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Studies on Cysteinyl Leukotriene Receptor 1 and 15-lipoxygenase-1 in Lymphomas

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Till mamma och pappa
Till David och Felicia

ABSTRACT

Classical Hodgkin lymphoma (cHL) is a malignant disorder with striking inflammatory features. Since cysteinyl leukotrienes (cysLTs) are potent inflammatory mediators it was of interest to study their potential role in the pathogenesis of cHL. We have shown that certain HL cell lines express functional $CysLT_1$ receptors, responding with a robust calcium signal upon leukotriene (LT) D_4 challenge. Stimulation of these cells with LTD_4 induced a $CysLT_1$ receptor-dependent release of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α. The malignant Hodgkin Reed-Sternberg (H-RS) cells in the majority of the primary cHL biopsies under study also expressed the $CysLT_1$ receptor. Since these cells are surrounded by cysLT-producing eosinophils, macrophages and mast cells, our results suggest that the cysLTs might be of importance for the pathogenesis of cHL **(paper I)**.

The cHL cell line L1236 has high expression of 15-lipoxygenase-1 (15-LO-1) and these cells were able to convert arachidonic acid to eoxin (EX) C_4, EXD_4 and EXE_4, pro-inflammatory metabolites recently discovered in human eosinophils and mast cells. Immunohistochemical studies of cHL tumor tissue demonstrated that 15-LO-1 was expressed in primary H-RS cells in the majority of the investigated biopsies. Thus, the expression of 15-LO-1 and the formation of eoxins by H-RS cells are likely to contribute to the inflammatory features of cHL. These findings may have important diagnostic and therapeutic implications **(paper II**).

The expression of the $CysLT_1$ receptor and 15-LO-1 was also elucidated in non-Hodgkin lymphomas (NHLs). The majority of the primary mediastinal B cell lymphomas under study, in contrast to other NHLs, expressed the $CysLT_1$ receptor. Furthermore, T cell-derived anaplastic large cell lymphoma was the only NHL entity shown to express 15-LO-1. Thus, the $CysLT_1$ receptor and 15-LO-1 are potential targets in lymphoma diagnostics and sub-classification **(paper III)**.

Finally, we have studied the post-translational regulation of 15-LO-1 in dendritic cells. In the presence of calcium, addition of phosphatidylinositol-4.5-bisphosphate or phosphatidylinositol-3.4-bisphosphate to 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. These results suggest that 15-LO-1 activity may also be regulated by the phospholipid constitution of membranes **(paper IV)**.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Schain F**, Tryselius Y, Sjöberg J, Backman L, Malec M, Porwit A, Xu D, Vockerodt M, Baumforth KRN, Wei W, Murray PG, Björkholm M and Claesson H-E. Evidence for a pathophysiological role of cysteinyl leukotrienes in classical Hodgkin lymphoma. *Int J Cancer*, in press

II. Claesson H-E, Brunnström Å, Griffith W, **Schain F**, Feltenmark S, Andersson E, Porwit A, Sjöberg J and Björkholm M. Hodgkin Reed-Sternberg cells express 15-lipoxygenase-1 and are putative producers of eoxins *in vivo*- novel insight into the inflammatory features of classical Hodgkin lymphoma. *European J Biochem*, 2008 Jul 16 [Epub ahead of print]

III. **Schain F**, Schain D, Mahshid Y, Liu C, Porwit A, Christer Sundström, Xu D, Claesson H-E, Björkholm M and Sjöberg J. Differential expression of cysteinyl leukotriene receptor 1 and 15-lipoxygenase-1 in non-Hodgkin lymphoma. *Submitted*

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

Related manuscripts not included in the thesis

Schain F, Sjöberg J, Brunnström Å, Andersson M, Xu D, Forsell P, Björkholm M, Claesson H-E. Inhibition of 15-lipoxygenase-1 during monocyte-derived dendritic cell differentiation generates dendritic cells with an impaired T-lymphocyte stimulating capacity *(manuscript)*

Liu C*, **Schain F***, Xu D, Andersson-Sand H, Forsell P, Claesson H-E, Björkholm M and Sjöberg J**.** Epigenetic and transcriptional control of the 15-lipoxygenase-1 gene in Hodgkin lymphoma cell-line L1236 *(submitted for publication)* *These authors contributed equally

Liu C, Xu D, **Schain F**, Björkholm M**,** Claesson H-E and Sjöberg J. 15-lipoxygenase-1 induces chemokine expression in cultured human lung epithelial cells *(submitted for publication)*

Liu C, Fang X, Xu D, **Schain F**, Yidong F, Claesson H-E, Björkholm M and Sjöberg J. Histone methyltransferase SMYD3 and histone demethylase SMCX regulate 15-lipoxygenase-1 gene expression through histone modifications *(manuscript)*

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LIST OF ABBREVIATIONS

5-LO	5-lipoxygenase
12-HETE	12-hydroxyeicosa-5E,8Z,10Z,14Z-tetraenoic acid
13-HODE	13-hydroxyoctadeca-9Z,11E-dienoic acid
15-HETE	15-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid
15-HPETE	15-hydroperoxy-eicosa-5Z,8Z,11Z,13E-tetraenoic acid
15-LO-1	15-lipoxygenase-1
ALA	α-linolenic acid
ALCL	Anaplastic large cell lymphoma
B-CLL	B cell chronic lymphocytic leukemia
BL	Burkitt lymphoma
CBA	Cytometric Bead Array
cHL	Classical Hodgkin lymphoma
CysLT	Cysteinyl leukotriene
DC	Dendritic cell
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DLBCL	Diffuse large B cell lymphoma
EET	Epoxieicosatrienoic acid
EIA	Enzyme immunoassay
EPA	Eicosapentaenoic acid
EX	Eoxin
FCDL	Follicular centre derived lymphoma
FITC	Fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
H-RS	Hodgkin Reed-Sternberg
IL	Interleukin
IFN-γ	Interferon-γ
JAK	Janus kinase
GM-CSF	Granulocyte macrophage colony-stimulating factor

GLA	γ-linolenic acid
LD	Lymphocyte depleted
LA	Linoleic acid
LR	Lymphocyte rich
LP	Lymphocyte predominant
LT	Leukotriene
LX	Lipoxin
MALT	Mucosa-associated lymphoid tissue
MC	Mixed cellularity
MCL	Mantle cell lymphoma
NHL	Non-Hodgkin lymphoma
NS	Nodular sclerosis
PE	Phosphatidylethanolamine
PG	Prostaglandin
PI	Phosphatidylinositol
PI(3.4)P_2	Phosphatidylinositol-3.4-bisphosphate
PI(4.5)P_2	Phosphatidylinositol-4.5-bisphosphate
PMBCL	Primary mediastinal B cell lymphoma
PTCL	Peripheral T cell lymphoma
PTX	Pertussis toxin
PUFA	Polyunsaturated fatty acids
SRS-A	Slow releasing substance of anaphylaxis
STAT6	Signal and activator of transcription 6
TCRBCL	T cell-rich B cell lymphoma
TNF-α	Tumor necrosis factor-α
TX	Thromboxane

BACKGROUND

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 signalling molecules. Based on structure and physical properties, lipids are classified into different subgroups such as fatty acids and phospholipids.

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. 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.

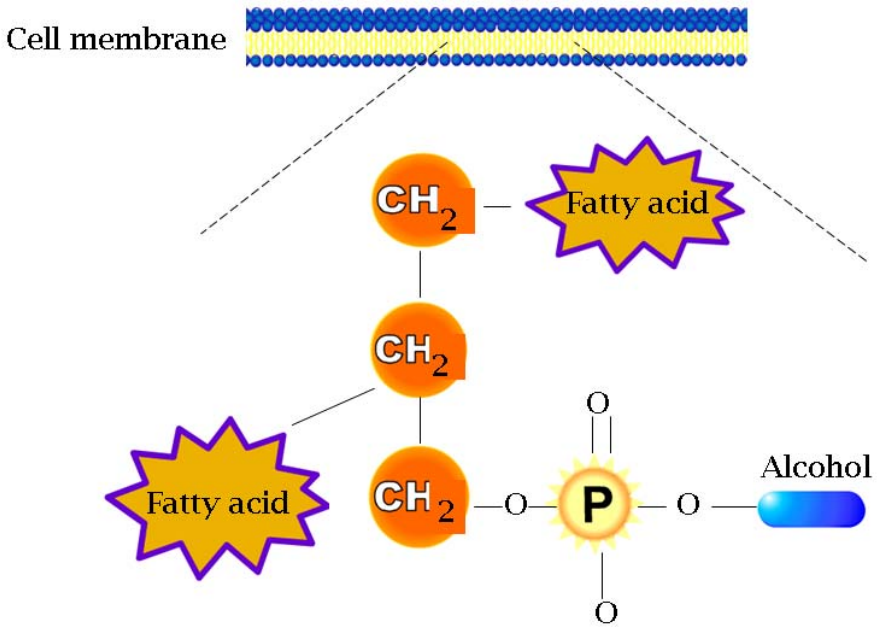

Figure 1. Structural picture of a phospholipid. Shown is a cellular membrane with a magnified picture of an incorporated phospholipid including a glycerol backbone, two fatty acids and one phosphate group coupled to a polar alcohol.

Fatty acids

Fatty acids, carboxylic acids with long-chain hydrocarbon groups, are referred to as saturated or unsaturated with respect to the absence or presence of doublebonds, respectively. The general formula for a fatty acid is CH_3-$(CH_2)_n$-COOH, where n is ≥ 6. Polyunsaturated fatty acids (PUFAs) contain ≥ 2 doublebonds. Arachidonic acid, a PUFA 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, abbreviated 20:4, indicating a total of 20 carbons (twenty in Greek is eicosi) and the presence of four doublebonds at the indicated positions (1). The PUFAs can be divided into different groups based on the position of the double bond closest to the methyl group. For instance, the ω-3 series PUFAs are derived from α-linolenic acid (ALA) and the ω-6 series PUFAs are derived from the linoleic acid (LA). Humans are not able to synthesize ALA and LA, and thus, these fatty acids are referred to as essential and needs to be obtained from dietary sources such as eggs, vegetable oils, whole-grain breads and walnuts (2). Figure 2 is a schematic picture demonstrating the metabolism of ALA and LA.

LIPOXYGENASES

Lipoxygenases are highly regulated enzymes that catalyze the introduction of molecular oxygen into PUFAs. They are classified with respect to their positional selectivity of arachidonic acid (3). Upon certain stimuli, arachidonic acid is released and metabolized to eicosanoids, defined as lipid mediators derived from 20 carbon PUFAs (1). Arachidonic acid is the quantitatively dominating eicosanoid precursor and the eicosanoid family includes lipoxygenase products (leukotrienes (LTs) and lipoxins) and cyclooxygenase products (thromboxanes and prostaglandins) (4).

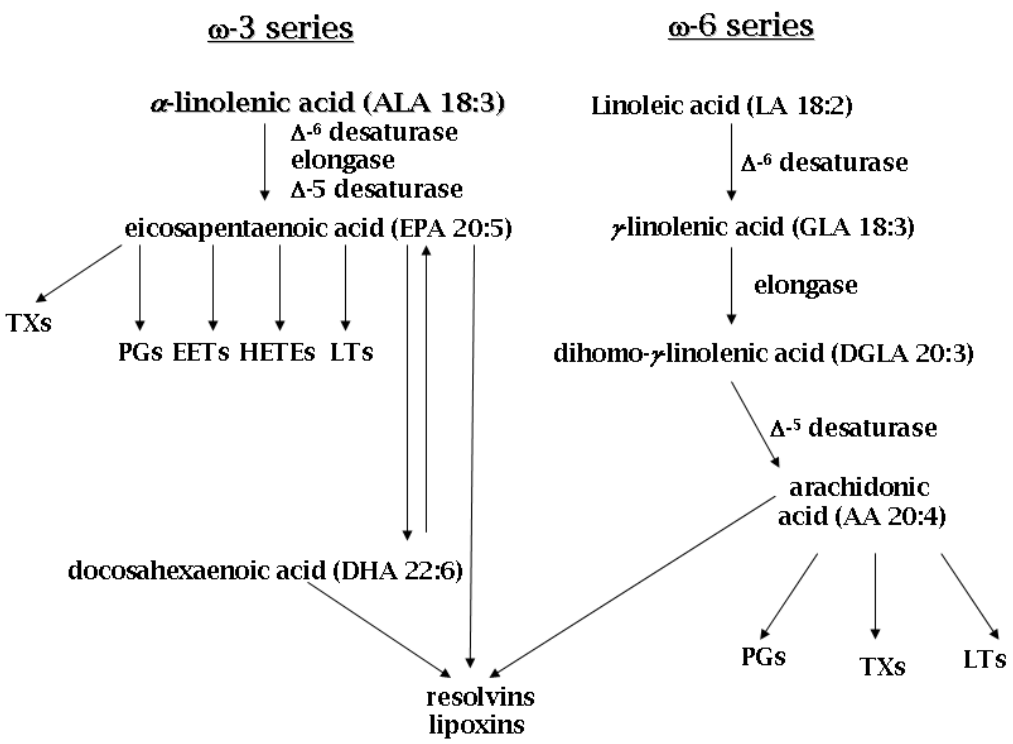

Figure 2. Metabolism of essential fatty acids. ALA and LA can be metabolized to a broad spectrum of potent pro-and antiinflammatory mediators. The designation in the parantheses refers to the number of carbons and doublebonds, respectively. TXs; thromboxanes, PGs; prostaglandins, LTs; leukotrienes, EETs; epoxieicosatrienoic acids, HETEs; hydroxy-eicosatetraenoic acid.

5-lipoxygenase

Since 5-lipoxygenase (5-LO) is the key enzyme for LT biosynthesis, the regulation of this enzyme has been well studied. Calcium is essential for the catalytic activity of 5-LO by promoting its translocation from the cytosol to membranes (5). Also, five-lipoxygenase activating protein (FLAP) is necessary for LT synthesis. The mechanism of FLAP action is however not completely clear although it is known that FLAP facilitates the 5-LO reaction and presents arachidonic acid to 5-LO (6). Furthermore, tyrosine residue phosphorylation of 5-LO has been suggested to increase LT synthesis (7).

15-lipoxygenase

The rabbit reticulocyte 15-lipoxygenase (15-LO-1) was discovered in the 1970´s (8), and some ten years later the human orthologue was

described in eosinophils (9). 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 (10). The orthologue to 15-LO-1 in animals is 12/15-LO, earlier called leukocyte 12-LO. Rabbit reticulocytes, however, also express 15-LO-1. The enzyme 12/15-LO has similar enzymatic properties, expression, distribution and regulation as the human 15-LO-1, but converts arachidonic acid mainly to 12-hydroxyeicosa-5E,8Z,10Z,14Z-tetraenoic acid (12-HETE) (10). 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 (11). 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 (10).

The expression of 15-LO-1 is strictly regulated at a transcriptional, translational, post-translational and epigenetical level (12,13). 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) signalling pathway (14). Furthermore, calcium-dependent membrane-association has been implicated as an important post-translational control mechanism (15).

Oxygenation of fatty acids

Three different enzymatic systems are able to enzymatically catalyze the oxygenation of arachidonic acid. These are the cyclooxygenases, the monooxygenases and the lipoxygenases. Lipoxygenases can efficiently catalyze the insertion of molecular oxygen into free fatty acids and

fatty acids esterified to membranes. Conjugated isomers including a hydroxyl group can also slowly, in comparison to enzymatically catalyzed reactions, be formed via autooxidation when molecular oxygen is non-enzymatically introduced into arachidonic acid. The reduction of these products results in hydroperoxy acids (including -OOH) and monohydroxy acids (including -OH). Lipoxygenases catalyzes the formation of a hydroperoxide product from a fatty acid by hydrogen atom abstraction from a bisallylic position followed by addition of oxygen. The hydrogen abstraction can take place due to a ferric hydroxide, with a remarkably high redox potential, residing in the lipoxygenase (16). Subsequently, radical rearrangement and oxygen insertion at carbon 15 in arachidonic acid is taking place. The resulting initial product formed is 15-hydroperoxy-eicosatetraenoic acid (15-HPETE).

The biological role of 15-lipoxygenase

The biological role of 15-LO-1 is at present unclear 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 (17). However, 12/15-LO knockout mice did not reveal impaired erythropoiesis, suggesting that at least in mice, the enzyme is not essential for erythropoiesis (18). The 15-LO-1 products 15-hydroxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid (15-HETE) and 13-hydroxyoctadeca-9Z,11E-dienoic acid (13-HODE) have been shown to exhibit both pro- and anti-inflammatory characteristics. In fact, 15-HETE has been shown to increase mucus secretion (19) and bronchial smooth muscle cell contraction (20), implicating 15-LO-1 in the pathogenesis of asthma. However, these results are contradicted by others (21). The degree of 15-LO-1 expression has been shown to correlate with disease progression in prostate carcinoma (22). In contrast, 15-LO-1 products suppressed cell proliferation and exhibited pro-apoptotic features in colon cancer (23). 15-LO-1 has also been implicated in immune regulation and peritoneal macrophages from mice lacking 12/15-lipoxygenase show impaired functions (24,25).

Lipoxins (LXs) is another group of lipid mediators which is partly formed via the 15-LO pathway (26). LX analogues exert anti-inflammatory effects in various biological systems (27,28).

Several studies support a pro-inflammatory role of the 15-LO pathway in lung. For instance, our group recently discovered novel pro-inflammatory arachidonic acid-derived metabolites referred to as eoxins (EXs) formed via the 15-LO-1 pathway in mast cells and eosinophils. These mediators were almost as potent as LTC_4 and LTD_4 and hundred times more potent than histamine, in increasing the permeability in an endothelial cell layer *in vitro* (29). Recent studies performed in our laboratory also demonstrated that lung epithelial cells over-expressing 15-LO-1 secreted elevated levels of chemokines, which led to increased recruitment of dendritic cells, mast cells and activated T-cells (Liu et al, submitted manuscript). Finally, studies on 12/15-LO knockout mice suggest that 12/15-LO-derived mediators play a pathophysiological role in allergen-induced airway inflammation and remodeling (30).

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 via the 5-LO pathway. SRS-A was later given the name leukotriene (1). LTs are 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 (31). Several cysteinyl leukotriene $(CysLT)_1$ receptor antagonists, such as zafirlukast, montelukast and pranlukast, are used in the treatment of asthma. These drugs are known to promote bronchodilatation (31) and decrease

the number of asthma exacerbations (31,32). Lately, a putative role for LTs in carcinogenesis has also been suggested (33,34).

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 (35) mainly produced by activated eosinophils, basophils, mast cells and macrophages (31). 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 (31). In concert with FLAP, 5-LO oxidizes free fatty acids to 5-hydroperoxy-eicosatetraenoic (5-HPETE), 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 (31) (Figure 3). The export of LTC_4 is energy-dependent and requires multidrug resistance protein 1 (36).

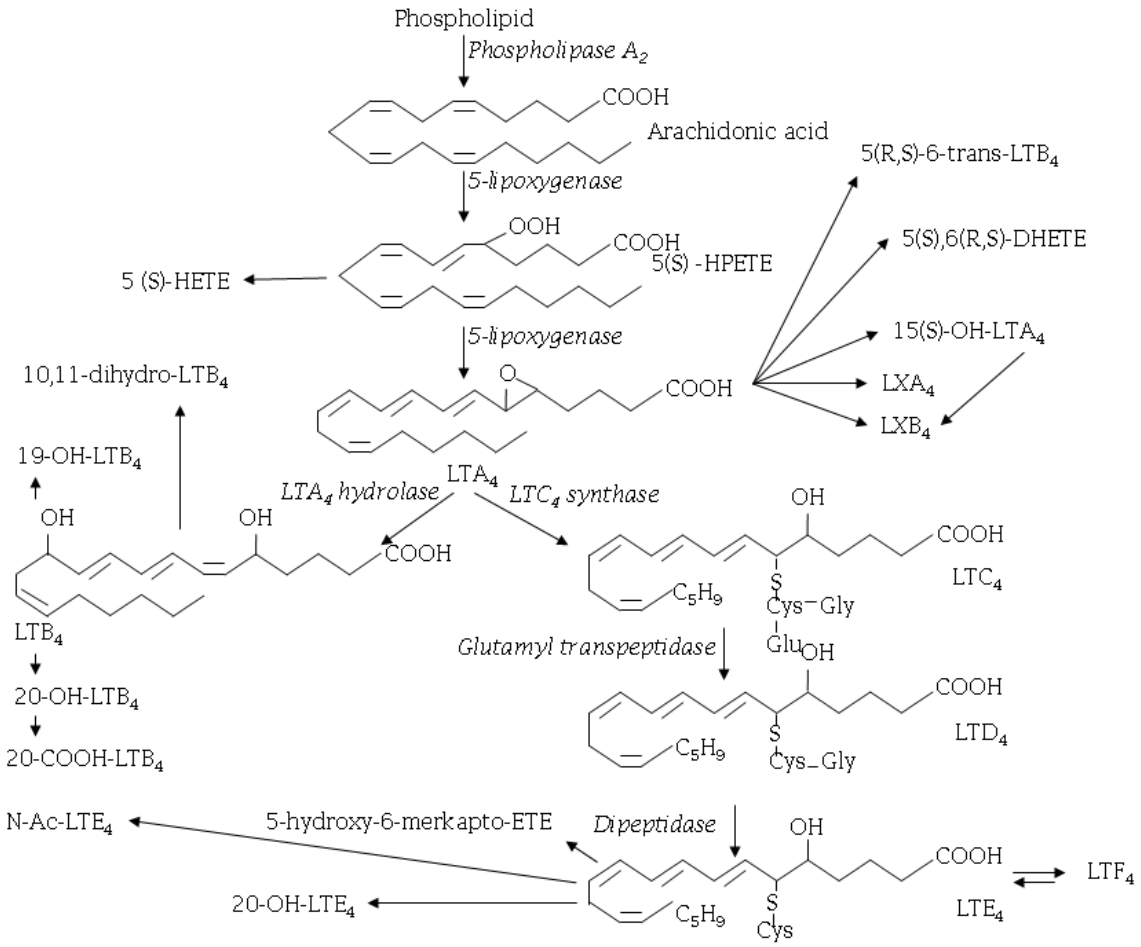

Figure 3. The 5-lipoxygenase metabolism pathway. LT; leukotriene, LX; lipoxin.

Cysteinyl leukotriene signalling

LTC_4, LTD_4 and LTE_4 are ligands for the CysLT receptors. CysLT signalling is mediated via the 7-transmembrane guanine nucleotide binding protein (G-protein) coupled receptors (GPCRs) $CysLT_1$, $CysLT_2$ and $CysLT_3$ (GPR17) (37). These receptors mediate intracellular signalling by utilizing heterotrimeric complexes including Gα, Gβ and Gγ subunits closely associated with the intracellular part of the GPCR. Given the fact that many genes are known to code for different G-proteins, it is not surprising that GPCRs elicit a diverse array of biological responses by triggering different downstream intracellular signalling molecules. In a GPCRs inactive state, the Gα subunit is bound to guanine diphosphate. Upon receptor activation, the guanine diphosphate is exchanged to a guanine triphosphate (38). In the last decade, the CysLT receptors have

been cloned and characterized (39). The $CysLT_2$ receptor is functionally defined as the receptor responsible for a CysLT response resistant to $CysLT_1$ receptor antagonists (40). The $CysLT_1$ and $CysLT_2$ receptor are expressed by mast cells, bronchial smooth muscle cells, monocytes, macrophages and eosinophils (41). The $CysLT_2$ receptor is also expressed by coronary smooth muscle cells, adrenal medulla cells, cardiac Purkinje cells (42) and endothelial cells (43). Recently, the $CysLT_3$ receptor was identified as a dual nucleotide and CysLT receptor with IC50 values similar to the $CysLT_1$ receptor. This receptor is mainly expressed in brain, kidney and heart (37). 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, the expression of the $CysLT_1$ receptor is 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 (44).

Leukotriene signalling and disease

LTD_4 signalling via the $CysLT_1$ receptor has been shown to contribute to the pathogenesis of aortic aneurysm by chemokine induction (45). Several studies have also demonstrated the role of LTs in vascular pathology and asthma due to their ability to affect smooth muscle cell behaviour (43,46-54), and LT antagonists are used for asthma therapy (32,56) as reviewed in (31). Furthermore, the proteins involved in cysLT signalling are abundantly expressed by the inflammatory cells in the upper airways of patients with allergic rhinitis (55). Leukotrienes have also been implicated in the pathogenesis of colon cancer (57,58) and hematological malignancies (33,34).

LYMPHOMA

Lymphomas are cancers originating from lymphoid cells. The tumor cells can spread to other lymphoid sites, to the spleen, as well as to extranodal organs such as skin, brain, lung and bone marrow. The lymphomas are divided into Hodgkin lymphoma (HL) and non-Hodgkin

lymphoma (NHL). Among the lymphomas, HL exhibits a distinct pattern of histopathology, epidemiology, immunophenotype, clinical features, and prognosis. The remaining lymphomas constitute a large heterogeneous group. The incidence of certain lymphomas have increased the last decade (59,60). The reason for this increase is not clear and can not be explained by the increased rate of HIV-infections (61). However, HIV-infected immunocompromised individuals may be predisposed to infections by other viruses and Epstein-Barr virus, human T cell leukemia/lymphoma virus (HTLV-1), hepatitis C and human herpes virus-8 have indeed been shown to play a role in the development of lymphomas (62,63).

Diagnosis and staging

The diagnosis is based on symptoms, histological examination of the affected tissue in combination with immunological and genetic analyses. Computed tomography of pelvis, abdomen and chest is routinely used for staging. A bone marrow biopsy is often performed to exclude or verify bone marrow infiltration. The complete blood count, serum lactate dehydrogenase and β2-microglobulin levels are determined since these variables are known to be important prognostic factors for some lymphomas.

The Cotswolds classification, a modified version of the Ann Arbor staging classification, is commonly used for staging of lymphomas (64) (Table I). Furthermore, HL patients can be divided into early stage favourable (stage I-II, no risk factors) early stage un-favourable (stage I-II, plus risk factors) or advanced stage (stage III-IV plus risk factors) disease. Risk factors include patient age ≥ 50 years, ≥ 3 involved regions, extranodal disease, large mediastinal mass (>1/3 of the greatest thorax section) and elevated erythrocyte sedimentation rate (65).

Table I. The Ann Arbor staging classification modified in Cotswalds

StageI	Involvement of a single lymph node region or extralymphatic organ
StageII	Involvement of ≥2 lymph node regions or localized involvement of extralymphatic organs on the same side of the diaphragm
StageIII	Involvement of lymph node regions or localized involvement of extralymphatic organs on both sides of the diaphragm
StageIV	Involvement of ≥1extralymphatic organs excluding that designated "E"
A	Absence of symtoms
B	Presence of any B-symtom; 1. Night sweats 2. Unexplained fever (>38°C) 3. Unexplained weight loss (>10% of the body weight in the last 6 months)
E	Involvement of a single extranodal site proximal to nodal site
X	Bulky disease (a peripheral lymph node >10 cm or a mediastinal mass >1/3 of the mediastinum at T5-6 level on a chest x-ray

Adapted from Carbone et al. Report of the Committee on Hodgkins Disease Staging Classification, *Cancer Res* 1971,31:1860-1.

Prognosis

The prognosis for lymphoma patients differs substantially. The prognosis for NHL patients is generally worse than for HL patients, with a 10 year survival period of less than 60 percent (66). The HL patients generally have a better prognosis with 80 percent of the patients experiencing long-term disease-free survival, although the prognosis is worse for elderly patients, patients with advanced stages and relapses. Additionally, serious treatment-related side-effects such as infertility, secondary malignancies and cardiopulmonary toxicity are common.

Classical Hodgkin lymphoma

HL was described in 1832 by Thomas Hodgkin and later Carl Sternberg and Dorothy Reed identified the malignant cells in HL. It was long debated whether HL was a malignant or infectious disease. It is now clear that the Hodgkin Reed-Sternberg (H-RS) cells are clonal and malignant (67). The annual incidence of HL in Sweden is approximately 200, and the disease is 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) cHL, nodular sclerosis (NS) cHL, lymphocyte depleted (LD) cHL and lymphocyte rich (LR) cHL (68). Clinically, cHL is characterized by enlargement of lymph nodes and spleen and 40 percent of the patients also suffer from B-symptoms (i.e. fever, night sweats or weight loss). cHL tumors are histologically characterized by a minority of H-RS cells interspersed among an abundant infiltrate of inflammatory cells such as mast cells, eosinophils and T-lymphocytes (69). The H-RS cells are CD30 positive mono- or multinucleated giant cells with a peculiar morphology (70). Although not exclusively expressed by H-RS cells (71), CD30 is diagnostically used for identification of H-RS cells in cHL.

Several studies suggest interdependency between H-RS cells and bystander cells and the wide array of cytokines and chemokines produced by the H-RS cells are thought to be 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 favouring persistence of the H-RS cells (72). The 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 (72).

Etiology and pathogenesis of Hodgkin lymphoma

The etiology of HL is still a mystery. Some studies clearly demonstrate that certain individuals are genetically predisposed for the development of HL (73). A potential correlation of different HLA genotypes with HL has been suggested, although HLA-independent genetic factors also have been described (63). Among children and young adolescents, HL seems to be more common among individuals with a high socio-economic status (74,75). However, similar studies on middle-aged individuals showed contradictory results, with an increased risk for both higher (76) and lower (77) socio-economic status. Interestingly, military staff affected by HL during the World War

II were shown to have significantly higher intelligence (78), as well as higher education (79) compared to the general army employee. Few siblings and well-educated mothers have also been suggested as risk factors, which indicate that a late exposure to a common infectious agent may increase the risk of HL development. It has been suggested that Epstein-Barr virus (EBV) is involved in the pathogenesis of HL in approximately half of all cases. The EBV-encoded protein LMP-1 has been demonstrated to up-regulate the anti-apoptotic bcl-2 and exhibit oncogenic potential (80, 81). EBV nuclear antigen 1 has been shown to induce the expression of CCL20 on HL cell lines and increase migration of regulatory T-cells (which are known to inhibit effector $CD4^+$ and $CD8^+$ T-cells) (82). Other risk factors that have been studied in this context include exposure to wood, exposure of healthcare personnel to HL patients, chemical exposure, cigarette smoking, drinking, diet and hormones (reviewed in (63). However, none of these factors seemed to correlate with HL.

Hodgkin lymphoma treatment

Chemotherapy, commonly ABVD (bleomycin, vinblastine, doxorubicin and dacarabazine) regimen, radiotherapy, or a combination of these constitute the foundation of HL treatment (83-86).

The origin of Hodgkin Reed-Sternberg cells

Mature B-cells expressing membrane-bound immunoglobulins are antigen-independently generated from lymphoid stem cells in the bone marrow. Every cell has a single antigenic specificity. Once mature, these naïve cells circulate in the blood and lymph and are eventually carried to the lymph nodes or the spleen. Upon antigen encountering, these cells undergo clonal expansion and differentiate into plasma cells and memory B-cells. During the differentiation process, B-cells migrate to certain lymph node compartments, secondary follicles, where they form germinal centres. These centres are sites for B-cell somatic hypermutations (i.e. strictly focused insertions, deletions and point mutations at a million-fold higher rate than normally observed). These

random events create a broad range of B-cell receptors with different antigen affinity, including a small proportion of cells expressing a high affinity B-cell receptor. For a long time the origin of the malignant H-RS cells remained mysterious. However, when the H-RS cells were shown to exhibit somatic mutations within the rearranged immunoglobulins, the germinal centre B-cell origin of these cells could be concluded (67,87). B cells exhibiting non-favourable mutations and thereby also a low-affinity B-cell receptor normally undergo FAS-mediated apoptosis. However, it is believed that the H-RS cells are rescued, at least in part by its high expression of c-FLIP, a potent inhibitor of FAS-mediated apoptosis (88). A schematic picture of the H-RS cell origin is depicted in Figure 4.

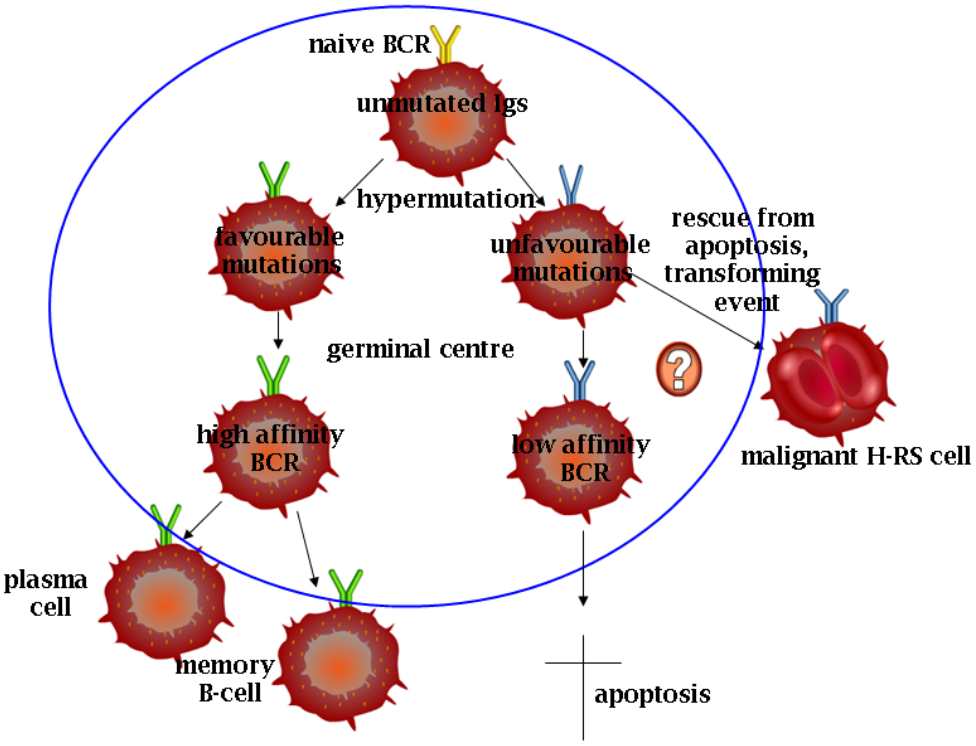

Figure 4. The origin of malignant cells in HL. Naive B-cells with unmutated immunoglobulins (Igs) form germinal centres within secondary follicles in lymph nodes. The cells undergo somatic hypermutations to create high-affinity B-cell receptors (BCR) on plasma cells and memory B-cells. Cells expressing a low affinity BCR normally undergo FAS-mediated apoptosis. However, H-RS cells may be generated if these cells are rescued from apoptosis followed by unknown transforming events.

Characteristics of Hodgkin Reed-Sternberg cells

Although H-RS cells are B cell-derived, these cells have major genotypic and phenotypic differences compared to B-cells. H-RS cells, in contrast to its normal counterpart, rarely express the B cell receptor. This may be related to the lack of the immunoglobulin-specific transcription factors OCT-2, BOB-1 and PU-1 expression in H-RS cells (89-91), aberrant rearrangements, unfavourable mutations in the immunoglobulin genes (87,92) or mutations in the promoter region of immunoglobulin genes (93-95). The H-RS cells express several B cell-atypical markers. These include CD15 and CD30 as well as the T cell associated proteins Notch 1, T-bet and GATA3 (96,97). Also, H-RS cells rarely express the typical B cell markers CD19, CD20 and CD79a. Thus, the H-RS cells have lost their B cell specific gene expression program (98,99).

Several aberrant signalling pathways contributing to the tumor growth have been demonstrated in H-RS cells. One of the most studied pathways is the constitutive activation of nuclear factor-κB (NF-κB) (100). NF-κB is a key regulator of the immune system and activates pro-proliferative and anti-apoptotic signalling pathways (101-103). The proliferation and survival of H-RS cells have been demonstrated to be dependent on NF-κB, and inhibition of this pathway by introduction of inhibitor of NF-κB (IκB) caused the H-RS cells to undergo apoptosis (104,105). Several factors contribute to the aberrant NF-κB signalling in H-RS cells, including CD30 signalling (106), CD40 signalling (107), mutations in the IκB genes, disturbed IκB kinase activity (108-110), NF-κB gene amplifications (111,112) and the EBV-encoded LMP-1 protein (113). Furthermore, several members of the signal transducer and activator of transcription (STAT) family, including STAT3, STAT5 and STAT6, have been implicated in the pathogenesis of HL (114-117).

Cytokines and chemokines in Hodgkin lymphoma

HL tissue is characterized by an aberrant cytokine and chemokine secretion pattern, and these proteins are important for the crosstalk

between H-RS cells and infiltrating bystander cells (72,118). IL-13 has been shown to promote H-RS cell proliferation in an autocrine manner (119-121), and some data, although less convincing, also point out IL-6, IL-7 and IL-9 as growth factors for H-RS cells (122-124). IL-10 and TGF-β are immunosuppressive cytokines (125-128) that significantly contribute to the immune evasion by H-RS cells, and elevated levels of IL-10 in sera of HL patients are considered to be an adverse prognostic factor (129,130). IL-8, MCP-4, IP-10, TARC, RANTES, MCP-1 and eotaxin, among other chemokines, are suggested to contribute to the typical cellular background in HL (131-137). A schematic picture of an H-RS cell and the typical cellular background is shown in Figure 5.

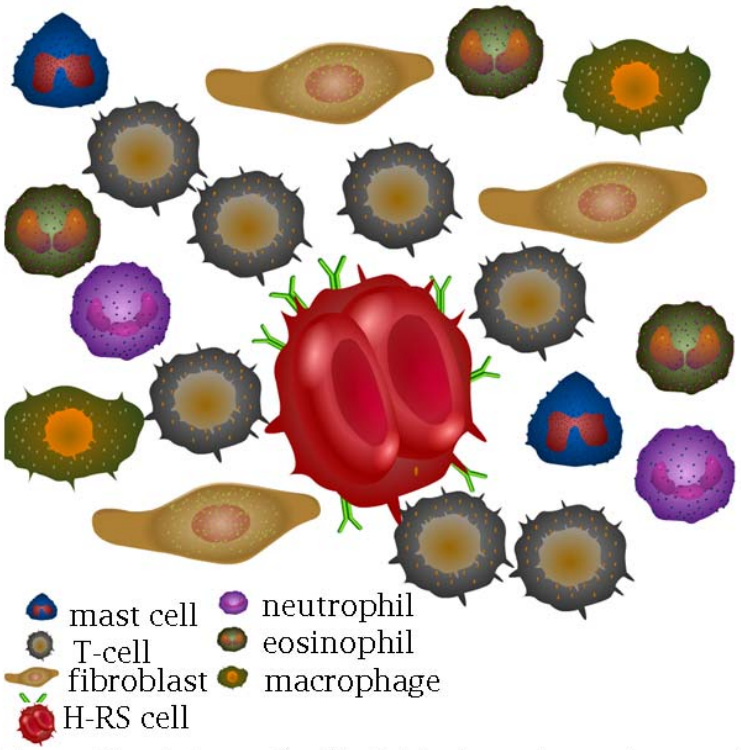

Figure 5. Schematic picture of a Hodgkin lymphoma tumor. The H-RS cells communicate with the inflammatory bystander cells via cytokines and chemokines. This interplay creates an enviroment that favours tumor growth.

Non-Hodgkin lymphoma

NHLs are heterogeneous malignancies classified according to the Revised European-American Lymphoma (REAL) classification (138) or the World Health Organization (WHO) (139) based on clinical

characteristics, morphology, genetics and immunology. The complex NHL WHO classification system (139) is summarized in Table II. Occasionally, lymphomas may transform, which is usually associated with clinical impairment, drug resistance, increase in tumor mass and elevated lactate dehydrogenase levels. The treatment strategies of the different NHLs may differ substantially, and therefore, an accurate diagnosis is crucial for the patient. However, in some cases the pathologist may experience difficulties in distinguishing the lymphomas from each other, and therefore, there is a need for additional biomarkers.

Non Hodgkin lymphoma treatment

Chemotherapy and radiotherapy are the main lymphoma treatment options. CHOP combination therapy, consisting of cyclophosamide, doxorubicin, vincristine and prednisolone, has been the most efficient NHL treatment for the last decades (140). Different modifications of the CHOP treatment has been performed in order to further improve the efficacy. The CNOP therapy (doxorubicin is replaced by mitoxantrone) has been shown to be inferior to the regular CHOP therapy among elderly patients with aggressive lymphomas (141). In the last decade, the addition of rituximab or mabthera (CD20 antibodies) to the conventional chemotherapy regimen has significantly contributed to an increased survival for follicular lymphomas and diffuse large B cell lymphoma (DLBCL) patients (142).

Table II

Non-Hodgkin lymphoma classification

B-lymphocyte malignancies

Lymphoma/Leukemia
- Precursor B-lymphoblastic leukemia/lymphoma
- Chronic lymphocytic leukemia
- Small lymphocytic lymphoma
- B-cell prolymphocytic leukemia
- Hairy cell leukemia
- Lymphoplasmocytoid lymphoma/ immunocytoma

Indolent nodal or extranodal lymphomas
- Marginal zone B-cell lymphoma
  - Mucosa-associated lymphoid tissue (MALT) lymphomas
  - Splenic marginal zone lymphoma
  - Nodal lymphomas
- Follicular lymphomas
  - Grade 1.-3
  - Cutaneous
  - Gastrointestinal
- Mantle cell lymphoma
  - Classic
  - With round cells
  - Blast
  - With large cells

Aggressive nodal or extranodal lymphomas
- Diffuse large B-cell lymphoma
  - Variants:Centroblastic lymphoma
    - Immunoblastic lymphoma
    - B-cell lymphoma rich in T-cells
    - B-cell lymphoma rich in histiocytes
    - Anaplastic large B-cell lymphoma
    - Burkitt-like lymphoma
    - Lymphomatoid granulomatosis
    - Pyothorax-associated lymphoma
  - Subtypes:Mediastinal lymphoma
    - Intravascular lymphoma
    - Serous lymphoma
- Burkitt´s lymphoma
  - With plasma cell differentiation

T-lymphocyte malignancies

Lymphoma/Leukemia
- Precursor T-lymphoblastic lymphoma/leukemia
- Prolymphocytic leukemia
- Large granular lymphocyte leukemia
  - T-cell type
  - NK-cell type
- NK-cell leukemia
- Mycosis fungoides
  - Pagetiod reticulosis
  - Follicular mucinosis
  - Granulomastous chalazodermia

Nodal or extranodal lymphomas
- T-cell, NK or γδ T-cell lymphomas
  - Nasal
  - Nasal-type
  - Subcutaneous apanniculitic T-cell lymphoma
  - Intestinal (enteropathy-associated) T-cell lymphoma
  - γδ T-cell hepatosplenic lymphoma
- Peripheral T-cell lymphomas
  - Lymphoepitheloid lymphoma
  - T-zone lymphoma
  - Angioimmunoblastic lymphoma
- Anaplastic large cell lymphoma (ALCL)
  - Lymphohistiocytic
  - Small cell
- CD30+ T-cell cutaneous lymphoproliferative syndromes
  - Lymphomatiod papulosis
  - Primary cutaneous lymphoma
  - Low-grade intermediate variants
- Lymphoma/Leukemia secondary to HTLV-1 infection
  - Acute variant
  - Lymphoma
  - Chronic variant
  - Sub-acute variant
  - Hodgkin-like variant

Adapted from Harris et al Blood 1994 and Harris et al Mod Pathol 2000

AIMS

I To elucidate the role of cysteinyl leukotrienes in the pathogenesis of classical Hodgkin lymphoma

II To elucidate the expression of the Cysteinyl leukotriene receptor 1 and 15-lipoxygenase-1 in different non-Hodgkin lymphoma entities

III To elucidate the expression pattern of 15-lipoxygenase-1 in primary Hodgkin Reed-Sternberg cells and map the production of 15-lipoxygenase-1-derived metabolites in Hodgkin lymphoma cell lines

IV To elucidate the membrane interaction and activity of human 15-lipoxygenase-1 in calcium ionophore stimulated dendritic cells

METHODOLOGICAL CONSIDERATIONS

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

Cell lines and biopsies (Papers I,II, III)

To this date there is no optimal animal model available for HL. Despite many thorough attempts it has been difficult to overcome problems such as low engraftment efficiency, requirement for immunosuppressive drugs as well as reproducibility difficulties (143-145). Thus, the research within this field is highly dependent on HL-derived cell lines. I have mainly worked with the B cell derived L1236 cell line since it has been molecularly proven to be of true H-RS cell origin (146,147). Included in the studies are also the HL cell lines L428 (mixed B- and T-cell phenotype), KMH2 (B-cell phenotype), and HDLM2 (T-cell phenotype). To better understand the pathogenesis of HL it would of course be desirable to also have an adequate animal model in the future.

Immunohistochemistry (Papers I-III)

Primary antibodies are produced by immunization of animals with certain antigens. The resulting specific antibodies could then be used to elucidate the presence of proteins in tissue biopsies. I have mainly used the avidin-biotin alkaline phosphatase method which includes labelling with a primary antibody, followed by a secondary biotin-conjugated antibody. Subsequently, alkaline phosphatase-conjugated avidin-biotin complexes bind to the secondary antibody and a substrate for alkaline phosphatase is utilized for visualization. If present, the protein of interest will be visible as red colour. To assure specific staining, proper controls are crucial. For all 15-LO-1 stainings, I used serum from pre-immunized rabbit as a specificity control. For the $CysLT_1$ and $CysLT_2$ receptor stainings, I used rabbit serum and omission of primary antibody to exclude binding of the secondary antibody directly to the

tissue. Finally, blocking peptide was pre-incubated with the corresponding antibody before application to the tissue to ascertain specific binding. The stainings were analyzed blinded by three persons; including one senior hematopathologist.

RESULTS AND DISCUSSION

The expression and function of cysteinyl leukotriene receptors in classical Hodgkin lymphoma (paper I)

Accumulating data suggest that the inflammatory bystander cells in cHL are important for the maintenance of the tumor (131,148). The bystander cells and the malignant cells are thought to communicate to create a favourable atmosphere for the tumor to grow. Elevated levels of the Th2 cytokines IL-5, IL-9 and IL-13 are commonly observed in the tumor (72), and the latter one has been shown to directly stimulate proliferation of the H-RS cells (149). Also other mediators are likely to be involved in this crosstalk, and therefore it was of interest to study the potential role of arachidonic acid-related metabolites in the pathogenesis of cHL.

Classical HL cell lines were screened against a library including a broad range of lipid-derived ligands and calcium mobilization assays were utilized to identify functional receptors. In this screening process, we found that the cHL cell lines L1236 and KMH2, but not HDLM2 and L428, responded with a robust calcium signal upon cysLT challenge. This signal was completely blocked by zafirlukast in the nanomolar range, suggesting that L1236 and KMH2 cells express functional $CysLT_1$ receptors. The $CysLT_1$ receptor expression was also confirmed by quantitative PCR and immunocytochemistry analyses.

We further investigated the $CysLT_1$ receptor protein expression by H-RS cells *ex vivo*. The expression of the $CysLT_1$ receptor was studied by immunohistochemistry in formalin-fixed paraffin-embedded sections from cHL lymph node biopsies with the avidin biotin alkaline phosphatase technique. The $CysLT_1$ receptor was expressed by the H-RS cells in 12 of 20 cHL tumors. Importantly, the presence of this receptor in primary H-RS cells could also be confirmed by microarray analysis, where laser captured primary H-RS cells were shown to express

substantially higher $CysLT_1$ receptor mRNA compared to the normal counterpart in five of nine tumors.

Since cHL is associated with an aberrant cytokine and chemokine production by the H-RS cells and the reactive infiltrate (72), we analyzed the effect of cysLTs on cytokine and chemokine release by L1236 and KMH2 cells by flow cytometry. Interestingly, LTD_4 significantly increased the secretion of TNF-α, ($p<0.001$), IL-6 ($p<0.001$) and IL-8 ($p=0.012$) by L1236 cells in a dose-dependent manner as compared to vehicle treated cells. This effect was indeed mediated via the $CysLT_1$ receptor, since the increased cytokine/chemokine protein secretion was abolished upon pre-treatment with zafirlukast (1μM). For KMH2 cells, IL-8 was markedly increased upon $CysLT_1$ receptor stimulation and this effect was reversed by zafirlukast (0.1 μM). Recently, the GPR17 receptor was identified as a dual nucleotide and CysLT receptor with IC50 values similar to the $CysLT_1$ receptor (37). Although the GPR17 receptor is also expressed by L1236 cells (unpublished data Schain, Claesson, Björkholm, Sjöberg, Abbracchio and Rosa), the IC50 values indicate that the observed calcium release by these cells is indeed $CysLT_1$ receptor-mediated. However, we cannot completely exclude a possible contribution from the GPR17 receptor.

The biological implications of these findings remain to be further elucidated since little is known regarding the role of TNF-α, IL-6 and IL-8 in the pathogenesis of cHL, mainly due to the lack of appropriate animal modes. One may however speculate that TNF-α-induced release of eotaxin by tumor-associated fibroblasts will attract eosinophils (132). These cells will constitute a source of additional cysLTs, and potentially contribute to maintenance of an inflammatory environment, favoring the persistence of the H-RS cells.

The stimulation of L1236 or KMH2 cells with LTD_4 did not affect the cell proliferation, although a significant increased DNA synthesis was noted in L1236 cells. The reason for these results is at present not

clear. However, cysLTs are probably not important growth-promoting factors in cHL. A schematic hypothetical model over cysLT signalling in cHL and its effects are shown in Figure 6.

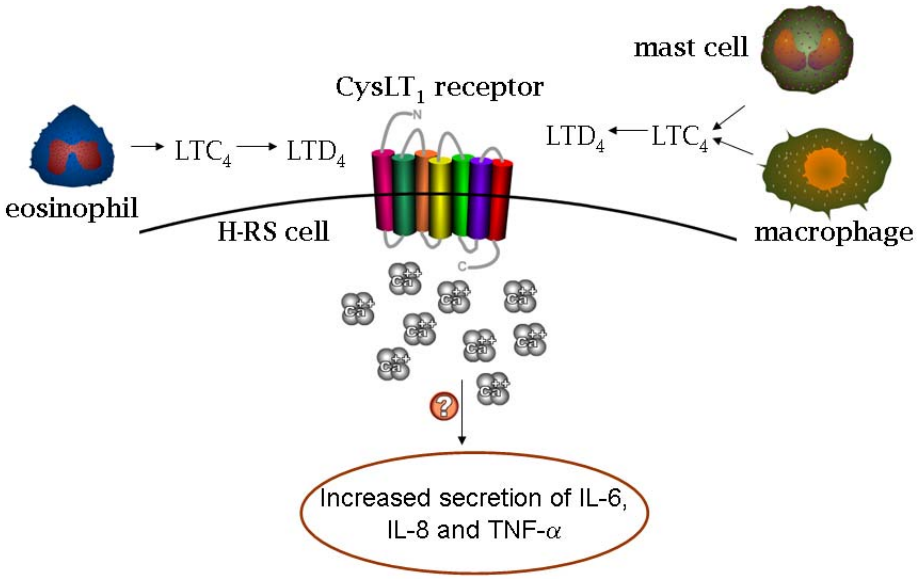

Figure 6. Hypothetical $CysLT_1$ receptor signaling in H-RS cells. Surrounding eosinophils, mast cells and macrophages are sources of LTC_4 which is released extracellularly and further metabolized by glutamyl transpeptidase to LTD_4. LTD_4 will bind to the $CysLT_1$ receptor expressed by H-RS cells, which will result in intracellular calcium release. By yet unidentified mechanisms, this will trigger increased release of IL-6, IL-8 and TNF-α by the H-RS cells.

Hodgkin Reed-Sternberg cells express 15-lipoxygenase-1 and produce eoxins (Paper II)

As previously mentioned, 15-LO-1 is an intriguing enzyme involved in inflammation (150) and cancer (22,151-160). Since inflammation is a characteristic feature of cHL it was of interest to study whether different HL cell lines express 15-LO-1. The metabolism of arachidonic acid was therefore investigated in four different cHL cell lines (i.e. L1236, L428, KMH2 and L570). These cell lines produced no or very low amounts of 5-HETE or LTs, indicating that these cells do not have any 5-LO activity. However, incubation of L1236 cells with arachidonic acid led to a major peak corresponding to 15-HETE and a minor peak corresponding to 12-HETE in a ratio 9:1. Chiral chromatography

analysis revealed that these products were exclusively 15 (S)-HETE and 12-(S)-HETE. L1236 cells were also able to produce 13-hydroxy-octaaadecadienoic acid (13-HODE) upon incubation with linoleic acid. Furthermore, RT-PCR analysis and immunocytochemistry confirmed a strong expression of 15-LO-1, but not 15-LO-2, in these cells. We also studied the subcellular localization of 15-LO-1 in L1236 cells in the presence and absence of calcium by immunocytochemistry and Western blot. In concordance with previous studies performed on other cell types (15,68), our studies demonstrated that 15-LO-1 was present mainly in the cytosol and that the enzyme translocated to membranes in the presence of calcium.

Our group recently described pro-inflammatory arachidonic acid-derived metabolites referred to as eoxins (EXs) formed via the 15-LO-1 pathway in mast cells and eosinophils. These mediators were almost as potent as LTC_4 and LTD_4 and hundred times more potent than histamine, in increasing the permeability in a endothelial cell layer *in vitro* (29). These data indicate that EXs may be important players in inflammatory responses. Interestingly, L1236 cells were also able to convert arachidonic acid to significant amounts of EXC_4, EXD_4 and EXE_4 as shown by HPLC. Shown in Figure 7 is an overview over the eoxin formation in L1236 cells.

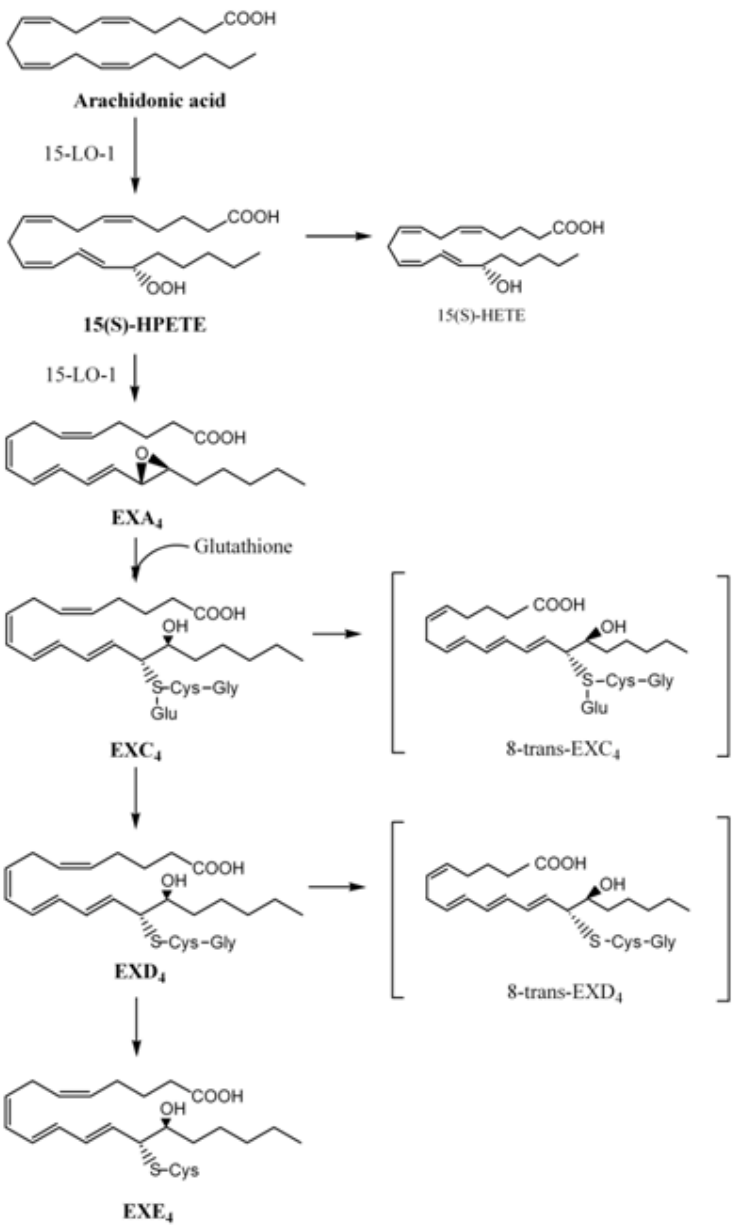

Figure 7. Overview of the metabolic pathway for the formation of eoxins in L1236 cells. EX; Eoxin

To elucidate the expression of 15-LO-1 protein in primary H-RS cells we performed immunohistochemical analyses on primary cHL biopsies. 15-LO-1 expression was noted in macrophages and eosinophils in the majority of the HL tumors and was thus used as internal positive controls. In 17 of 20 tumors, 15-LO-1 was also noted in the H-RS cells. The results from this study therefore suggest that H-RS cells might be able to produce eoxins and other 15-LO-1 derived metabolites also *in*

vivo. Future studies will reveal whether the eoxins are important players in the pathogenesis of cHL by a potential contribution to an inflammatory environment.

Since 15-LO-1 is strictly expressed by a limited number of cells, such as dendritic cells and eosinophils, these cells are commonly used to study the properties of 15-LO-1. These cells are however time-consuming and expensive to isolate and culture. Thus, L1236 cells may be a useful tool to study the properties and function of 15-LO-1.

Expression of the Cysteinyl Leukotriene Receptor 1 and 15-lipoxygenase-1 in non-Hodgkin lymphomas (paper III)

To study whether the $CysLT_1$ receptor and 15-LO-1 were expressed exclusively by the malignant cells in cHL, as opposed to other lymphoma entities, over 50 NHL tumors were examined for the presence of these proteins by immunohistochemistry. In contrast to all other NHLs under study, nine of ten primary mediastinal B-cell lymphomas (PMBCLs) exhibited malignant cells expressing the $CysLT_1$ receptor. The PMBCL-derived cell lines Med-B1 and Karpas-1106P responded to cysLTs with increased calcium mobilization in a $CysLT_1$-specific manner. Similarly to the HL data, stimulation of the $CysLT_1$ receptor did not cause any increased cell proliferation. We also elucidated whether Med-B1 or Karpas-1106P expressed 15-LO-1 by immunocytochemistry and HPLC analysis. The results showed that neither of these cell line had any 15-LO-1 protein or activity. Interestingly, when the Karpas-1106P cells were treated with IL-4 these cells were able to produce significant amounts of 15-LO-1-derived metabolites (unpublished data Andersson, Schain, Sjöberg, Björkholm, Forsell and Claesson). Additionally, one of four anaplastic large T cell lymphomas (ALCLs) strongly expressed 15-LO-1 *in vivo*. This is the first time T cell-originating cells have been shown to express this enzyme. The ALCL-derived cell lines L-82 and SU-DHL-1 were, however, not able to metabolize arachidonic acid or linoleic acid. Also, the expression of

15-LO-1 could not be detected by RT-PCR or immunohistochemistry in these cells. Thus, it appears that L-82 and SU-DHL-1 cells do not express 15-LO-1 mRNA or protein, although 80 percent of the malignant cells in one of four tumors strongly expressed this enzyme. Further studies including additional ALCL tumors are needed to clarify the expression of 15-LO-1 in this tumor entity. The findings in this study contribute to define the biological properties of lymphomas and, hypothetically, these molecules may be targets for therapeutical interventions or sub-classification of lymphomas.

Membrane interaction and activity of 15-lipoxygenase-1 in dendritic cells (paper IV)

15-LO-1 has been implicated in several pathophysiological conditions (20, 22). 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, dendritic cells were generated *in vitro* from peripheral blood monocytes according to standard protocols. Flow cytometry was used for dendritic cell immunophenotyping with antibodies directed against CD14, CD40, CD80 and DC-sign. The mature dendritic cells (i.e. $CD14^-$ $CD40^+CD80^+DC\text{-}sign^+$) were incubated in the absence or presence of calcium ionophore (2 µM) in 37°C. The cells were re-suspended in PBS without calcium or magnesium, and cytocentrifuged on glass slides. The dendritic cells were stained with a polyclonal 15-LO-1 antibody, visualized with immunofluorescence technique, and analyzed in a confocal microscope. In line with a previous study (15), 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 dendritic cells 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 (161).

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 the lipid composition 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 free fatty acids. 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.

SUMMARY AND CONCLUDING REMARKS

Certain HL cell lines express functional $CysLT_1$ receptors which respond to cysLTs with a robust calcium release. This receptor is also expressed by primary H-RS cells as shown by immunohistochemistry, microarray of lasercaptured cells and quantitative PCR. The stimulation of the $CysLT_1$ receptor with LTD_4 induced the HL cell line L1236 to secrete TNF-α, IL-6 and IL-8 and the KMH2 cell line to secrete IL-8. Thus, cysLT signalling may be an important player in the pathogenesis of cHL by significantly contributing to the aberrant cytokine and chemokine secretion by the H-RS cells.

The cHL cell line L1236 and primary H-RS cells express 15-LO-1 and the former cells produce eoxins via the 15-LO-1 pathway. Thus, this enzyme may contribute to the inflammatory features of cHL and these findings may have important both diagnostic and therapeutic implications.

The majority of the primary mediastinal B-cell lymphomas under study, in contrast to other NHLs, expressed the $CysLT_1$ receptor. Furthermore, one T cell-derived anaplastic large cell lymphoma was the only NHL shown to express 15-LO-1. The $CysLT_1$ receptor and 15-LO-1 are potential targets in lymphoma diagnostics and sub-classification.

In the presence of calcium, addition of $PI(3.4)P_2$ or $PI(4.5)P_2$ to vesicles containing arachidonic acid lead to a significantly increased formation of 15-HETE compared to vesicles without phosphoinositides. These results suggest that 15-LO-1 activity may also be regulated by the phospholipid constitution of membranes.

FUTURE PERSPECTIVES

Ongoing studies in our laboratory aim to elucidate the potential role of 15-LO-1 as a regulator of adaptive immune responses. Specifically, we have differentiated dendritic cells in the presence or absence of specific 15-LO-1 inhibitors and studied the resulting dendritic cell phenotype and function.

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 play an essential role in the adaptive immune response. Due to their high level of constitutively expressed class II major histocompatibility complexes (HLA in human) and co-stimulatory B7 membrane molecules, dendritic cells are the most potent antigen-presenting cell (162) and play a unique role in the defense against microorganisms. Upon antigen encountering in tissue or blood, immature dendritic cells migrate to lymphoid organs where they mature and not only promote expansion and differentiation of antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes (162), but also activate natural killer cells, macrophages and eosinophils (163,164). Mature dendritic cells 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 (165). Peripheral blood monocytes are relatively small cells (162) expressing 5-LO but not 15-LO (166). However, during *in vitro* differentiation these cells dramatically change phenotype to become large granulated cells (162) expressing 15-LO-1 but not 5-LO (166).

Figure 8 is a schematic picture illustrating the morphologic and phenotypic transformation of a monocyte during dendritic cell differentiation. The changes include loss of the monocyte marker CD14,

expression of co-stimulatory molecules (CD80, CD83, CD86), and DC-sign, HLA-DR and HLA-ABC.

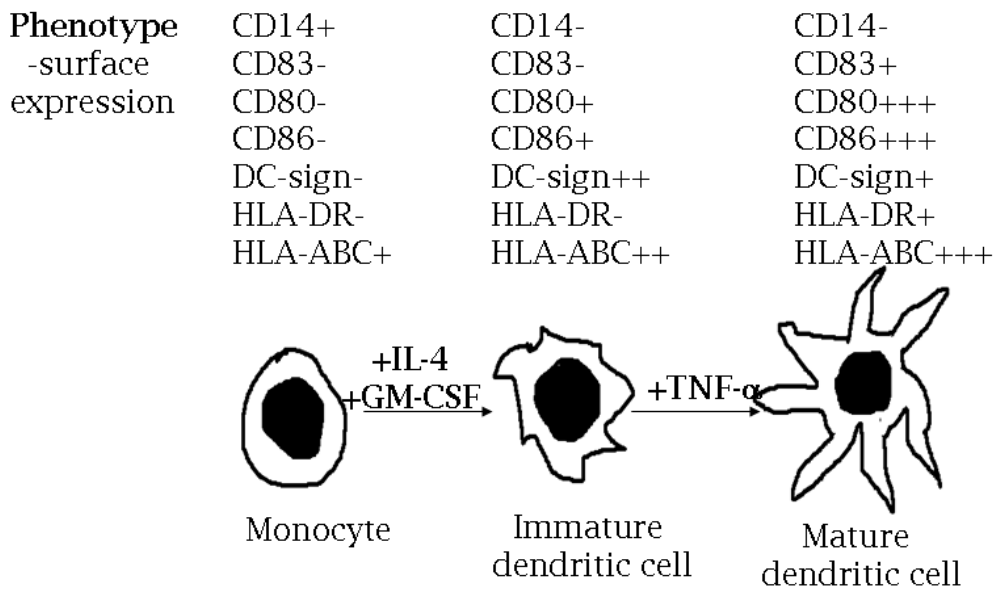

Figure 8. Dendritic cell differentiation-associated phenotypic changes. Mature dendritic cells can be generated *in vitro* from monocytes by the addition of IL-4, GM-CSF and TNF-α. Monocytes express CD14, but are negative for the dendritic cell-associated markers CD80, CD83, CD86, DC-sign and HLA-DR. In contrast, mature dendritic cells lack CD14 expression but are positive for CD80, CD83, CD86, DC-sign and HLA-DR.

Efficient T-cell activation requires dendritic cell HLA-peptide interaction with a T-cell receptor followed by a set of co-stimulatory events. CD80 and CD86, important co-stimulatory molecules expressed by dendritic cells, are known to interact with the CD28 receptor on T-cells. Additionally, CD83 is not only an important maturation marker for human dendritic cells, but also crucial for initiation of T-cell proliferation (167,168). CD14, a protein highly expressed by monocytes, is downregulated on mature dendritic cells (162).

To elucidate the role of 15-LO-1 in dendritic cell differentiation we have cultured monocytes for seven days in the presence or absence of 15-LO-1 inhibitors, followed by analysis of relevant cell surface markers by flow cytometry. Preliminary data indicate that the expression of HLA-

DR, HLA-ABC, CD83 and CD86 is decreased when the dendritic cells are differentiated in the absence of 15-LO-1. In contrast, the expression of CD80 and DC-sign is upregulated by 15-LO-1 inhibition. Thus, all alterations in surface marker expression, except for the up-regulation of CD80, point towards a more immature dendritic cell phenotype upon 15-LO-1 inhibition (162). Additionally, when the dendritic cells differentiated in the absence of 15-LO-1 activity was co-cultured with autologous peripheral blood mononuclear cells, the latter cells secreted markedly lower and higher levels of Th1 and Th2 cytokines, respectively. Further studies are needed to clarify the role of 15-LO-1 in dendritic cell differentiation and function.

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