DISSERTATION

INVESTIGATING NOVEL TREATMENT OPTIONS TO SUPPRESS EOSINOPHIL EFFECTOR RESPONSES

submitted by

Mag. farm.

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Graz, 03.02.2021

DISCLOSURES

This cumulative dissertation is based on the following papers:

1. **Eva Knuplez**[1], Sanja Curcic[3], Anna Theiler[1], Thomas Bärnthaler[1], Athina Trakaki[1], Markus Trieb[1,2], Michael Holzer[1,2], Akos Heinemann[1,2], Robert Zimmermann,[2,4] Eva M. Sturm[1], and Gunther Marsche[1,2]. Lysophosphatidylcholines inhibit human eosinophil activation and suppress eosinophil migration in vivo. *Biochim Biophys Acta Mol Cell Biol Lipids*. 2020;1865(7):158686.
2. **Eva Knuplez**[1], Melanie Kienzl[1,2], Athina Trakaki[1], Rudolf Schicho[1,2], Akos Heinemann[1,2], Eva M. Sturm[1], and Gunther Marsche[1,2]. The anti-parasitic drug miltefosine suppresses human eosinophil activation and ameliorates murine allergic inflammation *in vivo. Br. J. Pharmacol*. 178 (2021) 1234–1248.
3. **Eva Knuplez**[1,5], Rebecca Krier-Burris[5], Yun Cao[5], Gunther Marsche[1,2], Jeremy O'Sullivan[5], Bruce S Bochner[5]. Frontline Science: Superior mouse eosinophil depletion in vivo targeting transgenic Siglec-8 instead of endogenous Siglec-F: Mechanisms and pitfalls. *J Leukoc Biol*. 2020 Jul 5;108(1):43–58.
4. **Eva Knuplez**[1], Gunther Marsche[1,2]. An Updated Review of Pro- and Anti-Inflammatory Properties of Plasma Lysophosphatidylcholines in the Vascular System Vol. 21, *International Journal of Molecular Sciences*. MDPI AG; 2020. p. 1–18.

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Thomas Bärnthaler – carried out preliminary animal experiments, contributed to data analysis and manuscript editing.

Athina Trakaki – contributed to data interpretation and manuscript editing.

Markus Trieb – contributed to data interpretation and manuscript editing.

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Melanie Kienzl – helped carry out animal experiments, contributed to data analysis and manuscript editing.

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Jeremy O'Sullivan – contributed to data interpretation and manuscript editing.

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Eva M. Sturm – supervised animal experimentation and provided test subjects for animal models, contributed to data analysis, interpretation and manuscript editing.

Bruce S. Bochner – supervised Siglec-related experiments performed during the stay abroad, contributed to data interpretation and co-wrote the manuscript.

Gunther Marsche – planned and supervised the projects in Graz and co-wrote the manuscripts.

All co-authors have agreed to the inclusion of their published data in the dissertation and permission from respective publishers and the copyright holders for reproduction has been obtained.

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TABLE OF CONTENTS

ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
Akt	protein kinase B
BAL	broncho-alveolar lavage
CCL	CC chemokine ligand
EET	eosinophil extracellular trap
Erk	extracellular signal-regulated kinase
FFA	free fatty acid
GM 1	ganglioside M 1
GPCR	G-protein-coupled receptor
GPI	glycophosphatidylinositol
GTP	guanosine 5'-triphosphate
IFN	interferon
Ig	Immunoglobulin
IL	interleukin
LCAT	lecithin-cholesterol acyltransferase
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
PAF	platelet activating factor
PL	phospholipase
ROS	reactive oxygen species
Siglec	sialic acid-binding immunoglobulin-type lectins
sPLA	secretory phospholipase A
Th	T helper cell
TNF-α	tumour necrosis factor α
TLR	toll-like receptor

ZUSAMMENFASSUNG

Allergische Erkrankungen sind weltweit ein großes Gesundheitsproblem, das bis zu einem Drittel der Weltbevölkerung betrifft. Aufgrund ihrer zunehmenden Prävalenz sind sie zu einer der häufigsten chronischen Erkrankungen im Kindesalter geworden. Eine der Immunzellen, die stark mit einer fehlgesteuerten allergischen Reaktion in Verbindung gebracht wird, sind Eosinophile, die bei einer Überaktivierung Gewebeschäden und -umbau induzieren. Daher werden Eosinophile seit langem als neue Angriffspunkte für die Entwicklung von antiallergischen Therapeutika angesehen. In letzter Zeit wurden jedoch zusätzliche homöostatische Funktionen der Eosinophilen entdeckt, die die Entwicklung alternativer Behandlungsmöglichkeiten rechtfertigen, die auf ihre Überaktivierung und nicht auf ihre Eliminierung abzielen. Im Rahmen dieser Dissertation wurden daher neue Behandlungsmöglichkeiten untersucht, die auf die Effektor-Funktionen der Eosinophilen abzielen.

Im ersten Teil meiner Dissertation konnten wir zeigen, dass endogene bioaktive Mediatoren, so genannte Lysophosphatidylcholine (LPCs), die Überaktivierung und Efferktorfunktionen humaner Eosinophiler bei Aktivierung stark dämpfen. In ähnlicher Weise unterdrückten LPCs die Migration von Mäuse-Eosinophilen in der Lunge der Tiere. Somit haben unsere Daten LPCs als Substanzen mit starker modulatorischer Aktivität auf Eosinophile und als Leitsubstanzen für die Arzneimittelentwicklung identifiziert.

Ein Nachteil bei der Verwendung von LPCs ist, dass LPCs schnell metabolisiert werden. Daher testeten wir als nächstes ein metabolisch stabiles Strukturanalogon (Miltefosin) und seine Wirkungsweise auf Eosinophile. Miltefosin ist ein oral bioverfügbares Medikament, das für die Behandlung der parasitären Krankheit Leishmaniose zugelassen ist. In guter Übereinstimmung mit unseren Befunden mit LPC konnten wir auch bei der Behandlung mit Miltefosin eine vergleichbare Hemmung der Aktivierung humaner Eosinophile beobachten. Darüber hinaus haben wir Miltefosin erfolgreich in präklinischen Studien eingesetzt, wo es die Infiltration von Eosinophilen in Allergiemodellen signifikant reduzierte. Bemerkenswert war, dass die Anwendung von Miltefosin die Lungenfunktionsparameter nach einer Methacholin-Belastung bei Mäusen verbesserte, was seine Eignung als Medikamentenkandidat für die Behandlung von Eosinophil-assoziierten Krankheiten belegt.

Die jüngsten Fortschritte auf dem Gebiet des Drug Targeting von Eosinophilen wurden mit der Entwicklung eines spezifischen monoklonalen Antikörpers erzielt, der den Zell-Rezeptor Siglec-8 auf humanen Eosinophilen aktiviert und Eosinophile durch Antikörper-abhängige zelluläre Zytotoxizität eliminiert. Während meines Forschungsaufenthaltes bei Prof. Bruce Bochner an der Feinberg School of Medicine in Chicago, untersuchte ich die Rolle von Siglec-8 in einem neuartigen transgenen Mausstamm, bei dem Eosinophile humanes Siglec-8 exprimieren und murines Siglec-F fehlt. Wir konnten zeigen, dass diese Mauslinie große Vorteile hat, um Eosinophilen vollständig zu eliminieren.

Ein Teil meiner Dissertation bestand auch darin, die vorhandene und oft widersprüchliche Literatur zur Funktion von LPC im Gefäßsystem zu durchforsten und zu bewerten. Insbesondere heben wir in diesem Übersichtsartikel LPCs als bedeutende endogene Mediatoren hervor, die in allen Stadien der vaskulären Entzündung und der damit verbundenen Erkrankung beteiligt sind.

Zusammenfassend konnte ich während meiner Dissertation zeigen, dass Lipid-Raft-Modulatoren wie endogenes LPC oder sein synthetisches Analogon Miltefosin als alternative Ansätze zur Suppression der eosinophilen Überaktivierung dienen könnten. Darüber hinaus haben wir durch die Charakterisierung eines neuartigen transgenen Mausmodells mit humanähnlicher Siglec-Expression auf Eosinophilen Forschern ein neues Werkzeug für die präklinische Forschung an die Hand gegeben, um Eosinophile vollständig zu depletieren.

ABSTRACT

Allergic diseases are a major healthcare problem worldwide affecting up to one third of the global population. Due to their increasing prevalence, they have become one of the most common chronic conditions in childhood. One of the immune cells strongly linked to an aberrant allergic response are eosinophils, which induce tissue damage and remodelling upon their over-activation. Hence, eosinophils have long been considered as important drug targets for development of essential anti-allergic therapeutics. However, additional homeostatic functions of eosinophils have recently been discovered, which warrant the development of alternative treatment options aimed at their over-activation and not elimination. Accordingly, this PhD thesis investigates novel treatment options for targeting eosinophil effector responses as well as provides tools for studying their biology.

In the first part of my thesis I discovered that endogenous bioactive mediators and cleavage products of inflammatory secretory phospholipases lysophosphatidylcholines (LPCs) inhibit human eosinophil over-activation. We could show that saturated LPCs disrupted lipid-raft mediated signalling on the surface of eosinophils and thereby dampened their effector responses upon activation. Similarly, LPCs suppressed the migration of mouse eosinophils in lungs of animals upon eotaxin stimulation. Thus, our data have uncovered LPCs as compounds with strong modulatory activity on eosinophils and as potential lead compounds used for drug development.

A disadvantage of using LPCs is that LPCs are rapidly metabolised upon application. Therefore, we next tested a metabolically stable structural analogue (miltefosine) and its mode of action on eosinophils. Miltefosine is an orally bioavailable drug, registered for treatment of the parasitic disease leishmaniasis with proven immunomodulatory functions. Comparable to our findings with LPC, we could observe inhibition of human eosinophil activation upon miltefosine treatment. What is more, we have successfully used miltefosine in preclinical studies, where it significantly reduced the infiltration of eosinophils and other immune cells in models of allergy. Importantly, miltefosine application improved lung function parameters following methacholine challenge in mice proving its suitability as a drug candidate for treatment of eosinophil-associated diseases.

Recent progress in the field of drug targeting of eosinophils has been made with the development of a specific monoclonal antibody that activates the human eosinophil receptor

Siglec-8. Targeting Siglec-8 eliminates eosinophils through antibody-dependent cellular cytotoxicity. During my research stay in the USA at the Feinberg School of Medicine in Chicago (Lab of Prof. Bruce Bochner), I investigated the role of Siglec-8 in a novel transgenic mouse strain in which eosinophils express human Siglec-8 and murine Siglec-F is absent. We were able to show that this mouse strain has great advantages for transient eosinophil depletion. By characterising a novel transgenic mouse model with human-like Siglec expression on eosinophils, we have given research laboratories an entirely new tool to completely deplete eosinophils.

As part of my thesis I reviewed and evaluated the existing and often conflicting literature on the function of LPCs in the vasculature and summarised it in a review article. In the review article, we highlight LPCs as major endogenous mediators involved in all stages of vascular inflammation and associated diseases.

In summary, during my thesis I was able to show that lipid raft modulators such as endogenous LPC or its synthetic analogue miltefosine could serve as alternative approaches to target eosinophil overactivation. Furthermore, by characterising a novel transgenic mouse model with human-like Siglec expression on eosinophils, we have provided researchers with a novel tool for superior eosinophil elimination and for further exploration of the Siglec-8 biology.

1. INTRODUCTION

1.1 Allergic diseases

Allergic diseases have recently been identified as a major public health issue in terms of increasing prevalence and connected healthcare costs (1). The prevalence of asthma alone has increased 2-3 fold in the latter part of the 20th century (2). Today allergic diseases and other immune disorders are rapidly replacing infectious diseases (apart from novel coronavirus COVID-19) in number of hospitalizations and healthcare related costs in the developed world (3) and are reported to affect up to 30% of population world-wide (4).

Allergic diseases occur as a consequence of a dysregulated immune response, where the immune system strongly reacts towards an otherwise innocuous foreign substance (allergen) (4). Following exposure to a specific allergen inflammation occurs in sensitized subjects. Typically, a single exposure to an allergen produces an acute reaction called an early-phase reaction, which is characterized by Immunoglobulin E (IgE) - mediated local or systemic (anaphylaxis) response. IgE-induced degranulation of mast cells and basophils is considered a hallmark of an early phase reaction. Since basophils and mast cells release a variety of proinflammatory mediators, other immune cells are recruited to the site of inflammation and become activated, which contributes to the development of late-phase reactions. Late phase reactions are characterized by local recruitment and activation of T helper type 2 (T_H2) cells, eosinophils, basophils and over-activation of local resident cells (epithelial cells, mast cells). If the exposure to the allergen(s) persists, this may lead to chronic inflammation, where significant tissue remodelling and altered function of affected organs occurs (4,5).

The main allergic diseases include allergic asthma, allergic rhinitis, atopic eczema/dermatitis food allergy, and anaphylaxis (4). Asthma in particular has been identified as the most common chronic disease in children (6), resulting in a lower health-related quality of life from early stages of life. What is more, it has been recognized that asthma is not a single disease, but rather a complex of multiple separate syndromes that overlap, resulting in various endotypes and phenotypes (7,8). Due to the complexity and heterogeneity of the disease multiple treatment strategies are needed to enable therapy to be tailored to meet the needs of each individual patient (9).

1.2 Eosinophils – major players in allergy

Eosinophils are pleiotropic multifunctional leukocytes involved in initiation and propagation of diverse inflammatory responses, as well as modulators of innate and adaptive immunity (10). Eosinophils were first described by Paul Ehrlich in 1879, who identified a white blood cell whose granules stained red with eosin and other acidic dyes (11). Initially, eosinophils were deemed to be end-stage effector cells with the sole purpose of host defence against helminth infections (12). Soon thereafter their involvement and causative role in the pathological processes in response to allergens was proposed (13) and they furthermore started being regarded as crucial for asthma exacerbation (14) and lung connective tissue remodelling (15). In fact, eosinophilic airway inflammation is present in about 40-60% of asthmatics and eosinophil numbers in induced sputum are an established biomarker used to identify a type 2 asthma endotype, to assess disease severity and to select an appropriate treatment strategy (16–18).

Eosinophils are terminally differentiated granulocytes, which primarily develop in the bone marrow under the control of transcription factors such as GATA1, XBP1, PU.1, C/EBP and C/EBPα (19–21). Maturation of eosinophils from precursors occurs following exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 3 and IL-5 (22–24). Phenotypically mature eosinophils are then released from the bone marrow and are capable of being activated and recruited into the tissues (Figure 1) (25). Most prominent chemoattractants described for human eosinophils are IL-5 and the eosinophil-selective group of eotaxins (CCL11, CCL24 and CCL26) (26–28).

Normally, only a small number of eosinophils are released from the bone marrow into the blood stream, circulating for a brief amount of time (approx. 18 hours), before migrating to the thymus or gastrointestinal tract, where they reside under physiological conditions (29–31). However, eosinophil production and consequently their numbers can dramatically increase in T_H2-type inflammation characterized by elevated IL-5 and eotaxin-1 levels (32). Blood hypereosinophilia is defined when eosinophil counts in blood exceed 1500 cells / µl, which may result in eosinophil-induced organ damage diagnosed as hypereosinophilic syndrome (33). Interestingly, local in-situ eosinophilopoesis was recently described in lungs of severe asthmatics and allergic rhinitis patients (34,35). This is of particular importance, since local eosinophilopoesis was deemed a major factor for persistent airway eosinophilia and increased patient corticosteroid needs (34).

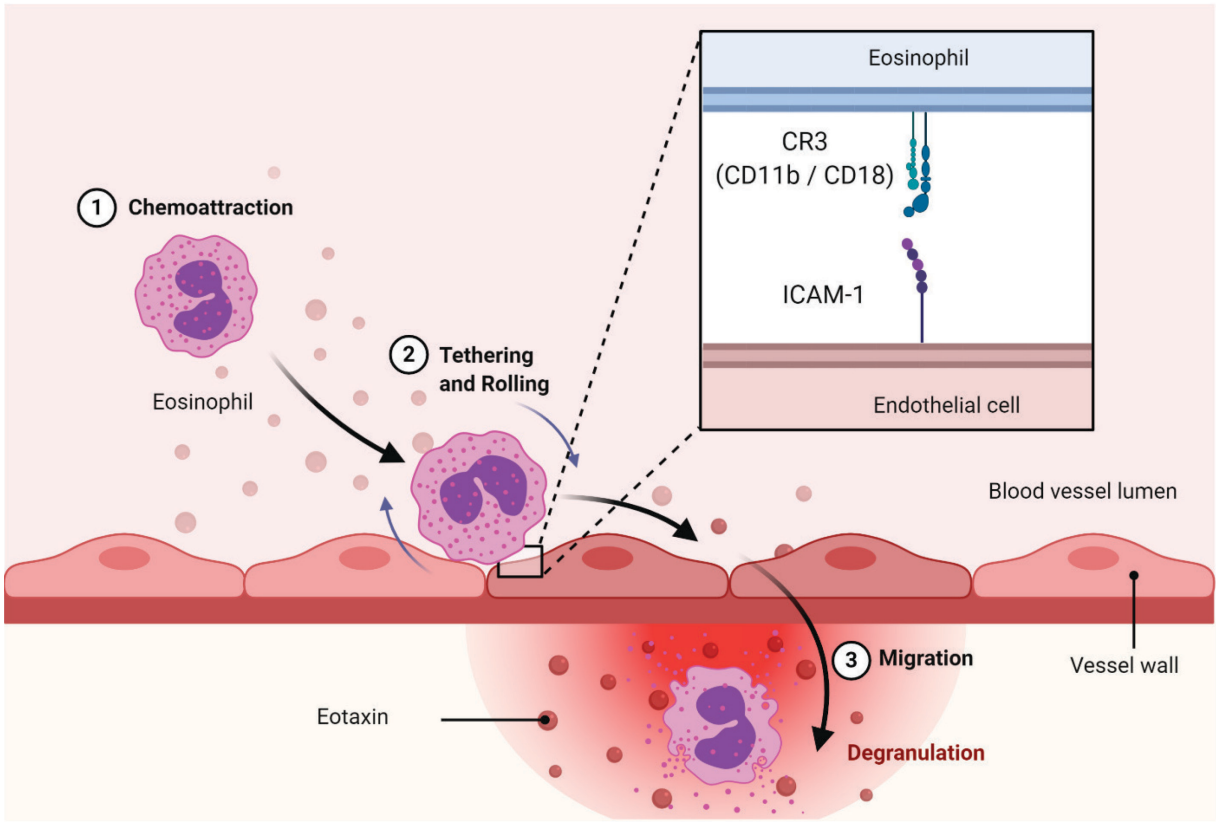

Figure 1: Eosinophil migration and activation. Abbreviations: CD, cluster of differentiation; CR3, complement receptor 3; ICAM-1, intercellular adhesion molecule 1. Figure created with BioRender.com.

Mature eosinophils express a variety of surface receptors ranging from previously mentioned chemoattractant receptors such as CCR3 -the receptor for eotaxin, to adhesion receptors and cytokine and growth factor receptors as well as receptors for lipid mediators (31). Some of these receptors such as the integrin CD11b (part of the Complement Receptor 3 or Macrophage-1 antigen adhesion receptor complex) may be used together with CCR3 as surface markers to detect eosinophils using flow cytometry in mouse and human samples (36).

Upon stimulation, eosinophils are capable of releasing their preformed granules in a process called degranulation. Various cytokines and chemokines such as interferon (IFN) γ and eotaxin-1 carefully regulate this process (31,37). Eosinophil secondary granules contain a crystalloid core of major basic protein, which is surrounded by a matrix of eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase (38). Once released, these proteins are highly toxic to the surrounding tissue and induce tissue damage and dysfunction such as the dysfunction of respiratory epithelium, smooth muscle constriction and increased vascular permeability (39,40). Eosinophil granules also store a variety of preformed cytokines

and other pro-inflammatory mediators, which can recruit and activate additional immune cells and thereby exacerbate the local inflammatory response (41). Moreover, it was recently discovered that purified eosinophil granules express receptors for IFN-γ (IFN-γR α chain), two cysteinyl leukotriene receptors (CysLT1R, CysLT2R), purinergic receptor P2Y12 and CCR3 and can release their content upon ligand-binding extracellularly (41,42).

Degranulation of eosinophils was once considered crucial for anti-helminth immunity. However, today additional, far more complex roles have been attributed to eosinophils in innate as well as in adaptive immunity (43). Eosinophils express pattern-recognition receptors such as toll-like receptors (TLRs), which enable them to sense and respond to specific pathogenic components or to endogenous molecules produced by injured or dying cells (44,45). So far they have been reported to recognize and react towards not only helminths, but also against certain viruses, bacteria and fungi (46–49). Particularly in response to bacteria and complement components, eosinophils have been shown to release so called eosinophil extracellular traps (EETs). EETs contain eosinophil mitochondrial DNA and together with eosinophil cationic protein and major basic protein form nets with bactericidal activity, resembling neutrophil extracellular traps (50,51). Of note, EETs have also been observed in lungs of severe asthmatics and in patients suffering from chronic obstructive pulmonary disease or eosinophilic esophagitis (52–54).

Through their effector functions and mediator release eosinophils have the ability to orchestrate the immune response and modulate local immune environment. Furthermore, eosinophils have the ability to act as antigen presenting cells since they express major histocompatibility complex class II (MHC II) and co-stimulatory molecules such as CD80 and CD86. Some of the effects of eosinophils on other immune cells include inducing antigen-specific T cell proliferation and polarization, mast cell secretion, dendritic cell maturation and activation, macrophage polarization and neutrophil activation (25,55–59). Moreover, eosinophils have been described as crucial for long term maintenance and plasma cell survival in the bone marrow (60).

Nowadays there is increasing evidence that eosinophils are involved in not only allergic diseases, but also in a plethora of other pathological conditions such as autoimmune diseases (61), thrombotic disease (62) and cancer (63). Additionally there have been recent discoveries illuminating important homeostatic functions of eosinophils in the local environment (64) as well as in the resolution of inflammation and promotion of homeostatic baseline (65).

1.2.1 Mouse eosinophils as a model for their human counterpart

Mouse models are being abundantly used to study human disease physiology (66). The wide spread use of the mouse in an experimental setting is facilitated by the relative ease of genetic manipulation, availability of transgenic models and an abundance of specific reagents needed to study phenotypic changes and cellular responses (66,67). However, using murine models of asthma and allergic disease to mimic human disease pathology has certain limitations. Mice do not spontaneously develop asthma; therefore, appropriate allergic phenotype has to be achieved with prolonged allergen sensitization and challenge, as well as by using adjuvants (e.g. aluminium). Such complete dependence on allergen stimulation results in different asthma etiology compared to humans, where other factors such as environmental factors and obesity contribute to the development of the disease (67,68). Moreover, even challenged murine models do not develop acute bronchoconstriction, which can only be achieved upon additional methacholine challenge (68). Other factors such as differences in leukocyte infiltration kinetics as well as the absence of eotaxin-3 (CCL26) in mice further complicate the use of murine asthma models in translational medicine (25,69–71).

Even though murine models do not fully recapitulate human allergic disease, mouse and human eosinophil biology, trafficking and activities are strikingly similar, which allows for extrapolation of data to human condition (65,72). Minor differences in eosinophil morphology and activation however, can still be observed between the two species. Mouse eosinophils are slightly smaller in diameter (9–12 μm versus 12–15 μm), and contain smaller and less densely packed granules in their cytoplasm. Primary granules composed of CLC / galectin-10 are surprisingly absent in mouse eosinophils. Moreover, human eosinophils degranulate easier and to a wider variety of stimuli *in vitro* (25,72). Degranulation, which is on one hand readily observed in the lungs of asthmatics, is on the other hand only sporadic or completely absent in the lungs of allergic mouse models (73–75).

In order to study eosinophil contribution to health and disease various transgenic mouse models were created, where eosinophil numbers are either decreased (e.g. Eotaxin-1$^{-/-}$ or CCR3$^{-/-}$ mice) or increased (IL5-Tg mice) (76–78). Furthermore, eosinophil deficient animals were created, where eosinophil deficiency is either congenital (PHIL or ΛdblGATA-1 mice) or inducible (iPHIL mice) (79–81). Additional specific manipulation of mouse eosinophils was achieved using cre-lox recombination. Here, mammalian *Cre* recombinase was inserted in the open reading frame for the *Epx* gene creating an eosinophil-lineage specific knock-in strain of mice (*eoCre*). This strain of mice enables eosinophil-specific gene targeting and overexpression in mice furthering our understanding of eosinophil biology and function (82).

Finally, efforts were made recently to establish so called ‘humanized’ mouse models, where immunodeficient mice are engrafted with human haematopoietic CD34+ stem cells. The differentiation of such stem cells is induced with a specific cytokine combination, which mimics the human immune system and enables the differentiation of stem cells into human-like end-stage effector cells. These mouse models are able to more broadly resemble aspects of human allergic inflammation and do not depend on manipulation of a single cell type (83,84).

1.2.2 Siglec-F versus Siglec-8 – two peas in a pod?

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are a family of type I membrane proteins, capable of recognizing glycan structures. Siglecs are mainly expressed on cells of the immune system and modulate both innate and adaptive immune responses through recognition of 'self' from 'non-self' (85,86). Siglecs display an extracellular amino-terminal V-set immunoglobulin like domain; which binds sialic acid, followed by a variable number of C2-set immunoglobulin domains. The cytosolic region of Siglecs usually contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), which can recruit tyrosine and inositol phosphatases (Figure 2). Therefore, Siglecs are able to function as inhibitory receptors by decreasing cell activation (85,86). A common feature of Siglecs is their ability to endocytose; either cycling constitutively between cell surface and endosomes or undergoing endocytosis upon multivalent ligand or antibody ligation (87,88). As of today, 14 Siglecs have been identified in humans and 9 in mice. All of them possess distinct expression pattern on immune cell subtypes and exhibit unique sialic acid linkage specificity making them important regulators of immune function in defence against pathogens, autoimmunity, cancer, neurodegeneration as well as allergic inflammation (85,89,90).

The significance of Siglecs and the field of glycobiology for allergic inflammation research became apparent following the discovery and characterization of Siglec-8 (also named sialoadhesin family 2) receptor (91,92). A unique feature of Siglec-8 is its expression pattern. It is expressed specifically on human eosinophils, mast cells and weakly on basophils; all important effector cells mediating allergic inflammation (91). Thus, Siglec-8 provides a rare targeting opportunity for development of new drugs combating allergy (93).

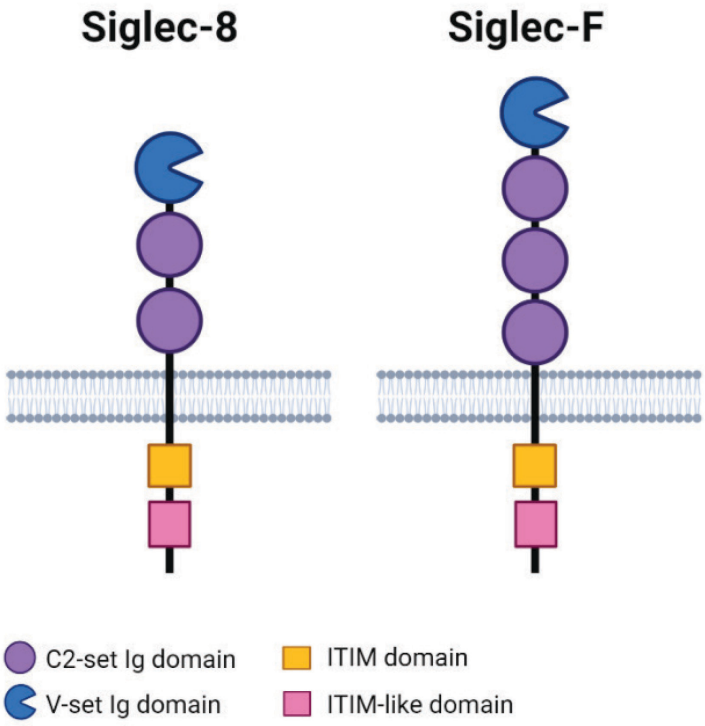

Figure 2: Schematic representation of Siglec-8 and Siglec-F. Abbreviations: Siglec, Sialic-acid binding immunoglobulin-like lectin; ITIM, Immunoreceptor Tyrosine-based Inhibitory Motif. Created with BioRender.com.

Siglec-F has been identified as the closest functional paralog of Siglec-8 on mouse eosinophils, recognizing and preferentially binding to the same ligand *in vitro* (94,95). Siglec-F is in contrast to Siglec-8 not expressed on mouse mast cells and basophils, yet can be found on lung resident alveolar macrophages as well as intestinal tuft and M cells (96–98). In inflammatory or pathological conditions Siglec-F can be additionally upregulated on the surface of other cell subpopulations like for example on lung cancer-infiltrating neutrophils (99).

Both Siglec-8 and Siglec-F are expressed late during eosinophil maturation and are absent on the surface of stem cells (100,101). Moreover, engagement of both receptors with respective specific antibodies results in internalization of the receptors and subsequent eosinophil cell death *in vitro* (102–105). Importantly, similar engagement of Siglec-F and Siglec-8 *in vivo* has led to reduced eosinophil numbers in both mice and human. Unequivocally demonstrating therapeutic efficacy and clinical symptom improvement in many disease models and clinical studies (106–110). Siglec-8 and Siglec-F are, due to their relatively specific and high expression, often used in combination with others as surface markers for identification of eosinophils by flow cytometry (72).

However, Siglec-8 and Siglec-F only share approximately 38% sequence similarity, which manifests in important structural and functional differences between the two receptors (Figure 2) (111). It was shown that Siglec-F expression is upregulated following allergen challenge in a mouse lung allergy model, while the expression of Siglec-8 remains constant and does not correlate with disease severity in healthy versus eosinophilic subjects (112,113). Further differences between receptors emerged upon the discovery of natural tissue ligands for Siglec-8 and Siglec-F in the airways. While the ligand for Siglec-F was found to be expressed on mouse epithelial cells, Siglec-8 ligand was identified to be a glycoprotein called 'deleted in malignant brain tumours' 1 (DMBT1) expressed on serous cells, a subpopulation of submucosal gland cells in the upper respiratory system (114,115). Last but not least, even though engagement of both Siglecs leads to eosinophil cell death, the mechanism and magnitude of cell killing differs between the two receptors (116). *In vitro* antibody ligation of Siglec-F results in modest, caspase dependent apoptosis, while ligation of Siglec-8 using crosslinking antibodies invokes reactive oxygen species (ROS) production and loss of mitochondrial membrane potential (117,118). Moreover, when human eosinophils are primed with IL-5, ligation of Siglec-8 results in a different biochemically regulated mode of cell death, which is more profound, omits the use for secondary crosslinking antibody and is characterized with MEK/ERK kinase activation, β2-integrin-dependent adhesion, granule release and enhanced ROS production (119,120).

Engagement of Siglec-F in mice with either polyclonal or specific monoclonal antibodies has often been used to transiently deplete eosinophils from mouse blood and tissues (110,121–126), providing an alternative approach to genetic models of eosinophil deficiency, which may have broader effects on the immune system due to homeostatic roles of eosinophils (127). However, eosinophil depletion following antibody ligation of Siglec-F is often incomplete (110,122,123,128,129). Additionally, even though Siglec-F has been shown to internalize following engagement, it is still consistently being used as a surface marker tracking eosinophil numbers following administration of Siglec-F antibodies (123–126).

In order to study Siglec-8-mediated eosinophil depletion *in vivo,* a novel transgenic mouse model was recently created, where Siglec-8 is specifically expressed on the surface of eosinophils. In these mice Siglec-8 antibody administration leads to profound and selective eosinophil depletion (105). Thus, allowing for translational studies of a crucial receptor expressed on human eosinophils mediating their survival in a *in vivo* experimental setting. Nevertheless, the differential effects of antibody induced cell death targeting Siglec-8 and Siglec-F *in vivo* in mice remain to be investigated in more depth.

1.3 Phospholipases and inflammation

1.3.1 Classification of phospholipases

Phospholipases are enzymes capable of cleaving phospholipids at certain ester bonds within the molecule, resulting in the production of fatty acids and other lipophilic substances (130). They were first discovered when components of pancreatic juice and animal venom (snake and bee) were found to hydrolyse egg lecithin in the early 1900s (131,132). Isolation of these components revealed small, water-soluble, rigid enzymes extensively cross linked with cysteine disulphide bonds (133). Since their discovery, they have been deemed crucial for normal functioning of numerous metabolic pathways (134). Furthermore, phospholipases (independent of their catalytic activity) and lipid mediators produced by them, have been shown to be involved in multiple pathologic processes in the human body (130,134,135).

The diverse superfamily of phospholipases can be broadly separated into two groups depending on the site of cleavage of the phospholipid molecule, where they act either as acylhydrolases or phosphodiasterases. Phospholipases A and phospholipases B are acylhydrolases, while phospholipases C and phospholipases D are phosphodiasterases (Figure 3) (136).

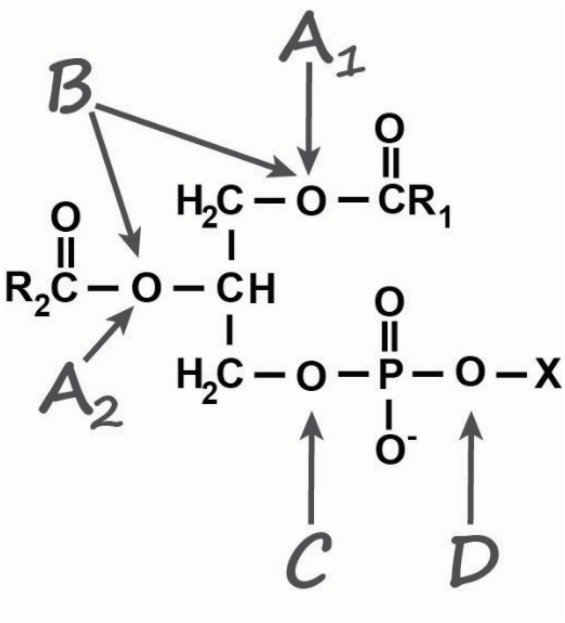

Figure 3: Cleavage sites of different phospholipase subgroups.

All of the phospholipase enzymes vary in their structure, physiological functions and regulatory mechanism, but they all exhibit a higher activity of substrate hydrolysis above their critical micellar concentration e.g. micelles or bilayer vesicles (136,137).

The most widely investigated group of phospholipases are phospholipases A_2 (PLA_2), which cleave the phospholipid molecule at the *sn*-2 position producing a free fatty acid and a lysophospholipid. The family of PLA_2s can be further subdivided into two major groups. The first group consists of small secreted calcium dependent enzymes using histidine as their primary catalytic residue. The second group, in contrast, consists of enzymes with calcium independent activity utilizing serine as their primary catalytic residue. Phospholipases such as cytosolic PLA_2s, intracellular calcium independent PLA_2s, platelet-activating factor acetylhydrolases and lysosomal PLA_2 belong to the second group (137–140). Additionally, a novel adipose-specific phospholipase A_2 has recently been identified, which utilizes histidine in its catalytic site, but does not depend on calcium for its activity (141). By producing lipid mediators such as lysolipids, free fatty acids necessary for inflammatory mediator (leukotriene, prostaglandin and thromboxane) production and platelet activating factor, PLA_2s are most recognized for their role in inflammation and inflammatory disorders (137,142).

1.3.2 Secreted phospholipases A_2 and inflammation

Secreted phospholipases A_2 ($sPLA_2$s) are small (14-19 kDa) enzymes found in organisms ranging from bacteria to mammals (143,144). They contain highly conserved functional motifs of their catalytic sites, calcium binding loop, disulphide bond pattern and cysteine residues, while the rest of the molecule including the surface residues varies greatly between the $sPLA_2$ types (145). As the name suggests, $sPLA_2$s are secreted and can therefore be found in fluids such as human plasma (146), tears (147), pancreatic juice (148) and seminal fluid (149) and are important components of certain animal venoms (150,151). As of today, 11 members of the $sPLA_2$ family have been discovered in humans and are as following: IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA and XIIB (152). Not only are these $sPLA_2$s differentially expressed in tissues, but they also exhibit unique substrate selectivity, suggesting functions other than the release of lipid mediators (153).

Of all the $sPLA_2$ family members - $sPLA_2$-IIA is uniquely induced under inflammatory conditions and is capable of increasing its activity by several orders of magnitude (154–156). Accordingly earning a designation as an acute phase protein (156–158). Originally characterized and isolated from the sera and synovial fluid of arthritis patients (159,160), it was later additionally

found to be highly expressed in platelets (161). Interestingly, $sPLA_2$-IIA is constitutively expressed in immune cells such as macrophages, eosinophils, neutrophils and mast cells and in organs of the immune system such as spleen, thymus and bone marrow (162–166). Its expression can be induced with pro-inflammatory mediators such as IL-1, IL-6, IFN-γ, tumour necrosis factor-α (TNF-α) or lipopolysaccharide (LPS) (167–169), while it is conversely suppressed by glucocorticoids (steroidal anti-inflammatory drugs) (170). Hence it comes as no surprise that $sPLA_2$-IIA inhibition has been extensively investigated as a possible therapeutic target for treatment of inflammation-related diseases such as psoriasis, atherosclerosis and cancer (171–174).

1.4 Lysophosphatidylcholine – an important phospholipase cleavage product and immune cell modulator

Lysophosphatidylcholine (LPC) is produced by enzymatic cleavage of phosphatidylcholine by PLA_2 and/or by the transfer of free fatty acids to free cholesterol by lecithin-cholesterol acyltransferase (LCAT) (175). Also formerly called lysolecithin, it is the most abundant lysolipid in human plasma where its concentration ranges from 125 µM to 145 µM under physiological conditions (176). Since LPC's production depends on the activity of $sPLA_2$, its concentration is likewise increased in inflammation-associated conditions and has been found to reach plasma concentrations of up to 300 µM in obesity, diabetes and ovarian cancer (176,177).

LPC was first discovered as the metabolic component of snake venom responsible for its toxic haemolytic activity in the 1940s, while knowledge of a lytic component in fresh snake venom exists since the early 1900s (178,179). The toxic effects were attributed to the structure of LPC (Figure 4), which can, if its concentration reaches above its critical micellar concentration, insert itself into membranes of susceptible cells disrupting their membrane structure resulting in direct cell lysis (180). The amphiphilic nature of the molecule containing a long hydrophobic fatty acid acyl chain and one large hydrophilic polar headgroup gives it surfactant and detergent-like properties. By possessing a large headgroup to acyl-chain ratio, it assumes the inverted conical shape (Figure 4), thereby favouring the bending of the membrane to a positive curvature (181). Importantly, LPC species with different acyl chain lengths and saturation do not exhibit equivalent lysis potential. In particular, unsaturated LPCs showed to be less potent, since higher concentrations and a longer preincubation time were needed to achieve the same effect (182). This has since been reproduced in various disease models and cell types, where distinct LPC species are able to act differently (183,184). Thus, making it hard to distinguish

individual LPC contribution in a physiological setting where a mixture of LPC species is present.

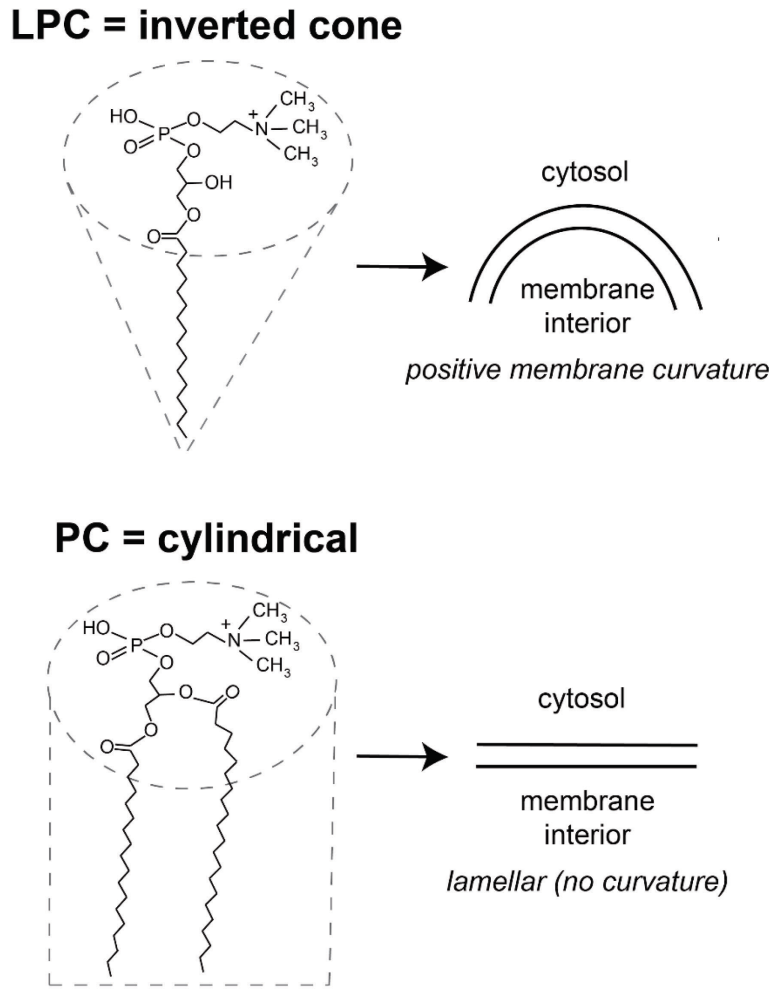

Figure 4: LPC structure and its effect on membrane curvature. Abbreviations: PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

However, even though toxic properties of LPC on membranes are observed in experimental settings, LPCs in the circulation have not been proven to be deleterious under homeostatic conditions. This indicates an existence of a buffering agent capable of sustaining a necessary ratio of inactive (bound) to active (free) LPC. Albumin has been identified as the main carrier of LPC in circulation and its potent inactivator (185,186). In fact, one molecule of albumin is able to bind up to five molecules of LPC (185). Moreover, lipoproteins have also been identified to bind and inactivate LPC (187). On one hand the cytotoxicity and biological effects of LPC

are greatly reduced when it is premixed with albumin or serum in an experimental setting (185,188,189). On the other hand conditions characterized with decreased albumin concentrations (190,191) or higher LPC production skewing the free / bound ratio may result in a greater impact of LPC on surrounding cells.

Even when the concentration of LPC does not surpass its critical micellar concentration, the bioactive lipid can still alter membrane structure and therefore affect cell functions. In particular, an increase in the ratio of lysophospholipids to phospholipids alters membrane properties modulating the lateral movement of membrane proteins and can influence the assembly of membrane-receptor complexes (192–194). Specifically owing to its structural properties LPC has been found to modulate the activity of cholesterol-rich signalling microdomains termed 'lipid rafts' (195,196). Lipid rafts are liquid-ordered domains mainly consisting of sphingolipids (such as ganglioside 1 – GM1) and cholesterol, but may additionally contain glycosylphosphatidylinositol (GPI)-anchored receptors, receptor tyrosine kinases, nonreceptor tyrosine kinases of the Src family, adapter and regulatory molecules of tyrosine kinase signalling cascade, heterotrimeric and small GTP-binding proteins, and cell adhesion molecules, thus acting as a signal transduction platform (197,198). Other receptors can upon intracellular or extracellular stimuli increase their affinity for lipid rafts (199–202). Importantly, small changes in lipid partitioning can result in initiation or inhibition of signalling cascades due to signal amplification (197,203,204). Some of the most widely recognized lipid raft-associated signalling events include T cell activation (205), IgE- mediated allergic response (206) and toll-like receptor (TLR) signalling (207,208).

Not only is LPC capable of altering membrane properties, but it is also reported to activate cells by specifically binding to G-protein-coupled receptors (GPCRs) (209,210). So far, LPC has been reported to specifically bind to and activate ˙G protein-coupled receptor G2A (GPR132) (211), G protein-coupled receptor 4 (GPR4) (212), GPR55 (213,214), GPR40 (free fatty acid receptor 1) (213) as well as GPR119 (215). However, the study reporting the binding of LPC to G2A was later withdrawn, due to inability of authors to distinguish between the membrane and receptor-specific effects of LPC (216). Furthermore, GPR4 receptor was later discovered to be pH sensitive (proton-sensing GPCR) and the specific effects of LPC upon binding could not be reproduced (217–219). Hence, GPR119, GPR55 and GPR40 are currently the only confirmed GPCRs capable of binding LPC. In these cases specific LPC binding elicits calcium flux and results in increased glucose stimulated insulin secretion (213,220). Moreover, LPC was also reported to modulate TLR- mediated signalling in

macrophages, but it remains to be seen whether the effects are receptor-mediated or result from altered membrane properties (221).

1.4.1 LPC as a pro- and anti-inflammatory mediator

In the context of inflammation, LPC is often overlooked in comparison to other products of $sPLA_2$ cleavage such as arachidonic acid. Moreover, the studies describing its functions in a variety of disease models and cell types are often contradictory, further baffling researchers (175). Initially, LPCs were considered potent pro-inflammatory mediators negatively influencing vascular reactivity either in their free form (222) or as components of oxidized LDL (223). They were discovered to activate endothelial cells and stimulate their production of proinflammatory cytokines (224), thereby acting as a damage-associated molecular patterns and attracting other immune cells to the site of inflammation (225,226). Furthermore, LPC was also reported to activate infiltrating immune cells resulting in increased oxidative stress and local damage to the endothelium (227,228). Taken together, LPCs were long considered to be crucial for the initiation and progression of atherogenic processes (229). Not only were LPCs investigated in the context of atherosclerosis, but also in other conditions characterized with an aberrant inflammatory response such as multiple sclerosis (230).

Contradicting these studies are the clinical data from recent highly sensitive lipidomic studies, where LPC levels were found to be decreased and inversely correlated with multiple pathologies. Namely decreased LPC levels were observed in sepsis (231), aging (232), rheumatoid arthritis (233), diabetes (234), asthma (235), polycystic ovary syndrome (236,237), Alzheimer disease (238,239), schizophrenia (240), and pulmonary arterial hypertension (241). Additionally, LPC levels were found to be associated with higher mortality in liver cirrhosis (242) and in-hospital mortality in pneumonia (243).

Importantly, LPCs were identified as possible biomarkers in cancer research and a prospective metabolomics study discovered that higher levels of saturated LPC 18:0 reduced the risk of most common cancers (244). Lower LPC levels were observed in most cancers such as hepatocellular carcinoma (245), colorectal cancer (246–248), cholangiocarcinoma (249), ovarian cancer (250,251), pancreatic and biliary tract cancer (252) as well as cervical cancer (253). Furthermore the concentration of LPC was lower in cancer, associated with weight loss and increased inflammation, where they also inversely correlated with C-reactive protein (CRP) levels in plasma (254). Possibly explaining these correlations are the discoveries of LPC reducing tumour cell migration and adhesion (255) and affecting the tumour

microenvironment. In fact, one of the first discoveries associated with LPC was its selective reduction of oxygen consumption of tumour cells resulting in their apoptosis (256). LPC was also found to prevent high mobility group box 1 (HMGB -1) protein production in macrophages and monocytes (257), which suppresses anti-tumour immunity and is therefore considered a drug target in cancer therapy (258). Moreover LPC formation was found to be vital for the functional cytotoxic activity of natural killer cells towards targeting tumour cells (259).

Not only were LPCs proven to be beneficial in inhibiting cancer development and spreading but their favourable effects on other immune cell types have now been demonstrated (260–262). What is more free LPC was proven to possess potent anti-aggregatory action on platelets (263,264) and to increase the cholesterol efflux from cholesterol-laden macrophages (265,266). Thus, this brings into question its previously described pro-atherogenic role.

The effects of LPC on the immune system could also prove to be advantageous in infections caused by different pathogens. In line with that notion, application of LPC improved survival in experimental models of sepsis. The observed results of improved survival were attributed to an increase in certain pro-inflammatory cytokines following LPC treatment and enhanced bacterial clearance (267,268). In addition, LPC reduced organ injury and dysfunction in models of gram-positive and gram-negative shock (269). Whilst in zymosan-A induced peritonitis LPC acted anti-inflammatory by reducing levels of TNF-α and IL-6 as well as by increasing the production of inflammation resolving lipoxin A (270,271). Aside from LPC activating the immune system to clear bacteria it also possesses intrinsic bactericidal activity (for example on methicillin-resistant Staphylococcus aureus (MRSA)) and can potentiate the bactericidal activity of gentamicin (272) and polymyxin antibiotics (273). Other actions of LPC on pathogens include a lytic effect on mycoplasma membranes (274) as well as inhibition of yeast growth and virulence (275). Owing to its membrane disrupting potential, LPC also exerts antiviral action in influenza and HIV by reversibly arresting exocytosis, viral membrane fusion and pore formation (276–278).

Other conditions where LPC or its analogues have recently demonstrated therapeutic effects include epilepsy (279), allergic diseases (280) and diabetes, where higher plasma levels of LPC were positively correlated with muscle insulin sensitivity index in diabetic patients (281) and inversely correlated with impaired fasting glucose and diabetes incidence (282–285).

1.5 Stable analogues of LPCs - Alkyl-lysophospholipids (ALPs)

The aforementioned pleiotropic mechanistic effects of LPC may be re-purposed for the treatment or manipulation of pathological conditions. The use of LPC in clinics is however limited, since the exogenously added lysophospholipids are metabolically not stable and are rapidly converted into lysophosphatidic acid via the action of lysophospholipase D termed autotaxin (286) or used as building blocks for synthesis of phosphatidylcholine (287). In order to avoid LPC degradation, synthetic alkyl ether lipids or alkyl-lysophospholipids (ALPs) were synthesized, which are metabolically stable and resistant against phospholipases and acetyltransferases (288).

1.5.1 Discovery and first generation of ALPs - edelfosine

The first synthesized prototype alkyl lysophospholipid was 1-O-octadecyl-2-Omethyl-rac-glycero-3-phophocholine (edelfosine). The molecular structure of edelfosine resembles that of LPC, however the ester bonds in the glycerol backbone (C1 and C2) are replaced by ether linkages (Figure 5). Edelfosine and other first generation LPC analogues were initially synthesized to mimic the immunomodulatory properties of LPC (288). Surprisingly it was discovered soon thereafter, that some of them possess potent and selective anti-tumour activity (289–291). This antitumoral activity was partly attributed to enhanced tumouricidal activity of macrophages and partly to direct cytotoxic effects on tumour cells (292). However, while preclinical data showed promise of a novel and effective drug candidate (293–295), edelfosine lacked the necessary efficacy and did not meet the required endpoints in human clinical trials (296,297). Edelfosine application was associated with significant gastrointestinal toxicity and haemolytic potential, limiting its use in humans (298). Moreover, residual platelet-aggregating effects could be observed, which were attributed to PAF-like action stemming from the structure similarity of both molecules (Figure 5) (297).

The mechanism of action reported for edelfosine and its analogues differed from traditional chemotherapeutics in which it did not target DNA and the replication machinery of the cell or the cytoskeleton. Instead ALPs were reported to specifically act on tumour cells by promoting programmed cell death – apoptosis (299,300). The long hydrocarbon chain of ALP is able to insert itself into the membrane and interfere with downstream signalling events such as calcium (301) and protein kinase C mobilization (302) as well as interfere with phosphatidylcholine synthesis (303,304).The best characterized target of edelfosine and subsequent analogues remains inhibition of protein kinase B (Akt), a serine threonine kinase (305,306). Edelfosine

was also found to permeabilize the cell membrane and increase its fluidity (307). The effects of edelfosine on membrane fluidity were further confirmed, when it was discovered that addition of cholesterol or endogenously higher cholesterol content in the cell membrane protects from edelfosine's cytotoxic action (308). Membrane fluidity of cancer cells is closely related to their proliferative and metastatic potential (309,310) and since normal resting cells have lower membrane fluidity, they are less sensitive to edelfosine's action. By altering the physical properties of the cell membrane, ALPs are additionally able to displace 'survival' signalling molecules from lipid rafts as well as reported to recruit the 'death receptor' Fas/CD95 (311). Finally, edelfosine was discovered to induce endoplasmic reticulum stress responses and a loss of mitochondrial membrane potential resulting in apoptosis (312,313). Of note, when edelfosine is used at high concentrations unspecific cell lysis of both normal and tumour cells occurs due to its cytotoxicity (314).

1.5.2. Second generation ALP and the path to clinical use - miltefosine

Based on the hypothesis, that phospholipase C is responsible for degradation of ALPs and generation of toxic metabolites (315), novel ALP analogues were synthesized and tested for their anti-tumoral activities. Unger et al. determined that the minimal structural requirements for anti-tumoral activity included a polar head and a hydrophobic acyl chain, and identified hexadecylphosphocholine (miltefosine) as a novel potent anti-tumour drug candidate (316). Miltefosine resembles the structure of first-generation ALPs, but lacks the glycerol backbone (Figure 5). Therefore, miltefosine is the first prototypic drug belonging to the class of alkylphosphocholines and not alkyllysophospholipids.

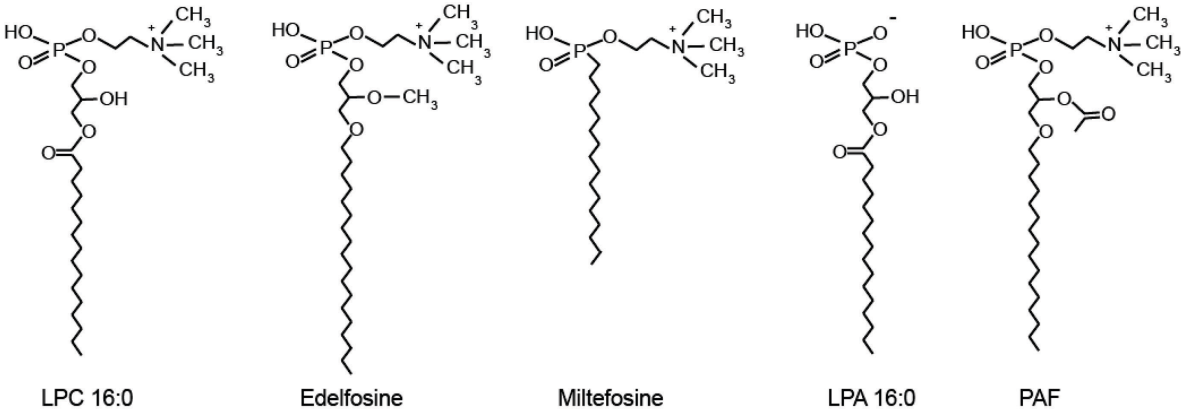

Figure 5: The molecular structure of LPC, its analogues and similar bioactive lipids. Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PAF, platelet activating factor.

The compound and its metabolites exhibit lower toxicity, compared to edelfosine upon topical or per os application (317). However, when applied intravenously, the drug still possesses significant haemolytic potential (318). Following encouraging preclinical studies, where miltefosine demonstrated high potency and selectivity, the topical solution of miltefosine - Miltex™ gained marketing approval for use against cutaneous metastases of breast cancer (319–321). Miltex™ therefore became the first topical solution specifically formulated for anti-cancer use (322).

Following its discovery miltefosine was not only found to effectively kill tumour cells but also proven to act against leishmania parasite *in vitro* (323,324). Since the oral drug formulation has already entered clinical studies for use in humans, a pilot study determining its effect in the rare disease leishmaniasis was performed promptly. The results of the study were promising (325), which lead to further successful clinical trials (326–328). Miltefosine received orphan drug designation and priority voucher review, when it was registered for the treatment of leishmaniasis in the USA (329). Moreover it was included on the 20th World Health Organization (WHO) Emergency medicines List (330). Today it remains the only orally bioavailable drug for the treatment of leishmaniasis (331). Affordable price, preferable oral route of application for patients resulting in increased patient compliance and mild to moderate side effects make it an attractive treatment option especially in third world countries (329). In 2017, miltefosine received orphan drug designation for another rare disease – Granulomatous amoebic encephalitis (332,333).

Similarly to LPC and edelfosine, miltefosine was also reported to act immunomodulatory by influencing the activation, cytokine secretion and mediator release from immune cells (334–338). Although studied in the context of cancer and leishmania parasite elimination, its effects on the immune system are likewise important for the disease course and therapy success (334,339). Following oral application miltefosine was found to accumulate in the peripheral blood mononuclear cells (PBMCs) with an intracellular concentration 2- to 3- fold higher than in plasma (340) impacting their activation and proliferation (334). Therefore, it comes as no surprise, that miltefosine treatment exhibited favourable results in models of certain T-cell mediated diseases such as inflammatory bowel syndrome (341), T-cell dependent dermal inflammation models (337) or atopic dermatitis (342). Furthermore, miltefosine was shown to inhibit the activation and degranulation of mast cells (343). The proposed mechanism being modulation of lipid rafts at the cell membrane and inhibition of protein kinase C resulting in inhibition of IgE receptor signalling and subsequent histamine release from mast cells (280,338). This was reflected in clinical studies where miltefosine was shown to be a safe and

effective treatment option for patients suffering from antihistamine-resistant chronic spontaneous urticaria (344). Altogether this suggests miltefosine might be a viable option for treatment of diseases characterized with an overactive immune system and particularly allergic diseases.

1.5.3 Third generation ALP– perifosine

Aiming to increase the therapeutic range of miltefosine the molecule was modified in the late 90s, where the choline moiety was replaced by the heterocyclic piperidin group yielding a new prototype ALP called perifosine. Perifosine was the first allosteric Akt inhibitor to enter clinical development for cancer treatment (345). According to ClinicalTrials.gov it has entered more than 40 clinical trials for different types of tumours with varying degrees of success. However, as of today the drug has not progressed further than Phase III trials for treatment of a number of cancers mainly due to lack of clinical activity (306,346). Since perifosine has proven to be safe and well-tolerated by patients while demonstrating promising *in vitro* anti-tumour activity, it is continuing to enter clinical studies either as combination therapy with other known anti-cancer drugs or as a radiosensitizer in radiation therapy (306,347–350).

Additional drug candidates such as erufosine and erucylphosphocholine were recently synthesized using miltefosine as a model structure. Erucylphospocholine contains a longer aliphatic chain (22 carbon atoms instead of 16) with a cis bond, while erufosine contains an additional methylene group in the phosphocholine headgroup (351). These relatively small modifications increase the hydrophobicity of the molecules, which in turn decreases their haemolytic potential, allowing for the molecules to be applied intravenously (306,351). This mode of administration omits gastrointestinal side effects as well as allows for 5 times lower doses compared to the oral route of administration. Furthermore, both molecules have been found to partially cross the blood-brain barrier and accumulate in brain tissue, thereby proving to be viable drug candidates for treatment of brain tumours (352–354).

1.6. LPC and its analogues in allergic inflammation

When compared to other circulating leukocytes, eosinophils express and secrete high levels of secretory phospholipases type X (355) and IIA (356). The involvement of both sPLA2s and their products was suggested in allergic inflammation, when it was discovered that their activity is elevated in bronchoalveolar (BAL) fluid following allergen challenge in patients with asthma and allergic rhinitis. Likewise their concentration and concentration of LPC was found to be elevated in allergic subjects (357–359). Earlier reports suggest a proinflammatory role of lysophosphatidylcholine mediating adhesion and activation of eosinophils as well as contributing to allergic inflammation by independently acting as a bronchoconstrictor (360,361). Prolonged models of LPC exposure in mice additionally suggested its role in allergic airway disease manifestation (362). Intriguingly, when a stable analogue of LPC miltefosine was used in models of allergic diseases it demonstrated potent anti-allergic activity by inhibiting mast cells or T cells (338,343,363). In fact, miltefosine was successfully tested in clinical trials as a treatment for urticaria- a mast cell mediated allergic disease (344).

The role of LPCs and especially synthetic alkyl lysophospholipids on eosinophil activation and function has so far been poorly investigated. The relationship between an important immune cell type involved in allergic responses and a highly prevalent bioactive lipid molecule present at sites of inflammation could not only offer insight in regulation of eosinophil homeostasis but also offer a novel orally bioavailable therapeutic option for the treatment of diseases characterized with eosinophil over-activation.

2. RESULTS

The results section is a short summary of the following scientific publications:

1. Knuplez E, Curcic S, Theiler A, Bärnthaler T, Trakaki A, Trieb M, et al. Lysophosphatidylcholines inhibit human eosinophil activation and suppress eosinophil migration in vivo. Biochim Biophys Acta - Mol Cell Biol Lipids. 2020 Jul 1;1865(7):158686.
2. Knuplez E, Kienzl M, Trakaki A, Schicho R, Heinemann A, Sturm EM, et al. The anti-parasitic drug miltefosine suppresses human eosinophil activation and ameliorates murine allergic inflammation in vivo. Br J Pharmacol. 2021 Mar 2;178(5):1234–48.
3. Knuplez E, Krier-Burris R, Cao Y, Marsche G, O'Sullivan J, Bochner BS. Frontline Science: Superior mouse eosinophil depletion in vivo targeting transgenic Siglec-8 instead of endogenous Siglec-F: Mechanisms and pitfalls. J Leukoc Biol. 2020 Jul 5;108(1):43–58.
4. Knuplez E, Marsche G. An updated review of pro-and anti-inflammatory properties of plasma lysophosphatidylcholines in the vascular system. Vol. 21, International Journal of Molecular Sciences. MDPI AG; 2020. p. 1–18.

Eosinophils are key effector cells involved in allergic inflammation and are contributing to many homeostatic processes in the organism (10,364). The aim of my thesis was to investigate novel potential therapeutic options targeting eosinophils.

Ad 1.) In my first project I investigated the effects of lysophosphatidylcholines (LPCs) on eosinophils. LPCs are important bioactive lipid mediators involved in a plethora of inflammatory conditions (175). We tested the effects of the most abundant LPCs on eosinophils and discovered that saturated LPCs 16:0 and 18:0 inhibited eosinophil activation and chemotaxis *in vitro* by disrupting lipid rafts on the surface of eosinophils. We could additionally show that saturated LPCs inhibited intracellular signalling as measured by decreased calcium flux and phosphorylation of extracellular signal-regulated kinase (Erk) and Akt kinase in eosinophils following stimulation. Importantly, LPC administration decreased the infiltration of eosinophils in the airways in a short *in vivo* migration model (365).

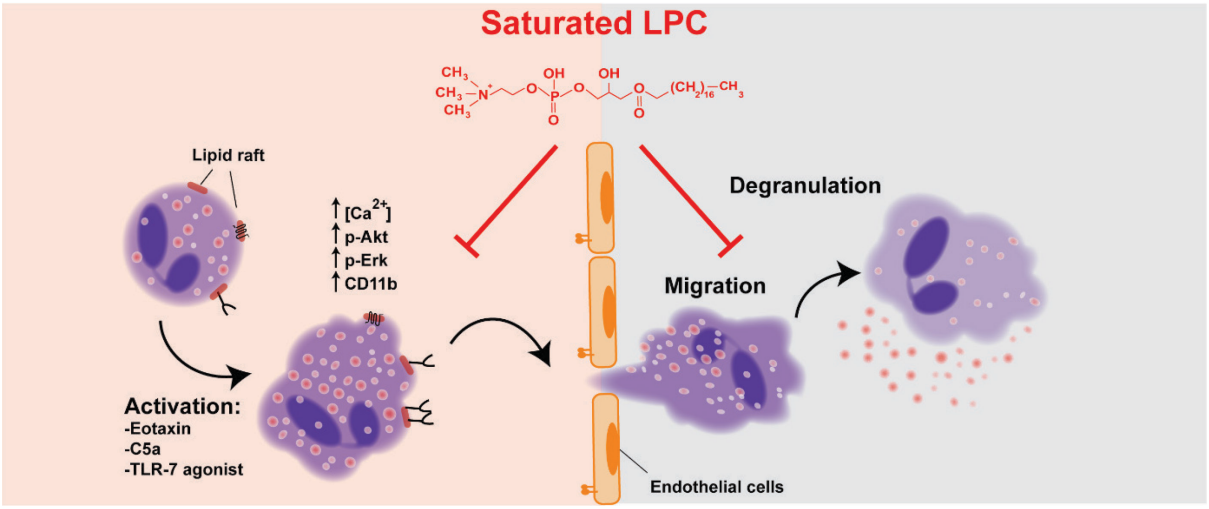

Figure 6: Graphical abstract Lysophosphatidylcholines inhibit human eosinophil activation and suppress eosinophil migration in vitro. Abbreviations: Akt, protein kinase B; Ca, calcium; C5a, complement component 5a; Erk, extracellular signal-regulated kinase; LPC, lysophosphatidylcholine; TLR, toll-like receptor. Image reproduced from Knuplez et al. (365) *under creative commons licence CC-BY-NC-ND.*

Ad 2.) In my second project I tested the effects of an anti-parasitic drug miltefosine on eosinophils and their effector functions. Synthetic alkyl lysophosphocholines (ALPs) such as miltefosine are stable analogues of LPC, which can be used to study effects of LPC in health and disease. Importantly, ALPs are safe drug compounds already used in clinical studies for treatment of allergic diseases (344,366). We found similar inhibitory actions of miltefosine on both human and mouse eosinophils *in vitro*. We observed that miltefosine decreased infiltration of eosinophils and other immune cells in BAL in a model of allergic inflammation, which resulted in improved murine lung function parameters. We additionally observed that miltefosine treatment significantly reduced the levels of eosinophil-derived cytokine IFN-γ. Of note, miltefosine did not significantly alter immune cell infiltration when eosinophil-deficient mice were exposed to the same allergic inflammation model (367).

Ad 3.) During my research stay abroad in the lab of Dr. Bochner I focused on investigating one of the novel eosinophil-depleting biologics targeting Siglec-8 and the monoclonal antibody targeting murine Siglec-F. Eosinophil depletion using Siglec-8 targeting antibody (lirentelimab) is currently extensively studied in clinical studies for eosinophil-associated diseases (108). In order to investigate mouse Siglec-F and human Siglec-8 targeting in preclinical studies, novel mouse models with human-like siglec expression were created and remain to be studied in depth. Through our work we have described a novel mouse model, where eosinophils express human Siglec-8 and lack mouse Siglec-F (368). We successfully characterized eosinophils

differentiated from bone marrow of these animals and shown that Siglec-8 expressed on their surface is functional. Furthermore, we demonstrated that eosinophils are rapidly and transiently depleted from blood, spleen and bone marrow upon Siglec-8 antibody application in these animals. When we compared the amount of eosinophil depletion to depletion induced with Siglec-F antibody in Siglec-F expressing animals, we observed a much stronger effect of the Siglec-8 targeting antibody. We could confirm the mouse IgG1 chain of the Siglec-8 antibody and the occurrence of antibody-dependent cellular cytotoxicity (ADCC) to be responsible for the observed profound depletion. With these experiments we additionally uncovered potential shortcomings of eosinophil-depletion with Siglec-F antibodies while utilizing Siglec-F as an eosinophil marker. We could show that Siglec-F gets internalized upon antibody engagement and therefore does not accurately identify the population of eosinophils with flow cytometry (368).

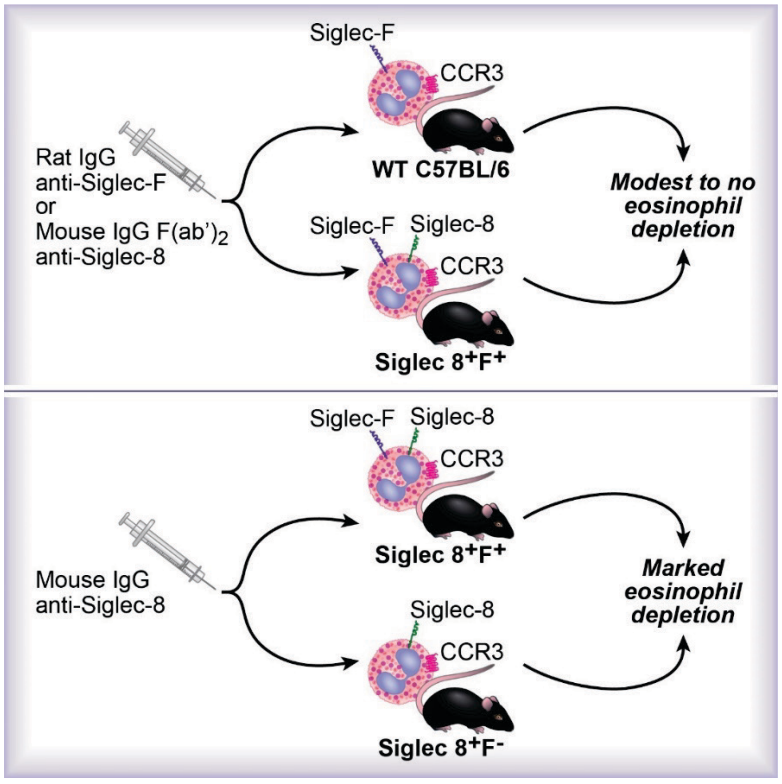

Figure 7: Graphical abstract Superior mouse eosinophil depletion in vivo targeting transgenic Siglec-8 instead of endogenous Siglec-F. Abbreviations: CCR3, C-C chemokine receptor type 3; IgG, immunoglobulin G; Siglec, sialic acid-binding immunoglobulin type lectin; WT, wild type. Image reproduced from Knuplez et a.l (368) *with permission from John Wiley and Sons.*

Ad 4.) Finally, I reviewed current and discordant literature pertaining to LPC and its functions in the vascular system (369). A lot of the earlier original studies describe LPC as pro-inflammatory, while more recent studies suggest potent anti-inflammatory actions of LPCs at the vascular-endothelium interface and on the activation and infiltration of immune cells (369). In addition, most recent highly sensitive metabolomics studies have observed decreased LPC levels in a variety of inflammatory conditions. These studies suggested that LPCs could serve as biomarkers in diseases such as cancer(244), schizophrenia (240), rheumatoid arthritis (233) and diabetes (370). Taken together we propose that LPCs are important homeostatic mediators involved in all stages of vascular inflammation (369).

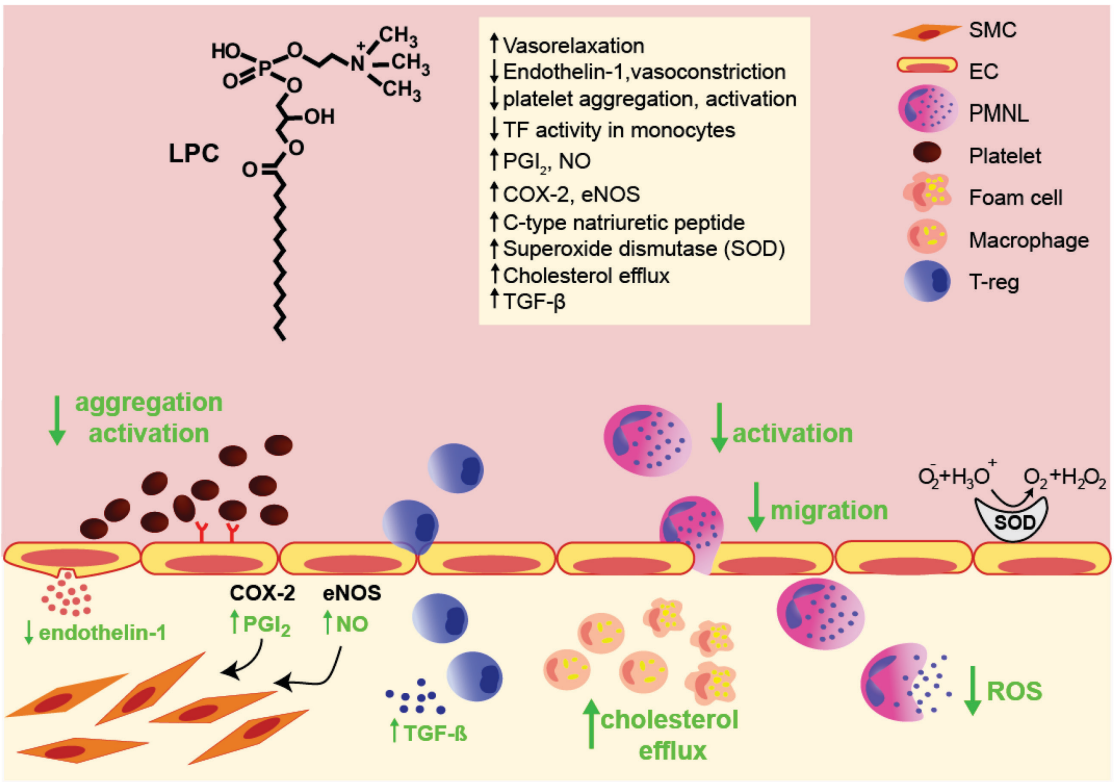

Figure 8: An updated review of pro-and anti-inflammatory properties of plasma lysophosphatidylcholines in the vascular system. Abbreviations: COX-2, cyclooxygenase-2; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SMC, smooth muscle cells; TF, tissue factor; TGF-β, transforming growth factor beta; T-reg, T regulatory cells. Image reproduced from Knuplez et al (369) *under creative commons licence CC-BY-NC-ND.*

Altogether these data uncover novel alternative means of targeting over-abundance and/or over-activation of eosinophils. Moreover, we describe a novel superior mouse model enabling the study of an important drug target in preclinical studies of eosinophil-related conditions.

3. DISCUSSION

During my PhD, I discovered alternative methods to combat the overabundance and/or overactivation of eosinophils – key effector cells in allergy. One of such is targeting lipid rafts on the eosinophil membrane either by using an endogenous bioactive mediator LPC or its synthetic analogue, an anti-parasitic drug miltefosine. We could demonstrate that both compounds significantly inhibited human eosinophil activation and migration. What is more, in preclinical models of allergy, oral application of miltefosine proved to inhibit immune cell infiltration in mouse lungs and improved clinical disease manifestation. Next, I characterized a novel transgenic mouse model expressing human Siglec-8 and lacking mouse Siglec-F on the surface of eosinophils. While doing so, we additionally discovered that targeting mouse Siglec-F with specific monoclonal antibodies, in order to induce eosinophil depletion, is often inadequate. Contrarily, by using the novel transgenic mouse model one could target human Siglec-8 in mice and achieve better eosinophil depletion through ADCC. Last but not least, by reviewing current literature on LPCs function in the vascular system we contributed a concise study examining this important homeostatic molecule from multiple perspectives. All in all, this dissertation elucidates the anti-inflammatory actions of LPC and miltefosine on eosinophils as well as betters our understanding of eosinophil biology through characterization of a human-like transgenic mouse model.

Asthma is the most common chronic lung disease in children with distinct and sometimes overlapping mechanistic pathways (endotypes) and variable clinical presentations (phenotypes) (371,372). While existing therapies enable good disease-management of most asthma endotypes, a subset of patients suffers from ‘difficult to manage’ or ‘severe’ asthma, which majorly impacts their quality of life (373). The most common and therefore best described asthma endotype is the disease associated with T_H2 cell-mediated allergic sensitization (18). One of the key effector immune cells involved in asthma and other diseases characterized with aberrant T_H2 cell response are eosinophils (25). In fact, persistent airway eosinophilia is present in 40-60% of severe asthma cases (374). It is of great importance to understand the interplay of immune cells and soluble mediators in asthma endotypes and other allergic diseases, in order to better our knowledge of the homeostatic mechanisms involved as well as to identify novel drug targets.

Eosinophils express high levels of secretory phospholipases, which produce bioactive mediators LPCs through phospholipid cleavage (355,356). It was discovered that the levels of LPCs are increased in the BAL fluid upon allergen challenge (358,359). Conversely, a novel unbiased metabolomics approach identified LPC to be significantly decreased in asthmatic versus non-asthmatic individuals (235). So far, the relationship between a key effector cell involved in allergic inflammation and an abundant endogenous lipid mediator has not been elucidated.

3.1 Lysophosphatidylcholines inhibit human eosinophil activation and suppress eosinophil migration in vivo (Biochim Biophys Acta - Mol Cell Biol Lipids. 2020 Jul 1;1865(7):158686).

LPCs are bioactive molecules that are present at sites of inflammation, as described above. In my initial research, we investigated possible effects of LPCs on eosinophil effector responses. We could demonstrate that preincubation with saturated LPC decreases the activation of human eosinophils *in vitro*. Our results showed that eosinophils dose-dependently upregulated the adhesion molecule and activation marker CD11b following eotaxin-2 stimulation and that this process was inhibited by LPC pre-treatment. It should be noted that engagement of eosinophil adhesion molecules activates expression of multiple proinflammatory genes within eosinophils (375). Moreover, involvement of CD11b was implicated in human eosinophil extracellular trap formation (376). Further, we discovered that chemotaxis of isolated human eosinophils towards eotaxin-2 was similarly inhibited by LPC 18:0 treatment (365). Eotaxin-2 has been found to be highly increased and correlated with persistent and late tissue eosinophilia in asthma as well with late eosinophilia in the skin of atopic dermatitis patients following allergen challenge (377,378). Our data hence indicate that circulating LPCs could prevent especially the delayed processes of eosinophil adhesion and migration and therefore decrease eosinophil tissue infiltration and chronicity of allergic diseases.

Following activation, eosinophils have the ability to degranulate and release a variety of pro-inflammatory mediators and toxic granule proteins (379). Accordingly, degranulation of eosinophils is considered to be a key pathogenic event in eosinophil-associated diseases (380). What is more, it was shown that while eosinophil numbers remain constant in some diseases like IBD, asthma or allergic rhinitis, their degranulation pattern changes, since eosinophil granule proteins can be detected in affected tissues (380). Our work uncovered that

saturated LPCs are able to attenuate the degranulation of isolated human eosinophils and the release of their toxic mediators (365).

Next, we set out to investigate the mechanism of the observed effects of LPC on eosinophils. So far LPC has been described to act on the membrane of other cell types, altering its properties, influencing the assembly of membrane-receptor complexes and modulating the activity of lipid-rafts (192,194,239,381). Furthermore, LPC was reported to bind to and activate certain GPCRs such as GPR119, GPR40 and GRP55 (213). With our experiments we could demonstrate, that saturated LPC destabilized lipid rafts by removing cholesterol from eosinophil cell membranes. Interestingly, we could show stronger LPC-induced cholesterol removal from the membrane of eosinophils, compared to neutrophil cell membranes, which may be due to different cell membrane composition. The membrane altering effects of LPC on eosinophils resulted in altered downstream signalling. In particular pre-treatment with saturated LPC decreased phosphorylation of kinases Akt and Erk (365). The former being involved in eosinophil survival (382), while the latter being crucial for eosinophil degranulation and cytokine production (383). We could show, that LPC inhibited calcium flux following stimulation with different agonists (eotaxin-1 and C5a). LPC's ability to alter eosinophil activation following stimulation with multiple agonists further suggests a non-receptor mediated mechanism of action. Of note, LPC treatment of eosinophils did not induce calcium flux in eosinophils on its own, which is reported to occur after LPC binds to GPR55, GPR40 or GPR119 (213). Therefore suggesting, that LPC's inhibitory effects are not a result of binding to the above-mentioned GPCRs.

Our *in vitro* experiments confirmed previously published observations of individual LPC species possessing different biological activity (183). In a physiological setting a mixture of LPCs is formed through phospholipid cleavage. However, data from our lab previously demonstrated that LPC 16:0 and LPC 18:0 are by far the most abundant LPC species formed by the action of inflammatory $sPLA_2$-IIA on HDL (263). We observed, that these saturated LPC 16:0 and 18:0 more potently inhibit eosinophil activation when compared to unsaturated LPC 18:1 (365). Similar results were obtained in the field of cancer research, where they discovered that in comparison to the physiological mix of LPCs, saturated LPCs alone increased membrane rigidity and prevented cancer cell metastasis (184). This observed effect may be due to the more conical shape of the saturated LPCs and their higher spontaneous curvature values compared to unsaturated LPCs (384).

Finally, we decided to test our hypothesis of LPC-induced eosinophil inhibition in an *in vivo* setting employing a rapid eosinophil *in vivo* migration model. In this model eotaxin-2 was

intranasally applied to IL-5Tg animals and eosinophils as well as other immune cells were enumerated in the BAL fluid shortly thereafter. We could observe a significant reduction in the number of infiltrating eosinophils as well as other immune cells such as neutrophils and monocytes in the animals treated with LPC 18:0. In our experimental setting we observed no change in tissue resident alveolar macrophages upon eotaxin challenge or LPC pretreatment (365).

It has to be noted, that our data disagrees with two previous studies performed *in vivo* testing LPC and its effects in allergy (362,385). In contrast to our findings the authors describe a pro-inflammatory role of LPC and demonstrate LPC's ability to recruit eosinophils to the lungs of animals and promote the development of allergic inflammation (362,385). Such LPC-induced eosinophilia was previously reported by Marathe et al. to be a consequence of PAF or PAF-like activity from contaminations in commercial preparations of LPC and not induced by LPC itself (386). Moreover, the authors used prolonged models of allergic inflammation where LPC was applied over a longer period of time (362,385). Since LPC is rapidly metabolized *in vivo* by the action of lysophospholipases and/or lysophospholipase D (also called autotaxin) generating the pro-inflammatory metabolite lysophosphatidic acid (LPA), the observed pro-inflammatory effects might be due to its metabolite LPA acting through G-protein coupled LPA receptors (LPA_{1-6}). In fact, both LPA and autotaxin have both been positively associated with allergic inflammation (387–390). Furthermore, LPA was proven to activate and recruit eosinophils to the lungs *in vivo* in multiple studies and models (391–394). Kwatia et al. further confirmed that LPC metabolites (and not LPC) contribute to manifestations of allergic lung disease. Their study demonstrated that the combined actions of secretory phospholipase and endogenous eosinophil lysophospholipase (converting LPC) are needed to induce pulmonary surfactant dysfunction implicated in asthma exacerbations (395).

Overall, we demonstrated that LPC attenuates eosinophil activation *in vitro* and *in vivo* by interfering with lipid raft-mediated signalling in eosinophils. Therefore, we propose that endogenous LPC is part of the negative feedback loop, helping to resolve inflammation acting similar to the resolvin or protectin group of bioactive mediators. Our discovery of LPC-induced targeting of lipid rafts could provide a mechanistic model for the discovery of novel drug compounds capable of attenuating eosinophilic inflammation. One of such is a stable analogue and structural mimetic of LPC 16:0 called miltefosine.

3.2 The anti-parasitic drug miltefosine suppresses human eosinophil activation and ameliorates murine allergic inflammation *in vivo*. (Br J Pharmacol. 2021 Mar 2;178(5):1234–48).

In my second project we studied the effects of miltefosine, an FDA-approved orphan-drug currently registered for the treatment of parasitic disease leishmaniasis (396). In addition to specifically killing the leishmania parasite, miltefosine has exhibited immunomodulatory properties, making it an interesting drug candidate for treatment of immune-mediated diseases (366). Consequently, in our subsequent project we aimed to explore the effects of the stable LPC analogue miltefosine on eosinophils.

First, we established that miltefosine in the concentrations of up to 20 μM was non-toxic and did not induce cell lysis or apoptosis of eosinophils *in vitro*. Upon pre-treating eosinophils with miltefosine we observed a similar inhibition of CD11b upregulation on eosinophils *in vitro* in comparison to saturated LPC. This comes as no surprise, since CD11b was reported to associate with CD66b in lipid rafts, which induces its clustering during eosinophil activation (397). Intercellular adhesion molecule-1 (ICAM-1) - the binding partner of CD11b on endothelial cells, has also been shown to associate with lipid rafts following leukocyte adhesion (398). What is more, miltefosine has been shown to downregulate endothelial adhesion molecule E-selectin involved in leukocyte adhesion (399). Therefore implying, that lipid raft-targeting could disrupt the adhesion process of eosinophils and the accompanying tissue infiltration on multiple levels.

Next, we demonstrated that miltefosine, like saturated LPC, inhibits chemotaxis and degranulation of isolated human eosinophils. Our data coincides with recent report of miltefosine action on mast cells; another crucial cell type involved in allergic reaction manifestation and allergic exacerbations (338,400). In this study, Rubikova et al. have shown that miltefosine is capable of inhibiting antigen-induced chemotaxis and degranulation of mast cells by modulating their activation at the plasma membrane and through PKC inhibition (338). Since mast cells and eosinophils have overlapping immunomodulatory roles in allergic inflammation, this indicates that miltefosine could effectively target and inhibit over-activation of both cell types simultaneously (400). However, in contrast to the above-mentioned study, we found that miltefosine is able to inhibit calcium flux following activation in eosinophils. Thus, signifying the existence of distinct downstream signalling interventions of miltefosine in different cell types. Of note, the observed calcium flux inhibition was rapid and occurred already after 1 minute of miltefosine addition. With a couple of preliminary experiments, we could

confirm that miltefosine, comparable to other cell types, is capable of inhibiting Akt phosphorylation in eosinophils (305).

Ultimately, we were interested to test miltefosine in preclinical models of allergic inflammation, in order to assess its viability as a potential drug candidate for treatment of allergic diseases. In all of our models we treated mice with miltefosine per os using a dosage regimen of 20 mg/kg/day, which was previously found to be safe and well tolerated (337). It should be noted that the *in vivo* migration assay was performed using IL-5Tg mice, which are characterized with blood eosinophilia. First, we repeated the short *in vivo* migration model, which revealed miltefosine treatment reducing eosinophil infiltration in the lungs *in vivo*. By using the instillation of eotaxin-2 in mice we could observe a similar inhibitory effect of eosinophil migration when compared to our *in vitro* chemotaxis results. These mice have been reported to have altered T cell and B cell numbers compared to wild type controls. In fact, IL-5 is reported to be an important cytokine for B cell maturation and differentiation. By overexpressing this cytokine, mice might have altered B cell responses (401,402).

Mouse models of allergic inflammation are, even with their limitations, widely accepted and used for the investigations into mechanisms of allergic diseases and drug discovery (67). However, we still wanted to explore whether the mechanism of miltefosine-induced eosinophil inhibition is similar in mice and human. For that we differentiated eosinophils from bone marrow of wild type mice and used them in *in vitro* experiments. We could demonstrate that miltefosine is capable of inhibiting calcium flux in mouse eosinophils in the same manner and with a similar potency as in human eosinophils, thereby validating the use of mouse eosinophils and mouse allergy models for testing of miltefosine's action *in vivo.*

We subsequently tested miltefosine in an acute model of allergic inflammation in wild type BALB/c mice using ovalbumin as a model allergen. In this model we could comparatively demonstrate that daily treatment with miltefosine reduces the infiltration of eosinophils in the lungs following allergen challenge. This in turn manifested in improved lung function parameters of mice as tested with methacholine challenge. However, an important shortcoming of this allergy model has to be noted. It utilizes aluminium as an adjuvant in order to achieve a strong allergic response (68). Moreover, ovalbumin is not a pathologically relevant allergen in humans as it does not induce an allergic response (403).

In both the ovalbumin model and the *in vivo* migration model the numbers of other infiltrating cell types were also partially reduced with miltefosine treatment. This can be explained on the one hand by the fact that activated and infiltrating eosinophils are capable of attracting other immune cells through the release of cytokines and bioactive mediators. For example, it was

shown that activated eosinophils attract neutrophils and B cells as well as release CCL17 and CCL22 thereby attracting T_H2 cells to the sites of inflammation (128,404–406). On the other hand, miltefosine could directly inhibit the infiltration of these cells, since it has been found modulate the response of multiple immune cell types in other disease models (337,341,344,407). To assess whether our findings are due to the action of miltefosine on eosinophils we repeated the ovalbumin model of allergic inflammation in eosinophil deficient Δdbl GATA-1 mice. We observed that in eosinophil deficient mice miltefosine did not significantly alter the numbers of other immune cell types. Our data from Δdbl GATA-1 mice thereby suggests, that the effect of miltefosine on eosinophils in wild type mice is responsible for the observed reduction in other cell types. Importantly, in all of our models the numbers of tissue resident alveolar macrophages were not altered by miltefosine treatment. Therefore, it is reasonable to think that by modulating eosinophil activation miltefosine specifically influences the pro-inflammatory infiltrating cells during allergen challenge.

To explore whether miltefosine inhibits the infiltration of cells directly or indirectly by altering the local cytokine milieu, we performed cytokine concentration measurements of the BAL fluid. Our key finding obtained from these measurements was that miltefosine significantly reduced the levels of IFN-γ in ovalbumin challenged wild-type animals. This finding goes hand in hand with the discovery of Verhaar et al., where they observed miltefosine-induced reduction in IFN-γ levels in a model of inflammatory bowel disease (341). What is more, we observed no reduction in IFN-γ levels in miltefosine treated Δdbl GATA-1 mice implicating eosinophils as the source of this cytokine in our experimental setting. There is controversy, however, about the role of IFN-γ in allergies. While some earlier studies describe its anti-inflammatory potential, it has recently received attention as an important pro-inflammatory cytokine increasing airflow obstruction and reducing lung function in asthma (408–410). Importantly, IFN-γ has been shown to induce steroid unresponsiveness in mice (411). Eosinophils in particular were shown to be a major source of IFN-γ, which is constitutively expressed and can be secreted in large quantities in response to T_H1, T_H2, and inflammatory stimuli (412). IFN-γ has been in turn demonstrated to activate eosinophils and promote their survival (413–415). Last but not least eosinophil-derived IFN-γ has been specifically recognized to induce airway hyperresponsiveness and lung inflammation even in the absence of lymphocytes (416).

When we examined the blood immune cell composition of treated IL-5Tg mice, we surprisingly observed an increase in the percentage of neutrophils in the animals treated with miltefosine. However, miltefosine treated wild-type animals did not vary in their neutrophil counts compared to their littermate controls at baseline. A study by Mukhopadhyay et al. showed an increase in

neutrophilic chemokine IL-8 in miltefosine-treated patients, but this observation remains to be confirmed in mice (339). An explanation for the observed neutrophilia, decreased IFN-γ levels and decreased airway inflammation could be miltefosine-induced endogenous corticosterone production (417,418). However, we observed no statistically significant differences in corticosterone levels in miltefosine treated animals and their littermate controls.

Altogether we were able to demonstrate the viability of miltefosine use in treatment of allergic and eosinophil-associated diseases. What is more, our discoveries of miltefosine's action on eosinophils and other immune cells has important implications for patients currently receiving the drug for parasitic infections. For example our work uncovered decreased infiltration of inflammatory monocytes, which have been reported to be detrimental in leishmania infections (419).

3.3 Knuplez E, Krier-Burris R, Cao Y, Marsche G, O'Sullivan J, Bochner BS. Frontline Science: Superior mouse eosinophil depletion *in vivo* targeting transgenic Siglec-8 instead of endogenous Siglec-F: Mechanisms and pitfalls. J Leukoc Biol. 2020 Jul 5;108(1):43–58.

Studies in the field of allergic inflammation, like ours, are often performed using various transgenic mouse models. Relative ease and accessibility of mouse genome manipulation enables researchers to recapitulate and study key aspects of human immune cell biology or disease pathology. The overall aim of mouse genetic manipulation is however, to closely resemble the human condition and therefore enable the translation of preclinical data to relevant clinical outcomes for the patient (66). Mouse and human eosinophils closely resemble one another in form and function yet important differences between the two exist (72). One of the pivotal differences lies in their distinct expression pattern of Siglec receptors, which have lately become recognized as important drug targets in a plethora of different immune-cell-mediated diseases (420,421). During my research stay abroad in the lab of Dr. Bochner at the Northwestern University of Chicago, we set out to explore and characterize a novel strain of mice expressing human Siglec-8 and lacking mouse Siglec-F on the surface of eosinophils.

First, we differentiated eosinophils from the novel Siglec8^{+}F^{-} strain of mice and tested the kinetics of surface Siglec-F and Siglec-8 expression during the differentiation process. We observed that Siglec-8 is expressed at approximately the same time of eosinophil differentiation compared to Siglec-F and before the late eosinophil differentiation marker CCR3 (105). Eosinophils from Siglec8^{+}F^{-} mice were considered mature on day 14 of the

differentiation process. Importantly, these eosinophils resembled eosinophils differentiated from wild type mice in their morphology and response to different stimuli. Moreover, we could demonstrate that engagement of Siglec-8 with its respective antibody on differentiated eosinophils resulted in CD11b upregulation. This could not be observed when Siglec-F was engaged on wild type or Siglec8^{+}F^{+} eosinophils, showcasing important signaling and functional differences between the two paralogs. Engagement of Siglec-8 on human eosinophils has been previously reported to be connected with increased integrin activation and cell adhesion (120), however this is the first report of a similar mechanism in mouse eosinophils expressing Siglec-8. During our experiments we observed the tendency of mouse eosinophils to upregulate CD11b to a lesser extent when compared to human, which might be due to inherent differences between mouse and human eosinophils. In particular it was shown, that mouse eosinophils respond to a lesser variety of stimuli and in a lesser manner when directly compared to isolated human cells (72).

Siglec-F and Siglec-8 engagement with their respective antibodies has been previously reported to result in specific cell death of eosinophils. On one hand *in vitro* antibody ligation of Siglec-F on murine eosinophils reportedly induces a modest caspase-dependent apoptosis of eosinophils (117). On the other hand, antibody engagement of Siglec-8 on human eosinophils primed with IL-5 results in a strong induction of non-apoptotic eosinophil cell death characterized with ROS production (120). *In vivo* application of high doses of Siglec-F antibody (sheep polyclonal) has similarly resulted in eosinophil cell death (110). In comparison to profound anti-Siglec-8 induced eosinophil depletion in humans, the eosinophil reduction in mice was shown to occur on a much smaller scale and was transient in nature (108,110). Of note, antibodies targeting Siglec-F proved to act specifically on murine eosinophils and did not alter the numbers of other cell types expressing Siglec-F such as alveolar macrophages (110). This has prompted the wide-spread use of Siglec-F antibodies for transient and specific depletion of eosinophils in order to study their contribution to health and disease in mice (123–126).

Since both Siglecs are very commonly used as a target for eosinophil depletion, we decided to investigate and compare the cell death-inducing effects of respective Siglec antibodies on eosinophils differentiated from mice with different Siglec expressing genetic backgrounds. We could observe only modest decreases in cell viability following anti-Siglec-F treatment *in vitro*, corroborating previous data (117). However, we observed the same modest effect following anti-Siglec-8 treatment on eosinophils expressing Siglec-8. ROS production is essential for anti-Siglec-8 induced cell death, since catalase (an extracellular superoxide scavenger) pre-

treatment of human eosinophils prevents the subsequent cell death of eosinophils. Because mouse eosinophils have a smaller tendency to degranulate *ex vivo* and they exhibit diminished capacity for ROS production, this may affect the killing capacity of Siglec-8 antibodies on murine eosinophils *in vitro* (72).

Next, we tested the efficacy of Siglec-F monoclonal antibodies for eosinophil depletion *in vivo*. We observed that a single application of a Siglec-F depleting antibody does not significantly reduce eosinophil numbers in blood and spleens of animals. We similarly observed no eosinophil depletion, when eosinophils were manually counted in blood smears. This finding comes as a surprise, since investigators have often used this exact acute method of eosinophil depletion in the past (124,422). The inconsistency in data could arise from difference in methods of eosinophil detection by flow cytometry between the research groups. While we used CCR3 as a surface marker, other have continued to use Siglec-F as a surface marker, even following application of a different Siglec-F antibody clone.

We further explored this irregularity and observed that anti-Siglec-F application *in vivo* profoundly reduces surface Siglec-F expression on eosinophils, thereby calling into question its use as a surface marker. With additional experiments we delineated that both Siglec-F antibody clones bind to the same cross-reactive epitope on the receptor, making accurate tracking of surface Siglec-F impossible. This finding has major implications for previously published work, where Siglec-F antibodies were used to reportedly completely deplete eosinophils. Our data suggests, that such depletion might have been incomplete, which brings their findings of eosinophil involvement into question (125,126,422,423). The observed rapid and potent effect of Siglec-F antibody application could also arise from actual Siglec-F targeting and not due to eosinophil depletion. Tumour promoting Siglec-F^{high} neutrophils have namely been described to act deleterious in lung cancer (99). What is more, Abdala-Valencia et al. propose the involvement of Siglec-F on eosinophils upon multistep recruitment into the airway following allergen challenge (424). The use of mice genetically deficient in Siglec-F could help elucidate the involvement of this receptor in the above-mentioned studies (113).

Importantly, following *in vivo* Siglec-F antibody application, other alternative methods to asses eosinophil depletion should be used, such as tissue MBP staining or microscopic eosin counting following staining with Discombe's fluid (128,425). Should flow cytometry be used as a preferred method, we suggest employing other surface markers like CCR3 to track eosinophil numbers. In our study we additionally showcase the potential of Siglec-8 expressed on the surface of eosinophils in Siglec8^{+}F^{+} mice as a marker for eosinophils.

After observing only modest eosinophil depletion following Siglec-F antibody application, we decided to investigate the level of depletion induced by specific Siglec-8 antibodies in Siglec-8 expressing mice. Remarkably, we could observe a rapid, profound and transient eosinophil depletion from blood, spleen and bone marrow following Siglec-8 antibody application. Demonstrating the superiority of Siglec-8 targeting in preclinical models of eosinophil depletion. This selective method of eosinophil depletion was comparable in effect to the use of IL-5 antibody treatment or to mice genetically deficient in eosinophils (426). Finally, in our last set of experiments we demonstrated that such profound eosinophil-depleting effect of Siglec-8 antibody (mouse IgG) compared to Siglec-F antibody (rat IgG) is at least in part due to the differences in the respective Fc isotype chains of the antibodies. In fact, the $F(ab')_2$ fragment of the Siglec-8 antibody, which lacks the Fc region of the mouse IgG chain, was ineffective in reducing the numbers of eosinophils. This finding in mice closely resembles the Siglec-8 targeting mechanism in humans. There, the Fc-region of a humanized, non-fucosylated IgG1 Siglec-8 antibody is capable of potent antibody-dependent cellular cytotoxic activity (ADCC) against eosinophils in the presence of natural killer cells (107).

In summary, we characterized a novel mouse model expressing human Siglec-8 and lacking mouse Siglec-F on the surface of eosinophils. As such, this model enables researchers to study human Siglec-8 biology in preclinical studies. What is more, we demonstrated the superiority of Siglec-8 targeting compared to Siglec-F targeting in mice with the purpose of eosinophil depletion. Last but not least, we highlighted the erroneous use of Siglec-F as an eosinophil surface marker following Siglec-F antibody application *in vivo*.

3.4 An updated review of pro-and anti-inflammatory properties of plasma lysophosphatidylcholines in the vascular system. (Vol. 21, International Journal of Molecular Sciences. MDPI AG; 2020. p. 1–18).

Lastly, our research work pertaining to the actions of LPC on eosinophils produced data conflicting with some of the earlier studies. This prompted up to further review the existing literature on LPC. Following extensive literature research in the field of LPC with the emphasis of LPC actions in the vascular system we uncovered major inconsistencies of the study design and data interpretation in many of the published works (369).

An important source of LPC produced during inflammation are secretory phospholipases A_2, which have long been considered important rate-limiting enzymes in inflammation (175). Phospholipid cleavage by $sPLA_2$ generates a lysophospholipid and a free fatty acid for

example the arachidonic acid – the precursor for the eicosanoid family of leukotrienes, prostaglandins, thromboxanes and lipoxins (427). The concentration of LPC species has been found to be drastically increased in inflammation (176,177). Similar to other products of $sPLA_2$ cleavage, LPCs were long considered to be toxic pro-inflammatory mediators exacerbating inflammation and activating immune cells (175). It was recently discovered that $sPLA_2$ group V exhibits the highest preference for cleavage of the large choline headgroup of phosphatidylcholine (428). Interestingly $sPLA_2$ group V exhibited potent anti-inflammatory action in the models of rheumatoid arthritis (429) and proved to act protective in models of aortic dissection (430), bringing the pro-inflammatory roles of its product LPC into question.

By inhibiting $sPLA_2$ one could potentially target the production of all these inflammatory mediators at once. Therefore, $sPLA_2$s have gained a lot attention as a possible drug target in a variety of inflammation-related pathologies (172). Therapeutic intervention in the form of $sPLA_2$ inhibitor varespladib (LY315920) targeting $sPLA_2$ groups IIA, V and X has recently been tested in a large clinical trial for the treatment of acute coronary syndrome as well as for the treatment of rheumatoid arthritis and asthma. Surprisingly, beneficial action of $sPLA_2$ inhibition could not be demonstrated in patients in any of the above mentioned studies (431–433). What is more, varespladib use was significantly associated with increased risk for myocardial infarction in patients with acute coronary syndrome (433). A subsequent Mendelian randomization study of 19 populations (>75000 test subjects) similarly found no association of lower $sPLA_2$-IIA levels and incidence of major vascular events (434). The clinical data thus demonstrated a more complex and yet to be elucidated role for $sPLA_2$ and its products in health and disease.

The current literature on LPCs effects often finds contradicting results even while studying them in the same disease or cell system. For example, while Englberger et al. demonstrate increased reactive oxygen species (ROS) production, Lin et al. show opposite effects of LPC inhibiting ROS production in neutrophils (228,260). In this case the authors demonstrate, that the solvent used for LPC preparation importantly influences the generated data (260). Moreover, most of the published studies use LPC in its free form without the addition albumin and serum. This results in membrane lysis at lower concentrations used and demonstrates LPC cytotoxicity in a non-physiological setting (185). Additional discrepancies in data ensue from using LPC mixtures or LPCs with varying degrees of alkyl chain length or saturation impacting their biological activity and function. Particularly some of the commercial LPC preparations were shown to stimulate leukocytes due to PAF or PAF-like activity from contaminating phospholipids (386). The authors demonstrated that once these commercial

preparations were exposed to PAF acetylhydrolase or saponification (targeting the *sn*-2 residue in PAF) the pro-inflammatory actions were abolished (386).

Recently, a number of novel high-throughput metabolomic studies identified LPC as a biomarker in different diseases (369). LPCs were found to be significantly decreased in a number of diseases and cancer types (244,435). Moreover, lower LPC concentration was associated with higher mortality in liver cirrhosis and sepsis patients (242,436).

It can now be concluded that LPCs exhibit both proinflammatory but also anti-inflammatory activities, and LPCs should be recognized as important homeostatic mediators involved in all stages of vascular inflammation. Taken together, the field of LPC research is constantly evolving and LPCs should not be simply described as potent proinflammatory mediators anymore.

4. CONCLUSION

The data from this thesis uncover anti-inflammatory actions of endogenous bioactive mediator LPC on human eosinophils. We could describe that saturated LPCs, which are excessively formed during inflammation, help prevent eosinophilic overactivation and tissue infiltration through membrane cholesterol removal and lipid raft modulation. Our data highlights a novel role for the previously considered deleterious product of inflammatory $sPLA_2$ cleavage. We furthermore validated our findings *in vivo* using a metabolically stable analogue of LPC a putative lipid raft modulator called miltefosine. Using several mouse models we could demonstrate the ability of miltefosine to prevent the infiltration of eosinophils in the BAL fluid. This consequently resulted in decreased levels of IFN-γ as well as in decreased infiltration of other immune cells. Importantly, miltefosine application lead to improved clinical parameters of lung function.

Altogether our data showcases, that the novel mechanism of targeting lipid raft assembly and signalling could be effectively used to target eosinophilic overactivation and therefore their detrimental effector functions in allergy and other diseases. What is more lipid raft modulation has previously been suggested as an important strategy in targeting immune cell activation in general (437). Since lipid rafts are involved in activation of many immune cell types and are associated with IgE-mediated signal transduction their targeting could effectively attenuate allergic inflammation on multiple levels (438). Such strategies aimed at targeting eosinophil over-activation are of special interest, since important homeostatic functions have recently been attributed to eosinophils. From that the LIAR (Local Immunity And/or Remodelling/Repair) hypothesis has been proposed, which identifies eosinophils as necessary for organismal homeostasis through their capacity for tissue remodelling and repair (439). Additionally, eosinophils have just recently been described to play a protective role in abdominal aortic aneurysm (440) and improve cardiac function after myocardial infarction (441). Thus, complete eosinophil depletion might have important homeostatic implications.

In cases where profound eosinophil depletion is necessary, novel biologic therapies selectively targeting eosinophils are rapidly becoming an attractive alternative treatment option (442). One of such is lirentelimab an antibody targeting inhibitory receptor Siglec-8 (108). As part of this thesis we have extensively described a novel mouse model with human-like Siglec expression on the surface of mouse eosinophils. Thus, facilitating the preclinical studies of Siglec-8 biology and targeting potential.

Through investigating alternative treatment strategies aimed either at eosinophil overactivation or eosinophil depletion, this thesis collectively advances our understanding of eosinophil homeostasis and contributes to future drug development.

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Lysophosphatidylcholines inhibit human eosinophil activation and suppress eosinophil migration *in vivo*

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ABSTRACT

Eosinophils are important multifaceted effector cells involved in allergic inflammation. Following allergen challenge, eosinophils and other immune cells release secreted phospholipases, generating lysophosphatidylcholines (LPCs). LPCs are potent lipid mediators, and serum levels of LPCs associate with asthma severity, suggesting a regulatory activity of LPCs in asthma development. As of yet, the direct effects of LPCs on eosinophils remain unclear. In the present study, we tested the effects of the major LPC species (16:0, 18:0 and 18:1) on eosinophils isolated from healthy human donors. Addition of saturated LPCs in the presence of albumin rapidly disrupted cholesterol-rich nanodomains on eosinophil cell membranes and suppressed multiple eosinophil effector responses, such as CD11b upregulation, degranulation, chemotaxis, and downstream signaling. Furthermore, we demonstrate in a mouse model of allergic cell recruitment, that LPC treatment markedly reduces immune cell infiltration into the lungs. Our observations suggest a strong modulatory activity of LPCs in the regulation of eosinophilic inflammation *in vitro* and *in vivo*.

1. Introduction

Eosinophils are regarded as key factors for asthma exacerbation [1] and lung connective tissue remodeling [2]. Increasing evidence suggests that eosinophils are involved not only in allergic diseases, but also in a plethora of other pathological conditions such as autoimmune diseases [3], thrombotic disease [4] and cancer [5]. Eosinophils, in comparison to other circulating leukocytes, express and secrete high levels of secreted phospholipases (sPLA2) type X [6] and IIA [7], which are able to cleave phospholipids into lysophospholipids and free fatty acids. In patients with asthma and allergic rhinitis, the activity and concentration of both sPLA2s [8,9] and lysophosphatidylcholines (LPCs) [10] was shown to be markedly elevated in bronchoalveolar (BAL) and nasal lavage fluid following allergen challenge.

Previous studies have shown that LPCs may alter the physiology of the vascular endothelium, pericytes, and neuron cells *in vitro* and *in vivo*, suggesting it may be a critical risk factor and may be associated with the pathogenesis and prognosis of cardiovascular diseases and neurodegenerative diseases [11–13]. However, more and more recent studies also show them as anti-inflammatory mediators [14–18] that even have positive therapeutic effects in sepsis models [19] and prevent neutrophil-mediated lung vessel injury in an *ex vivo* lung perfusion model [20]. Of particular interest, findings from recent clinical lipidomics studies showed an inverse relationship of LPCs with cardiovascular diseases [21–23]. In other studies, the LPC:PC ratio was found to be decreased in plasma as well as in cerebrospinal fluid from patients with Alzheimer's disease [24–26].

The seemingly contradictory pro- and anti-inflammatory effects of LPCs may be cell type specific effects or explained by the fast degradation of lysophospholipids, which are converted *in vivo* to pro-inflammatory lysophosphatidic acid [30]. Moreover, some of the ambiguity surrounding the results might stem from the reported toxicity of free LPC, which can be reversed by the addition of FBS or plasma proteins (*e.g.* serum albumin) [31]. In fact, even though LPC in human

Abbreviations: BAL, bronchoalveolar; C5a, complement component 5a; CCR, CC chemokine receptor; FACS, fluorescence-activated cell sorting; LPC, lysophosphatidylcholine; MβCD, methyl-beta-cyclodextrin; PI, propidium iodide; PMNL, polymorphonuclear leukocyte; sPLA2, secreted phospholipase A2

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serum can reach high micromolar concentrations [32–35], the majority of it is bound to albumin and lipoproteins. Therefore, the addition of albumin (or serum) to LPC before *in vitro* experiments is necessary to reduce the toxicity of the LPC and reflect the physiological conditions [31].

Surprisingly, little is known about the potential role of LPC in allergic diseases. Deciphering the relationship between an important immune cell type involved in allergic reactions and a highly prevalent bioactive lipid molecule at sites of inflammation could provide new insights into the regulation of eosinophilic homeostasis.

In the present study, we set out to study effects and mechanisms of action of the most abundant LPC species formed *in vivo* [36,37] on human eosinophil activation and function *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

All reagents were from Sigma (Vienna, Austria), unless otherwise specified. Eotaxin 1 (CCL11) and eotaxin 2 (CCL24) used *in vitro* assays were obtained from Immunotools (Friesoythe, Germany). Eotaxin 2 (CCL24) used for *in vivo* chemotaxis and recombinant human C5a were acquired from R&D systems (Minneapolis, MN, USA). Antibodies against phospho-Erk (Thr202/Tyr204) (#4370) and phospho-Akt (Ser 473) (#9271) were from Cell Signaling Technology, while secondary antibody (goat anti-rabbit Alexa fluoro-488 IgG) was from Life Technologies (ThermoFisher scientific, Waltham, MA, USA). Annexin V, Propidium Iodide, CD63-FITC, Siglec-F-PE (rat anti-mouse #552126), CD3-PE Cy5 (hamster anti-mouse #553065), CD11b-PE-Cy7 (rat anti-mouse #552850) and CD11c-BV421 (hamster anti-mouse #562782) were from BD Bioscience (Vienna, Austria). TruStain fcX CD 16/32 (anti-mouse #101320), Ly-6C–FITC (rat anti-mouse #128005), Ly-6G–APC (rat anti-mouse #127613) and Brilliant Violet 510™ I-A/I-E (rat anti-mouse #107635) were from Biolegend (San Diego, CA, USA). LPCs (16:0, 18:0, 18:1) were purchased from Avanti Polar Lipids (Birmingham, AL, USA), dissolved in chloroform/methanol and stored at −20 °C under argon atmosphere. Required amounts of LPC for assays were dried under a stream of argon and re-dissolved in phosphate buffered saline (PBS) before use.

All functional assays of eosinophils were performed in assay buffer (PBS with Ca^{2+} and Mg^{2+}, HEPES 10 mmol/L, glucose 10 mmol/L, bovine serum albumin 0.1%-1 mg/mL, pH 7.4). Following reconstitution in PBS, LPCs were dissolved in assay buffer to enable binding to albumin. LPC-albumin complexes were allowed to form for 10 min with occasional vortexing, prior to the addition to cells.

Fixative solution was prepared by adding 9 mL of distilled water and 30 mL of FACS-Flow to 1 mL of CellFix (BD, Vienna, Austria).

2.2. Blood sampling and eosinophil isolation

Blood sampling from healthy volunteers was approved by the Institutional Review Board of the Medical University of Graz (17-291 ex 05/06). All participants signed a written informed consent.

Polymorphonuclear neutrophil (PMNL) preparations were purified from whole blood as previously described [17]. In brief, platelet-rich plasma was separated by centrifugation of citrated whole blood. Erythrocytes and platelets were removed by dextran sedimentation. Preparations of PMNL were obtained by density gradient separation. Eosinophils were isolated from PMNL by negative magnetic selection using an Ab mixture (CD2, CD14, CD16, CD19, CD56, and glycophorin A) and colloidal magnetic particles from StemCell Technologies. Eosinophil purity was determined by morphological analysis of Kimura-stained cells and was typically >97%.

2.3. CD11b upregulation

Purified human eosinophils were stained with anti-CD11b-FITC (1:100), pretreated with vehicle or LPCs and stimulated with eotaxin-2 as indicated in the figure legend. CD11b upregulation was induced with 4 serial threefold dilutions of eotaxin-2 (starting at 30 nM). Eosinophil CD11b upregulation was measured as the geometric mean of the fluorescence in the fluorescein isothiocyanate (FITC) channel by flow cytometry and expressed as percent of vehicle response.

2.4. In vitro chemotaxis

Purified eosinophils were pretreated with vehicle or LPC 18:0 and were allowed to migrate to eotaxin-2 (in concentrations as indicated in the figure legends) in a HTS Transwell – 96 well plate with a 3 μm pore size polycarbonate membrane. Migrated eosinophils from the lower compartment were enumerated for 1 min by flow cytometric counting on a FACSCanto (BD Mountain View, CA, USA).

2.5. CD63 expression

Eosinophils were stained with anti-CD63-FITC (1:70) and pre-incubated with vehicle or LPCs. Afterwards cells were primed with cytochalasin B and stimulated with C5a [38]. Degranulation was analyzed by flow cytometry and expressed as fold increase in the fluorescence over indicated vehicle.

2.6. Calcium flux

Eosinophils were treated with 2 μM of Fluo-3 AM in the presence of 0.02% pluronic F-127 for 1 h at room temperature in the dark. Changes in $[Ca^{2+}]_I$ were detected as fluorescence in the FL1 channel by a FACSCalibur flow cytometer (BD, Mountain View, CA, USA), as was described previously [39].

2.7. Flow cytometry analysis of intracellular kinase phosphorylation

Eosinophils were pretreated with different LPCs or vehicle (15 min, RT). Following the pretreatment, cells were incubated with eotaxin 1 for 15 min. After stimulation, cells were fixed, permeabilized in 90% methanol, and stained with a rabbit anti-human p-Akt (pSer347, 1:50) or rabbit anti-human p-Erk (pThr202/Tyr204, 1:100) primary mAb followed by a goat anti-rabbit secondary Ab conjugated with Alexa Fluor-488 (1:500) for 30 min at RT. Cells were fixed, analyzed by flow cytometry and phosphorylation of Akt and Erk residues was quantified.

2.8. CC-chemokine receptor 3 (CCR3)

Isolated eosinophils were pretreated with vehicle, LPC 16:0 (30 μM) or phenylarsine oxide (4 μM) (15 min, RT), after which they were stimulated with eotaxin 2 at 37 °C for 4, 15 or 30 min. Following stimulation cells were put on ice, washed, stained with PE-anti-CD193 (CCR3) mAb (#310705) or PE-isotype control (#400311) (30 min, PBS + 2% FBS, on ice). Surface expression of CCR3 was quantified as the fluorescence in the PE fluorescence channel on FACSCanto and was compared to the fluorescence of unstimulated eosinophils.

2.9. Filipin III staining of unesterified cholesterol

Isolated PMNLs were stained with CD16-PE (1:200, 10 min, RT) and then preincubated with vehicle, positive control for cholesterol removal - methyl-beta-cyclodextrin (MβCD) (5 mg/mL) or LPC 16:0 (10 μM and 30 μM, 15 min, RT). After pretreatment, cells were washed with ice cold PBS, fixed with 1% formaldehyde at RT, washed and stained with Filipin III (50 μg/mL) in 10% FCS [17]. Cells were washed and analyzed by flow cytometry. Eosinophils were distinguished from neutrophils

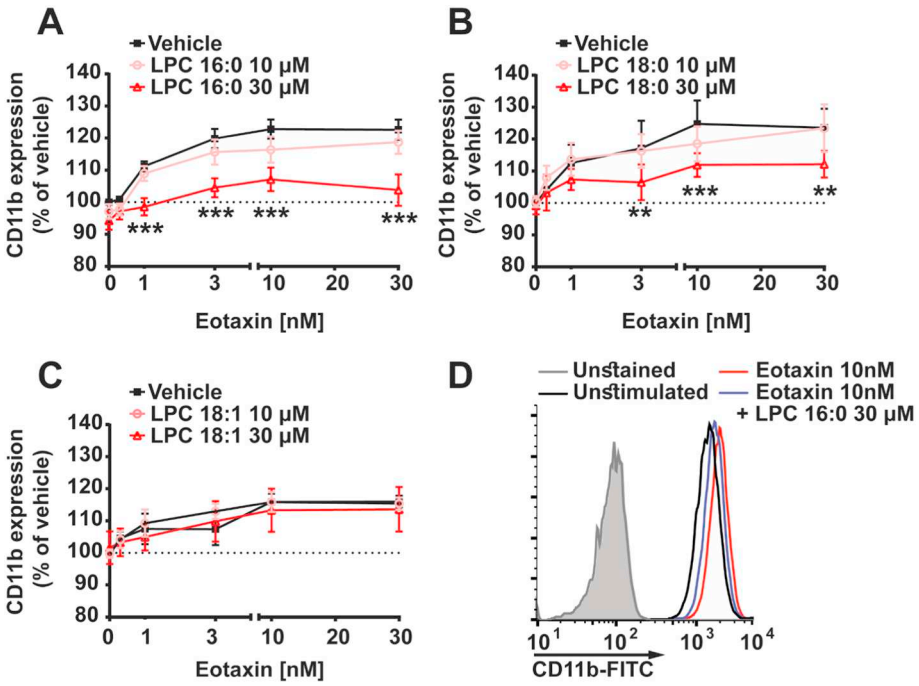

Fig. 1. Saturated LPCs concentration-dependently inhibit CD11b upregulation on eosinophils. Eosinophils were stained with anti-CD11b, followed by treatment with (A) LPC 16:0, (B) LPC 18:0, (C) LPC 18:1 or vehicle (15 min, RT). Subsequently, cells were stimulated with eotaxin-2 for 4 min at 37 °C and analyzed by flow cytometry. Eosinophil CD11b expression is expressed as percent of vehicle response. Data are shown as mean ± SEM from five individual experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, *vs* vehicle (two-way ANOVA with Bonferroni *post hoc* test). (D) Representative histogram of CD11b upregulation with LPC 16:0 pretreatment.

based on CD16 expression (neutrophils = CD16-pos, eosinophils = CD16-neg).

2.10. Cholesterol rich microdomains (lipid rafts) assessment

Eosinophils were preincubated with vehicle, LPC 16:0 (30 µM), or MβCD (5 mg/mL). Subsequently cells were washed with PBS and incubated with 1 µg/mL FITC-cholera toxin B for 1 h at room temperature. Cells were fixed and lipid raft abundance was measured by flow cytometry. For fluorescence microscopy, cells were pretreated with substances for 30 min, stained with FITC-cholera toxin B and spun on glass coverslips using a Cytospin 3 centrifuge (Shandon). The cells were mounted with Vectashield mounting medium including 4′6-diamidine-2′-phenylindole (DAPI) (Szabo Scandic, Vienna, Austria) and analyzed using OLYMPUS fluorescence microscope equipped with a Hamamatsu ORCA CCD camera, as described previously [17].

2.11. Apoptosis

Eosinophil survival after preincubation with the positive control formaldehyde (3,7%), LPCs (5–30 µM) after 30 min and 120 min was assessed by annexin V/propidium iodide staining, as described previously [40]. In addition, the apoptosis tests were performed using different LPC:bovine serum albumin (BSA) molar ratios and the time points of BSA addition.

2.12. In vivo chemotaxis

In vivo eosinophil migration was induced by intranasal application of 4 µg eotaxin-2 in eight-week-old male and female heterozygous interleukin-5 transgenic (IL-5 Tg) mice (BALB/c background). The mice were treated with vehicle or LPC 18:0 subcutaneously (20 mg/kg in PBS + 2% BSA) at time of intranasal application of eotaxin-2 and 2 h post-application.

BAL fluid was collected 4 h after experiment start. Migration of eosinophils was evaluated by flow cytometric counting of highly granular (high side scatter) $CD11c^-$/Siglec F^+ cells. The respective gating strategies for evaluation of other immune cells are shown in Fig. S4 and were as following: B cells ($CD11c^-/CD11b^-/MHCII^+$), T cells ($CD11c^-/CD11b^-/CD3^+$), alveolar macrophages (Siglec $F^+/CD11c^+$), neutrophils (Siglec $F^-/CD11b^+/Ly6G^+$), dendritic cells (Siglec F^-/$Ly6G^-/Ly6C^-/MHCII^+$) and inflammatory monocytes (Siglec F^-/$Ly6G^-/MHCII^-/LY6C^{++}$).

The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: BMWFW-66.010/0207-WF/V/3b/2017). The experimental procedure conforms to Directive 2010/63/EU, and was performed in accordance with national and international guidelines.

2.13. Statistics

Statistical analysis was preformed using the GraphPad Prism™ 6 software (GraphPad Software, Inc., CA, USA). Differences between groups were tested by one-way or two-way ANOVA followed by Dunnett's or Bonferroni posttest or Student's *t*-test. p values ≤ 0.05 were considered significant and are indicated as $^*p \leq 0.05$; $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

3. Results

3.1. LPCs inhibit activation of eosinophils

Chemotaxis of eosinophils involves expression and/or activation of adhesion molecules on the cell surface, which mediate the tethering/rolling and sticking/transmigration. The expression of the integrin $\alpha_M\beta_2$ (CD11b/CD18; Mac-1) is upregulated by the action of eotaxin and other chemokines [41].

In first experiments, we tested whether LPCs affect eotaxin-2-induced CD11b upregulation in human eosinophils. It is important to note that in all experiments LPC was complexed to albumin at molar ratio of maximum 2:1 (albumin contains 5 binding sites for LPC) [31]. When LPCs were preincubated with albumin (1 mg/mL) for 10 min, none of the tested LPC species induced cytotoxicity or apoptosis in eosinophils at the concentrations used in this study (Fig. S1).

At a concentration of 30 µM, LPC 16:0 (Fig. 1A) and LPC 18:0 (Fig. 1B) (diluted in buffer containing 1 mg/mL bovine serum albumin) significantly inhibited CD11b expression of eotaxin stimulated eosinophils, whereas unsaturated LPC 18:1 was ineffective at the

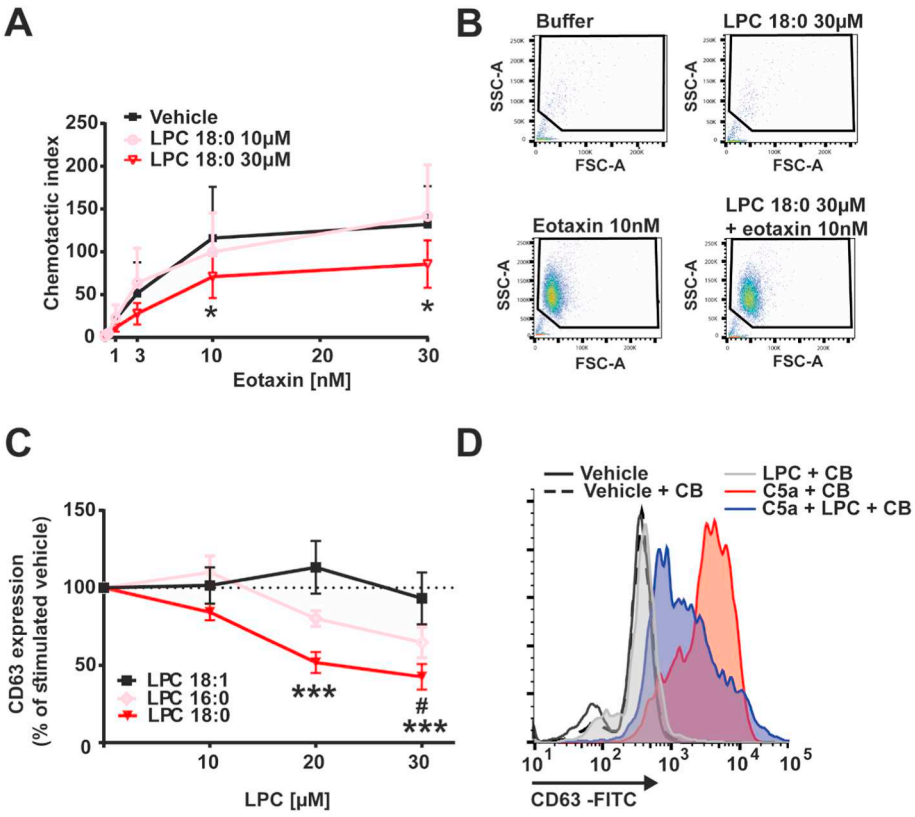

Fig. 2. LPC 18:0 inhibits chemotaxis and CD63 expression of eosinophils. (A–B) Purified human eosinophils were treated with LPC 18:0 (15 min, RT) or vehicle and allowed to migrate towards eotaxin-2 in Transwell plates (A). Migrated cells were enumerated by flow cytometry. Responses are expressed as chemotactic index (ratio of migrated cells over unstimulated vehicle – cells migrated to buffer). Results are expressed as mean ± SEM from five individual experiments. *$p < 0.05$ *vs* vehicle. (B) Representative scatter blots of cells migrating towards vehicle or eotaxin-2. (C–D) Eosinophils were stained with anti-CD63-FITC followed by incubation with LPCs or vehicle (15 min, RT). Subsequently, cells were primed with cytochalasin B (CB) and stimulated with C5a (100 nM) for 20 min at 37 °C. CD63 expression is expressed as percent of vehicle-stimulated cells. Results are expressed as mean ± SEM from five individual experiments. ***$p < 0.001$ LPC 18:0 *vs* vehicle; $^{\#}p < 0.05$, LPC 16:0 *vs* vehicle (two-way ANOVA with Bonferroni post-hoc test). (D) Representative histogram of CD63 staining following preincubation with LPC 18:0 (30 μM).

concentrations tested (Fig. 1C). Representative histograms of CD11b staining with and without LPC addition are shown in Fig. 1D. Preincubation of eosinophils with LPC in the absence of eotaxin (eotaxin 0 nM) showed no effect on CD11b expression.

3.2. LPCs inhibit chemotaxis of eosinophils

We next performed chemotaxis assays to assess whether LPC mediated inhibition of CD11b expression translates into a reduced migratory capability of eosinophils. Pretreatment of eosinophils with LPC 18:0 (30 μM) markedly suppressed chemotaxis of eosinophils towards eotaxin-2 (Fig. 2A, representative histograms are shown in B).

To test whether LPCs influence degranulation-associated processes in eosinophils, we determined the degranulation marker CD63, which is present in secretory granules and gets transported to the cell surface following degranulation [42]. Eosinophils were pretreated with LPC 16:0, LPC 18:0 and LPC 18:1 for 15 min and stimulated with recombinant human C5a, a strong inducer of degranulation [43]. Both, LPC 16:0 and LPC 18:0 dose-dependently inhibited degranulation by ~50% (Fig. 2C), whereas unsaturated LPC 18:1 had no effect (Fig. 2C). Representative histograms are given in (Fig. 2D).

3.3. LPC decreases Ca^{2+} flux in eosinophils

Prompted by the robust inhibitory activity of saturated LPCs, we performed calcium flux and kinase phosphorylation assays. Ca^{2+} flux in eosinophils was induced using eotaxin-1 and LPC 16:0 inhibited eotaxin-induced Ca^{2+} flux in a dose-dependent manner (Fig. 3A). LPC on its own did not affect Ca^{2+} flux in eosinophils, as shown in Fig. 3B. The saturated LPC species 16:0 and 18:0 caused stronger inhibition of calcium flux following stimulation with eotaxin (Fig. 3C–E) or C5a (Fig. 3F–H) when compared to LPC 18:1.

3.4. LPC inhibits phosphorylation of Akt and Erk

We next studied effects of LPCs on downstream signaling events in eosinophils following eotaxin activation, such as phosphorylation of Akt [27] and Erk (MAPK). pAkt is involved in cell survival and plays a role in asthma onset [28], while pErk is crucial for eosinophil migration and degranulation [29]. Both saturated LPC species 16:0 and 18:0 significantly inhibited phosphorylation of Akt (Fig. 4A) and Erk (MAPK) (Fig. 4B) following eotaxin-1 stimulation.

3.5. LPCs disrupt lipid rafts on cell membranes

Lipid rafts are cholesterol-rich signaling nanodomains integral to cellular signaling pathways involved in immune cell activation [44]. Disruption of these domains suppresses activation of eosinophils [45]. Given that LPCs are rapidly incorporated into cell membranes [46], we assessed whether LPCs affect lipid-raft abundance. We tested for two different components of lipid rafts – ganglioside GM1 (binds to cholera toxin B subunit) and unesterified cholesterol (binds to Filipin III). Methyl-beta-cyclodextrin (MβCD) was used as a positive control, due to its capability of depleting cholesterol and disrupting lipid rafts [47].

We pretreated eosinophils with either LPC or MβCD for 15 min and stained lipid rafts with cholera toxin B. Eosinophil lipid rafts were visualized by fluorescent microscopy (Fig. 5A) or by flow cytometry (Fig. 5B, C). Both MβCD and LPC decrease CTX-B staining compared to vehicle-treated cells, which becomes significant 15 and 30 min after treatment for MβCD and LPC, respectively. (Fig. 5B).

When using Filipin III for staining of cholesterol, we observed that MβCD and LPC remove cholesterol from cell membranes (Fig. 5D). Interestingly, LPC does not deplete cholesterol from neutrophils as efficiently as from eosinophils (Fig. 5E). We next tested whether LPC suppresses effector responses of receptors that directly interact with lipid rafts. Toll-like receptor 7 (TLR7) is localized in lipid rafts and is known to induce CD11b upregulation in eosinophils [48]. We activated TLR-7 with Resiquimod (R-848), a TLR7 agonist inducing CD11b

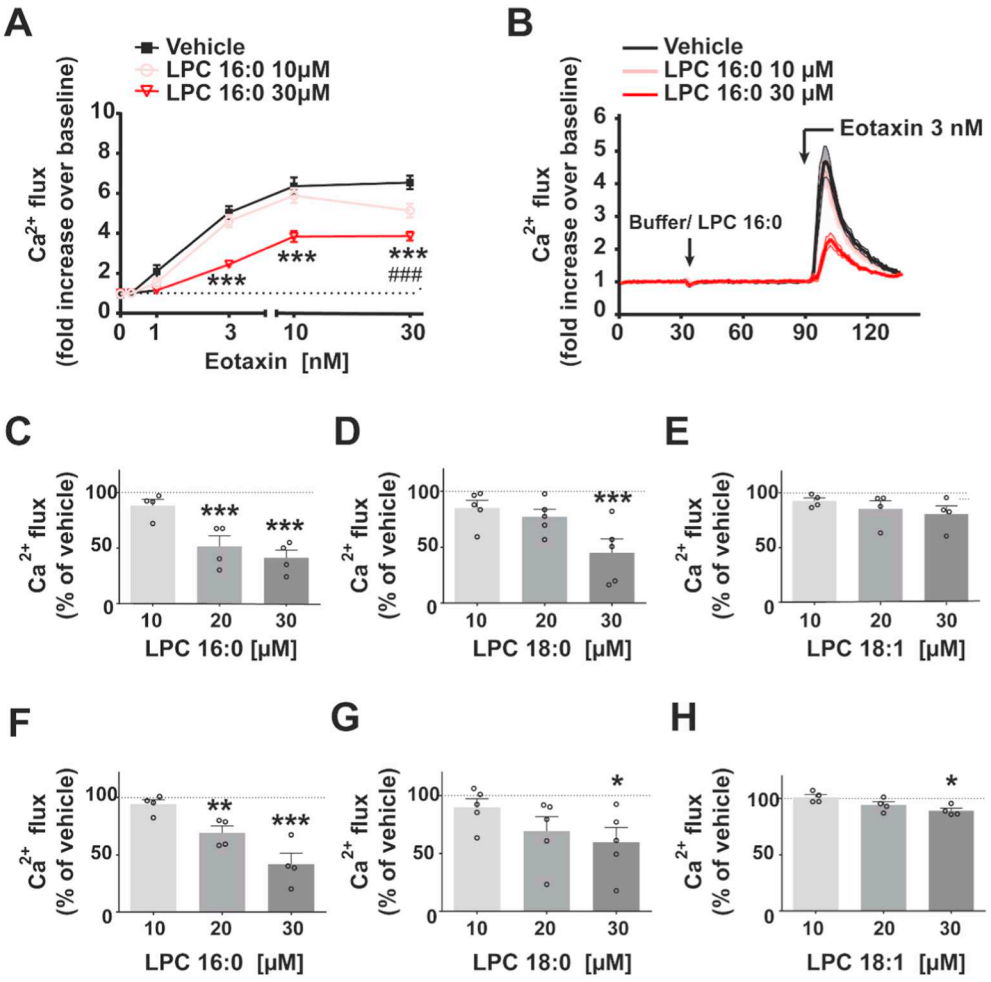

Fig. 3. LPC inhibits Ca^{2+} flux in eosinophils. (A) Concentration-response of eosinophils to eotaxin-1. (A) Eosinophils were labeled with Fluo-3 AM and changes in $[Ca^{2+}]_I$ were detected by flow cytometry. Eosinophils were stimulated with increasing concentrations of eotaxin-1 in the presence or absence of LPC 16:0 (30 μM). Results represent fold increase in $[Ca^{2+}]_I$ over vehicle. Data are shown as mean ± SEM from five individual experiments $^{***}p < 0.001$ LPC 16:0 (30 μM) *vs* vehicle; $^{###}p < 0.001$ LPC 16:0 (10 μM) *vs* vehicle (two-way ANOVA with Bonferroni post-hoc test). (B) Time course of Ca^{2+} flux in eosinophils stimulated with eotaxin-1. Following baseline measurement (30 s), LPC 16:0 or vehicle was added. After 1 min, eotaxin-1 (3 nM) was added to induce Ca^{2+} flux. Data are shown as mean ± SEM from five individual experiments. (C–H) Concentration-dependent effects of LPCs on eotaxin- and C5a-induced Ca^{2+} flux. Changes in $[Ca^{2+}]_I$ were expressed as % of vehicle response. Cells were pretreated with LPC 16:0 (C, F), LPC 18:0 (D, G), LPC 18:1 (E, H) or vehicle, followed by stimulation with C5a (0.2 nM) (C–E) or eotaxin-1 (10 nM) (F–H). Results are expressed as mean ± SEM from four to five individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* vehicle (One-way ANOVA with Dunnett's post-hoc test).

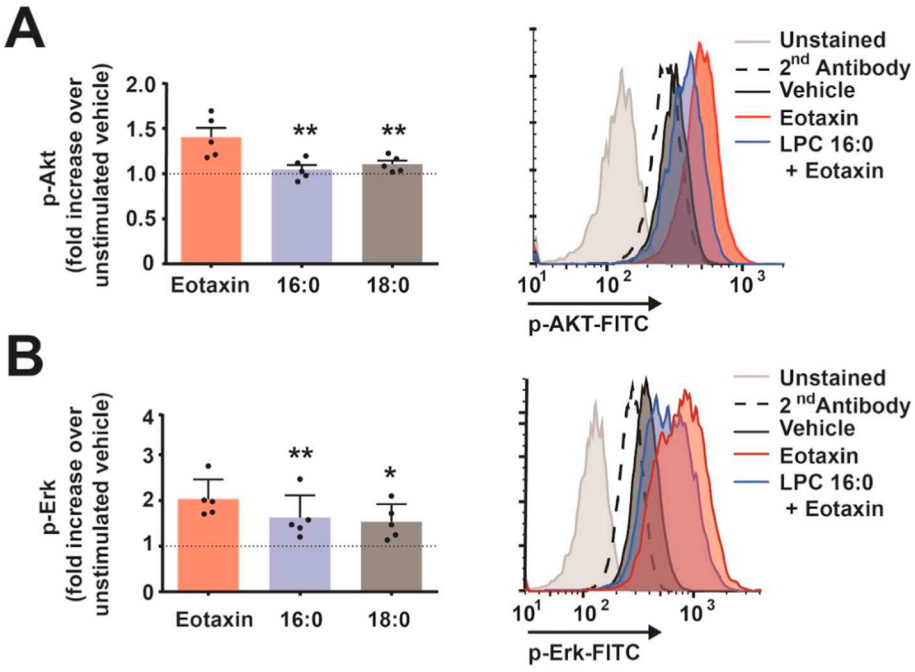

Fig. 4. Saturated LPCs inhibit phosphorylation of kinases. Eosinophils were pretreated with LPCs or vehicle (15 min, RT) followed by addition of eotaxin-1 (5 min, 37 °C). Subsequently, cells were fixed, permeabilized and stained against p-Akt or p-Erk with antibodies. Phosphorylation of Akt (A) or Erk (B) residues was quantified as the increase of fluorescence in the FITC fluorescence channel. Data are shown as mean ± SEM from five individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* vehicle (One-way ANOVA with Dunnett's post-hoc test).

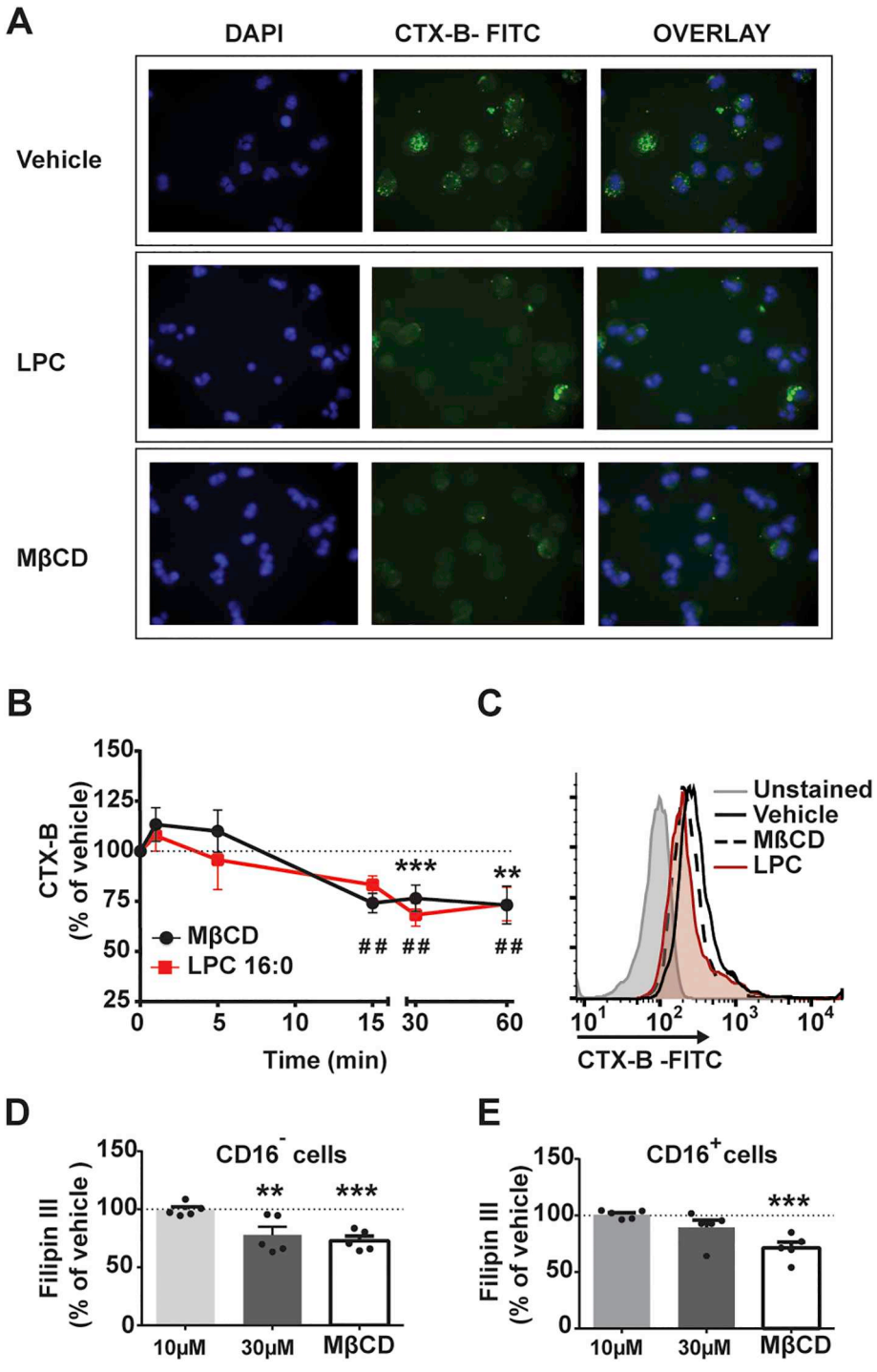

Fig. 5. LPC 16:0 disrupts cell membrane lipid rafts. (A) Eosinophils were pretreated with LPC 16:0 (30 μM), methyl-beta-cyclodextrin (MβCD) (5 mg/mL) or vehicle for 30 min. Lipid rafts were stained with FITC-cholera toxin B (CTX-B-FITC) (green) and nuclei with DAPI (blue). Representative images from three individual experiments are shown. (B) Lipid raft abundance of CTX-B-FITC stained eosinophils was quantified by flow cytometry. Control was set as 100% and values are expressed as % of vehicle control. Data are shown as mean ± SEM from four individual experiments. $^{\#\#}p < 0.001$ *vs* vehicle (two-way ANOVA with Dunnett's post-hoc test) (C) Representative histogram of CTX-B staining at time point 15 min. (D) Eosinophils (CD16 negative cells) and (E) neutrophils (CD16 positive cells) were stained with Filipin III and analyzed by flow cytometry. Vehicle control fluorescence was set as 100% and values are expressed as % of vehicle fluorescence. Data are shown as mean ± SEM from five individual experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* vehicle (one way ANOVA with Dunnett's post-hoc test).

upregulation in eosinophils in the presence or absence of LPC 18:0. Of note, LPC 18:0 effectively suppressed Resiquimod mediated CD11b upregulation (Fig. S2).

Receptor internalization is required for eotaxin-induced functional responses in human eosinophils [49]. Given that LPC disrupts lipid rafts, we hypothesized that LPC affects CCR3 surface expression and internalization. To inhibit CCR3 internalization, phenylarsine oxide was used as a control [49,50]. However, we observed that LPC pretreatment does not alter CCR3 expression on eosinophils (Fig. S3A) and receptor internalization after stimulation with eotaxin-2 (Fig. S3B).

3.6. LPC inhibits chemotaxis of eosinophils in vivo

Our results showed that LPC 18:0 inhibits the chemotaxis of human eosinophils *in vitro* (Fig. 2A). We next studied the inhibitory activity of LPC in an *in vivo* setting. Allergen-driven accumulation and mobilization of eosinophils into the airways involves the eotaxin/CCR3 pathway [51]. Directed movement of eosinophils into airways can be induced by intranasal administration of human eotaxin-2 in an IL-5 Tg mouse model. In IL-5 Tg mice eosinophils rapidly and selectively accumulate in the airways and can be enumerated in BAL fluid [52]. The treatment scheme of the *in vivo* migration assay is shown in Fig. 6A. Strikingly, we observed that two subcutaneous injections of LPC 18:0 (20 mg/kg) [19] were sufficient to inhibit the infiltration of immune cells, particularly

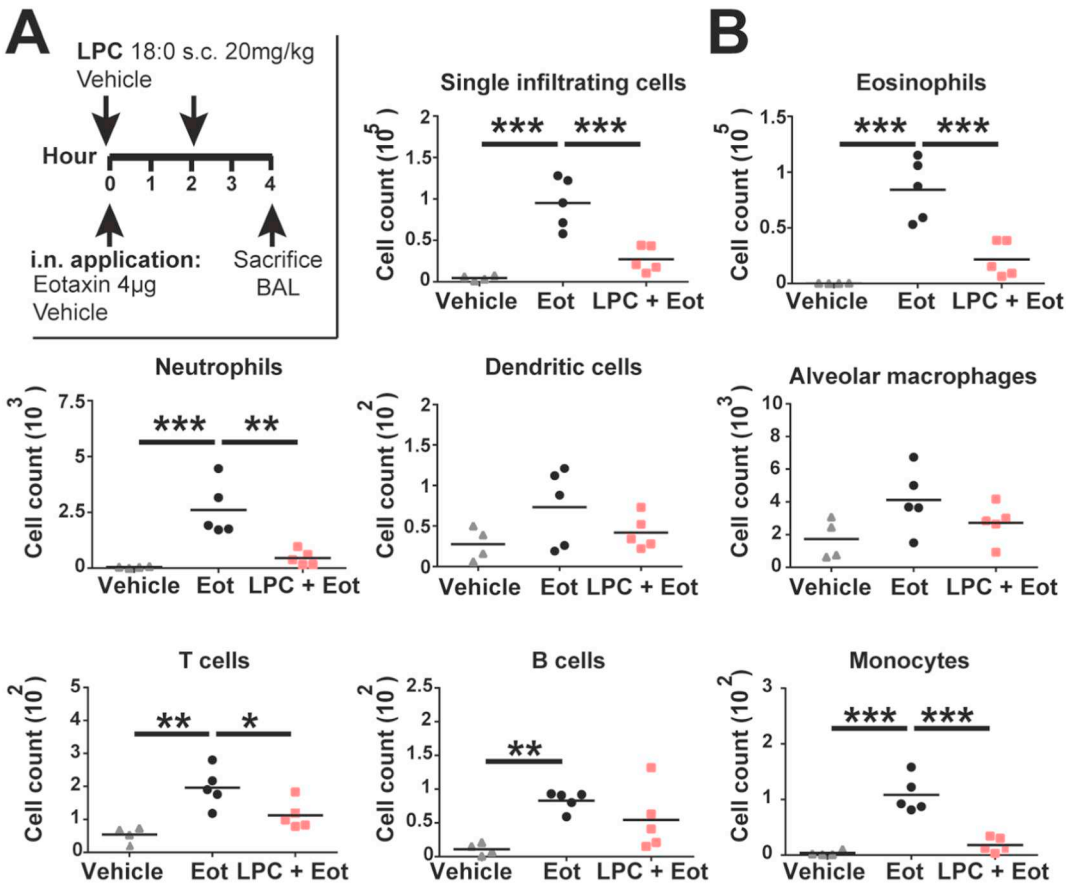

Fig. 6. LPC 18:0 inhibits migration of eosinophils *in vivo*. (A) Eight week old IL-5 Tg mice received either LPC 18:0 (20 mg/kg) or vehicle (PBS + 2% BSA) subcutaneously along with intranasal applied eotaxin-2 (Eot) or vehicle. Administration of LPC 18:0 was repeated after 2 h followed by sampling bronchoalveolar lavage (BAL) fluid after two additional 2 h. (B) Quantification of immune cell composition in the BAL fluid. Data are shown from 4 to 5 mice from two individual experiments. *p < 0.05, **p < 0.01, ***p < 0.001 *vs* Eot group (one-way ANOVA with Dunnett's post-hoc test).

eosinophils, into airways (Fig. 6B). Of note, LPC application also inhibited infiltration of neutrophils into airways. Immune cell composition was determined using the gating strategy as shown in Fig. S4.

Analysis of the blood immune cell composition revealed no change in eosinophils, dendritic cells, T and B cells and monocytes numbers (Fig. S5). However, we observed an increase in the percentage of neutrophils in blood following LPC administration, which is in accordance with previously published data [19].

To compare the effects of saturated and unsaturated LPCs on eosinophilic migration *in vivo*, we tested the inhibitory effect of LPC 18:1 (Fig. S6). Treatment with LPC 18:1 (applied at the same concentration as LPC 18:0) was less effective in comparison to LPC 18:0 and only tended to decrease ($p = 0.093$) eosinophilic infiltration into the lungs (Fig. S6B).

4. Discussion

In this study, we demonstrate for the first time that saturated LPC species strongly attenuate the activation of eosinophils. Saturated LPCs suppressed human eosinophil migration *in vitro* and the migration of eosinophils into the airways of IL-5 Tg mice *in vivo*. Our observations indicate that LPC destabilizes lipid rafts by removing cholesterol from cell membranes. Interestingly, we observed marked differences in the inhibitory activity of saturated LPCs at the highest used concentration (30 µM) compared to unsaturated LPC 18:1 *in vitro*, which may depend on the more conical shape of the saturated LPCs and their higher spontaneous curvature values compared to unsaturated LPCs [53]. Our *in vitro* results were also reflected *in vivo*, where LPC 18:0 significantly suppressed eosinophilic infiltration while LPC 18:1 was less effective. Similar results were reported in cancer research, where unsaturated LPC 18:1 was less effective than LPC 18:0 in preventing migration and spread of cancer cells and metastases [54]. Additionally, cells pretreated with LPC 18:0 showed higher membrane stiffness (*i.e.* lower lateral membrane mobility) as measured in photobleaching experiments [54].

Previous studies demonstrated that LPC incorporates into erythrocyte cell membranes within minutes [55] and insert into model membranes rapidly (rate constant 0.2 s^{-1}) [56], inducing membrane perturbations [57]. Our observations of LPC-induced disruption of lipid lifts are consistent with previous studies showing that LPC destabilizes membranes and cause a change in the conformational and/or motional properties of phospholipids [58]. It is generally accepted that lipid rafts serve as a platform for receptor signaling in different immune cells and that a disruption of their integrity alters the signaling of immune cells [44]. It was shown that the mobilization of CD11b in lipid rafts of eosinophils [45] and neutrophils [59] leads to cell activation.

In this context, it is particularly interesting that the integrity of lipid rafts is necessary for IgE-mediated signal transduction in mast cells and basophils [60], two other types of effector cells essential for allergic inflammation. Our results suggest LPC induced disruption of the integrity of lipid rafts suppresses activation of cells. In line with that assumption, we observed that LPC was able to suppress lipid raft located TLR-7 induced activation of eosinophils. However, the underlying molecular mechanism remains to be determined. LPC could interact directly with receptors (or other proteins) within the plasma membrane, interfering with eosinophilic activation. When using Filipin III for staining of cholesterol, we observed that LPC removes cholesterol from eosinophil cell membranes. This is of importance, given that lipid

rafts and cell membrane stability are closely related to the cholesterol content of the cell membrane. Statins induced cholesterol depletion of eosinophils suppresses chemotaxis and CCR3 expression [61], whereas high dietary cholesterol intake is linked to increased Th2 inflammation and IL-5 levels [62]. Of note, the involvement of cholesterol sensitive liver X receptors in Th2-driven allergic eosinophilic asthma has recently been demonstrated [63].

It is important to note that eosinophils are not only able to cause direct tissue damage, but also trigger Th2 immune responses through cytokine secretion and degranulation [64]. Aberrant Th2 cytokines are the driving force behind allergic inflammation [65] and continue to recruit eosinophils and other immune cells into the affected tissue. One of the potent cytokines produced by both Th2 cells and activated endothelium is eotaxin [66].

It should be noted that our results seem to contradict two earlier studies with guinea pigs and mice [67,68]. In these studies, the involvement of LPC in respiratory inflammation was described as pro-inflammatory by recruiting eosinophils into lungs [69] and airway allergic disease manifestation [70]. However, in these previous studies, the animals were treated with LPCs for up to 14 days. LPC is converted into proinflammatory lysophosphatidic acid (LPA) *in vivo* by the lysophospholipase D activity of autotaxin [30]. This is of particular importance, given that the activity of autotaxin is increased during allergic inflammation and allergen challenge, and lysophosphatidic acid was proven to act as a strong chemoattractant and activator of eosinophils [67]. Therefore, the pro-inflammatory effects of LPC observed *in vivo* [65] might be mediated through the actions of lysophosphatidic acid on its receptors. In contrast to the study design of these two studies investigating chronic effects of LPC treatment [64,65], we analyzed lung infiltration already 4 h after application and therefore studied an acute effect of LPC. In addition, we applied LPC subcutaneously instead of intranasally, a route of application suitable to prevent direct lung irritation.

Our study appears to contradict some of the findings by Zhu et al. [68], where LPC 16:0 activated eosinophils and promoted adhesion *via* a non-storage Ca^{2+} channel. The authors observed reduced eosinophilic viability at lower concentrations of LPC (4 μM) as used in our study. However, in contrast to our study, LPC was added in the absence of albumin (or serum), which probably explains the apparently contradictory results. This assumption is supported by the fact that we also observed toxic effects of LPC when added to eosinophils in the absence of albumin.

Some limitations of our study must be taken into account. We have only tested three major commercially available LPC species and their effects on eosinophils, while *in vivo* a combination of many other species is formed. Furthermore, our *in vivo* experiments were performed with a transgenic mouse model (IL-5 Tg mice), characterized by blood eosinophilia to facilitate eosinophilic enumeration in the BAL fluid of the animals.

In summary, our results clearly show that saturated LPCs have acute anti-inflammatory effects on human eosinophils. Furthermore, we have translated our *in vitro* data into an *in vivo* model of eosinophilic migration in which administration of LPCs significantly reduced the infiltration of immune cells into the lung. Our study provides new insights into a homeostatic activity of LPCs that attenuate eosinophilic inflammation.

CRediT authorship contribution statement

Eva Knuplez: Methodology, Investigation, Formal analysis, Writing - original draft. **Sanja Curcic:** Investigation, Formal analysis. **Anna Theiler:** Investigation, Formal analysis. **Thomas Bärnthaler:** Investigation, Formal analysis. **Athina Trakaki:** Investigation, Formal analysis. **Markus Trieb:** Investigation, Formal analysis. **Michael Holzer:** Formal analysis, Writing - review & editing. **Akos Heinemann:** Formal analysis, Writing - review & editing. **Robert Zimmermann:** Formal analysis, Writing - review & editing. **Eva M. Sturm:** Formal analysis, Writing - review & editing. **Gunther Marsche:** Conceptualization, Formal analysis, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

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RESEARCH PAPER

The anti-parasitic drug miltefosine suppresses human eosinophil activation and ameliorates murine allergic inflammation *in vivo*

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Background and Purpose: Miltefosine is an alkylphosphocholine drug with proven effectiveness against various types of parasites and cancer cells. Miltefosine is not only able to induce direct parasite killing but also modulates host immunity, for example by reducing the severity of allergies in patients. To date, there are no reports on the effect of miltefosine on eosinophils, central effector cells involved in allergic inflammation.

Experimental Approach: We tested the effect of miltefosine on the activation of human eosinophils and their effector responses *in vitro* and in mouse models of eosinophilic migration and ovalbumin-induced allergic lung inflammation.

Key Results: The addition of miltefosine suppressed several eosinophilic effector reactions such as CD11b up-regulation, degranulation, chemotaxis and downstream signalling. Miltefosine significantly reduced the infiltration of immune cells into the respiratory tract of mice in an allergic cell recruitment model. Finally, in a model of allergic inflammation, treatment with miltefosine resulted in an improvement of lung function parameters.

Conclusion and Implications: Our observations suggest a strong modulatory activity of miltefosine in the regulation of eosinophilic inflammation *in vitro* and *in vivo*. Our data underline the potential efficacy of miltefosine in the treatment of allergic diseases and other eosinophil-associated disorders and may raise important questions regarding the immunomodulatory effect of miltefosine in patients treated for leishmania infections.

KEYWORDS
allergic inflammation, eosinophils, miltefosine

Abbreviations: BMDE, bone marrow-derived eosinophil; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; FSC, forward scatter; PI, propidium iodide.

1 | INTRODUCTION

To date, miltefosine (Impavido®) is the only oral drug approved for the treatment of leishmaniasis with limited mild or moderate side effects (Pijpers et al., 2019). The development of miltefosine is a success story of public–private partnership, a breakthrough in medicine affordability and patient drug adherence, landing it on the World Health Organization (WHO)'s List of Essential Medicines (Berger et al., 2017; Sunyoto et al., 2018). Miltefosine disrupts membrane structures and affects phosphatidylcholine synthesis in susceptible promastigote cells (Pinto-Martinez et al., 2018; Rakotomanga et al., 2007). Due to its detergent-like properties, miltefosine is thought to interact with the mucosa of the gastrointestinal tract during oral use and cause its most commonly listed side effects—nausea, vomiting and diarrhoea (Bhattacharya et al., 2007). During prolonged treatment, the severity of the side effects was reported to decrease over time (8.2% during Week 1 to 3.2% during Week 4) (Bhattacharya et al., 2007).

Miltefosine exerts immunomodulatory effects on human cancer cells by inhibiting the **PI3K/Akt** signalling pathway (Ruiter et al., 2003), induces **IL-12**-dependent Th1 responses (Wadhone et al., 2009) and shows anti-inflammatory effects in endothelial cells, suppressing vascular inflammation (Fang et al., 2019). However, the immunomodulatory effects of miltefosine on primary human cells have so far only been described for T cells (Bäumer et al., 2010) and mast cells (Weller et al., 2009).

In plasma, miltefosine is mainly bound to albumin (96–98%) (Kip et al., 2018) and accumulates predominantly in cholesterol-rich microdomains of the cell membranes (lipid rafts) (Malta de Sá et al., 2015). Miltefosine increases membrane fluidity (Moreira et al., 2014), modulates lipid raft-dependent signalling (Weller et al., 2009) and could therefore be an attractive drug candidate for the treatment of diseases characterized by abundant lipid raft activation, such as allergic diseases (Dölle et al., 2010). Miltefosine attenuates allergic inflammation in T cell-dependent mouse models of dermal inflammation (Bäumer et al., 2010), improves local dermatitis in patients with atopic dermatitis (Dölle et al., 2010), inhibits activation and degranulation of mast cells, and significantly reduces allergic disease manifestation in patients (Magerl et al., 2013; Maurer et al., 2013; Rubíková et al., 2018).

Surprisingly, there are no reports on the effects of miltefosine on eosinophils, a key cell type involved in the initiation and propagation of immune responses in allergic diseases (Stone et al., 2010). Here, we studied in detail whether miltefosine exerts immunomodulatory effects on eosinophils *in vitro* and in mouse models of allergic lung inflammation.

What is already known

- Miltefosine is an orphan drug marketed for the treatment of leishmaniasis.
- Miltefosine reduces the severity of allergies in patients.

What this study adds

- Miltefosine inhibits activation of human eosinophils and suppresses human eosinophil effector responses.
- Miltefosine inhibits the infiltration of immune cells in the airways and improves animal lung function.

What is the clinical significance

- Miltefosine may serve as a potential candidate for the treatment of eosinophil-related diseases.
- Miltefosine treatment may influence eosinophil host responses in leishmania-infected patients.

2 | METHODS

2.1 | Materials

Unless otherwise indicated, all purchased reagents were from Sigma (Vienna, Austria). **Eotaxin-2 (CCL24)** used for *in vivo* chemotaxis and recombinant human **C5a** were acquired from R&D Systems (Minneapolis, MN, USA). **Eotaxin-1 (CCL11)** and eotaxin-2 (CCL24) used *in vitro* assays were obtained from ImmunoTools (Friesoythe, Germany). Antibody against phospho-Akt (Ser 473) (Cat#9271, RRID:AB_329825) was obtained from Cell Signaling Technology (Danvers, MA, USA), while secondary goat anti-rabbit Alexa Fluor 488 IgG antibody (Cat# A-11008, RRID:AB_143165) was from Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Annexin V, propidium iodide (PI) (Cat# 556547), CD63-FITC (Cat# 561924, RRID:AB_10894192), Siglec-F-PE (Cat# 552126, RRID:AB_394341), CD3-PE Cy5 (Cat# 553065, RRID:AB_394598), CD11b-PE-Cy7 (Cat# 552850, RRID:AB_394491) and CD11c-BV421 (Cat# 562782, RRID:AB_2737789) were from BD Biosciences (Vienna, Austria). TruStain fcX CD16/32 (Cat# 101320, RRID:AB_1574975), Ly-6C-FITC (Cat# 128005, RRID:AB_1186134), Ly-6G-APC (Cat# 127613, RRID:AB_1877163), CCR3-BV421 (Cat#144517, RRID:AB_2565743) and I-A/I-E-V510 (Cat# 107635, RRID:AB_2561397) were from BioLegend (San Diego, CA, USA). Aluminium hydroxide gel used as an adjuvant was acquired from InvivoGen (Toulouse, France). CD11b-FITC mouse anti-human antibody (Cat# IMO53OU) used for measuring CD11b up-regulation was obtained from Beckman Coulter (Krefeld, Germany). Miltefosine used in *in vivo* assays was purchased from Cayman Chemical (Ann Arbor, MI, USA). All functional assays of eosinophils were performed in assay buffer (PBS with Ca^{2+} and Mg^{2+}, HEPES 10 mM, glucose 10 mM and bovine serum albumin 0.1%, pH 7.4).

Fixative solution was prepared by adding 9 ml of distilled water and 30 ml of FACS sheath fluid (BD Biosciences) to 1 ml of CellFix (BD Biosciences, Vienna, Austria) as described previously (Knuplez, Curcic, et al., 2020).

2.2 | Mice

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All animal experiments were performed in the animal facilities of the Medical University of Graz. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: BMWFW-66.010/0207-WF/V/3b/2017); it conforms to Directive 2010/63/EU and was performed in accordance with national and international guidelines. BALB/c mice (RRID:IMSR_CRL:028) were either bred in-house or obtained from Charles River. Δdbl GATA-1 and interleukin-5 (IL-5) transgenic (IL-5Tg) mice (both BALB/c background) were initially obtained from Dr Helene Rosenberg (NIH, Bethesda, MD, USA) and bred in our facilities. IL-5Tg mice were originally generated by Lindsay A. Dent. A 10-kb genomic mouse Il5 sequence under the control of the dominant control region (DCR) from human CD2 was used for the transgene. Δdbl GATA mice were originally generated by C. Yu. A high-affinity, palindromic "double" GATA protein binding site in the Gata1 promoter presumed to mediate positive Gata1 autoregulation was replaced by a floxed Pgk-neo cassette; transient Cre recombinase expression in ES cells left a single loxP site flanked by two Not1 sites. The 21-bp deleted segment comprised nucleotides −691 through −671 upstream of the last nucleotide of the first haematopoietically expressed exon of Gata1. Mice were housed in plastic sawdust floor cages at constant temperature (22°C) and a 12:12-h light–dark cycle with free access to standard laboratory chow and water; 8- to 12-week-old male and female mice were included in experiments, where there were randomly divided in three groups (negative control—vehicle; positive control—ovalbumin or eotaxin stimulated and miltefosine pretreated and ovalbumin or eotaxin stimulated group). Experiments, where bronchoalveolar lavage fluid was collected, could not be performed blinded, due to investigator treating the mice prior to fluid collection. Lung function testing was performed blinded, since Investigator 1 treated the mice and Investigator 2 independently performed lung function testing on mice in a random order. For all animal experiments, at least five mice were included in each group and at least two repeat experiments were carried out. Experiments were designed to make sample sizes relatively equal and randomized among comparison groups. Sample sizes were determined according to previous studies with similar analyses (Knuplez, Curcic, et al., 2020; Theiler et al., 2019).

2.3 | Blood sampling and eosinophil isolation

Blood sampling from healthy volunteers was approved by the Institutional Review Board of the Medical University of Graz (17-291 ex 05/06). All participants signed a written informed consent.

Polymorphonuclear leukocytes preparations were purified from citrated whole blood as previously described (Curcic et al., 2015). Firstly, platelet-rich plasma was removed by centrifugation. Next, red blood cells and platelets were removed by dextran sedimentation and polymorphonuclear leukocytes preparations were obtained by density gradient separation. Eosinophils were isolated from polymorphonuclear leukocytes by negative magnetic selection using a cocktail of biotin-conjugated antibodies against **CD2**, **CD14**, CD16, **CD19**, **CD56 (neural cell adhesion molecule 1)**, CD123 (interleukin 3 receptor, α subunit) and CD235a (glycophorin A) as well as Anti-Biotin MicroBeads from Miltenyi Biotec (Bergisch Gladbach, Germany). Eosinophil purity was determined by morphological analysis of Kimura-stained cells and was typically greater than 97%.

2.4 | Eosinophil shape change

Eosinophil shape change was determined as described previously (Luschnig-Schratl et al., 2011). Approximately 5×10^4 eosinophils per sample were suspended in assay buffer with Ca^{2+} and Mg^{2+}, preincubated with miltefosine in different concentrations (15 min, room temperature [RT]) and then stimulated in water bath (4 min, 37°C) with eotaxin-1 (CCL11). Afterwards, cells were transferred to ice and ice-cold fixative solution was added to terminate the reaction and maintain the change in cell shape until analysis. The samples were analysed on a FACS Canto II flow cytometer (Becton Dickinson, Mountain View, CA, USA), where shape change was determined as the increase in the forward scatter (FSC) property of the cell and was normalized to unstimulated vehicle control.

2.5 | CD11b (integrin, alpha M subunit (complement component 3 receptor 3 subunit) up-regulation

CD11b up-regulation on eosinophils was determined as described in detail elsewhere (Knuplez, Curcic, et al., 2020). Briefly, eosinophils were stained with anti-CD11b–FITC, pretreated either with vehicle or miltefosine (20 μM) and stimulated with eotaxin-2 (CCL24) as indicated in the figure legend. Additionally, CD11b up-regulation assay was performed in the polymorphonuclear leukocytes fraction (see the Supporting Information), where cells were pre-stained with anti-CD16-PE to distinguish between eosinophil and neutrophil polymorphonuclear leukocytes fractions. CD11b up-regulation on neutrophils (CD16 + cells) was induced with *N*-formylmethionyl-leucyl-phenylalanine (**fMLP**). Eosinophil or neutrophil CD11b expression was determined by flow cytometry as the geometric mean of the fluorescence in the FITC channel and expressed as per cent of unstimulated vehicle response.

2.6 | CD63 expression

Eosinophils were stained with anti-CD63-FITC (1:100) and preincubated with vehicle or miltefosine (20 μM) (15 min, RT). Subsequently cells were primed with cytochalasin B (5 $\mu g \cdot ml^{-1}$) and

stimulated with C5a in two different concentrations (Schratl et al., 2006). Degranulation was analysed by flow cytometry and expressed as fold increase in fluorescence over indicated vehicle mean.

2.7 | Preparation of bone marrow-derived eosinophils (BMDEs)

Mouse eosinophils were derived from bone marrow of BALB/c mice as described before (Dyer et al., 2008; Kienzl et al., 2020; Knuplez, Krier-Burris, et al., 2020). Briefly, following the lysis of erythrocytes in bone marrow, the cells were cultured in RPMI + 20% HyClone FBS (GE Healthcare; # 10309433), 1% P/S, 25-mM HEPES (Thermo Fisher; # 15630–080), 1× non-essential amino acids (Thermo Fisher; # 11140–035), 1-mM sodium pyruvate (Thermo Fisher; # 11360–039) and 50-μM β-mercaptoethanol (Sigma-Aldrich; M3148) supplemented with 100-ng·ml^{-1} stem cell factor (PreproTech; # 250–03) and 100-ng·ml^{-1} FLT3L (PreproTech; # 250–31 L). On Day 4, medium was changed to media supplemented with 10-ng·ml^{-1} IL-5 (Bio-Techne; # 405-MI-005) only, to differentiate progenitors into eosinophils. On Day 14, purity and viability of bone marrow-derived eosinophils (BMDEs) was assessed by flow cytometry staining for mouse eosinophil markers CCR3 (CCR3-BV421) and Siglec-F (Siglec-F-PE), and PI respectively. Cytospins of BMDEs were prepared, stained with a Hemacolor Rapid staining of blood smear and imaged on an Olympus BX41 microscope (Olympus, Vienna, Austria). Day 14 BMDEs were used for further *in vitro* analyses.

2.8 | Calcium flux

Isolated human or differentiated mouse eosinophils were loaded with 2 μM of Fluo-3 AM in the presence of 0.02% pluronic F-127 for 1 h at RT in the dark. Individual samples were treated as indicated in the figure legend. Changes in $[Ca^{2+}]_I$ were detected as fluorescence in the FL1 channel by a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA), as described previously (Heinemann et al., 2003; Knuplez, Curcic, et al., 2020).

2.9 | *In vitro* chemotaxis

Purified eosinophils were pretreated with either vehicle or miltefosine in different concentrations (15 min, RT) and were allowed to migrate to 10-nM eotaxin-2 (CCL24) in an HTS Transwell 96-well plate with a 5-μm pore size polycarbonate membrane (1 h, 37°C). Eosinophils that have migrated to the lower compartment were enumerated for 1 min by flow cytometric counting on a FACS Canto II (Becton Dickinson, Mountain View, CA, USA) (Knuplez, Curcic, et al., 2020).

2.10 | Flow cytometric analysis of intracellular kinase phosphorylation

Isolated eosinophils were pretreated with either vehicle or miltefosine 20 (μM) (15 min, RT). Following the pretreatment, cells were incubated with 10-nM eotaxin-1 (CCL11) (3 min, 37°C). Subsequently, cells were fixed, permeabilized and stained as described previously (Knuplez, Curcic, et al., 2020). Phosphorylation of Akt residues in fixed eosinophils was quantified as the increase of fluorescence in the FITC fluorescence channel from unstimulated control.

2.11 | Apoptosis

Eosinophil survival after preincubation with vehicle, positive control formaldehyde (3.8%) or miltefosine (5–40 μM) for different time points at 37°C was assessed by annexin V/PI staining, as described previously (Heinemann et al., 2005; Knuplez, Curcic, et al., 2020).

2.12 | *In vivo* chemotaxis

In vivo eosinophil migration was induced by intranasal application of 4-μg eotaxin-2 CCL24 in 8-week-old male and female heterozygous IL-5 transgenic (IL-5Tg) mice (BALB/c background). The mice and their littermate controls received oral gavages of miltefosine (20 mg·kg^{-1} in 0.9% NaCl) or vehicle for three consecutive days before CCL24 application. Bronchoalveolar lavage fluid was collected 4 h after experiment had started. Migration of eosinophils was evaluated by flow cytometric counting of highly granular (high side scatter) CD11c$^-$/Siglec-F$^+$ cells, as described previously (Knuplez, Curcic, et al., 2020). The gating strategy for evaluation of other immune cells was previously published (Knuplez, Curcic, et al., 2020) and was as follows: alveolar macrophages (Siglec-F$^+$/CD11c$^+$), neutrophils (Siglec-F$^-$/CD11b$^+$/Ly6G$^+$), B cells (CD11c$^-$/CD11b$^-$/MHCII$^+$), T cells (CD11c$^-$/CD11b$^-$/CD3$^+$), dendritic cells (Siglec-F$^-$/Ly6G$^-$/Ly6C$^-$/MHCII$^+$) and inflammatory monocytes (Siglec-F$^-$/Ly6G$^-$/MHCII$^-$/LY6C^{++}).

2.13 | Mouse model of allergic lung inflammation

Eight-week-old male and female BALB/c or eosinophil-deficient (ΔdblGATA-1) mice were immunized by intraperitoneal injections of 20 μg of ovalbumin adsorbed to Al $(OH)_3$ on Days 0 and 7. Mice were challenged by an aerosol of ovalbumin (1 mg·ml^{-1} in 0.9% NaCl) on Days 14 and 16. During the last 10 days of the model, mice received daily oral gavages of miltefosine (20 mg·kg^{-1} in 0.9% NaCl) or vehicle. On Day 17, either airway hyperresponsiveness to methacholine was recorded with the FlexiVent system (SCIREQ, Montreal, QC, Canada) or bronchoalveolar lavage fluid was taken and analysed by flow cytometry. Bronchoalveolar lavage fluid supernatants were collected and stored at −70°C for further cytokine assessment. All animal subjects were randomized prior to inclusion in the experiments.

2.14 | Cytokine measurements in bronchoalveolar lavage fluid

Cytokine concentrations in stored bronchoalveolar lavage fluid supernatants from BALB/c and ΔdblGATA-1 mice subjected to ovalbumin/aluminium hydroxide were evaluated using the custom ProcartaPlex™ immunoassay (eBioscience, San Diego, CA, USA) according to the manufacturer's specifications. Fluorescent signals were quantified with the Bio-Plex 200 multiplex suspension array system equipped with Luminex® xMAP® technology combined with the Bio-Plex 5.0 software (Bio-Rad, Hercules, CA, USA). All cytokine concentrations were evaluated in duplicates.

2.15 | Corticosterone measurement in plasma

Corticosterone levels were assessed in plasma of BALB/c mice treated with oral gavages of miltefosine (20 $mg \cdot kg^{-1}$) once daily for 3 days. A blood sample was collected via cheek bleed 5 h after first miltefosine application on Day 1, as well as 4 h after last treatment on Day 3. Corticosterone levels were determined with a specific enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) with a sensitivity of 0.027 $ng \cdot ml^{-1}$ as previously described (Farzi et al., 2015) and according to the manufacturer's specifications.. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.16 | Statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Statistical analysis was performed using the GraphPad Prism™ 6 software (GraphPad Software, Inc., CA, USA). Data were normalized to baseline (1 or 100%) of the means of negative control in experiments performed with eosinophils isolated from human donors to reduce interindividual source of variation.

Statistical analysis was only performed for groups where $n \geq 5$. Additional preliminary data ($n = 3$) on p-Akt phosphorylation in eosinophils were included in the manuscript to suggest a mechanism previously shown for other cell types (Chugh et al., 2008; Ruiter et al., 2003). The group size given for each experiment is the number of independent values (individual human eosinophil donors or mice). Statistical analysis was performed using these independent values.

Data were tested for normality using D'Agostino and Pearson omnibus normality test. If normality was assumed, comparisons among multiple groups were performed with one-way ANOVA or two-way ANOVA. For these analyses, post hoc pairwise comparisons were performed using Bonferroni's multiple comparison test (or Dunnett's multiple comparison test, when comparing samples to the control group), only if a main effect for at least one factor or the interaction between two factors showed statistical significance and if there was no significant variance in homogeneity. Cytokine levels were compared using Mann–Whitney *U* test. Significance level for the analyses was set to $\alpha = 0.05$ and significant differences are indicated with the corresponding *P* value, $^*P \leq 0.05$.

2.17 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Miltefosine suppresses eosinophil activation *in vitro*

First, we tested the viability of eosinophils after pretreatment with different concentrations of miltefosine. Importantly, miltefosine (up to 20 μM, in the presence of 1-$mg \cdot ml^{-1}$ bovine serum albumin) showed no toxic effects on eosinophils (Figure S1).

During the state of allergic inflammation, elevated concentrations of cytokines and chemoattractants in the blood activate eosinophils, which leads to a rearrangement of their actin filaments (the so-called "shape change") (Willetts et al., 2014) and results in an up-regulation of the adhesion molecules integrins (e.g., CD11b/CD18 and Mac-1) on the cell surface (Jia et al., 1999). When human eosinophils were pretreated with miltefosine, we could observe a statistically significant inhibition of their shape change (by approx. 50%) induced by CCL11 stimulation (Figure 1a,b) when using the highest concentration of miltefosine (20 μM). Miltefosine addition did not alter eosinophil shape change in the absence of external stimuli (Figure S2). When isolated eosinophils were pretreated with 20-μM miltefosine, up-regulation of CD11b was reduced by about 50% (Figure 1c,d). Similarly, miltefosine suppressed CD11b expression of CCL11 activated eosinophils ($CD16^-$ cells) in the polymorphonuclear leukocytes fraction (Figure S3A). Notably, miltefosine did not alter CD11b expression of fMLP-stimulated neutrophils ($CD16^+$ cells) (Figure S3B).

To determine whether miltefosine has an effect on the chemotaxis of human eosinophils, we performed *in vitro* chemotaxis assays using eotaxin-2 (CCL24) as chemoattractant. Miltefosine significantly inhibited eosinophilic chemotaxis in a dose-dependent manner (Figure 2a,b).

We next assessed whether miltefosine affects degranulation-associated processes in eosinophils. For that purpose, eosinophils were pretreated with miltefosine and subsequently stimulated with recombinant C5a, a potent inducer of degranulation. Miltefosine effectively suppressed C5a (0.5 nM)-induced CD63 expression, a marker of eosinophilic degranulation (Carmo et al., 2016) (Figure 2c,d).

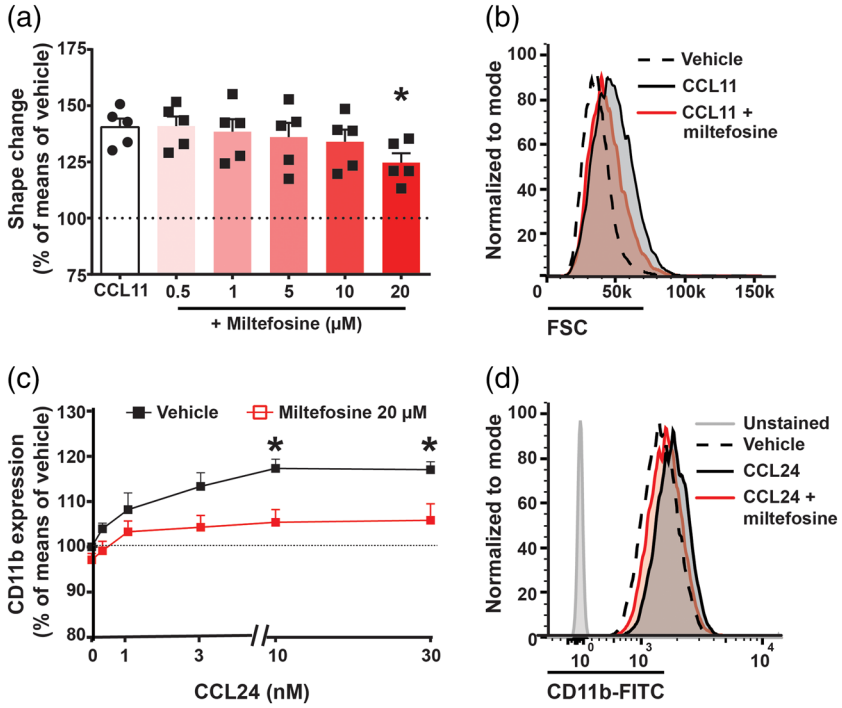

FIGURE 1 Miltefosine concentration-dependently inhibits eosinophil activation. (a, b) Eosinophils were pretreated with miltefosine in different concentrations (0.5–20 μM) (15 min, room temperature [RT]) and stimulated with 10-nM CCL11 (4 min, 37°C). Cells were fixed and the change in cell size (forward scatter [FSC]) was evaluated by flow cytometry. (a) Eosinophil shape change is expressed as per cent of unstimulated vehicle response. Data are shown as mean + SEM from five individual experiments. *P < 0.05 versus CCL11-stimulated vehicle (one-way ANOVA with Dunnett's post hoc test). (b) Representative histogram of eosinophil FSC with miltefosine (20 μM) pretreatment and CCL11 stimulation (10 nM). (c, d) Eosinophils were stained with anti-CD11b and treated with either miltefosine (20 μM) or vehicle control (15 min, RT). Subsequently, cells were stimulated with CCL24 (4 min, 37°C) and analysed by flow cytometry. Eosinophil CD11b expression is expressed as per cent of unstimulated vehicle response. Data are shown as mean + SEM from five individual experiments. *P < 0.05 versus vehicle (two-way ANOVA with Bonferroni post hoc test). (d) Representative histogram of CD11b up-regulation with miltefosine (20 μM) pretreatment and CCL24 (10 nM) stimulation

CD63 expression induced with very high concentrations of C5a (100 nM) was not affected by miltefosine (Figure 2e,f).

Previous studies have shown that miltefosine inhibits PI3K/Akt kinase signalling with an IC_{50} in the range of 5 to 35 μM, depending on the cell line tested (Kaleağasıoğlu et al., 2018; Rybczynska et al., 2001). Our preliminary results show a tendency of miltefosine (20 μM) inhibiting Akt phosphorylation (Figure 3a,b). Moreover, we could demonstrate that intracellular calcium flux in CCL11-stimulated eosinophils was inhibited by about 50% (Figure 3c,d) after only 1 min of miltefosine addition (20 μM).

3.2 | Miltefosine ameliorates ovalbumin-induced lung inflammation

Next, we investigated whether the *in vitro* results obtained with isolated human eosinophils are also relevant *in vivo*. We first performed Ca^{2+} flux assays using mouse bone marrow-derived eosinophils to test whether mouse eosinophils behave similar to human-isolated eosinophils (Figure 4a,b). For that purpose, eosinophils were differentiated from bone marrow cells of BALB/c mice following an established protocol (Kienzl et al., 2020), which yields a pure population of cultured eosinophils as determined by a single population positive for mouse eosinophil markers CCR3 and Siglec-F (Figure S4A). Microscopic analysis of cytospins of BMDEs shows a uniform population of cells exhibiting typical eosinophil staining and granule morphology (Figure S4B). These mature bone marrow eosinophils were used to perform Ca^{2+} flux assays under similar experimental conditions as isolated human eosinophils (Figure 4a,b). Our data show the level (approximately 50%) and kinetic of Ca^{2+} flux inhibition in mouse eosinophils resembling that of human eosinophils pretreated with miltefosine and stimulated with CCL24 (Figure 4a,b).

Next, we performed an *in vivo* eosinophilic migration test using IL-5Tg mice. This strain of mice is characterized by eosinophilia due to increased production of IL-5. Together, intranasal eotaxin application in IL-5-primed eosinophils results in abundant and eosinophil accumulation in the bronchoalveolar lavage fluid and lungs of animals (Ochkur et al., 2007). We treated IL-5Tg mice for three consecutive days perorally with miltefosine (20 $mg \cdot kg^{-1}$) (Figure 4c). We used a dosing regimen comparable with other studies in mice testing miltefosine (Bäumer et al., 2010; Dorlo et al., 2012). Remarkably, miltefosine

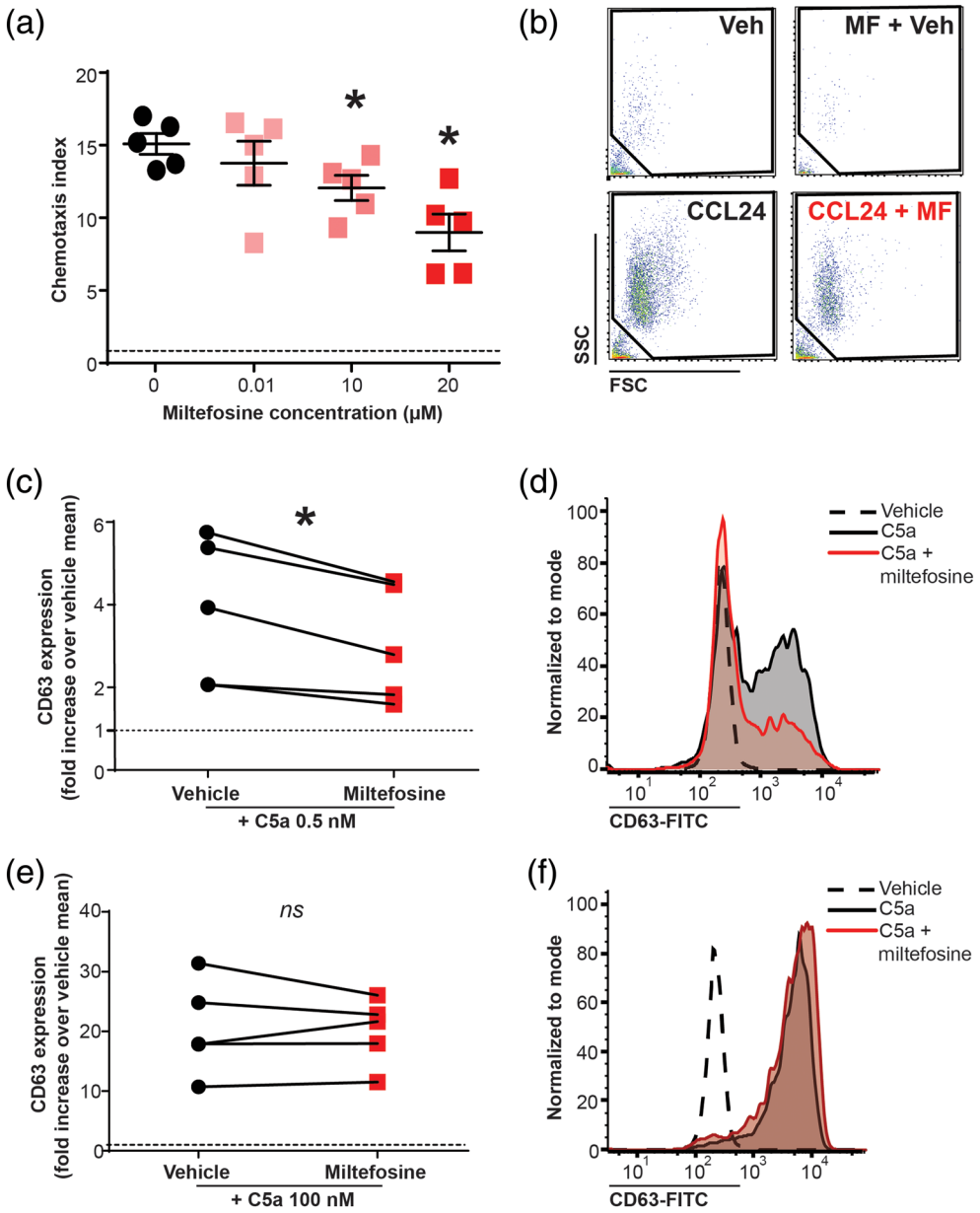

FIGURE 2 Miltefosine inhibits chemotaxis and CD63 expression of eosinophils. (a, b) Purified human eosinophils were treated with miltefosine in different concentrations (15 min, room temperature [RT]) or vehicle and allowed to migrate towards CCL24 (10 nM) in transwell plates (a). Migrated cells were enumerated by flow cytometry for 1 min. Results are presented as chemotactic index (migrated cell number/cell number migrated to unstimulated vehicle) and shown as mean ± SEM from five individual experiments. *$P < 0.05$ versus vehicle (one-way ANOVA with Dunnett's post hoc test). (b) Representative scatter blots of cells migrating towards vehicle/miltefosine (20 μM) in the bottom compartment or cells pretreated with vehicle or miltefosine (20 μM) migrating towards CCL24. (c–f) Eosinophils were stained with anti-CD63-FITC followed by incubation with miltefosine (20 μM) or vehicle (15 min, RT). Subsequently, cells were primed with cytochalasin B and stimulated with complement component 5a (C5a) 0.5 nM (c, d) or 100 nM (e, f) for 20 min at 37°C. CD63 expression is expressed as fold increase of fluorescence intensity over unstimulated vehicle for individual donors. *$P < 0.05$, miltefosine versus vehicle treated (paired Student's *t* test). CCL24 + MF, CCL24 + miltefosine; MF + Veh, miltefosine + vehicle; Veh, vehicle

significantly suppressed the migration of eosinophils to intranasal CCL24 into the bronchoalveolar lavage of animals (Figure 4d). A trend towards reduced infiltration of immune cells was observed for all detected cell types (Figure 4d). Analysis of the blood immune cell composition revealed an increase in the percentage of neutrophils in blood of IL-5Tg mice treated with miltefosine (Figure S5A); however, when BALB/c mice were treated with miltefosine, no increase in neutrophils was observed (Figure S5B). By testing plasma of BALB/c mice for their corticosterone levels, we observed no significant differences at both of the two tested time points (Figure S6A,B).

We next tested the efficacy of miltefosine in an acute model of allergic lung inflammation. Ovalbumin was used as a model allergen to

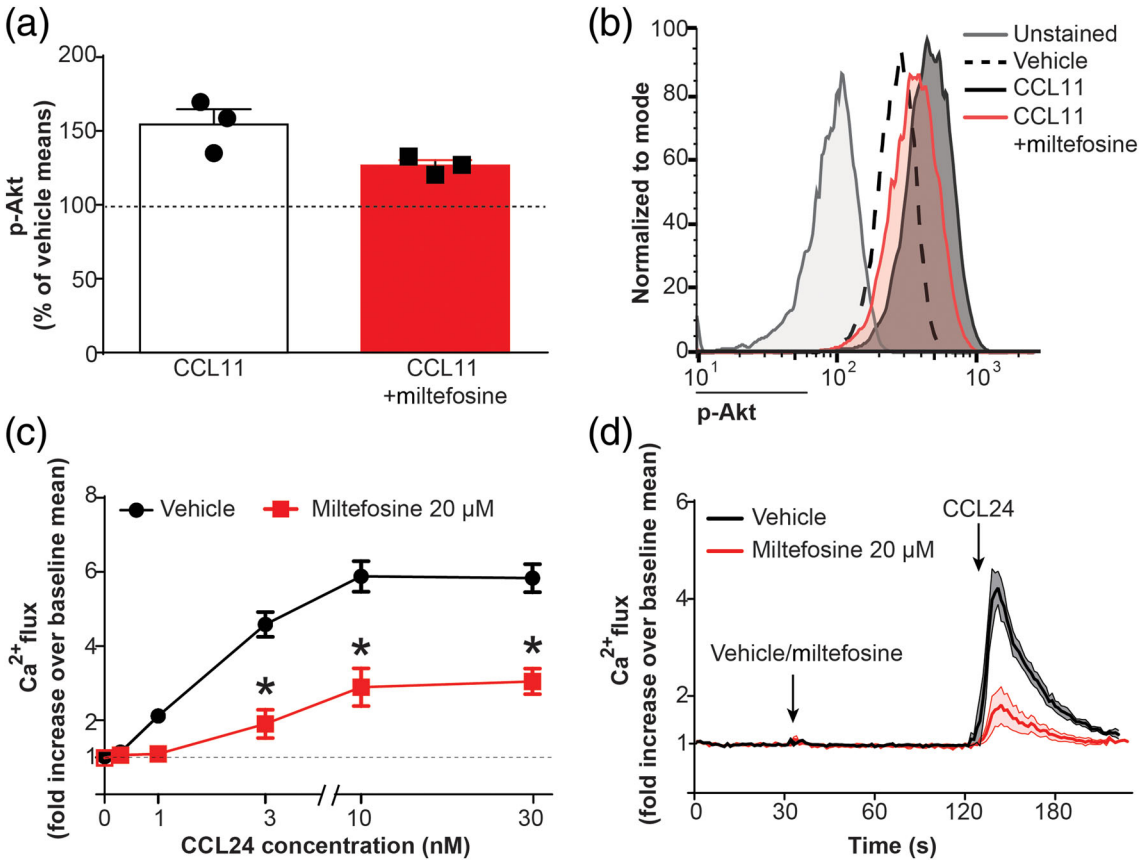

FIGURE 3 Miltefosine inhibits Akt phosphorylation and Ca^{2+} flux in human eosinophils. (a, b) Eosinophils were pretreated with miltefosine (20 µM) or vehicle (15 min, room temperature [RT]) followed by addition of CCL11 (3 min, 37°C). Subsequently, cells were fixed, permeabilized and stained. (a) Phosphorylation of Akt residues was quantified as the increase of fluorescence in the FITC fluorescence channel and expressed as per cent of unstimulated vehicle control. Data are shown as mean + SEM from three individual experiments. (b) Representative histogram of p-Akt staining following miltefosine pretreatment. (c, d) Eosinophils were labelled with Fluo-3 AM and changes in $[Ca^{2+}]_i$ were detected by flow cytometry. Eosinophils were stimulated with increasing concentrations of CCL24 (0–30 nM) in the presence or absence of miltefosine (20 µM). (c) Results represent fold increase in $[Ca^{2+}]_i$ over unstimulated vehicle. Data are shown as mean ± SEM from five individual experiments $^*P < 0.05$ miltefosine (20 µM) versus vehicle (two-way ANOVA with Bonferroni post hoc test). (d) Time course of Ca^{2+} flux in eosinophils. Following baseline measurement (30 s), miltefosine (20 µM) or vehicle was added. After 1 min, CCL24 (10 nM) was added to induce Ca^{2+} flux. Data are shown as mean ± SEM from five individual experiments

reproduce key features of clinical asthma, such as airway hyperresponsiveness to methacholine (Kumar et al., 2008). The treatment protocol of the model is shown in Figure 5a. We observed that daily peroral treatment with miltefosine markedly reduced the number of several infiltrating immune cells into airways of ovalbumin-challenged wild-type mice. Flow cytometric analysis of the composition of immune cells showed that the number of eosinophils as well as infiltrating T cells, B cells and dendritic cells was reduced by 50% upon miltefosine treatment (Figure 5b). Of note, mice treated with miltefosine showed significantly improved lung resistance and a trend towards improved lung compliance (Figure 5c). In order to test whether a decrease in eosinophil numbers was responsible for the reduction of other immune cells, eosinophil-deficient (Δdbl GATA-1) mice were exposed to the same ovalbumin-induced allergic model. In this mouse strain, treatment with miltefosine only had an impact on the number of dendritic cells (Figure 6), while other subgroups of immune cells were not affected.

Supernatants of bronchoalveolar lavage fluid of ovalbumin stimulated (vehicle) and miltefosine-treated and ovalbumin-stimulated BALB/c and Δdbl GATA-1 mice were further analysed for their cytokine content (Figures S7 and S8). We could observe significantly reduced levels of immunomodulatory cytokine IFN-γ in the bronchoalveolar lavage fluid of miltefosine-treated BALB/c mice (Figure S7A), while no such inhibition was observed in Δdbl GATA-1 mice (Figure S8A). Cytokine content of CCL11, TNF-α, IL-2 and IL-5 in bronchoalveolar lavage fluid was not significantly altered by miltefosine treatment in either BALB/c or Δdbl GATA-1 mice (Figures S7 and S8).

4 | DISCUSSION

In the present study, we show for the first time that the Food and Drug Administration (FDA)-approved drug miltefosine inhibits the

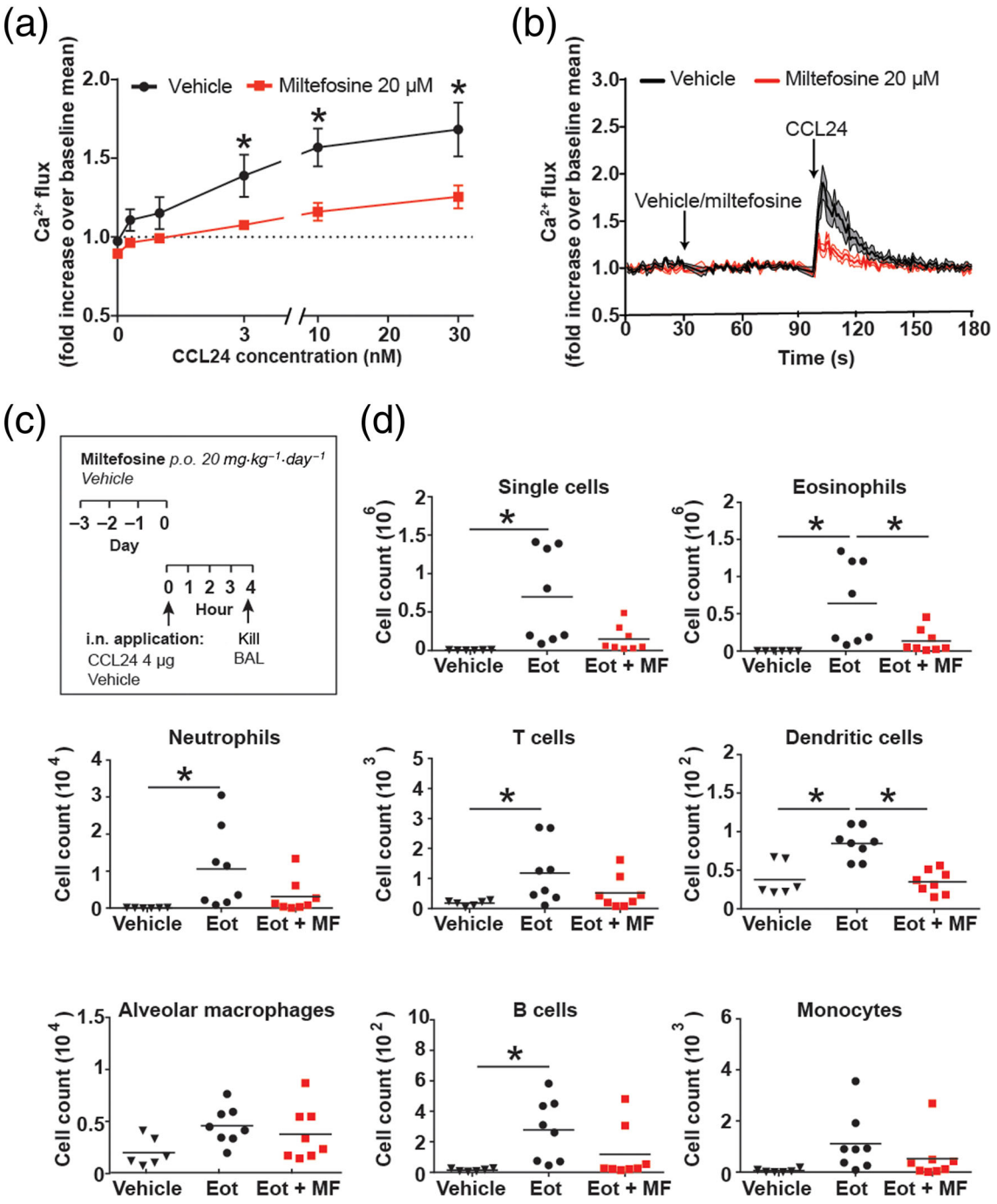

FIGURE 4 Miltefosine inhibits Ca^{2+} flux in mouse eosinophils and suppresses migration of eosinophils in vivo. (a, b) Bone marrow-derived mouse eosinophils were labelled with Fluo-3 AM and changes in $[Ca^{2+}]_i$ were detected by flow cytometry. Eosinophils were stimulated with increasing concentrations of CCL24 (0–30 nM) in the presence or absence of miltefosine (20 µM). (a) Results represent fold increase in $[Ca^{2+}]_i$ over unstimulated vehicle. Data are shown as mean ± SEM from five individual experiments $^*P < 0.05$ miltefosine (20 µM) versus vehicle (two-way ANOVA with Bonferroni post hoc test). (b) Time course of Ca^{2+} flux in eosinophils. Following baseline measurement (30 s), miltefosine (20 µM) or vehicle was added. After 1 min, CCL24 (10 nM) was added to induce Ca^{2+} flux. Data are shown as mean ± SEM from five individual experiments. (c, d) Eight-week-old IL-5Tg mice received either miltefosine (20 $mg \cdot kg^{-1}$) or vehicle (0.9% NaCl) per os for three consecutive days, followed by intranasal application of CCL24 (4 µg) or vehicle. After 4 h, mice were killed and bronchoalveolar lavage (BAL) fluid was collected. (b) Immune cell composition in BAL fluid was analysed by flow cytometry. Data are shown from six to eight mice from three individual experiments. $^*P < 0.05$ versus eotaxin (CCL24) (Eot) group (one-way ANOVA with Dunnett's post hoc test). Eot + MF, eotaxin (CCL24) + miltefosine

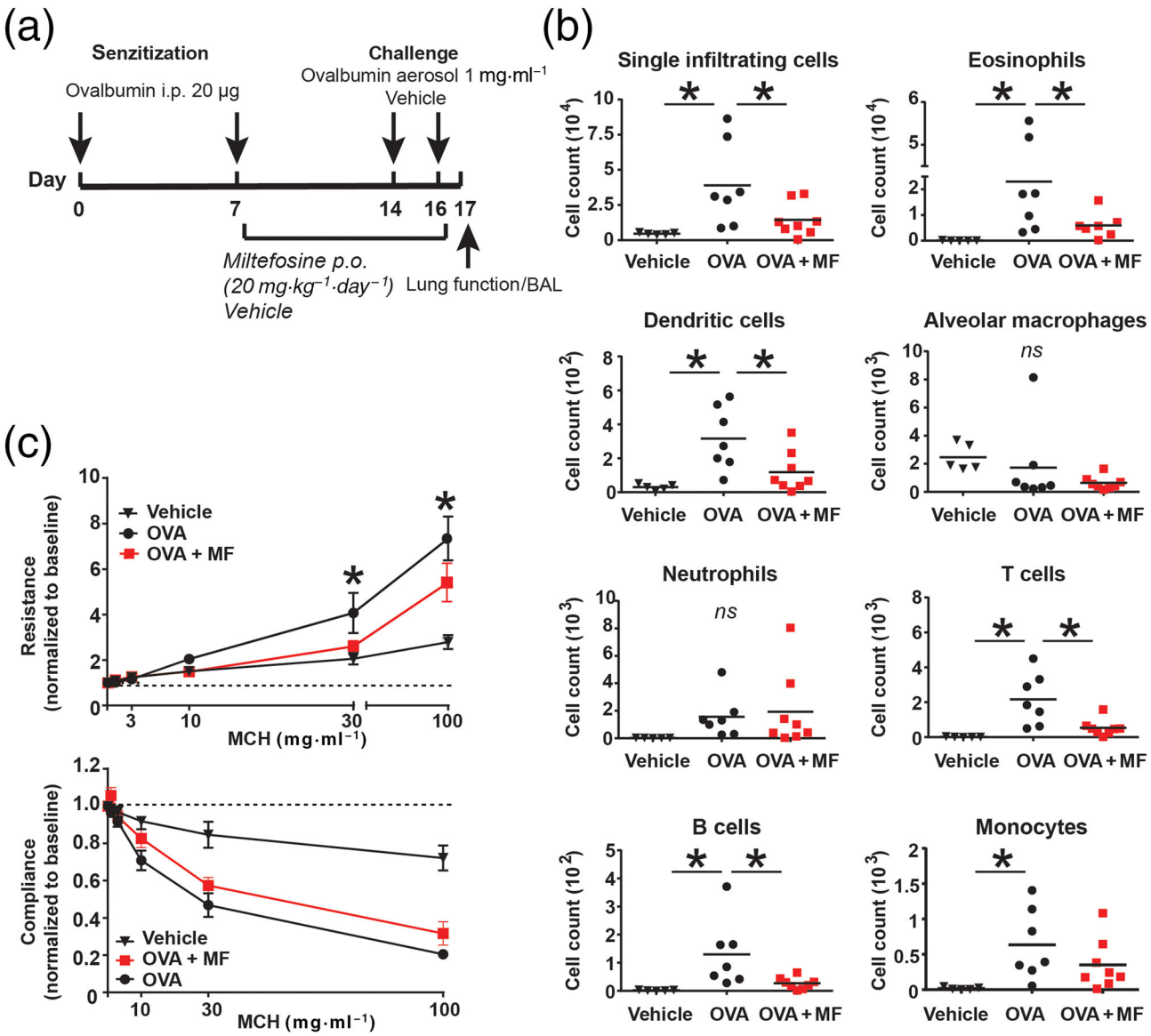

FIGURE 5 Miltefosine ameliorates ovalbumin-induced allergic lung inflammation. (a) Eight-week-old BALB/c mice were sensitized with ovalbumin intraperitoneal at Day 0 and at Day 7 and were subsequently treated with miltefosine (20 mg·kg^{-1}) or vehicle per os daily from Day 7 to Day 17. Afterwards, mice were challenged with an ovalbumin aerosol on Day 14 and Day 16 followed by lung function testing or sampling of bronchoalveolar lavage (BAL) fluid. (b) Immune cell composition in the BAL fluid was analysed by flow cytometry. Representative results from two individual experiments are shown. *P < 0.05 versus OVA group (one-way ANOVA with Dunnett's post hoc test). (c) Lung function of mice was assessed while applying increasing doses of methacholine (0–100 mg·ml^{-1}). Data are shown as mean ± SEM from two individual experiments performed with 5–8 mice. *P < 0.05, ovalbumin (OVA) + miltefosine (MF) versus OVA group (two-way ANOVA with Dunnett's post hoc test). MCH, methacholine

activation of human eosinophils. A short pretreatment with miltefosine suppressed human eosinophilic effector responses after stimulation with various agonists *in vitro*. We were able to transfer our *in vitro* findings to preclinically relevant endpoints in an *in vivo* model of eosinophilic migration and allergic inflammation. Most importantly, in a model of ovalbumin-induced allergic lung inflammation, peroral administration of miltefosine significantly reduced the infiltration of immune cells into the lung while improving lung function parameters.

The effects of miltefosine have previously been studied on some other immune cells. Notably, miltefosine was found to inhibit degranulation and antigen-induced chemotaxis of mast cells by modulating lipid rafts and by inhibiting cytosolic PKC (Rubíková et al., 2018). In contrast to our findings with eosinophils, calcium flux in mast cells was apparently not affected by miltefosine pretreatment, indicating cell type-specific differences. However, similar to mast cells, miltefosine led to an inhibition of effector functions and mediator release in eosinophils. In macrophages, miltefosine was found to

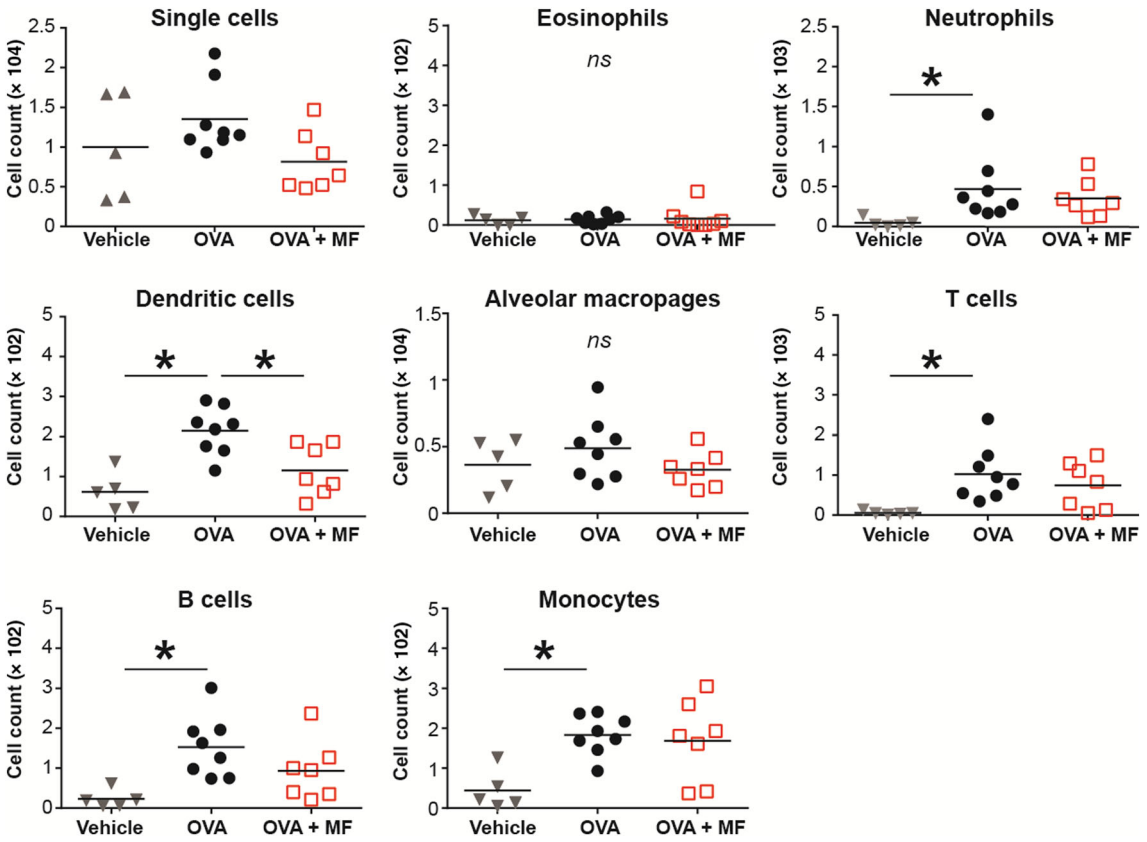

FIGURE 6 The effect of miltefosine treatment in eosinophil-deficient mice. Eight-week-old Δdbl GATA-1 mice were sensitized with ovalbumin intraperitoneal at Day 0 and at Day 7 and subsequently treated with miltefosine (20 mg·kg^{-1}) or vehicle per os daily from Day 7 to Day 17. Afterwards, mice were challenged with an ovalbumin aerosol on Day 14 and Day 16 followed by sampling of bronchoalveolar lavage (BAL) fluid. Immune cell composition in the BAL fluid was analysed by flow cytometry. Results from two individual experiments with 5–7 mice are shown. *$P < 0.05$ versus ovalbumin (OVA) group (one-way ANOVA with Dunnett's post hoc test). OVA + MF, ovalbumin + miltefosine

increase cholesterol release and phosphorylation of kinases associated with autophagy. Importantly, miltefosine decreased **toll-like receptor 4 (TLR-4)** recruitment to the cell surface of macrophages and dampened **IL-1β** release following stimulation with **lipopolysaccaride (LPS)** (Iacano et al., 2019). Given the fact that TLR-4 stimulation on eosinophils can help polarize macrophages towards pro- or anti-inflammatory phenotypes (Yoon et al., 2019), this finding further supports the evidence that miltefosine may influence the interplay and balance between various immune cell types during the state of inflammation.

It is noteworthy that in all our *in vitro* experiments, non-toxic concentrations of miltefosine were used to distinguish our results from the non-specific cytolytic effects of the drug. In particular, since homeostatic functions such as tissue remodelling and plasma cell survival (Jacobsen et al., 2012) have recently been attributed to eosinophils, we were mainly interested in inhibiting eosinophil overactivation, to prevent their potential tissue-damaging effector functions. For our *in vivo* experiments, we used a dosage regimen, comparable with other studies in mice testing miltefosine (Bäumer et al., 2010).

Results from our *in vivo* experiments show significantly decreased numbers of infiltrating eosinophils in miltefosine-treated animals compared with vehicle-challenged controls. The data correspond to our *in vitro* experiments in which miltefosine inhibited the activation, migration and up-regulation of adhesion molecules on eosinophils. Interestingly, we additionally discovered a trend towards decreased numbers of other infiltrating immune cells in miltefosine-treated animals, while the numbers of tissue resident alveolar macrophages remained the same across all treatment groups. In order to confirm whether infiltration of other immune cells is directly affected by miltefosine, we performed control experiments in eosinophil-deficient Δdbl GATA-1 mice. We discovered that the decreased infiltration of most immune cells was at least partially due to the decreased eosinophil numbers. This is not unexpected, since activated eosinophils are known to attract and activate other immune cell types such as neutrophils (Yousefi et al., 1995) or B cells (Chu et al., 2011). Moreover, eosinophil-derived **CCL17** and **CCL22** have proven to be crucial in attracting effector T cells in localized allergic inflammation (Jacobsen et al., 2008). Interestingly, we observed a decrease in dendritic cell ($CD11c^+/MHCII^+/Siglec\text{-}F^-/Ly6G^-/Ly6C^-$) numbers following miltefosine treatment compared with challenged controls both in wild-type and in eosinophil-deficient mice. It has been reported previously that combination therapy of paromomomycin/miltefosine can influence **TLR9** on dendritic cells and therefore modulate Th1 host

immune responses in leishmaniasis therapy (Das et al., 2014). As of yet, however, the direct effect of miltefosine on human dendritic cells remains unclear.

To assess whether the observed differences in immune cell count are a consequence of miltefosine directly inhibiting immune cell infiltration or rather indirectly altering the cytokine milieu in the lung, we additionally tested bronchoalveolar lavage supernatants from both BALB/c and ΔdblGATA-1 mice for cytokine expression. Cytokine concentrations of CCL11, **TNF-α**, IL-2 and IL-5 were not altered in miltefosine-treated and ovalbumin-stimulated mice of both genotypes. Interestingly however, we observed significantly reduced levels of the immunomodulatory cytokine **IFN-γ** in the miltefosine-treated group of BALB/c mice. These data corroborate previous findings from Verhaar et al. (2013), where they observed similarly reduced levels of IFN-γ in miltefosine-treated animals in a mouse model of inflammatory bowel disease. IFN-γ has on one hand long been considered to be beneficial in allergic inflammation as reviewed by Teixeira et al. (2005), while on the other hand, recent studies recognize its pro-inflammatory functions. Our findings of reduced IFN-γ expression in BALB/c mice and not in ΔdblGATA-1 mice are of particular interest, since it was discovered that eosinophil-derived IFN-γ induces airway hyperresponsiveness and lung inflammation even in the absence of lymphocytes (Kanda et al., 2009). Interestingly, IFN-γ was also found to up-regulate several eosinophil effector functions (Ishihara et al., 1997; Takaku et al., 2011) and promote their survival (Fujisawa et al., 1994).

When we examined the composition of immune cells in mouse blood, miltefosine-treated and CCL24-stimulated IL-5Tg animals showed an increased neutrophil count, yet miltefosine-treated BALB/c animals showed no altered neutrophil numbers at baseline. A previous study showed that patients treated with miltefosine exhibited increased levels of the neutrophilic chemokine **IL-8 (CXCL8)** (Mukhopadhyay et al., 2011. This finding remains to be confirmed in mice. Increased corticosterone levels in mice induced by miltefosine could be another plausible explanation for both increased neutrophil numbers (Liles et al., 1995) and decreased airway inflammation (Suqin et al., 2009). Furthermore, an inverse association between endogenous glucocorticoid and IFN-γ levels was observed in allergic lung inflammation (Suqin et al., 2009). Nonetheless, we observed no significant alterations in corticosterone levels in miltefosine-treated mice.

Fang et al. additionally showed that miltefosine acts on endothelial cells by down-regulating E-selectin, which is important for leukocyte adhesion and infiltration (Leung et al., 2007). Therefore, in our ovalbumin model of allergic inflammation, we cannot neglect additional anti-inflammatory effects of miltefosine.In another point, our work raises important questions regarding the immunomodulatory effect of miltefosine in patients treated for leishmania infections. So far, little has been reported about the drug's effect on the host responses responsible for fighting the infection. However, some *in vitro* findings report a strong reversal of Th2 responses of leishmania-infected macrophages towards Th1 type following miltefosine treatment (Wadhone et al., 2009). Since eosinophils are one of the primary cells recruited to the sites of leishmania infection (de Oliveira Cardoso et al., 2010) and have been shown to help control parasite load (Watanabe et al., 2004) in mice, it might be of interest to further investigate this issue in patients treated with miltefosine. In line with the present study, we have previously shown that saturated lysophosphatidylcholines, which are structurally similar to miltefosine, inhibit eosinophil effector responses (Knuplez, Curcic, et al., 2020; Knuplez, Krier-Burris, et al., 2020; Trieb et al., 2019).

A limitation of our work needs to be noted. Ovalbumin was used as a model allergen in our in vivo studies, albeit this model fails to completely reflect the aetiology of human asthma and its multi-step developmental process, including environmental factors associated with the disease. Further experiments with other physiological relevant antigens are needed to validate the relevance of our data in human disease setting.

In summary, we demonstrate the inhibitory effect of the orphan drug miltefosine on human eosinophils and its anti-inflammatory effect *in vivo* in a model of allergic inflammation. Our data highlight the potential efficacy of miltefosine or related molecules in the treatment of allergic diseases and other eosinophil-associated disorders.

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AUTHOR CONTRIBUTIONS

E.K. designed and performed the experiments, analysed the data, interpreted the results and wrote the manuscript. A.T. and M.K. performed the experiments, analysed the data and edited the manuscript. E.M.S., A.H. and R.S. interpreted the results and edited the manuscript. G.M. designed and supervised the study, interpreted the results and wrote and edited the manuscript.

CONFLICT OF INTEREST

A.H. received consultancy fees from AstraZeneca. The other authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

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

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HIGHLIGHTED ARTICLE

Frontline Science: Superior mouse eosinophil depletion in vivo targeting transgenic Siglec-8 instead of endogenous Siglec-F: Mechanisms and pitfalls

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Abstract

Eosinophils are important multifunctional granulocytes. When studying eosinophil function and its contribution to diseases, mouse models are often used. Mouse eosinophils selectively express sialic acid-binding immunoglobulin-like lectin (Siglec)-F. Its closest functional paralog on human eosinophils is Siglec-8. These Siglecs are being used to target eosinophils when exploring their mechanistic roles in disease and for potential therapeutic benefit. In order to facilitate preclinical studies of human Siglec-8, we developed transgenic mouse strains expressing human Siglec-8 only on the surface of eosinophils with or without endogenous Siglec-F and have begun characterizing various cellular functions in vitro and in vivo. Eosinophils from Siglec-8+ mice, with or without Siglec-F, responded to Siglec-8 antibody engagement in vitro by up-regulating surface CD11b, whereas Siglec-F antibody had no such effect. Engagement of Siglec-F or Siglec-8 with respective antibodies in vitro resulted in only modest increases in cell death. Administration of rat Siglec-F antibodies to mice led to a significant decrease in Siglec-F surface expression on eosinophils due to internalization, and thus appeared to decrease eosinophil numbers based on Siglec-F+ cells, but with proper gaiting strategies did not in fact result in significant eosinophil depletion. In marked contrast, administration of mouse Siglec-8 antibodies rapidly and effectively depleted eosinophils from blood and spleens of mice, but an $F(ab')_2$ version did not, indicating an Fc-mediated mechanism for eosinophil depletion in vivo. Siglec-8 expressing mice with or without endogenous Siglec-F will be useful to study Siglec-8-based therapeutics, and may be a preferred approach when acute or chronic eosinophil depletion is needed.

KEYWORDS

antibody-dependent cellular cytotoxicity, depletion, eosinophils, Siglec-8, Siglec-F

1 | INTRODUCTION

Eosinophils are innate immune granulocytes that contribute to a range of host defense, homeostatic, and disease-related responses.[1,2] Much of our knowledge of the biology of this cell comes from preclinical mouse models that employ mice congenitally or conditionally deficient in eosinophils.[3–6] Other strategies, such as those involving mouse, rat, or sheep antibody-based depletion by targeting sialic acid-binding immunoglobulin-like lectin (Siglec)-F, have also been used, with variable but consistently incomplete blood and tissue depletion.[7–11] In particular, administration of several commercial rat anti-mouse Siglec-F antibodies, or liposomes that selectively engage Siglec-F, also do a suboptimal job of depleting eosinophils.[12–16] Furthermore, Siglec-F is expressed on other cells including alveolar macrophages and intestinal cells[17,18] and, therefore, effects seen with these targeting strategies may not be eosinophil specific. Although ligation of its closest human paralog, Siglec-8,[7] on human eosinophils induces profound cell death in vitro, especially in cytokine primed cells,[19–21] ligation of Siglec-F on mouse eosinophils is a consistently poor inducer of cell death in vitro.[8,22,23] A similar response shared by Siglec-8 and Siglec-F is that both get internalized following ligand binding.[24–26] This makes it

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; PE, phycoerythrin; Siglec, sialic acid-binding immunoglobulin-like lectin; WT, wild-type.

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potentially problematic, without proper gating strategies and the use of additional eosinophil markers, to use surface expression Siglec-F to track eosinophils after systemic administration of Siglec-F antibody, even though this is frequently done.[27–30]

Recently, transgenic mice have been developed in which human Siglec-8 is expressed on the surface of mouse eosinophils and where effective eosinophil depletion is seen following systemic administration of mouse anti-human Siglec-8 mAb.[14,31] In humans, the most effective approaches for depleting eosinophils in vivo rely on antibody-dependent cellular cytotoxicity (ADCC) with humanized antibodies such as benralizumab or AK002, also called antolimab.[32–35] We therefore hypothesized that targeting of Siglec-8 with mouse IgG1 antibodies, rather than targeting Siglec-F with rat IgG antibodies, in mice transgenic for Siglec-8, will prove to be a more effective strategy for eliminating mouse eosinophils in vivo. If true, this could either be due to differences in mechanisms of ligand-induced cell death or because of differences in ADCC activity of the targeting antibody that is separate from of any ligation-induced death. Furthermore, we hypothesized that eosinophil tracking, by detecting either Siglec-8 or Siglec-F, whichever is not being targeted, will allow for more accurate assessment of blood and tissue depletion following mAb administration. Indeed, our experiments have identified potential pitfalls when attempting eosinophil depletion by targeting Siglec-F that are overcome by targeting Siglec-8.

2 | MATERIALS AND METHODS

2.1 | Mice

Adult male and female mice on a C57BL/6 genetic background were used. *SIGLEC-8^{Eo}* mice (Siglec-8$^+$F$^+$ mice) were previously characterized by O'Sullivan et al.[14] Siglec-8$^+$F$^+$ mice were bred with Siglec-F$^{-/-}$ mice[22] (a generous gift of Dr. Ajit Varki, University of California San Diego, [San Diego, CA, USA] back-crossed here to C57BL/6 (wild-type, WT) mice and re-derived to eliminate the Dock8 carrier mutation) generating Siglec-8$^+$F$^-$ mice. For a few experiments, a related strain of C57BL/6 mice in which Siglec-8 was selectively expressed on mouse mast cells, not eosinophils, was also used.[36] Mouse genotyping done on tail snips was outsourced to TransnetYX (Cordova, TN, USA). Surface expression of Siglec-F and Siglec-8 on eosinophils was determined by flow cytometric analysis of a blood sample prior to using mice in in vivo assays (see additional methods in the following sections). As a control, *SIGLEC-8^{LSL}* (lacking EoCre) age- and sex-matched littermate mice were used. Mouse studies were performed with the approval of the Institutional Animal Care and Use Committees of Northwestern University (Chicago, IL, USA; protocol number IS00007627).

2.2 | Eosinophil differentiation from bone marrow precursors and cell imaging

Eosinophils were generated from the bone marrow of WT, Siglec-8$^+$F$^+$, Siglec-8$^+$F$^-$, and Siglec-F$^{-/-}$ mice following a protocol previously described by Dyer et al.[37] The surface expression of Siglec-F, Siglec-8, and CCR3 on bone marrow cells throughout the differentiation process was measured with flow cytometry as done previously.[14] Cell viability was assessed either with DAPI (ThermoFisher Scientific, Waltham, MA, USA) or Ghost fixable viability dye (Tonbo Biosciences, San Diego, CA, USA). Mature eosinophils on day 14 of the differentiation protocol were used for functional assays or apoptosis testing. Cytospins of mature eosinophils were stained with a Diff-Kwik stain set (Shandon, ThermoFisher Scientific) and imaged on an Olympus DSU microscope (Olympus, Tokyo, Japan).

2.3 | Flow cytometric analyses

2.3.1 | Gating and Siglec surface detection

Single cell suspensions of peripheral blood, spleen, and bone marrow were depleted of erythrocytes by hypotonic lysis. FcR receptors on cells were blocked on ice using 1 μg/mL of anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA, USA). Unless otherwise stated, eosinophils in blood, spleen, and bone marrow were gated as single, viable, CD45$^+$ (phycoerythrin [PE]-Cy7, mAb clone 30-F11, Biolegend, San Diego, CA, USA), CD11b$^+$ (AF700, mAb clone M1/70, ThermoFisher Scientific), CCR3$^+$ (FITC, J073E5, Biolegend), high SSC cells and presented as percentage of CD45$^+$ cells (see representative gating strategy in Supporting Information Fig. S1A). Dead cells were excluded by DAPI. Details regarding all anti-Siglec-8 and anti-Siglec-F mAbs are listed in Table 1 for convenience and were used as follows. Anti-mouse Siglec-F PE (mAb clone E50-2440, rat IgG2a, BD Biosciences) was used to detect Siglec-F surface expression following depletion with anti-mouse Siglec-F (mAb clone 238047, rat IgG2a, R&D Systems, Minneapolis, MN, USA). In addition, to determine whether these two anti-Siglec-F antibodies recognized similar or different epitopes on Siglec-F, eosinophil suspensions were preincubated with rIgG isotype control or unconjugated anti-Siglec-F (mAb clone 238047) at different concentrations for 20 min on ice to prevent internalization. Following a washing step, cells were stained with antibodies used to distinguish eosinophils as listed above including an anti-Siglec-F PE detection mAb clone (E50-2440 or REA798, a recombinant human IgG1 mAb from Miltenyi Biotec, Auburn, CA, USA). Geometric mean of Siglec-F PE on eosinophils was analyzed by flow cytometry. In other experiments, the cell suspension was left unstained or was incubated with 5 μg/mL of one or both unconjugated anti-mouse Siglec-F mAb clones (E50-2440 and 238047) for 20 min. Following a washing step, a secondary polyclonal anti-rIgG conjugated with AF-488 (heavy and light chain, goat anti-rat, ThermoFisher Scientific) was added to all samples in order to detect bound anti-Siglec-F antibodies. Cells were washed and resuspended in buffer containing DAPI. Geometric mean of AF-488 (FITC) on viable high SSC cells was acquired with flow cytometry to analyze the additive effect of different mAb clone labeling.

2.3.2 | Eosinophil CD11b up-regulation assay

Mature bone marrow-derived eosinophils from 4 different genotypes mentioned above were collected from plates, washed, and resuspended in media containing 30 ng/mL of rmIL-5 for 4 h (priming step)

TABLE 1 Antibodies, clones and intended use for all anti-sialic acid-binding immunoglobulin-like lectin (Siglec)-8 and anti-Siglec-F mAb reagents

Antibody (species and subclass)	Clone name	Modifications, if any	Intended use and other information
Anti-Siglec-8 (mouse IgG1)	2C4	AF-647 conjugated, saporin conjugated or unconjugated	Detection or depletion; recognizes the same epitope as 2E2
Anti-Siglec-8 (mouse IgG1)	2E2	Unconjugated or F(ab')$_2$ fragment	Depletion; recognizes the same epitope as 2C4
Anti-Siglec-8 (mouse IgG1)	1H10	AF-647 conjugated	Detection; recognizes a different epitope from 2C4 and 2E2
Anti-Siglec-F (rat IgG2a)	E50-2440	Unconjugated or PE conjugated	Detection or depletion
Anti-Siglec-F (rat IgG2a)	238047	Unconjugated	Depletion
Anti-Siglec-F (human IgG1)	REA 798	PE conjugated	Detection
Anti-Siglec-F (rat IgG2b)	9C7	Saporin conjugated	Depletion

and seeded in flat bottom 96-well plates (200,000 cells/well). Vehicle, mIgG1 (mAb MOPC-21, Tonbo Biosciences), anti-Siglec-8 (mouse IgG1 mAb clones 2E2 and 2C4),[19] or anti-Siglec-F (mAb clone E50-2440, BD) were added to cells in a final concentration of 2.5 μg/mL for 2 h. Eosinophils were stained with anti-mouse CD11b-AF700 (mAb clone M1/70, 1:20, ThermoFisher Scientific) and CD11b expression levels were analyzed by flow cytometry. Mean fluorescence intensity of CD11b staining was acquired and normalized to vehicle control.

2.3.3 | Quantification of Siglec receptors on eosinophils

Quantitative analysis of Siglec-F and Siglec-8 receptors on mouse eosinophils in peripheral blood and spleen from various strains of mice was performed with Quantum Simply Cellular microspheres (Bangs Laboratories, Fishers, IN, USA) according to the manufacturer's protocol using anti-Siglec-F PE (mAb clone E50-2440) and anti-Siglec-8 (mAb clone 2C4-AF647).[38] The channel values and geometric means from five distinct populations of beads yielded a calibration curve, which, analyzed with QuickCal software, enabled the quantitative analysis of Siglec-F and Siglec-8 receptor numbers per cell.

2.4 | Chemotaxis and calcium flux assays

The in vitro chemotaxis assays were performed in HTS Transwell plates (Corning, Oneonta, NY, USA) with a 5 μm pore size polycarbonate membrane as previously described.[37] Mature bone marrow-derived eosinophils were diluted to a concentration of 10^6 cells/mL in rmIL-5-free differentiation medium. Recombinant mouse eotaxin-2 (R&D Systems) or platelet activating factor (PAF, Sigma, St. Louis, MO, USA) was prepared in 10-fold dilutions using the same differentiation medium. Then, 100 μL of cell suspension was added to the upper well and 100 μL of chemoattractant or media was added to the bottom well. Cell migration was performed for 1 h at 37°C. Migrated eosinophils obtained from the bottom compartment were enumerated by flow cytometric counting and normalized to vehicle (media without chemoattractant) control.

Calcium flux of mature bone marrow-derived eosinophils was measured with a Fluo-4 Direct Calcium Assay kit (ThermoFisher Scientific) following stimulation with mouse eotaxin-2. Assays were performed according to the manufacturer's instructions. Mature eosinophils were loaded with calcium dye at a concentration of 2.5×10^6 cells/mL for 60 min at 37°C. Cells were washed and resuspended in PBS containing Ca^{2+}. Changes in Ca^{2+} were detected in the FITC channel with an LSR II flow cytometer. Baseline fluorescence was acquired for 30 s, after which cells were stimulated either with vehicle or mouse eotaxin-2 at two different concentrations to induce Ca^{2+} flux.

2.5 | In vitro apoptosis assay

Viability of bone marrow-derived eosinophils was assessed with FITC-labelled Annexin V (BD Biosciences) and DAPI. Eosinophils were preincubated with anti-Siglec-F (clone E50-2440 or 238047), anti-Siglec-8 mAb (2C4) or saporin-conjugated antibodies and their respective isotype controls as indicated in the figure legend. Saporin conjugated anti-Siglec-F (clone 9C7, rat IgG2b, a generous gift of Dr. James Paulson, The Scripps Research Institute, La Jolla, CA, USA)[18] or anti-Siglec-8 (clone 2C4) or their isotype controls were custom produced by Advanced Targeting Systems (San Diego, CA, USA).[25]

2.6 | In vivo treatment of mice for studies of eosinophil depletion

In the "acute" eosinophil depletion protocol, Siglec-8^+F^-, Siglec-8^+F^+, or littermate control mice were given a single i.p. injection of the indicated amount of various anti-Siglec mAb (238047, 2C4, 2E2, or 2E2 F(ab')$_2$, the latter generously provided by Dr. Bradford Youngblood, Allakos, Inc., Redwood City, CA, USA), anti-Siglec-F or anti-Siglec-8 mAb conjugated to saporin (9C7 and 2C4, respectively) or matched isotype controls. Blood was collected at baseline by cheek bleed into EDTA-containing tubes (or in heparinized capillary tubes) and after 24–48 h yielding paired blood data for each mouse. For the "prolonged" model, mice (including Siglec-8+ eosinophil or mast cell mice, see text) were treated with anti-Siglec mAb or matched isotype control i.p. every 2 d. Before each consecutive treatment, a blood sample was obtained to follow eosinophil population percentages and Siglec-8 or Siglec-F surface expression over time. At the end of the protocol, mice were sacrificed, spleen and bone marrow were collected, and then processed as described earlier to generate a single cell suspension for analysis of cell depletion.[14] A total of 50 μL of blood was collected separately

into EDTA-coated tubes and absolute eosinophil numbers were determined after staining with Discombe's fluid.[39] For experiments examining depletion with anti-Siglec-8 (clone 2C4, mouse IgG1 mAb recognizing domain 1 of the extracellular region of Siglec-8), a different anti-Siglec-8-AF647 mAb (clone 1H10, mouse IgG1 recognizing domain 3 of the extracellular portion of Siglec-8[33] and thus not cross-reactive with mAb 2C4, also generously provided by Dr. Bradford Youngblood) was used to detect residual Siglec-8 surface expression. Fluorescence minus one (FMO) controls were used to determine proper population gating. Samples were acquired using a BD LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v.10 (TreeStar, Inc., Ashland, OR, USA).

2.7 | Statistical analysis

Statistical analysis was preformed using GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA). Differences between groups were tested by 1-way or 2-way ANOVA followed by Dunnett's or Sidak's posttest as indicated in the figure legends. When comparing two sets of data paired or unpaired Student's *t*-test was used as appropriate. All results are presented as mean ± SE of the means. *P*-values ≤0.05 were considered significant.

3 | RESULTS

3.1 | Phenotypic and functional characterization of WT and Siglec-8/Siglec-F-altered eosinophils differentiated from bone marrow of mice

In order to validate and compare eosinophils from mice with different *SIGLEC-8* and *SIGLEC-F* phenotypes beyond tail-snip genotyping, mouse eosinophils were differentiated from bone marrow cells ex vivo[37,40] as shown in Fig. 1A. The development of eosinophils was assessed by measuring the surface expression of Siglec-8, Siglec-F, and CCR3 by direct immunofluorescence and flow cytometry. As expected, the expression profile and kinetics of these surface markers on eosinophils differentiated from WT and Siglec-8$^+$F$^+$ mice mirrored published data (Fig. 1B-C).[14,37] Siglec-F and Siglec-8 expression followed a similar kinetic and were detected on the cell surface prior to CCR3. Also as expected, eosinophils differentiated from bone marrow of Siglec-8$^+$F$^-$ animals expressed Siglec-8, but lacked Siglec-F (Fig. 1D), whereas Siglec-F$^{-/-}$ eosinophils did not express Siglec-F or Siglec-8 (Fig. 1E). A representative overlay of Siglec-8 and Siglec-F expression from mature eosinophils of all four Siglec phenotypes is shown in Fig. 1F. Eosinophils were considered mature by day 14 of the protocol and exhibited typical mouse eosinophil morphology with ring-shaped nuclei and eosin-stained granules that was similar to cultures of WT eosinophils (Fig. 1G and data not shown).

The expression of Siglec-8 and Siglec-F was further quantified on eosinophils in peripheral blood and spleen of animals. Similar to bone marrow-derived eosinophils, eosinophils in blood and spleen of Siglec-8$^+$F$^-$ animals did not express Siglec-F (Fig. 1H, I). Expression of Siglec-8 was undetectable in blood and spleen of WT animals (Fig. 1J, K). The quantification of Siglec receptors showed ~5–10-fold higher numbers of Siglec-8 receptors per eosinophil (under the control of the CAG promotor)[14] compared to numbers of Siglec-F receptors per eosinophil, and about 5 times more Siglec-8 receptors per cell than what has been reported on human blood eosinophils.[34] Finally, transgenic manipulation of Siglec receptors did not result in any significant alterations of eosinophil responses to eotaxin-2 or PAF, such as chemotactic responses and calcium flux, as shown in Supporting Information Figure S2.

3.2 | Siglec-8 engagement increases CD11b expression on bone marrow-derived eosinophils

We next set out to investigate whether Siglec-8 expressed on the surface of mature mouse bone marrow eosinophils is functional. Previously published data showed that Siglec-8 mAb engagement induced rapid up-regulation of CD11b on the surface of human eosinophils.[21] Indeed, preincubation with two different clones of Siglec-8 mouse IgG1 mAb, 2C4 and 2E2, induced a modest, but significant increase in CD11b expression in mouse eosinophils expressing Siglec-8 (Fig. 2C-D), albeit less than that seen with human eosinophils,[21] but did not increase CD11b expression in eosinophils lacking Siglec-8 (Fig. 2A, B). In contrast, Siglec-F engagement (with rat IgG2a mAb E50-2440) under the same conditions had no effect on CD11b expression (Fig. 2A, C). Therefore, the CD11b up-regulation mediated by Siglec engagement was functional for Siglec-8 but not for Siglec-F in mouse eosinophils. Representative histograms of modest CD11b up-regulation following 2C4 stimulation on Siglec-F$^{-/-}$ and Siglec-8$^+$F$^-$ eosinophils are shown in Fig. 2E and F.

3.3 | Incubation with Siglec antibodies in vitro results in weak cell death responses in bone marrow-derived eosinophils

Engagement of Siglec-8 on human eosinophils in vitro can induce robust cell death responses, whereas this is much less remarkable following engagement of Siglec-F on mouse eosinophils.[8,19–23] Therefore, we examined whether Siglec mAb ligation leads to cell death of bone marrow-derived eosinophils. In accordance with previously published data,[23] Siglec-F mAb (clone E50-2440) induced a modest, but significant, WT eosinophil cell death in vitro after 48 h (Fig. 3A, B). As expected, there were no changes in cell viability following Siglec-F mAb incubation of Siglec-F$^{-/-}$ eosinophils (data not shown). Incubation of Siglec-8$^+$F$^+$ eosinophils with either Siglec-8 (2C4) or Siglec-F (E50-2440) mAb led to an equally modest decrease in cell viability (Fig. 3C) highlighting distinct differences between Siglec-8 engagement on mouse compared to human eosinophils.[8,19–23] A similarly small effect on cell death was observed following 24 h incubation of Siglec-8$^+$F$^-$ mouse eosinophils with Siglec-8 mAb (Fig. 3D). Moreover, even the addition of antibodies conjugated to saporin for 24 h did not enhance the cell death of mouse eosinophils (Fig. 3E), unlike what has been observed with human eosinophils,[25] suggesting that, at least for bone marrow-derived mouse eosinophils, Siglec-8 engagement

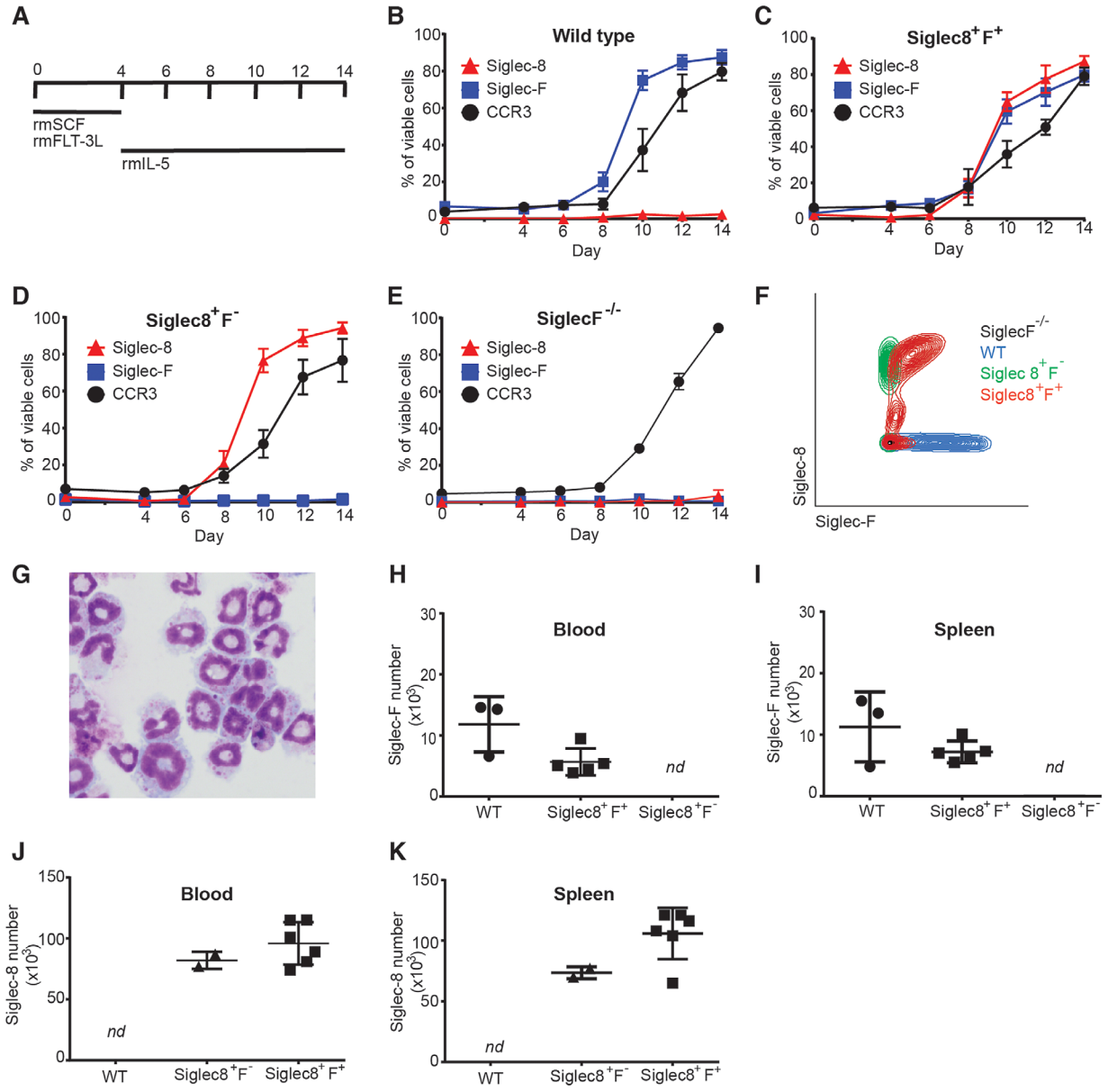

FIGURE 1 **Phenotypic characterization of bone marrow-derived eosinophils.** Eosinophils from mice of different genetic backgrounds were differentiated from bone marrow hematopoietic stem cells according to the protocol presented in (**A**). Surface expression of sialic acid-binding immunoglobulin-like lectin (Siglec)-8, Siglec-F and CCR3 was followed over time by flow cytometry in differentiating cells from wild-type (WT) (**B**), Siglec-8$^+$F$^+$ (**C**), Siglec-8$^+$F$^-$ (**D**), and Siglec-F$^{-/-}$ (**E**) mice, with a representative overlay of these results (**F**). Data from 4 to 5 mice per genetic model are shown. (**G**) Mouse Siglec-8$^+$F$^-$ eosinophils stained with Diff-Qwik on day 14 of differentiation. Siglec-F (**H-I**) and Siglec-8 (**J-K**) expression on mouse eosinophils was confirmed and quantified in peripheral blood and spleen of animals. Data from 2 to 6 mice per genetic model are shown. nd, not detected

results in much less cell death than with human eosinophils, even when involving internalization of toxic payloads.

3.4 | Systemic administration of Siglec-F antibody does not deplete eosinophils after a single dose, but does alter Siglec-F surface expression on eosinophils

After observing very modest Siglec mAb-mediated cell death in vitro (Fig. 3), the effect of Siglec-F administration on eosinophil depletion in vivo was explored. First, the effect of a single i.p. injection of a Siglec-F mAb (rat IgG2a clone 238047) on eosinophil percentages in blood and spleen (see representative gating strategy in Supporting Information Fig. S1A) was studied. After a single injection of Siglec-F mAb (15 μg) no significant decrease in eosinophils from baseline was observed when compared to isotype control (Fig. 4A, B). Furthermore, there was no reduction in manual eosinophil cell counts performed on blood samples as measured following staining with Discombe's fluid (Supporting Information Fig. S1E). This was unexpected, because an identical commercially available rat IgG2a Siglec-F mAb was

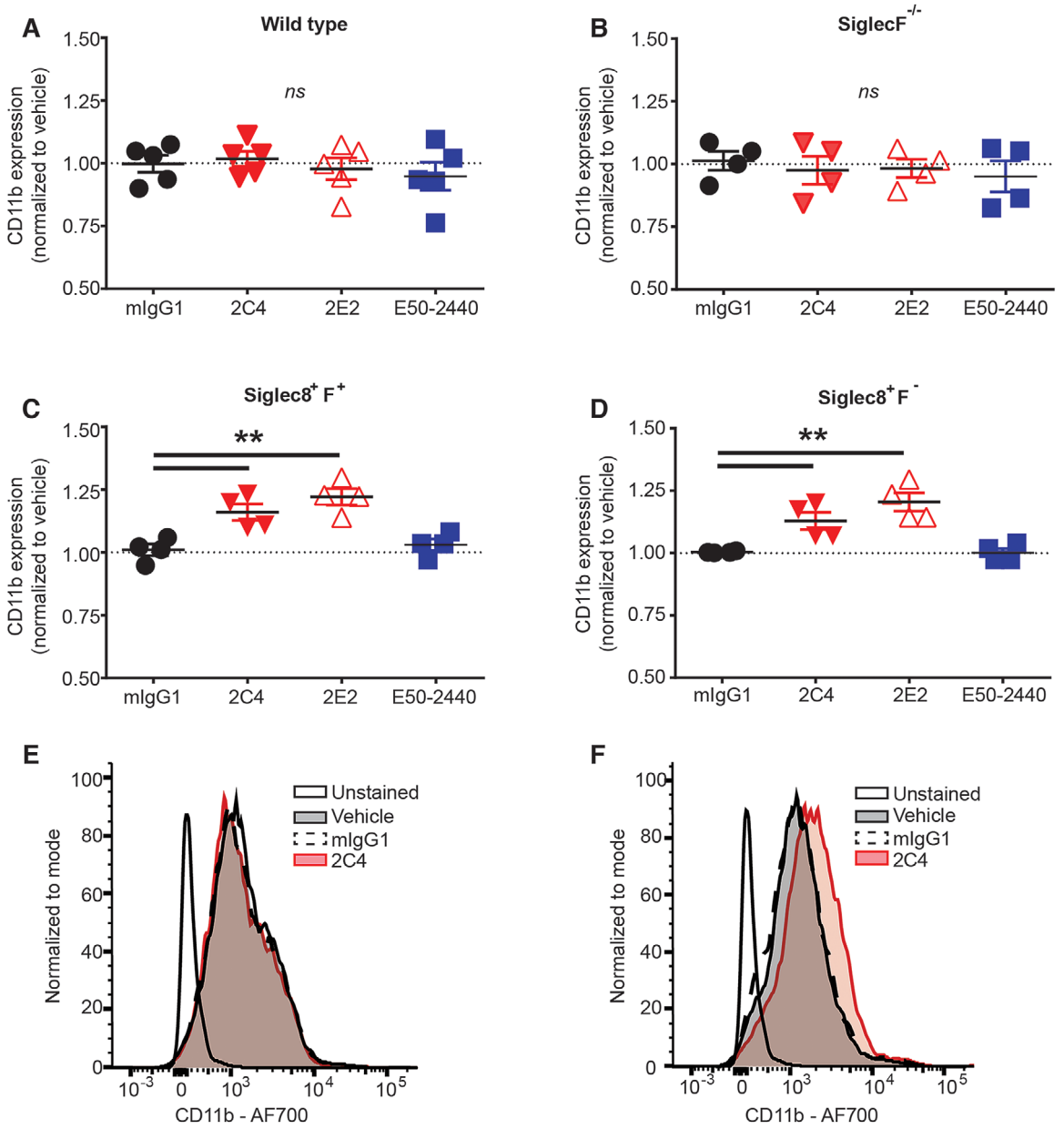

FIGURE 2 **CD11b expression on bone marrow-derived eosinophils following sialic acid-binding immunoglobulin-like lectin (Siglec) antibody engagement**. Mature bone marrow-derived eosinophils were primed with 30 ng/mL of recombinant mouse IL-5 for 4 h and afterward incubated with vehicle or 2.5 μg/mL of isotype control (mIgG1), anti-Siglec-8 (2C4 or 2E2), or anti-Siglec-F (E50-2440) for 2 h. CD11b expression was evaluated by flow cytometry and normalized to vehicle control. (**A**) Wild-type (WT) eosinophils, (**B**) Siglec-F$^{-/-}$ eosinophils, (**C**) Siglec-8^{+}F^{+} eosinophils, and (**D**) Siglec-8^{+}F^{-} eosinophils. Data from 4 to 5 biologic replicates per genetic model are presented and analyzed with 1-way ANOVA followed by Dunnett's posttest (antibody treated vs. isotype control). (**E-F**) Representative histograms of CD11b expression following 2C4 stimulation on Siglec-F$^{-/-}$ eosinophils (**E**) or Siglec-8^{+}F^{-} eosinophils (**F**). $^{**}P<0.01$; ns, not significant

previously used in studies to acutely deplete eosinophils.[28,41] In contrast to our study design, these investigators used Siglec-F mAb (clone E50-2440) after depletion with Siglec-F mAb (clone 238047) in order to detect eosinophils. However, following administration of the Siglec-F mAb, the expression of surface Siglec-F on circulating eosinophils was drastically decreased (Fig. 4C), consistent with either epitope overlap between these two antibodies and/or mAb-mediated receptor internalization as seen in vitro.[24] Siglec-F surface expression was also significantly reduced on eosinophils from the spleen of Siglec-F mAb-treated animals (Supporting Information Fig. S1C). As shown with representative flow cytometry data in Supporting Information Figure S1B, eosinophil percentages using Siglec-F as a marker are artifactually only about half of that detected when an additional eosinophil cell surface marker, CCR3, is included in the gating strategy (following pre-gating for single/viable/CD45^{+}/CD11b^{+} granulocytic cells).

In order to further study the interaction of these two mAb clones, an antibody additivity binding assay was performed, where whole blood leukocytes were incubated with either of the two clones alone, or both,

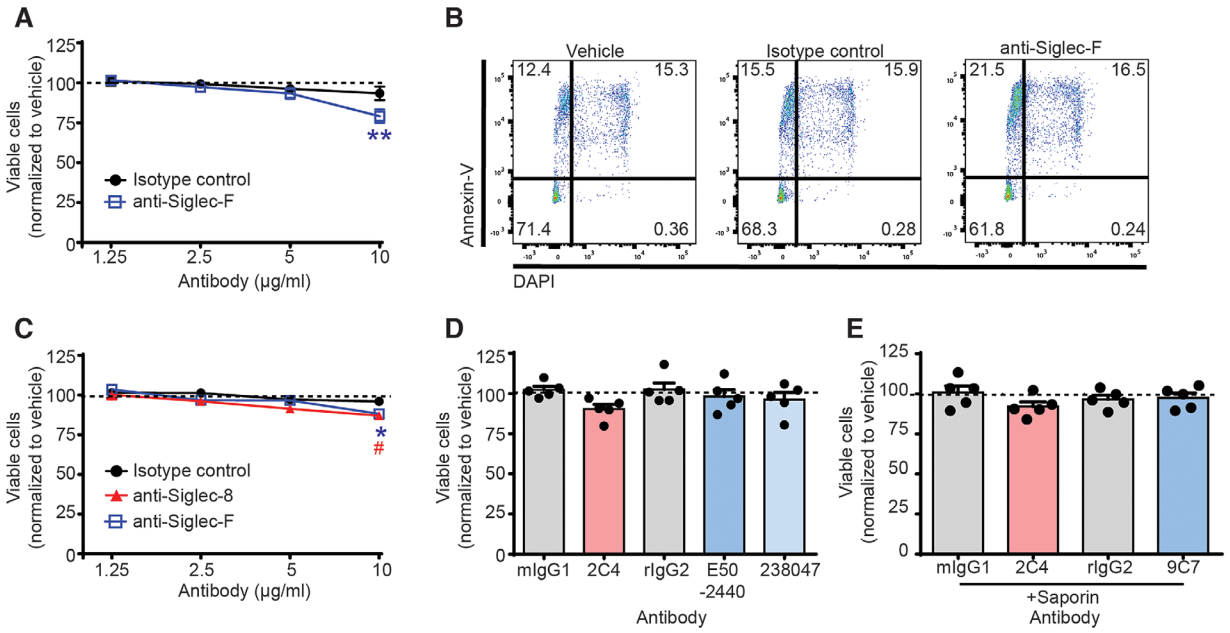

FIGURE 3 **Effect of sialic acid-binding immunoglobulin-like lectin (Siglec) engagement in vitro on eosinophil viability.** Cell viability of mature bone marrow eosinophils was assessed with Annexin V/DAPI staining. (**A**) Wild-type (WT) eosinophils were pretreated with isotype control or anti-Siglec-F (E50-2440, 1.25–10 μg/mL) for 48 h. (**B**) Representative histograms of Annexin V/DAPI staining of eosinophils in (**A**) treated with vehicle or isotype control and anti-Siglec-F at 10 μg/mL. (**C**) Siglec-8$^+$F$^+$ eosinophils were pretreated with 1.25–10 μg/mL isotype control, anti-Siglec-F (E50-2440), or anti-Siglec-8 (2C4) for 48 h. Data from 4 independent experiments performed in duplicates are shown and analyzed with 2-way ANOVA followed by Sidak's posttest. $^{**}P < 0.01$ anti-Siglec-F vs. isotype control; $^{*}P < 0.05$ anti-Siglec-F vs. isotype control, $\#P < 0.05$ anti-Siglec-8 vs. isotype control. (**D**) Siglec-8$^+$F$^-$ mouse eosinophils were pretreated with 10 μg/mL isotype controls (mouse IgG1 or rat IgG2), anti-Siglec-8 (2C4), or anti-Siglec-F (E50-2440 or 238047) for 24 h. (**E**) Siglec-8$^+$F$^-$ mouse eosinophils were pretreated with 10 μg/mL saporin-conjugated isotype controls (mouse IgG1 or rat IgG2), anti-Siglec-8 (2C4) or anti-Siglec-F (9C7) antibodies for 24 h

and bound antibody was detected with a fluorochrome-conjugated secondary anti-rat polyclonal antibody. As shown in Fig. 4D, the amount of secondary antibody binding was the same with preincubation of either mAb alone, or both Siglec-F antibodies, suggesting that the two mAb clones bind to a cross-reactive epitope. In fact, a progressive, dose-dependent decrease in anti-rat antibody binding was seen, suggesting that the binding of the first Siglec-F mAb interfered with the ability of the second mAb to bind (Fig. 4E, F). This was not due to receptor internalization by the first mAb, because the mAb incubations were performed on ice to block internalization.[24] Finally, this effect was also observed using a third clone of Siglec-F mAb (REA798, human IgG) for detection (Supporting Information Fig. S1D).

3.5 | Transgenic Siglec- 8 as a surface marker for tracking mouse eosinophil numbers in blood and spleen following Siglec-F antibody administration

To further test the extent of Siglec-F mAb-mediated eosinophil depletion and the kinetics of its surface expression on remaining eosinophils, a prolonged model of Siglec-F mAb (clone 238047, rat IgG2a isotype) administration was performed as shown in Fig. 5A. During the course of the protocol, the surface expression of Siglec-F and Siglec-8 on eosinophils was followed (identified by the previously mentioned gating strategy including CCR3 positivity) in Siglec-8$^+$F$^+$ animals. In Siglec-F mAb-treated animals, the percentage of eosinophils as assessed by detectable Siglec-F$^+$ cells decreased significantly after a single dose, and remained low with subsequent doses. This was remarkably lower than, and discordant with, the percentage of blood eosinophils as assessed by either CCR3$^+$ or Siglec-8$^+$ cell quantification, which were minimally altered by Siglec-F mAb administration (Fig. 5B). This discordance was further reflected in the levels of each of these surface markers, as assessed by flow cytometry. The geometric means for Siglec-F decreased in Siglec-F mAb treated animals when compared to control rat IgG treated mice (Fig. 5C), whereas the geometric means for CCR3 (data not shown) and Siglec-8 were comparable between the two mAb treatment groups (Fig. 5D). Furthermore, repeated administration of Siglec-F mAb significantly decreased the geometric mean of Siglec-F-PE mAb labeling of eosinophils in the spleen, but did not alter the geometric mean of Siglec-8 (Fig. 5E, F). These data strongly suggest that following systemic administration of Siglec-F mAb, the use of Siglec-F alone to track eosinophils significantly underestimates the number of eosinophils remaining in blood and spleen. By day 7 of the prolonged depletion protocol (4 repeated injections of Siglec-F mAb), eosinophils were partially and significantly depleted from spleen and blood of mice compared to control rat IgG treated animals (Fig. 5G, H). This was confirmed by performing manual microscopy counts from cytospins of the same blood samples (Supporting Information Fig. S1F).

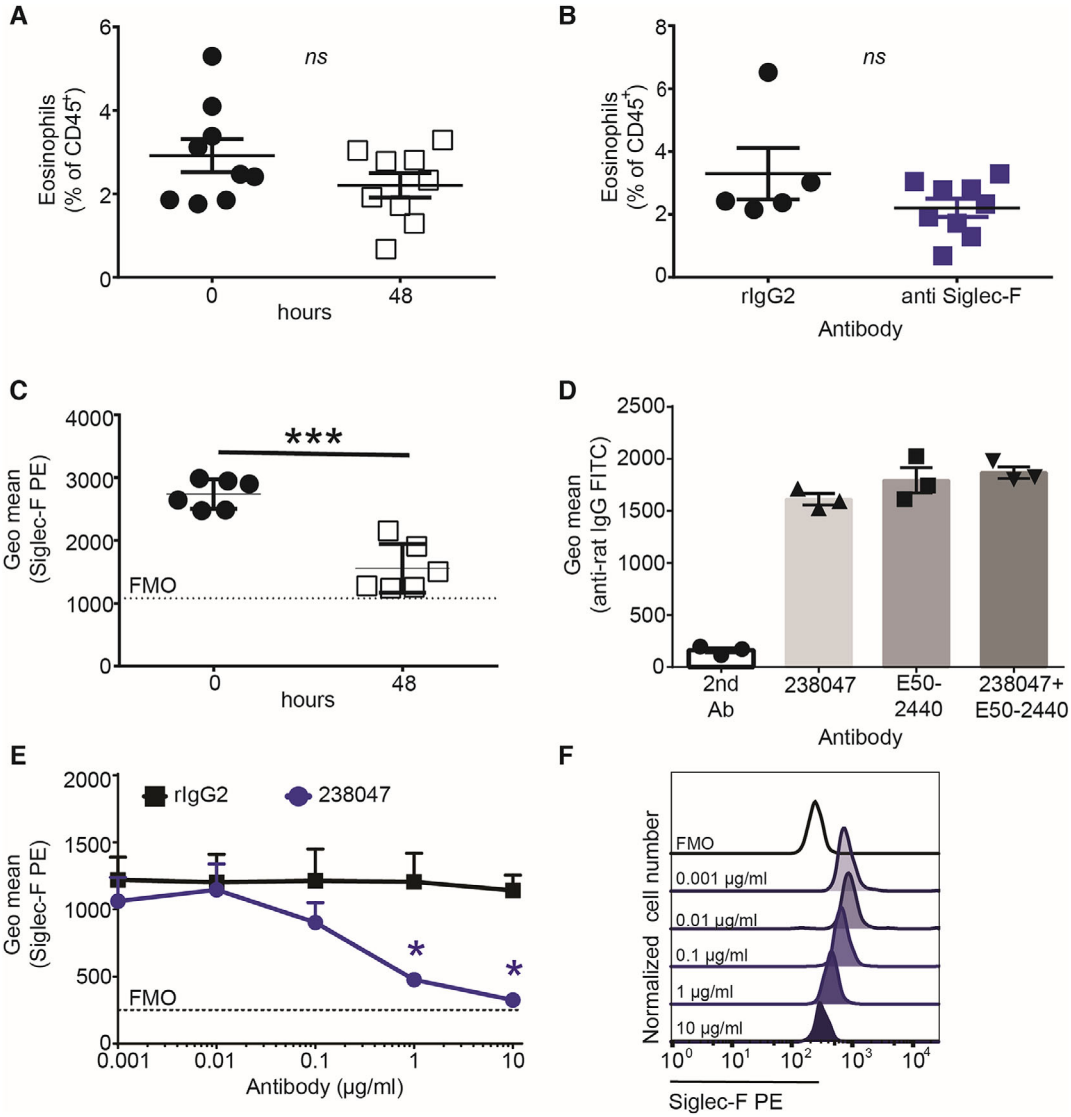

FIGURE 4 **Single administration of sialic acid-binding immunoglobulin-like lectin (Siglec)-F mAb in vivo decreases the surface expression of Siglec-F on eosinophils, but does not effectively deplete eosinophils.** Siglec-8$^+$F$^+$ mice received a single i.p. injection of 15 µg of isotype control or anti-Siglec-F (238047). (**A**) Percentage of eosinophils (analyzed as percentage of CD45$^+$ cells, see representative gating strategy in Supporting Information Fig. S1) at baseline and 48 h after the administration of anti-Siglec-F. (**B**) Eosinophil percentage in blood of isotype treated compared to anti-Siglec-F treated animals. Data from 2 independent experiments are shown. $n = 5–9$, ns (nonsignificant), unpaired Student's t-test. (**C**) Geometric mean of Siglec-F PE (clone E50-2440 used for detection) on blood eosinophils before and 48 h after anti-Siglec-F (clone 238047) administration. (**D**) Additivity trial of two Siglec-F mAb clones. Following red blood cell lysis, mouse blood leukocytes were incubated with vehicle or anti-Siglec-F (clone 238047 or clone E50-2440, 5 µg/mL) or both (5 µg/mL each). A secondary FITC-labelled polyclonal anti-rat IgG was then added to detect bound primary antibodies. 2nd Ab = vehicle treated cells incubated only with secondary antibody. (**E-F**) Following red blood cell lysis, mouse blood leukocytes were incubated with isotype control or anti-Siglec-F (clone 238047) at the indicated concentrations for 20 min, washed, and then incubated with a different anti-Siglec-F-PE clone. (**E**) Geometric mean of Siglec-F PE (staining clone E50-2440) is quantified with representative histograms of anti-Siglec-F pretreated cells shown in (**F**). Mean ± SEM from 2 to 3 independent experiments is shown. Data were analyzed with 2-way ANOVA followed by Sidak's posttest. *P<0.05, **P<0.01, ***P<0.001; ns, not significant

3.6 | Siglec-8 antibody administration leads to rapid and profound eosinophil depletion after a single injection

Having only obtained partial depletion of eosinophils when targeting Siglec-F in vivo, even after multiple injections of Siglec-F mAb, similar experiments were performed using Siglec-8 mAb (mAb clone 2C4, mouse IgG1). Remarkably, a single 10 µg i.p. injection was sufficient to cause near complete depletion of eosinophils from blood of Siglec-8$^+$ mice as assessed by gating on CCR3$^+$ or Siglec-F$^+$ cells (Fig. 6A). Administration of saporin-conjugated 2C4 (2C4-SAP) showed a similar, perhaps even more consistent and profound depletion effect (Fig. 6B). A single dose of 2C4 with or without saporin conjugation also partially depleted eosinophils from spleens and bone marrow of Siglec-8$^+$ mice (Fig. 6C–E). In order to elucidate the minimal dose needed, a range of mAb doses given as a single injection

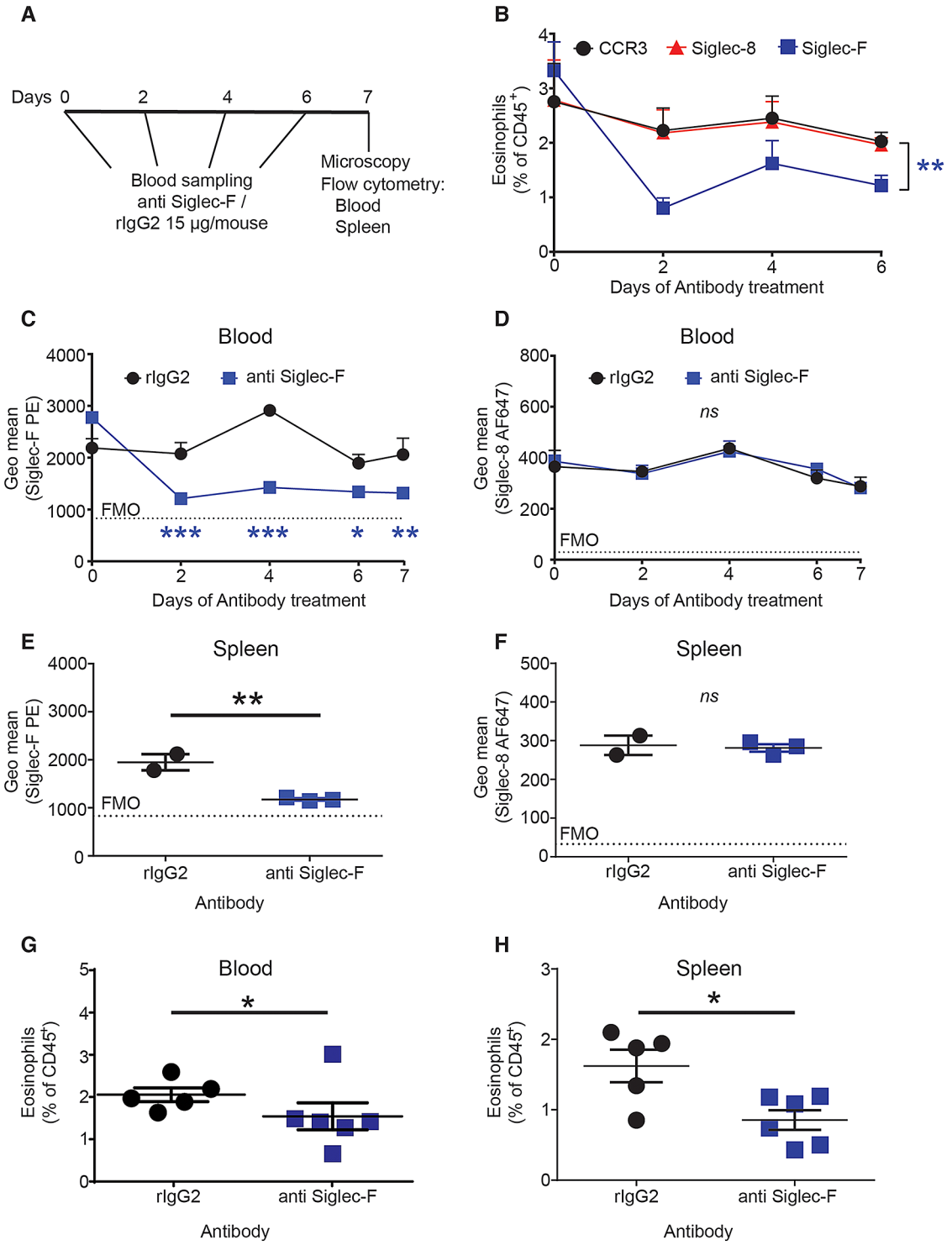

FIGURE 5 **Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 is a more reliable marker for detecting eosinophils than Siglec-F following systemic administration of Siglec-F antibody**. (**A**) Protocol used for the prolonged model of anti-Siglec-F treatment. (**B**) Eosinophil percentage in blood samples as determined by Siglec-F, Siglec-8 or CCR3 positivity over time. Mean ± SEM of 3 biologic replicates from 1 to 2 independent experiments are shown. $^{**}P < 0.01$ Siglec-F vs. CCR3 as a marker, 2-way ANOVA with Dunnett's posttest. (**C-D**) Geometric mean of Siglec-F-PE or Siglec-8-AF647 labeling of eosinophils in isotype vs. anti-Siglec-F treated animals over time. ns = nonsignificant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ analyzed with 2-way ANOVA followed with Sidak's posttest. (**E-F**) Geometric mean of Siglec-F-PE or Siglec-8-AF647 labeling of eosinophils from spleens of isotype and anti-Siglec-F treated animals. Data from 1 to 2 independent experiments are shown and analyzed with unpaired Student's *t*-test. $^{**}P < 0.01$, ns (nonsignificant, $P > 0.05$). (**G-H**) Repeated injections of anti-Siglec-F into Siglec-$8^{+}F^{-}$ mice partially deplete eosinophils. Eosinophil percentages in blood (**G**) and spleen (**H**) on day 7 of the protocol were determined with flow cytometry. Data from 5 to 6 biologic replicates per group from 2 independent experiments are shown and analyzed using unpaired Student's *t*-test ($^{*}P < 0.05$)

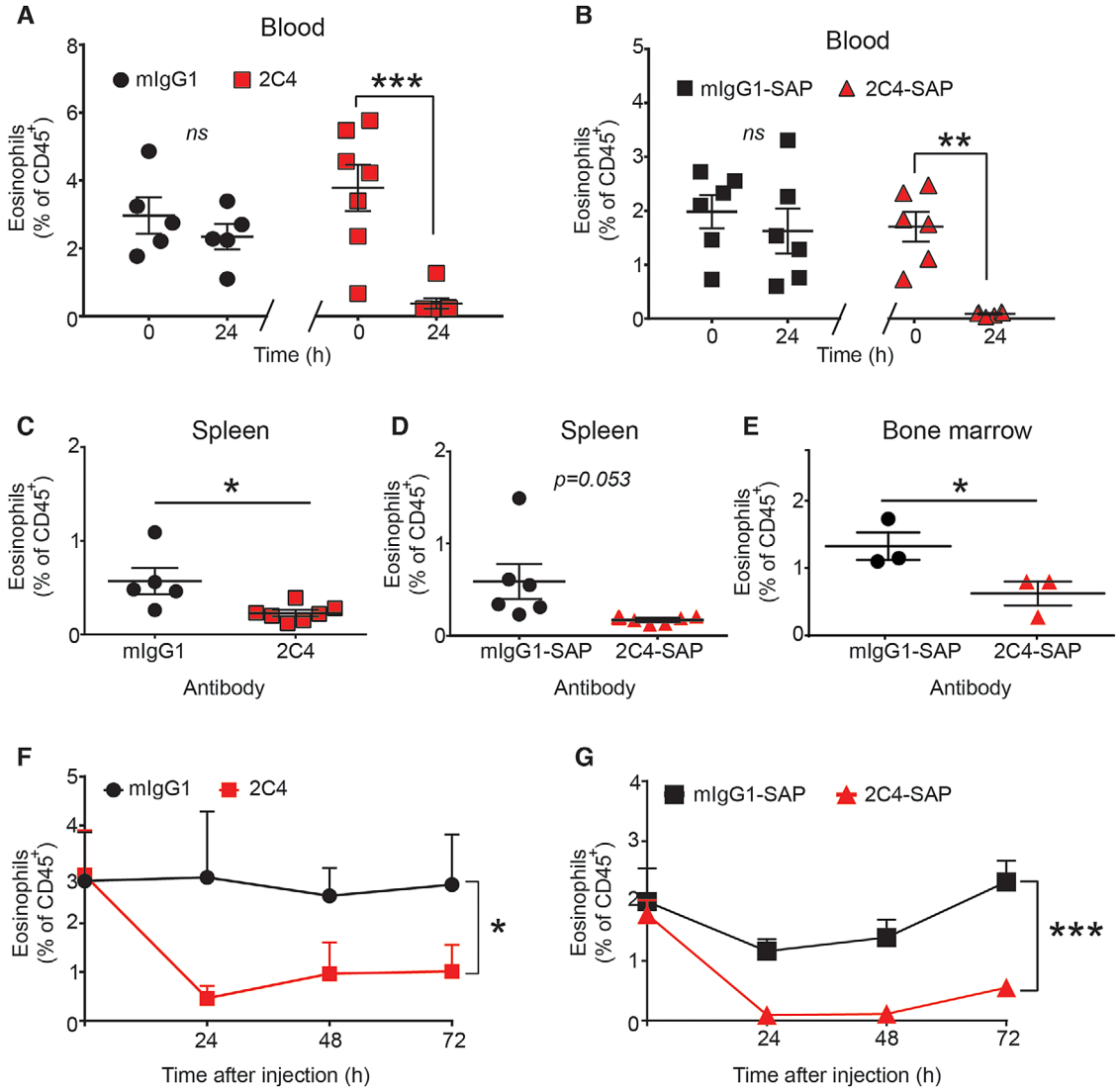

FIGURE 6 **A single dose of 2C4 or 2C4-saporin effectively depletes eosinophils in vivo**. A single application of anti-sialic acid-binding immunoglobulin-like lectin (Siglec)-8 (with or without saporin) effectively depletes eosinophils in vivo. (**A-B**) Eosinophil percentages at baseline and 24 h following a single i.p. injection of 10 μg anti-Siglec-8 (2C4 or 2C4-saporin) or isotype control (mIgG1 or mIgG1-saporin). Data from 5 to 7 mice per treatment are shown and analyzed with 2-way ANOVA followed by Sidak's posttest (0 h vs. 24 h). Eosinophil percentages in spleen (**C-D**) and bone marrow (**E**) 24 h after treatment are shown and compared to isotype control with unpaired Student's *t*-test. (**F-G**) Duration of anti-Siglec-8-mediated eosinophil depletion was followed with repeated blood sampling and is represented as eosinophil percentages in blood over time. Means ± SEM of 3 mice per treatment group are shown and analyzed with 2-way ANOVA followed by Dunnett's posttest (anti-Siglec-8 vs. isotype control treated mice). **P*<0.05, ***P*<0.01, ****P*<0.001; ns, not significant

of 2C4-saporin per mouse were tested. Paired data in blood (0–24 h) and percentages in spleen indicated that a 2.5 μg dose was the minimum dose needed for optimal depletion of eosinophils, because higher doses up to 10 μg/mouse were just as effective (Supporting Information Fig. S3).

To explore the duration of depletion, and to facilitate using 2C4 in Siglec-8$^+$ mice as a method of transient eosinophil depletion, mice received a single injection of 10 μg of 2C4 (Fig. 6F) or 2C4-saporin (Fig. 6G) and circulating eosinophil percentages were determined over time. Efficient depletion, especially with 2C4-saporin, was observed for up to 2 d, with recovery, albeit <50%, beginning by day 3 after injection.

3.7 | Repeated injections of 2C4 result in profound and selective eosinophil depletion from blood, spleen, and bone marrow

To further examine the extent of Siglec-8 mAb-mediated eosinophil depletion, 2C4 mAb was tested in a repeated-dose prolonged model, as shown in Fig. 7A. Mice were injected with 2C4 mAb or isotype control and eosinophil percentages were followed by tracking CCR3, Siglec-F, or Siglec-8. In contrast to Siglec-F targeting results, the eosinophil population was markedly depleted regardless of the gating strategy used (Fig. 7B). Multiple injections of 2C4 were more effective at reducing eosinophil numbers in bone marrow and spleen (Fig. 7C, D) compared

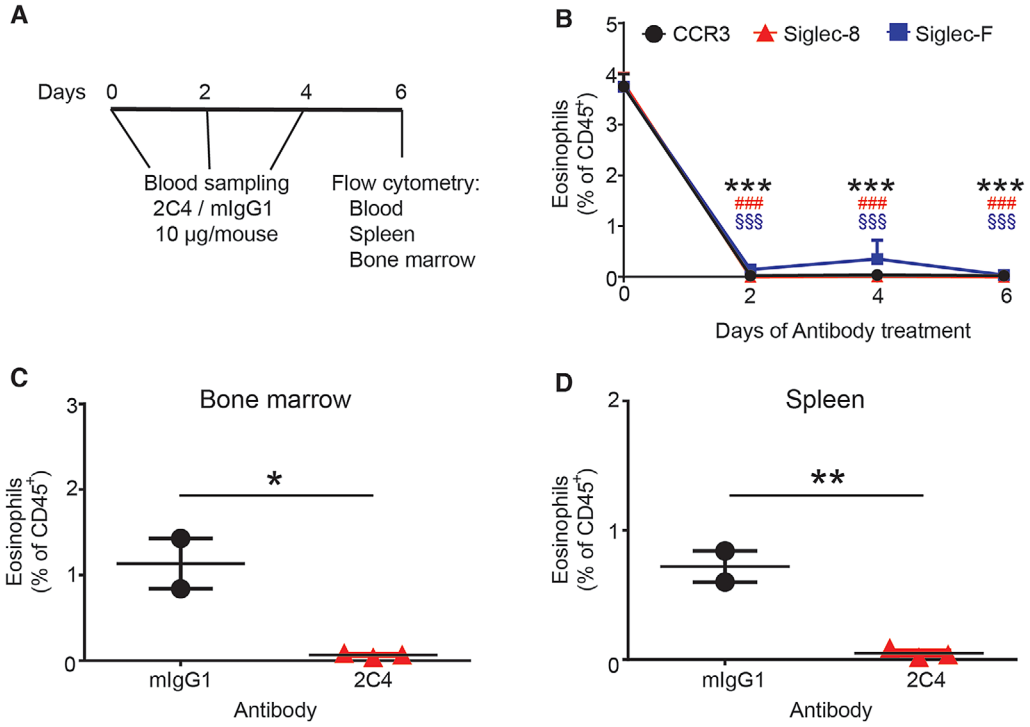

FIGURE 7 **Repeated dosing of 2C4 mAb effectively depletes eosinophils in vivo from multiple body compartments.** (**A**) Protocol used for anti-sialic acid-binding immunoglobulin-like lectin (Siglec)-8 (2C4) treatment. (**B**) Eosinophil percentage in blood samples as determined by Siglec-F, Siglec-8, or CCR3 positivity over time. Mean ± SEM of 3 biologic replicates. Two-way ANOVA with Sidak's posttest. ***$P < 0.001$ $CCR3^+$ population vs. time zero baseline, §§§$P < 0.001$ Siglec-F^+ population vs. time zero baseline, and ###$P < 0.001$ Siglec-8^+ population vs. time zero baseline. (**C**) Percentage of eosinophils in bone marrow on day 6 of the protocol. (**D**) Percentage of eosinophils in spleen on day 6 of the protocol. Differences between isotype treated animals ($n = 2$) and 2C4 treated animals ($n = 3$) were evaluated with unpaired Student's t-test. *P<0.05, **P<0.01 for panels C and D

to a single injection (Fig. 6D, E). No other cell types were depleted, and repeated injections were well tolerated (data not shown). In order to confirm that the observed effect was due to specific engagement of Siglec-8 on eosinophils, additional control experiments were performed in $EoCre^-$ animals (lacking Siglec-8) (Fig. 8A, B) and in Siglec-8+MCPT5+ animals (Fig. 8C, D) where Siglec-8 is expressed on mast cells instead of eosinophils.[36] As shown in Fig. 8, 2C4 mAb did not alter eosinophil numbers in these control experiments.

3.8 | Mechanism of Siglec-8 antibody-mediated eosinophil depletion in vivo: role of the Fc region of the mIgG1

Finally, pursuing the cause for discordance between the in vitro and in vivo effects seen with mouse IgG1 anti-Siglec-8 vs. rat IgG2a anti-Siglec-F antibodies, the contribution of Fc-mediated depletion, such as via ADCC, was investigated. Mice were injected with $F(ab')_2$ fragments of 2E2 mouse IgG1 anti-human Siglec-8 mAb, previously shown to induce human eosinophil death as effectively as the intact antibody.[21] Using the same gating strategies as for Fig. 7, the intact 2E2 mAb was found to deplete mouse eosinophils from Siglec-8^+ mice in a manner comparable to 2C4. In contrast, the administration of an identical dose of $F(ab')_2$ fragments of 2E2 had no depleting effect (Fig. 9A-C). The $F(ab')_2$ fragments of 2E2 were able to bind to mouse eosinophils in vivo because they internalized Siglec-8 (Fig. 9D; as detected by flow cytometry using a non-cross-reactive clone 1H10 Siglec-8 mAb). Taken together, these data demonstrate the distinctive mIgG1 Fc dependency of Siglec-8 mAb-mediated eosinophil depletion in vivo.

4 | DISCUSSION

In the present study we describe a novel strain of Siglec-8^+F^- mice generated from crossing Siglec-8^+F^+ and Siglec-F null strains. We demonstrate that eosinophils can be differentiated from bone marrow of these mice and that these eosinophils exhibit comparable functional responses to external migratory stimuli (Fig. 1 and Supporting Information Fig. S2). Moreover, eosinophils differentiated from Siglec-8+ mice increase their expression of CD11b on their surface following Siglec-8 (but interestingly not Siglec-F) antibody engagement (Fig. 2), an indication that the Siglec-8 present on the cell surface is functional and capable of downstream signaling.

It has previously been reported that antibody engagement of Siglec-8 on human eosinophils and its closest functional paralog Siglec-F on murine eosinophils, results in cell death in vitro. Cell death of human cytokine-primed eosinophils was quite marked and was shown to be dependent on ROS production,[19–21] whereas targeting Siglec-F on mouse eosinophils resulted in very modest levels of

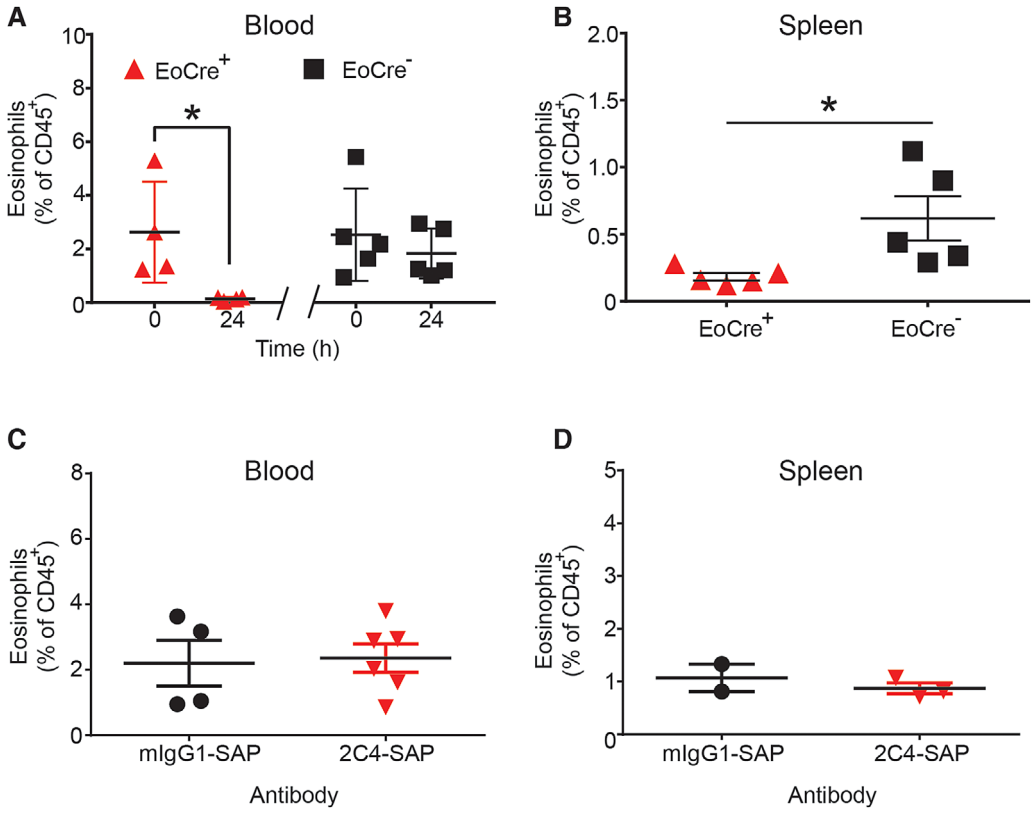

FIGURE 8 **Sialic acid-binding immunoglobulin-like lectin (Siglec)-8 expression on eosinophils is necessary for 2C4-saporin-mediated eosinophil depletion**. Mice were given a single 5 μg i.p. injection of 2C4-saporin. (**A-B**) Eosinophil percentages at baseline and 24 h after injection were analyzed in Siglec-8$^+$EoCre$^+$ mice (Siglec-8$^+$F$^+$) and Siglec-8$^+$EoCre$^-$ (Siglec-8$^-$F$^+$ littermate controls). (**A**) Paired blood sample data from 5 biologic replicates are shown and analyzed with 2-way ANOVA followed with Sidak's posttest, $^*P < 0.05$). (**B**) Eosinophil percentages in spleens harvested 24 h after injection from the same 5 animals per genotype as in (**A**) are shown and compared with unpaired Student's *t*-test, $^*P < 0.05$. (**C-D**) Mice with Siglec-8 expressed on mast cells (Siglec-8$^+$MCPT5$^+$ mice) were given a 10 μg i.p. injection of 2C4-saporin or mIgG1-saporin (isotype control). Eosinophil percentages in blood (**C**) and spleen (**D**) 24 h after treatment were analyzed with unpaired Student's *t*-test

caspase-dependent apoptosis.[8,22,23] Our data show that antibodies targeting Siglec-8 on mouse eosinophils from transgenic mice have a modest effect on cell viability in vitro. Whereas these studies have all been done with mAb clones 2E2 and 2C4, and have not been performed with others that are commercially available including mAb 7C9, all three are mouse IgG1 and recognize cross-reactive epitopes (unpublished observations); therefore, it seems reasonable to expect that all of them would share similar biologic activities. These modest in vitro murine eosinophil responses (cell death and CD11b up-regulation, Figs. 2 and 3) might be explained by the distinct mechanisms of mouse and human eosinophil activation and ROS production.[42] Importantly, modest in vitro death responses bring into question the mechanisms of eosinophil reductions sometimes seen following Siglec-F mAb administration in vivo.[7–16]

Previously published work has demonstrated that antibody binding to Siglec-F and Siglec-8 induces their rapid internalization in vitro and in vivo.[14,24,25] Additionally, we now demonstrate that there is complete cross-reactivity among several frequently used commercially available Siglec-F antibodies. For instance, the binding of one Siglec-F mAb clone can completely prevent the binding of another mAb clone. Taken together, these two phenomena can result in artifactual lowering of detectable Siglec-F levels on eosinophils, making the tracking of Siglec-F a fallible strategy for flow cytometric tracking of eosinophils following administration of Siglec-F antibodies. Instead, proper additional labeling and gating strategies that rely on detection of an alternative, nontargeted, specific eosinophil cell surface marker (e.g., CCR3 in WT mice) would be needed. Importantly, one of the benefits of our available Sig8+F+ mouse strain is that it enables tracking of eosinophil numbers by means of detecting Siglec-8, even when Siglec-F antibodies have been administered. Because Siglec-8 in *SIGLEC-8^{Eo}* mice is specifically expressed on the surface of eosinophils[14] and its surface expression is not affected by Siglec-F targeting (Fig. 4) it serves as an alternative flow cytometric marker to CCR3 for detecting eosinophils in murine blood and tissues. Furthermore, in the case of Siglec-8 targeting, a non-cross-reactive clone (1H10) is available that recognizes a separate receptor domain, enabling accurate tracking of Siglec-8 expression and internalization when Sigec-8 mAb is administered.

Using rat anti-mouse antibodies, whose IgG2a and IgG2b subclasses are not particularly effective at binding mouse FcγR, systemic administration of anti-Siglec-F did not result in robust reductions of eosinophil numbers after a single dose and only showed modest depletion effects after four repeated injections in a more prolonged model that resembled those used in prior publications, including those

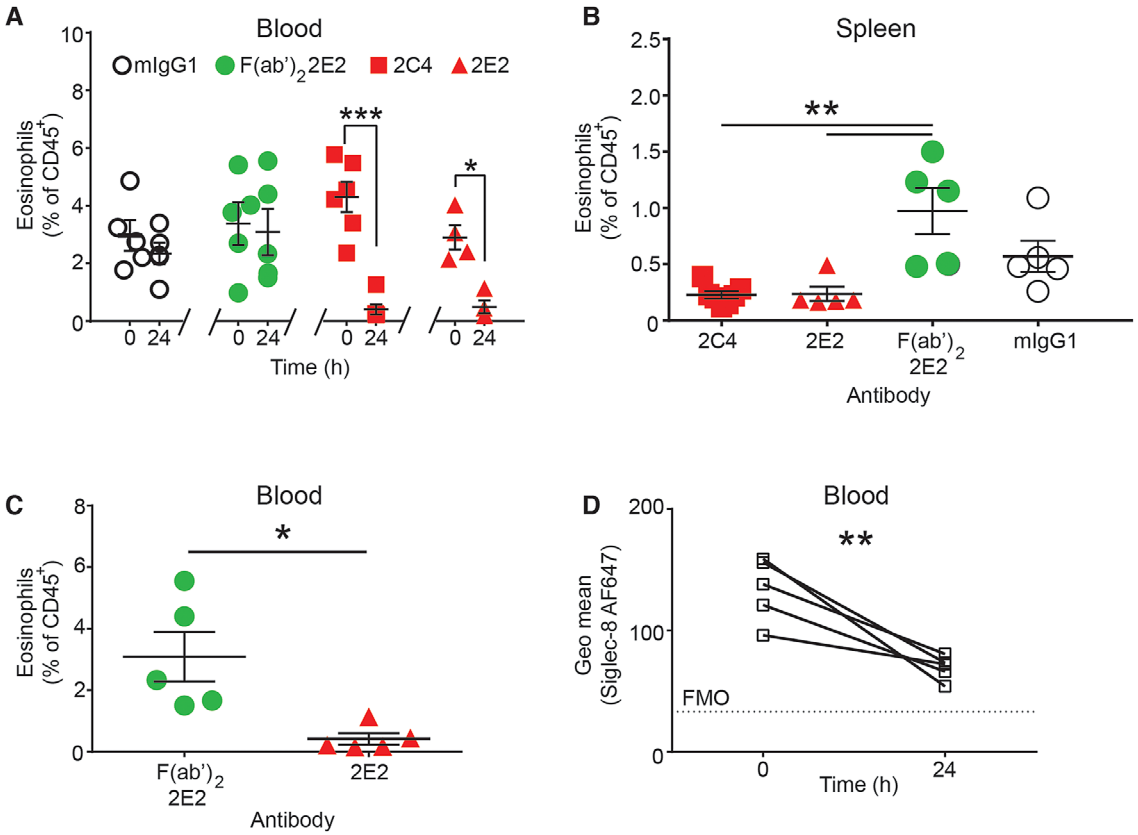

FIGURE 9 **The effect of 2C4-mediated eosinophil depletion in vivo is dependent on the Fc region of the mIgG1.** Sialic acid-binding immunoglobulin-like lectin (Siglec)-8$^+$F$^+$ mice were given a single i.p. injection of 10 μg isotype control, anti-Siglec-8 (2C4 or 2E2) or the F(ab′)$_2$ fragment of 2E2. (**A**) Eosinophil percentages (CCR3$^+$Siglec-F$^+$) were determined from blood samples at baseline and 24 h after treatment. Data from 5 to 7 biologic replicates per treatment are shown and analyzed with 2-way ANOVA followed with Sidak's posttest (baseline vs. 24 h). (**B**) Eosinophil percentages in spleen 24 h after treatment are presented. Data are analyzed by one-way ANOVA followed by Tukey's posttest. (**C**) Eosinophil percentages in blood of 2E2 and F(ab′)$_2$ 2E2 treated animals are shown and compared with unpaired Student's *t*-test. (**D**) Geometric mean of Siglec-8-AF647 (1H10 staining clone) labeling of eosinophils at baseline and 24 h following treatment with non-cross-reactive 2E2 F(ab′)$_2$. Data from 5 biologic replicates are shown and analyzed with paired Student's *t*-test. **P*<0.05, ***P*<0.01, and ****P*<0.001

that may have relied on detection of Siglec-F+ cells without additional gating.[16,27,41] This approach can be problematic as shown in Supporting Information Figure S1B. A more accurate assessment of Siglec-F antibody-mediated eosinophil reductions was accomplished with gating strategies using CCR3 and/or Siglec-8 as markers of eosinophils and by performing manual eosinophil counts by microscopy (Figs. 4 and 5).

Perhaps most remarkable in the present study was the finding that targeting Siglec-8 on eosinophils from Siglec-8$^+$F$^-$ and Siglec-8$^+$F$^+$ mice with a mouse IgG1 mAb, either alone or conjugated with the ribosomal toxin saporin, resulted in profound and sustained depletion in blood, spleen, and bone marrow of mice (Figs. 6 and 7). Siglec-8 mAb mediated-depletion was fully dependent on Siglec-8 expression on eosinophils (Fig. 8) without any detectable off-target effects against any other cells and was well tolerated by the mice. The saporin-conjugated mAb was slightly more efficacious in reducing eosinophil numbers, suggesting that mAb internalization and toxin delivery was at least partially responsible for the depleting effect. Although not compared directly, Siglec-8 antibody-mediated eosinophil depletion was faster and showed a similar or stronger effect as the use of iPHIL mice[43] or anti-IL-5 treatment (~50% reduction with 20 times the amount of antibody).[44,45] More importantly from a mechanistic perspective, by using F(ab′)$_2$ fragments of mouse IgG1 Siglec-8 mAb, we demonstrated that the Fc portion of the mAb was necessary for eosinophil depletion (Fig. 9). This strongly suggests that the greater eosinophil-depleting efficacy seen in vivo achieved via Siglec-8 targeting (with mouse IgG1) compared to anti-Siglec-F targeting (rat IgG2) is primarily, if not exclusively, due to Fc biology occurring in vivo via ADCC or ADCP (antibody-dependent cellular phagocytosis). It remains possible that some of the differing effects seen with various Siglec-F mAb clones are related to their subclass (e.g., rat IgG2a, rat IgG2b) and their engagement, or lack thereof, of FcγR on NK cells and others. Another possibility is that the 10-fold higher number of Siglec-8 receptors (≈100,000/cell) compared to Siglec-F receptors (≈10,000/cell) on the Siglec-8$^+$ mouse eosinophils (Fig. 1) contributed to differences in mAb mediated depletion, although we did not study eosinophils under inflammatory conditions, where higher Siglec-F levels can be observed. Regardless, the enhanced efficacy and consistency of eosinophil depletion in blood and tissues with Siglec-8 mAb targeting is striking.

One shortcoming of the present work is that we have not yet clarified whether NK cells or some other cell type is responsible for the Fc-mediated effects of anti-Siglec-8 eosinophil depletion. This could be tested with NK cell or FcγR deficient mice, but they would need to be bred with Siglec-8$^+$F$^+$ or Siglec-8$^+$F$^-$ mice. In order to directly compare the Fc receptor-mediated effects of anti-Siglec-F and anti-Siglec-8 mAb in vivo, we would need to create and test a mouse IgG anti-Siglec-F mAb and a rat IgG2 anti-Siglec-8 mAb for their relative depletion activity in vivo, neither of which is currently available. One prior publication used a mouse anti-mouse Siglec-F mAb to deplete eosinophils in WT mice, but even then the depletion was incomplete.[8] It is also unclear why repeated dosing is needed to maintain aggressive eosinophil depletion (Fig. 6), as we did not actually measure the half-life of any of the mouse or rat mAb used in our experiments. Finally, we have yet to explore the Siglec-8 mAb targeting on mast cell numbers in mast cell specific Siglec-8 knock in mice.

Our present study has important implications regarding past published work, where anti-Siglec-F mAb was used to "deplete" eosinophils in various in vivo models. For example, following the administration of anti-Siglec-F mAb (be it mouse anti-mouse, rat anti-mouse, or sheep anti-mouse polyclonal) the reductions of eosinophil numbers were often statistically significant, but transient or incomplete.[8] Moreover, a number of studies used Siglec-F as a flow cytometric marker to track eosinophil depletion after administration of anti-Siglec-F mAb.[27,41] As we have shown in Fig. 5 and Supporting Information Figures S1 and S2, this strategy may artifactually overestimate the extent of eosinophil depletion, whether due to Siglec-F internalization by the antibody treatment or simply antibody cross-reactivity between the antibody administered for depletion and the second anti-Siglec-F antibody used for detection ex vivo. In order to comprehensively track eosinophil depletion, methods other than Siglec-F-targeted flow cytometry should be used, such as tissue major basic protein staining, cytospins, and manual counts, or the use of a different anti-eosinophil antibody such as anti-CCR3.[9–12] Given the potential for less eosinophil depletion than anticipated, one wonders whether the biology observed in some of these prior studies might either be due to effects on other Siglec-F expressing cells (e.g., macrophages) or if Siglec-F and its ligand(s) play a direct role in the observed effects. If the latter is the case, the use of Siglec-F null mice that have normal numbers of eosinophils[22] might mirror the functional phenotype observed in anti-Siglec-F treated WT mice. Finally, the lack of effects of "eosinophil depletion" by administration of anti-Siglec-F mAb seen in some studies might simply be due to incomplete eosinophil depletion.

In summary, our study is the first to describe a novel mouse strain of Siglec-8$^+$F$^-$ eosinophils—a useful tool for studying human Siglec biology in preclinical models. We demonstrate that targeting Siglec-8 on the surface of these eosinophils in vivo results in rapid, selective, and extensive eosinophil depletion that was not simply due to Siglec-8 engagement and antibody-mediated Siglec-8 cross-linking, but instead due to mouse IgG1 Fc-mediated effects, likely ADCC or a similar Fc-dependent process. We incidentally identified potential shortcomings of using Siglec-F antibodies to deplete eosinophils in vivo, particularly with regard to solely using Siglec-F as a surface marker to detect eosinophils after anti-Siglec-F antibody administration. In contrast, by using nontargeted surface receptors such as endogenous CCR3 or transgenic Siglec-8 as specific eosinophil markers, we were able to accurately track eosinophil numbers in blood and tissues over time using flow cytometry, highlighting some important advantages of these novel Siglec-8 knock-in strains of mice.

ACKNOWLEDGMENTS

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AUTHORSHIP

E.K and B.S.B. were responsible for the conception and design and for analysis and interpretation; E.K., R.K.-B., Y.C., and J.O'S. performed the experiments; and E.K., R.K.-B., Y.C., G.M., J.O'S., and B.S.B. were responsible for drafting of the manuscript and for contribution of important intellectual content.

DISCLOSURES

B.S.B. receives remuneration for serving on the scientific advisory board of Allakos, Inc., and owns stock in Allakos. He receives publication-related royalty payments from Elsevier and UpToDate. He is a co-inventor on existing Siglec-8–related patents and thus may be entitled to a share of royalties received by Johns Hopkins University during development and potential sales of such products. B.S.B. is also a co-founder of Allakos, which makes him subject to certain restrictions under university policy. The terms of this arrangement are being managed by Johns Hopkins University and Northwestern University in accordance with their conflict of interest policies. The other authors have no competing financial interests.

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Review

An Updated Review of Pro- and Anti-Inflammatory Properties of Plasma Lysophosphatidylcholines in the Vascular System

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Abstract: Lysophosphatidylcholines are a group of bioactive lipids heavily investigated in the context of inflammation and atherosclerosis development. While present in plasma during physiological conditions, their concentration can drastically increase in certain inflammatory states. Lysophosphatidylcholines are widely regarded as potent pro-inflammatory and deleterious mediators, but an increasing number of more recent studies show multiple beneficial properties under various pathological conditions. Many of the discrepancies in the published studies are due to the investigation of different species or mixtures of lysophatidylcholines and the use of supra-physiological concentrations in the absence of serum or other carrier proteins. Furthermore, interpretation of the results is complicated by the rapid metabolism of lysophosphatidylcholine (LPC) in cells and tissues to pro-inflammatory lysophosphatidic acid. Interestingly, most of the recent studies, in contrast to older studies, found lower LPC plasma levels associated with unfavorable disease outcomes. Being the most abundant lysophospholipid in plasma, it is of utmost importance to understand its physiological functions and shed light on the discordant literature connected to its research. LPCs should be recognized as important homeostatic mediators involved in all stages of vascular inflammation. In this review, we want to point out potential pro- and anti-inflammatory activities of lysophospholipids in the vascular system and highlight recent discoveries about the effect of lysophosphatidylcholines on immune cells at the endothelial vascular interface. We will also look at their potential clinical application as biomarkers.

Keywords: lysophosphatidylcholine; inflammation; secreted phospholipases; biomarker; bioactive lipids

1. Introduction

Formerly known as lysolecithins, elevated plasma levels of lysophosphatidylcholines (LPCs) were discovered in the 1950s in certain pathological conditions and were identified as a metabolic product of snake venom [1,2]. In contrast to phospholipids, LPCs are "cone-shaped", with a polar "head" and a non-polar "tail" and therefore possess detergent-like properties [3]. The geometry of the LPC structure is also determined by the degree of saturation of the acyl chain. Combined, the saturation and length of the acyl chain is detrimental to its biophysical properties as well as its activity [4,5].

In addition to having non-specific membrane effects LPC was also reported to influence cell functions and activation status via binding to cell-specific G-coupled protein receptors (GPCRs) [6,7]. As of today, reports of LPC specifically binding to GPR119 [8], GPR40 (free fatty acid receptor 1) [9], GPR55 [9,10], GPR4 [11] as well as G2A [12] have been published. However, the study that reported binding of LPC to G2A [13] had to be withdrawn, because the authors could not demonstrate

whether LPCs mediated its effects on cells directly via the receptor or via indirect membrane effects. Moreover, GPR4 was later found to be pH sensitive (proton-sensing GPCR) and results with LPC could not be reproduced [14–16]. More recent studies support the finding that binding of LPC or its derivates to GPR119, GPR40 and GPR55 induces intracellular calcium mobilization and leads to increased glucose-stimulated insulin secretion in different cell systems [9,17]. Interestingly, the authors demonstrated modulation of GPR40, GPR55 and GPR119 receptor binding affinities using phosphorothioate modified endogenous LPC [9]. When LPC was modified with a covalently bound *P*-anisic acid at the *sn-1* position, this increased its stability and decreased its toxicity showing the potential of LPC modification as a therapeutic option, when enhanced insulin secretion is needed [17].

Concentration of LPC in plasma and body fluids is already high under physiological conditions and reaches 100–300 µM [5,18]. LPCs are bound mainly to albumin and to a lesser extent to lipoproteins [19–22]. Inflammation, cell damage and other pathophysiological conditions can profoundly alter the ratio of free to albumin bound LPC through increased production of LPC or decreased plasma levels of albumin [23–25].

Plasma LPCs are bioactive lipid metabolites of phosphatidylcholine, which are mainly produced by the action of secretory phospholipases A2 ($sPLA_2$) after removal of a fatty acid [26]. LPCs are also produced by the action of HDL-associated lecithin-cholesterol acyltransferase in the reverse cholesterol pathway [27], by the action of hepatic [28] and endothelial lipase [29] on lipoproteins as well as during lipoprotein oxidation [30]. The family of $sPLA_2$ enzymes contains 10 catalytically active isoforms (IB, IIA, IIC, IID, IIE, IIF, III, V, X), which are differentially expressed in tissues and exhibit unique substrate selectivity. Of these, $sPLA_2$-IIA is the only isoform detectable at higher concentrations in the bloodstream and is particularly elevated during inflammatory processes, triggering production of bioactive mediators of inflammation and resolution of inflammation [31,32]. One of the most well studied $sPLA_2$ cleavage product beside LPC is arachidonic acid, which can be further converted via enzymatic (cyclooxygenase-I,-II and lipoxygenase) or non-enzymatic (auto-oxidation with reactive oxygen species) metabolism into prostaglandins, lipoxins and resolvins [33].

Interestingly, most of the recent studies, in contrast to older studies, found lower LPC plasma levels associated with unfavorable disease outcomes. Decreased levels of LPC were observed in rheumatoid arthritis [34], diabetes [35], schizophrenia [36], polycystic ovary syndrome [37,38], Alzheimer disease [39,40], pulmonary arterial hypertension [41], aging [42], asthma [43] and liver cirrhosis, where they were associated with increased mortality risk [44].

2. The Complex Role of LPC in Vascular Inflammation

2.1. Postulated Pro-Inflammatory Action of LPC on Vascular Reactivity

Endothelial cell dysfunction and subsequent changes in vascular reactivity are one of the earliest changes associated with atherosclerotic cardiovascular disease [45]. Oxidized low-density lipoprotein (ox-LDL) modified by the action of secretory phospholipase was found to inhibit endothelium-dependent relaxations [46]. Similar observations were made using free LPC, which was able to produce a defect in endothelium-dependent vasomotor regulation [47]. This could be explained by the finding that both ox-LDL [48] and LPC reduce the production of prostaglandin PGI_2 in endothelial cells [49]. Subsequent research showed that reduced nitric oxide (NO) and not PGI_2 production in endothelial cells is inhibited and responsible for the defects in vasorelaxation [50,51]. Others describe the involvement of proconstricting prostanoids and superoxide anions in LPC-attenuated vasorelaxation [52]. Not only endothelium-dependent vasorelaxation is reportedly impacted by LPC, but ox-LDL enriched in LPC can also independently cause vasoconstriction [53] or potentiate angiotensin II induced vasoconstriction [54]. It must be noted that ox-LDL consists of a complex mixture of many oxidized lipids and protein oxidation products in addition to LPC. This yields inconsistent results because, as Rao et al. showed [52], the potency as well as the underlying mechanisms of LPC-dependent attenuation of vasorelaxation is heavily dependent on the LPC acyl chain length and degree of saturation.

2.2. Postulated Anti-Inflammatory Action of LPCs on Vascular Reactivity

In contrast to studies mentioned previously, reports of LPC inducing endothelium-dependent relaxation of smooth muscle cells via their non-specific membrane action have been published [55,56]. The induction of vasorelaxation was attributed to decreased endothelin-1 release, which acts as a potent vasoconstrictor [57]. Equally important was the finding that LPC induces cyclooxygenase-2 and endothelial nitric oxide synthase (eNOS) expression in endothelial cells, both of which can have vasoprotective effects either via production of prostacyclin or nitric oxide [58–61]. These observations indicate that LPC contributes to NO and endothelin-1 net balance, which regulates local vascular tone [62].

A recent study provided evidence that high-density lipoprotein (HDL) enriched with LPC (endothelial lipase modified HDL) increases eNOS activity by enriching the plasma membrane eNOS pool [63]. Moreover, LPC increased antioxidative capacity of HDL and protected LDL from oxidation [64]. In fact, contrarily to above mentioned older studies [48,49], in more recent studies LPC was shown to induce PGI_2 production in endothelial cells [65]. These reports were confirmed in vivo, where it was shown that vascular relaxation induced by LPC administration was dependent on functional and morphological integrity of the vascular wall [66]. Moreover, LPC administration increased coronary blood flow as well as decreased mean arterial pressure and total vascular resistance in rabbit [67]. An overview of potential anti-inflammatory actions of LPC at the vessel-endothelial interface is shown in Figure 1.

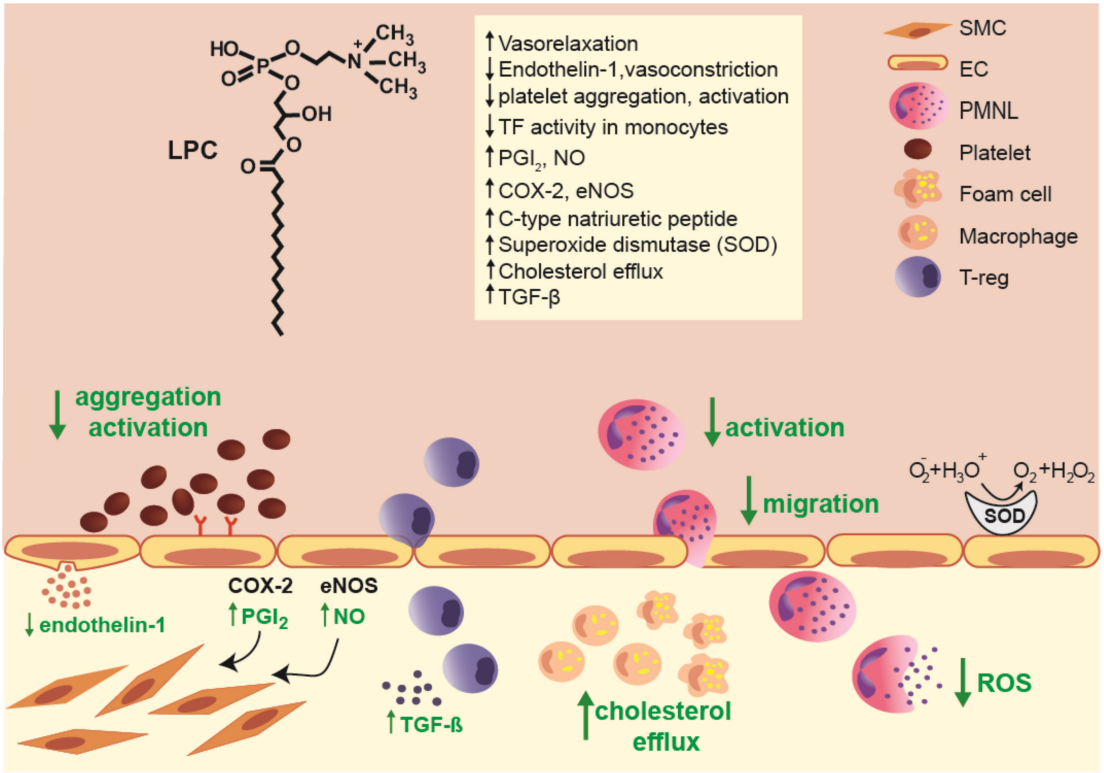

Figure 1. Overview of multiple anti-inflammatory effects of lysophosphatidylcholines (LPCs) at the vessel-endothelial interface. Abbreviations: COX-2, cyclooxygenase-2; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SMC, smooth muscle cells; TF, tissue factor; TGF-β, transforming growth factor beta; T-reg, T regulatory cells.

2.3. Investigating the Effects of LPCs on Immune Cells Involved in Vascular Inflammation

Immune cells are involved in all stages of atherosclerosis and are a major contributor of atherosclerosis progression [68,69]. While immune cells are normally present in the vascular system, their quantity and activation status are increased in atherosclerotic lesions. Through their action and production of cytokines they are able to alter the endothelial inflammatory phenotype and contribute to structural instability of atherosclerotic plaques [68,70]. Older studies reported that LPCs may directly contribute to immune cell infiltration during vascular inflammation by increasing the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) [71,72], vascular cell adhesion protein 1 (VCAM-1) [73] or P-selectin [74], the expression of damage associated molecular patterns (DAMP) and MHC-II receptors [75] and by production of chemokines such as monocyte chemoattractant protein (MCP-1), IL-8, and Chemokine (C-C motif) ligand 5 (CCL5), also known as RANTES from endothelial cells. Furthermore, LPC was found to act as a strong chemoattractant for monocytes [76,77], T cells [78] as well as natural killer (NK) cells [79], attracting them to sites of inflammation.

Similar to reports investigating the effects of LPC on vascular reactivity and endothelial activation, there are also contradictory reports on the effect of LPC on immune cells.

2.3.1. Effects of LPCs on Innate Immune Cells

Innate immune cells mainly consisting of monocytes and macrophages are the major players involved in the initiation and progression of vascular inflammation [80]. Studies performed on monocytes and macrophages on one hand show that LPC is able to activate macrophages, increase their phagocytic activity in the presence of T lymphocytes [81] and polarize them towards an M1-like phenotype [82]. Moreover, LPC was found to promote the release of arachidonic acid from monocytes [83] and regulate genes in the cholesterol synthesis pathway [84]. On the other hand, LPC was found to abrogate IL-6 release following lipopolysaccharide (LPS) stimulation [85] as well as to down-regulate platelet-activating factor (PAF) receptor expression and LPS-induced NF-κB translocation to the nucleus in monocytes [86]. A possible explanation for the seemingly confounding reports in monocytes was published by Carneiro et all where they proposed LPC serves as a dual-activity ligand molecule. LPC directly activates toll-like receptor (TLR) 4 and TLR-2-1 receptors in the absence of classical TLR ligands; however it inhibits TLR-mediated signaling in the presence of classical TLR ligands thereby acting as anti-inflammatory [87]. The surprising discovery of LPC mediated cholesterol efflux from cholesterol-laden macrophages—a well-known atheroprotective function of HDL—further suggested potent anti-atherogenic effects of LPC [88,89].

The granulocyte population of innate cells consisting of mast cells, neutrophils and eosinophils represents a smaller fraction of infiltrating immune cells during vascular inflammation [80]. However, their secreted granules contain factors capable of potentiating tissue damage and inflammation and proteases capable of modifying the surrounding extracellular matrix and locally deposited lipoproteins [90,91]. Moreover, it was shown that activated neutrophils and eosinophils form extracellular traps in the vessel wall, which are implicated in the clinical severity of the coronary lesion [90,92].

According to older studies performed in the 1980s, LPCs enhance the oxidative burst and reactive oxygen production in neutrophils [93]. However, the ability of LPC to prime neutrophils is heavily dependent on the length of the acyl chain of the molecule [93]. A more recent study performed by Lin et al. demonstrated that the most abundant LPC species in plasma (16:0, 18:0 and 18:1) in fact inhibit reactive oxygen production and activation of neutrophils. Furthermore, they demonstrated that the observed effect markedly varies on the solvent used to prepare LPC. Additionally, they demonstrated the anti-inflammatory effects of LPC in an ex vivo lung perfusion model where LPC prevented lung vascular injury mediated by neutrophils [94]. Curcic et al. likewise demonstrated that LPCs (16:0, 18:0 and 18:1) potently and rapidly inhibit neutrophil effector responses [95]. Similar results could be observed using eosinophils, where some studies found LPCs to increase cell migration [96] and

adhesion [97]. Others, using physiological LPC-albumin complexes, discovered the ability of saturated LPCs to inhibit the activation and migration of isolated human eosinophils in vitro and in vivo [98]. LPCs were also found to either potentiate mast cell activation and secretion [99] or to inhibit histamine release, serving as a endogenous membrane stabilizers [100]. Altogether, the multiple LPC-induced pro- or anti-inflammatory activities on immune cells make it difficult to draw clear conclusions at the moment. However, it appears that addition of LPC as physiological LPC-albumin or LPC-HDL complexes, as performed in more recent studies, in general demonstrate anti-inflammatory properties.

2.3.2. The Proposed Roles of LPC on the Adaptive Immune System

Adaptive immunity is defined by the presence of lymphocytes, consisting of T-cells and immunoregulatory cytokines, majorly influencing inflammation in the vascular wall and atherosclerosis disease activity and progression [101]. The involvement of T cells in atherosclerosis is supported by the discoveries that approximately 10% of T lymphocytes isolated from atherosclerotic lesions recognize oxidized LDL in an MHC-II class restricted manner [102] and that early lesions isolated from apolipoprotein E deficient mice show evidence of clonal T-cell expansion [103]. LPC, in contrast to other lysophospholipids, was found to specifically potentiate the activation of T lymphocytes, while having no effect on resting cells [104]. ROS production and chemokine receptor expression in human Jurkat T cells were similarly significantly increased upon LPC addition [105,106]. Furthermore, LPC enhanced IFN-γ secretion and gene expression in $CD4^+$ and $CD8^+$ T cells as well as increased CD40L and CXCR4 expression in $CD4^+$ T cells [107–109]. This enhanced activation of effector T cells, exhibited by increased OX40-Ligand (OX40L) and IFN-γ secretion by LPC may augment the inflammatory response in atherosclerotic lesions.

On the other hand, LPC enhances the suppressive function of human naturally occurring regulatory T cells through TGF-β production [110]. Several studies have demonstrated the atheroprotective role of T regulatory cells in murine models of atherosclerosis [111,112]. Moreover, the anti-inflammatory effects of TGF-β are supported by human clinical data showing patients with advanced atherosclerosis have less active TGF-β [113] and in experimental models, where application of anti-TGF-β blocking antibodies accelerated the development of atherosclerotic lesions [114]. Altogether LPC involvement in T-cell mediated inflammation is yet unclear. From augmenting the activation of effector T cells in the early stages of inflammation to aiding in immunosuppression by T regulatory cell activation, LPC might serve as an endogenous homeostatic factor potentiating specific T-cell responses as needed.

2.4. Additional Anti-Inflammatory Effects of LPC in Vascular Inflammation and Atherosclerosis Development

When activated, platelets adhere to the endothelial monolayer and set a variety of inflammatory mediators, promote atherogenesis and increase vascular inflammation [115,116]. Reports of LPC possessing potent anti-aggregatory effects on platelets originate from the 1960s [117]. More recent studies confirm that modification of lipoproteins by secretory phospholipases inhibit platelet activation and aggregation and identify LPC as playing an essential role in the observed effects [118,119]. Furthermore, different LPC species were found to dose-dependently inhibit platelet aggregation induced by different agonists [119]. Coagulation is a complex process [120] and LPC was found not just to inhibit platelet aggregation, but also to reduce tissue factor activity in monocytes thereby attenuating coagulation in atherosclerotic lesions [121]. Additional vasoprotective and anti-inflammatory effects of LPC include increased expression of extracellular superoxide dismutase, which is important for antioxidant capacity of vascular walls [122] and of C-type natriuretic peptide, which inhibits the migration and proliferation of vascular smooth muscle cells [123]. Finally, the ability of LPC to bind C-reactive protein (CRP) and therefore suppress its pro-atherogenic effect on macrophages and delay the progression of atherosclerosis was reported [124]. A list of potentially beneficial effects of LPCs in relation to vascular inflammation and tumor formation is given in Table 1.

Table 1. Proposed favorable functions of lysophosphatidylcholines related to vascular inflammation and tumor development.

Function/Action	Tissue/Cell Type Studied	LPC Species Examined
Inhibition of platelet aggregation [118,119]	platelets	Mixture [118]; LPC 16:0, LPC 18:0, LPC 18:1, LPC 18:2 [119]
Decreased tissue factor activity and NF-κB expression [121]; Increased expression of extracellular superoxide dismutase [122]; Suppression of IL-6 release following lipopolysaccharide (LPS) stimulation [85]; Down-regulation of platelet activating factor (PAF) receptor expression and NF-κB translocation to nucleus [86]; Decreased high-mobility group protein 1 (HMGB-1) production [125]	monocytes	LPC 16:0 [121]; Mixture [122]; Not listed [85]; Mixture [86]; LPC 18:0 [125]
Increase in cholesterol efflux [88,89]	macrophage foam cells	LPC 14:0, LPC 16:0, LPC 18:0 [88]; Not listed [89]
Vascular smooth muscle relaxation [55,56]; Decrease in mean arterial pressure and coronary, renal and total vascular resistance [67]	rabbit aortic strip [55,56] in vivo application in rabbit [67]	Mixture [55]; LPC 10:0, LPC 14:0, LPC 16:0, LPC 18:0, LPC 18:1 [56]; Not listed [67]
Suppression of endothelin-1 secretion [57]; Increased prostacyclin production [58,65]; Increase in NO production [60,61]	endothelial cells	LPC 16:0 [57,58,60]; LPC 16:0, LPC 18:1, LPC 20:4 [65]; Not listed [61]
Increased C-type natriuretic peptide expression [123]	vascular smooth muscle cells	Mixture
Promotion of dendritic cell maturation [126]; Reduction of cell motility and adhesion [127]	dendritic cells	Mixture [126]; LPC 18:0 [127]
(Potentiated) T-cell activation [104–109]; Maintenance of T-cell homeostatic turnover [128]; Enhanced suppressive function [110]	T cells [104,109] CD4+ T cells [105–108] CD8+ T cells [128] regulatory T cells [110]	Mixture [104,105]; LPC 16:0 > LPC 18:0 > LPC 14:0 > LPC 18:1 [106]; LPC 16:0 [107–109]; LPC 11:0 [128]; Not listed [110]
Increased cytotoxic activity towards tumor cells [129]	NK cells	Not listed [129]
Inhibition of histamine release [100]	mast cells	LPC 16:0
Increased bactericidal activity [130], increased reactive oxygen species (ROS) production [93,131]; Decreased ROS production [94]; Inhibition of activation and effector functions [95]	neutrophils (PMNL)	Mixture [93]; LPC 18:0 [130]; Mixture, LPC 14:0, LPC 16:0, LPC 18:0 [131]; LPC 16:0, LPC 18:0, LPC 18:1 [94]; LPC 18:0 [95]

Table 1. *Cont.*

Function/Action	Tissue/Cell Type Studied	LPC Species Examined
Inhibition of migration and effector functions [98]	eosinophils	LPC 16:0, LPC 18:0 [98]
Tumor cell apoptosis [132]; Reduction in tumor cell migration and adhesion [133]	tumor cells	Mixture [132]; LPC 16:0, LPC 18:0 [133]

3. Future Directions of LPC as a Biomarker

In contrast to older studies, most of the more recent studies using mass spectrometry to quantify LPC subspecies mainly reported that decreased plasma LPC levels are associated with unfavorable disease outcomes. Decreased levels of LPC were observed in rheumatoid arthritis [34], diabetes [35], schizophrenia [36], polycystic ovary syndrome [37,38], Alzheimer disease [39,40], pulmonary arterial hypertension [41], aging [42], asthma [43] and liver cirrhosis, where they were associated with increased mortality risk [44]. Correspondingly serum metabolic profiling of patients undergoing treatment for schizophrenia discovered an increase in LPC following successful pharmacologic intervention [134]. Similarly, plasma LPC levels are decreased in sepsis [135] and correlate inversely with sepsis mortality [136] and in-hospital mortality in pneumonia [137]. Additionally, higher blood concentrations of LPC are positively correlated with the muscle insulin sensitivity index in diabetic patients [138] and inversely correlate with impaired fasting glucose and diabetes incidence [139–142]. Importantly, a reduction in LPCs was associated with a risk of adverse outcome in chronic kidney disease patients [143].

In the context of cancer research decreased levels of certain LPCs were identified as potential biomarkers in colorectal cancer [144–146], hepatocellular carcinoma [147], ovarian cancer [148,149], cholangiocarcinoma [150], pancreatic and biliary tract cancer [151] as well as cervical cancer [152]. Of particular interest, LPC levels proved to correctly predict the recurrence of prostate cancer after surgery [153]. LPC levels are decreased in cancer, associated with weight loss and increased inflammation, where they inversely correlate with CRP levels in plasma [22]. In lung cancer, decreased LPC levels were observed in malignant compared to benign pleural effusion [154]. Furthermore, a prospective metabolomics study discovered that higher levels of saturated LPC 18:0 reduced the risk of most common cancers [155], while higher levels of 8 different LPCs correlated with lower risk of advanced stage prostate cancer [156]. To enable high-throughput and quantitative analysis of LPCs as cancer biomarkers a novel parylene matrix biochip was recently developed and validated for clinical diagnosis [157].

4. Conclusions

Research into bioactive LPCs often resulted in contradicting data, even from experiments performed in the same disease model or cell type. Older studies that suggested that LPC could negatively affect many inflammatory diseases led research to look for treatments to lower LPC levels. Plasma levels of $sPLA_2$-IIa correlate with cardiovascular risk and it is, therefore, thought to be involved in the pathogenesis of atherosclerosis [158–162]. This assumption suggests that a therapeutic intervention targeting $sPLA_2$ could lead to favorable therapeutic effects for the patients. Indeed, a large clinical trial using the $sPLA_2$ inhibitor varespladib (LY315920) targeting $sPLA_2$ groups IIa, V and X did decrease lipid biomarkers as expected, which theoretically should have translated to a lower propensity to plaque rupture in the 16 weeks following acute coronary syndromes [163]. However, the study was terminated early "for futility" after an interim analysis of the outcomes for only 212 of the 5012 randomized patients [163]. In contrast, the use of varespladib was found to increase the probability of myocardial infarction, stroke and mortality in patients with acute coronary syndrome and acute coronary disease [163]. Moreover, clinical trials using the $sPLA_2$ inhibitor varespladib found no evidence of beneficial effects for the treatment and prevention of sickle cell disease (NCT01522196),

asthma [164], rheumatoid arthritis [165] and acute coronary syndrome [163]. The use of Varespladib was even associated with higher events of the composite primary outcome (cardiovascular mortality, nonfatal myocardial infarction, nonfatal stroke or unstable angina requiring hospitalization) and the trial was terminated for potential harm [163]. Hence, a more unbiased approach is needed to understand LPC in the context of homeostasis and disease pathology.

Possible explanations for the discrepancy of data include use of different LPC species in regards to acyl chain length and saturation, which can impact their biological activity and function as shown by Frank et all in different models [52,59,65]. Since the free form of LPC is biologically most active [18], the results strongly depend on the presence and concentration of carrier proteins such as albumin or on the presence of lipoproteins in the experimental setup. Moreover, it was shown that a lot of reported pro-inflammatory effects attributed to LPC actually arise from PAF-like activity from contaminating phospholipids in some commercial preparations of LPC [166]. When these preparations were submitted to PAF acetylhydrolase or saponification (thereby targeting the susceptible *sn*-2 residue in PAF) the pro-inflammatory activity of the LPC preparations was abolished.

Another important, but often overlooked issue in older studies, is the rapid conversion of LPC in biological fluids, tissue and living cells to phospholipids [167] or to lysophosphatidic acid (LPA) through the action of autotaxin [168]. In fact, LPC is the main substrate for the production of LPA, which is able to signal through G protein-coupled receptors and is implicated in chronic inflammation, fibrotic diseases and thrombosis [169,170]. Of particular interest in this context are recently published studies that link the pro-atherogenic effects of LPC to the effect of LPA on its receptors. It may well be that LPA and not LPC is responsible for the reported effects of LPC and ox-LDL on the development of atherosclerosis. [171–175]. Small molecule inhibitors of autotaxin, a secreted phosphodiesterase that produces LPA from LPC, and thus increasing LPC levels - are a new promising therapeutic option. Autotoxin inhibitors, which are currently entering phase III clinical trials for idiopathic pulmonary fibrosis have been extensively reviewed elsewhere [176]. Results of future studies employing autotaxin inhibitors are eagerly awaited.

When interpreting the available clinical and biomarker data and many newer experimental studies, it is clear that LPCs cannot be described simply as pro-inflammatory mediators, as anti-inflammatory activities often predominate. We therefore suggest that LPCs should instead be recognized as important homeostatic mediators involved in all stages of vascular inflammation through their effect on vascular reactivity, endothelial activation and infiltration, and activation of immune cells. Like with everything in nature it is impossible to paint a completely black and white picture of LPC due to the complexity of its interactions with a plethora of immune cells and its involvement in various processes. Nonetheless, the advancement of methods designed with appropriate controls as well as the use of stable LPC analogues has clearly aided in greater understanding of LPC actions in health and disease.

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Abbreviations

eNOS	Endothelial Nitric Oxide Synthase
HDL	High-Density Lipoprotein
LPA	Lysophosphatidic Acid
LPC	lysophosphatidylcholine
Ox-LDL	Oxidized Low-Density Lipoprotein
$sPLA_2$	Secreted Phospholipase A2

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