Department of Medical Biochemistry and Biophysics
Karolinska Institutet
Stockholm, Sweden

Studies on the Regulation of Leukotriene Biosynthesis in Antigen Presenting Cells

Julia Esser

Karolinska Institutet

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Für Arndt

Die Verletzten sollen die Ärzte sein
Die Letzten sollen die Ersten sein
Die Ersten sehen als Letzte ein:
The geek shall inherit the earth (*Wir sind Helden*)

Summary

Leukotrienes (LTs) are pro-inflammatory lipid mediators with important roles in host defense and inflammatory disease. Leukotriene B_4 (LTB_4) is a potent chemoattractant for neutrophils and contributes to bacterial killing, while the cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) elicit increased vascular permeability, bronchoconstriction, eosinophil trafficking and mucus secretion. Due to their potency to trigger inflammatory responses, the biosynthesis of LTs is highly regulated. This regulation aids to proper pathogen clearance and simultaneously prevents the development of chronic inflammation. It was the aim of this thesis to contribute to a better understanding of the regulatory mechanisms that control LT biosynthesis.

5-lipoxygenase (5-LO), the first enzyme of the LT biosynthetic pathway, catalyzes the conversion of arachidonic acid (AA) to the intermediate leukotriene A_4 (LTA_4). Here, we describe a function of coactosin like protein (CLP) as a stabilizing chaperone for 5-LO. We found that the interaction of 5-LO with CLP, which protects the enzyme from inactivation, depends on lysine 131 in CLP and tryptophan 102 in the regulatory domain of 5-LO. Furthermore, we demonstrate co-localization of 5-LO and CLP in the human monocytic cell line Mono Mac 6 (MM6), implying a regulatory role for CLP in cellular LT formation.

The downstream enzymes of LT biosynthesis (LTA_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S)) further metabolize LTA_4 to the LTs B_4 and C_4, respectively. We studied the regulation of these enzymes during the differentiation of MM6 cells, human monocyte derived macrophages (MDMs) and dendritic cells (MDDCs). Interestingly, a prolonged exposure to the fungal cell wall preparation zymosan (48-96 h) potently downregulated the LTC_4S activity in MM6 cells, MDMs and MDDCs. Acetylsalicylic acid (ASA) and protein kinase inhibitors counteracted this downregulation. Further elucidation of the underlying signaling events indicated that the suppressive effect of zymosan involved toll like receptor 2 ligation, induction of PGE_2 synthesis and protein kinase A and C dependent inhibitory phosphorylation of LTC_4S. These mechanisms for control of cysLT biosynthesis may contribute the resolution of an acute inflammatory response. The counteracting effect of ASA on the suppression of cysLT formation might have implications for aspirin induced asthma.

Antigen presenting cells (APCs), such as macrophages and dendritic cells secrete nano-sized vesicles (exosomes), which serve as messengers in immunity. Here, we investigated if exosomes from MDMs and MDDCs might contain enzymes for LT biosynthesis. We demonstrate that exosomes from MDMs and MDDCs as well as from human plasma and bronchoalveolar lavage fluid (BALF) contain active LTA_4H and LTC_4S. In addition, we show that exosomes from APCs have the capacity to synthesize a variety of eicosanoids and to induce granulocyte migration. Furthermore, BALF exosomes from asthmatics could increase the release of pro-inflammatory mediators (interleukin-8 and cysLTs) from bronchial epithelial cells. Hence, our data implicate exosomes as novel players in LT biosynthesis with potential roles in inflammatory disorders, such as asthma.

1 List of Publications

This thesis is based on the articles as listed below, which are referred to in the text by their Roman numbers.

I. **Esser J***, Rakonjac M*, Hofmann B, Fischer L, Provost P, Schneider G, Steinhilber D, Samuelsson B, Rådmark O. *Coactosin-like protein functions as a stabilizing chaperone for 5-lipoxygenase: role of tryptophan 102.* Biochem J. 2009 Dec 14;425(1):265-74.

II. **Esser J**, Gehrmann U, Salvado MD, Wetterholm A, Haeggström JZ, Samuelsson B, Gabrielsson S, Scheynius A, Rådmark O. *Zymosan suppresses leukotriene C4 synthase activity in differentiating monocytes: antagonism by aspirin and protein kinase inhibitors* FASEB J. 2011 Jan; [Epub ahead of print]

III. **Esser J***, Gehrmann U*, D'Alexandri FL, Hidalgo-Estévez AM, Wheelock CE, Scheynius A, Gabrielsson S, Rådmark O. *Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration.* J Allergy Clin Immunol. 2010 Nov;126(5):1032-40, 1040.e1-4.

IV. Torregrosa Paredes P, **Esser J**, Admyre C, Claesson HE, Nord M, Rådmark O, Grönneberg R, Grunewald J, Eklund A, Scheynius A, Gabrielsson S. *Asthma patients display an altered profile of bronchoalveolar lavage fluid exosomes capable of leukotriene metabolism and induction of IL-8 production.* Manuscript

* equal contribution

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2 List of Abbreviations

12-HETE	12-hydroxy eicosatetraenoic acid
15-HETE	15-hydroxy eicosatetraenoic acid
15-LO	15-lipoxygenase
16HB14o	16 human bronchial 14o epithelial cell line
5-HETE	5-hydroxy eicosatetraenoic acid
5-LO	5-lipoxygenase
AA	arachidonic acid
AM	alveolar macrophage
APC	antigen presenting cell
ASA	acetylsalicylic acid
BEC	bronchial epithelial cell
BLT1	leukotriene B_4 receptor 1
cAMP	cyclic adenosine monophosphate
CLP	coactosin like protein
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
$CysLT_1$	cysteinyl leukotriene receptor 1
ERK	extracellular-signal regulated kinase
FLAP	5-lipoxygenase activating protein
GM-CSF	granulocyte macrophage colony stimulating factor
GPCR	guanine nucleotide binding protein coupled receptor
HPLC	high performance liquid chromatography
H_2S	hydrogen sulfide
IL	interleukin
LCs	Langerhans cells
LPS	lipopolysaccharide
LTA_4	leukotriene A_4
LXA_4	lipoxin A_4
LTA_4H	leukotriene A_4 hydrolase
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
LTC_4S	leukotriene C_4 synthase
MAMP	microbe associated molecular pattern
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen activated protein kinase
MDDCs	monocyte derived dendritic cells
MDMs	monocyte derived macrophages
MM6	mono mac 6 cell line
MRP-1	multi drug resistance protein-1
mTOR	mammalian target of rapamycin

NFAT	nuclear factor of activated T-cells
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
p70S6K	70-kDa ribosomal protein S6 kinase
PAF	platelet activating factor
PGE$_2$	prostaglandin E$_2$
PKC	protein kinase C
PI3K	phosphatidylinositol 3-phosphate kinase
PMNL	polymorphonuclear leukocytes
RANTES	regulated upon activation normal T-cell expressed and secreted
ROS	reactive oxygen species
RvE$_1$	resolvin E$_1$
Syk	spleen tyrosine kinase
TGFβ1	transforming growth factor beta 1
TNFα1	tumor necrose factor alpha
TLR	toll like receptor
T$_H$	T helper (cell)
Treg	regulatory T (cell)
WB	Western blot
VD$_3$	1α,25-dihydroxyvitamin D$_3$

3 Introduction

3.1 Inflammation

From birth on, we are exposed to an inconceivable variety of environmental factors. Many of these factors endanger the integrity of our body and threaten our survival. Thus, our body has evolved defense mechanisms to protect itself against physical as well as microbial insults, such as extreme temperatures, viruses or bacteria. "Inflammation" (Latin: *inflammare* = to set on fire) is the essential process that is started, when such an insult harms or penetrates any of our surface body barriers. The long recognized cardinal signs of inflammation are redness (*rubor*), increased heat (*calor*), swelling (*tumor*), pain (*dolor*) and loss of (tissue) function (*functio laesa*). These physiological reactions have as their common goal to ensure pathogen clearance and subsequent restoration of tissue homeostasis (Fig. 3.1).
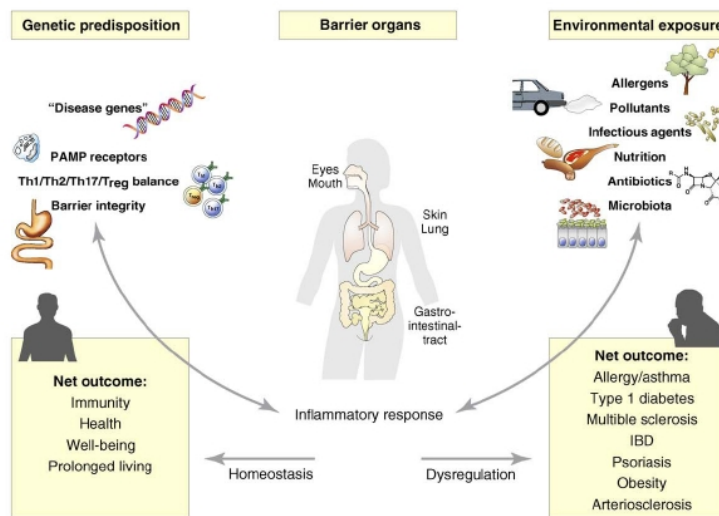
Figure 3.1: *The inflammatory process: determinants and outcomes* [78]

On the molecular level, inflammation is induced, amplified and resolved by a complex and dynamic network of mediators. The major sources of these mediators are white blood cells (leukocytes), which originate from the bone marrow and which can undergo several steps of differentiation, migration and maturation during their life span. The inflammatory pathway thus consists of "inducers" (e.g. infectious agents), "sensors" (tissue resident leukocytes such as macrophages), "mediators" (e.g. cytokines, see table 3.2) and target tissues (Fig. 3.2). To ensure efficient pathogen clearance but avoid persistence

of inflammation and permanent loss of tissue function, the synthesis and release of pro-
and anti-inflammatory mediators is tightly regulated.
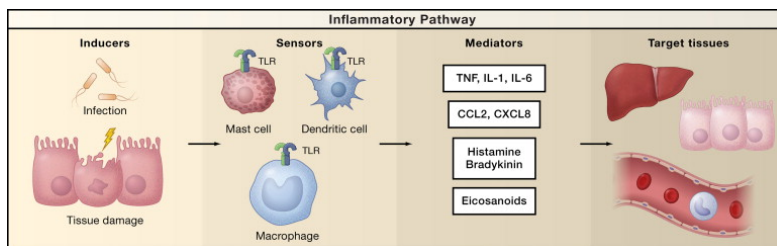
Figure 3.2: *The inflammatory pathway: inducers, sensors, mediators and target tissues*
[192]

3.2 Eicosanoids - biosynthesis and physiology

The accurate course of the inflammatory process depends on the chronological activa-
tion of signaling events, which are initiated by a variety of mediators. According to their
biosynthetic origin, these mediators can be divided into different classes:

mediators	chemical class	examples
cytokines	peptides, (glyco-)proteins	TNFα, IL-4, IL-10
chemokines	peptides, (glyco-)proteins	Eotaxin, RANTES, IL-8
gases	soluble gases	NO, H_2S
eicosanoids	lipids	PGE_2, LTB_4, LXA_4

Table 3.2: mediators of inflammation

Eicosanoids are lipid mediators, derived from arachidonic acid (AA), a polyunsaturated
omega-6 fatty acid with 20 carbon atoms and four *cis* double bonds in positions 5, 8,
11 and 14 (C20:4 (ω-6)) (see Fig. 3.3). AA can be metabolized by several enzymatic
cascades into distinct classes of eicosanoids with a broad range of physiological func-
tions (for some examples, see table 3.4) [282]. Thereby, the most well known pathway
is certainly the cyclooxygenase pathway, which gives rise to the prostanoids and which
is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic
acid (aspirin) [23], [333]. Prostanoids (prostaglandins, prostacyclins and thromboxanes)
contribute to the induction of essential physiological events such as fever, nociception,
uterine contraction, vasodilation and haemostasis [288]. An alternative enzymatic path-
way for conversion of AA are specialized cytochrome P450 epoxygenases [294], which
metabolize AA to epoxyeicosatrienoic acids (EETs). EETs are important regulators of
the vascular tone. Furthermore, AA can be oxygenated by lipoxygenase pathways, com-
prising 5-lipoxygenase and 12/15-lipoxygenase catalyzed reactions, giving rise to 5-, 12-
and 15- hydro(pero)xy eicosatetraenoic acids (H(P)ETEs) as well as to lipoxins [273].

eicosanoid	physiological role	references
5-KETE	granulocyte activation, eosinophil + basophil recruitment	[232], [211], [212], [203], [297]
LTB_4	neutrophil chemotaxis + adherence, stimulation of antimicrobial activity, T cell recruitment	[221], [271], [87], [109]
LTC_4	bronchoconstriction, increased vasopermeability, mucus secretion, airway remodeling, DC activation	[262], [67], [191], [261], [341]
LXA_4	inhibition of granulocyte trafficking and adhesion, stimulation of nonphlogistic phagocytosis	[38], [16], [107]
PGE_2	inhibition of leukocyte activation, modulation of DC maturation + Treg/T_H17 balance	[39], [127], [290], [158], [54]
PGD_2	bronchoconstriction, activation + chemotaxis of eosinophils, neutrophil recruitment	[126], [245], [96], [327]
$PGF_2\alpha$	uterine contraction, PMNL migration, ROS generation	[46], [281], [154]
TXA_2	platelet aggregation, vasoconstriction, modulation of APC-T cell interaction	[53], [151]

Table 3.4: Examples of eicosanoids and their physiological and immunological roles
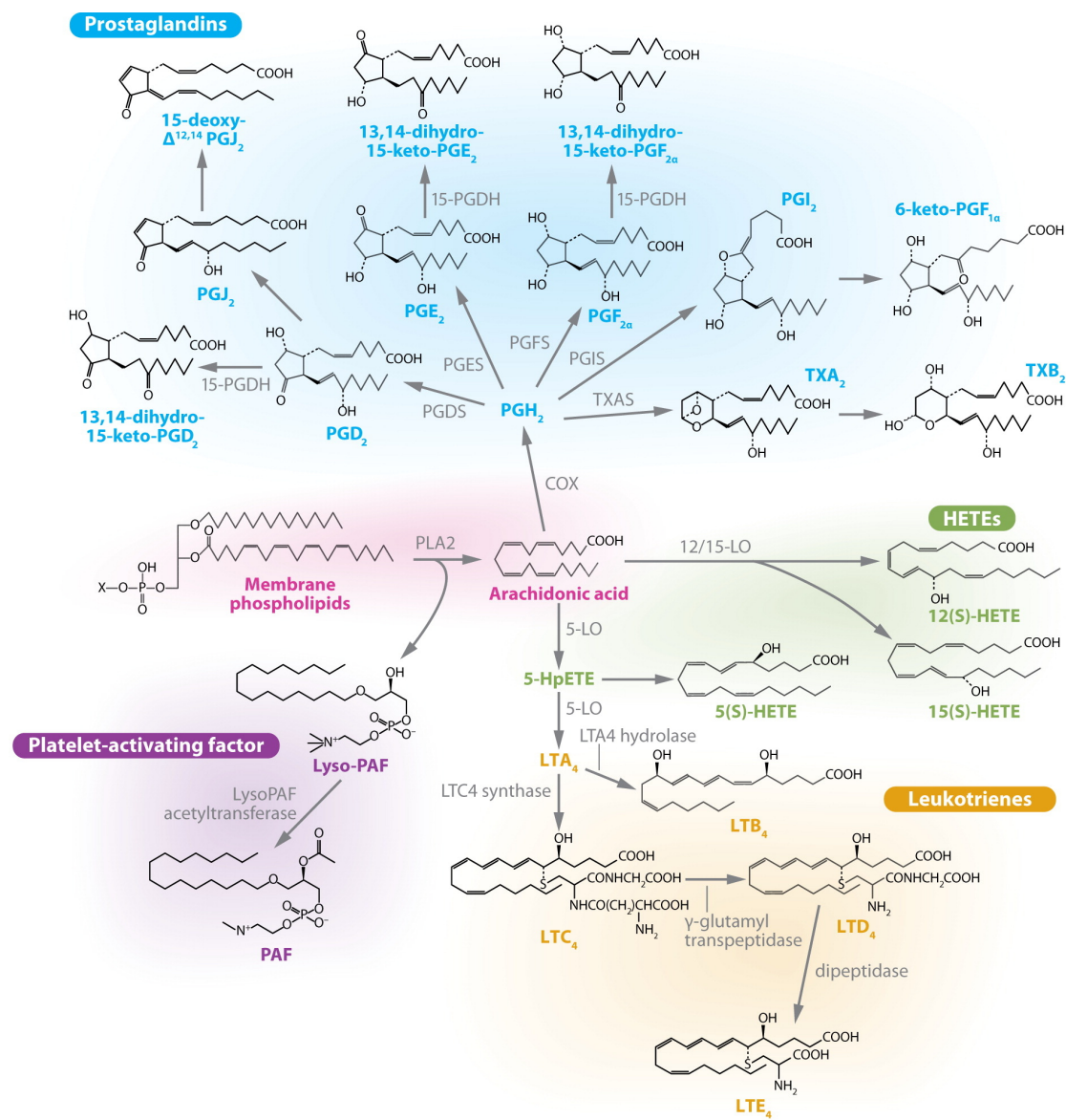
Figure 3.3: *Biosynthesis of PGs, LTs, HETEs and PAF* [282]

3.3 Leukotrienes - biosynthesis, metabolism and signaling

In order for leukotriene (LT) biosynthesis to start, AA has to be cleaved from membrane phospholipids by cytosolic phospholipase A_2 (cPLA$_2$), which hydrolyzes the ester bond in the sn2 position, yielding free AA and lysophospholipids. In the first step of LT biosynthesis, 5-lipoxygenase (5-LO) catalyzes the hydrogen abstraction at C-7 of AA and antarafacial incorporation of molecular oxygen into position five, resulting in formation of 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) (Fig. 3.4). In a second round of the 5-LO reaction cycle, hydrogen is abstracted at C-10 and 5-HPETE is dehydrated, giving rise to the instable epoxide intermediate LTA$_4$ [262], [204].
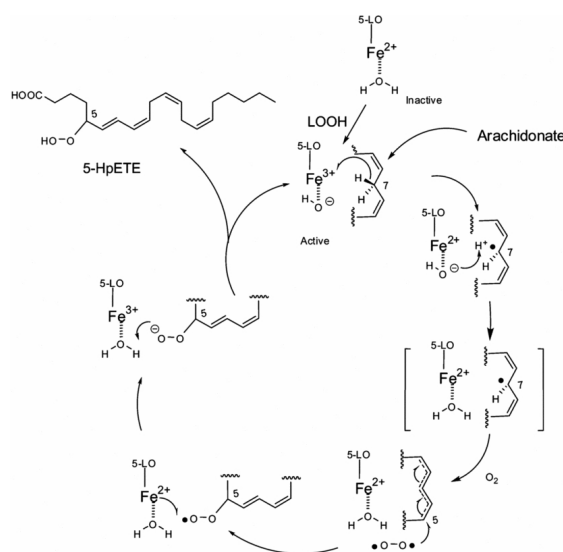
Figure 3.4: *Catalytic cycle for formation of 5-HPETE by 5-LO* [204]

The subsequent metabolism of LTA$_4$ by the downstream enzymes LTA$_4$ hydrolase (LTA$_4$H) or LTC$_4$ synthase (LTC$_4$S) will then yield the biologically active leukotrienes B$_4$ (LTB$_4$) and C$_4$ (LTC$_4$), respectively. LTA$_4$H is an epoxide hydrolase, which catalyzes the activation of the epoxide function of LTA$_4$ and the stereospecific addition of water in position 12 of the thereby formed carbocation intermediate [120]. The resulting LTB$_4$ can be metabolized by cytochrome P450 enzymes to 20-hydroxy and 20-carboxy LTB$_4$ [125]. Alternative to hydrolysis by LTA$_4$H, LTA$_4$ can be conjugated to glutathione (GSH) by LTC$_4$S. Based on the structure of LTC$_4$S [2], [182], a catalytic mechanism can be envisioned, starting with formation of a thiolate anion (GS$^-$) which then attacks the allylic carbon in position 6 of the epoxide function of LTA$_4$ [253]. The product of this reaction, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid (LTC$_4$), undergoes consecutive cleavage of its peptide moiety, resulting in generation of the other two cysLTs (LTD$_4$ and LTE$_4$) (Fig. 3.3).

In vivo, LTs have short half lives (sec-min). Both LTB$_4$ and LTE$_4$ are metabolized by ω-oxidation, which yields the corresponding 20-carboxy-derivative. Finally, 20-carboxy LTB$_4$ and 20-carboxy LTE$_4$ can be degraded by β-oxidation [204].

3.3.1 Structural properties of LT biosynthetic enzymes
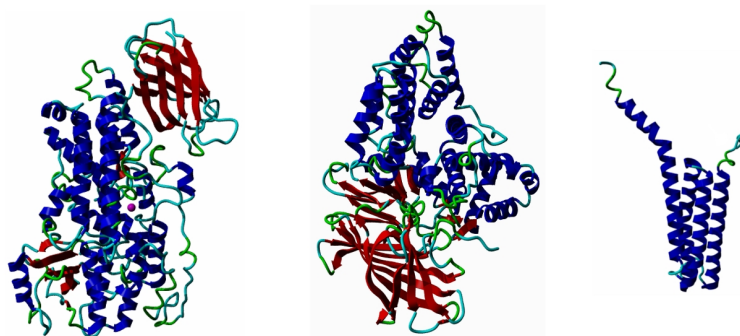
Figure 3.5: *Structures of 5-LO, LTA_4H and LTC_4S (monomer)*

5-lipoxygenase

5-LO is a 78 kDa enzyme, consisting of two domains: an N-terminal regulatory domain (residues 1-120) and a C-terminal catalytic domain (residues 121-673). These two domains are structurally distinct, with the regulatory domain showing a characteristic C2-like β-sandwich fold and the catalytic domain mainly consisting of α-helices [106] . The C-terminal domain comprises the active site with the prosthetic iron, while the N-terminal domain contains residues for binding of regulatory factors such as Ca^{2+}, membrane phospholipids and coactosin like protein (CLP) [244].

LTA_4 hydrolase

The 69 kDa protein LTA_4H consists of a C-terminal, a catalytic and an N-terminal domain, which are arranged in such a way that a hydrophobic pocket for substrate binding is formed. This hydrophobic cleft contains the active site with the catalytic zinc [324], [120].

LTC_4 synthase

LTC_4S is a trimeric, integral membrane protein, with a monomer size of 18 kDa [2], [182]. The LTC_4S monomer comprises four transmembrane α-helices and shares many structural features with FLAP and mPGES-1, two other members of the superfamily of membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) [143], [86], [146]. As the catalytic site of LTC_4S is directed towards the lumen of the outer nuclear membrane, the newly synthesized LTC_4 has to be exported into the cytoplasm via its carrier: multi drug resistance protein-1 (MRP-1) [58].

3.3.2 Regulation of leukotriene biosynthetic enzymes

5-lipoxygenase

The activity of 5-LO is controlled on several levels by various regulatory factors [250]. Interestingly, 5-LO shares some of its major regulators with $cPLA_2$ [244]. Both enzymes posses a C2 like domain, which can bind Ca^{2+}. Ca^{2+} binding confers association of $cPLA_2$ and 5-LO with the nuclear membrane, which is enriched in AA containing phospholipids [48]. Thus, stimuli increasing the intracellular Ca^{2+} concentration trigger translocation of $cPLA_2$ and 5-LO to the nuclear envelope. This allows for optimal substrate access and efficient LT generation due to the formation of the so-called "LT biosynthetic metabolon" together with FLAP and LTC_4S [340], [181], [180].

The 18 kDa integral membrane protein FLAP, which is required for cellular LT synthesis from endogenous substrate [73], binds AA and is believed to transfer it to 5-LO. In this way, FLAP increases AA utilization as well as conversion of 5-HPETE to LTA_4 [177], [1].

Another protein that influences the activity of 5-LO, is the 16 kDa F-actin binding protein Coactosin Like Protein (CLP) [246]. CLP has been shown to bind 5-LO [234] and to support 5-LO activity *in vitro* in two ways (summarized in Fig. 3.6): Firstly, CLP augmented 5-LO product formation in the absence of phosphatidylcholine (PC), which mimics the presence of nuclear membrane phospholipids and is needed for efficient 5-LO product formation in standard *in vitro* assays. Secondly, in presence of PC, CLP increased the capacity of 5-LO for synthesis of LTA_4. In addition, the apparent coordinated translocation of CLP and 5-LO in stimulated leukocytes allows speculations about a role of CLP as a promoting factor in cellular LT synthesis [244].
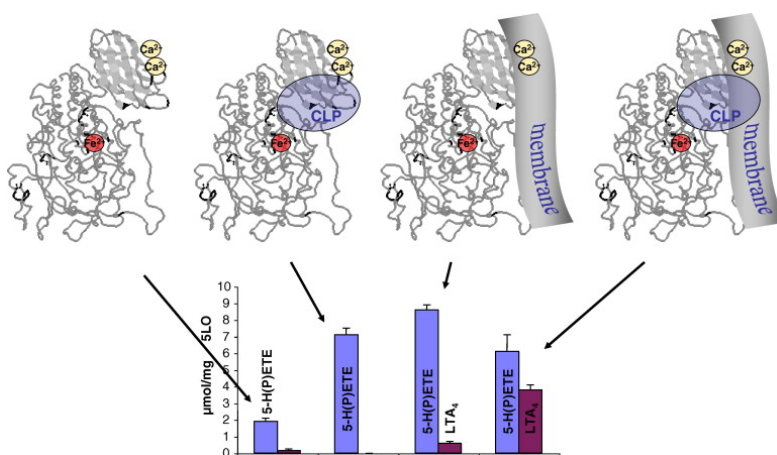
Figure 3.6: *Different activity modes of 5-lipoxygenase* [244]

Another crucial determinant of 5-LO activity and localization is the phosphorylation state of the enzyme. Several kinases can phosphorylate 5-LO at distinct sites, which will either up- or downregulate product formation and nuclear localization. Phosphorylation of 5-LO at Ser-663 by ERKs [336] as well as at Ser-271 by p38 kinase-dependent MAPKAP kinases [337] stimulated 5-LO activity and translocation to the nuclear membrane in PMNL and Mono Mac 6 cells [338], [336]. These reports are in line with the

observation that nuclear localization of 5-LO results in efficient LTB_4 synthesis [174]. Interestingly, differential ERK activation patterns in female *versus* male PMNL conferred differences in 5-LO localization, connected with a higher capacity for stimulated 5-LO product formation in female PMNL [227]. The effect of phosphorylation at Ser-271 appears to depend on the cell type: Nuclear export of 5-LO occurred in HEK293 cells, transiently expressing a 5-LO mutant, in which phosphorylation was mimicked by replacement of Ser-271 by glutamate [124]. However, in transfected NIH 3T3 cells, expressing a 5-LO, which is constitutively phosphorylated at Ser-271, the enzyme was exclusively localized in the nucleus [89]. These latter results are consistent with evidence from HeLa cells, which lack constitutive phosphorylation at Ser-271 and which exhibit no nuclear 5-LO.

In contrast to the stimulatory effects of ERKs and p38 kinase-dependent MAPKAP kinases, PKA mediated phosphorylation of 5-LO at Ser-523 conferred cytoplasmic localization and reduced 5-LO product synthesis [91], [175], [173]. Furthermore, the phosphorylation of 5-LO by PKA was reported to shift in the 5-LO product profile in rat myocardial cells from LTB_4 to 15-epi-LXA_4 [344].

Adhesion induced nuclear import of 5-LO was observed in human neutrophils and eosinophils [41], [40]. In neutrophils, translocation of 5-LO into the nucleus promoted LTB_4 formation [174], indicating that nuclear 5-LO, together with nuclear LTA_4H yields large amounts of LTB_4. In contrast, adhesion induced nuclear import of 5-LO suppressed LTC_4 generation in eosinophils without inhibiting LTC_4S activity [40]. These findings suggest that cytoplasmic 5-LO collaborates with LTC_4S to generate cysLTs, while nuclear 5-LO favors the synthesis of LTB_4 (Fig. 3.7). Thus, not only the enzymatic equipment but also 5-LO localization determines the cellular LT profile, which will then define the triggered effector functions and ultimately shape the nature of the inflammatory response.
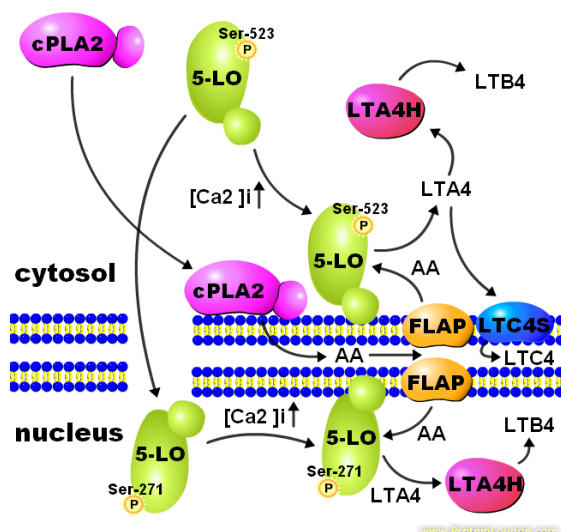

Figure 3.7: *Spatial organization of leukotriene biosynthesis in the cell*

In addition to these mechanisms for post-translational regulation of 5-LO, several factors modify the expression of the enzyme [250]. For example, 5-LO protein levels can change

dramatically during the differentiation of myeloid cells [235], [254], [63] often under the influence of cytokines and growth factors, such as TGFβ-1, 1α,25-dihydroxyvitamin D$_3$ and IL-4 [296], [43], [292]. Very recently, splice variants of 5-LO have been identified in PMNL and different leukemia cell lines. Interestingly, co-expression of these 5-LO isoforms with the active full length protein resulted in reduced 5-LO product formation [35]. These novel findings add yet another layer to the complex regulation of 5-LO activity.

LTC$_4$ synthase

As described for 5-LO, cellular LTC$_4$S activity is controlled both by transcriptional regulation as well as by postranslational modification. Activation of protein kinase C by treatment with phorbol esters decreased LTC$_4$ synthesis in HL-60 cells and human granulocytes [7], [153], [287]. The pan-kinase inhibitor staurosporine counteracted this PMA induced downregulation of the LTC$_4$S activity, suggesting an inhibitory phosphorylation of LTC$_4$S either by PKC itself or by a downstream kinase. Furthermore, phosphorylation of LTC$_4$S in whole cells could be demonstrated in immunoprecipitation experiments after radioactive ATP labeling [115]. These experiments indicated that the phosphorylation state of LTC$_4$S depends on the cell type as well as the level of cellular differentiation. Moreover, phosphorylation of LTC$_4$S was abrogated by specific PKC inhibitors [115].

Very recently, an alternative mechanism for control of LTC$_4$S activity has been described in mast cells: oxidative stress induced a reversible, covalent dimerization of LTC$_4$S, which suppressed the enzymatic activity to approximately 30 % of control [131]. Interestingly, both PGD$_2$ and PGE$_2$ inhibited LTC$_4$S activity in this model system. This inhibitory effect of the two PGs was apparently due to the ability of their cyclopentenone metabolites to decrease the intracellular GSH concentration.

Major LTC$_4$S expressing cells include innate immune cells, such as eosinophils, mast cells and monocytes/macrophages. Transcriptional regulation has mostly been studied in the monocytic cell line THP-1, in which LTC$_4$S mRNA was downregulated by TNFα and LPS [277], [278], while TGFβ upregulated LTC$_4$S on both mRNA and protein level [252]. However, also primary human bronchial fibroblasts and bronchial epithelial cells were shown to express LTC$_4$S and TGFβ-1 potently increased LTC$_4$S protein levels in these cells [145], [144].

LTA$_4$ hydrolase

While expression of 5-LO is mostly confined to leukocytes, LTA$_4$H is expressed by almost all cell types [95]. However, in spite of its wide tissue distribution, cellular LTA$_4$H expression can change considerably in response to a particular cytokine milieu or upon microbial stimulation. In freshly isolated human monocytes, the T$_H$2 cytokines IL-4 and IL-13 downregulated expression of LTA$_4$H, while the T$_H$1 cytokine interferon-γ increased LTA$_4$H mRNA and protein levels [198]. These effects, observed on mRNA and protein level, were accompanied by the according trend for LTB$_4$ formation. In contrast, in PMNL, IL-4 and IL-13 upregulated LTA$_4$H mRNA, protein and activity [349]. Furthermore, expression of LTA$_4$H was slightly increased in response to LPS, the calcium ionophore A23187 and bradykinin in human bronchial epithelial cells [144].

Compared to 5-LO and LTC_4S, much less is known about a regulatory role of phosphorylation for LTA_4H activity. Solely for endothelial cells, an inhibitory effect of LTA_4H phosphorylation has been demonstrated [258]. A well established principle for control of LTA_4H activity is suicide inactivation by its substrate LTA_4 [243], [219], [119]. Due to the limitation of LTA_4H activity in the activated neutrophil, LTA_4 is secreted, allowing for its conversion to either LTB_4 or LTC_4 by 5-LO negative cells such as erythrocytes, epithelial cells, platelets and endothelial cells [189], [30], [220], [83] (Fig. 3.8). The transcellular metabolism of LTs is a powerful strategy for fine tuning of LT synthesis in distinct physiological settings. Recent studies, using LT pathway knock-out mice in *in vivo* models of inflammation, have elegantly shown efficient transcellular LT metabolism during an acute inflammatory response [350], [259].

Hence, on the tissue level, the LT profile depends on the interactions of infiltrating or resident leukocytes with other tissue specific cell types. Together with the importance of the subcellular localization of the LT biosynthetic enzymes [209] transcellular metabolism adds extra complexity to the regulation of LT biosynthesis.

3.3.3 Leukotriene receptors

Once synthesized and released, LTs will bind to their specific G-protein coupled receptors on the target cell. Two receptors have been identified for LTB_4: the high affinity receptor BLT_1 [346] (K_d=1.1 nM), expressed mainly on leukocytes, and the low affinity receptor BLT_2 (K_d=23 nM) [347], showing wide tissue distribution [306]. Although at considerably higher concentrations (>10 μM), LTB_4 also activates peroxisome proliferator-activated receptor alpha (PPAR-α) [71]. It has been suggested that in this way, LTB_4 acts as an endogenous intracellular ligand, which can initiate its own degradation in a PPAR-α dependent manner [207].

For the cysLTs, the situation is more complex and the search for a selective LTE_4 receptor is currently ongoing [14]. However, it is well established that LTC_4 and LTD_4 bind to two major cysLT receptors: $CysLT_1$ and $CysLT_2$ [257]. Thereby, LTD_4 has the highest affinity for $CysLT_1$ (1-10 nM), which is predominantly expressed on leukocytes, spleen and smooth muscle of the lung and intestine. In contrast, LTC_4 and LTD_4 exhibit equal affinities for $CysLT_2$ ($K_d \approx$10 nM), which is expressed in leukocytes, spleen, kidney, heart and brain. LTE_4 is only a poor ligand for both of these receptors.

Depending on the cell type, LT receptors can couple to G proteins with different α subunits, resulting in a variety of downstream effects [282]. One major pathway of LT receptor signaling runs *via* $G\alpha_q$ proteins, triggering activation of phospholipase C, Ca^{2+} mobilization and ultimately responses such as MAPK activation and actin reorganization [101], [257]. The second crucial downstream pathway starts with the liberation of $G\alpha_i$ subunits, which inhibit adenlate cyclase, thereby lowering the cellular cAMP concentration. Since cAMP acts as a break signal for many innate immune cells, a decrease in the concentration of this second messenger will contribute to the activation of microbicidal effector mechanisms such as phagocytosis and ROS generation [272].

3.4 Leukotrienes - roles in immunity and inflammatory disease

As already adumbrated above, leukocytes are the most efficient producers of LTs. Innate immune cells such as neutrophils, eosinophils, basophils, mast cells and macrophages have high capacities for generation of LTB_4 and/or cysLTs (Fig. 3.8) [229]. Moreover, LTs exert potent auto- and paracrine effects on these cells, which are crucial for efficient immune responses against many infectious agents.
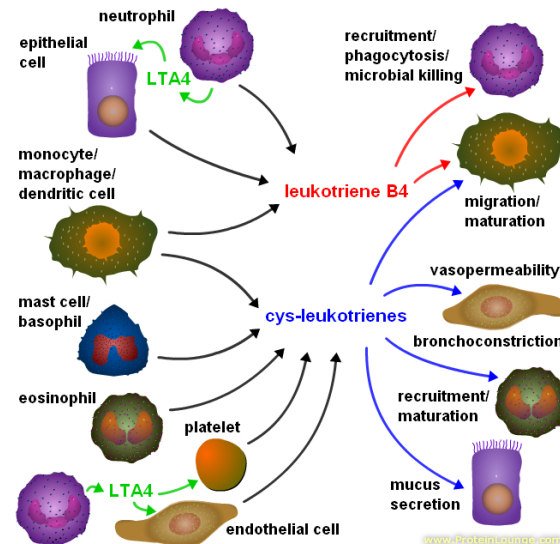
Figure 3.8: *Source and target cells of Leukotrienes*

3.4.1 Immunological triggers of LT release

Innate immune cells recognize microbial and viral intruders *via* pathogen recognition receptors (PRRs), which bind to microbe associated molecular patterns (MAMPs) such as bacterial lipopolysaccaride (LPS), peptidoglycan, lipoteichoic acid, flagellin or viral DNA and double stranded RNA [26], [307]. PRRs comprise the toll like receptors (TLRs), C-type lectin receptors (e.g. Dectin-1), Rig-I-like receptors and the NOD like receptors [138], [308].

Signaling of PRRs triggers the activation of kinases (e.g. ERK1/2, p38 or Syk) and transcription factors (e.g. NFκB or NFAT), which will increase the production of inflammatory mediators [110]. An overview of some PRRs and MAMPs that influence LT biosynthesis is presented in table 3.6.

PRR	ligand	effects	references
TLR4	LPS	priming for increased LTB_4 synthesis (neutrophils); $cPLA_2$ activation (macrophages)	[75], [239]
TLR2	zymosan, peptidoglycan	cysLT generation (Mast Cells); $cPLA_2$ activation (monocytes)	[188], [171]
β-glucan receptor (Dectin-1)	β-glucans, zymosan, *C. albicans*	AA release (macrophages, Mast Cells); cysLT release (Mast Cells, DCs)	[216], [217], [331], [304]
Mannose receptor (Dectin-2)	α-mannan, zymosan, *C. albicans*, *A. fumigatus*/ house dust mite extract	AA release and LT generation (macrophages, PMNL); cysLT production (DCs)	[50], [332], [304], [18]

Table 3.6: Some examples of PRRs and MAMPs with short time effects on LT biosynthesis

As mentioned above, stimuli that increase the intracellular Ca^{2+} concentration are potential inducers of LT formation. In macrophages and DCs, the TLR ligands LPS and peptidoglycan triggered Ca^{2+} mobilization *via* activation of phospholipase Cγ2 [4]. Similar to these innate immune mechanisms, also Fcγ receptor mediated recognition of immune complexes resulted in an increased intracellular Ca^{2+} concentration as well as LTB$_4$ release [328], [65].

However, in addition to microbial triggers, also physical stressors such as hyperosmolarity [285], hyperoxia [113] or heat [202] can provoke Ca^{2+} influx and LT biosynthesis.
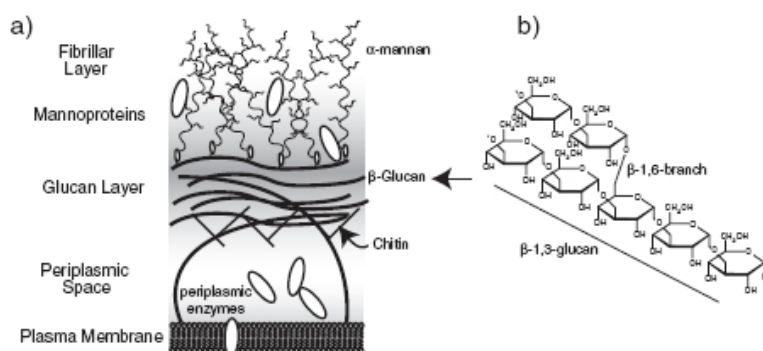
3.4.2 Roles of LTs in host defense

Figure 3.9: *Structure and composition of the yeast cell wall* [110]

LTs elicit a broad spectrum of effector mechanisms, which crucially contribute to pathogen clearance. Thereby, the cysLTs act mainly by blocking access and stimulating removal of pathogens, particularly in the respiratory tract, where they trigger bronchoconstriction and mucus hypersecretion [262]. Furthermore, by increasing vascular permeability and P-selectin expression on endothelial cells [68], cysLTs facilitate leukocyte infiltration to the site of infection. In addition to these immediate responses, cysLTs promote the expression of genes, which encode other host defense factors such as IL-8 [317], monocyte chemoattractant protein 1 (MCP-1) [141] and immunoglobulins [165].

As mentioned above, fungal cell wall constituents such as α-mannans and β-glucans (Figure 3.9) are potent inducers of acute cysLT generation in mast cells, macrophages and DCs (table 3.6). This allows speculations about a particular relevance of cysLTs in protective immunity against fungal infections.

The supporting effects of cysLTs on eosinophil differentiation [36], [242] and trafficking [92] suggest their critical involvement in immunity against parasitic infections [201], [136]. Interestingly, eosinophils, basophils and mast cells, which are major cysLT producing cells, play central roles in protective immunity against parasitic helminths [12].

In comparison to cysLTs, even more information exists about immune effector functions of LTB$_4$, which is involved at all levels of the microbial killing process: LTB$_4$ promotes leukocyte attraction [313], [284], FcγR mediated phagocytosis [47], ROS production [339] and the release of antimicrobial peptides [87], [334], [102]. Like the cysLTs, LTB$_4$ can induce a variety of host defense genes, resulting in increased expression of immune factors

such as TNFα, NO, IL-6, IL-8 and C-C chemokine receptor type 7 (CCR7) [309], [37], [233].

Due to these reinforcing effects on antimicrobial defense mechanisms, LTs contribute to protective immunity against infections with a variety of human pathogens. A direct role of cysLTs in immunity against infections was demonstrated in alveolar macrophages, where cysLTs efficiently enhanced phagocytosis of gram-negative bacteria [179]. Mice deficient in LTB$_4$ generation showed an increased susceptibility to *Klebsiella pneumoniae* and *Leishmania amazonensis* [15], [271]. Moreover, pharmacological inhibition of LT biosynthesis lead to exacerbation of pulmonary histoplasmosis [190] and increased mortality after infection with *Mycobacterium tuberculosis* [225]. The particular importance of LTs in pulmonary immunity is supported by the finding that intrapulmonary administration of LTB$_4$ improved host defense against pneumococcal pneumonia [178].

Evidence that 5-LO products play protective roles in other parts of the body as well comes from studies on 5-LO$^{-/-}$ mice and mice treated with the 5-LO inhibitor Zileuton, which were more susceptible to acute vesicular stomatitis virus (VSV) encephalitis [56]. This suggests that LTs also contribute to protective immunity in the central nervous system.

The evidence from infection models in mice with genetic ablation of LT biosynthesis might have some relevance for the human situation, where immunodeficiency due to malnutrition or HIV infection is connected to deficient LT generation [51], [318], [61], [187].

Therefore, application of aerosolized LTB$_4$ or biodegradable LTB$_4$-loaded microspheres has been suggested as a therapeutic approach for improving host defense against pulmonary infections, particularly in immuno-compromized individuals [210], [178].

3.4.3 Leukotrienes as mediators in asthma

The currently applied therapies in relation to LTs are designed to block rather than to boost the LT pathway in order to treat inflammatory conditions, which are characterized by an overproduction of LTs. The most studied condition with an established pathophysiological role for LTs is bronchial asthma [121]. Patients with diverse forms of asthma consistently show increased levels of LTs in exhaled breath condensate, broncho alveolar lavage fluid (BALF) and urine [335], [49], [98], [195].

The CysLT$_1$ antagonists montelukast, pranlukast and zafirlukast have been approved for the treatment of asthma and allergic rhinitis and are commonly used in combination therapy with topical glucocorticosteroids [183], [200]. Due to its hepatotoxic effects, the 5-LO inhibitor Zileuton is only applied in the treatment of severe and/or glucocorticosteroid resistant forms of asthma [22].

In asthma, inflammatory cells, such as mast cells and eosinophils, accumulate in the bronchi and bronchioli and release a diverse set of cytokines and lipid mediators, resulting in persistent inflammation (Fig. 3.10) [17].

LTC$_4$, LTD$_4$ and LTE$_4$, previously known as slow-reacting substance of anaphylaxis (SRS-A), have long been recognized as key mediators in asthma because of their potency to cause bronchial smooth muscle contraction, mucosal edema as well as mucus
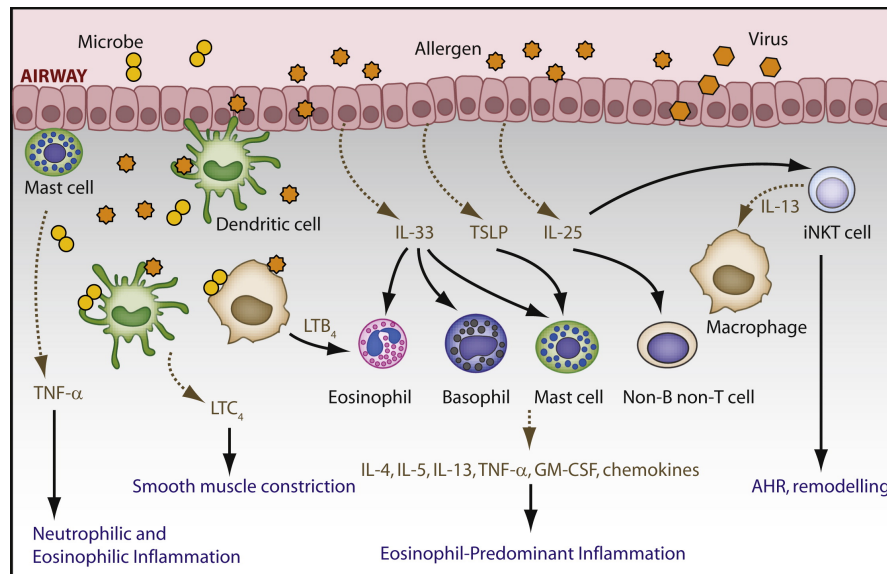
Figure 3.10: *Crucial cells and mediators in the pathogenesis of asthma* [17]

secretion [67]. In addition, cysLTs contribute to airway remodeling by eliciting eosinophil accumulation and survival [137], [168] and by triggering the release of the remodeling cytokines IL-13 and TGFβ1 [228], [121]. The outstanding importance of cysLTs for the pathology of asthma is further supported by their upregulating effect on T_H2 cytokine expression [60]. Interestingly, the capacity of the airway epithelium for cysLT biosynthesis is inversely related to the output of PGE_2, which exerts inhibitory effects on airway remodeling [137]. Furthermore, exogenous PGE_2 inhibited stimulated cysLT release from bronchial biopsy specimens [265]. This anti-inflammatory action of PGE_2 was more prominent for mucosal specimens from normal as compared to from inflamed lungs, indicating an abnormal regulation of cysLT biosynthesis in pulmonary inflammation.

While cysLTs trigger asthma symptoms directly, LTB_4 has mainly been implicated in the pathogenesis of the underlying airway inflammation and hyperresponsiveness. In mouse models of allergic asthma genetic ablation of the LTB_4 receptor BLT_1 protected against the development of airway hyperresponsiveness and T_H2-type immune responses [312], [196], [197].

In the human situation the LTB_4/BLT-1 axis might have implications for severe, glucocorticosteroid resistant asthma characterized by a predominance of neutrophilic inflammation [214], [199], [130]. Severe asthma and chronic rhinosinusitis, particularly if accompanied by aspirin intolerance, are associated with pronounced LT overproduction and a simultaneous decrease in the generation of anti-inflammatory lipoxin A_4 [330], [226].

Studies assessing polymorphisms of LT pathway proteins, which predispose to the development of asthma are inconsistent. However, particular polymorphisms in the promoter region of LTC_4S have repeatedly been linked to overexpression of the enzyme in certain groups of asthmatics [263], [155]. Furthermore, data from several groups suggest a correlation between increased expression of $CysLT_1$ and particular forms of asthma, e.g. aspirin intolerant asthma (AIA) or mild allergic asthma [159], [76], [352].

3.4.4 Leukotrienes in cardiovascular disease and cancer

In addition to its roles in asthma and other chronic inflammatory disorders such as rheumatoid arthritis [69], [55], [105] and colitis [280], [356], [66], the 5-LO pathway has been implicated in cardiovascular disease (CVD) and certain forms of cancer.

Investigations of human cancer tissue as well as murine cancer models suggest crucial roles of 5-LO products in chronic myeloid leukemia [57] and cancers of the prostate [116], colon [193] and pancreas [135].

Furthermore, there is a growing body of evidence describing genetic polymorphisms of the 5-LO pathway, which predispose to CVD [77], [134], [27], [93]. The pathological implication of LTs in atherosclerosis, aortic aneurysm and myocardial infarction has been confirmed in both animal models and tissue samples from patients [354], [240], [147], [104].

These studies imply that LT receptor antagonists or enzyme inhibitors should be explored as therapeutic options in the treatment and prevention of cancer and CVD [249], [21].

3.5 Endogenous suppressors of LT biosynthesis

As already adumbrated above, the biosynthesis by LTs can be controlled by endogenous compounds, such as PGE_2, which has long been recognized as a suppressor of leukocyte LT generation [122]. Further endogenous substances that can downregulate LT release are NO [44] as well as the autacoids adenosine and histamine [88], [90], which inhibited LTB_4 synthesis in macrophages and neutrophils, respectively. Most of these suppressive actions can be attributed to a receptor mediated elevation of the intracellular cAMP concentration, leading to subsequent activation of PKA. As described under 3.3.2, the activation of PKA results in reduced 5-LO translocation and product formation [91].

By contrast, distinct mechanisms were responsible for the inhibitory effects of sulphatides, endogenous selectin ligands, which prevented the translocation and activation of 5-LO in PMNL by causing rearrangement and redistribution of membrane lipids [301], [114].

Considering the therapeutic benefit of cortisol derivatives in the therapy of asthma, it is not surprising that also hydrocortisone (Cortisol) is an endogenous suppressor of LT biosynthesis [358], [283]. The inhibitory potential of hydrocortisone can most likely be attributed to its counteracting effect on $cPLA_2$ activation in response to various inflammatory stimuli [111].

3.6 Eicosanoids in antigen presenting cell biology

Professional antigen presenting cells (APCs) comprise dendritic cells (DCs), macrophages, B-cells and as recognized more recently and with some limitations also basophils [94], [289]. The common feature of professional APCs is their capacity to present antigens via MHC class II molecules and to activate naive T-cells. However, full T-cell activation requires antigen presentation in the presence of innate immune activation motives

such as MAMPs (see table 3.6). MAMPs induce APC maturation, expression of co-stimulatory molecules and cytokine secretion [142]. The specific cytokine profile of the activated APC results in the initiation of distinct types of adaptive immune responses, i.e. activation of regulatory T-cells, T_H2, T_H1 or T_H17 cells.

Thus, DCs and macrophages are of particular importance for the connection between innate and adaptive immunity. As sentinels, they sample their environment for foreign pathogens, which they phagocytose and process. In the following phases of their life cycle, APCs downregulate their capacity for antigen uptake, while at the same time up-regulating co-stimulatory molecules and molecules for antigen presentation [112]. Hence, proper APC function requires the successive induction of factors that facilitate antigen capture and processing, migration to lymph nodes, antigen presentation to T-cells and finally APC apoptosis [112].

This complex life cycle is controlled by cytokines and chemokines such as $TNF\alpha$, IL-4, IL-10 and CCL19 [20], [149]). Although often neglected, also eicosanoids can influence APC maturation, migration, antigen presentation and apoptosis [128]. Thereby, the prostaglandins E_2 and D_2 modify cytokine secretion as well as migratory and T-cell stimulatory properties of DCs [172], [152], [80], [10], [205], [314], [123]. The effects of PGD_2 and PGE_2 may differ considerably depending on the prostaglandin receptor sub-type, which is predominantly engaged [185]. Hence, PGs can shift the balance between regulatory T-cells, T_H2, T_H1 or T_H17 cells in a tissue specific manner [158], [33]. Compared to the complexity of PG mediated events, the effects of LTs on APC function are less diverse. Both cysLTs and LTB_4 act as stimulatory factors, which can trigger activation, migration and maturation of DCs [132], [215], [341], [74], [284], [70]. However, the magnitude of the LT triggered response may depend on the presence of other maturation stimuli such as TLR ligands, which can differentially modulate LT receptor expression on APCs [316].

In addition to their role as target cells of PGs and LTs, macrophages and DCs have long been recognized as potent eicosanoid producing cells, which can synthesize products of the cyclooxygenase and lipoxygenase pathways [85], [34], [167], [256]. It should be noted that the quantity and quality of the lipid mediator output of the different macrophage and DC subtypes is determined by their tissue localization and species origin. For example, alveolar macrophages (AMs) are considerably more efficient LTB_4 producers as compared to blood monocytes or peritoneal macrophages [29], [167].

Furthermore, LT release in response to diverse stimuli such as GM-CSF or house dust mite allergens differs clearly between macrophage and DC subsets [42], [18]. Many of the species and tissue specific factors, which may influence eicosanoid biosynthesis and signaling in APCs, still remain to be elucidated [186], [103]. Thus, evidence that has been obtained from studies of certain APC subtypes, cannot simply be extrapolated to other APCs.

One well established model system for human APCs are monocyte derived macrophages (MDMs) and dendritic cells (MDDCs), generated by one week culture of CD14$^+$ blood mononuclear cells in the presence of GM-CSF or GM-CSF plus IL-4, respectively [260] (Fig. 3.11). MDDCs represent an immature DC subset with high capacities for antigen capture and processing, but with very low endogenous eicosanoid synthesis due to suppression of cPLA$_2$ by IL-4 [351]. However, both MDDCs and MDMs can convert

exogenous AA to a variety of eicosanoids, including HETEs, prostanoids and LTs. Characterization of the MDDC lipoxygenase product profile revealed a clear predominance of the 15-LO *versus* the 5-LO pathway [292]. Interestingly, TGFβ1 treated MDDCs, resembling epidermal Langerhans cells (LCs), exhibited a clearly increased 5-LO activity, which is in accordance with the selective expression of 5-LO in skin LCs [293], [74].
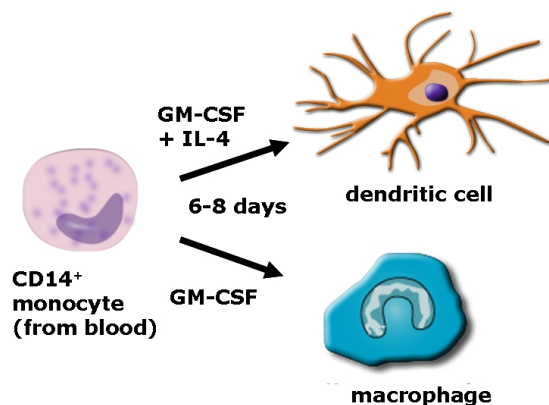

Figure 3.11: *Generation of monocyte derived macrophages and dendritic cells*

3.6.1 APCs and pro-resolving lipid mediators

APCs do not only participate in the initiation phase of the inflammatory response but also in the subsequent resolution steps, which require lipid mediator class switching from leukotrienes and prostaglandins to lipoxins and anti-inflammatory derivatives of ω-3 fatty acids [275], [176], [295] (Fig. 3.12). The non-phlogistic phagocytosis of apoptotic neutrophils by macrophages is a crucial event of the resolution phase, which could be promoted by lipoxins [107] and was accompanied by PGE_2 mediated inhibition of pro-inflammatory cytokine release [81]. As to DC function, lipoxin A_4 (LXA_4) suppressed IL-12 secretion and LPS induced maturation [8], [353].

Another class of anti-inflammatory lipid mediators with regulatory roles for APC function are the resolvins, which are synthesized from the ω-3 fatty acids eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA). Similar to LXA_4, the EPA derived resolvin E_1 (RvE_1) (Fig. 3.13) inhibited neutrophil infiltration and increased the uptake of apoptotic neutrophils by macrophages [269], [274], [213].

The anti-inflammatory properties of ω-3 fatty acid derivatives provide a biochemical basis for some of the beneficial effects of a high nutritional intake of ω-3 fatty acids, particularly in patients with chronic inflammatory diseases such as rheumatoid arthritis and bronchial asthma [99], [311].

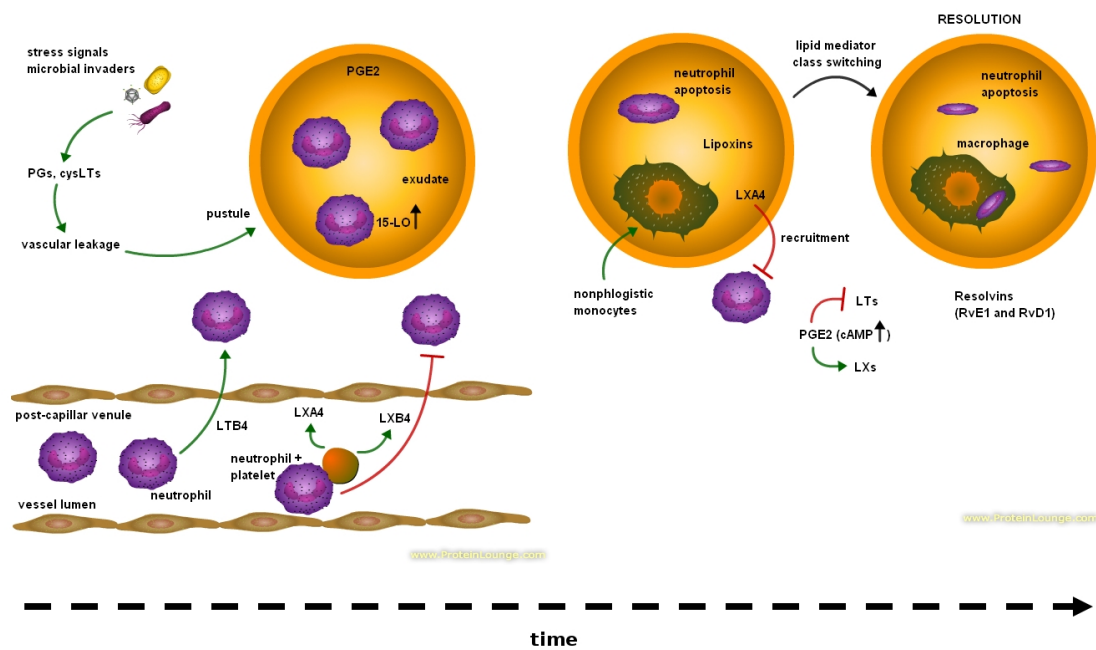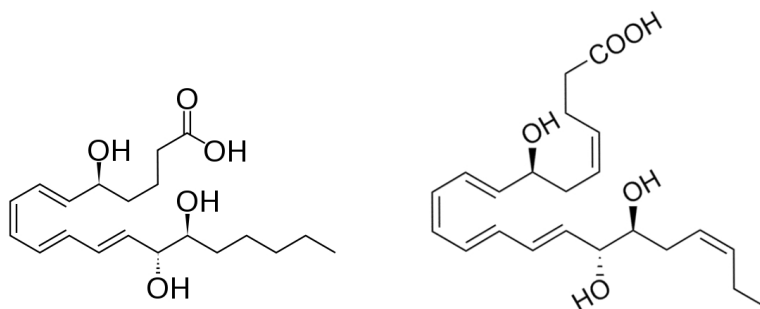Figure 3.12: *Lipid mediator class switching during the course of inflammation* [275]

Figure 3.13: *Chemical structures of lipoxin B$_4$ and resolvin D$_1$* [274]

3.7 Exosomes - generation and immunological roles

As discussed above, APCs are efficient producers of soluble mediators with diverse effects on the immune system. However, in recent years, another form of communication between APCs and their target cells has become increasingly recognized: Like many other cells, APCs secrete small membrane vesicles, so called exosomes, which are 40 to 100 nm in diameter. Exosomes contain a specific set of molecules, many of which are related to immunological functions (Fig. 3.14) [319], [59].
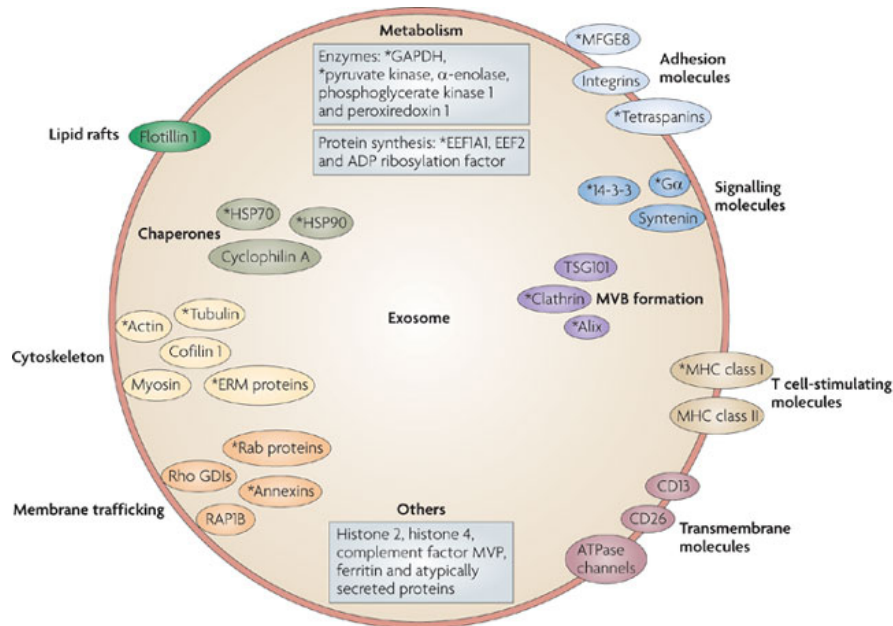

Figure 3.14: *Typical protein composition of exosomes* [319]

The protein and lipid composition of exosomes can be partially distinct from that of the parent cells, which is due to particular sorting events during exosome formation [79], [299]. Exosome biogenesis involves inward budding of late endosomal compartments, resulting in formation of multivesicular bodies (MVBs), which fuse with the plasma membrane to release the contained vesicles into the microenvironment [286], [184] (Fig. 3.15).

Exosomes can be isolated from body fluids or cell culture supernatants by ultra-centrifugation at 100,000 x g, often combined with a sucrose gradient, which yields highly purified exosomes [184]. The isolated vesicles can be visualized by electron microscopy, where they appear as cup-formed structures (Fig. 3.15).

Exosomes are stable in the circulation [310] and can long after their secretion be found at sites distinct from their origin. Moreover, the abundance of exosomes in various body fluids strongly suggests that exosome release is an important phenomenon, which occurs *in vivo* [52]. The presence of exosomes in body fluids was first described for malignant effusions from melanoma patients [11] and many tumor cells secrete massive amounts of these vesicles [267], [5]. Notwithstanding, exosomes can be secreted by most cell types.
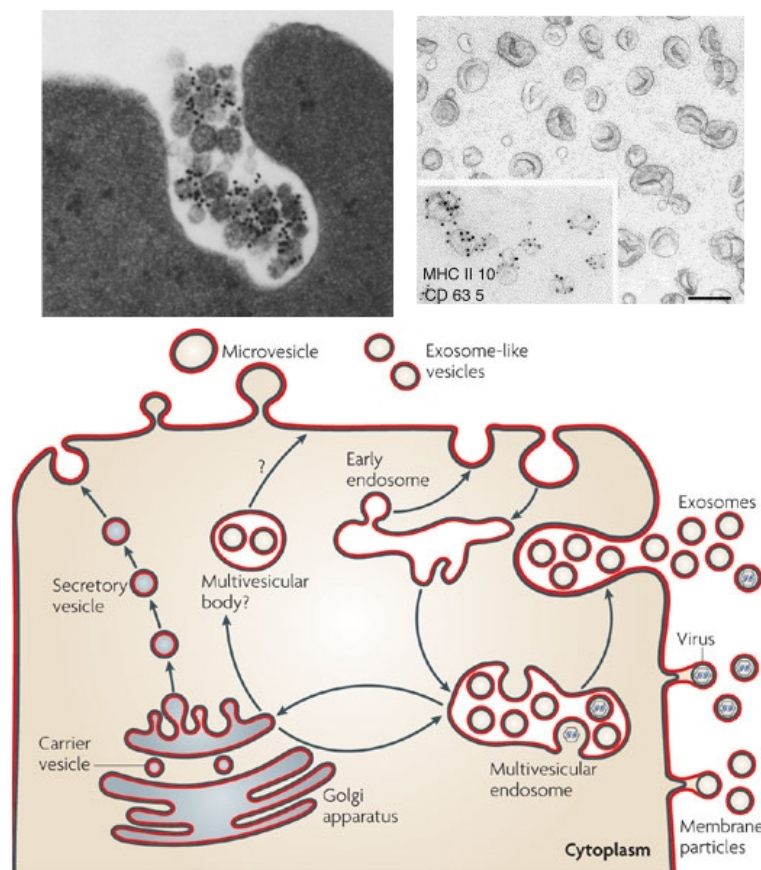
Figure 3.15: *Biogenesis of exosomes* - electron micrographs of exosomes during secretion (top, left) [222] and after purification (top, right) scale bar: 100 nm [97]; schematic representation of vesicle and exosome generation [319]

Up to date, it is still controversial if exosome release is mainly constitutive or if specific mechanisms regulate exosome output e.g. in response to danger signals or microbial stimuli. The latter conception is supported by the observation that exosome secretion by DCs or macrophages was increased by cognate T cell-DC interactions or by ATP and infection with *Mycobacterium tuberculosis*, respectively [45], [241], [247]. Intriguingly, exosome levels were clearly elevated in bronchoalveolar lavage fluid (BALF) from patients with the inflammatory lung disease sarcoidosis [236]. These findings suggest that exosome biogenesis and/ or release can be induced by pro-inflammatory factors.

Studies on APC derived exosomes have shown that the surface expression of co-stimulatory molecules and molecules for antigen presentation depends on the phenotype of the parent APC [150]. Antigen-loaded APC derived exosomes can transfer antigens to naive APCs or directly elicit T-cell responses [357], [320], [237], [319] (Fig. 3.16). Furthermore, exosomes, released by macrophages infected with intracellular pathogens, carried MAMPs and triggered pro-inflammatory cytokine secretion in naive bystander macrophages [28], [218].

Information about possible connections between lipid mediators and exosome biology is
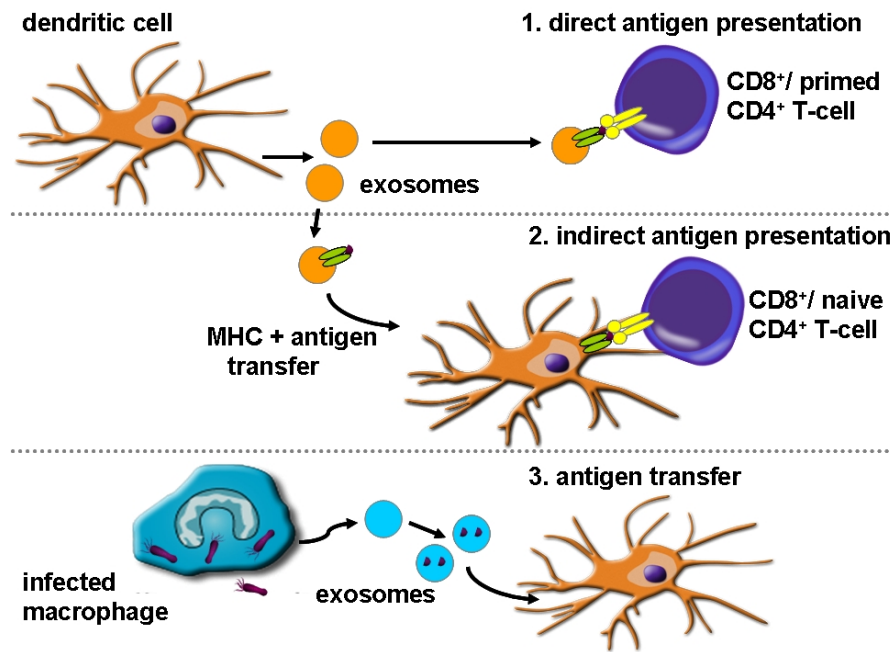
Figure 3.16: *Stimulatory immune functions of APC derived exosomes* [319]

very limited. Enrichment of phospholipases (PLs) in reticulocyte exosomes as well as an involvement of PLs and 15-LO in the exosome formation process have been reported [25], [166], [31]. However, only two studies have indicated that exosomes are carriers of bioactive eicosanoids: both exosomes from myeloid-derived suppressor cells (MDSCs) and from the basophil/ mast cell line RBL-2H3 were found to contain prostaglandins [342], [298]. The immuno-modulatory potential of exosomes as well as their high content of active PLAs and free fatty acids [298] suggest that they might be involved in lipid mediator biosynthesis.

Recent findings have demonstrated that nanovesicles are also carriers of LT receptors and might thus respond to LTs e.g. by releasing their content. Interestingly, cysLTs could trigger the secretion of pro-inflammatory factors from cell-free human eosinophil granules [208]. Taken together, these novel data allow speculations about previously unrecognized immunological settings, which are solely vesicle based.

4 Aims

The raising incidence of chronic inflammatory disease, seen today, demands a better understanding of how pro- and anti-inflammatory mediator production is regulated. It was the aim of this thesis to contribute some new pieces to the incomplete puzzle of the vast and complex network that controls inflammation.

The key roles of LTs as pro-inflammatory mediators in host defense and chronic inflammation motivated our studies on the regulation of LT biosynthesis on a molecular and cellular level as well as in completely new settings:

The objective of the first study, included in this thesis, was to characterize the role of coactosin like protein (CLP) for 5-LO activity [246] in more detail. Thereby, we focused on the individual impact of three Trp residues in the regulatory domain of 5-LO, which had previously been reported to confer membrane binding [164] as well as interaction with CLP [246]. As purified 5-LO is highly instable and rapidly inactivated upon exposure to oxygen or high temperatures, we also investigated a possible stabilizing effect of CLP.

The main purpose of the second study was to investigate potential long term effects of microbial stimulation on the LT output of macrophages and dendritic cells. Particularly, we aimed at investigating the regulation of the downstream enzymes for LT biosynthesis (LTA_4H and LTC_4S) during prolonged exposure to the fungal cell wall preparation zymosan. The identification of the key receptors and signaling pathways, involved in the observed responses, was another central goal of this study.

As the main objective of the last two studies, we analyzed exosomes from antigen presenting cells, blood plasma and brochoalveolar lavage fluid (BALF) regarding their unknown content of enzymes for LT biosynthesis.

A special focus of the first study on LT biosynthesis in exosomes was the comparison between the exosomal and cellular capacity for conversion of LTA_4 to the LTs B_4 and C_4. Furthermore, we were interested in the impact of $TGF\beta1$, previously reported to have major effects on lipoxygenase pathways in APCs [292], on the LT output of macrophages, DCs and their exosomes. Finally, we wanted to investigate potential roles of APC derived exosomes in transcellular LT metabolism and granulocyte trafficking.

The main purpose of our second study involving exosomes was the comparison of BALF exosomes from asthmatics and healthy individuals regarding possible differences in their content of pro-inflammatory molecules and in their capacity to trigger mediator release from bronchial epithelial cells. Moreover, we planned to assess potential changes in the characteristics of BALF exosomes from asthmatics before and after allergen challenge.

5 Materials and methods

5.1 Materials

Information about all materials that have been used during the experimental work for this thesis can be found in the "Materials and Methods" sections of the individual papers.

5.2 Methodology

The following methods were used to perform the studies described in this thesis:

1. High performance liquid chromatography (HPLC) (studies I, II, III, IV)

2. Cell isolation from blood and Buffy Coats (monocytes, PMNL) (studies II, III)

3. Cell culture (MM6 cells, MDMs, MDDCs, 16HB14o cells) (studies I, II, III, IV)

4. Exosome isolation from culture supernatants and body fluids (studies III, IV)

5. Flow cytometry (studies III, IV)

6. Cell incubations and solid phase extraction of lipid mediators (studies II, III, IV)

7. SDS polyacrylamide gel electrophoresis and Western blot (studies I, II, III, IV)

8. (Quantitative real time) polymerase chain reaction ((qRT)-PCR) (studies I, III)

9. Enzyme (linked) immuno(sorbent) assay (E(L)I(S)A) (studies II, IV)

10. *In vitro* chemotaxis assay (study III)

11. Electron microscopy (study III)

12. Expression and purification of recombinant proteins (studies I, II)

13. *In vitro* phosphorylation assay (study II)

For detailed descriptions of the experimental procedures, please refer to the "Materials and Methods" sections of the individual papers.

6 Results

6.1 Coactosin like protein functions as a chaperone for 5-LO

In the first study, we demonstrate that CLP can prevent the non-turnover related inactivation of 5-LO and protect the enzyme against heat inactivation (Fig.4, paper I). Further support for the stabilizing influence of CLP on 5-LO comes from the finding that degradation of 5-LO by the protease thermolysin was delayed in the presence of CLP (Fig. 7A, paper I).

6.1.1 Importance of 5-LO-Trp^{102} and CLP-Lys^{131} for the CLP-5-LO interaction

Previous studies had suggested a role of three tryptophan residues (Trp13, 75 and 102) in the regulatory domain of 5-LO for the interaction with CLP [246]. By separate mutagenesis of each of these tryptophans (replacement by alanine) we were able to identify 5-LO-Trp^{102} as the crucial residue for the activating and stabilizing effects of CLP (Figs. 1 and 5, paper I). Furthermore, we show that a lysine residue in CLP (Lys131), previously shown to be necessary for promotion of 5-LO product formation, is also essential for the stabilizing interaction between 5-LO and CLP (Fig. 6, paper I).

GST-pull down assays, performed to analyze the physical interaction of CLP with wt-5-LO, 5-LO-W13A, 5-LO-W75A and 5-LO-W102A, demonstrated that mutation of Trp102 abrogated binding of 5-LO to CLP (Fig. 3, paper I).

Computational docking led to the suggestion of a model for the CLP-5-LO interaction, which is based on the assumption that CLP and 5-LO are arranged in such a way that CLP-Lys^{131} and 5-LO-Trp^{102} are directed towards the respective partner protein (Fig. 6.1 or Fig. 9, paper I).

In such a complex, CLP-Lys^{131} and 5-LO-Trp^{102} could engage in a network, consisting of hydrogen bonds (black dashed lines) and cation-π interactions (red dashed lines) with 5-LO-Phe^{14} and 5-LO-Arg^{165} (Fig. 6.1, right panel).

6.1.2 CLP translocates together with 5-LO in MM6 cells

Upon stimulation of cells of the monocytic cell line MM6 with phorbol esters and the Ca^{2+} ionophore A23187, 5-LO translocates to the nuclear membrane, resulting in an increased capacity for LT biosynthesis [338]. In our study, we observed the same pattern of redistribution for CLP as for 5-LO: In undifferentiated MM6 cells, which are 5-LO negative, as well as in unstimulated differentiated (5-LO positive) cells, the major portion of CLP was found in the cytosolic fraction (Fig. 8, paper I). However, in differentiated
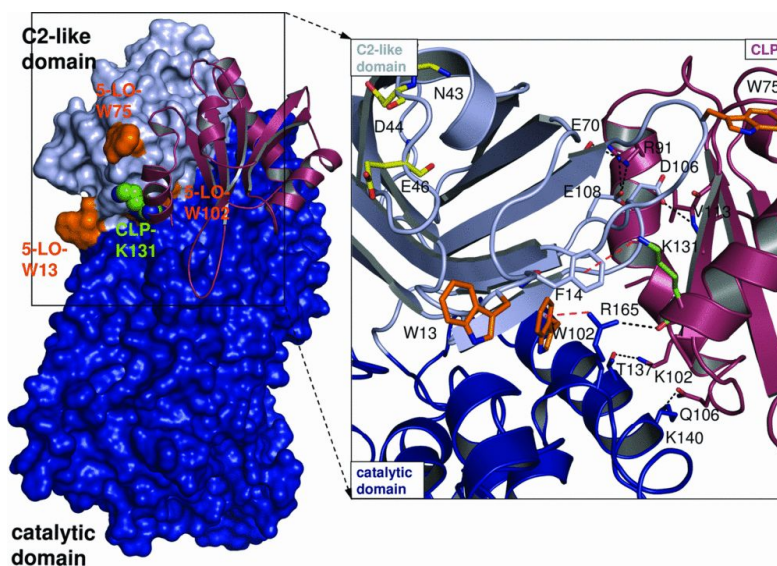
Figure 6.1: *Model complex of the interaction between 5-LO and CLP* (the graphic was prepared by Bettina Hofman, University of Frankfurt)

MM6 cells stimulation with phorbol ester and ionophore triggered the translocation of 5-LO to the nucleus, which was accompanied by increased levels of CLP protein in the nuclear fraction (Fig. 8B, paper I).

In summary, the results from this study support the hypothesis that CLP is a chaperoning scaffold factor for 5-LO, which may promote cellular LT biosynthesis.

6.2 Long-term zymosan stimulation suppresses LTC$_4$S activity

6.2.1 LTC$_4$S activity is upregulated during differentiation of MM6 cells

Already in the early nineties, several groups showed that cellular LTC$_4$S activity can be downregulated by the protein kinase C activator PMA in HL-60 cells, granulocytes and platelets, indicating phosphoregulation of LTC$_4$S [153], [7], [287], [326]. In a later study, Gupta et al. used [^{32}P]orthophosphate labeling followed by immunoprecipitation and autoradiography to obtain direct evidence for LTC$_4$S phosphorylation in whole cells [115]. In the same report, the authors demonstrated that phosphorylation of LTC$_4$S in response to PMA occurs in the human monocytic cell line THP-1.

In line with these studies, we found that a short pre-treatment with the pan-kinase inhibitor staurosporine upregulated LTC$_4$S activity in undifferentiated MM6 cells, which otherwise had a very low capacity for conversion of LTA$_4$ to LTC$_4$ (Fig. 2A, paper II). This observation provides further support for phosphoregulation of LTC$_4$S in monocytic cells.

TGFβ1 and VD$_3$ induced differentiation generated MM6 cells with a high capacity for conversion of LTA$_4$ to LTC$_4$ (Fig. 1A, paper II). In these cells, pre-incubation with staurosporine did not further increase the conversion of LTA$_4$ to LTC$_4$, suggesting that

the major part of LTC$_4$S was un-phosphorylated and fully active after differentiation in the presence of TGFβ1 and VD$_3$.

Even if less potently than staurosporine, also rapamycin, an inhibitor of the mTOR/p70 ribosomal S6 kinase (p70S6K) pathway, upregulated LTC$_4$S activity in undifferentiated MM6 cells (Fig. 2A, paper II). Furthermore, as previously shown for HL-60 cells [224], the phosphorylation and thus activity of p70S6K was time dependently decreased during differentiation of MM6 cells with TGFβ1 and VD$_3$ (Fig. 2B, paper II). By *in vitro* phosphorylation experiments, we demonstrated, that p70S6K can directly phosphorylate purified LTC$_4$S (Fig. 2C, paper II), thus identifying the mTOR/p70S6K pathway as a novel player in the regulation of LT biosynthesis. Finally, preliminary data from our group indicate that Resolvin E$_1$, which activates p70S6K [213], can attenuate LTC$_4$ formation in MM6 cells (unpublished data).

6.2.2 Prolonged zymosan exposure triggers inhibitory phosphorylation of LTC$_4$S

One hallmark of zymosan induced acute peritonitis, a commonly used *in vivo* model of acute inflammation, is the pronounced release of cysLTs, which can mostly be attributed to macrophages [162], [350]. Several groups have studied the receptors and signaling pathways involved in the acute (15-60 min) induction of LT biosynthesis in response to zymosan [217], [216], [331]. By contrast, we were interested in potential long-term effects of zymosan on the LT output of human monocytic cells.

The initial key finding in this study was the potent suppression of the LTC$_4$S activity after 48-96 hrs differentiation in the presence of zymosan, which coincided with a distinct upregulation of PGE$_2$ release (Figs. 1A, 1D, 3A, paper II). Interestingly, the pan-kinase inhibitor staurosporine and the PKA inhibitor H-89 partially restored the cellular capacity for LTC$_4$ biosynthesis (Figs. 1E, 2D, paper II). These results suggest that long term stimulation with zymosan triggers events, which lead to the inhibitory phosphorylation of LTC$_4$S.

Thereby, it appears that different kinases can contribute to phosphoregulation of LTC$_4$S in MM6 cells. While, in undifferentiated cells, p70S6K seems to play a role, PKA may mediate an important part of the suppressive zymosan effect in differentiating cells.

6.2.3 TLR2 mediates the zymosan induced downregulation of LTC$_4$S

Zymosan particles are prepared from the yeast cell wall (Fig. 3.9) and thus contain a mixture of α-mannans, β-glucans, glycolipids and lipopeptides. Two major receptors are implicated in the cellular responses to zymosan in macrophages and DCs: TLR2 and the β-glucan receptor (called Dectin-1 in mouse). These two receptors collaboratively trigger various acute inflammatory events [110]. However, the β-glucan receptor is the primary receptor for phagocytosis of zymosan as well as for induction of ROS production and NFAT activation [110]. In contrast, TLR2, which is recruited to zymosan-containing phagosomes [329], mediates activation of NFκB [100].

To identify the zymosan receptor upstream of the phosphorylation events for suppression of cysLT generation, we differentiated MM6 cells in the presence of a zymosan preparation ("depleted zymosan"), which binds to the β-glucan receptor, but not to TLR2. In both MM6 cells and MDDCs, a prolonged exposure to depleted zymosan did not affect the capacity to convert LTA$_4$ to LTC$_4$ (Fig. 4, paper II (data not shown for MDDCs)). In addition, in MM6 cells differentiated in the presence of the selective TLR2 agonist Pam2CSK4 or in the presence of zymosan together with the β-glucan receptor antagonist laminarin, LTC$_4$S activity was downregulated to a similar extent as observed with zymosan (Fig. 4, paper II). These findings indicate that the reduction of LTC$_4$ formation in response to zymosan depends on TLR2 rather than on the β-glucan receptor.

Long term exposure to α-mannan, a ligand of the mannose receptor (MR) and a constituent of zymosan (and the fungal cell wall (Fig. 3.9)), resulted in a weak but significant downregulation of the LTC$_4$S activity (Fig. 4, paper II). This suggests that TLR2- and MR-signaling might cooperate to induce the regulatory mechanisms for control of cysLT biosynthesis.

6.2.4 Zymosan triggered PGE$_2$ release leads to inhibition of LTC$_4$S

The prominent increase in PGE$_2$ release after 48-96 hrs of zymosan exposure (Fig. 3A, paper II) and the induction of COX-2 and mPGES-1 expression (Fig. 3B, paper II) correlated with the reduction of LTC$_4$S activity in the same time frame (Fig. 1A, paper II). Thus, we tested whether the non-selective COX inhibitor acetylsalicylic acid (ASA) or the COX-2 selective inhibitor etoricoxib might have an effect on the cysLT generation in zymosan treated MM6 cells. We also investigated a potential impact of exogenous PGE$_2$.

Interestingly, ASA counteracted the zymosan induced suppression of LTC$_4$S activity very efficiently, while etoricoxib had a more moderate effect (Fig. 3C, top panel, paper II). Furthermore, PGE$_2$ (100 nM, 24 h) significantly reduced LTC$_4$ generation from LTA$_4$ (Fig. 3D, paper II). These findings strongly implicate PGE$_2$ in the regulation of the LTC$_4$ output in human monocytic cells.

As discussed in paper II, we hypothesize a novel mechanism for control of cysLT biosynthesis during monocyte differentiation and in later phases of microbe triggered inflammatory responses (Fig. 6.2 or Fig. 5, paper II). We propose that this mechanism could constitute a component of the resolution phase and provide a biochemical explanation for the exaggerated cysLT release in conjunction with aspirin hypersensitivity.
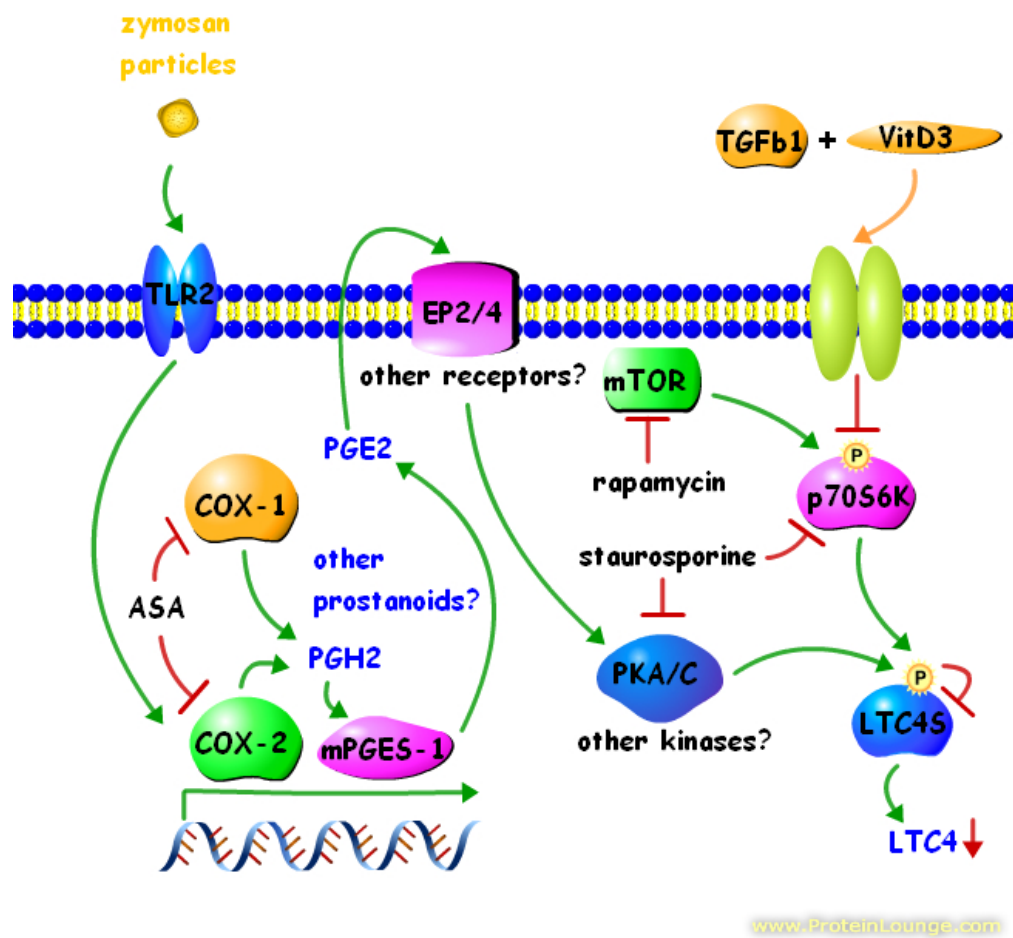
Figure 6.2: *Mechanisms for control of LTC$_4$ biosynthesis in monocytic cells*

6.3 Exosomes are novel players in LT biosynthesis

As membrane vesicles with immuno-modulatory functions [321] and a high content of polyunsaturated fatty acids (PUFAs) [298], exosomes appear to be well suited vehicles for lipid mediator synthesis. Hence, we were interested in exosomes as potential platforms for LT biosynthesis.

6.3.1 Exosomes from macrophages and DCs contain LTA_4H and LTC_4S

Initially, we investigated whether exosomes from human monocyte derived APCs (MDMs and MDDCs) contain proteins of the LT pathway and whether they are capable of converting LTA_4 to the bioactive LTs.

By using Western blotting, HPLC based activity assays and immuno-electron microscopy, we demonstrated that APC derived exosomes carry active LTC_4S and LTA_4H (Figs. 2A, B, 3, 4B, paper III). Thereby, exosomes showed an enrichment of LTC_4S protein and activity as compared to their parent cells (Figs. 2A, 4A, B, paper III).

We also studied the influence of TGFβ1, an important modulator of monocyte differentiation, on the LT output of cells and exosomes. Interestingly, TGFβ1 upregulated the cellular capacity for LT biosynthesis, but downregulated exosome secretion, particularly in MDMs (Figs. 4A, E2B, 1C, paper III). In earlier studies, TGFβ1 (together with VD_3) was shown to upregulate 5-LO expression in cells of the monocytic lineage [296], [292]. Here, we found that TGFβ1 also increased the expression of LTA_4H in monocytic cells (Fig. E1, paper III). In addition, LTA_4H protein levels and activities were higher in exosomes from TGFβ1-MDMs and TGFβ1-MDDCs as compared to their "non-TGFβ1" counterparts (Figs. 2B, 4B, paper III).

The high capacity of exosomes for conversion of LTA_4 motivated us to perform co-incubations of exosomes with PMNL, which are a rich endogenous source of LTA_4. Indeed, in AA and ionophore (A23187) incubations, PMNL that were co-incubated with exosomes, synthesized larger amounts of LTs as compared to PMNL alone (Fig. 4C, paper III). Exosomes alone did not convert exogenous AA to detectable levels of the LTs B_4 or C_4. However, in an LC-MS/MS analysis of extracts from AA and A23187 incubated MDM exosomes, we were able to detect a variety of AA metabolites, such as 5- and 15-HETE, 5- and 15-KETE as well as 20-carboxy-LTB_4 (table 1, paper III).

6.3.2 Exosomes promote granulocyte migration in vitro

Since several of the exosomal AA metabolites have chemotactic potential, we performed an *in vitro* migration assay with exosomes and PMNL. In this assay, we observed that exosomes could induce migration of PMNL, which was slightly increased in the presence of AA and A23187 (Fig. 5, paper III).

In summary, this study identifies exosomes as novel players in LT biosynthesis and leukocyte trafficking. Hence, exosomes might contribute to eicosanoid generation in inflammatory settings at sites remote from their parent cell.
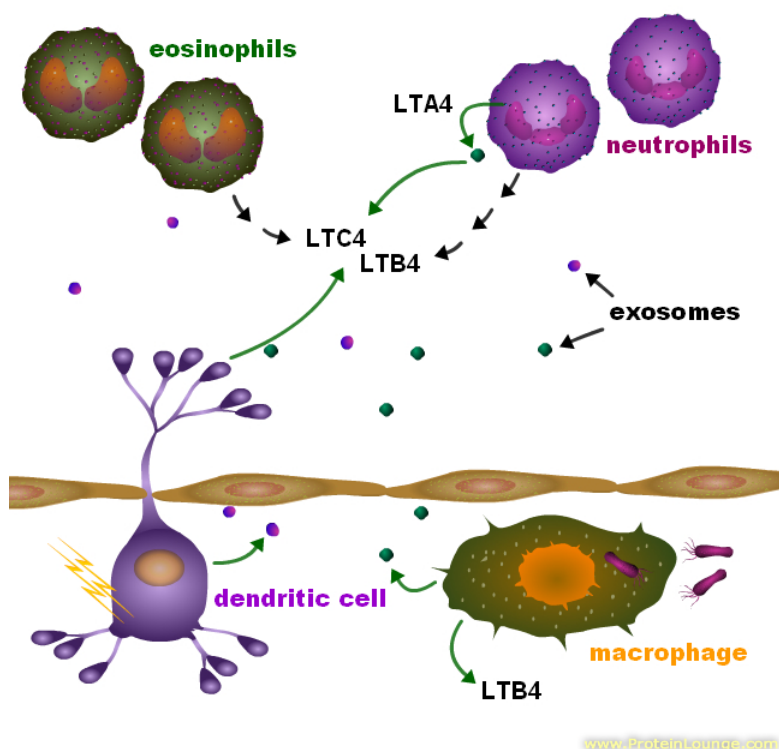
Figure 6.3: *Exosomes as amplifiers of LT biosynthesis and granulocyte chemotaxis*; green
arrows: synthetic capacity, multiple black arrows: chemotactic potential

6.3.3 Exosomes from BALF and plasma carry proteins for LT biosynthesis

To obtain a first insight into a potential *in vivo* relevance of exosome associated LT
synthesis, we performed Western Blot analysis and LTA_4 incubations of exosomes from
human plasma and bronchoalveolar lavage fluid (BALF) obtained from healthy individ-
uals. Western blots and activity assays revealed the abundance of active LTA_4H and
LTC_4S in exosomes from plasma (Fig. 6, paper III) and BALF (Fig. 4, paper IV).

Comparison of the LT biosynthetic capacities of exosomes from plasma and BALF indi-
cated that exosomes isolated from the lung were considerably more potent LT producers
(Figs. 6, paper III, 4B, paper IV). Interestingly, BALF exosomes had similar capacities
for conversion of LTA_4 as APC exosomes (Figs. 4B, paper III, 4B, paper IV), but they
displayed higher LTA_4H and lower LTC_4S activities.

6.4 Asthmatics display an altered profile of BALF exosomes capable of LT metabolism and induction of IL-8

The presence of LT biosynthetic enzymes in body fluid exosomes from healthy indi-
viduals sparked our interest in investigating exosomes from BALF of asthmatics. We
obtained BALF samples from a clinical study with mild birch pollen allergic asthmat-
ics, who underwent allergen challenge. Samples from the same patients were analyzed

in a previous study, which compared lymphocyte populations and cytokine levels in BALF from healthy controls and asthmatics before and 24 hrs after allergen challenge [323]. In accordance with earlier reports, T_H2 cytokine levels were found to be higher in BALF from asthmatics as compared to healthy controls and levels of IL-5, IL-6 and IL-9 further increased after allergen provocation. Moreover, characterization of BALF lymphocyte populations indicated increased numbers of activated T-cells, particularly regulatory T-cells, in BALF from allergen provoked asthmatics [323].

In our study, we assessed innate inflammatory cell populations (eosinophils, macrophages and mast cells) in BALF from healthy controls and asthmatics before and after birch pollen allergen exposure. We observed a significantly elevated eosinophil count after allergen challenge and a tendency towards increased mast cell numbers in asthmatics even at steady-state (Figs. 1A, C, paper IV). Interestingly, allergen provocation also resulted in a higher exosomal protein content in BALF (Fig. 1E).

6.4.1 BALF exosomes from asthmatics exhibit pro-inflammatory phenotypic characteristics

Since nothing was known about the phenotypic characteristics of BALF exosomes from asthmatic patients, we analyzed exosomal surface markers by flow cytometry. The tetraspanins CD63 and CD81 are classical exosome markers as they are enriched in late endosomes and multivesicular bodies, which are the cellular compartments for exosome biogenesis [322]. Characterization of BALF exosome surface marker expression showed increased levels of CD63 and CD81 on exosomes from asthmatics as compared to those from healthy controls (Figs. 2A, B paper IV). CD63 and CD81 are involved in processes like leukocyte activation and adhesion [170], [231] and CD63 is a marker of basophil activation, commonly used in allergy diagnostics [161]. Thus, BALF exosomes from asthmatics appear to carry a molecular signature, which reflects the inflammatory status of the lung.

6.4.2 BALF exosomes increase pro-inflammatory mediator release from BECs

In order to identify potential functional roles of exosomes in the lung, we used bronchial epithelial cells (BECs) as a model system. More specifically, we incubated BECs with BALF exosomes from healthy individuals and asthmatics and analyzed pro-inflammatory cytokine secretion.

Co-incubation of BECs with BALF exosomes from asthmatics resulted in a significantly increased secretion of IL-8 and cysLTs as compared to incubation with exosomes from healthy individuals (Figs. 6A, 5B, paper IV). Furthermore, in presence of the $CysLT_1$ receptor antagonist montelukast, BECs secreted less IL-8 (Fig. 7A, paper IV). This difference was particularly distinct for those exosomes from asthmatics, which elicited a clear increase in IL-8 release (Figs. 6A, 7A, paper IV).

Interestingly, the induction of IL-8 and cysLT secretion was most pronounced for BALF-exosomes from the patient that suffered the most severe response after allergen provocation (Figs. 5B, 6A, table 1, paper IV). The same patient displayed the highest inflam-

matory cell counts as well as exosomal protein content per BALF volume (Fig. 1, paper
IV).

In conclusion, our data suggest that in the asthmatic lung, exosomes may promote
pro-inflammatory mediator release by epithelial cells, thereby contributing to airway
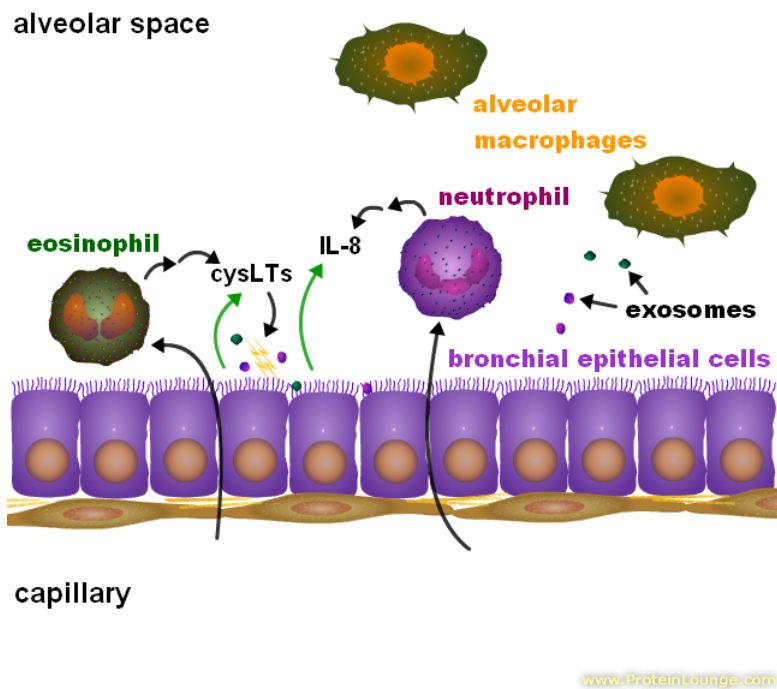inflammation.

Figure 6.4: *Suggested role of exosomes as promoters of pulmonary inflammation*; green
arrows: synthetic capacity, black arrows: chemotaxis

7 Discussion

The following chapter will discuss some important implications, limitations and open questions related to the studies described in this thesis.

7.1 Insights into the chaperone function of CLP for 5-LO

An attractive explanation for the protective effect of CLP against proteolysis (Fig. 7A, paper I) is that by binding to 5-LO, CLP renders the 5-LO structure more compact, and consequently bulky hydrophobic residues, preferred cleavage sites of thermolysin [133], may become less accessible. In soybean lipoxygenase-1, hydrophobic interactions contribute to association of the two domains [300] and hydrophobic residues are present between the two domains in 5-LO model structures [9]. The importance of 5-LO-W102 suggests that CLP binds at or near the domain interface of 5-LO. Possibly, CLP prevents access of thermolysin to a target site on hydrophobic interdomain areas of 5-LO.

The reliability of our model structure for the interaction between 5-LO and CLP (Fig. 9, paper I) is limited by the lack of an X-ray structure of 5-LO at the time, when our modeling was performed. It should be noted that also other binding modes could be envisioned: an intriguing possibility seems to be the CLP induced formation of a 5-LO dimer, which is more stable than the monomer (suggested by Prof. Elias Arnér, MBB, KI). In this case, Trp102 could be the essential residue for dimerization of 5-LO. This alternative is particularly appealing since dimeric structures have been described for other lipoxygenases [6] and Trp residues are often involved in the stabilizing stacking interactions in dimeric enzymes [13].

Our data suggest that CLP might have regulatory roles for 5-LO, similar to those, described for scaffolding proteins in the regulation of nitric oxide synthases [163]. Potentially, CLP is particularly relevant for 5-LO product formation in the cytosol [338], which occurs remote from the nuclear membrane protein FLAP. FLAP was shown to function as a 5-LO scaffold protein, which associates with 5-LO in IgE stimulated mast cells as well as in primary neutrophils from inflamed joints [180]. A potential interplay of CLP and FLAP in the support of 5-LO activity at the nuclear membrane remains to be elucidated.

Until now, all data that support the function of CLP as a 5-LO activity promoting chaperone have been obtained *in vitro*. Thus, the *in vivo* relevance of CLP for 5-LO product formation and stability remains to be shown. Knock-down strategies like genetic ablation of CLP could be applied to address this issue first in cell lines and ultimately in animal models.

7.2 Regulation of LT biosynthesis in human monocytic cells

When using cell lines or in *in vitro* generated cells, as it was the case in our studies, one should bear in mind that the obtained results might not entirely reflect the *in vivo* situation. As extensively discussed by Geissmann et al., *in vitro* generated macrophages and DCs are useful tools for studying APC biology, but do however not recapitulate the heterogeneity of mononuclear phagocytes *in vivo* [103]. *In vivo*, immune cells are exposed to a complex environment of differentiation factors, which depends on the state and cellular composition of the host tissue. However, all cells used in our studies were of human origin and share important characteristics with their primary *in vivo* counterparts [355], [260], [64].

In our studies on the regulation of LT biosynthesis in MM6 cells, MDMs and MDDCs, we focused on the cellular capacities for conversion of exogenous LTA_4 into the LTs B_4 and C_4. Thus, our findings might be particularly relevant in inflammatory settings, which involve large numbers of LTA_4 releasing neutrophils.

In contrast to MDDCs and undifferentiated MM6 cells, which are largely 5-LO negative, differentiated MM6 cells and TGFβ1-MDMs show high levels of 5-LO protein and product formation [296], (Figs. E1, E2, paper III (online repository)). In Western blots for 5-LO, two bands appeared in samples from MDMs and TGFβ1-MDDCs (Fig. E1, paper III), which displayed abundant 5-LO product formation (Fig. E2, paper III). By contrast, in lysates from MDDCs, synthesizing very low amounts of 5-HETE, the lower of the two bands was hardly detectable (Figs. E1, E2A, paper III). It is attractive to speculate that this lower band corresponds to active 5-LO, while the the upper band can be attributed to an inactive 5-LO splice variant. Several inactive isoforms of 5-LO have been identified very recently [35].

The treatment of monocytes with a particular cytokine combination (GM-CSF +/- IL-4 +/- TGFβ1 or TGFβ1 + VD_3) for four to eight days had a major impact on the cellular LTA_4 metabolism (Figs. 1A, paper II, 4A, paper III). In contrast to 5-LO and LTA_4H, LTC_4S protein levels were not influenced by the presence of TGFβ1 during differentiation (Figs. 2A, paper III, 1B, paper II). Hence, TGFβ1 should induce the observed increases in LTC_4S activity (Figs. 1A, paper II, 4A, paper III) by a post-translational mechanism, which we suggest might be the reduction of inhibitory phosphorylation (compare Fig. 6.2).

The capacity for conversion of LTA_4 to LTC_4 in differentiated MM6 cells was considerably (about 10fold) higher than in MDMs (Figs. 1A, D, paper II). This might be explained by the generation of MDMs in the presence of GM-CSF, which potently increases the phosphorylation of p70S6K [108]. As we have shown, p70S6K can directly phosphorylate LTC_4S (Fig. 2C, paper II). Thus, the activation of p70S6K by GM-CSF can be expected to result in a decreased cysLT output. Interestingly, TGFβ1 and VD_3 had a very weak influence on the LTC_4S activity in MDMs, suggesting a dominant suppressive effect of GM-CSF on cysLT generation in monocytic cells (unpublished observations).

The long-term effect of zymosan, to downregulate LTC_4S by inducing phosphorylation, occurred in connection with differentiation of the monocytic cells. This may resemble the phase of monocyte entry into tissues, where they differentiate under the influence

of the local cytokine milieu and where simultaneous encounter of MAMPs like those contained in zymosan might occur. A growing body of evidence suggests that different PRRs mediate the responses in early and late phases of pathogen recognition. This phenomenon also comprises the induction of eicosanoid production in response to yeast cell wall components: while the β-glucan receptor (Dectin-1) mediates immediate events like AA release and LT synthesis, later events, such as the induction of COX-2 often depend on TLR2 signaling [305], [223], [304]. These studies are in accordance with our results, which suggest that the late suppression of LTC$_4$S by zymosan is mainly mediated *via* TLR2 (Fig. 4, paper II).

7.3 PGE$_2$ mediated control of cysLT biosynthesis – clues for aspirin intolerance

In paper II, we hypothesize that COX product dependent control of cysLT generation *in vivo* is mediated by some of the kinase pathways that we have identified in monocytic cells *in vitro*. Furthermore, we suggest that the exaggerated release of cysLTs, observed in aspirin intolerant asthma (AIA) [194], might be explained by a failure of such regulatory mechanisms.

According to the "shunting hypothesis", which has been popular for a long time, the exacerbation of an acute asthma attack after intake of aspirin or other NSAIDs is due to "shunting" of AA from the COX into the 5-LO pathway. However, several studies indicate that the increased LT generation after aspirin intake, particularly in AIA, cannot entirely be explained by shunting [160], [82].

Importantly, in contrast to inhibition by other NSAIDs, which totally block COX activity [248], COX "inhibition" by ASA results in acetylation of the enzyme. Acetylated COX is not inactive, but instead shows an altered product profile: rather than to PGH$_2$, it converts AA to 15-H(P)ETE and aspirin triggered lipoxins (ATLs) [255], [279]. Thus, we cannot exclude that acetylated-COX products like ATLs play a role in the modulation of the LTC$_4$S activity by aspirin. Experiments with other NSAIDs (e.g. ibuprofen), which totally abrogate COX activity, could help to clarify this issue.

In contrast to aspirin and the other classical NSAIDs, AIA patients tolerate selective COX-2 inhibitors [270], [19], suggesting that the control of cysLT release in the airways of these patients depends on COX-1 activity [82]. This supports a possible relevance of our postulated control mechanism (Fig. 6.2) in the context of AIA, because ASA counteracted the suppression of LTC$_4$S activity more efficiently than the COX-2 selective inhibitor etoricoxib (Fig. 3C, top panel, paper II). However, even if etoricoxib showed a lower potency to inhibit PGE$_2$ release as compared to ASA (Fig. 3C, bottom panel, paper II), this rather small difference might not entirely explain the pronounced difference in the potency of ASA and etoricoxib to restore LTC$_4$ formation in zymosan treated cells (Fig. 3C, top panel, paper II). This discrepancy might be explained by two reasons: Firstly, ASA could influence LTC$_4$S activity *via* additional mechanisms, such as reduced generation of other prostanoids, induction of ATL synthesis (see above) or inhibition NFκB [118]. Secondly, as discussed in paper II, the contribution of COX-1 and COX-2 to PGE$_2$ synthesis might vary during earlier and later phases of monocyte differentiation in the presence of zymosan.

7.3. PGE₂ MEDIATED CONTROL OF CYSLT BIOSYNTHESIS - CLUES FOR ASPIRIN INTOLERANCE

PGE_2 can bind to four different PGE_2 receptors (EP1 to EP4) with distinct expression patterns and downstream signaling pathways (Fig. 7.1). Therefore, PGE_2 can trigger a diverse set of responses, often in a tissue specific manner [302], [169]. Immune cells of monocytic origin, which were in the focus of our study, express mRNA for EP2, EP3 and EP4 [140]. However, PGE_2 exerts most of its immunological functions *via* EP2 and EP4 [55], [33]. It appears that the suppressive effect of PGE_2 on LTC_4S activity is mediated *via* several PGE_2 receptors (Fig. 3C, top panel, paper II).
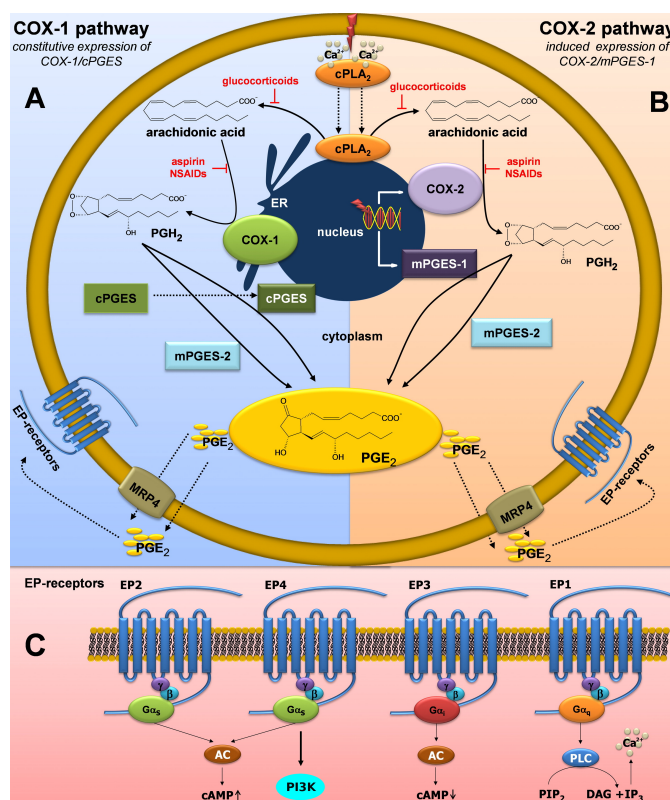

Figure 7.1: *Biosynthesis and signaling of PGE₂* [169], modified

The suppression of LTC_4S activity by PGE_2 is particularly intriguing in the context of transcellular LT metabolism, because PGE_2 also inhibits 5-LO product formation in neutrophils [122], [91], the major endogenous source of LTA_4. This underlines the importance of PGE_2 mediated control of LT generation at the site of inflammation with its complex milieu composed of structural cells and infiltrating leukocytes. Another mode of reduction of LT biosynthesis by PGE_2, which was described for mouse DCs, is IL-10 dependent downregulation of FLAP expression [129]. Interestingly, the combination of IL-10 and PGE_2 also downregulated expression of the $CysLT_1$ receptor in human MDDCs [315]. Thus, it appears that several components of the LT pathway can be inhibited *via* overlapping signaling mechanisms in different inflammatory cell types, thereby ensuring efficient "switch-off" of LT generation and effector functions in response to PGE_2.

The PGE_2 mediated suppression of cysLT biosynthesis is in accordance with clinical findings, describing reduced expression of EP-2 receptor or COX-1 and 2 in nasal mucosa or cultured bronchial epithelial cells from aspirin-intolerant patients, respectively [345], [230]. Furthermore, in comparison to aspirin tolerant asthmatics (ATA), aspirin intol-

erant asthmatics (AIA) display increased numbers of $CysLT_1$ expressing inflammatory leukocytes in their nasal mucosa [291]. This suggests that in aspirin intolerant respiratory disease, PGE_2 mediated control is lacking at several levels of the cysLT pathway, resulting in a vicious circle of overproduction, hyperresponsiveness and ultimately cysLT driven persistent inflammation. In a very recent study, targeted eicosanoid lipidomics showed a tendency towards lower levels of PGE_2 but higher levels of the stable PGE_2 metabolite (PGEM) in exhaled breath condensate of AIA versus ATA [264]. Thus, also abnormal degradation of PGE_2 might contribute to aspirin hypersensitivity.

7.4 Exosomal LTs as amplifiers of immunity and inflammation

We were the first to describe exosomes as carriers of enzymes for LT biosynthesis. Our studies also imply that exosomes can elicit trafficking of granulocytes (Fig. 5, paper III), a capacity, which might in part be mediated by chemotactic eicosanoids, contained or synthezised in the exosomes. Since this potential function of exosomal lipid mediators is based on *in vitro* experiments, future studies should assess immunological roles of exosomal eicosanoids *in vivo*. Moreover, the relative contribution of exosomes to LT biosynthesis in inflammatory settings is currently unknown. *In vivo* studies, using pharmacological inhibition or genetic ablation of LT biosynthetic enzymes in exosomes could shed light on the roles of exosomal LTs for physiology and immune functions.

Our findings support a role for exosomes in pulmonary host defense, which had previously been hypothesized based on a study on exosomes from human tracheobronchial epithelium (HTBE) [157]. In this study, HTBE exosomes were shown to contain sialic acid moieties, which allowed them to neutralize influenza virus. The presence of exosomal LTA_4H and LTC_4S might be of particular relevance in a situation, where neutrophils infiltrate the lung, e.g. in response to a microbial invader, and become activated to produce LTA_4. In such a context, neutrophil released LTA_4 might be converted by exosomal downstream enzymes (compare Fig. 4C, paper III) to LTs, which could contribute to pathogen clearance.

Moreover, it has recently been shown that BALF exosomes from sarcoidosis patients have pro-inflammatory features, suggesting a role for exosomes in the pathology of this inflammatory lung disease [236]. The results described in paper IV support such a pathophysiological role for exosomes in pulmonary inflammation. Interestingly, BALF exosomes from asthmatics and sarcoidosis patients exhibited similar pro-inflammatory characteristics, including increased tetraspanin levels (Fig. 2A, B, paper IV) and a pronounced capacity to induce IL-8 secretion in epithelial cells (Fig. 6A, paper IV) [236]. Our study on BALF exosomes from asthmatics implies exosomal LT generation as another pro-inflammatory pathway, by which exosomes might contribute to chronic inflammatory lung diseases.

The significance of our study has certainly been limited by the small number of subjects and the fact that BALF was only sampled at one time point (24 h) after allergen provocation. It is very likely that this time point represents a snap-shot from a rather late phase of the allergic response [32], when the resolution of inflammation has already started [275]. This is supported by the finding of increased numbers of regulatory T-cells

and induction of IL-10 release at this time point [323]. Possibly, more distinct phenotypic and functional characteristics of BALF-exosomes could have been observed at an earlier time point after allergen provocation.

In addition, it would have been interesting to compare the LT biosynthetic capacities of BALF exosomes from asthmatics to those of BALF exosomes from healthy controls. Unfortunately, the invasiveness of the sampling technique limited the access to patient samples and thus precluded such experiments. Notwithstanding, this cutting-edge study on BALF exosomes from mild allergic asthmatics indicates that it might be worthwhile to analyze LT pathways in BALF exosomes from patients with more severe pulmonary inflammation, such as in chronic obstructive pulmonary disease (COPD) or severe forms of asthma.

8 Future Perspectives

"Science may set limits to knowledge, but should not set limits to imagination." (Bertrand Russell, British author, mathematician and philosopher (1872 - 1970)). As I agree strongly with this statement, I allow myself some speculatory thoughts about implications and applications of the findings described in this thesis:

8.1 Interaction of CLP and 5-LO - a promising drug target in the treatment of cancer and auto-immunity

Overexpression of CLP is associated with an increased susceptibility to develop the autoimmune disorders rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [148]. Together with the implication of the 5-lipoxygenase pathway in the pathogenesis of these diseases [69], [117], [55], [105], this indicates a potential role for the CLP-5-LO interaction in the inflammatory processes related to autoimmunity.

Furthermore, CLP was identified as a tumor antigen, which is highly expressed in pancreatic cancer [206], a disease, which is also associated with increased expression of 5-LO [72], [135]. The pathological importance of the 5-LO pathway for pancreatic cancer is based on the promotion of pancreatic cancer cell growth by the 5-LO products 5-HETE and LTB_4 [72], [325]. Thus, 5-LO inhibitors are promising therapeutic options for an improved treatment of pancreatic cancer [156].

Due to these pathophysiological implications, future studies should address the role of CLP for LT generation in different cells and *in vivo* situations. In view of the roles of CLP and 5-LO in cancer, the chaperone function of CLP should be studied with regard to hypoxia or genotoxic stress.

It is our hope that interrupting the interaction between CLP and 5-LO might be a future therapeutic strategy for reduction of 5-LO product formation in diseases such as RA or pancreatic cancer. Interestingly, the natural compound Hyperforin, which inhibits 5-LO, was shown to counteract the association between CLP and 5-LO [84].

8.2 Regulation of cysLT biosynthesis by microbial products - chances for asthma therapy?

Our findings suggest that inhibitory phosphorylation of LTC_4S could be an important event at the turning point of the inflammatory process, when the resolution of inflammation and the return to homeostasis are initiated by a switch in mediator production (depicted in Fig. 3.12).

A malfunctioning of the mechanisms, which normally downregulate cysLT generation in the later phase of an immune response (Fig. 6.2), might contribute to the persistence of inflammation. Thus, in future studies, the phosphorylation state of LTC_4S in macrophages, mast cells and eosinophils from patients with allergies and chronic inflammatory diseases should be assessed. Such an analysis appears particularly indicated in primary leukocytes from the respiratory tract of AIA patients, the patient population with the strongest correlation between disease severity and overproduction of cysLTs [195].

The potent inhibitory effect of zymosan on the LTC_4S activity suggests that zymosan or similar microbial cell wall preparations could be attractive remedies in the treatment of asthma or other chronic inflammatory diseases. Needless to say, such a therapeutic application would require the previous characterization of the full spectrum of cellular responses, *in vivo*. It is promising though, that further anti-inflammatory and pro-resolving long term effects of zymosan have been described not only *in vitro* [3], [238], but also *in vivo* [24], [276].

8.3 Exosomal eicosanoids - novel tools for immuno-modulation, diagnostics and drug delivery

To elucidate the relative contribution of exosomal eicosanoid synthesis *in vivo* remains an important task of the future. Additionally, the lipid mediator profile of exosomes from different cells and body fluids should be analyzed, in order to identify possible roles of exosome associated eicosanoid release in settings such as host defense, tolerance or chronic inflammation.

Preliminary data from our group indicate the presence of metabolites of both arachidonic and linoleic acid (LA) in exosomes from human MDDCs and murine BMDCs (Lundstrom, Gehrmann, Esser, unpublished data). Thereby, the most abundant compounds, derived from AA, were 15-HETE, 15-oxo-ETE, 5-HETE, 12-oxo-ETE, 12-HETE, 5-oxo-ETE and 11-HETE. Albeit at much lower concentrations, exosomes from both, human and murine DCs also synthesized PGD_2 and PGE_2.

Hence, the amplification of granulocyte chemotaxis by exosomes (Fig. 5, paper III) might be related to their release of 5-oxo-ETE and PGD_2. DC exosomes could exert further immuno-modulatory functions mediated by e.g. PGE_2 (see table 3.4) or 15-HETE, which has suppressive effects on the expression of pro-inflammatory genes in macrophages and on DC maturation [251], [62], [268]. Interestingly, PGE_2 was identified as a major immuno-modulatory agent in tumor derived exosomes, which inhibited DC differentiation [348] and induced myeloid-derived suppressor cells [343].

A systematic analysis of the exosomal lipid (mediator) content might result in the application as a novel biomarker for disease states, e.g. in chronic inflammation. In this context, it would be intriguing to study potential changes in the exosomal eicosanoid profile after exposure to certain drugs, allergens or pathogens.

As exosomes can improve the selective delivery and efficacy of anti-inflammatory compounds, they have been suggested as novel drug delivery vehicles [303]. Hence, it appears possible that exosomes with the desired properties for a specific therapeutic application

will be engineered in the future. A tailored lipid composition could be an integral part of such therapeutic vesicles: For application in the treatment of inflammatory disease, exosomes could be loaded with anti-inflammatory lipids such as ω-3 fatty acids and their derivatives. In contrast, the potency of exosomes as vaccine delivery vehicles [139], [266] might be improved by the incorporation of immuno-stimulatory oxylipins such as LTB_4, as adjuvants. In view of the short half-lives of most eicosanoids, equipping exosomes with cyclooxygenase or lipoxygenase enzymes might however be a more efficient way to exploit exosomal eicosanoids in therapeutic applications.

In summary, there are many open questions to be answered and new options to be explored regarding the biosynthesis of leukotrienes and their implications in exosome biology. So, to close this chapter I avail myself of another quote (by George Bernard Shaw):" Science never solves a problem without creating ten more."

9 Popular scientific summary

9.1 English popular scientific summary

The constant exposure of our body to an environment full of threats like pathogenic microbes or extreme climate conditions requires a complex network of various mechanisms of defense and protection. If for example a bacterium penetrates into the tissue through our outer protection barrier (skin), it is recognized by specialized immune cells, which then initiate an inflammatory response. Thereby, the typical symptoms like redness, swelling and pain emerge. The major triggers of such responses are inflammatory mediators, which are produced by the immune cells. These mediators increase e.g. the permeability of small blood vessels (capillaries), so that additional immune cells can infiltrate into the tissue to combat the microbial intruder. Furthermore, these inflammatory mediators trigger the recruitment of further immune cells, an increased mucus production as well as a contraction of the bronchial musculature. Thus, it is easily conceivable that the very same mediators, which are essential for efficient defense against bacteria are also involved in the development of chronic inflammatory disease as e.g. asthma. To improve the therapy of such increasingly frequent diseases, a detailed understanding of the molecular mechanisms, which control inflammatory mediator production is indispensable. It was the aim of this thesis to make a small contribution to this improved understanding. In the studies described, I focused on the regulation of leukotriene production in specialized immune cells (phagocytes, antigen presenting cells). Leukotrienes are extremely potent mediators, which play a decisive role in the development of asthma. Leukotrienes are generated in several steps from the polyunsaturated fatty acid arachidonic acid. Each of these steps is performed by a particular protein (enzyme), the activity of which can be influenced by various factors. In the first study, we describe the stabilization of 5-lipoxygenase (the first enzyme of leukotriene synthesis) by its "protection protein" CLP. In the second study, we demonstrate that zymosan, a preparation from the yeast cell wall, inhibits leukotriene production in phagocytes, if these are stimulated for several days. Thereby, a particularly interesting finding is that aspirin can reverse this inhibition, which leads to an increased leukotriene production. We speculate that the molecular mechanisms, which we have unraveled in this study, are important for the downregulation of the leukotriene production following an acute inflammatory response. In the third study we show for the first time that small membrane vesicles (so called exosomes), which are released by cells, contain proteins for leukotriene production. Moreover, exosomes isolated from lung lavage fluid from asthmatics have an increased capacity to trigger the production of leukotrienes and other inflammatory mediators by lung epithelial cells. Our findings thus point to a role of exosomes as producers of leukotrienes and suggest that exosomes are involved in inflammatory processes.

9.2 Deutsche populärwissenschaftliche Zusammenfassung

Der ständige Kontakt unseres Körpers mit einer Aussenwelt voller Gefahren, wie krankheitserregenden Mikroorganismen oder extremen klimatischen Verhälnissen, erfordert ein kompliziertes Netzwerk verschiedenster Abwehr- und Schutzmechanismen. Dringt beispielsweise ein Bakterium durch eine Verletzung unserer äusseren Schutzschicht ((Schleim-)haut) ins Gewebe ein, so wird es dort von speziellen Immunzellen erkannt, die daraufhin eine Entzündungsreaktion in Gang setzen. Dabei kommt es zu den typischen Symptomen wie Rötung, Schwellung und Schmerz. Auslöser dieser Reaktionen sind von den Immunzellen produzierte Entzündungsbotenstoffe, die beispielsweise die Durchlässigkeit feiner Blutgefäße (Kapillaren) erhöhen, sodass weitere Immunzellen ins Gewebe einwandern können, um den eingedrungenen Mikroorganismus zu bekämpfen. Des Weiteren bewirken diese enztündlichen Botenstoffe ein Anlocken zahlreicher Immunzellen, eine erhöhte Schleimproduktion sowie eine Verengung der Bronchialmuskulatur. Es ist daher leicht vorstellbar, dass dieselben Botenstoffe, die zur effizienten Abwehr eines Bakteriums benötigt werden auch an der Entstehung chronisch entzündlicher Erkrankungen wie z.B. Asthma beteiligt sind. Um solche immer häufiger auftretendenden Erkrankungen besser therapieren zu können, ist ein detailiertes Verständnis der molekularen Mechanismen, die die Produktion entzündlicher Botenstoffe kontrollieren, unerlässlich. Das Ziel dieser Arbeit war es, einen kleinen Beitrag zu diesem besseren Verständnis zu leisten. In den beschriebenen Studien, habe ich mich auf die Regulation der Leukotrienproduktion in speziellen Immunzellen (Fresszellen, Antigen präsentierende Zellen) konzentriert. Leukotriene sind äußerst wirksame Botenstoffe, die vorallem bei der Enstehung von Asthma eine entscheidende Rolle spielen. Sie werden in mehreren Schritten aus der mehrfach ungesättigten Fettsäure "Arachidonsäure" hergestellt. Diese Schritte werden jeweils durch ein spezielles Protein (Enzym) durchgeführt, das in seiner Aktivität durch verschiedene Faktoren beeinflusst werden kann. In der ersten Studie beschreiben wir die Stabilisierung der 5-Lipoxygenase (das erste Enzym der Leukotriensynthese) durch ihr "Schutzprotein" CLP. In der zweiten Studie zeigen wir, dass ein Präparat aus Hefezellwänden, genannt Zymosan, die Leukotrienproduktion in Fresszellen hemmt, wenn man diese mehrere Tage stimuliert. Ein besonders interessantes Ergebnis ist dabei, dass Aspirin diese Hemmung rückgängig machen kann, was zu einer erhöhten Leukotrienproduktion führt. Wir vermuten, dass die molekularen Mechanismen, die wir in dieser Studie aufgeklärt haben, entscheidend für das Herunterregulieren der Leukotrienproduktion im Anschluss an eine akute Entzündunsreaktion sind. In der dritten und vierten Studie demonstrieren wir zum ersten Mal, dass kleine, von der Zelle ausgeschleuste Membranbläschen (sogenannte Exosomen), Proteine zur Leukotrienproduktion enthalten. Exosomen, die aus "Lungenwaschflüssigkeit" von Asthmatikern isoliert wurden, sind zudem verstärkt in der Lage, Lungenepithelzellen zur Produktion von Leukotrienen und anderen Entzündungsbotenstoffen anzuregen. Unsere Ergebnisse deuten somit auf eine Rolle von Exosomen als Leukotrienproduzenten hin und lassen vermuten, dass Exosomen an entzündlichen Prozessen beteiligt sind.

9.3 Svensk populärvetenskaplig sammanfattning

Det här är bara ett försök - ursäkta min dålig Svenska!

Den ständiga kontakten av vår kropp med en omvärld full av faror som smittsama mikroorganismer eller extrema klimatiska förhållanden kräver ett komplex nätverk av försvars- och skyddsmekanismer. Om till exempel en bakterium genomgår var utvändiga skyddsskikt ((slem-)hud), så erkänns den av speciella immunceller, som därimot startar en inflammatorisk reaktion. Därvid härstämma dem typiska symptomer som rödning, svullnader och ont. Dessa reaktioner utlösas av inflammatoriska signalsubstanser som produceras av immunceller och som höjer till exempel genomgänglighet av fina blodkärl (kapillarer), så att mer immunceller kan komma in i vävnad för att bekämpa mikroorganismen. Dessutom gör denna inflammatoriska signalsubstanser att fler immunceller rekruteras, att slemproduktionen höjs och att bronchialmuskulaturen kontrakteras. Det är därför lätt att föreställa sig att samma signalsubstanser som behövs för en efficient försvar imot en bakterie, är också inblandna i kroniska inflammatoriska sjukdomar som till exempel asthma. För att hitta bättre therapiemöjligheter för dessa allt vanligare sjukdomar, är det oumbärlig att utveckla en detailerad förståelse av dem molekularer mekanismer som kontrollerar produktionen av inflammatoriska signalsubstanser. Det var målet av denna avhandling att bidra en bit till denna förståelse. I dem beskrivna studier har ja fokuserat på regulationen av leukotrienproduktionen i speciella immunceller (antigen präsenterande celler). Leukotriener är väldigt potenta mediatorer som spelar en avgörande roll i utvecklingen av asthma. Dem produceras i flera steg från den fleromätat fettsyran "arakidonsyra". Varje av dessa steg utförs av ett specielt protein (enzym) som kan påverkas i sin aktivitet av olika faktorer. I första studien beskriver vi stabiliseringen av 5-lipoxygenas (första enzymen i leukotrienproduktionen) av sitt "skyddsprotein" CLP. I den andra studien visar vi att zymosan, ett preparat av jäst-cellväggar, hämmer leukotrienproduktionen i makrophager, om dessa stimuleras i flera dagar. En skärskilt interressant resultat är därvid att aspirin motverkar denna hämning så att leukotrienproduktionen ökas. Vi spekulerar att dem molekularer mekanismer som vi har uptäckt i denna studien är avgörande för minskingen av leukotrienproduktionen efter en akut inflammatorisk reaktion. I den tredje och fjärdje studien visar vi för första gången att lilla vesikler som avlossas av cellen (exosomer) innehåller proteiner för leukotrienproduktionen. Exosomer som har isolerats från "lungtvättsvätska" av astmatiker har en ökad kapacitet att stimulerar lungepithelceller att producera leukotriener och andra inflammatoriska signalsubstanser. Vår resultat tyder på en roll av exosomer som leukotrienproducenter och får oss att anta att exosomer deltar i inflammatoriska processer.

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