Dissertation

Role of Short Chain Fatty Acids (SCFA) in Allergic Inflammation

Submitted by

Anna THEILER, MSc. BSc.

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Otto Loewi Research Center

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Prof. Dr. Akos Heinemann

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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 organisations 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 "Guidelines of the Medical University of Graz on Good Scientific Practice".

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Disclosures

Most data presented in this thesis have been previously published:

Anna Theiler, Thomas Bärnthaler, Wolfgang Platzer, Georg Richtig, Miriam Peinhaupt, Sonja Rittchen, Julia Kargl, Trond Ulven, Leigh M. Marsh, Gunther Marsche, Rufina Schuligoi, Eva M. Sturm and Akos Heinemann.

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All co-authors, who actively contributed to the research in this thesis agreed to the use of their data:

Thomas Bärnthaler

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

Wolfgang Platzer

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

Georg Richtig

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

Miriam Peinhaupt

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

Sonja Rittchen

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

The following persons are not listed as co-authors, but provided technical assistance during the thesis as indicated in the result section and furthermore agree to the use of their data:

Kathrin Rohrer

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

Ilse Lanz

Otto Loewi Research Center (for Vascular Biology, Immunology and Inflammation) Division of Pharmacology, Medial University of Graz, Graz, Austria

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Abbreviations

AHR	Airway hyperresponsivenss
AM	Alveolar macrophages
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra-large
cAMP	Cyclic adenosine monophosphate
C5a	complement component C5a
CCR3	C-C chemokine receptor type 3
DC	Dendritic cell
EDN	Eosinophil-derived neurotoxin
ECP	Eosinophil cationic protein
EoE	Eosinophilic esophagitis
EoP	Eosinophil lineage–committed progenitors
EPO	Eosinophil peroxidase
ERK	Extracellular signal-regulated kinases
FAS (CD95)	apoptosis-mediating surface antigen
FASL (CD95L)	Fas ligand
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

fMLP	formyl-Methionyl-Leucyl-Phenylalanine
FoxP3	Forkhead-Box-Protein P3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptor
HDAC	Histone deacetylases
HDACi	Histone deacetylase inhibitors
HDM	House dust mite
HMVEC-L	Human pulmonary microvascular endothelial cells
IBD	Inflammatory bowel diseases
ICAM-1	Intercellular adhesion molecule 1
Ig	Immunoglobulin
ILC2	Innate lymphoid cells 2
IL	Interleukin
LIR	Leukocyte immunoglobulin-like receptor subfamily B member 3
LTB_4	Leukotriene B4
LTD_4	Leukotriene D4
MadCAM-1	Mucosal addressin cell adhesion molecule-1
MBP	Major basic protein
MCL-1	Myeloid cell leukemia 1
MCP	Monocyte chemotactic protein
MCT	Monocarboxylate-transporter
MMP ($\Delta\psi m$)	Mitochondrial membrane potential ($\Delta\psi m$)

NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
OVA	Ovalbumin
PAF	Platelet activating factor
PGD_2	Prostaglandin D2
PMNL	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand-1, CD162
PI	Propidium Iodide
ROS	Reactive oxygen species
SCFA	Short chain fatty acids
Siglec	Sialic acid-binding immunoglobulin-type lectins
SMCT	Sodium-coupled monocarboxylate transporter
TNFα	Tumor necrosis factor α
Treg	Regulatory T cells
TSA	Trichostatin A
VLA-4	Very late antigen 4
VCAM-1	Vascular cell adhesion molecule 1
ZVAD-FMK	N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone

Zusammenfassung – Rolle von kurzkettigen Fettsäuren in allergischer Entzündung

Eosinophile Granulozyten sind wichtige Effektorzellen, die im Rahmen von allergischem Asthma auftreten und an der Gewebeschädigung, sowie an der persistierenden Entzündung entscheidend beteiligt sind. Eosinophilie in Blut und Sputum von Patienten ist ein Indikator für die Schwere des Erkrankungsverlaufs und wird mit Hyperreaktivität der Atemwege in Verbindung gebracht. In den letzten Jahrzehnten ist es zu einem starken Anstieg von allergischem Asthma und anderen allergischen Entzündungserkrankungen in westlichen Ländern gekommen, was in Verbindung mit der Veränderung der Lebensumstände einhergehen könnte. Dies wird vom Begriff Hygiene-Hypothese beschrieben. Außerdem wurde ein Zusammenhang zwischen der Zusammensetzung der kommensalen Darmbakterien und des Vorhandenseins von kurzkettigen Fettsäuren (SCFA) als deren Hauptmetaboliten in der Prävention von allergischem Asthma und anderen Eosinophilen-meditierten Erkrankungen festgestellt. Die wichtigsten Vertreter der SCFA, die vom Darmmikrobiom produziert werden sind Acetat, Propionat und Butyrat, welche in den Blutkreislauf übertreten können und dadurch auch auf das periphere Gewebe und besonders Leukozyten wirken können. Die Wirkung von SCFA auf Eosinophile ist bisher unbekannt. Diese Dissertation hatte es deswegen zum Ziel, die Wirkung von SCFA auf die Funktion von humanen, peripheren Bluteosinophilen, sowie in einem Mausmodell für akutes allergisches Asthma zu untersuchen.

Eosinophile exprimieren GPR43 und GPR41 auf mRNA Ebene. Propionat und Butyrat induzierten Apoptose in humanen Eosinophilen, was jedoch nicht für Acetat nachgewiesen werden konnte. Dieser Effekt war unabhängig von der Aktivierung von GPR43 oder GPR41, ging aber mit der Acetylierung von Histonen einher und wurde von dem pan-HDAC Inhibitor TSA imitiert. Propionat und Butyrat verhinderten die Adhesion von Eosinophilen auf Endothelzellen und TSA zeigte einen ähnlichen Effekt, wohingegen Acetat keinen vergleichbaren Effekt zeigen konnte. Außerdem inhibierten Butyrat und TSA die Migration von Eosinophilen. Um zu überprüfen, ob diese Ergebnisse auch *in vivo* Relevanz haben, wurde die Wirksamkeit von Butyrat in einem Mausmodell für allergisches Asthma überprüft. Butyrat konnte die Lungeneosinohpilie

unterdrücken, verhinderte die Sekretion von Type 2 Zytokinen in den Bronchoalveolarraum und verbesserte die Hyperreaktivität der Atemwege. Diese Effekte waren bemerkenswerterweise spezifisch für Eosinophile, andere Leukozytenpopulationen wurden nicht beeinflusst.

Die Ergebnisse dieser Dissertation zeigen daher zum ersten Mal, dass SCFA, vor allem Butyrat, die Eosinophilenfunktion auf mehreren Stufen hemmen: i) Adhesion auf Endothelzellen, ii) Migration, sowie iii) das Überleben von Eosinophilen. Diese Ergebnisse haben *in vivo* Relevanz, da Butyrat im Mausmodell die allergische Entzündung in der Lunge verbessern konnte. Zusammenfassend konnte gezeigt werden, dass SCFA, besonders Butyrat einen direkten Effekt auf Eosinophile haben und deswegen eine neue Behandlungsform für bestehendes, allergisches Asthma und andere von Eosinophilen hervorgerufene Krankheiten haben könnte.

Abstract

Eosinophils are forceful effector cells in allergic asthma and have been implicated in tissue damage and the inhibition of resolution of inflammation. Blood and sputum eosinophilia is an indicator for disease severity and is linked to airway hyperresponsiveness. As the prevalence of asthma and other allergic inflammatory diseases have been increasing in western countries over the last decades and is accompanied with a change of life style, the hygiene hypothesis has emerged. Additionally, the composition of commensal gut microbiota and the presence of SCFA, as their major metabolic product have been demonstrated to be crucial in the prevention of allergic asthma and other inflammatory eosinophilic disorders. Acetate, propionate and butyrate are the major components in the group of SCFA, which are produced in the gut, but can enter circulation and thereby act on peripheral tissues, especially on leukocytes. However, the effect on eosinophils is not known until now. Therefore, this thesis aimed to elucidate the effect of SCFA on eosinophil effector function of human peripheral blood eosinophils and in a mouse model of acute allergic asthma.

Eosinophils express GPR43 and GPR41 on mRNA level. Propionate and butyrate strongly induced apoptosis in human eosinophils, whereas this was not mimicked by acetate. This effect was independent of the SCFA receptors GPR43 and GPR41 but was accompanied by histone acetylation and mimicked by the pan-HDAC inhibitor TSA. Additionally, propionate and butyrate hampered the adhesion of eosinophils to the endothelium and TSA mimicked this, whereas acetate was again ineffective. Accordingly, butyrate and TSA inhibited eosinophil migration. These finding proved to be relevant *in vivo* as systemic butyrate application, blunted lung eosinophilia, reduced the secretion of type 2 cytokines into the airways and improved airway hyperreactivity as determined by invasive spirometry. Strikingly, these effects were specific for eosinophils as other cell types remained unaffected.

This thesis clearly demonstrates for the first time that SCFA, especially butyrate, hamper eosinophil function at multiple stages including (1) adhesion to the endothelium, (2) migration, and (3) survival. These effects were confirmed to have *in vivo* relevance as butyrate alleviated allergic airway inflammation in mice. Collectively, these data suggest that SCFA, especially butyrate

directly target eosinophils and could represent a novel strategy in the therapy of already established forms of allergic asthma and other eosinophil-driven disorders.

1. Introduction

1.1 Eosinophils

1.1.1 Historical background

Eosinophils were first described in 19th century as "*granule blood cells*" by Thomas Wharton Jones (2). However, it took another 20 years until Paul Ehrlich published his paper "*Beiträge zur Kenntnis der granulierten Bindegewebszellen und der eosinophilen Leukocyten*" in 1879 where the term "*eosinophil*" was coined (3). Paul Ehrlich used aniline dyes, eosin, among others, which were originally synthesized for dying organic fabrics, to stain cells and tissues, especially blood smears (2,3). He realized that, by employing those staining technics, different cell types are stained in a unique way and he was thereby able to differentiate them (2). By that, he discovered the eosinophil, as its granules were brightly stained with eosin (3). This simple but specific approach made him able to develop blood cell count methods and further enabled him to draw first conclusions about eosinophil function and their involvement in pathologies, especially in asthma and helminthic infections (3). Later on, eosinophils were connected with anaphylaxis, as massive eosinophilia was found in the lung of anaphylactic guinea pigs (3). Since then, the scientific knowledge of eosinophils has grown, and the eosinophil is now regarded as a multifunctional leukocyte (3).

1.1.2 Eosinophilopoiesis

In humans, eosinophils are derived from eosinophil lineage-committed progenitors (EoP), which are defined by expressing CD34 and interleukin-5 receptor alpha subunit (IL-5RA). They represent a direct spinoff from the common myeloid progenitors (CMP) in humans and not as originally thought, being derived from granulocyte/macrophage progenitor cells (GMP) (4,5). In mice, however, EoP directly originate from GMP (6). The differentiation of eosinophils strongly relies on a distinct cytokine mixture and a tightly regulated expression of certain transcription factors (5). The lineage commitment, terminal maturation and survival of the eosinophil is strongly dependent on the presence of IL-5 (5). However, in addition to IL-5RA expression, EoP are also positive for IL-3RA and granulocyte-macrophage colony-stimulating receptor alpha subunit (GM-

CSF-RA) (5,7). While IL-5 is largely selective on eosinophils, IL-3 and GM-CSF are involved in the differentiation of other myeloid cells (5). During eosinophilopoiesis, EoP pool is expanded by IL-3 and GM-CSF, however, the expression of IL-5RA and the presence of IL-5 is a prerequisite of EoP proliferation and terminal eosinophil differentiation (5). Interestingly, basal eosinophil differentiation in mice seemingly does not rely on IL-5 as IL-5 knock-out mice produced eosinophils, but failed to develop tissue eosinophilia (8).

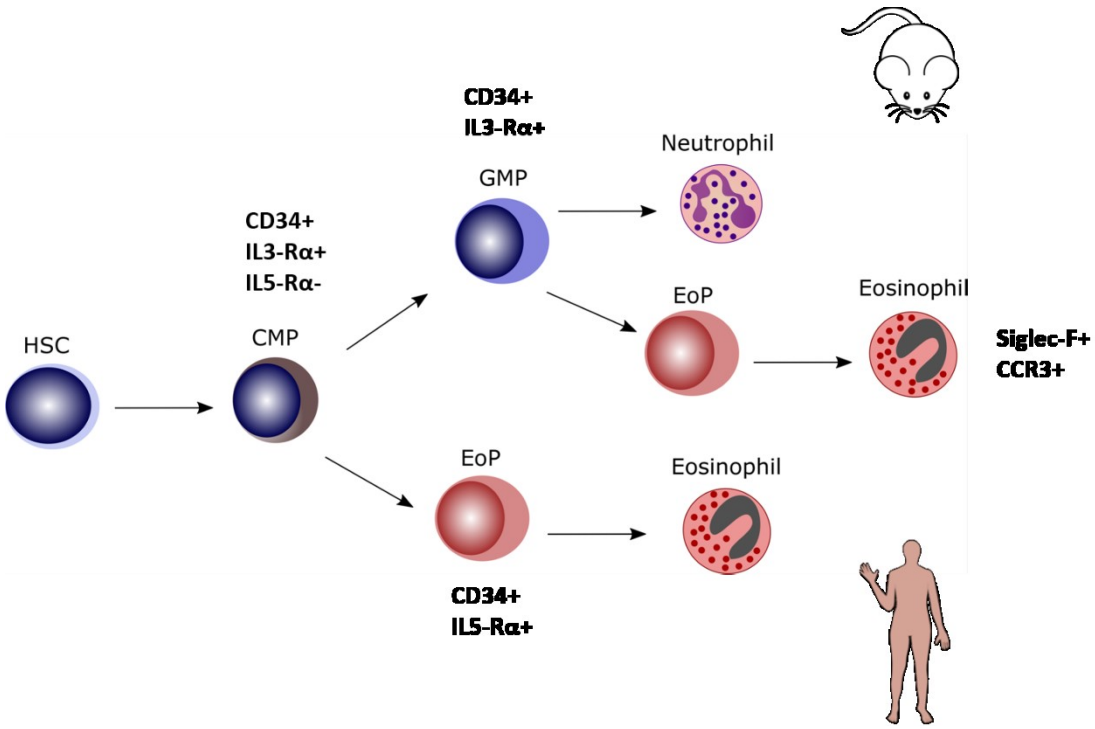

Figure 1. Eosinophilopoiesis. *Eosinophils originate from the common hematopoietic stem cell (HSC), which further differentiates into the common myeloid progenitor (CMP). In humans, eosinophil lineage-committed progenitors (EoP) arise from the CMP, whereas as in mice the EoP is derived from the granulocyte/macrophage progenitor (GMP).*

In general, eosinophilopoiesis takes place in the bone marrow, however, there is evidence that under certain inflammatory conduction (e.g. chronic allergen exposure) eosinophil progenitors can travel into the tissue and differentiate *in situ* (9–11). The differentiation of eosinophils is regulated via the co-expression of the transcription factors GATA, PU.1 and CCAAT/enhancer-binding protein (C/EBP), which is a unique combination for eosinophils, as macrophage and

neutrophil maturation is regulated by C/EBP family of transcription factors and the E-Twenty-Six (ETS) PU.1 while erythrocyte and megakaryocyte programming is dependent on GATA and friend of GATA protein 1 (FOG-1) which generally oppose each other (12). Strikingly, double GATA sites in promotor regions are unique to eosinophils, as ΔdblGATA mice are eosinophil deficient (13) and additionally, double GATA sites are found in the promotors regions of eosinophil specific genes, including C-C chemokine receptor type 3 (CCR3), Major basic protein (MBP), eosinophil peroxidase (EPO), and IL-5RA among others in humans (5,14).

1.1.3 Eosinophil survival and apoptosis

The life span of eosinophils is tightly regulated, and under physiological conditions eosinophils undergo spontaneous apoptosis in the absence of pro-survival signals, such as IL-5, IL-3 and GM-CSF (15). In circulation, eosinophils reside approximately 24 hours (16) before becoming apoptotic, which is most likely regulated by the B-cell lymphoma 2 (BCL-2) family member Bcl-2-associated X protein (BAX) (17) as peripheral blood eosinophils express high levels thereof (18). Tissue eosinophils are more persistent, as they can survive several days (19). In allergic inflammation the life span of eosinophils is enhanced by the presence of the aforementioned cytokines, which in turn promote the accumulation of eosinophils in the airways and their persistence (19,20). As mentioned above, IL-5, IL-3 and GM-CSF represent the most important survival signals for eosinophils (21) as they potently enhance eosinophil survival even with concentrations in the femtomolar range (22). Cytokine binding is achieved by the unique alpha chains of those receptors, however, they share a common β subunit, which enables receptor signaling (5). Signaling pathways triggered by IL-5/GM-CSF in eosinophils enclose Lyn/Syk–Ras–Raf-1–extracellular signal-regulated kinases (ERK) 1/2, Jak2-STAT1, and PI3K-Akt (18,23–28). In mouse eosinophils, IL-5 inhibited apoptosis dependent on PI3K, B-cell lymphoma-extra-large (BCL-XL) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) signaling (29). In human eosinophils, IL-5 counteracts the pro-apoptotic protein BAX, as it prevents the translocation of BAX into the mitochondria (24) which in turn inhibits mitochondrial membrane potential (ΔΨm) loss, and the release of cytochrome C, which causes pore formation and activation of the apoptotic machinery (17). Additionally, IL-5 and GM-CSF where shown to increase BCL-XL expression (18), whereas myeloid cell leukemia 1 (MCL-1), another BCL-2

family member of anti-apoptotic proteins expressed by eosinophils is only stabilized on basal expression levels by IL-5 (30).

In eosinophils, apoptosis induction is achieved by two pathways, the intrinsic (mitochondrial) and the extrinsic (receptor-mediated) apoptosis pathway (23). The intrinsic apoptosis pathway, which includes the spontaneous apoptosis in eosinophils, is tightly regulated by pro- and anti-apoptotic BCL-2 family members. Untreated eosinophils express high levels of the pro-apoptotic protein BAX (17), which is able to translocate into mitochondrial membrane, induces the formation of a pore complex, which results into loss of the mitochondrial membrane potential ($\Delta\Psi m$), cytochrome C release and the activation of effector caspases 3 and 6 (17,23,24). The extrinsic apoptosis pathway is initiated by ligand binding on death receptors, such as the apoptosis-mediating surface antigen (Fas, CD95) which is highly expressed on eosinophils (30). However, Fas-induced apoptosis seems to play a lesser role, as antibody-induced Fas activation had only a minor effect on eosinophil apoptosis *in vitro* (31). However, inhibition of apoptosis induction by using neutralization antibodies for the Fas ligand (CD95L) increased airway eosinophilia in an asthma model (23,32), which suggests a role for receptor-mediated apoptosis in activated eosinophils *in vivo* (15). Similar to the intrinsic pathway, effector caspases are recruited and the apoptotic machinery is induced (23). Apoptosis induction on eosinophils can be achieved by glucocorticoids (33), histone deacetylase (HDAC) inhibitors (34) and Sialic acid-binding immunoglobulin-type lectins (Siglec) 8 (35) among others.

1.1.4 Eosinophil recruitment

Eosinophil accumulation in the tissue is dependent on an interplay of cytokines (mainly IL-5), chemokine gradients, especially eotaxins, adhesion molecules and their respective receptors (36). Under steady state conditions, most of the eosinophil pool dwells in the gastrointestinal tract, with exception of the esophagus (37). However, in addition to gastrointestinal tract, eosinophils are also found in the thymus and other lymphoid organs, uterus and mammary gland (36,38). Under inflammatory conditions, e.g. allergic responses or helminth infections, eosinophils can be recruited to peripheral organs, e.g the lung (36).

Eosinophil adhesion to the endothelium

Rolling. A crucial step in eosinophil trafficking is their extravasation from blood flow into the homing tissue. Circulating eosinophils become loosely associated with the endothelium by selectins, which are glycoproteins, consisting of multiple domains (39). Purified eosinophils express L-selectin (CD62L), which has a pivotal function in the eosinophil rolling on the endothelium under flow conditions (39,40). Endothelial cells express ligands for L-selectin, including mucosal addressin cell adhesion molecule-1 (MadCAM-1) and CD34 among others (36). In addition to L-selectin, eosinophils express CD162 (P-selectin glycoprotein ligand-1, PSGL-1), which binds to P-selectin (39). Eosinophils from allergic-asthmatics have increased levels of PSGL-1, which results in augmented adhesion on IL-4 stimulated endothelium (41). Neutrophils display similar P-selectin binding properties as eosinophils (39). Interestingly, this was not the case when endothelial cells were stimulated with IL-13: Eosinophils were attached to the endothelium in a P-selectin dependent manner, whereas this was not observed for neutrophils (42).

Firm adhesion to the endothelium. The next step of eosinophil extravasation is the firm adhesion to the endothelium (i.e. arrest), which is mediated via integrins (39). Integrins are also transmembrane glycoproteins which consist of two, non-covalent chains: namely α and β (36). Both subunits are involved in the adhesion of eosinophils to ligands on other cells or matrix proteins (39). However, only the α subunit contributes to the binding affinity on eosinophils (43). The following integrins are expressed by freshly isolated eosinophils: α4β1, α6β1, αLβ2, αMβ2, αXβ2, αDβ2 and α4β7. α4β1 (very late antigen 4, VLA-4) mediates the binding of eosinophils to vascular cell adhesion molecule 1 (VCAM-1) expressed by endothelial cells (39) and is crucial for eosinophil migration to the lung or the conjunctiva during allergic inflammation (44,45). α6β1 acts as receptor for the extracellular matrixprotein laminin on human eosinophils (46), whereas αLβ2 (leukocyte function associated antigen-1, LFA-4), αMβ2 (Macrophage-1 antigen, MAC-1) and αXβ2 bind to intercellular adhesion molecule 1 (ICAM-1) with high affinity (36,39) and have been implicated to be crucial for eosinophil transmigration through the endothelium (47). Additionally, αDβ2 binds to VCAM-1 and ICAM-3 (48), whereas α4β7 interacts with MAdCAM-1 and mediates the eotaxin-1 induced eosinophil recruitment into the small intestine (36,37). Although the expression of some integrins and other adhesion molecules is increased on eosinophils from allergic donors and can be further stimulated via activation of the cells *in vitro,* adhesion is

primarily regulated by conformational changes, which affect integrin affinity (39). Strikingly, the affinity of integrins can be regulated by cytokines and chemokines, but also by the adhesion process itself (49). Interaction of integrins with their receptors such as VCAM-1 or ICAM-results in activation of intracellular signaling events. This process is called outside-in signaling (50). In the case of eosinophils this affects function and survival (39). Interaction of α4β1, αMβ2, and α4β7 with their counterparts enhance eosinophil survival in an autocrine/paracrine fashion via GM-SCF (39,51–53). Additionally, integrin binding induces degranulation, superoxide production and the release of cytokines and chemokines, which is mostly accounted to αMβ2 signaling (39,54,55). The activation of β1 and β2 integrins seems to be differentially regulated (39) as eotaxin-2 increases the affinity of β1 integrins (56), whereas β2 integrins are triggered by IL-5 (57), leukotriene D4 (LTD_4) (58) or histamine (59). Importantly, these processes are required for firm adhesion to the endothelium and are a prerequisite for transendothelial migration of eosinophils and thereby extravasation into the target tissue (39).

Transendothelial diapedesis and chemotaxis to the inflammatory site. As noted above the interaction of β1 integrins with VCAM-1 and β2 with ICAM-1 is crucial for the successful transmigration of eosinophils (39). Interestingly, α4β1/VCAM-1 interaction seems to be unique for eosinophils and might display a pre-selection step for eosinophils over other leukocytes for transendothelial migration under certain circumstances (60). Binding of α4β1 to VCAM-1 on endothelial cells induces the production of superoxide anions in endothelial cells (60) which in turn acts on the endothelium itself as it triggers gap formation by changes of the actin cytoskeleton (61,62) and promotes β2 expression on eosinophils (63), which is required for successful transmigration (47). Hence, the α4β1/VCAM-1 interaction does not only enable firm adhesion of eosinophils to the endothelium but also facilitates transmigration (63). Th2 cytokines like IL-4 and IL-13 further promote the extravasation of eosinophils as they increase the expression of P-selectin and VCAM-1, and further trigger the release of eotaxins from the endothelium (60). This is not only a requirement for eosinophil chemotaxis into the tissue but also increases eosinophil selectivity as it binds to C-C chemokine receptor type 3 (CCR3) expressed on endothelial cells (60,64) and induces the expression VCAM-1 and ICAM-1, but interestingly, inhibits the tumor necrosis factor α (TNF-α) triggered release of the neutrophil chemokine IL-8 (64). Furthermore,

eosinophil diapedesis through the basal membrane induces the expression of CD44, a receptor for hyaluronic acid, which facilitates the binding to tissue matrix (65).

1.1.5 Chemotaxis and eotaxins

Eosinophil recruitment to the inflammatory site is a multistep process, which is orchestrated by cytokines, and chemokines, with special focus on eotaxins, and is mediated by adhesion molecules (66). Eotaxins are a family of CC-chemokines, which specifically target eosinophils and enable them to migrate towards an inflammatory site (66). Eotaxin-1 (CCL11) was first purified from bronchoalveolar lavage (BAL) fluid of allergen-challenged guinea pigs and was shown to activate guinea pig and human eosinophils *in vitro* (67,68). Additionally to eotaxin-1, eotaxin-2 (CCL24) (69) and eotaxin-3 (CCL26) (70) have been described. Chemokine signaling is mediated by G protein-coupled receptors (66). On eosinophils, CCR3 is the most important chemokine receptor and is activated by all 3 eotaxins in humans (66). Other eosinophil chemokines include monocyte chemotactic proteins 2, 3 and 4 (MCP-2-4) and RANTES (CCL5), which all bind to CCR3, platelet activating factor (PAF), complement component C5a, leukotriene B4 (LTB_4), formyl-Methionyl-Leucyl-Phenylalanine (fMLP) (66) and prostaglandin D_2 (PGD_2) (71–73). In a model of allergic inflammation, eotaxin-1 deficient mice had 70 % less BAL eosinophils 18 h after ovalbumin (OVA) challenge when compared to wildtype, however, this effect was restored after 48 h, suggesting an involvement of eotaxin-1 in the early recruitment of eosinophils (74). Eotaxin-2, however, has been implicated to be involved in the late phase of eosinophil recruitment as demonstrated by eotaxin-2 deficient mice (75). Accordingly, CCR3 deficiency abrogated the eosinophil recruitment into the BAL of OVA challenged mice (75).

1.1.6 Inhibitory receptors on eosinophils

In addition to activating receptors, inhibitory receptors have been described for eosinophils which should prevent an overshooting immune reaction. These include FcRII-b, CD300a, CD300LF, Siglec 7-10 and LIR-3 for human eosinophils and CD33, Siglec F, leukocyte immunoglobulin-like receptor subfamily B member (LIR) 3 and LIR-4 on mouse eosinophils (76). However, only a few have been studied so far, including Siglec-8 and CD300a (76). Siglec 8 induces apoptosis in eosinophils in a caspase dependent manner even in the presence of IL-5 or

GM-CSF (77). Priming of eosinophils with IL-5 before Siglec-8 activation caused an even stronger apoptosis-inducing effect than Siglec-8 crosslinking alone (35). A recent study proposed a role for Siglec-7 in inhibiting eosinophil activation but not in promoting eosinophil apoptosis (78). CD300a is also known to interfere with eosinophil survival (76). Opposed to Siglec-8, this seems not to happen actively via apoptosis induction but merely by inhibiting the survival promoting signaling of IL-5 and GM-CSF (76,79). However, also activating receptors seem to be capable of sending inhibitory signals: Binding of monokine induced by interferon γ (MIG, CXCL9) on CCR3 has been reported to inhibit the recruitment of eosinophils in mice (80).

1.1.7 Eosinophils in asthma

Asthma is a chronic inflammatory disease of the airways which is defined by various symptoms including bronchial/airway hyperresponsiveness, chronic airway inflammation, cough, mucus hypersecretion and airflow obstruction (81). Up to 300 million people worldwide suffer from asthma (82). Allergic asthma is the predominant form in children and in around 50 % of the adults and is accompanied by allergic sensitization to inhaled or ingested allergens including pollen, fungal spores, house dust mite (HDM), animal dander or peanuts (82). Allergic sensitization is defined by the occurrence of serum IgE antibodies and or a positive skin prick test (82).

Eosinophils and asthma have been linked since the early 20th century. Lungs of patients who died from status asthmaticus showed mostly eosinophilic infiltrates in the airways and later on eosinophils were connected with clinical features of asthma (81). Asthmatics show increased blood and sputum eosinophilia in most cases, which is linked to disease severity (83) and also airway hyperresponsiveness (81). The latter is mostly accounted for the release of eosinophilic granules, which contain cytotoxic proteins including the major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and the eosinophil derived neurotoxin (EDN) (81), which are found in increased levels in BAL fluid of asthmatics and are responsible for eosinophil-induced epithelial tissue damage in the airways (81). Eosinophils also produce lipid mediators like leukotrienes LTD_4 and LTC_4, which both have been reported to be involved in increased bronchoconstriction and endothelial permeability in asthma (84). Not only Th2 cells or innate lymphoid cells (ILC) 2, but eosinophils themselves are capable of secreting Th2 cytokines and additionally also GM-CSF, IL-3 and TGF-β among others (39,81). Secretion of these factors

can increase the survival of eosinophils (85), promote their adhesion and migration into the tissue (60,86) and further contribute to airway remodeling (87,88). Accordingly, eosinophils are potent effector cells and significantly shape the pathology of asthma.

1.1.8 Eosinophil targeted therapy

The role of eosinophils in the pathogenesis of asthma is well established. The first-line treatment of asthma includes inhaled bronchodilators and corticosteroids, which directly counteract airway obstruction and inhibit the inflammatory cascade in asthma (89). However, some patients require a different therapeutic approach including leukotriene antagonists or theophylline (90). The last resort in asthma therapy was for a long time systemic application of corticosteroids (90). Due to their side effects, more selective therapeutic strategies were needed to treat especially severe uncontrolled asthma (89). Given that the eosinophil is a prominent effector cell in asthma, eosinophil targeted therapy has been in the focus (91). Currently, three therapeutic monoclonal antibodies, which either directly target IL-5 (mepolizumab and reslizumab) or the IL-5Rα subunit (benralizumab) are available (91). Both, mepolizumab and reslizumab improved lung function and reduced exacerbations (91–93). Moreover, a prednisone-sparing effect was demonstrated for mepolizumab (94). Although benralizumab also induces antigen antibody-dependent cellular cytotoxicity (ADCC) and in turn eosinophil apoptosis, its therapeutic effects were comparable to mepolizumab and reslizumab (91). Additionally, anti-immunoglobulin (Ig)E (Olamizumab) and anti-IL-4 receptor (Dupilumab) antibodies are approved for asthma treatment (91).

1.2 The biology and pharmacology of short chain fatty acids (SCFA)

Short chain fatty acids (SCFA) are the major product, which results from fermentation of dietary fibers in the colon by anaerobic commensal bacteria. This is indicated by germ free mice, which lack SCFA (95,96). Chemically, SCFA belong the group of saturated, aliphatic fatty acids, which contain 2-5 carbon atoms, comprising acetate (C2), propionate (C3), butyrate (C4) and valerate (C5) (97). However, the most abundant SCFA in the distant colon are acetate, propionate and butyrate, with concentrations of 60 mM, 20 mM and 20 mM (98), respectively. SCFA are rapidly absorbed by the enterocytes, enter the systemic blood circulation via the portal vein and are further distributed to peripheral tissues (98). A considerable amount of SCFA is taken up by the liver, which is also the primary site of SCFA metabolism (99). In the blood, the SCFA concentration is relatively low (low μM range), compared to the concentrations in the large intestine, with acetate found in highest amount (99). Butyrate serves as a predominant energy source for the enterocytes (100–102); therefore, almost none is secreted into circulation and thus detectable in the blood (99). Comparable to acetate, the largest portion of propionate vacates the intestine and enters the liver via the portal vein, where it is metabolized (97,99). Although bacterial colonization in the human lung is evident (103,104) and it was previously shown that bacteria obtained from the lung are capable of SCFA synthesis (105), SCFA concentrations in the lung are elusive. A recent study reported that the esophagus harbors bacteria recognized to be butyrate producing (106). Plus, in sputum of cystic fibrosis patients, SCFA reached concentrations up to 2 mM (107). Strikingly, SCFA can be detected in feces of neonates already 6 h after birth, suggesting a rapid bacterial colonialization (97,108).

SCFA exert various biological effects on the immune system, the gut, in metabolism and the nervous system as well as associated pathologies like asthma, inflammatory bowel diseases or cancer (97,109,110). These effects are mediated by the activation of G protein-coupled or the inhibition of histone deacetylases (HDAC) (97,109,110) and are described in more detail in the following.

1.2.1 SCFA production

SCFA are metabolized from indigestible polysaccharides and oligosaccharides, which are in general termed dietary fibers (97,111). The group of dietary fibers consist of large variety of carbohydrates including i) An insoluble group like cellulose and hemicellulose, ii) a soluble group including lignin, and finally iii) indigestible oligosaccharides, which are represented by inulin and resistant starch (97,111). Dietary fibers are contained in large amount in plants, especially in vegetables, fruit, legumes and grain (111). The recommended fiber intake is 25-38 g per day, however the average fiber intake of the American population is with 16 g per day way below the recommended amount (109,112).

Since humans are not equipped with the required enzymatic machinery to metabolize dietary fibers (97,113), these are transported into the large intestine, where they are further broken up by the gut microbiome in order to generate SCFA and energy in form of ATP (97,114). Besides others, SCFA are the main product of fiber fermentation and are predominantly produced by Bacteriodes, Firmicutes, Lachnospiraceae, and Ruminococcaceae, among others (110,115,116). However, the gut microbiome itself is shaped by diet (117,118), as high fiber diet fosters bacteria, which are associated with SCFA formation, whereas high fat and high sucrose diet can lead to their elimination (117,119). Additionally, butyrate can be directly taken up with butter and yogurt (120).

1.2.2 Transport of SCFA

After synthesis, SCFA are taken up by colonocytes, which express two types of SCFA transporters: the sodium-coupled monocarboxylate transporter 1 (SMCT-1, SLC5a8) and the monocarboxylate transporter-1 (MCT-1, SLC16a1) (121). Additionally, these transporters are found on immune cells, including lymphocytes and monocytes (122). Peripheral blood granulocytes, containing eosinophils and neutrophils, have been shown to express MCT-1 (122). Additionally, eosinophils express MCT-4 (SLC16a3) (123) which has been reported to be a butyrate transporter in a rat epithelial cell line (124). In contrast, SMCT-1 and SMCT-2 (SLC5a12), which have also been reported to transport propionate and butyrate (125,126) do not seem to be expressed by peripheral blood eosinophils (123). SCFA are transported by SMCT-1 with the following affinity: butyrate >propionate>acetate (127).

1.3 SCFA receptors, signaling and biological roles

1.3.1 SCFA receptors

SCFA predominately activate two G protein-coupled receptors, namely GPR43 and GPR41 (128–130), which are also termed free fatty acid receptor 2 (FFA2) and FFA3 (131), respectively. Interestingly, butyrate is also able to activate GPR109a, the natural receptor for niacin (132), which is important in an immunological context (133), but not discussed into depth here since GPR109a is absent on eosinophils (134).

Free fatty acid receptors (GPR43 and GPR41)

GPR43 and GPR41 were deorphanized in 2003 by three independent groups (128–130). Brown and colleagues utilized recombinant cell systems in order to prove that GPR43 is activated by SCFA (129). Le Poul et al. demonstrated that GPR43 receptor activation triggered intracellular Ca^{2+} release and also utilized a cyclic adenosine monophosphate (cAMP) release assay (128), whereas Nilsson and colleagues also employed a Ca^{2+} mobilization assay as well as a reporter gene-based system (130). Given the high sequence homology between GPR43 and GPR41 it was discovered that SCFA also activate GPR41 (128,129,135).

GPR43 (FFA2). GPR43 receptors show wide tissue distribution but are most pronounced on immune cells, including neutrophils, monocytes, lymphocyte, but also eosinophils (128–130,136). This is also reflected by its high expression by spleen and bone marrow at the mRNA level, which is most probably due to the present immune cells (128). Besides immune cells, GPR43 is found on the heart and skeletal muscle (130), the gut and adipose tissue (110). Activation of GPR43 can result in two different signaling events as GPR43 can be coupled on the one hand to $G_{q/11}$ subunit, which results in intracellular Ca^{2+} release (128,130) and on the other hand to an inhibitory G_i subunit which causes cAMP reduction (128). The rank order of potency of SCFA in terms of GPR43 activation is acetate=propionate>butyrate with $EC_{50} \sim 250\text{-}500\ \mu M$ for acetate and propionate (110,128).

GPR41 (FFA3). GPR41 on the other hand is mainly expressed on the epithelium of the colon but has also been reported to be expressed in spleen, pancreas and the lung (129,137) but to be absent on eosinophils at the protein level (123). However, the expression of GPR41 on adipocytes remains somewhat controversial (129,138–140). Additionally, GPR41 has recently

been reported to be expressed on a subtype of Th2 cells in eosinophilic esophagitis (EoE) (141). Opposite to the GPR43, which can produce different signaling events (128,130), GPR41 has been only reported to couple to pertussis sensitive G_i proteins (128). The GPR41 is activated by SCFA in the following rank order propionate>butyrate>>acetate with a EC_{50} ~12-274 μM for propionate (110,128).

1.3.2 Histone deacetylases (HDACs)

SCFA have been reported to act as HDAC inhibitors, with butyrate being the most potent compared to propionate or acetate (142–144). HDACs are enzymes which modulate the acetylation status of the chromatin and thereby regulate transcription (145). Hence, HDACs are mainly situated in the nucleus (class I), but there are certain groups, which can shift between nucleus and cytoplasm (class IIa) and a group, which act as scaffold proteins and are solely located within the cytoplasm (class IIb) (145). HDACs are involved in regulating a wide spectrum of cellular responses, namely adhesion, migration and survival (1,34,146–148). Importantly, the HDAC inhibitor trichostatin A (TSA) was reported to induce apoptosis in eosinophils (34) and furthermore, HDAC inhibitors were shown to be beneficial in mouse models of allergic airway inflammation (149,150).

1.3.3 Biological outcomes of SCFA action

Numerous biological functions of SCFA are evident due to the wide expression pattern of SCFA receptors (128–130,136,137) and their inhibitory capacities on HDACs (142–144). Therefore, SCFA have been implicated to have a function in regulating energy metabolism, cancer development and processes of the nervous system (97,109,110). In the present thesis, however, I will focus on SCFA in inflammation and immune cells, with particular focus on allergic airway inflammation.

Cellular responses

Eosinophils. Butyrate is frequently used to induce an eosinophilic phenotype from HL-60 leukemic cell lines (151–153). Additionally, butyrate was shown to cause IL-5RA induction in HL-60 cells (154). Although, GPR43 has been reported to be expressed on transcriptional level by eosinophils (155), the effect of SCFA on a functional level is still unclear.

Neutrophils. Peripheral blood neutrophils were among the first cell types which were engaged for functional assays under SCFA stimulation (128,156,157). This is no surprise given that GPR43 is highly expressed on neutrophils (128,129,156). Stimulation of peripheral blood neutrophils with acetate and propionate triggered intracellular Ca^{2+} release, whereas butyrate was ineffective (128,156). Furthermore, it was reported that acetate and propionate cause a rearrangement of the actin cytoskeleton (157) and also induce neutrophil chemotaxis (128). The literature regarding SCFA and ROS production in neutrophils is quite controversial as ROS inducing (155,158–160) and inhibitory effects of SCFA have been proposed (161–163). With regards to neutrophil survival, propionate and butyrate have been shown to induce apoptosis in neutrophils in a HDAC dependent fashion, whereas acetate was ineffective (146). However another group reported an apoptosis inducing potential of acetate on neutrophils, but used mouse neutrophils and higher acetate concentrations than the aforementioned study (164).

T cells. SCFA, in particular butyrate were reported to influence the fate of T cells. Numerous studies have provided evidence that SCFA promote tolerance by induction of regulatory T cells (Treg) (165–167) and the production of IL-10 (165,168). It was previously shown that SCFA regulate the number of colonic Treg as wells as their function in GPR43 dependent manner (165). Furusawa and colleagues reported that butyrate treatment resulted in increased acetylation of the forkhead-box-protein P3 (FoxP3) promotor and thereby enhanced the formation of colonic Tregs *in vitro* and *in vivo* (166). *In vitro* assays revealed that SCFA induced effector T cell function and regulatory T cell induction by the repression of HDAC and activation of the mTOR-S6K pathway (142).

Epithelium. As mentioned earlier, SCFA, especially butyrate is the major energy source for colonic epithelial cells and thereby involved in their homeostasis (100–102). *In vitro* studies using epithelial cells lines but also primary material suggested that SCFA strengthen the epithelial barrier function, via different pathways (102,169,170).

Short chain fatty acids in inflammation

As described above in this thesis, SCFA are potent regulators of inflammatory cells by regulating their numbers, activation and mediator release. In most studies, SCFA have been

attributed as anti-inflammatory mediators, particularly because of their ability to inhibit HDAC (142,143,146). Importantly, SCFA are crucial factors for maintaining gut homeostasis by strengthening of the epithelial barrier (169,170), induce tolerance by formation of Tregs (165–167), and the induction of IgA production (171), which might prevent autoimmune responses. Hence, loss of SCFA producing bacteria or decreased levels of SCFA in the gut can impair gut homeostasis and can promote inflammatory processes.

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease are characterized by infiltrating immune cells in the epithelium and gut leakiness (96,172). Importantly, it has been previously reported that IBD patients have lower concentrations of SCFA in feces (173,174) and also a lower representation of SCFA producing bacteria when compared to healthy controls (96,172). Additionally, data from animal studies provide evidence for the beneficial effects of SCFA in IBD (133,136,175), inflammatory arthritis (155), and food allergies (176,177). Oral butyrate application improved DSS induced colitis in mice (175). Similar results have been reported for acetate, which improved DSS colitis in a GPR43 (136) or GPR43 and GPR109a dependent fashion (133). In a model of inflammatory K/BxN serum induced arthritis, GPR43 deficient mice showed aggravated and non-resolving joint inflammation when compared to wildtype littermates (136). Eradication of commensal bacterial led to increased food allergen sensitization in mice (176). Additionally, Tan et al reported that a high fiber diet increased SCFA production, which protected against food allergy in a GPR43 and GPR109a dependent manner (177). Interestingly, intranasal butyrate application reduced the OVA induced allergic rhinitis in mice by reduction of IgE secretion, the reduction of goblet cells in the nasal mucosa, as well as a reduction of IL-4 levels (178). Strikingly, GPR41 was found on a subset of Th2 lymphocytes in patient samples of EoE and was significantly increased in patients with active disease (141). *In vitro* assays revealed that GPR41 is upregulated by IL-4 in anti-CD3-CD28 activated blood lymphocytes from EoE patients (141). Therefore, the authors concluded that GPR41 might play a pro-inflammatory role in EoE (141).

The importance of SCFA on the prevention of allergic airway inflammation has been reported (115,118,144,179,180). The latter is of utmost importance for the present thesis and will exclusively discussed in following section.

1.4 Short chain fatty acids in allergic inflammation

In the past few decades, a dramatic increase of incidence of asthma and allergies have been observed in western countries (181). This has been partially explained with the hygiene hypothesis, which outlines that reduction family size and improvement of personal hygiene, as well as the increased use of antibiotics leads to a reduced exposure to infectious agents, which thereby fosters an inappropriate immune response (109). Recently, there has been more focus on changes of diet in western countries, which in turn also affects the gut microbiome composition (117,119,182).

In general, a reduction of fiber consumption is evident in western countries (110), which are, when fermented by the commensal bacteria, the major source of SCFA. Strikingly, the microbiome from children from rural Burkina Faso, who traditionally consume a diet high in fiber, was rich in bacteria associated with SCFA production (117), whereas these were either reduced in abundance or completely absent in children from the European Union (EU) (117). Interestingly, this was also reflected by the increased amount of SCFA present in the feces from Burkina Faso children when compared to children from the EU (117). Noteworthy, the prevalence of asthma is remarkably low in Africa when compared to western countries (181). Another study outlined that infants, who showed a reduction of certain bacterial genera in the gut in the first three months of life were at higher risk in developing asthma (183). Strikingly, children with allergic sensitization had a microbiome in infancy, which lacked genes, encoding enzymes crucial for carbohydrate breakup and butyrate formation (184).

Adult asthmatics, who received a single soluble fiber meal, showed improved lung function and reduced inflammatory cells in sputum when compared to controls (185). Interestingly, there were no changes in eosinophil counts, but in exhaled nitric oxide (eNO), which the authors described as marker for eosinophils (185). Additionally, GPR43 and GPR41 expression was increased on sputum cells from patients who received soluble fibers (185). Strikingly, in an Australian cohort, severe asthmatics consumed significantly less fiber than healthy controls (186). This highlights the importance of an interplay between nutrition, gut microbiota composition and secreted metabolic products, especially SCFA, in the pathogenesis of asthma.

In order to pin point possible beneficial effects of gut microbiota, fiber-rich diet or SCFA in particular on asthma and allergic airway disease, animal models have been employed (115,118,144,179,180). Strikingly, germ-free mice showed exaggerated AHR, lung eosinophilia and polarization to type 2 immune response after OVA exposure, when compared to mice housed under specific-pathogen-free (SPF) conditions (179). This was prevented, when mice were recolonized with specific germs before the start of the OVA protocol (179).

Gollwitzer et al. compared the susceptibility of neonate mice from different age (3, 15 and 60 days) to HDM-mite induced airway inflammation (180). Mice of younger age were more prone to HDM-induced airway eosinophilia than older mice. This correlated with the bacterial colonization stage of the lung, which increased with age of the mice (180).

Previous studies have highlighted the positive effect of SCFA in experimental airway inflammation in mice (115,118,144,155,187). Vancomycin treatment depleted SCFA producers in mice, which in turn led to a reduced SCFA amount in the cecum of mice and resulted in a higher susceptibility to OVA-induced airway inflammation (115). Supplementation of a mixture of acetate, propionate and butyrate (67.5 mM, 25.9 mM and 40 mM, respectively) into the drinking water alleviated the infiltration of leukocytes into the airways and also improved airway eosinophilia (115). Interestingly, butyrate application alone prevented airway inflammation (115,188). These effects were dependent on reduced T cell and dendritic cell (DC) activity (115) or increased Treg numbers in the lung (188).

Not only the direct delivery of SCFA, but also high fiber diet of mice dampened the HDM induced airway inflammation, by improving airway eosinophilia, AHR and a reduction of type 2 cytokines in the bronchoalveolar lavage fluid (BALF) (118). Strikingly, low-fiber diet worsened the HDM-induced allergic airway inflammation (118). This was reflected by changes in bacteria phyla in cecum and furthermore the SCFA concentration, when comparing mice fed with high or low fiber diet to control mice (118). Propionate application mimicked the effect of high-fiber supplementation, via the activation of the GPR41 receptor but not GPR43 receptor as demonstrated by the use of GPR41 and GPR43 deficient mice (118). In contrast to that, Maslowski et al. reported, that GPR43 deficient mice are more prone to OVA evoked airway inflammation than their wildtype

littermates as they showed enhanced BALF cell count and an enhanced EPO activity in the lung tissue (155).

In a model of *Alternaria alternata*-induced experimental asthma, treatment with butyrate via the drinking water reduced airway eosinophilia and improved lung function by limiting the proliferation and function of ILC2 (144). Interestingly, this was a GPR43 and GPR41 independent effect but was due to HDAC inhibition. Similar results were obtained when butyrate was applied via the nose (144). This was in contrast to propionate as it affected the DC activation in the mediastinal lymph node and hematopoiesis in the bone marrow via GPR41 in HDM-exposed mice (118).

Notably, progeny from high-fiber or acetate fed mice during pregnancy were less prone to allergic airway inflammation evoked by HDM (187). Importantly, this was only evident when mice were fed with high-fiber diet or acetate *in utero*, as there was no effect when this diet regimen started during lactation or after birth (187). The authors showed that this was mediated via HDAC9 inhibition, which caused acetylation on FoxP3 promoter region and in turn resulted in enhanced production of Treg, cells that have been reported to be non-functional in asthmatics (187). Strikingly the same study suggested similar effects in humans as high acetate levels during pregnancy in mothers resulted in fewer general practitioner (GP) visits per year in their children in the first 12 month for cough and wheeze, which are predictors for asthma development later in life (187,189,190). Roduit et al. measured SCFA levels in feces from children in their first year and followed up on the development on atopic diseases (188). Interestingly, children with the highest propionate and butyrate quartile were less likely to develop asthma or food allergy later in life (188).

1.5 Aim of this thesis

It is hypothesized that the previous described beneficial effect of SCFA on allergic airway inflammation is a result of the impact of SCFA on eosinophil function and survival.

Therefore, this thesis aimed to:

- Confirm the expression of SCFA receptors GPR43 and GPR41 on human blood eosinophils
- Gain insight into the functional response of eosinophils upon direct SCFA treatment
- Elucidate the role of SCFA on eosinophil viability and the underlying mechanisms
- Determine the effect of butyrate as a treatment in allergic airway inflammation by using the ovalbumin mouse model of acute asthma

2. Materials and methods

Since parts of this thesis have been already published as an original article (1), the material and methods sections was partly adapted from this original article. Therefore, similarities in wording and content occur expectedly.

2.1 Reagents

All laboratory reagents were purchased from Sigma-Aldrich (Vienna, Austria) unless specified. Assay buffer was prepared by using PBS containing 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} (Thermo Fisher Scientific, Rockford, USA) and was supplemented with 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Eosinophil medium was prepared from RPMI 1640 (containing L-glutamine) and was supplemented with 10 % FCS and 1 % PenStrep (all from Thermofisher). Fixative solution was prepared by adding 9 ml of distilled water and 30 ml of FACS-Flow to 1ml of CellFix. CellFix and FACS-Flow were from BD (Vienna, Austria). Sodium acetate, sodium propionate or sodium butyrate are in the following declared as acetate, propionate or butyrate (1).

2.2 Methods

2.2.1 Blood donors

Blood was drawn after informed consent from healthy volunteers, and from patients in- and off-season, who reported themselves being allergic to aeroallergens or house dust mite according to an approved protocol from by the Ethics Committee of the Medical University of Graz (17-291 ex 05/06). Medical history and allergic symptoms were assessed using a standardized questionnaire asking for physician-diagnosed allergies and the presence, nature and frequency of allergic symptoms. This was previously reported (1).

2.2.2 Isolation of blood eosinophils

Peripheral blood eosinophils were isolated as previously described (191,192). In brief, 70 ml of whole blood was divided into two 50 mL falcons, containing 4.4 mL sodium citrate. Citrated blood was centrifuged at 400 x g for 20 min (low break, at room temperature) in order to remove

platelet-rich plasma. In the next step red blood cells were eliminated by adding dextran solution for 30 min to accomplish erythrocyte sedimentation. The remaining cell fraction, containing peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNL) was added to a Histopaque 1.077 gradient and separated by centrifugation. Further, the PMNL fraction was subjected to negative magnetic selection using the MACS cell separation system in order to isolate eosinophils, containing a cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a (Glycophorin A) (Eosinophil Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's protocol. Purity typically yielded >98 %. This eosinophil isolation procedure was described elsewhere (1)

2.2.3 Real-time PCR

Purified eosinophils or PMNL were pelleted via centrifugation (400 x g, 7 min) and carefully resuspended in 500 µL of TRI Reagent® (Sigma, Vienna, Austria) and incubated on ice for 10 min. 100 µL of chloroform was added followed by an incubation step (2-3 min at RT) in order to accomplish phase separation. The solution was spinned (12 000 rpm for 15 min at 4°C) and the aqueous phase was transferred to a new tube. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. In some experiments purified eosinophils were cultured in eosinophil medium for 3 h containing propionate (10 mM), butyrate (3 mM), IL-5 (50 pM) or the corresponding vehicle. Total RNA from parenchymal lung fibroblasts was a kind gift from the Ludwig Boltzmann Institute for Lung Vascular Research. Monocytes were purified as previously described (193). After total RNA was isolated, cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad, Vienna, Austria) according to the manufacturer's instruction. Real-time PCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix with PrimePCR™ SYBR® Green Assay primers for GPR41,GPR43, BCL2L1 (BCL-XL), MCL-1, CCR3, IL5RA, CD44, ITGA4 (CD49d) and RPS18, which was used as reference gene (all from Biorad). Samples were measured in duplicates and mRNA expression was quantified relative to the respective vehicle using the $^{2-\Delta\Delta}$CT method (194). First, the gene of interest was normalized to the housekeeping gene (RPS18) and then further normalized to vehicle control. Data are shown as percentage of vehicle control. GPR43 and GPR41 are shown as $^{\Delta}$CT relative to RPS18 as was already described in an original article (1).

2.2.4 Calcium (Ca^{2+}) flux

Isolated peripheral blood eosinophils (1 x10^6/mL) were resuspended in 1 mL of assay buffer, without Ca^{2+}/Mg^{2+}). Cells were loaded with the acetoxymethyl ester of Fluo-3 (2 µM final concentration) in the presence of 0.02% pluronic F-127 for 60 min at room temperature. Cells were washed, centrifuged and resuspended in 500 µL assay buffer containing Ca^{2+}/Mg^{2+}. Furthermore, PMNL were incubated (15 min at room temperature in the dark) with 5 µl of an anti-CD16 PE/Cy-5 antibody (Biolegend, San Diego, CA, USA) in order to separate eosinophils and neutrophils. Finally, cells were washed resuspended in assay buffer with Ca^{2+}/Mg^{2+} and equally divided to small reaction tubes. At first a stable baseline was recorded for 30 sec, followed by the addition of acetate, propionate or butyrate at indicated concentrations. In some experiments, cells were pre-treated with the GPR43 antagonist/inverse agonist CATPB (195) or the antagonist/negative allosteric modulator TUG-869 (196) (10 µM, for 10 min). Intracellular Ca^{2+} release was detected as fluorescence increase in the FL-1 channel of a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA) (1).

2.2.5 ROS production

Purified eosinophils were treated with serial dilutions of acetate, propionate, butyrate (prepared in assay buffer with Ca^{2+}/Mg^{2+}) or vehicle in the presence of dihydrorhodamine 123 (1 µM), for 30 min at 37°C. In order to stop the reaction samples were transferred to ice, fixative solution was added to each tube and samples were immediately analyzed by flow cytometry. Production of reactive oxygen species was detected as an increase of fluorescence in the FL-1 channel due to the oxidization of the non-fluorescent dye dihydrorhodamine 123 into fluorescent rhodamine 123. Data are expressed as fold change relative to vehicle control as previously described (1).

2.2.6 Shape change

PMNL were labeled with CD16 antibodies as described above. PMNL or isolated eosinophils were stimulated with serial dilutions (as indicated) of acetate, propionate butyrate, eotaxin-2 or the respective vehicle for 4 min at 37°C. All dilutions were prepared in assay buffer containing Ca^{2+}/Mg^{2+}. In an additional set of experiments, purified eosinophils were pre-treated

with acetate, propionate, butyrate or vehicle for 3 h before stimulation with eotaxin-2. After stimulation, samples were transferred on ice and fixed to stop the reaction. In the PMNL fraction eosinophils were distinguished from neutrophils as $CD16^{neg}$ cells. Shape change was detected as increase in forward scatter and normalized to vehicle treated cells (1,192,197).

2.2.7 Chemotaxis

Isolated eosinophils (2 x 10^6/mL) were pre-treated with acetate (10 mM), propionate (10 mM), butyrate (3 mM), TSA (330 nM) or vehicle for 3 h. After completing the incubation period, 50 µl of the cell suspension was placed into the top wells of 48-well micro-Boyden chamber with 3 µm pore-size PVP-free polycarbonate filter (NeuroProbe Inc, Gaithersburg, MD, USA) (198). Eosinophils were incubated at 37°C and were allowed to migrate towards increasing concentrations of eotaxin-2, placed into the lower wells of the Boyden chamber for 1 h. Cells that have migrated into the bottom wells of the chamber were counted by flow cytometry (1).

2.2.8 Annexin V/PI staining

Purified eosinophils were kept in RPMI 1640 (Thermo Fisher Scientific) containing 10 % FBS and 1% PenStrep at a density of 1 x10^6/mL. Cells were incubated with acetate, propionate, butyrate (1, 3, 10 mM, all). IL-5 (50 pM) or HDAC inhibitors at indicated concentrations and different periods at 37°C. In some experiments, eosinophils were pre-treated with the GPR43 antagonists CATPB (195) or GLP0974 (199) or the antagonists hydroxybutyrate and TUG-869 (196) (for 30 min) or inhibitors prior to SCFA incubation. Afterwards eosinophils were washed and collected via centrifugation and stained with annexin V and PI according to the manufacturer's protocol for 15 min (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen) at room temperature and subsequently analyzed on a FACSCalibur flow cytometer (1).

2.2.9 Wheat germ agglutinin (WGA) staining of human eosinophils

Isolated eosinophils were cultured in RPMI 1640 (supplemented with 10 % FCS and 1 % PenStrep) in the presence of propionate, butyrate, IL-5 (10 mM, 3 mM, 50 pM respectively) or vehicle for indicated time points. In an additional set of experiments, eosinophils were treated with a combination of either IL-5 and propionate or IL-5 and butyrate (concentrations as above). Afterwards, cells were washed and 3.7 % buffered formaldehyde was added for 15 min at room

temperature in order to fixate the cells. After a washing step, eosinophils were incubated with WGA- Alexa Fluor 594 (5 µg/ml) in HBSS, for 10 min in the dark at room temperature). After labelling was completed cells were washed, centrifuged and resuspended in 100 µL PBS and cytospins were prepared. Slides were mounted with Vectashield/DAPI mounting medium and images were taken utilizing the Olympus IX70 fluorescence microscope equipped with an Olympus UPlanApo-60x/14.2 oil immersion lens (1).

2.2.10 Caspase – 3/7 Glo assay

Eosinophils were seeded into 96-well plates (0.5 x 10^5/ per well) and cultured for either 3 h, 18 h or 24 h in RPMI 1640 containing 10 % FBS and 1% PenStrep supplemented with propionate or butyrate at indicated concentrations. In some experiments, eosinophils were pre-treated with either IL-5 prior to SCFA incubation or SCFA prior IL-5 stimulation for 3 h. Additionally, eosinophils from healthy donors were primed with IL-5 (30 ng/mL, 3h) in order to mimic an allergic phenotype followed by incubation with propionate (1 and 10 mM) or butyrate (3 and 10 mM) for a total of 24 h. Caspase Glo assay was performed as previously described (1,200).

2.2.11 JC-1 staining

Eosinophils (1x10^6 /mL) were kept in RPMI 1640 supplemented with 10 % FBS and 1% PenStrep and incubated with either propionate, butyrate (both 1, 3, 10 mM), IL-5 (50 pM), or the mitochondrial uncoupler FCCP (1 µM) or the respective vehicle control for indicated time points at 37°C. After washing and centrifugation, eosinophils were resuspended with the JC-1 dye (2 µg/mL, Thermo Fisher Scientific) for 20 min at 37°C. Mitochondrial depolarization was subsequently detected as decrease of red fluorescence (590 nM) on a FACSCalibur flow cytometer (1,201).

2.2.12 Western blot

Purified eosinophils (0.5 x 10^6 per condition) were kept in RPMI 1640 containing 10 % FBS and 1% PenStrep supplemented with propionate (10 mM), butyrate (3 mM) or vehicle. After a washing step cells were lysed in RIPA buffer containing protease inhibitor cocktail and incubated on ice for 15 min on ice. Samples were centrifuged (10 000 rpm, at 4°C for 10 min) and 6 x Laemmli buffer containing β-mercaptoethanol was added prior to protein denaturation (95°C for 5

min). Proteins were separated on 4-20% Tris-Glycine Gels (Thermo Fisher Scientific) (125 V, 2 h) and transferred to polyvinylidene difluoride membranes (iBlot™ Transfer Stack, PVDF) using the iBlot dry transfer system (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were blocked with Tris-buffered saline/Tween 20 buffer (TBST; 154 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.1% (v/v) Tween 20) containing 5% non-fat dry milk for 1 h at room temperature followed by incubation with primary antibodies (overnight, at 4°C): pan Acetyl H3 (1:1000, 06-599, Millipore, Merck, Vienna, Austria), Histone H3 (1:1000, #4499 CST) and GAPDH (1:1000, CST #5174, both from Cell Signaling Technology, Frankfurt am Main, Germany). After washing membranes were subjected to HRP conjugated secondary antibodies (1:5000 goat anti-rabbit, #111-036-045 Jackson ImmunoResearch, Westgrove, PA, USA) and incubated for 1 h at room temperature. Proteins of interest were visualized on a ChemiDoc Touch Imaging System using a HRP- detection substrate (both from Bio-Rad). Densitometric analysis of protein bands was performed with the Image Lab 5.2 software (Bio-Rad) (1,202).

2.2.13 Surface staining of human eosinophils

Purified eosinophils were kept in RPMI 1640, containing 10 % FCS, 1 % P/S in the presence of acetate, propionate (both 10 mM), butyrate (3 mM) or the corresponding vehicle. Cells were incubated for either 3 h or 18 h. After completed incubation period, eosinophils were washed with staining buffer, centrifuged (400 x g, 7 min) and the supernatant was decanted. Pelleted eosinophils were resuspended in Fc receptor blocking solution (human TruStain FcX™, 1:200, 10 min on ice) in order to prevent non-specific binding. After a washing step, eosinophils were subjected to the respective antibody solutions: (all anti human): CCR3 (1:100, Biolegend), CD49d (1:20), L-selectin (1:10), CD44 (1:20, all from BD) or respective isotype control antibodies for 30 min followed by a washing with PBS and centrifugation. Cell pellets were resuspended with a fixable viability dye ((Zombie NIR 1:2000, Biolegend) and incubated for 10 min in the dark. Finally, eosinophils were washed, pelleted by centrifugation and resuspended in fixative solution before analysis on a BD FACS Canto II flow cytometer (1).

2.2.14 Adhesion of eosinophils on HMVEC-L under flow conditions (Cellix)

Human pulmonary microvascular endothelial cells (HMVEC-L) were purchased as tertiary culture from Lonza (Basel, Switzerland) and were cultured in EGM-2 MV bullet kit media (Lonza) supplemented with 5% FCS as previously described (203,204). For adhesion measurements HMVEC-Ls were cultivated (0.75×10^5) on gelatin-coated Cellix biochips (Vena 8 Endothelial +, Cellix LTD, Dublin, Ireland) for 3 h. Purified human peripheral blood eosinophils were pre-treated with either acetate, propionate (10 mM), butyrate (3 mM), TSA (330 nM) or the corresponding vehicle (for 3 h) prior to stimulation with eotaxin-2 (10 nM) or vehicle for 10 min. Endothelial cell monolayers were superfused with eosinophils at 0.5 dyne/cm^2 for 5 min at 37 °C in a heated cage (OKOLAB H201-T1). Adhesion was monitored on a Zeiss Axiovert 40 CFL microscope equipped with a Zeiss A-Plan 5X/0.12 pol lens, using a Hamamatsu ORCA-03G digital camera and CellixVenaFlux software (205). Pictures were taken from at least 6 different regions of the channel, adherent eosinophil numbers were counted based on the taken pictures and a mean value was calculated. This method has also been reported in the original research article that resulted from this work (1).

2.2.15 CD11b upregulation

Whole blood was incubated with propionate (10 mM), butyrate (3 mM) or veh for 3 h before addition of various concentrations of eotaxin-2 for 30 min at 37°C. Cells were than stained with CD16-PE-Cy5 and anti-CD11b-FITC (both from Biolegend). CD11b upregulation was detected via flow cytometry. Eosinophils were identified as CD16^{neg} cells in granulocyte region of the FSC/SSC plot. CD11b upregulation is shown as percentage of vehicle (1).

2.2.16 CD63 expression

Human isolated eosinophils were incubated in RPMI 1640 containing 10 % FBS and 1% PenStrep with propionate (10 mM), butyrate (3 mM) or the corresponding vehicle for 3 h. Afterwards cells were resuspended at a concentration of 1×10^6 cells/ml in assay buffer (with Ca^{2+}/Mg^{2+}) in the presence of an anti-CD63-FITC antibody (BD Bioscience). Cytochalasin B (5 µg/mL) was added for 5 min at 37 °C to prime the eosinophils followed by incubation with different concentrations of C5a (20 min at 37°C) to induce degranulation. Cells were transferred on ice and

cold fixative solution was added to terminate the reaction. Changes in CD63 expression were detected by flow cytometry (1,192,206).

2.2.17 Animals

The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (BMWFW-66.10/0076-WF/V/3b/2017) and performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and ARRIVE guidelines (1,207).

Female BALB/c mice, 8-12 week old (body weight 16-20 g), were obtained from Charles River (Sulzfeld, Germany). Mice were housed in individually ventilated cages (4-5 per cage) under controlled conditions of temperature (set point 21°C), air humidity (set point 50%) and a 12 h light/dark cycle (lights on at 6:00 a.m.) and habituated to the environment for at least 1 week. Standard chow (altromin 1324 FORTI, Altromin, Lage, Germany) and water was provided ad libitum. Mice were randomly assigned before treatment (1).

2.2.18 Bronchoalveolar lavage

Mice were deeply anesthetized with ketamine (150 mg/kg) and xylazine (15 mg/kg) via intraperitoneal injection, trachea was exposed, blood was sampled and mice were exsanguinated by cutting the abdominal aorta. In total 3.5 mL of BAL buffer (PBS containing 1 mM EDTA) was instilled into the lung and BAL fluid was collected and stored on ice. BAL fluid was spinned in order to collect BAL fluid cells (400 x g, 4°C for 7 min). The cell free supernatant was stored on -80°C until further use (1).

2.2.19 Lung preparation

After BAL fluid was removed, the lung was flushed via the right heart using 10 mL of PBS to remove red blood cells. The whole lung was thoroughly minced, taken up in BAL buffer and further mechanically homogenized with an 18 G needle by harsh flushing through a syringe. The cell suspension obtained was strained through a 40 µM mesh in order to receive a single cell suspension, which was washed with BAL buffer and centrifuged (400 x g, 7 min) followed by red blood cell lysing using an ammonium chloride solution. After a washing step, the single cell

suspension was divided into three equal parts further subjected to subsequent staining protocols which are described below (1).

2.2.20 Ovalbumin (OVA)-induced pulmonary inflammation model

Eight week-old female BALB/c mice were immunized by i.p. injections of 20 µg OVA adsorbed to 1 mg aluminum hydroxide ($Al(OH)_3$) (Alhydrogel® adjuvant 2%, InvivoGen, Toulouse, France) on day 0 and 7. On day 18, 19 and 20 mice where challenged with 1 % (w/v) OVA dissolved in saline, or control mice with saline only, for 30 min. Mice received daily i.p. injections of butyrate (1 g/kg bodyweight) in saline or the corresponding vehicle from day 11 until the end of the experiment. On day 21 mice were deeply anesthetized via intraperitoneal injection as described above. BAL fluid, bone marrow and blood was collected, or airway hyperresponsiveness (AHR) to methacholine was recorded with the FlexiVent system (Scireq, Montreal,CA) (1,192).

2.2.21 Leukocyte staining from BAL and blood

Cells collected from BAL and blood were subsequently subjected to a staining protocol. In brief, erythrocytes were lysed using an ammonium chloride solution. After a washing step, non-specific binding sites were masked using TruStain fcX™ (anti-mouse CD16/32) antibody (Biolgend, 1:100) for 15 min on ice. Afterwards cells were washed and incubated with specific antibodies (all anti mouse): Siglec F (1:100), CD11b (1:200), CD11c (1:200) (all from BD Pharmingen), Ly6G (1:500) (all from Biolegend) for 30 min at 4°C. Cells were washed, fixed and analyzed by a BD FACS Canto II flow cytometer (1).

In detail, after doublet cells had been excluded, lymphocytes were identified as FSC/SSC^{low} $CD11b^{neg}/CD11c^{neg}$ cells. Remaining cells were further gated as $CD11c^{neg}$/Siglec F^{pos} eosinophils and $CD11c^{pos}$/Siglec F^{pos} alveolar macrophages (AM). CD11c/Siglec F double negative cells were characterized as $CD11b^{pos}/Ly6G^{pos}$ neutrophils. For blood leukocytes, the same gating strategy as for BAL fluid leukocytes was used (1).

In a second set of experiment, cells from BAL were either incubated with an antibody cocktail containing Siglec F (1:200), CD11b (1:200), and CD11c (1:200) followed by annexin V (all from BD Pharmingen) to identify apoptotic eosinophils, or with the same antibody solution

plus CCR3 (1:100, R&D Systems) or CD125 (IL-5Rα, 1:100) antibodies but not annexin V. For sample handling after completed staining, see section below. This method has also been reported in the original research article that resulted from this work (1).

2.2.22 Isolation of bone marrow

Bone marrow from femur was isolated as previously described (208,209). Briefly, bones were cut open and bone marrow was flushed out with 2 ml of BAL buffer each followed by centrifugation. Erythrocytes were lysed using an ammonium chloride solution (1). After a washing step, cells were equally divided and subjected to either the eosinophil antibody panel using Siglec F (1:100 and CCR3 (1:100, R&D Systems) or the eosinophil lineage–committed progenitor (EoP) antibody panel including linage marker (1:200, GR-1, Sca-1, both from Biolegend, CD3, BD Bioscience, CD4, CD8a, CD19, B220, all from Thermo Fisher), CD34 (1:100), CD125 (IL-5Rα) (1:100), both from BD Pharmingen, CD117 (c-Kit) (1:200), CD16/32 (1:400, both from Biolegend) and incubated for 30 min at 4°C. Cells were washed, and then incubated with a fixable viability dye (Zombie NIR, 1:2000, Biolegend) for 15 min at room temperature as previously described (210) and recorded on a BD FACS Canto II flow cytometer (1). EoP were characterized as $lineage^{neg}/CD34^{pos}/CD117^{int}/CD125^{pos}$ cells, accordingly granulocyte-macrophage progenitors (GMP) were defined as $lineage^{neg}/CD34^{pos}/CD117^{high}/CD125^{neg}/CD16/32^{pos}$ cells (1). PreEos were identified in the live cell population as Siglec $F^{pos}/CCR3^{neg}$ cells, whereas mature eosinophils were characterized as Siglec $F^{pos}/CCR3^{pos}$ cells. Corresponding fluorescence minus one (FMO) controls were used to identify the respective cell type. This experimental procedure has been published elsewhere (1).

2.2.23 Leukocyte staining from lung

Before subjection to different antibody staining protocols cells were incubated with TruStain fcX™ (anti-mouse CD16/32) antibody (Biolgend, 1:100) for 15 min on ice to mask non-specific binding sites. Cells which were stained with the regulatory T cell panel were treated differentially as described below (1).

After treatment with the FC block, cell suspensions were washed, centrifuged and resuspended in either CD45 (1:300, Biolegend), Siglec F (1:200), CD11b (1:200), CD11c (1:200) followed by annexin V (all from BD Pharmingen) to identify apoptotic eosinophils or the same antibody cocktail with CCR3 (1:100, R&D Systems) and CD125 (IL-5Rα, 1:100) but not annexin V (1).

For staining of regulatory T cells, the cells were first treated with a fixable viability dye (Zombie NIR 1:2000, Biolegend) for 15 min before permeabilization (Transcription Factor Buffer Set, BD) according to manufacturer's instructions. Cells were incubated with CD45 (1:300), CD4 (1:300, both Biolegend), CD25 (1:100, BD Pharmingen) and FoxP3 (1:40, Thermofisher) (1).

For all panels cells were incubated with antibody cocktail for 30 min at 4°C followed by a washing step. Cells were pelleted and resuspended in 200 µl fixative solution or, for the apoptosis panel, in 200 µl BAL buffer as previously published (1).

2.2.24 ILC2 staining in lung

Lung homogenate was prepared as described above. Unspecific binding sites were masked as outlined previously. Cells were washed and resuspended in an antibody cocktail containing: lineage marker (1:200, CD3, CD19, FcЄRI, F4/80, CD11b, CD11c and CD49b), CD45 (1:200) and CD90.2 and incubated for 30 min. ILC2 were identified as SSC^{low}/CD45pos/$lineage^{neg}$/$CD90.2^{pos}$ cells as previously described (144).

2.2.25 Assessment of lung function

Twenty four h after last OVA or vehicle challenge (as described above) airway hyperresponsiveness (AHR) to methacholine was recorded with the FlexiVent system (Scireq, Montreal,CA) (192). In brief, mice were anaesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg) by i.p. injection and after surgical anesthesia was confirmed, mice were tracheotomized and an 18 G cannula was inserted into the trachea. Mice were placed onto a heating pad (at 37°C), connected to the FlexiVent system and ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths per minute. Basal airway resistance (R) and compliance (C) before treatment and airway hyperresponsiveness after exposure to increasing concentrations of nebulized methacholine (1, 3, 10, 30 and 100 mg/mL) were recorded (1).

2.2.26 Detection of cytokines in BAL fluid

The first mL, which was obtained from BAL fluid was used for cytokine detection. Cytokines were determined by multiplex ELISA (Mouse Procarta 10-Plex, Thermo Fisher Scientific) according to the instructions. In brief, samples were incubated with magnetic beads followed by antigen specific detection antibodies. In the last step, streptavidin-PE (phycoerythrin) conjugates were added followed by detection with a Bio-Plex 200 (BioRad) (1).

2.2.27 Statistical analysis

All data are shown as mean + SEM for n observations. Statistical analyses were performed using GraphPad Prism software 6.0 (La Jolla, CA; USA). Groups were compared by one-way or two-way ANOVA followed by Bonferroni, Dunnett's or Tukey multiple comparison test or Student’s t-test. Probability values of $P < 0.05$ were regarded as statistically significant and are indicated as *P<0.05; **P<0.01; and ***P<0.001 (1).

3. Results

Most data presented in this thesis have been previously published:

Anna Theiler, Thomas Bärnthaler, Wolfgang Platzer, Georg Richtig, Miriam Peinhaupt, Sonja Rittchen, Julia Kargl, Trond Ulven, Leigh M. Marsh, Gunther Marsche, Rufina Schuligoi, Eva M. Sturm and Akos Heinemann.

Butyrate ameliorates allergic airway inflammation by limiting eosinophil trafficking and survival

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3.1 Short chain fatty acid receptors are present on transcriptional level on human eosinophils

SCFA activate G-protein coupled receptors, specifically GPR43 and GPR41. GPR43 mRNA has been previously reported to be expressed in various tissues, in particular in the gut and immune cells, also including eosinophils (128–130,211). GPR41 was shown to be abundant in the pancreas, adipose tissue, and spleen (129). Here, total RNA from purified peripheral blood eosinophils, neutrophils and monocytes was isolated and cDNA synthesis was performed. I found that GPR43 is expressed on eosinophils and neutrophils, while absent on parenchymal lung fibroblasts (Figure 2A). The GPR41 receptor on the other hand, was expressed by eosinophils and neutrophils, but not monocytes, which served as negative control (Figure 2B) (1).

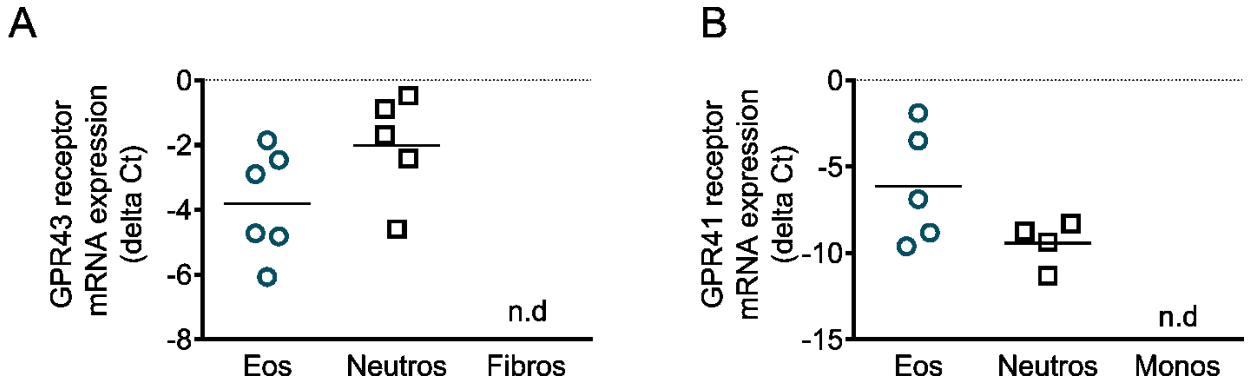

Figure 2. Peripheral blood eosinophils express SCFA receptors on transcriptional level*. Total RNA from purified eosinophils, neutrophils, monocytes and parenchymal lung fibroblasts was isolated and cDNA synthesis was performed. GPR43 is expressed on mRNA level on human eosinophils and neutrophils, whereas is absent on parenchymal lung fibroblasts (A). GPR41 mRNA is present on both eosinophils and neutrophils, but missing on monocytes (B). Data are shown as delta Ct. RSP18 was used as housekeeping gene. n.d.= not detectable. This figure was adapted from (1), with a right granted from Elsevier, as for all subsequent figures in this thesis, wherever applicable. Wolfgang Platzer performed real-time PCR experiment. Kathrin Rohrer isolated leukocytes from blood. Sonja Rittchen provided RNA from monocytes. Lung fibroblast RNA was obtained from the Ludwig Boltzmann Institute for Lung Vascular Research.*

3.2 GPR43 is functionally active on human eosinophils

GPR43 is coupled to $G_{q/11}$ subunit (128), which upon activation causes an increase of intracellular Ca^{2+}. I performed a flow cytometry based Ca^{2+} flux assay to elucidate whether SCFA can trigger Ca^{2+} release in eosinophils. In detail, eosinophils were loaded with the cell permeant Ca^{2+} indicator Fluo3-AM for one hour and were then resuspended in assay buffer, containing Ca^{2+}/Mg^{2+} before being analyzed by flow cytometry.

First, a stable baseline was recorded for 30 s, then eosinophils were stimulated with increasing concentrations of the SCFA acetate, propionate, butyrate (0.1 – 30 mM) or the corresponding vehicle. Acetate and propionate caused a concentration dependent Ca^{2+} flux in human eosinophils (Figure 3 A, B, left and middle panels), while butyrate application had no impact on eosinophil Ca^{2+} release (Figure 3 C, left and middle panel). In a next step, I was interested if the Ca^{2+} flux triggered by acetate and propionate is dependent on GPR43 receptor activation. For this purpose eosinophils were pre-treated with the GPR43 antagonist/inverse agonist CATPB (195) (10 μM) prior to addition of either acetate or propionate (both 10 mM, Figure 3 B, C, right panel). CATPB completely abolished the Ca^{2+} release in blood eosinophils down to baseline levels, whereas the GPR41 antagonist/negative allosteric modulator TUG-869 (196) was ineffective (data not shown) (1).

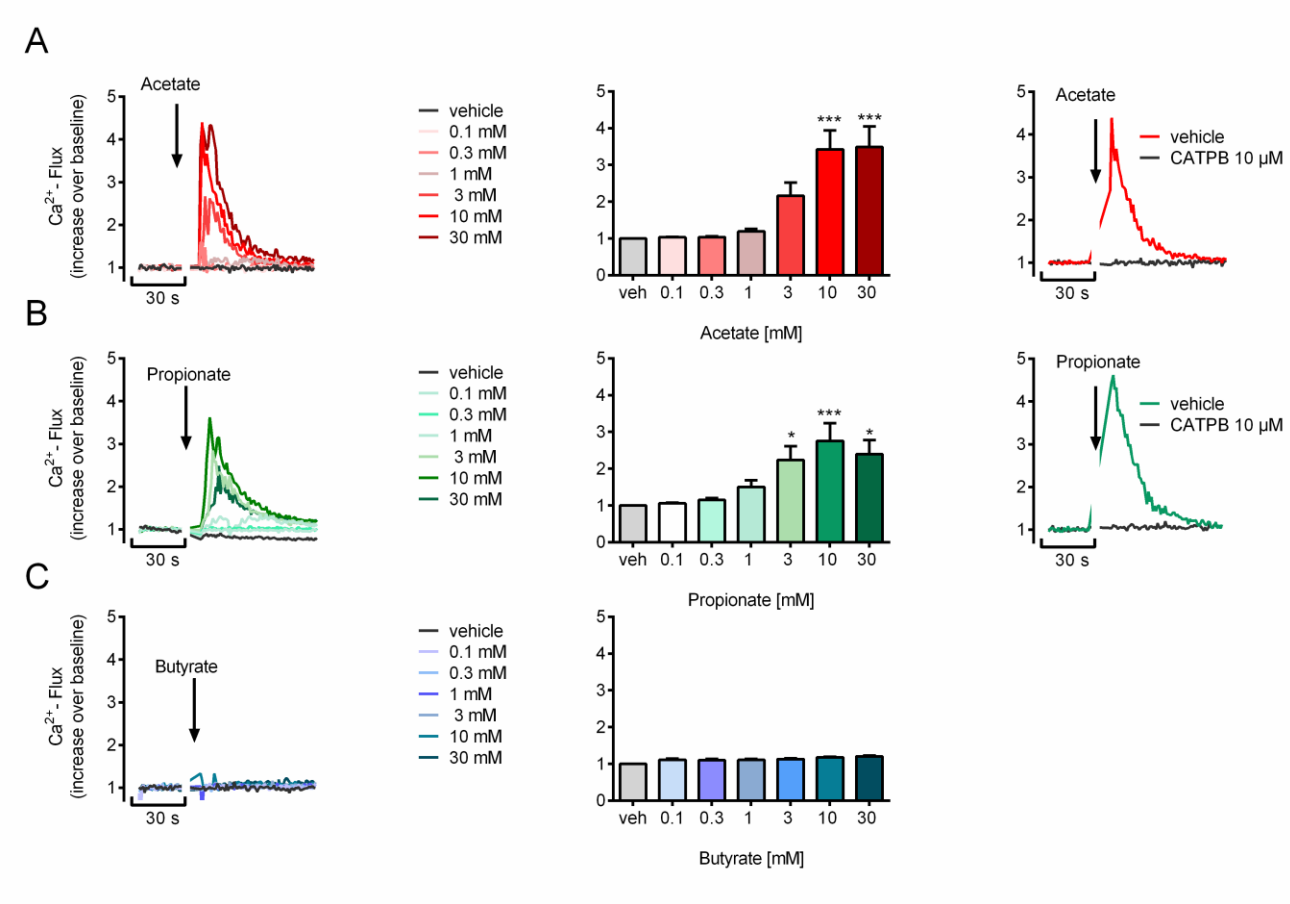

Figure 3. Acetate and propionate induce Ca^{2+} flux in human eosinophils, which is dependent on GPR43 receptor activation. *Purified eosinophils were resuspended in assay buffer and loaded with acetoxymethyl ester of Fluo-3. A stable baseline was recorded for 30 s before serial concentrations of SCFA were added. Acetate (A) and propionate (B) concentration-dependently induced an intracellular Ca^{2+} release in human eosinophils, which was dependent on the GPR43 receptor (right panels). Butyrate failed to induce a Ca^{2+} flux in human eosinophils (C). Data are shown as mean +SEM from 4-6 different donors, and were analyzed by one-way ANOVA, * P<0.05, *** P<0.001 vs. veh. This figure was adapted from (1). Experiment was partly performed by Kathrin Rohrer.*

Since I found that acetate and propionate were able to induce Ca^{2+} flux in human eosinophils, I was interested, if SCFA can modulate eosinophil function with regards to ROS production, eosinophil shape change or eosinophil migration. Therefore, eosinophils were treated

with serial dilutions of acetate, propionate and butyrate in the presence of dihydrorhodamine (DHR) 123. The oxidation of DHR 123 into the fluorescent rhodamine 123 due to ROS production was detected by flow cytometry. Both, acetate and propionate induced ROS in human eosinophils in a concentration-dependent fashion, with a maximal response at 10 mM (Figure 4 A). In agreement with the previous observation in the Ca^{2+} assay (Figure 3 C) butyrate was again unable to induce ROS production (Figure 4 A) (1).

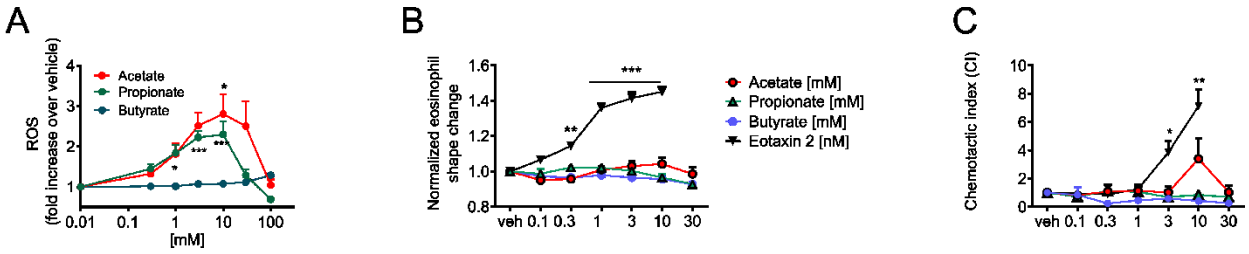

Figure 4. Acetate and propionate induce ROS production in human eosinophils. *Purified eosinophils were loaded with dihydrorhodamine 123 and treated with serial dilutions of acetate, propionate, butyrate or the corresponding vehicle (A). SCFA do not trigger shape change in human eosinophils (B) or eosinophil migration (C). Data are shown as mean + SEM, n=3-6 and were analyzed by one-way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, ***P<0.001 vs. veh. This figure was previously published (1). Kathrin Rohrer performed ROS production and chemotaxis assay.*

Having determined that acetate and propionate are able to trigger ROS production of human eosinophils, I was next interested, if SCFA activate human eosinophils. This was determined by employing the flow cytometry-based shape change assay (197). Upon stimulation, eosinophils change their appearance due to the formation of adhesive structures by rearrangement of the actin skeleton, which can be detected as an increase of the forward scatter by flow cytometry (197). Thus, eosinophils were again treated with serial dilutions of acetate, propionate, butyrate or vehicle. Eotaxin-2, a potent eosinophil activator served as positive control. Although acetate and propionate induced Ca^{2+} flux in human eosinophils, both SCFA were ineffective on eosinophil activation. As expected, butyrate had no effect on eosinophil shape change, whereas eotaxin-2 induced a pronounced eosinophil activation (Figure 4 B). Finally, an eosinophil migration assay, using micro Boyden chambers was performed. Eosinophils were placed on the top wells of the chamber and

were allowed to migrate towards serial concentrations of SCFA, eotaxin-2 or vehicle (concentrations as indicated) for an hour at 37°C. In accordance with my previous findings in the shape change assay (Figure 4 B), all SCFA failed to induce eosinophil migration. As expected, eotaxin-2 triggered the migration of eosinophils (Figure 4 C) (1).

3.3 Propionate and butyrate impair the viability of human eosinophils

Eosinophil viability is dramatically enhanced in allergic inflammation by the release of IL-3, IL-5 and GM-CSF, which in turn promotes the allergic response (19,20). Furthermore, it has been previously established that SCFA are able to induce apoptosis in neutrophils and other cells types including colon carcinoma cells (1,164,212). Therefore, I analyzed the apoptotic potential of the SCFA acetate, propionate and butyrate on human peripheral blood eosinophils. Eosinophils were cultured at 37°C in a humidified incubator in the presence of acetate, propionate butyrate (1, 3, 10 mM) or vehicle for the indicated time periods. IL-5 (50 pM) was used as positive control. After the completion of the incubation time, eosinophils were double stained with annexinV/PI in order to determine eosinophil viability and apoptosis. Interestingly, acetate failed to induce apoptosis in human eosinophils (Figure 5 A-E). Of note, acetate increased the living cell portion (annexin V-/PI-) of eosinophils and in turn reduced the early apoptotic starting 24 h of the initial treatment when compared to the vehicle treatment. This effect was still present 48 h after initial treatment (Figure 5 D). As expected, treatment with IL-5 improved eosinophil survival (Figure 5 B-E).

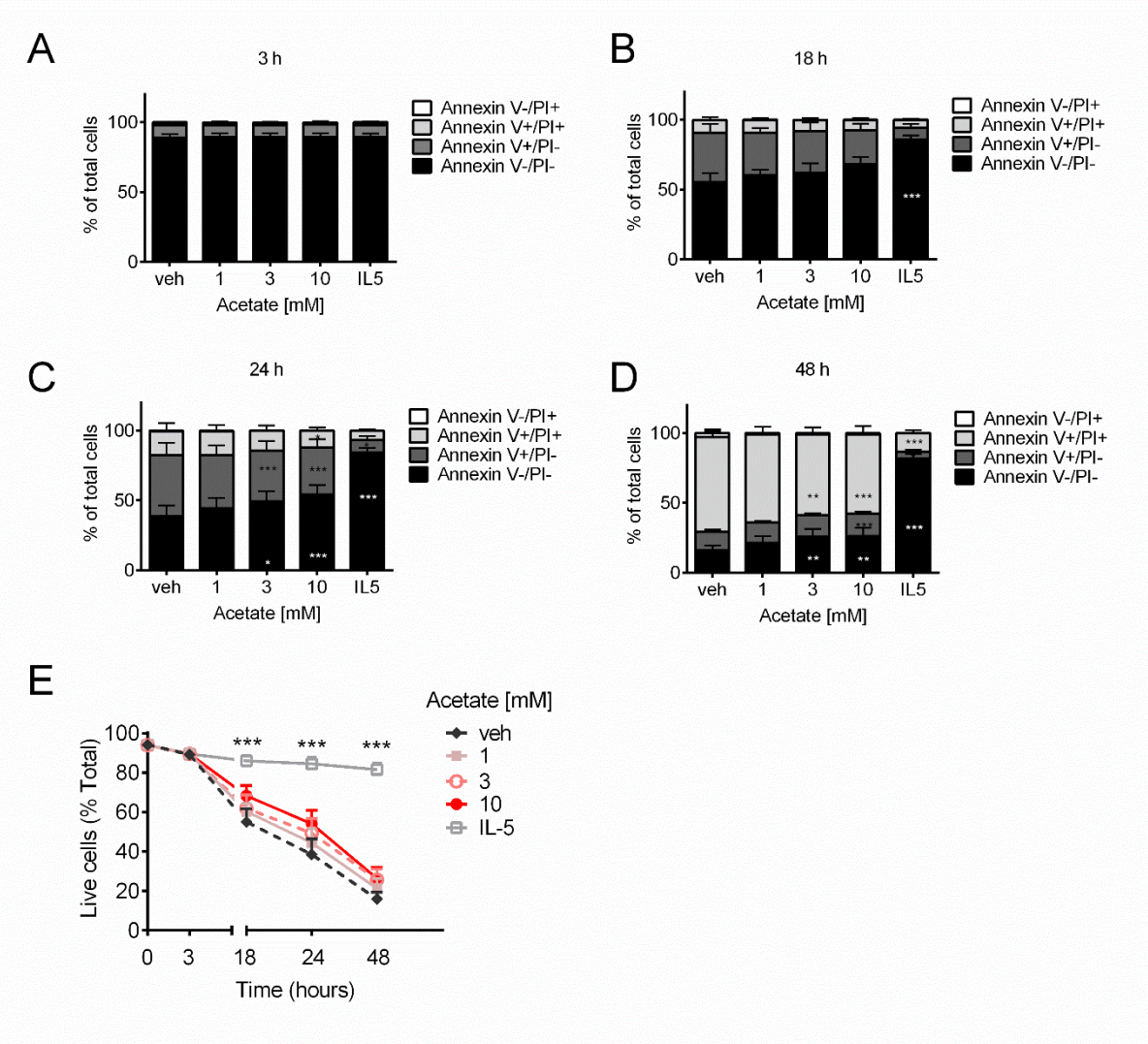

Figure 5. Acetate failed to induce apoptosis in human eosinophils. *Isolated eosinophils were incubated for the indicated time periods with acetate (1, 3, 10 mM), IL-5 (50 pM) or vehicle. Eosinophil viability was determined with flow cytometry based annexin V/PI assay. Representative dot plots from annexin V/PI staining at indicated time points and percentages of annexin V-/PI-, annexin V+/PI-, annexin V+/PI+, annexin V-/PI+ populations of total eosinophils are depicted. The living cell population over time is depicted (E). Data are shown as mean + SEM from 4 different donors and were analyzed by two-way ANOVA Tukey's post-test, *P<0.05, **P<0.01, ***P<0.001 vs. veh.* (1)

Notably, incubation with propionate (10 mM) or butyrate (3 mM and 10 mM) caused apoptosis induction starting after 18 h of initial treatment (Figure 6 A and Figure 7 A), but survival was not affected at earlier time points. I could observe the effect up to 48 h post treatment (Figure 6 A, B and Figure 7 A, B). Of note, propionate similar to acetate increased the viable (annexin V-/PI-) portion of the cells when incubated with 1 mM, but significantly augmented the early apoptotic population at 10 mM (Figure 6 A). Butyrate, however, did not favor eosinophil survival, but in contrast, significantly impaired the viable cell portion and accordingly augmented the early apoptotic (annexin V+/PI-) eosinophil population (Figure 7 A). Figures 6 B and 7 B show the live eosinophil, incubated with different concentrations of propionate (Figure 6 B), butyrate (Figure 7 B), vehicle or IL-5 over time (1).

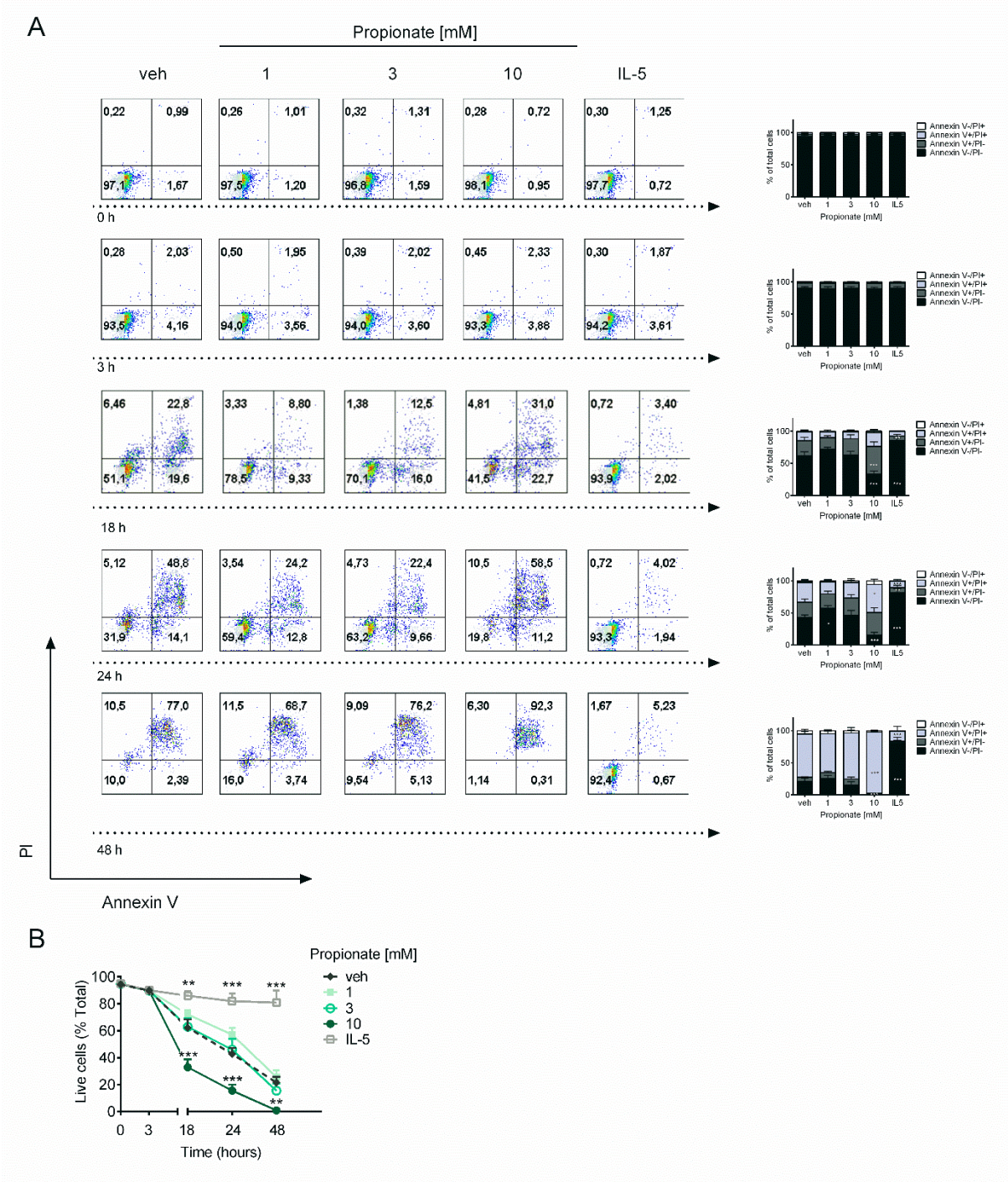

Figure 6. Propionate impairs the viability of human eosinophils. *Isolated eosinophils were incubated for the indicated time periods with propionate (1, 3, 10 mM), IL-5 (50 pM) or vehicle. Eosinophil viability was determined with flow cytometry based annexin V/PI assay. Typical flow*

*cytometry plots from annexin V/PI staining at indicated time points and percentages of annexin V-/PI-, annexin V+/PI-, annexin V+/PI+, annexin V-/PI+ populations of total eosinophils are shown (A). Decrease of the living eosinophil population starts 18 h after incubation with propionate (B). Data are shown as mean + SEM from 5-6 different donors and were analyzed by two-way ANOVA, Tukey's post-test *P<0.05, **P<0.01, ***P<0.001 vs. veh. This figure was adapted from (1).*

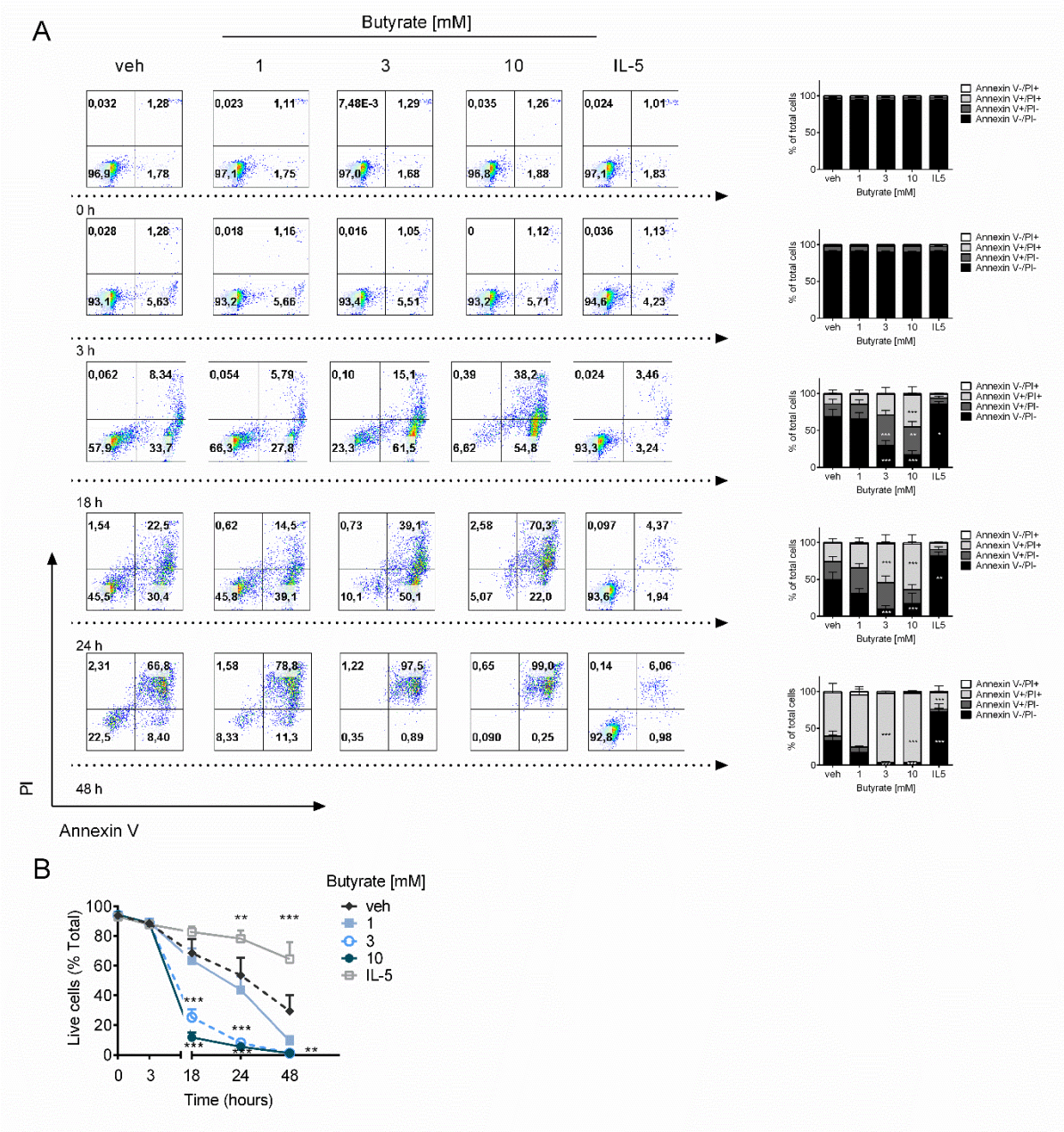

Figure 7. Butyrate compromises the survival of human eosinophils. *Isolated eosinophils were incubated for the indicated time periods with butyrate (1, 3, 10 mM), IL-5 (50 pM) or vehicle. Eosinophil viability was determined with flow cytometry based annexin V/PI assay. Typical flow cytometry plots from annexin V/PI staining at indicated time points and percentages of annexin V-/PI-, annexin V+/PI-, annexin V+/PI+, annexin V-/PI+ populations of total eosinophils are shown (A). Decrease of the living eosinophil population starts 18 h after incubation with butyrate (B). Data are shown as mean + SEM from 6 different donors and were analyzed by two-way ANOVA Tukey's post-test, *P<0.05, **P<0.01, ***P<0.001 vs. veh. This figure was adapted from (1).*

Next, we were interested if propionate and butyrate treatment affects the morphology of human eosinophils, which were cultured under the same conditions as described above in the annexin V/PI assay for 18 h. Cells were washed, fixed and were labelled with a wheat-germ-agglutinin- Alexa fluor 594 conjugate, which binds unspecifically to the plasma membrane. After the incubation period was completed, cytopsins were made, the nuclei were counter stained with DAPI and analyzed by fluorescence microscopy. We found that incubation with propionate (10 mM) or butyrate (3 mM) reduced cell size and cell number and that eosinophils lost their typical bilobed nuclei when compared with vehicle or IL-5 (50 pM) treatment (Figure 8) (1).

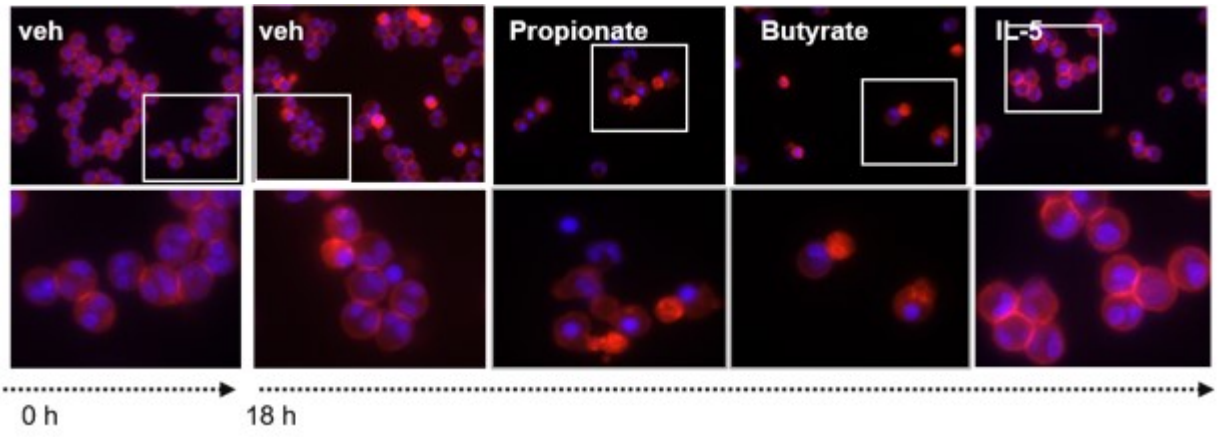

Figure 8. Propionate and butyrate treated eosinophils change their morphology. *Eosinophils were treated with propionate (10 mM), butyrate (3 mM), IL-5 (50 pM) or vehicle for the indicated time period. Cells were fixed and stained with WGA-AF 594 and spun onto glass coverslides. Eosinophil morphology in response to SCFA treatment is shown (60x, upper panel and 180x*

magnification, original micrographs were digitally enlarged by 3 X, lower panel). This figure was adapted from (1).

3.4 Apoptosis induction by SCFA is independent of GPR43 and GPR41 receptors

Since it is known that propionate and butyrate are able to activate the GPR43 and GPR41 receptors, I wanted to elucidate, whether the propionate and butyrate induced eosinophil apoptosis is also dependent on these receptors. First, I pre-treated eosinophils with the GPR43 antagonist CATPB (100 μM) for 30 min, followed by application of propionate for a total incubation time of 24 h. Interestingly, the GPR43 antagonist had no effect on the survival impairment of propionate on eosinophils (Figure 9 A, left panel). This was confirmed by pre-treatment with a second GPR43 antagonist (GLP097429, 100μM) under the same conditions (Figure 9 A, right panel). As I could show that the propionate-induced apoptosis is independent of GPR43 receptors, eosinophils were incubated with hydroxybutyrate, a purported GPR41 antagonist (213) (10 mM, 30 mins). Of note, hydroxybutyrate was also ineffective to reverse the propionate induced viability impairment of eosinophils (Figure 9 B, left panel). This was again confirmed by employing a second GPR41 receptors antagonist (TUG-86924, 10 μM) showing that the loss of viable eosinophils induced by propionate was not prevented by GPR41 inhibition (Figure 9 B, right panel) (1).

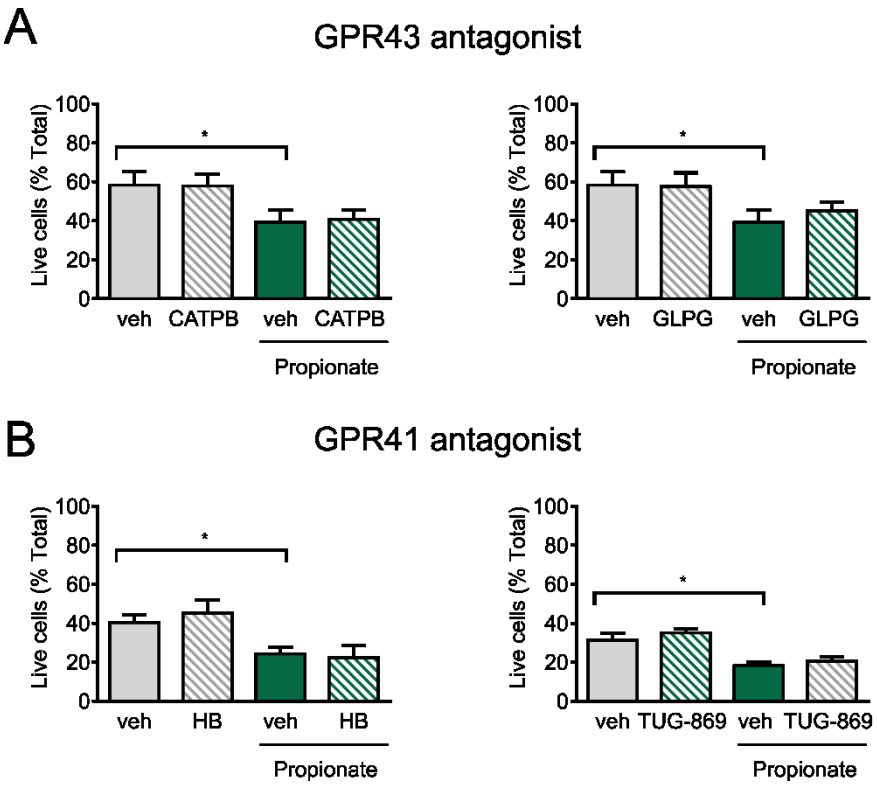

Figure 9. Propionate induced viability impairment is independent of SCFA receptors GPR43 and GPR41. *Purified eosinophils were pretreated with the GPR43 antagonists CATBP (100 µM) GLPG0974 (GLPG, 100 µM) (A), the GPR41 antagonists hydroxybutyrate (HB, 10 mM) or TUG-869 (10 µM) (B) or vehicle before addition of propionate (10 mM) or vehicle for a total of 24 h. The living cell population is shown. Data are shown as mean + SEM from 5 different donors. Treatment effects were analyzed by one-way ANOVA, Dunnett's post-test *P<0.05 vs. veh. This figure was adapted from (1).*

Having confirmed that the propionate induced eosinophil apoptosis is independent of GPR43 and GPR41 receptors, I was eager to decipher the mode of action regarding butyrate dependent eosinophil survival impairment. Eosinophils were pre-treated with GPR43 antagonist as described above. Similar to my previous findings, both GPR43 receptor antagonists were unable to protect from butyrate induced apoptosis (Figure 10 A), the same was found, when eosinophils were pre-treated with GPR41 receptor antagonists (Figure 10 B). In summary, my data clearly

suggest that propionate and butyrate induced apoptosis is independent of the SCFA receptors GPR43 and GPR41 (1).

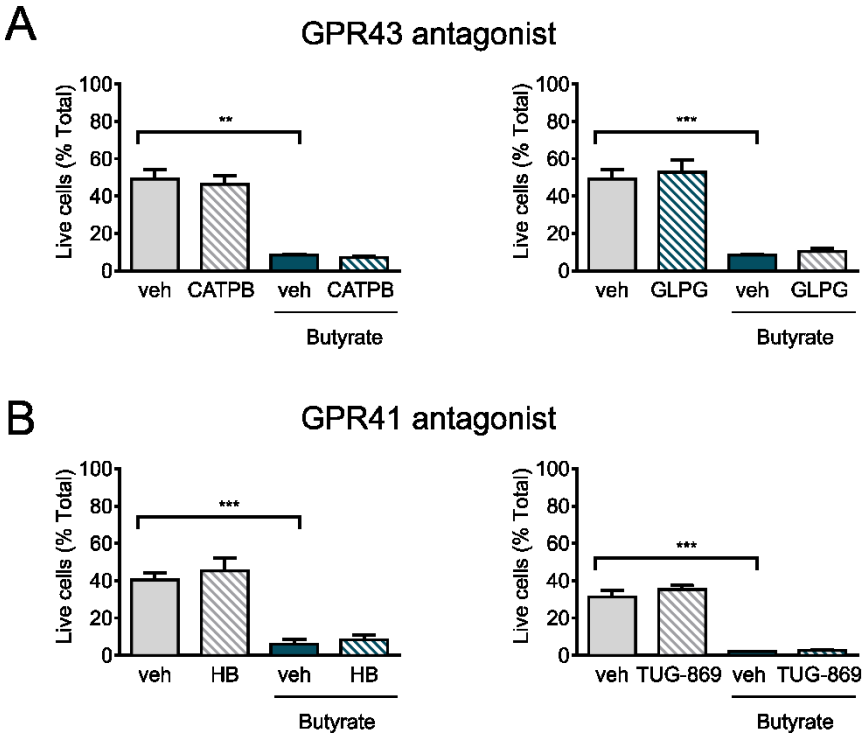

Figure 10. Butyrate induced viability impairment is independent of SCFA receptors GPR43 and GPR41. *Purified eosinophils were pretreated with the GPR43 antagonists CATBP (100 µM) GLPG0974 (GLPG, 100 µM) (A), the GPR41 antagonists hydroxybutyrate (HB, 10 mM) or TUG-869 (10 µM) (B) or vehicle before addition of butyrate (3 mM) or vehicle for a total of 24 h. The living cell population is shown. Data are shown as mean + SEM from 5 different donors. Treatment effects were analyzed by one-way ANOVA Tukey's post-test **P<0.01, ***P<0.001 vs. veh. This figure was adapted from (1).*

3.5 Propionate and butyrate induce apoptosis in human eosinophils via the intrinsic pathway

Apoptosis is a rigidly regulated process. Upon a pro-apoptotic stimulus, a loss of the mitochondrial membrane potential ($\Delta\Psi m$) occurs, which is accompanied by the release of cytochrome C which in turn results in subsequent activation of effector caspases (1). Therefore, I

tested whether propionate and butyrate are able to depolarize the ΔΨm of human eosinophils. I chose the same experimental conditions as in the annexin V/PI assay. In order to detect changes in the ΔΨm, I used a flow cytometry-based assay and labeled the eosinophils with the cationic JC-1 dye. In unimpaired mitochondria JC-1 forms aggregates which emit light at 590 nm (red fluorescence), whereas in mitochondria with defective ΔΨm JC-1 appears as monomers which is detectable by a shift in fluorescent spectrum to 530 nm (green fluorescence) (1,201).

SCFA treatment caused a loss in the red fluorescence (propionate at 10 mM 15 %, butyrate by 31 % with 3 mM and by 44 % with 10 mM as compared to vehicle) when incubated for 18 h, which is in agreement with mitochondrial impairment. Interestingly, lower SCFA concentrations did not alter the ΔΨm in human eosinophils, which is similar to the findings in the annexin V/PI assay. Incubation with IL-5 (50 pM), however, limited the mitochondrial membrane depolarization (Figure 11 A and B). Plus, treatment with FCCP, a potent uncoupler of the inner mitochondrial membrane (201) caused a massive loss in red fluorescence, when added for 30 min (Figure 12 A). SCFA evoked mitochondrial depolarization was evident after an incubation period of 18 h and prevailed up to 48 h (Figure 11 B and Figure 12) (1).

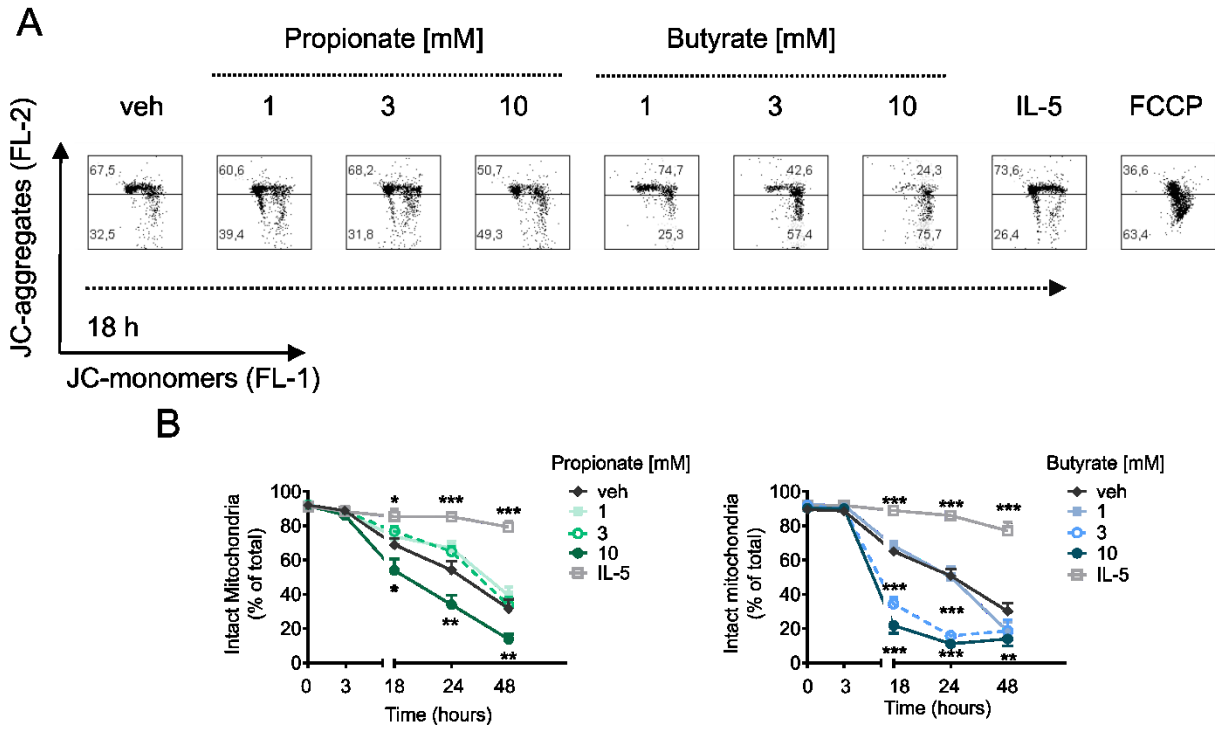

Figure 11. SCFA treatment causes mitochondrial depolarization in eosinophils. *Eosinophils were treated with propionate, butyrate or vehicle for the indicated time period. The mitochondrial membrane uncoupler FCCP served as a positive control. Eosinophils were loaded with the JC-1 dye. Representative dot plots are depicted (A). Loss of mitochondrial membrane potential started 18 h after SCFA treatment and is shown as a decrease in the FL-2 channel (B). Data are shown as mean +SEM from 4-7 different donors and were analyzed by two-way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, *** P<0.001 vs. veh. This figure was adapted from (1). JC-1 staining was partly performed by Georg Richtig.*

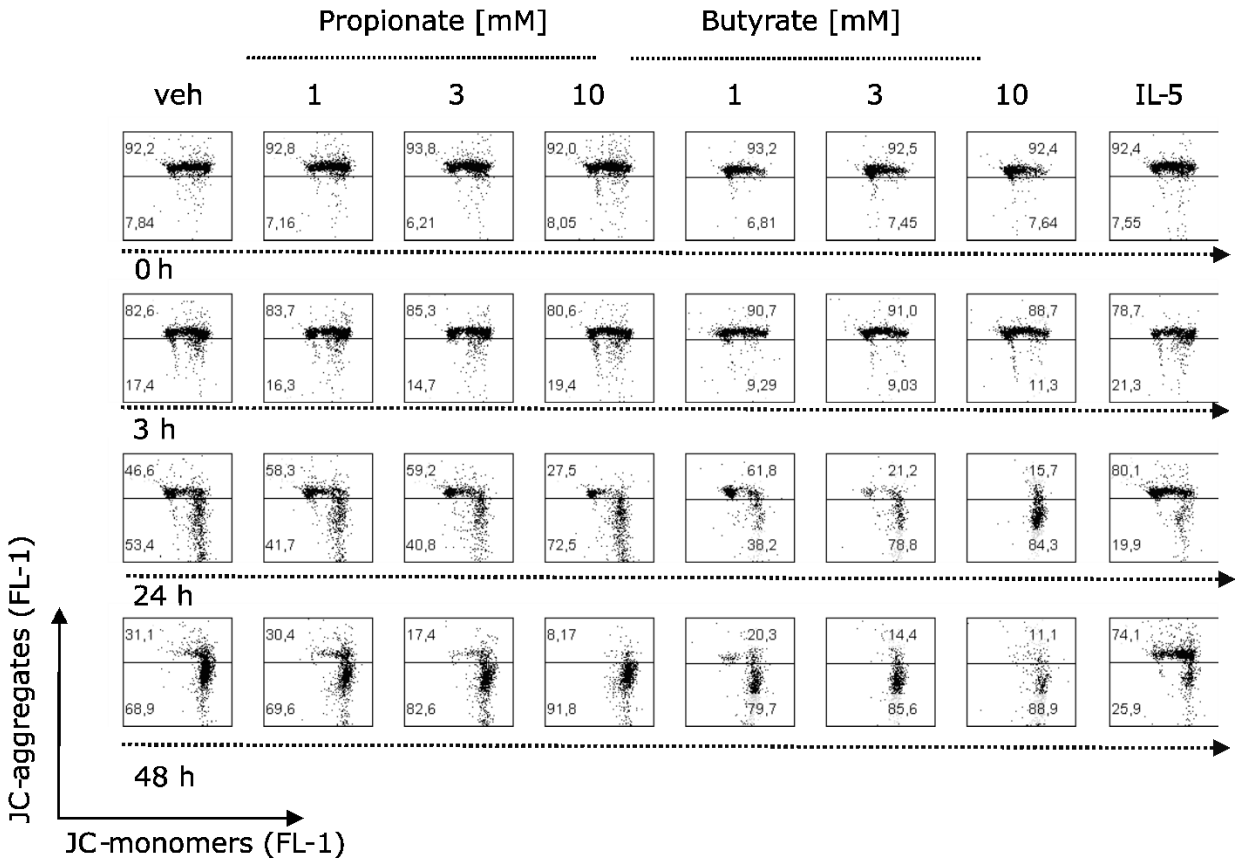

Figure 12. Loss of mitochondrial membrane potential after SCFA treatment. *Eosinophils were incubated with propionate, butyrate, IL-5 or vehicle (concentrations as indicated) and were*

harvested at indicated time points. Cells were loaded with the JC-1 dye and flow cytometric analysis was performed. Typical dot plots are depicted. This figure was previously published (1).

Having confirmed that propionate and butyrate treatment caused apoptosis induction and ΔΨm depolarization, I next sought to investigate whether this is dependent on caspase activation. For that purpose, eosinophils were treated with propionate, butyrate or vehicle (time periods and concentrations as indicated). Propionate (10 mM) induced caspase 3/7 activation starting 18 h after initial treatment and was detectable 24 h post treatment (Figure 13 A). Interestingly, low-concentration propionate (1 mM, 18 h) hampered the caspase 3/7 activation of human eosinophils (Figure 13 A, middle panel). In contrast to propionate, butyrate did not exert any life prolonging effects on human eosinophils, but strongly induced caspase 3/7 activation, 18 h after treatment (Figure 13 B). In order to confirm caspase dependence, eosinophils were pretreated with the pan-caspase inhibitor ZVAD-FMK (1000 μM, 30 min) before addition of propionate (10 mM) and butyrate (3 mM), which completely abolished activation of caspase by propionate or butyrate (Figure 13 C and D) (1).

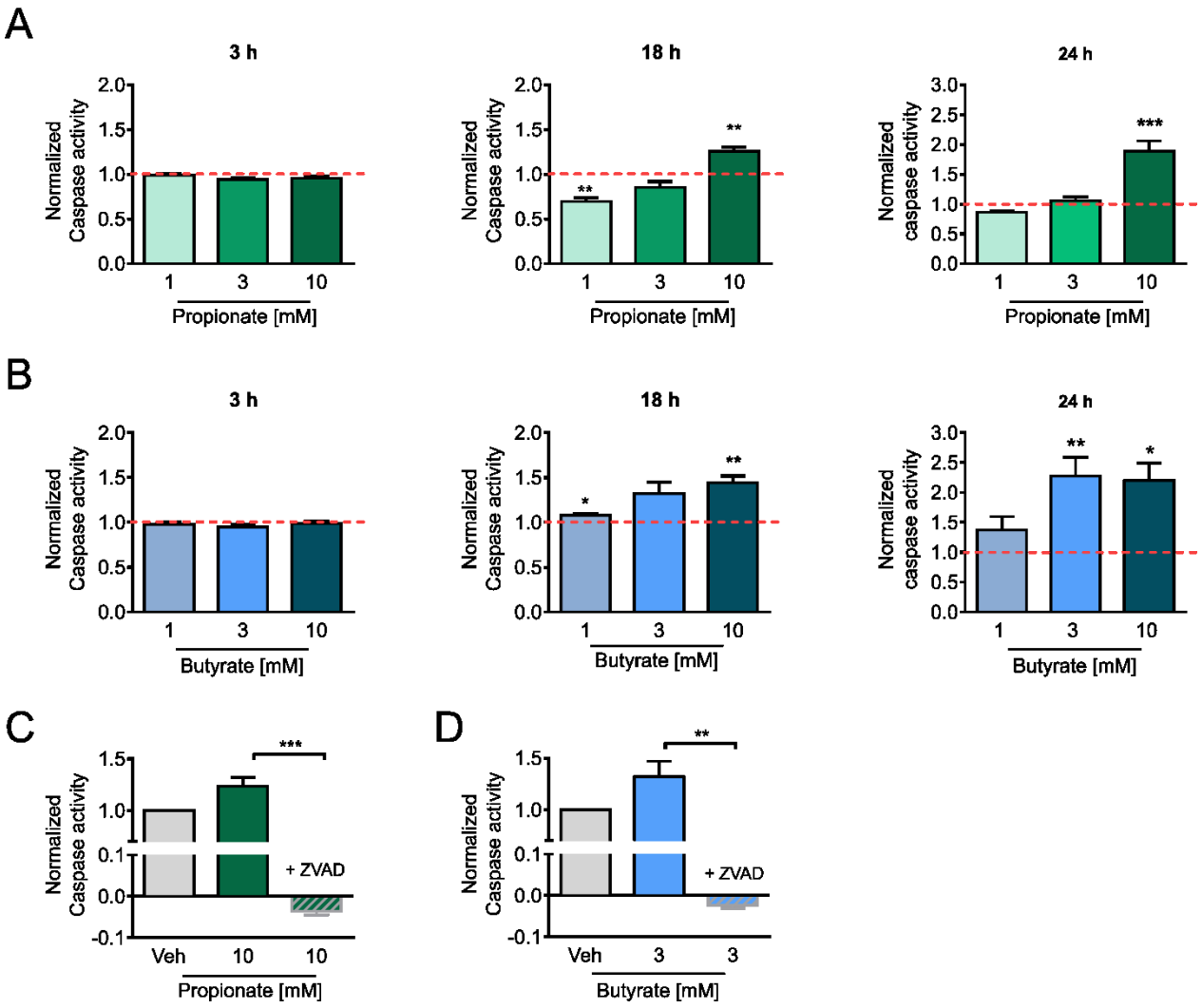

Figure 13. Propionate and butyrate activate effector caspases in peripheral blood eosinophils. *Purified eosinophils were treated with propionate (1, 3, 10 mM) (A) or butyrate (1, 3, 10 mM) (B) for indicated time points. Alternatively, eosinophils were pre-treated with the pan-caspase inhibitor ZVAD before addition of propionate (C) or butyrate (D). Activation of effector caspase was detected. Data are shown as mean +SEM from 5-7 different donors and were analyzed by one-way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, *** P<0.001 vs. veh. This figure was adapted from (1).*

Importantly, this was also observed, in annexin V/PI staining, again confirming that propionate induces apoptosis in a caspase-dependent manner (Figure 14 A and B).

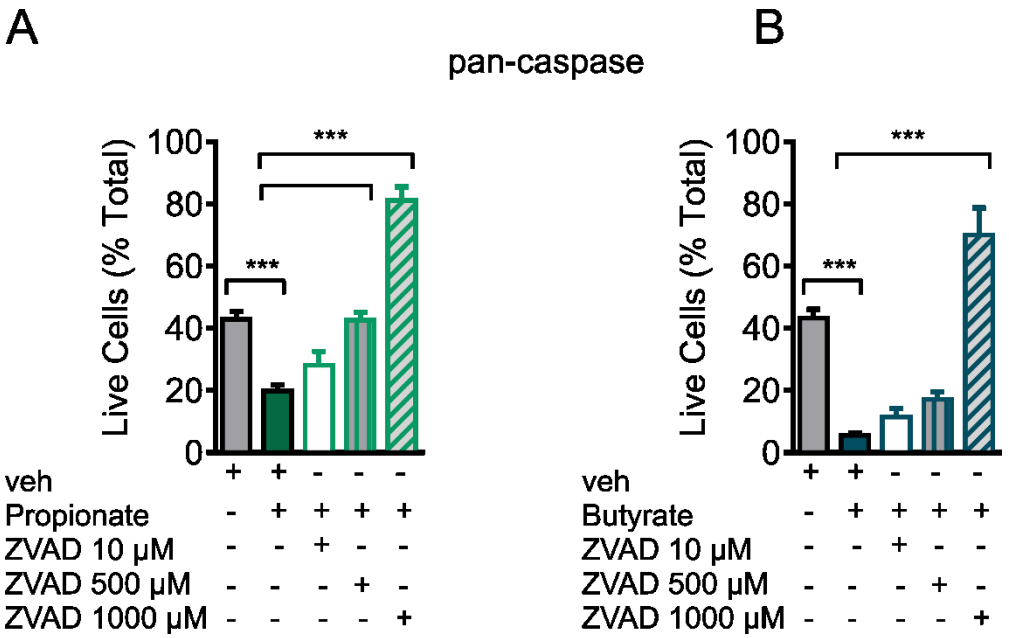

Figure 14. Propionate and butyrate induce apoptosis via caspase activation. *Eosinophils were pretreated with ZVAD-FMK (pan-caspase inhibitor, concentrations as indicated) followed by propionate (A) or butyrate (B). Cells were harvested 24 h after initial treatment and annexin V/PI staining was performed. Data are shown as mean +SEM from 5-9 different donors and were analyzed by one-way ANOVA, Tukey's post-test (D), *** P<0.001 vs. veh. This figure was adapted from (1).*

It has previously been shown that expression of the Bcl-2 family members BCL-XL and MCL-1 in eosinophils is associated with prolonged survival (1,30,214). Therefore I was interested whether apoptosis induction by propionate or butyrate in human eosinophils is due to downregulation of these two factors. I incubated eosinophils with propionate, butyrate (both 1, 3, 10 mM) or vehicle for 3 h and investigated changes of BCL-XL or MCL-1 expression at the mRNA level. IL-5 (50 pM) served as a control. According to literature (18), I found that IL-5 treatment increased the BCL-XL expression, but did not affect the MCL-1 expression (30) in human eosinophils (Figure 15 A and B). Propionate treatment (10 mM) significantly reduced BCL-XL expression (by 30 %), which was comparable to butyrate (10 mM, by 44 %), whereas lower SCFA concentrations had no decreasing effect (Figure 15 A). MCL-1 mRNA expression was also

significantly lowered by propionate (10 mM, by 31.5 %), while butyrate had an even stronger effect on MCL-1 expression (3 and 10 mM, 43 % and 65 %, respectively) (Figure 15 B) (1).

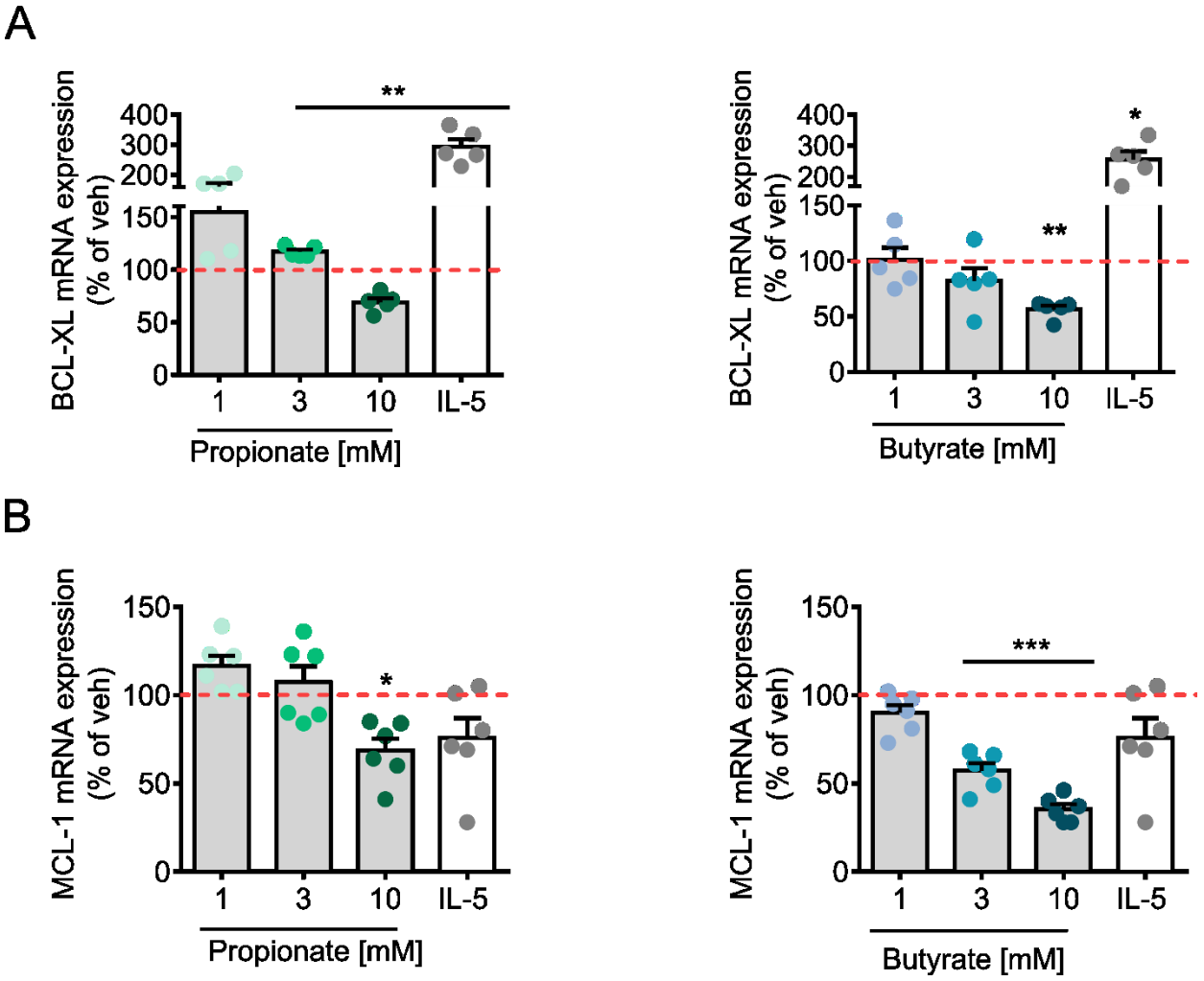

Figure 15. Short chain fatty acid treatment downregulates anti-apoptotic factors. *Purified eosinophils were isolated and treated with propionate or butyrate (concentrations as indicated) for 3 h. Total RNA was isolated and cDNA was synthesized. BCL-XL (A) and MCL-1 (B) expression is depicted. Data are shown as mean +SEM from 5-6 different donors, and were analyzed by one-way ANOVA, Tukey's post-test, * P<0.05, ** P<0.01, *** P<0.001 vs. veh (indicated as red dotted line). This figure was adapted from (1). Wolfgang Platzer performed real-time PCR experiment.*

Next, I was interested whether SCFA pre-treatment is able to abolish the survival promoting effect of IL-5. Therefore, eosinophils were pre-treated with propionate, butyrate or the corresponding vehicle before addition of IL-5 (50 pM). Strikingly, propionate (10 mM) and butyrate (3 and 10 mM) pre-treatment hampered the survival-promoting effect of IL-5 (Figure 16 A). This was also visualized in Figure 16 B by unspecific membrane labeling using the WGA staining (1).

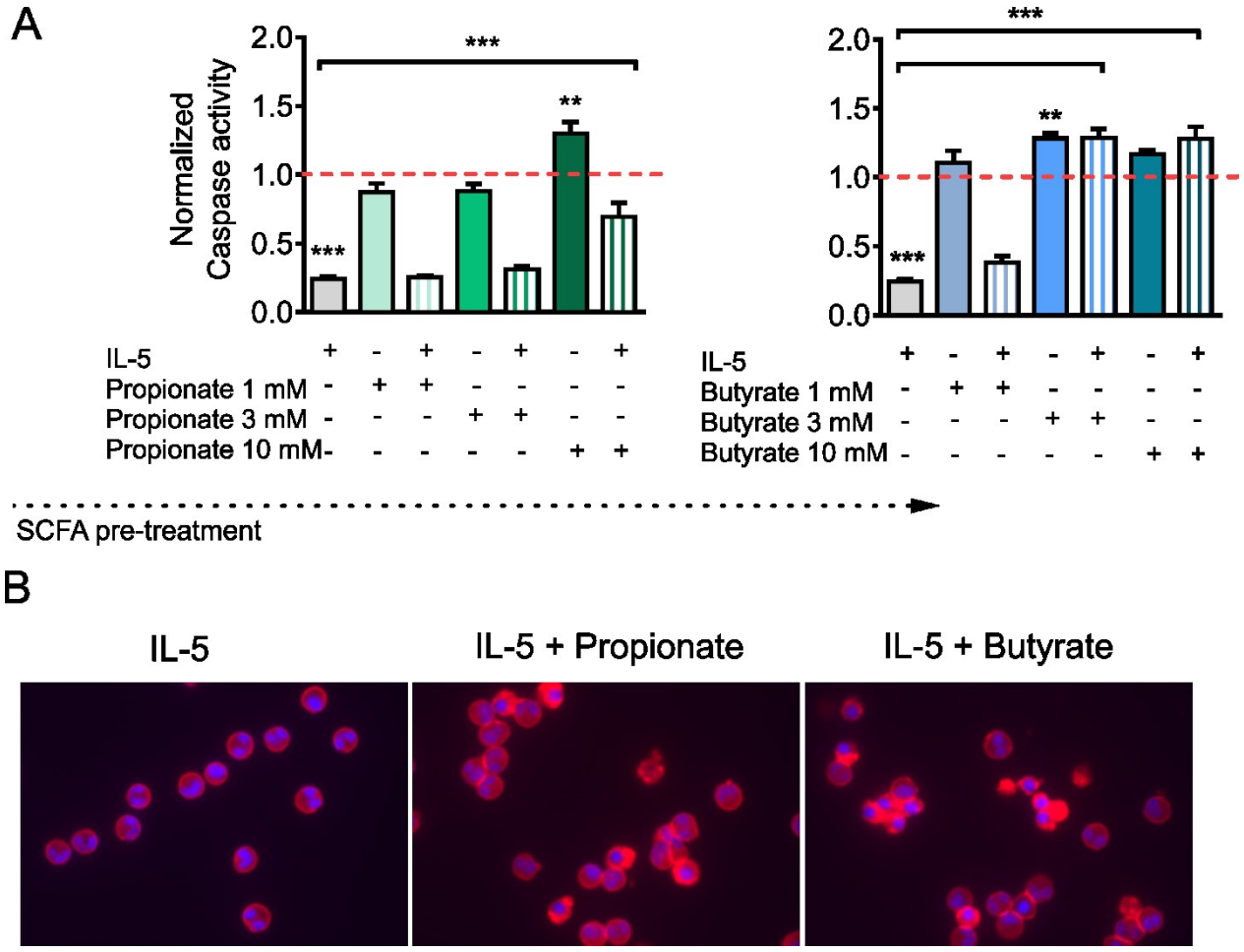

Figure 16. SCFA pre-treatment abolishes the survival promoting effect of IL-5 in eosinophils. *Eosinophils were pre-treated with SCFA (for 3 h) before addition of IL-5 and were cultivated for 24 h in total (A). Eosinophils treated with SCFA and IL-5 were visualized by WGA staining (B). Data are expressed as means + SEM from 5-7 different donors and were analyzed by one –way ANOVA, Tukey's post-test (A-E), **P<0.01, ***P<0.001. This figure was adapted from (1).*

The latter could also be observed when eosinophils were pre-treated with IL-5 (10 pM) before the addition of propionate (10 mM) or butyrate (3 and 10 mM) again already after 18 h till 24 h (Figure 17 A and B) (1).

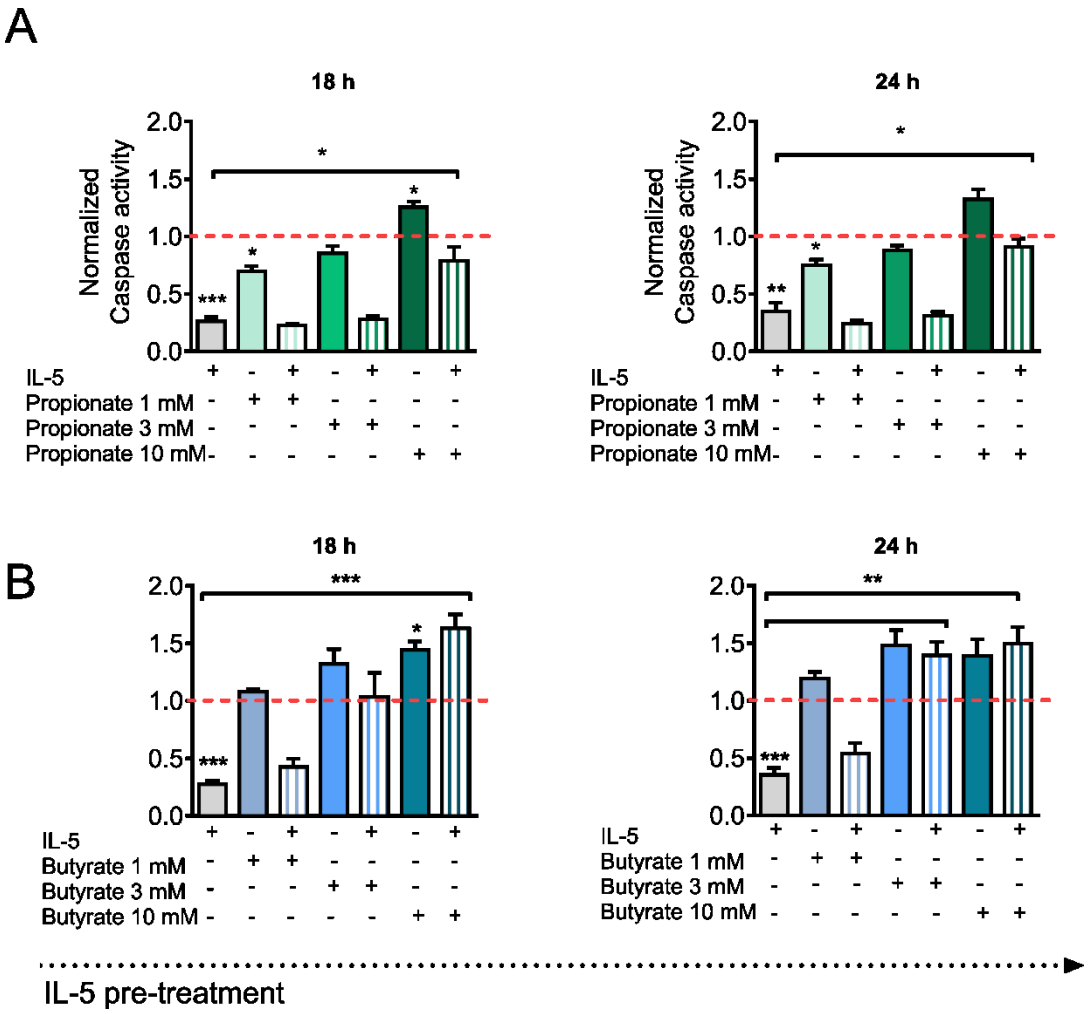

Figure 17. IL-5 pretreatment does not hamper caspase 3/7 activation by propionate and butyrate. *Purified eosinophils were pre-treated with IL-5 for 3 h before addition of propionate (A) or butyrate (B) as indicated, which still caused caspase activity. Data are expressed as means + SEM from 6-7 different donors and were analyzed by one –way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, ***P<0.001. This figure was adapted from (1).*

These findings clearly suggest that propionate and butyrate are potent inducers of eosinophil apoptosis, by activating the intrinsic apoptosis pathway also in the presence of the eosinophil survival factor IL-5.

In the previous experiments, only eosinophils from self-reported allergic donors were used. Hence, I sought to investigate whether there is a difference in SCFA-induced caspase activation between allergic and non-allergic individuals. Eosinophils from healthy and self-reported donors were treated with propionate, butyrate (both 1, 3, 10 mM, for 24 h) and caspase 3/7 activation was determined. Notably, both propionate (at 10 mM) and butyrate (3 mM) induced caspase activation only in eosinophils from allergic donors, whereas eosinophils from healthy individuals were unaffected (Figure 18 A) (1).

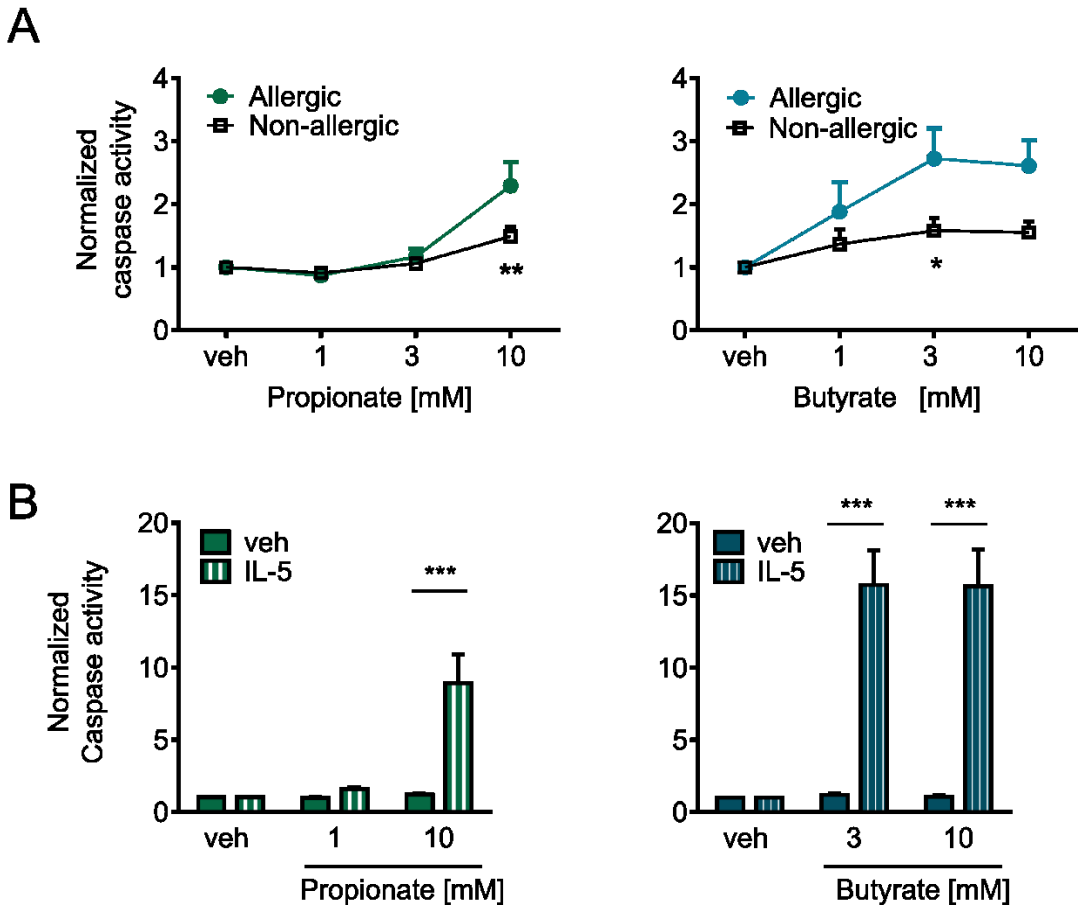

Figure 18. SCFA induce caspase activation in eosinophils from allergic donors. *Eosinophils from allergic and non-allergic individuals were treated with propionate (1, 3, 10 mM) or butyrate*

*(1, 3, 10 mM) for 24 h (A). Priming of eosinophils from non-allergic donors followed by propionate (1, 10 mM) or butyrate (3, 10 mM) treatment caused caspase activation (B). Data are shown as mean +SEM from 6 different donors and were analyzed by two-way ANOVA Tukey's or Bonferroni's post-test, *P<0.05, **P<0.01, *** P<0.001. This figure was adapted from (1).*

It was previously reported that Siglec 8 induced eosinophil apoptosis was enhanced when eosinophils were pre-activated with 30 ng/mL of IL-5 (35). Additionally, TNF-α treatment increased the butyrate-induced apoptosis in colon carcinoma cell lines (215,216). Hence I speculated that a cytokine, especially IL-5 rich environment was a mandatory factor for SCFA to induce caspase 3/7 activation in eosinophils. Hence, eosinophils from healthy donors were primed with IL-5 (30 ng/mL) (35) or vehicle before addition of propionate (1 and 10 mM) or butyrate (3 and 10 mM). Strikingly, caspase 3/7 activation by propionate (10 mM) and butyrate (3 and 10 mM) was restored by priming of eosinophils whereas this was absent in vehicle primed eosinophils (Figure 18 B), suggesting IL-5 activation is a prerequisite for SCFA-induced caspase activation (1).

3.6 Propionate and butyrate induce histone 3 (H3) acetylation in eosinophils

Apart from signaling via G protein-coupled receptors (128–130,211), butyrate and to a smaller degree propionate are known inhibitors of histone deacetylases (HDAC), which mostly accounts for their anti-inflammatory potential (142,143,146). HDACs are located intracellularly and it has been reported that SCFA can be transported into the cell via monocarboxylate transporters (MCT) (143). Hence, eosinophils were incubated with α-cyano-4-hydroxycinnamic acid (CHC, 30 min), a pan inhibitor for monocarboxylate transporters (MCT) before treatment with propionate or butyrate. Cells were harvested after 24 h and annexin V/PI staining was performed. Of note, 5 mM of CHC completely abolished the propionate-induced decrease in the live cell portion (Figure 19 A) but only partly diminished the butyrate-induced effect (Figure 19 B) (1).

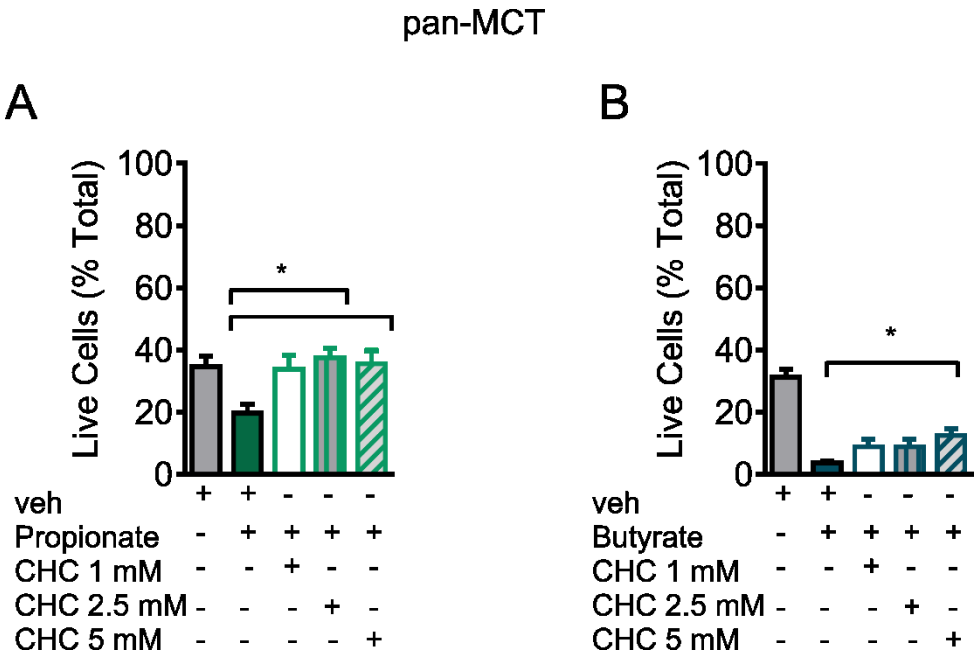

Figure 19. SCFA-induced apoptosis is dependent on MCT transporters. *Isolated eosinophils were pre-treated with CHC (pan-monocarboxylate transporter inhibitor), followed by propionate (A) or butyrate (B) treatment. Cells were harvested 24 h after initial treatment and annexin V/PI staining was performed. Data are shown as mean +SEM from 5-9 different donors and were analyzed by one-way ANOVA, Tukey's post-test (D), *P<0.05 vs. veh. This figure was adapted from (1).*

In order to confirm that propionate and butyrate exert HDAC inhibitory properties on human eosinophils, the cells were treated with propionate, butyrate or vehicle (10 mM, 3 mM) for the indicated time points and H3 acetylation as a measure of HDAC inhibition was determined (143) (Figure 20 A and B). I could detect acetylated H3 3 h after treatment with propionate or butyrate. H3 acetylation further increased after 18 h and 24 h post treatment, suggesting that both SCFA act as HDAC inhibitors on human eosinophils (Figure 20 A and B) (1).

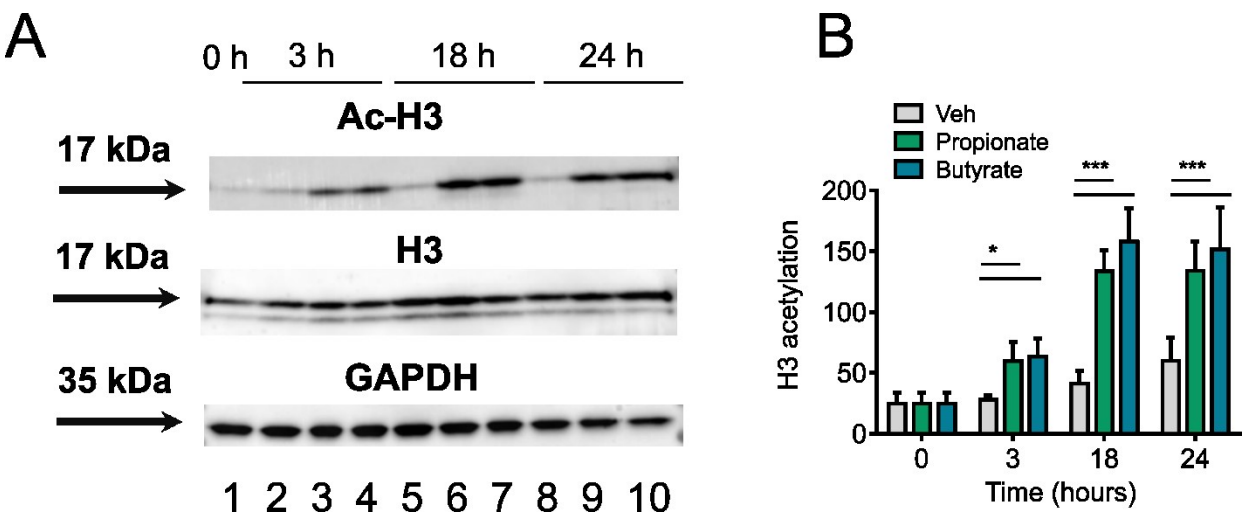

Figure 20. HDAC inhibition induced by propionate and butyrate was detected via H3 acetylation. *Eosinophils were incubated with propionate (lanes 3, 6, 9), butyrate (4, 7, 10) or vehicle (1, 2, 5, 8) for indicated time spans. Eosinophil lysates were probed for acetylated (ac)-H3, H3 and GAPDH. (A). H3 acetylation was quantified and normalized to GAPDH (B). Data are shown as mean +SEM from 5 different donors and were analyzed by two-way ANOVA, Tukey's post-test (D), *P<0.05, *** P<0.001 vs. veh. This figure was adapted from (1). Western blot was done by Wolfgang Platzer.*

The pan-HDACi trichostatin A (TSA) has been previously shown to promote eosinophil apoptosis (34); therefore, I set out to substantiate these results herein. In fact, TSA concentration-dependently decreased the live eosinophil population after 24 h treatment (Figure 21 A, right panel), whereas incubation for 3 h did not impair eosinophil survival (Figure 21 A, left panel). This was similar to propionate and butyrate and further supports the potential of HDACi in human eosinophils. Additionally, I tested several subtype-selective HDACi on eosinophil apoptosis and found that the HDAC class IIa selective inhibitor MC1568 significantly reduced eosinophil survival after 24 h incubation (Figure 21 B), whereas selective inhibitors of HDAC 2, 3, and 8 did not shorten the portion of live eosinophils (Figure 21 C-E) (1).

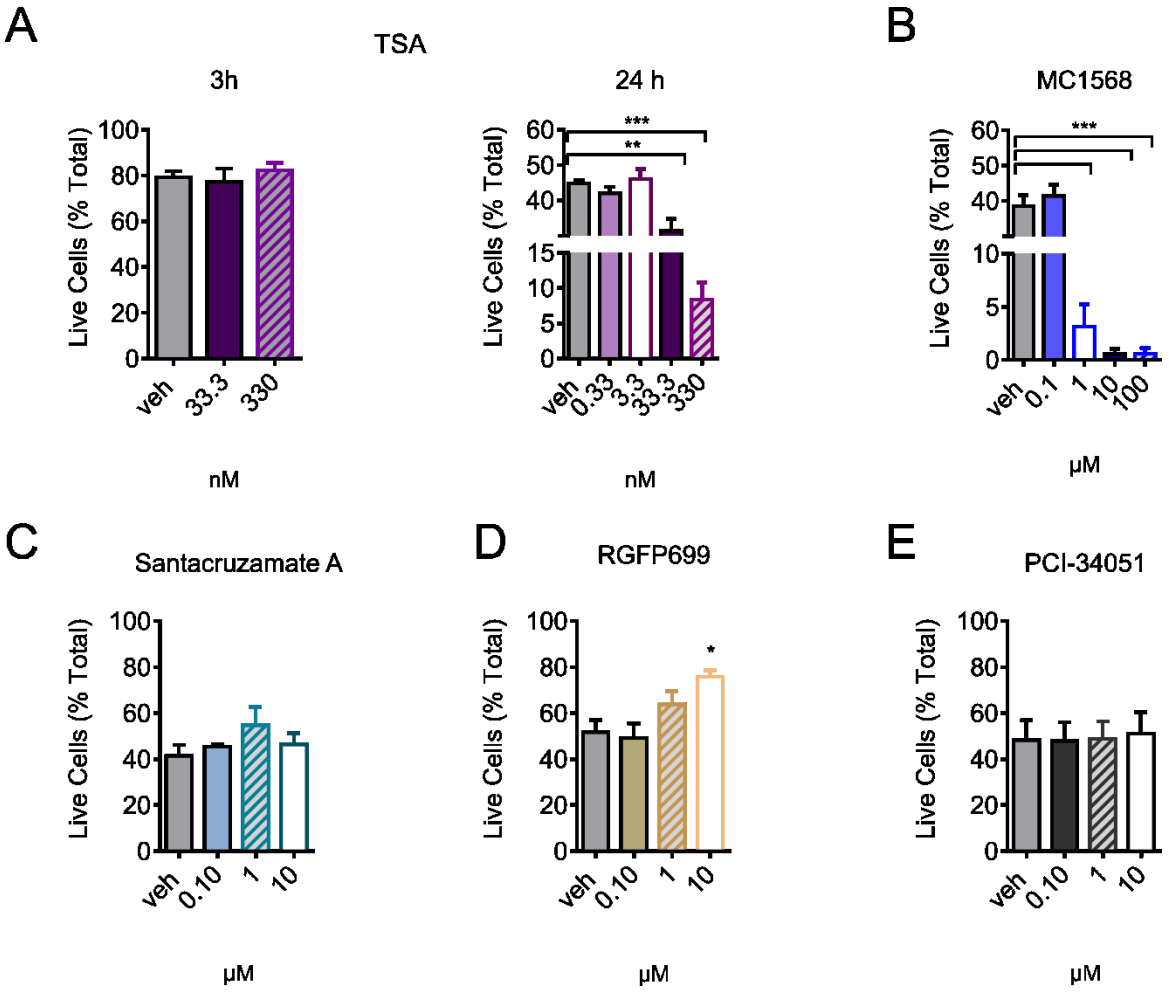

Figure 21. Effect of HDACi inhibitors on eosinophil survival. *Eosinophils were incubated with the pan-HDACi TSA for 3 h or 24h (A) the selective inhibitors for class II HDACs MC1568 (B) HDAC2 (C), HDAC3 (C) or HDAC8 (D) for 24 h. Annexin V/PI double staining was performed and analyzed by flow cytometry. The live cell population is shown as mean + SEM from 4-5 different donors. Data were analyzed by one-way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, *** P<0.001 vs. veh. This figure was adapted from (1).*

3.7 Propionate and butyrate regulate eosinophil transcription

Since my data clearly suggest that propionate and butyrate impair the survival of eosinophils from allergic donors by activating the intrinsic apoptosis pathway, I was eager to shed light on the regulatory potential of SCFA on the transcription of genes involved in adhesion,

migration and survival of human eosinophils. Eosinophils were incubated for 3 h with acetate, propionate, butyrate (all 1-10 mM), IL-5 (50 pM) or vehicle. Since I could detect histone acetylation, as a measure of HDAC inhibition induced by SCFA but no viability impairment at 3 h, I chose this time point for mRNA analysis. Integrin α-4 (CD49d) mRNA expression was significantly reduced with treatment of acetate or propionate (10 mM), whereas lower concentrations were ineffective. Likewise, butyrate effectively abrogated CD49d transcription at lower concentrations (1 mM), in comparison to vehicle. IL-5 also decreased the CD49d mRNA expression levels (Figure 22 A). Butyrate decreased the mRNA expression of the extracellular matrix receptor CD44 in a concentration-dependent fashion which, however, was unimpaired by incubation with acetate or propionate (Figure 22 B). Expression of CCR3, the crucial chemotactic receptor on eosinophils (1,217), was diminished by propionate (10 mM) and also by IL-5, while the effect of butyrate was again very potent and concentration-dependent. In contrast, acetate failed to blunt CCR3 expression of human eosinophils (Figure 22 C) (1).

Since I found that SCFA were particularly effective at inducing apoptosis in the presence of IL-5, I tested for changes of IL-5RA expression after SCFA treatment. Strikingly, my data showed that propionate and butyrate, but not acetate concentration-dependently decreased expression IL-5RA at the transcriptional level (Figure 22 D) (1).

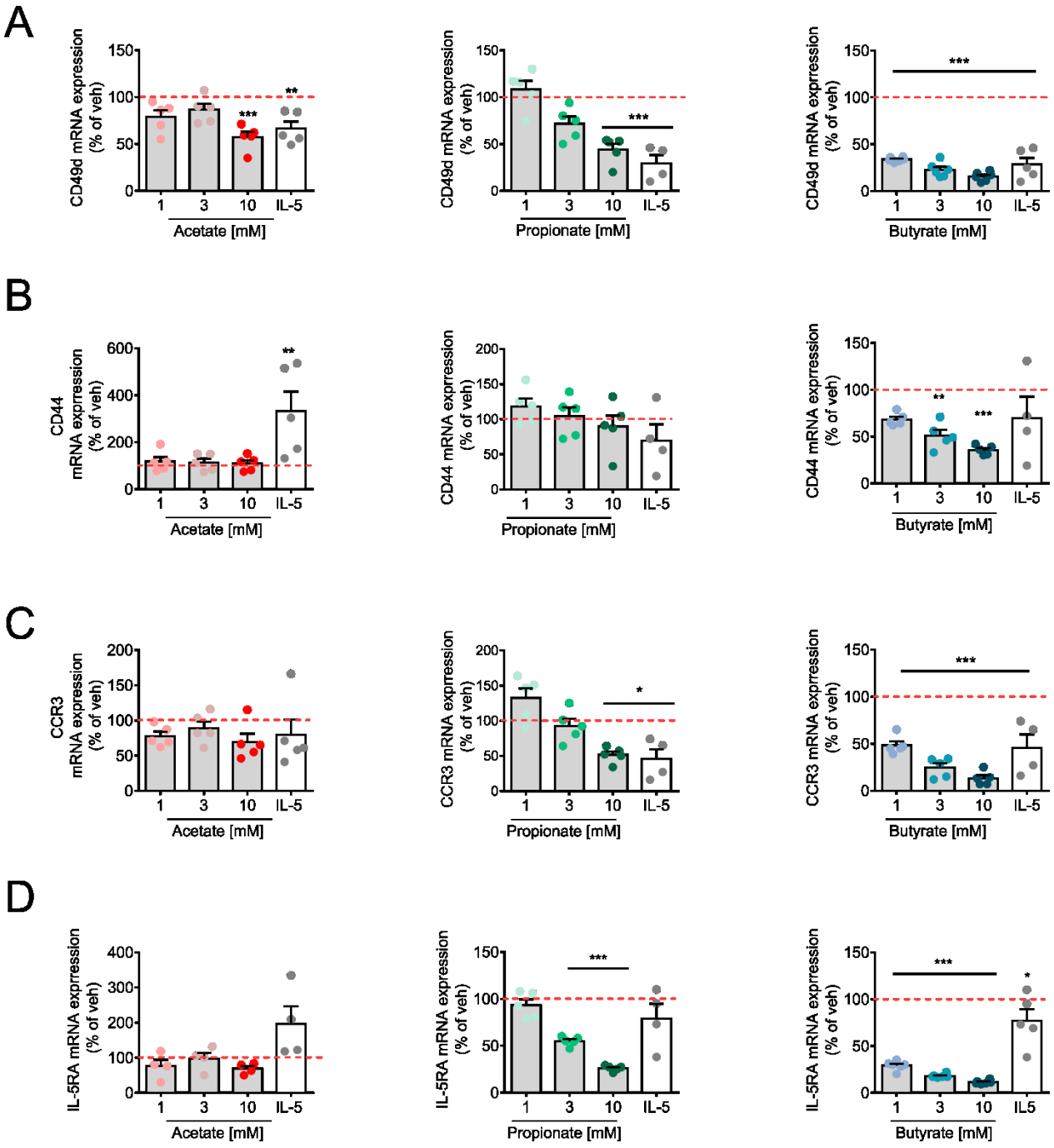

Figure 22. Expression of transcripts involved in eosinophil adhesion, migration and survival is regulated by SCFA. *Acetate, propionate and butyrate decrease the expression of CD49d mRNA (A). Butyrate, but not acetate or propionate also downregulated CD44 mRNA (B). CCR3 (C) and IL-5RA (D) mRNA expression was diminished by propionate and butyrate, whereas acetate could*

*not mimic this effect. Data are depicted as mean +SEM from 4-6 different donors, and were analyzed by one-way ANOVA, Tukey's post-test, *P<0.05, **P<0.01, *** P<0.001 vs. veh (indicated as red dotted line). This figure was adapted from (1). Wolfgang Platzer performed real-time PCR experiment.*

In order to further confirm my findings on mRNA level, I set out to investigate the surface expression of CD49d, CCR3 and CD44 on human eosinophils. Eosinophils were incubated with acetate, propionate (both 10 mM) or butyrate (3 mM) for 18 h. Gating on living eosinophils was performed (1).

CD49d surface expression was significantly decreased by propionate and butyrate treatment (Figure 23 A, right panel), whereas acetate was again ineffective (Figure 24 A, right panel). Of note, butyrate (3 mM) treatment reduced CCR3 and CD44 surface expression on eosinophils (Figure 23 B, C), whereas propionate (Figure 23 B, C) and acetate (Figure 24 B and C, right panels) failed to mimic this response (1).

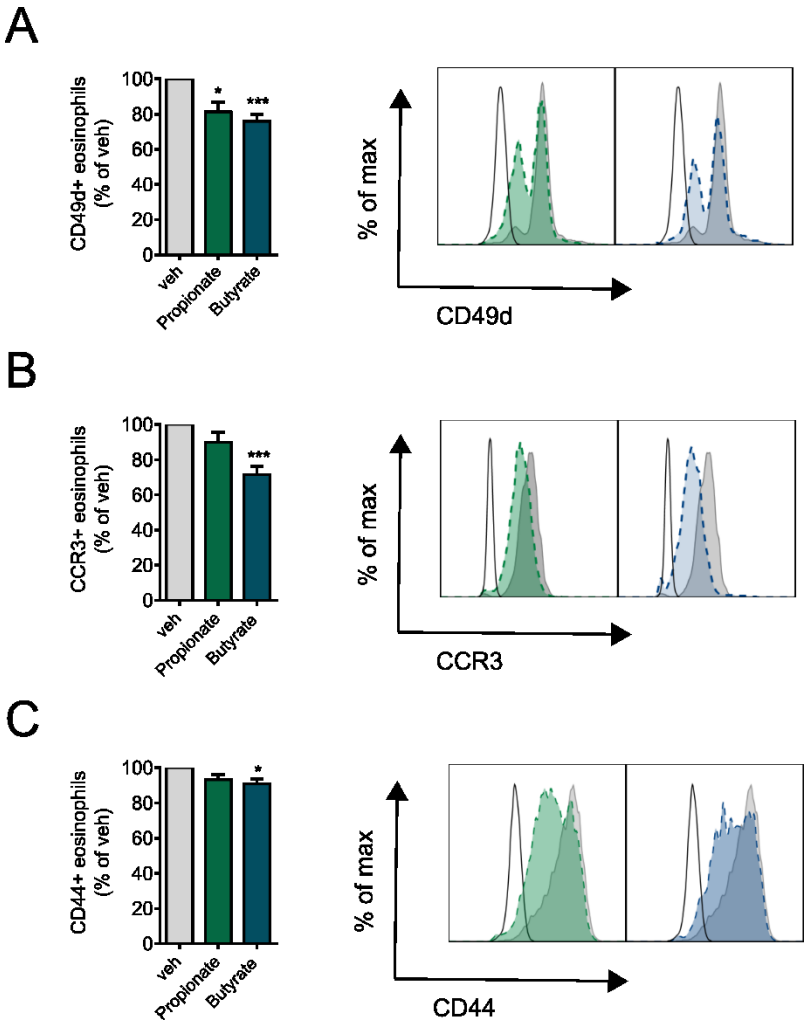

Figure 23. Surface expression of CD49d, CCR3 and CD44 under SCFA treatment. *Propionate and butyrate decrease the surface expression of CD49d (A). Butyrate, but not propionate also decreased CCR3 (B) and CD44 expression (C) on the cell membrane. Left panels, surface expression on eosinophils; right panels show typical flow cytometry plots. Gating on live eosinophils was performed for all flow cytometry stainings. Histograms: line: isotype, grey: vehicle, colored: respective treatment. Data are depicted as mean +SEM from 4-6 different donors, and were analyzed by one-way ANOVA, Tukey's post-test, * P<0.05, *** P<0.001 vs. veh. This figure was adapted from (1).*

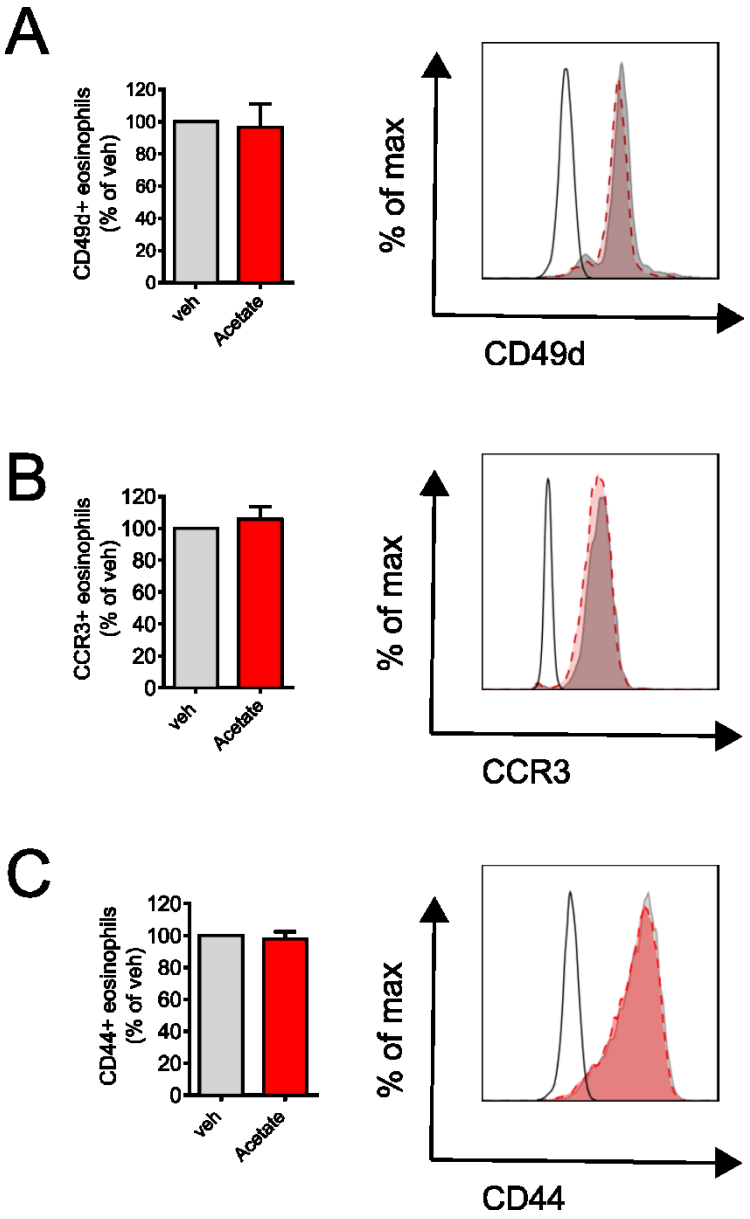

Figure 24. Surface expression of CD49d, CCR3 and CD44 under acetate treatment. Effect of acetate on eosinophil activation marker expression. *Acetate treatment of eosinophils alters surface expression of CD49d (A), CCR3 (B) or CD44 (C) The left panels illustrate the surface expression on eosinophils and the right panels depict typical flow cytometric plots. Gating on viable eosinophils was performed. Histograms: line: isotype, grey: vehicle, colored: respective treatment. Data are shown as mean +SEM from 4-6 different donors, and were statistically analyzed by one-way ANOVA, Tukey's post-test.*

Interestingly, L-selectin surface expression on eosinophils was unimpaired by SCFA application (Figure 25) (1).

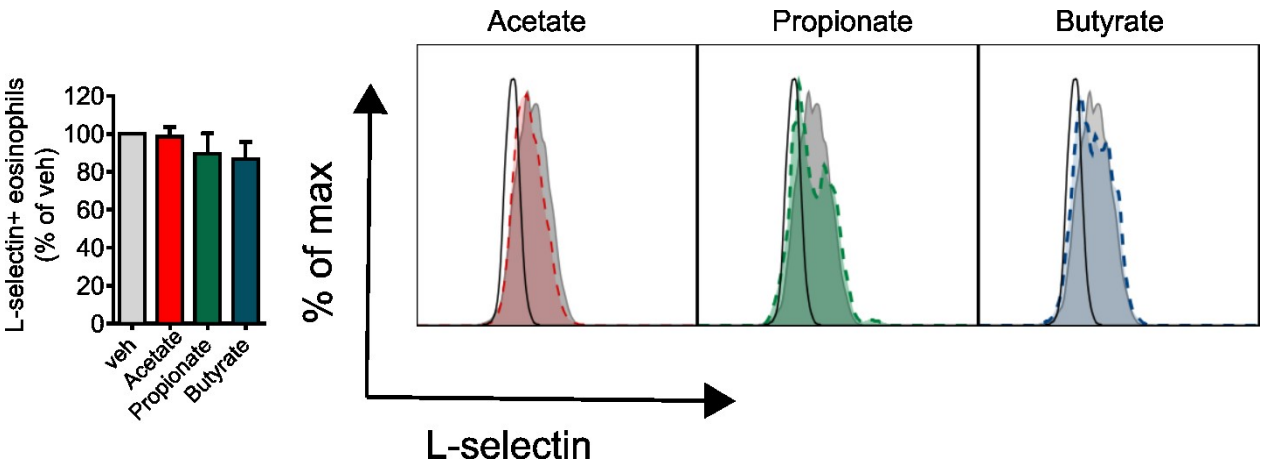

Figure 25. L-selectin surface expression after SCFA treatment. *Incubation of eosinophils with acetate (10 mM), propionate (10 mM) or butyrate (3 mM) had no impact on L-selectin surface expression. Gating on live eosinophils was performed. Histograms: line: isotype, grey: vehicle, colored: respective treatment. Data are shown as mean +SEM from 4-6 different donors, and were statistically analyzed by one-way ANOVA, Tukey's post-test. This figure was adapted from (1).*

3.8 Butyrate controls eosinophil locomotion, which is mimicked by TSA

Since I found that propionate and butyrate regulate molecules which are crucial in eosinophil adhesion and migration I consequently evaluated if this also translates into a functional level. Eosinophils were activated with eotaxin-2 (10 nM) or vehicle for 10 min and were superfused over pulmonary microvascular endothelial cell monolayer under physiological flow conditions in order to study eosinophil adhesion. As expected, eotaxin-2 induced a considerable adhesion of eosinophils to the endothelial monolayer. Crucially, pre-treatment with propionate (10 mM) or butyrate (3 mM) for 3 h significantly abolished this effect to control level (Figure 26 A and B) (1).

Consequently, I studied the role of propionate and butyrate on eosinophil migration. For that purpose a micro Boyden chamber assay was used. Eosinophils were pre-treated with propionate, butyrate or vehicle (same conditions as above) and allowed to migrate towards serial

dilutions of eotaxin-2 or vehicle. Interestingly, propionate had no effect on chemotaxis, while butyrate attenuated the eotaxin-2 induced migration (Figure 26 C and D) (1).

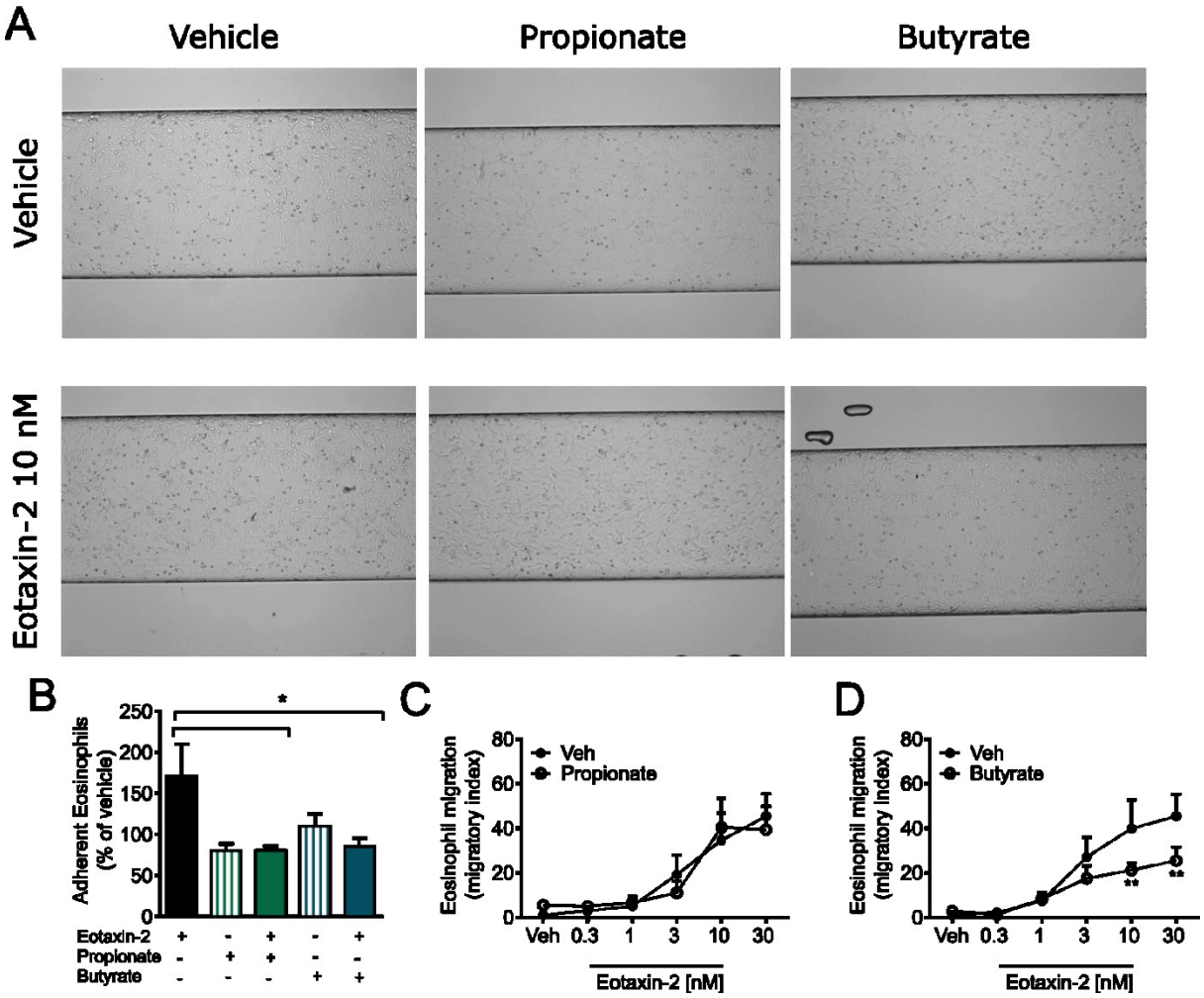

Figure 26. The eotaxin-2 induced eosinophil adhesion and chemotaxis is attenuated by butyrate. *Propionate and butyrate blunt the eotaxin-2 evoked adhesion of eosinophils to endothelial cells under physiological flow conditions (A, B). Pre-treatment with butyrate abated the eotaxin-2 induced migration (D), whereas propionate was ineffective (C). Data show means + SEM (5-6 different donors) and were analyzed by one-way (B) or two-way ANOVA, Tukey's post-test (C, D), *P<0.05, **P<0.01. Original micrographs (A) are shown in 5x magnification. This figure has been previously published (1). Eosinophil adhesion was assayed with the help of Miriam Peinhaupt. Kathrin Rohrer performed chemotaxis assay.*

Pre-treatment of eosinophils with acetate (10 mM) under the same experimental conditions as above could neither prevent from eotaxin-2 induced adhesion nor migration (Figure 27 A-C), which is in line with my previous findings (1).

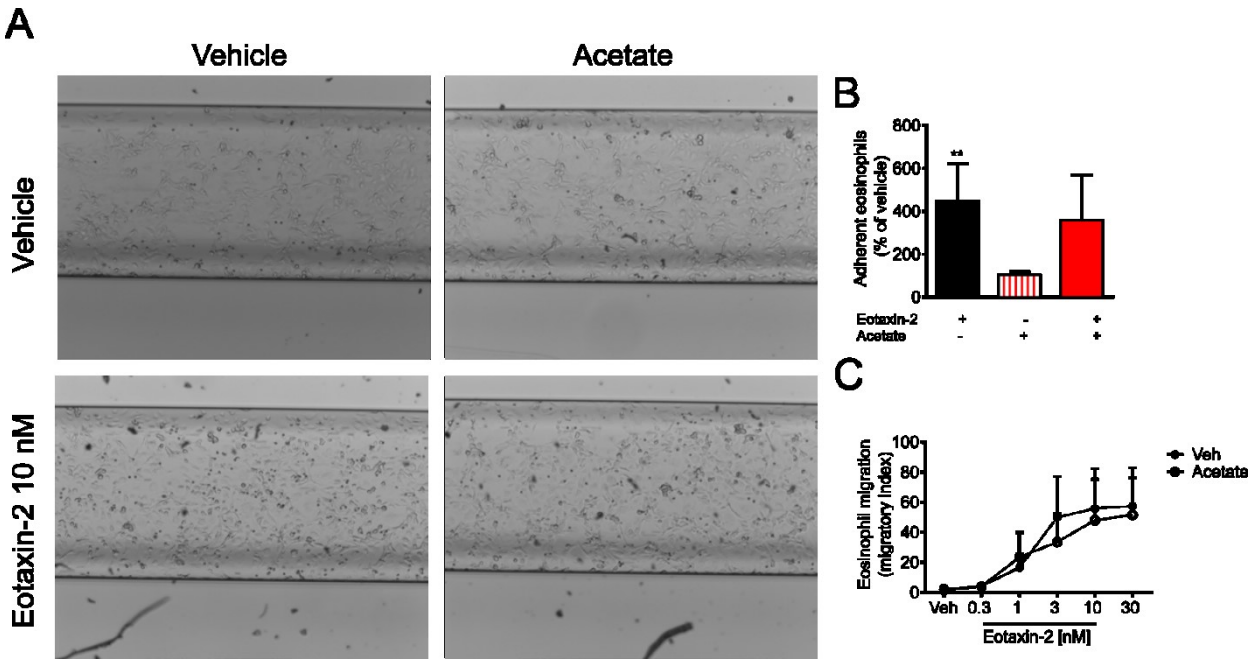

Figure 27. Acetate failed to impair eotaxin-2 induced adhesion and migration of eosinophils. *Acetate had no impact on the eotaxin-2 mediated adhesion of eosinophils on pulmonary endothelial cells under physiological flow conditions (A, B). Similarly, acetate failed to impair the eosinophil chemotaxis towards eotaxin-2 (C). Data show means + SEM (5-6 different donors) and were analyzed by Student's t-test (B) or two-way ANOVA, Tukey's post-test (C), **P<0.01. Original micrographs (A) are shown in 5x magnification. This figure has been previously published (1). Eosinophil adhesion was assayed with the help of Wolfgang Platzer. Kathrin Rohrer performed chemotaxis assay.*

Importantly, the pan-HDACi TSA (330 nM) mimicked butyrate on eosinophil adhesion and migration and completely attenuated the eotaxin-2 induced adhesion to the endothelium (Figure 28 A and B) and further curbed the eosinophil chemotaxis towards eotaxin-2 (Figure 28 C) (1).

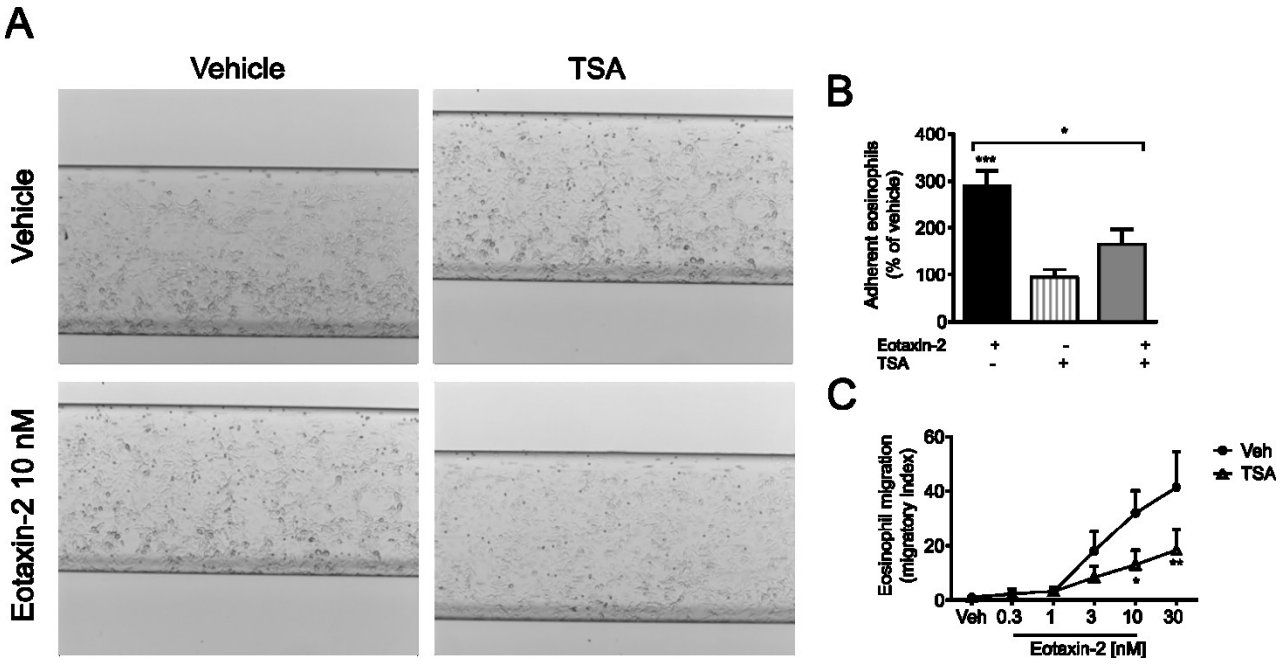

Figure 28. The pan HDAC inhibitor TSA mimics the effect of butyrate on eosinophil adhesion and migration. *TSA blunted the eotaxin-2 evoked adhesion of eosinophils to endothelial cells under physiological flow conditions (A, B). Pre-treatment with TSA abated the eotaxin-2 induced migration (C). Data show means + SEM (4-5 different donors) and were statistically analyzed by one-way (B) or two-way ANOVA, Tukey's post-test (C, D), *P<0.05, **P<0.01, *** P<0.001. Original micrographs (A) are shown in 5x magnification. This figure has been previously published (1). Eosinophil adhesion was assayed with the help of Wolfgang Platzer. Kathrin Rohrer performed chemotaxis assay.*

In contrast to my findings with regard to eosinophil adhesion and migration, propionate and butyrate failed to impair eosinophil shape change (Figure 29 A), CD11b upregulation (Figure 29 B) or degranulation as detected by CD63 expression (Figure 29 C) under the same experimental conditions (1).

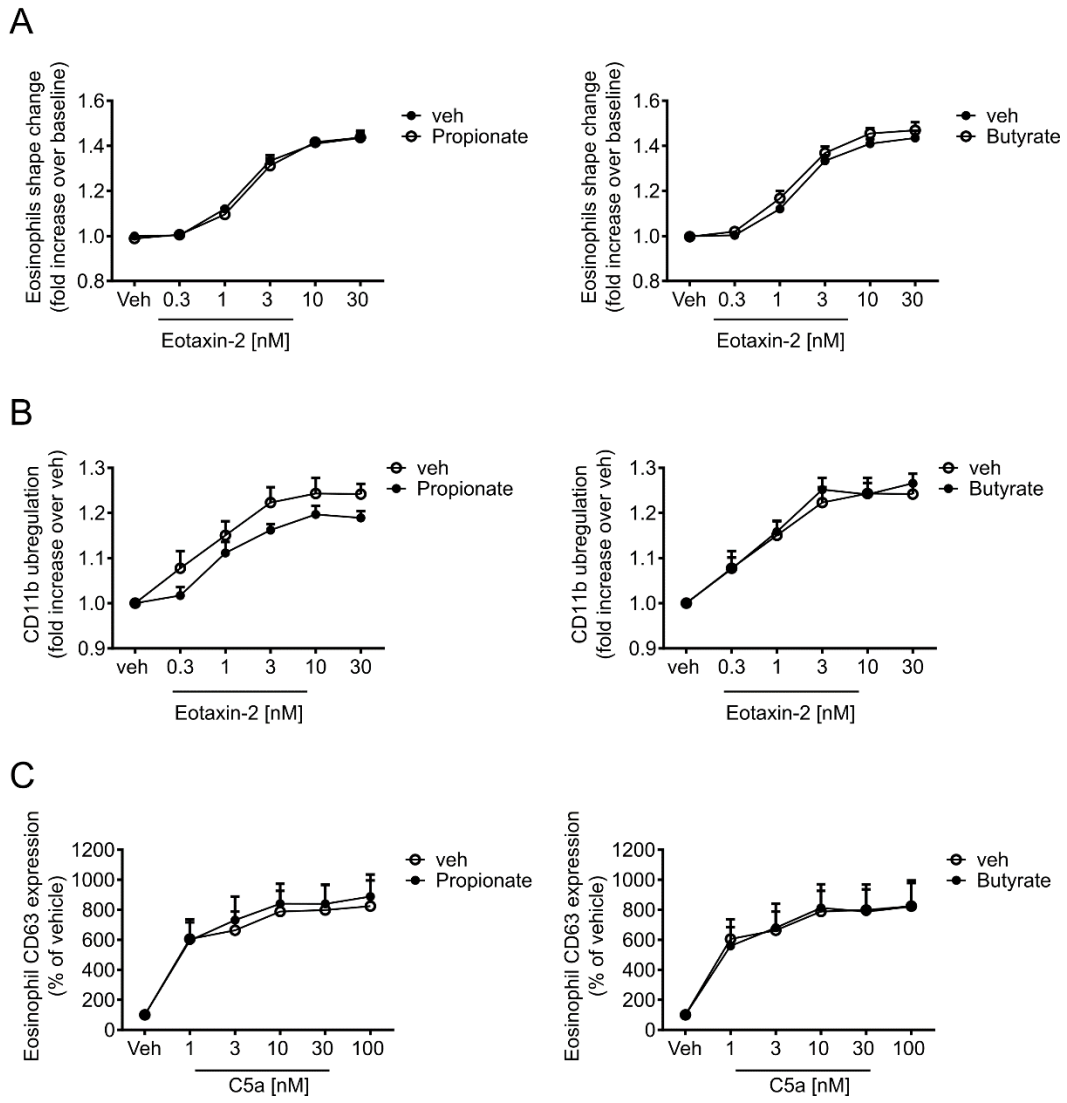

Figure 29. SCFA pre-treatment does not alter shape change, CD11b upregulation or CD63 expression on eosinophils*. Eosinophils were pre-incubated with vehicle (veh), propionate (left panel) or butyrate (right panel) for 3 h and were then stimulated with vehicle or eotaxin-2 (shape change, A, CD11b upregulation, B), or C5a (CD63, expression C) at indicated concentrations. Data are expressed as means +SEM from 4-6 different donors and were analyzed with two-way ANOVA, Tukey's post-test. This figure has been previously published (1).*

3.9 Butyrate alleviates airway eosinophilia induced by OVA in vivo

The gained *in vitro* data evidently demonstrate that SCFA, particularly butyrate, affect eosinophil recruitment and survival. Therefore, I sought to further investigate butyrate's *in vivo* relevance. The regulatory role of butyrate on ILC2 in allergic inflammation was recently reported (144). I chose the acute OVA-induced asthma mouse model. Female BALB/c mice were immunized to OVA adsorbed to $Al(OH)_3$ (day 0 and 7, 20 µg), and intraperitoneally treated with butyrate (1 g/kg) dailystarting from day 11 until day 20, which approximately produced a plasma concentration of 1.8 mM (1,218). From day 18-20 mice were challenged with OVA aerosol or vehicle. The experimental protocol was completed on day 21. Consequently, mice were sacrificed and BAL, blood and bone marrow were taken, lung homogenate was prepared or invasive spirometry was performed. The experimental procedure is depicted in Figure 30 (1).

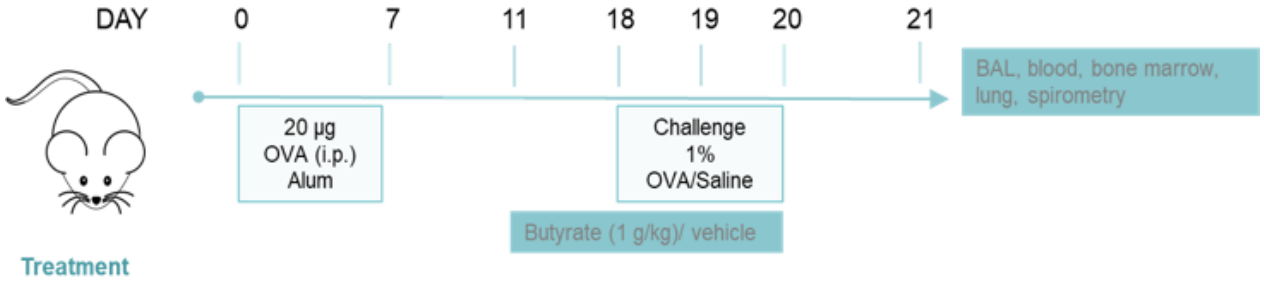

Figure 30. Schematic presentation of the in vivo OVA model. *Female BALB/c mice were immune with i.p. injection of OVA adsorbed to alum on day 0 and 7, followed by daily injection of butyrate or saline starting from day 11 until day 20. Mice were challenged on day 18-20 with OVA aerosol or saline before being sacrificed on day 21. Organ collection was performed with help of Thomas Bärnthaler and Ilse Lanz.*

OVA challenge caused a massive influx of immune cells, the majority being eosinophils, into bronchoalveolar space, which was strongly impeded by systemic butyrate application (Figure 31 A). Butyrate without OVA exposure did not affect the cell numbers (Figure 31 A and B) in BAL. Importantly, I observed no changes on other cell types upon butyrate treatment, including lymphocytes, alveolar macrophages or neutrophils (Figure 31 C). The gating strategy is provided in Figure 32 (1).

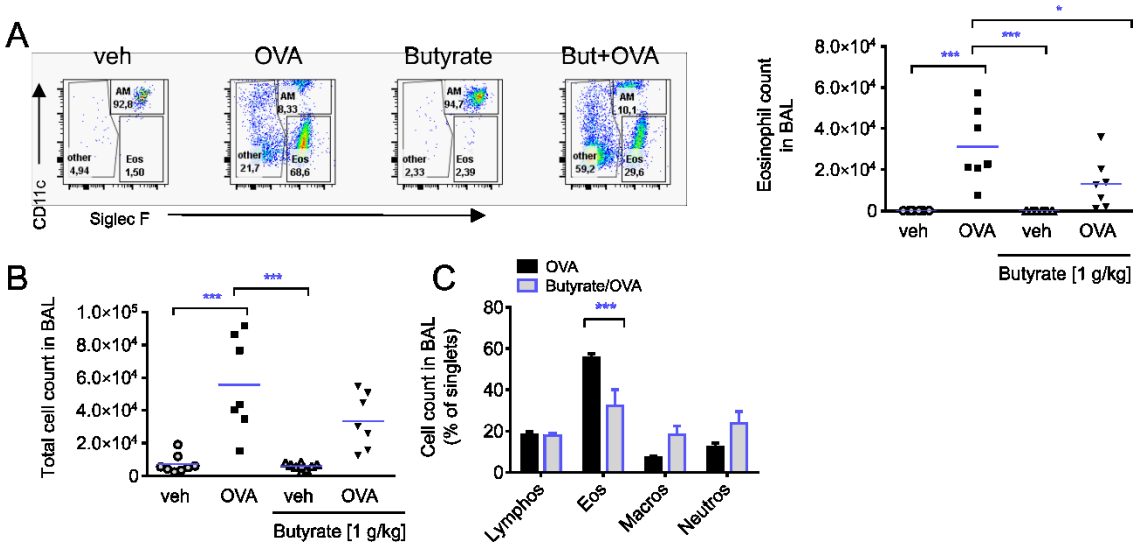

Figure 31. Butyrate ameliorates airway eosinophilia in BAL fluid of OVA exposed mice. *Systemic butyrate application diminished eosinophil influx into the airways (A). Total cell count (B) and differential cell count from BAL (C) is depicted. Data are expressed as means or means +SEM, 7-8 mice per group. Statistical differences were calculated with one-way (B, C) or two-way ANOVA, Tukey's post-test (D, E), *P<0.05, ***P<0.001. Figure adapted from (1).*

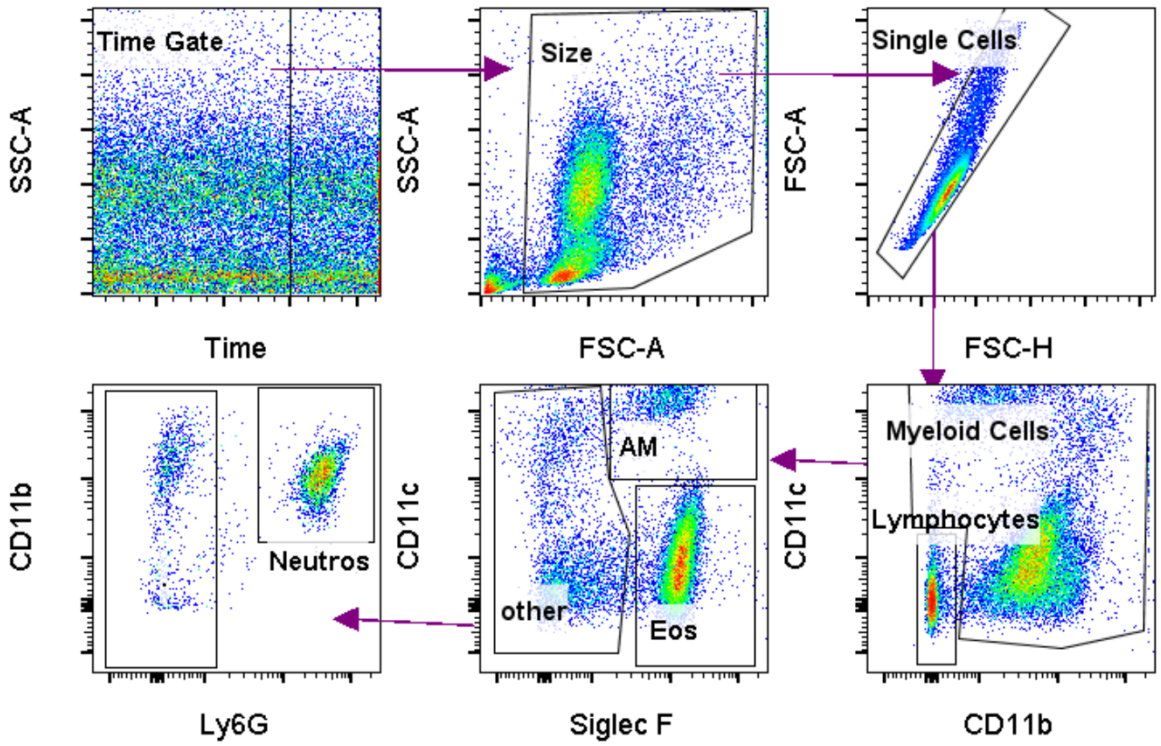

Figure 32. Gating strategy for BAL leukocytes. *BALB/c mice were sensitized and exposed to OVA or vehicle. From day 11 until the end of the experiment mice were injected with butyrate or vehicle. BAL fluid was taken on day 21. The gating strategy for lymphocytes, eosinophils (Eos), alveolar macrophages (AM) and neutrophils (Neutros) is shown. Cells counted for 30 s (represented in the time gate) were used for analysis. This figure has been previously published (1).*

It has been previously reported that SCFA treatment increases the barrier function of epithelial cells (169). To exclude the possibility that the butyrate-dependent reduction of eosinophils in bronchoaveolar space is due to increased epithelial barrier integrity and not due to reduced eosinophil recruitment, I also harvested the mouse lung in a different set of experiments and determined cell numbers in lung tissue. Figure 33 depicts the gating strategy for lung eosinophils.

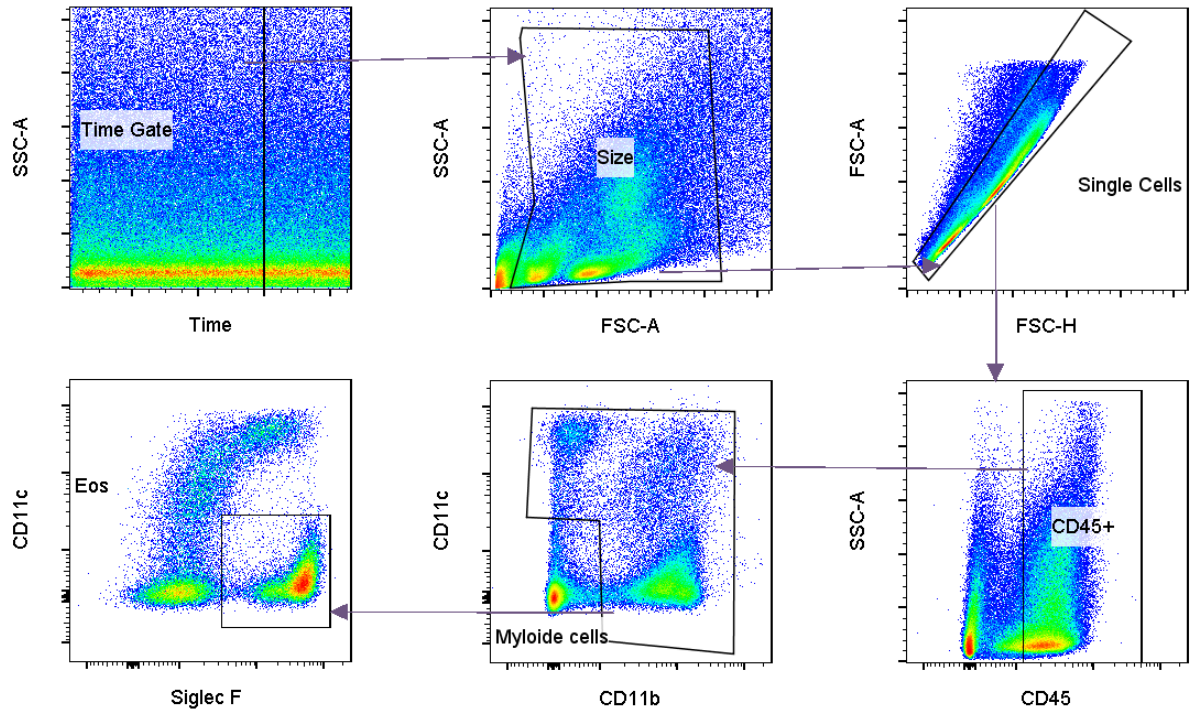

Figure 33. Gating strategy for lung eosinophils. *Female BALB/c mice were sensitized and exposed to OVA or vehicle. From day 11 until the end of the experiment mice were injected with butyrate or vehicle. The lung was taken on day 21 and tissue homogenization was performed. The gating strategy for eosinophils (Eos) is shown. Cells counted for 30 s (represented in the time gate) were used for analysis.*

In fact, butyrate treatment significantly reduced the influx of eosinophils into the lung tissue of OVA challenged mice (Figure 34 A), whereas I could not detect any differences in the $CD45^{pos}$ leukocytes population (Figure 34 B). These findings clearly highlight a direct effect of butyrate on eosinophils (1).

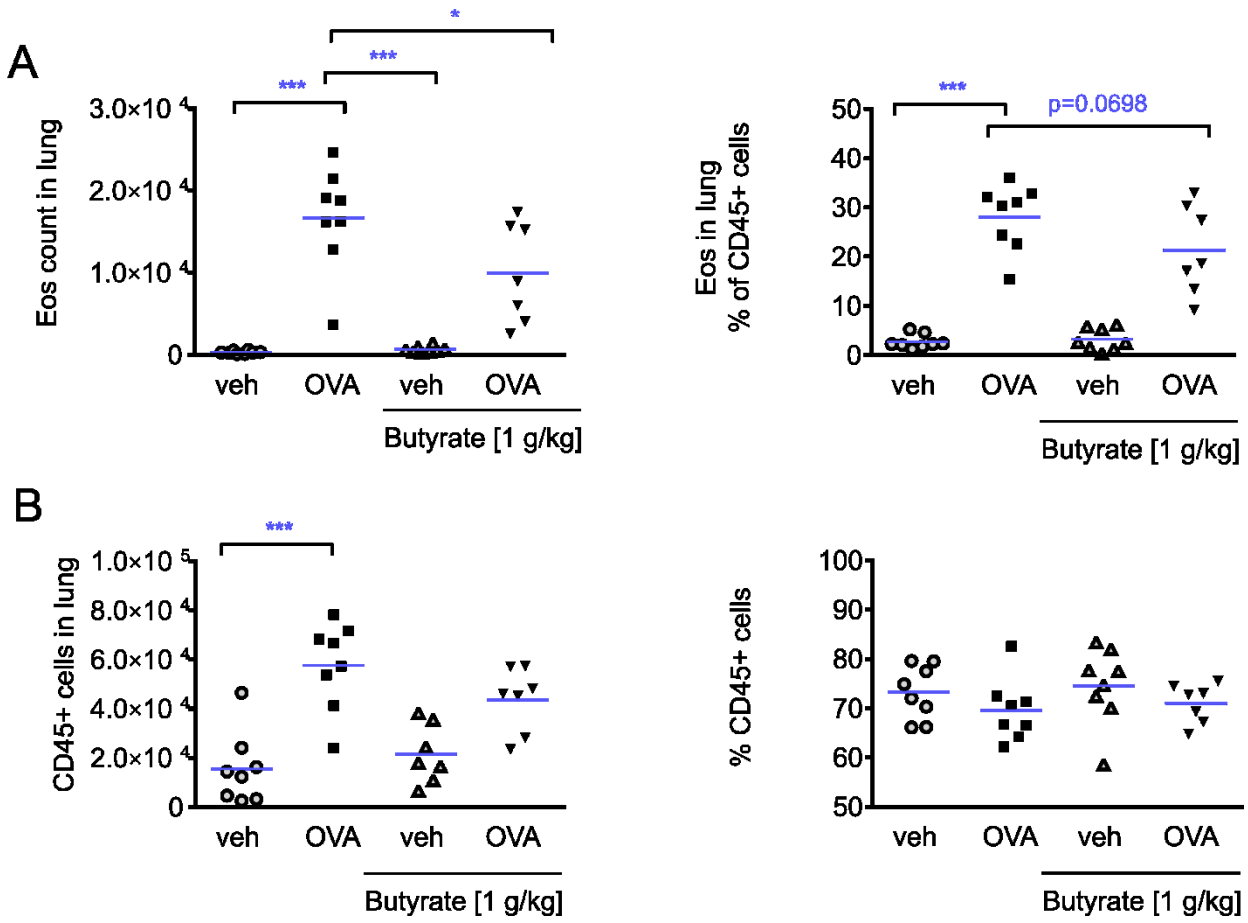

Figure 34. Lung eosinophil numbers are reduced by butyrate. *Systemic butyrate treatment of OVA-exposed mice reduced absolute eosinophil numbers and tended to lower relative eosinophil numbers (left panel) in lung homogenate, whereas CD45^{pos} positive leukocytes (right panel) were unaffected (A). Data are expressed as means or means + SEM from 7-8 animals per group and were analyzed by one-way ANOVA, Tukey's post-test. *p<0.05, ***p<0.001. Figure adapted from (1).*

Next, I was interested, whether butyrate application also induces changes in blood leukocyte population of OVA challenged mice. The same antibody cocktail and gating strategy as for the BAL leukocytes was used. In agreement with my findings regarding BAL leukocytes, I was not able to detect any changes in blood lymphocytes or neutrophils. In addition, blood eosinophils were unchanged in the butyrate group when compared to the vehicle group (Figure 35) (1).

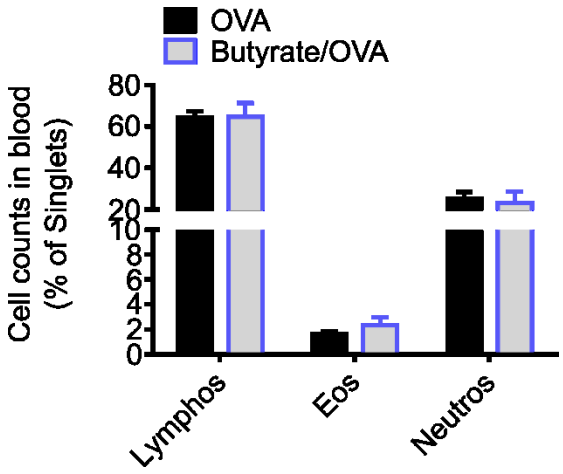

Figure 35. Blood leukocytes are not changed by butyrate treatment. *Blood was taken from OVA challenged mice on 21, which were injected with butyrate or vehicle from day 11 until the end of the experiment. Differential cell count in blood is shown. Data are expressed as means +SEM, 7-8 mice per group and were analyzed with two-way ANOVA, Tukey's post-test.*

As I could show that butyrate strongly attenuated the influx of eosinophils into lung of allergen exposed mice, but had no effect on eosinophils in circulation, I was next interested whether systemic butyrate application has an impact on eosinophilopoiesis. Hence, I took bone marrow on day 21 of the OVA protocol and subjected it to two different staining protocols. First, I checked on changes of eosinophil lineage-committed progenitors (EoP) (lineage^{neg}/CD34^{pos}/CD117^{int}/CD125^{pos}) and granulocyte-monocyte progenitors (GMP) (lineage^{neg}/CD34^{pos}/CD117^{high}/CD125^{neg}/CD16/32^{pos}), the gating strategy is provided in Figure 36 A, in the bone marrow. Secondly, I also stained for pre-eosinophils (Siglec F^{pos}/CCR3^{neg}) and mature eosinophils (Siglec F^{pos}/CCR3^{pos}), Figure 36 B. In agreement with my previous findings regarding blood eosinophils, I could not observe any changes in mature bone marrow eosinophils nor their progenitors (Figure 36 C). Of note, GMP were also unchanged (Figure 36 C) (1).

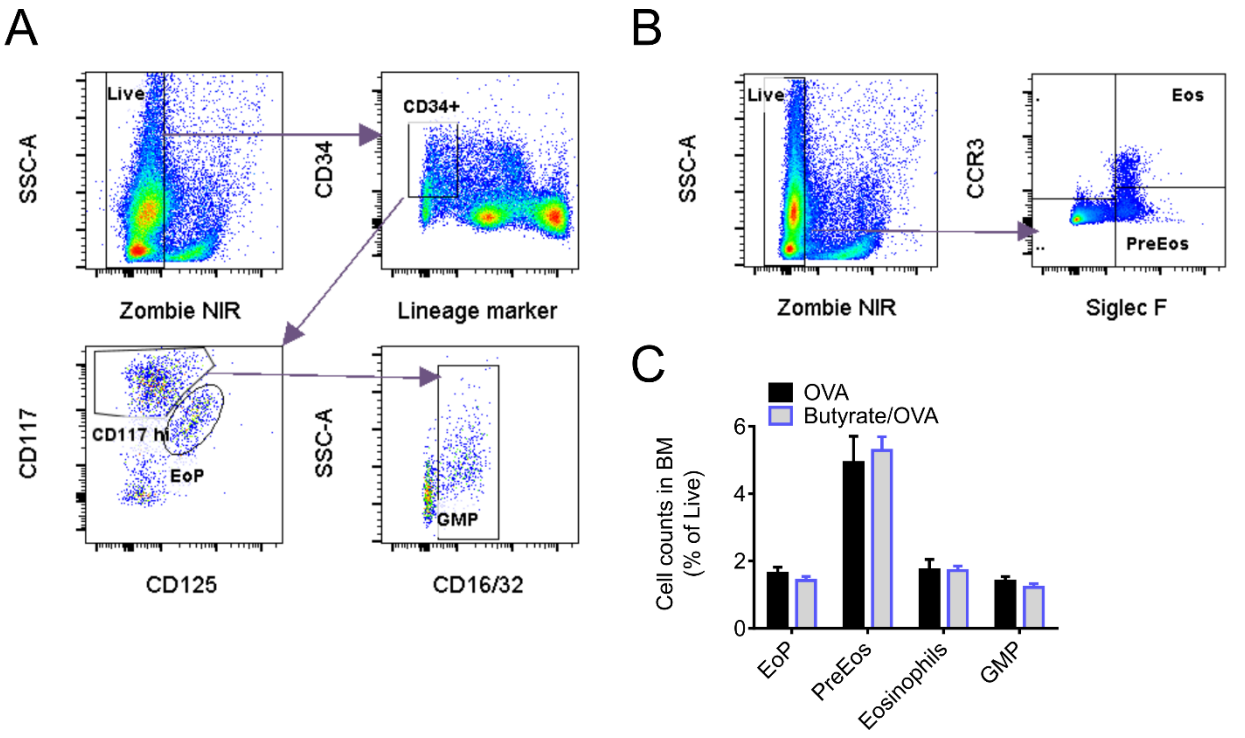

Figure 36. Eosinophilopoiesis is unaffected by butyrate in OVA exposed mice. *BALB/c were sensitized and exposed to OVA or vehicle and treated with butyrate or vehicle from day 11 until day 20. Bone marrow was taken on day 21. The gating strategies for eosinophil lineage-committed progenitors (EoP) and granulocyte-monocyte progenitors (GMP) (A) or eosinophils (B) is shown. Differential cell count in bone marrow is depicted (C). Data are expressed as means +SEM, 7-8 mice per group. Data were analyzed with two-way ANOVA, Tukey's post-test. This figure has been previously published (1).*

Others have previously reported the regulatory potential of butyrate on regulatory T cells (142,166,188), however I could not detect any changes of CD4/CD25/FoxP3 Treg numbers in the lung tissue (Figure 37 B and C). The gating strategy is provided in Figure 37 A (1).

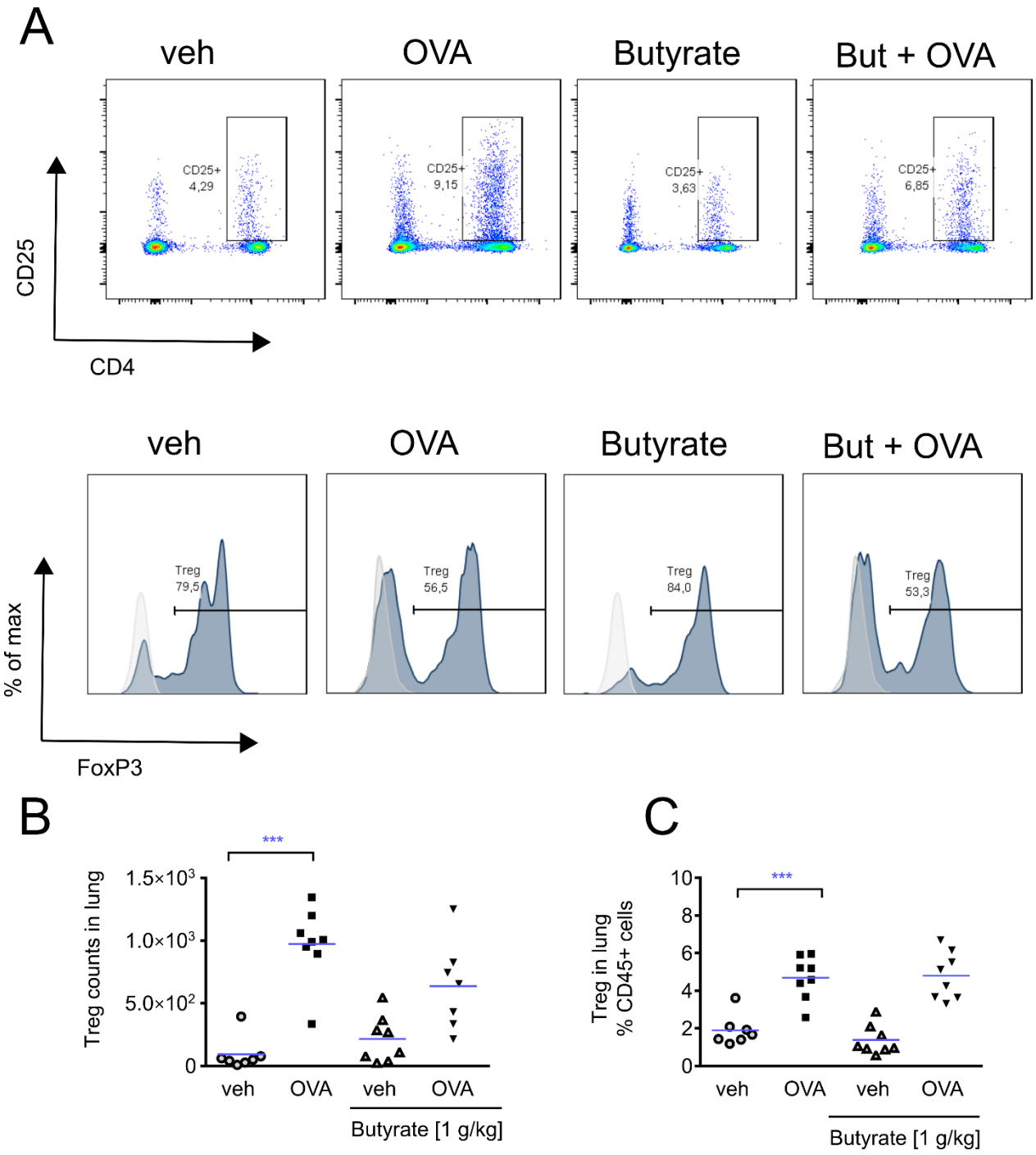

Figure 37. Butyrate treatment does not affect regulatory T cells in the lung. *The lung from OVA exposed mice was taken. The gating strategy for Tregs (A). Total Treg counts (B) and relative Treg counts (C) are depicted. Histograms: grey: FMO colored: stained. FMO (fluorescence minus one, full antibody cocktail, without FoxP3), Data are expressed as means from 7-8 animals per group*

*and were analyzed by one-way ANOVA, Tukey's post-test, ***p<0.001. This figure has been previously published (1).*

Besides the reported induction of Tregs, a recent study from Thio et al. demonstrated that butyrate inhibits ILC2 function in *Alternaria alternata*-induced allergic inflammation. Since ILC2 are potent inducers of type 2 inflammation (144), I wanted to elucidate whether this cell type was also affected in the present model. Mouse lungs were harvested, homogenized and underwent a staining protocol as previously reported (144). In brief, ILC2 were identified as $SSC^{low}/CD45^{pos}/lineage^{neg}/CD90.2^{pos}$ cells (Figure 38 A). OVA challenge trended to increase the ILC2 population in the lung, but in my hands, I observed no differences in mice when treated with butyrate compared to the OVA-only group. (Figure 38 B). Therefore, these data clearly suggest, that butyrate exerts eosinophil-selective effects in the lung of OVA challenged mice.

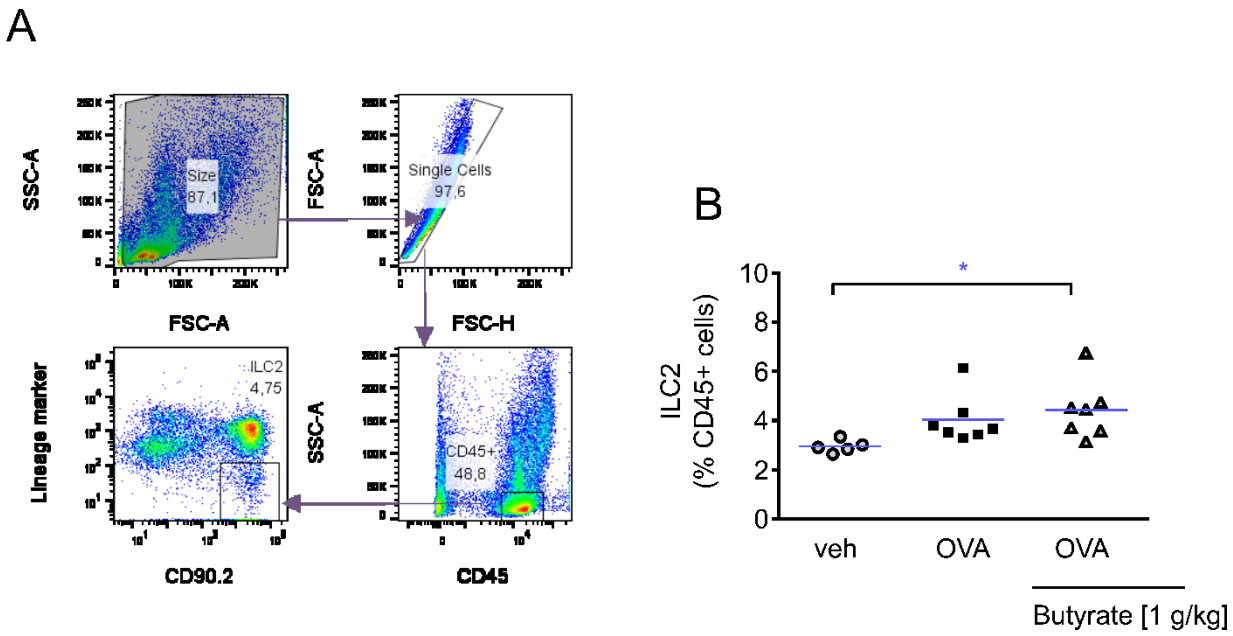

Figure 38. ILC2 in lungs are unaffected by butyrate treatment. *Female BALB/c mice were sensitized and exposed to OVA or vehicle. From day 11 until the end of the experiment mice were injected with butyrate or vehicle. The lung was taken on day 21 and tissue homogenization was performed. The gating strategy for ILC2 ($lineage^{neg}/CD90.2^{pos}$) is shown (A). Frequency of ILC2 cells in the lung homogenate is shown. Data are expressed as means or means + SEM from 7-8 animals analyzed by one-way ANOVA, Tukey's post-test. *p<0.05.*

Having confirmed that butyrate targets eosinophils in the OVA induced asthma model, I set out to determine whether this was dependent on apoptosis induction or changes in expression levels of chemokine or cytokine receptors of eosinophils. Again, I harvested BAL, blood and lungs from mice and identified annexin V^{pos} eosinophils in all three preparations. However, I could not detect an increased annexin V binding on blood eosinophils in the butyrate group (Figure 39), whereas when analyzing BAL eosinophils, butyrate increased annexin V binding, which did not reach significance (Figure 40) (1).

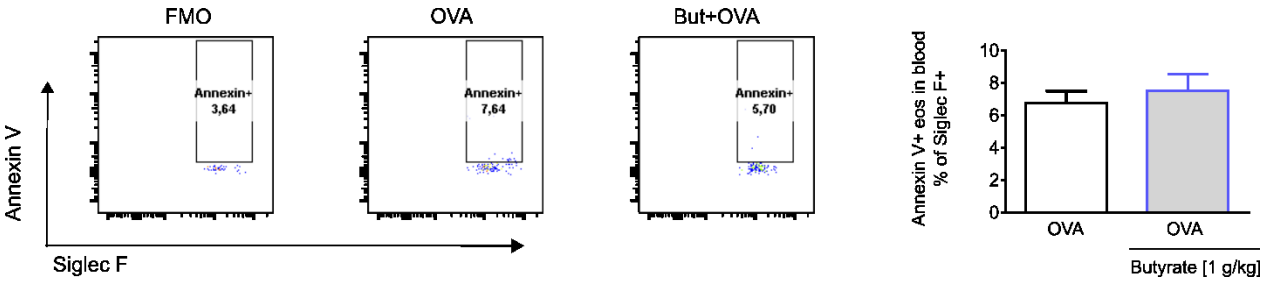

Figure 39. Annexin V binding on blood eosinophils of allergen-exposed mice. *Representative stainings are depicted on the left panel. FMO: Fluorescence minus one (full antibody cocktail without annexin V). Histograms: grey: OVA, colored: OVA + butyrate. MFI: mean fluorescence intensity. Data are expressed as means or means + SEM from 7-8 animals per group and were analyzed by Student's t test.*

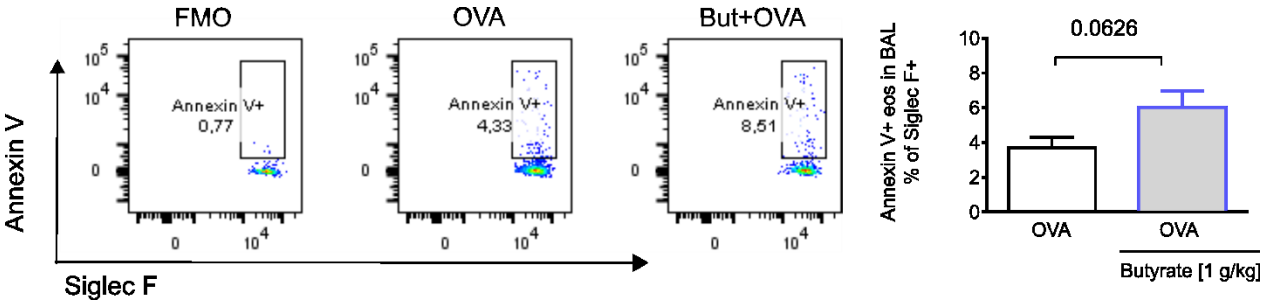

Figure 40. BAL eosinophils show increased annexin V binding under butyrate application. *Annexin+ BAL eosinophils of OVA exposed mice are shown. The left panel depicts the gating strategy. FMO: Fluorescence minus one (full antibody cocktail without annexin V). Histograms:*

grey: OVA, colored: OVA + butyrate. Data are shown as means or means + SEM from 7-8 animals per group and were analyzed student's t test. This figure was adapted from (1).

Similar to the BAL eosinophils, lung eosinophils trended to increased annexin V positivity, suggesting an apoptotic phenotype in butyrate treatment group (Figure 41) (1).

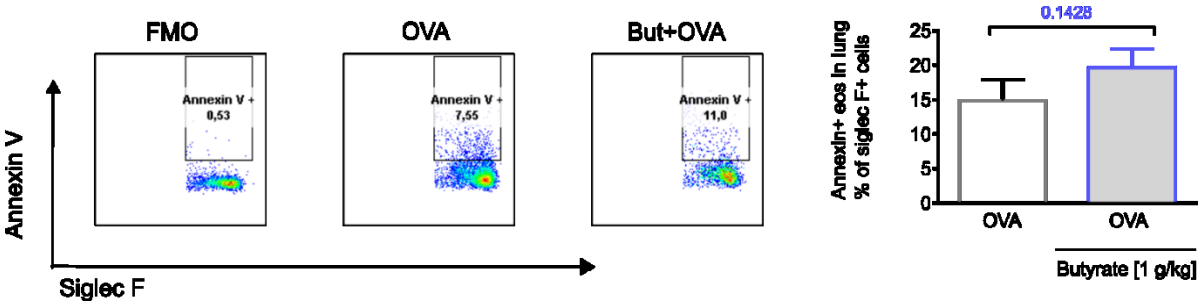

Figure 41. Induced apoptosis of lung eosinophils tended to rise upon butyrate treatment. *Typical stainings+ are depicted on the left panel. FMO: Fluorescence minus one (full antibody cocktail without annexin V). Histograms: grey: OVA, colored: OVA + butyrate. Data are expressed as means or means + SEM from 7-8 animals per group and were analyzed by Student's t test. This figure was adapted from (1).*

My previous data clearly show that butyrate affects eosinophil survival and recruitment. *In vitro*, butyrate downregulated expression of CCR3 and IL-5RA on mRNA level. Hence, I tested if this was also the case for eosinophil from blood, BAL or lung of OVA challenged mice.

I found that butyrate treatment did not affect the CCR3 surface expression on eosinophils (Figure 42 A), but interestingly increased the IL5Rα expression on blood eosinophils (Figure 42 B) (1).

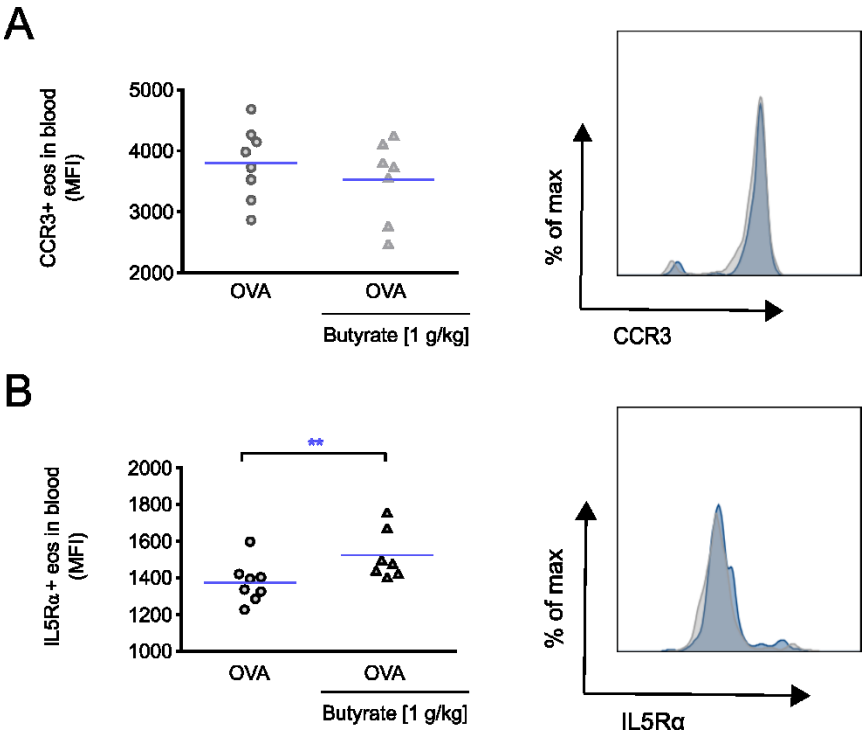

Figure 42. Butyrate treatment increased IL5Rα expression on blood eosinophils. *Butyrate had no effect on CCR3 expression levels (left panel). (A). Expression of IL5Rα increased (right panels) upon butyrate treatment (B). Histograms: grey: OVA, colored: OVA + butyrate. Data are expressed as means or means + SEM from 7-8 animals per group and were analyzed by Student's t test. **p<0.01.*

This was similar to lung eosinophils, where the CCR3 expression was unaffected by butyrate application. However, I found a decrease of Siglec F^{pos}/$CCR3^{pos}$ eosinophils in the lung (Figure 43 A). $IL5R\alpha^{pos}$ lung eosinophil numbers were unchanged, again the IL5Rα expression was increased by butyrate in OVA challenged mice (Figure 43 B) (1).

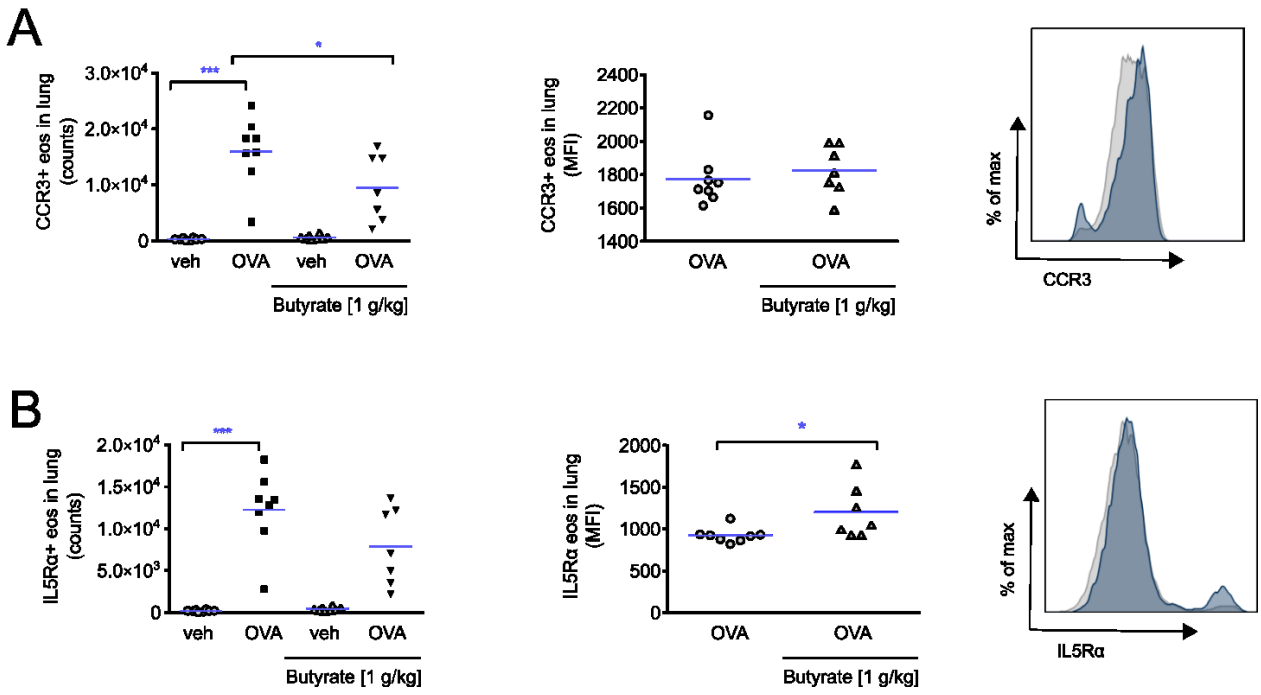

Figure 43. Butyrate treatment increased IL5Rα expression on lung eosinophils. *Butyrate diminished absolute numbers of CCR3+ eosinophils in the lung (left panel) but was ineffective on CCR3 surface expression (right panels) (A). On the contrary absolute numbers of IL5Rα + lung eosinophils were unaffected by butyrate application (left panels) but the surface expression of IL5Rα was elevated (right panels) (B). Histograms: grey: OVA, colored: OVA + butyrate. Data are expressed as means or means + SEM from 7-8 animals per group and were analyzed by one-way ANOVA, Tukey's post-test (A, B) or Student's t test (C, D). *p<0.05, ***p<0.001. This figure was adapted from (1).*

Interestingly, the situation was slightly different in BAL eosinophils, as both CCR3 and IL5Rα expression was not impaired by butyrate treatment (Figure 44 A and B).

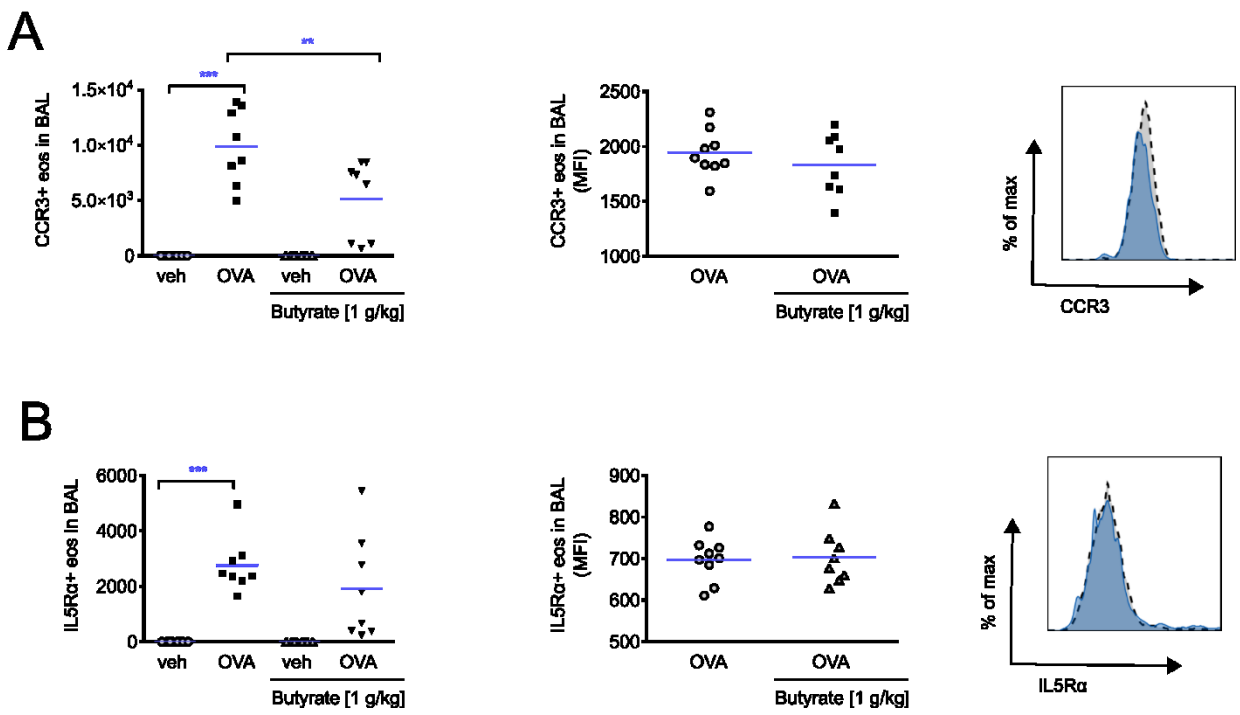

Figure 44. Butyrate treatment does not affect CCR3 and IL-5RA surface expression of BAL eosinophils. *Mice were sensitized and exposed to OVA or vehicle. Absolute numbers of CCR3+ eosinophils were abated by butyrate application (left panel) whereas CCR3 and IL5Rα surface expression on BAL eosinophils remained unaltered (right panel) (A, B). Histograms: grey: OVA, colored: OVA + butyrate. MFI: mean fluorescence intensity. Data are shown as means or means + SEM from 7-8 animals per group and were analyzed by one-way ANOVA, Tukey's post-test or student's t test **p<0.01, ***p<0.001. This figure was adapted from (1).*

3.10 Airway hyper responsiveness (AHR) is alleviated by butyrate

Airway hyper responsiveness is a common feature of asthma (1,219). Hence, I studied the effect of butyrate treatment via invasive spirometry in order to confirm my obtained data on a more functional level. In brief, mice were deeply anesthetized and connected to a Flexivent device. Mice were then nebulized with serial dilutions of methacholine in order to induce bronchoconstriction and lung function was determined. Firstly, OVA strongly induced AHR, which was completely reversed by butyrate (Figure 45 A). Secondly, OVA challenge impaired the airway compliance, which was also significantly improved by butyrate (Figure 45 B) (1).

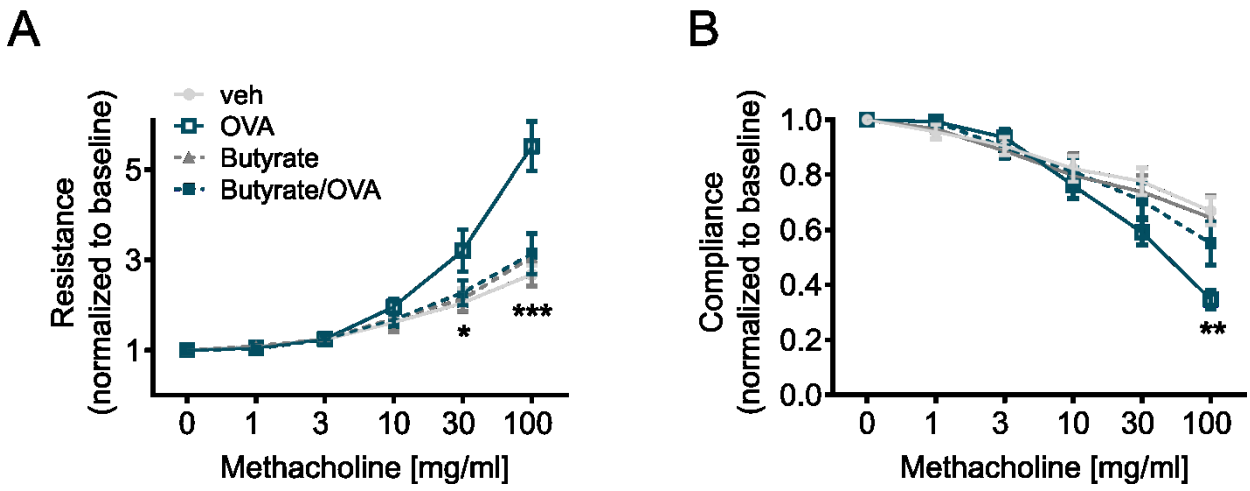

Figure 45. Methacholine induced AHR is improved in butyrate administered mice. *Invasive spirometry was performed on OVA or vehicle sensitized mice on day 21. Mice received daily i.p, injection of butyrate or vehicle. On day 21, mice were anesthetized and connected to a Flexivent device. Airway resistance (A) and airway compliance (B) were calculated and normalized to baseline. Data are expressed as means +SEM, 5-12 mice per group. Statistical differences were calculated with two-way ANOVA, Tukey's post-test *P<0.05, **p<0.01, ***P<0.001. This figure was adapted from (1). Kathrin Rohrer assisted during lung function measurements.*

Allergic airway inflammation is accompanied by increased levels of the type 2 cytokines, IL-4, IL-5 and IL-13, which are released by TH2 cells, ILC2 but also eosinophils (82). These factors foster the allergic response by promoting eosinophil accumulation and persistence in the airways. Accordingly, IL-4, IL-5 and IL-13 levels in the BALF were detected by multiplex ELISA. As expected, OVA challenge significantly induced type 2 cytokine release into the bronchoalveolar space (Figure 46), which was counteracted by butyrate (1).

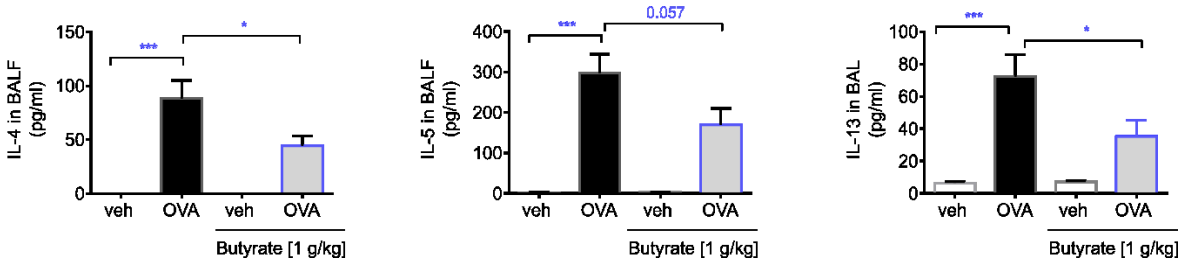

Figure 46. Butyrate treatment decreased type 2 cytokine levels in BALF of OVA challenged mice. *BALF was taken at day 21, 24 h after the last OVA challenge. IL-4, IL-5 and IL-13 levels were detected in the cell free BALF, using a multiplex ELISA kit. Statistical differences were calculated with one-way ANOVA, Tukey's post-test, *P<0.05, ***P<0.001. This figure was adapted from (1).*

4. Discussion

Parts of this discussion resemble the discussion section already published in an original article, which was a result of this present thesis (1).

Since the 1960s the prevalence of asthma and allergic disease has been increasing dramatically in Western countries (181) and until now is affecting up to 300 million people worldwide (82). Asthma is characterized by bronchial/airway hyperresponsiveness, chronic airway inflammation, cough, mucus hypersecretion and airflow obstruction (81)

Eosinophilia is a hallmark of asthma and eosinophils are key effector cells in the pathology of allergic inflammatory diseases, which are involved in mediator secretion and tissue damage (89). The increase in the prevalence of asthma and allergic disorder coincides with changes of lifestyle in the Western countries, which includes the improvement of personal hygiene, the increased use of antibiotics and changes in family size which might favor a shift towards dysregulation of the immune system and inappropriate immune response (109). However, the hygiene hypothesis has been questioned, and has been extended also to include dietary aspects. Asthma and allergies are associated with Westernized diet (140). This diet is particular defined by consumption of high sugar, high fat and high sodium as opposed to a diet that is considered healthy comprising high-fiber foodstuff such as vegetables, fruits, legumes and grain (140). In Africa, the asthma prevalence is remarkably low (181) and children from rural Burkina Faso, who traditionally consume a diet rich in fiber, show increased bacterial richness in their feces, when compared to European children, which was also reflected in an impaired production of SCFA in European children (117).

SCFA are crucial signaling molecules, which enable communication of the gut microbiome with its host and are indispensable for human health (110). Recent studies have highlighted the favorable effect of SCFA as major metabolite from the gut microbiome but also dietary fibers as their precursor molecules in the pathogenesis of asthma and allergic disease. Up to now, eosinophils have been neglected in this area with regard to the microbiome-SCFA-axis in allergic inflammatory diseases.

Accordingly, this thesis aimed to elucidate the effect of SCFA on eosinophil effector function and survival and furthermore to determine the effect of butyrate as a therapeutic regimen

in a mouse model of acute allergic asthma. This thesis describes for the first time, that SCFA directly affect human peripheral blood eosinophils in terms of (1) adhesion to the endothelium (2), recruitment (3) survival (4) and - importantly - ameliorating allergic airway inflammation in a mouse model of acute asthma (1).

Herein I describe that the GPR43 and GPR41 receptors are expressed by human eosinophils and that GPR43 is functionally active on human eosinophils as propionate and acetate, but not butyrate trigger a GPR43-dependent Ca^{2+} flux in eosinophils. Moreover, propionate and butyrate induce apoptosis in human eosinophils, but acetate failed to mimic this effect. Interestingly, the apoptosis induction was independent of the SCFA receptors GPR43 and GPR41, but was mediated by the intrinsic apoptotic pathway. Strikingly, treatment of eosinophils with HDAC inhibitors resembled the effect of SCFA on eosinophil apoptosis. Furthermore, propionate and butyrate potently inhibited the adhesion of eosinophils to pulmonary microvascular endothelial cells. This was again mimicked by the HDAC inhibitor TSA and again acetate failed in this context. Additionally, I provide evidence that butyrate and TSA abrogate the eotaxin-2 induced migration of eosinophils, whereas both, propionate and acetate had no impact. In agreement with these findings, systemic butyrate application dampened lung eosinophilia and AHR in allergen challenged mice. Strikingly, this effect was specific for mature eosinophils as other cell types proved to be unaffected (1).

GPR43 and GPR41 transcripts have been previously reported to be expressed by eosinophils and neutrophils (155) which corresponds with the findings in this thesis. However, the eosinophil proteome was recently published, which suggested that peripheral blood eosinophils express GPR43 but lack GPR41 (123). Admittedly, mRNA expression levels of GPR41 in eosinophils are rather modest and this study lacks data regarding of GPR41 expression on protein level. Therefore, I have to admit that the GPR41 receptor might not be expressed by peripheral blood eosinophils. Acetate and propionate triggered an intracellular Ca^{2+} release, whereas butyrate lacked thereof. Pre-incubation of eosinophils with a GPR43 antagonist revealed GPR43 dependency in the acetate- and propionate-induced increase of intracellular Ca^{2+} levels. Comparable results have been proposed for neutrophils (128,156). Although acetate and propionate triggered intracellular Ca^{2+} levels in eosinophils, they failed to induce eosinophil activation with

regards to eosinophil shape change and migration. This is opposed to neutrophils, where acetate and propionate were reported to trigger rearrangements of the actin cytoskeleton and migration (128,157). GPR43 is prominently expressed by neutrophils compared to other cells (128,129). In my hands, I could detect a more pronounced Ca^{2+} increase in neutrophils than eosinophils (data not shown). With regards to the trend to a less prominent GPR43 mRNA expression of eosinophils than neutrophils, I would assume that the Ca^{2+} release induced by acetate and propionate on eosinophils is too small to trigger activation and migration (1). The inability of butyrate to stimulate Ca^{2+} flux might be explained with limited potency of butyrate to activate GPR43 (128).

Additionally, I tested the potential of SCFA to induce ROS production in purified, human eosinophils. Production of ROS by eosinophils is believed to be involved in eosinophil-induced tissue damage (81). I found that acetate and propionate stimulated ROS production in human eosinophils, whereas butyrate was ineffective.

SCFA are known apoptosis inducers in neutrophils (146,155,164) and malignant cells (212). Hence, I tested the apoptosis-inducing potential of acetate, propionate and butyrate on peripheral blood eosinophils. Accordingly, I could demonstrate that both propionate and butyrate impair the survival of human eosinophils, independent of GPR43 and GPR41, which was demonstrated by the usage of the respective receptor antagonists (1).

An increase of intracellular Ca^{2+} levels can occur during the course of apoptosis in eosinophils (220,221). However although propionate and acetate induce Ca^{2+} flux in human eosinophils, it is suggested by the present results that apoptosis induction occurs independently of intracellular Ca^{2+} levels as i) acetate induces calcium flux but not apoptosis and ii) I could block the acetate and propionate-induced Ca^{2+} flux with the GPR43 antagonist, which was not the case with propionate-induced apoptosis. A similar conclusion might be drawn with regards to ROS production and apoptosis as acetate and propionate induce ROS production in human eosinophils, whereas butyrate was unable. Given that propionate and butyrate induce apoptosis in human eosinophils and only acetate and propionate induced ROS production in eosinophils one could speculate that ROS production is, similar to Ca^{2+} flux dispensable for apoptosis induction by SCFA in eosinophils.

However, in addition to GPR43 and GPR41 dependent signaling, SCFA, in particular butyrate, have been shown to act as HDAC inhibitors. Interestingly, a similar mechanism of SCFA induced apoptosis has been proposed for neutrophils (146) as I have found herein for human eosinophils. Correspondingly, HDAC inhibitors have been revealed to promote apoptosis in eosinophils and neutrophils (34). This notion was further supported in my study as propionate- and butyrate-induced apoptosis on peripheral blood eosinophils was accompanied by histone acetylation, as measure of HDAC inhibition (1). Furthermore, the HDAC inhibitor mimicked SCFA in their apoptosis-inducing potential in human eosinophils and displayed similar kinetics (1).

As HDACs are located intracellularly (145), SCFA require transport into the cell. This is facilitated by monocarboxylate transporters. Eosinophils express MCT-1 and MCT-4. Therefore, I employed a pan-MCT inhibitor in the apoptosis assay. In fact, CHC was able to completely inhibit the survival impairment by propionate and this was at least partially true for butyrate. Additionally, butyrate might passively cross the cell membrane (143) or be engaged by an unknown transporter. These data clearly suggest that propionate and butyrate induce apoptosis in eosinophils by HDAC inhibition (1).

Eosinophils have been reported to express HDAC1, HDAC2, HDAC3, HDAC4, HDAC6, HDAC7, HDAC8 and HDAC10 at the mRNA level (34). The same study also suggested that the TSA-mediated apoptosis in eosinophils might be HDAC3 dependent, as the HDAC inhibitor MC-275 only at higher concentrations induced apoptosis in eosinophils. MC-275 has been reported to target HDAC1 at lower concentrations (0.1-1 μM), whereas it inhibits HDAC3 at higher concentrations. In this present study, I employed subtype-specific inhibitors for HDAC2, HDAC3 and HDAC8, as well as a class IIa HDAC inhibitor. Incubation of eosinophils with inhibitors for HDAC2, HDAC3 and HDAC8 did not impair eosinophil survival, whereas the class IIa HDAC inhibitor MC 1568 dose-dependently reduced the live portion of human eosinophils. Although MC 1568 is reported to be specific for class IIa HDACs, which includes HDAC4, HDAC5 and HDAC7 (222), it was also reported to inhibit HDAC6 in cancer cell lines (223). However, which HDAC subtype is targeted by propionate or butyrate in human eosinophils needs further clarification.

Additionally, butyrate was reported to induce apoptosis in a PPARγ-dependent manner in colon cancer cells (224). However, this seems not true for eosinophils, as a PPARγ antagonist was not able to abolish the viability impairment by butyrate (data not shown) (1).

Interestingly, butyrate was also shown to act as a ligand on the niacin receptor GPR109a as it induces apoptosis in a GPR109a-dependent manner in breast cancer cells (225). Given the fact that eosinophils lack GPR109a (134) this possibility is highly unlikely for eosinophils (1).

Acetate was previously shown to induce apoptosis in neutrophils (164). However, a concentration of 25 mM was used in this study. In the present thesis, I used acetate up to 10 mM, hence it might be possible that higher concentrations of acetate also induce apoptosis in eosinophils (1).

Propionate and butyrate have been previously reported to activate the intrinsic apoptosis pathway (226,227). This is also the case for eosinophils and implicated by the present data, as I could show that propionate and butyrate promote the mitochondrial membrane potential disruption and induce the activation of effector caspases. As a proof of principle, a pan-caspase inhibitor was employed in the annexin V/PI apoptosis to substantiate the caspase dependency of propionate- and butyrate-induced apoptosis. In fact, pre-treatment with the pan-caspase inhibitor prevented from apoptosis induced by propionate and butyrate in human eosinophils (1).

BCL-XL and MCL-1, as anti-apoptotic members of the Bcl-2 family have been implicated in prolonging the survival of eosinophils (30,214). Correspondingly, propionate and butyrate impaired the mRNA expression of BCL-XL and MCL-1 in peripheral blood eosinophils. Additionally, it has been reported that the TSA-induced apoptosis pathway in a lung cancer cell line is very similar to the one I found induced by propionate and butyrate and requires the downregulation of BCL-2 family members and activation of effector caspases (228). This further supports the notion that the propionate- and butyrate-induced apoptosis in eosinophils is dependent on HDAC inhibition (1).

Eosinophil survival is sensitively enhanced by survival promoting factors, including IL-5, which are present in high amounts during allergic inflammation. This in turn fosters eosinophil persistence and impairs resolution (1,19,20). Therefore, my next aim was to elucidate, whether

propionate and butyrate are able to impair eosinophil survival in the presence of IL-5. Eosinophils were pre-incubated with IL-5 before SCFA application or vice versa, and then caspase 3/7 activation assay was performed. Strikingly, IL-5 was not able to prevent from propionate and butyrate induced caspase activation. This corresponds to my finding that propionate and butyrate downregulate IL-5RA expression at the mRNA level, which in turn might impair the IL-5 responsiveness. Conversely, butyrate was demonstrated to procure IL-5RA expression on protein level in the HL-60 eosinophilic cell line (154). However, this proves to be difficult to be compared with the present study, as I used primary blood eosinophils, different butyrate concentrations and shorter incubation periods in comparison to the abovementioned study (1).

For all previous experiments, eosinophils from self-reported allergic donors were used. Therefore, I was interested whether there is a difference in effector caspase activation by propionate and butyrate from allergic in comparison to non-allergic donors. Strikingly, both propionate and butyrate induced effector caspase activation in allergic donors, whereas this effect was absent in eosinophils from non-allergic individuals. Interestingly, colon cancer cells are more sensitive to apoptosis induction by butyrate, when they are exposed to TNF-α (215,216). Accordingly, it was previously shown that Siglec-8 induced eosinophil apoptosis was augmented when eosinophils were pre-activated with IL-5 (35). Therefore, I speculated that a cytokine-rich milieu, which is present in allergic individuals, is required for propionate and butyrate to induce caspase activation. Strikingly, after priming of eosinophils from non-allergic donors with IL-5 rendered them responsive to SCFA in terms of effector caspase activation (1).

Importantly, these results suggest that propionate and butyrate induce HDAC inhibition, which was detected by histone acetylation. Butyrate, as well as propionate have been reported to act as HDAC inhibitors in several cell types (142,143). HDACs are involved in regulating a wide spectrum of cellular responses, namely survival, adhesion and migration (1,34,146–148). Therefore my next aim was to determine whether SCFA can impair eosinophil trafficking. Pre-treatment of eosinophils with propionate or butyrate prevented eosinophils, which had been activated by eotaxin-2, from becoming adherent on a monolayer of pulmonary microvascular endothelial cells under flow conditions. Strikingly, this was mimicked by the pan-HDAC inhibitor TSA, but not acetate. After extravasation, eosinophils need to migrate to the target tissue, which is enabled by a

cytokine gradient (39). Hence, a chemotaxis assay was performed to study the effect of SCFA on eosinophil migration. Butyrate abolished the eosinophil migration towards eotaxin-2 and TSA again mimicked this. Interestingly, acetate and propionate acted differently, as they were not able to prevent the eotaxin-2 induced migration (1). These findings were in line with other results of this thesis as butyrate diminished the mRNA expression of the adhesion molecules CD49d, the integrin α subunit of VLA-4 and CD44 as well as their surface expression. VLA-4 regulates the migration of eosinophil into the homing tissue and has been reported to be critical for the accumulation of eosinophils into the lung and conjunctiva during allergic inflammation (44,45). Similarly, butyrate and other HDAC inhibitors were reported to impair the expression of CD44 and VLA-4 in different cell types (147,229–231).

Additionally to α4β1 integrins, αMβ2 is crucial to eosinophil trafficking and extravasation as they meditate endothelial transmigration via the interaction of their receptor ICAM-1 on the endothelium (60). Hence, I tested whether propionate and butyrate are able to inhibit the CD11b upregulation by eotaxin-2, which is the αM subunit of the αMβ2 integrin. However, CD11b upregulation by eotaxin-2 was unaffected by propionate or butyrate treatment. Interestingly, SCFA treatment did not affect L-selectin surface expression on eosinophils.

Furthermore, propionate and butyrate decreased the expression of CCR3, the receptor for eotaxins and other chemokines (232) at the mRNA level and protein level, which might be a reason for the decreased responsiveness of eosinophils to eotaxin-2 in terms of adhesion and migration. Surprisingly, propionate had a different effect than butyrate, as it only hampered eosinophil adhesion but failed to impair chemotaxis towards eotaxin-2. Additionally, acetate was unable to abrogate eosinophil adhesion or migration. This might be explained by observations that i) propionate induces an intracellular Ca^{2+} elevation in eosinophils, which in turn might abolish its own antagonistic effect on chemotaxis and additionally ii) it diminished CCR3 expression less potently than butyrate (1). Interestingly, pre-incubation of eosinophils with propionate or butyrate did not inhibit eosinophil shape change induced by eotaxin-2, suggesting that both SCFA are not able to inhibit eosinophil activation by eotaxin-2 but rather affect the CCR3-mediated effect (1).

As the previously described *in vitro* results point to an inhibitory effect of SCFA, especially, butyrate on human eosinophil function, I set out to verify this on a more translational level.

Therefore, I employed OVA-induced acute asthma model in mice. Additionally, the obtained *in vitro* data suggested a more pronounced effect of butyrate in comparison to propionate, therefore only butyrate was used for the in vivo experiments. Plus, propionate might also exert some activating effects on eosinophils, with regards to Ca^{2+} elevation and ROS production. In brief, female BALB/c mice were immunized to OVA on day 0 and 7 and were then challenged with OVA aerosol or vehicle on three consecutive days. From day 11 until the end of experiment mice received daily i.p. injections of butyrate (1 g/kg bodyweight). This concentration was previously used by other groups for butyrate and other SCFA for *in vivo* models (118,136,233,234) and produces an estimated plasma concentration of approximately 1.8 mM (1,218,235). Butyrate application i) blunted eosinophil trafficking into the lung, ii) alleviated airway eosinophilia, iii) reduced airway hyperresponsiveness and iv) reduced Th2 cytokine secretion as anticipated by the previously described *in vitro* data (1). Strikingly, systemic butyrate application only affected eosinophils as other leukocyte populations were not altered. Additionally, I could not detect any changes in eosinophil progenitors in the bone marrow by butyrate treatment, which suggests that butyrate selectively targets mature eosinophils in this experimental setting (1).

In detail, OVA challenge caused an immense influx of leukocytes into the airways, most of them being eosinophils. This was inhibited by systemic butyrate application, whereas alveolar macrophages, lymphocytes and neutrophils in the airways were unaffected. Comparable results were obtained for lung eosinophils, whereas other leukocytes, gated as $CD45^{+}$ cells were unaffected (1). However, blood eosinophil numbers were not affected by butyrate application, which was also reflected by the fact that I could not detect any changes in eosinophil progenitors after butyrate application. This might be explained by the fact that in spite of OVA challenge, blood eosinophils and eosinophil progenitors in the bone marrow represent the minority of cells, which makes it difficult to detect changes upon butyrate treatment.

Since I have demonstrated that butyrate induces apoptosis in peripheral blood eosinophils *in vitro* I was interested whether this is also the case *in vivo*. In fact, I could detect a trend to increased annexin V binding in BAL eosinophils, and to a lesser extent in lung tissue eosinophils of OVA challenged mice that received butyrate treatment. However, this effect was absent in blood eosinophils. These findings suggest that butyrate application might be able to induce apoptosis in

eosinophils also *in vivo* and thereby also might reduce eosinophil persistence in the lung. However, the apoptosis inducing effect of butyrate might be enhanced by a more local application route, e.g. intranasally or by inhalation.

As a next step, surface expression levels of IL-5Rα and CCR3 on blood, lung and BAL eosinophils were determined in OVA challenged mice. CCR3-positive eosinophils were reduced in numbers in lung and BAL eosinophils, however, CCR3 expression at the cellular level was unaffected in all three cells fractions. Since, CCR3 is a specific marker for mouse eosinophils (236), the decrease in CCR3-positive BAL and lung eosinophils can be explained by the decrease in total eosinophil numbers by butyrate treatment. Interestingly, I could detect an increased surface expression of IL-5Rα in blood and lung eosinophils but not on BAL eosinophils upon butyrate treatment in OVA challenged mice. It was previously reported that upon IL-5 binding the IL-5Rα is internalized, which is reflected by decreased surface expression *in vitro* (237). Similar results have been obtained for BAL eosinophils after segmental allergen challenge (238). I could detect a trend towards decreased IL-5 secretion into the BALF of OVA-challenged mice upon butyrate treatment. A decreased IL-5 concentration could then lead to reduced IL-5Rα activation and in turn to less receptor internalization, which might explain the increased surface expression of IL-5Rα by butyrate treatment in OVA challenged mice. However, since I have not analyzed IL-5 levels in blood and the lung tissue, this remains rather speculative and would require further clarification (1).

Previous publications have shed light on the importance of gut microbiota and their most important metabolic products, SCFA, in the prevention of the asthma onset, as high fiber feeding or SCFA application was performed before allergen contact (144,182). Therefore, I aimed to determine the interventional potential of butyrate in my experiments as butyrate application was commenced after initial allergen sensitization. Hence, these findings suggest that butyrate has a promising therapeutic capacity in already established asthma and allergic inflammation (1). Considering the high concentration of SCFA, especially butyrate used in this study, modification are required to improve the potency and bioavailability in patients. Therefore, my findings strongly suggest that butyrate could be regarded as a lead compound in the development of novel drugs suitable for treatment of eosinophilic disease (1).

The effect of SCFA, in particular butyrate on Tregs has been repeatedly demonstrated (142,165–167,188). Hence, I sought to determine the numbers of CD4/CD25/FoxP3$^+$ Treg in the lung tissue in the OVA model. Strikingly, Treg numbers were unchanged by butyrate treatment. Correspondingly, Thio et al. described similar results regarding butyrate and Treg in the lung tissue of *Alternaria alternata* exposed mice (144). However, in their study, butyrate inhibited ILC2 function in an HDAC-dependent manner which in turn alleviated lung eosinophilia and improved lung function (144). In order to exclude the possibility, that the reduction of airway eosinophilia is just a secondary effect due to impaired ILC2 response, I determined ILC2 in the lung tissue of OVA mice. Again, I could not find any changes in ILC2 by butyrate. This further suggests, that eosinophils are specifically targeted by butyrate in the current model. This notion is further supported by the fact that the BAL lymphocyte population was unaltered by butyrate application, which also contains ILC2. Additionally, the CD45$^+$ population in the lung was not changed by butyrate, whereas I found a decrease in eosinophil counts in the lung tissue.

Th2 cytokines are secreted during allergic response and thereby facilitate the persistence of inflammation (19). IL-4, IL-5 and IL-13 were increased by OVA exposure in mice. Strikingly, butyrate application reduced the IL-4 and IL-13 secretion into the BAL fluid and I further observed a trend towards IL-5 reduction by butyrate. ILC2 are potent Th2 cytokine producers in allergic inflammation (1,82,144). However, those factors are also stored by eosinophilic granules and can be released upon stimulation (36).

Furthermore, butyrate has been shown to strengthen the epithelial barrier, at least of colon epithelial cells (102,169,170), which might also explained the reduced eosinophil number in the BAL. However, this seems of minor importance in the present thesis as I could demonstrate that lung eosinophils were also reduced by butyrate treatment in OVA challenged mice whereas numbers of CD45$^+$ cells were unaltered. These data suggest that at least in this model the barrier-promoting effect of butyrate seems to be less important and again the effect seems to be specific for eosinophils (1).

4.1 Conclusion

In conclusion, the results presented in this thesis strongly suggest a novel role for SCFA, in particular for butyrate, in controlling fundamental steps of eosinophil function and life cycle. SCFA, especially butyrate, hamper the recruitment and persistence of eosinophils in tissue. This was proven to be true *in vitro* and in a mouse model of acute allergic airway inflammation, where butyrate specifically targeted eosinophils. Therefore, butyrate could represent a novel therapeutic strategy in already established forms of allergic asthma and other eosinophilic diseases.

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Appendix

Table 1. Reagents and concentrations

Agonists & Antagonists	*Experimental concentration or dilution*	*Stock concentration & solvent*	*Supplier*
Sodium acetate	0.3 -100 mM	1 M/A dest.	Sigma
Sodium propionate	0.3 -100 mM	1 M/A dest.	Sigma
Sodium butyrate	0.3 -100 mM; 1 g/kg (*in vivo*)	1 M/A dest	Sigma
Hydroxybutyrate	10 mM	1 M/A dest.	Sigma
CTABP	10, 100 µM	10 mM/DMSO	Trond Ulven
GLPG0974	10, 100 µM	10 mM/DMSO	Trond Ulven
TUG-869	10 µM	100 mM/ DMSO	Trond Ulven
Inhibitors			
α-cyano-4-hydroxycinnamic acid	1.25, 2.5, 5 mM	100 mM/DMSO	Sigma
ZVAD-FMK	100, 500, 1000 µM	100 mM/DMSO	Selleckchem
TSA	0.3 – 300 nM	10 mM/DMSO	Cayman Chemicals
Santacruzamate A	0.1 -100 µM	10 mM/DMSO	Selleckchem
MC1568	0.1 -100 µM	10 mM/DMSO	Selleckchem
RGFP-699	0.1 -100 µM	10 mM/DMSO	Selleckchem
PCI-34051	0.1 -100 µM	10 mM/DMSO	Selleckchem
Cytokines and Chemokines			
rh-eotaxin-2 (CCL-24)	0.3 -30 nM	1 µM/A.dest	Immunotools
rh-IL-5	10 pM/50 pM	5 µM/A.dest	Immunotools
rh-C5a	1-100 nM	100 µM/ Adest.	Biomedica

Other substances			
FCCP	1 µM	10 mM/DMSO	Abcam
Wheat-germ-agglutinin Alexa fluor 594	5 µg/mL	1 mg/mL/A. dest	Thermo Fisher
JC-1 dye	2 µg/ml	5mg/mL/DMSO	Thermo Fisher
Zombie NIR	1:2000	DMSO	Biolegend
Fluo-3,AM	2 µM	2 mM/DMSO	Thermo Fisher
DHR 123	1 µM	2 mM/DMSO	Sigma

Table 2. Antibodies and concentrations

Antibodies		
Anti-human		
CD16 PE-Cy5	1:100	Biolegend
Histone H3	1:1000	Cell Signaling Technology
Pan-acetyl H3	1:1000	Millipore, Merck
GAPDH	1:1000	Cell Signaling Technology
Secondary AB (HRP) conjugated goat anti rabbit	1:5000	Jackson ImmunoResearch
CD16 PE-Cy5	1:100	Biolegend
CD11b FITC	1:50	Biolegend
CD63 FITC	1:50	BD Bioscience
CD44 PE	1:20	BD Pharmingen
CD49d PE	1:20	BD Pharmingen
CCR3 BV 421	1:100	Biolegend
L-selectin FITC	1:10	BD Pharmingen
Anti-mouse		
TruStain fcX™ (anti-mouse CD16/32)	1:100	Biolegend

Siglec F PE	1:100	BD Pharmingen
CD11b PE-Cy™7	1:200	BD Pharmingen
CD11c BV 421	1:200	BD Pharmingen
Ly6G APC	1:500	Biolegend
CCR3 FITC	1:100	R&D
GR-1	1:200	Biolegend
Sca-1	1:200	Biolegend
CD3	1:200	Thermo Fisher
CD4	1:200	Thermo Fisher
CD8a	1:200	Thermo Fisher
CD19	1:200	Thermo Fisher
B220	1:200	Thermo Fisher
CD34 FITC	1:100	BD Pharmingen
CD125 PE	1:100	BD Pharmingen
CD117 BV 421	1:200	Biolegend
CD16/32 PE-Cy™7	1:400	Biolegend
CD45 BV 510	1:300	Biolegend
CD4 FITC	1:300	Biolegend
CD25 APC	1:100	BD Pharmingen
FoxP3 PE	1:40	Thermofisher
Siglec F BV421	1:200	BD Pharmingen
CD11c APC	1:200	BD Pharmingen
CD45 APC	1:200	Biolegend
CD90.2 BV 214	1:400	Biolegend
FcƐRI-PE-Cy5.5	1:200	Biolegend
F4/80 PE-Cy5	1:200	Biolegend
CD11b PerCP-Cy5.5	1:200	BD Pharmingen
CD11c PerCP-Cy5.5	1:200	BD Pharmingen
CD49b PerCP Cy5.5	1:200	Biolegend

Table 3. Equipment

CDX Connect™ Real-Time PCR detection system	Bio Rad
FACSCalibur flow cytometer	BD Biosciences
BD FACS Canto II flow cytometer	BD Biosciences
48-well micro-Boyden chamber	NeuroProbe Inc
Olympus IX70 fluorescence microscope	Olympus
iBlot™	Thermo Fisher
ChemiDoc Touch Imaging System	Bio Rad
Zeiss Axiovert 40 CFL microscope	Carl Zeiss Microscopy GmbH, Germany
VenaFlux™ Microfl uidic Platform	Cellix Ltd.
FlexiVent system	Scireq
Bio-Plex 200	Bio Rad