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Toll-like Receptor-mediated Regulation of Leukotriene Biosynthesis in Human Monocytes

Dissertation

Toll-like Receptor-mediated Regulation of Leukotriene Biosynthesis in Human Monocytes

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ABBREVIATIONS

5-HEDH	5-hydroxyeicosanoid dehydrogenase
5-HETE	5-hydroxyeicosatetraenoic acid
5-HpETE	5-hydroperoxyeicosatetraenoic acid
5-LO	5-lipoxygenase
5-oxo-ETE	5-oxo-eicosatetraenoic acid
13-HpODE	13-hydroperoxyoctadecadienoic acid
AA	arachidonic acid
aa	amino acid
Ala	alanine (letter code: **A**)
AP-1	activator protein 1
APC	antigen presenting cell
Arg	arginine (letter code: **R**)
Asn	asparagine (letter code: **N**)
Asp	aspartic acid (letter code: **D**)
ATP	adenosine triphosphate
BEL	bromoenollactone
BLT	B leukotriene receptor
BSA	bovine serum albumine
C1P	ceramide-1-phosphate
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
CBL	Ca^{2+} binding loop
CLP	coactosin-like protein
COX	cyclooxygenase
CpG	cytidine-phosphate-guanosine
cPLA$_2$	cytosolic phospholipase A_2
Cys	cysteine (letter code: **C**)
CysLT	cysteinyl leukotriene
DAG	diacylglyceride
DAMP	danger-associated molecular pattern
DC	dendritic cell
DHA	docosahexaenoic acid
DiC8	1,2-dioctanoyl-*sn*-glycerol
DNA	desoxyribonucleic acid
DOG	1,2-dioctanoyl-*sn*-glycerol
dsRNA	double stranded ribonucleic acid
DTT	dithiotreitol
EAG	1-*O*-hexadecyl-2-acetyl-*sn*-glycerol
EBV	Epstein-Barr-Virus
EDTA	ethylene diamine tetraacetate
EPA	eicosapentaenoic acid
ER	endoplasmatic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2 (= p42/44 MAPK)
FACS	fluorescence-activated cell sorting
FAF-BSA	fatty acid free bovine serum albumine
FLAP	5-LO activating protein
fMLP	*N*-formyl-leucyl-phenylalanine
FSL-1	synthetic diacylated lipopeptide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gln	glutamine (letter code: **Q**)
Glu	glutamic acid (letter code: **E**)

Gly	glycine (letter code: **G**)
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
GPx	glutathione peroxidase
HA	hemagglutinin
His	histidine (letter code: **H**)
HPLC	high performance liquid chromatography
Hsp	heat shock protein
IFN	interferon
IgG	immunoglobulin G
IgE	immunoglobulin E
IKK	IκB kinase
IL	interleukine
Ile	isoleucine (letter code: **I**)
iPLA$_2$	Ca^{2+} independent phospholipase A_2
IRAK	interleukin-1 receptor-associated kinase
JNK	c-jun N-terminal kinase
kDA	kilo Dalton
LA	linoleic acid
LBP	lipopolysaccharide-binding protein
Leu	leucine (letter code: **L**)
LRR	leucine-rich repeat
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LTA$_4$	leukotriene A_4
LTB$_4$	leukotriene B_4
LTC$_4$	leukotriene C_4
LTD$_4$	leukotriene D_4
LTE$_4$	leukotriene E_4
LT	leukotriene
Lys	lysine (letter code: **K**)
MAPEG	membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	mitogen-activated protein kinase
Met	methionine (letter code: **M**)
MGST2/3	microsomal glutathione *S*-transferase type 2/3
MK2	MAPK-regulated protein kinase 2
MM6	Mono Mac 6
Mnk-1	MAPK-interacting kinase 1
MRP	multidrug resistance protein
MyD88	myeloid differentiation primary-response protein 88
NEMO	NF-κB essential modifier
NF-κB	nuclear factor κB
NK cell	natural killer cell
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
NP-40	nonylphenoxylpolyethoxylethanol
OAG	1-oleoyl-2-acetyl-*sn*-glycerol
ODN	oligodeoxynucleotide
OG	1-*O*-oleyl-*rac*-glycerol
p	phospho
PA-P	phosphatidic acid phosphatase
PAF	platelet activating factor
PAF-AH	PAF acetylhydrolase
Pam$_3$CSK$_4$	synthetic triacylated lipopeptide
PAMP	pattern-associated molecular pattern

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	phosphatidylcholine
PG	phosphatidylglycerol
PGC buffer	PBS containing glucose and $CaCl_2$
PGN	peptidoglycan
PGE_2	prostaglandin E_2
Phe	phenylalanine (letter code: **F**)
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethanesulfonylfluoride
Poly I:C	polyinosinic-polycytidylic acid
Pro	proline (letter code: **P**)
PRR	pattern recognition receptor
PS	phosphatidylserine
PYR	pyrrolidine-1
R-848	resiquimod
RBL-1	rat basophilic leukemia cells
RLR	retinoic acid-inducible gene I (RIG-I)-like receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine (letter code: **S**)
$sPLA_2$	secreted phospholipase A_2
ssPolyU	single stranded poly-uridine
ssRNA	single stranded ribonucleic acid
STI	soybean trypsin inhibitor
TAB	TAK-binding proteins
TAK	transforming growth factor beta-activated kinase
TFA	trifluoroacetic acid
TG	thapsigargin
TGFβ	transforming growth factor beta
Thr	threonine (letter code: **T**)
TIRAP	TIR domain-containing adapter protein
TIR domain	toll/IL-1 receptor domain
TLR	toll-like receptor
TNFα	tumor necrose factor alpha
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR domain-containing adapter protein inducing interferon beta
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophane (letter code: **W**)
Tyr	tyrosine (letter code: **Y**)
UFA	unsaturated fatty acid
Val	valine (letter code: **V**)
VSMC	vascular smooth muscle cells

1 INTRODUCTION

1.1 Leukotrienes

1.1.1 Leukotrienes – a family of eisosanoids

Eicosanoids (greek *eicosi* for "twenty") constitue a group of lipid mediators that are oxygenated metabolites of one common precursor, the arachidonic acid (AA). This essential 20-carbon polyunsaturated fatty acid is esterified at the *sn*-2 position of cellular membrane phospholipids and can be released by phospholipases via enzymatic deacylation for further conversion. A wide variety of cell types synthesize various eicosanoids by distinct enzymatic pathways, which are referred to by the name of its initial oxygenase enzyme. Thus, prostanoids are formed by the cyclooxygenase (COX) pathway, and the 5-lipoxygenase (5-LO) pathway leads to production of leukotrienes (LTs). Furthermore, 12- and 15-hydroxyeicosatetraenoic acids (12- and 15-HETE) and epoxyeicosatrienoic acids are formed by the 12-LO, 15-LO and cytochrome P450 oxygenation pathways, respectively [1].

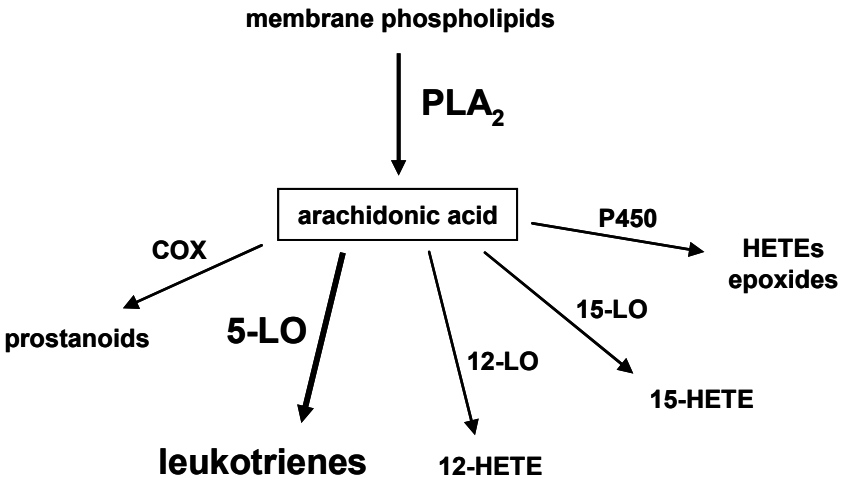


Figure 1: Overview of eicosanoid biosynthesis. AA is released from membrane phospholipids by PLA_2 enzymes and is further metabolized by different enzymes including COX, 5-LO, 12-LO, 15-LO and cytochrome P450 to produce various eicosanoids. Figure according to [1].

Structural studies carried out in the laboratory of Dr. Bengt Samuelsson in the late 1970s led to the discovery and characterization of LTs as novel AA metabolites [2-4], which were

described to exert profound proinflammatory activities including smooth muscle contraction [5] or neutrophil chemotaxis [6]. These investigations finally resulted in the elucidation of the LT biosynthesis pathway catalyzed by 5-LO. The term “leukotrienes” indicates the presence of three conjugated double bonds within the 20-carbon structure of AA (“trienes”) and points out that these compounds are derived from leukocytes (greek *leuko* for “white”) [7-9].

1.1.2 The leukotriene biosynthesis pathway

5-LO catalyzes the first two steps within the LT biosynthesis pathway. The first enzymatic step is catalyzed by the oxygenase activity of 5-LO and involves the abstraction of a hydrogen from C-7 of AA followed by the insertion of molecular oxygen at position C-5 to form 5-hydroperoxyeicosatetraenoic acid (5-HpETE). A second enzymatic step is catalyzed by the LTA_4 synthase activity of 5-LO and involves removal of a hydrogen from C-10, resulting in the formation of the conjugated triene C-5,6 epoxide leukotriene A_4 (LTA_4) by dehydration. The further metabolization of the unstable intermediate LTA_4 is dependent on the cell type and the enzymes present (see Figure 2): it is either converted into LTB_4 by LTA_4 hydrolase or it can be conjugated with glutathione to form LTC_4, which is catalyzed by LTC_4 synthase and the LTC_4 synthase isoenzymes microsomal glutathione *S*-transferase type 2 (MGST2) and type 3 (MGST3) [8-12]. LTC_4 is exported from the cell by the ATP-dependent multidrug resistance proteins, including MRP1 and MRP2 [13]. Subsequently it is metabolized by the enzymatic removal of the gamma glutamyl portion of glutathione to yield LTD_4, which is further converted to LTE_4 by the cleavage of glycin [1, 8]. Also LTB_4 is exported by a protein carrier, which has not yet been identified [1, 14].

Besides LT formation, the 5-LO pathway is also involved in the production of several other AA metabolites. The first intermediate of AA conversion by 5-LO, 5-HpETE, can alternatively be rapidly reduced to the corresponding alcohol 5-hydroxyeicosatetraenoic acid (5-HETE). Thereby, the ratio of LTA_4 to 5-HETE production is dependent on intracellular conditions and, in experimental models, on the terms of the experimental assay. Cellular glutathione peroxidases, but also a pseudoperoxidase activity of 5-LO have been accounted for the reduction of 5-HpETE [11, 15, 16]. Further oxidation of 5-HETE by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) is stimulated by activation of the respiratory burst and by oxidative stress, and leads to the formation of 5-oxo-eicosatetraenoic acid (5-oxo-ETE). 5-oxo-ETE in turn can also be further converted into several additional eicosanoids by both enzymatic and nonenzymatic pathways [1, 17, 18]. Lipoxins comprise another group of AA metabolites that are generated through the combined action of 5-LO and

15-LO, 12-LO or aspirin acetylated COX-2 (COX-2/ASA). These bioactive trihydroxytetraene derivatives of AA appear to function contrarily to LTs as stop signals for inflammatory responses [1, 19, 20].

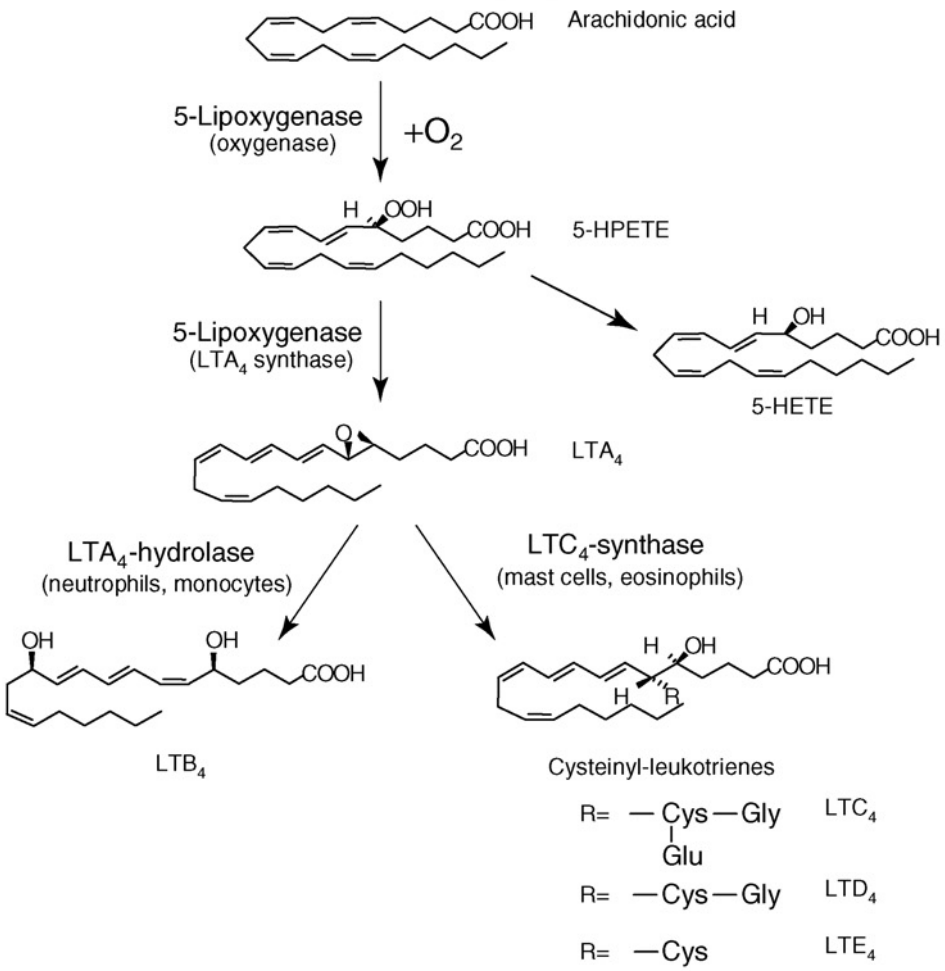


Figure 2: 5-Lipoxygenase reactions. 5-LO catalyzes the conversion of AA to 5-HpETE followed by the formation of the epoxide LTA_4. Enzymatic hydrolysis of LTA_4 by LTA_4 hydrolase leads to LTB_4, whereas LTC_4 synthase catalyzes the conjugation with glutathione to generate LTC_4 [16].

The output of cellular LT biosynthesis depends on the availability of free AA, which is controlled by the enzymatic phospholipid deacylation and reacylation process, and on the regulation of expression, intracellular compartimentalization and catalytic activity of each of the proteins of the 5-LO pathway [1, 8].

1.1.3 Phospholipases A_2 – the release of arachidonic acid

The phospholipase A_2 family

Enzymes of the phospholipase A_2 (PLA_2) superfamily catalyze the hydrolysis of the *sn*-2 ester bond in a variety of different glycerophospholipids present in cellular membranes. This results in the production of a free fatty acid such as arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) on the one hand, as well as a lysophospholipid, which both can serve as precursors for the generation of lipid signaling molecules that exert important biological functions [21, 22].

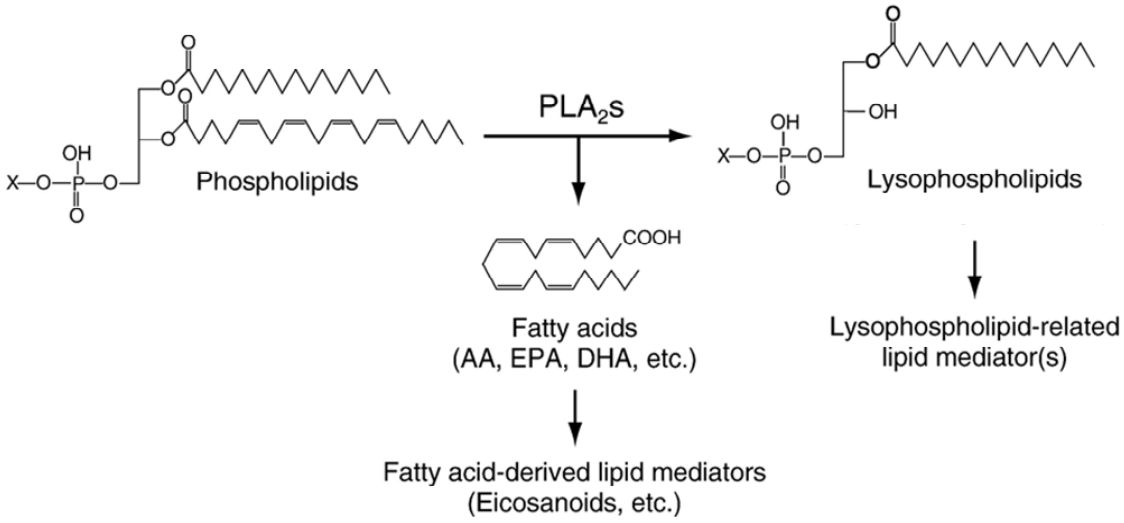


Figure 3: Phospholipase A_2 reaction. PLA_2s catalyze the hydrolysis of the sn-2 ester bond in membrane phospholipids. Figure according to [23].

Different PLA_2 enzymes have been classified into 15 separate groups with subgroups according to their primary structures, molecular weight, disulfide bonding patterns, localization and properties such as Ca^{2+} requirement. These groups are assigned to five main types of PLA_2s, namely the secreted PLA_2s ($sPLA_2$s), cytosolic group IV PLA_2s ($cPLA_2$s), Ca^{2+} independent group VI PLA_2s ($iPLA_2$s), platelet activating factor acetylhydrolases (PAF-AHs) and finally a lysosomal PLA_2 [21, 24].

The $sPLA_2$ family comprises group I-III, groupV and group IX-XIV PLA_2s, which are small secreted proteins that are characterized by their low molecular weight (14-17 kDa), the requirement of millimolar concentrations of Ca^{2+} for catalysis, the presence of six conserved disulfide bonds with one or two variable additional disulfide bonds, and a His/Asp dyad in the

active site. Mammalian $sPLA_2$s do not show a distinct preference for the release of certain fatty acids, but they generally seem to prefer anionic phospholipid substrates [21, 24-26]. Upon secretion, they may act in a paracrine as well as in an autocrine fashion to release fatty acids including AA, which can subsequently be converted into eicosanoids. However, recent studies suggested that certain $sPLA_2$s also function intracellularly to release AA, before being secreted [24, 27-31].

The group IV PLA_2s ($cPLA_2$ family) is comprised of six enzymes, which are named group IVA to IVF PLA_2s or alternatively described as $cPLA_2\alpha$, β, γ, δ and $cPLA_2\zeta$. They are large cytosolic proteins with variable sizes (61-114 kDa), which function through the action of a Ser/Asp dyad. The $cPLA_2$ enzymes do not have the same disulfide bonding network as $sPLA_2$s and require only micromolar concentrations of Ca^{2+} for activity. One exception is group IVC PLA_2, which acts in a Ca^{2+}-independent manner. The different $cPLA_2$s show different specificity for fatty acids in the *sn*-2 position of glycerophospholipids. Group IVA PLA_2 ($cPLA_2\alpha$) is specific for AA containing phospholipids and plays a major role in eicosanoid generation. Group IVB and C, in contrast, have very little specificity. Group IVD seems to favorably address linoleic acid (LA) containing membrane lipids, while group IVE and F hydrolyze both AA and LA [21, 32-36].

The members of the $iPLA_2$ family do not require Ca^{2+} for catalytic activity and act through a catalytic serine in the active site. Moreover, fatty acid specificity has not been observed in any of the group VI PLA_2 enzymes. The $iPLA_2$s are presumed to play a major role in phospholipid remodeling [21, 37]. The PAF acetylhydrolase family also does not require Ca^{2+} for catalysis and likewise contains an active site serine. PAF-AHs hydrolyze the acetyl group from the *sn*-2 position of platelet activating factor [21, 38]. Finally, the recently discovered lysosomal PLA_2, also named 1-*O*-acylceramide synthase, was found to acylate the hydroxyl group in the C-1 position of ceramide using the acyl group from the *sn*-2 position of phospholipids as substrate [21, 39, 40].

Although different PLA_2s, especially $sPLA_2$s and $iPLA_2$s have been shown to be involved in the release of AA, $cPLA_2\alpha$ is widely recognized as the key enzyme for the initiation of eicosanoid metabolism.

The structure of cytosolic phospholipase A_2 alpha ($cPLA_2\alpha$)

The crystal structure of $cPLA_2\alpha$, which was solved in 1999, revealed the presence of an N-terminal C2 domain connected to a C-terminal catalytic domain by a short 5-amino acid flexible linker [41].

The C-terminal region of cPLA$_2$α contains two homologous catalytic domains A and B interspaced with an isoform-specific sequence. The lipase consensus sequence, GXSGS, is localized in the N-terminal region of domain A. The active site dyad is composed of Ser-228 and Asp-549, which mediates the hydrolysis of phospholipid substrate. Furthermore, a conserved arginine (Arg-200) seems to play an important role by binding the charged headgroups present in membrane glycerophospholipids [21, 22, 41-44]. The Ser/Asp active site is located at the bottom of a narrow funnel lined by hydrophobic residues and covered by a lid (residues 413-457). It has been suggested that anionic residues on the cPLA$_2$α catalytic domain play a role in displacing the lid by electrostatic repulsion with anionic lipids on the surface of cellular membranes. Removal of the lid exposes hydrophobic residues (Ile-339, Leu-400, Leu-552) proximal to the active site, which facilitates membrane penetration and allows the fatty acid chain of a phospholipid substrate to enter the active site [41, 45, 46]. The cPLA$_2$α was shown to preferentially hydrolyze AA from the *sn*-2 position of membrane phospholipids, which seems to be due to the architecture of the active site funnel. In contrast to oleic acid (C18:1) and linoleic acid (C18:2), which are poorly released, cPLA$_2$α also hydrolyzes α-linoleic acid (C18:3) and eicosapentaenoic acid (C20:5), which are found in rather low quantities in cellular membranes, though [37, 41]. The cPLA$_2$α catalytic domain contains a phosphatidylinositol 4,5-biphosphate (PIP$_2$) binding site located at a cluster of basic residues (Lys-485, -541, -543, -544), which may regulate membrane binding through association with anionic phospholipid [45, 47]. Furthermore, a tryptophane residue (Trp-464) within the catalytic domain seems to be involved in a calcium-independent mechanism of cPLA$_2$α membrane association [48]. Finally, several serine residues are present in the catalytic domain, which can be phosphorylated by signal-activated protein kinases [37].

At its N-terminus, cPLA$_2$α contains a Ca^{2+} binding C2 domain, which is a conserved structure present in several lipid binding proteins such as protein kinase C (PKC) and promotes Ca^{2+}-dependent interaction of proteins with membranes. C2 domains are composed of approximately 120 amino acids, which form an eight-stranded anti-parallel β sheet [21, 46, 49, 50]. The cPLA$_2$α C2 domain binds two Ca^{2+} ions through a cluster of aspartic acid residues in the four calcium binding loops (CBLs) of the membrane binding surface. In the C2 domains of several other proteins, Ca^{2+} binding neutralizes the anionic residues and, due to the presence of basic amino acids in the membrane binding region, the potential switches to electropositive, which allows binding to anionic phospholipids in membranes. In the cPLA$_2$α C2 domain, in contrast, Ca^{2+} binding facilitates hydrophobic residues in CBL1 (Phe-35, Leu-39) and CBL3 (Tyr-96, Val-97) to preferentially associate with charge-neutral

phosphatidylcholine (PC) and to penetrate into the membrane interface [51-59]. As another feature, finally, a specific site in the cPLA$_2$α C2 domain (Arg-57, Lys-58, Arg-59) was identified to bind ceramide-1-phosphate (C1P) [60].

Regulation of cPLA$_2$α expression

The cPLA$_2$α is ubiquitously and constitutively expressed in most cells and tissues, yet expression levels can be increased by certain pro-inflammatory cytokines and growth factors in several cell-types. Regulation of cPLA$_2$α expression by cytokines can be mediated through transcriptional mechanisms as well as post-transcriptional effects on mRNA stability. Glucocorticoids, on the other hand, were shown to repress the expression of cPLA$_2$α and production of eicosanoids. Interestingly, mature T and B lymphocytes are an exception in that they do not seem to contain detectable levels of cPLA$_2$α [61-66].

Regulation of cPLA$_2$α enzymatic activity

As the initial and rate-limiting step of eicosanoid biosynthesis, the cellular liberation of AA by cPLA$_2$α is tightly regulated, mainly by Ca^{2+} mediated membrane association, binding of secondary lipid messengers and by phosphorylation on serine residues.

Ca^{2+} binding is not required for catalysis itself, as it is the case for the enzymes of the sPLA$_2$ family, but an increase of the intracellular Ca^{2+} level induces cPLA$_2$α transolcation to and association with intracellular membrane surfaces, which is mediated through the C2 domain as described above. The PC binding specificity of cPLA$_2$α thereby plays a role in regulating the subcellular targeting of cPLA$_2$α to distinct intracellular membranes. In cells exposed to Ca^{2+}-mobilizing agents, cPLA$_2$α translocates primarily to the nuclear envelope, endoplasmatic reticulum (ER) and the Golgi apparatus, and it has been suspected that this may be due to an enrichment of PC in these membranes. In detail, cPLA$_2$α redistributes to Golgi in response to physiological short-duration elevations of the intracellular Ca^{2+} exceeding 100-125 nM, and to Golgi, the ER and the nuclear envelope at an intracellular Ca^{2+} concentration of greater than 210-280 nM. Sustained elevations of the intracellular Ca^{2+} thereby stabilize cPLA$_2$α membrane association resulting in the release of AA [37, 46, 67-73]. However, cPLA$_2$α activation has been also reported without any change in the intracellular Ca^{2+} level and cPLA$_2$α displayed also Ca^{2+}-independent membrane binding, which may be regulated by hydrophobic binding of the catalytic domain, by anionic lipids and by phosphorylation [47, 74-78].

Anionic phospholipids, particularly phosphatidylinositol 4,5-biphosphate (PIP_2) and phosphatidylinositol 3,4,5-triphosphate (PIP_3) can promote $cPLA_2\alpha$ binding to lipid vesicles and increase its activity *in vitro* in a Ca^{2+}-independent manner. Interestingly, even though the PIP_2 binding site is located in the catalytic domain, PIP_2 activation requires the presence of the C2 domain, indicating its decisive role in $cPLA_2\alpha$ interfacial binding. It has been suggested that PIP_2 binding may induce a conformational change that leads to optimized positioning of the catalytic domain on the membrane or that may cause the increase of the catalytic efficiency. Furthermore, it has been suggested that PIP_2 facilitates $cPLA_2\alpha$ translocation to perinuclear membranes at low intracellular Ca^{2+} concentrations by serving as an anchor molecule [22, 45, 47, 76, 79-82]. As another lipid, ceramide-1-phosphate (C1P) can bind $cPLA_2\alpha$ via the C2 domain and activates the enzyme in a Ca^{2+}-dependent manner. It was suggested that C1P may function to recruit $cPLA_2\alpha$ to intracellular membranes and to decrease the dissociation constant from membrane surfaces [60, 83-85].

The catalytic domain of $cPLA_2\alpha$ contains several serine residues that can be phosphorylated depending on the cell type and the agonist used for activation. In detail, Ser-505 is addressed by mitogen-activated protein kinases (MAPKs), namely ERK1/2 or p38 MAPK, Ser-515 serves as a target of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) and Ser-727 is phosphorylated by MAPK-activated protein kinases such as MAPK-interacting kinase Mnk-1. Phosphorylation of $cPLA_2\alpha$ on either Ser-505, Ser-515 or Ser-727 was found to increase enzymatic activity 2-3 fold *in vitro* [86-90]. On the other hand, it was suggested that phosphorylation may influence $cPLA_2\alpha$ membrane binding affinity, rather than playing a role strictly in the regulation of catalytic activity. In this regard, Ser-505 and Ser-727 phosphorylation of $cPLA_2\alpha$ played a role in cellular AA release under conditions of transient Ca^{2+} increases, but was less important with higher sustained elevation of the intracellular Ca^{2+} concentration. Furthermore, there are studies revealing that phosphorylation on Ser-505 increased the phospholipid binding affinity of $cPLA_2\alpha$ particularly at low Ca^{2+} concentrations. It was shown that Ser-505 phosphorylation promotes membrane penetration of hydrophobic residues of the $cPLA_2\alpha$ catalytic domain, presumably by inducing a conformational change of the protein. These enhanced hydrophobic interactions facilitate sustained membrane interaction in response to transient Ca^{2+} elevations. As phosphorylated $cPLA_2\alpha$ (Ser-505) fails to release AA in the total absence of an increase of intracellular Ca^{2+}, it seems that phosphorylation per se is insufficient, but plays an augmentative role in $cPLA_2\alpha$ mediated cellular AA release [37, 46, 77, 87, 91-93]. The treatment of $cPLA_2\alpha$ expressing Sf9 insect cells with the protein phosphatase inhibitor okadaic acid results in phosphorylation of Ser-727

and triggers AA release yet without an increase in the intracellular Ca^{2+} level. Thus, Ser-727 phosphorylation may facilitate a membrane association of cPLA$_2$α in a Ca^{2+}-independent manner, but the functional relevance has not yet been elucidated [22, 93-95]. A role of CamKII in cPLA$_2$α phosphorylation has been reported in norepinephrine and angiotensin II stimulated vascular smoothe muscle cells (VSMC) and in fetal bovine serum treated U-937 myeloid leukemia cells. In VSMC, CamKII was required for cPLA$_2$α activation and directly bound to the enzyme resulting in the phosphorylation of Ser-515 and an increase of the enzymatic activity. Moreover, Ser-515 phosphorylation was required for norepinephrine-induced translocation of cPLA$_2$α to the nuclear envelope in VSMC [88, 96-98]. Importantly, it should be taken in account that the three serine residues may also be interactive, as Mnk-1 is activated by members of the MAPK familiy, and because ERK activation can occur downstream from CamKII [22, 99, 100]. Further phosphorylation sites have been reported at Ser-437 and Ser-454 in Sf9 cells, but further studies have yet to be carried out to clarify the impact of phosphorylation at these residues [95]. Taken together, phosphorylation events may function to regulate catalytic activity and membrane binding of cPLA$_2$α.

As another regulating factor, the specific subcellular localization of cPLA$_2$α plays an important role in AA release and subsequent eicosanoid production. Besides Ca^{2+}-dependent translocation of the enzyme from the cytosol to the perinuclear region, cPLA$_2$α has also been reported to localize inside the nucleus. In subconfluent endothelial cells, a truncated form of cPLA$_2$α was found in the nucleoplasm, which translocated to the nuclear envelope from its intranuclear side in response to cellular stimulation. Although cPLA$_2$α lacks classical nuclear localization signals, it has been observed that nuclear import can occur through association with binding proteins. In this regard, cPLA$_2$α associates with the nuclear transcription factor B-Myb containing nuclear localization signals and directing the nuclear import of cPLA$_2$α [101, 102]. During phagocytosis of opsonized zymosan by macrophages, cPLA$_2$α was found to translocate to the forming phagosome and also to membrane ruffles at the plasma membrane [103]. Also in neutrophils, cPLA$_2$α has been reported to translocate to the plasma membrane upon stimulation with *N*-formyl-leucyl-phenylalanine (fMLP), opsonized zymosan or phorbol-12-myristate-13-acetate (PMA). It is recruited to this site by the association with components of the NADPH oxidase system and releases AA, thereby assisting in the production of superoxide [104]. Finally, cPLA$_2$α colocalizes with MAPKs at cytoplasmic AA-rich lipid bodies in human leukemic U937 cells [105].

Several interaction partners of cPLA$_2$α have been identified, which may also play a role in the cellular regulation of cPLA$_2$α. Vimentin, the major component of the intermediate filaments,

is enriched in the perinuclear region and binds to the C2 domain of cPLA$_2$α in the presence of Ca^{2+}, which might possibly stabilize the binding of cPLA$_2$α to the membrane. The cPLA$_2$α – interacting protein PLIP interacts with the N-terminal region of cPLA$_2$α. In renal mesangial cells, cPLA$_2$α colocalizes with PLIP in the nucleus, and overexpression of PLIP enhances cPLA$_2$α-mediated PGE_2 synthesis. The p11/calpactin light chain, a member of the S100 protein family, interacts with the C-terminal region of cPLA$_2$α and inhibits enzymatic activity *in vitro*. In this regard, the induction of p11 protein expression seems to account for the inhibitory effect of dexamethasone on the release of AA. Finally, also some annexins can function as negative regulators of cPLA$_2$α. The C-terminal region of annexin I binds to the C2 domain of cPLA$_2$α inhibiting enzymatic activity by specific interaction, whereas annexin V inhibits cPLA$_2$α activity mainly by substrate depletion [46, 106-111].

1.1.4 5-Lipoxygenase (5-LO) – key enzyme for leukotriene biosynthesis

Structure of 5-LO

The three-dimensional structures of several plant and animal lipoxygenases, including soy bean 15-LO, coral 8-LO and rabbit reticulocyte 15-LO, have been resolved successively in various studies. The structure of human 5-LO as one of six human lipoxygenases, in contrast, has not been determined up to now. However, computational models based on the crystal structure of rabbit reticulocyte 15-LO indicate that human 5-LO is composed of a C-terminal catalytic and an N-terminal regulatory domain with characteristics similar to that of cPLA$_2$α [9, 12, 16, 112-116].

The catalytic C-terminal domain (residues 121-673) has a mainly helical structure and contains a non-heme iron in its active site, which plays a decisive role in 5-LO catalytic activity. The iron of the isolated, inactive enzyme was found to be in the ferrous state (Fe^{2+}), whereas the conversion into the active, ferric form (Fe^{3+}) requires the interaction with fatty acid hydroperoxides. Importantly, the iron cycles between the ferric (active) and the ferrous (inactive) form during 5-LO catalytic action [12, 117-120]. As it is a typical feature of mononuclear non-heme Fe^{2+} enzymes, the iron of the 5-LO active site is coordinated by a 2-His-1-carboxylate facial triad (His-372, His-550 and Ile-673), but also His-367 and Asn-554 seem to function as additional ligands. The catalytic domain is subject to regulation by phosphorylation of several serine residues (Ser-271, Ser-663 and Ser-523) [11, 12, 16, 118, 121-123].

The smaller regulatory N-terminal domain (residues 1-114) shows some structural and functional similarities to the C2 domains, which are found in several proteins such as protein kinase C (PKC) or cytosolic phospholipase A_2 ($cPLA_2$) as described above. However, due to differences in the arrangement of β-strands in the N-terminal domain of 5-LO this region is referred to as C2-like domain. The 5-LO C2-like β sandwich contains typical ligand-binding loops, which have been found to bind various lipids (phosphatidylcholine [PC], glycerides or lipid membranes), Ca^{2+} or Mg^{2+} ions (two per 5-LO protein), and also coactosin-like protein (CLP), which is known as an F actin-binding protein. These factors positively regulate 5-LO catalysis by improving access to the substrate AA and oxidation of ferrous to the active ferric 5-LO, or they may lead to stabilization of an active 5-LO structure [12, 16, 124-127].

Regulation of 5-LO protein expression

Most of the cells known to express 5-LO are of myeloid origin and the capability to express 5-LO seems to be acquired during cell maturation. Thus, 5-LO can be mainly found in mature leukocytes including granulocytes, monocytes / macrophages, mast cells and dendritic cells, but also in B lymphocytes, whereas platelets, T lymphocytes and erythrocytes are 5-LO negative. It is believed that 5-LO expression is up-regulated during leukocyte differentiation in the bone marrow, but also when blood monocytes enter tissues, an increase of 5-LO expression occurrs in the course of differentiation into macrophages [9, 12, 16]. Accordingly, differentiation inducers such as dimethylsulfoxide (DMSO), retinoic acid, 1α,25-dihydroxyvitamin D_3 and transforming growth factor β (TGF β) have been shown to stimulate 5-LO mRNA and protein expression in several studies [128-132]. Although the expression of 5-LO is restricted to a certain subset of cells, the promoter of the 5-LO gene has been characterized to show similarity to typical house-keeping genes [133]. 5-LO transcription was found to be regulated by DNA methylation and histone deacetylation [134-137]. 5-LO up-regulation during the cell differentiation process, however, does not seem to occur due to changes in DNA methylation. In fact, other mechanisms involving regulatory elements within the coding sequence of the 5-LO gene, and processes resulting in transcript elongation, seem to account for 5-LO expression in response to the differentiation inducers 1α,25-dihydroxyvitamin D_3 and TGF β [16, 138-140].

Regulation of 5-LO catalytic activity in cell-free assays

As aforementioned, the presence of hydroperoxides is an important parameter for 5-LO activity, as they regulate the redox state of the active-site iron. Various lipid hydroperoxides

such as 5-, 12- or 15-Hydroperoxyeicosatetraenoic acid (5-,12- or 15-HpETE) and 13-Hydroperoxyoctadecadienoic acid (13-HpODE) have been reported to activate 5-LO in crude leukocyte homogenates, whereas hydrogen peroxide and several other organic peroxides were not effective [118, 120]. In this regard, addition of glutathione peroxidase-1 (GPx-1) to cell-free 5-LO activity assays resulted in an inhibition of 5-LO product formation due to reduction of lipid hydroperoxides [127]. Although lipid hydroperoxides are needed to generate active ferric enzyme, they may, in the course of their formation during the 5-LO catalytic reaction, also be responsible for rapid inactivation of 5-LO. After initiation of 5-LO catalysis by conversion of 5-LO to its active state, the further reaction profile is characterized by a linear propagation phase with maximal conversion rates, and an irreversible turnover dependent inactivation, which is also referred to as suicide inactivation of 5-LO [11, 141].

Secondly, Ca^{2+} ions are key determinants of 5-LO enzymatic activity and activate 5-LO purified from human leukocytes [142]. In mutagenesis studies it was shown that Ca^{2+} binds to the N-terminal C2-like domain of 5-LO via certain residues in the ligand-binding loop 2 (Asn-43, Asp-44 and Glu-46), and that these residues are important for Ca^{2+}-stimulated 5-LO activity. In equilibrium dialysis studies a K_d of 6 μM was determined and a 2:1 stoichiometry of Ca^{2+} binding to 5-LO was suggested [125, 143]. Furthermore, half-maximal activation of purified 5-LO was achieved with 1-2 μM Ca^{2+}, and 4-10 μM Ca^{2+} were required for maximal activation of the enzyme [144, 145]. Interestingly, with a lower efficiency, Mg^{2+} ions can substitute for Ca^{2+} regarding *in vitro* activation of 5-LO, and bind to 5-LO via a similar site as Ca^{2+} [146]. Several mechanisms of Ca^{2+}-induced 5-LO activiation have been suggested. Ca^{2+} appears to reduce the requirement for activating lipid hydroperoxides and a Ca^{2+}-mediated increase of 5-LO affinity to AA was observed [147, 148]. Moreover, it was shown that Ca^{2+} increases the hydrophobicity of 5-LO, and thus facilitates 5-LO binding to phosphatidylcholine (PC) vesicles and association with cellular membranes. In fact, activation of 5-LO by Ca^{2+} requires the presence of PC or also CLP [126, 143, 145, 149-151].

In this regard, phospholipids represent a third important factor for 5-LO activity. Enzymatic activation was shown to be up-regulated by membrane preparations that could be replaced by PC vesicles. Three tryptophane residues (Trp-13, Trp-75 and Trp-102) located within the Ca^{2+} binding loops confer the selective binding of the C2-like domain of 5-LO to PC. Thereby, a higher affinity for zwitterionic PC than for anionic phosphatidylserine (PS) and phosphatidylglycerol (PG) vesicles was detected, and it was suggested that the PC selectivity accounts for the binding of 5-LO to the PC rich nuclear membrane [126, 152, 153].

Various glycerides like 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), 1-*O*-oleyl-*rac*-glycerol (OG), 1,2-dioctanoyl-*sn*-glycerol (DOG) and 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (EAG) directly stimulate 5-LO catalysis *in vitro* and it was found that OAG directly binds to the 5-LO C2-like domain via the three tryptophane residues, which constitue the PC binding pocket. Ca^{2+} and phospholipids or membranes, however, prevent the stimulatory effect of OAG. Thus, it was suspected that uncharged glycerides bind to the C2-like domain in the absence of charge neutralization or changes in side chain orientations, which are considered important for Ca^{2+}-induced 5-LO binding to PC. Similar to Ca^{2+}, OAG was also found to render 5-LO activity resistant against inhibition by GPx activity and seems to increase the affinity of 5-LO towards lipid hydroperoxides [16, 127].

The coactosin-like protein (CLP), as another factor influencing 5-LO activity, was reported to bind and up-regulate 5-LO activity via the phospholipid binding site of the C2-like β sandwich. *In vitro* studies revealed, that CLP up-regulates Ca^{2+} induced 5-LO activity in the absence of PC and also increases LT formation, when PC is present. It was suggested that CLP, similar to membranes, can function as a scaffold for 5-LO, stabilizes the enzyme and prevents inactivation of 5-LO *in vitro* [124, 154, 155].

Finally, ATP has been shown to bind and stimulate purified 5-LO, but the binding site has not yet been resolved. Interestingly, ATP hydrolysis is not required for the activating effect. It appears that 5-LO is stabilized in the presence of ATP. Thus, ATP affinity can be utilized for purification of 5-LO [16, 156-158].

Regulation of cellular 5-LO catalysis

In the cellular context an even more complex picture arises. In addition to the previously mentioned parameters, several cellular factors and conditions have an influence on the regulation of LT biosynthesis.

Because lipid hydroperoxides are crucial for the initial oxidation of the iron in the 5-LO active site, the cellular redox tone is decisive for cellular 5-LO product formation. Thus, formation of reactive oxygen species (ROS), inhibition of glutathione peroxidases (GPx) or depletion of glutathione can result in an up-regulation of LT formation, whereas the presence of highly active GPx enzymes suppress 5-LO activity by the reduction of peroxides. In monocytes and the monocytic cell-line Mono Mac 6 (MM6), GPx-1 is responsible for the rather strong suppression of cellular 5-LO activity in these cells, whereas GPx-4 is involved in 5-LO inhibition in the B lymphocytic cell line BL41-E95-A and immature HL-60 cells [159-162].

Early studies revealed that stimulation of cells with Ca^{2+} ionophores triggers a pronounced Ca^{2+} influx into the cell and effectively activates cellular LT formation [2]. Also, other Ca^{2+} mobilizing agents such as thapsigargin (TG) or the soluble agonists platelet activating factor (PAF), LTB_4, *N*-formyl-leucyl-phenylalanine (fMLP), the cytokine IL-8 or phogocytic particles such as zymosan, urate or phosphate crystals are used to induce cellular 5-LO activation and LT biosynthesis. Naturally occurring inflammatory stimuli like fMLP, C5a, PAF or LTB_4 are rather poor activators of 5-LO metabolism, which appears to be due to the rather moderate Ca^{2+} influx that can be achieved compared to ionophores [11, 12, 163]. However, in intact cells, lower Ca^{2+} concentrations (200-300 nM) seem to be sufficient for 5-LO activation than in cell free systems (1-10 μM), indicating that cellular events can relativize the Ca^{2+} requirement [16, 164, 165]. Recent studies indicated that endogenous diacylglycerides (DAGs), formed upon cellular activation of LT synthesis with Ca^{2+} ionophore or TG, might reduce the requirement for Ca^{2+} and it was suggested that in the absence of DAGs, elevation on intracellular Ca^{2+} is not sufficient to facilitate 5-LO metabolism [166]. On the other hand, cellular 5-LO activation might not necessarily involve Ca^{2+} under certain conditions. Thus, addition of exogenous AA was sufficient for 5-LO product formation in Ca^{2+} depleted human neutrophils or RBL-1 cells, and simultaneous induction of cell stress or osmotic shock further enhanced 5-LO activity in the absence of Ca^{2+} [167, 168].

Besides the intracellular Ca^{2+} level, certain kinase pathways have an important inpact on cellular 5-LO product formation. Mitogen-activated protein kinase - regulated protein kinase 2 (MAPKAPK2, MK2), a downstream kinase of p38 MAPK, as well as ERK1/2 were found to phosphorylate 5-LO *in vitro* at Ser-271 and Ser-663, respectively, and activation of these kinase pathways mediates cellular activation of 5-LO. Interestingly, the *in vitro* 5-LO phosphorylation efficiency of these kinases is havily dependent on the presence of unsaturated fatty acids [169-171]. As touched in the previous section, stimulation of MK2 by cell stress or osmotic shock can activate 5-LO without an increase in the intracellular Ca^{2+} level in certain cell types, but can also support Ca^{2+}-induced activation in cells. It is yet debatable if phosphorylation by MAP kinases directly affects 5-LO enzymatic activity or rather regulates nuclear membrane association of 5-LO, modulates its interaction with other cellular components or lowers the requirement for Ca^{2+} in the cellular context. Recent data suggest an effect of 5-LO phosphorylation on 5-LO subcellular localization [12, 16, 172, 173]. Furthermore, protein kinase A (PKA) can phosphorylate 5-LO at Ser-523, which directly inhibits 5-LO activity *in vitro*, as well as in the cell, which may be due to allosteric changes

that lead to decreased enzymatic activity. Moreover, phosphorylation of Ser-523 was shown to affect the cellular localization of 5-LO, resulting in the accumulation of the enzyme in the cytoplasm [174, 175]. PKA activation is stimulated by agents that rise intracellular cAMP (e.g. PGE_2 or adenosine), which have been observed to down-regulate cellular LT biosynthesis and association of 5-LO with the nuclear envelope. Polyunsaturated fatty acids prevent cAMP-mediated inhibition of 5-LO product synthesis in activated neutrophils [16, 176].

In addition to increased intracellular Ca^{2+} and 5-LO phosphorylation, a third determinant of cellular 5-LO catalysis was described very recently. The endogenous generation of diacylglycerides (DAGs) via the phospholipase D (PLD) / phosphatidic acid phosphatase (PA-P) pathway was shown to be critical for cellular 5-LO activation and its redistribution to the nuclear membrane in ionophore- and TG-stimulated neutrophils. Inhibition of PLD or PA-P potently suppressed 5-LO product synthesis, which could be reversed by the addition of OAG, apparently substituting for endogenous DAGs. It was suggested that in agonist-stimulated cells, DAGs are required for association of 5-LO with the nuclear membrane, and it seemed that in the absence of DAGs, elevation of intracellular Ca^{2+} is not sufficient to enable cellular 5-LO metabolism. However, also DAGs alone seemingly are insufficient for 5-LO product formation and nuclear membrane association. Possibly, DAGs may reduce the Ca^{2+} requirement for the activation of 5-LO [166].

The subcellular localization of 5-LO is an important parameter for cellular LT biosynthesis, and therefore is clearly regulated. In resting cells, 5-LO occurs as a soluble enzyme. Dependent on the cell type, 5-LO is either found in the cytosol (e.g. in neutrophils, eosinophils or peritoneal macrophages) or it is localized within the nucleus associated with chromatin (e.g. in alveolar macrophages, Langerhans cells or rat basophilic leukemia cells) [12, 16]. Nuclear import sequences, which are rich in basic amino acids, are present both in the N-terminal and also in the C-terminal domain of 5-LO, and also nuclear export sequences have been identified. Elicitation of resting cells by glycogen, cytokines or by cell adhesion, was found to cause nuclear import of 5-LO, which occurs without activation of the enzyme. However, the capacity for LT generation seems to be higher, when 5-LO is located in the nucleus [173, 177-179]. Upon stimulation with various agonists, which lead to cellular LT formation, 5-LO translocates from the soluble compartment to the nuclear envelope. Thereby, cytoplasmic 5-LO associates with the endoplasmatic reticulum (ER) and the outer nuclear membrane, whereas intranuclear 5-LO was observed to translocate to the inner nuclear envelope [12, 16, 173].

Finally, 5-LO interactions with other cellular proteins have to be considered as factors that can influence 5-LO catalysis in the cell. As mentioned previously, CLP binds and up-regulates 5-LO activity *in vitro*. In resting cells, CLP is localized in the cytosol, but both CLP and 5-LO are found in the nuclear fraction of human neutrophils after stimulation with ionophore, and also in stimulated MM6 cells, the same pattern of subcellular redistribution was found for CLP as for 5-LO. It was suggested that the two proteins permanently form a complex, which may facilitate the formation of 5-HpETE and 5-HETE in the cytosol, when activated by Ca^{2+} in the presence of AA from exogenous sources. Moreover, CLP and 5-LO as a complex may associate with the nuclear membrane in a coordinated fashion, which is a prerequisite for LTA_4 formation and metabolization from endogenously AA in stimulated cells [16, 124, 155]. Another cellular interaction partner of 5-LO is the 5-LO activating protein (FLAP), which is a member of the MAPEG protein family (membrane-associated proteins in eicosanoid and glutathione metabolism) that also includes LTC_4 synthase, mPGE synthase-1 and MGST-1, -2 and -3 [180, 181]. FLAP is located in the nuclear envelope as a permanently membrane-bound protein. A concomitant expression of FLAP and 5-LO has been detected in several blood cells [182-184]. In cell transfection experiments and in studies using FLAP-deficient macrophages from knock-out mice, the requirement of FLAP for cellular LT biosynthesis from endogenous AA clearly became evident [184-186]. In contrast, FLAP is dispensable for catalytic 5-LO activity in cell homogenates and in cells that are stimulated in presence of exogenous AA. It was found that FLAP can bind AA, but no enzymatic activity of FLAP is known up to now [187, 188]. There is some evidence that FLAP and 5-LO may functionally interact, as FLAP facilitates the usage of 12- or 15-HETE as substrate by 5-LO. Moreover, recent data suggest that FLAP can form a homodimer in neutrophils, and also a complex consisting of 2 FLAP and 2 5-LO molecules seems possible [189-191]. Up to now it is generally accepted that FLAP may assist in the provision of substrate to 5-LO at the nuclear membrane [16]. In addition to the mentioned proteins, further 5-LO interacting proteins such as TGFβ receptor I associated protein 1 (TRAP-1) or Dicer may affect and modulate cellular 5-LO activity [12].

1.1.5 Summary of the concerted cellular LT biosynthesis

Stimuli capable of inducing cellular LT formation by elevating the intracellular Ca^{2+} concentration, inducing the release of DAGs and / or activating MAPKs, cause activation of both 5-LO and $cPLA_2\alpha$. In a generally accepted model, 5-LO and $cPLA_2\alpha$ comigrate from a soluble compartment to the nuclear membrane, where AA is released by the catalysis of

cPLA$_2$α from membrane phospholipids. Then AA is provided to 5-LO via the assistance of FLAP, and LTA$_4$ is formed and further metabolized depending on the cell type [12, 16]. Human neutrophils, dendritic cells, B lymphocytes primarily produce LTB$_4$, and human monocytes and macrophages synthesize more LTB$_4$ than LTC$_4$. Human mast cells, in contrast, release more LTC$_4$ than LTB$_4$, and human eosinophils, finally, are LTC$_4$ secreting cells [192]. If 5-LO negative nonleukocyte cells do express LTA$_4$ metabolizing enzymes they can take up LTA$_4$ for the formation of bioactive LTs in a process termed "transcellular biosynthesis" [8, 9, 20].

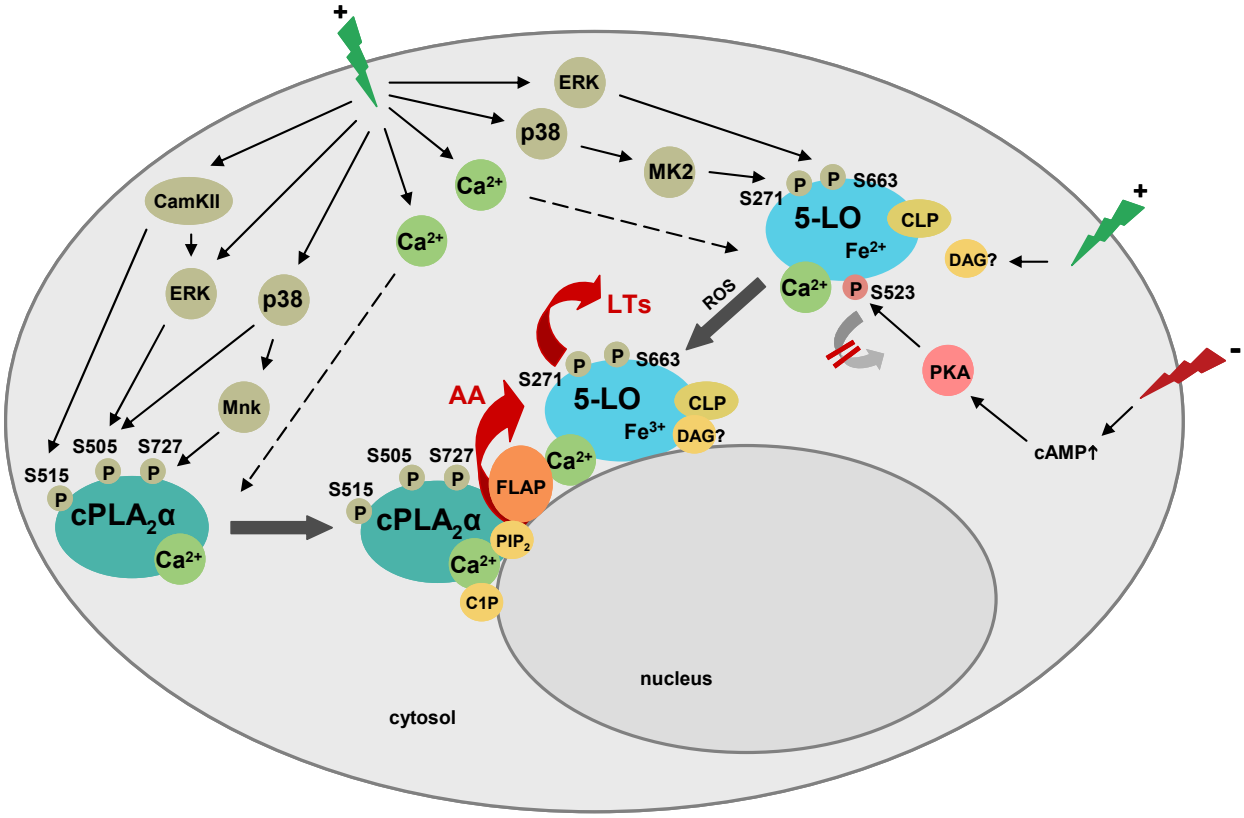


Figure 4: Summary of the concerted cellular LT biosynthesis.

1.1.6 Biological activity of leukotrienes

LT receptors

LTs act on leukocytes, epithelial cells, smooth-muscle cells or also on endothelial cells by interaction with LT receptors, which are seven transmembrane-spanning rhodopsin-type G-protein coupled receptors (GPCRs) expressed on the surface of the target cells. LT receptors can activate G_q proteins resulting in increased intracellular Ca^{2+}, and / or they activate G_i

proteins leading to decreased intracellular cyclic AMP (cAMP). These events trigger the activation of downstream kinase cascades, which influence several cellular and tissue responses. LTs thereby are analogous to hormones that act locally, as they can act on neighboring cells in a paracrine fashion or can, in an autocrine manner, even affect the source cell itself [8, 192].

LTB_4 exerts its effects via the B leukotriene receptors BLT_1 and BLT_2. BLT_1 is expressed primarily on leukocytes and binds LTB_4 with high specifity, as competitive binding studies show displacement only with 20-hydroxy LTB_4 and 12-epi LTB_4. It is a high-affinity receptor for LTB_4, responding optimally at concentrations of 1-100 nM. Interaction with LTB_4 leads to activation of G_i proteins suppressing cAMP signaling or G_q proteins activating PLC [8, 192-196]. BLT_2 shows sequence similarities to BLT_1, but is expressed more ubiquitously in various tissues. Human BLT_2 is activated by a broader range of eicosanoids including 20-hydroxy LTB_4, 12-epi LTB_4, 12-HETE, 15-HETE, 12-HpETE and 15-HpETE. Moreover, BLT_1 has a lower affinity for LTB_4 responding at concentrations greater than 10 nM with a maximal effect at 0.1-1 μM [8, 193, 194, 197]. Interestingly, besides its action on BLTs, LTB_4 was also found to serve as a ligand for the peroxisome proliferator-activated receptor α (PPARα), which is a nuclear hormone receptor that plays a role in regulating lipid metabolism. LTB_4 binds PPARα *in vitro* and activates it in cells [192, 198, 199].

The effects of the different cyteinyl LTs are mediated by the cysLT-binding G-protein coupled receptors $CysLT_1$ and $CysLT_2$. $CysLT_1$ is expressed rather ubiquitously in human organ tissues. It preferentially binds LTD_4 with high affinity (1-10 nM), whereas LTC_4 and LTE_4 binding occurs with approximately 200-fold less affinities. Also $CysLT_2$ shows a rather broad distribution, but was found to bind LTC_4 and LTD_4 with equal affinity (K_d about 10 nM) followed by LTE_4. Activation of $CysLT_1$ or $CysLT_2$ initiates Ca^{2+} influx through pertussis toxin (PTX)-resistant G_q proteins [8, 193, 200-204]. Recently, GPCR17 was discovered as a novel receptor that binds both uracil nucleotides and CysLTs, which increased Ca^{2+} influx and inhibited adenylyl cyclase in cells overexpressing GPCR17 [205].

Biological activities of LTs

LTs are potent biological mediators in inflammatory reactions and immune processes.

LTB_4 plays an important role in initiating inflammatory responses. As a brief outline, when LTB_4 is produced by tissue leukocytes in response to infection or stress stimuli, it promotes adherence of leukocytes to the endothelium, chemotaxis, and additionally also activation of neutrophils or other leukocytes, which triggers important functions. Thus, LTB_4 leads to

degranulation, release of superoxide and lysosomal enzymes or augments phagocytosis of neutrophils and macrophages. By this means, LTB_4 drives the recruitment of leukocytes from the blood stream into tissues, with a concomitant activation of leukocytes, which results in a dramatic increase in tissue cellularity, a characteristic of inflammation [6, 192, 206-210].

CysLTs account for the biological activity that led to the previous term "slow-reacting substance of anaphylaxis". They are best known for their bronchoconstricting effects as a result of the contraction of airway smooth muscle cells and the induction of mucus secretion by bronchial mucosa. CysLTs can also promote the constriction of arterial vascular smooth muscle and thus are involved in the regulation of vasoconstriction. Moreover, endothelial cells are affected by CysLTs to produce vascular leak of plasma into tissues resulting in edema. Finally, CysLTs are also involved in the attraction and activation of leukocytes, and by this way also contribute to inflammatory processes [8, 211-216].

Biological activities of other 5-LO products

5-oxo-ETE, which is formed by the oxidation of 5-HETE by 5-HEDH, is a potent chemoattractant for granulocytes and elicits actin polymerization, Ca^{2+} mobilization, integrin expression and degranulation in these cells. Also monocytes have been described to be attracted in response to 5-oxo-ETE. It acts via the G_i-coupled OXE-receptor and may play important roles in asthma and allergic diseases. Moreover, it has been found to promote the survival of tumor cells [18, 217-219].

Lipoxins, in contrast, act to reduce and resolve inflammation and promote wound healing. They were shown to control the entry of neutrophils to sites of inflammation, reduce vascular permeability and stimulate non-phlogistic uptake of apoptotic neutrophils by macrophages. Furthermore, lipoxins are chemoattractants for monocytes, cells that appear to be required for wound healing. Finally, lipoxins inhibit neutrophil adhesion, chemotaxis, degranulation and superoxide formation, but stimulate monocyte adherence and chemotaxis without causing degranulation or release of ROS [19, 220-227].

1.1.7 Pathophysiological role of leukotrienes

Soon it became clear that LTs play an important role in inflammatory and allergic disorders. Particularly, bronchial asthma is presumed to be the major LT-associated disease. Accordingly, anti-LT therapies have been developed and are approved for the treatment of asthma including the $CysLT_1$ antagonists montelukast, zafirlukast and pranlukast (approval in Japan only) or the 5-LO inhibitor zileuton (approval in the United States only). These drugs

are effective in reducing both the early and late phase of bronchospasm and decrease airway hyperreactivity [228, 229]. In the first instance, LTs have been identified to be involved in further disorders including rheumatoid arthritis, inflammatory bowel disease, psoriasis and allergic rhinitis, but ongoing research revealed that the influence of LTs in the pathophysiology of inflammatory bowel disease and rheumatoid arthritis seems of minor importance, and also in psoriasis, LTs appeared to have a negligible impact [12, 230]. Instead, the 5-LO pathway has recently been associated with the pathogenesis of cardiovascular diseases like atherosclerosis, aortic aneurysm, stroke or myocardial infarction (MI). In this regard, several studies revealed the considerable expression of LT producing enzymes and LT receptors in diseased tissues, and increased LT formation has been correlated with cardiovascular disorders in animal models as well as in patients [231-238]. Genetic studies with knockout mice support the link between cardiovascular disease and the 5-LO pathway, and even human population genetic studies correlated certain genotypes of LT forming proteins to cardiovascular disease [239-244]. Recently, clinical studies with the FLAP inhibitor DG-031 (veliflapon) have provided promising effects on the level of biomarkers associated with MI risk in MI patients carrying at-risk variants in the FLAP or LTA_4 hydrolase gene [245]. As another novel indication, the pathogenesis of osteoporosis has been linked to certain effects of 5-LO products, which appear to positively modulate bone-resorbing osteoclasts, but exert a negative impact on the bone forming capacity of osteoblasts [12]. Finally, the 5-LO pathway appears to play a role in certain types of cancer including prostate, pancreas and breast cancer, as its enzymes and products were found to be present in transformed cells and tissues versus non-transformed cells, and 5-LO products seemingly can stimulate cancer cell proliferation and survival, whereas pharmacological or genetic inhibition of the 5-LO pathway results in cancer cell growth and inhibition of apoptosis. Very recently the 5-LO gene (*Alox5)* was identified as essential for leukemia stem cells to evolve into a chronic myeloid leukemia (CML), as *Alox5* deficinecy caused a failure of CML development. This suggested 5-LO as a specific target gene for the inhibition of cancer stem cells and possibly a treatment of cancer [12, 230, 246].

1.1.8 Leukotrienes and host defense

Besides their role as promoters of inflammation and their disadvantageous role in the pathogenesis of several diseases, LTs yet are also noticed as highly relevant mediators of innate immunity against pathogens [192, 247].

Thus, *in vitro* LT generation has been observed in response to bacteria, mycobacterial species, *Toxoplasma gondii*, *Pneumocystis carinii*, *Histoplamsa capsulatum*, influenza A virus or Epstein-Barr-Virus (EBV) [209, 248-254], and elevated levels of LTs have been reported *in vivo* in patients during bacterial and viral infections such as bacterial or syncytical virus pneumonia and infection with *Vibrio cholerae*, *Helicobacter pylori* or rhinovirus [255-259]. Moreover, studies using 5-LO deficient mice or pharmacological LT synthesis inhibitors support the involvement of LTs in host defense against infectious agents. In this regard, 5-LO deficient mice exhibited increased susceptibility to gram-negative pneumonia, and higher levels of bacteria were found in the peritoneal cavities of 5-LO$^{-/-}$ mice compared to wild-type controls in a model of peritonitis and sepsis. Also, pharmacological inhibition of LT synthesis impaired peritoneal clearance of *E. coli* in a murine model of infectious peritonitis. As another example, intravenous injection of LTB_4 reduced viral loads in mice infected with cytomegalovirus, whereas 5-LO$^{-/-}$ mice or mice treated with a FLAP inhibitor had increased viral loads compared to wild-type or untreated mice [260-263]. LTs exert distinct effector functions that account for their important role in fighting bacterial, fungal or viral infections. First, LTs facilitate leukocyte recruitment through a number of mechanisms. LTB_4, as a potent chemoattractant, recruits neutrophils and monocytes to the site of infection and up-regulates expression of integrins on the surface of leukocytes, thus enhancing their adherence to endothelial cells in the vasculature of infected tissues. Also CysLTs may support leukocyte recruitment by enhancing microvascular permeability, which allows the movement of cells through the endothelium into tissues. LTs also contribute to effector T cell recruitment and dendritic cell (DC) trafficking following antigen stimulation and thus might play a role in directing the adaptive immune response [6, 264-271]. Secondly, LTs augment the ability of macrophages and neutrophils to phagocytose microoganisms as observed in numerous studies [209, 272-274]. Thirdly, LTs play a role in microbial killing by LTB_4 mediated increase of lysosomal enzyme release and release of α-defensins or the induction of NO synthesis and ROS production, which can be triggered by LTB_4 and CysLTs [275-279]. Finally, LTs can induce the release of preformed proinflammatory mediators or initiate gene expression of cytokines, chemokine receptors and transcription factors that in turn augment mechanisms of host defense and immunity [192].

1.2 Toll-like receptors

1.2.1 Function and classification of pattern recognition receptors (PRRs)

Innate immunity acts as a sentinel for the immune system and is immediately activated after recognition of invading microbial pathogens. This is mediated via various pattern-recognition receptors (PRRs) expressed on immune and non-immune cells. They detect highly conserved structures of microbial pathogens, which are considered to be essential components for the survival of the pathogen and which are unique to microorganisms. They are termed as pathogen-associated molecular patterns (PAMPs). The detection of PAMPs by PRRs triggers the development of an appropriate immune response for host defense [280-282].

PRRs can be functionally classified into two classes, the non-signaling and the signaling PRRs [283]. Non-signaling PRRs include soluble factors such as acute-phase proteins (e.g. C-reactive protein or lectins), or transmembrane proteins such as scavenger receptors. They can bind to microorganisms and thus facilitate phagocytosis or recognition by the complement system, but do not principally activate signaling cascades in innate immune cells. The signaling PRRs likewise comprise transmembrane and cytosolic proteins. The nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are cytosolic signaling PRRs [284], and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) as well belong to this category of PRRs [285]. Toll-like receptors (TLRs), finally, are representatives of transmembrane signaling PRRs. The detection of PAMPs by TLRs triggers signaling pathways that lead to the release of inflammatory cytokines and chemokines or type1 interferons (IFNs). These responses are important not only to eliminate pathogens, but also to develop pathogen-specific B- and T cell mediated adaptive immunity by up-regulation of co-stimulatory molecules on antigen-presenting cells, especially macrophages and dendritic cells (DCs) [282, 286, 287].

1.2.2 The family of toll-like receptors (TLRs)

Initially, a so called Toll protein was identified in fruitflies (*Drosophila*) and was described to play an important role in the immune response against fungal infection with *Aspergillus fumigatus* in flies [288]. Ongoing studies led to the discovery of homologues of the Toll protein in humans and mice through data base searches, and were referred to as the TLRs [289-291]. To date, 13 members of the TLR family have been identified in mammals. Of these, TLR1-9 are conserved between humans and mice, whereas TLR10 is not functional in mice and TLR11-13 are lost in human genomes [281, 292].

The structure of TLRs

TLRs are type I integral membrane glycoproteins composed of three major domains. The extracellular N-terminal domain, which mediates the recognition of microorganisms, consists of approximately 16-28 tandem leucin-rich repeats (LRRs), and each LRR consists of 20-30 amino acids with the conserved motif LXXLXLXXN. Some ligands induce homodimer or heterodimer complexes of TLRs, such as TLR3-TLR3, TLR4-TLR4 or TLR1-TLR2. Molecular detail on ligand recognition has recently emerged from the crystal structures of the TLR1-TLR2-ligand complex, the ectodomain mouse TLR4-MD-2 complex and of the complex of TLR3 ectodomain with its ligand. The heterodimer or homodimer of the respective LRR domains show a horseshoe-like structure and consists of both a concave and a convex surface. These surfaces are responsible for ligand binding and ligand-induced TLR dimerization [280, 281, 293-295]. The transmembrane domain connects the extracellular domain to the intracellular C-terminal domain, which is termed Toll/IL-1 receptor (TIR) domain due to its homology with that of the IL-1 receptor. This domain is required for the recruitment of various adapter molecules, which also contain a TIR domain for their interaction with the respective TLR leading to activation of downstream signaling pathways [296, 297].

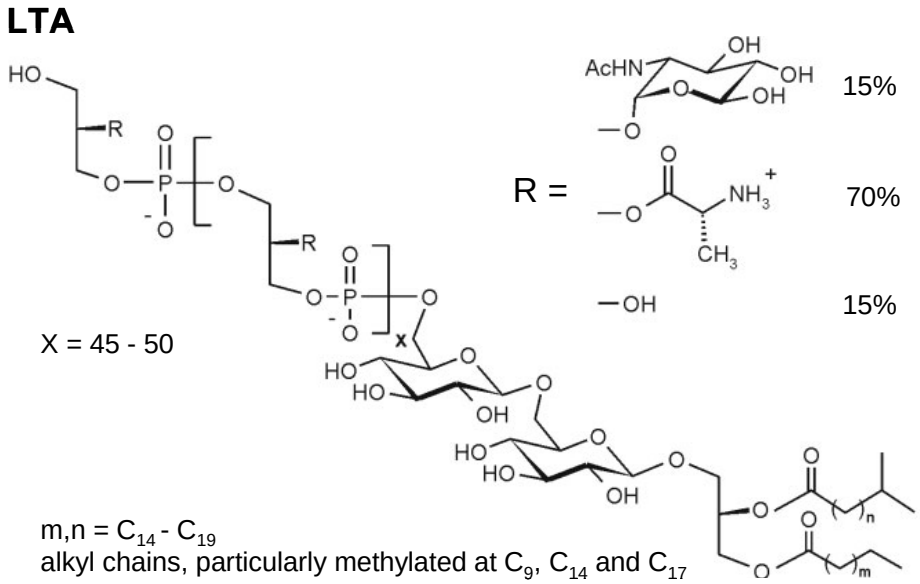
Expression and localization of TLRs

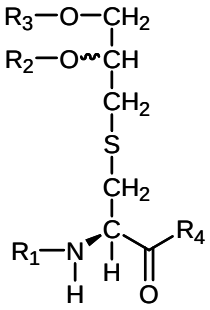
The TLR family members are expressed on various immune and non-immune cells including B lymphocytes, natural killer (NK) cells, DCs, macrophages, fibroblast cells, epithelial cells and endothelial cells [280]. Two subpopulations of TLR subtypes can be distinguished with regard to their subcellular localization. TLR1, 2, 4, 5, and TLR6 are expressed on the cell surface and recognize microbial membrane components such as lipids, lipoproteins and proteins, whereas TLR3, 7, 8 and TLR9 are localized in intracellular vesicles such as the ER and the endosome. Endolysosomes constitute the first site, where pathogen-derived nucleic acids are released after degradation of the microorganism in lysosomes. Thus, intracellular TLRs predominantely recognize various microbial nucleic acid species and particularly trigger anti-viral innate immune responses. Moreover, this intracellular localization of nucleic acid sensing TLRs is also important for the discrimination of non-self from self nucleic acids. In this regard, ectopic expression of TLR9 on the macrophage cell surface was shown to facilitate its response to DNA derived from self. Abnormal recognition of self-DNA, however, has been associated with the pathogenesis of autoimmune diseases [281, 286, 298].

1.2.3 TLR subtypes and their ligands

TLR2

TLR2 is able to form a homodimer with another TLR2 molecule or heterodimers with TLR1, TLR6 or non-TLR molecules such as CD36, CD14 and dectin-1 to discriminate the molecular structures of a wide range of PAMPs derived from various pathogens ranging from bacteria, fungi, parasites and viruses. These ligands include triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan (PGN) and lipoteichoic acid (LTA) from gram-positive bacteria, porin from *Neisseria*, lipoarabinomannan from mycobacteria, zymosan (consisting of β-glucan, mannans, chitin, lipid and protein) from fungi, *Trypanosoma* glycosylphosphatidylinositol (GPI)-mucin and hemagglutinin protein from measles virus [281]. Regarding lipopeptide detection, the TLR2-TLR6 heterodimer was identified to generally recognize mycobacterial diacylated lipopeptides, but also LTA and zymosan, whereas TLR2-TLR1 appears to detect bacterial triacylated lipopeptides. However, there are still diacylated lipopeptides such as Pam_2CSK_4, which were shown to signal in a TLR6 independent manner, and it has been proposed that TLR2 forms a TLR2/TLR2 homodimer when recognizing Pam_2CSK_4. CD36, a member of a class II scavenger receptor, is involved in sensing some of the TLR2 ligands, including TLR2-TLR6 activators. CD14, a GPI-linked protein containing LLRs, plays a role in the recognition of diacylated lipopeptide and lipoarabinomannan. Finally, Dectin-1, an immunoreceptor tyrosine-based activation motif (ITAM)-containing C-type lectin receptor, is required for TLR2 mediated inflammatory responses induced by β-glucan [281, 299-304].





Pam_3CSK_4

R_1, R_2, R_3 = palmitoyl
R_4 = SKKKK

FSL-1

R_1 = H
R_2, R_3 = palmitoyl
R_4 = GDPKHPKSF

Figure 5: TLR2 ligands. LTA: TLR2/6 ligand; chemical structure of LTA from S.aureus; figure according to [305]. Pam_3CSK_4: synthetic triacylated lipopeptide recognized by TLR2/1 [306]. FSL-1: synthetic diacylated lipopeptide recognized by TLR2/6 [307].

TLR4

TLR4 is essential for responses to lipopolysaccharides (LPS), a major component of the outer membrane of gram-negative bacteria, which has long been known as a potent immunostimulatory molecule that can cause septic shock. TLR4 tightly associates with the cell surface protein MD-2, and this complex is required for a substantial induction of inflammatory cytokines. Additionally, the so called LPS-binding protein (LBP) and CD14 are involved in the detection of LPS. LBP is present as a soluble protein or a plasma membrane protein and binds LPS. CD14 in turn binds LBP and delivers LPS-LBP to the TLR4-MD-2 complex [281, 292, 308].

Further TLR subtypes

TLR5, also localized on the cell surface, recognizes a highly conserved central site of flagellin, a protein component of bacterial flagella [281, 309]. Moreover, various types of nucleic acids play a role in the activation of endosomal TLRs. TLR3 recognizes a synthetic analogue of dsRNA polyinosinic-polycytidylic acid [Poly (I:C)], genomic RNA purified from dsRNA viruses such as reovirus, and dsRNA produced during the replication process of ssRNA viruses such as RSV, encephalomyocarditis virus (EMCV) and West Nile virus (WNV) [281, 310, 311]. TLR7 was originally observed to respond to imidazoquinoline derivatives such as imiquimod and resiquimod (R-848) and guanine analogues such as loxoribine, all of which have anti-viral and anti-tumor properties. Subsequently, guanosine-rich and uridine-rich ssRNA derived from HIV or influenza virus and synthetic polyuridine

ssRNA were identified to function as ligands for TLR7. TLR8 has a phylogenetically high similarity with TLR7. Thus, human TLR8 was found to preferentially recognize R-848, bacterial RNA as well as ssRNA derived from HIV, VSV and influenza A virus [281, 312-314].

TLR subtype	Adapters	PAMPs/Activators	Species
TLR2-TLR1	MyD88 - TIRAP	Triacyl lipopeptides	Bacteria
TLR2-TLR6	MyD88 - TIRAP	Diacyl lipopeptides LTA Zymosan	Mycoplasma Bacteria Fungus
TLR2	MyD88 - TIRAP	PGN Lipoarabinomannan Porins tGPI-mucin HA protein	Bacteria Mycobacteria Bacteria *(Neisseria)* Parasites *(Trypanosoma)* Virus (Measles virus)
TLR3	TRIF	dsRNA	Virus
TLR4	MyD88 - TIRAP TRIF - TRAM	LPS Envelope proteins	Bacteria Virus (RSV, MMTV)
TLR5	MyD88	Flagellin	Bacteria
TLR7	MyD88	ssRNA	RNA virus
TLR8	MyD88	ssRNA	RNA virus
TLR9	MyD88	CpG DNA DNA Malaria hemozoin	Bacteria DNA virus Parasites

Table 1: Overview of TLR subtypes, utilized adapters and respective TLR ligands. According to [281].

TLR9 was initially identified to recognize unmethylated 2´-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs that are typically present in bacteria, but are rare in the host, where these sequences are highly methylated at the cytosine base and therefore exert poor stimulatory activity. Recent studies revealed that TLR9 recognition of natural DNAs occurs independently of the base sequence. The sugar backbone 2´-deoxyribose of DNA appeared to be sufficient to induce signaling, which was enhanced by DNA bases, even short CpG motifs. Three structurally distinct classes of synthetic oligodeoxynucleotides (ODNs) containing CpG

motifs are used experimentally for TLR9 activation. A-type (D-type) CpG ODNs show strong ability to induce type I IFN production from plasmacytoid DCs (pDCs), but weaker potency to activate B cells or macrophages. They contain a single CpG motif, and the activity requires a mixed backbone of phosphodiester-phosphorothioate. Especially the region around the CpG motif should be palindromic and phosphodiester-linked. In addition, a poly-G tail should be included at the 3´end. B-type (K-type) CpG ODNs has the potent ability to activate B cells and induce cytokine production from macrophages. This type has multiple CpG motifs, and the activity is stabilized by a phosphorothioate backbone. C-type CpG DNA, finally, exhibit type I IFN-inducing, as well as B cell-activating functions. It is comprised of a phosphorothioate backbone with multiple CpG motifs and a TCG dimer at the 5´end. In addition to DNA, hemozoin derived from *Plasmodium falciparum* activates TLR9 and triggers the production of inflammatory cytokines and chemokines [281, 283, 315-318].

1.2.4 TLR signaling pathways

Signaling pathways via TLRs culminate in the activation of NF-κB and / or MAPKs including ERK1/2, p38 and c-jun N-terminal kinases (JNKs), which phosphorylate the activator protein 1 (AP-1) family of transcription factors. Both variants, finally, regulate the expression of numerous immune and inflammatory genes. Several TLRs that recognize viral PAMPs additionally activate members of the IRF family of transcription factors, which induce the expression of type I IFNs and inflammatory genes [281, 285, 292].

MyD88-dependent TLR2 signaling pathway

After ligand-induced homo- or heterodimer formation of TLR2, myeloid differentiation primary-response protein 88 (MyD88), which serves as an adapter protein, is recruited to the receptor complex through the assistance of a second sorting adapter, TIRAP (TIR domain-containing adapter protein). MyD88 in turn recruits the IL-1R-associated kinase (IRAK) family protein kinases IRAK4, IRAK1 and IRAK2. The activation of IRAKs results in TNF receptor-associated factor (TRAF)6 activation. TRAF6 forms a complex with the E2 ubiquitin-conjugating enzyme complex Ubc 13 and Uev1A to induce the synthesis of lysine 63-linked polyubiquitin chains, which in turn activate TGFβ-activated kinase 1 (TAK1). TAK1, in combination with TAK1-binding proteins TAB1, TAB2 and TAB3, subsequently activates the two pathways involving the IκB kinase (IKK) complex or MAPK. The IKK complex, which comprises the catalytic subunit IKKα and IKKβ and a regulatory subunit NF-κB essential modifier (NEMO, also known as IKKγ), catalyzes the phosphorylation of IκB

proteins that results in the degradation of IκBs and the subsequent nuclear translocation of NF-κB [281, 292, 319].

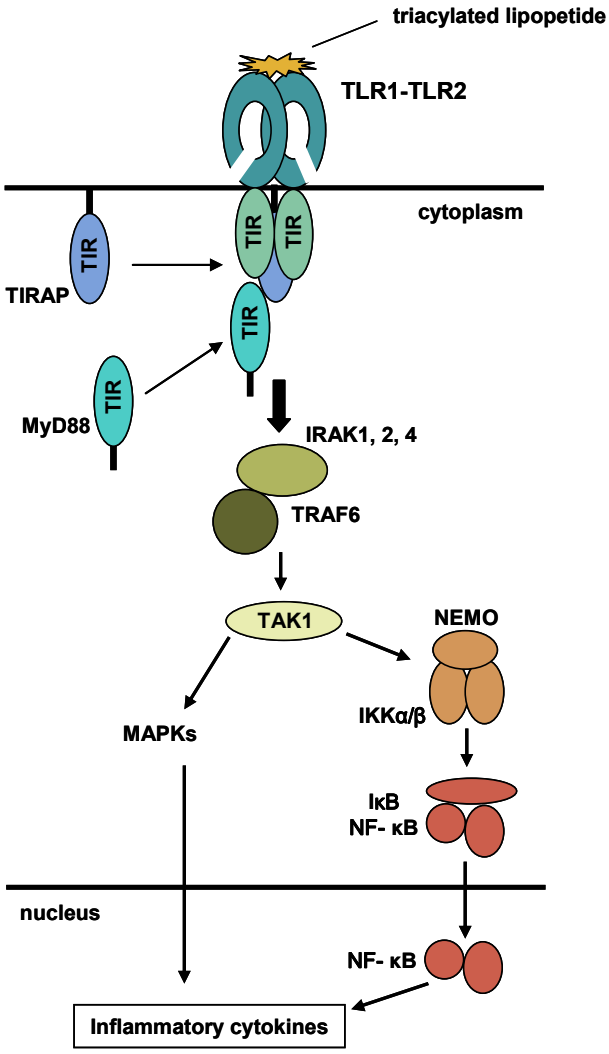


Figure 6: MyD88-dependent TLR2 signaling pathway. Figure according to [281].

Further TLR signaling pathways

Although all TLRs, except TLR3, are known to utilize the MyD88-dependent pathway, individual TLRs additionally can trigger partially different signaling pathways leading to diverse responses depending on the PAMP present. These differences in signaling output were explained by the utilization of individual Toll/IL-1R (TIR) domain containing adapter proteins, which link activated receptors to the downstream kinases that define a given signaling pathway. Besides MyD88, which in most cases drives NF-κB and MAPK activation to induce a pro-inflammatory response, another three adapter proteins have been discovered. TIRAP, which functions as a sorting adapter to recruit MyD88, is utilized by TLR2 and TLR4, but not by the other TLRs for MyD88 recruitment. TRIF (TIR-domain-containing

adapter protein inducing IFN-β), is an adapter that initiates an alternative pathway leading to IRF3, as well as NF-κB and MAPK activation to induce type I IFN and inflammatory cytokines. TRIF is utilized by TLR3 and TLR4. TRAM (TRIF-related adapter molecule) in turn serves to link TRIF to TLR4, but not to TLR3. An even more complex picture arises, when considering the fact that MyD88 is also capable of inducing type I IFN production from some TLRs such as TLR7, 8 and TLR9. Because of the complexitiy of the pathways, TLR signaling is commonly categorized into MyD88-dependent and TRIF-dependent pathways [281, 286, 320-326].

1.2.5 Functional role of TLRs in innate and adaptive immunity

The main function of TLRs is the induction of inflammation and the establishment of adaptive immunity. On the one hand, TLR signaling can trigger substantial production of inflammatory cytokines such as IL-6 and TNFα, which in turn activate surrounding cells to express chemokines or adhesion molecules. Consequently, various inflammatory cells are recruited into the sites of infection. Recruited macrophages or neutrophils are activated and ingest invading pathogens through internalizing PRRs, which induces microbial killing by production of NO, ROS or defensins. Thus, inflammation is an important local response to resolve infection. However, inflammation needs guarded regulation, because excessive amounts of cytokines can be lethal for the host, as in the case of endotoxin shock. Aside from the direct innate immune response, TLR-activated antigen presenting cells (APCs) enhance the expression of surface molecules and, together with antigen-presentation, mediate T cell activation. $CD4^+$ T cells can differentiate into T_H1 or T_H2 cells. Whereas T_H1 cells produce IFNγ and mediate antiviral or antibacterial immunity, T_H2 cells secrete IL-4, IL-13 or both and play a role in allergic reactions or immunity against helminthes. TLR2 activation was found to provoke T_H2 immune responses under certain conditions. Furthermore, LPS can induce T_H2 responses at low dose or when inhaled. Most TLR ligands, however, stimulate APCs to release T_H1-inducing cytokines such as IL-12 and IL-18 supporting T_H1-driven immune responses. In particular, TLR9 signaling activated by CpG DNA has the strongest activity to induce T_H1 cell differentiation [283, 327-330].

1.2.6 Pathophysiological role of TLR2

Besides their crucial role in infectious diseases, much evidence also points to a role for TLRs in further immune and inflammatory disorders and increasingly in cancer. Not only PAMPS, but even also endogenous ligands, so called danger associated molecular patterns (DAMPs),

which may be expressed or accumulated at sites of tissue injury or tissue remodeling might be involved in TLR activation under such conditions. However, recently it was suggested that these molecules mediate their pro-inflammatory effects not by direct stimulation of TLRs, but rather by enhancing the sensitivity of local cells to PAMPs or other inflammatory mediators involved. The repetory of proposed endogenous agonists / assistants for TLR2 includes Hsp60, Hsp70, gp96, HMGB1, minimally modified LDL as well as biglycan [331-333].

TLR2 and noninfectious diseases

TLR2 has been implicated in several inflammatory conditions, and its role in the pathogenesis of disorders like arthritis, inflammatory bowel disease or psoriasis has been supported by reports of elevated TLR2 expression in diseased tissue in patients and responsiveness of respective cell types to TLR2 activation, as well as by reports of TLR2 polymorphisms in humans linked to these diseases [332, 334-338]. Moreover, growing evidence points to a critical role for TLR2 in atherosclerosis. TLR2 expression in endothelial cells from atherosclerotic lesions was shown to be upregulated compared to endothelial cells of normal arteries. Also in atherosclerosis mouse models, atherosclerotic plaques and circulating monocytes exhibited increased expression of TLR2 compared to control mice, and administration of a TLR2/TLR1 ligand increased the disease burden, whereas TLR2 dificiency led to a reduction in ligand induced atherosclerosis and plaque formation or reduced the levels of circulating monocyte chemoattractant protein-1 (MCP-1), which is suggested to play a critical role in the progression of atherosclerosis. Finally, a report suggests a TLR2 polymorphism to be associated with atherosclerosis, but results in this regard yet remain contradictory [339-344]. Furthermore, asthma and atopy have been linked to TLR2 polymorphisms in some human population genetic studies [345, 346], also ischemia / reperfusion injury of the kidney and heart seems to importantly involve TLR2 [347-349], and a role of TLR2 in autoimmune diabetes is under discussion [350]. Finally, there is also some evidence that suggests a role of TLR2 for the development of certain types of cancer. However, it has also been shown that beneficial T cell-dependent immunity to some forms of tumor can depend on the stimulation of TLRs, particularly TLR2, expressed by macrophages and DCs [351-353].

TLR2 as drug target

The essential involvement of TLRs in a variety of diseases has opened the possibility to develop new approches using TLRs as therapeutical targets. TLR ligands are investigated as

adjuvants for the improvement of vaccines, are tested for cancer immunotherapy and seem to rebalance allergic immune responses. TLR antagonists instead could be beneficial for treatment of endotoxin shock as well as inflammatory and autoimmune diseases. Ongoing clinical trials include studies with several agents targeting TLR3, TLR4, TLR7 and TLR9 for various applications [354].

Some approaches targeting TLR2 as well are currently under preclinical and clinical investigation. A TLR2 neutralizing antibody has been developed which demonstrates therapeutic potential for the treatment of sepsis in a murine model [355]. In the field of cancer immunotherapy, a three-component vaccine composed of a TLR2 agonist, together with a peptide T-helper epitope and a tumor-associated glycopeptides was found to elicit high titers of IgG antibodies in mice [356]. In a phase I/II trial, the TLR2/6 agonist MALP-2 was successfully tested as an immunological adjuvant in patients with pancreas carcinomas [357]. Additionally, MALP-2 seems promising as a pharmaceutical agent in the field of allergy [358, 359], as a vaccine adjuvant [360] as well as in the field of wound healing [361].

2 AIM OF THE PRESENT STUDY

Leukotrienes (LTs) as pro-inflammtory mediators play a significant role in promoting inflammatory reactions and immune processes. They have been shown to be released from leukocytes in response to bacterial and viral infections and appear to substantially contribute to an effective immune reaction for host defense [247]. The mechanisms for innate immune pathogen recognition and signaling have been subject of intense research activity since the discovery of the TLR family of pattern recognition receptors approximately 10 years ago. By now it is well established that TLRs play a key role in the detection of invading pathogens and for subsequent initiation of the appropriate immune response [281]. Upon activation, they trigger the activation of signaling pathways such as especially the MAP kinase network, which as well is known to be involved in the regulation of cellular leukotriene biosynthesis.

In this regard, it seemed conceivable that the release of LTs might be regulated in the course of TLR activation. Thus, the present studies were undertaken in order to verify and characterize a possible influence of TLR activation on the biosynthesis of LTs, and furthermore to identify the involved signaling pathways and underlying mechanisms.

Few studies provided a first hint that TLR activation may be linked to LT formation. Thus, bacterial PGN and yeast zymosan, but not *E. coli* LPS or triacylated lipopeptide, had been found to directly induce LTC_4 generation in human mast cells [362]. Furthermore, PGN had been noted to enhance anti-IgE antibody stimulated LTC_4 release in human basophils [363]. The mechanisms, however, had not been studied, respectively. LPS, furthermore, had long been known to prime leukocytes for enhanced LT release upon stimulation [364-367]. It had not yet been asked, though, whether this effect was mediated via TLR4 activation and signaling.

Monocytes / macrophages constitute key actors in host defense, which exert important effector functions like phagocytosis and microbial killing, as well as the modulation of innate immune responses [368]. The present investigations, accordingly, were carried out in various human monocytic cell systems.

3 MATERIALS AND METHODS

3.1 Cells and cell culture

3.1.1 Cell lines

Mono-Mac-6

Mono-Mac-6 is a human cell line which was established from the peripheral blood of a 64-year-old man with acute monocytic leukemia. Mono-Mac-6 cells constitutively express phenotypic and functional features of mature monocytes [369]. Treatment of Mono-Mac-6 cells with hTGFβ_1 and 1α,25-dihydroxyvitamin D_3 leads to expression of 5-LO protein with substantial enzymatic activity [132]. Cells were kindly provided by Prof. Dr. med. Dr. rer. nat. G. Geisslinger, Institute of Clinical Pharmacology, University Hospital Goethe-University Frankfurt.

THP-1

THP-1 is a human cell line which was established from the blood of a 1-year-old boy with acute monocytic leukemia [370]. THP-1 cells are negative for several markers that are specific for mature monocytes [369]. After treatment with hTGFβ_1 and 1α,25-dihydroxyvitamin D_3 substantial 5-LO activity can be measured. Cells were obtained from Prof. Dr. med. J. Pfeilschifter, Institute of General Pharmacology and Toxicology, University Hospital Goethe-University Frankfurt.

3.1.2 Cell culture

Mono-Mac-6 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 µg/mL streptomycin, 100 U/mL penicillin, 1 mM oxaloacetic acid, 1 × MEM non-essential amino acids and 10 µg/mL human insulin (kindly provided by Sanofi-Aventis, Frankfurt a. M.) at 37°C, in an humified atmosphere and at 5% CO_2. Mono Mac 6 cells were seeded at a density of 0.2×10^6 cells. For induction of 5-LO expression cells were differentiated with 1 ng/mL natural hTGFβ_1 (R&D Systems GmbH, Wiesbaden, Germany) and 50 nM 1α,25-dihydroxyvitamin D_3 (Sigma-Aldrich, Schnelldorf, Germany) for 4 days, then harvested by centrifugation (200×g, 10 min, room temperature (RT)) and washed once in PBS pH 7.4.

THP-1 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin at

37°C, in humified atmosphere, and at 5% CO_2. THP-1 cells were also seeded at a density of 0.2×10^6 cells. Also THP-1 cells were treated with 1 ng/mL natural hTGFβ_1 and 50 nM 1α,25-dihydroxyvitamin D_3 for 4 days, then harvested by centrifugation (200×g, 10 min, RT) and washed once in PBS pH 7.4.

3.1.3 Isolation of human PBMC from buffy coats

Human peripheral blood mononuclear cells (hPBMC) were freshly isolated from buffy coats obtained from Municipal Hospital Frankfurt-Hoechst as follows. Buffy coats were diluted with PBS pH 7.4 and erythrocytes were depleted by dextran sedimentation (dextran 1% (w/v), 30 min). The supernatant was subjected to density centrifugation (LSM 1077 Lymphocyte Separation Medium, PAA Laboratories GmbH, 800×g, 10 min, RT). The mononuclear cells including lymphocytes and monocytes appear as a layer on the separation medium after centrifugation. Human PBMC were washed twice with PBS pH 7.4 and the monocyte content was elevated by adherence in RPMI 1640 medium supplemented with 2 mM L-glutamine and 25% human plasma for 3 hours (37°C, 5% CO_2). Thus, lymphocytes in suspension were removed, and adhered monocytes were gently detached, resuspended in PBS pH 7.4 and harvested by centrifugation (200×g, 10 min, RT). Cell types were kindly determined by Dr. Ulrike Koehl, Pediatric Hematology and Oncology, University Hospital Goethe-University Frankfurt, by FACS analysis: CD14+ monocytes 30.2-52.9%, CD19+ B lymphocytes 3.6-5.1%, CD3+CD56+ NK cells 7.5-9.0%, CD3+ T lymphocytes 25.2-43.8%.

3.2 Determination of metabolites of the 5-LO pathway

3.2.1 Cellular leukotriene biosynthesis

Differentiated MM6 cells (3×10^6), differentiated THP-1 cells (3×10^6) or hPBMC (5×10^6) were resuspended in 1 mL endotoxin free PGC buffer (PBS containing 1 mg/mL glucose and 1 mM $CaCl_2$). In some experiments cells were pre-treated with inhibitors at 37°C for the indicated times. In the majority of cases cells were primed with TLR ligands (InvivoGen, Toulouse, France) or with PMA (Calbiochem, Merck, Darmstadt, Germany) as indicated. Then cellular leukotriene formation was induced by addition of ionophore A23187 or fMLP (both from Sigma-Aldrich, Schnelldorf, Germany) at the indicated concentrations. Alternatively, for determination of cellular 5-LO activity, leukotriene formation was induced by ionophore A 23187 in presence of exogenous AA (20-40 µM), in order to circumvent endogenous AA supply and to render biosynthesis of leukotrienes independent from PLA_2

activity. For purified 5-LO enzyme in the presence of Ca^{2+} it was described that maximum amounts of 5-LO products are formed within 6-8 min, remaining constant until ~13-15 min [127], implying that a 10 min incubation is suitable to assess cellular 5-LO activity. Thus, after 10 minutes at 37°C, the reaction was stopped with 1 mL methanol.

3.2.2 Product formation of recombinant 5-LO (test of a potential 5-LO inhibitor)

Recombinant 5-LO proteins, expressed in *E. coli* BL21 and purified using a ATP-agarose column as described previously [127], were kindly provided by Ann-Kathrin Haefner / Prof. Dr. D. Steinhilber, Institute for Pharmaceutical Chemistry, Goethe-University Frankfurt. 5-LO enzyme (corresponding to an activity of 1 µg 5-LO products per amount of protein) was resuspended in 1 mL PBS containing 1mM EDTA and 1 mM ATP. After pre-incubation of the potential 5-LO inhibitor (test of different concentrations as indicated) for 15 minutes at 4°C, the samples were pre-warmed for 30 s at 37°C and the 5-LO reaction was started by the addition of 20 µM AA in presence of 2 mM Ca_2Cl. After 10 minutes at 37°C, the reaction was stopped with 1 mL methanol.

3.2.3 Solid phase extraction and HPLC

For further sample preparation, 30 µL 1N HCl, 500 µL PBS and 200 ng prostaglandin B_1 as internal standard were added. After centrifugation (800×g, 10 min, RT) the samples were applied to C-18 solid-phase extraction columns (Clean-up® Extraction Columns from UCT, Bristol, PA, USA), preconditioned with 1 mL methanol and 1 mL water. The columns were washed with 1 mL water and 1 mL methanol 75% (v/v). Subsequently, metabolites of the 5-LO pathway were eluted with 300 µL methanol, diluted with 120 µL water and analyzed by HPLC as described [371] using a C-18 RP column (Novapak C-18 Radial-Pak column, 100 mm 5 mm I.D., 4 µm, Waters). The mobile phase was composed of methanol 72% (v/v), water 28% (v/v) and TFA 0.007% (v/v). Metabolites were detected at 280 nm for the first 8 min, then at 235 nm for the remaining 22 min. The flow was 1.2 mL/min. In samples derived from intact cells, product formation is expressed as ng 5-LO products per 10^6 cells, in samples derived from recombinant 5-LO, activity is measured as ng 5-LO products per amount of protein and inhibition of 5-LO activity is expressed as % of the control without inhibitor. Total 5-LO products include leukotriene B_4 (LTB_4; only in samples derived from intact cells), its all-*trans* isomers and 5(S)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE). Cysteinyl-LTs were not detected and oxidation products of LTB_4 were not determined.

3.3 Determination of [^{3}H]AA release

MM6 cells were resuspended at 2 × 10^6/mL in pre-warmed RPMI 1640 medium containing 0.5 µCi/mL [^{3}H]AA (specific activity 7.4 MBq/µmol, from Biotrend, Cologne, Germany), and incubated for 3 hours at 37°C, 5% CO_2. Thereafter, cells were collected by centrifugation (1000×g, 10 min, RT), washed with PBS containing 2 mg/mL low endotoxin fatty acid free bovine serum albumin (FAF-BSA, Sigma-Aldrich, Schnelldorf, Germany; ≤ 0.1 ng/mg endotoxin according to manufacturer´s specification) to remove unincorporated [^{3}H]AA, and resuspended in PGC buffer / FAF-BSA at 3 × 10^6/0.5 mL or 2 × 10^5/0.2 mL. After labeling, MM6 cells were in some experiments pre-treated with inhibitors and in the majority of cases primed with TLR ligands as indicated. [^{3}H]AA release was induced by the addition of ionophore A23187 2.5 µM for 10 min at 37°C unless otherwise noted. To stop the reaction, samples were placed on ice. After centrifugation (200×g, 5 min, 4°C), 100 µL of the supernatants were mixed with 3 mL of a liquid scintillation cocktail (Optiphase Hisafe 3, Perkin Elmer, Shelton, CT, USA), and analyzed with the Wallac 1409 liquid scintillation counter (Perkin Elmer). [^{3}H]AA release is expressed as decays per minute (dpm) per 10^6 cells.

3.4 Calcium imaging

MM6 cells (3 × 10^7/mL) were resuspended in PBS containing 1 mg/mL glucose and incubated with 2 µM Fura-2/AM for 30 min at 37°C. After washing, 5 × 10^6 cells/mL PBS (containing 1 mg/mL glucose) were pre-warmed in a thermally controlled (37°C) fluorometer cuvette in a spectrofluorometer (Aminco-Bowman series 2; Thermo Electron Corporation, Waltham, MA, USA) with continuous stirring. Then, 1 mM Ca^{2+} was added and 2 min later measurement was started. After 30 s, cells were stimulated with TLR2 ligands, PMA or ionophore A23187 as indicated. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively, and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [372]. F_{max} (maximal fluorescence) was determined after lysing the cells with 1% Triton X-100 (w/v) and F_{min} after chelating Ca^{2+} with 10 mM EDTA.

3.5 Determination of protein phosphorylation

3.5.1 Incubation and sample preparation

MM6 cells (3 × 10^6) were resuspended in 100 µl PGC buffer. For time course analyses, cells were stimulated with TLR ligands for different periods of time at 37°C as indicated. Alternatively, MM6 cells were stimulated with TLR ligands or ionophore A18237 for 15

minutes at 37°C. In some experiments, cells were pre-treated with inhibitors as indicated before stimulation. Incubations were stopped by addition of 100 µL of 2× SDS-PAGE sample loading buffer (20mM Tris / HCl pH 8.0, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol) and placed on ice. Samples were heated at 95°C for 6 minutes and subsequently sonified (10 seconds).

3.5.2 SDS PAGE and Western Blot

Total cell lysates corresponding to 0.3×10^6 cells in 20 µL were mixed with 3 µL of glycerol / 0.1% bromophenol blue (1:1, v/v) and analyzed by SDS-PAGE using a Mini Protean system (Bio-Rad, Munich, Germany) on a 10% gel. After electroblot on nitrocellulose membrane (Amersham Hybond C, GE Healthcare Europe GmbH, Freiburg, Germany) and blocking with Odyssey® Blocking Buffer (*LI-COR*® Biosciences, Bad Homburg, Germany) for 1 hour / RT, membranes were incubated with primary antibodies overnight at 4°C.

Primary Antibodies

Description	source	dilution	producer
phospho-p38 MAPK (Thr180/Tyr182)	rabbit pAby	1:1000	NEB, Frankfurt a.M.
p38 MAPK (C-20)	goat pAby	1:1000	Santa Cruz, Heidelberg
phospho-5-LO (Ser271)	rabbit pAby	1:1000	NEB, Frankfurt a.M.
5-LO (catalytic domain)	mouse mAby, IgG1 (clone 6A12)	1:100	Prof. Dr. D. Steinhilber, Inst. f. Pharm. Chem., Goethe-University, Frankfurt a.M.
phospho-p44/p42 MAPK (Thr202/Tyr204)	mouse mAby; IgG1 (E10)	1:1000	NEB, Frankfurt a.M.
p44/42 MAPK	rabbit pAby	1:1000	NEB, Frankfurt a.M.
phospho-Mnk-1 (Thr197/202)	rabbit pAby	1:1000	NEB, Frankfurt a.M.
Mnk-1 (C-20)	goat pAby	1:200	Santa Cruz, Heidelberg
phospho-cPLA$_2$ (Ser505)	rabbit pAby	1:1000	NEB, Frankfurt a.M.
cPLA$_2$ (C-20)	goat pAby	1:200	Santa Cruz, Heidelberg
IκB-α	rabbit pAby	1:1000	NEB, Frankfurt a.M.

The respective antibody solutions contained an antibody specific for the phosphorylated form of the investigated protein, together with a second antibody, detecting the unphosphorylated and the phosphorylated form of the protein (derived from different species). Membranes were washed with PBS pH 7.4 / Tween 0.1% (v/v) and incubated with infrared dye conjugated secondary antibodies (IRDye®, *LI-COR*® Biosciences) for 45min / RT under protection from light. Two differentially labeled secondary antibodies (emission wavelength 680 nm and 800

nm, respectively) were used for simultaneous detection of the phosphorylated form and the total amount of the investigated protein.

Secondary Antibodies

Description	source	dilution	producer
IRDye® 800CW	donkey anti-rabbit IgG	1:5000	*LI-COR*®, Bad Homburg
IRDye® 680	donkey anti-goat IgG	1:5000	*LI-COR*®, Bad Homburg
IRDye® 800CW	goat anti-rabbit IgG	1:5000	*LI-COR*®, Bad Homburg
IRDye® 680	goat anti-mouse IgG	1:5000	*LI-COR*®, Bad Homburg

Membranes were washed with PBS pH 7.4 / Tween 0.1% (v/v), and finally with PBS pH 7.4. Visualization and quantitative analysis of protein bands was carried out with the Odyssey® Infrared Imaging System (*LI-COR*® Biosciences). For quantification, the intensities of bands representing the phosphorylated protein were corrected by band intensities of the total protein (loading control). Enhancement of phosphorylation was calculated by relating band intensities received after cell stimulation, to band intensities derived from unstimulated cell samples.

3.6 5-LO translocation assay

3.6.1 Preparation of nuclear and nonnuclear cellular fractions

MM6 cells ($5 – 10 \times 10^6$) were resuspended in 1mL PGC buffer and incubated as indicated at 37°C. In several cases, cells were primed with TLR2 ligands or with PMA for 5-15 min before stimulation with ionophore A18237 for 5-10 min, in one of these experimental series the incubations were carried out in presence of p38 MAPK inhibitor SB203580. Otherwise, cells were separately treated with TLR2 ligands or ionophore A18237 at the indicated conditions. After incubation, the samples were chilled on ice for 5 min and centrifuged (200×g, 5 min, 4°C). Pellets were then suspended in 300 µL ice-cold NP-40-lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 60 µg/mL STI and 10 µg/mL leupeptin), vortexed (3×5 s), kept on ice for 10 min and centrifuged (800×g, 10 min, 4°C). Resulting supernatants containing cytosol, plasma membrane, ER, Golgi apparatus and cytoskeletal proteins (nonnuclear fractions) were transferred to a new tube, and pellets containing intact nuclei (nuclear fractions) were resuspended in 300 µL ice-cold relaxation buffer (50 mM Tris-HCl pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 µg/mL STI and 10 µg/mL leupeptin). Both nuclear and nonnuclear fractions were centrifuged again (800×g, 10 min,

4°C) for further purification. Nuclei in relaxation buffer were disrupted by sonication (3 × 5 s). Aliquots of nuclear and nonnuclear fractions were mixed with the same volume of 2× SDS-PAGE sample loading buffer (20mM Tris / HCl pH 8.0, 2 mM EDTA, 5% SDS (w/v), 10% β-mercaptoethanol) and heated at 95°C for 6 min.

3.6.2 Preparation of membrane and cytosolic fractions

MM6 cells (5×10^6) were resuspended in 1mL PGC buffer and primed with TLR2 ligands or with PMA for 15 min / 37°C at the indicated concentrations before stimulation with 2.5 µM ionophore A18237 for another 10 min at 37°C. Then the samples were chilled on ice for 5 min and centrifuged (200×g, 5 min, 4°C). Pellets were suspended in 300 µL ice-cold NP-40-lysis buffer, sonicated for 8 s, kept on ice for 10 min and 275 µL of this mixture were subjected to an ultracentrifugation step (100,000×g, 70 min, 4°C). Resulting supernatants containing cytosolic proteins (cytosolic fractions) were transferred to a new tube, and pellets containing cellular membranes (membrane fractions) were resuspended in 275 µL ice-cold relaxation buffer and resuspended by sonication (10 s). Aliquots of cytosolic and membrane fractions were mixed with the same volume of 2× SDS-PAGE sample loading buffer and heated at 95°C for 6 min.

3.6.3 Analysis and assay control by SDS PAGE and Western Blot

Aliquots (20 µL) of the subcellular fractions were mixed with 3 µL of glycerol / 0.1% bromophenol blue (1:1, v/v) and pair-wise samples (nonnuclear / nuclear or cytosolic / membrane) corresponding to identical cell numbers were processed separately by SDS PAGE on a 10% gel and subsequent electroblot on nitrocellulose membrane. For analyses with the Odyssey® Infrared Imaging System, blocking was carried out with the Odyssey® Blocking Buffer, in case of protein visualization by alkaline phosphatase reaction, blocking was done with 5% nonfat dry milk in TBS (50 mM Tris-HCl pH 7.4 and 100 mM NaCl) for 1 hour / RT. Then, membranes were washed with TBS and incubated with the 5-LO antibody overnight at 4°C.

Primary Antibodies

Description	source	dilution	producer
5-LO (catalytic domain)	mouse mAby, IgG1 (clone 6A12)	1:100	Inst. f. Pharm. Chem., Frankfurt a.M.
GAPDH	rabbit mAby, IgG (14C10)	1:2000	NEB, Frankfurt a.M.
Lamin A/C	rabbit pAby	1:1000	NEB, Frankfurt a.M.

Primary antibody solutions contained two additional antibodies detecting the cytosolic marker protein GAPDH and also lamin A/C, which are ubiquitous proteins exclusively present in the nuclear membrane. By the analysis of both markers in each of the subcellular fractions, the samples were tested for correct fractionation. Furthermore, these marker proteins were used as loading controls in the respective subcellular fractions. In case of infrared imaging, membranes were washed with PBS pH 7.4 / Tween 0.1% (v/v) and incubated with infrared dye conjugated secondary antibodies for 45min / RT under protection from light. Membranes were washed with PBS pH 7.4 / Tween 0.1% (v/v), finally with PBS pH 7.4, and were analyzed with the Odyssey® Infrared Imaging System. For quantification, band intensities of the 5-LO protein were corrected by band intensities of the loading control. Results are given as relative band intensities in the respective fraction after cell incubation, related to band intensities detected in the respective fraction of untreated cells.

Secondary Antibodies

Description	source	dilution	producer
IRDye® 800CW	donkey anti-mouse IgG	1:5000	*LI-COR*®, Bad Homburg
IRDye® 680	donkey anti-rabbit IgG	1:5000	*LI-COR*®, Bad Homburg
Anti-mouse IgG (whole molecule) – alkaline phosphatase	goat	1:1000	Sigma, Schnelldorf

Otherwise, after incubation of the primary antibody, membranes were washed with TBS and incubated with alkaline phosphatase-conjugated anti-mouse IgG for 2 h / RT. After washing with TBS and TBS plus 0.1% NP40, 5-LO protein was visualized with the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$).

3.7 Statistics

Results are given as mean + S.D., $n \geq 3$, except as noted otherwise. Statistical analysis was carried out by Student's unpaired t test (one-tailed), except as noted otherwise. Differences were considered as significant for $p<0.05$ (indicated as * $p<0.05$ or ** $p<0.01$). IC_{50} values were determined by non-linear regression using GraphPad Prism 5.0 and a sigmoid concentration – response model.

4 RESULTS

4.1 The influence of TLR ligands on leukotriene biosynthesis in human monocytes

4.1.1 Enhancement of leukotriene biosynthesis by TLR ligands in MM6 cells

In order to evaluate the effects of TLR ligands on LT biosynthesis in human monocytes, several activators of different TLR subtypes were screened for their impact on 5-LO product formation in differentiated MM6 cells. Therefore, cells were pre-incubated with commonly used concentrations of the respective TLR ligands and subsequently stimulated for leukotriene formation with calcium ionophore A23187. The two different types of CpG ODN as TLR9 activators, TLR7 ligand Loxoribine and TLR3 ligand Poly (I:C) did not show any impact on the biosynthesis of 5-LO metabolites. As expected due to former publications, pre-incubation with LPS, which is an activator of TLR4, enhanced the biosynthesis of 5-LO products. However, also the TLR2/6 ligands LTA and FSL-1, as well as TLR2/1 ligand Pam_3CSK_4 led to an approximate twofold increase of ionophore-induced 5-LO product formation.

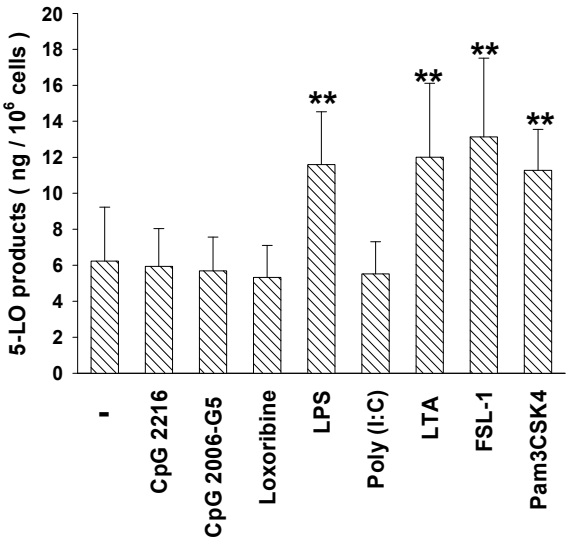


*Figure 7: Enhancement of 5-LO product formation by TLR ligands in differentiated MM6 cells. Differentiated MM6 (3×10^6 cells) were primed with TLR ligand or solvent as indicated 20 min / 37°C (CpG 2216 1 µM and CpG 2006-G5 1 µM, TLR9; Loxoribine 100 µM, TLR 7; LPS 10 µg/mL, TLR4; Poly (I:C) 25 µg/mL, TLR3; LTA 2 µg/mL, TLR2/6; FSL-1 1 µg/mL, TLR2/6; Pam_3CSK_4 1 µg/mL, TLR2/1) before stimulation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC. Student´s t test; **p<0.01;*

4.1.2 Characterization of TLR2 ligand mediated priming of MM6 cells

The following studies focus on TLR2 ligand induced effects and provide a more detailed characterization of the observed enhancement of 5-LO product formation in MM6 cells.

Determination of the optimal ionophore concentration

In order to determine the optimal concentration of calcium ionophore within the experimental setting, 5-LO product formation was stimulated with 0.1 to 5 µM of A23187 after pre-incubation with the three different TLR2 ligands, respectively. Relative 5-LO product formation was augmented with increasing concentrations of ionophore up to 2.5 µM, which was defined as optimal concentration for subsequent experiments. With a further increase of ionophore concentrations beyond 2.5 µM, the enhancement of 5-LO product formation by TLR2 ligands was diminished again.

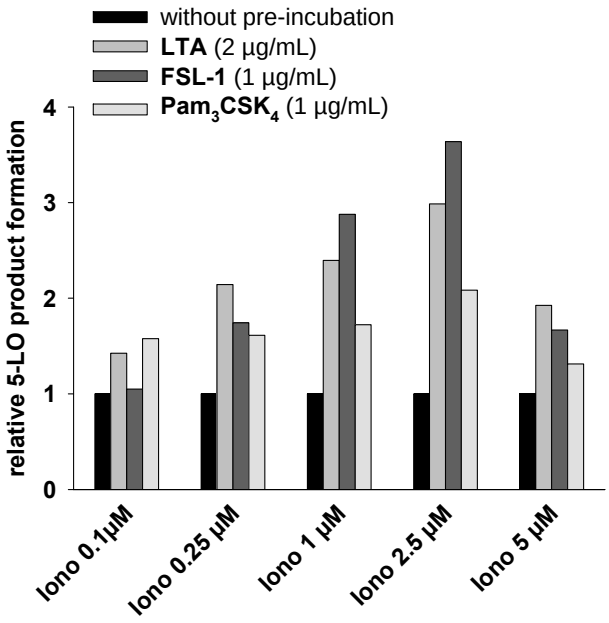


Figure 8: Determination of the optimal ionophore concentration. Differentiated MM6 (3 × 10^6 cells) were pre-incubated with TLR ligands or solvent as indicated 20 min / 37°C before stimulation of 5-LO product formation with different concentrations of ionophore A23187 (Iono) for another 10 min / 37°C. 5-LO products were determined by HPLC. Relative increase of products was calculated in relation to product formation without TLR ligand pre-incubation, respectively. The data represent one out of two experiments.

Determination of the optimal TLR2 ligand concentration

Next, further dose response characteristics were determined. MM6 cells were pre-treated with increasing concentrations of LTA, FSL-1 or Pam_3CSK_4 before stimulation with ionophore.

Each of the ligands showed concentration-dependent effects. LTA and FSL-1 exhibited a maximum effect at 0.5 µg/mL, followed by a decreasing response beyond this concentration. Only at very high concentrations of LTA and FSL-1, a second enhancement of 5-LO metabolites could be detected. In contrast, Pam_3CSK_4 consistently enhanced cellular LT formation with increasing concentrations. 5 µg/mL Pam_3CSK_4 evoked a comparable increase of 5-LO products as the other two ligands at 0.5 µg/mL, suggesting a weaker potency of Pam_3CSK_4. LTA 0.5 µg/mL, FSL-1 0.5 µg/mL and Pam_3CSK_4 5 µg/mL were regarded as optimal concentrations for the following studies.

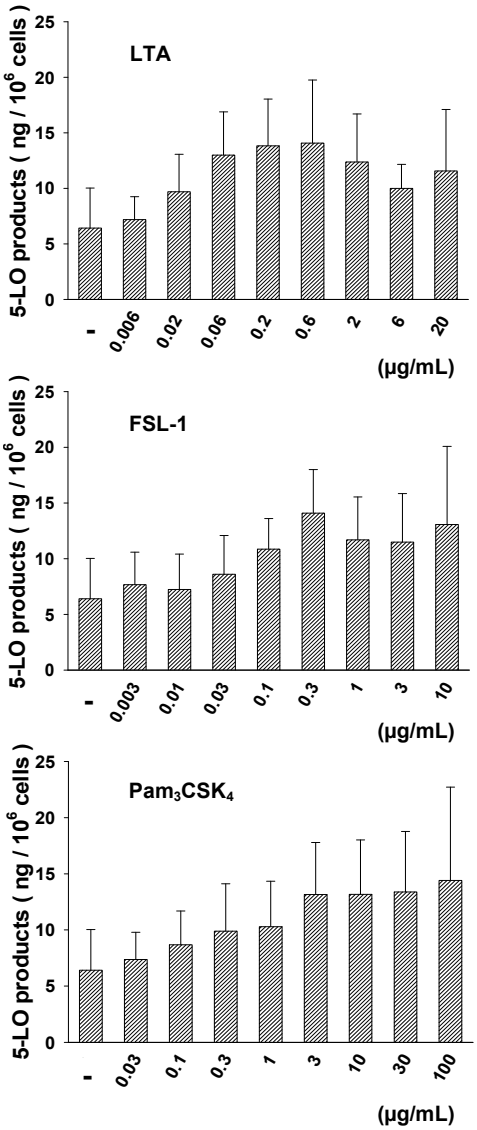


Figure 9: Determination of the optimal TLR2 ligand concentration. Differentiated MM6 (3 × 10^6 cells) were pre-incubated with solvent or TLR ligands at the indicated concentrations 20 min / 37°C before stimulation of 5-LO product formation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

Time dependence of TLR2 ligand-induced effects

For further characterization, time course experiments were performed. Therefore, cells were pre-incubated with TLR2 ligands for different periods of time before ionophore stimulation. All ligands showed comparable time-dependent characteristics. The maximum response was obtained with a pre-incubation time of 15 minutes. However, the effect disappeared when pre-incubation was further extended up to 90 minutes. Pre-incubation of the ligands was essential, as no enhancement of LT formation was detectable when MM6 cells were treated with TLR2 ligands and ionophore simultaneously. These observations indicated that TLR2 ligands seemed to act as priming agents on human MM6 cells for an enhanced response to ionophore stimulation, but did not seem to directly act as costimuli.

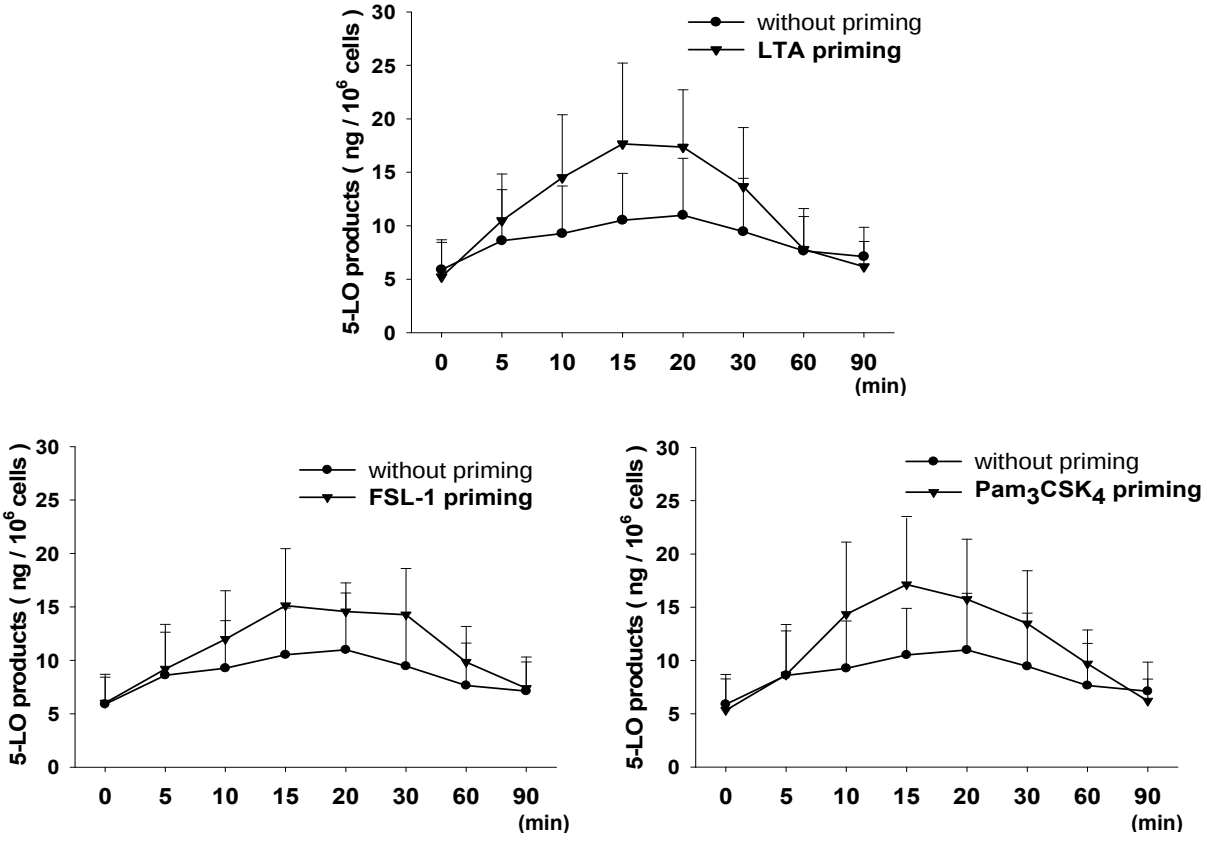


Figure 10: Time dependence of TLR2 ligand-induced effects. Differentiated MM6 (3 × 10^6 cells) were pre-incubated with solvent or TLR ligands at their optimal concentrations (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) for different periods of time / 37°C before stimulation of 5-LO product formation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

TLR2 ligand priming: a synergistic mechanism

The aim of the following experiment was to further clarify, if TLR2 ligands are able to directly stimulate the formation of LTs. Within the optimal time frames of incubation, the effects of the three TLR2 ligands and of ionophore were investigated separately, and were then compared to the effects evoked by the optimal combination of the agents. Ionophore stimulation of MM6 cells for 10 minutes after 15 minutes of pre-incubation with solvent led to a distinct, but low 5-LO product formation (approximately 1.2 ng 5-LO products per 10^6 cells). The response after incubation of MM6 cells with LTA and FSL-1 for 15 minutes with a subsequent incubation period of 10 minutes without ionophore was marginal, and Pam_3CSK_4 only weakly induced a slight direct response. Thus, LT formation upon TLR2 ligand incubation is too low to characterize them as direct stimulators of the LT biosynthesis pathway. However, after combination of at least LTA or FSL-1 with ionophore, the extent of 5-LO product formation was higher than the sum of the single effects of the ligands and of ionophore, respectively (up to 4.5 ng 5-LO products per 10^6 cells). From this it was concluded that these TLR2 ligands in a synergistic manner prime MM6 cells for enhanced biosynthesis of 5-LO metabolites.

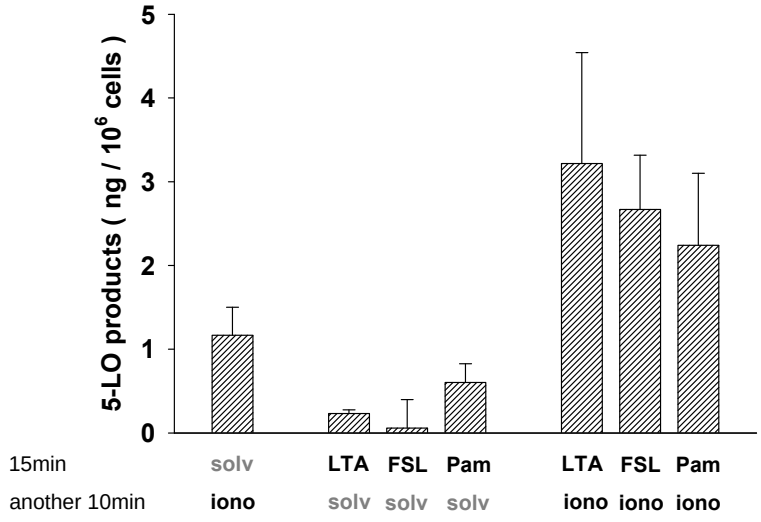


Figure 11: TLR2 ligand priming: a synergistic mechanism. Differentiated MM6 (3×10^6 cells) were incubated with solvent or TLR ligands as indicated (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) at 37°C. After 15 min, ionophore A23187 2.5 µM or solvent was added for another 10 min / 37°C. 5-LO product formation was determined by HPLC.

4.1.3 Confirmation of the priming effects of TLR2 ligands in hPBMC

In order to confirm the observed effects of TLR2 ligands in MM6 cells and to exclude cell-line specific effects, human primary cells were tested under analogous conditions. Human peripheral blood mononuclear cells (hPBMC) were primed with TLR2 ligands followed by stimulation with calcium ionophore. A significant increase of 5-LO products was detected when hPBMC were stimulated with 0.025 µM of ionophore after priming. Preliminary tests had revealed that the use of high concentrations of calcium ionophore (2.5µM) indeed led to substantial 5-LO product formation, which was not significantly enhanced by TLR2 ligands though. In a further experiment, *N*-formyl-leucyl-phenylalanine (fMLP) at 1µM was used as a physiological stimulus. In analogy, TLR2 activators led to a significant enhancement of 5-LO product release after fMLP stimulation. Taken together, TLR2 ligands showed a similar impact on hPBMC as on MM6 cells, particularly upon weak stimulation.

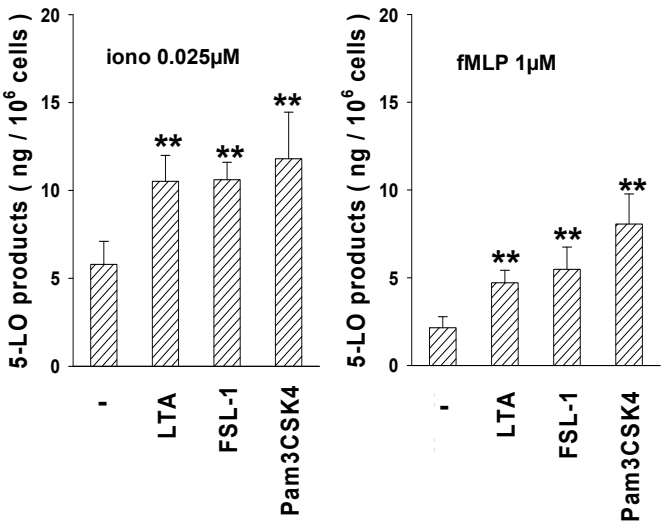


*Figure 12: Confirmation of the priming effects of TLR2 ligands in hPBMC. PBMC (5 × 10^6 cells) were primed with TLR2 ligands or solvent (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) for 15 min/37°C. Then 5-LO product formation was induced by ionophore A23187 (iono) 0.025 µM or fMLP 1 µM for another 10min / 37°C. 5-LO products were determined by HPLC. Student´s t test; ** p<0.01;*

4.1.4 Priming activities of further TLR ligands in MM6 cells

The following tests complement initial screening experiments and test another two TLR ligands for their priming properties in MM6 cells. After pre-incubation with TLR5 ligand flagellin or TLR8 ligand ssPolyU, cells were stimulated with ionophore. As a positive control,

phorbol-12-myristate-13-acetate (PMA) was used, which is known as a potent priming agent in MM6 cells that enhances 5-LO product formation upon ionophore stimulation [170, 373]. As it is shown in the figure below, also flagellin led to a significant increase of 5-LO metabolites in MM6 cells. In contrast, for TLR8 activator ssPolyU no clear priming effect was detected.

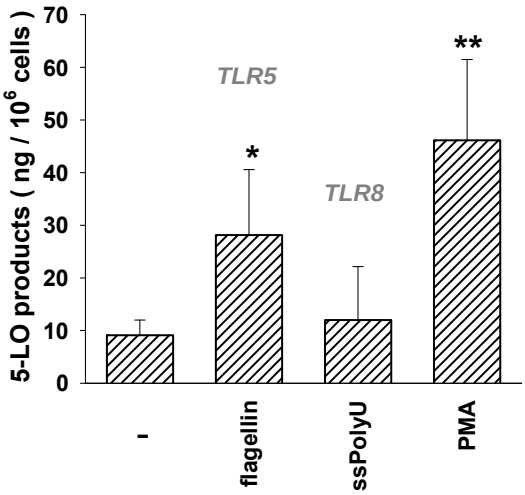


*Figure 13: Priming activities of further TLR ligands in MM6 cells. Differentiated MM6 (3 × 10^6 cells) were primed with solvent, PMA 100 nM or TLR ligand (flagellin 5 µg/mL, TLR5; ssPolyU/LyoVec 5µg/mL, TLR8) as indicated 15 min / 37°C before stimulation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC. Student´s t test; ** p<0.01, * p<0.05;*

4.2 The influence of TLR ligands on 5-lipoxygenase in human monocytes

After description and characterization of TLR ligand induced priming of human monocytic cells for enhanced 5-LO product formation, the following studies concentrate on understanding the underlying mechanisms of the observed effects.
At first, the influence of TLR ligands on 5-LO, the key enzyme in the biosynthesis of leukotrienes, was investigated in detail.

4.2.1 The influence of TLR ligands on 5-lipoxygenase activity

The aim of subsequent investigations was to elucidate if TLR ligands have an impact on cellular 5-LO activity, which may account for enhanced leukotriene formation after priming. For this purpose, cells were stimulated in presence of an overabundant amount of exogenous AA, respectively. Under such conditions, formation of 5-LO metabolites is no longer dependent on endogenous AA release and 5-LO activity can be measured irrespectively of PLA_2 activity.

Stimulation of 5-LO activity by TLR ligands in MM6 cells

In order to find out if TLR ligands can stimulate 5-LO activity in MM6, cells were pre-incubated with LTA as a representative of TLR2 ligands or with flagellin, before an excess of exogenous AA was added for another 10 minutes. Compared to the control, neither LTA nor flagellin further stimulated 5-LO activity in MM6 cells. In fact, LTA even showed an inhibitory effect in this experimental setting.

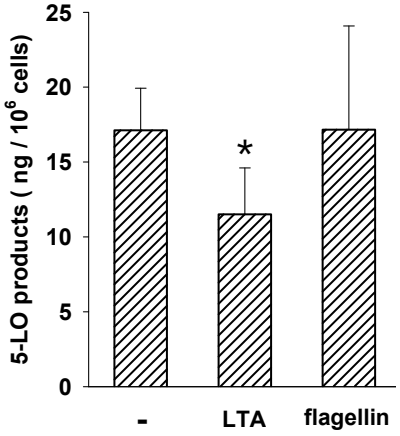


*Figure 14: Direct stimulation of 5-LO activity by TLR ligands in MM6 cells. Differentiated MM6 (3×10^6 cells) were pre-incubated with solvent, LTA 0.5 µg/mL or flagellin 5 µg/mL 15 min / 37°C. Then AA 40 µM was added for another 10 min / 37°C. 5-LO product formation was determined by HPLC. Student´s t test; * $p<0.05$;*

Priming effect on 5-LO activity by TLR ligands in MM6 cells and THP-1 cells

Next, TLR ligand priming for an increase of ionophore-stimulated 5-LO activity was analyzed in MM6 cells, and in THP-1 cells as a second monocytic cell line. MM6 or THP-1 cells were primed with LTA, flagellin or ssPolyU, which had not been identified as a priming agent, and then stimulated with ionophore in presence of exogenous AA. Biosynthesis of 5-HETE, which is independent from LTA_4 hydrolase activity and also independent from subcellular localization of 5-LO, was used as a readout for 5-LO activity in these experiments. None of the TLR ligands led to an increase of 5-LO activity, neither in MM6 cells nor in THP-1 cells. Repeatedly, a slight trend to an inhibitory influence of TLR ligands was noticed in MM6 cells.

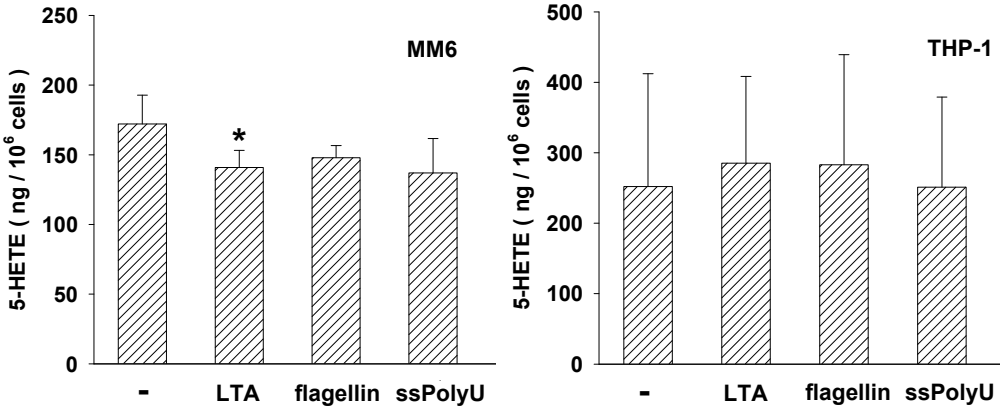


*Figure 15: Priming effect on 5-LO activity by TLR ligands in monocytic cell lines. Differentiated MM6 (3×10^6 cells) or differentiated THP-1 cells (3×10^6 cells) were primed with solvent, LTA 0.5 µg/mL, flagellin 5 µg/mL or ssPolyU/LyoVec 5µg/mL as indicated 15 min / 37°C. Then ionophore A23187 2.5 µM plus AA 40 µM were added for another 10 min / 37°C. 5-HETE formation was determined by HPLC. Student´s t test; * $p<0.05$;*

Priming effect on 5-LO activity by TLR2 ligands and PMA in MM6 cells

In addition to the antecedent data, all of the three TLR2 ligands were tested for a potential enhancement of 5-LO activity under analogous conditions. In a second experiment, priming properties of LTA were compared to PMA-mediated priming. LTA, FSL-1 and Pam_3CSK_4 did not show an enhancing impact on 5-LO activity in MM6 cells. PMA, however, led to a significant increase of 5-LO product formation, as it was expected on the basis of corresponding publications, which report an increase of 5-LO activity after PMA priming [170, 373]. By comparison, LTA seemed to marginally inhibit 5-LO activity by trend.

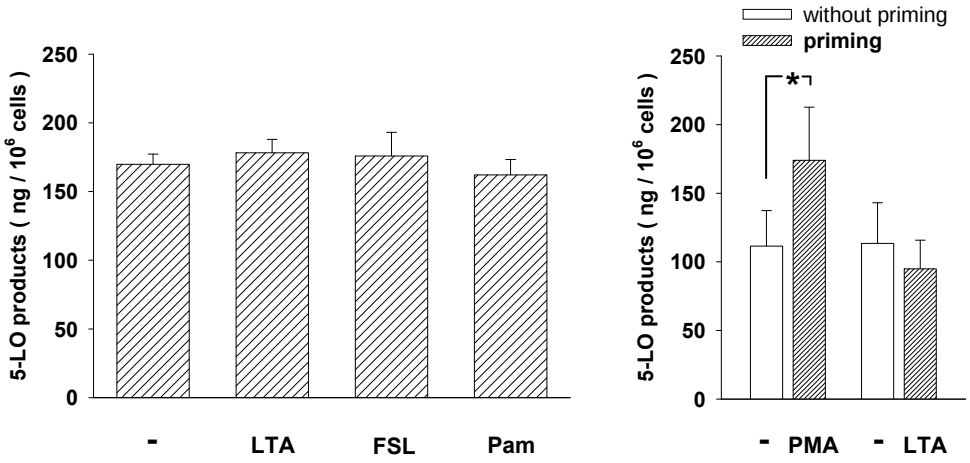


*Figure 16: Priming effect on 5-LO activity by TLR2 ligands in MM6 cells. Differentiated MM6 (3 × 10^6 cells) were primed with solvent, PMA 100 nM or TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) as indicated 15 min / 37°C. Then ionophore A23187 2.5 µM and AA 20 µM were added for another 10 min / 37°C. 5-LO product formation was determined by HPLC. Student´s t test; * p<0.05;*

4.2.2 Influence of TLR2 ligands on 5-lipoxygenase translocation in MM6 cells

The regulation of the subcellular distribution of 5-LO plays a crucial role in cellular LT formation from endogenous AA. Upon cell stimulation, 5-LO is activated and essentially translocates to the nuclear envelope, where a biosynthetic complex is assembled that facilitates AA release from membrane phospholipids by $cPLA_2$ and subsequent transfer of AA to 5-LO via FLAP [16, 374].

The influence of TLR2 ligands on subcellular 5-LO localization was investigated in MM6 cells. The aim was to analyze if the increase of LT formation after priming can be traced back to enhanced 5-LO translocation to a membrane compartment, where the enzyme would have a greater access to free AA.

Influence of LTA on 5-LO translocation

The following translocation assays test TLR2 ligand LTA for its impact on subcellular distribution of 5-LO. Furthermore, the results compare the properties of LTA and PMA, which is known to enhance ionophore-induced 5-LO translocation in MM6 cells [373]. Cells were incubated with ionophore or with LTA, or they were primed with LTA or PMA before stimulation with ionophore. For each sample a nuclear fraction containing intact nuclei, and a nonnuclear fraction containing cytosol, plasma membrane, ER, Golgi apparatus and

cytoskeletal proteins were prepared from the cells. The subcellular fractions were then analyzed separately by 5-LO immunoblotting.

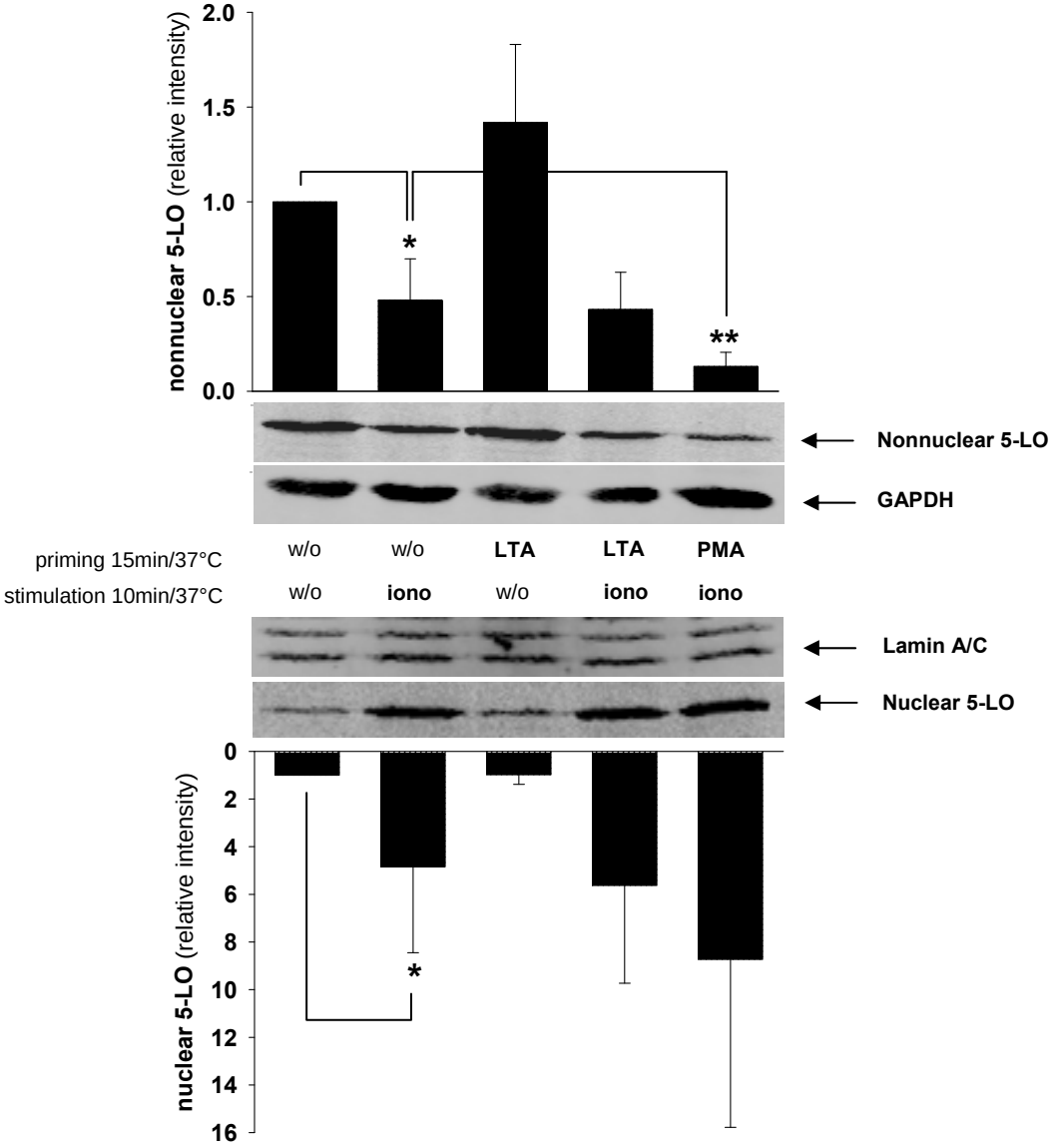


*Figure 17: Influence of LTA on 5-LO translocation. Differentiated MM6 (10 × 10^6 cells) were primed with solvent, PMA 100nM or LTA 0.5 µg/mL as indicated 15 min / 37°C. Then, solvent or ionophore A23187 2.5 µM was added for another 5 min / 37°C. After cell lysis, nonnuclear and nuclear fractions were separated by centrifugation and analyzed separately by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System). For quantification, band intensities of 5-LO were corrected by band intensities of specific loading controls for the nonnuclear (GAPDH) and the nuclear fraction (Lamin A/C), respectively. The amount of 5-LO protein is related to the level of 5-LO protein in the negative control. Pair-wise samples (nonnuclear, nuclear) correspond to identical cell numbers. Student´s t test (paired; one-tailed); ** p<0.01, * p<0.05;*

Quantification of 5-LO band intensities in the respective fractions revealed that in resting cells 5-LO was mainly located in the nonnuclear fraction, shown by a strong band in the nonnuclear and a weak band in the nuclear fraction. After stimulation with ionophore, 5-LO partially translocated from the nonnuclear fraction to the nucleus. The ionophore induced shift of 5-LO protein to the nuclear fraction was considerably enhanced by PMA priming, as it was shown in prior studies [373]. In contrast, after LTA priming no significant enhancement of ionophore induced 5-LO translocation was detected and subcellular distribution of 5-LO in primed and in unprimed cells was similar. The direct impact of LTA on 5-LO localization was investigated as well. Interestingly, a tendency towards an oppositional distribution of 5-LO was observed. After incubation of MM6 cells with LTA, an insignificant increase of 5-LO protein in the nonnuclear fraction was detected.

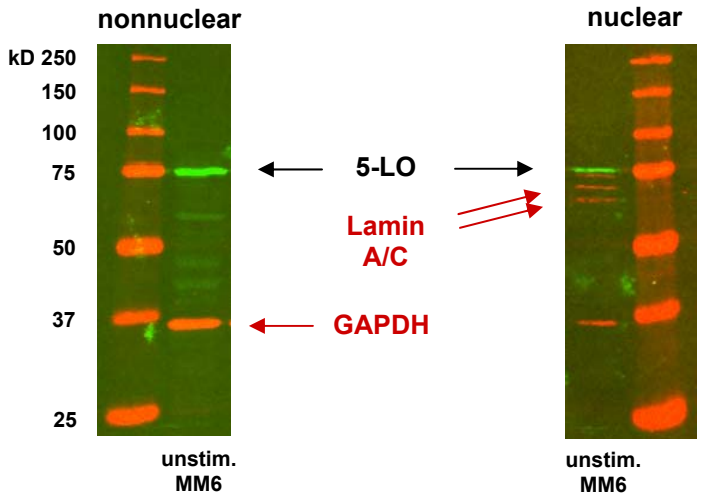


Figure 18: Assay control: Unstimulated MM6 cells (10 × 10^6 cells) were lysed, nonnuclear and nuclear fractions were separated by centrifugation and analyzed separately by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System). In each of the fractions both, the nonnuclear and the nuclear loading control proteins were measured, respectively.

For each experiment, the cytosolic marker GAPDH and the nuclear marker protein lamin were measured simultaneously in each fraction, respectively. This was used as an assay control, in order to check an adequate separation of nonnuclear from nuclear compartments. The figure shows one representative example of these control experiments carried out with unstimulated MM6 cells. Besides the signal of the 5-LO protein, a strong band of GAPDH, but no lamin was found in the nonnuclear fraction. In the nuclear fraction, a weak band of 5-LO and a distinct signal of lamin A/C could be detected. In comparison to the nonnuclear fraction, a low amount of GAPDH was found also in the nuclear fraction, which indicated a

slightly incomplete separation of the nonnuclear compartment from the nuclei. This was regarded as neglibigle, though, in terms of 5-LO translocation analyzes, and overall, the assay procedure appeared to provide a sufficient separation of fractions.

In summary, subcellular distribution analyzes revealed that LTA priming did not result in an enhanced 5-LO translocation, which accordingly does not seem to account for enhanced LT biosynthesis after TLR2 ligand pre-incubation.

Direct stimulation of 5-LO translocation by TLR2 ligands?

In further translocation assays also the lipopeptides FSL-1 and Pam_3CSK_4 were tested regarding their impact on subcellular 5-LO localization in MM6 cells and their influence was checked against that of LTA. The following experiments were performed to exclude a direct stimulation of 5-LO translocation for each of the three TLR2 ligands.

Therefore, MM6 cells were incubated with TLR2 ligands before analysis of nuclear and nonnuclear cell fractions by 5-LO immunoblotting. As a positive control experiment for strong 5-LO translocation, cells were stimulated with ionophore after priming with PMA. For resting MM6 cells, a predominant localization of 5-LO in nonnuclear compartments of the cell could be confirmed. PMA priming and subsequent ionophore stimulation again led to a clear shift of 5-LO protein to the nuclear fraction. Yet FSL-1 and Pam_3CSK_4 and again LTA did not directly induce 5-LO translocation to the nucleus in MM6 cells. However, from these analyzes it was not possible to detect reliably if 5-LO tends to redistribute into the nonnuclear compartment after TLR2 ligand incubation, as it was seen after LTA stimulation in previous translocation assays.

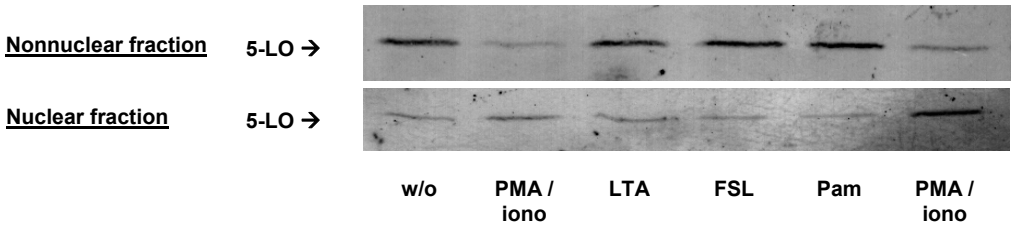


Figure 19: Direct stimulation of 5-LO translocation by TLR2 ligands. Differentiated MM6 (5×10^6 cells) were incubated with solvent or TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) as indicated 20 min / 37°C. As positive control MM6 were primed with PMA 100 nM 10min / 37°C, before ionophore A23187 5 µM was added for another 10 min / 37°C. After cell lysis, nonnuclear and nuclear fractions were separated by centrifugation and analyzed separately by SDS PAGE and immunoblotting (alkaline phosphatase reaction). Pair-wise samples (nonnuclear, nuclear) correspond to identical cell numbers. Similar results were obtained in 2 additional experiments.

Enhancement of ionophore induced 5-LO translocation by TLR2 ligands?

The influence of TLR2 ligand priming on ionophore induced 5-LO translocation was investigated, now including also the properties of FSL-1 and Pam_3CSK_4. After priming of MM6 cells and subsequent ionophore stimulation, nuclear and nonnuclear fractions were analyzed by 5-LO immunoblotting. Particularly the 5-LO bands of the nonnuclear fraction show that priming with none of the three TLR2 activators enhanced ionophore induced 5-LO translocation in comparison to unprimed cells. As a positive control, PMA priming again resulted in an augmented translocation of 5-LO. These results confirmed that besides LTA, also FSL-1 and Pam_3CSK_4 did not seem to enhance LT biosynthesis by increasing redistribution of 5-LO to the nucleus.

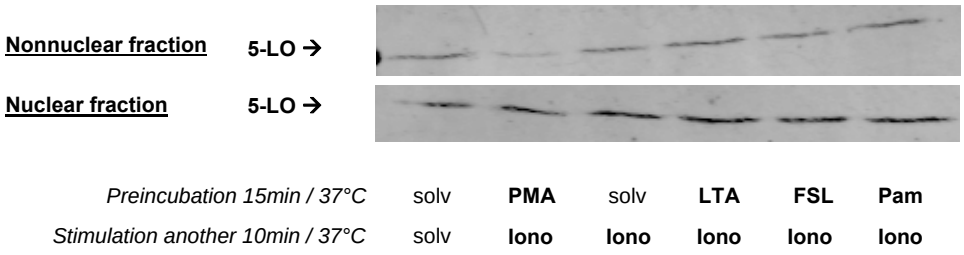


Figure 20: Enhancement of ionophore induced 5-LO translocation to the nucleus by TLR2 ligands. Differentiated MM6 (5 × 10^6 cells) were primed with solvent, PMA 100 nM or TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) as indicated. Solvent or ionophore A23187 2.5 µM was added for another 10 min / 37°C. Nonnuclear and nuclear fractions were separated by centrifugation and analyzed by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System). Pair-wise samples correspond to identical cell numbers. Similar results were obtained in 3 additional experiments.

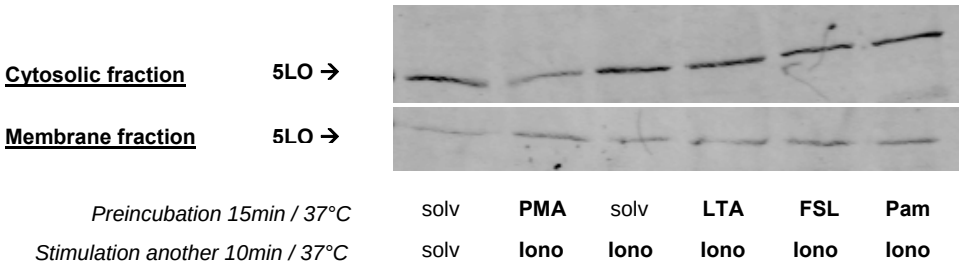


Figure 21: Enhancement of ionophore induced 5-LO translocation to cellular membranes by TLR2 ligands. Differentiated MM6 (5 × 10^6 cells) were incubated as described above. After cell lysis, cytosolic and membrane fractions were separated by centrifugation and analyzed separately by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System). Pair-wise samples correspond to identical cell numbers. Results reflect preliminary data.

Additionally it was excluded that 5-LO redistribution might occur to other cellular membranes than simply to the nuclear envelope. Thus, a cytosolic and a membrane fraction containing all the cellular membranes were prepared by ultracentrifugation of cell lysates, after priming and stimulation of MM6 cells. Separate analysis revealed that TLR2 ligand priming did not result in a definite increase of 5-LO translocation and again subcellular localization of 5-LO after stimulation was comparable in primed and in unprimed cells. Collectively, it was concluded that TLR2 ligand priming does not regulate cellular redistribution of 5-LO, neither to the nuclear envelope, nor to other cellular membranes.

4.2.3 Excursus: Observation of the "5-HETE effect" after priming

The following section contains a more detailed analysis of the product rates of 5-LO metabolites formed by primed and unprimed human monocytic cells after ionophore stimulation.

Product rates after TLR ligand priming in MM6 cells

At first, the data of the initial screening experiment, which was already discussed in an earlier section (4.1.1), were evaluated in more detail. For this purpose, various metabolites of the LT biosynthesis pathway were analyzed separately and their induction by TLR ligand priming was calculated.

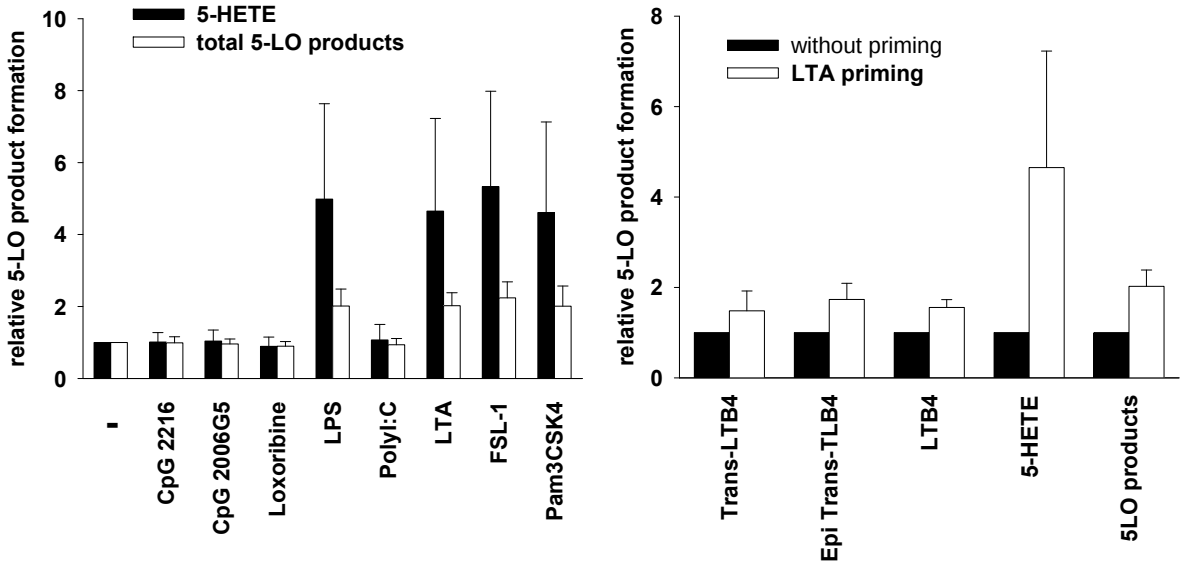


Figure 22: "5-HETE effect" after priming with TLR2 ligands. Differentiated MM6 (3 × 10^6 cells) were primed with TLR ligand or solvent as indicated 20 min / 37°C (CpG 2216 1 µM and CpG 2006-G5 1 µM, Loxoribine 100 µM, LPS 10 µg/mL, Poly (I:C) 25 µg/mL, LTA 2 µg/mL, FSL-1 1 µg/mL, Pam_3CSK_4 1 µg/mL) before stimulation of 5-LO product formation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

An approximate 2-fold induction by priming was observed for the entirety of the detected products including LTB_4, its all-*trans* isomers and 5-HETE. However, separate analysis of 5-HETE revealed an up to 5-fold enhancement after priming in comparison to unprimed cells. This "5-HETE effect" was observed after LPS priming, as well as after priming with TLR2 ligands. The formation of LTB_4 and its all-*trans* isomers after priming with LTA was further analyzed, but only a 1.5 – 2-fold enhancement of these metabolites was found.

5-HETE formation in MM6 cells after priming with LTA or PMA

Further studies were undertaken in order to clarify if the preferential increase of 5-HETE occurred as a result of poor 5-LO translocation in MM6 cells even upon priming with TLR2 ligands and subsequent stimulation. The underlying idea was that in case of sufficient substrate supply, 5-HETE yet might be formed in the cytosol, even if the interplay of 5-LO with LTA_4 metabolizing enzymes and the assembly of the biosynthetic complex at the nuclear envelope is not optimal. Therefore, MM6 cells were primed either with TLR2 ligand LTA or with PMA for different periods of time before stimulation with ionophore, and 5-HETE formation was analyzed. Surprisingly, a "5-HETE effect" was not only observed after LTA priming, but also after priming with PMA, which had been shown to enhance 5-LO translocation potently. These results indicated that the "5-HETE effect" did not seem to reflect low 5-LO translocation under these conditions, and that a predominant induction of the 5-HETE formation does not occur specifically after TLR2 ligand priming.

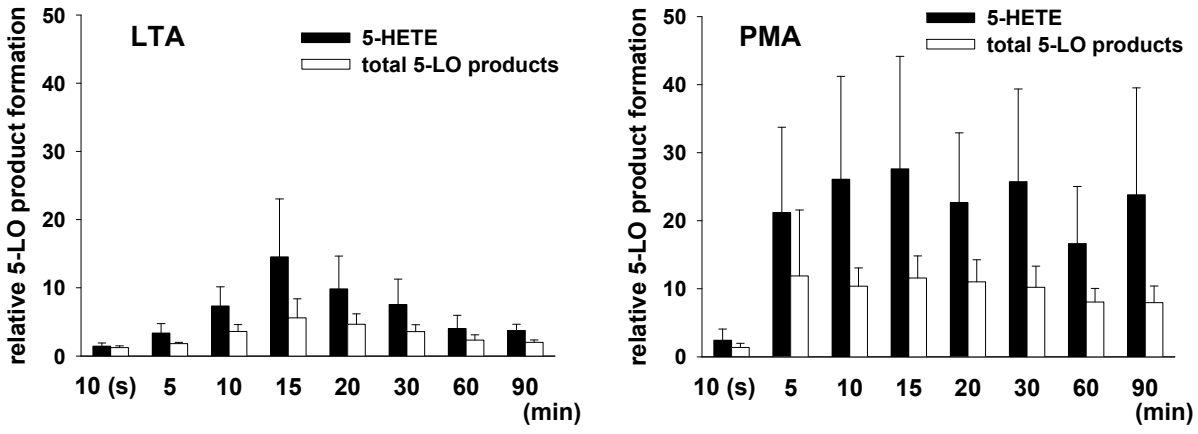


Figure 23: 5-HETE formation in MM6 cells after priming with LTA or PMA. Differentiated MM6 (3×10^6 cells) were primed with LTA 0.5 µg/mL or PMA 100 nM different periods of time / 37°C before stimulation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

Interestingly, the curves showed different characteristics after LTA and PMA priming of MM6 cells, respectively. An up to 20-fold increase of 5-HETE was detected with LTA priming for 15 minutes, whereas PMA enhanced 5-HETE formation 30 to 40-fold already with shorter priming times of 5 to 10 minutes. Furthermore, PMA effects persisted on this high level, even if priming times were extended up to 90 minutes. In contrast, LTA effects decreased considerably, when priming times exceeded 15 minutes.

5-HETE formation in THP-1 cells after priming with LTA or PMA

In order to exclude that the observed "5-HETE effect" was specific only for MM6 cells, analogous time course experiments were repeated with THP-1 cells, another human monocytic cell line. Thus, THP-1 cells were primed with LTA or PMA for different periods of time and then stimulated with ionophore. Again for both agents, priming had a greater effect on the formation of 5-HETE than of the other metabolites, which confirmed the findings in MM6 cells.

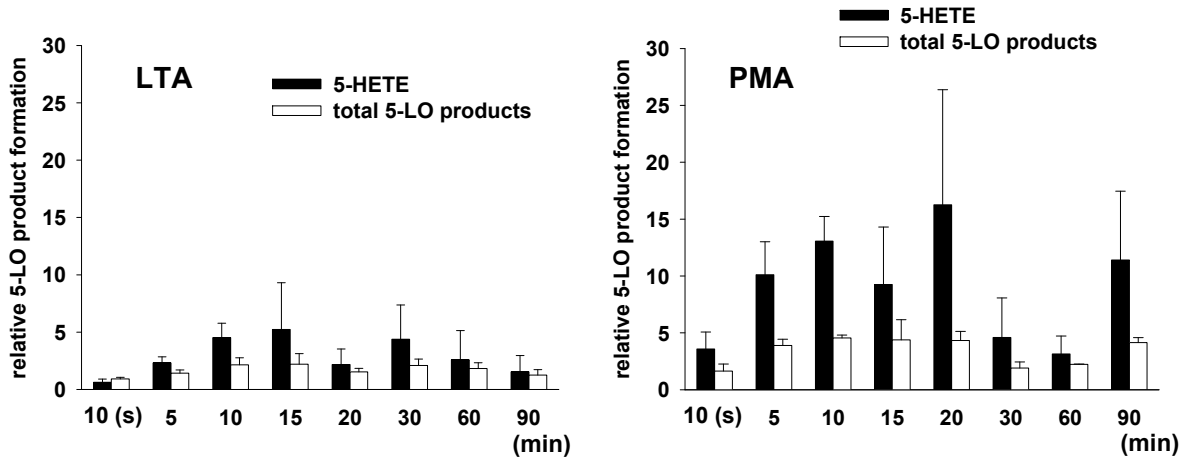


Figure 24: 5-HETE formation in THP-1 cells after priming with LTA or PMA. Differentiated THP-1 (3×10^6 cells) were primed with LTA 0.5 µg/mL or PMA 100 nM different periods of time / 37°C before stimulation with ionophore A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

The kinetics of priming in THP-1 cells differed from that in MM6 cells, though. Two phases of enhanced 5-LO product formation became apparent after LTA and also PMA priming. LTA induced a first maximum with a priming time of 15 minutes and a second peak with a pre-incubation for 30 minutes. PMA enhanced 5-LO product formation by priming times up to 20 minutes. With further temporal extension of priming, the PMA effect disappeared, but appeared again with a priming time of 90 minutes. 5-HETE was enhanced approximate 9-fold by LTA, and up to 25-fold by PMA at its maximum, respectively.

5-HETE formation in hPBMC after priming with LTA or PMA

Finally, time courses of LTA and PMA priming were once more analyzed also in hPBMC. After different times of priming, human primary cells were stimulated with a low concentration of ionophore (0.025 µM) as it was assessed as optimal in previous studies. A "5-HETE effect" was detected also in this cellular system, although it was not as pronounced after LTA priming as it was found in both monocytic cell lines. Pre-incubation with LTA for 10 minutes resulted in an approximate 7-fold enhancement of 5-HETE and notably also a 5-fold increase of total 5-LO products. Interestingly, PMA did not show a stronger priming effect than LTA in hPBMC.

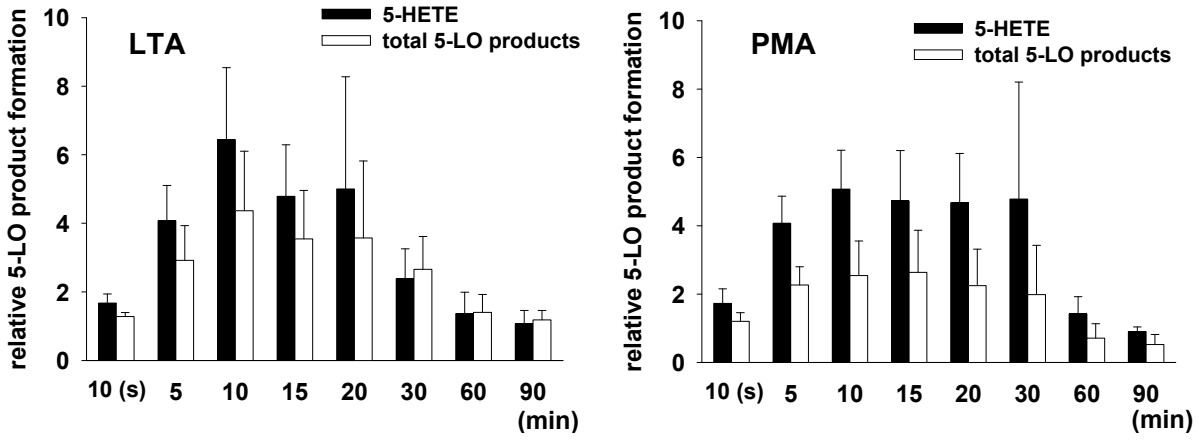


Figure 25: 5-HETE formation in hPBMC after priming with LTA or PMA. Human PBMC (5 × 10^6 cells) were primed with LTA 0.5 µg/mL or PMA 100 nM different periods of time / 37°C before stimulation with ionophore A23187 0.025 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

In summary, again it was concluded that a particular increase of 5-HETE biosynthesis does not seem to be a specific event after TLR2 ligand priming, and apparently is observed generally after priming of human monocytes.

4.2.4 Comparison of priming properties of LTA and PMA in MM6 cells

So far, investigations concerning the underlying mechanisms of TLR2 ligand priming revealed that the ligands do not seem to enhance activity and translocation of 5-LO as opposed to PMA, a well-established priming agent [373]. The following studies were undertaken to analyze and understand the differing modes of action of LTA compared to PMA.

Influence of PKA inhibitors on priming effects

In previous sections an insignificant decrease of 5-LO activity was found in MM6 cells after LTA priming and subsequent stimulation in presence of exogenous AA. Additionally, a redistribution of 5-LO into nonnuclear compartments was noticed after incubation with LTA. These observations led to the assumption that LTA might trigger cellular signals resulting in an inhibition of 5-LO and counteracting an increase of 5-LO activity after LTA priming. Therefore, the role of PKA as a negative regulator of 5-LO was determined under priming conditions, and LTA and PMA effects on 5-LO activity were analyzed in presence of the PKA inhibitors H-89 and 14-22 amide. First, the results confirmed once more that there was no enhancement of 5-LO activity after LTA priming in MM6 cells. Both PKA inhibitors caused a minor decrease of the leukotriene formation level, respectively. However, even in presence of H-89 as well as 14-22 amide, no LTA priming effect was detectable and also PMA priming was not affected in the control experiment. Thus it was reasoned that a possible PKA activation does not seem to play a role during priming with LTA.

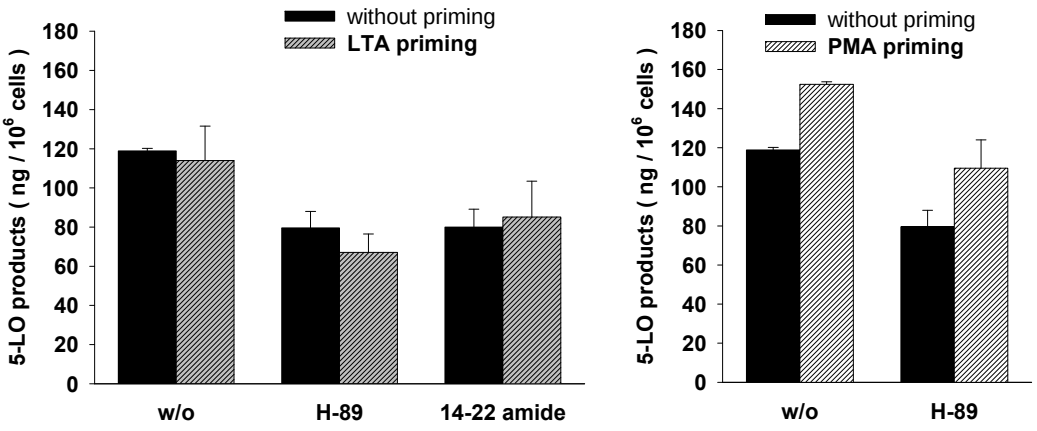


Figure 26: Influence of PKA inhibitors on priming effects. Differentiated MM6 cells (3 x 10^6) were pre-incubated without or with the PKA inhibitors 14-22 amide 100µM or H-89 10µM 30 min / 37°C. After priming with LTA 0.5 µg/mL or PMA 100 nM 15 min / 37°C, cells were stimulated with A23187 2.5 µM and AA 20 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

In influence of LTA and PMA on the intracellular calcium concentration

The intracellular Ca^{2+} concentration is a key parameter for 5-LO activity and regulates the association of 5-LO with cellular membranes [11]. Therefore it was of interest to test and to compare the influence of LTA and PMA on the intracellular Ca^{2+} level, in order to elucidate

possible differences in the mechanisms of priming. MM6 cells were incubated with LTA or PMA and intracellular Ca^{2+} was measured, respectively. As a positive control, ionophore 0.25 µM strongly induced a Ca^{2+} influx and its concentration rose approximately to 250 nM. However, neither LTA nor PMA had an impact on intracellular Ca^{2+}, which remained on the basal level similar to the negative control. Thus, different priming properties of LTA and PMA are not predicated on differences concerning their influence on intracellular Ca^{2+} levels.

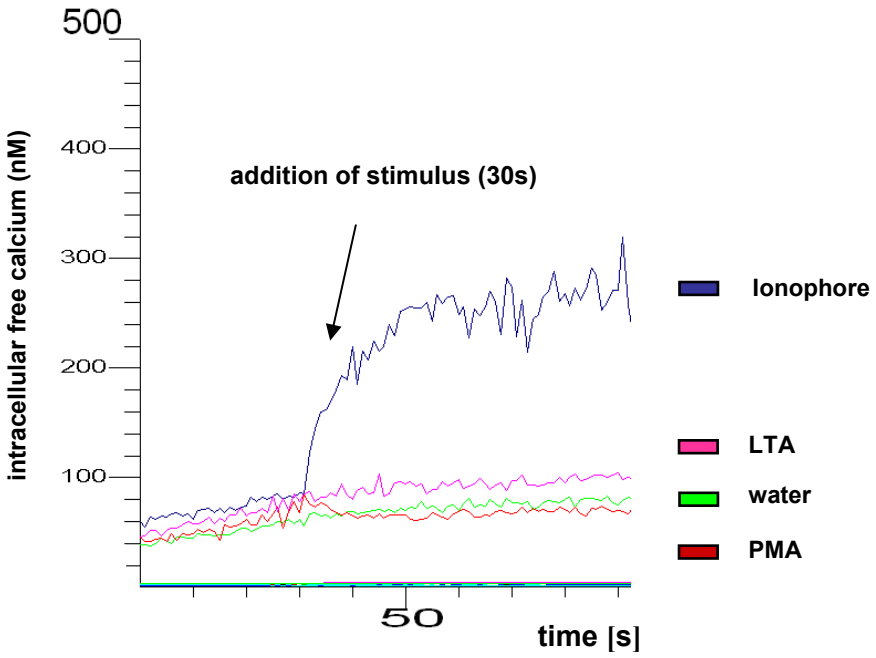


Figure 27: Influence of LTA and PMA on the intracellular calcium concentration. Differentiated MM6 (5 x 10^6 cells) were loaded with Fura-2/AM 2 µM 30 min / 37°C and afterwards resuspended in 1 mL PBS containing 1 mM Ca^{2+}. Then solvent, LTA 0.5 µg/mL, PMA 100 nM or ionophore A23187 0.25 µM were added as indicated. Fluorescence was measured and intracellular free Ca^{2+} was calculated. The curve shows one typical experiment out of 3.

Presence of arachidonic acid during priming

The p38 MAPK and ERK pathways also play an important role in the regulation of 5-LO and may trigger a stimulation or an increase of 5-LO activity by phosphorylation [16]. MAP kinases are activated by various stress stimuli, phorbol esters or also as a result of TLR activation [16, 319]. It was demonstrated that efficient phosphorylation of 5-LO by ERK1/2 required the presence of unsaturated fatty acids in vitro and that in MM6 cells PMA priming was essential for ERK1/2 activation and efficient phosphorylation of 5-LO [170]. In the following experiment, MM6 cells were primed with LTA in presence of various concentrations of AA, before ionophore and an excess of AA was added to stimulate LT

formation. The addition of 1-10 µM AA during the period of pre-incubation had no impact on the level of 5-LO product formation in comparison to the controls that were pre-treated without AA. Irrespective of the absence or presence of AA during pre-incubation, LTA priming again caused a slight decrease of 5-LO activity compared to unprimed cells. The addition of 30 µM AA at least resulted in a general reduction of the product formation level appearing to a comparable extent in primed as well as in unprimed cells. In summary, no enhancement of 5-LO activity became evident after priming of MM6 cells with LTA in presence of low AA concentrations. Accordingly, these conditions do not seem appropriate to imitate conditions of PMA priming and thus did not help to scent out different mode of actions of LTA and PMA.

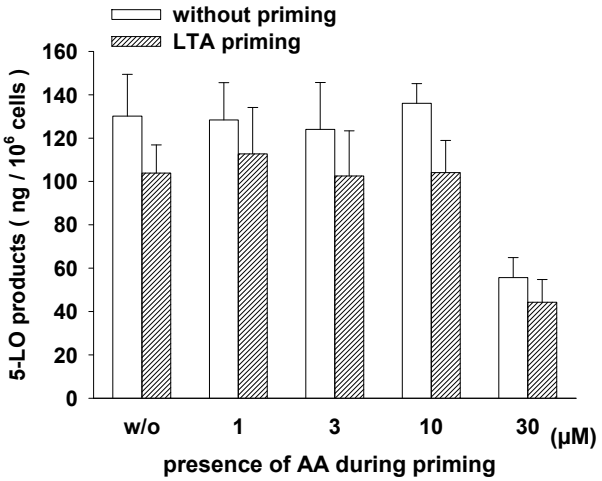


Figure 28: Presence of arachidonic acid during priming. Differentiated MM6 cells (3 x 10^6) were primed with or without LTA 0.5 µg/mL in presence or absence of different concentrations of AA as indicated 15 min / 37°C. Then cells were stimulated with A23187 2.5 µM and AA 20 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

Comparative analysis of p38 MAPK activation

In former publications it was demonstrated that inhibition of ERK signalling by U0126 abolished the 5-LO upregulation effects of PMA in MM6 cells [170]. Hence, ERK pathways seem to play a decisive role in the mechanism of priming by PMA. Therefore it seemed promising to analyze LTA and PMA induced activation of the relevant MAP kinase pathways in more detail, to possibly get an idea about the differing modes of action of the two priming agents. At first, a comparative analysis of p38 activation was carried out. By immunoblotting, p38 phosphorylation was determined in MM6 cell lysates after treatment with LTA or PMA for different periods of time. Two clearly different p38 activation profiles were found. LTA

induced a substantial activation of p38 MAP kinase (up to 17-fold in comparison to untreated cells) already after 5 minutes. This activation level was detectable up to 15 minutes of incubation, but with further extension of incubation times p-p38 signals decreased again to the basic level after 90 minutes of incubation. PMA in comparison induced p38 phosphorylation only very weakly. After 5 minutes a maximal 2-fold activation became visible, but almost no activation of p38 was found for longer periods of incubation. In summary, LTA revealed to be a strong p38 activator as opposed to PMA. It was concluded that activation of p38 MAP kinase pathways may not be of major importance for a regulation of 5-LO by priming.

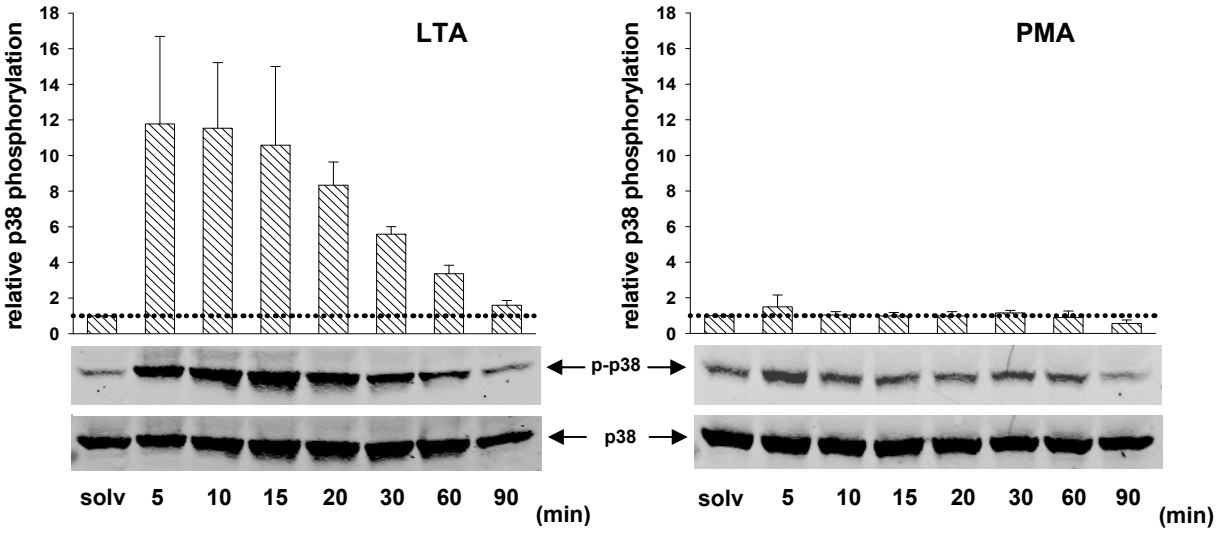


Figure 29: Comparative analysis of p38 MAP kinase activation. Differentiated MM6 (3 × 10^6 cells) were incubated with LTA 0.5 µg/mL or PMA 100 nM for different periods of time / 37°C as indicated. As negative control, cells were treated with solvent for 15min / 37°C (solv 15). Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect p38 MAPK activation, an antibody specific for the dually phosphorylated form of the protein was used. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was determined. For quantification, band intensities of the phosphorylated proteins were corrected by band intensities of both forms. Phosphorylation levels are related to the negative control (dotted lines).

5-LO phosphorylation at Ser-271

MK-2/3 lie downstream of p38 MAP kinase and were shown to phosphorylate 5-LO at Ser-271 resulting in a positive regulation of 5-LO activity [16]. In a preliminary immunoblotting experiment, phosphorylation of 5-LO at Ser-271 was investigated in MM6 cell lysates after incubation with LTA or PMA. As expected due to the antecedent results, after PMA treatment almost no 5-LO phosphorylation was measurable, only a slight band of p-5-LO may be sensed

after 5 minutes. After incubation with LTA, however, phosphorylated 5-LO was detected after 5 to 15 minutes of incubation. Nevertheless, these findings did not seem to have a relation to the lack of enhanced 5-LO activity observed after priming with LTA in contrast to PMA priming. Therefore studies were not further pursued.

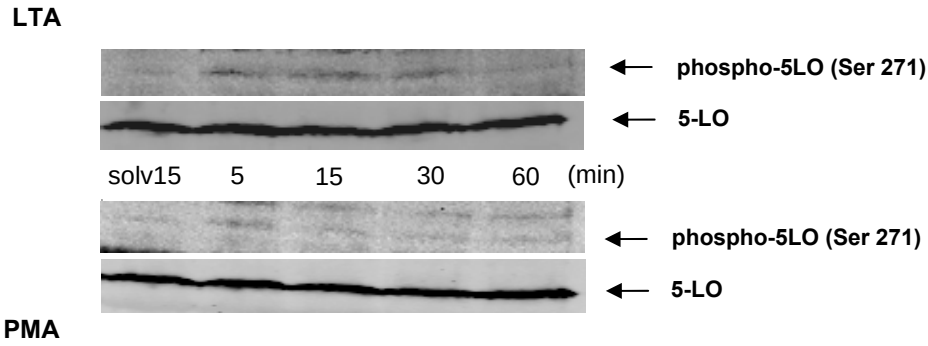


Figure 30: 5-LO phosphorylation at Ser-271: Differentiated MM6 (3 × 10^6 cells) were incubated with LTA 0.5 µg/mL or PMA 100 nM for different periods of time / 37°C as indicated. As negative control, cells were treated with solvent for 15min / 37°C (solv 15). Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect 5-LO phosphorylation, an antibody specific for the phosphorylated form of the protein was used. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was detected. The results reflect preliminary data.

Comparative analysis of ERK1/2 activation

It seemed of major relevance to investigate and to compare the activation of ERK1/2 pathways after stimulation of MM6 cells with LTA or PMA. Phosphorylation of ERK1/2 was analyzed by immunoblotting. In comparison to the p38 MAP kinase activation profile, a converse picture emerged that might have a relation to different priming properties of LTA and PMA. As expected, PMA was identified as a strong activator of ERK1/2 pathways. Already after 5 minutes of incubation an up to 80-fold increase of p42 phosphorylation was measured in comparison to untreated cells. With extended periods of incubation p-p42 band intensities decreased again and were yet weak after 60 to 90 minutes of PMA treatment. LTA in contrast, turned out to be a rather weak ERK activator. The increase of phosphorylated p42 was low and only very transient compared to PMA. LTA induced ERK1/2 activation was detectable after 5 minutes (approximately 10-fold), declined fast and was no more visible after a treatment for 20 minutes.

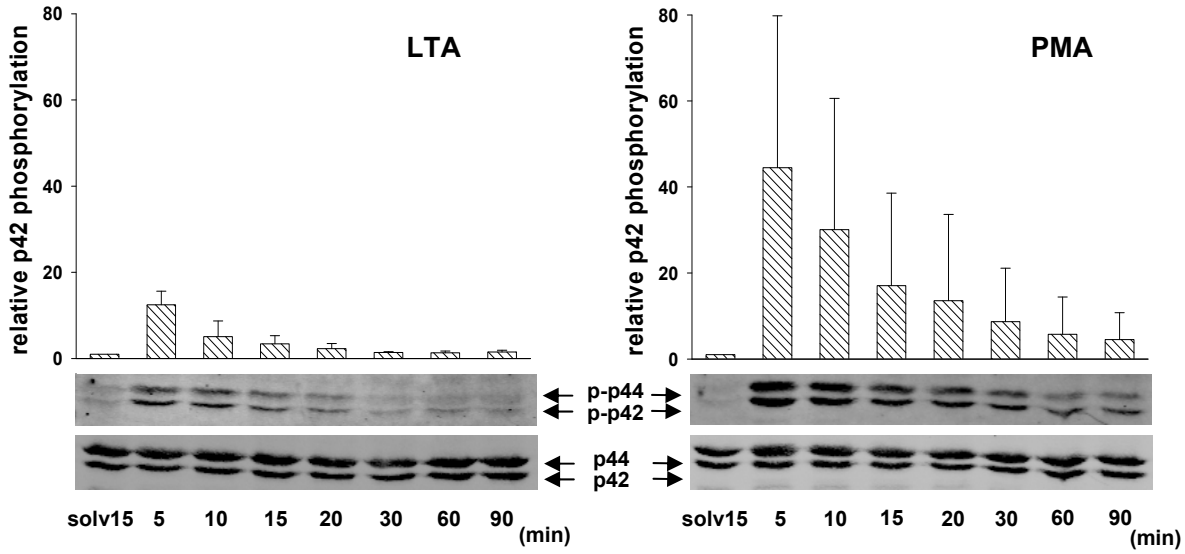


Figure 31: Comparative analysis of ERK1/2 activation. Differentiated MM6 (3×10^6 cells) were incubated with LTA 0.5 µg/mL or PMA 100 nM for different periods of time / 37°C as indicated. As negative control, cells were treated with solvent for 15min / 37°C (solv 15). Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect ERK1/2 activation, an antibody specific for the dually phosphorylated form of the protein was used. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was determined. For quantification, band intensities of the phosphorylated proteins were corrected by band intensities of both forms. Phosphorylation levels are related to phosphorylation levels in the negative control.

Kinetics of priming for enhanced 5-LO activity

Considering the respective ERK activation profiles it was suspected that an appropriate intensity and persistence of ERK activation might be required for an upregulation of 5-LO by priming in MM6 cells. After incubation with LTA for 5 minutes, a distinct p42 phosphorylation had been detectable. Hence the question came up, if a shorter period of priming (5 minutes instead of 15 minutes) would result in an upregulation of 5-LO activity by LTA. Therefore, another time course experiment was undertaken. MM6 cells were primed with LTA or PMA for different time periods, and 5-LO activity was measured after stimulation with ionophore in presence of AA. In fact, 5 minutes of LTA priming led to a distinct, yet not significant enhancement of 5-LO activity. With priming for 15 minutes, again a slight decrease of product formation was determined compared to unprimed cells. 5-LO upregulation after PMA priming was also maximal (about 2-fold) with a short incubation time for 5 minutes. When pre-incubation times were further extended, the enhancement of LT formation decreased again and was not yet measurable with 90 minutes of priming. This kinetics showed a strong analogy to the respective ERK activation profile. The time course

characteristics of LTA priming also showed a first induction of 5-LO activity, followed by a consistent decrease of activity values similar to the respective ERK activation profile. However, with longer periods of LTA priming, product formation dropped under the product level of unprimed cells. Yet with priming for 90 minutes, LT synthesis was similar in primed as well as in unprimed cells again.

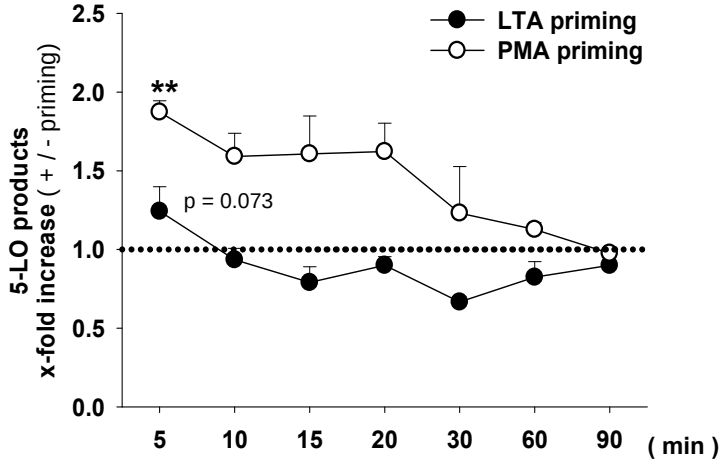


*Figure 32: Kinetics of priming for enhanced 5-LO activity. Differentiated MM6 (3 × 10^6 cells) were primed with solvent, with LTA 0.5 µg/mL or PMA 100 nM different periods of time / 37°C as indicated, before stimulation of 5-LO product formation with ionophore A23187 2.5 µM plus AA 20 µM for another 10 min / 37°C. 5-LO products were determined by HPLC. The increase of 5-LO products by LTA or PMA priming is related to the product formation level in cells that were analogously pre-incubated with solvent, respectively. Student´s t test (paired; one-tailed); ** p<0.01, * p<0.05;*

Enhanced ERK activation during blockade of p38 MAP kinase pathways

In the following experiment MM6 cells were incubated with the TLR2 ligands LTA, FSL-1 or Pam_3CSK_4 in absence or presence of p38 MAP kinase inhibitor SB203580, and ERK1/2 activation was determined. In absence of the inhibitory agent, a weak p42 phosphorylation was detectable after treatment with LTA or FSL-1. After Pam_3CSK_4 incubation, ERK1/2 activation was marginal. The presence of SB203580, however, considerably enhanced TLR2 ligand induced activation of ERK1/2, and also after Pam_3CSK_4 incubation p42 phosphorylation was substantial. These findings were harnessed in subsequent investigations.

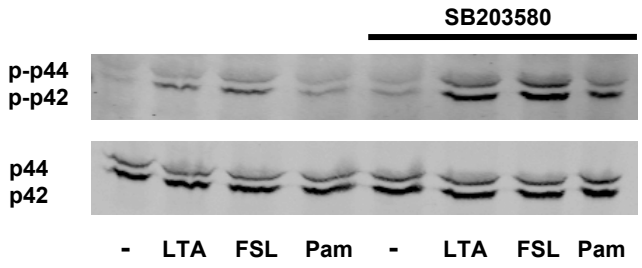


Figure 33: Influence on ERK1/2 activation during blockade of the p38 MAPK pathway. Differentiated MM6 (3 × 10^6 cells) were pre-incubated with or without SB203580 (10 µM) 15 min / 37°C and stimulated with or without TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) as indicated for another 15 min / 37°C. Total cell lysates were analyzed by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System).

The influence of priming on 5-LO translocation during blockade of p38 MAPK

The consequences of a shorter period of LTA priming for ionophore-induced 5-LO translocation in MM6 cells were assessed in the following studies. The preceding Western Blot results had shown a stronger activation of ERK pathways, when p38 MAPK signalling was blocked. Thus, translocation assays with 5 minutes of priming were additionally carried out in presence of SB203580, in order to provoke higher ERK activation levels after incubation with LTA and to render a potential ERK-mediated LTA effect detectable. Again, after incubation of MM6 cells nuclear and nonnuclear fractions were prepared and analyzed separately by 5-LO immunoblotting. Calculations of relative band intensities revealed that also after reduction of the period of pre-incubation from 15 to 5 minutes no significant enhancement of 5-LO translocation by LTA priming was detected, neither in the nonnuclear nor in the nuclear fraction. Even PMA mediated upregulation was not significant. The presence of SB203580 seemed to generally increase the degree of 5-LO translocation after stimulation of MM6 cells: in absence of SB203580, the decrease of 5-LO protein in the nonnuclear fraction after ionophore stimulation was approximately 25% in comparison to untreated cells, whereas in presence of the p38 inhibitor a reduction of 5-LO of about 50% was measured in the nonnuclear fraction compared to the untreated control. Complementary, a significant increase of 5-LO in the nuclear fraction was found after ionophore stimulation in presence of SB203580. Furthermore, PMA enhanced 5-LO translocation significantly in presence of the p38 inhibitor. Nevertheless, blockade of p38 MAPK pathways did not result in a significant increase of ionophore-induced 5-LO translocation after LTA priming for 5 minutes.

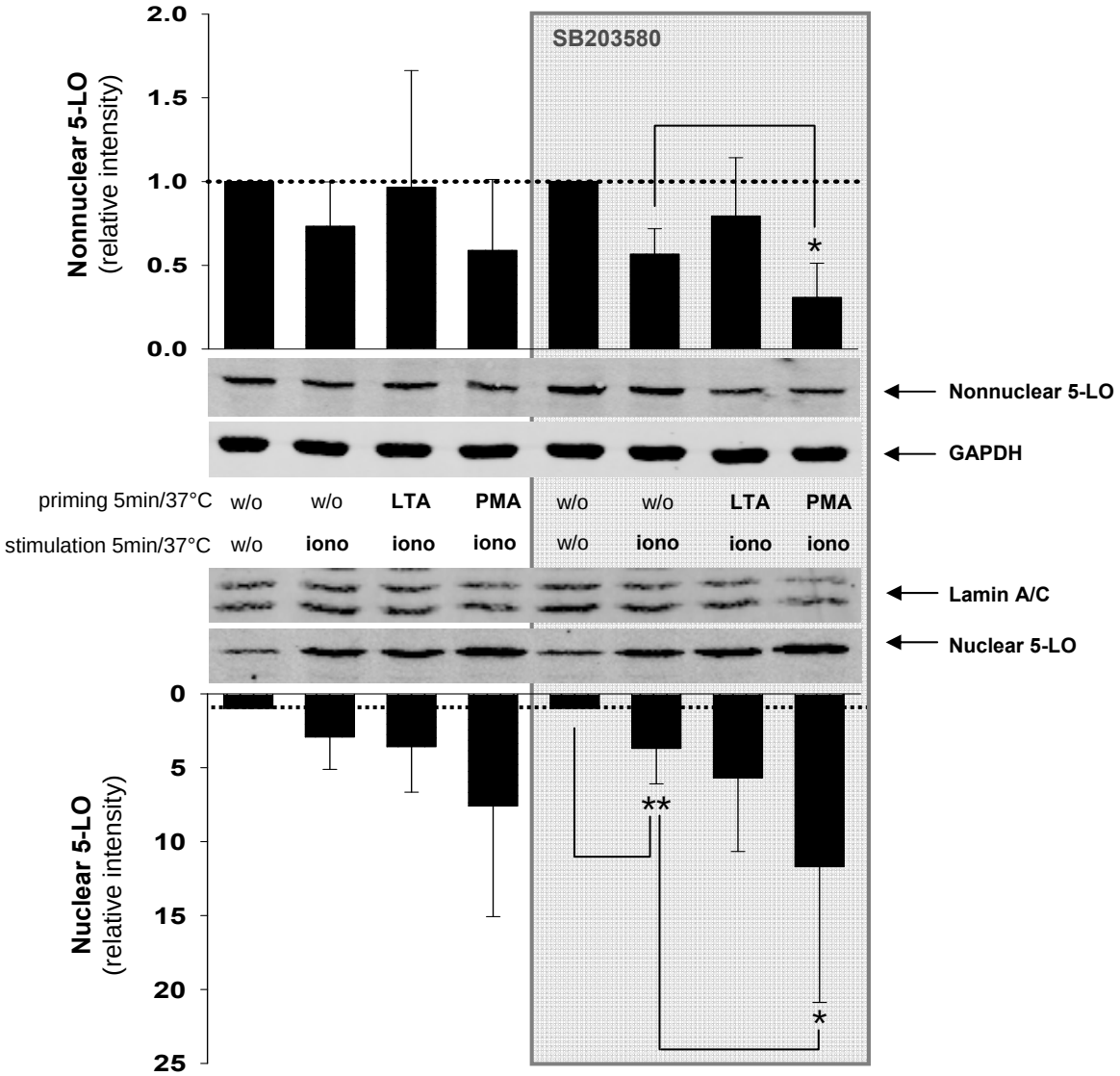


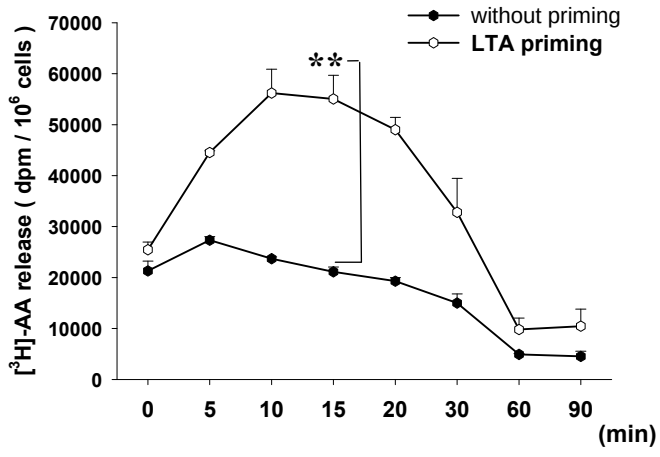
*Figure 34: Influence on 5-LO translocation during blockade of the p38 MAPK pathway. Differentiated MM6 (10 × 10^6 cells) were pre-incubated with or without SB203580 (10 µM) 15 min / 37°C and were primed with solvent, PMA 100nM or LTA 0.5 µg/mL as indicated 5 min / 37°C. Solvent or ionophore A23187 2.5 µM was added for another 5 min / 37°C. After cell lysis, nonnuclear and nuclear fractions were separated by centrifugation and analyzed separately by SDS PAGE and immunoblotting as previously described (Odyssey® Infrared Imaging System). For quantification, band intensities of 5-LO were corrected by band intensities of specific loading controls for the nonnuclear (GAPDH) and the nuclear fraction (Lamin A/C), respectively. The amount of 5-LO protein is related to the level of 5-LO protein in the negative controls, respectively (dotted line). Pair-wise samples (nonnuclear, nuclear) correspond to identical cell numbers. Student´s t test (paired; one-tailed); ** p<0.01, * p<0.05;*

4.3 The influence of TLR2 ligands on arachidonic acid release in MM6 cells

This chapter furthermore deals with the elucidation of the underlying mechanisms of TLR2 mediated priming of MM6 cells for an increase of ionophore-induced 5-LO product formation. The results of the preceding sections (4.2) suggested that enhanced biosynthesis of 5-LO products apparently does not result from an upregulation of 5-LO activity or translocation after TLR2 ligand priming. Hence, further investigations focussed on the impact of TLR2 ligands on the ionophore induced release of AA, which serves as substrate for LT biosynthesis via the 5-LO pathway.

4.3.1 Time-dependent enhancement of AA release

At first it was investigated, if enhanced TLR2 ligand mediated leukotriene formation is due to an increased availability of AA in MM6 cells. Thus, for time course analyses, MM6 cells were pre-treated with increasing concentrations of LTA, FSL-1 or Pam_3CSK_4 before stimulation with ionophore and AA release was determined in comparison to unprimed, but ionophore-stimulated cells. The results were similar to the time course experiments of 5-LO product formation after TLR2 ligand priming (4.1.2.3). Simultaneous addition of TLR2 ligands with ionophore did not evoke an enhancement of ionophore induced AA release. Priming of the cells with TLR2 ligands, however, led to a time dependent increase in free AA, peaking after pre-incubation of 10 to 15 minutes. With further extension of pre-incubation times the response decreased again and almost completely disappeared with priming times of 60 to 90 minutes. For all ligands, the kinetics of enhanced AA generation was comparable, even though the response upon Pam_3CSK_4 priming was slightly weaker.



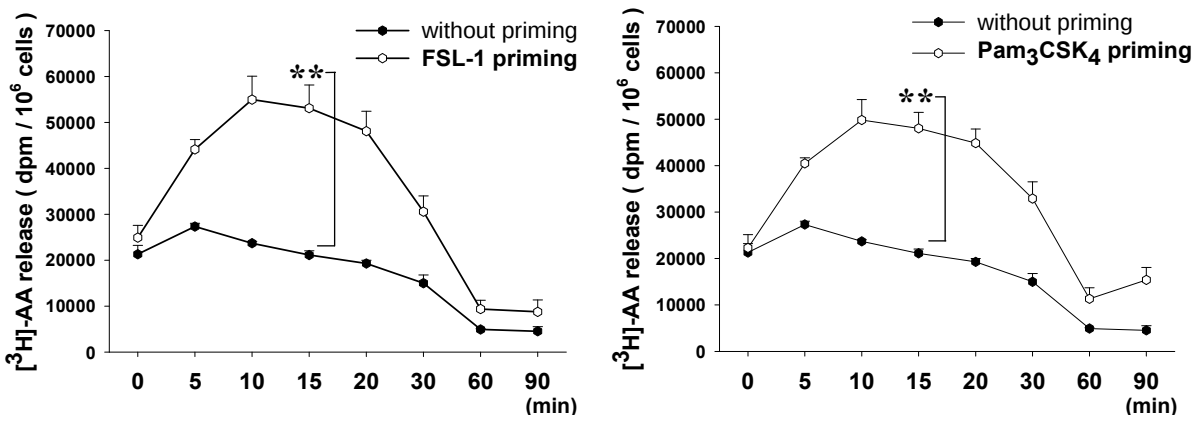


*Figure 35: Time-dependent enhancement of AA release. Differentiated MM6 cells (2 × 10^6 cells/mL) were pre-labeled with 0.5µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2mg/mL fatty acid free BSA (3 × 10^6/0.5ml). [^{3}H]AA-labeled MM6 cells were primed with TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) or solvent for different periods of time before induction of arachidonic acid release with ionophore A23187 2.5 µM for another 10min / 37°C and free [^{3}H]AA was analyzed. Student´s t test; ** p<0.01;*

4.3.2 Direct stimulation of AA release

Given that simultaneous addition of TLR2 ligands together with ionophore did not raise the levels of free AA compared to ionophore alone, it was expected that TLR2 ligands by themselves do not induce AA release.

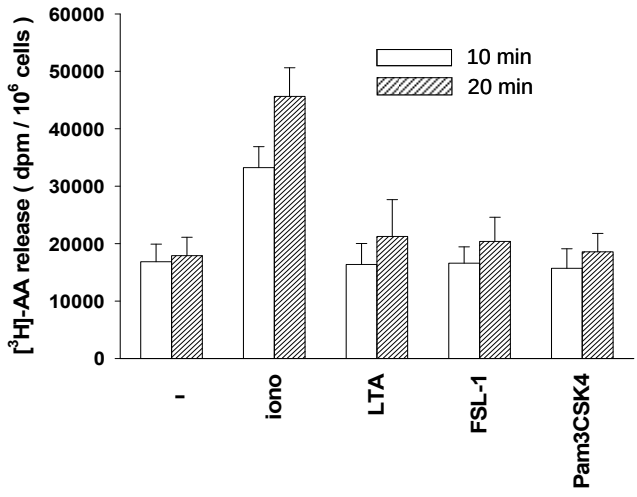


Figure 36: Direct stimulation of AA release. Differentiated MM6 cells (2 × 10^6 cells/mL) were pre-labeled with 0.5µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2mg/mL fatty acid free BSA (3 × 10^6/0.5ml). [^{3}H]AA-labeled MM6 cells were incubated with either solvent, ionophore A23187 (iono) or TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) for 10 or 20 min / 37°C, and free [^{3}H]AA was analyzed.

In order to confirm this, MM6 cells were stimulated either with ionophore or with LTA, FSL-1 or Pam_3CSK_4. Determination of free AA revealed that TLR2 ligands did not directly induce a significant release of AA after 10 or 20 minutes of incubation. Therefore, increased LT formation in MM6 cells by TLR2 ligand priming seemed to be due to an enhancement of AA release.

4.3.3 The influence of TLR2 ligands on the intracellular calcium concentration

Similar to the regulatory properties of 5-LO, AA releasing PLA_2 enzymes are tightly regulated by Ca^{2+}, and a direct stimulation of cellular AA release requires an increase of the intracellular Ca^{2+} level [16, 37]. Supplementary to the preceding experiment, the influence of the three TLR2 ligands on the intracellular Ca^{2+} concentration in MM6 cells was investigated. Ionophore as a positive control expectedly stimulated a substantial increase of the intracellular Ca^{2+} level. As it had been observed also in former analyzes (4.2.3), LTA incubation did not result in an alteration of the intracellular Ca^{2+} concentration, which remained on a basal level comparable to the negative control. This could also be confirmed for FSL-1 and Pam_3CSK_4, which also did not evoke a cellular Ca^{2+} influx. As expected, TLR2 ligands did not show an influence on intracellular Ca^{2+} and accordingly did not directly stimulate AA release.

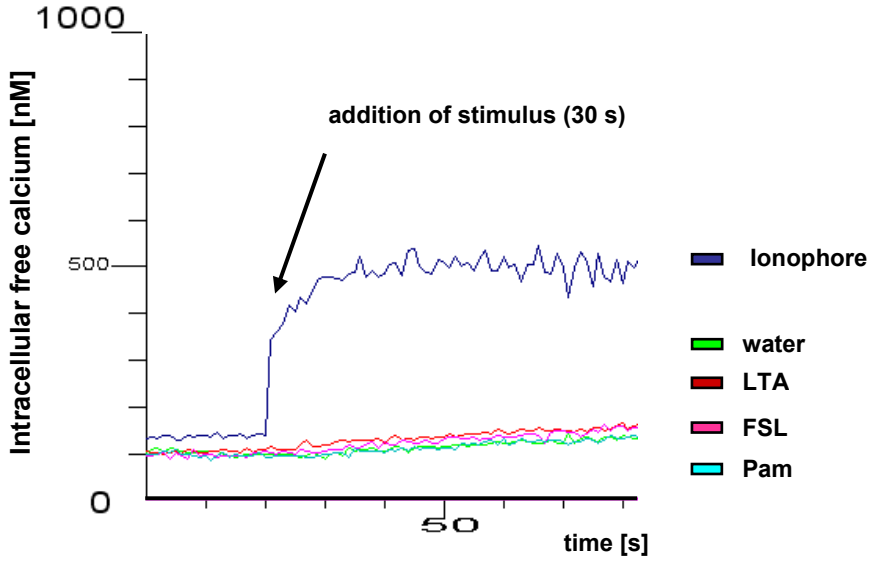


Figure 37: Influence of TLR2 ligands on the intracellular calcium concentration. Differentiated MM6 (4 x 10^6 cells) were loaded with Fura-2/AM 2 µM 30 min / 37°C and resuspended in 1 mL PBS containing 1 mM Ca^{2+}. Then solvent, LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL or ionophore A23187 0.5 µM were added as indicated. Fluorescence was measured and intracellular free Ca^{2+} was calculated. The curve shows one typical experiment out of 2.

4.3.4 Identification of the involved PLA_2 enzymes

AA release is catalyzed preferentially by group V secretory PLA_2s ($sPLA_2$) and by group IV cytosolic PLA_2s ($cPLA_2$) [37]. The following inhibitor studies were undertaken to identify the PLA_2 enzymes that are involved in TLR2 ligand mediated enhancement of AA release.

Effects of PLA_2 inhibitors on enhanced AA release

MM6 cells were treated either with $iPLA_2$ inhibitor bromoenollactone (BEL), the $sPLA_2$ inhibitor dithiotreitol (DTT) or with the selective $cPLA_2$ inhibitor pyrrolidine-1 (PYR) before priming and subsequent stimulation. As expected, BEL virtually did not repress AA release, neither in unprimed nor in primed MM6 cells, indicating an insignificant role of $iPLA_2$ for AA release under these conditions. Inhibition of $sPLA_2$ diminished AA release considerably, in primed as well as in unprimed cells. However, the magnitude of the TLR2 ligand mediated priming effect remained constant despite elimination of $sPLA_2$ activity. Inhibition of $cPLA_2$ almost completely prevented AA release upon ionophore stimulation in MM6 cells, pointing to a crucial role of this enzyme for AA generation under these conditions. The residual PLA_2 activity was very low and TLR2 ligand mediated increase was not detectable any more. These data suggested that the effects of TLR2 ligands on AA are mediated by $cPLA_2$.

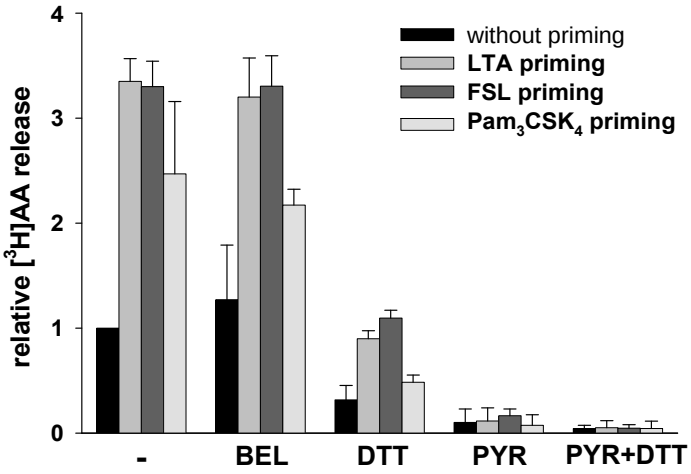


Figure 38: Effects of PLA_2 inhibitors on enhanced AA release. Differentiated MM6 (2×10^6 cells) were pre-labeled with 0.5 µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2 mg/mL fatty acid free BSA. [^{3}H]AA-labeled MM6 cells were pre-treated with or without PLA_2 inhibitors (bromoenollactone (BEL) 10 µM, dithiotreitol (DTT) 1 mM, pyrrolidine-1 (PYR) 10 µM) as indicated 15 min / 37°C. Subsequently cells were primed with TLR2 ligands (LTA 0.5µg/mL, FSL-1 0.5µg/mL, Pam_3CSK_4 5µg/mL) or treated with solvent 15 min / 37°C, and thereupon stimulated for AA release with A23187 2.5 µM for another 10 min / 37°C.

Effects of PLA_2 inhibitors on enhanced LT biosynthesis

The aim of subsequent inhibitor studies was to verify a link between enhanced 5-LO product formation and the $cPLA_2$ mediated increase of AA release by TLR2 ligand priming. Consequently, the effect of PLA_2 inhibitors on the biosynthesis of 5-LO products was tested. The results were in line with the data for AA release. The $sPLA_2$ inhibitor DTT clearly reduced 5-LO metabolites in primed and unprimed cells, but without affecting TLR2 ligand mediated increase of 5-LO product formation. The $cPLA_2$ inhibitor completely abolished leukotriene biosynthesis. The data suggested that the effects of TLR2 ligands on leukotriene release were mediated by $cPLA_2$.

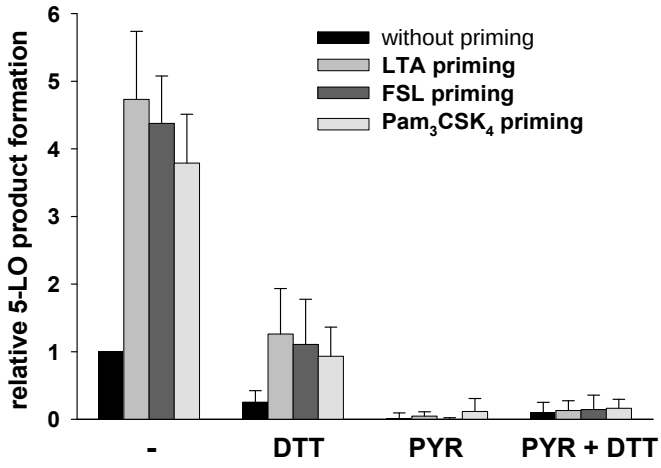


Figure 39: Effects of PLA_2 inhibitors on enhanced LT biosynthesis. Differentiated MM6 cells (3 x 10^6) were pre-incubated without or with PLA_2 inhibitors (dithiotreitol (DTT) 1 mM, pyrrolidine-1 (PYR) 10 µM or both) as indicated 15 min / 37°C. After priming with TLR2 ligands (LTA 0.5µg/mL, FSL-1 0.5µg/mL, Pam_3CSK_4 5µg/mL) or solvent 15 min / 37°C, cells were stimulated with A23187 2.5 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

Excursus: Effects of PLA_2 inhibitors on 5-LO activity

Extended investigations were carried out to confirm the antecedent data and to exclude other biological effects of the PLA_2 inhibitors on the 5-LO pathway that would distort the preceding results. Once more MM6 cells were incubated with DTT or with pyrrolidine-1 before TLR2 ligand priming. Subsequently, cells were stimulated in presence of exogenous AA, to keep the assay conditions independent from endogenous substrate supply. Thus, the influence of the inhibitors on 5-LO activity was determined. Surprisingly, a moderate increase of 5-LO activity by 1 mM DTT, and a remarkable inhibition of 5-LO activity by 10 µM of the $cPLA_2$

inhibitor was observed, which called the selectivity of the inhibitors at least at the used concentrations into question. Moreover, it was concluded that the effects of these two PLA_2 inhibitors on LT biosynthesis were not only due to PLA_2 inhibition, but were superposed by additional effects on 5-LO activity.

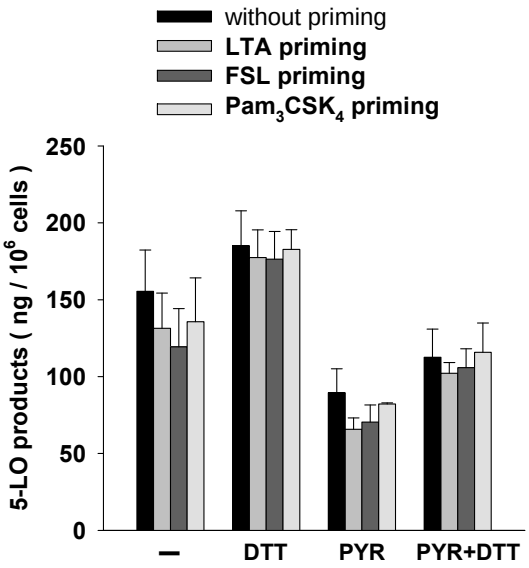


Figure 40: Effects of PLA_2 inhibitors on 5-LO activity. Differentiated MM6 cells (3 x 10^6) were pre-incubated without or with PLA_2 inhibitors (dithiotreitol (DTT) 1 mM, pyrrolidine-1 (PYR) 10 µM or both) as indicated 15 min / 37°C. After priming with TLR2 ligands (LTA 0.5µg/mL, FSL-1 0.5µg/mL, Pam_3CSK_4 5µg/mL) or solvent 15 min / 37°C, cells were stimulated with A23187 2.5 µM plus AA 40 µM for another 10 min / 37°C. 5-LO products were determined by HPLC.

Excursus: $cPLA_2$ inhibitor pyrrolidine-1 as a direct 5-LO inhibitor

Aside from the actual investigations on TLR2 ligand priming, the role of $cPLA_2$ inhibitor pyrrolidine-1 as a direct 5-LO inhibitor was evaluated in more detail. 5-LO was expressed in *E. coli* and the purified enzyme was pre-incubated with the inhibitor, before 5-LO activity was stimulated by addition of AA in presence of calcium. Notably, pyrrolidine-1 directly inhibited the 5-LO enzyme in a concentration dependent manner with an IC_{50} value of 2.25 µM. Hence, this unspecific side effect has to be taken into account, when pyrrolidine-1 is used as a $cPLA_2$ inhibitor.

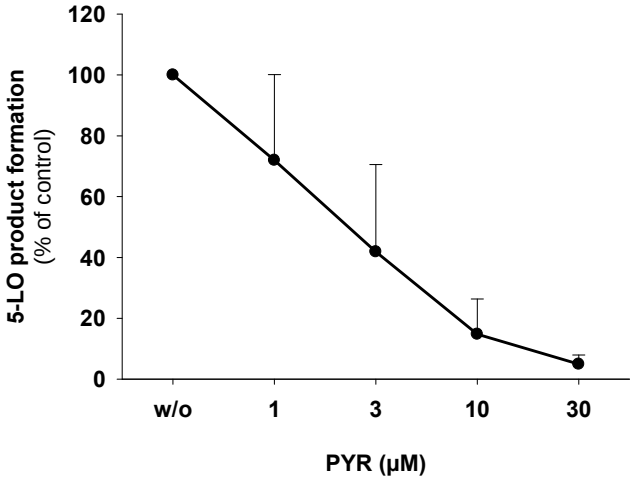


Figure 41: cPLA$_2$ inhibitor pyrrolidine-1 as a direct 5-LO inhibitor. 5-LO was expressed in E. coli. Purified enzymes were diluted in PBS plus 1 mM EDTA, 1 mM ATP and pre-incubated with the indicated concentrations of pyrrolidine-1 (PYR) 15 min / 4°C. Then samples were pre-warmed, 2 mM $CaCl_2$ and then 20 µM AA was added for 10 min / 37°C. 5-LO products were determined by HPLC.

4.3.5 Activation of MAPK pathways by TLR2 ligands

In successive studies the mechanisms of TLR2 ligand priming for enhanced cPLA$_2$ activity were surveyed. The activity of cPLA$_2$α has been shown to be regulated not only by Ca^{2+}, but also by phosphorylation leading to enhanced cPLA$_2$α activity *in vitro* [22, 37]. The following experiments analyzed TLR2 ligand induced activation of MAP kinase pathways that play a role in cPLA$_2$α phosphorylation.

Time-dependent p38 MAPK activation by TLR2 ligands

It has been demonstrated that activated p38 MAPK can phosphorylate cPLA$_2$α at Ser-505 [22]. As it was already described in a former section (4.2.4), activation of p38 MAPK upon incubation with LTA was again analyzed in MM6 cell lysates by immunoblotting, but was now compared to p38 MAPK activation by FSL-1 and Pam_3CSK_4. An increase of phosphorylated p38 MAPK was detectable for each of the three TLR2 ligands. In this experiment, the ligand LTA significantly induced phospho-p38 MAPK 2-4 fold during 15 minutes of incubation in comparison to unstimulated cells, whereas FSL-1 and Pam_3CSK_4 led to a 1.5 to 3.5 fold and to a 1.5 to 2.5 fold increase, respectively. Expectedly, activation of p38 MAPK was dependent on the incubation time. Time courses of p38 MAPK phosphorylation showed similar characteristics for each of the three TLR2 ligands with a

maximal activation between 5 minutes and 15 minutes, followed by a decline to the initial level.

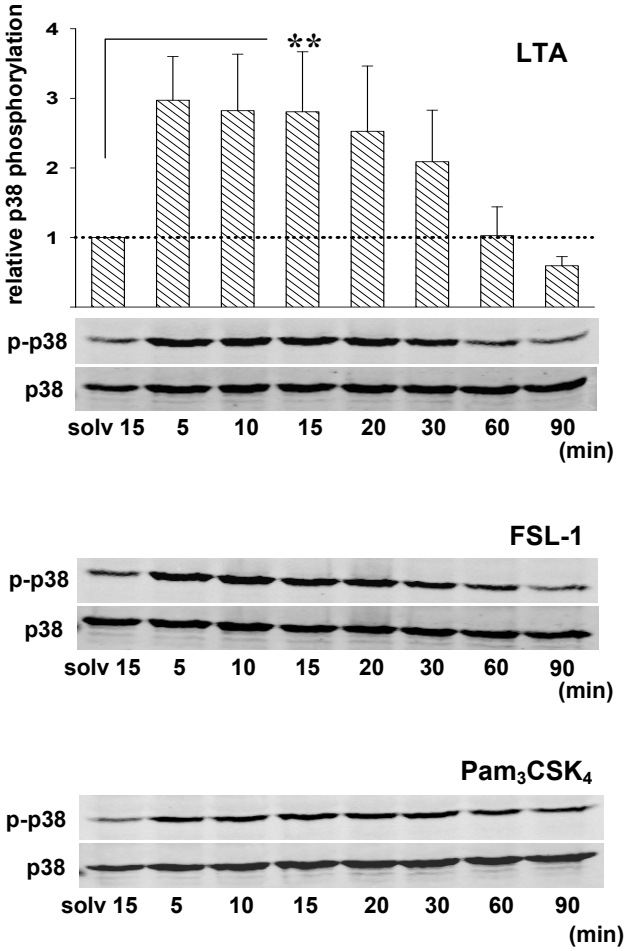


*Figure 42: Time-dependent p38 MAPK activation by TLR2 ligands. Differentiated MM6 (3 × 10^6 cells) were incubated with LTA 0.5 µg/mL, FSL-1 0.5µg/mL or Pam_3CSK_4 5µg/mL for different periods of time / 37°C as indicated. As negative control, cells were treated with solvent for 15min / 37°C (solv 15). Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect p38 MAPK activation, an antibody specific for the dually phosphorylated form of the protein was used. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was determined. For quantification, band intensities of the phosphorylated proteins were corrected by band intensities of both forms. Phosphorylation levels are related to the negative control (dotted line). Student´s t test; ** p<0.01;*

Time-dependent ERK1/2 activation by TLR2 ligands

ERK1/2 activation has also been reported to result in phosphorylation of $cPLA_2\alpha$ at Ser-505 [22]. Analysis of the ERK1/2 activation profiles after TLR2 ligand incubation of MM6 cells revealed a rapid ERK1/2 phosphorylation, which peaked within 5 minutes. However, differences in the time course characteristics were observed between the TLR2 ligands, with respect to phospho-p42 (ERK2) induction. The profile of p42 phosphorylation by LTA was

slightly divergent from former analyzes (4.2.4), since in these experiments the effect was stable over 15 minutes. The initial state of phosphorylation was reached again after 30 minutes of incubation. Phosphorylation of p42 was induced up to 13-fold by LTA in comparison to the control, which was similar to previous observations. Comparable peak inductions (12-fold) were obtained with FSL-1, but the induction was even less sustained. The ligand Pam_3CSK_4 evoked a very transient response, that peaked at an about 14-fold increase after 5 minutes and was almost completely abrogated after 10 minutes.

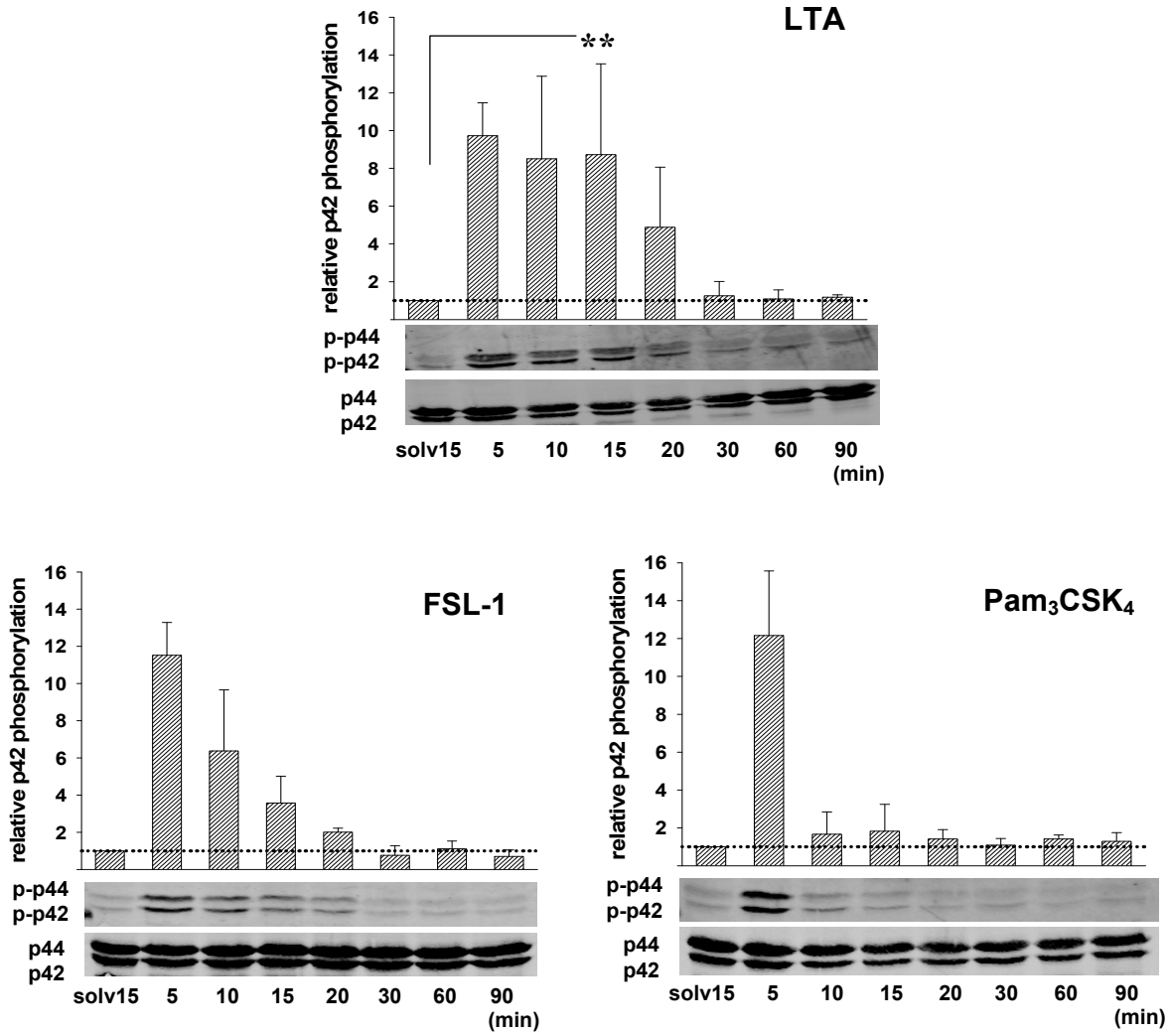


*Figure 43: Time-dependent ERK1/2 activation by TLR2 ligands. Differentiated MM6 (3 × 10^6 cells) were incubated as described above. Total cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect ERK1/2 activation, an antibody specific for the dually phosphorylated form of the protein was used. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was determined. For quantification, band intensities of the phosphorylated proteins were corrected by band intensities of both forms. Phosphorylation levels are related to the negative control (dotted lines). Student´s t test; ** p<0.01;*

Mnk-1 activation by TLR2 ligands

Since p38 MAPK-activated protein kinase Mnk-1 is also known to influence cPLA$_2$α activity by Ser-727 phosphorylation [37], Mnk-1 activation was investigated as well. It was observed that Mnk-1, which is a downstream target of MAP kinases, was also activated by the TLR2 ligands. The ligands LTA and FSL-1 induced Mnk-1 phosphorylation to a comparable extent, whereas Pam$_3$CSK$_4$ again displayed weaker activity.

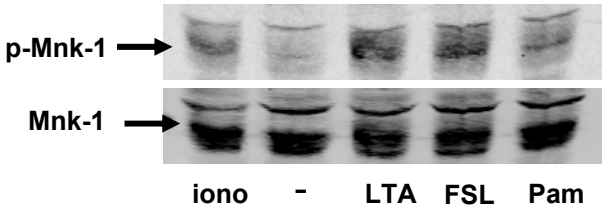


Figure 44: Mnk-1 activation by TLR2 ligands. Differentiated MM6 cells (3 × 10^6 cells) were incubated with ionophore A23187 (iono) 5µM (positive control), solvent (negative control) or TLR2 ligands (LTA 0.5µg/mL, FSL-1 0.5µg/mL, Pam$_3$CSK$_4$ 5µg/mL) for 15min / 37°C. Total cell lysates were prepared and then analyzed by SDS-PAGE and immunoblottin (Odyssey® Infrared Imaging System). Mnk-1 activation was determined by using an antibody specific for the dually phosphorylated form of the protein. As loading control, the total amount of the protein (phosphorylated and unphosphorylated form) was determined.

4.3.6 Time-dependent cPLA$_2$ (Ser-505) phosphorylation by TLR2 ligands

Next, the question was addressed, if TLR2 ligands induce cPLA$_2$α phosphorylation at Ser-505. MM6 cell lysates were analyzed for phospho-cPLA$_2$α (Ser-505) after treatment with the TLR2 activators. Simultaneously, activation of the TLR2 signaling pathway was tested by analysis of IκBα degradation. Each ligand evoked time dependent phosphorylation of cPLA$_2$α. Quantitative analysis of phospho-cPLA$_2$α signals after LTA treatment revealed an up to 2-fold increase of cPLA$_2$α phosphorylation. Extended incubation led to a decrease of phospho-cPLA$_2$α after 60 to 90 minutes. Conversely, LTA and FSL-1 initially led to IκBα degradation over time with a recovery of IκBα protein after 60 to 90 minutes. In contrast, no definite IκBα degradation could be determined after Pam$_3$CSK$_4$ treatment, indicating a poor activation of the TLR2 signaling pathway in comparison to the other ligands.

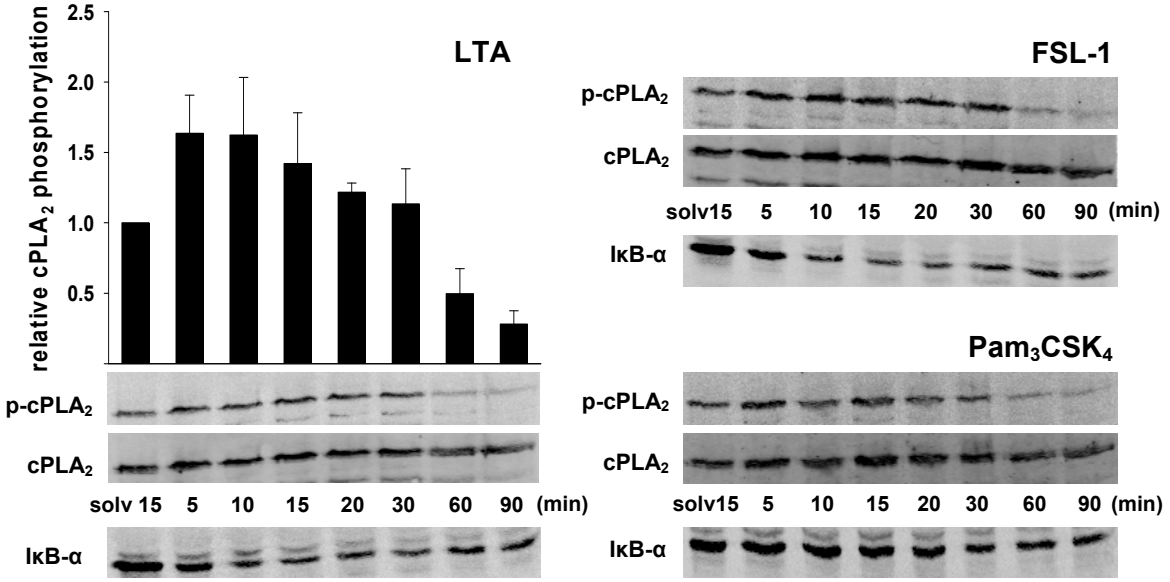


Figure 45: Time-dependent $cPLA_2$ phosphorylation (Ser-505) by TLR2 ligands. Differentiated MM6 (3×10^6 cells) were incubated with TLR2 ligands (LTA 0.5µg/mL, FSL-1 0.5µg/mL, Pam_3CSK_4 5µg/mL) for different periods of time / 37°C as indicated, or cells were treated with solvent for 15 min / 37°C (solv 15). Total cell lysates were analyzed by SDS-PAGE and immunoblotting (Odyssey® Infrared Imaging System). To detect $cPLA_2$ phosphorylation by p38 MAPK and ERK1/2, an antibody specific for phosphorylated $cPLA_2$ (Ser-505) was used. As a control for the activation of TLR signaling pathways, IκB-α degradation was analyzed over time. Simultaneous detection of total $cPLA_2$ served as loading control. For quantification, band intensities of phosphorylated $cPLA_2$ were corrected by band intensities of the total $cPLA_2$ protein. Phosphorylation levels are related to the negative control.

4.3.7 Influence of kinase inhibitors on $cPLA_2$ phosphorylation and AA release

Furthermore, it was investigated if phosphorylation of $cPLA_2\alpha$ (Ser-505) and the increase of AA release was linked to TLR2 ligand triggered activation of p38 MAPK and ERK1/2 in MM6 cells.

The role of MAP kinases in $cPLA_2$ phosphorylation

The following studies comprise the comparison of the increase of phospho-$cPLA_2\alpha$ in stimulated and unstimulated cells, in presence or absence of p38 MAPK inhibitor SB203580, or of PD98059, which selectively inhibits ERK1/2 signaling. In line with the results of the preceding experiment, an up to 2-fold increase of $cPLA_2\alpha$ phosphorylation upon TLR2 ligand stimulation of MM6 cells was found. In presence of SB203580 or PD98059, however, LTA and also FSL-1 mediated increase of phospho-$cPLA_2\alpha$ was reduced, but neither of the two inhibitors abolished the increase completely. Obviously, the two MAPK pathways were

functionally redundant concerning cPLA$_2$α phosphorylation. Only the presence of both inhibitors led to a total prevention of phospho-cPLA$_2$α increase, which was significant for each of the three TLR2 ligands. Hence, both MAPK signaling pathways seem to play a role in TLR2 ligand induced cPLA$_2$α phosphorylation at Ser-505. Control experiments analyzing IκBα protein confirmed the activation of TLR signaling pathways by LTA, FSL-1, and essentially to a weaker extent by Pam$_3$CSK$_4$.

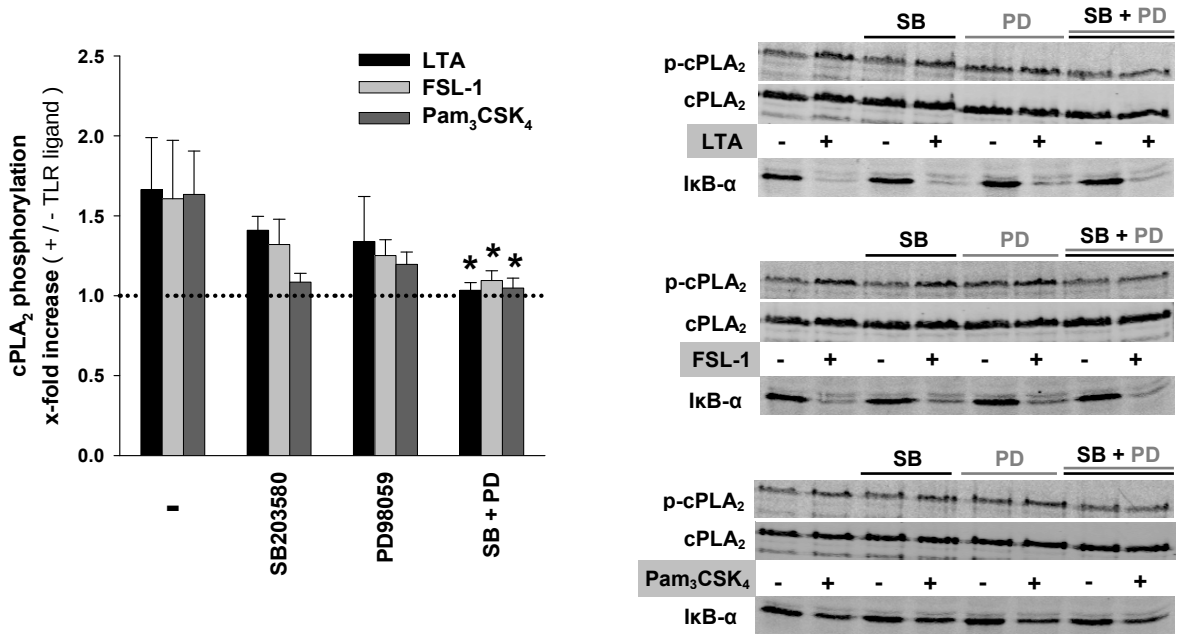


*Figure 46: The role of MAP kinases in cPLA$_2$ phosphorylation. Differentiated MM6 (3 × 10^6 cells) were treated with SB203580 (SB) 10 µM, PD98059 (PD) 50 µM, with both inhibitors or without inhibitor as indicated for 15 min / 37°C. Then cells were stimulated with or without TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam$_3$CSK$_4$ 5 µg/mL) for another 15 min / 37°C. After termination of the incubation, total cell lysates were analyzed by SDS PAGE and immunoblotting (Odyssey® Infrared Imaging System). The increase of cPLA$_2$ phosphorylation by TLR2 ligands is related to the cPLA$_2$ phosphorylation level without TLR activation (dotted line), respectively. Student´s t test; * p<0.05;*

The role of MAP kinases in enhanced AA release

By means of analogous inhibitor studies, it was determined if MAPK mediated phosphorylation of cPLA$_2$α accounts for induced AA release after TLR2 ligand priming. Therefore, the inhibitory influence of SB203580 and of PD98059 on TLR2 ligand mediated increase of free AA was determined in MM6 cells. The results were in accordance with the antecedent data on cPLA$_2$α phosphorylation. Only a combined use of both inhibitors completely blocked TLR2 ligand effects. Thus, activation of two MAPK pathways and

subsequent phosphorylation of cPLA$_2$α seemed to account for the enhancement of ionophore stimulated AA release.

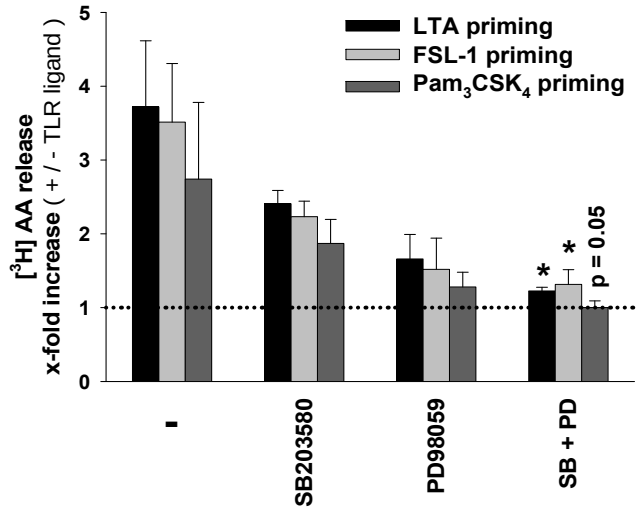


*Figure 47: The role of MAP kinases in enhanced AA release. Differentiated MM6 (2 × 10^6 cells) were pre-labeled with 0.5 µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2mg/mL fatty acid free BSA. Cells were pre-treated with SB203580 (10 µM), PD98059 (50 µM), with the combination of both, or without inhibitor as indicated 15 min / 37°. Thereupon, MM6 cells were primed with TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam$_3$CSK$_4$ 5 µg/mL) or treated with solvent 15 min / 37°C and subsequently stimulated for AA release with A23187 for another 10 min / 37°C. The increase of AA release by TLR2 ligands is related to the AA release level without TLR2 ligand priming (dotted line), respectively. Student´s t test; * p<0.05;*

The role of Mnk-1 and CamKII for enhanced AA release

Apart from p38 MAPK and ERK1/2, two more protein kinases have been suggested to regulate cPLA$_2$α activity by phosphorylation [37]. Mnk-1 accounts for cPLA$_2$α phosphorylation at Ser-727, and Ca^{2+}/Calmodulin kinase II (CamKII) was found to phosphorylate cPLA$_2$α on Ser-515 [22]. Specific inhibitors for Mnk-1 and CamKII were used, respectively, to investigate the role of these two cPLA$_2$ kinases in the enhancement of AA release in MM6 cells. However, neither of the two inhibitors showed a detectable impact on the effects of LTA, FSL-1 and Pam$_3$CSK$_4$, which indicated that these kinases do not seem to be involved in upregulation of cPLA$_2$α activity by TLR2 ligand priming.

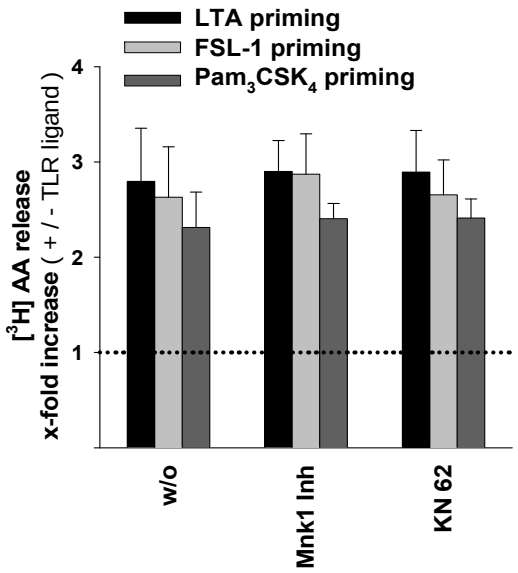


Figure 48: The role of Mnk-1 and CamKII in enhanced AA release. Differentiated MM6 (2 × 10^6 cells) were pre-labeled with 0.5 µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2mg/mL fatty acid free BSA. Cells were pre-treated with Mnk-1 inhibitor (Mnk1 Inh) 20 µM, with CaMKII inhibitor KN-62 (KN 62) 10 µM or without inhibitor as indicated 15 min / 37°. Then, MM6 cells were primed with TLR2 ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) or treated with solvent 15 min / 37°C and subsequently stimulated for AA release with A23187 for another 10 min / 37°C. The increase of AA release by TLR2 ligands is related to the AA release level without TLR2 ligand priming (dotted line), respectively.

4.3.8 TLR dependence of enhanced AA release and LT biosynthesis

As a final study, it was tested whether the enhancing effect of LTA, FSL-1 and Pam_3CSK_4 on AA release is TLR2 dependent. Additionally, it was clarified if TLR4 was also involved in the initiation of the observed effects on AA release and LT formation in MM6 cells.

The role of TLR 4 in TLR2 ligand induced priming effects

The first experiments were undertaken to exclude that the augmentation of AA and LT formation was triggered by LPS contaminations activating TLR4. Therefore, MM6 cells were primed with TLR2 ligands or alternatively with LPS in presence or absence of LPS binding reagent polymyxin B. After subsequent ionophore stimulation, free AA or 5-LO product formation were measured. The addition of polymyxin B significantly blocked the LPS mediated increase of AA release and also of 5-LO product formation in MM6 cells. TLR2 ligand induced increase, however, was not affected by the inhibitor of TLR4 signaling. From that it was concluded that TLR4 was not involved in TLR2 ligand priming.

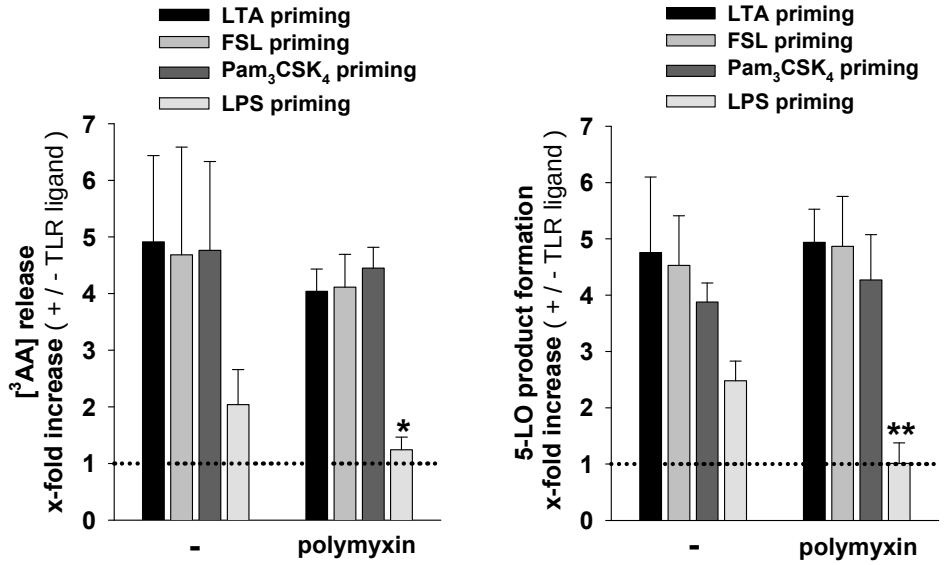


*Figure 49: The role of TLR4 in TLR2 ligand induced priming effects. TLR2 ligands: LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL; TLR4 ligand: LPS 10 µg/mL; stimulus: ionophore A23187 2.5 µM; the increase of AA release or 5-LO product formation by TLR ligands is related to the response level without TLR ligand priming (dotted line), respectively. To measure AA release, differentiated MM6 (2×10^6) were pre-labeled with 0.5 µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 2 mg/mL fatty acid free BSA. [^{3}H]AA-labeled MM6 cells were pre-treated with polymyxin B 100 µg/mL or with solvent 15 min / 37°C. Subsequently cells were primed with TLR ligands or solvent as indicated 15 min / 37°C and then stimulated for AA release with A23187 for another 10 min / 37°C. To measure 5-LO product formation, differentiated MM6 cells (3×10^6) were pre-incubated with or without polymyxin B 100 µg/mL 15 min / 37°C. After priming with TLR ligands or treatment with solvent 15 min / 37°C, cells were stimulated with A23187 for another 10 min / 37°C. Student's t test; ** $p<0.01$, * $p<0.05$;*

TLR 2 dependence of enhanced AA release

Secondly, analyzes were undertaken to prove that the enhancement of AA release by LTA, FSL-1 and Pam_3CSK_4 was mediated by TLR2. In measurements of AA release, it was observed that a neutralizing anti-hTLR2 antibody significantly inhibited the TLR2 ligand induced priming effects in MM6 cells and almost completely blocked LTA, FSL-1 and Pam_3CSK_4 induced enhancement of ionophore induced AA release. Taken together, the investigations confirmed that priming of MM6 cells by LTA, FSL-1 and Pam_3CSK_4 for enhanced AA release is dependent on TLR2 activation.

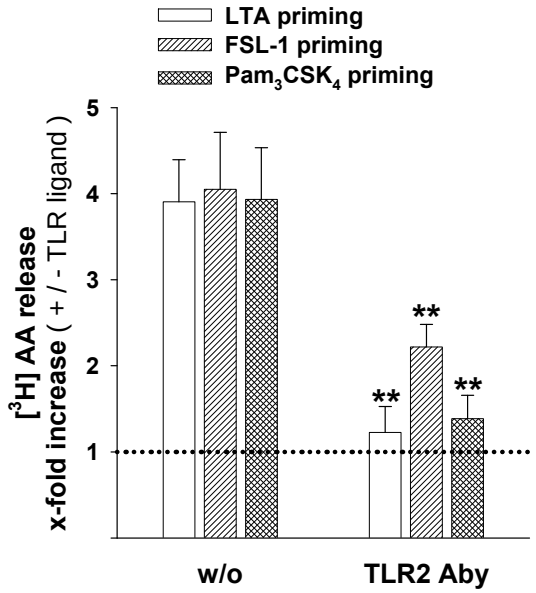


*Figure 50: TLR2 dependence of enhanced AA release. Differentiated MM6 (2×10^6) were pre-labeled with 0.5 µCi/mL [^{3}H]AA for 3 h / 37°C. Unincorporated [^{3}H]AA was removed and cells were resuspended in PGC buffer containing 1 mg/mL fatty acid free BSA. [^{3}H]AA-labeled MM6 cells were pre-treated with anti-hTLR2 antibody (TLR2 Aby) 250 µg/mL or with solvent 15 min / 37°C. Subsequently cells were primed with TLR ligands (LTA 0.5 µg/mL, FSL-1 0.5 µg/mL, Pam_3CSK_4 5 µg/mL) or solvent as indicated 15 min / 37°C and then stimulated for AA release with A23187 2.5 µM for another 10 min / 37°C. The increase of AA release by TLR ligands is related to the response level without TLR ligand priming (dotted line), respectively. Student´s t test; ** $p<0.01$;*

5 DISCUSSION

The results of the present project revealed an enhancement of the cellular LT biosynthesis by TLR ligand priming of human monocytes. Besides TLR4 ligand LPS and TLR5 ligand flagellin, the TLR2 ligands LTA, FSL-1 and Pam_3CSK_4 led to an increase of ionophore- or fMLP-induced 5-LO product formation in monocytic cell-lines and primary human monocytes. Investigations comprised a characterization of TLR2 ligand mediated effects and an analysis of the underlying mechanisms. The influence of TLR2 signaling on two key enzymes of the LT biosynthesis pathway, 5-LO and $cPLA_2\alpha$, was investigated. This section comments on the findings and provides a discussion of present results against the background of published knowledge in related research topics.

5.1 Priming effect of TLR ligands on LT biosynthesis in human monocytes

5.1.1 Overview: enhancement of LT biosynthesis by priming agents

Enhanced LT biosynthesis in leukocytes after pre-treatment of these cells with so-called priming agents has been widely described. To provide an overview about numerous reports, a simplified classification of such priming substances into three groups is suggested in the following section: priming by various cytokines, by diacylglycerol-related compounds and furthermore by pathogen-derived molecules [11].

Early reports refer enhanced biosynthesis of 5-LO metabolites in PMNL upon stimulation with fMLP or complement factor C5a, or also upon stimulation with a Ca^{2+} ionophore, with PAF or alternatively also with LTB_4 or IL-8, if cells had been pre-incubated with GM-CSF 30 min – 2 h prior to stimulation [375-379]. Not only in isolated PMNL, but also in human blood [380], in human primary monocytes [381], and in peripheral blood eosinophils from allergic rhinitis patients [382], GM-CSF priming for enhanced 5-LO product formation has been reported. TNFα has been studied as a priming agent for PMNL and human blood to enhance fMLP-, PAF-, C5a-, zymosan- or Ca^{2+} ionophore - induced biosynthesis of LTB_4 and 5-HETE [380, 383, 384]. IL-3 and IL-5 enhanced Ca^{2+} ionophore-stimulated LTB_4 production in RBL-1 cells [385], IL-3 primed human primary monocytes for enhanced 5-HETE and LTB_4 formation [381] and IL-5 increased fMLP induced LTC_4 synthesis in human eosinophils [386]. Recent studies furthermore revealed priming activities of the C-C chemokines

RANTES and eotaxin, which enhanced ionophore-induced LTC_4 biosynthesis in human eosinophils and basophils [387], and also IL-16 was shown to augment LTC_4 release from human eosinophils [388].

Diacylglycerols and phorbol esters constitute another group of priming agents. Among the diacylglycerols, 1-oleoyl-2-acetylglycerol (OAG) and 1,2-dioctanoylglycerol (DiC8) have been characterized as priming agents for enhanced 5-LO product synthesis in PMNL upon stimulation with fMLP, Ca^{2+} ionophore or zymosan [384, 389-391], and also in rat peritoneal macrophages, formation of 5-LO metabolites was augmented by OAG [392]. Among the phorbol esters, phorbol-12-myristate-13-acetate (PMA) is known to enhance ionophore- or fMLP-induced LTB_4 release in PMNL [390, 391, 393] and 5-LO product formation in rat alveolar and peritoneal macrophages upon stimulation with Ca^{2+} ionophore [392]. Furthermore, MM6 cells and PMNL were shown to be primed by PMA and its analogue mezerein for an increase of 5-LO metabolites [373]. PAF was also described to prime human PMNL for enhanced Ca^{2+} ionophore - induced formation of LTB_4 in one study [394].

Lipopolysaccharides (LPS), a component of the outer membrane of gram-negative bacteria, are potent activators of host defense mechanisms during infections and have early been recognized as priming agents for augmented LT formation. First studies described LPS priming of peritoneal mouse macrophages for 60 minutes, which resulted in enhanced formation of 5-LO metabolites upon stimulation with zymosan, immune complexes, PMA, Ca^{2+} ionophore or latex particles [395]. Successive reports described an increase of zymosan-, PMA- or ionophore-induced, and furthermore fMLP-induced LTB_4 synthesis from human PMNL after priming with LPS [364, 365, 367]. Also in human PBMC and in whole blood, LPS priming effects have been detected [365, 366]. Not only LPS as bacterial product, but also Epstein-Barr virus (EBV) was discovered to exert priming activity on human primary monocytes that were stimulated for LT formation with Ca^{2+} ionophore, fMLP or zymosan [254]. In very recent investigations finally, priming for LT generation was linked to TLR activation for the first time [363, 396, 397]. PGN was found to enhance LTC_4 release from human basophils stimulated with an anti-IgE antibody [363]. Buczynski et al. investigated TLR4 priming of RAW264.7 murine macrophages with Kdo_2-lipid A, an LPS substructure with endotoxin activity, which resulted in enhanced 5-LO product formation after ionophore- or ATP-stimulation [397]. Furthermore, TLR7/8 ligand resiquimod was shown to act as a priming agent on human neutrophils via TLR8 activation, leading to enhanced LTB_4 formation upon stimulation with fMLP, PAF and ionophore [396].

	priming agent	cell type	agonist	reference
cytokines	**GM-CSF**	human PMNL	C5a	[377, 379]
			fMLP	[377-379, 382]
			ionophore	[376, 378, 379]
			PAF	[375, 379]
			LTB_4	[379]
			IL-8	[379]
		human blood	C5a, fMLP, ionophore, PAF, zymosan	[380]
		human monocytes	ionophore	[381]
		human eosinophils	fMLP	[382]
	TNFα	human PMNL	fMLP	[383]
			zymosan	[384]
		human blood	C5a, fMLP ionophore, PAF, zymosan	[380]
	IL-3	human monocytes	ionophore	[381]
		RBL-1	ionophore	[385]
	IL-5	RBL-1	ionophore	[385]
		human eosinophils	fMLP	[386]
	RANTES, eotaxin	human eosinophils, basophils	ionophore	[387]
	IL-16	human eosinophils	ionophore	[388]
diacylglycerol - related	**OAG**	human PMNL	fMLP	[389, 390]
			ionophore	[389, 391]
		rat peritoneal macrophages	ionophore	[392]
	DiC8	human PMNL	fMLP	[390]
			zymosan	[384]
	PMA	human PMNL	fMLP	[390]
			ionophore	[373, 391, 393]
		MM6	ionophore	[373]
		rat peritoneal and alveolar macrophages	ionophore	[392]
	mezerein	human PMNL	ionophore	[373]
		MM6	ionophore	[373]
	PAF	human PMNL	ionophore	[394]
pathogen - related		**LPS**	mouse peritoneal macrophages	zymosan, immune complexes, PMA, ionophore, latex particles	[395]
			human PMNL	zymosan, PMA ionophore	[364]
				fMLP	[365, 367]
			human PBMC	fMLP	[365, 366]
			whole blood	fMLP, C5a, IL-8	[365]
		EBV	human monocytes	ionophore, fMLP, zymosan	[254]
	TLR priming	**PGN**	human basophils	anti-IgE antibody	[363]
		Kdo_2-lipid A	RAW264.7	ATP, ionophore	[397]
		resiquimod	human PMNL	fMLP, PAF, ionophore	[396]

Table 2: Priming agents that increase 5-LO product formation upon subsequent agonist stimulation. Extended according to [11].

The reported priming events showed some consistent characteristics, which have been described repeatedly throughout all studies. First, priming agents commonly do not have the ability to directly stimulate LT biosynthesis or only marginally induce the formation of 5-LO products [254, 364-367, 375, 377, 378, 383, 393, 395, 396], but pre-incubation of leukocytes with these agents synergistically augments the cellular response upon subsequent agonist stimulation. Thereby, priming has an extensive impact on LT biosynthesis, particularly if weak physiological agonists or suboptimal concentrations of the highly effective Ca^{2+} ionophores are used for stimulation [254, 364, 373, 378, 379, 391, 393, 396]. Secondly, the extent of increased LT formation was dependent on the priming agent concentration as assessed in many cases [364, 365, 375, 377, 380, 387, 388, 391, 394, 396]. Furthermore, the priming effect is decisively dependent on the pre-incubation time. Several reports describe priming as a short-time effect that becomes visible after minutes, reaches its maximum with pre-incubation times for 15 – 120 minutes (depending on the assay conditions) and disappears with extended periods of pre-incubation [254, 364-367, 373, 375, 379, 380, 396].

5.1.2 Characterisation of TLR2 ligand priming in human monocytes

In initial screening experiments of the present study it was shown that ligands for TLR2/6 (LTA and FSL-1), for TLR2/1 (Pam_3CSK_4), and expectedly also TLR4 activator LPS displayed enhancing effects on 5-LO product formation in MM6 cells. Furthermore, TLR5 ligand flagellin was found to increase 5-LO products in an additional experiment. In contrast, no influence on ionophore-induced LT levels was detected after pre-incubation with TLR3 ligand Poly (I:C), TLR7 ligand Loxoribine, TLR8 ligand ssPolyU and type A or type B CpG ODNs, which are activators of TLR9. In analyses of TLR mRNA expression patterns in cellular subsets of hPBMC, it was shown that high expression of TLR2 was characteristic for monocytes [398]. These studies furthermore revealed considerable levels of TLR1 and TLR4, moderate levels of TLR5, TLR6 and TLR8, and weak, but detectable TLR9 expression in monocytes isolated from hPBMC [398]. TLR7 and TLR3, however, were absent in monocytes, and despite low expression of TLR9, monocytes did not respond to TLR9 activators in the absence of plasmacytoid dendritic cells (pDC) [398, 399]. The TLR expression patterns described for monocytes are compatible with present observations that no response to TLR7 ligand Loxoribine, TLR3 ligand Poly(I:C) and to TLR9 activators CpG ODNs was detected in MM6 cells. It is not clear, though, why MM6 cells were not responsive to TLR8 ligand ssPolyU. In this regard it should be noted that MM6 cells only reacted on

ligands that activate TLRs localized on the cellular surface. TLR8, however, is located in the endosome. According to the manufacturer, ssPolyU/LyoVec^{TM} is a lyophilized preparation of single-stranded poly-uridine complexed with the cationic lipid LyoVec^{TM}, to protect it from degradation and facilitate its uptake, but nevertheless, TLR8 may possibly not be reached efficiently by its ligand within 15 minutes of pre-incubation.

Toll-like receptor 2 ligand-mediated effects on leukotriene biosynthesis were characterized in terms of their concentration-dependence. As it was found in several reports mentioned in the previous section, the increase of LT formation was clearly dependent on the TLR ligand concentration. For LTA and FSL-1 an optimal concentration of about 0.5 µg/mL was defined resulting in a maximal enhancement of 5-LO products. A second maximum was observed at concentrations higher than 10 µg/mL, which may be due to additional, less specific effects or effects on low affinity receptors. Pam_3CSK_4, in contrast, enhanced 5-LO products continuously with increasing concentrations without an intermediate maximum. For Pam_3CSK_4 it was reported in studies of lipopeptide-induced B-cell activation that a concentration of 80 µg/mL induced a moderate increase of intracellular calcium and it was speculated that this may be ascribed to ionophore-like membrane interactions [400]. Furthermore, Pam_3CSK_4, at concentrations greater than 25 µg/mL, was shown to activate superoxide formation and lysozyme release in human neutrophils and it was suggested that Pam_3CSK_4 acts through G-proteins in a similar way as fMLP [401]. Therefore, TLR2 dependent effects of Pam_3CSK_4 on LT formation may be enhanced by additional mechanisms at concentrations greater than 10 µg/mL. Thus, the use of 5 µg/mL Pam_3CSK_4 was defined as optimal to enhance 5-LO products to a comparable extent as with LTA and FSL-1 at their optimal concentrations. Overall, Pam_3CSK_4 seemed to have a weaker potency, as a 10-fold higher concentration of Pam_3CSK_4 compared to FSL-1 was required to evoke similar response intensities. It can be suspected that this may be attributed to a lower affinity of Pam_3CSK_4 to its heterodimeric receptor, compared to possibly higher receptor-binding affinities of FSL-1 or LTA.

A TLR2 ligand induced enhancement of 5-LO product formation was also found in human primary cells. The preparations of hPBMC in large parts consisted of monocytes (30.2 – 52.9 %), which are known to highly express TLR2 [398]. Human PBMC also included proportions of T-lymphocytes (25.2 – 43.8%) and minor amounts of B-lymphocytes (3.6 – 5.1%) and NK cells (7.9 – 9.0%) as determined by FACS analysis. Whereas T cells are considered to lack 5-lipoxygenase [16], B cells were shown to express 5-LO. It was demonstrated, however, that in B-lymphocytes calcium ionophore is not sufficient to activate 5-lipoxygenase [402].

Moreover, lymphocytes are not responsive to fMLP [366, 403]. Preliminary experiments furthermore indicated, that human primary NK cell preparations do not seem to synthesize leukotrienes upon stimulation with calcium ionophore (data not shown in present studies). Thus, enhanced 5-LO product synthesis upon TLR2 ligand pre-treatment in PBMC was regarded as a monocytic response, even though it cannot be excluded, that other cell-types containing the LTA_4 hydrolase enzyme may contribute to LTB_4 synthesis through transcellular metabolism of LTA_4 [366]. In summary it was concluded, that TLR2 ligands act on human primary monocytic cells in a similar manner as on MM6 cells, and that MM6 cells seem to be an appropriate model to investigate TLR2 mediated effects on 5-LO product formation in human monocytes.

TLR2 ligands enhanced LT formation in PBMC, particularly upon activation by low ionophore concentrations and the weak agonist fMLP, whereas strong stimulation by high concentrations of ionophore was not further enhanced. Also in MM6 cells it was observed that TLR2 ligand effects were strong upon stimulation with suboptimal concentrations of ionophore (1-2.5 μM), and were reduced again with high stimulatory concentrations of ionophore (>5 μM). Apparently, the influence of TLR2 ligands seems to be of importance, particularly if subsequent stimulation of 5-LO product formation is moderate. This is consistent with previous data that describe substantial LT formation or AA release upon stimulation with normally ineffective concentrations of weak stimuli like PAF or of ionomycin, when PMNL had been pre-treated with GM-CSF [375, 378]. Also PMA enhanced 5-LO product formation in PMNL particularly upon low-dose ionophore stimulation [373], and PMNL priming with LPS or R-848 could not be demonstrated, when optimal stimulatory concentrations of ionophore were used [364, 396].

Time course experiments revealed that TLR2 ligand-mediated increase of 5-LO products is a transient short-time effect with a maximal enhancement with 15 minutes of pre-incubation. All of the three curve progressions showed that with pre-incubation times exceeding 15 minutes the increase of 5-LO products was reduced. These findings very well correspond with previous reports that demonstrated similar kinetics of GM-CSF, LPS, EBV or R-848 priming [254, 365-367, 379, 396]. In analyses of 5-LO products of Kdo_2-lipid A primed RAW264.7 macrophages, a synergistic mode of action was determined for Kdo_2-lipid A and the subsequently added Ca^{2+} agonist, by calculation of a synergistic activation ratio [397]. In the present studies, pre-incubation of TLR2 ligands was essential for the enhancement of LT formation, and simultaneous addition of TLR2 ligands together with ionophore did not evoke augmented 5-LO product synthesis. These characteristics indicated that TLR2 ligands do not

directly stimulate LT biosynthesis in MM6 cells and that enhancement of leukotriene formation cannot be explained as a simple additive activation by two individual stimuli. This suggestion was confirmed by the observation that 5-LO product formation after TLR2 ligand pre-incubation and subsequent stimulation was higher than the sum of products formed by TLR2 ligand and ionophore stimulation separately. However, Pam_3CSK_4 showed a weak direct stimulatory potency on 5-LO product formation and the synergistic effect of Pam_3CSK_4 priming with ionophore stimulation was not as pronounced as of the other ligands. It seems conceivable that Pam_3CSK_4 may additionally induce other weak cellular effects regardless from TLR activation, which may slightly interfere with the synergistic mechanism of priming. Essentially, it was concluded though that TLR2 ligands can synergistically prime human monocytes for an enhanced response upon stimulation and that they show typical priming characteristics, which are similar to the properties of previously known priming agents.

5.2 Investigations on the underlying mechanisms of TLR2 ligand priming

5.2.1 The influence of TLR ligands on the 5-lipoxygenase enzyme

As 5-LO is the key enzyme for the biosynthesis of leukotrienes, the enhancement of LT formation by TLR ligand priming could be a result of a ligand-mediated augmentation of cellular 5-LO activity. If cellular LT biosynthesis is stimulated in presence of an overabundant amount of exogenous AA, the proportion of 5-LO products formed from cellular AA becomes insignificant [170]. Thus, 5-LO product formation – for the decisive part – occurs independent from endogenous substrate supply and can be investigated irrespective from PLA_2 enzyme activity. Furthermore, it was assumed that exogenous substrate is also converted efficiently by 5-LO regardless of 5-LO redistribution to the nuclear envelope [373, 404]. Therefore, measurement of 5-LO product formation in presence of exogenous AA should be independent from efficient 5-LO translocation and assembly of the LT biosynthetic complex at the nuclear membrane. Accordingly, a TLR ligand priming effect under such conditions would point to an enhancement of 5-LO enzymatic activity as a mechanism of priming. 5-HpETE is the first product formed from AA by the 5-lipoxygenase reaction. On the one hand it is further metabolized to LTA_4 by 5-LO, but it can also be reduced to the corresponding hydroxyacid 5-HETE by cellular glutathione peroxidases [16]. As an early product of the 5-LO pathway, 5-HETE is formed independent from downstream enzymes of LT biosynthesis (e.g. LTA_4 hydrolase). Thus, an increase of 5-HETE formation as a result of

priming may also indicate an enhancement of 5-LO activity. However, a priming effect of LTA, flagellin and expectedly ssPolyU on 5-HETE formation was not detectable in presence of AA, neither in differentiated MM6 cells, nor in differentiated THP-1 cells. Additionally, no enhancement of total 5-LO product formation became visible after priming of differentiated MM6 cells with the three different TLR2 ligands LTA, FSL-1 or Pam_3CSK_4 and subsequent stimulation with ionophore and AA. Surprisingly, TLR ligands rather exerted a slightly inhibitory influence on 5-HETE formation in MM6 cells under such conditions, which was only significant after LTA pre-treatment, though. Total 5-LO product formation also seemed weakly reduced after LTA priming. Therefore it was concluded that compared to PMA, which was used as a control priming agent [373], TLR ligands do not seem to prime human monocytes for enhanced LT formation by augmenting cellular 5-LO activity. By contrast, in former studies an influence on 5-LO activity indeed has been observed for several priming agents. Thus, GM-CSF or LPS pre-incubation of PMNL, and EBV pre-incubation of PBMC enhanced the conversion of the model substrate 15-HpETE into 5,15-diHETE upon stimulation of PMNL with PAF or fMLP, or of PBMC with ionophore [254, 367, 375]. Furthermore, PMA priming resulted in an increase of ionophore-induced 5-LO product formation in presence of exogenous AA in MM6 cells and PMNL [373].
In additional investigations, LTA and flagellin pre-incubation per se also did not result in a further stimulation of LT biosynthesis after addition of AA, which itself only marginally induces 5-LO product formation in MM6 cells compared to other cell-types [167]. Also simultaneous addition with AA had no effect (preliminary data not shown). Instead, LTA pre-treatment again reduced 5-LO products in MM6 cells compared to the control. Therefore, a stimulatory influence on cellular 5-LO enzyme activity by TLR ligands was excluded. For some priming agents, in contrast, also direct stimulatory properties for 5-LO were shown. PAF on the one hand has been used as a priming agent in PMNL [394], but was also capable of weakly inducing 5-LO product formation as a stimulus in several studies [375, 379, 380, 396]. Furthermore, OAG did not only prime for enhanced LT biosynthesis upon stimulation with ionophore or fMLP [389-392], but also by itself stimulated 5-LO activity, and a direct interaction of OAG with the 5-LO enzyme was demonstrated [127, 405].

Not only enzymatic activity of 5-LO, but also its effective translocation to the nuclear envelope upon stimulation is a critical parameter for LT biosynthesis from endogenous substrate. In former studies, priming agents were demonstrated to upregulate 5-LO translocation, which may account for enhanced LT formation. It was reported that priming of

MM6 cells with the phorbol esters PMA or mezerein augmented ionophore-induced association of cellular 5-LO with the nuclear membrane and similar results were obtained for PMNL stimulated with low dose ionophore [373]. Furthermore, 5-LO translocation to the nuclear fraction was observed after fMLP stimulation of LPS-primed PMNL, but not of unprimed cells [367] and also TLR7/8 ligand resiquimod promoted 5-LO translocation in PMNL to cellular membranes, which was blocked by $cPLA_2\alpha$ inhibitor pyrrophenone and FLAP inhibitor MK-0591 pointing to a FLAP-dependent mechanism [396]. In present investigations, however, no increase of 5-LO redistribution from the nonnuclear to the nuclear compartment was detected in TLR2 ligand primed MM6 cells compared to unprimed cells upon stimulation with ionophore. PMA mediated enhancement of 5-LO translocation, in contrast, could be confirmed as it was described for MM6 cells [373]. Similar to earlier observations, 5-LO was predominantly found in the nonnuclear fraction of resting cells, but divergent to the former report, ionophore stimulation per se in fact significantly induced a partial redistribution of 5-LO into the nuclear fraction of MM6 cells [373]. Dissimilar handling of the fractionation technique and different sensitivities of protein detection possibly might contribute to these divergent results, which are not contradictory in principle, though. Control experiments for adequate cellular fractionation revealed a weak signal of the cytosolic marker GAPDH in the nuclear fraction indicating that also a small proportion of nonnuclear 5-LO was possibly detected in the nuclear fraction. However, this observation was neglected due to the minor extent of this inaccuracy. Moreover, this does not detract the findings that no enhancement of 5-LO translocation could be found in TLR2 ligand treated cells. TLR2 ligand incubation of MM6 cells without subsequent addition of ionophore also did not stimulate 5-LO translocation, but LTA surprisingly seemed to exert opposite effects, as a slight increase of 5-LO in the nonnuclear fraction was detected. In an additional experiment, 5-LO translocation to cellular membranes in general was analyzed in MM6 cells, but TLR2 ligand priming did not result in enhanced translocation of 5-LO from the cytosol to the membrane fraction. Interestingly, also other sites of 5-LO product synthesis than the nuclear envelope have been discussed for primed leukocytes. PAF mediated enhancement of ionophore-induced LTB_4 release and also chemokine-enhanced LTC_4 production was correlated with PAF- or chemokine-induced lipid body formation in PMNL or eosinophils and basophils [387, 388, 394]. Moreover, cytoplasmic lipid bodies were shown to be the site for enhanced LT synthesis in eosinophils [387, 406]. In very recent studies, leukocytes from *Histoplasma capsulatum (Hc)* infected mice were shown to produce lipid bodies, which correlated with enhanced generation of LTB_4 upon stimulation with ionophore. Moreover, *Hc*-induced lipid

body formation in murine alveolar macrophages was dependent on TLR2 [407]. Also *Mycobacterium leprae*-induced lipid body formation was observed in human mononuclear phagocytes and mouse peritoneal macrophages, and an involvement of TLR2/6 activation was suggested. Furthermore, increased levels of PGE_2 were detected temporally coinciding with lipid body formation [408]. Up to the present, there is no information available, if lipid body formation can also occur in MM6 cells, and present data do not provide any information, if TLR ligand enhanced LT formation might occur at such structures in MM6 cells.

During analyses of TLR2 ligand mediated priming of MM6 cells, a more prominent enhancement of 5-HETE in comparison to total 5-LO products was noticed, which is referred to as "5-HETE effect" in this dissertation. Assuming sufficient substrate supply, it was speculated as a first hypothesis, if such a prominent 5-HETE formation might occur in the cytosol independent from effective interaction of LT forming enzymes within the biosynthetic complex, and thus might reflect poor 5-LO translocation to the nuclear envelope under TLR2 ligand priming conditions. However, also the noticeable generation of the non-enzymatically formed all-*trans* isomers of LTB_4 would have been expected under such conditions, which yet was not observed. Furthermore, if translocation of 5-LO in MM6 cells is relevant for effective co-localization with LTA_4 hydrolase and their metabolic coupling in the conversion of LTA_4 to LTB_4, is not clear. LTA_4 hydrolase was described as a soluble enzyme, which was found in the cytosolic fraction of rat neutrophils [409, 410]. Moreover, different subcellular distribution of LTA_4 hydrolase and co-localization with 5-LO was found depending on the cell type. Thus, in rat basophilic leukemia cells and rat primary alveolar macrophages, LTA_4 hydrolase together with 5-LO was detected in the soluble nuclear fraction of the cells. In human PMNL, in contrast, LTA_4 hydrolase and 5-LO were located in the cytosol, but only 5-LO subcellular redistribution into the nuclear fraction was observed in activated neutrophils [411]. Hence, if minor enhancement of LTB_4 in TLR2 ligand primed MM6 cells is linked to ineffective 5-LO translocation, is not known. Nevertheless, in order to check the hypothesis, 5-HETE formation in LTA primed cells was compared to PMA primed cells, which do show substantial 5-LO translocation upon stimulation with ionophore. However, the "5-HETE effect" was detectable in LTA primed and likewise in PMA primed MM6 cells, and these results were confirmed in THP-1 cells and to a lesser extent also in PBMC. Therefore it was concluded that the "5-HETE effect" does not seem to be characteristic particularly for TLR2 ligand primed cells, and does not only occur in differentiated monocytic cell lines, but also in primary monocytes. In comparison, OAG priming of fMLP stimulated PMNL did not

increase 5-HETE to a greater extent than LTB_4 [389, 390]. Kdo_2-lipid A priming of RAW264.7 murine macrophages, in contrast, resulted in a stronger enhancement of 5-HETE than of LTC_4 formation upon stimulation with ionomycin or ATP [397]. Monocytic cells, in contrast to neutrophils or B-lymphocytes, were shown to contain glutathione peroxidase-1, and MM6 cells were found to have high glutathione peroxidase activities [162], which possibly could also be true for monocytes / macrophages in general. Therefore, a considerable 5-HETE formation might be characteristic for monocytic cells, which may have a greater capactity to reduce 5-HpETE to 5-HETE than other cell types. Nevertheless, the reason for a greater enhancement of 5-HETE compared to other 5-LO products in primed monocytes is not clear. It can only be speculated that priming may exert an enhancing effect on glutathione peroxidase activity or might possibly alter the velocity of single reaction steps catalyzed by 5-LO.

5.2.2 A comparison of priming properties of LTA and PMA in MM6 cells

Present investigations revealed differences in the mechanism of priming of LTA compared to several other priming agents, which in contrast to LTA up-regulate 5-LO activity and translocation. As particularly PMA was shown to exert such effects in MM6 cells [373], the question came up, how differing modes of action of LTA and PMA would account for these differences regarding the mechanism of priming.

The phorbol ester PMA is a PKC activator that directly binds PKC at its C1 domain within the regulatory domain, thus substituting for diacylglycerol, which is generated endogenously by PLC consequent to receptor-ligand interactions [392, 412]. Activation of PKC is regulated by DAG or phorbol ester binding in the presence of a phospholipid cofactor and also by Ca^{2+}, which binds at the C2 domain of PKC and mediates its association to membranes [412]. In early reports, PMA was shown to rescue ionophore-induced LTB_4 synthesis and AA release that had been blocked by PKC inhibitors in PMNL [393], and it was demonstrated that PKC activation was accompanied with an up-regulation of the ionophore stimulated 5-LO pathway in resident rat alveolar and peritoneal macrophages [392]. Thus, a role of PKC for LT biosynthesis was ascertained. In MM6 cells, in contrast to PMNL, ionophore stimulation is not sufficient to induce pronounced LT biosynthesis, and previous PMA priming was required for substantial 5-LO translocation and 5-LO product formation from endogenous AA [373]. Both processes were blocked by PKC inhibitor Calphostin C, pointing to a role of PKC in PMA priming. Furthermore, in PMA primed MM6 cells, a Calphostin C sensitive up-

regulation of ionophore-induced MAPK-activated protein kinase 2 (MK2) activity was found. MK2 had been shown to phosphorylate 5-LO at Ser-271 *in vitro*, which is believed to positively regulate 5-LO catalysis [169, 171]. 5-LO was also found to be phosphorylated by ERK1/2 at Ser-663 *in vitro*, and in MM6 cells PMA priming was essential for ERK1/2 activation and considerable 5-LO phosphorylation [170]. Moreover, enhancement of ionophore-induced 5-LO product formation in presence of exogenous AA was reversed by ERK activation inhibitor U0126 in PMA primed MM6 cells, but U0126 hardly affected product formation in unprimed cells [170]. Thus, 5-LO phosphorylation by MK2 and ERK1/2 could contribute to increased 5-LO activity by PMA priming. Phosphorylation has been speculated to possibly lower the concentration of Ca^{2+} needed to activate 5-LO in the cell [16]. A direct connection between 5-LO kinase activation and 5-LO translocation is still unclear. 5-LO phosphorylation may govern nuclear redistribution [16], but phosphorylation of other proteins important for 5-LO activity could also be involved [373].
TLR ligands are known to induce MAP kinase activation via the MyD88-dependent signalling pathway, which is common to all TLR subtypes except TLR3 [282, 286], and TLR2 activation by LTA has been implicated in p38 MAPK and ERK1/2 activation in monocytic cells or macrophages in numerous studies [292, 413, 414]. Hence, LTA induced activation of the p38 MAPK and ERK1/2 pathways seemed likely also in MM6 cells, and consecutive phosphorylation of 5-LO seemed conceivable. Furthermore, LTA has also been described as a PKC activator, at rather high concentrations (1-50 µg/mL) though, in some studies. LTA was suggested to activate phosphatidylcholine-PLC (PC-PLC) and PC-PLD, but not PIP_2-PLC, to induce PKC activation in a human pulmonary epithelial cell line (A549) [415]. In RAW 264.7 macrophages and human tracheal smooth muscle cells, in contrast, LTA-induced PKC activation seemed to be mediated by PC-PLC and by PIP_2-PLC [416, 417].

As a positive regulation of 5-LO was not apparent after LTA priming, three possible causes were hypothesized. First, TLR2 signalling may additionally induce cellular processes that have an inhibitory impact on 5-LO, which counterbalance enhancing effects by a supposable MAP kinase activation. Second, besides MAP kinase activation, PMA may trigger further cellular processes, which essentially contribute to its priming effect, but which are not induced by LTA. Third, despite both priming agents do activate PKC and MAP kinase pathways, there might be a decisive difference regarding intensity and kinetics.

Regarding the first hypothesis, LTA had displayed weak inhibitory effects on 5-LO activity in some experiments or seemed to have an opposed influence on 5-LO translocation, as mentioned previously. PKA mediated phosphorylation of 5-LO at Ser-523 was shown to inhibit its activity *in vitro* and in intact cells, but ionophore-induced 5-LO translocation was not affected by PKA activation [174]. Nevertheless, LTA-induced PKA activation was taken in account. However, neither H-89, nor the myristoylated protein kinase inhibitor peptide (PKA inhibitor 14-22 amide), both selective and cell permeable inhibitors of cAMP-dependent PKA, did unmask LTA priming for enhanced 5-LO activity, and expectedly also PMA priming was not affected. LTA (10µg/mL) induced NO synthase expression in RAW 264.7 macrophages, and this was shown to involve COX-2 induction, PGE_2 release and subsequent PKA activation [418], but no reports were found regarding short-time PKA activation by LTA. That supports the present data, suggesting that PKA activation does not play a role in the transient process of LTA priming, which could have explained differences between LTA and PMA priming. PMA has scarcely been implicated in PKA activation. Similarly to LTA, PMA was reported to activate PKA indirectly by inducing the formation of PGE_2, which then activates PKA via adenylyl cyclase linked cell surface receptors [419]. Such processes, however, might carry no weight in comparison to the rather strong up-regulation of 5-LO. Another possibility for an inhibitory impact of LTA might be the enhancement of glutathione peroxidase activity. As it had already been considered in a previous section, such a process may on the one hand result in enhanced reduction of 5-HpETE to 5-HETE, but may also further reduce cellular hydroperoxide levels and thus impair 5-LO activation.

As a second hypothesis it was speculated, if undefined PMA-induced cellular events besides 5-LO kinase activation essentially contribute to the positive regulation of 5-LO, which in contrast may not be induced by LTA priming. In former publications it was reported that the presence of unsaturated fatty acids (UFAs) was required for effective *in vitro* phosphorylation of 5-LO at Ser-271 and Ser-663, and that 5-LO intrinsically turned out to be a rather poor substrate for MK2 and ERK1/2, respectively [170, 171]. It was assessed that UFAs like AA, oleic acid (C18:1) or linoleic acid (18:2) enhanced 5-LO phosphorylation, whereas saturated fatty acids, oxygenated metabolites of UFAs or esterified fatty acids were much less, or hardly ever effective. Conformational changes of 5-LO and the resulting exposure of phosphorylation sites due to fatty acid binding were presumed as an explanation of at least AA triggered upregulation of 5-LO phosphorylation. In connection with present

investigations it seemed conceivable that PMA priming, in contrast to LTA priming, may trigger cellular events, which result in the release of free UFAs also in the cellular context and therefore facilitate effective 5-LO phosphorylation. This could be mediated for instance by a slight elevation of the intracellular calcium level during the period of priming, which then may weakly activate $cPLA_2$ and induce the release of an adequate amount of AA. Also oleic acid release was shown to be mediated by $cPLA_2$ activity in macrophages in few reports [420, 421], but this occurred during long-term cell stimulation and therefore may not be relevant during 15 minutes of priming. LTA was not able to significantly induce AA release in present studies, as it was investigated in a posterior experiment and will be discussed later-on. PMA on its own seems to liberate AA only in certain cell types such as macrophages and neutrophils [94], but interestingly, reports about a direct activation of AA release by PMA in such cells are contradictory [390, 391].

Even slight elevations of the intracellular calcium are sufficient to induce the release of AA. At $[Ca^{2+}]_i$ greater than 210-280 nM, $cPLA_2\alpha$ translocates to the endoplasmatic reticulum and the nuclear envelope, but already sustained $[Ca^{2+}]_i$ greater than 100-125 nM can induce the translocation of $cPLA_2\alpha$ to the Golgi apparatus. Duration of $[Ca^{2+}]_i$ stabilizes the association with the membrane, leading to AA release [37]. Rather high Ca^{2+} concentrations are necessary in MM6 cells to activate LT biosynthesis, as half-maximal 5-LO product formation in MM6 cells was measured at about 325 nM intracellular calcium [422]. Thus, it seems possible that a slight release of AA could be activated by moderate calcium elevation without concomitant induction of 5-LO product formation during the period of priming. However, neither LTA nor PMA clearly affected the basal intracellular calcium level in MM6 cells, and $[Ca^{2+}]_i$ did not rise above 100 nM upon LTA or PMA treatment in present investigations. In fact, PMA seemed to rather lower the intracellular calcium concentration by trend. This is in accordance with publications reporting that PMA does not increase $[Ca^{2+}]_i$ in macrophages [94], or even diminishes stimulus-induced calcium mobilization in several cell types [423-428]. In contrast to present observations, some studies, however, revealed the TLR ligand induced elevation of $[Ca^{2+}]_i$ as it will be described in a posterior section. As aforementioned, LTA has been identified as an activator of PLC pathways, which are known to result in Ca^{2+} mobilization from intracellular stores. In human tracheal smooth muscle cells, high LTA concentrations (50-250 µg/mL) were found to transiently increase $[Ca^{2+}]_i$, and an involvement of PC-PLC and PIP_2-PLC in LTA-induced responses was suggested [417]. The lower LTA concentration (0.5 µg/mL) used during present priming studies, however, appears to be insufficient to induce an effective raise of $[Ca^{2+}]_i$ in MM6 cells.

As PMA was described to induce arachidonic acid release without increasing $[Ca^{2+}]_i$ via an alternative mechanism [94], a second experimental approach was used to survey the speculation, that PMA priming, but not LTA priming, may effect the presence of UFAs facilitating effectual 5-LO phosphorylation. LTA priming of MM6 cells was carried out in presence of various AA concentrations to compensate a possible lack of UFAs in the cell, but also under such conditions LTA priming was not able to enhance 5-LO activity.

Therefore, besides 5-LO kinase activation, other additional cellular events than the release of UFAs might be activated by PMA priming, but not by LTA priming. It is conceivable, that PMA can activate also yet undefined signal transduction pathways, which contribute to 5-LO activation. Furthermore, it should be considered, that in addition to Ca^{2+} and 5-LO phosphorylation, the endogenous generation of diacylglycerols (DAGs) was recently identified as a decisive determinant for cellular 5-LO activation in response to ionophore in presence of exogenous AA in PMNL. Besides elevation of $[Ca^{2+}]_i$, ionophore induced DAG formation via the phospholipase D / phosphatidic acid phosphatase (PLD / PA-P) pathway was found to be required for 5-LO translocation and substantial 5-LO product formation [166]. In MM6 cells, in contrast, ionophore is not capable of substantially inducing 5-LO translocation, 5-LO product formation and also no substantial ERK1/2 activation could be detected [170, 373]. Thus, it was speculated that in MM6 cells ionophore cannot activate certain kinase cascades, as it is the case in PMNL, and that endogenous signal molecules such as DAGs, whose action can be mimicked by PMA, might not be adequately generated [373]. DAGs on the one hand are activators of PKC signalling pathways, but DAGs were also shown to influence the formation of 5-LO products apart from activating PKC / MAPK pathways [166]. 1-Oleoyl-2-acetylglycerol (OAG) functions as direct agonist of 5-LO product formation in PMNL [405], and was found to directly stimulate 5-LO catalysis *in vitro* by acting at the C2-like domain of 5-LO [127]. It was suggested that OAG renders 5-LO activity resistant against the inhibitory influence of glutathione peroxidases (GPx) by increasing the affinity towards hydroperoxides. Furthermore, OAG itself had no influence on cellular redistribution of 5-LO in PMNL [405], but was able to restore ionophore induced 5-LO translocation during blockade of endogenous DAG generation [166]. This indicates that DAGs could play a role for membrane association of 5-LO, as they do for PKC. PMA, in comparison, does not directly stimulate 5-LO activity and it seems daring to speculate that PMA, besides PKC activation, may in parts mimic also direct effects of DAGs. It is very interesting however, that there are some publications reporting PMA induced PLD activation via PKC activation, which indeed could result in DAG generation [429-431]. Interestingly,

for LTA (30 µg/mL) PLD activation was reported [415], but PLC / PLD / PKC pathways do not seem sufficiently activated by LTA in present investigations, which could be related to the lower LTA concentrations that were used for optimal MM6 priming in present studies.

The third hypothesis is based on the assumption that ERK1/2 activation and subsequent 5-LO phosphorylation are the key events of PMA priming effects on 5-LO. This was suggested, as U0126, an inhibitor of ERK1/2 signalling pathways, abrogated this 5-LO up-regulation in MM6 cells [170]. Against this background it was assumed that intensity or duration of ERK1/2 activation might be decisive for 5-LO up-regulation, and differences herein may explain the lack of such effects after LTA priming compared to PMA priming.
The comparison of p38 and ERK1/2 activation profiles by PMA and LTA, firstly revealed that LTA is a strong activator of p38 MAPK, and that PMA mediated p38 MAPK activation, in comparison, was rather moderate. Downstream 5-LO phosphorylation at Ser-271 was investigated as well, and according to the p38 MAPK activation profiles, a slight phosphorylation seemed apparent after LTA incubation, but was hardly visible after PMA treatment. These data, however, may be of minor importance regarding the underlying mechanism of PMA versus LTA priming. It seems that p38 MAPK pathways can be activated by priming, but may have no decisive relevance for a positive regulation of 5-LO in this case. Nevertheless, the detection of 5-LO phosphorylation by immunoblotting is worthy of particular attention. Earlier studies have reported technical difficulties in providing evidence for in vivo 5-LO phosphorylation [170]. In HL-60 cells 5-LO phosphorylation indeed was shown, but only a very small amount of 5-LO was phosphorylated [432], which might explain certain difficulties with reliable detection methods. Immunoblotting using phospho-specific antibodies, which are available for relevant 5-LO phosphorylation sites, may possibly help to resolve such problems. Admittedly, 5-LO phosphorylation at Ser-663 could yet not be detected successfully by immunoblotting in present studies.
Of greater interest than the analysis of p38 MAPK profiles, was the observation that PMA induced ERK1/2 activation was strong and persistent up to 20 minutes, whereas only a moderate and very transient ERK1/2 activation was seen after 5 minutes of LTA treatment. It seemed consistent, that this difference may account for lacking 5-LO up-regulation effects after 15 minutes of LTA priming. Further experiments were undertaken to corroborate this assumption. Firstly, analysis of 5-LO activity after various periods of PMA and LTA priming revealed that the characteristics of enhanced 5-LO activity over time coincided with the respective ERK1/2 activation profiles. Whereas PMA clearly enhanced 5-LO activity after

priming for 5 up to 20 minutes, a weak LTA effect was detected only after 5 minutes of priming, which was yet not significant. Intriguingly, 5-LO activity dropped below the level of unprimed cells with increasing times of LTA treatment. In a second approach it was attempted to boost LTA mediated ERK1/2 activation by blockade of the p38 MAPK signalling pathway, which was found to shift MAPK activation towards a more pronounced ERK1/2 activation, to render 5-LO up-regulation detectable. However, 5 minutes of LTA priming in presence of SB203580, a selective inhibitor of p38 MAPK signalling that acts on MK2, did also not result in a significant enhancement of 5-LO activity, which could have been reversed by an ERK inhibitor in a subsequent approach (data not shown). Moreover, blockade of p38 MAPK signalling seemed to generally promote 5-LO nuclear translocation, but did not lead to a significant enhancement of ionophore-induced cellular resdistribution of 5-LO by LTA priming for 5 minutes.

In summary, LTA mediated ERK1/2 activation via the TLR2 pathway may be too weak and too transient to positively regulate 5-LO. Nevertheless it seems, that inadequate ERK1/2 activation may not be the only decisive factor for missing LTA priming effects on 5-LO. Possibly, LTA concentrations that were determined as optimal for the TLR2-mediated enhancement of LT formation in these studies, might not be capable of effectively inducing PLC and PKC activation, which would on the one hand promote a more pronounced MAPK activation, but might possibly also trigger PKC mediated PLD activation and release of DAGs, which were shown to importantly determine 5-LO activity. Furthermore, some observations indicated that an undefined inhibitory influence on 5-LO may superpose and counteract LTA priming effects on 5-LO.

5.2.3 The influence of TLR2 ligands on the release of AA

Besides cellular 5-LO activity, the regulation of AA release is a crucial determinant for LT formation. The amount of free AA and its availability as a substrate for 5-LO thus is an important parameter for the extent of LT biosynthesis. In a set of experiments it was investigated, if an increased supply of AA accounts for TLR2 ligand mediated enhancement of LT formation in MM6 cells.

Numerous reports indeed had described an increase of free AA after pre-treatment of agonist stimulated leukocytes with priming agents. Namely, the cytokines GM-CSF [376, 378] and

TNFα [376, 383], as well as OAG [389, 390, 392] and PMA [373, 390-392], EBV [254], and also LPS [365-367, 395, 433] have been reported to exert such effects. Moreover, TLR dependent priming for enhanced LT formation by resiquimod and Kdo_2-lipid A has been linked to an augmentation of AA release [396, 397]. Also in present investigations, the TLR2 ligands LTA, FSL-1 and Pam_3CSK_4 mediated an increase in ionophore induced supply of AA, and the respective time course characteristics of enhanced AA release and of increased 5-LO product formation showed great coincidence. The maximal effect of TLR2 ligands on AA release occurred somewhat earlier (10 to 15 minutes of priming) than on 5-LO product formation (15 to 20 minutes of priming), probably reflecting the fact that the release of AA must occur prior LT synthesis. Some differences were observed regarding intensity. Whereas LT formation was enhanced up to twofold, the release of AA was at least three times higher in primed than in unprimed cells. This may be related to the fact that cells of the monocytic lineage contain high levels of GPx-1 [162], and 5-LO activation is therefore hampered in such cells. Moreover, AA is expected to be metabolized also by other lipoxygenases (12-, 15-LO), by cyclooxygenases and cytochrome P450 enzymes, or may be partially re-acylated into membrane phospholipids by lysophospholipid acyltransferase [1]. Finally, observations regarding intensity are in line with previous results, showing that TLR2 ligand priming did not promote, but rather seemed to inhibit 5-LO activity. As it had been detected for 5-LO product formation, enhanced AA levels were no longer apparent after extended priming for 60 to 90 minutes. Furthermore, AA release was not increased, when TLR2 ligands were added simultaneously with ionophore without pre-incubation. These observations suggested that TLR2 ligands by themselves do not directly stimulate AA release, which could be confirmed in a respective experiment. Additionally, neither LTA, as already discussed previously, nor FSL-1 and Pam_3CSK_4 were capable of increasing the intracellular Ca^{2+} level, which is an important factor for the stimulation of an immediate AA release. However, a couple of studies are known, which indeed report TLR ligand mediated Ca^{2+} mobilization, and reports about TLR mediated AA release are partially conflicting. Not only LTA greater than 50 µg/mL directly evoked a peak of $[Ca^{2+}]_i$ in tracheal smooth muscle cells [417], but also other TLR2 and TLR4 ligands have been implicated in Ca^{2+} mobilization. Thus, TLR2 ligands Pam_3CSK_4 at concentrations greater than 15 µg/mL [400, 434], and peptidoglycan (PGN) at 50 - 100 µg/mL [435, 436] were shown to stimulate cellular Ca^{2+} influx, and also LPS stimulation (1-100 µg/mL) resulted in an increase of intracellular Ca^{2+} levels [435, 437, 438]. In accordance with present results, Pam_3CSK_4 (1 µg/mL) failed to stimulate AA release within 20 minutes in murine mast cells and this was ascribed to the lack of a respective Ca^{2+} signal [439]. Rather

high concentrations of ligands seem required to induce Ca^{2+} mobilization by activation of cell surface TLRs, and such concentrations may possibly also be able to trigger direct stimulatory effects on rapid AA release and 5-LO product formation, whereas lower concentrations are sufficient for priming. On the other hand, LPS (10 μg/mL) failed to induce AA release in human PMNL, and this was also reported for LTA and Pam_3CSK_4 even at high concentrations [440]. Furthermore, TLR8 ligand resiquimod, which was identified as a priming agent, indeed triggered a direct release of AA in human PMNL, but the underlying mechanism was not investigated [396]. Besides concentration, also cell type specific differences may explain diverging effects of TLR ligands on the mobilization of Ca^{2+} and AA. Interestingly, in murine macrophages and mast cells, a Ca^{2+} independent direct AA release by TLR ligands, which was apparent after hours of stimulation, has been reported in several studies [397, 441-446]. As a mechanism, TLR mediated MAPK activation and $cPLA_2$ phosphorylation [442-446] have been suggested, also PIP_2 or C1P mediated redistribution of $cPLA_2$ to cellular membranes have been discussed [397, 443] and a role for PA-P and PKC for $cPLA_2$ activation were found [441]. Additionally, enhanced expression of $cPLA_2$ did not seem to play a role for the stimulation of AA release in these investigations [397, 443]. In summary, whereas TLR ligands may be capable of directly stimulating AA supply in certain cell-types and species, this was not detected in MM6 cells and does not play a role for the mechanism of priming.

Nevertheless, present results suggested that the TLR2 ligand mediated enhancement of AA release is the critical step for the enhancement of LT formation, and further analyses were undertaken to identify the phospholipase A_2 isoenzymes involved in this process. The $sPLA_2$ and $cPLA_2$ family has been implicated in eicosanoid generation, whereas other PLA_2s such as the $iPLA_2$ are mainly involved in other phospholipid related processes [37]. Among the $cPLA_2$ familiy, $cPLA_2\alpha$ is considered as a key component of the initiation of AA metabolism. Present inhibitor studies revealed, that $iPLA_2$ inhibitor bromoenollactone (BEL) [447, 448] expectedly did not affect AA release significantly, neither in primed nor in unprimed cells. Thus, it was concluded that $iPLA_2$ does not contribute to the supply of AA under present conditions. The commonly used $sPLA_2$ inhibitor DTT, however, which inactivates $sPLA_2$, but not $cPLA_2$ [449, 450], led to a considerable reduction of AA release in unprimed as well as in primed cells, indicating that $sPLA_2$ is involved in AA release in ionophore stimulated MM6 cells. However, the priming effect of at least LTA and FSL-1 was not affected considerably by DTT, which indicates that $sPLA_2$ does not seem to play a decisive role for the mechanism

of priming. It was assumed, that it is not the $sPLA_2$ activity, which is enhanced, and that $sPLA_2$ does also not mediate enhanced AA release. Accordingly, the activity of $cPLA_2$ must be augmented by TLR2 ligands. Moreover, the $cPLA_2\alpha$ isoenzyme was found to be crucial under present experimental conditions, since its inhibition by the specific $cPLA_2\alpha$ inhibitor pyrrolidine-1 [451] almost completely prevented AA release upon ionophore stimulation, and no appreciable $sPLA_2$ activity remained detectable in presence of the $cPLA_2\alpha$ inhibitor. Other publications support these findings, as $cPLA_2$ was found to mediate AA release after fMLP stimulation of LPS-primed PMNL [367], as well as in EBV-primed mononuclear leukocytes after stimulation with ionophore [254], and a role of $cPLA_2\alpha$ in TLR8 mediated priming of fMLP stimulated PMNL was demonstrated [396]. Furthermore, there is evidence of a functional coupling of $sPLA_2$ and $cPLA_2$ activation. Thus, it was demonstrated that activation of $sPLA_2$ succeeds $cPLA_2$ activation, as it is dependent on previous AA release by $cPLA_2$ in LPS primed, PAF stimulated $P388D_1$ macrophages [452, 453]. Such a sequential activation may serve as an explanation, why inhibition of $cPLA_2\alpha$ activity completely blocked AA release in present investigations without $sPLA_2$ being activated. Interestingly, LTA and also PMA priming of THP-1 cells, which has been discussed in a previous section, resulted in a biphasic enhancement of LT formation. It seems possible that this may arise from a successive activation of different PLA_2 isoenzymes, which may occur in a retarded fashion and thus is discriminable in THP-1 cells, but not in MM6 cells. In this regard, it was reported that Pam_3CSK_4 stimulated biphasic eicosanoid generation was mediated by initial activation of $cPLA_2\alpha$ within 5 minutes, which was further amplified by $sPLA_2$ through its regulation of sequential phosphorylation of ERK1/2 and $cPLA_2\alpha$ in a second phase of AA release after about 15 minutes [444]. However, $sPLA_2$ dependent amplification of AA release does not seem to underlie the present priming effect by LTA and FSL-1, as an enhancement was still apparent during $sPLA_2$ inactivation. Admittedly, Pam_3CSK_4 mediated priming was slightly reduced in presence of DTT. Therefore, a role of $sPLA_2$ may not be definitely excluded in this case. Successive experiments revealed comparable effects of the PLA_2 inhibitors on primed LT formation as on primed AA release suggesting that enhancement of LTs in fact is due to an increase of free AA. Surprisingly, control experiments yet revealed that $cPLA_2$ inhibitor pyrrolidine-1 is not absolutely specific for the inhibition of $cPLA_2\alpha$, but also functions as a direct 5-LO inhibitor. This characteristic necessarily has to be taken in account in future investigations and may exclude its suitability in certain functional analyses of endogenous LT formation. Nevertheless, in the present case these findings actually do not challenge the

assumption that enhanced AA release triggers the increase of LTs in TLR2 ligand primed MM6 cells.

The following analyses focused on the question of how TLR2 ligand priming can trigger an increase of cellular cPLA$_2$α activity. Several studies have demonstrated that the activation of cPLA$_2$α is not only regulated by Ca^{2+} but also by phosphorylation [37, 46, 94, 454-456]. It has been shown, that cPLA$_2$α is a target of MAP kinases. Phosphorylation at Ser-505 by p42 or p38 MAPK led to an increase in catalytic activity *in vitro* and mutation of this serine residue markedly reduced cPLA$_2$α mediated AA release in cells stimulated with physiological stimuli [37, 86, 87, 89, 90, 457]. In general, phosphorylation at Ser-505 increases cPLA$_2$α activation, but phosphorylation per se is considered as insufficient to induce cPLA$_2$α mediated cellular AA release, and a concomitant increase in the intracellular calcium level seems required [37]. It was suggested that phosphorylation may influence calcium dependent membrane binding affinity, and may induce conformational changes optimizing interaction of cPLA$_2$α catalytic domain with membrane substrates for catalytic activity [46]. Besides Ser-505 phosphorylation, cPLA$_2$α activity was also positively regulated via phosphorylation of Ser-727, which is a target of MAPK interacting kinase 1 (Mnk-1), a downstream kinase of p38 MAPK and ERK1/2 [87, 95, 99, 458]. As a third serine residue playing a role for increased enzymatic activity, Ser-515 of cPLA$_2$α was found to be phosphorylated by Ca^{2+}/calmodulin kinase II (CaMKII) in norepinephrine stimulated human vascular smooth muscle cells (VSMC) [88]. Besides, it was suggested that CaMKII activation leads to activation of ERK1/2 in rabbit or rat VSMC, which in turn can trigger Ser-505 phosphorylation of cPLA$_2$α [96, 100, 459-461]. Toll-like receptor stimulation is known to result in activation of MAP kinases (ERK, JNK, p38) [282, 286]. Accordingly, in present studies it was demonstrated that phosphorylation of ERKs and p38 MAPK is detectable also in MM6 cells challenged with TLR2 ligands, moreover occurring within the time-frame of enhanced AA release and LT formation. Maximal activation of both MAPKs appeared fast within 5 minutes, and thus seems to precede AA generation and successive LT biosynthesis. In comparison to p38 MAPK activation (maximal increase about 3-fold), p42 phosphorylation was induced to a greater extent (about 12-fold). Phosphorylation of p42 was more transient and displayed slightly different characteristics depending on the respective TLR2 ligand. In this respect, FSL-1 induced ERK1/2 activation was less sustained (5-15 min) than the response triggered by LTA (5-20 min), and Pam_3CSK_4 mediated ERK1/2 phosphorylation was even more transient occurring and disappearing within just about 5 minutes. It seems inconsistent that the

intensity of LTA induced p38 MAP kinase activation was clearly weaker than in precedent analyses (4.2.4). This may be attributed to a different responsiveness of cells or may depend on an unnoticed variation of assay or detection conditions between these two experimental series. Therefore, these data cannot be directly compared. Nevertheless, experiments within a narrow time-frame provided consistent data and thus seem to allow for valid comparative analyses.

As shown in further investigations, time-dependent cPLA$_2$α phosphorylation at Ser-505 was evoked by TLR2 ligands, which maximally occurred short after MAPK activation, but slightly preceded the intracellular release of AA. Interestingly, a high initial basal level of phosphorylated cPLA$_2$α was found, as observed also by others [462], which could be possibly due to early stress-related MAPK activation by harvesting and handling the cells within the assay procedure. In following studies, the combined use of a p38 MAPK and a MAP kinase kinase (MEK) inhibitor led to complete prevention of TLR2 ligand induced increase of phospho-cPLA$_2$α (Ser-505) as well as of TLR2 ligand mediated enhancement of AA release. This suggests, that cPLA$_2$α phosphorylation via both, the p38 MAPK and ERK1/2 signaling pathway, is crucial for an increase in cPLA$_2$α activity after TLR2 ligand priming. It cannot be excluded though that also other MAPK mediated cellular processes contribute to enhance AA release. Similar to present results, enhanced phospho-cPLA$_2$α was detected in EBV primed human PBMC [254], and also TLR7/8 ligand resiquimod stimulated cPLA$_2$α phosphorylation in PMNL [396]. LPS priming of PMNL as well resulted in a phosphorylation of cPLA$_2$α, but this was considered as not sufficient for the enhancement of AA release and additionally a role of increased cPLA$_2$α nuclear translocation was suggested [367]. In the present study, the involvement of other cPLA$_2$α kinases in increasing AA release was tested. Although Mnk-1 activation, presumably induced via p38 MAPK and ERK1/2 pathways, was detected in present studies upon TLR2 ligand treatment, a Mnk-1 inhibitor did not affect enhanced AA release under priming conditions. This suggests, that phosphorylation of Ser-727 does not substantially contribute to enhanced cPLA$_2$α activity after priming with TLR2 ligands. Furthermore, also CamKII inhibitor KN-62 had no effect on primed AA release in MM6 cells, and it was concluded that neither CamKII triggered Ser-515 phosphorylation, nor a CamKII mediated ERK1/2 activation plays a role for the mechanism of priming. This was expected, as CamKII is activated by Ca^{2+} influx as well as by Ca^{2+} released from intracellular stores [463], which was not induced by TLR2 under present conditions. In summary, MAPK mediated cPLA$_2$α phosphorylation at Ser-505 seems to account for enhanced AA release and successive LT formation in TLR2 ligand primed MM6 cells.

Following experiments confirmed that the observed priming effect is mediated by TLR2. As a first evidence, TLR2 ligand stimulated IκB-α degradation had been observed in previous WB analyses (4.3.6 and 4.3.7.1), indicating that TLR signaling pathways were active. A second approach demonstrated that neutralization of TLR2 by the use of an anti-hTLR2 antibody essentially abolished the priming effects of LTA and Pam_3CSK_4 on AA release, and also the response to FSL-1 was significantly reduced. This clearly indicated the involvement of TLR2, mediating the effects of the three ligands. Possibly TLR6 as part of the heterodimeric TLR2/6 receptor complex is responsible for the remainig activity after FSL-1 priming in presence of the TLR2 antibody, as it may be still functional and capable of partly transducing an activation by FSL-1 under these conditions. In an additional experiment, the presence of polymyxin B, which functions as a LPS neutralizing polypeptide [464], did not alter the impact of TLR2 ligands on AA and LT release, assuring that LPS contaminations, possibly via TLR4 activation, do not contribute to the observed effects. In summary, these data clearly confirmed that the ligand effects are dependent on TLR2 activation.

5.3 Concluding remarks

In the present study, short-term effects of TLR activation on the biosynthesis of LTs in mononuclear leukocytes were addressed. It was demonstrated that activation of TLR2 in human monocytes does not directly stimulate, but increase the formation of 5-LO products. This effect is due to an enhanced cellular AA supply, which arises from a MAPK mediated phosphorylation and up-regulation of $cPLA_2\alpha$ activity. Thereby, TLR2 ligand priming before stimulus-induced activation of LT formation turned out to be essential. The anticipated phosphorylation of $cPLA_2\alpha$ seems to prepare the enzyme for a boosted response upon subsequent Ca^{2+} elevation. The 5-LO enzyme, however, was not positively regulated by LTA induced TLR activation under these conditions. In fact, LTA seemed to exert slight inhibitory effects on 5-LO activity. Present investigation revealed a priming mechanism that only in part corresponds with previous findings. Besides enhanced availability of AA, some studies also reported an up-regulation of 5-LO, and it was suggested that, in this case, priming increases the overlap of AA release and 5-LO activation, which leads to a greater capacity to synthesize 5-LO products [367]. In Kdo_2-lipid A primed mouse macrophages, however, a completely different mechanism was postulated. As priming, in this case, did not change $cPLA_2$ activity and no $cPLA_2$ phosphorylation was detectable, PIP_2 or C1P mediated effects on $cPLA_2$ membrane localization and activity were proposed [397]. It seems likely that more complex

mechanisms underlie the effects of priming in general, affecting several cellular events depending on the priming agent, the cell type and the priming conditions.

TLR dependent enhancement of LT biosynthesis represents an interesting link between activation of innate immune receptors and the rapid formation of proinflammatory lipid mediators. On the one hand, this seems to support the role of LTs in host defence and infectious deseases, but may also be relevant in pathophysiological processes, which involve TLRs as well as LTs, as it has been shown for the pathogenesis of atherosclerosis or allergic diseases. In this regard, Hsp70 was lately shown to stimulate LT synthesis through activation of TLR4 in mast cells, which are central effector cells in allergic responses [465]. Very recent studies, furthermore, demonstrate that TLR ligands can induce inflammatory cell functions such as PMN migration via the formation of LTB_4 and PAF [466]. Thus, in the meanwhile more and more reports are emerging that support a role for TLR activation in the regulation of LT release in the context of infection as well as inflammatory diseases.

6 SUMMARY

Leukotrienes (LTs) are pro-inflammatory lipid mediators that belong to the group of eicosanoids, which are oxygenated metabolites of one common precursor, the aracidonic acid (AA). This polyunsaturated fatty acid is esterified at the *sn*-2 position of cellular membrane phospholipids and can be released by phospholipase A_2 (PLA_2) enzymatic deacylation [1]. Among the PLA_2 enzyme familiy, the group IVA PLA_2 ($cPLA_2\alpha$) plays the key role for cellular release of AA, which then can be further converted into LTs by the catalytic reaction of 5-lipoxygenase (5-LO). Enzymatic catalysis of $cPLA_2\alpha$ and of 5-LO are tightly regulated. Key determinants for the activation of both, $cPLA_2\alpha$ as well as of 5-LO, are an increase of the intracellular Ca^{2+} concentration and / or phosphorylation of serine residues by signal-activated protein kinases. For 5-LO activation, the presence of diacylglycerols (DAGs) appears to constitute another essential factor, as described in recent studies [166]. In response to cellular stimuli that elevate the intracellular Ca^{2+} level, induce the release of DAGs and / or activate MAP kinase pathways, $cPLA_2\alpha$ and 5-LO comigrate from a soluble cell compartment (mainly the cytosol) to the nuclear membrane, where AA is released und converted into LTs [16, 21]. LTs play a significant role in promoting inflammatory reactions and immune processes. They have been shown to be released from leukocytes in response to bacterial and viral infections and substantially contribute to an effective immune reaction for host defense [247].
The mechanisms for innate immune pathogen recognition and signaling have been subject of intense research since the discovery of the Toll-like receptor (TLR) family of pattern recognition receptors. Within the last decade, 10 human TLR subtypes have been identified, all of which recognize distinct highly conserved microbial structures (pathogen-associated molecular patterns, PAMPs). As an example, TLR2-TLR6 heterodimers were identified to recognize lipoteichoic acid (LTA) from gram-positive bacteria or mycobacterial diacylated lipopeptides, whereas heterodimeric TLR2-TLR1 appears to be responsive to bacterial triacylated lipopetides. Receptor activation triggers the induction of signaling pathways that lead to the expression of numerous immune and inflammatory genes. Generally, TLR signaling culminates in the activation NF-κB and / or the MAP kinase network, which as well is known to be involved in the regulation of cellular leukotriene biosynthesis [281].

In this regard, it seemed conceivable that the release of LTs might be regulated in the course of TLR activation. Thus, present studies were undertaken in order to verify and characterize a

possible influence of TLR activation on the biosynthesis of LTs, and furthermore to identify the involved signaling pathways and underlying mechanisms. Investigations were carried out in human monocytic cell lines and primary human monocytes, which constitute key effector cells in the first line of innate immune host defense.

First screening experiments revealed that pre-incubation of differentiated Mono Mac 6 (MM6) cells with TLR4 ligand LPS, TLR5 ligand flagellin, as well as TLR2/6 ligands LTA and FSL-1 (synthetic diacylated lipopeptide) and the TLR2/1 ligand Pam_3CSK_4 (synthetic triacylated lipopeptide) led to an about 2-fold enhancement of Ca^{2+} ionophore-induced LT biosynthesis, whereas ligands of other TLR subtypes had no influence. This observation could also be confirmed in primary human monocytes stimulated with ionophore or fMLP. With focus on the TLR2 ligands, further studies were carried out to characterize the observed effect in MM6 cells. It was demonstrated that the enhancement of LT formation was more pronounced, when suboptimal concentrations of ionophore were used for stimulation, which is an experimental scenario that more closely imitates physiologically relevant conditions. Furthermore, the extent of LT formation was dependent on the ligand concentration used, as well as on the pre-incubation time. In this regard, 15 minutes of ligand pre-incubation turned out to be optimal, whereas prolonged incubation decreased LT formation again. Moreover, simultaneous addition of TLR2 ligands with ionophore did also not result in enhanced LT formation. These observations indicated that TLR2 ligands act as priming agents on human monocytes for an enhanced response upon ionophore stimulation, but do not directly act as costimuli. Further experiments confirmed that TLR2 ligands were not capable of directly stimulating the biosynthesis of LTs.

To analyze the underlying mechanism, the influence of the TLR2 ligands on the two key enzymes of the LT biosynthesis pathway, $cPLA_2\alpha$ and 5-LO, was investigated.

In this regard, it could be shown that TLR2 ligands enhanced ionophore-induced release of AA in MM6 cells, which occurred in the same time frame and with a similar time-course as LT formation, but displaying a prior maximum at 10 minutes of pre-incubation. A direct stimulation of AA release, however, could not been detected, which was corroborated by the observation that TLR2 ligands had no influence on the intracellular Ca^{2+} level. Inhibitor studies revealed $cPLA_2\alpha$ to be essential for AA release in TLR2 ligand primed, ionophore-stimulated MM6 cells, but also $sPLA_2$ was found to be involved. However, the priming effect of TLR2 ligands appeared to be mediated exclusively by $cPLA_2\alpha$. Western Blot analyses indicated that p38 MAP kinase, as well as ERK1/2, become activated in MM6 cells in

response to TLR2 ligands, and also Ser-505 phosphorylation of cPLA$_2$α was detected. A cPLA$_2$α phosphorylation at Ser-505 is known to be induced by MAP kinases and was shown to increase cPLA$_2$α activity *in vitro* [37]. Interestingly, the time courses of these phosphorylations again coincided with LT biosynthesis in primed MM6 cells. Maximal phosphorylation occurred at 5-10 minutes of TLR2 ligand incubation, slightly preceding maximal AA release at 10 minutes and maximal LT formation at 15 minutes of priming. The combined use of a specific p38 MAPK inhibitor with an inhibitor of the ERK1/2 signaling pathway resulted in a complete prevention of cPLA$_2$α phosphorylation, and also TLR2 ligand mediated enhancement of AA release was abolished. These results indicated that both MAPK pathways seem to play a role for TLR2 ligand mediated upregulation of AA release. An impact of other kinases such as Mnk-1 and CamKII, which can also regulate cPLA$_2$α by phosphorylation, was excluded. Further studies were undertaken to exclude that LPS contaminations via TLR4 may trigger the observed effects. However, the LPS binding reagent polymyxin B did not affect the TLR2 ligand priming effects on AA or LT release. Furthermore, an anti-hTLR2 antibody significantly reduced enhanced AA release, confirming the priming effects to be dependent on TLR2 activation.

5-LO, in contrast, could not been shown to be positively regulated by TLR ligand priming in 5-LO activity assays. 5-LO activity was stimulated in presence of exogenous AA, in order to determine 5-LO activation independent from PLA$_2$-mediated endogenous AA release. Neither a direct stimulation, nor an enhancement of 5-LO activity by TLR ligands was detectable in MM6, as well as in THP-1 cells. In fact, a slight inhibitory influence seemed to occur after LTA pre-incubation. Similarly, LTA did not enhance ionophore-induced 5-LO translocation to the nuclear membrane, but rather seemed to promote 5-LO redistribution into the cytosol. These results revealed differences in the mechanism of priming of LTA compared to established priming agents such as the phorbol ester PMA, which, in contrast, do upregulate 5-LO activity and translocation presumably through the envolvement of MAPK pathways (particularly ERK1/2 pathways) and potentially by 5-LO phosphorylation [373]. Three possible hypotheses were tested, to analyze and understand the different modes of action between LTA and PMA. First, an involvement of protein kinase A (PKA), which can negatively regulate 5-LO by phosphorylation, was assumed to counteract an LTA priming effect on 5-LO, but PKA inhibitors did not unmask LTA priming. Secondly, it was hypothesized that PMA, but not LTA, might facilitate the release of polyunsaturated fatty acids, which are known to be required for efficient 5-LO phosphorylation by MAP kinases. However, supplementation of AA during LTA priming had no effect. Finally, comparison of

the MAPK activation profiles of LTA and PMA suggested that ERK1/2 activation in response to LTA might be too weak and too transient to up-regulate 5-LO catalysis, compared to PMA. Nevertheless it seems that inadequate ERK1/2 activation may not be the only decisive factor for missing LTA priming effects on 5-LO. Possibly, PMA may trigger further cellular processes, which essentially contribute to its priming effect, but which are not induced by LTA. Furthermore, some observations indicated that an undefined inhibitory influence on 5-LO may superpose and counteract LTA priming effects on 5-LO.

In summary, it was concluded that the increase of LT biosynthesis by TLR2 ligand priming is considerably due to an enhanced cellular AA supply, which arises from a MAPK mediated phosphorylation and up-regulation of $cPLA_2\alpha$.
TLR dependent enhancement of LT biosynthesis represents an interesting link between activation of innate immune receptors and the rapid formation of proinflammatory lipid mediators. On the one hand, this seems to support the role of LTs in host defence and infectious deseases, but may also be relevant in pathophysiological processes, which involve TLRs as well as LTs, as it has been shown for the pathogenesis of atherosclerosis or allergic diseases.

7 ZUSAMMENFASSUNG

Leukotriene sind Entzündungsmediatoren aus der Gruppe der Eicosanoide, welche sich von der Arachidonsäure (AA) als ihre gemeinsame Vorstufe ableiten. Diese mehrfach ungesättigte Fettsäure ist in der Zelle an der *sn*-2 Position von Membranphospholipiden verestert, und kann enzymatisch durch Phospholipase A_2 (PLA_2)-Enzyme, insbesondere durch die cytosolische $PLA_2\alpha$ ($cPLA_2\alpha$), freigesetzt werden [1]. In einem zweiten Schritt wird die AA enzymatisch durch die 5-Lipoxygenase (5-LO) zu Leukotrienen umgesetzt. Die Enzymaktivität der $cPLA_2\alpha$ und der 5-LO sind sehr ähnlich reguliert. Eine Aktivierung beider Enzyme wird durch einen Anstieg des intrazellulären Ca^{2+}-Spiegels und / oder durch Phosphorylierungen der Enzyme durch Proteinkinasen ausgelöst, wobei für die Aktivierung der 5-LO nach neuesten Erkenntnissen zusätzlich auch die Freisetzung von Diacylglycerolen (DAGs) eine entscheidende Rolle zu spielen scheint [166]. Zelluläre Stimuli also, die zu einem zellulären Ca^{2+}-Einstrom, der Freisetzung von DAGs und / oder der Aktivierung von Mitogen-aktivierten Proteinkinasen (MAPKs) führen, lösen eine Aktivierung, und die konzertierte Translokation beider Enzyme aus dem Zytosol zur Kernmembran aus, wo die Freisetzung der AA und die Leukotrien-Biosynthese erfolgt [16, 21]. Leukotriene spielen eine wichtige Rolle in Entzündungsreaktionen und Immunprozessen. Sie werden von Leukozyten beispielsweise bei bakteriellen und viralen Infektionen gebildet, und tragen zur Aktivierung entsprechender Immunabwehrmechanismen bei [247].

Die Pathogenerkennung und Signalweiterleitung durch das angeborene Immunsystem ist seit der Enteckung der Toll-like Rezeptoren (TLRs), die eine Schlüsselrolle für die Detektion von Mikroorganismen im Organismus spielen, Gegenstand intensiver Forschung. Inzwischen sind 10 verschiedene humane Subtypen der TLR-Familie bekannt, die jeweils definierte hoch konservierte pathogen-assoziierte molekulare Strukturen (PAMPs) erkennen, wodurch Signaltransduktionswege aktiviert werden, die zur Expression entzündungsrelevanter Proteine und somit zur Entwicklung einer Immunantwort führen. Beispielsweise erkennt das TLR2-TLR6-Heterodimer Lipoteichonsäuren (LTA) aus gram-positiven Bakterien oder diacylierte Lipopeptide aus Mycobakterien, während TLR2-TLR1 bakterielle triacylierte Lipopeptide detektiert. Der dadurch aktivierte TLR-Signaltransduktionsweg führt im Allgemeinen zur Aktivierung des Transkriptionsfaktors NF-κB, aber auch zur Aktivierung von MAP-Kinasen, deren Rolle in der Regulation der Leukotrien-Biosynthese bereits erwähnt wurde [281].

Vor diesem Hintergrund schien es denkbar, dass im Zuge einer TLR-Aktivierung auch eine Regulation der Leukotrienbildung erfolgen könnte. Ziel dieser Arbeit war es, diesen Zusammenhang zu verifizieren, den Einfluss von TLR-Liganden auf die Biosynthese von Leukotrienen zu charakterisieren und die zugrundeliegenden Mechanismen aufzuklären.

Erste Versuche haben in der Tat gezeigt, dass die Vorbehandlung differenzierter Mono Mac 6 (MM6) Zellen mit dem TLR4-Ligand LPS, dem TLR5-Ligand Flagellin, aber auch mit den TLR2/6-Liganden LTA und FSL-1 (diacyliertes Lipopeptid), und dem TLR2/1-Ligand Pam_3CSK_4 (triacyliertes Lipopeptid) etwa zu einer Verdopplung der Leukotrien-Biosynthese führte, die durch Ca^{2+}-Ionophor stimuliert worden war. Die Liganden anderer TLR-Subtypen zeigten dagegen keine Wirkung. Der verstärkende Effekt konnte in primären humanen Monozyten ebenfalls bestätigt werden. Die weitere Charakterisierung des beobachteten Effektes in MM6-Zellen erfolgte anhand der drei ausgewählten TLR2-Liganden. Die verstärkende Wirkung der Liganden war abhängig von der Liganden-Konzentration. Außerdem war sie besonders ausgeprägt, wenn die nachfolgende Stimulation durch Ionophor bei einer suboptimalen Konzentration erfolgte, was eine Stimulation unter physiologischen Bedingungen mit schwächeren Agentien gut imitieren dürfte. Weiterhin spielte die Dauer der Vorinkubationszeit eine entscheidende Rolle. Eine 15-minütige Vorbehandlung mit den Liganden erwies sich als optimal, während eine Verlängerung der Inkubationsdauer zu einer Abschwächung bis hin zum Verschwinden des Effektes führte. Wurden die TLR2-Liganden zusammen mit Ionophor inkubiert, war ebenfalls keine Verstärkung der Leukotrienbildung messbar. Diese Beobachtungen führten zu der Annahme, dass die TLR2-Liganden zwar als sogenannte Priming-Agenzien in der Lage sind, die Stimulation der Leukotrienbildung zu verstärken, selbst jedoch nicht direkt aktivieren können, was in Folgeexperimenten auch bestätigt werden konnte.

Zur Aufdeckung des zugrundeliegenden zellulären Mechanismus wurde der Einfluss der TLR2-Liganden auf die $cPLA_2\alpha$, als auch auf die 5-LO untersucht.

Zunächst konnte gezeigt werden, dass TLR2-Liganden in MM6 Zellen auch die Ionophor-induzierte Freisetzung von AA verstärken. Hierbei war, wie für die Leukotrienbildung, eine analoge Zeitabhängigkeit des Effektes mit einer optimalen Vorinkubationszeit der Liganden von etwas 10 Minuten feststellbar. Auch in diesem Fall erfolgte keine direkte Stimulation der AA-Freisetzung durch die TLR2-Liganden, die dementsprechend auch keinen Einfluss auf die intrazelluläre Ca^{2+}-Konzentration zeigten. In Inhibitorstudien stellte sich heraus, dass sowohl die $cPLA_2\alpha$ als auch $sPLA_2$ an der AA-Freisetzung in vorbehandelten, und mit Ca^{2+}-Ionophor

stimulierten MM6-Zellen beteiligt sind. Der Verstärkungseffekt der Liganden schien jedoch allein durch die cPLA$_2$α vermittelt zu sein. Weiterhin konnte in MM6-Zellen nach Inkubation mit TLR2-Liganden sowohl die Aktivierung der p38 und der p42/44 MAP-Kinase (ERK1/2), als auch die Phosphorylierung der cPLA$_2$α an Ser-505 nachgewiesen werden. Aus der Literatur ist bekannt, dass die Phosphorylierung des Ser-505 durch MAP-Kinasen erfolgt, und *in vitro* zur Aktivitätssteigerung der cPLA$_2$α führt [37]. Der zeitliche Verlauf der Phosphorylierungen deckte sich interessanterweise wiederum mit der Zeitkurve der Leukotrienbildung in geprimten, Ca^{2+}-Ionophor-stimulierten Zellen. Nach etwa 5-minütiger Behandlung riefen die Liganden maximale Phosphorylierung hervor, die somit einer maximalen AA-Freisetzung bei 10-minütigem Priming und einer maximalen Leukotrienbildung bei 15-minütigen Priming vorauszugehen schien. Die Kombination von Inhibitoren des p38 und des p42/44 MAP-Kinase Signalweges führte schließlich zur vollständigen Aufhebung sowohl der beobachteten cPLA$_2$α-Phosphorylierung, als auch der Verstärkung der AA-Freisetzung. Beide MAP-Kinasewege scheinen somit eine Rolle für diesen Verstärkungseffekt der TLR2-Liganden zu spielen. Der Einfluss weiterer für die Regulation der cPLA$_2$α relevanter Kinasen (Mnk-1 und CamKII) konnte mittels Inhibitorstudien ausgeschlossen werden. Weiterhin konnte abschließend gezeigt werden, dass die beobachtete Steigerung der AA-Freisetzung (und der Leukotriensynthese) nicht aufgrund von LPS-Kontaminationen durch eine TLR4-Aktivierung, sondern tatsächlich durch Aktivierung des TLR2 vermittelt wird.

Im Gegensatz zur cPLA$_2$α ließ sich für die 5-LO keine positive Regulation durch Priming von MM6-Zellen mit TLR2-Liganden nachweisen. Um die 5-LO-Aktivität unabhängig von der endogenen AA-Freisetzung messen zu können, wurde die Leukotrienbildung in Gegenwart exogen zugegebener AA stimuliert. Hierbei konnte weder in MM6-Zellen, noch in THP-1-Zellen nach Inkubation mit TLR-Liganden eine direkte Stimulation, und auch keine Verstärkung der Ionophor-induzierten 5-LO-Aktivität detektiert werden. Der TLR2-Ligand LTA schien sogar eher einen inhibitorischen Einfluss auf die Aktivität der 5-LO auszuüben. Weiterhin zeigte LTA auch keine Verstärkung der Ionophor-induzierten 5-LO-Translokation zur nukleären Membran, sondern förderte eher die Akkumulation der 5-LO im Zytosol. Diese Ergebnisse standen im Widerspruch zu den Eigenschaften bekannter Priming-Agenzien wie z.B. des Phorbolesters PMA, der in MM6 Zellen tatsächlich in der Lage ist, sowohl die 5-LO-Aktivität, als auch deren Translokation wahrscheinlich durch Aktivierung von MAP-Kinasen (vor allem der p42/44 MAP-Kinasen) und möglicherweise durch 5-LO-Phosphorylierung positiv zu regulieren [373]. Um die unterschiedliche Wirkungsweise von LTA und PMA zu

verstehen, wurden drei denkbare Hypothesen überprüft. Zum einen schien es möglich, dass eine LTA-vermittelte Aktivierung der PKA, welche die 5-LO durch Phosphorylierung negativ regulieren kann, einem Primingeffekt von LTA entgegenwirken bzw. diesen aufheben könnte. Jedoch führte die experimentelle Hemmung der PKA nicht zu einer Demaskierung eines möglicherweise vorhandenen LTA-Effekts. Zweitens schien es denkbar, dass PMA im Gegensatz zu LTA möglicherweise die zelluläre Freisetzung ungesättigter Fettsäuren stimulieren könnte. In der Literatur wurde berichtet, dass freie ungesättigte Fettsäuren die 5-LO-Phosphorylierung durch MAP-Kinasen erheblich begünstigen und verstärken. Priming von MM6-Zellen mit LTA unter Zusatz exogener AA konnte jedoch eine Steigerung der Ionophor-induzierten 5-LO-Aktivität ebenfalls nicht provozieren. Schließlich führte ein direkter Vergleich der MAP-Kinase-Aktivierung (Ausmaß und Dauer) durch PMA bzw. LTA zu der Beobachtung, dass die Aktivierung der p42/44 MAP-Kinasen durch LTA wesentlich schwächer und transienter erfolgte als durch PMA, was für die fehlende LTA-Wirkung auf die 5-LO mitverantwortlich sein könnte. Dennoch ist es denkbar, dass PMA im Vergleich zu LTA zusätzlich zelluläre Prozesse aktiviert, die ebenfalls essentiell zur positiven Regulation der 5-LO erforderlich sind. Weiterhin scheinen auch unbekannte inhibitorische Effekte eine mögliche Verstärkung der 5-LO Aktivierung durch LTA zu überlagern oder zu verhindern.

Zusammenfassend lässt sich feststellen, dass die TLR2-vermittelte Verstärkung der Ionophor-induzierten Leukotrien-Biosynthese in MM6-Zellen hauptsächlich auf eine vermehrte Freisetzung von AA zurückzuführen ist. TLR2-Liganden induzieren die Aktivierung von MAP-Kinasen, die die Phosphorylierung und die verstärkte Aktivierung der $cPLA_2\alpha$, und damit die vermehrte AA-Bereitstellung vermitteln.

Die TLR-abhängige Verstärkung der Leukotrienbildung stellt einen interessanten Zusammenhang zwischen der Aktivierung von Rezeptoren des angeborenen Immunsystems und der kurzfristigen Freisetzung pro-inflammatorischer Lipidmediatoren her, der die Bedeutung der Leukotriene für die Immunabwehr unterstreicht. Weiterhin spielen sowohl TLRs, als auch Leukotriene in der Pathogenese allergischer Erkrankungen oder beispielsweise der Atherosklerose eine wichtige Rolle. Auch hier könnte der beschriebene Zusammenhang von Bedeutung sein.

8 REFERENCES

1. Brock, T.G., Peters-Golden, M. (2007) Activation and regulation of cellular eicosanoid biosynthesis. *ScientificWorldJournal* **7**, 1273-1284.
2. Borgeat, P., Samuelsson, B. (1979) Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. U S A* **76**, 2148-2152.
3. Borgeat, P., Samuelsson, B. (1979) Metabolism of arachidonic acid in polymorphonuclear leukocytes. Structural analysis of novel hydroxylated compounds. *J. Biol. Chem.* **254**, 7865-7869.
4. Murphy, R.C., Hammarström, S., Samuelsson, B. (1979) Leukotriene C: a slow-reacting substance from murine mastocytoma cells. *Proc. Natl. Acad. Sci. U S A* **76**, 4275-4279.
5. Orange, R.P. (1977) The formation and release of slow-reacting substance of anaphylaxis. *Monogr. Allergy* **12**, 231-240.
6. Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., Smith, M.J. (1980) Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. *Nature* **286**, 264-265.
7. Samuelsson, B., Borgeat, P., Hammarström, S., Murphy, R.C. (1979) Introduction of a nomenclature: leukotrienes. *Prostaglandins* **17**, 785-787.
8. Peters-Golden, M., Henderson, W.R., Jr. (2007) Leukotrienes. *N. Engl. J. Med.* **357**, 1841-1854.
9. Murphy, R.C., Gijón, M.A. (2007) Biosynthesis and metabolism of leukotrienes. *Biochem. J.* **405**, 379-395.
10. Schröder, O., Sjostrom, M., Qiu, H., Stein, J., Jakobsson, P.J., Haeggström, J.Z. (2003) Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. *Biochem. Biophys. Res. Commun.* **312**, 271-276.
11. Werz, O. (2002) 5-lipoxygenase: cellular biology and molecular pharmacology. *Curr. Drug Targets Inflamm. Allergy* **1**, 23-44.
12. Werz, O., Steinhilber, D. (2006) Therapeutic options for 5-lipoxygenase inhibitors. *Pharmacol. Ther.* **112**, 701-718.
13. Leier, I., Jedlitschky, G., Buchholz, U., Cole, S.P., Deeley, R.G., Keppler, D. (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C4 and structurally related conjugates. *J. Biol. Chem.* **269**, 27807-27810.
14. Lam, B.K., Gagnon, L., Austen, K.F., Soberman, R.J. (1990) The mechanism of leukotriene B4 export from human polymorphonuclear leukocytes. *J. Biol. Chem.* **265**, 13438-13441.
15. Riendeau, D., Falgueyret, J.P., Guay, J., Ueda, N., Yamamoto, S. (1991) Pseudoperoxidase activity of 5-lipoxygenase stimulated by potent benzofuranol and N-hydroxyurea inhibitors of the lipoxygenase reaction. *Biochem. J.* **274 (Pt 1)**, 287-292.
16. Rådmark, O., Werz, O., Steinhilber, D., Samuelsson, B. (2007) 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem. Sci.* **32**, 332-341.
17. Powell, W.S., Gravelle, F., Gravel, S. (1992) Metabolism of 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid and other 5(S)-hydroxyeicosanoids by a specific dehydrogenase in human polymorphonuclear leukocytes. *J. Biol. Chem.* **267**, 19233-19241.
18. Powell, W.S., Rokach, J. (2005) Biochemistry, biology and chemistry of the 5-lipoxygenase product 5-oxo-ETE. *Prog. Lipid. Res.* **44**, 154-183.

19. Serhan, C.N. (2002) Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an update and role in anti-inflammation and pro-resolution. *Prostaglandins Other Lipid. Mediat.* **68-69**, 433-455.
20. Folco, G., Murphy, R.C. (2006) Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses. *Pharmacol. Rev.* **58**, 375-388.
21. Burke, J.E., Dennis, E.A. (2009) Phospholipase A2 biochemistry. *Cardiovasc. Drugs Ther.* **23**, 49-59.
22. Hirabayashi, T., Murayama, T., Shimizu, T. (2004) Regulatory mechanism and physiological role of cytosolic phospholipase A2. *Biol. Pharm. Bull.* **27**, 1168-1173.
23. Kita, Y., Ohto, T., Uozumi, N., Shimizu, T. (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A2s. *Biochim. Biophys. Acta* **1761**, 1317-1322.
24. Schaloske, R.H., Dennis, E.A. (2006) The phospholipase A2 superfamily and its group numbering system. *Biochim. Biophys. Acta* **1761**, 1246-1259.
25. Six, D.A., Dennis, E.A. (2000) The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. *Biochim. Biophys. Acta* **1488**, 1-19.
26. Singer, A.G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., Gelb, M.H. (2002) Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. *J. Biol. Chem.* **277**, 48535-48549.
27. Reddy, S.T., Herschman, H.R. (1996) Transcellular prostaglandin production following mast cell activation is mediated by proximal secretory phospholipase A2 and distal prostaglandin synthase 1. *J. Biol. Chem.* **271**, 186-191.
28. Mounier, C.M., Ghomashchi, F., Lindsay, M.R., James, S., Singer, A.G., Parton, R.G., Gelb, M.H. (2004) Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A(2) occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A(2)-alpha. *J. Biol. Chem.* **279**, 25024-25038.
29. Ni, Z., Okeley, N.M., Smart, B.P., Gelb, M.H. (2006) Intracellular actions of group IIA secreted phospholipase A2 and group IVA cytosolic phospholipase A2 contribute to arachidonic acid release and prostaglandin production in rat gastric mucosal cells and transfected human embryonic kidney cells. *J. Biol. Chem.* **281**, 16245-16255.
30. Murakami, M., Kambe, T., Shimbara, S., Kudo, I. (1999) Functional coupling between various phospholipase A2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J. Biol. Chem.* **274**, 3103-1315.
31. Wijewickrama, G.T., Kim, J.H., Kim, Y.J., Abraham, A., Oh, Y., Ananthanarayanan, B., Kwatia, M., Ackerman, S.J., Cho, W. (2006) Systematic evaluation of transcellular activities of secretory phospholipases A2. High activity of group V phospholipases A2 to induce eicosanoid biosynthesis in neighboring inflammatory cells. *J. Biol. Chem.* **281**, 10935-10944.
32. Ohto, T., Uozumi, N., Hirabayashi, T., Shimizu, T. (2005) Identification of novel cytosolic phospholipase A(2)s, murine cPLA(2){delta}, {epsilon}, and {zeta}, which form a gene cluster with cPLA(2){beta}. *J. Biol. Chem.* **280**, 24576-24583.
33. Pickard, R.T., Strifler, B.A., Kramer, R.M., Sharp, J.D. (1999) Molecular cloning of two new human paralogs of 85-kDa cytosolic phospholipase A2. *J. Biol. Chem.* **274**, 8823-8831.
34. Song, C., Chang, X.J., Bean, K.M., Proia, M.S., Knopf, J.L., Kriz, R.W. (1999) Molecular characterization of cytosolic phospholipase A2-beta. *J. Biol. Chem.* **274**, 17063-17067.
35. Chiba, H., Michibata, H., Wakimoto, K., Seishima, M., Kawasaki, S., Okubo, K., Mitsui, H., Torii, H., Imai, Y. (2004) Cloning of a gene for a novel epithelium-specific

cytosolic phospholipase A2, cPLA2delta, induced in psoriatic skin. *J. Biol. Chem.* **279**, 12890-12897.
36. Clark, J.D., Lin, L.L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N., Knopf, J.L. (1991) A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043-1051.
37. Kudo, I., Murakami, M. (2002) Phospholipase A2 enzymes. *Prostaglandins Other Lipid. Mediat.* **68-69**, 3-58.
38. Tjoelker, L.W., Eberhardt, C., Unger, J., Trong, H.L., Zimmerman, G.A., McIntyre, T.M., Stafforini, D.M., Prescott, S.M., Gray, P.W. (1995) Plasma platelet-activating factor acetylhydrolase is a secreted phospholipase A2 with a catalytic triad. *J. Biol. Chem.* **270**, 25481-25487.
39. Abe, A., Shayman, J.A. (1998) Purification and characterization of 1-O-acylceramide synthase, a novel phospholipase A2 with transacylase activity. *J. Biol. Chem.* **273**, 8467-8474.
40. Hiraoka, M., Abe, A., Shayman, J.A. (2002) Cloning and characterization of a lysosomal phospholipase A2, 1-O-acylceramide synthase. *J. Biol. Chem.* **277**, 10090-10099.
41. Dessen, A., Tang, J., Schmidt, H., Stahl, M., Clark, J.D., Seehra, J., Somers, W.S. (1999) Crystal structure of human cytosolic phospholipase A2 reveals a novel topology and catalytic mechanism. *Cell* **97**, 349-360.
42. Sharp, J.D., Pickard, R.T., Chiou, X.G., Manetta, J.V., Kovacevic, S., Miller, J.R., Varshavsky, A.D., Roberts, E.F., Strifler, B.A., Brems, D.N., et al. (1994) Serine 228 is essential for catalytic activities of 85-kDa cytosolic phospholipase A2. *J. Biol. Chem.* **269**, 23250-23254.
43. Pickard, R.T., Chiou, X.G., Strifler, B.A., DeFelippis, M.R., Hyslop, P.A., Tebbe, A.L., Yee, Y.K., Reynolds, L.J., Dennis, E.A., Kramer, R.M., Sharp, J.D. (1996) Identification of essential residues for the catalytic function of 85-kDa cytosolic phospholipase A2. Probing the role of histidine, aspartic acid, cysteine, and arginine. *J. Biol. Chem.* **271**, 19225-19231.
44. Huang, Z., Payette, P., Abdullah, K., Cromlish, W.A., Kennedy, B.P. (1996) Functional identification of the active-site nucleophile of the human 85-kDa cytosolic phospholipase A2. *Biochemistry* **35**, 3712-3721.
45. Das, S., Cho, W. (2002) Roles of catalytic domain residues in interfacial binding and activation of group IV cytosolic phospholipase A2. *J. Biol. Chem.* **277**, 23838-23846.
46. Ghosh, M., Tucker, D.E., Burchett, S.A., Leslie, C.C. (2006) Properties of the Group IV phospholipase A2 family. *Prog. Lipid. Res.* **45**, 487-510.
47. Six, D.A., Dennis, E.A. (2003) Essential Ca(2+)-independent role of the group IVA cytosolic phospholipase A(2) C2 domain for interfacial activity. *J. Biol. Chem.* **278**, 23842-23850.
48. Han, S.K., Kim, K.P., Koduri, R., Bittova, L., Munoz, N.M., Leff, A.R., Wilton, D.C., Gelb, M.H., Cho, W. (1999) Roles of Trp31 in high membrane binding and proinflammatory activity of human group V phospholipase A2. *J. Biol. Chem.* **274**, 11881-11888.
49. Nalefski, E.A., Falke, J.J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. *Protein Sci.* **5**, 2375-2390.
50. Cho, W. (2001) Membrane targeting by C1 and C2 domains. *J. Biol. Chem.* **276**, 32407-13240.
51. Malkova, S., Long, F., Stahelin, R.V., Pingali, S.V., Murray, D., Cho, W., Schlossman, M.L. (2005) X-ray reflectivity studies of cPLA2{alpha}-C2 domains adsorbed onto Langmuir monolayers of SOPC. *Biophys. J.* **89**, 1861-1873.

52. Murray, D., Honig, B. (2002) Electrostatic control of the membrane targeting of C2 domains. *Mol. Cell.* **9**, 145-154.
53. Perisic, O., Paterson, H.F., Mosedale, G., Lara-González, S., Williams, R.L. (1999) Mapping the phospholipid-binding surface and translocation determinants of the C2 domain from cytosolic phospholipase A2. *J. Biol. Chem.* **274**, 14979-14987.
54. Bittova, L., Sumandea, M., Cho, W. (1999) A structure-function study of the C2 domain of cytosolic phospholipase A2. Identification of essential calcium ligands and hydrophobic membrane binding residues. *J. Biol. Chem.* **274**, 9665-9672.
55. Perisic, O., Fong, S., Lynch, D.E., Bycroft, M., Williams, R.L. (1998) Crystal structure of a calcium-phospholipid binding domain from cytosolic phospholipase A2. *J. Biol. Chem.* **273**, 1596-1604.
56. Nalefski, E.A., Falke, J.J. (1998) Location of the membrane-docking face on the Ca2+-activated C2 domain of cytosolic phospholipase A2. *Biochemistry* **37**, 17642-17650.
57. Xu, G.Y., McDonagh, T., Yu, H.A., Nalefski, E.A., Clark, J.D., Cumming, D.A. (1998) Solution structure and membrane interactions of the C2 domain of cytosolic phospholipase A2. *J. Mol. Biol.* **280**, 485-500.
58. Nalefski, E.A., McDonagh, T., Somers, W., Seehra, J., Falke, J.J., Clark, J.D. (1998) Independent folding and ligand specificity of the C2 calcium-dependent lipid binding domain of cytosolic phospholipase A2. *J. Biol. Chem.* **273**, 1365-1372.
59. Malmberg, N.J., Van Buskirk, D.R., Falke, J.J. (2003) Membrane-docking loops of the cPLA2 C2 domain: detailed structural analysis of the protein-membrane interface via site-directed spin-labeling. *Biochemistry* **42**, 13227-13240.
60. Stahelin, R.V., Subramanian, P., Vora, M., Cho, W., Chalfant, C.E. (2007) Ceramide-1-phosphate binds group IVA cytosolic phospholipase a2 via a novel site in the C2 domain. *J. Biol. Chem.* **282**, 20467-20474.
61. Clark, J.D., Schievella, A.R., Nalefski, E.A., Lin, L.L. (1995) Cytosolic phospholipase A2. *J. Lipid. Mediat. Cell. Signal.* **12**, 83-117.
62. Kita, Y., Ohto, T., Uozumi, N., Shimizu, T. (2006) Biochemical properties and pathophysiological roles of cytosolic phospholipase A2s. *Biochim. Biophys. Acta.* **1761**, 1317-1322.
63. Sharp, J.D., White, D.L. (1993) Cytosolic PLA2: mRNA levels and potential for transcriptional regulation. *J. Lipid. Mediat.* **8**, 183-189.
64. Dolan-O'Keefe, M., Chow, V., Monnier, J., Visner, G.A., Nick, H.S. (2000) Transcriptional regulation and structural organization of the human cytosolic phospholipase A(2) gene. *Am. J. Physiol. Lung. Cell. Mol. Physiol.* **278**, L649-657.
65. Tay, A., Maxwell, P., Li, Z.G., Goldberg, H., Skorecki, K. (1994) Cytosolic phospholipase A2 gene expression in rat mesangial cells is regulated post-transcriptionally. *Biochem. J.* **304 (Pt 2)**, 417-422.
66. Gilbert, J.J., Stewart, A., Courtney, C.A., Fleming, M.C., Reid, P., Jackson, C.G., Wise, A., Wakelam, M.J., Harnett, M.M. (1996) Antigen receptors on immature, but not mature, B and T cells are coupled to cytosolic phospholipase A2 activation: expression and activation of cytosolic phospholipase A2 correlate with lymphocyte maturation. *J. Immunol.* **156**, 2054-2061.
67. Hirabayashi, T., Kume, K., Hirose, K., Yokomizo, T., Iino, M., Itoh, H., Shimizu, T. (1999) Critical duration of intracellular Ca2+ response required for continuous translocation and activation of cytosolic phospholipase A2. *J. Biol. Chem.* **274**, 5163-5169.
68. Evans, J.H., Gerber, S.H., Murray, D., Leslie, C.C. (2004) The calcium binding loops of the cytosolic phospholipase A2 C2 domain specify targeting to Golgi and ER in live cells. *Mol. Biol. Cell.* **15**, 371-383.

69. Stahelin, R.V., Rafter, J.D., Das, S., Cho, W. (2003) The molecular basis of differential subcellular localization of C2 domains of protein kinase C-alpha and group IVa cytosolic phospholipase A2. *J. Biol. Chem.* **278**, 12452-12460.
70. Evans, J.H., Spencer, D.M., Zweifach, A., Leslie, C.C. (2001) Intracellular calcium signals regulating cytosolic phospholipase A2 translocation to internal membranes. *J. Biol. Chem.* **276**, 30150-30160.
71. Glover, S., de Carvalho, M.S., Bayburt, T., Jonas, M., Chi, E., Leslie, C.C., Gelb, M.H. (1995) Translocation of the 85-kDa phospholipase A2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J. Biol. Chem.* **270**, 15359-15367.
72. Peters-Golden, M., Song, K., Marshall, T., Brock, T. (1996) Translocation of cytosolic phospholipase A2 to the nuclear envelope elicits topographically localized phospholipid hydrolysis. *Biochem. J.* **318 (Pt 3)**, 797-803.
73. Schievella, A.R., Regier, M.K., Smith, W.L., Lin, L.L. (1995) Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *J. Biol. Chem.* **270**, 30749-30754.
74. Gijón, M.A., Spencer, D.M., Siddiqi, A.R., Bonventre, J.V., Leslie, C.C. (2000) Cytosolic phospholipase A2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A2 regulation. *J. Biol. Chem.* **275**, 20146-20156.
75. Balsinde, J., Balboa, M.A., Li, W.H., Llopis, J., Dennis, E.A. (2000) Cellular regulation of cytosolic group IV phospholipase A2 by phosphatidylinositol bisphosphate levels. *J. Immunol.* **164**, 5398-5402.
76. Leslie, C.C., Channon, J.Y. (1990) Anionic phospholipids stimulate an arachidonoyl-hydrolyzing phospholipase A2 from macrophages and reduce the calcium requirement for activity. *Biochim. Biophys. Acta.* **1045**, 261-270.
77. Das, S., Rafter, J.D., Kim, K.P., Gygi, S.P., Cho, W. (2003) Mechanism of group IVA cytosolic phospholipase A(2) activation by phosphorylation. *J. Biol. Chem.* **278**, 41431-41442.
78. Evans, J.H., Leslie, C.C. (2004) The cytosolic phospholipase A2 catalytic domain modulates association and residence time at Golgi membranes. *J. Biol. Chem.* **279**, 6005-6016.
79. Mosior, M., Six, D.A., Dennis, E.A. (1998) Group IV cytosolic phospholipase A2 binds with high affinity and specificity to phosphatidylinositol 4,5-bisphosphate resulting in dramatic increases in activity. *J. Biol. Chem.* **273**, 2184-2191.
80. Hixon, M.S., Ball, A., Gelb, M.H. (1998) Calcium-dependent and -independent interfacial binding and catalysis of cytosolic group IV phospholipase A2. *Biochemistry* **37**, 8516-8526.
81. Subramanian, P., Vora, M., Gentile, L.B., Stahelin, R.V., Chalfant, C.E. (2007) Anionic lipids activate group IVA cytosolic phospholipase A2 via distinct and separate mechanisms. *J. Lipid. Res.* **48**, 2701-2708.
82. Casas, J., Gijon, M.A., Vigo, A.G., Crespo, M.S., Balsinde, J., Balboa, M.A. (2006) Phosphatidylinositol 4,5-bisphosphate anchors cytosolic group IVA phospholipase A2 to perinuclear membranes and decreases its calcium requirement for translocation in live cells. *Mol. Biol. Cell.* **17**, 155-162.
83. Subramanian, P., Stahelin, R.V., Szulc, Z., Bielawska, A., Cho, W., Chalfant, C.E. (2005) Ceramide 1-phosphate acts as a positive allosteric activator of group IVA cytosolic phospholipase A2 alpha and enhances the interaction of the enzyme with phosphatidylcholine. *J. Biol. Chem.* **280**, 17601-17607.

84. Kitatani, K., Oka, T., Murata, T., Hayama, M., Akiba, S., Sato, T. (2000) Acceleration by ceramide of calcium-dependent translocation of phospholipase A2 from cytosol to membranes in platelets. *Arch. Biochem. Biophys.* **382**, 296-302.
85. Huwiler, A., Johansen, B., Skarstad, A., Pfeilschifter, J. (2001) Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. *FASEB J.* **15**, 7-9.
86. Börsch-Haubold, A.G., Bartoli, F., Asselin, J., Dudler, T., Kramer, R.M., Apitz-Castro, R., Watson, S.P., Gelb, M.H. (1998) Identification of the phosphorylation sites of cytosolic phospholipase A2 in agonist-stimulated human platelets and HeLa cells. *J. Biol. Chem.* **273**, 4449-4458.
87. Hefner, Y., Börsch-Haubold, A.G., Murakami, M., Wilde, J.I., Pasquet, S., Schieltz, D., Ghomashchi, F., Yates, J.R., 3rd, Armstrong, C.G., Paterson, A., Cohen, P., Fukunaga, R., Hunter, T., Kudo, I., Watson, S.P., Gelb, M.H. (2000) Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *J. Biol. Chem.* **275**, 37542-37551.
88. Muthalif, M.M., Hefner, Y., Canaan, S., Harper, J., Zhou, H., Parmentier, J.H., Aebersold, R., Gelb, M.H., Malik, K.U. (2001) Functional interaction of calcium-/calmodulin-dependent protein kinase II and cytosolic phospholipase A(2). *J. Biol. Chem.* **276**, 39653-39660.
89. Lin, L.L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A., Davis, R.J. (1993) cPLA2 is phosphorylated and activated by MAP kinase. *Cell* **72**, 269-278.
90. Kramer, R.M., Roberts, E.F., Um, S.L., Börsch-Haubold, A.G., Watson, S.P., Fisher, M.J., Jakubowski, J.A. (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J. Biol. Chem.* **271**, 27723-27729.
91. Evans, J.H., Fergus, D.J., Leslie, C.C. (2002) Inhibition of the MEK1/ERK pathway reduces arachidonic acid release independently of cPLA2 phosphorylation and translocation. *BMC Biochem.* **3**, 30.
92. Magne, S., Couchie, D., Pecker, F., Pavoine, C. (2001) Beta(2)-adrenergic receptor agonists increase intracellular free Ca(2+) concentration cycling in ventricular cardiomyocytes through p38 and p42/44 MAPK-mediated cytosolic phospholipase A(2) activation. *J. Biol. Chem.* **276**, 39539-39548.
93. Gijón, M.A., Spencer, D.M., Kaiser, A.L., Leslie, C.C. (1999) Role of phosphorylation sites and the C2 domain in regulation of cytosolic phospholipase A2. *J. Cell. Biol.* **145**, 1219-1232.
94. Qiu, Z.H., Gijón, M.A., de Carvalho, M.S., Spencer, D.M., Leslie, C.C. (1998) The role of calcium and phosphorylation of cytosolic phospholipase A2 in regulating arachidonic acid release in macrophages. *J. Biol. Chem.* **273**, 8203-8211.
95. de Carvalho, M.G., McCormack, A.L., Olson, E., Ghomashchi, F., Gelb, M.H., Yates, J.R., 3rd, Leslie, C.C. (1996) Identification of phosphorylation sites of human 85-kDa cytosolic phospholipase A2 expressed in insect cells and present in human monocytes. *J. Biol. Chem.* **271**, 6987-6997.
96. Muthalif, M.M., Benter, I.F., Uddin, M.R., Malik, K.U. (1996) Calcium/calmodulin-dependent protein kinase IIalpha mediates activation of mitogen-activated protein kinase and cytosolic phospholipase A2 in norepinephrine-induced arachidonic acid release in rabbit aortic smooth muscle cells. *J. Biol. Chem.* **271**, 30149-30157.
97. Muthalif, M.M., Ljuca, F., Roaten, J.B., Pentapaty, N., Uddin, M.R., Malik, K.U. (2001) Ca2+/calmodulin-dependent protein kinase II and cytosolic phospholipase A2 contribute to mitogenic signaling in myeloblastic leukemia U-937 cells. *J. Pharmacol. Exp. Ther.* **298**, 272-278.

98. Fatima, S., Yaghini, F.A., Ahmed, A., Khandekar, Z., Malik, K.U. (2003) CaM kinase IIalpha mediates norepinephrine-induced translocation of cytosolic phospholipase A2 to the nuclear envelope. *J. Cell. Sci.* **116**, 353-365.
99. Waskiewicz, A.J., Flynn, A., Proud, C.G., Cooper, J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16**, 1909-1920.
100. Muthalif, M.M., Benter, I.F., Karzoun, N., Fatima, S., Harper, J., Uddin, M.R., Malik, K.U. (1998) 20-Hydroxyeicosatetraenoic acid mediates calcium/calmodulin-dependent protein kinase II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *Proc. Natl. Acad. Sci. U S A* **95**, 12701-12706.
101. Sierra-Honigmann, M.R., Bradley, J.R., Pober, J.S. (1996) "Cytosolic" phospholipase A2 is in the nucleus of subconfluent endothelial cells but confined to the cytoplasm of confluent endothelial cells and redistributes to the nuclear envelope and cell junctions upon histamine stimulation. *Lab. Invest.* **74**, 684-695.
102. Tashiro, S., Sumi, T., Uozumi, N., Shimizu, T., Nakamura, T. (2004) B-Myb-dependent regulation of c-Myc expression by cytosolic phospholipase A2. *J. Biol. Chem.* **279**, 17715-17722.
103. Girotti, M., Evans, J.H., Burke, D., Leslie, C.C. (2004) Cytosolic phospholipase A2 translocates to forming phagosomes during phagocytosis of zymosan in macrophages. *J. Biol. Chem.* **279**, 19113-19121.
104. Shmelzer, Z., Haddad, N., Admon, E., Pessach, I., Leto, T.L., Eitan-Hazan, Z., Hershfinkel, M., Levy, R. (2003) Unique targeting of cytosolic phospholipase A2 to plasma membranes mediated by the NADPH oxidase in phagocytes. *J. Cell. Biol.* **162**, 683-692.
105. Yu, W., Bozza, P.T., Tzizik, D.M., Gray, J.P., Cassara, J., Dvorak, A.M., Weller, P.F. (1998) Co-compartmentalization of MAP kinases and cytosolic phospholipase A2 at cytoplasmic arachidonate-rich lipid bodies. *Am. J. Pathol.* **152**, 759-769.
106. Nakatani, Y., Tanioka, T., Sunaga, S., Murakami, M., Kudo, I. (2000) Identification of a cellular protein that functionally interacts with the C2 domain of cytosolic phospholipase A(2)alpha. *J. Biol. Chem.* **275**, 1161-1168.
107. Sheridan, A.M., Force, T., Yoon, H.J., O'Leary, E., Choukroun, G., Taheri, M.R., Bonventre, J.V. (2001) PLIP, a novel splice variant of Tip60, interacts with group IV cytosolic phospholipase A(2), induces apoptosis, and potentiates prostaglandin production. *Mol. Cell. Biol.* **21**, 4470-4481.
108. Wu, T., Angus, C.W., Yao, X.L., Logun, C., Shelhamer, J.H. (1997) P11, a unique member of the S100 family of calcium-binding proteins, interacts with and inhibits the activity of the 85-kDa cytosolic phospholipase A2. *J. Biol. Chem.* **272**, 17145-17153.
109. Yao, X.L., Cowan, M.J., Gladwin, M.T., Lawrence, M.M., Angus, C.W., Shelhamer, J.H. (1999) Dexamethasone alters arachidonate release from human epithelial cells by induction of p11 protein synthesis and inhibition of phospholipase A2 activity. *J. Biol. Chem.* **274**, 17202-17208.
110. Kim, S., Ko, J., Kim, J.H., Choi, E.C., Na, D.S. (2001) Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model. *FEBS Lett.* **489**, 243-248.
111. Mira, J.P., Dubois, T., Oudinet, J.P., Lukowski, S., Russo-Marie, F., Geny, B. (1997) Inhibition of cytosolic phospholipase A2 by annexin V in differentiated permeabilized HL-60 cells. Evidence of crucial importance of domain I type II Ca2+-binding site in the mechanism of inhibition. *J. Biol. Chem.* **272**, 10474-10482.
112. Minor, W., Steczko, J., Bolin, J.T., Otwinowski, Z., Axelrod, B. (1993) Crystallographic determination of the active site iron and its ligands in soybean lipoxygenase L-1. *Biochemistry* **32**, 6320-6323.

113. Boyington, J.C., Gaffney, B.J., Amzel, L.M. (1993) The three-dimensional structure of an arachidonic acid 15-lipoxygenase. *Science* **260**, 1482-1486.
114. Skrzypczak-Jankun, E., Amzel, L.M., Kroa, B.A., Funk, M.O., Jr. (1997) Structure of soybean lipoxygenase L3 and a comparison with its L1 isoenzyme. *Proteins* **29**, 15-31.
115. Gillmor, S.A., Villasenor, A., Fletterick, R., Sigal, E., Browner, M.F. (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. *Nat. Struct. Biol.* **4**, 1003-1009.
116. Hemak, J., Gale, D., Brock, T.G. (2002) Structural characterization of the catalytic domain of the human 5-lipoxygenase enzyme. *J. Mol. Model.* **8**, 102-112.
117. Riendeau, D., Denis, D., Choo, L.Y., Nathaniel, D.J. (1989) Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation. *Biochem. J.* **263**, 565-572.
118. Hammarberg, T., Kuprin, S., Rådmark, O., Holmgren, A. (2001) EPR investigation of the active site of recombinant human 5-lipoxygenase: inhibition by selenide. *Biochemistry* **40**, 6371-6378.
119. Chasteen, N.D., Grady, J.K., Skorey, K.I., Neden, K.J., Riendeau, D., Percival, M.D. (1993) Characterization of the non-heme iron center of human 5-lipoxygenase by electron paramagnetic resonance, fluorescence, and ultraviolet-visible spectroscopy: redox cycling between ferrous and ferric states. *Biochemistry* **32**, 9763-7971.
120. Rouzer, C.A., Samuelsson, B. (1986) The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS Lett.* **204**, 293-296.
121. Rådmark, O. (1995) Arachidonate 5-lipoxygenase. *J. Lipid. Mediat Cell. Signal.* **12**, 171-184.
122. Hegg, E.L., Que, L., Jr. (1997) The 2-His-1-carboxylate facial triad--an emerging structural motif in mononuclear non-heme iron(II) enzymes. *Eur. J. Biochem.* **250**, 625-629.
123. Hammarberg, T., Zhang, Y.Y., Lind, B., Rådmark, O., Samuelsson, B. (1995) Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase. *Eur. J. Biochem.* **230**, 401-407.
124. Rakonjac, M., Fischer, L., Provost, P., Werz, O., Steinhilber, D., Samuelsson, B., Rådmark, O. (2006) Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production. *Proc. Natl. Acad. Sci. U S A* **103**, 13150-13155.
125. Hammarberg, T., Provost, P., Persson, B., Rådmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. *J. Biol. Chem.* **275**, 38787-38793.
126. Kulkarni, S., Das, S., Funk, C.D., Murray, D., Cho, W. (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. *J. Biol. Chem.* **277**, 13167-13174.
127. Hörnig, C., Albert, D., Fischer, L., Hörnig, M., Rådmark, O., Steinhilber, D., Werz, O. (2005) 1-Oleoyl-2-acetylglycerol stimulates 5-lipoxygenase activity via a putative (phospho)lipid binding site within the N-terminal C2-like domain. *J. Biol. Chem.* **280**, 26913-26921.
128. Reid, G.K., Kargman, S., Vickers, P.J., Mancini, J.A., Leveille, C., Ethier, D., Miller, D.K., Gillard, J.W., Dixon, R.A., Evans, J.F. (1990) Correlation between expression of 5-lipoxygenase-activating protein, 5-lipoxygenase, and cellular leukotriene synthesis. *J. Biol. Chem.* **265**, 19818-19823.
129. Dixon, R.A., Jones, R.E., Diehl, R.E., Bennett, C.D., Kargman, S., Rouzer, C.A. (1988) Cloning of the cDNA for human 5-lipoxygenase. *Proc. Natl. Acad Sci. U S A* **85**, 416-420.

130. Bennett, C.F., Chiang, M.Y., Monia, B.P., Crooke, S.T. (1993) Regulation of 5-lipoxygenase and 5-lipoxygenase-activating protein expression in HL-60 cells. *Biochem. J.* **289 (Pt 1)**, 33-39.
131. Brungs, M., Rådmark, O., Samuelsson, B., Steinhilber, D. (1994) On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGF beta. *Biochem. Biophys. Res. Commun.* **205**, 1572-1580.
132. Brungs, M., Rådmark, O., Samuelsson, B., Steinhilber, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D3. *Proc. Natl. Acad. Sci. U S A* **92**, 107-111.
133. Funk, C.D., Hoshiko, S., Matsumoto, T., Rådmark, O., Samuelsson, B. (1989) Characterization of the human 5-lipoxygenase gene. *Proc. Natl. Acad Sci. U S A* **86**, 2587-2591.
134. Uhl, J., Klan, N., Rose, M., Entian, K.D., Werz, O., Steinhilber, D. (2002) The 5-lipoxygenase promoter is regulated by DNA methylation. *J. Biol. Chem.* **277**, 4374-4379.
135. Klan, N., Seuter, S., Schnur, N., Jung, M., Steinhilber, D. (2003) Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. *Biol. Chem.* **384**, 777-785.
136. Schnur, N., Seuter, S., Katryniok, C., Rådmark, O., Steinhilber, D. (2007) The histone deacetylase inhibitor trichostatin A mediates upregulation of 5-lipoxygenase promoter activity by recruitment of Sp1 to distinct GC-boxes. *Biochim. Biophys. Acta* **1771**, 1271-1282.
137. Katryniok, C., Schnur, N., Gillis, A., von Knethen, A., Sorg, B.L., Looijenga, L., Rådmark, O., Steinhilber, D. Role of DNA methylation and methyl-DNA binding proteins in the repression of 5-lipoxygenase promoter activity. *Biochim. Biophys. Acta.* **1801**, 49-57.
138. Sorg, B.L., Klan, N., Seuter, S., Dishart, D., Rådmark, O., Habenicht, A., Carlberg, C., Werz, O., Steinhilber, D. (2006) Analysis of the 5-lipoxygenase promoter and characterization of a vitamin D receptor binding site. *Biochim. Biophys. Acta.* **1761**, 686-697.
139. Seuter, S., Sorg, B.L., Steinhilber, D. (2006) The coding sequence mediates induction of 5-lipoxygenase expression by Smads3/4. *Biochem. Biophys. Res Commun* **348**, 1403-1410.
140. Stoffers, K.L., Sorg, B.L., Seuter, S., Rau, O., Rådmark, O., Steinhilber, D. Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. *J. Mol. Biol.* **395**, 884-896.
141. Aharony, D., Redkar-Brown, D.G., Hubbs, S.J., Stein, R.L. (1987) Kinetic studies on the inactivation of 5-lipoxygenase by 5(S)-hydroperoxyeicosatetraenoic acid. *Prostaglandins* **33**, 85-100.
142. Rouzer, C.A., Samuelsson, B. (1985) On the nature of the 5-lipoxygenase reaction in human leukocytes: enzyme purification and requirement for multiple stimulatory factors. *Proc. Natl. Acad. Sci. U S A* **82**, 6040-6044.
143. Hammarberg, T., Rådmark, O. (1999) 5-lipoxygenase binds calcium. *Biochemistry* **38**, 4441-4447.
144. Percival, M.D., Denis, D., Riendeau, D., Gresser, M.J. (1992) Investigation of the mechanism of non-turnover-dependent inactivation of purified human 5-lipoxygenase. Inactivation by H2O2 and inhibition by metal ions. *Eur. J. Biochem.* **210**, 109-117.
145. Noguchi, M., Miyano, M., Matsumoto, T., Noma, M. (1994) Human 5-lipoxygenase associates with phosphatidylcholine liposomes and modulates LTA4 synthetase activity. *Biochim. Biophys. Acta* **1215**, 300-306.

146. Bindu, P.H., Sastry, G.M., Sastry, G.N. (2004) Characterization of calcium and magnesium binding domains of human 5-lipoxygenase. *Biochem. Biophys. Res. Commun.* **320**, 461-467.
147. Bürkert, E., Arnold, C., Hammarberg, T., Rådmark, O., Steinhilber, D., Werz, O. (2003) The C2-like beta-barrel domain mediates the Ca2+-dependent resistance of 5-lipoxygenase activity against inhibition by glutathione peroxidase-1. *J Biol. Chem.* **278**, 42846-42853.
148. Aharony, D., Stein, R.L. (1986) Kinetic mechanism of guinea pig neutrophil 5-lipoxygenase. *J. Biol. Chem.* **261**, 11512-11519.
149. Rouzer, C.A., Samuelsson, B. (1987) Reversible, calcium-dependent membrane association of human leukocyte 5-lipoxygenase. *Proc. Natl. Acad. Sci. U S A* **84**, 7393-7397.
150. Rouzer, C.A., Kargman, S. (1988) Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* **263**, 10980-10988.
151. Wong, A., Hwang, S.M., Cook, M.N., Hogaboom, G.K., Crooke, S.T. (1988) Interactions of 5-lipoxygenase with membranes: studies on the association of soluble enzyme with membranes and alterations in enzyme activity. *Biochemistry* **27**, 6763-6769.
152. Rouzer, C.A., Shimizu, T., Samuelsson, B. (1985) On the nature of the 5-lipoxygenase reaction in human leukocytes: characterization of a membrane-associated stimulatory factor. *Proc. Natl. Acad. Sci. U S A* **82**, 7505-7509.
153. Puustinen, T., Scheffer, M.M., Samuelsson, B. (1988) Regulation of the human leukocyte 5-lipoxygenase: stimulation by micromolar Ca2+ levels and phosphatidylcholine vesicles. *Biochim. Biophys. Acta* **960**, 261-267.
154. Provost, P., Samuelsson, B., Rådmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. U S A* **96**, 1881-1885.
155. 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.* **425**, 265-274.
156. Rådmark, O., Samuelsson, B. (2005) Regulation of 5-lipoxygenase enzyme activity. *Biochem. Biophys. Res Commun* **338**, 102-110.
157. Ochi, K., Yoshimoto, T., Yamamoto, S., Taniguchi, K., Miyamoto, T. (1983) Arachidonate 5-lipoxygenase of guinea pig peritoneal polymorphonuclear leukocytes. Activation by adenosine 5'-triphosphate. *J. Biol. Chem.* **258**, 5754-5758.
158. Falgueyret, J.P., Denis, D., Macdonald, D., Hutchinson, J.H., Riendeau, D. (1995) Characterization of the arachidonate and ATP binding sites of human 5-lipoxygenase using photoaffinity labeling and enzyme immobilization. *Biochemistry* **34**, 13603-13611.
159. Werz, O., Szellas, D., Steinhilber, D. (2000) Reactive oxygen species released from granulocytes stimulate 5-lipoxygenase activity in a B-lymphocytic cell line. *Eur. J. Biochem.* **267**, 1263-1269.
160. Weitzel, F., Wendel, A. (1993) Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone. *J. Biol. Chem.* **268**, 6288-6292.
161. Werz, O., Steinhilber, D. (1996) Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells. The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells. *Eur J Biochem* **242**, 90-7.

162. Straif, D., Werz, O., Kellner, R., Bahr, U., Steinhilber, D. (2000) Glutathione peroxidase-1 but not -4 is involved in the regulation of cellular 5-lipoxygenase activity in monocytic cells. *Biochem. J.* **349**, 455-461.
163. McDonald, P.P., McColl, S.R., Naccache, P.H., Borgeat, P. (1991) Studies on the activation of human neutrophil 5-lipoxygenase induced by natural agonists and Ca2+ ionophore A23187. *Biochem. J.* **280 (Pt 2)**, 379-385.
164. Schatz-Munding, M., Hatzelmann, A., Ullrich, V. (1991) The involvement of extracellular calcium in the formation of 5-lipoxygenase metabolites by human polymorphonuclear leukocytes. *Eur. J. Biochem.* **197**, 487-493.
165. Wong, A., Cook, M.N., Foley, J.J., Sarau, H.M., Marshall, P., Hwang, S.M. (1991) Influx of extracellular calcium is required for the membrane translocation of 5-lipoxygenase and leukotriene synthesis. *Biochemistry* **30**, 9346-9354.
166. Albert, D., Pergola, C., Koeberle, A., Dodt, G., Steinhilber, D., Werz, O. (2008) The role of diacylglyceride generation by phospholipase D and phosphatidic acid phosphatase in the activation of 5-lipoxygenase in polymorphonuclear leukocytes. *J. Leukoc. Biol.* **83**, 1019-1027.
167. Bürkert, E., Szellas, D., Rådmark, O., Steinhilber, D., Werz, O. (2003) Cell type-dependent activation of 5-lipoxygenase by arachidonic acid. *J. Leukoc. Biol.* **73**, 191-200.
168. Werz, O., Bürkert, E., Samuelsson, B., Rådmark, O., Steinhilber, D. (2002) Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. *Blood* **99**, 1044-1052.
169. Werz, O., Klemm, J., Samuelsson, B., Rådmark, O. (2000) 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. *Proc. Natl. Acad. Sci. U S A* **97**, 5261-5266.
170. Werz, O., Bürkert, E., Fischer, L., Szellas, D., Dishart, D., Samuelsson, B., Rådmark, O., Steinhilber, D. (2002) Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes. *Faseb J.* **16**, 1441-1443.
171. Werz, O., Szellas, D., Steinhilber, D., Rådmark, O. (2002) Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPK-activated protein kinase 2 (MK2). *J. Biol. Chem.* **277**, 14793-14800.
172. Hanaka, H., Shimizu, T., Izumi, T. (2005) Stress-induced nuclear export of 5-lipoxygenase. *Biochem. Biophys. Res. Commun.* **338**, 111-116.
173. Luo, M., Jones, S.M., Peters-Golden, M., Brock, T.G. (2003) Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B4 synthetic capacity. *Proc. Natl. Acad. Sci. U S A* **100**, 12165-12170.
174. Luo, M., Jones, S.M., Phare, S.M., Coffey, M.J., Peters-Golden, M., Brock, T.G. (2004) Protein kinase A inhibits leukotriene synthesis by phosphorylation of 5-lipoxygenase on serine 523. *J. Biol. Chem.* **279**, 41512-41520.
175. Luo, M., Jones, S.M., Flamand, N., Aronoff, D.M., Peters-Golden, M., Brock, T.G. (2005) Phosphorylation by protein kinase a inhibits nuclear import of 5-lipoxygenase. *J. Biol. Chem.* **280**, 40609-40616.
176. Flamand, N., Surette, M.E., Picard, S., Bourgoin, S., Borgeat, P. (2002) Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Mol. Pharmacol.* **62**, 250-256.
177. Luo, M., Pang, C.W., Gerken, A.E., Brock, T.G. (2004) Multiple nuclear localization sequences allow modulation of 5-lipoxygenase nuclear import. *Traffic* **5**, 847-854.
178. Brock, T.G. (2005) Regulating leukotriene synthesis: the role of nuclear 5-lipoxygenase. *J. Cell. Biochem.* **96**, 1203-1211.

179. Hanaka, H., Shimizu, T., Izumi, T. (2002) Nuclear-localization-signal-dependent and nuclear-export-signal-dependent mechanisms determine the localization of 5-lipoxygenase. *Biochem. J.* **361**, 505-514.
180. Lam, B.K., Penrose, J.F., Freeman, G.J., Austen, K.F. (1994) Expression cloning of a cDNA for human leukotriene C4 synthase, an integral membrane protein conjugating reduced glutathione to leukotriene A4. *Proc. Natl. Acad. Sci. U S A* **91**, 7663-7667.
181. Jakobsson, P.J., Morgenstern, R., Mancini, J., Ford-Hutchinson, A., Persson, B. (2000) Membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG). A widespread protein superfamily. *Am. J. Respir. Crit. Care. Med.* **161**, S20-S24.
182. Vickers, P.J. (1995) 5-Lipoxygenase-activating protein (FLAP). *J. Lipid. Mediat Cell. Signal.* **12**, 185-194.
183. Steinhilber, D. (1994) 5-Lipoxygenase: enzyme expression and regulation of activity. *Pharm. Acta. Helv.* **69**, 3-14.
184. Peters-Golden, M., Brock, T.G. (2003) 5-lipoxygenase and FLAP. *Prostaglandins Leukot. Essent. Fatty Acids* **69**, 99-109.
185. Dixon, R.A., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., Miller, D.K. (1990) Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. *Nature* **343**, 282-284.
186. Byrum, R.S., Goulet, J.L., Griffiths, R.J., Koller, B.H. (1997) Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses. *J. Exp. Med.* **185**, 1065-1075.
187. Abramovitz, M., Wong, E., Cox, M.E., Richardson, C.D., Li, C., Vickers, P.J. (1993) 5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase. *Eur. J. Biochem.* **215**, 105-111.
188. Mancini, J.A., Abramovitz, M., Cox, M.E., Wong, E., Charleson, S., Perrier, H., Wang, Z., Prasit, P., Vickers, P.J. (1993) 5-lipoxygenase-activating protein is an arachidonate binding protein. *FEBS Lett.* **318**, 277-281.
189. Hill, E., Maclouf, J., Murphy, R.C., Henson, P.M. (1992) Reversible membrane association of neutrophil 5-lipoxygenase is accompanied by retention of activity and a change in substrate specificity. *J. Biol. Chem.* **267**, 22048-22053.
190. Mancini, J.A., Waterman, H., Riendeau, D. (1998) Cellular oxygenation of 12-hydroxyeicosatetraenoic acid and 15-hydroxyeicosatetraenoic acid by 5-lipoxygenase is stimulated by 5-lipoxygenase-activating protein. *J. Biol. Chem.* **273**, 32842-32847.
191. Plante, H., Picard, S., Mancini, J., Borgeat, P. (2006) 5-Lipoxygenase-activating protein homodimer in human neutrophils: evidence for a role in leukotriene biosynthesis. *Biochem. J.* **393**, 211-218.
192. Flamand, N., Mancuso, P., Serezani, C.H., Brock, T.G. (2007) Leukotrienes: mediators that have been typecast as villains. *Cell. Mol. Life Sci.* **64**, 2657-2670.
193. Kim, N., Luster, A.D. (2007) Regulation of immune cells by eicosanoid receptors. *ScientificWorldJournal* **7**, 1307-1328.
194. Yokomizo, T., Kato, K., Hagiya, H., Izumi, T., Shimizu, T. (2001) Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. *J. Biol. Chem.* **276**, 12454-12459.
195. Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., Shimizu, T. (1997) A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* **387**, 620-624.
196. Gaudreau, R., Le Gouill, C., Métaoui, S., Lemire, S., Stankovà, J., Rola-Pleszczynski, M. (1998) Signalling through the leukotriene B4 receptor involves both alphai and alpha16, but not alphaq or alpha11 G-protein subunits. *Biochem. J.* **335 (Pt 1)**, 15-18.

197. Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., Shimizu, T. (2000) A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J. Exp. Med.* **192**, 421-432.
198. Lin, Q., Ruuska, S.E., Shaw, N.S., Dong, D., Noy, N. (1999) Ligand selectivity of the peroxisome proliferator-activated receptor alpha. *Biochemistry* **38**, 185-190.
199. Devchand, P.R., Keller, H., Peters, J.M., Vazquez, M., Gonzalez, F.J., Wahli, W. (1996) The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature* **384**, 39-43.
200. Takasaki, J., Kamohara, M., Matsumoto, M., Saito, T., Sugimoto, T., Ohishi, T., Ishii, H., Ota, T., Nishikawa, T., Kawai, Y., Masuho, Y., Isogai, T., Suzuki, Y., Sugano, S., Furuichi, K. (2000) The molecular characterization and tissue distribution of the human cysteinyl leukotriene CysLT(2) receptor. *Biochem. Biophys. Res. Commun.* **274**, 316-322.
201. Lynch, K.R., O'Neill, G.P., Liu, Q., Im, D.S., Sawyer, N., Metters, K.M., Coulombe, N., Abramovitz, M., Figueroa, D.J., Zeng, Z., Connolly, B.M., Bai, C., Austin, C.P., Chateauneuf, A., Stocco, R., Greig, G.M., Kargman, S., Hooks, S.B., Hosfield, E., Williams, D.L., Jr., Ford-Hutchinson, A.W., Caskey, C.T., Evans, J.F. (1999) Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* **399**, 789-793.
202. Sarau, H.M., Ames, R.S., Chambers, J., Ellis, C., Elshourbagy, N., Foley, J.J., Schmidt, D.B., Muccitelli, R.M., Jenkins, O., Murdock, P.R., Herrity, N.C., Halsey, W., Sathe, G., Muir, A.I., Nuthulaganti, P., Dytko, G.M., Buckley, P.T., Wilson, S., Bergsma, D.J., Hay, D.W. (1999) Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Mol. Pharmacol.* **56**, 657-663.
203. Nothacker, H.P., Wang, Z., Zhu, Y., Reinscheid, R.K., Lin, S.H., Civelli, O. (2000) Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. *Mol. Pharmacol.* **58**, 1601-1608.
204. Heise, C.E., O'Dowd, B.F., Figueroa, D.J., Sawyer, N., Nguyen, T., Im, D.S., Stocco, R., Bellefeuille, J.N., Abramovitz, M., Cheng, R., Williams, D.L., Jr., Zeng, Z., Liu, Q., Ma, L., Clements, M.K., Coulombe, N., Liu, Y., Austin, C.P., George, S.R., O'Neill, G.P., Metters, K.M., Lynch, K.R., Evans, J.F. (2000) Characterization of the human cysteinyl leukotriene 2 receptor. *J. Biol. Chem.* **275**, 30531-30536.
205. Ciana, P., Fumagalli, M., Trincavelli, M.L., Verderio, C., Rosa, P., Lecca, D., Ferrario, S., Parravicini, C., Capra, V., Gelosa, P., Guerrini, U., Belcredito, S., Cimino, M., Sironi, L., Tremoli, E., Rovati, G.E., Martini, C., Abbracchio, M.P. (2006) The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. *EMBO J.* **25**, 4615-4627.
206. Gimbrone, M.A., Jr., Brock, A.F., Schafer, A.I. (1984) Leukotriene B4 stimulates polymorphonuclear leukocyte adhesion to cultured vascular endothelial cells. *J. Clin. Invest.* **74**, 1552-1555.
207. Claesson, H.E., Feinmark, S.J. (1984) Relationship of cyclic-AMP levels in leukotriene B4-stimulated leukocytes to lysosomal enzyme release and the generation of superoxide anions. *Biochim. Biophys. Acta* **804**, 52-57.
208. Cunningham, F.M., Shipley, M.E., Smith, M.J. (1980) Aggregation of rat polymorphonuclear leucocytes in vitro. *J. Pharm. Pharmacol.* **32**, 377-380.
209. Mancuso, P., Standiford, T.J., Marshall, T., Peters-Golden, M. (1998) 5-Lipoxygenase reaction products modulate alveolar macrophage phagocytosis of Klebsiella pneumoniae. *Infect. Immun.* **66**, 5140-5146.
210. Mancuso, P., Nana-Sinkam, P., Peters-Golden, M. (2001) Leukotriene B4 augments neutrophil phagocytosis of Klebsiella pneumoniae. *Infect. Immun.* **69**, 2011-2016.

211. Dahlén, S.E., Hedqvist, P., Hammarström, S., Samuelsson, B. (1980) Leukotrienes are potent constrictors of human bronchi. *Nature* **288**, 484-486.
212. Marom, Z., Shelhamer, J.H., Bach, M.K., Morton, D.R., Kaliner, M. (1982) Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am. Rev. Respir. Dis.* **126**, 449-451.
213. Michelassi, F., Landa, L., Hill, R.D., Lowenstein, E., Watkins, W.D., Petkau, A.J., Zapol, W.M. (1982) Leukotriene D4: a potent coronary artery vasoconstrictor associated with impaired ventricular contraction. *Science* **217**, 841-843.
214. Schellenberg, R.R., Foster, A. (1984) Differential activity of leukotrienes upon human pulmonary vein and artery. *Prostaglandins* **27**, 475-482.
215. Drazen, J.M., Austen, K.F., Lewis, R.A., Clark, D.A., Goto, G., Marfat, A., Corey, E.J. (1980) Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc. Natl. Acad. Sci. U S A* **77**, 4354-4358.
216. Dahlén, S.E., Björk, J., Hedqvist, P., Arfors, K.E., Hammarström, S., Lindgren, J.A., Samuelsson, B. (1981) Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. U S A* **78**, 3887-3891.
217. Grant, G.E., Rokach, J., Powell, W.S. (2009) 5-Oxo-ETE and the OXE receptor. *Prostaglandins Other Lipid. Mediat.* **89**, 98-104.
218. Ghosh, J., Myers, C.E. (1998) Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. *Proc. Natl. Acad. Sci. U S A* **95**, 13182-13187.
219. Hosoi, T., Koguchi, Y., Sugikawa, E., Chikada, A., Ogawa, K., Tsuda, N., Suto, N., Tsunoda, S., Taniguchi, T., Ohnuki, T. (2002) Identification of a novel human eicosanoid receptor coupled to G(i/o). *J. Biol. Chem.* **277**, 31459-31465.
220. Chiang, N., Arita, M., Serhan, C.N. (2005) Anti-inflammatory circuitry: lipoxin, aspirin-triggered lipoxins and their receptor ALX. *Prostaglandins Leukot. Essent. Fatty Acids* **73**, 163-177.
221. Schwab, J.M., Serhan, C.N. (2006) Lipoxins and new lipid mediators in the resolution of inflammation. *Curr. Opin. Pharmacol.* **6**, 414-420.
222. Lee, T.H., Horton, C.E., Kyan-Aung, U., Haskard, D., Crea, A.E., Spur, B.W. (1989) Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Clin. Sci. (Lond)* **77**, 195-203.
223. Colgan, S.P., Serhan, C.N., Parkos, C.A., Delp-Archer, C., Madara, J.L. (1993) Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J. Clin. Invest.* **92**, 75-82.
224. Papayianni, A., Serhan, C.N., Brady, H.R. (1996) Lipoxin A4 and B4 inhibit leukotriene-stimulated interactions of human neutrophils and endothelial cells. *J. Immunol.* **156**, 2264-2272.
225. Hachicha, M., Pouliot, M., Petasis, N.A., Serhan, C.N. (1999) Lipoxin (LX)A4 and aspirin-triggered 15-epi-LXA4 inhibit tumor necrosis factor 1alpha-initiated neutrophil responses and trafficking: regulators of a cytokine-chemokine axis. *J. Exp. Med.* **189**, 1923-1930.
226. Maddox, J.F., Serhan, C.N. (1996) Lipoxin A4 and B4 are potent stimuli for human monocyte migration and adhesion: selective inactivation by dehydrogenation and reduction. *J. Exp. Med.* **183**, 137-146.
227. Maddox, J.F., Hachicha, M., Takano, T., Petasis, N.A., Fokin, V.V., Serhan, C.N. (1997) Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. *J. Biol. Chem.* **272**, 6972-6978.

228. McGill, K.A., Busse, W.W. (1996) Zileuton. *Lancet* **348**, 519-524.
229. Claesson, H.E., Dahlen, S.E. (1999) Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs. *J. Intern. Med.* **245**, 205-227.
230. Werz, O. (2007) Inhibition of 5-lipoxygenase product synthesis by natural compounds of plant origin. *Planta Med.* **73**, 1331-1357.
231. Spanbroek, R., Grabner, R., Lotzer, K., Hildner, M., Urbach, A., Ruhling, K., Moos, M.P., Kaiser, B., Cohnert, T.U., Wahlers, T., Zieske, A., Plenz, G., Robenek, H., Salbach, P., Kuhn, H., Rådmark, O., Samuelsson, B., Habenicht, A.J. (2003) Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. *Proc. Natl. Acad. Sci. U S A* **100**, 1238-1243.
232. De Caterina, R., Mazzone, A., Giannessi, D., Sicari, R., Pelosi, W., Lazzerini, G., Azzara, A., Forder, R., Carey, F., Caruso, D., et al. (1988) Leukotriene B4 production in human atherosclerotic plaques. *Biomed. Biochim. Acta.* **47**, S182-185.
233. Sjöström, M., Johansson, A.S., Schröder, O., Qiu, H., Palmblad, J., Haeggström, J.Z. (2003) Dominant expression of the CysLT2 receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **23**, e37-41.
234. Lötzer, K., Funk, C.D., Habenicht, A.J. (2005) The 5-lipoxygenase pathway in arterial wall biology and atherosclerosis. *Biochim. Biophys. Acta* **1736**, 30-37.
235. Allen, S., Dashwood, M., Morrison, K., Yacoub, M. (1998) Differential leukotriene constrictor responses in human atherosclerotic coronary arteries. *Circulation* **97**, 2406-2413.
236. Allen, S.P., Dashwood, M.R., Chester, A.H., Tadjkarimi, S., Collins, M., Piper, P.J., Yacoub, M.H. (1993) Influence of atherosclerosis on the vascular reactivity of isolated human epicardial coronary arteries to leukotriene C4. *Cardioscience* **4**, 47-54.
237. Aiello, R.J., Bourassa, P.A., Lindsey, S., Weng, W., Freeman, A., Showell, H.J. (2002) Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice. *Arterioscler. Thromb. Vasc. Biol.* **22**, 443-449.
238. Subbarao, K., Jala, V.R., Mathis, S., Suttles, J., Zacharias, W., Ahamed, J., Ali, H., Tseng, M.T., Haribabu, B. (2004) Role of leukotriene B4 receptors in the development of atherosclerosis: potential mechanisms. *Arterioscler. Thromb. Vasc. Biol.* **24**, 369-375.
239. Helgadottir, A., Manolescu, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorsteinsdottir, U., Samani, N.J., Gudmundsson, G., Grant, S.F., Thorgeirsson, G., Sveinbjornsdottir, S., Valdimarsson, E.M., Matthiasson, S.E., Johannsson, H., Gudmundsdottir, O., Gurney, M.E., Sainz, J., Thorhallsdottir, M., Andresdottir, M., Frigge, M.L., Topol, E.J., Kong, A., Gudnason, V., Hakonarson, H., Gulcher, J.R., Stefansson, K. (2004) The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.* **36**, 233-239.
240. Mehrabian, M., Allayee, H., Wong, J., Shi, W., Wang, X.P., Shaposhnik, Z., Funk, C.D., Lusis, A.J. (2002) Identification of 5-lipoxygenase as a major gene contributing to atherosclerosis susceptibility in mice. *Circ. Res.* **91**, 120-126.
241. Zhao, L., Moos, M.P., Gräbner, R., Pédrono, F., Fan, J., Kaiser, B., John, N., Schmidt, S., Spanbroek, R., Lötzer, K., Huang, L., Cui, J., Rader, D.J., Evans, J.F., Habenicht, A.J., Funk, C.D. (2004) The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. *Nat. Med.* **10**, 966-973.
242. Dwyer, J.H., Allayee, H., Dwyer, K.M., Fan, J., Wu, H., Mar, R., Lusis, A.J., Mehrabian, M. (2004) Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis. *N. Engl. J. Med.* **350**, 29-37.
243. Helgadottir, A., Gretarsdottir, S., St Clair, D., Manolescu, A., Cheung, J., Thorleifsson, G., Pasdar, A., Grant, S.F., Whalley, L.J., Hakonarson, H.,

Thorsteinsdottir, U., Kong, A., Gulcher, J., Stefansson, K., MacLeod, M.J. (2005) Association between the gene encoding 5-lipoxygenase-activating protein and stroke replicated in a Scottish population. *Am. J. Hum. Genet.* **76**, 505-509.

244. Helgadottir, A., Manolescu, A., Helgason, A., Thorleifsson, G., Thorsteinsdottir, U., Gudbjartsson, D.F., Gretarsdottir, S., Magnusson, K.P., Gudmundsson, G., Hicks, A., Jonsson, T., Grant, S.F., Sainz, J., O'Brien, S.J., Sveinbjornsdottir, S., Valdimarsson, E.M., Matthiasson, S.E., Levey, A.I., Abramson, J.L., Reilly, M.P., Vaccarino, V., Wolfe, M.L., Gudnason, V., Quyyumi, A.A., Topol, E.J., Rader, D.J., Thorgeirsson, G., Gulcher, J.R., Hakonarson, H., Kong, A., Stefansson, K. (2006) A variant of the gene encoding leukotriene A4 hydrolase confers ethnicity-specific risk of myocardial infarction. *Nat. Genet.* **38**, 68-74.
245. Hakonarson, H., Thorvaldsson, S., Helgadottir, A., Gudbjartsson, D., Zink, F., Andresdottir, M., Manolescu, A., Arnar, D.O., Andersen, K., Sigurdsson, A., Thorgeirsson, G., Jonsson, A., Agnarsson, U., Bjornsdottir, H., Gottskalksson, G., Einarsson, A., Gudmundsdottir, H., Adalsteinsdottir, A.E., Gudmundsson, K., Kristjansson, K., Hardarson, T., Kristinsson, A., Topol, E.J., Gulcher, J., Kong, A., Gurney, M., Stefansson, K. (2005) Effects of a 5-lipoxygenase-activating protein inhibitor on biomarkers associated with risk of myocardial infarction: a randomized trial. *JAMA* **293**, 2245-2256.
246. Chen, Y., Hu, Y., Zhang, H., Peng, C., Li, S. (2009) Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. *Nat. Genet.* **41**, 783-792.
247. Peters-Golden, M., Canetti, C., Mancuso, P., Coffey, M.J. (2005) Leukotrienes: underappreciated mediators of innate immune responses. *J. Immunol.* **174**, 589-594.
248. Claesson, H.E., Lindgren, J.A., Gustafsson, B. (1985) Opsonized bacteria stimulate leukotriene synthesis in human leukocytes. *Biochim. Biophys. Acta* **836**, 361-367.
249. Coffey, M.J., Phare, S.M., Peters-Golden, M. (2004) Role of leukotrienes in killing of Mycobacterium bovis by neutrophils. *Prostaglandins Leukot. Essent. Fatty Acids* **71**, 185-190.
250. Locksley, R.M., Fankhauser, J., Henderson, W.R. (1985) Alteration of leukotriene release by macrophages ingesting Toxoplasma gondii. *Proc. Natl. Acad. Sci. U S A* **82**, 6922-6926.
251. Castro, M., Morgenthaler, T.I., Hoffman, O.A., Standing, J.E., Rohrbach, M.S., Limper, A.H. (1993) Pneumocystis carinii induces the release of arachidonic acid and its metabolites from alveolar macrophages. *Am. J. Respir. Cell. Mol. Biol.* **9**, 73-81.
252. Wolf, J.E., Massof, S.E., Peters, S.P. (1992) Alterations in murine macrophage arachidonic acid metabolism following ingestion of nonviable Histoplasma capsulatum. *Infect. Immun.* **60**, 2559-2564.
253. Hennet, T., Ziltener, H.J., Frei, K., Peterhans, E. (1992) A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. *J. Immunol.* **149**, 932-939.
254. Gosselin, J., Borgeat, P. (1997) Epstein-Barr virus modulates 5-lipoxygenase product synthesis in human peripheral blood mononuclear cells. *Blood* **89**, 2122-2130.
255. Hopkins, H., Stull, T., Von Essen, S.G., Robbins, R.A., Rennard, S.I. (1989) Neutrophil chemotactic factors in bacterial pneumonia. *Chest* **95**, 1021-1027.
256. Volovitz, B., Welliver, R.C., De Castro, G., Krystofik, D.A., Ogra, P.L. (1988) The release of leukotrienes in the respiratory tract during infection with respiratory syncytial virus: role in obstructive airway disease. *Pediatr. Res.* **24**, 504-507.
257. Qadri, F., Raqib, R., Ahmed, F., Rahman, T., Wenneras, C., Das, S.K., Alam, N.H., Mathan, M.M., Svennerholm, A.M. (2002) Increased levels of inflammatory mediators in children and adults infected with Vibrio cholerae O1 and O139. *Clin. Diagn. Lab. Immunol.* **9**, 221-229.

258. Kasirga, E., Coker, I., Aydogdu, S., Yağci, R.V., Taneli, B., Gousseinov, A. (1999) Increased gastric juice leukotriene B4, C4 and E4 concentrations in children with Helicobacter pylori colonization. *Turk. J. Pediatr.* **41**, 335-339.
259. Gentile, D.A., Fireman, P., Skoner, D.P. (2003) Elevations of local leukotriene C4 levels during viral upper respiratory tract infections. *Ann. Allergy Asthma Immunol.* **91**, 270-274.
260. Bailie, M.B., Standiford, T.J., Laichalk, L.L., Coffey, M.J., Strieter, R., Peters-Golden, M. (1996) Leukotriene-deficient mice manifest enhanced lethality from Klebsiella pneumonia in association with decreased alveolar macrophage phagocytic and bactericidal activities. *J. Immunol.* **157**, 5221-5224.
261. Benjamim, C.F., Canetti, C., Cunha, F.Q., Kunkel, S.L., Peters-Golden, M. (2005) Opposing and hierarchical roles of leukotrienes in local innate immune versus vascular responses in a model of sepsis. *J. Immunol.* **174**, 1616-1620.
262. Malaviya, R., Abraham, S.N. (2000) Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. *J. Leukoc. Biol.* **67**, 841-846.
263. Gosselin, J., Borgeat, P., Flamand, L. (2005) Leukotriene B4 protects latently infected mice against murine cytomegalovirus reactivation following allogeneic transplantation. *J. Immunol.* **174**, 1587-1593.
264. Matsukawa, A., Hogaboam, C.M., Lukacs, N.W., Lincoln, P.M., Strieter, R.M., Kunkel, S.L. (1999) Endogenous monocyte chemoattractant protein-1 (MCP-1) protects mice in a model of acute septic peritonitis: cross-talk between MCP-1 and leukotriene B4. *J. Immunol.* **163**, 6148-6154.
265. Zimmerman, B.J., Holt, J.W., Paulson, J.C., Anderson, D.C., Miyasaka, M., Tamatani, T., Todd, R.F., 3rd, Rusche, J.R., Granger, D.N. (1994) Molecular determinants of lipid mediator-induced leukocyte adherence and emigration in rat mesenteric venules. *Am. J. Physiol.* **266**, H847-H853.
266. Maekawa, A., Austen, K.F., Kanaoka, Y. (2002) Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. *J. Biol. Chem.* **277**, 20820-20824.
267. Robbiani, D.F., Finch, R.A., Jäger, D., Muller, W.A., Sartorelli, A.C., Randolph, G.J. (2000) The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell* **103**, 757-768.
268. Tager, A.M., Bromley, S.K., Medoff, B.D., Islam, S.A., Bercury, S.D., Friedrich, E.B., Carafone, A.D., Gerszten, R.E., Luster, A.D. (2003) Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. *Nat. Immunol.* **4**, 982-990.
269. Ott, V.L., Cambier, J.C., Kappler, J., Marrack, P., Swanson, B.J. (2003) Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. *Nat. Immunol.* **4**, 974-981.
270. Prinz, I., Gregoire, C., Mollenkopf, H., Aguado, E., Wang, Y., Malissen, M., Kaufmann, S.H., Malissen, B. (2005) The type 1 cysteinyl leukotriene receptor triggers calcium influx and chemotaxis in mouse alpha beta- and gamma delta effector T cells. *J. Immunol.* **175**, 713-719.
271. Parameswaran, K., Liang, H., Fanat, A., Watson, R., Snider, D.P., O'Byrne, P.M. (2004) Role for cysteinyl leukotrienes in allergen-induced change in circulating dendritic cell number in asthma. *J. Allergy Clin. Immunol.* **114**, 73-79.
272. Demitsu, T., Katayama, H., Saito-Taki, T., Yaoita, H., Nakano, M. (1989) Phagocytosis and bactericidal action of mouse peritoneal macrophages treated with leukotriene B4. *Int. J. Immunopharmacol.* **11**, 801-808.

273. Mancuso, P., Peters-Golden, M. (2000) Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor-mediated and protein kinase C-dependent. *Am. J. Respir. Cell. Mol. Biol.* **23**, 727-733.
274. Canetti, C., Hu, B., Curtis, J.L., Peters-Golden, M. (2003) Syk activation is a leukotriene B4-regulated event involved in macrophage phagocytosis of IgG-coated targets but not apoptotic cells. *Blood* **102**, 1877-1883.
275. Serhan, C.N., Radin, A., Smolen, J.E., Korchak, H., Samuelsson, B., Weissmann, G. (1982) Leukotriene B4 is a complete secretagogue in human neutrophils: a kinetic analysis. *Biochem. Biophys. Res Commun.* **107**, 1006-1012.
276. Flamand, L., Borgeat, P., Lalonde, R., Gosselin, J. (2004) Release of anti-HIV mediators after administration of leukotriene B4 to humans. *J. Infect. Dis.* **189**, 2001-2009.
277. Lärfars, G., Lantoine, F., Devynck, M.A., Palmblad, J., Gyllenhammar, H. (1999) Activation of nitric oxide release and oxidative metabolism by leukotrienes B4, C4, and D4 in human polymorphonuclear leukocytes. *Blood* **93**, 1399-1405.
278. Talvani, A., Machado, F.S., Santana, G.C., Klein, A., Barcelos, L., Silva, J.S., Teixeira, M.M. (2002) Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. *Infect. Immun.* **70**, 4247-4253.
279. Serezani, C.H., Aronoff, D.M., Jancar, S., Mancuso, P., Peters-Golden, M. (2005) Leukotrienes enhance the bactericidal activity of alveolar macrophages against Klebsiella pneumoniae through the activation of NADPH oxidase. *Blood* **106**, 1067-1075.
280. Kumar, H., Kawai, T., Akira, S. (2009) Pathogen recognition in the innate immune response. *Biochem. J.* **420**, 1-16.
281. Kawai, T., Akira, S. (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int. Immunol.* **21**, 317-337.
282. Kumar, H., Kawai, T., Akira, S. (2009) Toll-like receptors and innate immunity. *Biochem. Biophys. Res Commun.* **388**, 621-625.
283. Kaisho, T., Akira, S. (2006) Toll-like receptor function and signaling. *J. Allergy Clin. Immunol.* **117**, 979-987; quiz 988.
284. Inohara, N., Nunez, G. (2003) NODs: intracellular proteins involved in inflammation and apoptosis. *Nat. Rev. Immunol.* **3**, 371-382.
285. Kawai, T., Akira, S. (2006) Innate immune recognition of viral infection. *Nat. Immunol.* **7**, 131-137.
286. Barton, G.M., Kagan, J.C. (2009) A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat. Rev. Immunol.* **9**, 535-542.
287. Pasare, C., Medzhitov, R. (2004) Toll-like receptors and acquired immunity. *Semin. Immunol.* **16**, 23-26.
288. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983.
289. Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397.
290. Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**, 2085-2088.
291. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are

hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* **162**, 3749-3752.
292. Akira, S., Uematsu, S., Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783-801.
293. Jin, M.S., Kim, S.E., Heo, J.Y., Lee, M.E., Kim, H.M., Paik, S.G., Lee, H., Lee, J.O. (2007) Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell* **130**, 1071-1082.
294. Liu, L., Botos, I., Wang, Y., Leonard, J.N., Shiloach, J., Segal, D.M., Davies, D.R. (2008) Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science* **320**, 379-381.
295. Kim, H.M., Park, B.S., Kim, J.I., Kim, S.E., Lee, J., Oh, S.C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O.J., Lee, J.O. (2007) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* **130**, 906-917.
296. Akira, S., Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* **4**, 499-511.
297. O'Neill, L.A., Bowie, A.G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat. Rev. Immunol.* **7**, 353-364.
298. Barton, G.M., Kagan, J.C., Medzhitov, R. (2006) Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. *Nat. Immunol.* **7**, 49-56.
299. Takeuchi, O., Sato, S., Horiuchi, T., Hoshino, K., Takeda, K., Dong, Z., Modlin, R.L., Akira, S. (2002) Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J. Immunol.* **169**, 10-14.
300. Takeuchi, O., Kawai, T., Muhlradt, P.F., Morr, M., Radolf, J.D., Zychlinsky, A., Takeda, K., Akira, S. (2001) Discrimination of bacterial lipoproteins by Toll-like receptor 6. *Int. Immunol.* **13**, 933-940.
301. Buwitt-Beckmann, U., Heine, H., Wiesmuller, K.H., Jung, G., Brock, R., Akira, S., Ulmer, A.J. (2005) Toll-like receptor 6-independent signaling by diacylated lipopeptides. *Eur. J. Immunol.* **35**, 282-289.
302. Buwitt-Beckmann, U., Heine, H., Wiesmüller, K.H., Jung, G., Brock, R., Akira, S., Ulmer, A.J. (2006) TLR1- and TLR6-independent recognition of bacterial lipopeptides. *J. Biol. Chem.* **281**, 9049-9057.
303. Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zahringer, U., Beutler, B. (2005) CD36 is a sensor of diacylglycerides. *Nature* **433**, 523-527.
304. Gantner, B.N., Simmons, R.M., Canavera, S.J., Akira, S., Underhill, D.M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* **197**, 1107-1117.
305. Morath, S., von Aulock, S., Hartung, T. (2005) Structure/function relationships of lipoteichoic acids. *J. Endotoxin Res.* **11**, 348-356.
306. Akira, S., Sato, S. (2003) Toll-like receptors and their signaling mechanisms. *Scand. J. Infect. Dis.* **35**, 555-562.
307. Farhat, K., Riekenberg, S., Heine, H., Debarry, J., Lang, R., Mages, J., Buwitt-Beckmann, U., Roschmann, K., Jung, G., Wiesmuller, K.H., Ulmer, A.J. (2008) Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J. Leukoc. Biol.* **83**, 692-701.
308. Jiang, Z., Georgel, P., Du, X., Shamel, L., Sovath, S., Mudd, S., Huber, M., Kalis, C., Keck, S., Galanos, C., Freudenberg, M., Beutler, B. (2005) CD14 is required for MyD88-independent LPS signaling. *Nat. Immunol.* **6**, 565-570.

309. Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099-1103.
310. Alexopoulou, L., Holt, A.C., Medzhitov, R., Flavell, R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738.
311. Wang, T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., Flavell, R.A. (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* **10**, 1366-1373.
312. Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., Akira, S. (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* **3**, 196-200.
313. Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., Reis e Sousa, C. (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-1531.
314. Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S. (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303**, 1526-1529.
315. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740-745.
316. Haas, T., Metzger, J., Schmitz, F., Heit, A., Muller, T., Latz, E., Wagner, H. (2008) The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation. *Immunity* **28**, 315-323.
317. Klinman, D.M. (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* **4**, 249-258.
318. Coban, C., Ishii, K.J., Kawai, T., Hemmi, H., Sato, S., Uematsu, S., Yamamoto, M., Takeuchi, O., Itagaki, S., Kumar, N., Horii, T., Akira, S. (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* **201**, 19-25.
319. Kawai, T., Akira, S. (2007) TLR signaling. *Semin. Immunol.* **19**, 24-32.
320. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., Janeway, C.A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol. Cell.* **2**, 253-258.
321. Horng, T., Barton, G.M., Medzhitov, R. (2001) TIRAP: an adapter molecule in the Toll signaling pathway. *Nat. Immunol.* **2**, 835-841.
322. Horng, T., Barton, G.M., Flavell, R.A., Medzhitov, R. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature* **420**, 329-333.
323. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., Takeda, K., Akira, S. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* **420**, 324-329.
324. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., Akira, S. (2002) Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* **169**, 6668-6672.
325. Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**, 640-643.

326. Fitzgerald, K.A., Rowe, D.C., Barnes, B.J., Caffrey, D.R., Visintin, A., Latz, E., Monks, B., Pitha, P.M., Golenbock, D.T. (2003) LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J. Exp. Med.* **198**, 1043-1055.
327. Abbas, A.K., Murphy, K.M., Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383**, 787-793.
328. Redecke, V., Hacker, H., Datta, S.K., Fermin, A., Pitha, P.M., Broide, D.H., Raz, E. (2004) Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. *J. Immunol.* **172**, 2739-2743.
329. Eisenbarth, S.C., Piggott, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A., Bottomly, K. (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196**, 1645-1651.
330. Kim, S.K., Ragupathi, G., Musselli, C., Choi, S.J., Park, Y.S., Livingston, P.O. (1999) Comparison of the effect of different immunological adjuvants on the antibody and T-cell response to immunization with MUC1-KLH and GD3-KLH conjugate cancer vaccines. *Vaccine* **18**, 597-603.
331. Miyake, K. (2007) Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin. Immunol.* **19**, 3-10.
332. Pålsson-McDermott, E.M., O'Neill, L.A. (2007) The potential of targeting Toll-like receptor 2 in autoimmune and inflammatory diseases. *Ir. J. Med Sci.* **176**, 253-260.
333. Erridge, C. Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J. Leukoc. Biol.*
334. Seibl, R., Birchler, T., Loeliger, S., Hossle, J.P., Gay, R.E., Saurenmann, T., Michel, B.A., Seger, R.A., Gay, S., Lauener, R.P. (2003) Expression and regulation of Toll-like receptor 2 in rheumatoid arthritis synovium. *Am. J. Pathol.* **162**, 1221-1227.
335. Pierer, M., Rethage, J., Seibl, R., Lauener, R., Brentano, F., Wagner, U., Hantzschel, H., Michel, B.A., Gay, R.E., Gay, S., Kyburz, D. (2004) Chemokine secretion of rheumatoid arthritis synovial fibroblasts stimulated by Toll-like receptor 2 ligands. *J. Immunol.* **172**, 1256-1265.
336. Lee, E.Y., Yim, J.J., Lee, H.S., Lee, Y.J., Lee, E.B., Song, Y.W. (2006) Dinucleotide repeat polymorphism in intron II of human Toll-like receptor 2 gene and susceptibility to rheumatoid arthritis. *Int. J. Immunogenet.* **33**, 211-215.
337. Wang, F., Tahara, T., Arisawa, T., Shibata, T., Nakamura, M., Fujita, H., Iwata, M., Kamiya, Y., Nagasaka, M., Takahama, K., Watanabe, M., Hirata, I., Nakano, H. (2007) Genetic polymorphisms of CD14 and Toll-like receptor-2 (TLR2) in patients with ulcerative colitis. *J. Gastroenterol. Hepatol.* **22**, 925-929.
338. Baker, B.S., Ovigne, J.M., Powles, A.V., Corcoran, S., Fry, L. (2003) Normal keratinocytes express Toll-like receptors (TLRs) 1, 2 and 5: modulation of TLR expression in chronic plaque psoriasis. *Br. J. Dermatol.* **148**, 670-679.
339. Edfeldt, K., Swedenborg, J., Hansson, G.K., Yan, Z.Q. (2002) Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* **105**, 1158-1161.
340. Schoneveld, A.H., Hoefer, I., Sluijter, J.P., Laman, J.D., de Kleijn, D.P., Pasterkamp, G. (2008) Atherosclerotic lesion development and Toll like receptor 2 and 4 responsiveness. *Atherosclerosis* **197**, 95-104.
341. Liu, X., Ukai, T., Yumoto, H., Davey, M., Goswami, S., Gibson, F.C., 3rd, Genco, C.A. (2008) Toll-like receptor 2 plays a critical role in the progression of atherosclerosis that is independent of dietary lipids. *Atherosclerosis* **196**, 146-154.
342. Mullick, A.E., Tobias, P.S., Curtiss, L.K. (2005) Modulation of atherosclerosis in mice by Toll-like receptor 2. *J. Clin. Invest.* **115**, 3149-3156.

343. Lepper, P.M., von Eynatten, M., Humpert, P.M., Triantafilou, M., Triantafilou, K. (2007) Toll-like receptor polymorphisms and carotid artery intima-media thickness. *Stroke* **38**, e50.
344. Labrum, R., Bevan, S., Sitzer, M., Lorenz, M., Markus, H.S. (2007) Toll receptor polymorphisms and carotid artery intima-media thickness. *Stroke* **38**, 1179-1184.
345. Eder, W., Klimecki, W., Yu, L., von Mutius, E., Riedler, J., Braun-Fahrlander, C., Nowak, D., Martinez, F.D. (2004) Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J. Allergy Clin. Immunol.* **113**, 482-488.
346. Kormann, M.S., Depner, M., Hartl, D., Klopp, N., Illig, T., Adamski, J., Vogelberg, C., Weiland, S.K., von Mutius, E., Kabesch, M. (2008) Toll-like receptor heterodimer variants protect from childhood asthma. *J. Allergy Clin. Immunol.* **122**, 86-92, 92 e1-8.
347. Leemans, J.C., Stokman, G., Claessen, N., Rouschop, K.M., Teske, G.J., Kirschning, C.J., Akira, S., van der Poll, T., Weening, J.J., Florquin, S. (2005) Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J. Clin. Invest.* **115**, 2894-2903.
348. Shigeoka, A.A., Holscher, T.D., King, A.J., Hall, F.W., Kiosses, W.B., Tobias, P.S., Mackman, N., McKay, D.B. (2007) TLR2 is constitutively expressed within the kidney and participates in ischemic renal injury through both MyD88-dependent and -independent pathways. *J. Immunol.* **178**, 6252-6258.
349. Favre, J., Musette, P., Douin-Echinard, V., Laude, K., Henry, J.P., Arnal, J.F., Thuillez, C., Richard, V. (2007) Toll-like receptors 2-deficient mice are protected against postischemic coronary endothelial dysfunction. *Arterioscler. Thromb. Vasc. Biol.* **27**, 1064-1071.
350. Hugues, S., Mougneau, E., Ferlin, W., Jeske, D., Hofman, P., Homann, D., Beaudoin, L., Schrike, C., Von Herrath, M., Lehuen, A., Glaichenhaus, N. (2002) Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. *Immunity* **16**, 169-181.
351. Boraska Jelavic, T., Barisic, M., Drmic Hofman, I., Boraska, V., Vrdoljak, E., Peruzovic, M., Hozo, I., Puljiz, Z., Terzic, J. (2006) Microsatelite GT polymorphism in the toll-like receptor 2 is associated with colorectal cancer. *Clin. Genet.* **70**, 156-160.
352. Yang, H.Z., Cui, B., Liu, H.Z., Mi, S., Yan, J., Yan, H.M., Hua, F., Lin, H., Cai, W.F., Xie, W.J., Lv, X.X., Wang, X.X., Xin, B.M., Zhan, Q.M., Hu, Z.W. (2009) Blocking TLR2 activity attenuates pulmonary metastases of tumor. *PLoS One* **4**, e6520.
353. Curtin, J.F., Liu, N., Candolfi, M., Xiong, W., Assi, H., Yagiz, K., Edwards, M.R., Michelsen, K.S., Kroeger, K.M., Liu, C., Muhammad, A.K., Clark, M.C., Arditi, M., Comin-Anduix, B., Ribas, A., Lowenstein, P.R., Castro, M.G. (2009) HMGB1 mediates endogenous TLR2 activation and brain tumor regression. *PLoS Med* **6**, e10.
354. Akira, S. (2009) Pathogen recognition by innate immunity and its signaling. *Proc. Jpn. Acad. Ser. B. Phys Biol. Sci.* **85**, 143-156.
355. Meng, G., Rutz, M., Schiemann, M., Metzger, J., Grabiec, A., Schwandner, R., Luppa, P.B., Ebel, F., Busch, D.H., Bauer, S., Wagner, H., Kirschning, C.J. (2004) Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. *J. Clin. Invest.* **113**, 1473-1481.
356. Ingale, S., Wolfert, M.A., Gaekwad, J., Buskas, T., Boons, G.J. (2007) Robust immune responses elicited by a fully synthetic three-component vaccine. *Nat. Chem. Biol.* **3**, 663-667.
357. Schmidt, J., Welsch, T., Jager, D., Muhlradt, P.F., Buchler, M.W., Marten, A. (2007) Intratumoural injection of the toll-like receptor-2/6 agonist 'macrophage-activating lipopeptide-2' in patients with pancreatic carcinoma: a phase I/II trial. *Br. J. Cancer* **97**, 598-604.

358. Weigt, H., Nassenstein, C., Tschernig, T., Muhlradt, P.F., Krug, N., Braun, A. (2005) Efficacy of macrophage-activating lipopeptide-2 combined with interferon-gamma in a murine asthma model. *Am. J. Respir. Crit. Care Med.* **172**, 566-572.
359. Fuchs, B., Knothe, S., Rochlitzer, S., Nassimi, M., Greweling, M., Lauenstein, H.D., Nassenstein, C., Muller, M., Ebensen, T., Dittrich, A.M., Krug, N., Guzman, C.A., Braun, A. (2009) A Toll-Like Receptor 2/6 Agonist Reduces Allergic Airway Inflammation in Chronic Respiratory Sensitisation to Timothy Grass Pollen Antigens. *Int. Arch. Allergy Immunol.* **152**, 131-139.
360. Lührmann, A., Tschernig, T., Pabst, R., Niewiesk, S. (2005) Improved intranasal immunization with live-attenuated measles virus after co-inoculation of the lipopeptide MALP-2. *Vaccine* **23**, 4721-4726.
361. Deiters, U., Barsig, J., Tawil, B., Muhlradt, P.F. (2004) The macrophage-activating lipopeptide-2 accelerates wound healing in diabetic mice. *Exp. Dermatol.* **13**, 731-739.
362. McCurdy, J.D., Olynych, T.J., Maher, L.H., Marshall, J.S. (2003) Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *J. Immunol.* **170**, 1625-1629.
363. Bieneman, A.P., Chichester, K.L., Chen, Y.H., Schroeder, J.T. (2005) Toll-like receptor 2 ligands activate human basophils for both IgE-dependent and IgE-independent secretion. *J. Allergy Clin. Immunol.* **115**, 295-301.
364. Doerfler, M.E., Danner, R.L., Shelhamer, J.H., Parrillo, J.E. (1989) Bacterial lipopolysaccharides prime human neutrophils for enhanced production of leukotriene B4. *J. Clin. Invest.* **83**, 970-977.
365. Surette, M.E., Palmantier, R., Gosselin, J., Borgeat, P. (1993) Lipopolysaccharides prime whole human blood and isolated neutrophils for the increased synthesis of 5-lipoxygenase products by enhancing arachidonic acid availability: involvement of the CD14 antigen. *J. Exp. Med.* **178**, 1347-1355.
366. Surette, M.E., Nadeau, M., Borgeat, P., Gosselin, J. (1996) Priming of human peripheral blood mononuclear cells with lipopolysaccharides for enhanced arachidonic acid release and leukotriene synthesis. *J. Leukoc. Biol.* **59**, 709-715.
367. Surette, M.E., Dallaire, N., Jean, N., Picard, S., Borgeat, P. (1998) Mechanisms of the priming effect of lipopolysaccharides on the biosynthesis of leukotriene B4 in chemotactic peptide-stimulated human neutrophils. *FASEB J.* **12**, 1521-1531.
368. Kantari, C., Pederzoli-Ribeil, M., Witko-Sarsat, V. (2008) The role of neutrophils and monocytes in innate immunity. *Contrib. Microbiol.* **15**, 118-146.
369. Ziegler-Heitbrock, H.W., Thiel, E., Fütterer, A., Herzog, V., Wirtz, A., Riethmuller, G. (1988) Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer* **41**, 456-461.
370. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K. (1980) Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* **26**, 171-176.
371. Steinhilber, D., Herrmann, T., Roth, H.J. (1989) Separation of lipoxins and leukotrienes from human granulocytes by high-performance liquid chromatography with a Radial-Pak cartridge after extraction with an octadecyl reversed-phase column. *J. Chromatogr.* **493**, 361-366.
372. Grynkiewicz, G., Poenie, M., Tsien, R.Y. (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.
373. Werz, O., Klemm, J., Samuelsson, B., Rådmark, O. (2001) Phorbol ester up-regulates capacities for nuclear translocation and phosphorylation of 5-lipoxygenase in Mono Mac 6 cells and human polymorphonuclear leukocytes. *Blood* **97**, 2487-2495.
374. Haeggström, J.Z., Wetterholm, A. (2002) Enzymes and receptors in the leukotriene cascade. *Cell. Mol. Life Sci.* **59**, 742-753.

375. McColl, S.R., Krump, E., Naccache, P.H., Poubelle, P.E., Braquet, P., Braquet, M., Borgeat, P. (1991) Granulocyte-macrophage colony-stimulating factor increases the synthesis of leukotriene B4 by human neutrophils in response to platelet-activating factor. Enhancement of both arachidonic acid availability and 5-lipoxygenase activation. *J. Immunol.* **146**, 1204-1211.
376. DiPersio, J.F., Billing, P., Williams, R., Gasson, J.C. (1988) Human granulocyte-macrophage colony-stimulating factor and other cytokines prime human neutrophils for enhanced arachidonic acid release and leukotriene B4 synthesis. *J. Immunol.* **140**, 4315-4322.
377. Dahinden, C.A., Zingg, J., Maly, F.E., de Weck, A.L. (1988) Leukotriene production in human neutrophils primed by recombinant human granulocyte/macrophage colony-stimulating factor and stimulated with the complement component C5A and FMLP as second signals. *J. Exp. Med.* **167**, 1281-1295.
378. Schatz-Munding, M., Ullrich, V. (1992) Priming of human polymorphonuclear leukocytes with granulocyte-macrophage colony-stimulating factor involves protein kinase C rather than enhanced calcium mobilisation. *Eur. J. Biochem.* **204**, 705-712.
379. McDonald, P.P., Pouliot, M., Borgeat, P. (1993) Enhancement by GM-CSF of agonist-induced 5-lipoxygenase activation in human neutrophils involves protein synthesis and gene transcription. *J. Lipid Mediat.* **6**, 59-67.
380. Palmantier, R., Surette, M.E., Sanchez, A., Braquet, P., Borgeat, P. (1994) Priming for the synthesis of 5-lipoxygenase products in human blood ex vivo by human granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. *Lab. Invest.* **70**, 696-704.
381. Ring, W.L., Riddick, C.A., Baker, J.R., Munafo, D.A., Bigby, T.D. (1996) Lymphocytes stimulate expression of 5-lipoxygenase and its activating protein in monocytes in vitro via granulocyte macrophage colony-stimulating factor and interleukin 3. *J. Clin. Invest.* **97**, 1293-1301.
382. Nagata, M., Sedgwick, J.B., Busse, W.W. (1995) Differential effects of granulocyte-macrophage colony-stimulating factor on eosinophil and neutrophil superoxide anion generation. *J. Immunol.* **155**, 4948-4954.
383. Bauldry, S.A., McCall, C.E., Cousart, S.L., Bass, D.A. (1991) Tumor necrosis factor-alpha priming of phospholipase A2 activation in human neutrophils. An alternative mechanism of priming. *J. Immunol.* **146**, 1277-1285.
384. Steadman, R., Petersen, M.M., Williams, J.D. (1996) CD11b/CD18-dependent stimulation of leukotriene B4 synthesis by human neutrophils (PMN) is synergistically enhanced by tumour necrosis factor alpha and low dose diacylglycerol. *Int. J. Biochem. Cell Biol.* **28**, 771-776.
385. Matsumoto, S., Hamasaki, Y., Ichimaru, T., Miyazaki, S. (1995) IL-3 and IL-5 enhance the production of LTB4 stimulated by calcium ionophore in rat basophilic leukemia cells. *Prostaglandins Leukot. Essent. Fatty Acids* **52**, 417-422.
386. Bates, M.E., Green, V.L., Bertics, P.J. (2000) ERK1 and ERK2 activation by chemotactic factors in human eosinophils is interleukin 5-dependent and contributes to leukotriene C(4) biosynthesis. *J. Biol. Chem.* **275**, 10968-10975.
387. Bandeira-Melo, C., Phoofolo, M., Weller, P.F. (2001) Extranuclear lipid bodies, elicited by CCR3-mediated signaling pathways, are the sites of chemokine-enhanced leukotriene C4 production in eosinophils and basophils. *J. Biol. Chem.* **276**, 22779-22787.
388. Bandeira-Melo, C., Sugiyama, K., Woods, L.J., Phoofolo, M., Center, D.M., Cruikshank, W.W., Weller, P.F. (2002) IL-16 promotes leukotriene C(4) and IL-4 release from human eosinophils via CD4- and autocrine CCR3-chemokine-mediated signaling. *J. Immunol.* **168**, 4756-4763.

389. Bauldry, S.A., Wykle, R.L., Bass, D.A. (1988) Phospholipase A2 activation in human neutrophils. Differential actions of diacylglycerols and alkylacylglycerols in priming cells for stimulation by N-formyl-Met-Leu-Phe. *J. Biol. Chem.* **263**, 16787-16795.
390. Rosenthal, M.D., Franson, R.C. (1994) Separation of agonist-stimulated arachidonate mobilization from subsequent leukotriene B4 synthesis in human neutrophils: different effects of oleoylacetylglycerol and phorbol myristate acetate as priming agents. *J. Cell. Physiol.* **160**, 522-530.
391. Liles, W.C., Meier, K.E., Henderson, W.R. (1987) Phorbol myristate acetate and the calcium ionophore A23187 synergistically induce release of LTB4 by human neutrophils: involvement of protein kinase C activation in regulation of the 5-lipoxygenase pathway. *J. Immunol.* **138**, 3396-3402.
392. Peters-Golden, M., McNish, R.W., Sporn, P.H., Balazovich, K. (1991) Basal activation of protein kinase C in rat alveolar macrophages: implications for arachidonate metabolism. *Am. J. Physiol.* **261**, L462-471.
393. McIntyre, T.M., Reinhold, S.L., Prescott, S.M., Zimmerman, G.A. (1987) Protein kinase C activity appears to be required for the synthesis of platelet-activating factor and leukotriene B4 by human neutrophils. *J. Biol. Chem.* **262**, 15370-15376.
394. Bozza, P.T., Payne, J.L., Goulet, J.L., Weller, P.F. (1996) Mechanisms of platelet-activating factor-induced lipid body formation: requisite roles for 5-lipoxygenase and de novo protein synthesis in the compartmentalization of neutrophil lipids. *J. Exp. Med.* **183**, 1515-1525.
395. Aderem, A.A., Cohen, D.S., Wright, S.D., Cohn, Z.A. (1986) Bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites. *J. Exp. Med.* **164**, 165-179.
396. Hattermann, K., Picard, S., Borgeat, M., Leclerc, P., Pouliot, M., Borgeat, P. (2007) The Toll-like receptor 7/8-ligand resiquimod (R-848) primes human neutrophils for leukotriene B4, prostaglandin E2 and platelet-activating factor biosynthesis. *FASEB J.* **21**, 1575-1585.
397. Buczynski, M.W., Stephens, D.L., Bowers-Gentry, R.C., Grkovich, A., Deems, R.A., Dennis, E.A. (2007) TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. *J. Biol. Chem.* **282**, 22834-22847.
398. Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdörfer, B., Giese, T., Endres, S., Hartmann, G. (2002) Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* **168**, 4531-4537.
399. Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L.P., Allavena, P., Mantovani, A. (2000) Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* **164**, 5998-6004.
400. Hauschildt, S., Lückhoff, A., Langhorne, J., Wiesmüller, K.H., Jung, G., Bessler, W., Cambier, J.C. (1991) Increase in the intracellular free calcium concentration is not an obligatory early event in lipopeptide-induced B-cell activation. *Immunology* **73**, 366-368.
401. Seifert, R., Schultz, G., Richter-Freund, M., Metzger, J., Wiesmüller, K.H., Jung, G., Bessler, W.G., Hauschildt, S. (1990) Activation of superoxide formation and lysozyme release in human neutrophils by the synthetic lipopeptide Pam3Cys-Ser-(Lys)4. Involvement of guanine-nucleotide-binding proteins and synergism with chemotactic peptides. *Biochem. J.* **267**, 795-802.

402. Jakobsson, P.J., Shaskin, P., Larsson, P., Feltenmark, S., Odlander, B., Aguilar-Santelises, M., Jondal, M., Biberfeld, P., Claesson, H.E. (1995) Studies on the regulation and localization of 5-lipoxygenase in human B-lymphocytes. *Eur. J. Biochem.* **232**, 37-46.
403. Elsner, J., Kaever, V., Emmendörffer, A., Breidenbach, T., Lohmann-Matthes, M.L., Roesler, J. (1992) Heterogeneity in the mobilization of cytoplasmic calcium by human polymorphonuclear leukocytes in response to fMLP, C5a and IL-8/NAP-1. *J. Leukoc. Biol.* **51**, 77-83.
404. Sala, A., Zarini, S., Folco, G., Murphy, R.C., Henson, P.M. (1999) Differential metabolism of exogenous and endogenous arachidonic acid in human neutrophils. *J. Biol. Chem.* **274**, 28264-28269.
405. Albert, D., Buerkert, E., Steinhilber, D., Werz, O. (2003) Induction of 5-lipoxygenase activation in polymorphonuclear leukocytes by 1-oleoyl-2-acetylglycerol. *Biochim. Biophys. Acta* **1631**, 85-93.
406. Bozza, P.T., Yu, W., Penrose, J.F., Morgan, E.S., Dvorak, A.M., Weller, P.F. (1997) Eosinophil lipid bodies: specific, inducible intracellular sites for enhanced eicosanoid formation. *J. Exp. Med.* **186**, 909-920.
407. Sorgi, C.A., Secatto, A., Fontanari, C., Turato, W.M., Belangér, C., de Medeiros, A.I., Kashima, S., Marleau, S., Covas, D.T., Bozza, P.T., Faccioli, L.H. (2009) Histoplasma capsulatum cell wall {beta}-glucan induces lipid body formation through CD18, TLR2, and dectin-1 receptors: correlation with leukotriene B4 generation and role in HIV-1 infection. *J. Immunol.* **182**, 4025-4035.
408. Mattos, K.A., D'Avila, H., Rodrigues, L.S., Oliveira, V.G., Sarno, E.N., Atella, G.C., Pereira, G.M., Bozza, P.T., Pessolani, M.C. (2009) Lipid droplet formation in leprosy: Toll-like receptor-regulated organelles involved in eicosanoid formation and Mycobacterium leprae pathogenesis. *J. Leukoc. Biol.*
409. Rådmark, O., Shimizu, T., Jornvall, H., Samuelsson, B. (1984) Leukotriene A4 hydrolase in human leukocytes. Purification and properties. *J. Biol. Chem.* **259**, 12339-12345.
410. Evans, J.F., Dupuis, P., Ford-Hutchinson, A.W. (1985) Purification and characterisation of leukotriene A4 hydrolase from rat neutrophils. *Biochim. Biophys. Acta* **840**, 43-50.
411. Brock, T.G., Maydanski, E., McNish, R.W., Peters-Golden, M. (2001) Co-localization of leukotriene a4 hydrolase with 5-lipoxygenase in nuclei of alveolar macrophages and rat basophilic leukemia cells but not neutrophils. *J. Biol. Chem.* **276**, 35071-35077.
412. Ron, D., Kazanietz, M.G. (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* **13**, 1658-1676.
413. Sadeghi, K., Berger, A., Langgartner, M., Prusa, A.R., Hayde, M., Herkner, K., Pollak, A., Spittler, A., Förster-Waldl, E. (2007) Immaturity of infection control in preterm and term newborns is associated with impaired toll-like receptor signaling. *J. Infect. Dis.* **195**, 296-302.
414. Pinheiro da Silva, F., Gallo, R.L., Nizet, V. (2009) Differing effects of exogenous or endogenous cathelicidin on macrophage toll-like receptor signaling. *Immunol. Cell. Biol.* **87**, 496-500.
415. Lin, C.H., Kuan, I.H., Lee, H.M., Lee, W.S., Sheu, J.R., Ho, Y.S., Wang, C.H., Kuo, H.P. (2001) Induction of cyclooxygenase-2 protein by lipoteichoic acid from Staphylococcus aureus in human pulmonary epithelial cells: involvement of a nuclear factor-kappa B-dependent pathway. *Br. J. Pharmacol.* **134**, 543-552.
416. Kuo, C.T., Chiang, L.L., Lee, C.N., Yu, M.C., Bai, K.J., Lee, H.M., Lee, W.S., Sheu, J.R., Lin, C.H. (2003) Induction of nitric oxide synthase in RAW 264.7 macrophages

by lipoteichoic acid from Staphylococcus aureus: involvement of protein kinase C- and nuclear factor-kB-dependent mechanisms. *J. Biomed. Sci.* **10**, 136-145.
417. Lee, C.W., Chien, C.S., Yang, C.M. (2004) Lipoteichoic acid-stimulated p42/p44 MAPK activation via Toll-like receptor 2 in tracheal smooth muscle cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **286**, L921-930.
418. Chang, Y.C., Li, P.C., Chen, B.C., Chang, M.S., Wang, J.L., Chiu, W.T., Lin, C.H. (2006) Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E2, protein kinase A, p38 MAPK, and nuclear factor-kappaB pathways. *Cell. Signal.* **18**, 1235-1243.
419. Baillie, G., MacKenzie, S.J., Houslay, M.D. (2001) Phorbol 12-myristate 13-acetate triggers the protein kinase A-mediated phosphorylation and activation of the PDE4D5 cAMP phosphodiesterase in human aortic smooth muscle cells through a route involving extracellular signal regulated kinase (ERK). *Mol. Pharmacol.* **60**, 1100-1111.
420. Balsinde, J., Balboa, M.A., Yedgar, S., Dennis, E.A. (2000) Group V phospholipase A(2)-mediated oleic acid mobilization in lipopolysaccharide-stimulated P388D(1) macrophages. *J. Biol. Chem.* **275**, 4783-4786.
421. Akiba, S., Yoneda, Y., Ohno, S., Nemoto, M., Sato, T. (2003) Oxidized LDL activates phospholipase A2 to supply fatty acids required for cholesterol esterification. *J. Lipid. Res.* **44**, 1676-1685.
422. Bürkert, E., Rådmark, O., Steinhilber, D., Werz, O. (2002) Monocyte-derived soluble protein confers 5-lipoxygenase activity Ca2+-dependent. *Biochem. Biophys. Res. Commun.* **295**, 985-991.
423. Yang, C.M., Sung, T.C., Ong, R., Hsieh, J.T., Luo, S.F. (1994) Effect of phorbol ester on phosphoinositide hydrolysis and calcium mobilization in cultured canine tracheal smooth muscle cells. *Naunyn. Schmiedebergs Arch. Pharmacol.* **350**, 77-83.
424. Yang, C.M., Ong, R., Chen, Y.C., Hsieh, J.T., Tsao, H.L., Tsai, C.T. (1995) Effect of phorbol ester on phosphoinositide hydrolysis and calcium mobilization induced by endothelin-1 in cultured canine tracheal smooth muscle cells. *Cell. Calcium* **17**, 129-140.
425. Luo, S.F., Tsao, H.L., Ong, R., Hsieh, J.T., Yang, C.M. (1995) Inhibitory effect of phorbol ester on bradykinin-induced phosphoinositide hydrolysis and calcium mobilization in cultured canine tracheal smooth muscle cells. *Cell Signal.* **7**, 571-581.
426. Ishikawa, S., Saito, T. (1991) Inhibition by phorbol ester of cellular adenosine 3',5'-monophosphate production and cellular free calcium mobilization in response to arginine vasopressin in rat renal papillary collecting tubule cells in culture. *Endocrinology* **128**, 786-91.
427. Balsinde, J., Fernandez, B., Diez, E. (1990) Regulation of arachidonic acid release in mouse peritoneal macrophages. The role of extracellular calcium and protein kinase C. *J. Immunol.* **144**, 4298-4304.
428. Naccache, P.H., Molski, T.F., Borgeat, P., White, J.R., Sha'afi, R.I. (1985) Phorbol esters inhibit the fMet-Leu-Phe- and leukotriene B4-stimulated calcium mobilization and enzyme secretion in rabbit neutrophils. *J. Biol. Chem.* **260**, 2125-2131.
429. Park, S.K., Provost, J.J., Bae, C.D., Ho, W.T., Exton, J.H. (1997) Cloning and characterization of phospholipase D from rat brain. *J. Biol. Chem.* **272**, 29263-29271.
430. Hammond, S.M., Jenco, J.M., Nakashima, S., Cadwallader, K., Gu, Q., Cook, S., Nozawa, Y., Prestwich, G.D., Frohman, M.A., Morris, A.J. (1997) Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-alpha. *J. Biol. Chem.* **272**, 3860-3868.

431. Chen, J.S., Exton, J.H. (2004) Regulation of phospholipase D2 activity by protein kinase C alpha. *J. Biol. Chem.* **279**, 22076-22083.
432. Lepley, R.A., Muskardin, D.T., Fitzpatrick, F.A. (1996) Tyrosine kinase activity modulates catalysis and translocation of cellular 5-lipoxygenase. *J. Biol. Chem.* **271**, 6179-6184.
433. Glaser, K.B., Asmis, R., Dennis, E.A. (1990) Bacterial lipopolysaccharide priming of P388D1 macrophage-like cells for enhanced arachidonic acid metabolism. Platelet-activating factor receptor activation and regulation of phospholipase A2. *J. Biol. Chem.* **265**, 8658-8664.
434. Chun, J., Prince, A. (2006) Activation of Ca2+-dependent signaling by TLR2. *J. Immunol.* **177**, 1330-1337.
435. Aki, D., Minoda, Y., Yoshida, H., Watanabe, S., Yoshida, R., Takaesu, G., Chinen, T., Inaba, T., Hikida, M., Kurosaki, T., Saeki, K., Yoshimura, A. (2008) Peptidoglycan and lipopolysaccharide activate PLCgamma2, leading to enhanced cytokine production in macrophages and dendritic cells. *Genes Cells.* **13**, 199-208.
436. Supajatura, V., Ushio, H., Nakao, A., Akira, S., Okumura, K., Ra, C., Ogawa, H. (2002) Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.* **109**, 1351-1359.
437. Zhou, X., Yang, W., Li, J. (2006) Ca2+- and protein kinase C-dependent signaling pathway for nuclear factor-kappaB activation, inducible nitric-oxide synthase expression, and tumor necrosis factor-alpha production in lipopolysaccharide-stimulated rat peritoneal macrophages. *J. Biol. Chem.* **281**, 31337-31347.
438. Kim, Y., Moon, J.S., Lee, K.S., Park, S.Y., Cheong, J., Kang, H.S., Lee, H.Y., Kim, H.D. (2004) Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates the expression of iNOS through IKK and NF-kappaB activity in LPS-stimulated mouse peritoneal macrophages and RAW 264.7 cells. *Biochem. Biophys. Res. Commun.* **314**, 695-703.
439. Qiao, H., Andrade, M.V., Lisboa, F.A., Morgan, K., Beaven, M.A. (2006) FcepsilonR1 and toll-like receptors mediate synergistic signals to markedly augment production of inflammatory cytokines in murine mast cells. *Blood* **107**, 610-618.
440. Valera, I., Vigo, A.G., Alonso, S., Barbolla, L., Crespo, M.S., Fernandez, N. (2007) Peptidoglycan and mannose-based molecular patterns trigger the arachidonic acid cascade in human polymorphonuclear leukocytes. *J. Leukoc. Biol.* **81**, 925-933.
441. Grkovich, A., Armando, A., Quehenberger, O., Dennis, E.A. (2009) TLR-4 mediated group IVA phospholipase A(2) activation is phosphatidic acid phosphohydrolase 1 and protein kinase C dependent. *Biochim. Biophys. Acta* **1791**, 975-982.
442. Noor, S., Goldfine, H., Tucker, D.E., Suram, S., Lenz, L.L., Akira, S., Uematsu, S., Girotti, M., Bonventre, J.V., Breuel, K., Williams, D.L., Leslie, C.C. (2008) Activation of cytosolic phospholipase A2alpha in resident peritoneal macrophages by Listeria monocytogenes involves listeriolysin O and TLR2. *J. Biol. Chem.* **283**, 4744-4755.
443. Qi, H.Y., Shelhamer, J.H. (2005) Toll-like receptor 4 signaling regulates cytosolic phospholipase A2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. *J. Bio.l Chem.* **280**, 38969-38975.
444. Kikawada, E., Bonventre, J.V., Arm, J.P. (2007) Group V secretory PLA2 regulates TLR2-dependent eicosanoid generation in mouse mast cells through amplification of ERK and cPLA2alpha activation. *Blood* **110**, 561-567.
445. Lee, J.G., Lee, S.H., Park, D.W., Yoon, H.S., Chin, B.R., Kim, J.H., Kim, J.R., Baek, S.H. (2008) Toll-like receptor 9-stimulated monocyte chemoattractant protein-1 is mediated via JNK-cytosolic phospholipase A2-ROS signaling. *Cell. Signal.* **20**, 105-111.

446. Ruipérez, V., Astudillo, A.M., Balboa, M.A., Balsinde, J. (2009) Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A2s. *J. Immunol.* **182**, 3877-3883.
447. Hazen, S.L., Zupan, L.A., Weiss, R.H., Getman, D.P., Gross, R.W. (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A2. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A2. *J. Biol. Chem.* **266**, 7227-7232.
448. Ackermann, E.J., Conde-Frieboes, K., Dennis, E.A. (1995) Inhibition of macrophage Ca(2+)-independent phospholipase A2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.* **270**, 445-450.
449. Bauldry, S.A., Wooten, R.E., Bass, D.A. (1996) Activation of cytosolic phospholipase A2 in permeabilized human neutrophils. *Biochim. Biophys. Acta* **1299**, 223-234.
450. Seeds, M.C., Jones, D.F., Chilton, F.H., Bass, D.A. (1998) Secretory and cytosolic phospholipases A2 are activated during TNF priming of human neutrophils. *Biochim. Biophys. Acta* **1389**, 273-284.
451. Ghomashchi, F., Stewart, A., Hefner, Y., Ramanadham, S., Turk, J., Leslie, C.C., Gelb, M.H. (2001) A pyrrolidine-based specific inhibitor of cytosolic phospholipase A(2)alpha blocks arachidonic acid release in a variety of mammalian cells. *Biochim. Biophys. Acta* **1513**, 160-166.
452. Balsinde, J., Balboa, M.A., Dennis, E.A. (1998) Functional coupling between secretory phospholipase A2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A2. *Proc. Natl. Acad. Sci. U S A* **95**, 7951-7956.
453. Balsinde, J., Dennis, E.A. (1996) Distinct roles in signal transduction for each of the phospholipase A2 enzymes present in P388D1 macrophages. *J. Biol. Chem.* **271**, 6758-6765.
454. Qiu, Z.H., de Carvalho, M.S., Leslie, C.C. (1993) Regulation of phospholipase A2 activation by phosphorylation in mouse peritoneal macrophages. *J. Biol. Chem.* **268**, 24506-24513.
455. Lin, L.L., Lin, A.Y., Knopf, J.L. (1992) Cytosolic phospholipase A2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. U S A* **89**, 6147-6151.
456. Kramer, R.M., Roberts, E.F., Manetta, J.V., Hyslop, P.A., Jakubowski, J.A. (1993) Thrombin-induced phosphorylation and activation of Ca(2+)-sensitive cytosolic phospholipase A2 in human platelets. *J. Biol. Chem.* **268**, 26796-26804.
457. Nemenoff, R.A., Winitz, S., Qian, N.X., Van Putten, V., Johnson, G.L., Heasley, L.E. (1993) Phosphorylation and activation of a high molecular weight form of phospholipase A2 by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.* **268**, 1960-1964.
458. Tian, W., Wijewickrama, G.T., Kim, J.H., Das, S., Tun, M.P., Gokhale, N., Jung, J.W., Kim, K.P., Cho, W. (2008) Mechanism of regulation of group IVA phospholipase A2 activity by Ser727 phosphorylation. *J. Biol. Chem.* **283**, 3960-3971.
459. Pavicevic, Z., Leslie, C.C., Malik, K.U. (2008) cPLA2 phosphorylation at serine-515 and serine-505 is required for arachidonic acid release in vascular smooth muscle cells. *J. Lipid. Res.* **49**, 724-737.
460. Abraham, S.T., Benscoter, H.A., Schworer, C.M., Singer, H.A. (1997) A role for Ca2+/calmodulin-dependent protein kinase II in the mitogen-activated protein kinase signaling cascade of cultured rat aortic vascular smooth muscle cells. *Circ. Res.* **81**, 575-584.
461. Muthalif, M.M., Benter, I.F., Uddin, M.R., Harper, J.L., Malik, K.U. (1998) Signal transduction mechanisms involved in angiotensin-(1-7)-stimulated arachidonic acid

release and prostanoid synthesis in rabbit aortic smooth muscle cells. *J. Pharmacol. Exp. Ther.* **284**, 388-398.
462. Balboa, M.A., Balsinde, J., Dennis, E.A. (2000) Phosphorylation of cytosolic group IV phospholipase A(2) is necessary but not sufficient for Arachidonic acid release in P388D(1) macrophages. *Biochem. Biophys. Res. Commun.* **267**, 145-148.
463. Braun, A.P., Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu. Rev. Physiol.* **57**, 417-445.
464. Duff, G.W., Atkins, E. (1982) The inhibitory effect of polymyxin B on endotoxin-induced endogenous pyrogen production. *J. Immunol. Methods* **52**, 333-340.
465. Mortaz, E., Redegeld, F.A., Dunsmore, K., Odoms, K., Wong, H.R., Nijkamp, F.P., Engels, F. (2007) Stimulation of cysteinyl leukotriene production in mast cells by heat shock and acetylsalicylic acid. *Eur. J. Pharmacol.* **561**, 214-219.
466. Lefebvre, J.S., Marleau, S., Milot, V., Levesque, T., Picard, S., Flamand, N., Borgeat, P. Toll-like receptor ligands induce polymorphonuclear leukocyte migration: key roles for leukotriene B4 and platelet-activating factor. *FASEB J.* **24**, 637-647.

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Funk, D., Sorg, B.L., Lindner, S.C., Schmeiser, H.H. ^{32}P-postlabelling analysis of DNA adducts formed by Leukotriene A$_4$ (LTA$_4$). *Environ Mol Mutagen. 2010 May; 51(4):338-43.*

Zagryazhskaya, A.N., Lindner, S.C., Galkina, S.I., Grishina, Z.V., Steinhilber, D., Sud'ina, G.F. Nitric oxide mediates distinct effects of various LPS chemotypes on phagocytosis and leukotriene synthesis in human neutrophils. *Int J Biochem Cell Biol. 2010 Jun; 42(6):921-31. Epub 2010 Feb 1.*

Posterpräsentationen

Lindner, S.C., Sorg, B.L., Steinhilber, D. Influence of toll-like receptor ligands on leukotriene biosynthesis in a monocytic cell-line.*CFS, DPhG & HSPS Joint Meeting, Marburg 2006*

Lindner, S.C., Steinhilber, D., Sorg, B.L. TLR2 ligand lipoteichoic acid enhances calcium-induced leukotriene release in Mono Mac 6 cells. *Leopoldina Symposium Lipid Signalling, Frankfurt / Main 2008*

Lindner, S.C., Köhl, U., Maier, T.J., Steinhilber, D., Sorg, B.L. TLR2 ligand lipoteichoic acid enhances calcium-induced leukotriene release in Mono Mac 6 cells. *Excellence Cluster Cardio-pulmonary System (ECCPS) – Retreat, Bad Nauheim2009*

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