenotypic and functional differences between lung (r)EOS and (i)EOS, we used flow cytometry and histological techniques to assess surface receptor expression. Flow cytometry analysis showed that (i)EOS expressed a variety of molecules associated with pro-inflammatory responses in addition to CD98 and CD101, including TLR-4, which induces inflammatory responses by sensing the bacterial component LPS (Thoburn et al., 2016), and CD69, which represents an early activation marker (Wang et al., 2004) (Fig. 3.2D). Conversely, (r)EOS expressed higher levels of molecules associated with regulatory functions than (i)EOS, such as the co-stimulatory molecule CD86 (Woerly et al., 1999), and the transcription factor STAT5, which is phosphorylated in response to IL-5 (Buitenhuis et al., 2003) (Fig. 3.2E). Despite similar expression of the eotaxin receptor CCR3 (Fig. 3.2F), (r)EOS showed greater expression of IL-5Rα compared to (i)EOS from HDM-treated mice (Fig. 3.2G), suggesting that, although (i)EOS recognise IL-5, (r)EOS are more responsive to it.

During allergic lung inflammation eosinophils can secrete various inflammatory mediators, such as cytokines and cytotoxic proteins (McBrien and Menzies-Gow, 2017; Bandeira-Melo and Weller, 2005). To understand whether expression of pro-inflammatory molecules on (i)EOS was correlated with enhanced release of pro-inflammatory mediators, sorted (r)EOS and (i)EOS were stimulated with a combination of IL-5, GM-CSF and IL-33, cytokines that are known to support eosinophil survival and activation (Melo et al., 2013; Johnston et al., 2016 and 2017) or with the Ca^{2+} ionophore A23187. The latter functions as a less physiologic, but established stimulus to induce eosinophil activation by increasing intracellular Ca^{2+} levels (Kirino et al., 2000; Adamko et al., 2004). Stimulation with the cytokine cocktail and A23187 led to an enhanced release of IL-4 and IL-6 by (i)EOS compared to (r)EOS, which were almost refractory to stimulation (Fig 3.2H). (i)EOS also released higher levels of the granule protein eosinophil peroxidase (EPX) compared to (r)EOS but only in response to A23187 (Fig. 3.2H). These results suggest that (i)EOS are more activated than (r)EOS and actively participate in the immune response to allergen inhalation in the lung.

As previously described by Mesnil et al., (2016), we also detected differences in the morphology and tissue location of (r)EOS and (i)EOS. The latter population displayed more segmented nuclei and larger cytoplasmic areas, compared to (r)EOS showing typical ring-shaped nuclei and restricted cytoplasmic areas (Fig. 3.2I). Mesnil et al. (2016) also showed that (i)EOS upregulated expression of ALOX15 and hypoxia-inducible factor-1-alpha (HIF-1α) when compared to (r)EOS (Supplementary Fig. 2A). The gene ALOX15 encodes for the enzyme arachidonate-15 lipoxygenase (15-LO), which is a member of the lipoxygenase family that metabolizes arachidonic acid (AA) to 15(S)-hydroperoxy-eicosatetraenoic acid (15-(S)-HETE)

and is mainly expressed in eosinophils, activated monocytes, epithelial cells and mast cells (Claesson et al., 2008). 15-LO activity in eosinophils has been associated with production of the pro-inflammatory mediators eoxins (Feltenmark et al., 2008) and has been suggested to promote eosinophil infiltration in eosinophilic inflammatory diseases, such as eosinophilic esophagitis and asthma (Matoso et al., 2014; Chu et al., 2002). HIF-1α is a transcription factor activated by hypoxia and upregulates transcription of several pro-inflammatory cytokines, chemokines and adhesion molecules. (Biddlestone et al., 2015). Like ALOX15, expression of HIF-1α has been associated with enhanced inflammatory responses in asthma, since genetic and pharmacologic inhibition of its signalling resulted in diminished eosinophilia, airway remodelling and inflammation in mouse models (Huerta-Yepez et al., 2011; Dewitz et al., 2017; Ning et al., 2019). In our study, staining for the eosinophil-specific granule protein EPX allowed us to identify all lung eosinophils in naïve and HDM-treated mice (Fig. 3.2J), clearly showing eosinophilia in the lungs of HDM-treated mice. Additionally, co-staining of EPX with either ALOX-15 or HIF-1α allowed us to identify (i)EOS, which were bright for both markers, and to localise them mainly around the bronchi in lung tissue sections (Fig. 3.2J).

Altogether these data show that (i)EOS represent a subset of (r)EOS with pro-inflammatory functions that correlate with acquired responsiveness to a variety of inflammatory mediators, as well as alterations in their morphology and tissue location.
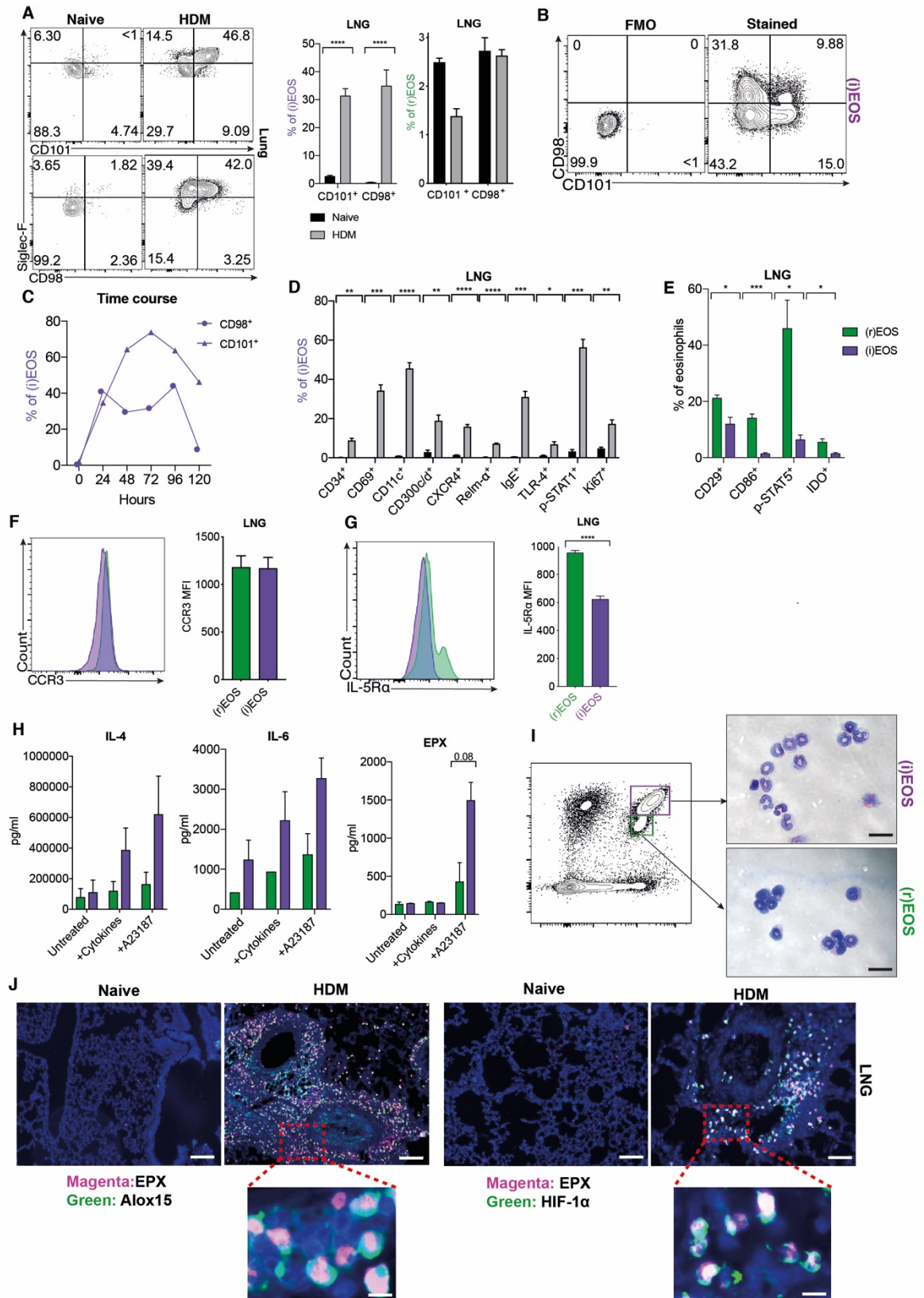
Figure 3.2: (i)EOS express high levels of CD98 and are more activated than (r)EOS
A) The percentage of eosinophils expressing CD101 and CD98 was quantified by flow cytometry in the lung of naive and HDM-treated mice. B) The percentage of (i)EOS expressing CD101 and CD98 was quantified by flow cytometry in the lung HDM-treated mice. C) The percentage of (i)EOS expressing CD101 and CD98 was quantified by flow cytometry in the lung of HDM-treated mice 24 to 120 hours after the last challenge. D) The

percentage of (i)EOS expressing CD34, CD69, CD11c, CD300c/d, CXCR4, Relm-α, IgE, TLR4, pSTAT1 and Ki67 was quantified by flow cytometry in the lung of naïve and HDM-treated mice. E) The percentage of (r)EOS and (i)EOS expressing CD29, CD86, pSTAT5 and IDO was quantified by flow cytometry in the lung of HDM-treated mice. F) Expression of CCR3 by lung (r)EOS and (i)EOS from HDM-treated mice was quantified by flow cytometry and expressed as MFI. G) Expression of IL-5Rα by lung (r)EOS and (i)EOS from HDM-treated mice was quantified by flow cytometry and expressed as MFI. H) Levels of IL-4, IL-6 and EPX were determined by ELISA from the supernatant of sorted lung (r)EOS and (i)EOS cultured in the presence or absence of IL-33, GM-CSF and IL-5 or with A23187 for 24 hours. I) Cytospins of sorted lung (r)EOS and (i)EOS were stained with Diff-Quik (scale bar 10 µm). J) Representative staining of lung tissue for EPX (magenta), ALOX15 or HIF-1α (green) and counterstained with DAPI (blue) (scale bar 100 µm and 10 µm). n = 3-6 per experiment. n = 1 for C. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: $p < 0.05$; $**p < 0.01$; $***p < 0.001$

3.3.3 Factors enriched in the lung environment support upregulation of Siglec-F and expression of CD98 on eosinophils

Release of a variety of factors from the airway epithelium and pulmonary immune cells is known to occur during allergic lung inflammation (Bartemes and Kita, 2012; Barnes, 2008). Several cytokines including the canonical Th2-associated cytokines IL-4, IL-13, IL-5 and IL-9, the pro-inflammatory cytokine IL-18, the growth factors GM-CSF and TGF-β, as well as the alarmin IL-33, are known to be involved in promoting Th2 responses in the airways as well as eosinophil recruitment, survival and activation (McBrien and Menzies-Gow, 2017). To address if some of these factors could induce characteristic features of (i)EOS, such as expression of CD98 and Siglec-F upregulation, we firstly incubated BM eosinophils from naïve and HDM-treated mice with the above-mentioned cytokines for 24 hours at 37°C. This determined that IL-5, GM-CSF and IL-33 to a greater extent upregulated Siglec-F expression on BM eosinophils with no differences between naïve and HDM-treated mice (Fig. 3.3A), whereas other mediators such as TGF-β had no effect (Fig. 3.3A).

Since we identified IL-5, GM-CSF and IL-33 as being responsible for Siglec-F upregulation on BM eosinophils, we combined all the cytokines to understand whether together they induced a higher increase in Siglec-F expression compared to each cytokine alone. Interestingly, the results showed no cumulative effect when looking at Siglec-F expression on eosinophils (Fig. 3.3B). Platelet activating factor (PAF) has also been largely used to activate eosinophils *in vitro* and to stimulate their degranulation (Kroegel et al., 1988; Bartemes et al., 1999; Dyer et al., 2010). Therefore, we investigated the effects of eosinophil stimulation with PAF. BM eosinophils were stimulated *in vitro* using a procedure similar to Dyer et al., 2010, which involved incubation with PAF alongside IL-33 and the Ca²⁺ ionophore Ionomycin for 3 hours at 37°C. Interestingly, the results showed that this treatment induced the generation of a Siglec-F^{int} and a Siglec-F^{hi} population of BM eosinophils, reminiscent of (r)EOS and (i)EOS seen in the lungs of allergic mice (Fig. 3.3C). To better understand whether the combination of these factors or each factor alone was responsible for such effect, we cultured BM eosinophils with PAF, IL-33 or Ionomycin alone, PAF with Ionomycin or IL-33 with Ionomycin. Surprisingly, the results showed that only

the combination of IL-33 and Ionomycin was able to induce the generation of Siglec-F^{int} and Siglec-F^{hi} populations of BM eosinophils, whereas each factor alone did not (Supplementary Fig. 3). This suggests that PAF has no impact on Siglec-F expression and that a shorter stimulation together with the addition of Ionomycin to IL-33 affected Siglec-F expression differentially compared to a 24 hours stimulation with IL-33, GM-CSF and IL-5. Since Ionomycin increases intracellular Ca^{2+} concentrations (Dedkova et al., 2000), the results suggest that changes in Siglec-F expression may be dependent on Ca^{2+} influx into the cell.

Since we detected eosinophilia in the BM and blood and (i)EOS in the circulation (Fig. 3.1C), we aimed to establish whether eosinophils at those distal sites exhibited the same phenotype identified in the lung. Flow cytometry analysis showed that both eosinophils in the BM and in the blood expressed very little CD98 (Fig. 3.3D) and did not express CD101 (data not shown) compared to lung (i)EOS. This suggests that together with upregulation of Siglec-F expression on eosinophils, CD98 expression may also be driven by changes occurring in the lung environment following allergen exposure. Considering the effects seen so far on Siglec-F expression, we investigated whether IL-33, GM-CSF and IL-5 could also upregulate expression of CD98 on eosinophils. Given that there was no difference on Siglec-F expression on BM eosinophils from naïve or HDM-treated mice, we stimulated BM eosinophils from HDM-treated mice with each cytokine for 24 hours. Surprisingly, GM-CSF and IL-33 to a greater extent had the ability to enhance CD98 expression on BM eosinophils, whereas IL-5 alone did not (Fig. 3.3E). In support of this, *Il-33–LacZ* gene trap (Gt) reporter strain (Il-33$^{Gt/Gt}$), which lack endogenous IL-33 (Pichery et al., 2012), treated with HDM showed a significant reduction in CD98 expression on lung (i)EOS compared to Il-33$^{+/Gt}$ heterozygous controls (Fig. 3.3F).

Together these data suggest that eosinophil phenotype can be modulated by exposure to specific factors independently of *in vivo* priming or tissue of origin. In this case, IL-33 together with GM-CSF and IL-5, whose levels are known to be elevated in the airways during allergic responses, may be able to shape eosinophil phenotype and function by upregulating their expression of Siglec-F and CD98.
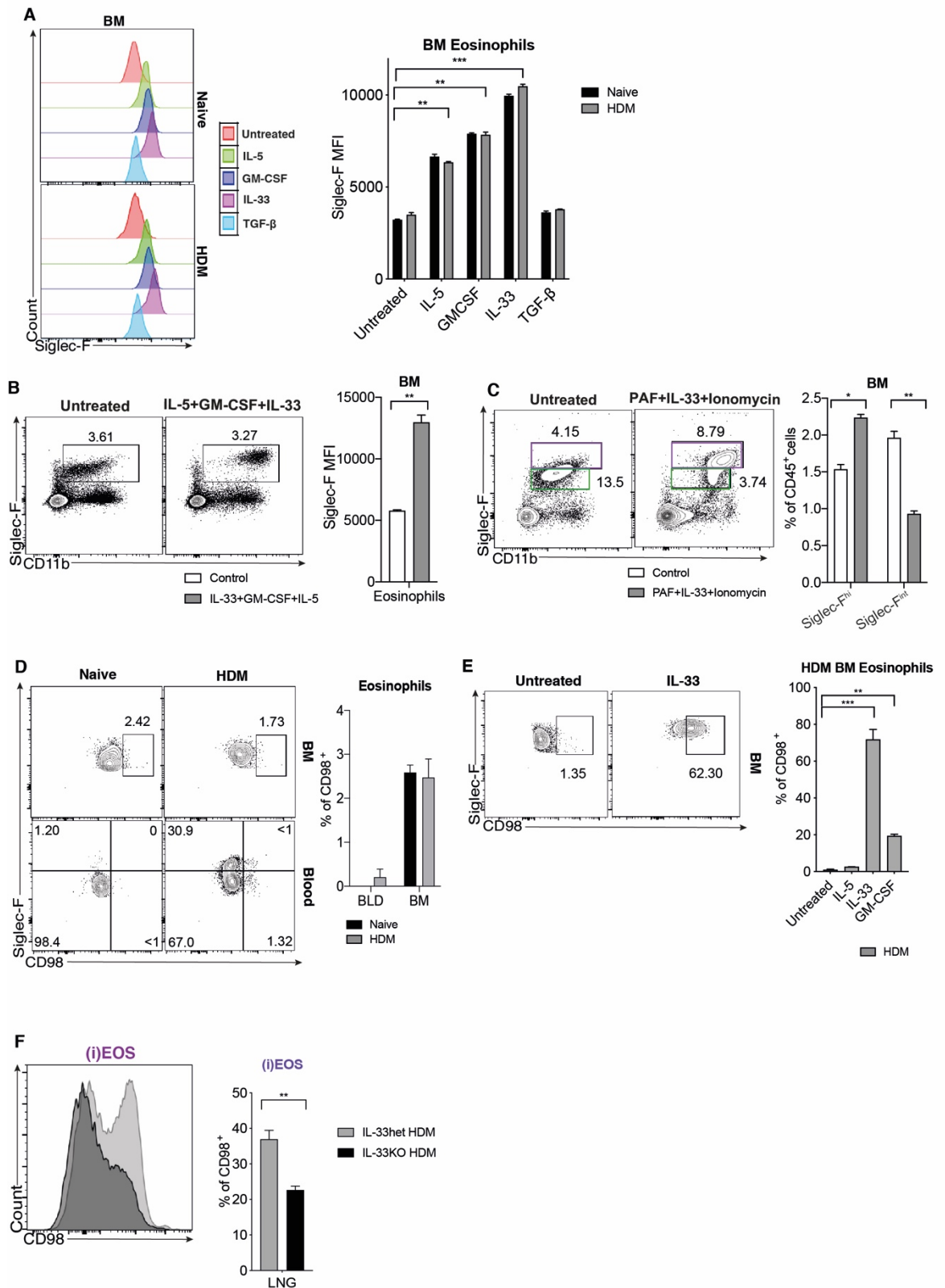
Figure 3.3: The lung environment supports activation of (i)EOS and expression of CD98

A) Expression of Siglec-F by BM eosinophils from naïve and HDM-treated mice cultured in the presence or absence of IL-5 or GM-CSF or IL-33 or TGF-β for 24 hours was quantified by flow cytometry and expressed as MFI. B) Expression of Siglec-F by BM eosinophils from naïve mice cultured in the presence or absence of IL-5, GM-CSF and IL-33 for 24 hours was quantified by flow cytometry and expressed as MFI. C) Siglec-F[hi] and Siglec-F[int] eosinophils were quantified by flow cytometry from the BM of naïve mice cultured in the presence or absence of PAF+IL-33+Ionomycin for 3 hours and expressed as a percentage of CD45^{+} cells. D) The

percentage of eosinophils expressing CD98 was quantified by flow cytometry in the BM and blood of naive and HDM-treated mice. E) The percentage of BM eosinophils from HDM-treated mice cultured in the presence or absence of IL-33 or GM-CSF or IL-5 for 24 hours expressing CD98 was quantified by flow cytometry. F) The percentage of (i)EOS expressing CD98 was quantified by flow cytometry in the lung of Il-33$^{Gt/Gt}$ and Il-33$^{+/Gt}$ HDM-treated mice. Data shown are representative from at least two independent experiments. n = 2-6 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (B, C, D, F) or one-way ANOVA (A, E): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.4 CD98 expression on (i)EOS correlates with increased nutrient uptake and metabolic activity

As mentioned previously (section 3.3.2), CD98 facilitates amino acid transport into cells. It does so by covalently binding large amino acid transporter 1 (LAT1), also known as SLC7A5, allowing its stability and insertion into the cell membrane. This allows the functional transport of neutral amino acids, such as leucine, isoleucine and arginine in exchange for glutamine (Cantor and Ginsberg, 2012). Intake of extracellular amino acids activates the serine and threonine kinase mammalian target of rapamycin (mTOR), which initiates a cascade of phosphorylation events that plays a central role in cell growth and survival (Nguyen and Merlin, 2012). To investigate whether CD98 expression on (i)EOS correlated with enhanced nutrient uptake and metabolic activation, (i)EOS were firstly examined by flow cytometry for expression of phosphorylated mTOR and S6. The latter is a ribosomal protein activated downstream of mTOR involved in regulating protein translation and the eukaryotic initiation factor 3 (eIF3) translation complex (Yerlikaya et al., 2016). (i)EOS showed elevated expression of both phosphorylated mTOR and S6 compared to (r)EOS, which displayed little expression of either molecule (Fig. 3.4A). These observations suggest that HDM-induced CD98 expression on (i)EOS is likely correlated with activation of the mTOR signalling pathway.

To further elucidate whether activation of the mTOR signalling pathway is correlated with increased nutrient uptake, we examined amino acid and glucose uptake by incubating lung cell suspensions with kynurenine and the glucose analogue 2-NBDG. Kynurenine is the product of tryptophan metabolism and is known to be transported across the cell membrane by LAT-1/CD98 complex (Sinclair et al., 2018). The amino acid lysine does not affect LAT-1 transport of amino acids, therefore it was used as positive control. 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), which is a System L transporter inhibitor, was used as a negative control (Sinclair et al., 2018). The results showed that although both (r)EOS and (i)EOS were capable of taking up kynurenine, kynurenine uptake was increased in (i)EOS compared to (r)EOS (Fig. 3.4B). Kynurenine uptake was unaltered by lysine and inhibited by BCH, confirming that its transport is mediated by LAT-1/CD98 complex (Fig. 3.4B). Incubation with 2-NBDG also showed that both (r)EOS and (i)EOS could uptake glucose but (i)EOS displayed greater intake of the molecule (Fig. 3.4C).

To verify whether increased nutrient uptake by (i)EOS is correlated with higher metabolic activity, we performed a glycolytic stress test on sorted lung eosinophils. The assay analyses the rate of glucose catabolism into lactate by measuring the acidification rate of the extracellular medium (ECAR) (Mookerjee et al., 2016). Cells were firstly incubated with glucose, which triggers glycolysis, then with oligomycin, which inhibits the mitochondrial ATP synthase and further drives glycolysis-mediated ATP production, and finally with 2-Deoxy-D-glucose (2-DG), which inhibits glycolysis (Mookerjee et al., 2016). This allows the measurement of glycolysis, thus the conversion of glucose into lactate, glycolytic capacity, defined as the maximum rate of glycolysis, and glycolytic reserve, which represents the difference between glycolytic capacity and glycolysis (Mookerjee et al., 2016). Our results firstly showed that (r)EOS from HDM-treated mice exhibited a ~4% increase in glycolysis, glycolytic capacity and reserve compared to (r)EOS from naïve mice (Fig. 3.4D). Strikingly, (i)EOS showed the greatest increase in glycolysis, glycolytic capacity and reserve (~10%) compared to both (r)EOS populations (Fig. 3.4D).

CD98 has not only been associated with metabolic functions but it has also been linked with control of cell growth, survival and proliferation by binding to integrins, such as CD29 (integrin-β1) (Rintoul et al., 2002). CD29 expression was upregulated on (i)EOS after HDM exposure and co-stained with CD98 (Fig. 3.4E). This suggests that CD98 may have functions beyond metabolic control of (i)EOS, such as modifying their tissue retention via regulation of integrin functions.

These data confirm that HDM exposure leads to metabolic alterations to lung eosinophils, with changes to the inflammatory population in particular.
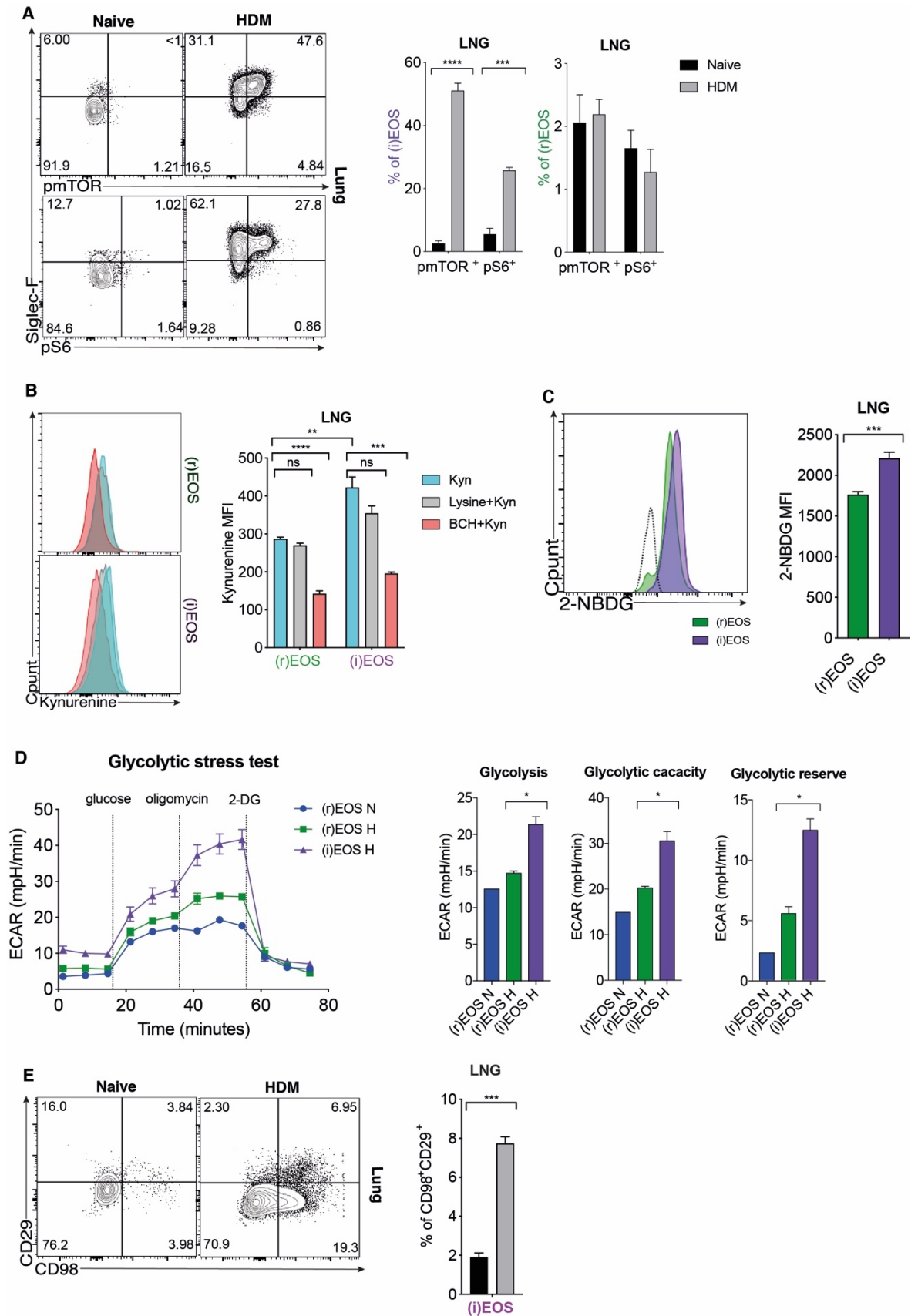
Figure 3.4: CD98 expression on (i)EOS correlates with increased nutrient uptake and metabolic activity
A) The percentage of (i)EOS and (r)EOS expressing pmTOR and pS6 was quantified directly *ex vivo* by flow cytometry in the lung of naive and HDM-treated mice. B) The percentage of (i)EOS and (r)EOS uptaking kynurenine following treatment with kynurenine alone or with lysine or BCH was quantified by flow cytometry in

the lung of HDM-treated mice and expressed as MFI. C) The percentage of (i)EOS and (r)EOS uptaking 2-NBDG was quantified by flow cytometry in the lung of HDM-treated mice and expressed as MFI. D) ECAR of sorted lung eosinophils (Siglec-F^{hi}CD11b^{hi} and Siglec-F^{int}CD11b^{int}) from naïve and HDM-treated mice at baseline and after sequential treatment (vertical lines) with glucose, oligomycin or 2-DG to measure glycolysis, glycolytic reserve and glycolytic capacity. E) The percentage of (i)EOS expressing CD98 and CD29 was quantified by flow cytometry in the lung of HDM-treated mice. Data shown are representative from at least two independent experiments. n = 4-5 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (A, C, E) or one-way ANOVA (D) or two-way ANOVA (B): *p < 0.05; **p < 0.01; ***p < 0.001.

3.3.5 (i)EOS and CD98 expression are a common feature of lung inflammation

To our knowledge, the presence of two distinct populations of eosinophils in the lung has only been reported in mouse models of allergic asthma (Mesnil et al., 2016; Abdala Valencia et al., 2015). For this reason, we investigated whether they were also present in other murine models of lung inflammatory diseases.

Following infection with Influenza virus, an increase in the percentage of (i)EOS in the lung was seen, with a large proportion of these cells expressing CD98 (Fig. 3.5A-B). However, compared to our allergy model, Influenza infection did not lead to increased proportions of eosinophils in the airways, compared to naïve mice (Fig. 3.5A).

Helminth infections are known to induce systemic Th2 responses (Mishra et al., 2014). We infected mice with three different helminth parasites to assess changes in lung eosinophils. *Heligmosomoides polygyrus bakeri* resides in the small intestine throughout its lifespan, but is known to provoke systemic inflammation, including in the lung (Mohrs et al., 2005; Filbey et al., 2019). At day 7 after infection, both (r)EOS and (i)EOS were indeed present in the lung and a significant proportion of (i)EOS expressed CD98 in infected mice (Fig. 3.5C-D). The proportions of total eosinophils increased in the lung of mice infected with the parasite compared to naïve mice, similarly to our allergy model (Fig. 3.5C).

Nippostrongylus brasiliensis hookworm larvae enter the circulation through the skin and migrate to the lung where they cause epithelial damage and blood loss (Harvie et al., 2010). This triggers immune responses mainly mediated by ILCs, Th2 cells and macrophages aimed at protecting the airways from infection and repairing damage (Bouchery et al., 2015). Maturing larvae are then transported up the respiratory tract where they are ingested to finally reach the small intestine, where they mate before being expelled 7-9 days after infection (Bouchery et al., 2017). Both (r)EOS and (i)EOS were found in the lung 6 days after infection with *N. brasiliensis* and again, a significant proportion of (i)EOS expressed CD98 (Fig. 3.5E-F).

Schistosoma mansoni larvae (cercariae) also migrate via the lungs but enter the hepatic portal system to reach the bladder where the adult worms reside (Butrous, 2019). *Schistosoma* eggs

can become trapped in areas of venous drainage in the lung and liver causing aggregation of inflammatory cells (termed granulomas), such as macrophages, lymphocytes and eosinophils, which drive generation of Th2 inflammatory responses (Mickael and Graham, 2019). In our study, mice were infected with 20 cercariae on day 0 and 7 and subsequently with 80 cercariae on day 14. This infection regime aimed to mirror natural repeated exposure to the parasite in humans (Colley and Secor, 2014). Tissues were analysed 28 days after the last infection to avoid egg-induced immune responses clouding over larvae-induced immune responses. Mice infected with *S. mansoni* developed significant eosinophilia, with both (r)EOS and (i)EOS being present in the lung. Also in this case, (i)EOS showed high expression of CD98 (Fig. 3.5G-H).

Together these data show that generation of (i)EOS is not restricted to allergic lung disease but represent a feature of inflammatory responses in the lung irrespectively of the upstream cause. Moreover, expression of CD98, therefore increased metabolic activation, is also a commonly acquired characteristic of (i)EOS during lung inflammation. This suggests that (i)EOS and CD98 expression may be considered as common bio-markers to identify lung inflammatory responses.
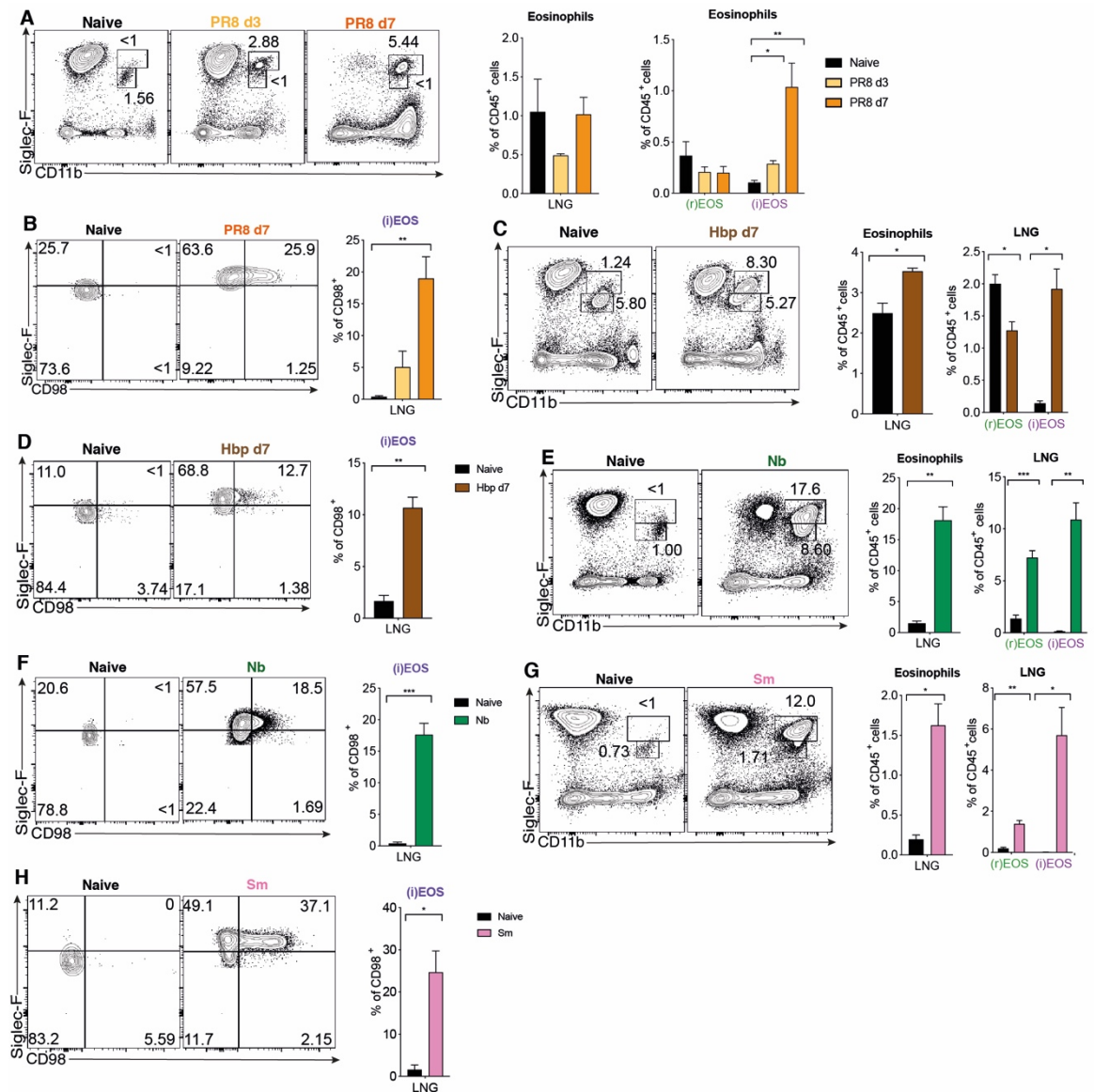
Figure 3.5: (i)EOS and CD98 expression are a common feature of lung inflammation
A) Eosinophils were quantified by flow cytometry in the lungs of naïve and Influenza (PR8 strain)-infected mice and expressed as a percentage of CD45$^+$ cells. B) The percentage of eosinophils expressing CD98 was quantified by flow cytometry in the lungs of naive and Influenza (PR8 strain)-infected mice. C) Eosinophils were quantified by flow cytometry in the lungs of naïve and *H. polygyrus*-infected mice and expressed as a percentage of CD45$^+$ cells. D) The percentage of eosinophils expressing CD98 was quantified by flow cytometry in the lungs of naive and *H. polygyrus*-infected mice. E) Eosinophils were quantified by flow cytometry in the lungs of naïve and *N. brasiliensis*-infected mice and expressed as a percentage of CD45$^+$ cells. F) The percentage of eosinophils expressing CD98 was quantified by flow cytometry in the lungs of naive and *N. brasiliensis*-infected mice. G) Eosinophils were quantified by flow cytometry in the lungs of naïve and *S. mansoni*-infected mice and expressed as a percentage of CD45$^+$ cells. H) The percentage of eosinophils expressing CD98 was quantified by flow cytometry in the lungs of naive and *S. mansoni*-infected mice. Data shown are from one experiment. n = 3-5. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (C, D, E, F, G, H) or one-way ANOVA (A): *p < 0.05; **p < 0.01; ***p < 0.001.

3.3.6 Lung (i)EOS express high levels of GPR109a

Our results identifying (i)EOS as pro-inflammatory cells with higher metabolic activity, prompted us to investigate whether this population had specific features that could inform mechanisms for

therapeutic manipulation. Short chain fatty acids (SCFAs) generated by the gut microbiota, including acetate, propionate and butyrate, have recently been shown to have immunomodulatory and anti-inflammatory effects in intestinal and lung inflammatory diseases (Dang and Marsland, 2019). These effects can be mediated by SCFAs binding to the G-protein coupled receptors (GPCRs) GPR41, GPR43 or GPR109a or by intracellular inhibition of histone deacetylases (HDACs) (Dang and Marsland, 2019). In the context of allergic asthma, SCFAs have been shown to indirectly induce such effects by targeting antigen-presenting cells (APCs) in the BM in a receptor-mediated manner (GPR41 and GPR43 may both be involved) (Maslowski et al., 2009; Trompette et al., 2014). Nevertheless, it is not clear whether SCFAs may directly affect cell populations present in the lung, such as eosinophils, during allergic lung inflammation.

To this end, we firstly sorted (r)EOS and (i)EOS from the lung of HDM-treated mice and assessed their expression of SCFA GPCRs. Blood neutrophils have been reported to express GPR109a, whereas blood monocytes are not thought to express the receptor (Kostylina et al., 2008, Theiler et al., 2019). Therefore, we sorted lung neutrophils and monocytes alongside eosinophils, and used them as positive and negative controls respectively. Interestingly, immunofluorescence staining showed that (i)EOS displayed higher expression of the SCFA receptors GPR109a compared to (r)EOS and, as expected, the receptor was also expressed by neutrophils and not expressed by monocytes (Fig. 3.6A). Moreover, the microarray sequencing from Mesnil et al. (2016) also showed that *Hcar2*, the gene encoding for GPR109a, was upregulated on (i)EOS compared to (r)EOS from naïve and allergic mice (Supplementary Fig. 4). (i)EOS also showed higher expression of GPR43 compared to (r)EOS and in contrast with the literature, monocytes also displayed GPR43 expression (Fig. 3.6B). On the other hand, (i)EOS showed lower expression of GPR41 compared to (r)EOS and monocytes showed negligible staining (Fig. 3.6C).

Flow cytometry analysis confirmed that lung (i)EOS expressed higher levels of GPR109a compared to (r)EOS in allergic mice but, interestingly, blood (i)EOS, (r)EOS and BM eosinophils expressed very low levels of the receptor (Fig. 3.6D). This suggests that factors enriched in the lung environment may support expression of GPR109a on eosinophils during allergic inflammation. Whilst GPR41 and GPR43 can be bound by all SCFAs, GPR109a is selective for butyrate (Kasubuchi et al., 2015), suggesting that butyrate in particular could have important effects in modulating eosinophil phenotype and/or function. Butyrate is not the only agonist of GPR109a - β-hydroxybutyrate and niacin, also known as nicotinic acid or vitamin B3, can also bind and activate the receptor (Singh et al., 2014). Since we demonstrated that IL-33, GM-CSF and IL-5 induce upregulation of CD98 on BM eosinophils from naïve mice, we used the same cytokines to test whether they may support expression of GPR109a on eosinophils. This time,

we also wanted to establish whether addition of the GPR109a agonists butyrate or niacin may affect GPR109a expression. The results showed that cytokine stimulation and addition of niacin in particular slightly enhanced expression of GPR109a on BM or lung eosinophils (Fig. 3.6E).

These data show for the first time that only a subset of murine eosinophils expresses higher levels of the butyrate and niacin receptor GPR109a in the lung but not in the blood or BM of allergic mice. Moreover, expression of this receptor on eosinophils may be dictated by the factors present in the lung environment, rendering such cells more responsive to stimulation with butyrate or niacin.
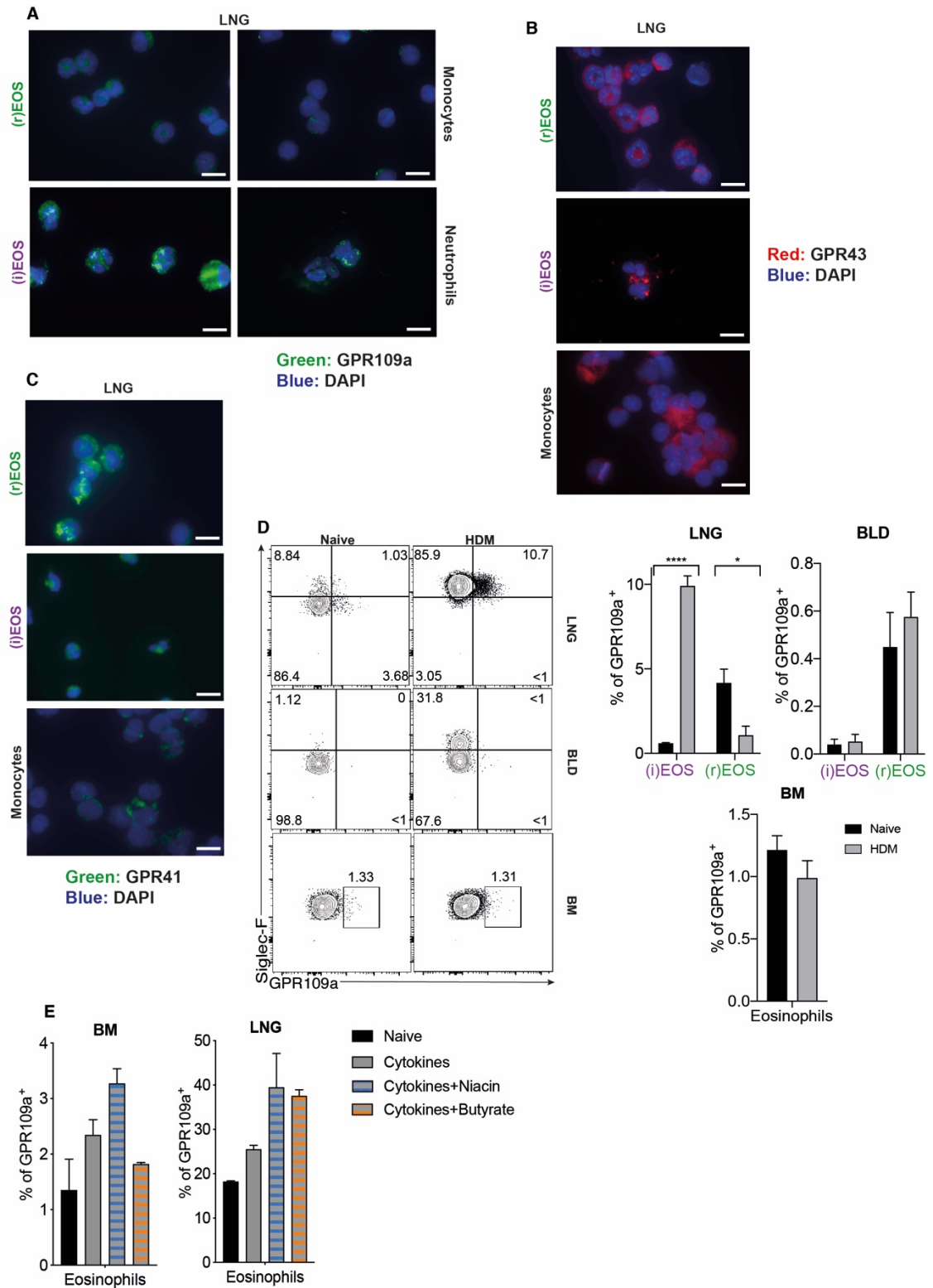
Figure 3.6: Lung (i)EOS express high levels of GPR109a

A) Representative staining of sorted (r)EOS, (i)EOS, monocytes and neutrophils from the lungs of HDM-treated mice for GPR109a (green) and counterstained with DAPI (blue) (scale bar 10 μm). B) Representative staining of sorted (r)EOS, (i)EOS and monocytes from the lungs of HDM-treated mice for GPR43 (red) and counterstained with DAPI (blue) (scale bar 10 μm). C) Representative staining of sorted (r)EOS, (i)EOS and monocytes from the lungs of HDM-treated mice for GPR41 (green) and counterstained with DAPI (blue) (scale bar 10 μm). D) The percentage of eosinophils expressing GPR109a was quantified by flow cytometry in the lungs, BM and blood of naive and HDM-treated mice. E) The percentage of lung and BM eosinophils from naïve

mice expressing GPR109a following culture in the presence or absence of IL-33, GM-CSF and IL-5 alone or with butyrate or niacin for 24 hours was quantified by flow cytometry. Data shown are from at least two representative experiments. n = 2-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (D) or one-way ANOVA (E): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.7 Oral administration of butyrate ameliorates BM and lung eosinophilia following exposure to HDM

SCFAs are the products of dietary fibre fermented by intestinal commensal bacteria, and are therefore present in high amounts in the intestines (Tan et al., 2014). There they maintain homeostasis by fortifying barriers of defence and by their anti-inflammatory effects (Koh et al., 2016). Despite SCFAs being largely concentrated in the gut, they can be absorbed into the bloodstream and there is evidence for SCFA-mediated anti-inflammatory effects in the periphery (Dang and Marsland, 2019). Importantly, several studies suggest that SCFAs are beneficial in reducing allergic inflammatory responses (Maslowski et al., 2009; Trompette et al, 2014; Cait et al., 2018). Although Theiler et al. (2019) has recently shown that butyrate may affect eosinophil survival and migration, the full spectrum of effects on lung eosinophil populations still has to be addressed.

Our data showed that (i)EOS express higher levels of the butyrate-selective GPR109a compared to (r)EOS. To firstly understand whether the gut microbiota and derived metabolites could affect eosinophil phenotype at steady-state we looked at lung eosinophils from specific pathogen free (SPF) mice i.e. with a normal microbiome, and germ-free (GF) mice. Interestingly, GF mice showed reduced levels of eosinophils in the BM, whereas increased proportions were found in the lung (Fig. 3.7A). Closer examination identified both (r)EOS and (i)EOS in the lungs of GF mice whereas, as also shown in previous experiments, SPF mice have very low proportions of (i)EOS at steady-state (Fig. 3.7A). Comparable to our mouse model of allergic asthma, expression of CD98 and CD101 was also present on (i)EOS from naïve GF mice (Fig. 3.7B). These data therefore suggest that the gut microbiome or derived metabolites can affect the lung environment and impact eosinophil phenotype and function.

The results from GF mice prompted us to investigate whether administration of the SCFA butyrate could have beneficial effects in reducing allergic inflammation in our model and whether this was reflected by changes in (r)EOS and (i)EOS. To this end, we orally administered butyrate in the drinking water to BALB/c mice for a period of 2 weeks prior and then during HDM treatment (Fig. 3.7C). Allergic mice treated with butyrate showed that this SCFA significantly decreased the frequencies of eosinophils and (i)EOS in particular (Fig. 3.7D). Butyrate not only decreased (i)EOS proportions in the lung but also their activation, as shown by reduced expression of both CD98 and CD101 (Fig. 3.7E). HDM-induced eosinophilia in the BM was also decreased by butyrate, suggesting that oral treatment with the SCFA may target eosinophils at several sites

(Fig. 3.7D). The anti-inflammatory effects of butyrate on eosinophils were also extended to the whole lung environment, as shown by reduced mRNA expression of Th2-associated inflammatory mediators (Fig. 3.7F). We measured expression of IL-13 and IL-4, the key cytokines involved in orchestrating Th2 responses (Junttila, 2018), Relm-α and Ym-1, effector molecules known to be abundantly released during Th2 inflammatory responses (Sutherland et al., 2018), CCL11, an eosinophil-selective chemokine (Broide and Sriramarao, 2001), and Muc5ac, a mucin expressed on goblet cells within the tracheal and bronchial epithelium known to be highly expressed during asthma development (Bonser and Erle, 2017). Allergen exposure upregulated expression of all transcripts analysed, which was significantly reduced by treatment with butyrate, suggesting that this SCFA can induce broad anti-inflammatory effects in the lung tissue.

In summary, our data support the idea that the microbiota and derived SCFAs, with butyrate in particular, are important in balancing immune responses at mucosal sites. In this case, GF mice showed that the gut microbiome may have an important role in promoting tolerance and homeostasis not only in the intestine but also in the airways. Moreover, we showed that the anti-inflammatory effects of butyrate were evidenced by reduced eosinophilia and eosinophil activation as well as expression of Th2 mediators in the lungs of HDM-treated mice. Therefore, butyrate reduced lung inflammation via direct targeting of eosinophils, indirect changes to the inflamed environment, or both.
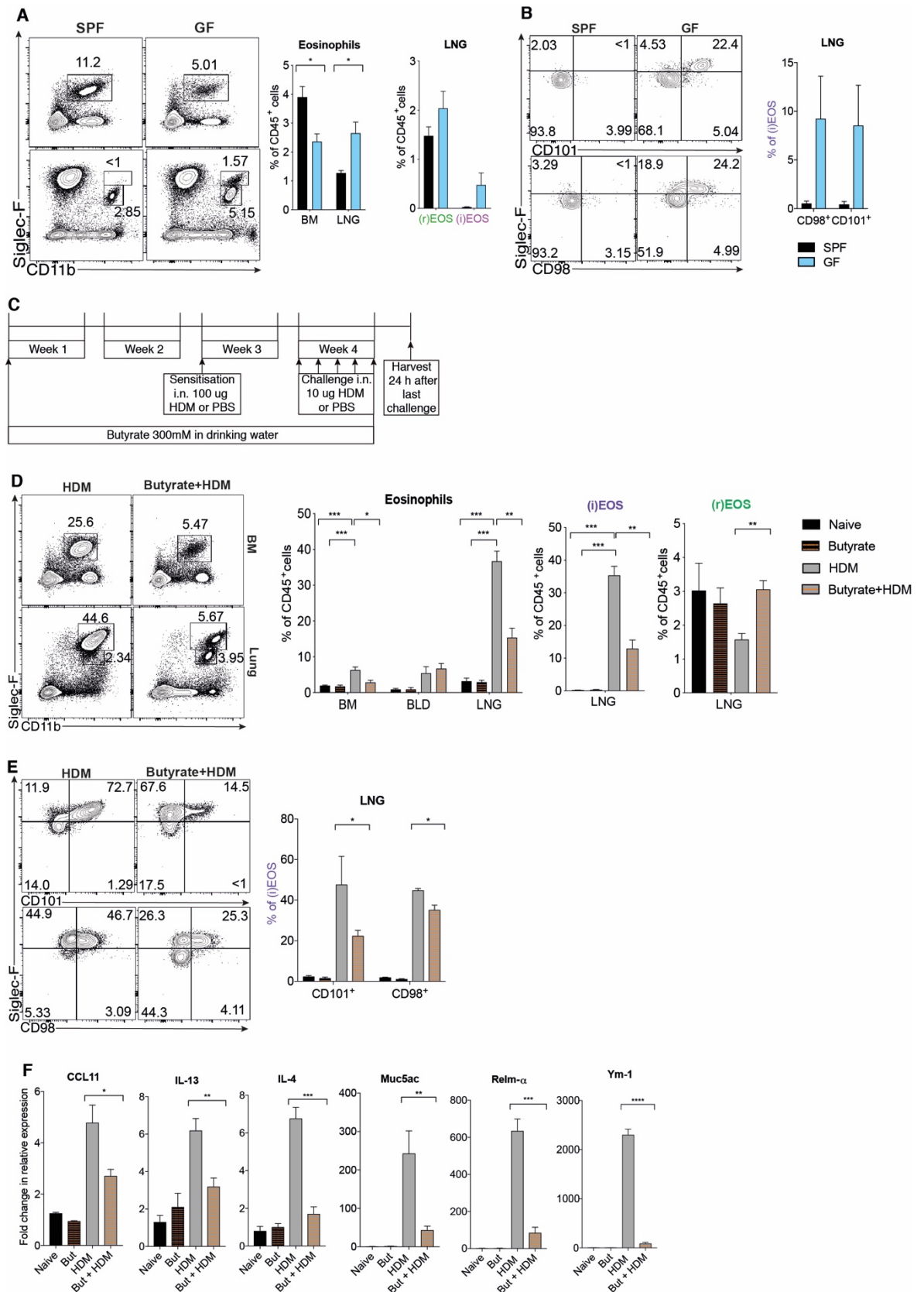
Figure 3.7: Oral administration of butyrate ameliorates BM and lung eosinophilia following exposure to HDM

A) Eosinophils were quantified by flow cytometry in the lung of SPF and GF mice and expressed as a percentage of CD45+ cells. B) The percentage of eosinophils expressing CD101 and CD98 was quantified by flow cytometry in the lung of SPF and GF mice. C) Experimental timeline. 8-12 week old female BALB/c WT mice were

administered 300 mM butyrate in the drinking water for two weeks prior and during HDM treatment. Mice were euthanised 24 hours after the last challenge with HDM (on day 12). D) Eosinophils were quantified by flow cytometry in the lung and BM of naive mice and mice treated with HDM or butyrate alone and HDM with butyrate and expressed as a percentage of $CD45^+$ cells. E) The percentage of eosinophils expressing CD101 and CD98 was quantified by flow cytometry in the lung and BM of naive mice and mice treated with HDM or butyrate alone and HDM with butyrate. F) Expression of IL-4, IL-13, Ym-1 Relm-α, Muc5ac and CCL11 was established in lung of naive mice and mice treated with HDM or butyrate alone and HDM with butyrate following RNA extraction and qPCR. Data shown are from at least two representative experiments. n = 3-5 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (A, B) or one-way ANOVA (D, E, F): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.8 Intranasal administration of butyrate ameliorates lung eosinophilia following exposure to HDM in a GPR109a-dependent manner

Butyrate is thought to provide anti-inflammatory effects mostly through inhibition of HDACs (Park et al., 2015, Chang et al., 2014; Aoyama et al., 2010; Thio et al., 2018; Theiler et al., 2019). However, our data suggest that agonism of GPR109a may be crucially important in allergic lung inflammation. To further evaluate whether butyrate-induced anti-inflammatory effects on lung (i)EOS are mediated by GPR109a, we intranasally administered butyrate or the specific GPR109a agonist niacin for 2 weeks prior and throughout the HDM administration protocol (Fig. 3.8A). Treatment with both butyrate and niacin decreased HDM-induced lung eosinophilia (Fig. 3.8B) but did not affect BM eosinophils as with oral treatment (data not shown), suggesting that priming of the BM is not necessary for butyrate-induced anti-inflammatory effects. Intranasal administration of butyrate and niacin also reduced eosinophil activation, which was evidenced by decreased expression of CD98 as well as phosphorylated mTOR (Fig. 3.8C).

Activation of GPR109a, which is coupled to $G_{i/o}$, leads to inhibition of cAMP production and downstream phosphorylation and activation of the extracellular signal-regulated kinases (ERK)1/2 (Li et al., 2011). We therefore assessed the expression of phosphorylated ERK1/2 in (i)EOS to further understand whether niacin and butyrate may directly induce similar effects through binding of GPR109a. The results showed that both butyrate and niacin significantly increased expression of phosphorylated ERK1/2 on (i)EOS following HDM exposure, suggesting that butyrate-induced effects may indeed be mediated through binding of GPR109a (Fig. 3.8D).

Although we detected expression of GPR109a on lung neutrophils, DCs, AMs and $CD4^+$ T cells (data not shown), we saw no significant differences in the proportions of these cells in the lung or in their expression of activation-associated markers following treatment with butyrate or niacin, suggesting that these agents may better target eosinophils (Fig. 3.8E).

Our results showed no differences in the proportions of $CD4^+$ T cells following treatment with butyrate or niacin (Fig. 3.8E), however SCFA-induced anti-inflammatory effects have been

associated with increased levels of activated Tregs (CD4$^+$Foxp3$^+$ T cells) in several studies (Singh et al., 2014; Smith et al., 2013; Zaiss et al., 2015). For this reason, we investigated whether butyrate and niacin treatment indirectly mediated anti-inflammatory effects on eosinophils via Treg induction. Neither butyrate or niacin increased proportions of Tregs as percentages of CD45$^+$ cells (Fig. 3.8F) or as percentages of CD4$^+$ T cells (data not shown), suggesting that butyrate does not induce Treg expansion in our experiments.

Similar to oral treatment with butyrate, intranasal administration of butyrate or niacin induced anti-inflammatory effects in the whole lung environment, as shown by reduced mRNA expression of Th2-associated inflammatory mediators (Fig. 3.8G).

Since we established that intranasal administration of butyrate or niacin prior to and during HDM treatment induced anti-inflammatory effects on eosinophils in the lung, we aimed to understand whether limiting the administration of butyrate or niacin to only the HDM challenge period would induce the same effects (Supplementary Fig. 5A). The results showed that both butyrate and niacin were still effective in reducing eosinophilia and eosinophil activation in the lung when given only during the HDM challenge (Supplementary Fig. 5B), suggesting that pre-treatment during the sensitisation period is not necessarily required to dampen inflammatory responses in the lung. As before, treatment with butyrate or niacin during the challenge with HDM also decreased mRNA expression of Th2 mediators (Supplementary Fig. 5C), suggesting that the anti-inflammatory effect is extended to the whole lung tissue.

To further demonstrate that agonism of GPR109a mediates butyrate- and niacin-induced anti-inflammatory effects on eosinophils, we also administered a specific GPR109a agonist developed by GSK. Due to the compound's properties, the agonist could only be administered orally, therefore mice were given the drug by oral gavage during the challenge phase with HDM. The results showed that oral administration of the agonist was effective in decreasing lung eosinophilia and eosinophil activation in the lung of HDM-treated mice, although not as dramatically as with intranasal administration with butyrate or niacin (Supplementary Fig. 5D). Although oral administration of this compound might not be the most effective method of delivery, these data suggest that agonism of GPR109a is beneficial in reducing Th2 inflammatory responses in the lung.

Overall, these results illustrate that butyrate significantly decreases inflammation in the lungs of allergic mice and that this is associated with decreased eosinophil activation. We showed that the GPR109a agonist niacin as well as the GSK-developed GPR109a agonist also mirrored butyrate-induced reductions in (i)EOS numbers and activation reinforcing the hypothesis that these effects are mediated by GPR109a expressed on (i)EOS.
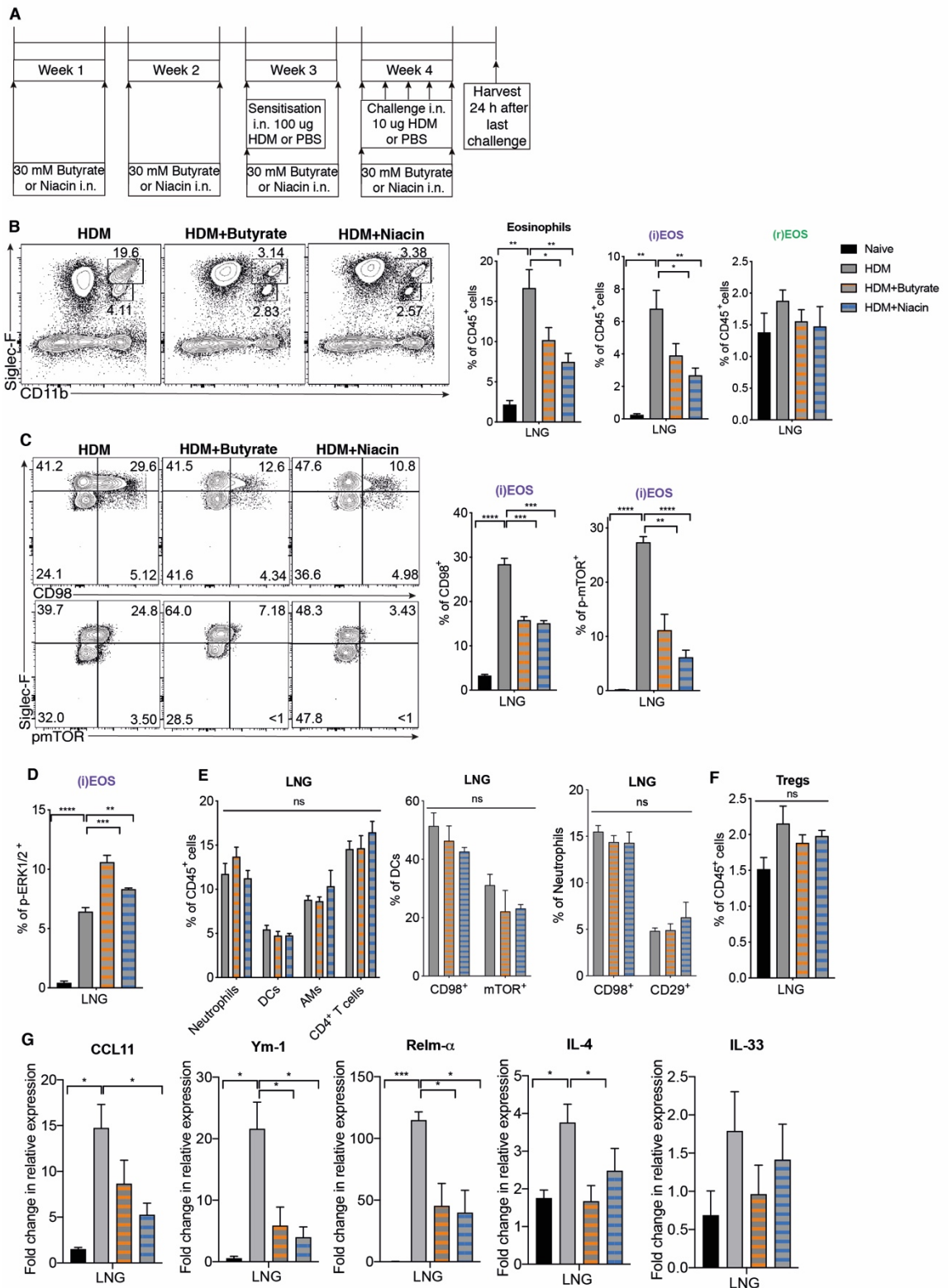
Figure 3.8: Intranasal administration of butyrate ameliorates lung eosinophilia following exposure to HDM in a GPR109a-dependent manner

A) Experimental timeline. 8-12 week old female BALB/c WT mice were administered 30 mM butyrate or niacin i.n. twice a week for two weeks prior and during HDM treatment. Mice were euthanised 24 hours after the last challenge with HDM (on day 12). B) Eosinophils were quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with butyrate or niacin and expressed as a percentage of CD45$^+$ cells. C) The percentage of eosinophils expressing CD101 and pmTOR was quantified by flow cytometry in the lung of naive

mice and mice treated with HDM alone or with butyrate or niacin. D) Tregs (CD4+Foxp3+ cells) were quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with butyrate or niacin and expressed as a percentage of CD45+ cells. E) The percentage of (i)EOS expressing pERK1/2 was quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with butyrate or niacin. F) Neutrophils, DCs (Siglec-F-CD11c+CD11b+ cells) and AMs (Siglec-F+CD11c+CD11b- cells) as a percentage of CD45+ cells, the percentage of DCs expressing CD98 and pmTOR, and the percentage of neutrophils expressing CD98 and CD29 were quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with butyrate or niacin. G) Expression of IL-4, IL-33, Ym-1, Relm-α and CCL11 was established in lung of naive mice and mice treated with HDM alone or with butyrate or niacin following RNA extraction and qPCR. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

3.3.9 Agonism of GPR109a is effective in reducing inflammation in a mouse model of neutrophilic asthma

Allergic asthma has been determined to be a heterogeneous disease with several distinct pathologic phenotypes (Borish, 2016; Brussino et al., 2018). Although eosinophilic asthma is more prevalent, there is evidence for neutrophils being the predominant cellular infiltrate in cases of severe and persistent asthma, asthma exacerbations and corticosteroid-resistant asthma (Wang et al., 2016). Although the knowledge about the mechanisms, phenotypes and treatments for asthma has increased over the last decades, severe asthma and asthma control in particular are the main unmet needs in the field (Varsano et al., 2017).

We previously showed that not only eosinophils but also lung neutrophils express GPR109a; for these reasons, we decided to investigate whether agonism of GPR109a could also be beneficial in ameliorating neutrophilic asthma. To this end, we developed a mouse model of mixed granulocytic asthma by administering LPS (Yu and Chen, 2018), together with HDM on days 7, 9 and 11 of our challenge model (Fig. 3.9A). The results showed that addition of LPS to our HDM model dramatically increased neutrophil levels in the lungs (Fig. 3.9B) as well as their expression of CD98 (Fig. 3.9C). The mechanisms underlying neutrophilic asthma are still unclear, however there is evidence for increased levels of neutrophil chemotactic and activating mediators, such as IL-8, IL-17A and TNF-α, in the airways (Yang et al., 2018). We therefore also measured IL-17A and TNF-α expression in CD4+ and CD8+ T cells, in addition to IL-13, IL-5 and IFN-γ, all of which were increased in mice treated with HDM+LPS (Fig. 3.9D). Similarly to our previous results, intranasal administration of butyrate or niacin during the challenge phase with HDM and LPS was effective in reducing lung neutrophilia, CD98 expression on neutrophils as well as cytokine release from T cells (Fig. 3.9B-D), suggesting that agonism of GPR109a may also be beneficial in this asthmatic phenotype.

As before, we also administered the GPR109a agonist developed by GSK to this mouse model to further demonstrate that agonism of GPR109a may mediate butyrate- and niacin-induced anti-inflammatory effects on neutrophils. Oral administration of the agonist was effective in

decreasing neutrophilia as well as neutrophil expression of CD98 also in this model (Supplementary Fig. 6A). The anti-inflammatory effects of the GPR109a agonist were also evidenced by decreased lung mRNA expression of IL-17A, TNF-α, IFN-γ and Th2 cytokines (Supplementary Fig. 6B).

Importantly, these data suggest that agonism of GPR109a can be beneficial in ameliorating allergic lung inflammation when either eosinophils or neutrophils are the major inflammatory infiltrates.
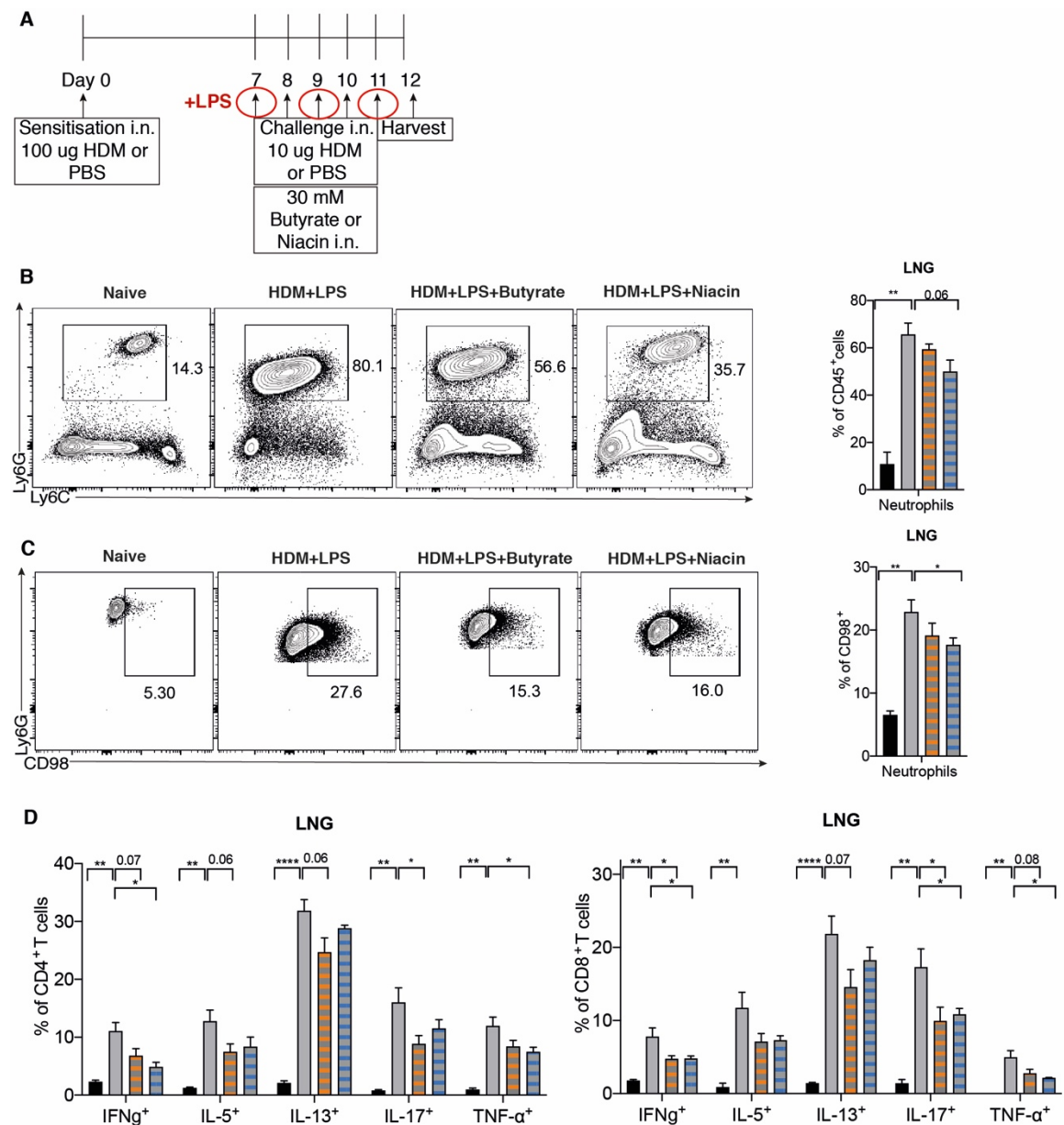
Figure 3.9: Agonism of GPR109a is also effective in reducing inflammation in a mouse model of neutrophilic asthma

A) Experimental timeline. 8-12 week old female BALB/c WT mice were administered 30 mM butyrate or niacin i.n. during the challenge phase of HDM treatment. 5 μg LPS were also administered i.n. with HDM on day 7-9-11. Mice were euthanised 24 hours after the last challenge with HDM (on day 12). B) Neutrophils were quantified

by flow cytometry in the lung of naive mice and mice treated with HDM+LPS alone or with butyrate or niacin and expressed as a percentage of CD45$^+$ cells. C) The percentage of neutrophils expressing CD98 was quantified by flow cytometry in the lung of naive mice and mice treated with HDM+LPS alone or with butyrate or niacin. D) The percentage of T cells expressing IL-17A, TNF-α, IFN-γ, IL-5 and IL-13 was quantified by flow cytometry in the lung of naive mice and mice treated with HDM+LPS alone or with butyrate or niacin. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

3.3.10 Asthmatic patients also have two populations of eosinophils in the lung and blood with a similar phenotype to mouse eosinophils

To investigate the potential existence of separate eosinophil populations in humans, we used the gating strategy showed in Supplementary Fig. 7A to identify eosinophils by assessing the surface expression of Siglec-8 (i.e. the human ortholog of murine Siglec-F), CCR3, IL-5Rα, CD98 and CD101 on blood and lung samples from asthmatic patients (APs) and healthy controls (HCs). As with eosinophils from allergic mice, eosinophils from the blood and lungs of APs upregulated expression of Siglec-8 compared to HCs, resulting in the potential identification of two different populations of eosinophils that could be distinguished as Siglec-8^{hi}CD11b$^+$ and Siglec-8^{int}CD11b$^+$ (Fig. 3.10A). In contrast to murine lung eosinophils, about 20% of human lung eosinophils expressed CD101 already in HCs, together with low expression of CD98 and GPR109a (Fig. 3.10B). However, similarly to murine lung eosinophils, the expression of these molecules was upregulated in APs (Fig. 3.10B). Human blood eosinophils from HCs expressed very low levels of CD101, CD98 and GPR109a, as we also showed in the murine counterparts (Fig. 3.10B). Due to limited sample availability, we could not establish expression of GPR109a on eosinophils from the blood of APs but we identified higher expression of CD101 and CD98 (Fig. 3.10B), suggesting phenotypic similarities in eosinophils from allergic mice and humans.

In order to understand whether the lung environment might support eosinophil activation and expression of CD101 and CD98 in humans, we exposed isolated blood eosinophils to IL-33, GM-CSF and IL-5, as we did with murine eosinophils. We also assessed whether addition of butyrate on niacin would also affect GPR109a expression as we detected on murine eosinophils. To this end, we isolated blood eosinophils from the blood of HCs using enrichment columns and identified a CD16$^-$CD66b$^+$cell population in an enriched granulocyte suspension. The purity of the sorted eosinophil population was close to 100%, as assessed by flow cytometry and cytospins (Supplementary Fig. 7B-C). Moreover, immunofluorescence staining with the eosinophil-specific granule EPX (magenta) showed expression of the protein on the isolated cells (Supplementary Fig. 7D). We then exposed the isolated eosinophils to IL-33, GM-CSF and IL-5 alone or with niacin or butyrate. The results showed that, similarly to our results from mice experiments, cytokine stimulation increased expression of CD98 on blood eosinophils, which however was only slightly reduced by niacin (Fig. 3.10C). Importantly, cytokine stimulation with

niacin induced an increase in GPR109a expression above the levels on untreated cells (Fig. 3.10C).

Our data potentially show the existence of two different populations of eosinophils in asthmatic patients, which can be distinguished by expression of Siglec-8 and increased expression of CD98 and CD101 as in allergic mice. Similarly to our *in vitro* experiments with murine eosinophils, we showed that IL-33, GM-CSF and IL-5 may enhance expression of CD98 and GPR109a. Additionally, treatment with butyrate or niacin also affected CD98 and GPR109a expression, therefore suggesting that human eosinophils may also be targeted by agonism of GPR109a.
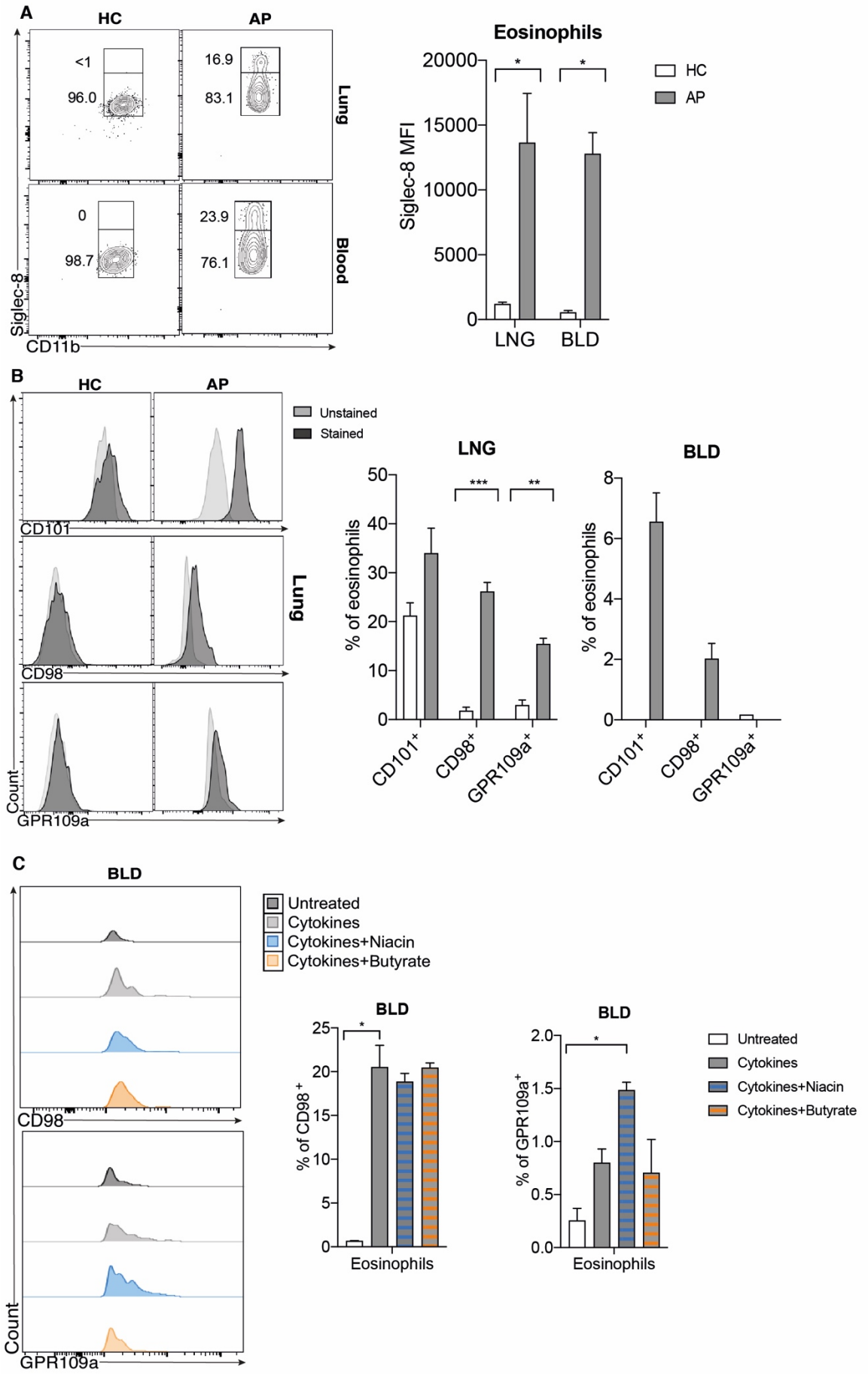
Figure 3.10: Asthmatic patients also show two populations of eosinophils in the lung and blood with similar phenotype to mouse eosinophils

A) Siglec-8 expression was quantified on eosinophils (Siglec-8$^+$CD11b$^+$ cells) from the blood and lung of HCs and APs and expressed as MFI. B) The percentage of eosinophils expressing CD101, CD98 and GPR109a was quantified by flow cytometry in the lung and blood of HCs and APs. C) The percentage of blood eosinophils from HCs expressing CD98 and GPR109a following culture in the presence or absence of IL-33, GM-CSF and IL-5 alone or with butyrate or niacin for 24 hours was quantified by flow cytometry. Data shown are representative from at least two independent experiments. n = 1-2 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test (A, B) one-way ANOVA (C): *p < 0.05; **p < 0.01; ***p < 0.001.

3.4 DISCUSSION

Here we have shown that (i)EOS (Siglec-F^{hi}CD11b^{hi} eosinophils) and (r)EOS (Siglec-F^{int}CD11b^{int} eosinophils) represent two phenotypically distinct populations of eosinophils found in the airways of allergic mice. Factors enriched in the lung environment supported eosinophil activation during allergic responses, showing that (i)EOS were pro-inflammatory and more metabolically active than (r)EOS and this correlated with expression of CD98. Importantly, we identified Siglec-F^{hi}CD98$^+$ eosinophils being a common feature of lung inflammation. For the first time, we have also shown that lung (i)EOS upregulate expression of the receptor GPR109a and that agonism of this receptor can directly affect eosinophil phenotype and function *in vitro* and *in vivo*. Finally, we also provide evidence for the potential existence of two populations of eosinophils in the lungs and blood of asthmatic patients that may express GPR109a and be responsive to agonism of this receptor.

Our results firstly demonstrated that mice exposed to the allergen HDM developed airway, blood and BM eosinophilia. However, we detected two populations of eosinophils, identified as (i)EOS (Siglec-F^{hi} eosinophils) and (r)EOS (Siglec-F^{int} eosinophils) only in the airways and blood of allergic mice, suggesting that these cells are generated following egress from the BM or the lungs. (i)EOS in the lung and BAL showed upregulation of CD11b, whereas (i)EOS in the blood did not show upregulation of this molecule. Previous studies have documented the existence of two separate populations of eosinophils in murine models of allergic asthma (Mesnil et al., 2016; Abdala Valencia et al., 2015). (r)EOS were reported not to be affected by allergen exposure and to have homeostatic functions, whereas (i)EOS to increase in response to allergen inhalation and to be pro-inflammatory (Mesnil et al., 2016). Conversely, our results showed a simultaneous decrease in the levels of (r)EOS with an increase in (i)EOS following HDM administration, which was also concomitant to transition from a Siglec-F^{int}CD11b^{int} to a Siglec-F^{hi}CD11b^{hi} phenotype. Upregulation of the integrin CD11b has been associated with cell activation and a pro-inflammatory phenotype in humans and mice (Duan et al., 2016; Arnold et al., 2018). The function of Siglec-F on eosinophils remains a controversial topic. On the one hand, Siglec-F engagement has been shown to induce eosinophil apoptosis, suggesting that upregulation of Siglec-F on eosinophils represents a negative feedback mechanism to regulate pro-

inflammatory responses in an autocrine manner (Zhang et al., 2007; Zimmermann et al., 2008). On the other hand, Siglec-F upregulation on eosinophils has been associated with increased eosinophil survival and cytokine release (Willebrand and Voehringer, 2016), suggesting that this receptor may actually possess both inhibitory and activatory properties. These observations together with our results, suggest that recruitment of eosinophils from the BM is enhanced by allergen exposure and that, once in the lung, BM-derived eosinophils are shaped by local changes in the environment. Although at steady-state eosinophils are defined as tissue-resident cells (Weller and Spencer, 2017), with the use of lung-shielded chimeras and intravascular staining we found that lung eosinophils are not long-lived self-renewing cells like AMs (Guilliams et al., 2013). Conversely, lung eosinophils require constant replenishment from the BM to maintain their tissue pool. We also observed that HDM exposure increased the frequencies of host-derived eosinophils in the lung. This is concomitant with the appearence of (i)EOS in the airways, normally absent at steady-state, leading to the hypothesis that (i)EOS may be generated from local BM-derived (r)EOS. Administration of low doses of HDM supported our hypothesis by revealing that this treatment regime was not sufficient to trigger systemic or airway eosinophilia, but it induced local generation of (i)EOS. Unaltered proportions of EoPs in the BM, blood and lungs of HDM-treated mice compared to controls suggested the possibility that (i)EOS are not generated from local progenitor cells. However, we cannot fully rule out that changes in EoPs occurred at different time points we did not analyse or that (i)EOS are being generated in the circulation prior to entering the lung. Nevertheless, these results importantly suggest that eosinophils are plastic cells susceptible to changes in the local tissue microenvironment, which modulates their phenotype to potentially favour their adaptation.

Phenotypically, (i)EOS, but not (r)EOS, have been reported to express CD101 (Mesnil et al., 2016), which we confirmed in our analysis. In addition, we saw that (i)EOS expressed elevated levels of CD98, which has not been reported before. Interestingly, the majority of (i)EOS did not co-express the two molecules, allowing identification of four further subpopulations. It would therefore be interesting to provide more in-depth analysis of such populations to improve our understanding of eosinophil biology. CD98 is known to support cell growth and survival by multiple mechanisms: it allows amino acid transport by coupling with LAT1 and it associates with integrins regulating their functions (Cormerais et al., 2016). Our data showed that CD98 expression by (i)EOS indeed correlated with increased amino acid uptake and concurrently activation of the mTOR signalling pathway, as well as with glucose uptake, which was associated with increased glycolytic activity. Moreover (i)EOS positive for the integrin CD29 co-stained with CD98, suggesting that CD98 may fulfil its role of a pleiotropic molecule on eosinophils. Cellular metabolism is essential in determining immune cell function and metabolic changes are crucial in dictating how cells respond to a variety of signals. Our data show that (i)EOS are indeed more pro-inflammatory and responsive to stimulation than (r)EOS as they

released higher amounts of IL-4 and IL-6, together with the granule protein EPX. Altogether these data further support the hypothesis that (i)EOS represent a subset of (r)EOS more sensitive to changes in the tissue microenvironment, involving their metabolic reprogramming which may be necessary to adapt to such alterations.

Although little is known about eosinophil metabolism, our results are in agreement with Jones et al., (2019), who showed that human blood eosinophils are plastic cells able to upregulate glycolysis and the TCA cycle upon activation. Moreover, data from Porter et al., (2018) suggest that, although both human eosinophils and neutrophils use glycolysis, eosinophils also access additional pathways such as glucose oxidation and mitochondrial oxidative phosphorylation. This supports the idea that eosinophils show significant metabolic flexibility, which allows them to better adapt to tissue requirements. Inflamed tissues are often hypoxic, due to increased cellular oxygen demand and reduced oxygen availability. Hypoxia is a pro-inflammatory stimulus, which activates HIF-1α signalling, resulting in a metabolic shift from oxidative phosphorylation to glycolysis and we showed that lung (i)EOS can be identified by their expression of HIF-1α. Enhanced glycolysis in eosinophils may therefore allow them to perform their functions in environments with limited oxygen availability (Porter et al., 2018).

In vitro assays further led us to identify IL-33, IL-5 and GM-CSF as factors capable of increasing CD98 and Siglec-F expression on eosinophils over a period of 24 hours. These observations are also supported by Jones et al. (2019), who showed that IL-5, GM-CSF and IL-3 induce eosinophil glycolysis. Although IL-5, GM-CSF and IL-3 are known as 'eosinophilopoietins' (Melo et al., 2013), a role for IL-33 in supporting eosinophil development, survival and activation has only emerged recently (Johnston et al., 2016 and 2017; Willebrand and Voehringer, 2016). Our results indicate the importance of IL-33 in driving metabolic adaptation of (i)EOS since IL-33 deficient mice displayed reduced expression of CD98 compared to heterozygous controls. Although IL-33 is reported to indirectly affect eosinophils through GM-CSF or IL-5, our data show that IL-33 can activate eosinophils in the absence of other cytokines. In fact, there is evidence for IL-33R (ST2) expression on eosinophils, which can directly mediate effects of IL-33 on these cells (Stolarski et al., 2010). Furthermore, eosinophil stimulation with IL-33 or IL-5 is correlated with increased surface expression of CD11b, increased adhesion and granule protein release (Angulo et al., 2019). However, the combination of IL-33, IL-5 and GM-CSF did not lead to release of EPX in our study, which was achieved only using a non-specific Ca^{2+} ionophore. This could be because eosinophil activation can vary at different time points (Esnault and Kelly, 2016), or because not all granule proteins may be released following stimulation with those cytokines (Angulo et al., 2019). Exposure of BM eosinophils to IL-33 and the Ca^{2+} ionophore Ionomycin but not either factor alone for a shorter period of 3 hours showed that this treatment induced the generation of a Siglec-F^{int} and a Siglec-F^{hi} population of BM eosinophils,

reminiscent of (r)EOS and (i)EOS seen in the lungs of allergic mice. This suggests that triggering of Ca^{2+} influx into eosinophils over a shorter period of time led us to capture eosinophils in a transition state i.e. from Siglec-F^{int} to Siglec-F^{hi} as shown following a 24 hours stimulation.

Further phenotypic analysis of (i)EOS and (r)EOS confirmed previous studies in that (i)EOS displayed more segmented nuclei and were localised around the bronchi in the lung, whereas (r)EOS showed typical ring-shaped nuclei and were located in the parenchyma (Mesnil et al., 2016; Abdala Valencia et al., 2015). Although we did not see differences in CCR3 expression between (i)EOS and (r)EOS as previously reported, we saw that (r)EOS expressed higher levels of IL-5Rα compared to (i)EOS together with STAT5, suggesting that (i)EOS decrease responsiveness to the cytokine. Accordingly, other studies have shown that eosinophils decrease expression of IL-5Rα in response to antigen challenge and that this is partly mediated by proteolytic cleavage of membrane-bound IL-5Rα, which in turn contributes to the presence of soluble IL-5Rα (Liu et al., 2002; Wilson et al., 2011). Additionally, (r)EOS expressed greater levels of other molecules associated with regulatory functions compared to (i)EOS, including the integrin CD29, the co-stimulatory molecule CD86 and the immunomodulatory enzyme IDO. (r)EOS have indeed been associated with homeostatic functions, such as inhibition of BM-derived DC-mediated activation of Th2 cells (Mesnil et al., 2016). Conversely, (i)EOS expressed additional proteins associated with pro-inflammatory functions and cell activation, such as the LPS receptor TLR-4, membrane-bound IgE, the transcription factor STAT1, the activation marker CD69, the adhesion molecule CD34, the receptor CD300c/d and the integrin CD11c. Increased expression of the latter on lung eosinophils from allergic mice was previously reported by Abdala Valencia et al. (2015), showing increased capacity of these cells to interact with the respiratory mucosa through fibrinogen binding.

Although we and others have pointed out changes in eosinophil phenotype in allergic asthma, it is not known whether similarities or differences in eosinophils exist across murine models of lung inflammation. Surprisingly, in three different models of parasite-induced lung inflammation as well as after Influenza infection, we detected (i)EOS and (r)EOS in the lungs, with (i)EOS expressing CD98. These data reveal that eosinophil transition from (r)EOS into (i)EOS with an activated phenotype are a common characteristic of lung inflammatory responses. Moreover, these changes depend on the nature of the inflammatory response but not on the causing stimulus. This is significant as (i)EOS expressing CD98 may be considered as a common biomarker to identify lung inflammatory responses.

Interestingly, our phenotypic analysis of lung eosinophils suggested that (i)EOS expressed higher levels of the SCFA receptors GPR109a and GPR43, but not GPR41, compared to (r)EOS. SCFAs are the metabolic product of undigestible fibres fermented by the intestinal

microbiota, with the most abundant being acetate, propionate and butyrate. While GPR43 and GPR41 are activated by all three SCFAs, GPR109a is activated only by butyrate (Macia et al., 2015). GPR109a is known to be expressed in adipocytes and immune cells, including macrophages, DCs and neutrophils (Tunaru et al., 2003; Singh et al., 2015; Zandi-Nejad et al., 2013) but, to our knowledge, this is the first time that a subset of murine eosinophils is reported to express GPR109a. In contrast to our findings, Kostylina et al. (2008) reported that mature neutrophils but not eosinophils express GPR109a. However, their study was based on analysis of human blood rather than cells from tissues. Our data have shown that the signals present in the tissue microenvironment are crucial in the modulation of eosinophil phenotype, suggesting that the properties of circulatory eosinophils are not representative of eosinophils from different tissue compartments.

Several studies suggest that SCFAs have beneficial effects on inflammatory disease pathogenesis, including allergic asthma (Maslowski et al., 2009; Trompette et al, 2014; Cait et al., 2018). We observed that GF mice, which are devoid of the intestinal microbiota, had both lung (r)EOS and (i)EOS at steady-state. This is in comparison to normal SPF mice which only have (r)EOS, thus supporting the idea that the microbiota and their derived metabolites, such as butyrate, play an important role in the regulation of immune responses in the lung and may prevent the generation of aberrant inflammatory responses to inhaled particulates. In support of this, Cahenzli et al. (2013) found that naïve GF mice had higher serum IgE levels compared to naïve SPF mice and showed that appropriate microbial exposure during early life has to occur in order to induce immune-regulatory mechanisms able to maintain serum IgE levels at baseline. Interestingly, full colonization of the lung by eosinophils occurs a week after birth in mice, suggesting that this may be correlated to simultaneous development of the gut microbiota (Mesnil et al., 2016). Further evidence for a link between commensals and eosinophils comes from both murine and human studies showing that reduced microbial diversity in the gut leads to increased susceptibility to developing eosinophilia and allergic airway inflammation (Stokes et al., 2015; Wesemann and Nagler, 2016; Zhang et al., 2020).

Although butyrate has recently been shown to affect eosinophil survival and migration (Theiler et al., 2019), it is still not known whether lung eosinophils can be directly targeted by SCFAs during allergic lung inflammation. Our experiments firstly revealed that lung (i)EOS but not blood (i)EOS or BM eosinophils expressed high levels of the butyrate-selective GPR109a and that its expression on eosinophils could be induced by cytokine stimulation and enhanced by addition of the GPR109a agonists butyrate or niacin. Based on these data, we hypothesised that butyrate could directly mediate anti-inflammatory effects on eosinophils through binding of GPR109a. Oral or intranasal administration of the SCFA was indeed effective in ameliorating lung inflammation in allergic mice, and it showed remarkable inhibition of the (i)EOS phenotype.

Administration of niacin also showed comparable effects on (i)EOS phenotype. Butyrate or niacin also enhanced expression of phosphorylated ERK1/2, which is activated downstream of GPR109a (Li et al., 2011). Further experiments using GPR109a KO mice will be required to fully confirm the effects of agonism of GPR109a on lung eosinophils. Nevertheless, to our knowledge, this is the first time that the effecs of GPR109a agonists have been shown in the context of allergic asthma.

Tregs have been implicated in mediating the anti-inflammatory effects of SCFA in several studies (Singh et al., 2014; Smith et al., 2013; Zaiss et al., 2015). In contrast, our experiments showed that the frequencies Tregs were not not altered by either butyrate or niacin following HDM challenge. Moreover, butyrate or niacin did not affect the phenotype and activation of other cell types in the lung, including CD4$^+$ T cells, neutrophils, DCs and AMs. These data therefore suggest that eosinophils can be specifically targeted by GPR109a agonists in our model and strongly suggest that agonism of this receptor can directly affect eosinophil phenotype and activation. Further experiments involving transfer of BM eosinophils from GPR109a KO mice into eosinophil-deficient mice will enable us to better establish the specific effects that agonism of GPR109a has on eosinophils.

It is increasingly recognised that asthma is a very heterogeneous disease and in patients with severe asthma neutrophils represent the predominant inflammatory infiltrate. Importantly, patients with neutrophilic asthma are often resistant to corticosteroid treatment and lack alternative personalised treatment options (Fajt and Wenzel, 2015). Since we established that lung neutrophils express GPR109a, we administered butyrate or niacin to a mouse model of mixed granulocytic lung allergy to understand whether agonism of the receptor mediated anti-inflammatory effects. Treatment with butyrate or niacin was anti-inflammatory also in this model, resulting in decreased neutrophilia, neutrophil activation and cytokine release from T cells. These data therefore suggest that agonism on GPR109a may ameliorate both Th2 and non-Th2 allergic inflammatory responses.

In the majority of human studies, eosinophils are obtained from the circulation or sputum due to sample accessibility and availability. However, there is evidence for the existence of eosinophils not only in the lungs of APs but also in healthy individuals (Kraft et al., 1999; Willetts et al., 2011). Our data confirm the presence of eosinophils in the lung and blood of HCs and APs, the latter showing that these cells can be distinguished into Siglec-8^{hi}CD11b$^+$ and Siglec-8^{int}CD11b$^+$ similarly to our results from murine studies. This is in contrast to Mesnil et al. (2016) who concluded that lung eosinophils cannot be distinguished based on expression of Siglec-8. In the same study, lung eosinophils were shown to express CD62L, whereas BAL eosinophils expressed IL-3R but not CD101. Neither CD62L or IL-3R were expressed by eosinophils in our

study, but notably, we detected significant expression of CD98, CD101 and GPR109a on eosinophils from APs. Similarly to their murine counterparts, we also showed that cytokine stimulation enhanced expression of CD98 and GPR109a on blood eosinophils, suggesting that the lung environment has a major role in dictating eosinophil phenotype and function also in humans. Moreover, we showed that treatment with niacin or butyrate enhanced expression of GPR109a on cytokine-stimulated eosinophils, suggesting that these cells may also be responsive to agonism of the receptor. This opens up the possibility that agonism of GPR109a may represent a suitable treatment option for patients with Th2 and non-Th2 asthma.

One limitation of our study is that the samples received were all from asthmatic subjects with cancer that were not selected on the basis of a defined medical history or treatment plan. Only asthmatic patients on immunosuppressant drugs or with autoimmune diseases were excluded from our study. As such, we analysed a small heterogeneous cohort of patients whose disease origins and severity varied considerably. Nevertheless, our results suggest the existence of two populations of eosinophils in the human lung and blood with remarkably similar characteristics to the murine counterpart. A larger number of samples as well as further immunophenotyping with the use of flow cytometry and immunohistochemistry would therefore be required to better establish the reliability of our preliminary data.

In conclusion, this study indicates that lung eosinophils are short-lived plastic cells that can quickly adapt to tissue requirements by profoundly changing their phenotype and functional characteristics during Th2 lung inflammatory responses. In addition, we highlight a crucial role for the microbiome in regulating lung eosinophils and suggest that GPR109a may represent an intriguing target for therapeutic interventions to slow the progression of lung inflammatory diseases.

3.5 SUPPLEMENTARY FIGURES
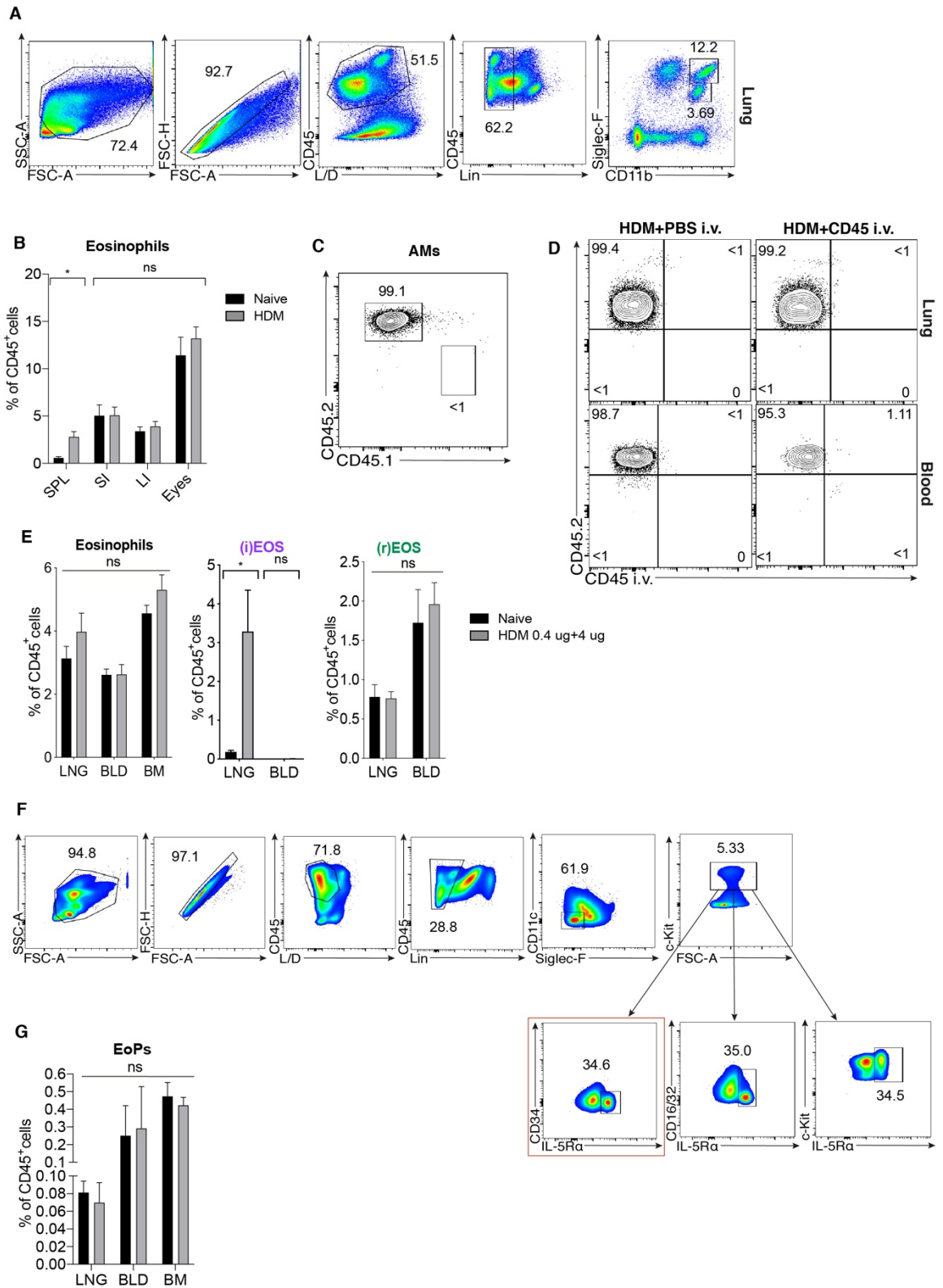
Figure 3.11: Supplementary figure 1.
A) Gating strategy to identify lung eosinophils. Cells were identified as follows: SSC-A versus FSC-A to remove cellular debris; FSC-H versus FSC-A for doublet exclusion; Zombie Live-Dead versus CD45 to identify live haematopoietic populations; lineage versus CD45 to exclude contaminating populations (lineage defined as

TCRβ, B220, NK1.1, Ly6C and Ly6G to exclude T cells, B cells, NK cells, monocytes/macrophages and neutrophils respectively); Siglec-F versus CD11b to identify eosinophils (CD11b+Siglec-F+) and exclude alveolar macrophages (AMs) (Siglec-F+CD11b-). B) Eosinophils were quantified by flow cytometry in the spleen, small intestine, large intestine and eyes of naïve and HDM-treated mice and expressed as a percentage of CD45+ cells. C) The percentage of AMs expressing CD45.1 and CD45.2 was quantified by flow cytometry in the lung of naïve and HDM-treated lung shielded chimeric mice. D) The percentage of (i)EOS expressing AF700-conjugated CD45 administered i.v and CD45.2 was quantified by flow cytometry in the lung and blood of HDM-treated mice. E) Eosinophils were quantified by flow cytometry in the lung, BM and blood of naïve and HDM-treated mice (0.4 μg and 4 μg) and expressed as a percentage of CD45+ cells. F) Gating strategy to identify EoPs. Cells were identified as follows: SSC-A versus FSC-A to remove cellular debris; FSC-H versus FSC-A for doublet exclusion; Zombie Live-Dead versus CD45 to identify live haematopoietic populations; lineage versus CD45 to exclude contaminating populations (lineage defined as TCRβ, B220, NK1.1, Ly6C, Ly6G and FcεRI to exclude T cells, B cells, NK cells, monocytes, neutrophils and basophils and mast cells respectively); CD11c versus Siglec-F to exclude eosinophils and CD11c+ cells; c-Kit versus FSC-A to identify c-Kit+ cells; IL-5Rα versus CD34 or CD16/32 or c-Kit to identify EoPs. G) EoPs were quantified by flow cytometry in the lung, BM and blood of naïve and HDM-treated mice and expressed as a percentage of CD45+ cells. Data shown are representative from at least two independent experiments. n = 3-6 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05, **p < 0.01; ***p < 0.001.

Figure 3.12: Supplementary figure 2.
A) Gene expression analysis of the microarray gene sequencing from Mesnil et al. (2016). Data are expressed as arbitrary units (A.U.). B) t-SNE map representing t-SNE guided gating of fcs files from the lungs of HDM-treated mice run with the markers Siglec-F, CD11b, CD101 and CD98. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *p < 0.05, **p < 0.01; ***p < 0.001.

Figure 3.13: Supplementary figure 3.

Siglec-F^{hi} and Siglec-F^{int} eosinophils were quantified by flow cytometry from the BM of naïve mice cultured in the presence or absence of PAF, IL-33 or Ionomycin alone or PAF+Ionomycin and IL-33+Ionomycin for 3 hours and expressed as a percentage of CD45$^+$ cells. Data shown are representative from two independent experiments. n = 2 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *p < 0.05, **p < 0.01; ***p < 0.001.
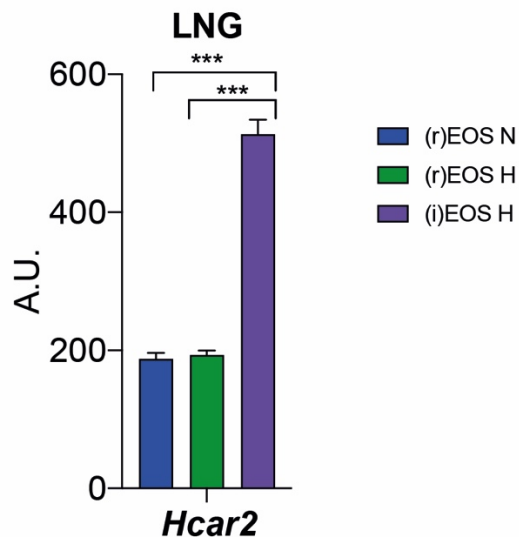
Figure 3.14: Supplementary figure 4.

Gene expression analysis of the microarray gene sequencing from Mesnil et al. (2016). Data are expressed as arbitrary units (A.U.). Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *p < 0.05, **p < 0.01; ***p < 0.001.
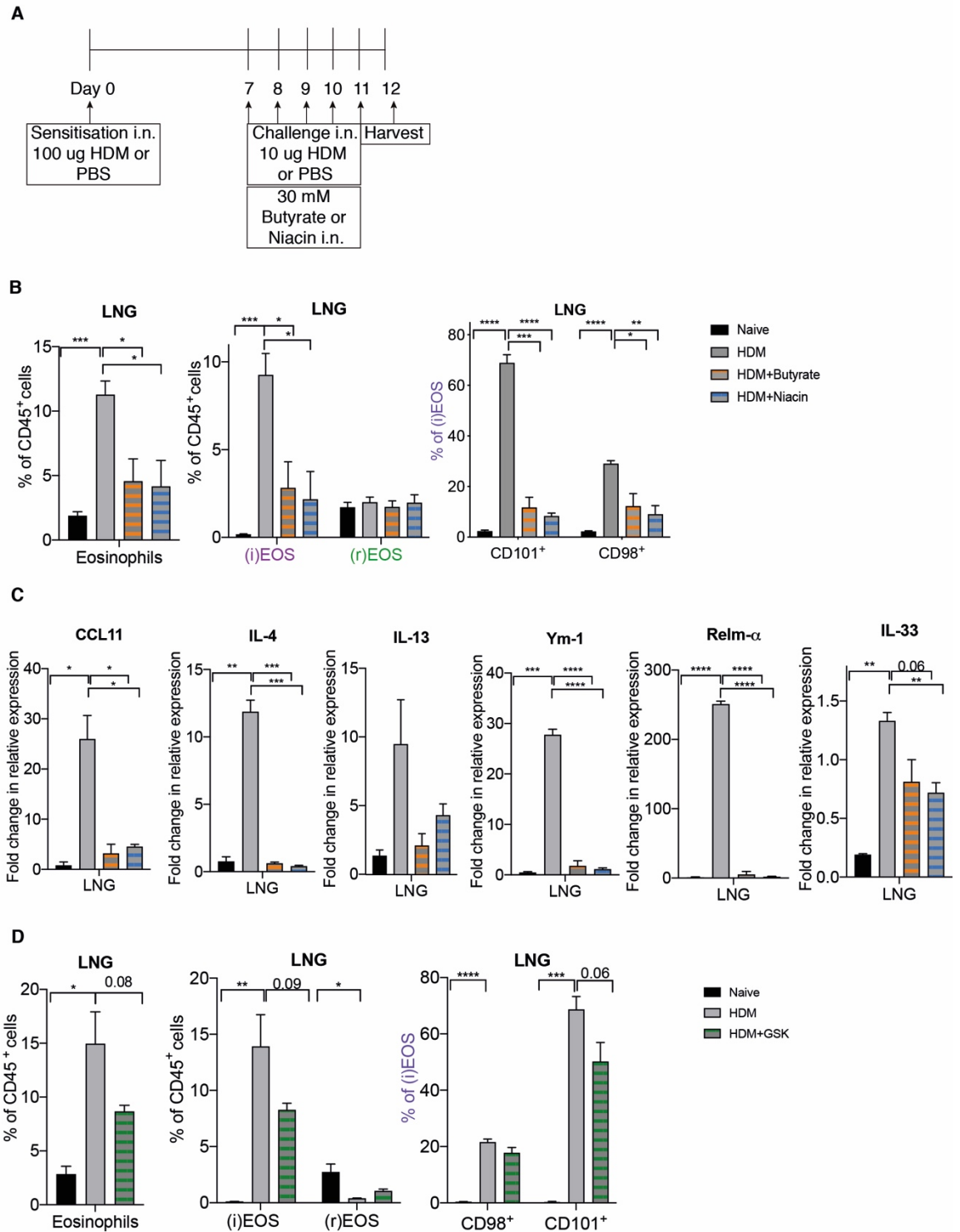
Figure 3.15: Supplementary figure 5.

A) Experimental timeline. 8-12 week old female BALB/c WT mice were administered 30 mM butyrate or niacin i.n. during the challenge phase of HDM treatment. Mice were euthanised 24 hours after the last challenge (on day 12). B) Eosinophils as a percentage of CD45+ cells and their expression of CD101 and CD98 were quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with butyrate or niacin. C) Expression of IL-4, IL-13, IL-33, Ym-1, Relm-α and CCL11 was established in lung of naive mice and mice treated with HDM alone or with butyrate or niacin following RNA extraction and qPCR. D) Eosinophils as a percentage of CD45+ cells and their expression of CD101 and CD98 were quantified by flow cytometry in the lung of naive mice and mice treated with HDM alone or with the GSK developed GPR109a agonist. Data shown

are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *p < 0.05; **p < 0.01; ***p < 0.001.
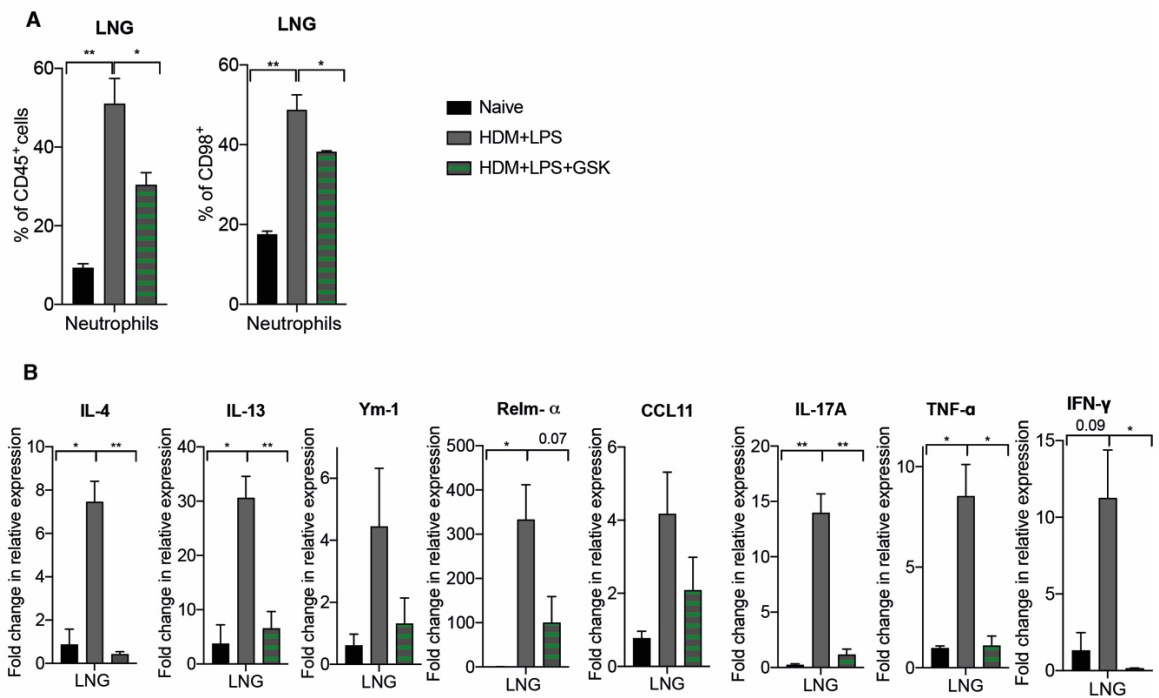
Figure 3.16: Supplementary figure 6.
A) Neutrophils as a percentage of CD45^{+} cells and their expression of CD98 were quantified by flow cytometry in the lung of naive mice and mice treated with HDM+LPS alone or with the GSK developed GPR109a agonist. B) Expression of IL-4, IL-13, IL-33, Ym-1, Relm-α, CCL11, TNF-α, IFN-γ and IL-17A was established in lung of naive mice and mice treated with HDM+LPS alone or with the GSK developed GPR109a agonist following RNA extraction and qPCR. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *p < 0.05; **p < 0.01; ***p < 0.001.
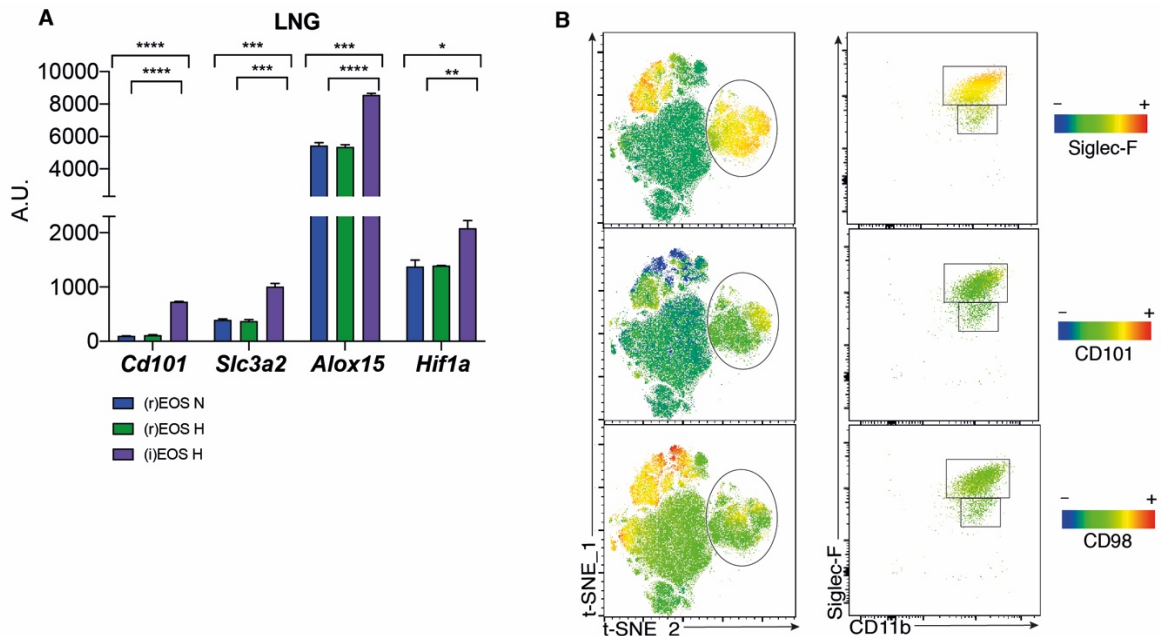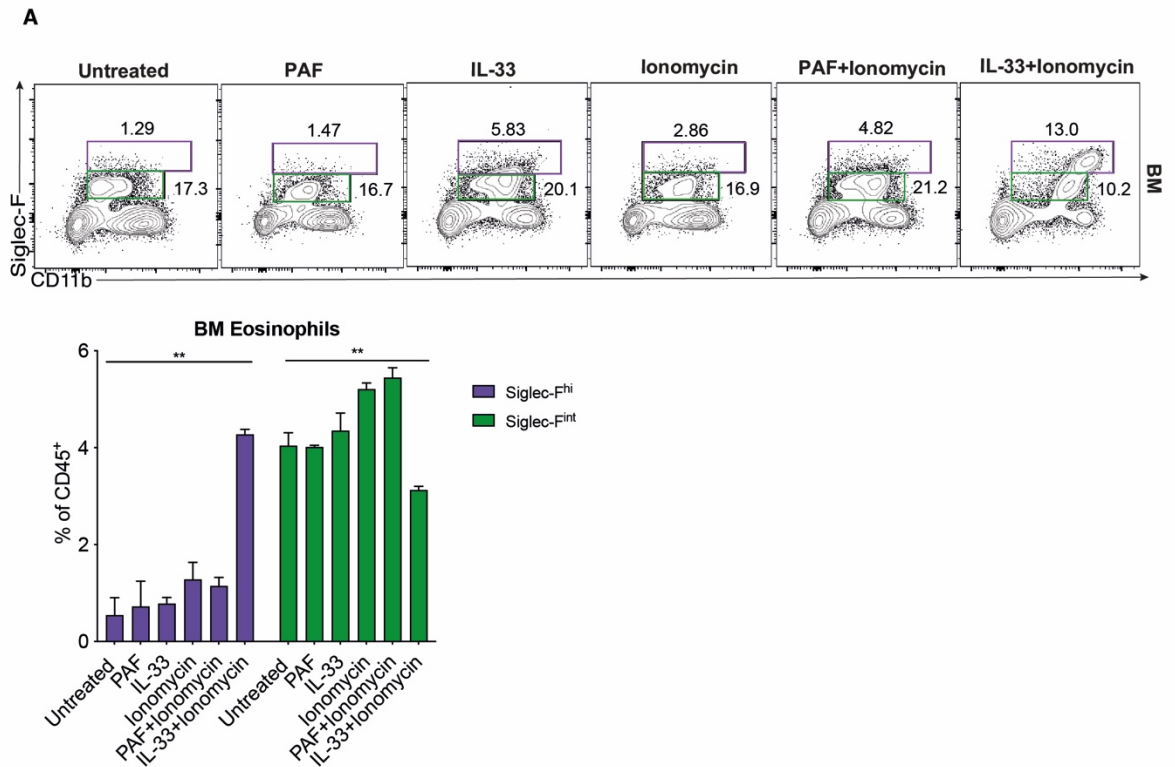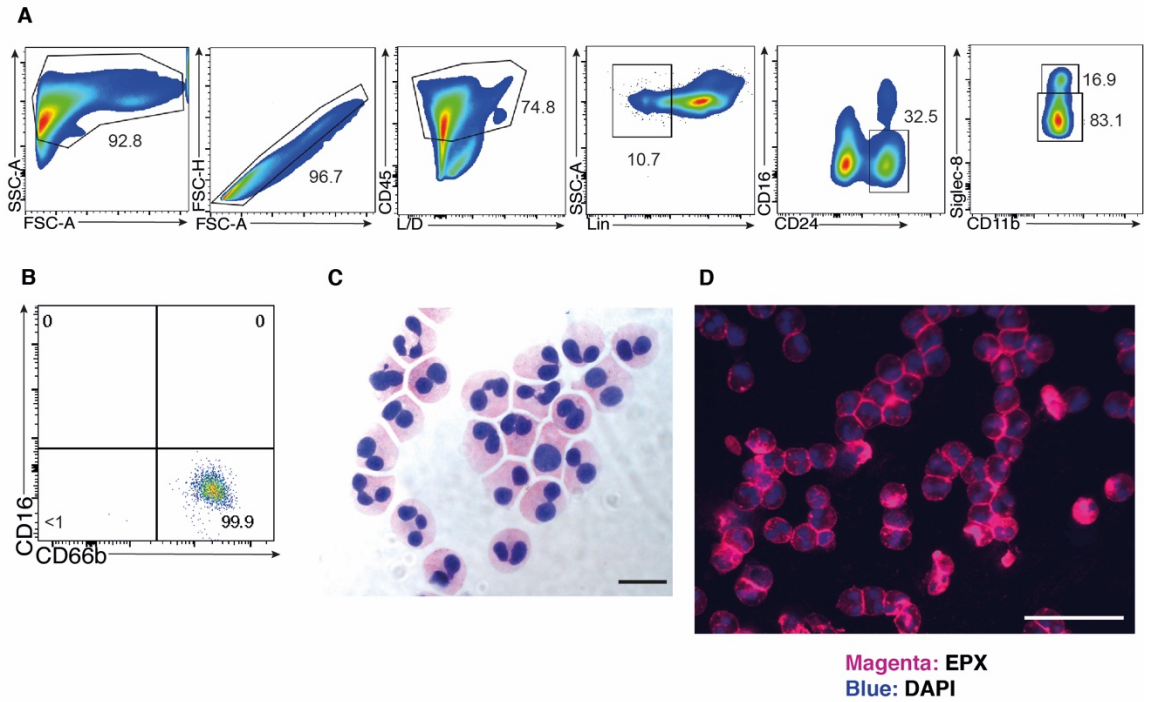
Figure 3.17: Supplementary figure 7.
A) Gating strategy to identify eosinophils in human lung or blood. Cells were identified as follows: SSC-A versus FSC-A to remove cellular debris; FSC-H versus FSC-A for doublet exclusion; Zombie Live-Dead versus CD45 to identify live haematopoietic populations; lineage versus SSC-A to exclude contaminating populations (lineage defined as TCRβ, CD56, CD14, CD20, CD117 and CD123 to exclude T cells, NK cells, monocytes, B cells, mast cells and basophils respectively); CD16 versus CD24 to exclude neutrophils and CD24⁻ cells; Siglec-8 versus CD11b to identify eosinophils. B) Expression of CD16 and CD66b was quantified by flow cytometry on Isolated human blood eosinophils. C) Cytospin of isolated human blood eosinophils stained with Diff-Quik (scale bar 10 μm). D) Representative staining of isolated human blood eosinophils for EPX (magenta) and counterstained with DAPI (blue) (scale bar 50 μm). Data shown are representative from at least two independent experiments.

CHAPTER 4 – PAPER 2

Lung-initiated Th2 inflammation is associated with development of intestinal type 2 responses

4.1 ABSTRACT

Recent studies have reported increased incidence of gut inflammation in patients with pulmonary diseases, supporting a bi-directional relationship between these distal organs. Nevertheless, the underlying mechanisms are still poorly understood. This study investigated the impact of acute allergen exposure in the lung on the frequency and phenotype of immune cells within the gut. We revealed that intranasal administration of house dust mite (HDM) led to development of Th2 responses not only in the lung but also in the small (SI) and large intestine (LI). In the SI, eosinophils dominated the inflammatory response, whereas Th2 T cells and ILC2s were the main immune populations in the LI. Importantly, this resulted in structural and biochemical changes in the LI microenvironment, as well as shifts in the composition of the gut microbiota. These findings broaden our current understanding of inter-organ communication and may play a role in the development of novel therapies for asthmatic patients.

4.2 INTRODUCTION

Patients affected by chronic inflammatory diseases of the airways, such as asthma and chronic obstructive pulmonary disease (COPD), often show concomitant chronic inflammation of the gut (Marsland et al., 2015).

Patients with inflammatory bowel disease (IBD) but no history of chronic lung disease often display airway inflammation or impaired lung function (Wang et al., 2013; Luo et al., 2008). Conversely, many asthmatic patients show alterations in their gut similar to those in their bronchial mucosa, including cellular atrophy, fibrosis, leukocyte infiltration and elevated cytokine levels (Vieira and Pretorius, 2010). Communication between the gut and the lung has also been evidenced by animal models of allergic asthma or ulcerative colitis (UC), showing simultaneous inflammatory responses in the colon and mesenteric lymph nodes (Huffnagle, 2010; Jung et al., 2019) or lung respectively (Liu et al., 2013).

Recently, alterations in the composition of the gut microbiota have been associated with predisposition to the pathogenesis of asthma both in humans and murine models (Hufnagl et al., 2020). Microbial dysbiosis in the first months of life has especially been linked with increased risk of developing asthma (Wesemann and Nagler, 2016; Zhang et al., 2020). This is clearly shown in GF mice, which are more prone to generate inflammatory responses and display impaired induction of oral tolerance (Frati et al., 2019).

The gut and the lung are very similar organs, as they share the same embryonic origin, structure, as well as exposure to commensals and pathogens (Budden et al., 2017). This further supports the evidence for a bi-directional relationship between these two sites. Nevertheless, the underlying mechanisms are still poorly understood. Moreover, the causality between lung and gut inflammation is also not clear (Tulic et al., 2016). Further mechanistic insight into the pathways and mediators are therefore necessary to improve our understanding of the lung-gut axis and inform the development of novel therapies.

Using a mouse model of HDM-induced allergic lung inflammation we found that allergen inhalation triggered Th2 responses in the gut, that could be distinguished between the SI and LI. In the LI we detected increased proportions of T cells and ILC2s secreting Th2 cytokines, whereas eosinophils dominated the Th2 response in the SI. We showed that IL-33 inhalation did not induce intestinal Th2 responses, suggesting that they may be antigen dependent. Moreover, we showed that HDM did not directly trigger the generation of Th2 responses in the gut and provided evidence for T cells migrating from the lung to the gut by upregulating gut-homing receptors. In addition, cell-mediated Th2 responses affected the whole intestinal environment by inducing changes in goblet cell structure, mucin glycosylation as well as increasing HIF-1α expression and levels of Th2 mediators, with IL-33 being significantly elevated in the LI compared to the SI. Cell-mediated Th2 responses were reduced in the absence of IL-33, suggesting that this mediator is important in regulating Th2 immunity in the LI. Importantly, HDM-treated mice also displayed alterations in the composition of the gut microbiota, with a decrease in the relative abundance of *Bacteroidetes* and an increase in *Firmicutes,* suggesting that microbiome-mediated metabolic shifts may result from ongoing Th2 responses in the lung. Together these data further support the existence of a lung-gut axis, in which Th2 responses at one mucosal site can prime for similar responses in distant mucosal sites, and this may inform about therapeutic development for asthmatic patients.

4.3 RESULTS

4.3.1 Allergen inhalation triggers Th2 responses in the gut

Inhalation of allergens, such as HDM, is widely known to cause allergic lung inflammation both in humans and animal models (Daan de Boer et al., 2013). The inflammation is characterised by Th2 responses involving airway infiltration of antigen-specific CD4^{+} T cells, primed by antigen presenting cells (APCs) in lung-draining lymph nodes, and airway eosinophilia. This leads to elevated production of Th2 cytokines, such as IL-4, IL-13 and IL-5, mucus hypersecretion and airway remodelling (Gregory and Lloyd, 2011). Recently, allergic responses in the lung have been correlated with concomitant gut inflammation and pathogenesis of diseases such as inflammatory bowel disease (IBD) i.e. ulcerative colitis (UC) and Crohn's disease (CD) (Liu et

al., 2013; Wang et al, 2013). Changes in the gut microbiota in early life have also been suggested to play a major role in predisposing the development of allergic lung disease (Frati et al., 2019). Nevertheless, the mechanisms underlying lung-gut cross communication are still poorly understood (Tulic et al., 2016).

To establish whether inhalation of HDM caused simultaneous inflammatory responses in the lung and gut, we exposed WT BALB/c mice to a 11-day sensitisation and challenge protocol using 100 µg and 10 µg of HDM respectively and, 24 hours after the last challenge, tissues were collected for histological and flow cytometry analysis (Fig. 4.1A). As mentioned previously, cellular infiltration and activation are characteristic pathological features of lung allergic asthma and are observed in both central and peripheral airways. Cellular infiltration is normally assessed using histological staining techniques, such as Haematoxylin and Eosin (H&E) (Hamid, 2003). To firstly verify whether changes in cellular infiltration occurred both in the lung and the gut after HDM administration, we performed H&E staining of paraffin-embedded lung and gut tissue sections. This showed increased cellular infiltrate not only in the lungs of HDM-exposed mice compared to naïve controls, but also in their SI and LI (Fig. 4.1B). Considering that CD4$^+$ T cells are the main mediators of Th2 inflammatory responses in the lung during allergic asthma, we aimed at understanding whether numbers of these cells were increased in the gut mucosa following administration of HDM. Immunofluorescence analysis showed increased numbers of CD4$^+$ cells, most likely T cells, in the LI but not in the SI of HDM-treated mice compared to naïve controls (Fig. 4.1C). Flow cytometry analysis also showed that CD4$^+$ T cells were increased in the LI as a percentage of CD45$^+$ cells (Fig. 4.1D). Although the SI lamina propria (LP) did not show an increase in the number of CD4$^+$ T cells, their percentage was increased in the Peyer's Patches (PPs) of HDM-treated mice compared to naïve controls (Fig. 4.1E). Interestingly, flow cytometry analysis also showed that the proportions of CD8$^+$ T cells and ILC2s were increased in the LI of HDM-treated mice compared to naïve controls (Fig. 4.1D). Both CD4$^+$ T cells and CD8$^+$ T cells in the LI (gating strategy shown in Supplementary Fig. 1A) were activated and polarised towards a Th2 phenotype as shown by increased production of the Th2 cytokines IL-13, IL-4, IL-5 and IL-33 (Fig. 4.1F-G) and expression of the transcription factor GATA3 (Supplementary Fig. 1B). Nevertheless, both cell types also upregulated release the Th1 cytokine IFN-γ (Fig 4.1F-G) and expression of the transcription factor T-bet (Supplementary Fig. 1B). CD4$^+$ T cells in the PPs also showed increased release of Th2 and Th1 cytokines following HDM administration (Fig. 4.1I). ILCs have been categorized into three groups based on their developmental pathways, being group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) (Bal et al., 2020). Like Th2 cells, ILC2s depend on the transcription factor GATA3 for their development and regulation of *Il5* and *Il13* gene expression (Zhu, 2017). Like T cells, also ILC2s in the LI (gating strategy shown in Supplementary Fig. 1C)

displayed upregulated expression of Th2 cytokines in addition to GATA3 and Ki67, the latter being a marker used to indicate cellular proliferation (Li et al., 2017) (Fig. 4.1H).

These results indicate that allergen inhalation not only induces Th2 inflammatory responses in the lung but also in the gut, especially in the LI LP compared to the SI LP. Our data then suggest that these responses are mediated by activated T cells- and ILC2s-producing Th2 cytokines. These data further support the idea that inter-organ communication occurs between the lung and the gut and provide additional evidence for Th2 responses taking places at mucosal sites distal from the primary site of inflammation.
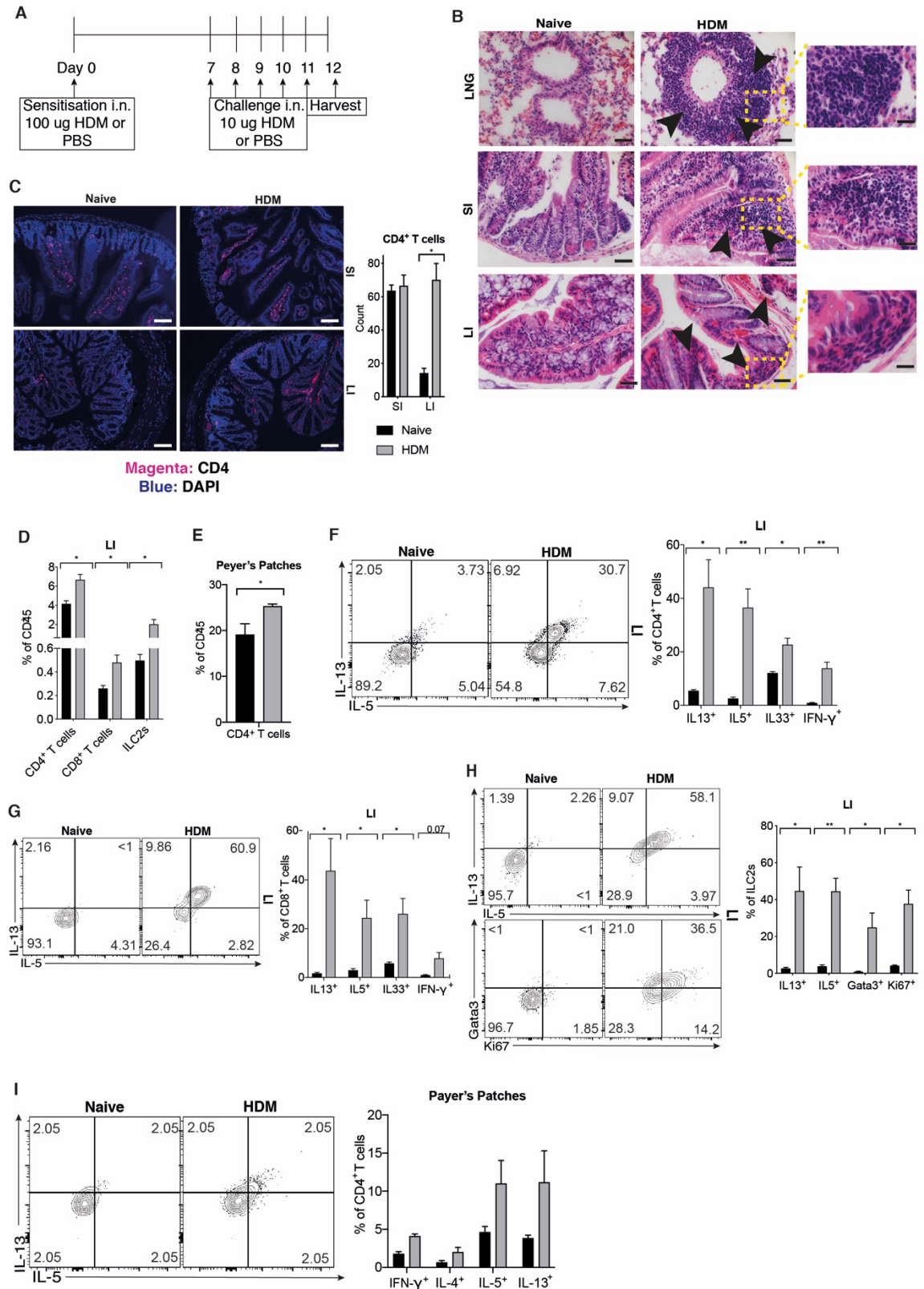
Figure 4.1: Th2 cells are increased and activated in the gut

A) Experimental timeline. 8-12 week old female BALB/c WT mice were sensitised on day 0 with 100 µg HDM and challenged on day 7-11 with 10 µg HDM. Control mice sensitised and challenged with PBS. Mice were euthanised 24 hours after the last challenge (on day 12). B) Cellular infiltration was determined with H&E-stained sections of the lung, SI and LI from naïve and HDM-treated mice (scale bar 100 µm and 10 µm). C) CD4⁺ T

cells were quantified by IF staining of CD4 on gut tissue from naïve and HDM-treated mice (scale bar 100 µm and 10 µm). D) T cells and ILC2s were quantified by flow cytometry in the LI of naïve and HDM-treated mice and expressed as a percentage of CD45$^+$ cells. E) CD4$^+$ T cells were quantified by flow cytometry in the PPs of naïve and HDM-treated mice and expressed as a percentage of CD45$^+$ cells. F) The percentage of CD4$^+$ T cells expressing IL-13, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the LI of naive and HDM-treated mice. G) The percentage of CD8$^+$ T cells expressing IL-13, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the LI of naive and HDM-treated mice. H) The percentage of ILC2s expressing IL-13, IL-5, GATA3 and Ki67 was quantified by flow cytometry in the LI of naive and HDM-treated mice. I) The percentage of CD4$^+$ T cells expressing IL-13, IL-4, IL-5 and IFN-γ was quantified by flow cytometry in the PPs of naive and HDM-treated mice. Data shown are representative from at least two independent experiments. n = 3-5 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001

4.3.2 Allergen inhalation increases eosinophils and their activation in the SI

Together with T cells, increased levels of activated eosinophils are a characteristic feature of the allergic response in the lung (Fahy, 2009). Therefore, we sought to determine whether eosinophils also played a role in the Th2 response generated in the gut. Immunofluorescence analysis of the gut showed increased numbers of eosinophils, identified by expression of the granule major basic protein (MBP), in the SI but not in the LI of HDM-treated mice compared to naïve controls (Fig. 4.2A). Flow cytometry analysis also showed increased proportions of eosinophils in the SI and PPs but not in the LI of HDM-treated mice compared to controls (Fig. 4.2A-B). Interestingly, eosinophils were not increased in the LP of the SI but in its intraepithelial (IE) compartment (Fig. 4.2B), where they displayed higher expression of CD11b, compared to LP eosinophils, even at steady-state (Fig. 4.2C). We have previously shown that inflammatory eosinophils, or (i)EOS in the lung, upregulate expression of CD101 and CD98 following HDM administration (see Paper 1). Therefore, we looked to see whether eosinophils in the gut expressed such molecules and whether their expression was modulated by HDM treatment. Both eosinophils from the LP and IE compartment of the SI expressed CD101 and CD98 at steady-state, but only IE eosinophils upregulated both molecules following HDM treatment (Fig. 4.2D). Similar to LP eosinophils, eosinophils from the LI expressed CD101 and CD98 at steady-state but their expression was unaltered by HDM (data not shown). Interestingly we also noted a marked increase in IL-4$^+$ eosinophils in the PPs of HDM-treated mice compared to controls (Fig. 4.2E), suggesting that these cells may be a major source of this Th2 cytokine in the SI following allergen inhalation.

These data suggest that eosinophil-mediated Th2 responses are predominant in the SI compared to the LI, where we saw that T cells are the main immune populations. Moreover, we showed that eosinophil heterogeneity is not only present in the lung during inflammation, but that different populations of eosinophils may exist in the SI where their phenotype and function can be modulated by inhalation of HDM.
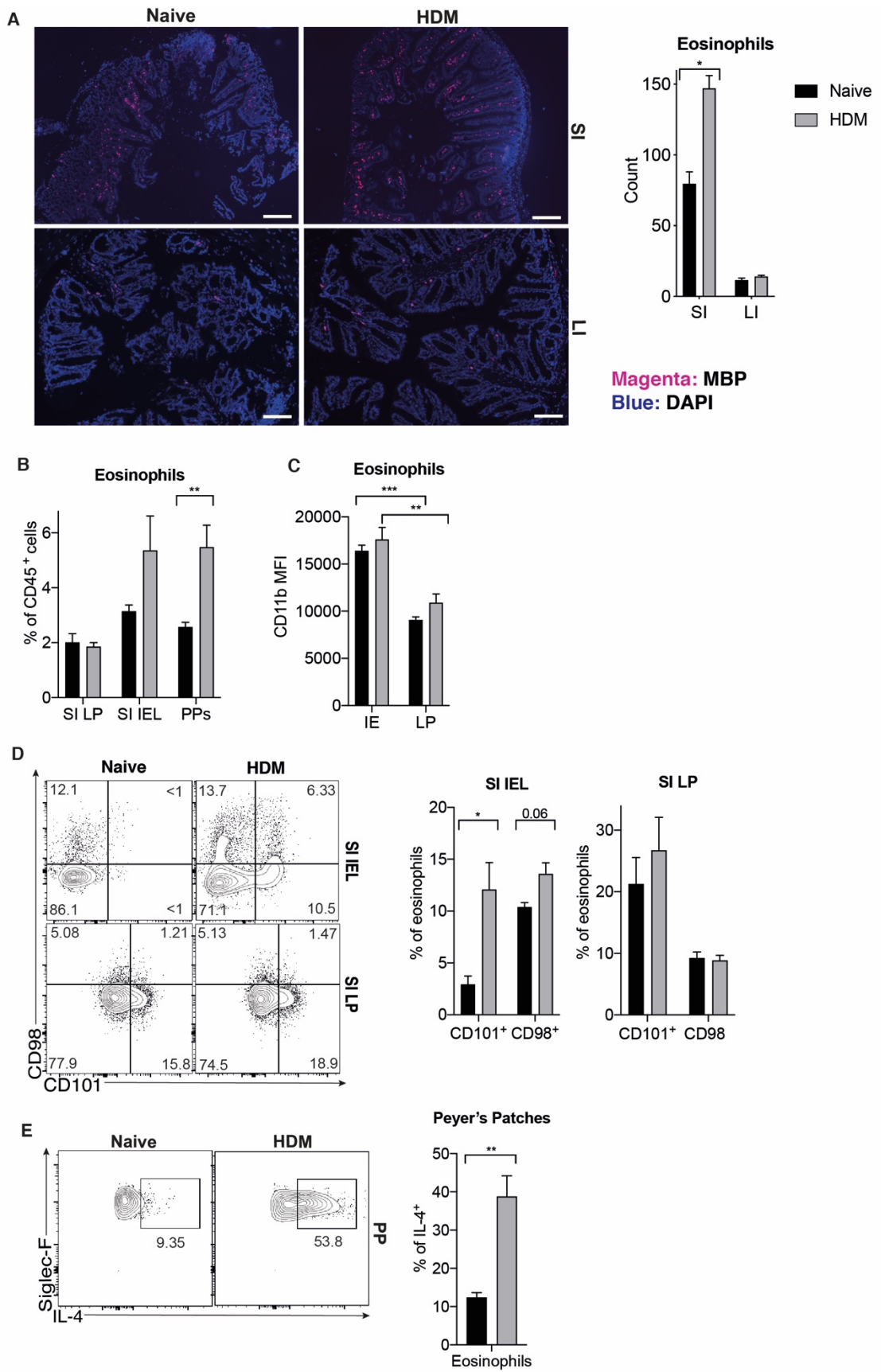
Figure 4.2: Eosinophils are increased and activated in the SI and PPs
A) Eosinophils were quantified (count per crypt over 20 crypts) by IF staining of major basic protein (MBP) on gut tissue from naïve and HDM-treated mice (scale bar 100 µm). B) Eosinophils were quantified by flow

cytometry in the SI and PPs of naïve and HDM-treated mice and expressed as a percentage of CD45$^+$ cells. C) Expression of CD11b was quantified by flow cytometry on eosinophils from the IE and LP of the SI of naïve and HDM-treated mice and expressed as MFI. D) The percentage of eosinophils expressing CD101 and CD98 was quantified by flow cytometry in the IE and LP of the SI of naïve and HDM-treated mice. E) The percentage of eosinophils expressing IL-4 was quantified by flow cytometry in the PPs of naive and HDM-treated mice. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001

4.3.3 Th2 responses in the gut are indirectly induced by HDM

Since intranasal administration may lead to inadvertent delivery of allergen to the GI tract, we aimed to understand whether Th2 responses in the gut were either directly or indirectly caused by intranasal exposure to HDM. To this end, we firstly administered HDM intratracheally, which, compared to intranasal administration, involves direct delivery of the antigen to the airways, as we showed following administration of Evan's blue (Supplementary Fig. 3A). Similar to intranasal administration, intratracheal administration of HDM induced a Th2 response in the gut, demonstrated by increased frequencies of CD4$^+$ and CD8$^+$ T cells and their production of Th2 cytokines both in the LI and PPs (Fig. 4.3A-E). To provide further evidence that HDM does not directly trigger an inflammatory response in the gut, we labelled the antigen with AF647 Antibody Labelling Kit (Invitrogen) and delivered it intranasally or by oral gavage to establish whether APCs underwent antigen uptake in the lung, mediastinal (MED) lymph nodes (LNs), mesenteric (MES) LNs or SI. Flow cytometry analysis showed that ~40% and ~20% of DCs in the lung and MED LNs respectively were positive for HDM when delivered intranasally, indicating that these cells performed antigen uptake (Fig. 4.3F-G). Conversely, DCs in the SI and MES LNs did not show HDM uptake when delivered intranasally (Fig. 4.3F-G). Likewise, when HDM was orally administered, DCs did not uptake the antigen neither in the gut nor in the lung (Fig. 4.3F-G). These results were matched by histological analysis of lung and SI tissue, showing that when HDM was delivered intranasally and not orally, only cells in the lung but not in the SI were labelled (Fig. 4.3I). These data suggest that HDM does not reach the gut mucosa when either intranasally or orally administered and, consequently, APCs like DCs do not perform antigen uptake. Flow cytometry analysis also revealed that cells other than DCs, including AMs, eosinophils and B cells had the ability to uptake HDM in the lung (Fig. 4.3H), suggesting the possibility of them acting as APCs or directly responding to antigen challenge.

These results therefore imply that Th2 responses found in the gut are not the result of direct contact with HDM but may be induced indirectly.
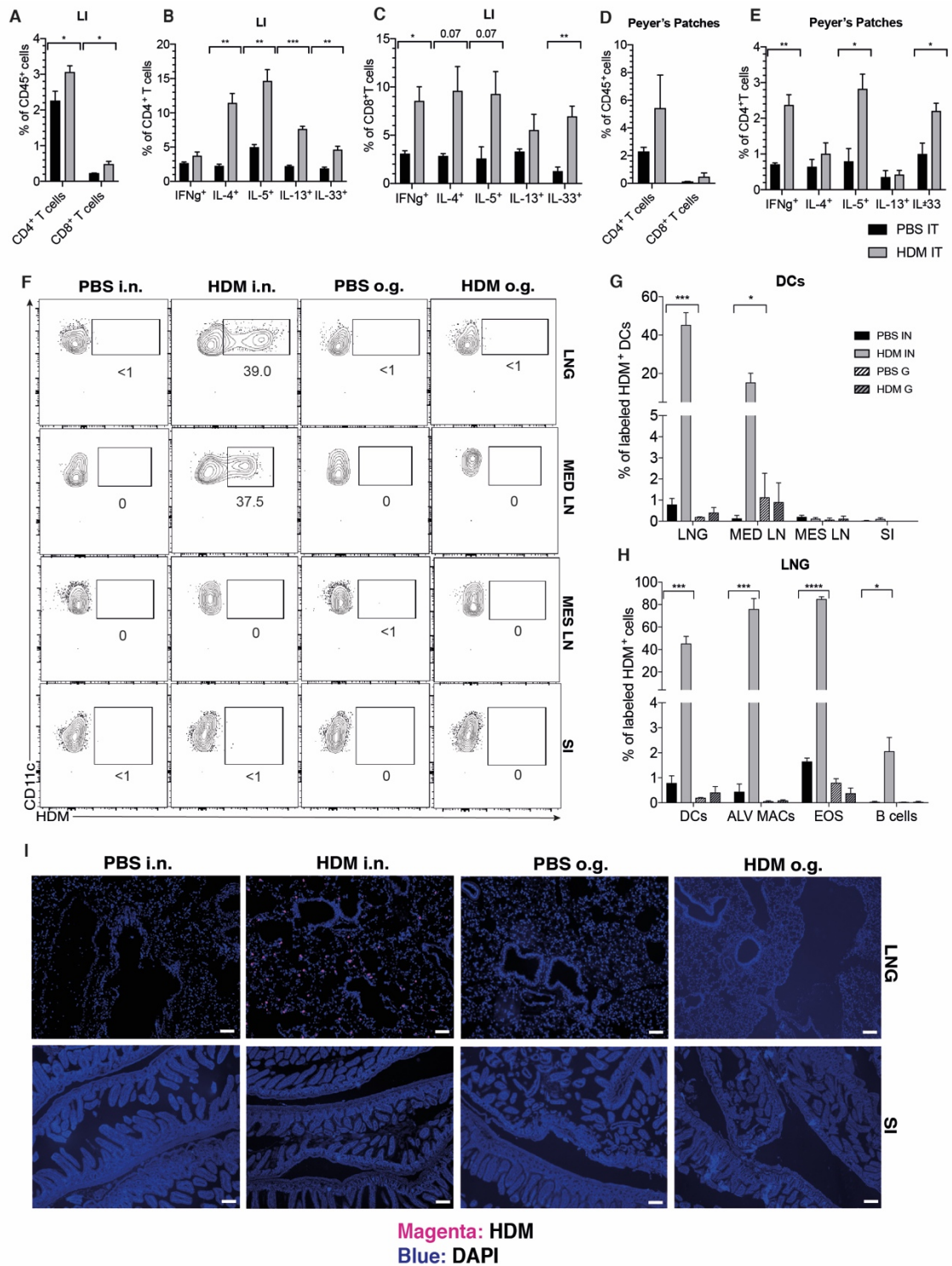
Figure 4.3: Th2 responses in the gut are indirectly induced by allergen inhalation

A) T cells were quantified by flow cytometry in the LI of naive and HDM i.t.-treated mice and expressed as percentage of CD45$^+$ cells. B-C) The percentage of CD4$^+$ and CD8$^+$ T cells expressing IL-13, IL-4, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the LI of naive and HDM i.t.-treated mice. D) T cells were quantified by flow cytometry in the PPs of naive and HDM i.t.-treated mice and expressed as percentage of CD45$^+$ cells. E) The percentage of CD4$^+$ T cells expressing IL-13, IL-4, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the PPs of naive and HDM i.t.-treated mice. F-G) The percentage of HDM$^+$ DCs (CD11c$^+$major histocompatibility complex (MHC)-II$^+$) was quantified by flow cytometry in the lung, MED LNs, MES LNs and SI of mice treated with PBS or HDM either administered intranasally or orally. H) The percentage of HDM$^+$ cells was quantified by flow cytometry in the lung of mice treated with PBS or HDM either administered intranasally

or orally. I) Representative staining of lung and SI tissue for HDM^{+} cells (magenta) and counterstained with DAPI (blue) (scale bar 100 μm). Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with one-way ANOVA: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

4.3.4 Intranasal administration of IL-33 does not induce Th2 responses in the gut

Following allergen inhalation, DCs uptake antigens in the airways and subsequently colocalise with naïve CD4^{+} T cells in lung-draining LNs. There, DCs present antigens to T-cells via MHC-II and, via expression of co-stimulatory molecules, lead to T cell activation and differentiation into antigen-specific Th2 cells (Grinnan et al., 2006). Having established that HDM does not enter the GI tract after intranasal or intratracheal administration, we explored whether antigen non-specific activation could account for the Th2 responses we detected in the gut. To this end, instead of HDM we intranasally administered the cytokine IL-33, which is known to trigger allergic asthma in mice (Kurowska-Stolarska et al., 2009; Chan et al., 2019). Increased levels of activated eosinophils are a characteristic feature of the allergic response in the lung (Fahy, 2009), and we showed that inhalation of IL-33 triggered the same features as HDM in the lung, namely eosinophilia, generation of (i)EOS and their upregulation of CD101 and CD98 (Fig. 4.4A-B and see Paper 1). However, flow cytometry analysis of the LI showed that IL-33 inhalation neither resulted in increased frequencies of CD4^{+} T cells or ILC2s (Fig. 4.4C) nor in their activation (Fig. 4.4D-E) compared to administration of HDM.

Taken together, these data suggest that antigen delivery, and subsequent generation of antigen specific T cells, may be necessary for the generation of Th2 responses in the gut mucosa, which were not generated following cytokine-induced allergic lung inflammation.
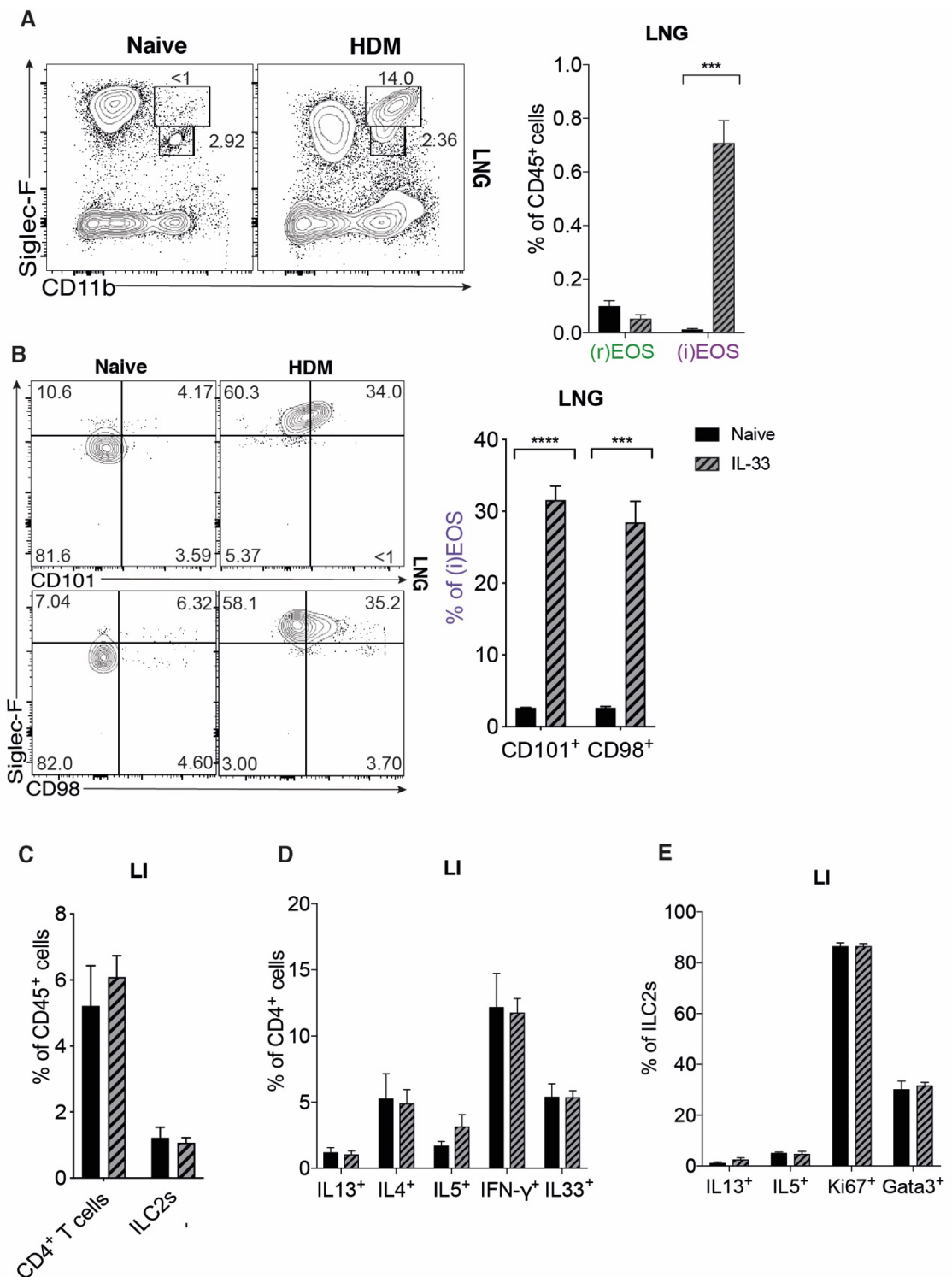
Figure 4.4: IL-33 i.n. does not induce gut inflammation
A) Eosinophils were quantified by flow cytometry in the lungs of naive and IL-33-treated mice and expressed as percentage of CD45$^+$ cells. B) The percentage of (i)EOS positive for CD101 and CD98 was quantified by flow cytometry in the lungs of of naive and IL-33-treated mice. C) CD4$^+$ T cells and ILC2s were quantified by flow cytometry in the LI of naive and IL-33-treated mice and expressed as percentage of CD45$^+$ cells. D) The percentage of CD4$^+$ T cells expressing IL-13, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the LI of naive and IL-33-treated mice. E) The percentage of ILC2s expressing IL-13, IL-5, Gata3 and Ki67 was quantified by flow cytometry in the LI of naive and IL-33-treated mice. Data shown are from one experiment. n = 3-5. Error

bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001

4.3.5 T cells in the lung, blood and MED LNs upregulate gut-homing receptors

In addition to their role as initiators of the adaptive immune response, there is evidence that lung DCs can induce expression of gut-homing receptors on antigen-specific T cells allowing their migration from the lung to the gut following intranasal immunisation (Ruane et al., 2013; Jung et al., 2019). Integrin α4β7 (or LPAM-1) and CCR9 have been identified as SI-homing receptors (Berlin et al., 1995, Zabel et al., 1999), whereas more recent studies identified GPR15, GPR55 and GPR18 to be involved in cell migration within or to the LI (Sumida et al., 2018, Wang et al., 2014, Kim et al., 2013). We therefore hypothesised that circulating T cells could migrate from the lung to the gut and be involved in the generation of a Th2 response following HDM administration. To verify our hypothesis, HDM-treated mice or naïve controls received intraperitoneal administration of FTY720, which is an inhibitor of lymphocyte egress from LNs, and frequencies of T cells from the lung, MED LNs, blood and MES LNs were determined. Expression of the SI-homing receptors α4β7 and CCR9, as well as the LI-homing receptors GPR15, GPR55 and GPR18 was also investigated. Our results showed that HDM treatment increased the frequencies of CD4$^+$ (Fig. 4.5A) and CD8$^+$ (data not shown) T cells in the MED LNs compared to naïve controls, whereas it decreased their frequencies in the MES LNs (Fig. 4.5A). As expected, treatment with FTY720 markedly decreased frequencies of CD4$^+$ (Fig. 4.5A) and CD8$^+$ (data not shown) T cells in the lung and circulation of HDM-treated mice but increased their frequencies in the MES LNs (Fig. 4.5A). HDM treatment decreased the frequencies of CD4$^+$CD8$^+$ T cells in the MED and MES LNs, which was reversed by FTY720 (Fig. 4.5B). Our results also showed that HDM treatment decreased expression of SI- and LI-homing receptors on CD4$^+$ and double positive T cells from the lung, blood and MED LNs compared to naïve controls but increased their expression on CD4$^+$ and double positive T cells from the MES LNs (Fig. 4.5C-E). Treatment with FTY720 increased expression of SI- and to a greater extent LI-homing receptor on CD4$^+$ (Fig 4.5C-D) and CD8$^+$ (data not shown) T-cells from the lungs, MED LNs and blood. Upregulated expression of CCR9, GPR18 and α4β7 was detected on lung T cells, whereas T cells in the MED LNs upregulated expression of CCR9, GPR18 and GPR55 (Fig. 4.5C); T cells in the blood also showed upregulation of GPR18, α4β7 and GPR55, in addition to GPR15 (Fig. 4.5D). Conversely, expression of CCR9, GPR18 and GPR55 on CD4$^+$ T-cells from the MES LNs was downregulated following treatment with FTY720 (Fig. 4.5D). CD4$^+$CD8$^+$ T cells displayed the highest expression of CCR9 in all tissues (data not shown) and this was significantly upregulated by FTY720 in the MED LNs (Fig. 4.5E). HDM induced expression of CCR9, GPR55 and GPR18 on CD4$^+$CD8$^+$ T cells in the MES LNs, but again their expression was reduced by FTY720 (Fig. 4.5E).

These results suggest that following HDM administration T cells acquire expression of gut homing receptors in the lung, blood and MED LNs, which may license their migration to the gut. High expression of CCR9 on $CD4^+CD8^+$ T cells compared to single positive T cells may also suggest that double positive T cells preferentially migrate to the SI. Moreover, upregulation of gut-homing receptors was not uniformly induced on T cells but may be regulated in a receptor- and tissue-specific manner.
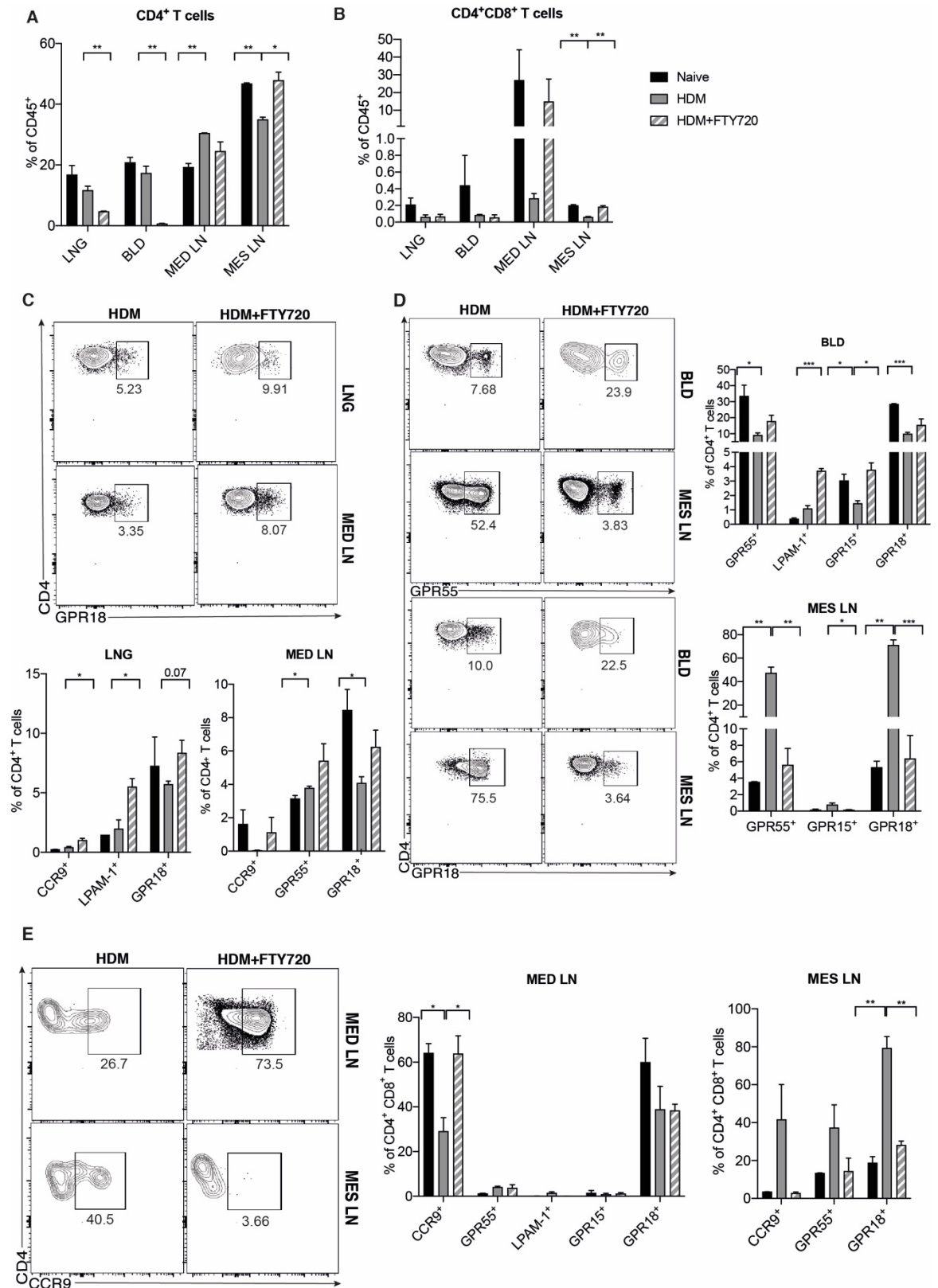
Figure 4.5: T cells may mediate the lung-gut cross talk
A-B) T cells were quantified by flow cytometry in the lung, MED LNs, blood and MES LNs of naïve mice and mice treated with HDM alone or with HDM+FTY720 and expressed as percentage of CD45^{+} cells. C-D) The percentage of CD4^{+} T cells expressing CCR9, GPR55, LPAM-1, GPR15 and GPR18 were quantified by flow cytometry in the lung, MED LNs, blood and MES LNs of naïve mice and mice treated with HDM alone or with HDM+FTY720. E) The percentage of CD4^{+}CD8^{+} T cells expressing CCR9, GPR55, LPAM-1, GPR15 and GPR18 were quantified by flow cytometry in the MED and MES LNs of naïve mice and mice treated with HDM

alone or with HDM+FTY720. Data shown are from one experiment. n = 3-4. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001.

4.3.6 HDM-induced Th2 responses in the gut affect the whole intestinal environment

After having investigated the cell-specific nature of the Th2 inflammation being generated in the gut following inhalation of HDM, we wanted to understand whether these responses were associated with development of downstream type 2 effector molecules. To answer this question, we analysed the cytokine milieu of the whole SI and LI and compared this to the lung. The results showed that mRNA expression of cytokines known to be associated with Th2 responses, such as IL-4, IL-13, as well as Relm-α and Ym-1 (Sutherland et al., 2018), were not only upregulated in the lung following HDM administration but also in the SI and to a lesser extent in the LI (Fig. 4.6A). In contrast, mRNA expression of IL-33 was higher in the LI compared to the SI (Fig. 4.6A). mRNA expression of the chemokine CCL11 which is involved with eosinophil recruitment (Broide and Sriramarao, 2001), was also upregulated in the SI but not in the LI (Fig. 4.6A). Analysis of secreted cytokines in the supernatant of lysed tissues confirmed the presence of type 2 associated factors in the gut, evidenced by high levels of IL-4, IL-13, Relm-α, Ym-1 and IL-33. Of note, IL-4, Relm-α and Ym-1 were predominant in the SI, whereas IL-13 and IL-33 were highest in the LI (Fig. 4.6B). Immunofluorescence staining of the lung revealed that HDM induced Ym-1 (red) production by cellular infiltrates scattered throughout the alveoli, whereas Relm-α (green) was mainly released by epithelial cells around the bronchi (Fig. 4.6C). HDM also induced expression of Relm-α and Ym-1 in the SI and LI compared to controls (Fig. 4.6C). However, in contrast to the lung, these molecules were co-expressed by the same cells in the gut (Fig. 4.6C). Neither CD4$^+$ T cells nor eosinophils stained for Relm-α or Ym-1 (data not shown), suggesting that additional cell types contribute to the Th2 response in the gut.

IL-33 is known to play a major role in the activation of ILC2s (Moro et al., 2010) and Th2 cells (Schmitz et al., 2005). Since we detected higher expression of IL-33 mRNA and protein in the LI compared to the SI of HDM-treated mice, we investigated the role of this cytokine in the activation of T cells and ILC2s by using *Il-33–LacZ* gene trap (Gt) reporter strain (Il-33$^{Gt/Gt}$), which lack endogenous IL-33 (Pichery et al., 2012). The results showed that the HDM-induced increase in the proportions of T cells or ILC2s that we detected in comparison to naïve mice (Fig. 1C-D) was reduced in the LI in the absence of IL-33 (Fig. 4.6D). IL-33 deficiency also decreased HDM-induced activation of both CD4$^+$ T cells and ILC2s compared to controls (Fig. 4.6E-F).

These results suggest that Th2 responses in the gut may lead to polarision of the whole intestinal environment by increasing the expression and levels of Th2 cytokines. Moreover, IL-33 may be

involved in the generation of such responses and may have a role in lymphocyte recruitment and activation in the LI.
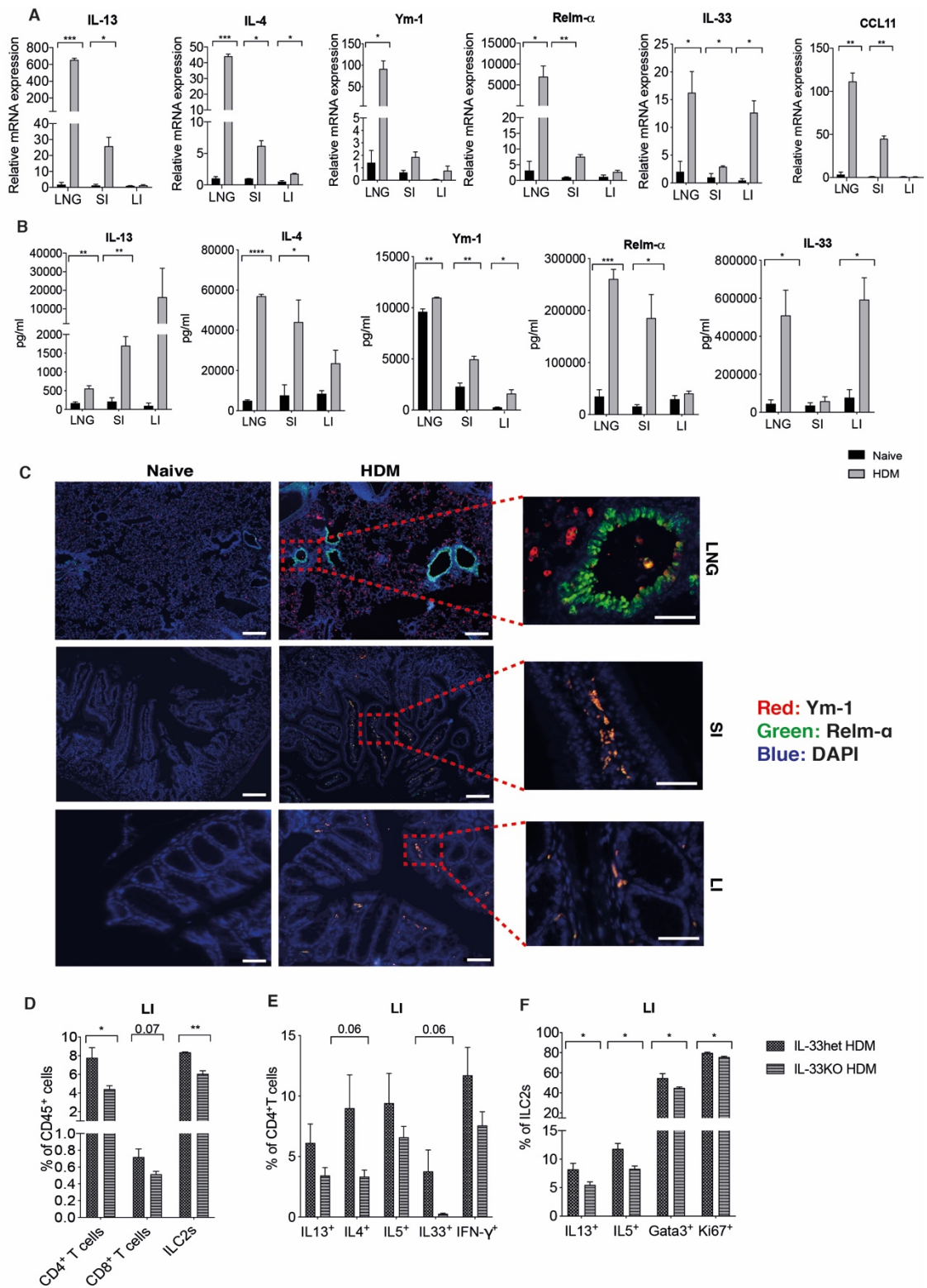
Figure 4.6: Effects of Th2 inflammation on the gut environment
A) Expression of IL-4, IL-13, Ym-1 Relm-α, IL-33 and CCL11 was established in lung, SI and LI of naive and HDM-treated mice following RNA extraction and qPCR. B) Levels of IL-4, IL-13, Ym-1 Relm-α and IL-33 were determined by ELISA in the supernatant of lysed lung, SI and LI tissue of naïve and HDM-treated mice. C)

Representative staining of lung, SI and LI tissue for Ym-1 (red), Relm-α (green) and counterstained with DAPI (blue) (scale bar 100 μm and 50 μm). D) T cells and ILC2s were quantified by flow cytometry in the LI of HDM-treated Il-33$^{Gt/Gt}$ and Il-33$^{+/Gt}$ mice and expressed as percentage of CD45$^+$ cells. E) The percentage of CD4$^+$ T cells expressing IL-13, IL-5, IL-33 and IFN-γ was quantified by flow cytometry in the LI of HDM-treated Il-33$^{Gt/Gt}$ and Il-33$^{+/Gt}$ mice. F) The percentage of ILC2s expressing IL-13, IL-5, Gata3 and Ki67 was quantified by flow cytometry in the LI of HDM-treated Il-33$^{Gt/Gt}$ and Il-33$^{+/Gt}$ mice. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4.3.7 HDM-induced structural changes in the LI may be correlated with alterations in the composition of the gut microbiota

Goblet cell hyperplasia is another characteristic pathological feature of lung allergic asthma and is mainly induced by high levels of IL-13 (Junttila, 2018). Since we established that Th2 cytokines significantly increased in the gut following HDM administration, with IL-13 particularly in the LI, we evaluated whether changes in goblet cells were also present. To do so, lung and gut sections were stained with PAS-Alcian blue, which showed goblet cells being more filled and intensely stained in the lung. This was also identified in the gut but it was predominant in the LI compared to the SI (Fig. 4.7A).

Together with IL-13, HIF-1α signalling has also been found to promote goblet cell hyperplasia (Polosukhin et al., 2011). Therefore, we also assessed cellular expression of HIF-1α by immunofluorescence staining. Our results showed an increase in the number of cells expressing HIF-1α (Fig. 4.7B), suggesting that its signalling might contribute to the changes seen in goblet cells.

Alterations in goblet cell structure have been correlated with changes in mucin glycosylation (Kim and Khan, 2013), therefore we investigated whether this was also present in our model. To this end, we performed dual immunofluorescence labelling for Muc2 (green), the major component of the intestinal mucus (Birchenough et al., 2015), and lectins (red), as specific molecular probes to detect mucin carbohydrates (Kawano et al., 1984). The most striking differences were observed in the expression of soybean agglutinin (SBA), which recognises α-D-N-acetyl-galactosamine (GalNAc) and α and β galactose (Gal) (Rao et al., 1998), and peanut agglutinin (PNA), which recognises D-Gal(1-3)GalNAc (Natchiar et al., 2007). Increased binding of these lectins was detected in the LI of mice exposed to HDM compared to controls (Fig. 4.7C), suggesting that changes in goblet cell structure are accompanied by alterations in their glycosylation pattern.

Defective goblet cell activity and structure have not only been correlated with inflammation but also with dysbiosis of the gut microbiome (Corfield, 2018). We therefore investigated whether the alterations in goblet cells we identified were associated with changes in the composition of

the gut microbiome. To this end, we determined the abundance of the phyla *Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia* and *Proteobacteria,* and the species *Segmented filamentous bacterium (SFB), Lactobacillae, F. prausnitzii (Firmicutes), Helicobacter (Proteobacteria), Veillonella* and *Prevotella (Bacteroidetes)* relative to 16S by qPCR from the faeces of control and HDM-treated mice. The results showed decreased abundance of *Bacteroidetes, Veillonella* and *β Proteobacteria,* and unaltered *Lactobacillae* in HDM-treated mice, whereas all the other phyla and species analysed showed a trend towards an increase (Fig. 4.7D).

Together, these data suggest that exposure to HDM not only induces cell-specific changes in phenotype and function, but affects the overall gut environment, including mucus production and mucin glycosylation, that may potentially cause subsequent alterations in the composition of the microbiome.

A

Naive · HDM — LNG, SI, LI

Goblet cells

Count

LI ·· SI

■ Naive
■ HDM

*** · 0.09

B

Naive · HDM — LI

Green: HIF-1α
Blue: DAPI

LI

Count

HIF-1α⁺ cells

*

C

Naive · HDM — LI

PNA · SBA

Red: PNA or SBA
Green: Muc2
Blue: DAPI

LI

% of goblet cells

PNA⁺ · SBA⁺

** · **

D   **Relative mRNA expression**

Bacteroidetes
Firmicutes
Actinobacteria
Verrucomicrobia
SFB
Helicobacter
Veillonella
Lactobacillae
F. prausnitzii
γδ Proteobacteria
β Proteobateria
Prevotella

Day 12 PBS   Day 12 HDM

Bacteroidetes

Relative mRNA expression

Faeces

****

Firmicutes

Relative mRNA expression

Faeces

● Day 12 PBS
■ Day 12 HDM

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Figure 4.7: HDM inhalation induces changes in goblet cells and microbial dysbiosis in the gut
A) Goblet cells were quantified (count per crypt over 20 crypts) using PAS and Alcian-blue stained sections of lung, SI and LI from naive and HDM-treated mice (scale bar 100 μm). B) HIF-1α expressing cells were quantified (count per crypt over 20 crypts) by IF staining of HIF-1α on on sections of LI from naive and HDM-treated mice (scale bar 100 μm). C) Goblet cells and lectin^{+} goblet cells were quantified by IF staining of Muc2, SBA or PNA on sections of LI from naive and HDM-treated mice (scale bar 100 μm). D) The relative abundance of *Bacteroidetes, Firmicutes, Actinobacteria, Verrucomicrobia, SFB, Helicobacter, Veillonella, Lactobacillae, F. prausnitzii, β Proteobacteria, γδ Proteobacteria* and *Prevotella* was quantified by qPCR following DNA extraction from faeces of naïve and HDM-treated mice. Data shown are representative from at least two independent experiments. n = 3-4 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001.

4.4 DISCUSSION

Our results show that allergen inhalation not only triggered type 2 immune responses in the lung but it also induced the generation of such responses in the gut mucosa. Th2 responses in the gut were associated with activated T cells and ILC2s in the LI and eosinophils in the SI. Cytokine-induced lung allergy did not induce type 2 immune responses in the gut, suggesting that these inflammatory responses may be antigen dependent. We showed that T cells may mediate such responses by migrating from the lung to the gut as they upregulated expression of gut homing-receptors in the lung, circulation and MED LNs. HDM-induced inflammatory responses affected the whole gut microenvironment, which was characterised by increased expression and levels of Th2 cytokines, increased numbers of cells expressing HIF-1α, changes in mucus production and mucin glycosylation, as well as alterations in the composition of the commensal microbiota.

Several studies suggest the existence of a lung-gut axis, in which these tissues can communicate during the development of inflammatory disease originating from either organ (Bienenstock et al., 1978; Camus, et al., 1993; Tulic et al., 2016; Keely et al., 2012; West et al., 2015). Although many explanations for this cross-communication have been proposed, no definitive conclusions have been drawn yet. Moreover, whether the inflammatory process shifts from the gut to the airways or vice versa is still not known (Wang et al., 2013). Our results firstly support the idea that tissue cross-communication occurs during inflammatory responses, since inhalation of HDM triggered Th2 inflammation in both the lung and the gut. Inflammatory responses in the LI involved increased proportions of activated T cells and ILC2s secreting Th2 cytokines in the LI. IL-4, IL-13 and IL-5 are the central cytokines involved in orchestrating Th2 responses (Junttila, 2018), whereas IL-33 has been shown to be involved in initiating and amplifying Th2 responses mainly by targeting Th2 cells and ILC2s (Paul and Zhu, 2010; Löhning et al., 1998; Hodzic et al., 2017; Wynn, 2015).

Enhanced release of the Th1 cytokine IFN-γ by CD4^{+} and CD8^{+} T cells in the LI was also detected in our experiments. The role of IFN-γ release during Th2 responses is controversial,

as it has been shown to either promote homeostasis or contribute to the inflammatory responses both in the lung (Huang et al., 2001; Randolph et al., 1999; Nakagome et al., 2009; Hansen et al., 1999) and in the gut (Brasseit et al., 2018; Eriguchi et al., 2018; Ost et al., 2017; Farin et al., 2014). Interestingly, our data show that CD8^{+} T cells also predominantly secreted Th2 cytokines and a smaller amount of IFN-γ. This agrees with other studies showing that in the presence of IL-4, CD8^{+} T cells may convert into Th2 cytokine producing cells, which have been identified in Th2-driven diseases (Erard et al., 1993; Coyle et al., 1995; Jia et al., 2013; Seneviratne et al., 2007). These data therefore suggest that T cells and ILC2s were involved in the generation of inflammatory responses in the LI by secreting high levels of Th2 cytokines, whereas release of IFN-γ may either promote the ongoing inflammatory response or be associated with balancing Th2 responses to avoid extensive tissue damage.

Together with T cells, eosinophils are also important mediators of Th2 responses (Spencer and Weller, 2010). Although we did not detect changes in eosinophils in the LI, our results showed an increase in eosinophil numbers and proportions in the SI of HDM-treated mice compared to controls. Similar to Olbrich et al. (2020) and Xenakis et al. (2018), we also noticed that eosinophils isolated from the IE compartment of the SI expressed higher levels of CD11b compared to eosinophils from the LP. Upregulation of CD11b and CD11c on eosinophils has been associated with inflammation, cellular activation and trans-epithelial migration (Mesnil et al., 2016, Abdala Valencia et al., 2016), thereby suggesting that IE eosinophils display a more activated phenotype compared to LP eosinophils already at steady-state. Our analysis also showed that IE eosinophils but not LP eosinophils upregulated expression of CD101 and CD98 in HDM-treated mice compared to controls. We have previously demonstrated that inflammatory eosinophils in the airways of allergic mice upregulate such molecules (see Paper 1), suggesting that IE eosinophils are more prone to activation than their LP counterparts. Like CD4^{+} T cells, eosinophils are also a major source of IL-4 (Bjerke et al., 1996) and our analysis highlighted the presence of IL-4^{+} eosinophils in the PPs of allergic mice, suggesting that, together with IE eosinophils, they could mediate a Th2 response in the SI. These data firstly support the idea that eosinophils are plastic cells able to adapt and change their phenotype depending on the inflammatory response and tissue milieu they are localised in. Secondly, these results suggest that HDM-induced Th2 responses can be distinguished between the LI and SI: T cells and ILC2s may be the main mediators in the LI, whereas eosinophils in the SI.

To investigate the mechanisms underlying communication between the lung and the gut following allergen inhalation, we firstly demonstrated that HDM when intratracheally, intranasally or orally administered did not reach the gut or MES LNs. Unlike intranasal administration, which may lead to inadvertent delivery of the allergen to the GI tract, we confirmed that intratracheal instillations were restricted to the airways. We then showed that intratracheal delivery of HDM

still induced Th2 responses in the gut, shown by increased frequencies of T cells and enhanced cytokine release. Although HDM labelling and fluorescence analysis might not be sensitive enough to detect small amounts of antigen, we also showed that uptake of labelled HDM by APCs only occurred in the lung and MED LNs following intranasal delivery of HDM, suggesting that Th2 responses in the gut are generated via indirect mechanisms. Although we cannot completely rule out that some of the antigen may reach the GI tract, other studies have shown that intranasal or oral administration of substances is confined to the airways or stomach respectively (Olbrich et al., 2020; Lundholm et al., 1999; Trolle et al., 2000).

Previous studies suggest that inflammatory responses in the gut following intranasal immunisation are caused by lung-derived antigen-specific T cells migrating to the gut (Ruane et al., 2013; Jung et al., 2019). Accordingly, our results showed that, in contrast to antigen-driven inflammation induced by HDM, cytokine-driven Th2 responses induced by IL-33 were restricted to the airways and did not occur in the gut mucosa, suggesting that Th2 responses taking place in the gut may be antigen dependent.

To provide further evidence, we performed experiments to block the egress of T cells from LNs using FTY720. This resulted in a drop in the frequency of T cells in the lung and blood but accumulation of CD4$^+$ and CD8$^+$ T cells in the MES LNs and CD4$^+$CD8$^+$ T cells in the MED LNs, confirming that expansion of T cells occurred at lymphoid sites. More importantly, we observed that FTY720 induced upregulation of LI-, and to a lesser extent, SI-homing receptors on both CD4$^+$ and CD8$^+$ T cells, whereas CD4$^+$CD8$^+$ T cells highly expressed and upregulated the SI-homing receptor CCR9 in the lung, circulation and MED LNs. By contrast, expression of these receptors was significantly reduced in the MES LNs, suggesting that circulating T cells may migrate to the gut via upregulation of gut-homing receptors and be involved in the generation of Th2 responses in the LI.

Importantly, we not only identified cell-specific changes in the gut, but the Th2 response generated following inhalation of HDM had profound effects on the whole intestinal environment. This was shown by elevated mRNA expression and levels of Th2 cytokines, namely IL-4, IL-13, IL-5, IL-33, Relm-α and Ym-1, with IL-13 and IL-33 being predominantly increased in the LI compared to the SI. IL-4 and IL-13 signal through the IL-4 receptor α-chain (IL-4Rα) inducing phosphorylation of signal transducer and activator of transcription 6 (STAT6) (Minton, 2008). The receptor formed by IL-4/IL-4Rα with γc is a type I IL-4 receptor and the IL-4/IL-4Rα complex binding IL-13Rα1 is a type II IL-4 receptor (Nelms et al., 1999). In fact, based on the tissue distribution and cellular expression of type I and II IL-4R, IL-4 and IL-13 exert different functions. IL-4 mainly regulates lymphocyte functions, such as Th2 differentiation and B-cell IgG1 and IgE class switch from IgM, whereas IL-13 acts as effector cytokine inducing smooth muscle

contraction, goblet cell hyperplasia and increased mucus production (Junttila, 2018). Goblet cell hyperplasia, thus hypersecretion of stored mucins, leads to goblet cell depletion, resulting in defective mucin synthesis and barrier functions (Kim and Ho, 2010). Our results showed marked goblet cell hyperplasia in the LI and to a lesser extent in the SI, which could be attributed to the presence of higher levels of IL-13 in the LI (Kondo et al., 2006; Zhu et al., 1999; Finkelman et al., 2004). Together with IL-13, HIF-1α signalling has also been found to promote goblet cell hyperplasia (Polosukhin et al., 2011). HIF-1α is activated and stabilised in hypoxic conditions, and, although the gut normally is under a relative level of hypoxia, inflammation is frequently characterised by reductions in tissue oxygen levels (Bartels et al., 2013). Our results showed an increase in the number of cells expressing HIF-1α in the LI of HDM-treated mice compared to controls, suggesting it being a consequence of the ongoing inflammatory responses and a potential contributor to the changes seen in goblet cells.

Following allergen exposure, IL-33 is produced at high levels by epithelial and endothelial cells in the lung (Hammad et al., 2009) and has been shown to exacerbate antigen-induced asthma (Sjöberg et al., 2017). Gut inflammatory responses also involve increased production of IL-33 by endothelial cells (Sedhom et al., 2013), which has been shown to worsen inflammation, promote fibrosis (Pastorelli et al., 2011; Lopetuso et al., 2012) and induce goblet cell hyperplasia through IL-13 production by ILC2s (Waddell et al., 2019). Although non-haematopoietic cells are the main producers of IL-33, there is evidence that myeloid cells, such as AMs and mast cells, can also secrete this cytokine (Chang et al., 2011; Shimokawa et al., 2017). Our data firstly confirmed that allergen exposure in the lung is associated with upregulation of IL-33 and that levels of the cytokine were also highly upregulated in the LI but not in the SI, suggesting that IL-33 may be particularly important in regulating Th2 responses in this region of the gut. With the use of IL-33 deficient mice, we further showed that IL-33 may be one of the key factors involved in initiating Th2 responses in the LI, as absence of the cytokine significantly reduced HDM-induced Th2 cell and ILC2 activation. Interestingly, our data also indicate that T cells and eosinophils released IL-33, which has not been reported before. This suggests that these cells may contribute to secretion of IL-33 to maintain the ongoing inflammatory response in the gut. Nevertheless, further work will be required to confirm this.

Our results also showed that HDM induced upregulation of the chemokine CCL11 in the lung and SI but not in the LI. CCL11 specifically recruits eosinophils to tissues by binding to CCR3 expressed on their surface (Klion, 2017), therefore suggesting that eosinophils are recruited to the lung and SI following HDM exposure but not to the LI. This is in agreement with our data showing that numbers of eosinophils and their activation are increased in the SI but not in the LI of HDM-treated mice.

Relm-α and Ym-1 are effector molecules known to be abundantly released during Th2 inflammatory responses (Sutherland et al., 2018). Upregulation of such molecules occurs following binding of IL-4/IL-13 to IL-4Rα in several cell types, including eosinophils, macrophages and epithelial cells (Holcomb et al., 2000; Raes et al., 2002; Nair et al., 2009). The functions of Relm-α and Ym-1 are still not completely understood, as there is evidence for these molecules inducing both pro- and anti-inflammatory effects (Munitz et al., 2008 and 2012; Nair et al., 2009; Sutherland et al., 2014). Although we did not investigate the specific functions of Relm-α and Ym-1 in our study, we confirmed that these molecules are highly expressed and produced during Th2 responses in both the lung and the gut. Interestingly, cellular expression of Relm-α and Ym-1 differed between the two tissues. In the lung, cells localised throughout the alveoli solely expressed Ym1 and these cells have been identified to be AMs or neutrophils (Sutherland et al., 2018). On the other hand, Relm-α was only expressed by epithelial cells surrounding the bronchi. In the gut, Relm-α and Ym-1 were co-expressed by the same cell type, suggesting that expression of such molecules is regulated in a tissue- and cell-dependent manner.

Importantly, we not only identified goblet cell hyperplasia in the LI of HDM-treated mice, but changes in mucin glycosylation and composition of the commensal microbiome were also highlighted in this study and these are characteristic features of several inflammatory diseases, such as IBD, cancer, asthma and obesity (Arrieta et al., 2015; Fujimura et al., 2016; Wesemann and Nagler, 2016; Zhang et al., 2020; Morampudi et al., 2016; Kim and Ho, 2010).

The protein backbone of mucins contains tandem repeat units of varying length consisting of the amino acids proline, serine, and threonine, which create sites for O-glycosylation by O-linked oligosaccharides. The majority of these O-linked oligosaccharides are composed of N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), galactose (Gal), fucose (Fuc), and are often terminated by sialic acids (Grondin et al, 2020). The formation of these oligosaccharide chains is governed by several glycosyltransferases, each of which is specific for every link of the chain (Shirazi et al., 2000; Argüeso et al., 2003). Lectin histochemistry has been used in the evaluation of glycoprotein expression and changes in glycoproteins have been suggested to be responsible where the patterns of lectin binding were modified (Fiorentino et al., 2018). We detected increased binding of the lectin SBA and PNA on the goblet cells of HDM-treated mice compared to controls. Binding of SBA and PNA, that recognise GalNAc residues, therefore indicates an increase in these terminals on the mucin oligosaccharide chains and alterations in the activity of glycotransferases may therefore be responsible for this. Non-enzymatic mechanisms might also contribute to altered glycosylation; intestinal inflammation causes oxidative stress, which has been shown to induce degradation of glycans or glycoconjugates (Song et al., 2016).

The mucus layer in the gut has the important role of keeping commensals and luminal contents away from the apical mucosal cell surface. Moreover, it represents an essential source of nutrients, together with undigested dietary carbohydrates, for the intestinal microbiota. In turn the bacteria reciprocally modulate mucin synthesis and secretion, as well as epithelial cell functions. Disruption in these balanced interactions may therefore result in a defective mucus barrier, inflammation or injury of the intestinal mucosa. Together with goblet cell hyperplasia, changes in the carbohydrate content of mucins has also been correlated with a defective mucus barrier as well as with microbial dysbiosis, by altering the expression of binding sites of specific bacteria and the availability of nutrients. Cleavage of glycans found on Muc2 by enzymes expressed by several bacterial species is in fact an essential mechanism for their nourishment (Allaire et al., 2018; Alemao et al., 2020). The most abundant phyla found in the gut microbiota are the *Firmicutes* and *Bacteroidetes*, with fewer *Proteobacteria, Verrucomicrobia,* and *Actinobacteria*. As mentioned above, together with changes in goblet cells, dysbiosis of the gut microbiome has been identified in several inflammatory diseases. Despite some inconsistencies among studies, patients with IBD show a trend towards depletion of *Firmicutes* and *Bacteroidetes* with concomitant increases in *Proteobacteria* and *Actinobacteria* (Kim and Ho, 2010; Pozuelo et al., 2015). Asthmatic patients have also been reported to show decreased *Bacteroidetes* but increased *Firmicutes* (Martinez and Guerra, 2018). Other studies have also shown that microbial dysbiosis occurring in infancy increases the likelihood to develop asthma in adulthood, involving significant decreases in the species of *Lachnospira, Veillonella, Faecalibacterium, Rothia* and *Akkermansia a*s well as reduced levels of the short chain fatty acid (SCFA) acetate (Arrieta et al., 2015; Arrieta et al., 2018; Barcik et al., 2020). In obese mice and humans, a diminished population of *Bacteroidetes* with a higher proportion of *Firmicutes* have also been observed (Xu et al., 2020). This is in agreement with other studies demonstrating that consumption of a diet high in fat and low in fibres results in increased *Firmicutes* and decreased *Bacteroidetes* (Wang and Jia, 2016; Schroeder et al., 2018). In contrast, high-fibre intake and consequent increased SCFA production are beneficial for mucosal health. SCFAs are the result of undigestible carbohydrates being fermented by several commensal species, and the most abundant SCFAs in the gut are butyrate, acetate and propionate. Butyrate is produced mainly by *Firmicutes*, whereas acetate and propionate by *Bacteroidetes* (Macfarlane, 2003).

Our results showed an increase in the relative abundance of *Firmicutes* and a decrease in *Bacteroidetes* in the faeces of HDM-treated mice compared to controls, therefore suggesting that butyrate-producing bacteria could be dominant. Although all SCFAs have been shown to provide anti-inflammatory effects via receptor binding or inhibition of histone deacetylase (HDAC) activity, they differ in their function and tissue distribution, with butyrate being used preferentially as an energy source by colonocytes, propionate contributing to gluconeogenesis

in the liver and acetate achieving the highest systemic concentrations in blood (Morrison and Preston, 2016). Not only changes in nutrient supply but also alterations in the pH and oxygen availability in the gut environment play a role in shaping the gut microbiota (Walker et al., 2015). Decreased luminal pH to 5.5 and oxygen consumption are associated with increased butyrate-producing bacteria (Walker et al., 2025; Louis and Flint, 2017). Increased tissue hypoxia, thus expression of HIF-1α, has also been associated with decreased pH (Kumar et al., 2020). We can therefore hypothesise that following inhalation of HDM, increased inflammatory responses in the LI may have caused upregulation of HIF-1α and the generation of a more hypoxic and acidic environment, that, together with alterations in goblet cells and mucus production, may have affected the composition of the gut microbiome, preferentially selecting butyrate-producing bacteria. In fact, butyrate has been reported to enhance oxygen consumption and HIF-1α stabilisation, that promote barrier integrity and reduce inflammation in the gut (Taylor and Colgan, 2007).

In conclusion, this study provides further mechanistic evidence for the existence of lung-gut communication during allergic lung inflammation. Importantly, our results showed how Th2 inflammatory responses generated in the gut could be distinguished between the SI and LI and resulted in profound changes to the gut microenvironment. Moreover, we observed that inflammatory responses were associated with dysbiosis of the gut microbiota and hypothesised that this reprogramming was achieved to promote restoration of barrier integrity and gut homeostasis.
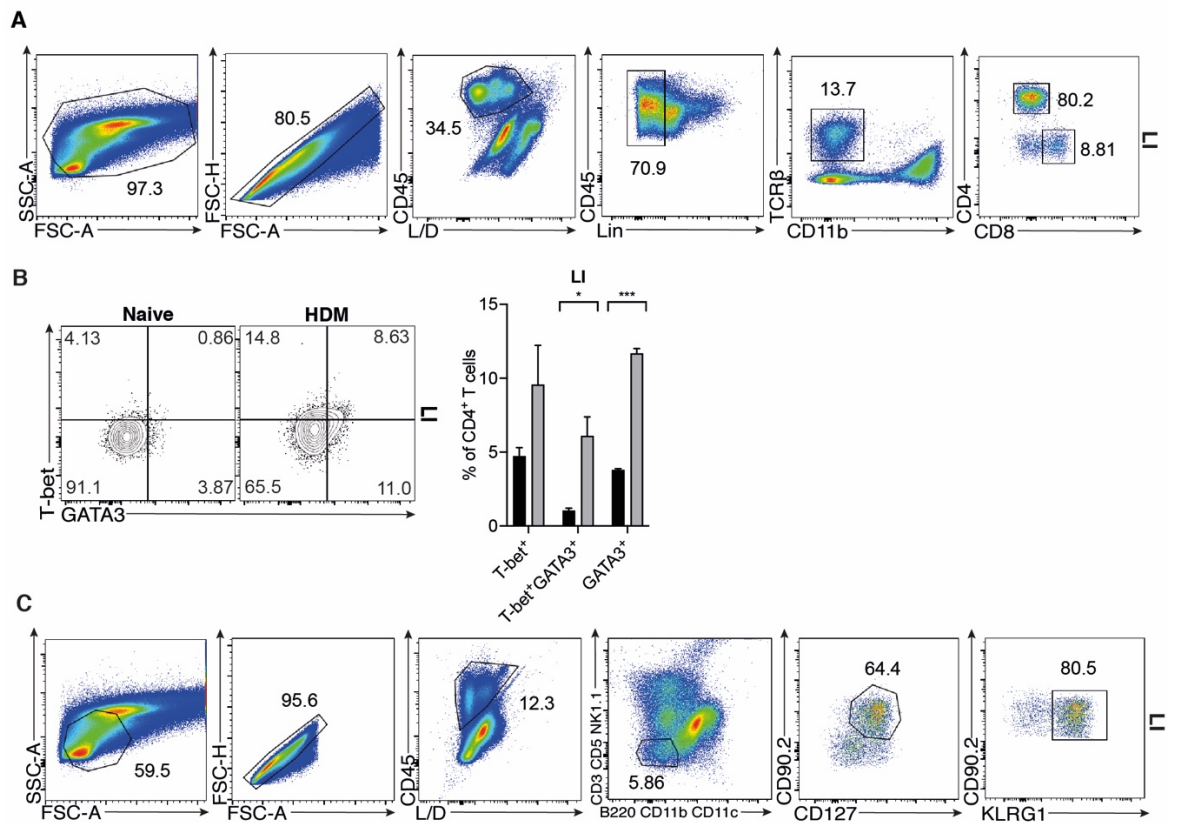
Figure 4.8: Supplementary Figure 1.

A) Gating strategy to identify CD4^{+} and CD8^{+} T cells in murine LI. Cells were identified as follows: SSC-A versus FSC-A to remove cellular debris; FSC-H versus FSC-A for doublet exclusion; Zombie Live-Dead versus CD45 to identify live haematopoietic populations; lineage versus CD45 to exclude contaminating populations (lineage defined as B220, NK1.1, Ly6C, Ly6G and Siglec-F to exclude B cells, NK cells, monocytes, neutrophils and eosinophils respectively); TCRβ versus CD11b to identify TCRβ^{+} T cells; CD4 versus CD8 to identify CD4^{+} and CD8^{+} T cells. B) The percentage of CD4^{+} T cells expressing GATA3 and T-bet was quantified by flow cytometry in the LI of naive and HDM-treated mice. C) Gating strategy to identify ILC2s in murine LI. Cells were identified as follows: SSC-A versus FSC-A to remove cellular debris and identify lymphocytes; FSC-H versus FSC-A for doublet exclusion; Zombie Live-Dead versus CD45 to identify live haematopoietic populations; CD3 CD5 NK1.1 versus B220 CD11b CD11c to exclude contaminating cells; CD90.2 versus CD127 to identify ILCs; CD90.2 versus KLRG1 to identify ILC2s. Data shown are representative from at least two independent experiments. n = 2-3 per experiment. Error bars show ± SEM. Statistical comparisons were performed with Student's t test: *p < 0.05; **p < 0.01; ***p < 0.001
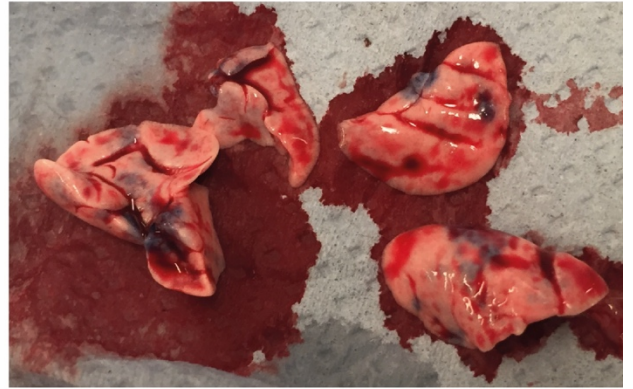
Figure 4.9: Supplementary Figure 2.
A-C) Pictures taken following intratracheal administration of Evan's blue. The dye is localised in the lungs but not in the stomach or gut.

CHAPTER 5 – GENERAL DISCUSSION

Asthma is a chronic obstructive disease involving inflammation of the respiratory tract (Kim et al., 2013). Although inhalation of allergens is a major cause of asthma pathology, a combination of genetic predisposition and environmental factors may promote development of the disease (van Tilburg Bernardes and Arrieta, 2017).

There has been a sharp increase in the global prevalence of asthma and approximately 300 million people worldwide currently have the disease, with its prevalence increasing by 50% every decade. Poor diagnosis and undertreament of the disease have contributed to increasing asthma-associated morbidity, mortality and economic burden over the last 40 years, resulting in considerable healthcare costs and loss of work productivity (Braman, 2006). Asthma onset is most common among children and recently this has been shown to be correlated with alterations in the infant gut microbiota pre- and post-natally (Liu, 2015). Several studies over the last 50 years have indeed demonstrated cross-communication between the gut and lung despite being anatomically distant from each other (Bienenstock et al., 1978; Camus, et al., 1993; Tulic et al., 2016; Keely et al., 2012; West et al., 2015). Nevertheless, mechanistic evidence of how cross-talk between these distal tissues occurs is still lacking.

Although the use of inhaled bronchodilators and corticosteroids are useful and effective in treating asthma over a short period of time, long-term therapy causes significant side effects, including increased AHR and impaired growth (van Schayck, 2013; Dunlap and Fulmer, 1984). Moreover, it is increasingly recognised that asthma is a very heterogenous disease with different phenotypes and endotypes i.e. observable combinations of clinical, biological and physiological characteristics with associated molecular mechanisms (Kuruvilla et al., 2019). Asthma has long been characterised by aberrant Th2 responses, involving significant infiltration of the bronchial mucosa by Th2 lymphocytes and eosinophils, goblet cell hyperplasia and increased serum IgE levels (Nathan et al., 2009). Although Th2 asthma is more prevalent, the heterogeneous nature of the disease has led to the discovery that non-Th2 inflammation can underlie its development, with neutrophils and Th1/Th17 lymphocytes being the predominant cellular infiltrates (Borish, 2016; Brussino et al., 2018). Despite similar clinical symptoms, patients may therefore respond very differently to the same therapeutic interventions and this awareness has increased the need for more tailored treatments (Kuruvilla et al., 2019). Biologic therapies have been developed to target specific inflammatory mediators and despite these therapeutics showing efficacy in reducing asthma symptoms and eosinophilia, they are still not curative. Additionally, patients with neutrophilic asthma are often resistant to corticosteroid treatment and lack alternative personalised treatment options (Fajt and Wenzel 2015).

Considering the relationship between the gut and the lung, manipulation of the microbiota and derived metabolites, such as SCFAs, has recently been investigated as a potential strategy to provide therapeutic benefits in gut and lung inflammatory diseases. The most abundant SCFAs in the gut are butyrate, acetate and propionate and all have been shown to mediate anti-inflammatory effects by binding to their receptors GPR43, GPR41 or GPR109a or by directly modulating gene transcription via inhibition of HDACs (Dang and Marsland, 2019). Moreover, despite SCFAs being largely concentrated in the gut, there is evidence for SCFA-mediated anti-inflammatory effects in the periphery and in the context of allergic lung inflammation (Maslowski et al., 2009; Trompette et al, 2014; Cait et al., 2018). Nevertheless, whether eosinophils or neutrophils can be directly targeted by SCFAs during allergic asthma remains unexplored.

Recently, two different populations of eosinophils have been identified in the airways of allergic mice and shown to possess homeostatic and pro-inflammatory features, hence defined as resident - (r)EOS and inflammatory - (i)EOS respectively (Mesnil et al., 2016). Nevertheless, it remains unclear how and where these two populations of eosinophils originate, and how their phenotype correlates to their function. The factors that may dictate properties of (r)EOS and (i)EOS have also not been defined. Moreover, whether it is possible to identify (r)EOS and (i)EOS in the human lung and target one of the two populations from a therapeutic stand point has not been addressed.

Thus, the overarching aims of the PhD were to better elucidate the precise effects of Th2 inflammation on eosinophils and whether these responses extend to peripheral organs in order to understand whether SCFAs can be used as eosinophil-targeted therapies in allergic asthma.

A combination of *in vivo* and *in vitro* models was employed to assesses the local and distal effects of lung Th2 inflammatory responses on granulocyte and lymphocyte populations in the lung, circulation, BM and intestines, and the consequences of SCFA treatment on such cells and tissue microenvironments. The work illustrated in **Paper 1** focussed on: 1. assessing the effects of Th2 inflammation on lung (r)EOS and (i)EOS and how this may dictate their phenotype and function and 2. establishing whether SCFAs can be used as eosinophil-targeted therapies utilising flow cytometry, histology and transcriptional analysis.

In agreement with previous reports, administration of the allergen HDM caused allergic inflammation of the airways, which was characterised by marked Th2 responses, airway and BM eosinophilia (Gregory and Lloyd, 2011; Buday and Plevkova, 2014; Jacobsen et al., 2014; Inman, 2000) as well as generation of Siglec-F^{hi}CD11b^{hi} (i)EOS in the airways and circulation (Mesnil et al., 2016) but not in the BM of allergic mice. This led us to hypothesise that, contrary to current assumptions, (r)EOS and (i)EOS are not terminally differentiated eosinophil

populations arising in the BM. With the use of lung-shielded chimeras and intravascular staining we demonstrated that lung eosinophils are not long-lived tissue-resident cells, such as AMs (Guilliams et al., 2013), and that (i)EOS may potentially arise locally from (r)EOS when in the lung, suggesting that environmental factors contribute to the process. Further work using a BM-transfer model, in which eosinophils from WT mice are transferred i.n. in the lungs of allergic eosinophil-deficient ΔdblGATA recipients should clarify whether (i)EOS are lung- or blood-borne.

Further phenotypic analysis of (r)EOS and (i)EOS allowed us to suggest that (r)EOS are more responsive to IL-5 compared to (i)EOS and to confirm the pro-inflammatory nature of (i)EOS. For the first time, we showed that (i)EOS were characterised by marked expression of CD98 with associated metabolic activation, involving increased nutrient uptake and enhanced glycolysis. Importantly, blood (i)EOS expressed very low levels of CD98 compared to lung (i)EOS, reinforcing our hypothesis that the lung environment could have a major role in shaping eosinophil phenotype and function. This led us to identify GM-CSF, IL-5 and IL-33 in particular as specific factors able to induce upregulation of Siglec-F and CD98 on BM or lung eosinophils. More importantly, we determined that (i)EOS expressing CD98 were not only present in allergic lung inflammation but represented a characteristic feature of other murine models of lung inflammation.

The first step to evaluate whether treatment with SCFAs could target eosinophils and be therapeutic in allergic asthma involved assessment of SCFA receptor expression on lung eosinophils. Interestingly, we discovered that (i)EOS expressed higher levels of the butyrate-selective GPR109a when compared to (r)EOS, which led to the hypothesis that butyrate may directly target (i)EOS. As for CD98, blood (i)EOS expressed very low levels of the receptor compared to the lung counterpart, highlighting the importance of the tissue microenvironment in determining eosinophil characteristics. Oral or intranasal treatment with the SCFA or niacin, another agonist of GPR109a, led us to find out that agonism on this receptor reduced proportions of (i)EOS and their activation, together with Th2 inflammatory responses in the lung. Moreover, we determined that lung neutrophils expressed GPR109a and that agonism on the receptor was also effective in reducing neutrophilic and Th1/Th17 responses in a mouse model of mixed granulocytic asthma. Further work involving the use of GPR109a KO mice will clearly determine whether agonism of GPR109a mediates anti-inflammatory effects on granulocyte and lymphocyte populations.

Finally, assessment of our findings from mouse models in humans, provided some evidence for lung and blood eosinophils from APs to upregulate Siglec-8, CD98 and GPR109a expression, like their murine counterparts. However, more samples and further phenotyping would be required to confirm the reliability of these preliminary results.

The findings of this work are important in that they could inform the development of novel therapies for asthma. Clinical trials have shown that treatment with eosinophil-targeted biologics, aimed at disrupting IL-5 signalling in eosinophils, do not provide significant clinical improvement in APs (Borish, 2016). This may be explained because those therapeutics were designed to target all eosinophils, without distinguishing them into separate populations. We showed that (r)EOS may respond better to IL-5 compared to (i)EOS, suggesting that IL-5 neutralisation may predominantly affect homeostatic (r)EOS and less so pro-inflammatory (i)EOS. With this work we provide evidence for alternative druggable pathways that could be antagonised to prevent or reduce eosinophil activation, including IL-33 and CD98-mTOR signalling, and glycolysis. We also highlight the importance of the gut microbiota and derived metabolites in influencing peripheral immunity and show that agonism on the butyrate-selective GPR109a may be able to counteract granulocyte and lymphocyte-mediated inflammatory responses in Th2 and non-Th2 asthma.

In **Paper 2**, examination of the systemic effects of HDM-induced lung allergic inflammation allowed us to further expose the complex relationship between the lung and the gut. Here we focussed on: 1. describing the systemic effects of allergic lung inflammation on immune cells in distal tissues with particular focus on the gut and 2. investigate the mechanisms underlying such inter-organ communication.

The gut is a very similar organ to the lung, as they share the same embryonic origin, comparable functions, structure and exposure to bacteria, and there is now robust evidence suggesting a bi-directional relationship between these distal organs (Tulic et al., 2016). Several studies have shown development of GI disorders in asthmatic subjects and vice versa; however, how this occurs mechanistically is still poorly understood (Tulic et al., 2016).

Our results broadly support the idea that cross-communication between the lung and the gut occurs during allergic asthma, but we provide additional mechanistic evidence that may enhance our understanding of how this occurs. Importantly, we showed that inflammatory responses generated in the gut were distinct when comparing the SI and LI. We detected an eosinophil-dominated Th2 response in the SI and showed heterogeneity of eosinophil populations according to their location and expression of CD11b, CD101 and CD98 as we previously documented for lung eosinophils. We also measured increased frequencies of eosinophils in the spleen of allergic mice and it would be interesting to investigate the significance of this. Olbrich et al., 2020 showed that following epicutaneous immunisation increased levels of eosinophils can be found in the lungs. It would be interesting to investigate whether the reverse occurs in our model i.e. lung inflammation promotes eosinophil infiltration in the skin, together with the SI. In the LI, T cells and ILC2s dominated the Th2 response, which was characterised by high levels of IL-13 and IL-33, that we associated with marked goblet cell

hyperplasia and initiation or maintenance of such responses respectively. Importantly, we determined that Th2 responses in the gut were not the result of direct contact with the antigen but were potentially mediated by circulating T cells upregulating gut-homing receptors in distal sites, which has already been reported (Ruane et al., 2013; Jung et al., 2019). Further work involving the use of an HDM-specific tetramer should better define whether antigen-specific T cells are indeed present in the gut of allergic mice. Ruane et al., 2013 specifies that lung DCs induce expression of the SI-homing receptor LPAM-1 on T cells in a TGF-β and RA-dependent manner and that this provided protective immunity in the GI tract. It would be interesting to determine whether lung DCs mediate upregulation of gut-homing receptors and required TGF-β and RA in our model and whether this occurs for both SI- and LI-gut homing receptors. Although Jung et al. (2019) also showed migration of T cells following i.n. immunisation, this induced colitis mediated by Th1 inflammatory responses. We also detected increased release of IFN-γ by T cells in our study, however levels of Th2 cytokines were predominant. Jung et al. (2019) administered OVA i.p. and i.t. to C57BL/6 mice in their study whereas we administered HDM i.n. to BALB/c mice, suggesting that differences in the antigen, mode of delivery or mouse strain may be responsible for changes in the nature of the inflammatory response.

We also showed that Th2 responses altered the mucosal microenvironment of the LI by increasing expression of HIF1-α and altering the glycosylation patterns on mucins. Microbial dysbiosis has been associated with such changes in gut and lung inflammatory diseases (Arrieta et al., 2015; Fujimura et al., 2016; Wesemann and Nagler, 2016; Zhang et al., 2020; Morampudi et al., 2016; Kim and Ho, 2010) and our study also identified a relative increase in the abundance of *Firmicutes* and a decrease in *Bacteroidetes* in the faeces of allergic mice. Although 16s sequencing would have provided a more complete identification of changes in bacterial phyla and species, our results led us to hypothesise that an increase in *Firmicutes* may be favoured due to their butyrate-producing activity. As we mentioned previously, all SCFAs have been reported to provide anti-inflammatory effects, but butyrate is used preferentially as an energy source by colonocytes compared to acetate or propionate (Morrison and Preston, 2016). Moreover, butyrate enhances oxygen consumption in the gut, which promotes barrier integrity and reduces inflammation (Taylor and Colgan, 2007). It would be useful to supplement this study with measurements of SCFA levels in the gut and circulation to better establish whether our hypothesis is reliable. Another possibility is that alterations in the gut microbiome may promote an overall metabolic state that could support Th2 responses at other sites, which could be beneficial for protection (e.g. against helminths), but that would be detrimental in allergic asthma. Assessment of whether the changes observed here are exacerbated or totally altered in a mouse model of chronic asthma would also be useful.

Although more work is required to better understand whether these findings can be translated into humans, this study provides new insights into the organ cross-talk relevant to allergic

disease progressions and highlights the differences and similarities with respect to the nature of the inflammatory response at each site.

Together, through the application of *in vivo* and *in vitro* allergy models, the data presented here shed more light on the complex inter-organ relationship between the lung and the gut, where one influences the immune properties of the other and vice-versa. With a focus on allergic asthma, we showed that allergic lung inflammation triggers Th2 responses in the intestines and can significantly modulate eosinophil phenotype and function at both sites. Intestinal Th2 responses potentially result in a metabolic shift allowing for more butyrate to be produced, that in turn may promote anti-inflammatory effects in the lung by binding to GPR109a expressed on lung eosinophils (Fig. 5.1).
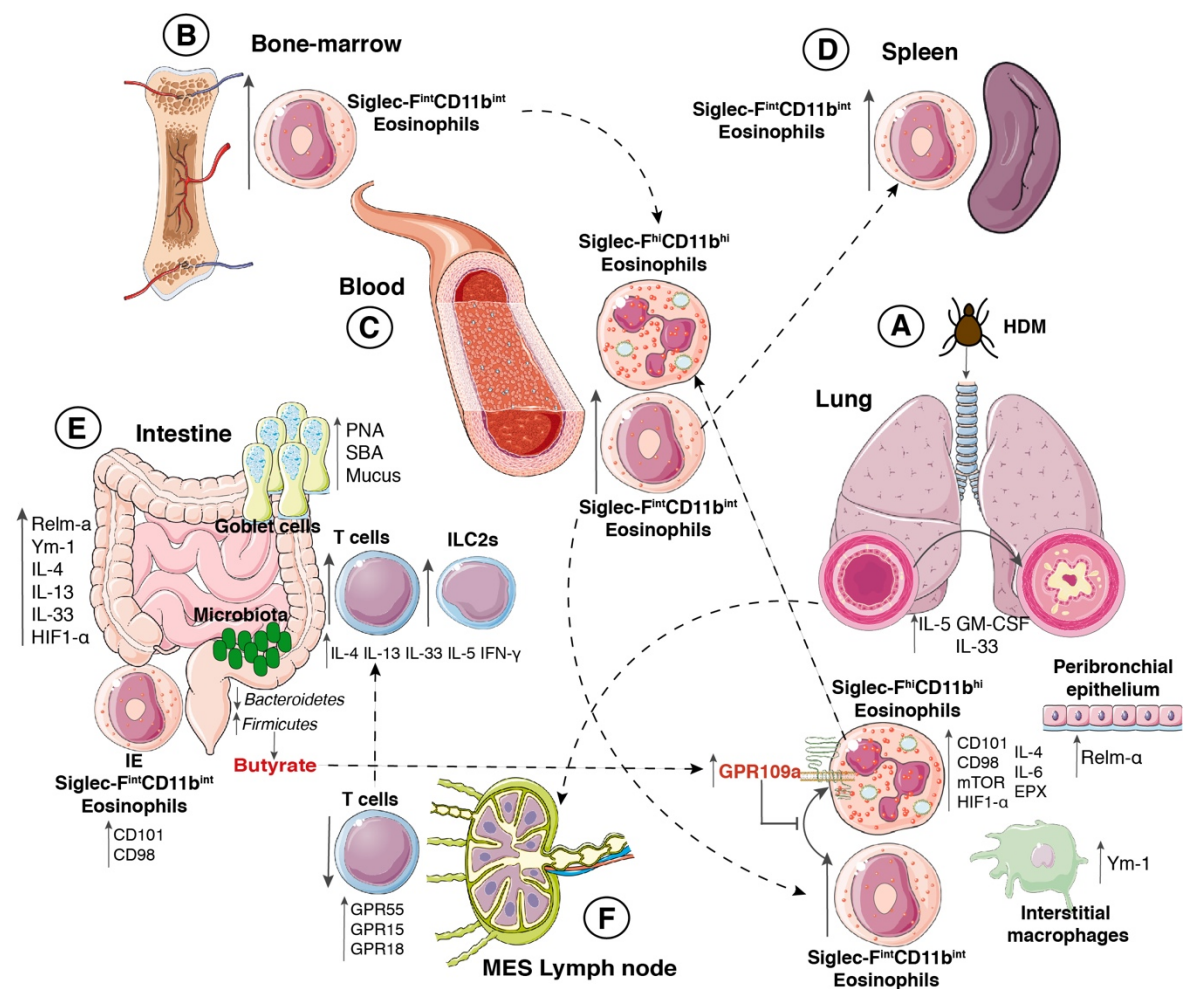
Figure 5.1: Inter-organ communication between the lung and the gut during allergic asthma and its effects on eosinophils.

A) Inhalation of HDM triggers development of Th2 responses in the lung, which are characterised by increased infiltration of lymphocytes and eosinophils, mucus production, expression of Relm-α by the peribronchial epithelium and Ym-1 by interstitial macrophages. Th2 responses also involve increased expression and levels of IL-5, GM-CSF and IL-33, which can modulate BM-derived eosinophil phenotype by increasing Siglec-F, CD98 and GPR109a expression. These cells, showed further pro-inflammatory characteristics, including increased

expression of CD11b, CD101 and HIF-1α, metabolic activity, release of EPX, IL-4 and IL-6 and morphological changes. B) Allergen inhalation triggers BM haematopoiesis and increased release of Siglec-F^{int}CD11b^{int} eosinophils in the circulation. C) BM-derived Siglec-F^{int}CD11b^{int} eosinophils in the circulation are recruited to the lung where they are exposed to Th2 signals and undergo phenotypical and functional changes. Siglec-F^{hi}CD11b^{hi} eosinophils may then extravasate in the circulation. D) BM-derived Siglec-F^{int}CD11b^{int} eosinophils in the circulation are also recruited to the spleen. E) Allergen inhalation also induces Th2 responses in the gut which can be distringuished between the SI and LI. In the SI they were characterised by increased numbers of Siglec-F^{int}CD11b^{int} eosinophils showing increased expression of CD98 and CD101 in the IE compartment and increased tissue expression and levels of Relm-α, Ym-1 and IL-4. In the LI, Th2 responses featured increased frequencies of T cells- and ILC2s-secreting Th2 cytokines, goblet cell hyperplasia, changes in mucin glycosylation as well as shifts in the composition of the microbiome favouring butyrate-producing *Firmicutes* over *Bacteroidetes.* Butyrate is known to provide anti-inflammatory effects and is the only SCFA able to bind GPR109a. We propose that agonism of GPR109a expressed on lung Siglec-F^{hi}CD11b^{int} eosinophils by GPR109a agonists such as butyrate (or the vitamin niacin) is beneficial in reducing the inflammatory eosinophil phenotype and lung inflammation during allergy. F) T cells circulating from the lung and MED LNs to the gut via the MES LNs may mediate Th2 responses through upregulation of gut-homing receptors.

CHAPTER 6 – REFERENCES

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