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BIOSYNTHESIS OF LEUKOTRIENE B_4 IN HEMATOLOGICAL MALIGNANCIES

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ABSTRACT

Leukotrienes (LT) are biologically active metabolites of the fatty acid arachidonic acid (AA). After liberation of AA by phospholipase A_2 (PLA_2), this fatty acid can be converted to leukotrienes, lipoxins, prostaglandins or thromboxane. The conversion of AA to LTA_4 is catalyzed by five-lipoxygenase (5-LO). LTA_4 can then be further metabolized to LTB_4 or LTC_4 by the catalytic action of LTA_4 hydrolase or LTC_4 synthase, respectively. Cellular leukotriene synthesis is dependent on 5-LO activating protein (FLAP), a membrane protein that binds AA and facilitates the 5-LO reaction. LTB_4 is produced in myeloid cells and B-lymphocytes. Besides a role in various immunological and inflammatory reactions, several reports indicate that LTB_4 may have a role in the proliferation of myeloid and lymphoid cells.

Cells from patients with precursor B-acute lymphoblastic leukemia (B-ALL) were studied. All eight investigated clones expressed the genes for FLAP, LTA_4 hydrolase and cyclooxygenase-1 (COX-1). Seven out of eight clones expressed COX-2. Four of the more mature cell clones expressed 5-LO but not $cPLA_2$ and the cells also produced LTB_4. The remaining four clones expressed $cPLA_2$ but not 5-LO and hence produced no leukotrienes. On the basis of the expression of $cPLA_2$ and 5-LO, two biologically different subsets of B-ALL were identified. This finding may be of clinical relevance in future treatment of B-ALL.

Splice variants of the cytosolic calcium independent PLA_2 ($iPLA_2$) have been observed in myeloid and lymphoid cells and are suggested to regulate enzyme activity. The expression of $iPLA_2$ and its role in leukotriene synthesis was studied in immature myeloid cells and granulocytes. One additional splice variant, believed to function as a negative regulator of enzyme activity, was expressed in acute myeloid leukemia (AML) and HL-60 cells but not in granulocytes. Results obtained with inhibitors, suggest that $iPLA_2$ is involved in leukotriene synthesis in granulocytes.

The majority of studied AML clones were found to express 5-LO, FLAP and LTA_4 hydrolase proteins. Only three of 16 clones produced similar amounts of leukotrienes as granulocytes upon calcium ionophore A23187 stimulation. Addition of exogenous AA and/or diamide, a redox active substance, resulted in activation of leukotriene synthesis. The AA release in AML cells was two to ten times less than that observed in granulocytes after calcium ionophore activation. The expression of $cPLA_2$ was high in all investigated clones. However, $cPLA_2$ may be activated by other mechanisms than calcium influx in AML cells, and contribute to proliferation in AML, and thus be a putative target in this disease.

B-cell chronic lymphocytic leukemia (B-CLL) cells produced low amounts of LTB_4 after stimulation with A23187 and AA but similar amounts in homogenates as granulocytes. B-CLL cells expressed the high affinity LTB_4 receptor BLT1. Cultivation of B-CLL cells with CD40-ligand-transfected fibroblasts stimulated DNA synthesis and the expression of the activation markers CD23, CD150 and the adhesion molecule ICAM-1 (CD54). The specific leukotriene biosynthesis inhibitors MK886 and BWA4C counteracted this stimulation. Addition of exogenous LTB_4 (150 nM) almost completely reversed the effect of the inhibitors.

In summary, these studies indicate that there are several enzymes and receptors in the arachidonic acid cascade that might be putative drug targets. Such drugs may have a therapeutic role in certain malignant hematological diseases.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals.

I. Feltenmark S**., Runarsson G**., Larsson P., Jakobsson P. J., Björkholm M. and Claesson H-E. Diverse expression of phospholipase A_2, 5-lipoxygenase and prostaglandin H synthase 2 in acute pre-B-lymphocytic leukemia cells.
British Journal of Hematology 1995; 90: 585-594.

II. Larsson Forsell P. K. A., **Runarsson G**., Ibrahim M., Björkholm M. and Claesson H-E. On the expression of cytosolic calcium independent phospolipase A_2 (88 kDa) in immature and mature myeloid cells and its role in leukotriene synthesis in human granulocytes.
FEBS Letters 1998; 434: 296-299.

III. **Runarsson G**., Feltenmark S., Forsell P., Björkholm M. and Claesson H-E. The expression of cytosolic phospholipase A_2 and biosynthesis of leukotriene B_4 in acute myeloid leukemia cells.
Manuscript.

IV. **Runarsson G**., Liu A., Mahshid Y., Feltenmark S., Pettersson A., Klein E., Björkholm M. and Claesson H-E. Leukotriene B_4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells.
Blood 2005; 105: 1274-1279.

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LIST OF ABBREVIATIONS

5-LO	Five-lipoxygenase
AA	Arachidonic acid
$AACOOF_3$	Arachidonyl trifluoromethylketone
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BEL	(E)-6-(bromomethylene)-3-(1-naphtaenyl)-2H-tetrahydropyran-2-one
BLT	Leukotriene B_4 receptor
C2	Constant region 2
CaLB	Calcium dependent phospholipid binding domain
Cam kinase II	Calcium calmodulin kinase II
CD	Clusters of differentiation
cDNA	Complementary DNA
CLL	Chronic lymphocytic leukemia
CMKRL	Chemoattractant receptor, chemoattractant receptorlike
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
Diamide	Azodicarboxylic acid bis [dimethylamide]
ERK	Extracellulary regulated kinase
FAB	French-American-British (classification of leukemia)
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FLAP	Five lipoxygenase activating protein
FMLP	Formyl-leucine-methionine-phenylalanine
GPCR	G-protein-coupled receptor
HSC	Hematopoietic stem cell
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
$iPLA_2$	Calcium independent phospholipase A_2 (group VIA)
LT	Leukotriene
LX	Lipoxine
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MAPK	Mitogen activated protein kinase
MAPKAP	Mitogen-activated protein kinase-activated protein kinase
MNK-1	MAPK-interacting kinase
mPGES	Microsomal prostaglandin E synthase-1
mRNA	Messenger RNA
NSAID	Non-steroidal antiinflammatory drug
ORF	Open reading frame
PA	Phosphatidic acid
PAF	Platelet activating factor
PAP-1	PA-phosphohydrolase
PC	Phosphatidyl choline

PG	Prostaglandin
PGHS	Prostaglandin H synthase
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLL	Prolymphocytic leukemia
PMNL	Polymorphonuclear leukocytes
PPARα	Peroxisome proliferator receptor α
SCID	Severe combined immune deficiency
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLAM	Signaling lymphocytic activating molecule
Sn	Stereoscopic nomenclature
SP	Side population
$sPLA_2$	Secreted PLA_2
SRS	Slow releasing substance
WBC	White blood cell

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CONTENTS

1 INTRODUCTION

A fundamental aspect of all living cells is the capacity to sense and react to stimuli. Cells use various types of molecules to communicate with their surroundings. The cell membranes create the essential barrier between the environment and internal compartments in the cells. The phospholipid bilayer is a fundamental building structure of all cell membranes. Besides that, important signal molecules can be generated from the fatty acids in the lipid bilayer. Arachidonic acid (AA) can be converted to leukotrienes, lipoxins, prostaglandins and thromboxanes. AA and its metabolites exert various types of effects but are best known for their role in various inflammatory reactions. In addition these compounds have a role in proliferation and apoptosis. This thesis has mainly focused on the generation and effects of leukotriene B_4 in hematological malignancies in order to elucidate the biological role of LTB_4 in these diseases.

1.1 PHOSPHOLIPIDS

The cell membranes contain phosphoglycerides. The phosphoglycerides consist of a glycerol backbone with two fatty acid chains and a phosphorylated alcohol. This gives the phospholipids amphipathic characteristics, i.e. they contain both the hydrophilic alcohol and the hydrophobic fatty acids. Phosphoglycerides form structures such as lipid bilayer and micelles in aqueous solution. The polar head groups attract by electrostatic forces and face the aqueous solution whereas the fatty acid chains interact by hydrophobic forces [1, 2]. Phosphoglycerides are essential constituents of cell membranes and cellular compartments and thus essential for life.

A stereoscopic nomenclature (sn) is used for the phosphoglycerides, based on the position on the glycerol backbone as outlined (figure 1) [3, 4]. The two fatty acid chains are positioned in the sn 1 and sn2 position whereas the phosphorylated alcohol group is in the sn3 position.

Phosphoglycerides can be divided into different classes based on the constituents of the side groups. The simplest form phosphatidic acid (PA), has no alcohol esterified to the phosphorous group, in contrast to the more common types. Various fatty acid chains can be attached at the sn1 and 2 positions. Saturated fatty acid chains are usually at the sn1 positions and unsaturated forms at the sn2 position [5]. Phosphatidylcholine (PC) has phosphocholine attached at the sn-3 position. It exists in three forms, 1,2-diacyl-glycero-3-phosphocholine, 1-O-alkyl-2-acyl-glycero-3-phosphocholine and 1-alkyl-1´-enyl-2-acyl-glycero-3-phosphocholine [6]. AA is enriched in the 1-O-alkyl-2-acyl-glycero-3-phosphocholine and this form can account for 30-70% of the total PC contents of some myeloid cells [5, 7].

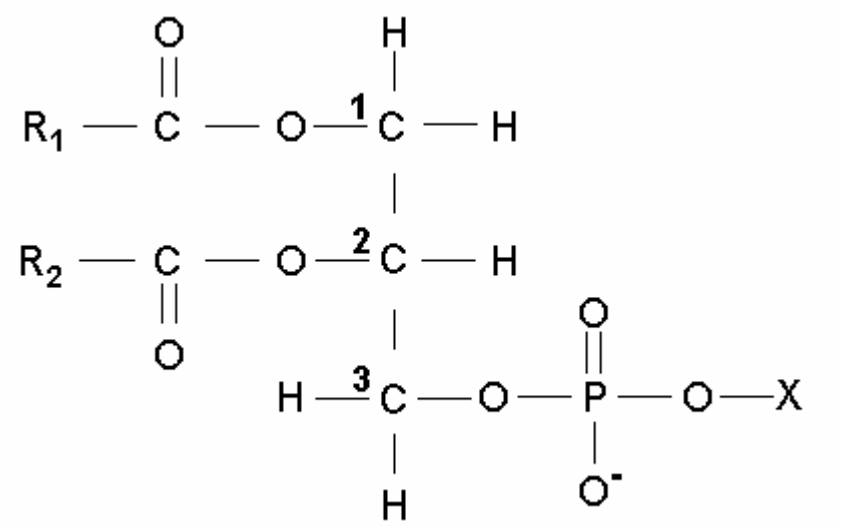

Figure 1. *Phosphoglycerides and the sn numbering of the glycerol backbone R_1 andR_2 refers to the fatty acids and X to the alcohol group.*

1.2 ARACHIDONIC ACID

AA is a polyunsaturated fatty acid esterified to the phospholipids in the cell membranes. It also plays a central role as a precursor for the production of signal molecules that mediate proinflammatory and proliferative responses. The majority of AA is bound in the membrane phospholipids in resting cells but can be released upon activation of the cells [8].

1.3 EICOSANOIDS

"Eicosanoids" is a common term used for unsaturated fatty acids derivatives that consist of 20 carbons (eicosi is the word for twenty in Greek). The family of eicosanoids includes the cyclooxygenase products (i.e. prostaglandins (PG) and thromboxanes (TX)) as well as the lipoxygenase products (i.e. leukotrienes (LT) and lipoxins (LX)). Dihomo-γ-linolenic acid (20:3 ω6), AA (20:4 ω6) and eicosapentaenoic acid (20:5 ω3) are 20 carbon fatty acids that contain three, four and five double bounds respectively. Linoleic acid (18:2 ω6) is the precursor of AA and dihomo-γ-linolenic acid, whereas linolenic acid (18:3 ω3) is the precursor of eicosapentaenoic acid. Linoleic acid and linolenic acid are essential fatty acids that must be provided in the diet, since mammals lack the enzymes necessary to introduce double bonds at carbons beyond C-9 in the fatty acid chain. Consequently, the composition of the diet can influence the production of leukotrienes, prostaglandins and thromboxanes. An example is a diet with a high proportion of ω-3 fatty acids, such as fish oils, rich in eicosapentaenoic acid, resulting in increased formation of the less potent inflammatory mediator LTB_5 instead of LTB_4 [9, 10].

Eicosanoids are synthesized de novo from AA and other 20 carbon fatty acids, released from the membrane phospholipids after stimulation by various signals. The steady state amount of AA in cells is low [11, 12]. The half-life of free arachidonate in cells is

short. If it is not metabolized to eicosanoids, it is quickly reacetylated to membrane phospholipids [12]. Eicosanoids are locally active hormones that exert their effects in the cells where they are formed or in adjacent cells. Eicosanoids can be formed by transcellular metabolism by a conserted action of two celltypes and even at subcellular locations. They are quickly formed upon appropriate stimuli and can thus be described as early mediators of inflammation.

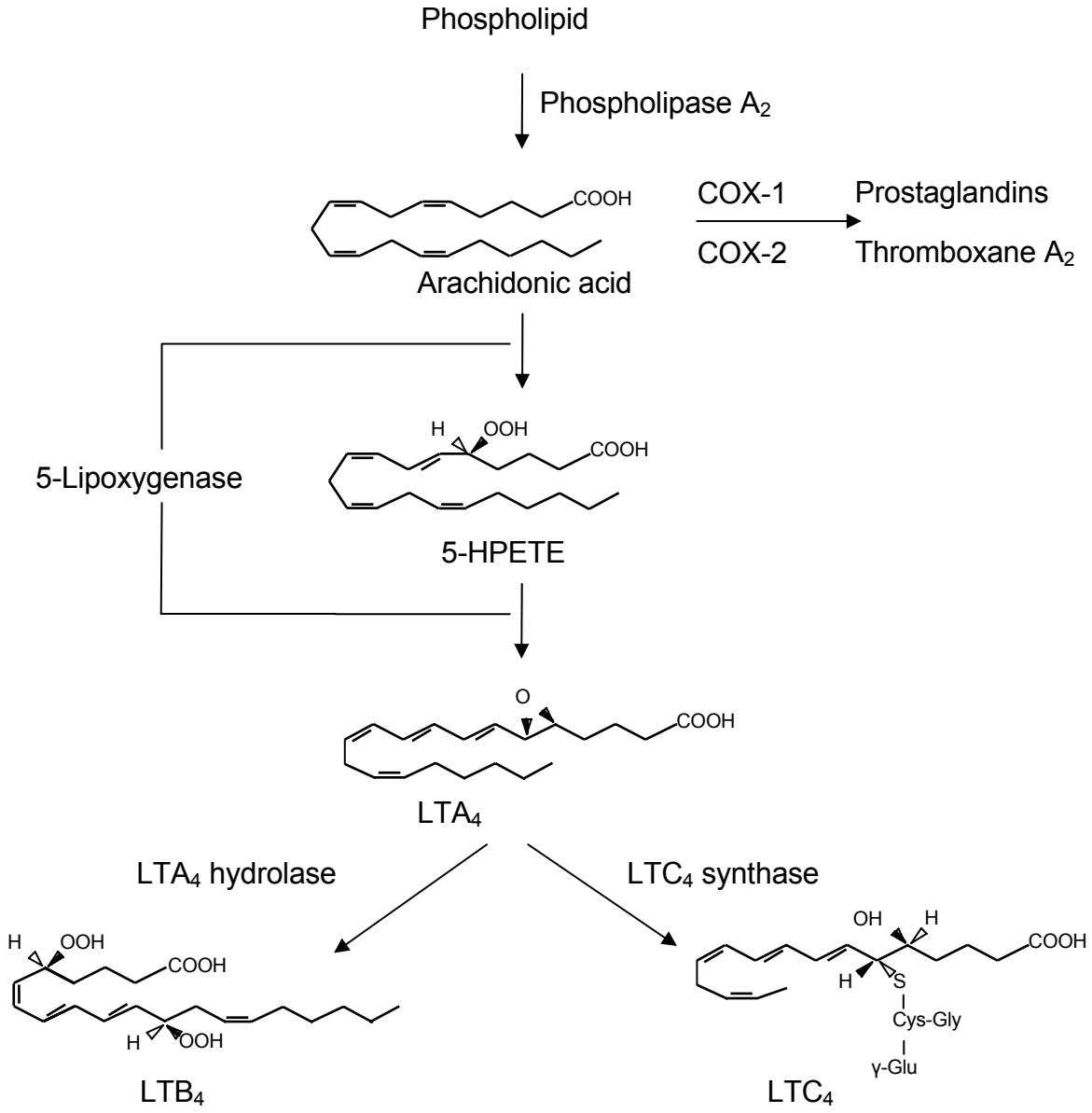

Figure 2. *Illustration of the formation of leukotrienes and prostaglandins.*

1.3.1 Cellular leukotriene synthesis

Leukotrienes are produced by mature myeloid cells in response to stimuli such as bacteria and FMLP (formyl-met-leu-phe), or by calcium ionophore A23187 [13]. The first step in leukotriene synthesis is the release of AA, a reaction catalyzed by $cPLA_2$. After activation, $cPLA_2$ translocates to the nuclear membrane by a Ca^{2+}-dependent

mechanism and releases AA from the sn-2 position in the membrane phospholipids [14, 15]. Thereafter, AA can be converted to leukotriene A_4 in a 2-step reaction catalysed by 5-lipoxygenase, the key enzyme in leukotriene synthesis [16]. Upon an increase in cytoplasmic calcium, 5-LO moves to the nuclear envelope where it associates with FLAP, a protein necessary for cellular leukotriene synthesis, and converts AA to the intermediate leukotriene LTA_4 [15, 17-19]. Leukotriene A_4 can be further metabolised to LTB_4 by the catalytic action of LTA_4 hydrolase [16]. Another metabolic fate of LTA_4 is conversion to the cysteinyl leukotriene, LTC_4, catalysed by LTC_4 synthase [20]. In contrast to myeloid cells, B-lymphocytes require exogenously added AA in addition to calcium ionophore to produce leukotrienes but still release low amounts of leukotrienes. To fully activate leukotriene synthesis in B-cells requires the further addition of a thiol reactive substance such as azodicarboxylic acid bis [dimethylamide] (diamide).

1.3.2 Phospholipase A_2

Phospholipase A_2 (PLA_2) is an enzyme that catalyses the hydrolysis of the sn-2 acylesterbond in phospholipids, leading to the formation of a free fatty acid and a lysophospholipid. Several phospholipases A_2 have been characterized and they can be categorized into several groups based on sequence, structure, localization during catalysis and on calcium dependency (for details see review) [21]. Based upon the Ca^{++} requirement for enzyme activity, the PLA_2s can be divided into three categories: secretory ($sPLA_2$s), cytosolic ($cPLA_2$s) and calcium-independent PLA_2s ($iPLA_2$s). The $sPLA_2$s are secreted extracellulary from granules or directly after synthesis in response to certain stimuli [22-24]. They require millimolar concentrations of Ca^{++} for activity [25]. The $cPLA_2$s are located in the cytosol, require micromolar or submicromolar concentrations of Ca^{++} for activity and translocate into the membrane upon activation. The $iPLA_2$s are located both in the cytosol and in membrane fractions. The group IVα 85 kDa cytosolic PLA_2 and the group VIA calcium-independent PLA_2 will be briefly discussed here.

1.3.2.1 Cytosolic phospholipase A_2

The calcium dependent cytosolic $PLA_2\alpha$ (here referred to as $cPLA_2$), which is classified as a group IV PLA_2, preferably liberates AA and has thus received much attention. The enzyme was first isolated from the monocytic cell line U937 and THP-1 [15, 26, 27] and later from other sources. The enzyme is widely distributed in human tissues except lymphocytes [28, 29]. The human $cPLA_2$ gene is located on chromosome 1q25 and encodes a 85 kDa protein [30-32]. On SDS polyacrylamide gel electrophoresis (SDS-PAGE) $cPLA_2$ migrates approximately as a 90-110 kDa protein. The purified enzyme demonstrates maximal activity at micromolar or submicromolar concentrations of Ca^{++} [15, 33-35]. The $cPLA_2$ contains a calcium dependent phospholipid binding domain (CaLB), similar to C2 domains in enzymes such as phospholipase Cγ (PLCγ) and protein kinase C (PKC) [30, 31, 36]. This C2 domain in $cPLA_2$ is considered important for the translocation of $cPLA_2$ and embedding in the nuclear membrane where its substrate is [31, 37]. Studies on the catalytic site by site directed mutagenesis studies and by active site directed inhibitors have suggested that ser-228 is catalytically active [38-40]. $cPLA_2$ is completely inactivated by thiol modifying agents and cys-331 has been found to be involved in the loss of enzyme

activity [41, 42]. $cPLA_2$ contains several phosphorylation sites [43, 44]. At least three sites are functionally important, ser-505, ser-515 and ser-727 which are phosphorylated by mitogen activated protein kinases (MAPK), calcium-calmodulin kinase II (Cam kinase II) and MAPK-interacting kinase (MNK1), respectively [44-46]. Elevated protein expression of $cPLA_2$ has been shown in certain human malignancies such as colon [47-49], small bowel [47] and lung cancer [50]. The results of a recent study on U937 cells suggests that fetal bovine serum (FBS) induced proliferation of U937 cells is mediated by activation of $cPLA_2$ by CaM kinase II and generation of lysophophatidylcholine [51]. In addition to the phospholipase A_2 activity of $cPLA_2$, the enzyme also has significant lysophospholipase activity and a weak transacylase activity [52].

1.3.2.2 Calcium independent phospholipase A_2

The classical cytosolic calcium-independent phospholipase A_2 ($iPLA_2$) is a group VI (now termed VIA) PLA_2. It was initially purified from the mouse macrophage-like cell line $P388D_1$ [53] and subsequently cloned from a variety of cell types and species [54-57]. The human $iPLA_2$ gene encodes five splice variants with 7-8 ankyrin repeats at the N-terminus and has a molecular weight of 85-88 kDa [54, 56, 57]. The enzyme contains a lipase consensus sequence (GXSTG) and a putative ATP binding sequence [54-57]. It has been suggested that $iPLA_2$ is a tetramer in active form [53]. Ankyrin repeats are common motifs in proteins and mediate protein-protein interaction (for review, see [58]) and can be responsible for the oligomerization of $iPLA_2$ [54]. Two catalytically active splice variants of $iPLA_2$ have been identified, termed group VIA-1 and 2 [54, 56, 57]. The active isoforms can be found both in the cytosol and membrane in resting cells. Group VIA-3 has the consensus lipase part, but lacks the C-terminus and the function of this isoform is unknown [57]. Two other isoforms exist that terminate at the C-terminus prior to the consensus lipase sequence (group VIA ankyrin-1 and 2). They are not catalytically active but may act as negative inhibitors of enzyme activity by interfering with formation of catalytically active tetramers [57]. The enzyme is regulated by protein kinase C (PKC) [59], calmodulin [60, 61] and by stimuli such as zymosan, thrombin and reactive oxygen species [59, 62, 63]. Although the $iPLA_2$ does not require Ca^{++} for activity, it is activated by substances which deplete intracellular calcium stores, such as thapsigargin [60]. It has been suggested that calmodulin regulates $iPLA_2$ in association with the depletion of intracellular calcium stores [60]. In addition to a PLA_2 activity [53, 54], $iPLA_2$ possesses lysophospholipase activity [64], PAF hydrolase activity [54] and a weak transacylase activity [64].
Inhibitors of $iPLA_2$ have been developed, such as bromoenol lactone (BEL), methyl arachidonyl fluorophosphonate (MAFP) and arachidonyl trifluoromethyl ketone ($AACOCF_3$) [65, 66]. MAFP and $AACOCF_3$ are both potent inhibitors of $cPLA_2$, but have also been shown to inhibit $iPLA_2$. BEL does not inhibit $cPLA_2$, but markedly inhibits $iPLA_2$. However, BEL also inhibits phosphatidate phosphohydrolase 1 (PAP-1) with similar IC_{50} values. Since PAP-1 increases the generation of diacylglycerol that is hydrolyzed by diacylglycerol lipase to release AA, the interpretation of results using these inhibitors can be difficult [67, 68]. The activity of PAP-1 can be inhibited by use of specific inhibitors such as propranolol, but BEL has also been shown to interact with several proteins and inhibit group VIB PLA_2 [69, 70].

Studies with antisense olionucleotides and $iPLA_2$ inhibitors have suggested a housekeeping role in phospholipid remodeling. It involves the generation of lysophospholipids as acceptors for the incorporation of AA into membranes [67, 71, 72]. Several studies have indicated a role for $iPLA_2$ in signal transduction, involving AA release and eicosanoid generation (paper II) [59, 73, 74]. Furthermore, it has been suggested that $iPLA_2$ may play an amplifying role in the Fas receptor induced apoptosis [75].

1.3.3 Five-lipoxygenase

Five-lipoxygenase (5-LO) is the key enzyme in leukotriene synthesis. It catalyzes the conversion of AA to the intermediate leukotriene LTA_4 in a two-step reaction. First, free arachidonate is oxidized to 5-hydroxyperoxy-eicosatetraenic-acid (5-HPETE). Subsequently, 5-HPETE is dehydrated to yield the epoxide intermediate LTA_4 [13]. 5-LO is primarily expressed in myeloid cells such as monocytes, macrophages, polymorphonuclear leukocytes (PMNL), eosinophils, mast cells and in B- lymphocytes [76, 77]. 5-LO is a soluble 78 kDa monomeric enzyme and it contains a non-heme iron atom critical for enzyme activity [78, 79]. 5-LO is activated by a variety of different stimuli that lead to an increase in intracellular Ca^{++}. Upon activation, 5-LO translocates to the nuclear membrane, where it associates with a membrane protein, 5-lipoxygenase activating protein (FLAP), and becomes catalytically active [19]. The activation of 5-LO is still poorly understood. For maximal activity, 5-LO requires several stimulatory factors, including Ca^{++}, ATP, lipid hydroperoxides and microsomal membranes/phosphatidylcholine vesicles. The binding of Ca^{++} or ATP to the enzyme is still not defined, since strict homologies to well defined ATP or Ca^{++} binding sites have not been identified [78, 80]. The activation of 5-LO is also controlled by phosphorylation. A mitogen-activated protein kinase-activated protein kinase (MAPCAP) phosphorylates the enzyme at ser-271 and ERK½ phosphorylates the enzyme at ser-663 [81, 82]. Phosphorylation of 5-LO at ser-523 by PKA inhibits enzyme activity [83]. Furthermore, selenium dependent peroxidases expressed in immature myeloid cells and B-lymphocytes can have a suppressing effect on 5-LO [84].

1.3.4 Five-lipoxygenase activating protein

Five-lipoxygenase activating protein (FLAP) is an 18 kDa membrane associated protein located in the nuclear membrane [18]. The gene coding for FLAP in humans is located on chromosome 13 and comprises 31 kb. It consists of five small exons and four large introns. The promotor region contains a TATA box [85]. FLAP is a member of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) protein superfamily [86, 87]. FLAP was discovered as a result of the observation that an inhibitor of LT biosynthesis, MK886, inhibited leukotriene synthesis in intact cells but not in cell homogenates. Subsequent studies showed that MK886 bound to a membrane associated protein (FLAP) and could be isolated by affinity chromatography with MK886 [88, 89]. It is now assumed that FLAP is a substrate transfer protein, i.e., presents the arachidonate released from the membrane to 5-LO. FLAP is considered necessary for cellular leukotriene synthesis [18, 90]. Human osteosarcoma cells transfected with 5-LO require additional transfection with FLAP in order to activate 5-LO upon stimulation with calcium ionophore A23187 [17].

Furthermore, MK886 inhibits leukotriene synthesis in intact cells, but not in cell homogenates, and not in cell free systems or in intact cells when AA is exogenously added [17].

1.3.5 Leukotriene A_4 hydrolase

The final step in the biosynthesis of LTB_4, the conversion of LTA_4 to LTB_4 is a reaction catalyzed by LTA_4 hydrolase. LTA_4 hydrolase is an epoxide hydrolase, that hydrolyses the 5,6 trans epoxide on LTA_4 [91]. The gene encoding the enzyme in humans is located on chromosome 12q22. It comprises 35 kb DNA and has 19 exons and 18 introns. The promotor region contains several transcription factor-binding sites, but no TATA box [92]. LTA_4 hydrolase is widely distributed, and has been detected in most cells and organs in humans, as well as in other species [93]. LTA_4 hydrolase activity has been detected in neutrophils [94, 95], monocytes [96, 97], lymphocytes [98-100], mast cells [101] and erythrocytes [102]. Since LTA_4 hydrolase is widely distributed, it can be involved in the transcellular metabolism of LTA_4 [102]. However, it can be expressed for other purposes, since LTA_4 hydrolase also displays an aminopeptidase activity [103]. LTA_4 hydrolase is a zinc-binding protein like other aminopeptidases [104]. The zinc atom is necessary for both of its enzymatic activities [105].

1.3.6 Leukotriene C_4 synthase

Another metabolic pathway for LTA_4 is the conjugation to glutathione to form 5(S)-hydroxy-6(R)-S-glytathionyl-7, 9-trans-11, 14-cis-eicosatetraenoic acid (LTC_4). The enzyme LTC_4 synthase catalyzes this reaction [20]. LTC_4 synthase is an integral membrane protein and is enzymatically active as a homodimer of two 18 kd subunits. The enzyme catalyses the conjugation of glutathione with LTA_4 [106, 107]. LTC_4 synthase is a member of the MAPEG superfamily, like FLAP, and the microsomal prostaglandin E synthase-1 (mPGES-1) and the recently identified microsomal glutathione S-transferases, MGST2 and MGST3, which both exhibit LTC_4 synthase activity [86, 108-110]. Two thirds of the LTC_4 synthase N-terminal part exhibit 44% identity to FLAP in amino acid sequence, and the FLAP inhibitor MK886 also inhibits the enzyme [111]. LTC_4 synthase activity has been described in eosinophils, mast cells, monocytes and macrophages, as well as in cells that apparently do not express 5-LO, such as platelets and endothelial cells [112-115], and in vascular smooth muscle cells, synoviocytes and lung, kidney and brain tissue (for review, see [116]).

1.3.7 Cyclooxygenases

The cyclooxygenase pathway of AA metabolism will only be briefly mentioned here. Cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS), converts AA to prostaglandin H_2 (PGH_2) in a two-step reaction through the generation of a PGG_2 intermediate in a reaction that introduces two O_2 molecules. The hydroperoxide group is then reduced to a hydroxyl group to yield PGH_2. This compound can then be further metabolized into PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2. Cyclooxygenase exists as two isoenzymes encoded by two separate genes. COX-1 is constitutively expressed in most tissues. The enzyme principally is involved in basal prostaglandin production to maintain normal physiological functions (for review, see [117]. In contrast, COX-2 is a

highly inducible enzyme upon proinflammatory and mitogenic stimuli, resulting in increased prostaglandin synthesis and release [118, 119]. The cyclooxygenases are the targets for non-steroidal anti-inflammatory drugs (NSAID), such as aspirin. The NSAIDs are extensively used for their anti-inflammatory properties. They interact with the active site of COX-1 and –2. NSAIDs are well known for their side effects on the gastric mucosa, causing gastric ulcers and sometimes, fatal gastrointestinal lesions. The gastric mucosa predominantly contains COX-1 that is involved in production of mucosa-protective prostaglandins. This has led to the development and marketing of specific COX-2 inhibitors with less gastrointestinal side effects. However, recent reports indicate that these substances increase the risk of myocardial infarction and stroke.

The first indication of the role of cyclooxygenase in cancer was demonstrated in epidemiological studies that showed a reduced risk of colon cancer in chronic aspirin users (se review [120]). Later studies have shown increased amounts of prostaglandins in colonic adenomas and malignant tumors, concomitant with the increased expression of COX-2 but not COX-1 in these tumors [121-123]. In a mouse model of familial adenomatous polyposis (FAP), COX-2 inactivation lead to a significant decrease in number of intestinal polyps [124]. Human trials in FAP patients have also shown a reduction in the number of polyps after NSAID treatment [125]. Recent studies on the role of COX-2 in cancer and carcinogenesis have indicated that COX-2 is involved in inhibition of apoptosis, tumor angiogenesis, metastatic potential and tumor induced immunosuppression (for review see [126]). COX-2 overexpression has been reported in many cancer types, such as lung, pancreas, bladder, gastric and breast cancer, and in gliomas and mesotheliomas and in many cases is related to poor prognosis [127-133].

1.4 LEUKOTRIENES AND PROSTAGLANDINS

1.4.1 Leukotriene B_4

Leukotriene B_4 (LTB_4) is a potent chemoattractant produced by myeloid cells and B-lymphocytes. At nanomolar concentration, LTB_4 acts as a chemoattractant for PMNL [100, 134-137]. Furthermore, LTB_4 stimulates several functions of PMNL, such as activation, adherence and aggregation [134], Ca^{++} mobilisation [138, 139], production of superoxide anions, degranulation and release of lysosomal enzymes [134, 140, 141].

LTB_4 stimulates certain function of monocytes and macrophages, and is important in host defence against infections [142, 143]. Alveolar macrophages from LT deficient mice require exogenously added LTB_4 for effective phagocytosis and killing of bacteria [142]. LTB_4 is also a key mediator of PAF-induced shock [144-146].

LTB_4 modulates B-lymphocyte activation and proliferation, and has a stimulatory role in antibody production [147]. It stimulates T-lymphocyte activation, proliferation and T-cell migration [148-151]. LTB_4 produced by myeloid cells at inflammatory sites is involved in early recruitment of activated CD4+ and CD8+ T-cells, prior to chemokine-mediated T-cell recruitment [152-154]. Figure 3 illustrates the effects of LTB_4 in the immune system.

LTB_4 has been implicated in various inflammatory diseases and autoimmune disorders. It is involved in allergic asthma, rheumatic arthritis, psoriasis, inflammatory bowel disease and postischemic reperfusion injury (reviewed by Tager) [155]. Studies in mouse models and humans have implicated a role for leukotrienes in atherosclerosis. In the gene encoding FLAP, a four single nucleotide polymorphism haplotype was shown

to nearly double the risk of myocardial infarction and stroke. The at-risk haplotype had a stronger association to disease in males, and neutrophils from male carriers generated more LTB_4 upon ionomycin stimulation [156]. Recently, variant 5-LO promoter genotypes have been shown to identify a subpopulation with increased atherosclerosis [157]. Studies in hyperlipidemic $apoE^{-/-}$ or $LDL^{-/-}$ mice have indicated a significant role of leukotrienes in atherosclerosis [158, 159].

The enzymes 5-LO and LTA_4 hydrolase, and the products of the 5-LO pathway, such as 5-HETE and LTB_4, have been implicated in development of cancer. 5-LO is expressed in pancreas cancer cells and LTB_4 seems to stimulate growth and survival in these cells. LTA_4 hydrolase is overexpressed in oesophageal adenocarcinoma in a rat model. Human colon carcinoma cell lines have been shown to express 5-LO and overexpression of LTA_4 hydrolase has been shown to increase the proliferation rate of colon carcinoma cell lines. LTB_4 and LTC_4 stimulate myelopoiesis and studies with leukotriene synthesis inhibitors have indicated a stimulatory role of leukotrienes in both normal and malignant myelopoiesis [160-163].

The effect of LTB_4 on immune cells

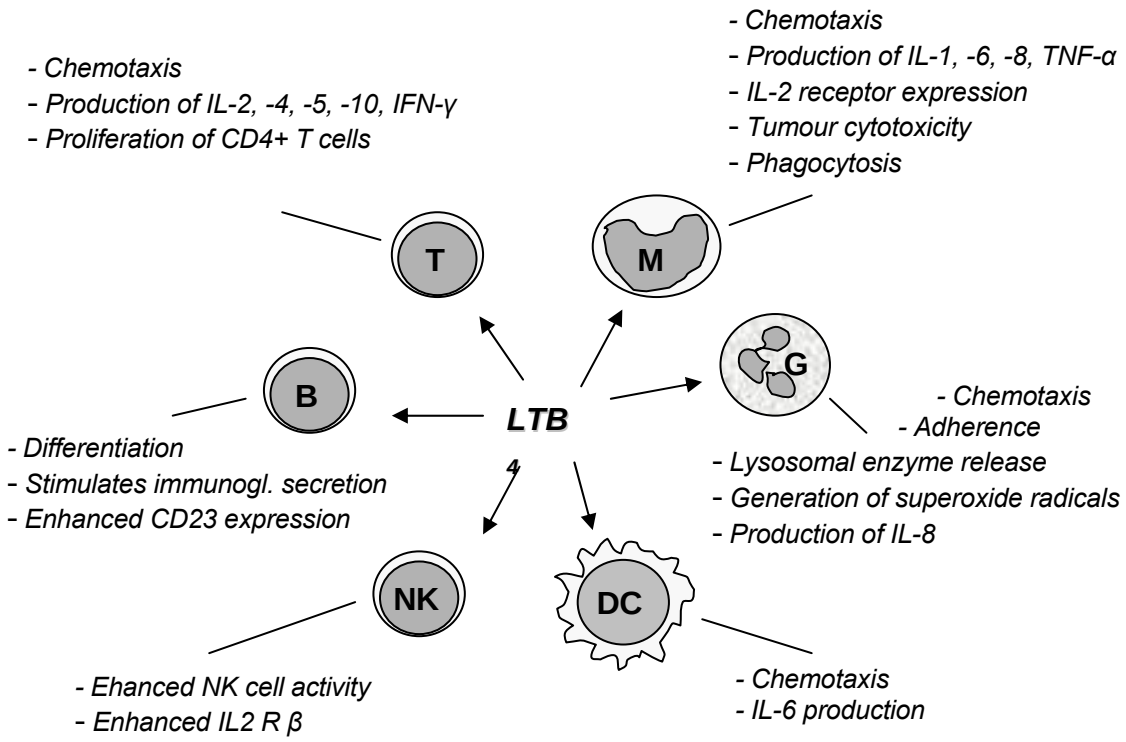

Figure 3. *The effects of LTB_4 on cells in the immune system. T: T-lymphocytes, B: B-lymphocytes, NK: NK cells, M: monocytes/macrophages, G: granulocytes, DC: dendritic cells.*

1.4.2 The cysteinyl leukotrienes

The cysteinyl leukotrienes, i.e. LTC_4, LTD_4 and LTE_4, were previously known as the slow reacting substance of anaphylaxis (SRS-A) [164, 165]. They are made by mast cells, macrophages and eosinophils, and also by transcellular metabolism through the

concerted action of PMNL and endothelial cells/platelets [13]. They induce smooth muscle contraction in bronchi and in capillary walls, increase vascular permeability in postcapillary venules, stimulate mucus secretion of epithelial cells and recruit inflammatory cells [164-167]. The cysteinyl leukotrienes have been implicated in the pathogenesis of asthma, allergic rhinitis and psoriasis [13]. The cysteinyl leukotrienes have also been shown to stimulate myelopoiesis [162].

1.4.3 Prostaglandins and thromboxanes

The conversion of AA to prostaglandin H_2 is catalysed by cyclooxygenase (also known as prostaglandin H_2 synthase or prostaglandin endoperoxide synthase). Prostaglandin H_2 can then be converted to prostaglandins and thromboxane A_2 [168, 169]. Almost all cells in the human body form prostaglandins, such as monocytes, mast cells and endothelial cells, whereas thromboxanes are formed mainly by platelets. In addition to their role in several homeostatic biological functions and inflammation, prostaglandins have also been implicated in cancer development and growth (see also chapter on COX-2) [170-172].

1.5 LTB_4 RECEPTORS

To date, three specific LTB_4 receptors have been described. LTB_4 was identified in 1979, and a few years later, two stereospecific LTB_4 receptors were shown to exist on PMNL [173-175]. By characterization of ^{3}H-LTB_4 binding to PMNL, the presence of a high-affinity and a low-affinity LTB_4 receptor were shown with a mean of 4400 and 270,000 copies per cell, respectively. Although initially believed to represent two different functional states of the same receptor, it was not until about 15 years later that BLT1 and BLT2 were identified as the high- and low-affinity LTB_4 receptors. The nuclear receptor peroxisome proliferator -activated receptor α (PPARα) was in the meantime the first LTB_4 receptor identified.

1.5.1 BLT1

The high affinity LTB_4 receptor BLT1 was first definitely identified and cloned in 1997 by use of a cDNA subtraction strategy in retinoic acid differentiated HL-60 cells [176]. BLT1 had previously been cloned from rat aortic smooth muscle cell cDNA library and characterized as a purinoreceptor [177, 178]. BLT1 had also been cloned from human B-lymphoblast cDNA library and characterized as a putative chemoattractant receptor, chemoattractant receptorlike 1 (CMKRL1) [179, 180]. BLT1 is a G-protein coupled receptor (GPCR). The dissociation constant K_d for LTB_4 binding to BLT1 is in the subnanomolar range [176]. The human gene encoding BLT1 is located on chromosome 14q11.2-q12 and consists of three exons. The open reading frame (ORF) contains no introns, like some other GPCRs [179]. BLT1 is mainly expressed on leukocytes, including neutrophils, monocytes [176, 179, 180], peripheral B-lymphocytes, T-lymphocyte subsets ($CD16^+$ subset of $CD8^+$) [181], on activated $CD4^+$ and $CD8^+$ T-cells [152-154] and in certain B-cell malignancies (IV).

1.5.2 BLT2

A second GPCR for LTB_4 was discovered during the analysis and evaluation of the genomic structure and transcriptional regulation of BLT1. The receptor was a second

low affinity receptor for LTB_4, termed BLT2. The open reading frame (ORF) of BLT2 was located in the promoter region of BLT1and was the first example of a "promoter in ORF" in mammals [182, 183]. BLT2 has a 45.2% sequence identity to BLT1 at the amino acid level. The Kd value of LTB_4 binding to the receptor is 23 nM, which is about 20 times higher than for BLT1 [182]. The mRNA expression of BLT2 revealed by northern blot analysis showed high expression in liver, spleen, ovary and leukocytes, and a weak signal in almost all other human tissues.

1.5.3 Peroxisome proliferator-activated receptor α

Peroxisome proliferators are a group of diverse compounds that stimulate the increase in hepatic and renal peroxisomes that metabolize fatty acids by β-oxidation. A high-fat diet or compounds such as chlorinated hydrocarbons, herbicides and the lipid lowering clofibrates can elicit an increase in peroxisomes. PPARα is a receptor for LTB_4 and is believed to have a role in a feed-back mechanism that induces genes involved in degradation of LTB_4 [184, 185].

1.6 LEUKOCYTES

1.6.1 Hematopoietic progenitor cells

Human hematopoietic stem cells (HSC) reside in the bone marrow in adult life. They have the capacity for self-renewal and the pluripotential capacity to differentiate into lineage specific blood cell progenitors and mature blood cells. They have been characterized by their ability to support hematopoiesis in humans receiving myeloablative chemotherapy, or to reconstitute human hematopoiesis in immunodeficient mice. HSCs have traditionally been characterized by the surface expression of the CD34 antigen and absence of expression of lineage specific antigens and CD38 [186]. However, in recent years, evidence indicates that CD34+ lineage negative cells may not precisely define the stem cell compartment. It was first shown in 1996 that murine HSC could be CD34-, and later that human CD34- cells may contribute to reconstitution of human hematopoiesis in SCID mice [187, 188]. A highly enriched stem cell fraction, termed "side population" (SP), was defined by the ability to efflux the fluorescent dye Hoechst 33342 [189]. Human SP cells derived from bone marrow can engraft in immunosuppressed mice, whereas SP cells from peripher blood have poor engrafting capacity [190]. It has been postulated that CD34 may be up or down regulated in HSC in association with the activation status, and can thus be both CD34+ and CD34-.

1.6.2 Myeloid cells

Myeloid cells are derived from the myelomonocytic lineage of bone marrow cells. They are divided into PMNL, eosinophils, basophils, monocytes and macrophages. The leukotriene synthesis in myeloid cells has been extensively studied.

1.6.3 B-lymphocytes

B-lymphocytes are derived from the B-cell precursor lineage in the bone marrow. They proliferate and reach a certain maturation stage in the bone marrow. There they rearrange their immunoglobulin heavy chain and enter the blood circulation as B-

lymphocytes expressing surface immunoglobulins. Upon activation in the lymph nodes they maturate further. In this milieu B-cells proliferate, rearrange their light chains, undergo heavy chain class switching and develop into memory cells or immunoglobulin-producing plasma cells.

1.6.4 Acute leukemia

Acute leukemia is characterized by a neoplastic expansion of immature hematopoietic cells (blasts) in the bone marrow. The immature blasts accumulate in the bone marrow, leading to suppression of normal hematopoiesis. This leads to anemia, neutropenia and thrombocytopenia, resulting in fatigue, infections and bleeding disorders. The leukemic blasts enter the circulation to a varying degree, and leukemic infiltration in organs can occur, with concurrent symptoms. Hyperleukocytosis (extremely high white blood cell count) can occur, resulting in disturbed microcirculation with multiorgan failure and bleeding disorders.

1.6.5 Precursor B-cell acute lymphoblastic leukemia

Precursor B-cell acute lymphoblastic leukemia (B-ALL) is a neoplasm of lymphoblasts committed to the B-cell lineage. In adults, the incidence of ALL is in the range between 0.7-1.8/100,000 [191]. ALL is more common in children than adults and has a peak incidence in the age of 3-4 years (5.7/100,000/year). ALL account for about 75%-80% of all childhood leukemias [192]. The majority of adult ALL cases (about 75%) exhibit the B-ALL phenotype.
Morphologically, B-ALL blasts are small to medium sized and categorized as L1 and L2 in the French-American-British (FAB) classification [193]. The diagnosis and classification of ALL relies on morphology and immunophenotyping. The clusters of differentiation (CD) antigens are markers of differentiation and lineage and are used to define the lineage and differentiational stage in ontogeny of normal hematopoesis. These markers are often retained in leukemic cells and can thus be used for diagnosis and classification [194]. In the WHO classification, the B-ALL can be subdivided into, three, maturity related groups based on immunophenotyping. They generally express terminal deoxynucleotidyl transferase (TdT), HLA-DR, CD19 and cytoplasmic CD79a as observed in the earliest stage, the early precursor B-ALL (or pre-pre-B). The expression of CD10 delineates the intermediate stage, termed common ALL. The most mature stage, termed pre-B-ALL is characterized by the expression of cytoplasmic mu chains (cyt-mu) [195].

1.6.6 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a neoplasm of immature myeloid precursor cells (myeloblasts). AML is a heterogeneous disease, showing variability in the degree of differentiation and lineage in the expanding clone. The overall incidence of AML is approximately 2.4/100,000. The incidence increases progressively with age and at 65 years of age and above, it is 12.6/100,000 [196]. The incidence of AML in Sweden is about 4/100,000 [197].
The FAB classification of AML [193], with later modifications [198, 199], is based on morphology, cytochemistry and immunophenotyping. In the FAB classification, the AML clones are classified according to stage of maturation and lineage.

The more recent WHO classification [195], attempts to define biologically homologous entities with clinical relevance. This classification is based on morphology, cytochemistry, immunophenotype, cytogenetics and clinical features.

1.6.7 B-cell chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (B-CLL) is a neoplasm of monomorphic small B-lymphocytes in the bone marrow, peripheral blood and lymph nodes. It is admixed with the larger prolymphocytes, and in bone marrow and lymph nodes, pseudofollicles with admixed paraimmunoblasts. B-CLL is usually characterized by a slow accumulation of malignant cells, which escape apoptosis by a variety of mechanisms [200]. B-CLL is the most common leukemia in the Western world. As a disease of the elderly the majority of patients are more than 50 years old and the median age at presentation is about 65 years. The incidence in Sweden is about 5/100,000 and the disease is two times more common among men than women.

The diagnosis of B-CLL is based on morphological features and immunophenotyping, i.e., expression of CD19, CD5, CD20, CD23 and weak surface-membrane immunoglobin [201].

B-CLL follows an extremely variable clinical course. About one third of patients are symptomatic at diagnosis and require treatment. Another third have an initially indolent course, followed by progress requiring treatment, and the remaining third never require treatment and die from unrelated causes [202]. The clinical stage of patients according to the classifications introduced by Rai et al. and Binet et al. [203, 204] still gives important prognostic information, and is a basis for therapeutic decisions [205]. There is, however, considerable variation, even within each clinical stage, which has led to a search for new and more apt predictors of prognosis. Many additional prognostic factors have now been identified, based on cytogenetic and laboratory factors (for review, see [206]).

2 AIMS OF THE STUDIES

- The overall aim of these studies was to delineate the capacity of cells, isolated from patients with hematological malignancies, to generate and respond to leukotriene B_4 in order to elucidate the biological role of LTB_4 in these diseases.

  More specifically:
- To characterize the expression of enzymes involved in the metabolism of AA in precursor B-ALL and AML, in relation to the stage of differentiation and to elucidate the capacity of the leukemic cells to produce LTB_4.
- To define the expression of $cPLA_2$ and $iPLA_2$ in myeloid cells and B-cells of different maturation stages and evaluate the role of $iPLA_2$ in leukotriene synthesis in human polymorphonuclear granulocytes.
- To explore the synthesis of leukotrienes in B-CLL cells and the effect of specific inhibitors of leukotriene synthesis on the activation of B-CLL cells.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Diverse expression of cytosolic phospholipase A_2, 5-lipoxygenase and prostaglandin H synthase 2 in acute pre B lymphocytic leukaemia cells.

When this study was designed, the capacity of B-lymphocytes to produce LTB_4 had recently been described [100]. In addition to a role in inflammatory and immunological reactions, LTB_4 has been reported to stimulate lymphocyte proliferation [207], myelopoiesis [160, 162] and the proto-oncogenes fos and jun [208]. In this study, we investigated the gene expression of the enzymes involved in the synthesis of LTB_4 in precursor B-ALL and the capacity of the ALL cells to produce leukotrienes. We also studied the gene expression of COX-1 and COX-2 (PGHS-1 and 2), since at that time there was no convincing evidence for COX-1 expression in lymphocytes. The COX-2 gene had recently been described and appeared to be a primary response gene, induced by mitogens and inhibited by glucocorticoids [118, 119, 209, 210]. Furthermore, aspirin, an inhibitor of COX-1 and COX-2, had been reported to reduce the risk of colon cancer, suggesting a role of the COX enzymes in malignancy [172].

Previously frozen blasts from eight patients were used in this study. Three samples (A, B and C) were from patients with the more immature early pre-B-ALL phenotype (CD19+, CD10-), the other five samples (D-H) were of the more mature common ALL (cALL) phenotype (CD19+, CD10+). The expression of $cPLA_2$ was detected in four leukemic clones (patients A, B, C and D), while the remaining tumor samples (patients, E, F, G and H) did not express $cPLA_2$ mRNA. The signal pattern of 5-LO was, however, the opposite: samples E, F, G and H, that did not express $cPLA_2$, expressed the gene coding for 5-LO, and the clones that expressed $cPLA_2$ did not express 5-LO. An immunoblot for 5-LO protein, performed on samples from two patients, confirmed that the expression of the 5-LO transcripts correlated with the expression of 5-LO protein. In contrast to the diverse expression of $cPLA_2$ mRNA and 5-lipoxygenase mRNA, all cell clones expressed the genes coding for FLAP and LTA_4 hydrolase. The capacity to generate LTB_4 was also investigated. Four of the six ALL cell clones studied produced similar amounts of LTB_4 as tonsillar B-lymphocytes, monoclonal B-cells, monocytes and PMNL [77, 100, 114, 211]. The ALL cells produced LTB_4 under the same conditions as B-cells, i.e., intact cells required externally added AA plus diamide in addition to calcium ionophore A23187. Furthermore, LTB_4 generation was also observed in sonicates of these cell clones. The capacity of the ALL cells to generate leukotrienes was in good agreement with the expression of the enzymes of the 5-LO pathway, i.e., the expression of 5-LO, FLAP and LTA_4 hydrolase. Based on the diverse expression of 5-LO and $cPLA_2$ that mainly seems to be maturation related, two biological subsets of precursor B-ALL were defined. This biological difference might be of importance in the future management of patients with this disease.

Rather surprisingly, COX-1 expression was detected in all eight ALL clones, as well as in B-CLL and the Burkitt lymphoma derived B-cell line BL41-E95-A used in this study, but only a faint signal was detected in human tonsillar B-cells. A strong signal for COX-

2 was detected in six of the ALL clones, a weak signal in one clone, and in one clone COX-2 was not detected.
The expression of $cPLA_2$ observed in this study was interesting, since $cPLA_2$ appeared only in ALL clones that did not express 5-LO. Analysis of more mature B-cells such as B-CLL and tonsillar B-cells that expressed 5-LO revealed that $cPLA_2$ was not expressed in these cells. It thus appears that $cPLA_2$ is of no importance in leukotriene synthesis in B-lymphocytes. The question arises whether $cPLA_2$ has any role in ALL. In a subsequent study (III), we have observed the expression of $cPLA_2$ in hematopoietic CD34+ cells and a strong expression of this enzyme in AML cells. In addition, recent data suggests that $cPLA_2$ might be involved in the proliferation of the myeloblastic leukemia cell-line U937 [51]. Additional studies are needed to elucidate whether $cPLA_2$ is a therapeutic target in ALL.
In this study, we described for the first time the expression of 5-LO and leukotriene synthesis in B-cell precursors. 5-LO was not expressed in the more immature ALL clones, but appeared to be expressed at a certain stage of differentiation, i.e., about the stage when CD10 expression appears. The exact role and regulation of leukotriene synthesis in B-cells is still unclear.
The expression of COX-2 in ALL deserves some attention. In recent years, COX-2 has been linked to carcinogenesis and several aspects of cancer growth in many different cancer types (see chapter on COX-2 for details). In light of the association of COX-2 to mitogen stimuli, we later studied the expression of COX-2 protein by immunobloting in precursor B-ALL, but no COX-2 protein was detected (unpublished data). Thus, the COX-2 mRNA is apparently not translated to protein.

3.2 PAPER II

On the expression of cytosolic calcium-independent phospholipase A_2 (88 kDa) in immature and mature myeloid cells and its role in leukotriene synthesis in human granulocytes.

The aim of this study was to investigate the expression of $iPLA_2$ in myeloid cells of different maturity and the role of this enzyme in leukotriene synthesis in human granulocytes. The gene encoding the human $iPLA_2$ gene had recently been cloned and the expression of multiple splice variants had been described. Two of these splice variants lacked the active site but contained ankyrin repeats, and it was postulated that they could act as inhibitors of the enzyme [57]. Studying the expression of $iPLA_2$ in myeloid cells of different stages of maturation could therefore possibly elucidate whether expression of splice variants might be a way to regulate enzyme activity. The murine $iPLA_2$ was postulated to be mainly involved in phospholipid remodeling [67, 71]. However, there were reports indicating that $iPLA_2$ might also be involved in release of AA in smooth muscle cells, murine macrophages and rat neutrophils [60, 212, 213]. It was therefore of interest to investigate if $iPLA_2$ could be involved in leukotriene synthesis in granulocytes.
We analyzed the expression of $iPLA_2$ splice variants in CD34+ hematopoietic stem cells, five AML clones, the HL-60 cell line, blood granulocytes and the monoclonal B-cell line RAJI. Expression of $iPLA_2$ and both ankyrin-$iPLA_2$-1 and ankyrin-$iPLA_2$-2 was detected in CD34+ cells and AML cells, whereas the more mature leukemic cells HL-60 and granulocytes expressed $iPLA_2$ and only ankyrin-$iPLA_2$-1 mRNA.

The expression of splice variants of $iPLA_2$ is thus different in mature and immature myeloid cells. In light of the regulatory effect of the splice variants on native $iPLA_2$ activity [57], up- and down-regulation of ankyrin-iPLA-1 and 2 can be a way of controlling $iPLA_2$ activity in myeloid cells.
By liquid chromatography of the cytosolic fraction from homogenates of granulocytes (mainly neutrophils), a fraction could be resolved with calcium independent PLA_2 activity that was inhibited by the $iPLA_2$ inhibitor BEL (10 μM). To determine the involvement of $iPLA_2$ in leukotriene synthesis in granulocytes, intact cells were incubated with or without BEL prior to stimulation with the calcium ionophore A23187. An inhibitory effect of BEL on leukotriene synthesis was observed at low doses of A23187 (0.10-0.15 μM) in a dose dependent manner. At 1μM concentration of A23187, the inhibitory effect of BEL was not as prominent. At 5μM concentrations of BEL, leukotriene synthesis was inhibited by 69%. BEL also inhibited leukotriene synthesis in cells stimulated with opsonized zymosan. In a broken cell assay, BEL had no direct inhibitory effect on leukotriene synthesis. Furthermore, the release of ^{3}H-AA after stimulation with A23187 (0.1-0.15 μM) was inhibited by BEL. To exclude that the effect of BEL was not due to inhibition of phosphatidic acid phosphohydrolase (PAP), the effect of the PAP inhibitor propranolol was tested and found to have no effect on leukotriene synthesis.
In this study, BEL inhibited AA release and leukotriene synthesis. BEL does not inhibit $cPLA_2$ [67], and BEL had no effect on 5-LO activity in cell homogenates in our study. The effect of BEL in this study thus appears to be mediated by inhibition of a PLA_2 enzyme. Recently, BEL has been shown to interact with several unknown proteins, and also inhibit group VIB PLA_2 [69, 70]. Thus, the role of $iPLA_2$ in leukotriene synthesis in granulocytes still needs to be regarded with caution.

3.3 PAPER III

The expression of cytosolic phospholipase A_2 and biosynthesis of leukotriene B_4 in acute myeloid leukemia cells (paper III).

The reason for initiating this study was earlier reports that indicated a stimulatory role of leukotrienes in normal and malignant myelopoiesis [160-162]. We selected 16 AML clones that represented different maturation stages according to the FAB classification (AML M0-M5), and included normal peripheral blood CD34+ pluripotent stem cells in the study.
The $cPLA_2$ but not 5-LO transcript and protein were expressed in CD34+ cells. FLAP and LTA_4 hydrolase transcripts were also expressed in CD34+ cells. In the AML samples, the expression of $cPLA_2$ was high, and in most cases higher than in PMNL. In contrast, the expression of 5-LO, FLAP and LTA_4 hydrolase was lower than that in PMNL. The expression of 5-LO was to some extent related to the maturation stage of the clones.
Since release of AA is required for leukotriene synthesis, we studied the effect of calcium ionophore A23187 on [1-^{14}C] labelled AA release from 11 of the 16 AML clones. The AML clones released two to 10 times less AA than PMNL. It is apparent from these results, that the activation of $cPLA_2$ upon A23187 stimulation is impaired in

AML, bearing in mind the relatively strong expression of $cPLA_2$ in AML compared to PMNL.

A PLA_2 assay was performed to elucidate if high expression of $cPLA_2$ led to high PLA_2 activity. Interestingly, the PLA_2 activity of 5 out of 6 AML samples was higher than in PMNL. The $cPLA_2$ inhibitor $AACOCF_3$ (arachidonyl trifluoromethylketone) (10 μM) inhibited the enzyme activity.

A similar finding of high $cPLA_2$ expression and poor response to A23187 was found in U937 cells. The reason for this was apparently a higher phosphorylated proportion of the enzyme than in mature cells. In addition, the cells also required activation of a G-protein for optimal $cPLA_2$ activation [15, 214]. AML cells had a higher PLA_2 activity than PMNL. However, the experiment was not specific for $cPLA_2$, since $AACOOF_3$ also inhibits $iPLA_2$ and $iPLA_2$ is expressed both in AML and PMNL (II). Furthermore, other PLA_2 enzymes may have contributed to the activity in AML. Overexpression of $cPLA_2$ has been observed in certain cancers [47-50], but this increase has been associated with high COX-2 expression. A recent study on $cPLA_2$ in U937 suggests that the enzyme is involved in fetal bovine serum (FBS) induced proliferation, and the proliferative signals are mediated by lysophospholipids generated by the enzyme [51].

In cell homogenates, generation of LTB_4 and/or 5-HETE was detected in all samples that had a detectable 5-LO expression. The capacity to synthesise leukotrienes in AML was generally much lower than in PMNL, except for the more mature clones. This can mainly be explained by the expression pattern of 5-LO.

The biosynthesis of LTB_4 and/or 5-HETE after stimulation with A23187 was only detected in six of the 16 clones. Only three of the most mature clones produced leukotrienes in amounts similar to those produced by normal granulocytes. This different capacity to produce leukotrienes could not be explained by difference in enzyme expression or AA release. Instead, it was apparently due to impaired activation of 5-LO. The capacity to produce leukotrienes was apparently more closely related to the maturation of the cells than to the amounts of 5-LO protein.

When the AML cells were stimulated with AA or AA plus the thiol reactive substance diamide, biosynthesis of leukotrienes was observed in most of the clones. Furthermore, diamide had an additional stimulatory effect on the production of leukotrienes in most cases. Thus, the addition of AA with or without diamide stimulated leukotriene synthesis in AML, but the capacity to produce leukotrienes was still low, except in the most mature AML phenotypes.

Our findings indicate that the 5-LO pathway is inactive in most AML clones, in spite of expression of 5-LO. This might be explained by defects in 5-LO activation by protein kinases [81, 82, 215, 216], inhibition by phosphorylation at ser-523 by PKA [83] or presence of inhibiting factors such as selenium dependent peroxidases, shown to be expressed in immature myeloid cells and B-lymphocytes [84]. Production of significant amounts of LTB_4 was only observed in the most mature AML clones. This finding is in line with observations made by others that myeloid cells gain the capacity to produce leukotrienes upon maturation [217-219]. The enhancing effects of exogenously added AA and diamide were in agreement with earlier observations [77, 220].

The results obtained in this study, do not support the idea that leukotriene synthesis has an intrinsic role in AML. However, $cPLA_2$ might be a putative target in the treatment of AML.

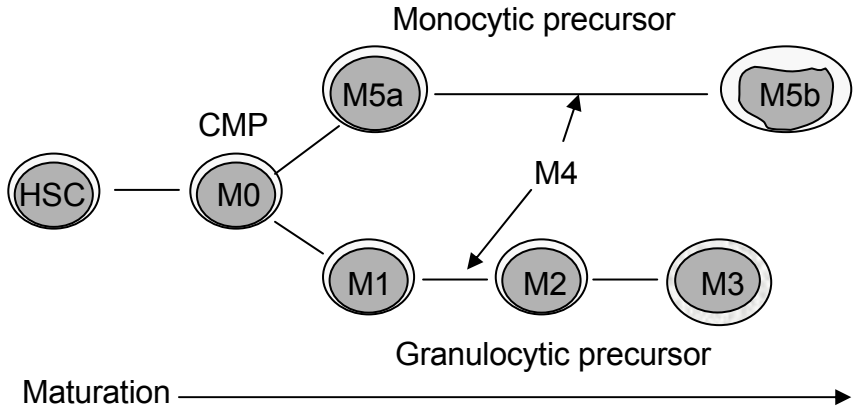

Figure 4. *This illustration shows the maturation of the AML clones in paper III. HSC: hematopoietic stem cells, CMP: common myeloid progenitor.*

3.4 PAPER IV

Leukotriene B_4 plays a pivotal role in CD40 dependent activation of chronic B lymphocytic leukemia cells (paper IV)

The synthesis and biological role of leukotrienes in B-lymphocytes has not been well characterized. In contrast to myeloid cells, B-lymphocytes do not express $cPLA_2$ and do not produce leukotrienes after challenge with calcium ionophore A23187 alone [77, 100]. Although B-cells have been shown to have phospholipase A_2 activity [221], how or if B-cells release AA for leukotriene synthesis is unclear. LTB_4 has been reported to activate B-cells and enhance activation, proliferation and antibody production in tonsillar B-lymphocytes [147, 207, 222, 223]. Microarray studies had shown abundant expression of the 5-LO gene in B-CLL [224]. It was therefore of interest to explore the leukotriene synthesis in B-CLL and the effects of specific inhibitors of leukotriene synthesis on the activation of B-CLL cells.

Intact B-CLL cells produced low amounts of LTB_4 (mean 2.6 ± 0.8 pmol/10^6 cells) after stimulation with A23187 in the presence of exogenously added AA. Preincubation of intact cells with the thiol-reactive agent diamide, prior to addition of calcium ionophore and AA, led to a markedly increased production of LTB_4 (mean 33.5 ± 1.2 pmol/10^6 cells). Similar amounts of LTB_4 (mean 34.8 ± 1.7 pmol/10^6 cells) were produced in sonicated cells, incubated with AA. No cell clones produced detectable amounts of leukotrienes after challenge with either calcium ionophore A23187 or AA alone. The results demonstrated that all investigated B-CLL clones had the capacity to produce LTB_4 in similar amounts as myeloid cells and that all B-CLL clones contained substantial amounts of 5-LO, which could be activated under certain conditions. These results are in agreement with earlier reports [77, 221].

Flow cytometry analysis of cells from six patients with B-CLL and two patients with B-PLL (prolymphocytic leukemia (a more aggressive form of B-cell leukemia than B-CLL)) revealed that all six B-CLL clones analyzed expressed the LTB_4 receptor, BLT1. The expression varied from 15% to 85% in the investigated B-CLL clones (average

expression: 42%). The average expression of BLT1 on B-PLL cells was 74%. In peripheral blood samples from healthy donors, virtually all monocytes and PMNL expressed BLT1, but no expression of BLT1 was observed on peripheral non-activated CD4+ and CD8+ T-lymphocytes. In contrast to T-cells, 30%-50% of peripheral B-lymphocytes stained positive for BLT1. The results obtained here on peripheral blood leukocytes are in agreement with observations of others, except for the expression of BLT1 on a small subset of CD8+ T-cells [181]. The presence of BLT1 on B-CLL cells suggests that LTB_4 might influence the function of B-CLL cells in an autocrine and/or paracrine manner.

Cultivation of B-CLL cells, together with CD40L-L cells, resulted in an increased DNA synthesis of the CLL cells, measured as ^{3}H-thymidine incorporation during the final eight hours of four days cultures. In the presence of control L cells, only minimal ^{3}H-thymidine incorporation was observed. In the presence of MK-886 (a specific FLAP inhibitor) [88], the inhibitory action of 1 μM and 100 nM MK886 on thymidine incorporation was 46 and 38 %, respectively. The specific 5-LO inhibitor BWA4C was even more potent than MK-886 in blocking DNA synthesis. A significant inhibitory effect of BWA4C on thymidine incorporation was observed at 10 nM. LTB_4 (final concentration 150 nM) alone did not amplify CD40-induced thymidine incorporation. However, exogenously added LTB_4 almost completely reversed the inhibitory effect of MK-886 and BWA4C on thymidine incorporation. The cell survival after four days cultivation was about 80 % in all B-CLL cultures stimulated with CD40L-L, both in the absence and presence of inhibitor or LTB_4. Taken together, these specific inhibitors of leukotriene synthesis caused a pronounced inhibition of DNA synthesis, which could be reversed by addition of exogenous LTB_4.

These results suggest that endogenous leukotriene synthesis stimulates DNA synthesis in B-CLL under these experimental conditions. However, addition of LTB_4 did not further stimulate DNA synthesis, suggesting that endogenous LTB_4 caused maximal effects. MK-886 (100 nM) has been found to inhibit DNA synthesis in a subset of AML cells [161]. Furthermore, MK-886 at 100 nM concentration has an antiproliferative effect and induces apoptosis in HL-60 cells, an effect that could be reversed by addition of exogenously added LTB_4 [163].

FACS analysis demonstrated that CD40-CD40L interactions caused an increased expression of CD23, CD54 and CD150. MK-886 and BWA4C, at a concentration of 100 nM, markedly counteracted the CD40-induced expression of CD23, CD54 and CD150. Leukotriene B_4 alone did not cause any significant effect on the antigen expression pattern. However, addition of exogenous LTB_4 (150 nM) almost completely reversed the effect of the inhibitors on antigen expression.

B-CLL cells express CD23, and high amounts of CD23 in serum (sCD23), are associated with high tumor burden and a shorter time to progression in B-CLL [225, 226]. CD23 is a low-affinity receptor for IgE (FceRII), and is involved in the feedback regulation of IgE synthesis. CD23 has been proposed to be involved in cell viability and proliferation [227]. The expression of CD23 on B-CLL cells was inhibited by the leukotriene biosynthesis inhibitors and reversed by LTB_4.

B-lymphocytes from CLL patients have an increased expression of CD54 compared to normal B-cells [228]. High expression is associated with poor prognostic features, including increased tumor burden and sometimes with a short lymphocyte doubling time [228, 229]. Soluble CD54 (sICAM-1) levels are high in patients with advanced clinical stage/high tumor burden [230]. CD54/intercellular adhesion molecule-1

(ICAM-1) is a single chain membrane glycoprotein, which is expressed on many types of cells, such as leukocytes, endothelial and epithelial cells. Both the FLAP inhibitor and the 5-LO inhibitor counteracted the stimulatory action of CD40-CD40L interaction on the expression of CD54. In these experiments, exogenously added LTB_4 (150 nM) also reversed these effects of the inhibitors. In agreement with these findings, LTB_4 has been reported to stimulate the expression of CD54 on endothelial cells and CD23 on B cells [147, 222, 231].

CD150 or signaling lymphocytic activating molecule (SLAM) is involved in bi-directional stimulation of B/B and B/T lymphocytes, and has been shown to enhance B-lymphocyte proliferation [228, 229, 232].

In summary, this study demonstrates that LTB_4 plays an important role in the activation of B-CLL cells. Inhibitors of leukotriene synthesis have so far only been used for treatment of asthma. The present report indicates that leukotriene biosynthesis inhibitors, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL.

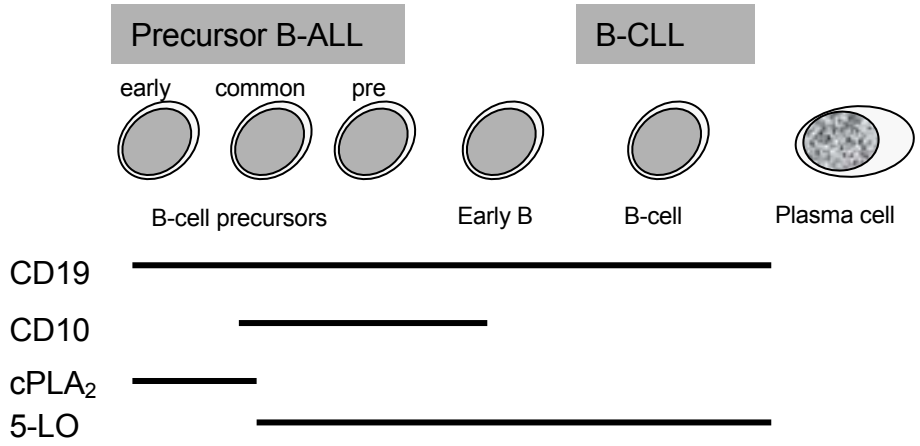

Figure 5. *Scheme of lymphoid differentiation in relation to precursor B-ALL and B-CLL. Expression of CD antigens and $cPLA_2$ and 5-LO in patient samples is shown.*

4 SUMMARY AND CONCLUSIONS

- $cPLA_2$ is highly expressed in AML and precursor B-ALL and may be a putative target in these diseases.
- Leukotriene synthesis in precursor B-ALL is activated in a similar way as in normal B-lymphocytes but the possible role of leukotriene syntehesis in this disease needs to be studied further.
- The expression of splice variants of $iPLA_2$ is different in mature and immature myeloid cells. Up- and down-regulation of ankyrin-$iPLA_2$-1 and 2 can be a way of controlling $iPLA_2$ activity in myeloid cells.
- The results indicate that $iPLA_2$ is involved in the leukotriene synthesis in granulocytes. B-lymphocytes express $iPLA_2$ and the enzyme might be involved in leukotriene synthesis in B-lymphocytes as well.
- The results obtained in this study do not support the idea that leukotriene synthesis has an intrinsic role in AML.
- LTB_4 plays an important role in the activation of B-CLL cells. The results indicate that leukotriene biosynthesis inhibitors, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL.
- In conclusion, these studies indicate that there are several enzymes and receptors in the arachidonic acid cascade that might be putative drug target. These drugs can have a therapeutic role in the treatment of certain malignant hematological diseases.

5 METHODOLOGY

For experimental details, see the Material and Methods sections in papers I-IV.

6 SAMMANFATTNING PÅ SVENSKA

Mitt avhandlingsarbete berör frågeställningar kring bildning och funktion av leukotriener inom den normala hematopoesen och vid olika hematologiska maligniteter. Fynden har relaterats till cellernas mognadsgrad och jag har även studerat leukotrienernas roll vid aktivering och celltillväxt av vissa maligna blodceller.

Leukotriener är lokalt verkande hormoner som bildas från den fleromättade fettsyran arakidonsyra som finns bunden i cellmembranernas fosfolipider. Cytosolärt fosfolipas A_2 ($cPLA_2$) och kalciumoberoende fosfolipas A_2 ($iPLA_2$) spjälkar av arakidonsyra från cellmembranernas fosfolipider. Arakidonsyra kan omvandlas till leukotriener (LT), lipoxiner, prostaglandiner och tromboxaner. Första steget i leukotriensyntesen, bildningen av leukotrien A_4 (LTA_4**)**, katalyseras av enzymet 5-lipoxygenas (5-LO). FLAP (5-lipoxygenase activating protein) är ett protein som behövs för den cellulära syntesen av leukotriener. LTA_4 kan sedan omvandlas till LTB_4, katalyserat av enzymet LTA_4 hydrolas.

6.1 STUDIE 1.

Syfte: I den här studien undersöktes om celler isolerade från patienter med akut B-lymfatisk leukemi (ALL) hade samma kapacitet att bilda leukotriener (LT) som mogna B-lymfocyter. Vi studerade också uttrycket av enzymer som är involverade i leukotriensyntesen och prostaglandinsyntesen (cyklooxygenaser (COX)-1 och COX-2).
Resultat: RT-PCR analys visade att de mest omogna ALL-cellerna uttryckte $cPLA_2$ men inte 5-LO och de mer mogna uttryckte 5-LO men inte $cPLA_2$. Alla kloner uttryckte FLAP och LTA_4-hydrolas samt COX-1. Sju av åtta cellkloner uttryckte COX-2. Uttrycket av 5-LO på proteinnivå överensstämde med RT-PCR resultaten. Celler som uttryckte 5-LO bildade leukotriener.
Konklusion: Förmågan att bilda leukotriener i B-cell prekursor ALL förefaller vara relaterad till tumörcellernas differentieringsgrad. ALL-celler som uttrycker 5-LO bildar leukotriener under samma betingelser som mogna B-celler. Det cytosolära PLA_2 som uttrycks i de mest omogna klonerna är nedreglerat i celler som uttrycker 5-LO, i likhet med mogna B-lymfocyter och förefaller därför inte vara kopplad till leukotriensyntesen hos B-lymfocyter. Baserat på genuttrycket av $cPLA_2$ och 5-LO kunde patienterna delas in i olika undergrupper. Ytterligare studier behövs för att klarlägga om dessa fynd kan kopplas till kliniska faktorer som exempelvis svar på behandling med cytostatika.

6.2 STUDIE 2.

Syfte: I det här arbetet studerades uttrycket av det då nyligen beskrivna kalciumoberoende fosfolipas A_2 ($iPLA_2$) i omogna myeloida celler och mogna granulocyter samt enzymets eventuella roll i leukotriensyntesen i granulocyter. Humana CD34+ hematopoetiska stamceller, akut myeloisk leukemi (AML) celler och granulocyter användes i studien. RT-PCR användes för analys av uttrycket av $iPLA_2$ och dess splice-varianter.
Resultat: Alla analyserade celler uttryckte $iPLA_2$mRNA men dessutom observerades förekomst av två splice-varianter som saknar den aktiva delen av enzymet. Dessa varianter anses kunna ha hämmande effekt på $iPLA_2$ genom att störa protein-protein interaktioner. En av dessa varianter amplifierades huvudsakligen i de mest omogna

AML typerna samt i CD34+ stamceller. Granulocyter hade fosfolipasaktivitet som delvis kunde hämmas av en specifik hämmare av $iPLA_2$, bromoenollakton (BEL), ett fynd som tyder på att $iPLA_2$-aktivitet finns i granulocyter. BEL minskade också leukotriensyntesen i granulocyter och effekten var mest uttalad vid låga doser av kalciumjonofor A23187.
Konklusion: Omogna och mogna myeloida celler uttrycker $iPLA_2$. De mest omogna cellklonerna uttrycker flera splice-varianter som kan ha hämmande effekt på $iPLA_2$-aktiviteten. Förekomsten av splice-varianter i omogna myeloida celler talar för att aktiviteten av $iPLA_2$ är nedreglerat. Resultaten visar att granulocyter har $iPLA_2$ aktivitet och $iPLA_2$ är involverat i bildningen av leukotriener i humana granulocyter.

6.3 STUDIE 3.

Syfte: Studera bildningen av LTB_4 i AML-celler. Vi analyserade leukotrienbildningen, och proteinuttrycket av enzymer involverade i leukotriensyntesen i AML-celler och i CD34+ hematopoetiska stamceller. I studien användes dessutom AML-klonernas mognadsgrad som modellsystem för att studera LT syntesen i omogna myeloida celler
Resultat: CD34 positiva hematopoetiska stamceller uttrycker $cPLA_2$ men inte 5-LO. De flesta AML-celler har ett starkt uttryck av $cPLA_2$ men arakidonsyrafrisättningen är låg med hänsyn till proteinuttrycket, jämfört med mogna granulocyter. Uttrycket av 5-LO är lägre än i granulocyter och verkar vara relaterat till mognadsgraden av cellerna. FLAP och LTA_4 hydrolas uttrycks generellt i lägre mängder i AML-celler än i granulocyter. Efter stimulering med kalciumjonofor kunde LTB_4-bildning endast detekteras i 5/16 AML-kloner och i betydande mängder i endast tre av de mest mogna klonerna. Tillsats av arakidonsyra stimulerade leukotriensyntesen i de flesta AML-klonerna. Skillnaderna i leukotriensyntesen kunde inte förklaras enbart av olikheter i 5-LO uttrycket, utan föreföll främst relatera till mognadsgraden.
Konklusion: Hematopoetiska stamceller uttrycker inte 5-LO. Kapaciteten att bilda leukotriener vid AML förefaller vara relaterad till klonernas mognadsgrad, vilket tyder på att leukotriensyntesen inte spelar någon betydande roll tidigt i myelopoesen eller i omogna AML-kloner. AML celler har högt uttryck av $cPLA_2$. Ytterligare studier krävs för att utröna om $cPLA_2$ kan ha betydelse vid AML.

6.4 STUDIE 4.

Syfte: Att studera bildningen av leukotriener, uttrycket av högaffinitetsreceptorn för LTB_4 (BLT1) och vilken effekt leukotrienesynteshämmare har på cellaktivering och DNA syntes i celler isolerade från patienter med kronisk B-cells lymfatisk leukemi (B-KLL).
Resultat: Alla sex B-KLL kloner bildade LTB_4 efter stimulering med kalciumjonofor A23187 och arakidonsyra. Resultaten visade att KLL-celler har lika hög kapacitet som granulocyter att bilda leukotriener. Flödescytometri visade att samtliga KLL kloner uttryckte BLT1 men i varierande grad. Stimulering av KLL-celler med CD40-ligand-transfekterade fibroblaster (CD40L-L), ett system som har likheter med T-cells stimulering av B-celler, ökade DNA-syntesen, vilken blockerades av specifika hämmare av leukotriensyntesen (MK886 och BWA4C). Tillsats av LTB_4 upphävde nästan helt den hämmande effekten av MK886 och BWA4C vilket visar att effekten av hämmarna inte var ospecifik. KLL-celler odlade i CD40L-L systemet visade

uppreglering av aktivitetsmarkörerna CD23, CD150 och adhesionsmolekylen CD54. Denna uppreglering av antigenerna hämmades också av leukotriensynteshämmarna. Tillsats av LTB_4 motverkade effekten av hämmarna.
Konklusion: Aktiverade B-KLL-celler har förmåga att bilda lika stora mängder leukotriener som granulocyter. B-KLL-celler uttrycker högaffinitetsreceptorn BLT1. CD40-ligand stimulering av B-KLL-celler ger en LTB_4-beroende ökning av DNA-syntesen samt stimulerar uttrycket av CD23, CD54 och CD150. Resultaten indikerar att leukotriensynteshämmare kan ha en framtida plats i behandlingen av patienter med B-KLL.

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