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STUDIES ON 15-LIPOXYGENASE IN DENDRITIC CELLS AND LEUKOTRIENE RECEPTORS IN HODGKIN LYMPHOMA

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ABSTRACT

Classical Hodgkin lymphoma (cHL) is histologically characterized by a minority of malignant cells, the so-called Hodgkin-Reed Sternberg (H-RS) cells, surrounded by inflammatory cells such as T lymphocytes, eosinophils, macrophages and mast cells. It is generally believed that various molecules released by the H-RS cells are of major importance in the pathophysiology of cHL. In **paper I,** we report a link between inflammatory cell-derived arachidonic acid metabolites, the cysteinyl leukotrienes (CysLTs), to tumor cell growth and function in this disease entity. Two HL cell lines, L1236 and KMH2, were shown to express functional $CysLT_1$ receptors, responding with a robust calcium signal upon challenge with LTD_4. Incubation of L1236 cells with LTD_4 not only stimulated DNA synthesis but also the transcription and protein release of tumor necrosis factor-α, interleukin-6 and -8. Importantly, all these LTD_4-induced effects were blocked by the $CysLT_1$ receptor-specific antagonist zafirlukast. Immunohistochemical studies of classical HL biopsies and microarray analysis of laser captured cells revealed that the $CysLT_1$ receptor is expressed also by the H-RS cells *ex vivo*. Since these cells are surrounded by CysLT-producing eosinophils, macrophages and mast cells, our results suggest the CysLTs as novel mediators in the pathogenesis of cHL by contributing to the growth and the cytokine features of this tumor.

Lipoxygenases are highly regulated enzymes that catalyze the introduction of molecular oxygen in polyunsaturated fatty acids. The lipoxygenases are classified with respect to their positional selectivity of the substrate arachidonic acid. 15-lipoxygenase-1 (15-LO-1), an enzyme implicated in several pathophysiological conditions, possesses the ability to oxygenate free fatty acids and fatty acids bound to membrane phospholipids. The regulation of the enzymatic activity of membrane associated 15- LO-1 is poorly understood. In **paper II** we demonstrate that calcium ionophore stimulates the translocation of 15-LO-1 to the plasma membrane in human dendritic cells. Furthermore, as shown in a protein-lipid overlay assay, 15-LO-1 was shown to interact with several phosphoinositides. In the presence of calcium, addition of phosphatidylinositol-4.5-bisphosphate ($PI(4.5)P_2$) or phosphatidylinositol-3.4-bisphosphate ($PI(3.4)P_2$) to the vesicles containing arachidonic acid led to a significantly increased formation of 15- hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15-HETE) compared to vesicles without phosphoinositides. Kinetic studies further revealed that vesicles containing $PI(4.5)P_2$ or $PI(3.4)P_2$ lowered the Km value (substrate concentration required for maximum enzymatic velocity) for 15-LO-1, whereas Vmax (the maximum enzymatic velocity) was unaffected. These results suggest that 15-LO-1 activity also might be regulated by the phospholipid constitution of membranes.

LIST OF PUBLICATIONS

I. **F Schain**, Y Tryselius, J Sjöberg, L Backman, M Malec, A Porwit-MacDonald, D Xu, Karl RN Baumforth, Paul G Murray, M Björkholm and H-E Claesson. Evidence for a pathophysiological role of cysteinyl leukotrienes in classical Hodgkin lymphoma. *Submitted*

II. E Andersson, **F Schain**, M Svedling, H-E Claesson and P Forsell. Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity. *Biochimica et Biopfysics Acta*, 2006. 1761(12): p. 1498-505

CONTENTS

LIST OF ABBREVIATIONS

12-HETE	12-hydroxyeicosa-5E,8Z,10Z,14Z-tetraenoic acid
15-HETE	15-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid
13-HODE	13-hydroxyoctadeca-9Z,11E-dienoic acid
5-LO	5-lipoxygenase
15-LO-1	15-lipoxygenase-1
cHL	Classical Hodgkin lymphoma
CysLT	Cysteinyl leukotriene
DC	Dendritic cell
FITC	Fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
H-RS	Hodgkin-Reed Sternberg
IL	Interleukin
JAK	Janus kinase
GM-CSF	Granulocyte macrophage colony stimulating factor
LT	Leukotriene
MC	Mixed cellularity
NS	Nodular sclerisis
PE	Phosphatidylethanolamine
PG	Prostaglandin
PI	Phosphatidylinositol
$PI(3.4)P_2$	Phosphatidylinositol-3.4-bisphosphate
$PI(4.5)P_2$	Phosphatidylinositol-4.5-bisphosphate
PTX	pertussis toxin
SRS	Slow releasing substance
STAT6	Signal and activator of transcription 6
TNF	Tumor necrosis factor

1 INTRODUCTION

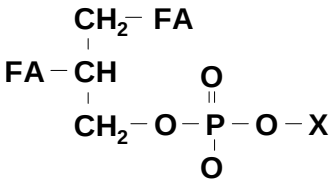
1.1 LIPIDS

Lipids are important hydrophobic molecules involved in an array of diverse biological functions. They not only constitute the foundation for all biological membranes, but also serve as energy stores as well as intra- and intercellular signaling molecules. Based on structure and physical properties, lipids are classified into different subgroups such as fatty acids and phospholipids. Fatty acids, carboxylic acids with long-chain hydrocarbon groups, are referred to as saturated or unsaturated with respect to the absence or presence of double bonds, respectively. The general formula for a fatty acid is CH_3-$(CH_2)_n$-COOH, where n is ≥ 6.

Arachidonic acid, a polyunsaturated fatty acid of particular interest to this study, is mainly esterified to phospholipids in cell membranes. The systemic name of arachidonic acid is 5,8,11,14-eicosatetraenoic acid, symbolized as $C_{20:4}$, indicating a total of 20 carbons (twenty in Greek is eicosi) and the presence of four double bonds at the indicated positions [1]. Upon certain stimuli, arachidonic acid is released and metabolized to eicosanoids, defined as lipid mediators derived from 20 carbon polyunsaturated fatty acids [1]. Arachidonic acid is the quantitatively dominating eicosanoid precursor. The eicosanoid family includes lipoxygenase products (leukotrienes (LTs) and lipoxins) and cyclooxygenase products (thromboxanes and prostaglandins) [2].

Most phospholipids consist of a glycerol backbone, two esterified fatty acids, and a polar alcohol (Figure 1). If the alcohol is ethanolamine or inositol, the phospholipid is referred to as phosphatidylethanolamine (PE) or phosphatidylinositol (PI), respectively. Furthermore, if two phosphate groups are present at position 3 and 4 in a PI, it is referred to as phosphatidylinositol-3.4-bisphosphate or PI(3.4)P_2.

Fig 1



FA; fatty acid
X; alcohol

1.2 LIPOXYGENASES

Lipoxygenases are highly regulated enzymes that catalyze the introduction of molecular oxygen in polyunsaturated fatty acids. The lipoxygenases are classified with respect to their positional selectivity of arachidonic acid [3].

Since 5-lipoxygenase (5-LO) is the key enzyme for leukotriene (LT) biosynthesis, the regulation of this enzyme has been well studied. Calcium is known to be essential for the catalytic activity of 5-LO by promoting its translocation from the cytosol to membranes [4]. Also, five-lipoxygenase activating protein (FLAP) is necessary for LT synthesis. The mechanism of FLAP action is however not completely clear although it has been suggested that FLAP acts by presenting arachidonic acid to 5-LO [5]. Furthermore, tyrosine residue phosphorylation of 5-LO has been suggested to increase LT synthesis [6].

The rabbit reticulocyte 15-lipoxygenase (15-LO-1) was discovered in the 1970´s [7], and some ten years later the human orthologue was described in eosinophils [8]. Two types of human 15-LO have been cloned, possessing a low degree of sequence homology and different tissue distribution. The 15-LO-1 is mainly expressed by epithelial cells in the upper airways, dendritic cells, reticulocytes and eosinophils, whereas the epidermis 15-LO (15-LO-2) is primarily found in lung, hair root, cornea and prostate [9]. The orthologue to 15-LO-1 in animals, except for rabbits, is the so called leukocyte 12-LO. This enzyme has similar enzymatic properties, expression, distribution and regulation as the human 15-LO-1, but

converts arachidonic acid mainly to 12-HETE. The enzyme is therefore often called 12/15-LO [9]. The crystal structure of rabbit 15-LO-1 reveals an N-terminal domain with a presumed lipase activity, as well as a C-terminal catalytic domain. With the methyl group first, the fatty acid is thought to slide into the substrate binding pocket of 15-LO-1. The interaction between the enzyme and the substrate has been suggested to occur at both the methyl and the carboxyl terminus [10]. Since no oxygen cavity has been identified in the rabbit 15-LO-1, it is unlikely that the enzyme specifically positions the oxygen in close proximity to the substrate, rather, the rate of oxygenation is thought to be controlled by oxygen diffusion [9].

The expression of 15-LO-1 is strictly regulated on a transcriptional, translational and post-translational level [11]. Interleukin (IL)-4 and IL-13 have been shown to induce 15-LO-1 transcription via the signal and activator of transcription (STAT) 6/ Janus kinase (JAK) signaling pathway [12]. Furthermore, calcium-dependent membrane-association has been implicated as an important post-translational control mechanism [13].

The biological role of 15-LO-1 is at present not clear, although a role in cell differentiation has been described. For instance, the enzyme is thought to be important during erythropoiesis by contributing to mitochondrial degradation [14]. However, 12/15-LO knockout mice did not reveal impaired erythropoiesis, suggesting that at least in mice, 15-LO is not essential for erythropoiesis [15]. The15-LO-1 products 15-HETE and 13-HODE have been shown to exhibit both pro- and anti-inflammatory characteristics. In fact, 15-HETE has been shown to increase mucus secretion [16] and bronchial smooth muscle cell contraction [17], implicating 15-LO-1 in the pathogenesis of asthma. However, these results are contradicted in other studies [18]. Furthermore, 15-LO-1 expression has been shown to correlate with disease progression in prostate carcinoma [19]. In contrast, 15-LO-1 products were shown to suppress cell proliferation and exhibit pro-apoptotic features in colon cancer [20].

1.3 DENDRITIC CELLS

The immune system can be divided into two interdependent branches; the nonspecific innate immunity, constituting the first line of defense upon pathogen encountering, and the adaptive immunity, which exhibit features of antigenic specificity, immunologic memory, diversity and the ability to distinguish between self and non-self. Dendritic cells (DCs), essential players in the adaptive immune response, have in mice been shown to derive from both myeloid and lymphoid cells. Due to their high level of constitutively expressed class II major histocompatibility complexes and co-stimulatory B7 membrane molecules, DCs are the most potent antigen-presenting cells [21]. Upon antigen encountering in tissue or blood, immature DCs migrate to lymphoid organs where they mature and not only promote expansion and differentiation of antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes [21], but also activate natural killer cells, macrophages and eosinophils [22-23]. Mature DCs can be generated *in vitro* from peripheral blood monocytes cultured in the presence of granulocyte macrophage colony stimulating factor (GM-CSF), IL-4 and additional pro-inflammatory mediators [24]. Peripheral blood monocytes are relatively small $CD14^+CD80^-CD83^-$DC-$sign^-$ cells [21] expressing 5-LO but not 15-LO [25]. However, during *in vitro* differentiation these cells dramatically change phenotype to become large granulated $CD14^-CD80^+CD83^+$DC-$sign^+$ cells [21]. Simultaneously, the expression of 5-LO is down-regulated with the reciprocal up-regulation of 15-LO [25]. The role of 15-LO in the differentiation process from monocyte to DC still remains to be elucidated.

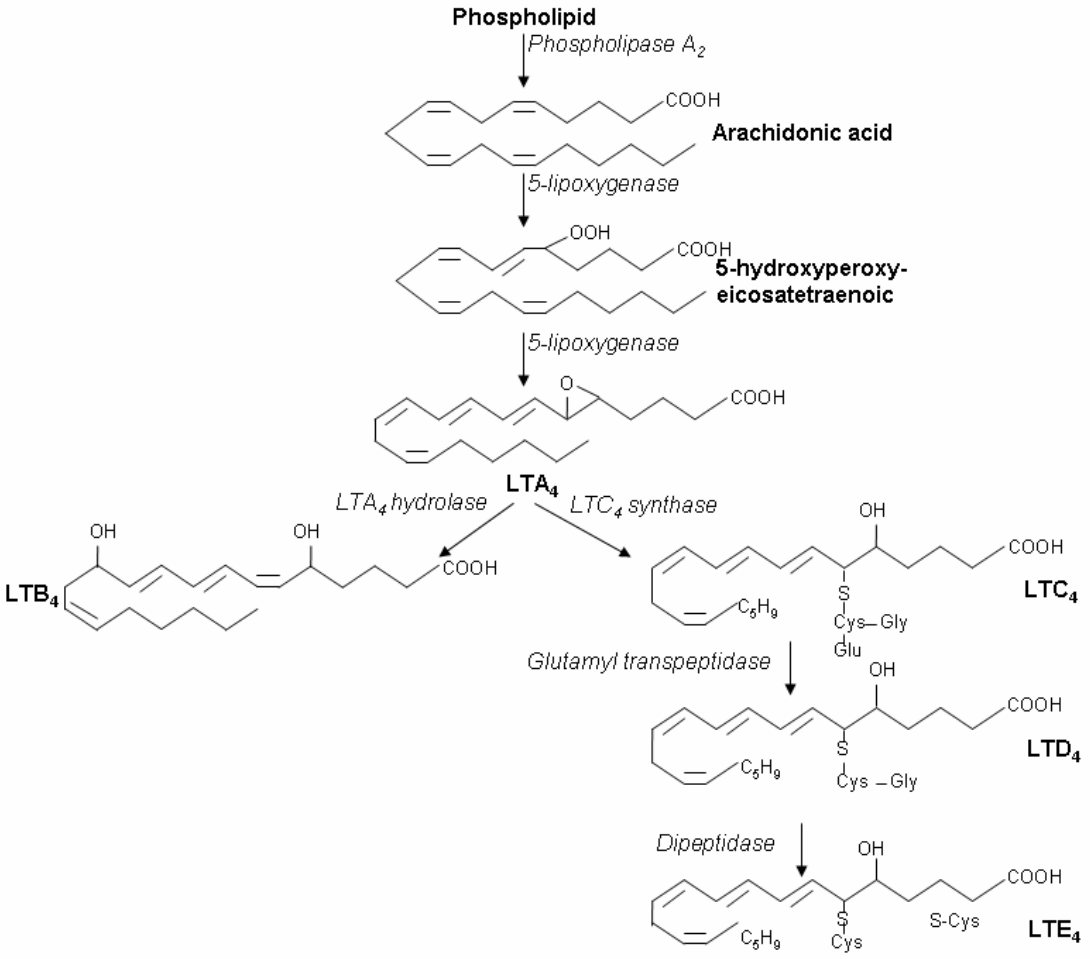
1.4 LEUKOTRIENES

Slow releasing substance of anaphylaxis (SRS-A) was identified in the lungs of guinea pigs and described as a smooth muscle contracting compound as early as 1938. The chemical structure remained unknown until the late seventies when it was shown that SRS-A was derived from arachidonic acid, presumably via the lipoxygenase pathway. SRS-A was later given the name leukotriene [1]. Traditionally, LTs have been considered to be key players in

inflammatory diseases. They are potent bronchoconstrictors, known to promote granulocytic infiltration, vascular permeability, edema formation and airway remodeling, all important features of asthma [26]. Several $CysLT_1$ receptor antagonists, such as zafirlukast and pranlukast, are used in the treatment of asthma [35]. These drugs have been shown to promote bronchodilatation [26] and decrease the number of asthma exacerbations [26-27]. A putative role for LTs in carcinogenesis has also been suggested [28-29].

The LTs are divided into dihydroxy LTs and cysteinyl LTs (CysLTs) with regard to the absence or presence of a cysteine group, respectively. The CysLTs LTC_4, LTD_4 and LTE_4 are arachidonic acid-derived lipid mediators [30] mainly produced by activated eosinophils, basophils, mast cells and macrophages [26]. Cytosolic phospholipase A_2, the enzyme initiating the biosynthesis of LTs in response to for instance calcium mobilization, catalyzes the release of arachidonic acid from membrane phospholipids [26]. In concert with FLAP, 5-LO oxidizes free fatty acids to 5-hydroxyperoxy-eicosatetraenoic, followed by subsequent dehydration to yield LTA_4. This compound can be further metabolized to LTB_4 by LTA_4 hydrolase. Alternatively, LTA_4 can be converted by LTC_4 synthase to LTC_4, which is exported extracellularly for conversion to LTD_4 and LTE_4, via glutamyl transpeptidase or dipeptidase, respectively [26] (Figure 2). The export of LTC_4 is energy-dependent and requires multidrug resistance protein 1 [31].

Fig 2



1.5 CYSTEINYL LEUKOTRIENE SIGNALING

CysLT signaling is mediated by the 7-transmembrane guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs) $CysLT_1$ and $CysLT_2$. GPCRs mediate intracellular signaling by utilizing heterotrimeric complexes including Gα, Gβ and Gγ subunits closely associated with the intracellular part of the GPCR. Given the fact that over 30 genes are known to code for different G-proteins, it is not surprising that GPCRs are able to elicit a diverse array of biological responses by triggering different downstream intracellular signaling molecules. In a GPCRs inactive state, the Gα subunit is bound to guanine diphospate. Upon receptor activation, the guanine diphospate is exchanged to a guanine triphosphate [32]. In the

last decade, the CysLT receptors have been cloned and characterized [33,49-51] The $CysLT_2$ receptor is functionally defined as the receptor responsible for a CysLT response resistant to $CysLT_1$ receptor antagonists [34]. LTC_4, LTD_4 and LTE_4 are ligands for the $CysLT_1$ receptor which is mainly expressed by bronchial smooth muscle cells, monocytes, macrophages and eosinophils [36]. The gene for the human $CysLT_1$ receptor is located at Xq13.2-21.1 and consists of five exons variably spliced. In human monocytes and macrophages, expression of the $CysLT_1$ receptor has been shown to be transcriptionally induced by IL-4 and IL-13 through activation of STAT6 and its subsequent binding to a response element in the proximal $CysLT_1$ receptor promoter [37].

1.6 CLASSICAL HODGKIN LYMPHOMA

Hodgkin lymphoma (HL) was described in 1832 by Thomas Hodgkin and some 70 years later Carl Sternberg and Dorothy Reed identified the malignant cells in HL. The annual incidence of HL in Sweden is approximately 200, and it is considered to be one of the most common malignancies among young adults. According to WHO, HL comprise two entities, namely nodular lymphocyte predominant HL and classical HL (cHL). Based on cellular background, cHL is further divided into mixed cellularity (MC) HL, nodular sclerosis (NS) HL, lymphocyte-depleted HL and lymphocyte-rich HL [38]. Clinically, cHL is characterized by enlargement of lymph nodes and spleen as well as fever, night sweats and weight loss. Classical HL tumors are histologically characterized by a minority of malignant Hodgkin-Reed Sternberg (H-RS) cells interspersed among an abundant infiltrate of inflammatory cells such as mast cells, eosinophils and T-lymphocytes [39]. The H-RS cells are CD30 positive mono- or multinucleated giant cells with a peculiar morphology [40]. Although not exclusively expressed by H-RS cells [41], CD30 is diagnostically used for identification of H-RS cells in cHL.

Several studies suggest interdependency between the H-RS cells and the bystander cells, contributing to the specific features of the tumor. In this respect, the wide array of cytokines and chemokines produced by the H-RS cells are of particular importance. These molecules not only participate in the attraction of inflammatory cells to the tumor site, but also affect tumor-associated cells, ultimately leading to a microenvironment favoring persistence of the H-RS cells [42]. Furthermore, expression of a numerous cytokine and chemokine receptors by the H-RS cells themselves constitutes the foundation of autocrine loops promoting tumor survival and progression [42].

Probably due to the scarcity of malignant cells in cHL, it has been difficult to establish reliable cHL cell lines. In fact, the MC HL-derived cell line L1236 is the only cell line today proven to be of H-RS cell origin [43], which was confirmed when L1236 cells and H-RS cells of the same patient's bone marrow were demonstrated to exhibit identical Ig gene rearrangements [44].

2 AIMS OF THE STUDY

I To elucidate the role of CysLTs in the pathogenesis of cHL.

II To elucidate the membrane interaction and activity of human 15-LO-1 and the subcellular localization of the enzyme in calcium ionophore stimulated DCs.

3 RESULTS AND DISCUSSION

3.1 PUBLICATION I

Evidence for a pathophysiological role for cysteinyl leukotrienes in classical Hodgkin lymphoma. *Submitted*

Classical HL is a disease biologically resembling asthma in many aspects. For instance, both diseases have a skewed Th1/Th2 balance towards a Th2 response, including elevated expression levels of IL-13, IL-9 and IL-5 [42,46] and it has been suggested that IL-13 plays a pivotal role in the pathogenesis of both cHL and asthma [45-46]. LTs are also known to be key players in asthma [26], but their potential role in cHL has not yet been studied. The H-RS cells themselves do not produce LTs due to the lack of 5-LO expression (unpublished data). However, since these cells are surrounded by LT producing cells such as mast cells, eosinophils and macrophages, we hypothesized that LTs could be of importance also in cHL. Therefore, the HL cell lines L1236, KMH2, HDLM and L428 were screened for the $CysLT_1$ and $CysLT_2$ receptor mRNA expression using real-time PCR. Total RNA was reversed transcribed and cDNA was synthesized according to standard protocols. The results revealed that L1236 cells and KMH2 cells, but not HDLM2 cells and L428 cells, express $CysLT_1$ receptor mRNA. The $CysLT_2$ receptor mRNA was found to be expressed by L1236 cells and HDLM2 cells, in contrast to KMH2 cells and L428 cells. To elucidate the protein expression of CysLT receptors in HL cell lines, immunohistochemical studies were performed using polyclonal rabbit antibodies raised against the human $CysLT_1$ and $CysLT_2$ receptor, respectively. For detection, the avidin biotin alkaline phosphatase technique was used. In concordance with the mRNA expression, L1236 cells and KMH2 cells were strongly and weakly positive, respectively, for the $CysLT_1$ receptor protein. The $CysLT_2$ receptor protein expression was only found in L1236 cells. This signal was only partly quenched after pre-incubation with excessive amounts of the corresponding peptide, and we therefore consider

the protein expression of the $CysLT_2$ receptor in the HL cell lines as indefinite. Also, the antibody raised against the $CysLT_2$ receptor did not work well on formalin fixed paraffin embedded cHL tumor tissue, and therefore, it remains to be elucidated whether this receptor is expressed by H-RS cells in cHL. However, microarray analysis revealed elevated levels of $CysLT_2$ receptor mRNA when primary H-RS cells were compared to germinal center B cells (the presumed progenitors of H-RS cells).

To exclude the possibility that these findings were cell line artefacts, we further investigated $CysLT_1$ receptor protein expression by H-RS cells *ex vivo*. The co-expression of CD30 and the $CysLT_1$ receptor was studied by immunohistochemistry in formalin fixed paraffin embedded consecutive sections from cHL lymph node biopsies with the avidin biotin alkaline phosphatase technique. The tumors studied were of subtype NS HL (n=8) and MC HL (n=8), according to the WHO classification [38]. At least a fraction of H-RS cells positive for the $CysLT_1$ receptor protein were detected in 12 of 16 tumors. In two of these, a very strong expression was noted. The expression was generally more frequent and usually stronger in H-RS cells from tumors of the NS subtype. Importantly, the $CysLT_1$ receptor expression by H-RS cells was confirmed by microarray analysis, where laser captured primary H-RS cells from three patients were shown to express substantially higher $CysLT_1$ receptor mRNA compared to germinal center B cells.

To elucidate whether the identified CysLT receptors were functional, exogenous LTs were added and the HL cell lines where screened in a calcium mobilization assay. All CysLTs, but not LTB_4, were capable to evoke a calcium response in KMH2 and L1236 cells, whereas L428 and HDLM2 cells did not respond to any LT. In L1236 cells, the rank order of potency to mount a calcium release was LTC_4=LTD_4>LTE_4. This rank order has been reported for $CysLT_1$ [47] but could also be valid for a $CysLT_2$ response [48-50]. This makes it tempting to speculate that the L1236 cells express either a $CysLT_1$-$CysLT_2$ receptor dimer, or alternatively, the recently discovered CysLT receptor GPR17 [54]. However, the CysLT-

induced calcium responses were completely abolished if the cells were pre-treated with zafirlukast or montelukast at concentrations selectively inhibiting the $CysLT_1$ receptor, strongly suggesting this receptor to be solely responsible for the CysLT-mediated response. The corresponding rank order for KMH2 cells was LTD_4>LTC_4=LTE_4, which is in line with a $CysLT_1$ receptor specific response [33,51]. Exogenously added LTs did not mount a calcium release in L428 cells or HDLM2 cells. The discrepancy noted between the different HL cell lines in this study might be explained by the fact that L1236 and KMH2 are B-lymphocyte derived cell lines of MC subtype. In contrast, HDLM2 is of NS subtype and exhibit a T-lymphocyte phenotype, and L428 is a B-cell derived HL cell line of NS subtype. Importantly, as mentioned earlier, L1236 is still the only cell line proven to be of true H-RS cell origin [43].

Classical HL is associated with an aberrant cytokine and chemokine production by the H-RS cells and the reactive infiltrate [42]. Therefore, we performed a real-time PCR analysis for different cytokines and chemokines transcribed in L1236 cells cultured in the presence of LTD_4 (100 nM) for 4 hours. TNF-α, IL-6, IL-8 and IL-13 mRNA were shown to be up-regulated compared to control cells upon LTD_4 treatment. To further elucidate the effect of LTD_4 on L1236 cells, the cytometric bead array technique was used. ANOVA with Fisher statistics was used for statistical analysis. Supernatants from L1236 cells cultured in the absence or presence of LTD_4 (100 nM) for 4, 11, and 25 hours, were analyzed for different cytokines and chemokines. Interestingly, the results showed that LTD_4 significantly increased the secretion of TNF-α ($p<0.001$), IL-6 ($p<0.001$) and IL-8 ($p=0.018$) by L1236 cells as compared to control cells. The increase of TNF-α and IL-6 secretion was shown to be dose-dependent (1-300 nM LTD_4) and $CysLT_1$ specific, since the increased protein secretion was abolished upon pre-treatment with zafirlukast (1 and 10 μM). The dose-dependency of IL-8 was difficult to evaluate due to values in close proximity to the detection limit. Since IL-13 is a cytokine of major importance in cHL [45], the release of IL-13 upon LTD_4 treatment was separately investigated using the quantitative sandwich enzyme immunoassay technique.

However, LTD_4 did not seem to have a major impact of the IL-13 secretion, although the data were difficult to interpret due to levels below the limit for detection.

When comparing the mRNA data to the corresponding data for protein release it becomes evident that the increased secretion of TNF-α, in contrast to IL-6 and IL-8, occurred with a relatively low transcriptional induction. These results indicate that the elevated TNF-α protein release was primarily due to post-transcriptional regulation (i.e. increased translation or elevated protein-stability) or increased release of pre-formed proteins, rather than *de novo* synthesis. Furthermore, a plateau was reached in the presence of LTD_4, indicating that pre-formed TNF-α is released faster upon LTD_4 stimulation compared to control cells, and that the pre-formed pool might become exhausted. The biological implications of these findings remain to be further elucidated. In fact, little is known regarding the role of these cytokines in the pathogenesis of cHL, mainly due to the lack of an appropriate *in vitro* or animal model. One may however speculate that TNF-α-induced release of eotaxin by tumor-associated fibroblasts will attract eosinophils [52]. These cells will not only constitute a source of additional CysLTs, but also contribute to maintenance of an inflammatory environment, favoring persistence of the H-RS cells. Given the fact that cHL is characterized by a Th2 dominating profile, one may anticipate that eotaxin with its Th2 lymphocyte-attracting properties, in concert with IL-6 and its Th1 suppressive features, may further promote this imbalance.

In order to delineate whether exogenously added LTD_4 could influence H-RS cell DNA synthesis, thymidine incorporation was performed. In fact, LTD_4 was able to stimulate DNA synthesis in L1236 cells in a dose-dependent manner (LTD_4 30 nM *vs* control, p=0.039; LTD_4 300 nM *vs* control, p=0.008). Furthermore, the LTD_4-induced DNA synthesis was abolished by pre-incubation with 1 μM zafirlukast (p=0.013).

In summary, this study supports a role of CysLTs in the pathogenesis of cHL by contributing to the growth of the malignant cells and significantly increased release of TNF-α, IL-6 and IL-8 by these. $CysLT_1$ receptor antagonists may have a potential role in the treatment of cHL.

3.2 PUBLICATION II

Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity. *Biochimica et Biopfysics Acta,2006, 1761(12): p. 1498-505.*

15-LO-1 has been implicated in several pathophysiological conditions [17,19]. It is therefore highly interesting to improve the understanding of how the activity of this enzyme is regulated. In order to study the subcellular localization of 15-LO-1 upon calcium ionophore stimulation, DCs were generated *in vitro* from peripheral blood monocytes according to standard protocols. Flow cytometry was used for DC immunophenotyping with antibodies directed against CD14, CD40, CD80 and DC-sign. The mature DCs were incubated in the absence or presence of calcium ionophore (2 μM) for 5 minutes in 37°C. After centrifugation, the cells were resuspended in PBS without calcium or magnesium, and cytocentrifuged on glass slides. The DCs were stained with a polyclonal 15-LO-1 antibody, visualized with immunofluorescence technique, and analyzed in a confocal microscope. In line with previous studies, we found 15-LO-1 located mainly in the plasma membrane but also in the cytosol after calcium ionophore stimulation. However, a significant amount of 15-LO-1 was found in the plasma membrane also in the absence of calcium ionophore. These results were also confirmed by Western blot where DCs were fractionated into cytosolic and membrane fractions. When the extracellular calcium was chelated with EGTA, the translocation was reversed and most 15-LO-1 was found in the cytoplasm, although a significant part still could be detected in the membrane. These findings suggest a calcium-dependent as well as a calcium-independent mechanism for 15-LO-1 translocation. This is in concordance with a previous study on membrane association of cytosolic phospholipase A_2 [53].

A protein-lipid overlay assay was performed to elucidate whether 15-LO-1 preferentially binds to certain phospholipids upon calcium challenge. An array of different phospholipids attached to membranes was incubated with 15-LO-1 protein. The results showed that 15-LO-1 specifically bound the following phospholipids with decreasing intensity: $PI(3.5)P_2 \geq PI(3.4)P_2 > PI(4)P > PI(5)P > PI > PI(4.5)P_2$. This system should however be considered as a highly artificial qualitative method, lacking the complex structure of biological membranes.

Since we found that 15-LO-1 preferentially bound certain phospholipids we further investigated whether this could influence the enzymatic activity of 15-LO-1. A vesicle assay was set up and the enzyme activity was measured as monohydroxy fatty acid formation using HPLC. The vesicles consisted of a lipid bilayer and free arachidonic acid, simulating the *in vivo* situation of fatty acids released by lipases. Additionally, one mole percent of different phospholipids was also included in the vesicles. When $PI(3.4)P_2$ or $PI(4.5)P_2$ was present in the vesicles, the enzymatic activity of 15-LO-1 was elevated as seen by significantly increased 15-HETE and 12-HETE formation. Upon calcium chelating, these differences were reversed suggesting calcium dependency of the increased 15-LO-1 activity caused by these phospholipids. The positional specificity seemed to be independent of the lipid composition since the ratio of 15-HETE and 12-HETE was 9:1 in all samples. The same trend, although less pronounced, was seen if the substrate was changed to linoleic acid as measured by a significant increased 13-HODE formation. Kinetic vesicle assays also revealed that the addition of $PI(3.4)P_2$ or $PI(4.5)P_2$ did not affect Vmax (the maximum enzymatic velocity). However, since the Km (the substrate concentration required for maximum enzymatic velocity) was significantly lower compared to control vesicles these data suggest that the increased arachidonic acid turnover was due to higher affinity for the substrate. The reason for why 15-LO-1 bind $PI(3.4)P_2$ and $PI(4.5)P_2$ with higher affinity compared to other PIs is not clear, however, one may speculate that 15-LO-1 has a specific binding site for these structurally similar molecules.

4 SUMMARY AND CONCLUSIONS

I In this study we report the expression of functional $CysLT_1$ receptors in the HL cell lines L1236 and KMH2 as seen by a significant calcium signal upon challenge with CysLTs. Culturing of L1236 cells in the presence of LTD_4 not only stimulated DNA synthesis but also the transcription and protein release of TNF-α, IL-6 and IL-8. Importantly, all these LTD_4-induced effects were abolished by the $CysLT_1$ receptor-specific antagonist zafirlukast. Immunohistochemical studies of cHL biopsies and microarray analyses of laser captured cells demonstrated $CysLT_1$ receptor expression also by the H-RS cells *ex vivo*. Thus, by contributing to the growth and the disturbed cytokine features typical of this tumor, CysLTs might have a potential role in the pathogenesis of cHL.

II We herein demonstrate that 15-LO-1 translocates to the plasma membrane in DCs upon calcium ionophore stimulation. Furthermore, 15-LO-1 was shown to preferentially bind certain phospholipids. In a vesicle assay, the inclusion of $PI(3.4)P_2$ and $PI(4.5)P_2$ significantly increased the formation of 15-HETE in a calcium dependent manner, indicating that lipid constitution might be an additional regulatory mechanism for 15-LO-1 activity. Kinetic studies further revealed that vesicles containing these phospholipids lowered the Km value for 15-LO-1, whereas Vmax was unaffected. Thus, the increased 15-LO-1 activity seemed to be due to higher substrate affinity of 15-LO-1.

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