Division of Physiological Chemistry II
Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

BIOSYNTHESIS AND BIOLOGICAL ROLE OF LEUKOTRIENES IN HUMAN LYMPHOCYTES

Yilmaz Mahshid

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Yesterday is history, tomorrow is a mystery. But today is a gift, that is why they call it 'the present'. *Alice Morse Earle 1851-1911*

ABSTRACT

Leukotrienes (LT) are biologically active metabolites of arachidonic acid. The key enzyme in leukotriene biosynthesis is 5-lipoxygenase (5-LO). Leukotriene B_4 exerts its biological effect mainly via BLT1, the high affinity receptor for LTB_4. Leukotriene B_4 is a potent proinflammatory and chemotactic mediator.

Myeloid cells are the main LT producing cells in humans and the role of LTB_4 in these immune cells is well established. In contrast, the role of LTs in lymphocytes is not fully understood. B lymphocytes express 5-LO and BLT1, and activated T cells express BLT1. In this thesis the biosynthesis and biological function of LTs in human lymphocytes were studied.

The role of 5-LO and LTB_4 in chronic B lymphocytic leukemia cells (B-CLL) was investigated. Treatment of B-CLL cells with CD40-ligand resulted in cellular activation as measured by increased expression of cell surface markers (CD23, CD54, CD150) and thymidine incorporation. This activation could be inhibited by LT biosynthesis inhibitors. Addition of exogenous LTB_4 counteracted the inhibitory action of the inhibitors on B-CLL cells.

To elucidate the expression of 5-LO in various subtypes of normal B cells, B cells from tonsils were isolated. Using Western blot, immunohistochemistry and RT-PCR, it was shown that mantle zone B cells and memory B cells expressed high amounts of 5-LO. In contrast, germinal center B cells and plasma cells contained low or undetectable amounts of 5-LO, respectively. Further studies in tumor biopsies from mantle cell lymphoma (MCL) patients demonstrated high expression of 5-LO. MCL cell lines also expressed 5-LO.

Studies on the chemical properties of 5-LO protein in MCL cell lines (Granta519, JEKO1, Rec1) showed that the enzyme was phosphorylated on serine 523. In contrast, native 5-LO in neutrophils was not phosphorylated on serine 523. Phosphorylated 5-LO was purified from Rec1 cells using an ATP-agarose column, and the purified enzyme could be dephosphorylated with alkaline phosphatase. The MCL cell lines constitutively expressed phosphorylated 5-LO and this phosphorylation could be induced by activation of protein kinase A. Western blot analysis of biopsies and peripheral blood from patients suffering from MCL or B-CLL demonstrated that these cells also expressed pSer523-5-LO.

We have studied the role of LTB_4 in cellular immune responses. Due to the lack of EBV specific cellular memory, cord blood mononuclear cells (CBMC) are well suited to study immunological interactions during primary EBV infections. Polysaccharide K (PSK) and thioredoxin 80 (Trx80) are immunostimulating compounds and have been demonstrated to activate T and NK cells which inhibit the proliferation of EBV infected B cells. We have found that LTB_4 activated T cells which in turn inhibited the proliferation of EBV infected B cells. We have also shown that LTB_4 alone is as good as PSK and Trx80 to inhibit proliferation of EBV infected B cells.

In conclusion, these studies demonstrate the expression of 5-LO in various subtypes of B cells and for the first time we demonstrate a chemical difference between 5-LO in B cells (express pSer523-5-LO) and neutrophils. Furthermore, the importance of LTB_4 in activation of B-CLL cells and in cellular immune responses to EBV infections is also shown.

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Differential expression of cysteinyl leukotriene receptor 1 and 15-lipoxygenase-1 in non-Hodgkin lymphomas.

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CONTENTS

LIST OF ABBREVIATIONS

12(S)-HETE	12(S)-hydroxy-5,8,14-cis-10-trans-eicosatetraenoic acid
12(S)-HPETE	12(S)-hydroperoxy-5,8,14-cis-10-trans-eicosatetraenoic acid
12-HHT	12(S)-hydroxy-5-cis-8,10-trans-heptadecatrienoic acid
15(S)-HETE	15(S)-hydroxy-5,8,11-cis-13-trans-eicosatetraenoic acid
5,6-DHETE	5(S),6(R,S)-dihydroxy-7,9,11,14-eicosatetraenoic acid
5-HETE	5(S)-hydroxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-HPETE	5(S)-hydroperoxy-8,11,14-cis-6-trans-eicosatetraenoic acid
5-LO	5-Lipoxygenase
AA	Arachidonic acid
ALCL	Anaplastic large-cell lymphoma
ARDS	Acute respiratory distress syndrome
ATP	Adenosine 5'-triphosphate
B-CLL	Chronic B lymphocytic leukemia
B-PLL	prolymphocytic B cell leukemia
CaMKII	calcium/calmodulin-dependent kinase II
cAMP	Adenosine 3',5'-cyclic monophosphate
CBMC	Cord blood mononuclear cells
CDNB	1-chloro-2,4-dinitrobenzene
CLP	Coactosin-like protein
CMKRL1	Chemoattractant receptor-like 1
COPD	Chronic obstructive pulmonary disease
$cPLA_2$	Cytosolic phospholipase A_2
Diamide	Azodicarboxylic acid bis(dimethylamide)
EC50	The molar concentration of an agonist required to achieve 50% of maximum effect
ERK	extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FDC	Follicular dendritic cells
FL	Follicular lymphoma
FLAP	5-Lipoxygenase activating protein
fMLP	f-Met-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine
GC	Germinal center
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vein endothelial cells
IBD	Inflammatory bowel disease
IL	Interleukin
IFN	Interferon
Ig	Immunoglobulin
IM	Infectious mononucleosis
LPS	Lipopolysaccharide
LT	Leukotriene
LTA_4	Leukotriene A_4, 5,6-epoxy-7,9-trans-11,14-cis-eicosatetraenoic acid

LTB_4	Leukotriene B_4, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid
LTC_4	Leukotriene C_4, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTD_4	Leukotriene D_4, 5(S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid
LTE_4	Leukotriene E_4, 5(S)-hydroxy-6(R)-S-cyteinyl-7,9-trans-11,14-cis-eicosatetraenoic acid
MAP kinase	Mitogen activated protein kinase
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
MCL	Mantle cell lymphoma
MGST	Microsomal glutathione s-transferase
MK2	Mitogen activated protein kinase-activated protein kinase 2
MMTS	Methyl methanethiosulfonate
MPP	Multipotent progenitor cell
MS	Multiple sclerosis
NEM	N-ethylmaleimide
NES	Nuclear export signal
NK cell	Natural killer cell
NLS	Nuclear leading signal
PAF	Platelet-activating factor
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PMNL	Polymorphonuclear leukocytes
PSK	Polysaccharide K
PTX	*Bordetella pertussis* toxin
PUFA	Polyunsaturated fatty acid
RA	Rheumatoid arthritis
SAC	*Staphylococccus aureus* Cowan I
SAP	SLAM-associated protein
SLAM	Signalling lymphocyte-activation molecule
SRS-A	Slow-reacting substance of anaphylaxis
Trx80	Thioredoxin 80
TNF-α	Tumor necrosis factor-α
UDP	Uridine diphosphate
UFA	Unsaturated fatty acid

1 INTRODUCTION

1.1 HISTORICAL BACKGROUND

In the first half of the last century several physiological observations were made of extracts from prostate gland and human semen. In 1913 Battez and Boulet[1] found that injection of extracts from prostate gland into dogs lowered the blood pressure and contracted the urinary bladder. In the 1930s, Kurzrok and Lieb[2] found in *in vivo* and *in vitro* experiments that human semen affected human uterus by contraction and relaxation. Further investigations were carried out by Goldblatt[3,4] and von Euler[5-7] who were able to perform biochemical characterizations of these unknown substances. Von Euler's observations led to the name "prostaglandin"[6], substances from the prostate gland. Bergström and Sjövall managed in late 50´s and early 60´s to isolate and elucidate the structures of prostaglandin E and F[8-10]. In 1964, van Dorp's and Bergström's groups demonstrated the enzymatic formation of prostaglandin E_2 from arachidonic acid[11,12].

At the same time other researchers were interested in biologically active compounds from the lung. Harkavy reported in 1930[13] that sputum from patients suffering from asthma contained a compound that led to contraction of intestinal smooth muscle cells. In 1938, Feldberg and Kellaway published a study about the effects of cobra venom on perfused lungs. The effluent from the lungs induced a slow and long lasting contraction of guinea-pig intestinal smooth muscle cells[14]. This observation led to the creation of the name slow-reacting substance (SRS). The biological action of SRSs were further demonstrated between 1940[15] to 1960[16]. During this period SRS were renamed as SRS-A (slow-reacting substance of anaphylaxis) since most observations were made in experiments were anaphylactic shock was induced. In the 70's it was shown that SRSs contained conjugated double bonds[17], sulfur[18] and could be released upon stimulation with calcium ionophore[19]. Samuelsson and coworkers finally resolved the structures of SRSs. It was shown to be a mixture of three compounds, leukotriene C_4, D_4 and E_4, later named as cysteinyl containing leukotrienes (cys-LTs)[20].

1.2 EICOSANOIDS

The word eicosanoid is derived from the Greek word *eicosi*, meaning 20. It is a common name for the classification of compounds derived from polyunsaturated fatty acids containing 20 carbons. Eicosanoids are biologically active mediators acting in a paracrine and autocrine manner *in vivo*. Due to the inability of the human being to introduce double bonds beyond carbon nine, some of the eicosanoid precursors can only be intaken dietary. The polyunsaturated fatty acids linoleic acid and α-linolenic acid are therefore regarded as essential fatty acids. Both linoleic acid and α-linolenic acid are 18 carbon long and can further be metabolized through elongation to eicosatetraenoic acid or eicosapentaenoic acid, respectively (figure 1)[21]. Arachidonic acid (AA), a.k.a. cis-5,8,11,14-eicosatetraenoic acid, is an ω-6 fatty acid, (denoted by calculating the first double bond from the carbon on the opposite side of the carboxylgroup), and can be metabolized to prostaglandins and thromboxanes of the 2-series and leukotrienes and lipoxins of the 4-series. This is the main pathway by which prostaglandins and leukotrienes are produced in the human body. Accordingly, eicosapentaenoic acid is an ω-3 fatty acid and can also be metabolized to prostaglandins and thromboxanes of the 3-series and leukotrienes and lipoxins of the 5-series. ω-3 fatty acids are common in fish oil and investigations have shown diets in high ω-3 gives rise to less potent eicosanoids[22-25].

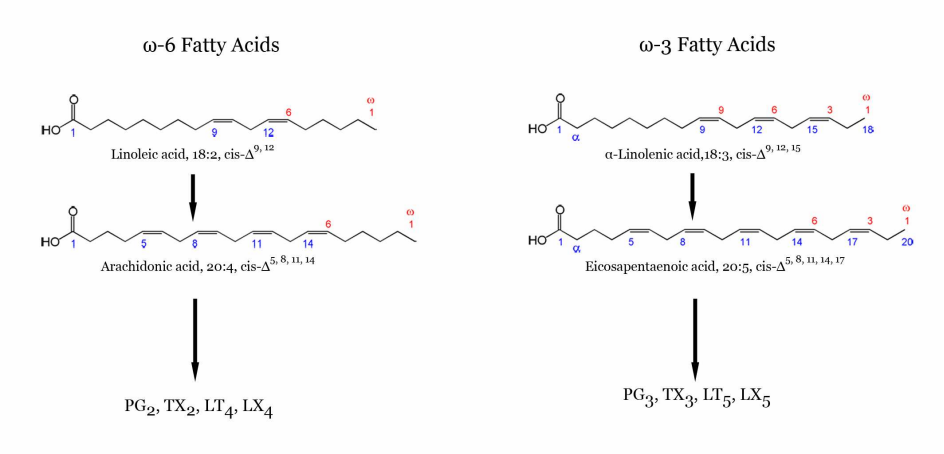

Figure 1. *Precursors of eicosanoids. PG – Prostaglandins, TX – Thromboxane, LT – Leukotriene and LX – Lipoxin.*

2 LEUKOTRIENES

2.1 BIOSYNTHESIS OF LEUKOTRIENES

Biosynthesis of leukotrienes requires free AA. Arachidonic acid is found in the sn-2 position of membrane phospholipids and $cPLA_2$ translocates upon cell activation from the cellular cytosol to the nuclear membrane and liberates AA[26,27]. Conversion of free AA to leukotrienes is catalyzed by 5-lipoxygenase (5-LO). This 78 kDa monomeric enzyme contains a non-heme iron atom pivotal for enzyme activity[28,29]. In the first catalytic step 5-LO converts AA to 5-HPETE (figure 2) and 5-HPETE can further be, enzymatically or non-enzymatically, converted to 5-HETE. Alternatively 5-HPETE can be enzymatically converted to LTA_4. To enable cellular 5-LO activity, the presence of 5-LO activating protein (FLAP) is necessary. This 18 kDa membrane associated protein is thought to support the leukotriene biosynthesis by presenting free AA to 5-LO[30,31]. Leukotriene A_4 is an unstable intermediate in the leukotriene biosynthesis and can either enzymatically or non-enzymatically be converted to 12-epi-6-trans-LTB_4, 5,6-DHETE, LTB_4 and LTC_4. To convert LTA_4 to LTB_4 (figure 2) the enzyme LTA_4 hydrolase is necessary and this enzyme acts by hydrolyzing the epoxide of LTA_4[32]. Leukotriene C_4 synthase conjugates a glutathione molecule to LTA_4, thereby producing LTC_4[33].

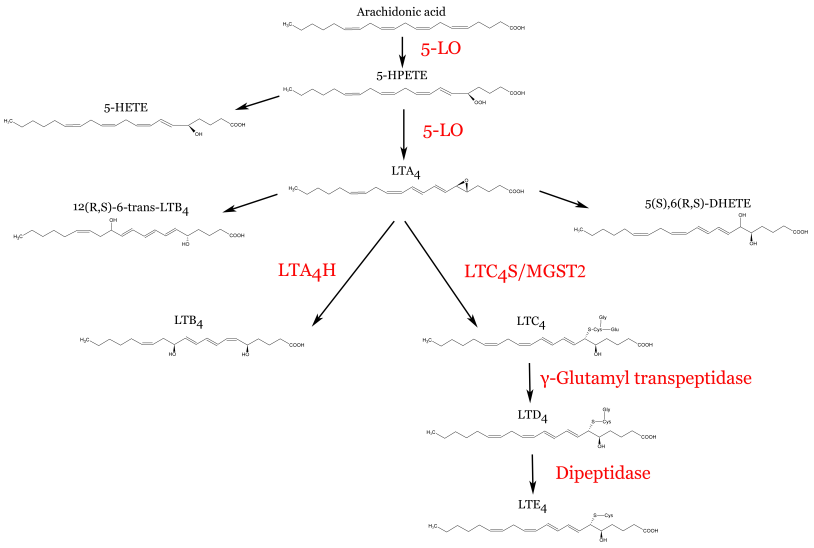

Figure 2. *Biosynthesis of leukotrienes. LTA_4H – LTA_4 hydrolase, LTC_4S – LTC_4 synthase, MGST2 – microsomal glutathione S-transferase 2.*

The molecular weight of LTC_4 synthase is, like FLAP, 18 kDa and both proteins are members of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily[34]. The crystal structure of FLAP and LTC_4 synthase have been elucidated and show a homotrimeric protein complex[35-37]. Two other MAPEG proteins, microsomal glutathione s-transferase 2 (MGST2) and MGST3, have also been shown to convert LTA_4 to LTC_4[34,38,39]. To yield LTD_4 from LTC_4, the action of γ-glutamyl transpeptidase is required[40,41], and LTE_4 is formed from LTD_4 catalyzed by a dipeptidase[42-44].

2.2 ACTIVATION OF LEUKOTRIENE BIOSYNTHESIS IN HUMAN CELLS

In 1976 it was found that rabbit polymorphonuclear cells could transform AA to 5-HETE[45]. Three years later it was found that AA could give rise to LTB_4 in the same cells[46]. Today we know that 5-LO is primarily expressed in human mature myeloid cells e.g. monocytes, macrophages, neutrophils, eosinophils, mast cells and B lymphocytes[47,48]. Myeloid cells can be triggered to produce LTs for example with bacteria, fMLP or calcium ionophore (A23187)[49]. To trigger the production of LTB_4 in

intact B lymphocytes a redox active agent, exogenous AA and A23187 is required[48]. Leukotriene A_4 hydrolase is expressed in most tissues even in cells lacking 5-LO e.g. endothelial cells, erythrocytes, fibroblasts and T cells. Despite the lack of 5-LO, these cells can produce LTB_4 when provided with LTA_4 from another cell, so called transcellular metabolism[50-53]. Other cells lacking 5-LO and have been described to produce LTC_4 are those expressing LTC_4 synthase, MGST2 or MGST3, e.g. platelets, endothelial cells, vascular smooth muscle cells, lung tissue and kidney tissue[39,49-51,54-56].

2.3 RECEPTORS OF LEUKOTRIENE B_4

In 1996 two independent groups cloned an orphan G-protein coupled receptor (GPCR) that was named R2 and chemoattractant receptor-like 1 (CMKRL1)[57,58]. One year later the LTB_4 receptor was fully cloned from differentiated HL60 cells[59]. The receptor was first named BLTR and renamed to BLT1 when the second LTB_4 receptor was discovered. The K_d was found to be between 0.39-1.5 nM for LTB_4[60-62]. Northern blot experiments have demonstrated the expression of BLT1 primarily to leukocytes but was also found in a much lower extent in spleen, thymus, bone marrow, lymph nodes, heart, skeletal muscle, brain and liver[63]. The BLT1 affinity for different ligands relative to LTB_4 is, LTB_4 >12-oxo-LTB_4 = 20-OH-LTB_4 >> 20-COOH-LTB_4[59].

The second LTB_4 receptor BLT2 was cloned year 2000 by four independent groups[64-67]. Like BLT1, BLT2 is a GPCR receptor. The similarity between the two human receptors is 45.2%, similar to the homology in mice, 44.6%. The similarity between BLT2 in human and mice is high as 92.7%. BLT2 has been referred to as the low affinity receptor of LTB_4 due to the 20-fold higher K_d[64], this was also confirmed by intracellular calcium measurements and in chemotaxis assays[64]. Many other eicosanoids have also been described as ligands for BLT2; LTB_4> 12(S)-HETE> 12(S)-HPETE> 15(S)-HETE> 20-OH-LTB_4[68]. Today it is known that the high affinity ligand of BLT2 is 12-HHT[69]. BLT2 expression in human is significantly different from BLT1. Most human tissues express BLT2, and highest expression has been found in spleen, liver, ovary, and peripheral leukocytes. There is evidence that the receptors couple through different G proteins (both PTX-sensitive and –insensitive G proteins) but also within the same cell different signaling cascades occur[63]. Several pharmacological active antagonists have been developed against the receptors, some are BLT1 or BLT2 specific and some binding both receptors[63,70].

2.4 RECEPTORS OF CYSTEINYL LEUKOTRIENES

The biological response of cys-LTs has been believed to be elicited through two receptors, CysLT1 and CysLT2[66,71-74]. Recently, a third receptor for cys-LTs was identified, GPR17[75]. All three receptors are, as LTB_4-receptors, G-protein coupled receptors.

Human CysLT1 receptor mRNA has been found in several cells and tissues e.g. spleen, PBL, lung, placenta and colon[73,74,76,77]. The receptors affinity for cys-LTs is between 2.5 nM – 240 nM, with highest affinity for LTD_4 and lowest for LTE_4[73]. In addition to cys-LTs, acetyl choline and UDP have also been reported as ligands for CysLT1 and homology with P2Y has been identified[78,79].

CysLT2 receptor mRNA seems to be ubiquitously expressed with highest expression in heart, brain, spleen, PBL, placenta, lymph node, and adrenal gland[71,72,74]. The affinity of CysLT2 is similar for LTC_4 and LTD_4 with EC50 values ranging from 2-10 nM. As for CysLT1, LTE_4 have the lowest affinity for the receptor with EC50 values between 50-330 nM[72,74].

The latest member of the cys-LT receptor family is GPR17. This GPCR was deorphanized in the beginning of 2006 and determined by phylogenic analysis to be at intermediate position, between P2Y receptor and cys-LT receptors. It was shown to have nanomolar affinity for cys-LTs and micromolar affinity to uracil nucleotides. GPR17 is expressed at mRNA levels in rat and human brain, heart and kidney[75], recently it was suggested to have a role as a sensor of brain damage[80].

3 BIOLOGICAL EFFECTS OF LEUKOTRIENES

3.1 LEUKOTRIENE B_4

Leukotriene B_4 is a potent inflammatory mediator and exerts its biological effects primarily on leukocytes. Neutrophils are affected by LTB_4 at nano- to micromolar concentrations by chemotaxis, adherence to the endothelial walls, aggregation, production of superoxide anions, degranulation and release of lysozomal enzymes[81-86]. Upon LTB_4 treatment, neutrophil survival is prolonged by preventing apoptosis[87]. Mice lacking the 5-LO gene required exogenously added LTB_4 to activate neutrophils and alveolar macrophages for effective phagocytosis and killing of bacteria[88,89]. These mice respond to endotoxin shock in the same way as wild type mice but they do not suffer lethal effects from PAF-induced shock and have less severe symptoms from ear inflammation induced by AA[90,91].

Leukotriene B_4 has been shown to be an agonist for the nuclear transcription factor PPARα, implicating a feedback mechanism of LTB_4 and/or lipidmetabolism[92]. Monocytes treated with LTB_4 affects the production of IL-6, IL-1 and TNF-α[93,94]. A positive effect of LTB_4 in myelopoiesis and immunoregulation has been reported[95,96]. Moreover, LTB_4 is considered to affect proliferation and differentiation of B cells and also the expression of CD23 and secretion of immunoglobulins[97,98]. It has also been proposed to induce NK cell activity, IL-2Rβ expression and sensitivity to IL-2[93,99,100]. LTB_4 does not only activate human leukocytes but also carcinoma cell lines and endothelial cells[101-104]. Treatment of endothelial cell monolayer with LTB_4 increases their binding of neutrophils, and promotes the trans-endothelial migration of neutrophils[102,103]. Increased proliferation rates were observed in human colon carcinoma cell lines and human pancreatic cell lines, upon stimulation with LTB_4[101,105]. Furthermore, LTB_4 has been implicated in several inflammatory and autoimmune diseases, e.g. cystic fibrosis[106,107], chronic obstructive pulmonary disease (COPD)[108,109], asthma[49], acute respiratory distress syndrome (ARDS)[110], multiple sclerosis (MS)[111], rheumatoid arthritis (RA)[112], psoriasis[113,114], inflammatory bowel disease (IBD)[115,116], and chronic B lymphocytic leukemia (B-CLL)[117]. Human diseases where LTB_4 has been implicated is summarized in table 1.

Disease	Reference
Cystic fibrosis	106,107
Chronic obstructive pulmonary disease	108,109
Asthma	49
Acute respiratory distress syndrome	110
Multiple sclerosis	111
Rheumatoid arthritis	112
Psoriasis	113,114
Inflammatory bowel disease	115,116
Chronic B lymphocytic leukemia	117

Table 1. *LTB_4 and human diseases.*

Recently it was discovered that androgens have effects on 5-LO in neutrophils. It was shown that 5-LO in neutrophils treated with androgens had decreased ability to synthesize LTB_4[118].

As mentioned above, LTB_4 exerts its biological effects mainly through the BLT1 receptor. Many cell and mouse models examining the biological role of BLT1 have been established. Resting neutrophils and macrophages in mice barely express BLT1 but sodium casein injection into the peritoneum dramatically induce BLT1 in these cells[119]. Stimulation of RAW 264.7 macrophage cell line by IFNγ and human peripheral neutrophils by dexamethasone also induced BLT1 expression[119,120]. Upregulation of BLT1 has also been demonstrated to be one mechanism through which glucocorticoids prolong neutrophil survival[120]. Due to the proinflammatory effects of LTB_4, it is intriguing that anti-inflammatory agents e.g. dexamethasone and glucocorticoids induce BLT1.

Treatment of endothelial cells with a LTB_4 receptor antagonist almost completely abolished LTB_4 induced neutrophil transmigration[121]. Reduced phagocytosis was observed in human neutrophils pre-treated with a LTB_4 receptor antagonist. Several human pancreatic cancer cell lines have been shown to induce apoptosis *in vitro*, upon treatment with an LTB_4 receptor antagonist[122]. *In vivo* treatment of athymic mice receiving human pancreatic carcinoma xenografts with an LTB_4 receptor antagonist also induced apoptosis of these tumors[122]. Recently it was shown that BLT1 and BLT2 are differentially expressed in activated HUVEC. LPS or LTB_4 treatment of HUVEC

led to a specific induction of BLT1 while TNF-α treatment primarily induced BLT2 expression[123].

In BLT1 deficient, mice neutrophil chemotaxis, and activation as well as LTB_4 induced calcium flux is abrogated[124,125], indicating that these functions are mediated through BLT1. Furthermore, edema and protein extravasation in response to topically applied AA is reduced and female BLT1 deficient mice have reduced mortality in a model of immediate hypersensitivity induced by intravenous injection of PAF[125]. The observed sex differences in this model have not yet been elucidated. In a chitin induced lung inflammation in mice, resembling a parasite infection, mice deficient of BLT1 had significantly attenuated eosinophil and basophil recruitment to the lung. This was shown to be macrophage dependent[126]. BLT2 deficient mice have yet not been created, therefore little is known about the specific biological effects mediated through this receptor. The high degree of similarity across species of BLT2 suggests that this receptor has an important biological function. Recently a double BLT1/BLT2 knock out mice was created. In an arthritis model with collagen, these mice were equally good protected from disease development as the single BLT1 knock out mice[127].

LTB_4 receptor antagonists have been used with success in several inflammatory animal models i.e. amelioration or complete improvements has been observed. The BLT2 antagonist LY255283 significantly improved systemic arterial hypotension, pulmonary arterial hypertension, pulmonary edema and arterial hypoxemia in a porcine model of shock and ARDS[128]. In a study with anaplastic large-cell lymphoma (ALCL), LY293111 (non-specific BLT antagonist) treatment inhibited proliferation and induced G1-S cell cycle arrest[129]. Significant improvements with CP-105696 (BLT1 antagonist) were seen in collagen induced arthritis, experimental allergic encephalitis (model of MS), airway hyperresponsiveness (model of Asthma), cardiac allograft rejection (model of cardiac transplantation) and atherosclerosis[130-134]. ONO4057 (non-specific BLT antagonist) significantly prolonged allograft survival in a rat model of transplantation[135]. SC-53228 improved the disease outcome in a murine model of IBD[136].

3.2 CYSTEINYL LEUKOTRIENES

Cysteinyl leukotrienes (cys-LT) are produced by mast cells, macrophages and eosinophils but also through transcellular metabolism between PMNLs and endothelial cells/platelets[49]. These substances are potent constrictors of human bronchial smooth muscle cells both *in vitro* and *in vivo*[137-144]. On concentration basis, cys-LTs are 100-1000 times more potent than histamine[145]. Elevated levels of LTE_4 are found in the urine of atopic asthmatics after allergen challenge and in the urine of aspirin intolerant asthmatics after lysine-aspirin challenge. These and previously presented data support the concept of cys-LTs being mediators of asthma[49,146-149]. Cysteinyl LTs also increase vascular permeability in postcapulary venules, stimulate mucus secretion of epithelial cells and recruit inflammatory cells[150-152]. Cysteinyl leukotrienes have also been implicated in the pathogenesis of allergic rhinitis and psoriasis[49]. There is also evidence that cys-LTs stimulate myelopoiesis and are involved in the pathogenesis of atherosclerosis, cerebral ischemia and myocardial ischemia as well [95,153]. As mentioned earlier implications of cys-LTs and their receptors have primarily been in the pathogenesis of allergic and asthmatic diseases[49,154], and CysLT1 antagonists are used in the treatment of asthma.

4 THE ROLE OF 5-LO AND LTB_4 IN THE IMMUNE SYSTEM; B LYMPHOCYTES, T LYMPHOCYTES AND MONOCYTES

4.1 B LYMPHOCYTES

In the 80's there was big controversy whether or not B lymphocytes possessed the ability to produce LTs. The ability of human B lymphocytes to convert AA to LTB_4 was described in 1991. This was achieved in cell homogenates incubated with ATP, calcium and AA[155]. At the same time, studies published by another research group indicated that B cells could produce 5-HETE[156,157]. In 1992, the transcript and the protein expression of 5-LO was demonstrated in B lymphocytes[48]. It was also shown that intact B cells could biosynthesize LTB_4. The activation of LTB_4 biosynthesis was achieved by stimulation of cells with glutathione depleting agents (Diamide and CDNB) plus AA and calcium ionophore (A23187)[48]. Further investigations were made on the pre-requisite of glutathione depleting agents for the ability of intact B cells to synthesize LTB_4. In these experiments, it was found that also methyl methanethiosulfonate, N-ethylmaleimide and H_2O_2 stimulated LT synthesis. All three compounds were equally potent as Diamide to activate 5-LO. Furthermore, primary malignant B cells (B-CLL) were also found to express 5-LO[158].

Several reports have described the biological effects of LTB_4 on B lymphocytes, before the discovery of BLT1 and its expression on B cells. Already in 1989, the synergistic stimulating effect of LTB_4 and B cell stimulating factors (BSF-MP6, IL-2, IL-4 and protein A) on B cell activation was shown, measured by increased CD23 expression, thymidine incorporation and cell numbers[97]. Leukotriene B_4 also induced the secretion of IgG and IgM from B cells exposed to SAC[97]. These results have been confirmed, and also extended, to show the enhancing effect of LTB_4 on release of soluble CD23 and increased expression of MHC class II[159].

4.2 T LYMPHOCYTES

Whether or not T lymphocytes can express 5-LO under certain conditions is still an open question. There are conflicting reports about the expression of 5-LO in T cells. We have not found any evidence of 5-LO expression in T cells although these cells express FLAP and LTA_4 hydrolase[48,93,160-164].

On the other hand it has clearly been shown that different subtypes of T cells express a LTB_4 receptor[93]. The expression and function of BLT1 on mouse T cells was simultaneously published in three papers. These reports showed that activated wild-type $CD8^+$ T cells were more efficient in migrating to inflamed tissue in comparison to activated T cells from BLT1-deficient mice[165]. Activated $CD4^+$ T cells also expressed BLT1 which resulted in more effective migration into the airways of an active immunization asthma model[166]. The expression of BLT1 was increased on activated $CD8^+$ T cells which resulted in migration towards LTB_4 produced by activated mast cells[167].

Since then many groups have published reports linking the expression of BLT1 on activated T cells mainly to airway hyperresponsiveness (AHR) and airway inflammation, but also to other immunologically complex disorders e.g. EBV-infection and organ transplantation. It has been reported that activated $CD8^+$ effector T cells are essential for the AHR in an allergen induced model with CD8-deficient mice[168]. The same group used an allergen-induced AHR model to investigate the role of BLT1. They concluded that BLT1-deficient mice developed significantly lower AHR compared to wild type mice. This was correlated to $CD8^+$ T cells expressing BLT1 and IL-13[169]. Another group showed similar results in an ovalbumin-induced bronchial asthma model. Conclusions were made that BLT1-deficient mice did not develop AHR, had less inflammation and reduced IgE production, which were linked to attenuated Th2-type immune response[170]. One publication in 2006 investigated the importance of LTB_4 produced by mast cells in a $CD8^+$ T cell-mediated allergic response in the airways. In these experiments T cells from CD8-deficient and BLT1-deficient mice were used to elucidate the AHR in OVA-sensitized mice. Results showed the importance of BLT1 expression on activated $CD8^+$ T cells in a mast cell dependent allergen induced AHR-model[171]. The importance of BLT1 expression on T cells for lung rejection and obliterative bronchiolitis have been shown. It was demonstrated that disruption of BLT1 signaling in $CD8^+$ T cells reduces lung inflammation and mortality in a murine model of acute lung rejection. Moreover, the paper also demonstrates reduced fibrosis and AHR in BLT1-deficient mice that undergo tracheal transplants, along with

defective T cell recruitment[172]. The same group also published another paper showing the increase of BLT1 on $CD8^+$ EBV-specific T cells in response to an acute EBV infection[173].

4.3 MONOCYTES

It has been known for more than two decades that monocytes have the ability to synthesize LTB_4 upon stimulation with calcium ionophore (A23187 is the most commonly used ionophore for stimulation of cellular LT formation), but discussions were originally ongoing whether contamination of cell preparations had given rise to misleading conclusions. To clarify this issue, Borgeat and colleagues published, in 1984, investigations on purified mononuclear cell preparations. Stimulation of these cell preparations with calcium ionophore gave rise to high amounts of LTB_4, which were abolished when the purified mononuclear cells were depleted on monocytes[174]. A second way of activating monocytes were published the same year showing that adherent monocytes could be triggered to synthesize LTB_4 upon stimulation with zymosan particles[175]. Two years later it was reported on activation of 5-LO and production of LTB_4 in monocytes stimulated with fMLP after pretreatment with cytochalsin B[176]. Another publication investigated the effects of concanavalin A on purified monocytes. It was found that treatment of monocytes with concanavalin A and PMA, in combination, resulted in LTB_4 release in similar amounts as produced upon calcium ionophore stimulation[177]. It has been shown that IL-4 induced IgE production by PBMC was significantly enhanced by LTB_4. This stimulating effect of LTB_4 was solely dependent on the presence of monocytes[98].

Except for the ability of monocytes to produce LTB_4, it has also been shown that LTB_4 induces chemotaxis, surface CD11b expression and phagocytosis of these cells[178]. In accordance with these results, a paper was published describing the expression of BLT1 on $CD14^{++}CD16^-$ monocytes, which are the primary monocytic population in blood[179]. It has been shown in an atherosclerotic mouse model, that treatment with a BLT1 antagonist (CP-105,696) inhibits monocytic foam cell migration and the expression of CD11bn[134].

5 CELLULAR REGULATION AND LOCALIZATION OF 5-LO

Human 5-lipoxygenase (swissprot accession number P09917) is a 78 kDa protein consisting of 674 amino acids (aa). The enzyme contains a catalytic active non-heme iron, which is active in its ferric form (Fe^{3+})[180]. Several factors assist 5-LO in the leukotriene formation either by direct induction of enzyme activity or by stabilizing the enzyme (indirect induction of enzyme activity). These factors are:
1. Phosphatidylcholine (PtdCho), which is required for basal 5-LO activity *in vitro* and has also been suggested to promote the translocation of 5-LO to the nuclear membrane[181]. 2. Coactosin-like protein (CLP) binds to 5-LO but does not activate the enzyme alone. Induction of 5-LO activity occurs in synergy with phosphatidylcholine and/or calcium[182]. 3. Calcium ions have been shown to induce 5-LO activity in combination with phosphatidylcholine or coactosin-like protein[180]. 4. Glycerides alone can activate 5-LO *in vitro* and in cells, and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) has been determined to be the most potent glyceride[183]. Based on mutational studies, the above mentioned factors bind to 5-LO on the C2-like domain[180]. 5. A fifth factor that is known to stabilize 5-LO and help to facilitate high enzyme activity is ATP. (See table 2 for a summary).

Effect on 5-LO	Factor
↑ activity *in vitro* Promotes translocation to the nuclear membrane	Phosphatidylcholine
↑ activity in synergy with PtdCho/Ca^{2+} Enzyme stability	Coactosin-like protein
↑ activity in synergy with PtdCho/CLP	Ca^{2+}
↑ activity	Glycerides
Enzyme stability	ATP

Table 2. *Factors affecting 5-LO.*

Many efforts have been made to produce a protein crystal and solve the 3-dimensional structure of the human enzyme, but so far no successful reports have been published. However, a putative 3-dimensional structure has been created on the basis of the crystal structure of rabbit reticulocyte 15-lipoxygenase [184]. The putative structure model of the human enzyme consists of a smaller N-terminal domain (aa residues 1-114) (a.k.a. C2-like domain), and a larger catalytic domain (aa residues 121-673).

Cellular distribution of 5-LO has been extensively investigated. The enzyme has been reported to reside in the cytosol, nucleus, and nuclear membranes. In a study on the subcellular localization of phospholipase A_2 (PLA_2), 5-LO and FLAP in rat peritoneal macrophages, it was shown that both cytosolic PLA_2 and 5-LO redistributed from the cytosol fraction in resting cells to the nuclear fraction in activated cells. At the same time it was shown that FLAP was localized predominantly in the nuclear fraction of both resting and activated cells[185]. Another report demonstrated that FLAP was located on the nuclear envelope in both resting and ionophore stimulated cells. This is in contrast to 5-LO, which is found in the nuclear envelope, only in stimulated cells. In resting cells 5-LO could not be detected along the nuclear envelope[186]. Further investigations have demonstrated that 5-LO localization is cell type dependent. In experiments with rat basophilic leukemia (RBL) cells, it was found that 5-LO was present in both the nucleus and the cytosol[187]. Similar results were found in human alveolar macrophages but with a predominant presence of 5-LO within the nucleus[188]. In both cell types, stimulation with A23187 directed the cellular distribution of 5-LO to the nuclear envelope[187,188]. Studies on the localization of 5-LO in B cells (peripheral B cells, chronic B lymphocytic leukemia cells and BL41-E95-A) showed a predominant expression of 5-LO within the nucleus, which did not alter upon cell activation (with Diamide plus A23187 and AA)[158]. Another publication on 5-LO in B cells (BL41-E95-A) identified 5-LO in the non-nuclear fraction[189]. A publication investigated the hypothesis whether or not neutrophil recruitment into sites of inflammation can alter the subcellular distribution of 5-LO. It was shown that 5-LO in neutrophils, both from rats and humans, translocated from the cytosol to the nuclear membrane upon adherence[190]. A summary over the cellular localization of 5-LO is presented in table 3.

Cell type	Resting cells	Activated cells	Method	Reference
Rat peritoneal macrophages	Cytosol	Nucleus	Western blot	185
Human monocytes and neutrophils	Cytosol	Nuclear membranes	Electron microscope	186
Rat basophilic leukemia cells,	Cytosol and Nucleus	Nuclear membranes	Western blot and Immunofluorescent microscopy	187
Rat alveolar macrophages	Cytosol and Nucleus	Cytosol and Nuclear membranes	Western blot and Immunofluorescent microscopy	187
Human alveolar macrophages	Cytosol and Nucleus	Cytosol and Nuclear membranes	Western blot, Electron microscopy, Immunofluorescent microscopy	188
Human peripheral B cells, B-CLL, BL41-E95-A	Nucleus	-	Immuno-histochemistry	158
B-CLL	Nucleus	Nucleus	Western blot	158
BL41-E95-A	Cytosol	Cytosol	Western blot	189

Table 3. *Distribution of 5-LO in different cell types.*

Except for the factors mentioned above, phosphorylation has been reported to be another way of altering the subcellular distribution of 5-LO and enzyme activity. Three sites of phosphorylation have been identified which can affect 5-LO activity either by inducing or inhibiting the enzyme. The effects can be a direct effect of the enzyme activity or an indirect effect, which has been correlated to the intracellular localization of 5-LO. This has been shown using in-gel kinase assays of total cell lysates of PMNL and mono mac 6 cells that 5-LO is a substrate for mitogen-activated protein kinase-activated protein kinase 2 (MK2). Bioinformatic searches suggest that Ser271 is the motif being phosphorylated. This phosphorylation motif has also shown to be involved in the activation of the enzyme[191]. It has been demonstrated that various cell stress inducing agents (e.g. sorbitol, NaCl, hydrogen peroxide and Diamide) can activate 5-LO in B cells. This occurred via the p38 MAP kinase pathway, where MK2 is one of the down stream substrates, indicating 5-LO activation via phosphorylation at

Ser271[189]. In another paper, more evidence on the putative Ser271 phosphorylation site was shown[192]. Serine 271 was mutated to alanine and studied using in-gel and *in vitro* kinase assays. It was shown using radioactive phosphate in an *in vitro* kinase assay with MK2, that mutated 5-LO could not be phosphorylated when activated with AA. It was also shown that calcium/calmodulin-dependent kinase II (CaMKII) and protein kinase A (PKA) can phosphorylate 5-LO, but phosphorylation of 5-LO by these kinases could not be induced with AA. The increased activation of 5-LO, induced by phosphorylation at Ser271 was dependent on unsaturated fatty acids (UFAs)[192]. Furthermore *in vitro* kinase strategies were used to elucidate a new phosphorylation motif on 5-LO. It was shown that Ser663 is phosphorylated by extracellular signal-regulated kinase 2 (ERK2). In a similar fashion as MK2, phosphorylation at Ser663 was regulated by UFAs[193]. The third phosphorylation site that has been demonstrated is Ser523, which will be described more detailed below.

One publication reported on the importance of cAMP in regulating 5-LO in PMNL[194]. They discovered that a variety of intracellular cAMP elevating agents inhibit LT biosynthesis and 5-LO translocation to the nucleus. Experiments with PKA inhibitors prevented the inhibitory effect of cAMP on 5-LO activity and translocation. It was also shown that intracellular cAMP increase inhibited phosphorylation of p38 MAP kinase in activated PMNL[194]. Histamine is usually regarded as a pro-inflammatory mediator, therefore it was intriguing when it was published to have an inhibitory effect on LTB_4 formation in activated PMNL[195]. The effect of histamine was shown to be mediated through the H_2 receptor by inhibiting 5-LO translocation to the nuclear membrane and inhibiting release of AA. Specific inhibitors of PKA prevented the inhibitory effect of histamine, showing the importance of this kinase in modulating 5-LO activity[195]. A biochemical explanation for the inhibitory effect of cAMP and PKA on LT biosynthesis was published in 2004 when it was shown using *in vitro* kinase assays and co-transfection of 5-LO with the catalytic subunit of PKA that PKA could phosphorylate 5-LO and significantly reduce the LT biosynthesis. Mutational studies of 5-LO were in agreement with the observed phosphorylation effects[196].

Several groups have investigated the mechanism regulating the cellular distribution of 5-LO. One paper reported the existence of nuclear localization signals (NLS) and nuclear export signals (NES) based upon GFP fusion proteins and inhibitors of NES-dependent transport[197]. Another paper summarized the three known NLSs (Leu111-

Asp121, Asp156-Asp166 and Val514-Leu535) of 5-LO and their complex interaction in regulating the subcellular distribution of 5-LO[198]. It was demonstrated that phosphorylation of Ser523, which is situated in one of the NLS of 5-LO, resulted in a predominant cytoplasmic distribution of 5-LO (figure 3). Furthermore, it was concluded that intracellular cAMP levels affect PKA, which induces the phosphorylation[199]. In the context of cAMP inhibition of 5-LO activity and translocation, it was found that exogenous AA completely prevented the cAMP induced effects in PMNL. These results were not AA specific because 18- and 20-carbon polyunsaturated fatty acids (PUFAs) containing at least three double bonds caused the same phenotype[200]. A recently published paper explored previously known data about phosphorylation of Ser271 and its effect on cellular localization of 5-LO. 5-LO is constitutively phosphorylated on Ser271 in NIH-3T3 cells overexpressing 5-LO. This phosphorylation on 5-LO in NIH-3T3 cells is associated with nuclear accumulation of the enzyme (figure 3). Mutation of Ser271 to alanine altered the cellular distribution in NIH-3T3 cells, but did not affect enzyme activity or association to the nuclear membrane. Moreover, no phosphorylation of Ser271 could be observed on 5-LO overexpressed in HeLa cells[201].

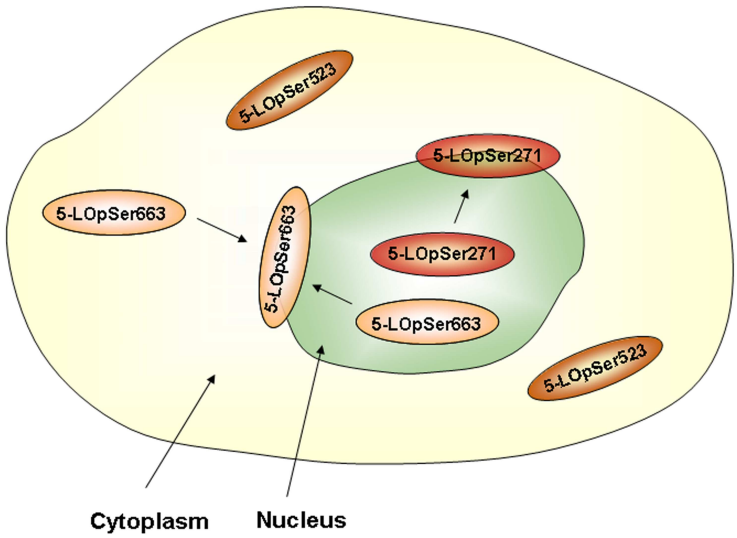

Figure 3. *Phosphorylation dependent localization of 5-LO.*

6 B LYMPHOCYTES

6.1 DEVELOPMENT OF NORMAL B LYMPHOCYTES

B lymphocytes are derived from the bone marrow. The cells mature in the spleen and lymph nodes and express surface immunoglobulin (Ig) receptors. The final step in the B cells life cycle is to either differentiate to plasma cells or memory cells[202]. The research field of lymphocytes began to grow in the 1960´s, when B and T cells were first discovered[202].

The life of a B lymphocyte begins in the bone marrow, starting with the hematopoietic stem cell (HSC) which via the multipotent progenitor cell (MPP) becomes the common lymphoid progenitor cell (CLP) (figure 4)[203]. Common lymphoid progenitor cells are the origin of all lymphoid derived cells including natural killer cells, T cells and B cells. A schematic picture of the development of B lymphocytes is depicted in figure 4.

B cells that leave the bone marrow are referred to as mature but immunologically naïve B cells [204]. These cells migrate to the peripheral lymphoid tissue e.g. spleen, tonsil and lymph nodes, where they encounter and respond to T-cell dependent foreign antigens bound to follicular dendritic cells (FDC). FDCs can attract B cells and aid in their differentiation into either plasma or memory cells.

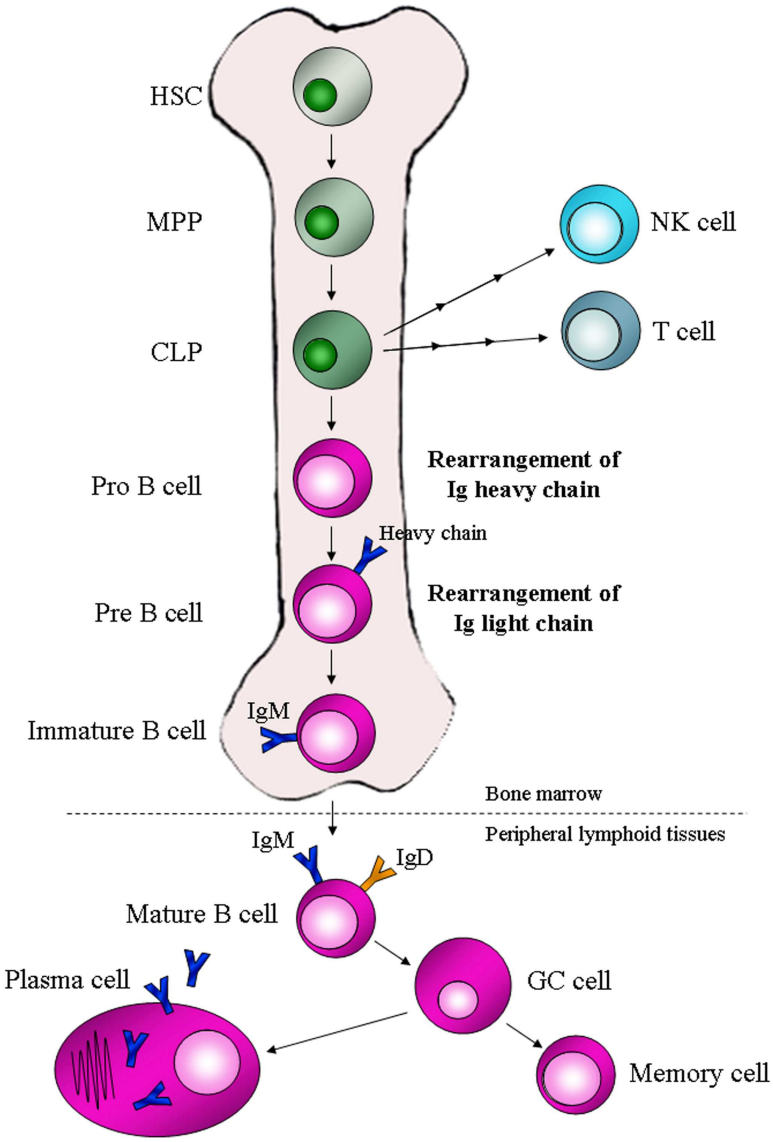

Figure 4. *Development of B lymphocytes. HSC – hematopoietic stem cell, MPP – multipotent progenitor cell, CLP – common lymphoid progenitor cell, Pro B cell – progenitor B cell, Ig – immunoglobulin, GC – germinal center.*

Lymph nodes have a defined architecture where cell types are segregated (figure 5). A lymph node consists primarily of B and T cells, which compartmentalize into a follicle (consisting of B cells) and a T cell zone (consisting of T cells). A follicle can be divided into two main areas, the mantle zone and the germinal center. The germinal center is the center of the follicle, which is surrounded by the mantle zone. B cells that reside in the mantle zone are naïve B cells (non-activated) and are also called mantle zone B cells. B cells that are found in the germinal center are activated B cells which undergo a differentiation process that includes somatic hypermutation, affinity maturation and isotype class switching. The activated B cells will differentiate into either memory B cells or plasma cells. During this differentiation process proliferating B cells reduce their expression of immunoglobulins, particularly IgD.

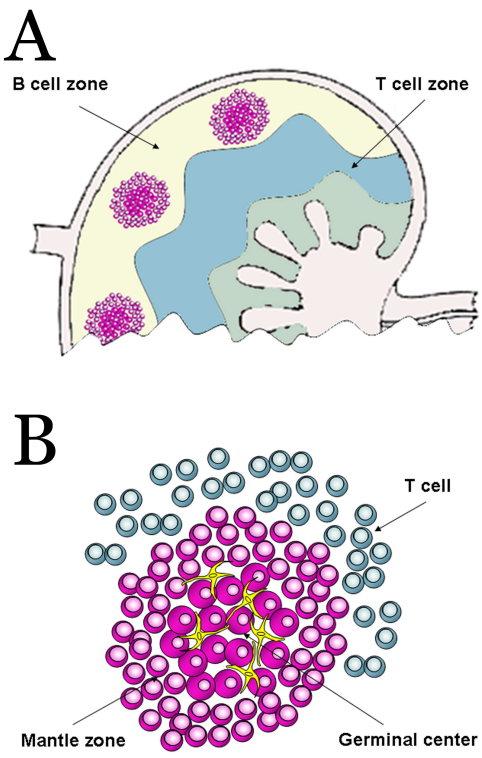

Figure 5. *Schematic picture of a lymph node. Figure 5A depicts an overview of a lymph node with marked B cell zone and T cell zone. Figure 5B depicts the two main areas (germinal center and mantle zone) of a lymphoid follicle.*

6.2 CHRONIC B LYMPHOCYTIC LEUKEMIA

Chronic B lymphocytic leukemia (B-CLL) is a type of cancer originating from the bone marrow. The disease is characterized by a slow accumulation of malignant B cells, which escape apoptosis via a variety of mechanisms[205]. B-CLL is most common in individuals over 50 years of age with a median age of approximately 65 years. It is the most common leukemia in the Western part of the world and is two times more common in men than in woman. The incidence in Sweden is about 5 cases per 100,000 individuals.

B-CLL is diagnosed by morphological examinations and analysis of cell specific antigens including CD5, CD19, CD20, CD23 and surface membrane immunoglobulin[206].

The disease is very heterogeneous with variable clinical outcome. Classification of the different clinical stages are made according to guidelines set by Rai and Binet[207,208], which are the basis for therapeutic decisions. Approximately one third of the patients display symptoms (e.g. weight loss, fatigue, and fever) and require immediate treatment. Another third have an indolent course which can progress. The remaining third never require treatment and die from other causes[209]. Recently many new prognostic features have been identified which may in the future be helpful when deciding on a therapeutic approach[210].

6.3 MANTLE CELL LYMPHOMA

Mantle cell lymphoma (MCL) is a well-defined cancer compared to many other lymphomas. It is predominant in elderly men and represents 5-10% of all non-Hodgkins lymphomas[211,212]. Commonly, the disease is very aggressive with short responses to treatment and frequent relapses. Currently available therapies are sub-optimal and few patients are cured[211,212]. The primary event in the pathogenesis of the tumor is the constitutively overexpression of cyclin D1, which is not normally expressed in B lymphocytes. Cyclin D1 is involved in the cell cycle mechanism, facilitating progression of the cell through G1 into S phase proliferation[211,212]. Overexpression of cyclin D1 occurs due to a t(11;14)(q13;q32) translocation. This translocation results in movement of the cyclin D1 gene to the Ig heavy chain gene enhancer region, thereby causing its overexpression[211,212]. The neoplastic cells in MCL are thought to arise from mature naïve B lymphocytes, which normally express surface

expression markers such as IgD and CD5[211,212]. These cells normally reside in the mantle zone of the lymphoid follicle.

7 EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) is named after Anthony Epstein and Yvonne Barr. They discovered the virus in collaboration with Bert Achong in 1964 from African tumor specimens sent to them by Denis Burkitt[213]. EBV belongs to the family of herpes viruses and is also called human herpes virus 4. EBV is considered a very successful virus infecting more than 90% of the world's adult population. When infected with EBV, the virus persists in the host for life, like all herpes viruses. Commonly the virus doesn't cause any outbreak of disease, but in some primary infections an outbreak of infectious mononucleosis (IM) can occur[214]. Infectious mononucleosis commonly occurs in young adults and is characterized by lymphadenopathy, fever and pharyngitis. Hematological features of IM include blood lymphocytosis that consists mainly of EBV-specific $CD8^+$ T cells and increased numbers of EBV-infected B cells[214].

8 RESULTS AND DISCUSSION

8.1 PAPER I

Leukotriene B_4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells

The biological role of leukotrienes in B lymphocytes is poorly understood in comparison to myeloid cells. B lymphocytes express 5-LO as myeloid cells, but do not produce leukotrienes upon challenge with calcium ionophore (A23187). To activate the biosynthesis of leukotrienes in intact B cells, stimulation with Diamide (a thiol-reactive reagent) is needed together with AA and A23187[93]. It has been reported that LTB_4 affect B cells in different ways e.g. activation, proliferation and increased antibody production[93,98,159]. Microarray studies have shown 5-LO to be one of the most abundant expressed genes in chronic B lymphocytic leukemia (B-CLL)[215]. It was therefore of interest to investigate whether B-CLL cells had the capacity to produce leukotrienes and which role 5-LO plays in the function of these cells.

Low amounts of leukotrienes were detected when B-CLL cells were challenged with AA and A23187. B-CLL cells produced similar amounts of LTB4 as neutrophils after treatment with Diamide (as described above) and also in sonicated cells incubated with AA, ATP and calcium.

B-CLL cells were subjected to flow cytometry analysis to investigate if they expressed the BLT1 receptor. Cells from six patients with B-CLL and two patients with prolymphocytic B cell leukemia (B-PLL) were investigated; average expression of BLT1 was 42% and 74% of the cells, respectively. As a comparison, 30-50% of the normal B lymphocytes did also express the BLT1 receptor.

In order to determine whether leukotrienes could affect the proliferation of B-CLL cells, CD40-ligand expressing L cells (CD40L-L) were used to activate the B-CLL cells in combination with LTB_4 and two leukotriene biosynthesis inhibitors. The proliferation rate was determined with thymidine incorporation assay. When B-CLL cells were cultivated with control L cells (96 hrs) only minimal thymidine incorporation was observed in contrast to cultivation with CD40L-L cells (96 hrs) where a significant increase was observed. In the presence of 100 nM MK-886 (a specific FLAP inhibitor)[216] a significant inhibition of thymidine incorporation was seen. BWA4C, a specific 5-LO inhibitor, also significantly inhibited the proliferation at 10 nM.

Exogenously added LTB_4 (150 nM) almost completely reversed the inhibitory action of MK-886 and BWA4C. LTB_4 alone did not further amplify the CD40-induced thymidine incorporation.

Flow cytometric analysis demonstrated that CD40-CD40L interactions caused an increased expression of CD23, CD54 and CD150. MK-886 (100 nM) and BWA4C (100 nM) markedly counteracted the antigen expressions. Addition of exogenous LTB_4 (150 nM) almost completely reversed the effects of MK-886 and BWA4C. LTB_4 alone did not further induce CD23, CD54 and CD150 expression.

In this paper we provide evidence that 5-LO in B-CLL cells can, as in normal B lymphocytes, be activated during certain conditions[48,155]. CD40-CD40L interaction caused a pronounced increased proliferation of B-CLL cells detected by thymidine incorporation assay and antigen expressions analyzed using flow cytometry. MK-886 (a specific FLAP inhibitor) and BWA4C (a specific 5-LO inhibitor) markedly inhibited the proliferation both on DNA synthesis and expression of CD23, CD54 and CD150. Interestingly, exogenous LTB_4 reversed the effects of the inhibitors. These results are in agreement with earlier findings that LTB_4 affects the activation and proliferation of B cells[93]. It is known that normal B cells barely express CD54, while B cells from CLL patients have an increased expression[217]. High expression is associated with poor prognostic features, including increased tumor burden and sometimes a short lymphocyte doubling time[217,218]. CD23 has been proposed to be involved in cell viability and proliferation[219]. High serum levels of soluble CD23 was associated with high tumor burden and shorter time to progression in B-CLL[220,221]. It has also been reported that LTB_4 stimulate the expression of CD54 on endothelial cells and CD23 on B cells[97,159,222]. 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, LTA_4 hydrolase inhibitors, or BLT1 antagonists, alone or in combination with conventional therapy, might also be useful in the treatment of B-CLL cells.

8.2 PAPER II

Leukotriene B_4 activates T cells that inhibit B-cell proliferation in EBV-infected cord blood-derived mononuclear cell cultures

In this paper we used cord blood-derived mononuclear cells (CBMC) to determine the importance of LTB_4 during EBV-infections. EBV-specific cellular immunity is not transferred from mother to child, which makes CBMC an excellent system to study primary EBV-infections. To elucidate LTB_4 specific effects, inhibitors of LTB_4 biosynthesis (MK886, a specific FLAP inhibitor and BWA4C, a specific 5-LO inhibitor) were used.

Addition of the immunomodulators polysaccharide K (PSK) and thioredoxin 80 (Trx80) to EBV-infected cultures activated T and NK cells which was measured by increased expression of signalling lymphocyte-activation molecule-associated protein (SAP)[223]. Interestingly, addition of MK886 (1 μM) or BWA4C (100 nM) to these cultures reduced the expression of SAP. Reduction of SAP expression was restored by the addition of exogenous LTB_4 (100 nM).

It has previously been shown that addition of PSK and Trx80 to the EBV-infected CBMC cultures induces the amount of IL-15 and IL-12 present in the cultures, respectively. MK886 and BWA4C reduced the level of these cytokines, which was restored when exogenous LTB_4 was added. As a consequence, addition of immunomodulators inhibited EBV-induced B cell proliferation, which was counteracted with MK886 and BWA4C. Again, addition of exogenous LTB_4 reversed the effects of the inhibitors. A more pronounced inhibition of activation was observed when inhibitors were used simultaneously.

In agreement with these findings, LTB_4 was detected in EBV-infected CBMC cultures primed with immunomodulators. Monocytes were found to be the source of LTB_4. The cytokine interferon-γ (IFN- γ) was also detected in the virus infected cell cultures. The presence of IFN- γ was also dependent upon the stimulation with immunomodulators. Furthermore, it was shown that neutralizing cytokines (IL-15, IL-12, IFN- γ) with specific antibodies markedly reduced the amount of LTB_4 that could be detected in the cell cultures.

We explored the expression of BLT1 (the high affinity expression of LTB_4) in the lymphocytic population. BLT1 was expressed in *ex vivo* B lymphocytes, but was

abolished in the cell cultures. On the other hand, T cells in infected cultures did express BLT1, while T cells in non-infected cultures did not express BLT1.

Next, we investigated if LTB_4 had the same immunostimulating effect as PSK and Trx80 in EBV-infected CBMC cultures. We showed, by SAP expression, that LTB_4 (100 nM) activated NK and T cells in EBV-infected cultures. The stimulating effect of LTB_4 was specific, since neither LTC_4, LTD_4 nor 5,12-DiHETE resulted in increased SAP expression. Activation of T cells resulted in decreased B cell proliferation. IFN- γ and IL-18 were detected in LTB_4-activated CBMC cultures. Depletion of monocytes from these cultures resulted in decreased IFN-γ and abolished the presence of IL-18 in the cell cultures.

To understand the effect of LTB_4 in EBV-infected CBMC cultures, we added exogenous LTB_4 to the cell cultures depleted on monocytes. This activated T and NK cells and inhibited the B cell proliferation. The stimulatory effect of cytokines (IL-18, IL-15 and IL-12) on T and NK cells were also found to be important. To determine the source of SAP expression, which was used as the marker for T and NK cell activation, CBMC cultures were depleted of T cells. Stimulation of these cultures with LTB_4 did not result in any increase in SAP expression, providing further evidence of the specific effect of LTB_4 on T cells.

The composition of cells was compared in EBV-infected CBMC cultures stimulated with LTB_4 versus EBV-infected CBMC cultures without any LTB_4 stimulation. $CD4^+$ T cells were the dominant cell type in the LTB_4 stimulated cultures. In contrast, B lymphocytes were the dominant cell type in the non-stimulated culture.

Transformation of B lymphocytes by EBV initiates events leading to activation of T and NK cells. Activation of T and NK cells results in a complex interplay between B cells, T cells, NK cells and monocytes. We found that LTB_4 has an important function in the response against EBV-infected B lymphocytes. The effect of LTB_4 was mediated by BLT1 which was expressed on activated T cells and monocytes. Monocytes were the source of LTB_4 and cellular activation also resulted in production of other cytokines (IFN-γ and IL-18). Use of specific leukotriene biosynthesis inhibitors led to increased B cell proliferation. Our results suggest the importance of LTB_4 for EBV initiated infections and also as a link between the innate and adaptive immunity.

8.3 PAPER III

High expression of 5-lipoxygenase in normal and malignant mantle zone B lymphocytes

The expression of 5-LO in peripheral and malignant B lymphocytes has been described[48,117,158,224]. However, the expression of 5-LO in different subtypes of tonsillary B cells is not known. In this paper we have investigated the expression of 5-LO in mantle zone B cells, germinal center (GC) B cells, plasma cells, memory cells, mantle cell lymphoma (MCL) and follicular lymphoma (FL).

The gene expression of proteins involved in the leukotriene biosynthesis pathway was investigated using RT-PCR. This was performed on isolated RNA from different subtypes of tonsillary B cells. 5-Lipoxygenase, FLAP and LTA_4 hydrolase were expressed in tonsillary B cells ($CD19^+$), mantle zone B cells ($CD19^+$, $CD38^+$, IgD^+), memory B cells ($CD19^+$, $CD38^+$, IgD^-), and GC B cells ($CD19^+$, $CD38^{++}$, IgD^-). Mantle zone B cells were found to have the highest relative expression of 5-LO, in contrast to GC B cells where the lowest relative expression was found. None of the investigated subtypes expressed the gene for LTC_4 synthase.

To confirm the gene expression results, the protein expression of 5-LO was investigated using Western blot. The expression of 5-LO was detected in mantle zone B cells, GC B cells and memory B cells. High expression was found in mantle zone B cells and memory B cells, and low expression in GC B cells. In contrast, plasma cells ($CD19^+$, $CD38^{+++}$, IgD^-) did not express any 5-LO.

Tonsils obtained from tonsillectomies were investigated with immunofluorescence for the expression of 5-LO in tissue. Antibodies against CD38, CD138, and IgD were used to differentiate the B cell subtypes and elucidate the 5-LO protein expression. High expression of 5-LO was found in mantle zone B cells, but very low expression in GC B cells and no expression in plasma cells. These results were in agreement with obtained Western blot results.

It has been proposed that the cell of origin in MCL is mantle zone B cells[211,212]. Follicular lymphoma (FL) originate from germinal center B cells[211]. Consequently, we investigated if the 5-LO expression in these lymphomas resembled 5-LO expression seen in non-malignant B lymphocytes. In total, 10 MCL and 10 FL biopsies were immunohistochemically investigated for 5-LO expression. It was found that most of the MCL samples expressed high amounts of 5-LO, whilst most of the FL samples did not

express 5-LO. Thus, the expression of 5-LO was similar in primary non-malignant B lymphocytes and corresponding malignant B lymphocytes.

In order to investigate the capacity of mantle zone B cells to produce leukotrienes, cyclin D1 positive prolymphocytic B cell leukemia cells (B-PLL, which is considered to be the leukemized form of MCL[225-227]) were investigated. B-PLL cells stimulated with Diamide plus AA and A23187 produced similar amounts of LTB_4 as human neutrophils[228]. Sonicated cells also readily produced LTB_4. Stimulation with A23187 alone resulted in about 1 pmol LTB_4 / 10^6 cells, indicating the low contamination of neutrophils in these preparations. Similar results were obtained with three cell lines derived from MCL. The MCL cell lines Granta519, JEKO1 and Rec1 produced LTB_4, upon stimulation with Diamide plus AA and A23187. The 5-LO expression in the MCL cell lines was confirmed with Western blot, showing the presence of 5-LO protein in all three cell lines.

It has been known for almost 20 years that B lymphocytes express 5-LO, but the mechanism of activation of the enzyme is still not clarified. In this paper we have characterized the 5-LO expression in subsets of B lymphocytes and found 5-LO is mainly expressed in mantle zone B cells. Taken together, this study and earlier reports demonstrate that 5-LO is expressed in relatively immature B cells, peripheral B cells, various malignant B cells, mantle zone B cells and memory B cells but not in plasma cells and the most immature B cells[48,158,224]. However, it is still not known under which physiological conditions 5-LO in B lymphocytes is active. Our findings indicate that the enzyme might have a function in B cells before terminal differentiation into plasma cells.

8.4 PAPER IV

Constitutive phosphorylation of Serine 523 on 5-lipoxygenase in B lymphocytes

Three different sites of phosphorylation have previously been described on 5-LO, Ser271, Ser523 and Ser663[192,193,196]. Phosphorylation of 5-LO has been shown to affect subcellular localization and activity of 5-LO. So far, phosphorylation of 5-LO has been reported in *in vitro* experiments or in cells transfected with 5-LO. Our paper describes phosphorylation of 5-LO on Ser523 in mantle cell lymphoma cells and B-CLL cells.

Protein preparations from three MCL cell lines (Granta519, JEKO1 and Rec1) and primary PMNLs were investigated using Western blot. 5-Lipoxygenase expression was observed in all four samples. Western blot analysis using the antiserum raised against pSer523-5-LO, demonstrated a band at the expected size in the MCL cell lines but not in PMNL.

5-Lipoxygenase isolated from Rec1 cells was purified using an ATP-agarose column on an ÄKTA Purifier and analysed with Western blot. Eluted fractions containing 5-LO was analysed with pSer523-5-LO antibody. This immunoblot displayed a single immunoreactive band, which was assumed to be pSer523-5-LO.

Phosphorylation of 5-LO was confirmed as follows. Purified enzyme was dephosphorylated with alkaline phosphatase. Alkaline phosphate treated and untreated protein samples were analyzed using Western blot and pSer523-5-LO antibody. An immunoreactive band in the untreated sample was observed which was absent in the treated sample.

Protein kinase A (PKA) has been reported to stimulate phosphorylation of Ser523 on 5-LO[196]. To investigate if this could be the case in MCL cells, we preformed studies using a cAMP analogue. Granta519 and JEKO1 cells were treated with IBMX (a phosphodiesterase IV inhibitor), to inhibit the degradation of cAMP, prior to addition of 8-Br-cAMP for 2, 4, 8 and 24h. This combination of treatment markedly increased the phosphorylation of Ser523 which was time course dependent. Treatment with 8-Br-cAMP resulted in approximately doubled phosphorylation levels in Granta519 and JEKO1 cells after 8 and 24 hours, respectively. Prostaglandin E_2, which is known to increase intracellular cAMP levels, also induced phosphorylation of Ser523 in JEKO1 cells after 8 hours of treatment.

To elucidate if expression of pSer523-5-LO is MCL cell line specific or if it also can be detected in *ex vivo* cells from patients with MCL or B-CLL, primary cells from

two MCL patients and three B-CLL patients were investigated using Western blot. All the *ex vivo* samples investigated displayed an immunoreactive band at the right molecular size of 5-LO using pSer523-5-LO antibody.

The expression of 5-LO and the capacity to produce LTB_4 in B cells has been known for almost 20 years, but the biological role in B lymphocytes is still not fully understood. It is known that the cellular oxidative status is of importance for 5-LO activation in B cells. Thus, stimulation with calcium ionophore alone does not activate 5-LO in B lymphocytes, in contrast to myeloid cells where high amounts of leukotrienes can be detected after calcium ionophore stimulation. As far as we know this paper describes for the first time phosphorylation of native 5-LO, both *in vitro* and *ex vivo*. This is also the first description of a chemical difference between 5-LO in myeloid and B cells.

9 CONCLUSION

Leukotrienes and 5-LO have been known and studied by the scientific community for almost 30 years. The expression of 5-LO can mainly be found in myeloid cells and B cells. The pro-inflammatory role of LTB_4 is well established. Leukotriene B_4 activates and induces chemotaxis of leukocytes. Leukotriene B_4 has been shown, in several animal models, to be important for the host defense[88,89]. The majority of the studies published have focused on myeloid cells and much less is known about the function of 5-LO and LTB_4 in lymphocytes. Although, 18 years have passed since the discovery of 5-LO in B lymphocytes, the biological role of this enzyme in B cells is not well understood.

Results in paper I demonstrated that inhibitors of the LT biosynthesis pathway inhibited the activation of chronic B lymphocytic leukemia cell. This inhibitory effect was counteracted by addition of exogenous LTB_4.

The role of LTB_4 in the immune response against EBV-infection was examined in paper II. We found that the expression of BLT1 was down-regulated in infected B cells, but up-regulated in activated T cells. LTB_4 in these cultures, produced by activated monocytes, modulated T cells to inhibit the proliferation of EBV-infected B cells. This study highlights the importance of LTB_4 in host defense against EBV-infections.

The expression of 5-LO in various subtypes of B cells was investigated in paper III. Subtypes of B cells from tonsils were separated and analyzed for 5-LO expression. High levels of 5-LO expression were found in mantle zone B cells. In accordance with these results, the expression of 5-LO was also found in mantle cell lymphoma cell lines and mantle cell lymphoma biopsies.

Phosphorylation of serine 523 (Ser523) on 5-LO in B lymphocytes was investigated in paper IV. Evidence of native pSer523-5-LO was found in mantle cell lymphoma cell lines using Western blot. The presence of pSer523-5-LO was confirmed by purification of the enzyme and dephosphorylation experiments. Activation of protein kinase A induced pSer523-5-LO expression. The expression of pSer523-5-LO was not only restricted to cell lines but was also demonstrated in primary cells *ex vivo* in samples derived from patients with B-CLL or mantle cell lymphoma.

Taken together, LTB_4 is shown to be important for the activation of lymphocytes in the host defense against EBV-infection. The expression of 5-LO has been elucidated

in subtypes of tonsillary B lymphocytes and showed high expression in mantle zone B cells. Our results, together with previously published data demonstrate the expression of 5-LO in relative immature B cells ($CD10^+$ acute pre-B lymphocytic cells), peripheral B cells, IgD^+ mantle zone B cells, and $CD38^+$ memory B cells (figure 6)[48,117,163,224]. We demonstrate that native 5-LO in malignant B cells is constitutively phosphorylated on Ser523. This is the first demonstration of a chemical difference of 5-LO in myeloid cells and B cells.

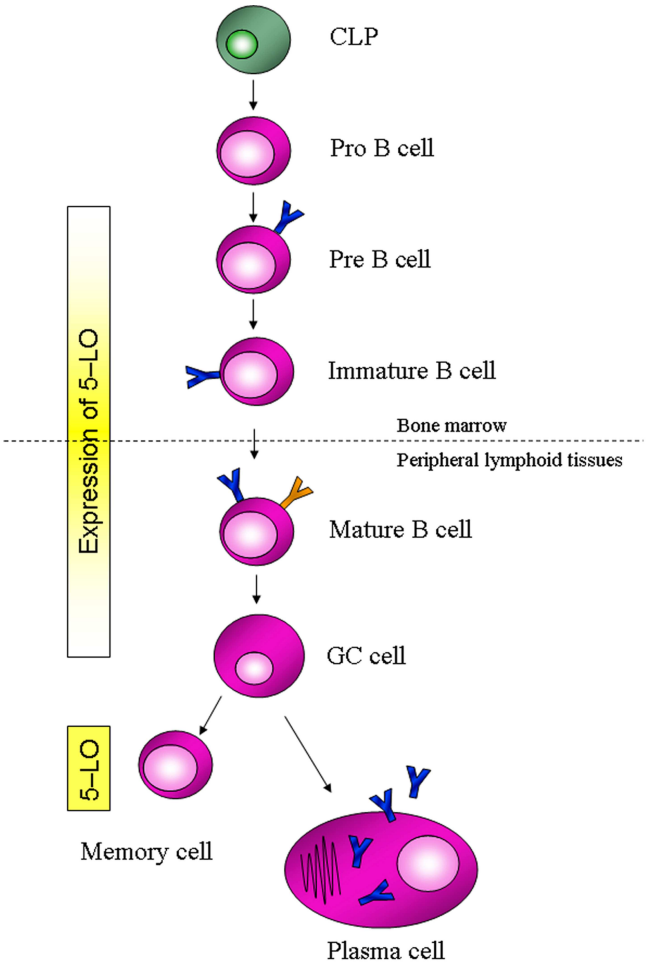

Figure 6. *Expression of 5-lipoxygenase in B lymphocytes. The expression of 5-LO is shown from immature B cells to mature B cells. CLP – common lymphoid progenitor cell, Pro B cell – progenitor B cell, GC – germinal center.*

10 METHODOLOGY

Methodologies used in this thesis are established methods within the fields of biochemistry, immunology and molecular biology. They are listed and described in the respective paper.

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☺

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