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STUDIES ON THE REGULATION AND FUNCTION OF LIPOXYGENASES IN HODGKIN LYMPHOMA

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ABSTRACT

Hodgkin lymphoma (HL) is a unique entity among the lymphomas, with a minority of malignant Hodgkin and Reed-Sternberg (H-RS) cells surrounded by a broad range of infiltrating cells. The infiltration of certain inflammatory cells has been reported to predict prognosis of the disease. In HL tumor microenvironment, the primary H-RS cells and those inflammatory cells interact interdependently. The aberrant cytokine production of H-RS cells has been suggested to contribute to this interdependency. However, little is known in terms of the mechanisms involved in the abnormal cytokine secretion by H-RS cells. Previous studies suggested that several pro-inflammatory molecules likely contribute to the aberrant cytokine secretion of HL, including cysteinyl-leukotrienes receptor type I ($CysLT_1R$) and 15-lipoxygenase-1(15-LOX-1) that are highly expressed in primary H-RS cells and cultured HL-derived L1236 cells.

Previous and present studies in cultured HL cells demonstrate that $CysLT_1R$ mediates transcription and secretion of cytokines, including interleukin (IL)-6, IL-8 and tumor necrosis factor-α, upon stimulation by leukotriene D_4 (LTD_4). This lipid mediator is formed from arachidonic acid through the 5-lipoxygenase (5-LOX) pathway and several types of inflammatory cells surrounding H-RS cells can produce cysteinyl-leukotrienes. To depict the intracellular signaling pathways that bridge the LTD_4-$CysLT_1R$ ligation to cytokine induction, a mechanistic study was carried out in L1236 cells. The results demonstrated that the transcription factor early growth response (EGR)-1 is involved in the LTD_4-triggered cytokine transcriptional induction.

The regulatory mechanisms implicated in 15-LOX-1 trans-activation in HL have been obscure. This study has also assessed the epigenetic modulation of 15-LOX-1 in different aspects. The results revealed that signal transducer and activator of transcription (STAT)-6 positively regulates 15-LOX-1 transcription by binding to its promoter, in which three putative STAT-6 binding motifs are identified to be required for full activation. The accessibility of STAT-6 to the 15-LOX-1 promoter is controlled by DNA methylation and histone modification. The histone H3 lysine (K)-4 specific methyltransferase SMYD3 was found to exhibit an important role in this multi-step regulation. Although the H3K27me3 demethylase UTX mediates 15-LOX-1 trans-activation by H3K27 demethylation upon IL-4 stimulation in lung carcinoma A549 cells, a crucial histone H3K27-demethylase-independent role of UTX in 15-LOX-1 transcriptional regulation in L1236 cells was demonstrated.

In conclusion, this study has evaluated the biology of HL by using *in vitro* models, focusing on lipoxygenases regulation and function. The results not only demonstrated a signaling pathway that hypothetically bridges 5-LOX activity to the striking inflammatory microenvironment in HL, but also uncovered epigenetic regulation mechanisms involved in 15-LOX-1 expression in HL-derived cells. Our findings suggest that lipid signaling pathways might play critical roles in HL pathogenesis, thus warranting further HL research.

LIST OF PUBLICATIONS

I. **Han H***, Xue-Franzén Y*, Miao X, Nagy E, Xu D, Sjöberg J, Björkholm M, Claesson HE. *The transcription factor Early Growth Response mediates leukotriene D4-induced expression of cytokines in the Hodgkin lymphoma Cell Line L1236*, Submitted

II. Liu C*, Schain F*, **Han H***, Xu D, Andersson-Sand H, Forsell P, Claesson HE, Björkholm M, Sjöberg J. *Epigenetic and transcriptional control of the 15-lipoxygenase-1 gene in a Hodgkin lymphoma cell line*. Exp Cell Res, 2012. 318(3): p 169-76.

III. Liu C, Xu D, **Han H**, Fan Y, Schain F, Xu Z, Claesson HE, Björkholm M, Sjöberg J. *Transcriptional regulation of 15-lipoxygenase expression by histone h3 lysine 4 methylation/demethylation*. PLoS One, 2012.7(12): p e52703.

IV. **Han H**, Xu D, Liu C, Claesson HE, Björkholm M, Sjöberg J. *Interleukin-4-mediated 15-Lipoxygenase-1 trans-activation requires UTX recruitment and H3K27Me3 demethylation at the promoter in A549 cells*. PLoS One, in press.

* contributed equally

CONTENTS

LIST OF ABBREVIATIONS

15(S)-HETE	15(S)-hydroxy Eicosatetraenoic acid
15-LOX-1	15-lipoxygenase-1
5-Aza	5-Aza-2'-deoxycytidine
AA	Arachidonic acid
AP-1	Activator protein 1
BCR	B cell receptor
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
c-FLIP	FLICE-like inhibitory protein
ChIP	Chromatin immunoprecipitation
CREB	cAMP response element-binding protein
CRISPR	Clustered regularly interspaced shortpalindromic repeats
CSIF	Cytokine synthesis inhibitory factor (IL-10)
CysLTs	Cysteinyl-leukotrienes
DAPI	4′, 6-diamino-2-phenylindol
DC	Dendritic cell
DDMs	DNA demethylases
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
EDF	Eosinophil differentiation Factor
EGR-1	Early Growth Response 1
ELISA	Enzyme-linked immunosorbent assay
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA	GATA-binding protein
GC	Germinal center
HATs	Histone Acetyltransferases
HDACs	Histone deacetylases
HDMs	Histone demethylases
HL	Hodgkin lymphoma
HMTs	Histone methyltransferases
HnKm	Histone Hn lysine m
HnKmme2	Histone Hn lysine m di-methylation
HnKmme3	Histone Hn lysine m tri-methylation
H-RS	Hodgkin Reed-Sternberg
IFNB2	Interferon beta-2
IgG	Immunoglobulin G
IL	Interleukin
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

JMJD3	Histone Demethylase Jumonji D3
KAT3B	Histone acetyltransferase lysine (K)-acetyltransferase 3B
KDM3A	Lysine-Specific Demethylase 3A
LPS	Lipopolysaccharides
LT	Leukotriene
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MDC	Macrophage-derived chemokine
MIP	Macrophage inflammatory protein
MSP	Methylation-specific PCR
MUM-1	Multiple Myeloma Oncogene 1
NAB-2	NGFI-A binding (NAB) proteins member 2
NCBI	National Center for Biotechnology Information
NEAA	Non-Essential Amino Acids
NF	Nuclear factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NLPHL	Nodular lymphocyte predominant Hodgkin lymphoma
NR4A3	Steroid-thyroid hormone-retinoid receptor Nuclear Receptor Subfamily 4, Group A, Member 3
Pax-5	Paired box family of transcription factors, member 5
PcG	Polycomb group
PCR	Polymerase chain reaction
PRCs	Polycomb-Repressive Complexes
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative Real-time PCR
RANK	Receptor Activator of Nuclear Factor κ B
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAHA	Suberoylanilide hydroxamic acid
SCC	Squamous cell carcinoma
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SMCX	Smcy Homolog, X-Linked
SMYD3	SET And MYND Domain-Containing Protein 3
STAT	Signal transducer and activator of transcription
TARC	Thymus and activation regulated chemokines
TNF-α	Tumor necrosis factor-α
TRIB-1	Tribbles homolog 1
TrxG	Trithorax group
TSA	Trichostatin A
UTX	Ubiquitously Transcribed Tetratricopeptide Repeat Protein X-Linked
XIAP	X-linked inhibitor of apoptosis
β2m	Beta-2 microglobulin

1 INTRODUCTION

1.1 HODGKIN LYMPHOMA:

A cancer with an inflammatory signature

In 1832, Hodgkin lymphoma (HL) was first described by Thomas Hodgkin[1], and in 1865, it was re-described by Samuel Wilks in further detail and later to be called Hodgkin's disease[2]. About 70 years later, Carl Sternberg and Dorothy Reed identified a type of giant cells, later termed Hodgkin Reed-Sternberg (H-RS) cells [3]. These cells are now considered as the malignant cells in HL. According to the World Health Organization classification, HL comprises two entities, nodular lymphocyte predominant HL (NLPHL) and classical HL. The classical HL can be further subdivided into four subtypes, including nodular sclerosis, mixed cellularity, lymphocyte-depleted, and lymphocyte-rich [4, 5]. Hereafter HL refers to classical HL unless stated otherwise.

The identification of HL malignant H-RS cells provides an opportunity for the HL biologic and therapeutic research. In this respect, the origin of H-RS cells became crucial since it could lead us to understand the pathogenesis of this disease. Although the origin of H-RS cells is now identified to be the germinal center (GC) B cells [4, 6-9], the origin of these particular cells has confused researchers for decades, mainly due to the limited number of H-RS cells in HL tumor tissue. The HL tumor tissues contain only 1-3% H-RS cells surrounded by abundant inflammatory cells [10]. Moreover, mixed hematopoietic cell markers are expressed in H-RS cells, such as the dendritic cell associated molecules: restin, fascin and thymus and activation regulated chemokines (TARC); Granulocyte and monocyte (CD15), B cells (Pax-5), plasma cells (MUM-1, CD138)[4]. Finally, when H-RS cell immunoglobulin rearrangements was analyzed by using microdissection and single-cell PCR, the origin of these cells were clarified as pre-apoptotic GC B-cells because they were carrying destructive V-gene mutations[6, 7, 11], although rare cases are derived from T cells[12-15]. The origin of H-RS cells and lymphocytic and histiocytic cells in NLPHL was described schematically by Thomas et al[5](figure 1). In GC, most B cells with low B cell receptor (BCR) affinity are programmed to die after positive selection, and this cell programmed death is mediated by the TNF

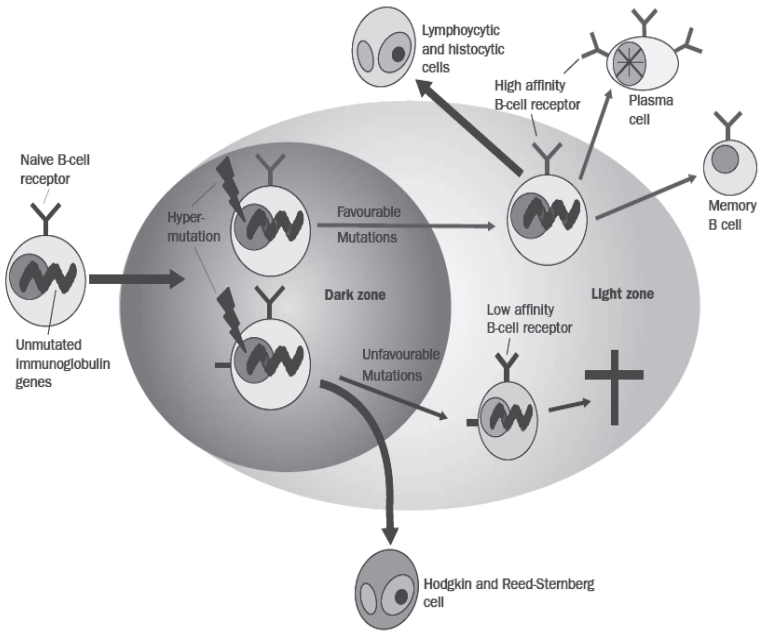

Figure 1 The origin of the H-RS cell (adapted from RK Thomas, *et al*.2004. With permission)

super-family member Fas[16]. However, the H-RS cells survive in absence of selection for antigen and lack of expression of the BCR, indicating that these particular cells are Fas resistant, which was confirmed previously in cultivated H-RS cells[17]. The mechanisms implicated in Fas resistance may potentially be therapeutically approachable. The apoptosis escaping mechanism of H-RS cells has been an attractive topic. However, addressing somatic mutations of the Fas gene seems not to be a main strategy because only 3 of 32 examined cases of HL with proven GC B-cell derivation were found to harbor deleterious FAS gene mutations[18, 19]. Several molecules were demonstrated to be involved in the anti-apoptotic feature of H-RS cells. Among those, X-linked inhibitor of apoptosis (XIAP) [20] and anti-apoptotic c-FLIP [19, 21] has been suggested to play important roles in apoptosis resistance. Additionally, certain pro-survivor factors such as cytokines and transcription factors are also implicated in H-RS cell survival (discussed later).

A feature that makes this lymphoma unique is the minority of neoplastic H-RS cells interspersed in an abundant reactive background of inflammatory cells (figure 2), including non-neoplastic B and T lymphocytes, mast cells, eosinophils, plasma cells,

and histiocytes/macrophages. Fibroblast-like cells and reticulum cells are also found detectable [22]. Among these infiltrating cells, CD4 positive T lymphocytes usually predominate and form rosette-like communications with H-RS cells[23]. The H-RS cells and the surrounding inflammatory cells are interdependent, and the infiltration of macrophages, eosinophils and mast cells has been reported to predict prognosis in some but not all studies[24-29]. Indeed, soluble factors such as cytokines, chemokines as well as lipid mediators together with surface receptors mediate the specific cellular composition of the reactive infiltrate and promote H-RS cell survival[30-33] .

1.2 THE H-RS CELL

A master of cytokine production

Cytokines are small cell-released proteins that have specific effects on the communications between cells, including leukocyte recruitment. Several lines of evidence suggest that the reactive infiltrating cells in the HL microenvironment are recruited by cytokines and chemokines released by H-RS cells, contributing to a favorable microenvironment [30, 34-36]. Indeed, H-RS cells and the HL cell lines produce a wide array of cytokines and chemokines, which are summarized in table 1.

1.3 THE MOLECULAR SIGNATURE OF H-RS CELLS

1.3.1 Signal transducer and activator of transcription-6, constitutively activated in H-RS cells and the L1236 cell line, critical for cell survival

The Signal transducer and activator of transcription (STAT) family, comprises seven different members including STAT-1, STAT-2, STAT-3, STAT-4, STAT-5 (STAT-5A and STAT-5B) and STAT-6[37]. In primary H-RS cells and HL cell lines, STAT-3[38-40], STAT-5[41, 42] and STAT-6[43, 44] are commonly activated, at least in part likely due to the multiple cytokines produced by these cells. Among these activated transcription factors, STAT-6 is constitutively phosphorylated in 5 of 5 tested HL cell lines and in H-RS cells from 25 of 32 (78%) evaluated classical HL cases[44].

STAT-6 is a mediator involved in the IL-4/IL-13 cascade. Briefly, in response to IL-4/IL-13, STAT-6 is phosphorylated by the receptor associated Janus kinase, and then

forms STAT-6 homo-dimers that translocate to the nucleus where target genes are bound and activated (figure 3). IL-13 and the IL-13-specific receptor chain (IL-13Rα1) are frequently co-expressed in H-RS cells and HL-derived cell lines[45, 46], suggesting that an autocrine loop triggers and maintains constitutively activated STAT-6, which has been proven by significant decrease of STAT-6 phosphorylation upon IL-13 neutralizing antibody treatment of HL cell lines[47]. In the same study, the cell proliferation was also affected by antibody-mediated neutralization of IL-13, indicating that the IL-13/STAT-6 signaling pathway acts as an important proliferation signal in H-RS cells and HL cell lines. Lentiviral shRNA-mediated STAT-6 inhibition significantly suppressed proliferation and induced apoptosis in L1236 cells [43], further demonstrating the crucial role of STAT-6 in HL pathogenesis.

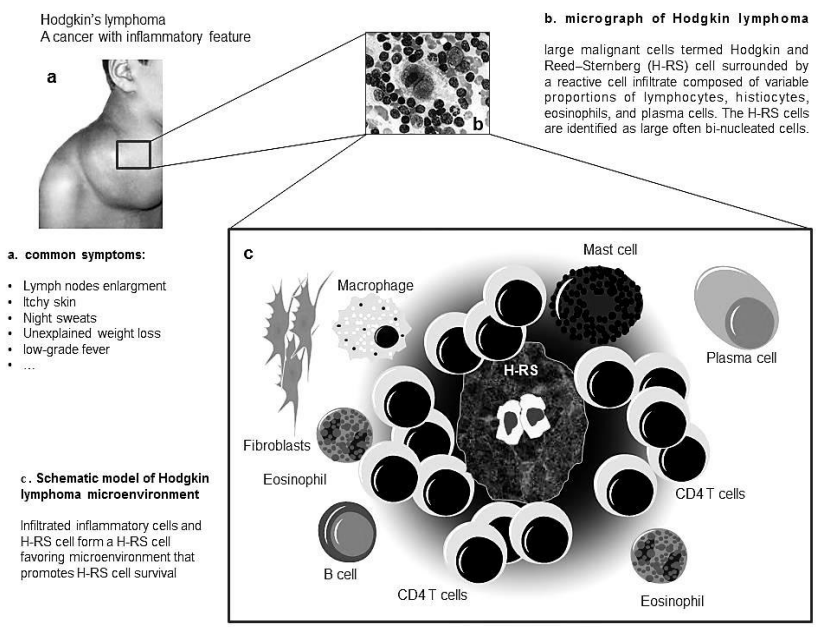

Figure 2 Clinical symptoms and histologic feature of HL

Additionally, STAT-6 is thought to contribute to the inflammatory features of HL. Our preliminary data (not shown) revealed changes of cytokine expression pattern upon STAT-6 inhibition, indicating that STAT-6 directly contributes to cytokine transcription. Further, STAT-6 is a common transcription factor involved in immune cell differentiation including Th2 cells, macrophages and dendritic cells, triggered by IL-4 stimulation. In human macrophages and dendritic cells, STAT-6 mediates

Table 1 Cytokines and Chemokines involved in HL

Cytokines	Aliases	General Function	HL tumor tissue	HL-derived cell lines	Functions in HL
IL-1α /IL-1β	IL-1A /IL-1B	Pro-inflammation, involved in host defense[48-50]	H-RS cells frequently express IL-1α (58%-75%), rarely IL-1β (<5%)[51-55]	HDLM2 secrets IL-1α, KM-H2 mainly produces IL-1β[56, 57]	Fibrosis and sclerosis induction[52, 55]
IL-5	EDF	Eosinophil activation[22, 58]	Highly expressed in HL tissue (95%)[35, 59]	Expressed in L-428, KM-H2[57, 60]	Associated with tumor eosinophilia[36, 61]
IL-6	IFNB2	B cell and plasma cell differentiation[62], pro-inflammation[63]	Frequently (from 65% to 100%)[64-68]	L1236, L428, KM-H2, HDLM2[57, 65, 69]	Pro-inflammatory infiltration formation
IL-7	/	Growth factor for B and T cells, involved in activation of the immune response[70-72]	IL-7 mRNA expression was demonstrated in HL (77%)[73]	IL-7 was detectable in L-1236, HDLM-2 and KM-H2 cells; very low levels in L-540 and L-428 cells[73, 74]	Promotes proliferation of regulatory T cells[74]
IL-9	P40	T-cell and mast cell growth factor [75]	Positive (50%)[35, 76]	In L428 by stimulation with PHA/PMA[77]	Growth factor for H-RS, infiltration of eosinophils and mast cells [77, 78]
IL-10	CSIF	Anti-inflammatory cytokine, inhibits Th1 response[79, 80]	Detected in a variable percentage of H-RS (21% to 36%)[81, 82]	Expressed in L1236 and Ho[69, 81]	Associated with EBV+ HL[81, 82]
IL-13		B-cell maturation and activation[83]	IL-13 expressed in H-RS cells (86% to 93%)[46, 60, 84]	Positive in HDLM2, L1236, L428 and KM-H2[44, 60]	Autocrine growth factor for H-RS cells[35, 44-47]
TNF-α		Involved in inflammation, tissue remodeling, and wound healing[85]	Detected in primary H-RS cells (69%)[51, 52, 67, 86-88]	Expressed in L1236, L428, KM-H2, HDLM2 and L540[32, 57, 67, 69, 87]	Autocrine effects, collagen synthesis[89]
LT-α		Pro-inflammatory cytokine[90, 91]	LT-α was detected in HL (77%)[35, 67, 86]	Detected in HDLM-1, KMH2, L428 and L540[67, 87, 88]	Autocrine factor for H-RS[3]
TGF-β		B- and T-cell suppression; fibroblast proliferation and collagen synthesis[92, 93]	Expression in H-RS cells (61%), mainly in NSHL[94, 95]	Detected in L1236 and L428[96, 97]	Associated with nodular sclerosis subtype, suppresses T-cell activation [95]
IFN-γ		Pro-inflammatory cytokine, Macrophage differentiation and activation; NK and CD8 T-cell activation[98, 99]	Expressed in H-RS cells (47% tumors) [66, 100-102]	Expressed in RS cell lines L1236, HDLM2, L591 and L1236 but is absent in L428[69, 101]	Stimulates CXCL10[22]
CCL1	I-309	Monocyte, NK cells, B cells chemotaxis[103, 104]	Expressed in HL tissue[105]	mRNA was Detected in L540, HDLM2 and L428[105, 106]	Might contribute to the pro-inflammatory microenvironment
CCL3 /CCL4	MIP-1α /MIP-1β	Recruit Th1 cells , monocytes and dendritic cells[107, 108]	Detected in HL tissue, higher in MCHL subtype[102, 109]	mRNA detected in L1236 cells (unpublished data)	Might contribute to the pro-inflammatory microenvironment
CCL5	Rantes	Recruits monocytes, eosinophils, mast cells and T cells[110, 111]	Protein expression in the H-RS cells[105]	All Hodgkin cell lines express [105, 106, 112]	Contributes to the recruitment of the reactive cells in HL[109, 112, 113]
CCL11	Eotaxin	Attracts eosinophils, monocytes, and T-cells[114, 115]	Expressed by fibroblasts in HL tissue (63%)[116]	Positive in L1236 cells[116]	Eosinophil accumulation, produced by fibroblasts[116]
CCL13	MCP-4	Recruits monocytes, eosinophils, and T-cells[117]	Low protein expression in H-RS cells[105]	Positive in L1236 cells[105]	Might contribute to the pro-inflammatory microenvironment in HL
CCL17	TARC	Attracts Th2 cells, regulatory T-cells, basophils, and monocytes[118]	Highly expressed in H-RS cells (88%)[119, 120]	Expressed in all HL cell lines[119]	Likely contributes to Th2-like and Treg T cells enriched surrounding in HL[119, 121]
CCL22	MDC	Th2 response and regulatory T cell induction[22]	Most HL cases, expression by H-RS cells (87%),higher in NSHL[102, 122]	Detected in L1236, L428 and L591[105, 106]	Likely contributes to Th2-like and Treg T cells enriched surrounding in HL[3, 22]
CXCL8	IL-8	Neutrophils recruitment[123]	Rarely observed in H-RS cells, but positive in reactive cells[124]	Detected in L1236 cells upon LTD_4 stimulation[32]	Correlated with Neutrophils accumulation[3]
CXCL9 /CXCL10	MIG /IP-10	Th1 cell recruitment[125]	Expressed at higher levels in HL compared to benign lymphoid tissues[35]	Detected in L1236 cells and L591[3]	Associated with EBV+ HL infiltration[35]

$CysLT_1$ receptor[126] and 15-LOX-1 [127] induction upon IL-4 stimulation. Both the $CysLT_1$ receptor and 15-LOX-1 are pro-inflammatory molecules, which will be discussed in more detail.

Apart from STAT family members STAT-3, STAT-5 and STAT-6, several other transcription factors were also frequently found in H-RS cell and HL cell lines, including NF-κB, GATA-2, NOTCH1 and AP-1 (table 2); among which the NF-κB pathway has been well studied and its aberrant activity is likely attributed to mutations in the IκBα gene, amplification of the NF-κB/REL locus, and autonomously active CD30, CD40, RANK, as well as Notch 1 signaling pathways[128-137].

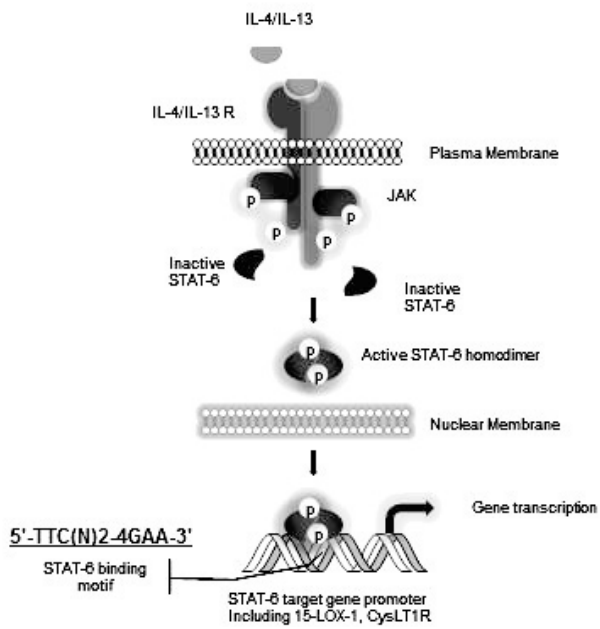

Figure 3 Schematic model of STAT-6 activation

1.3.2 The CysLT1 receptor mediates the crosstalk between H-RS cells and microenvironment

LTC_4, LTD_4, and LTE_4, collectively called CysLTs, are lipid mediators derived from arachidonic acid (AA) through the 5-LOX pathway. 5-LOX and LTC_4 synthase catalyze in sequence the conversion of AA to LTC_4, which in turn is transported out of the cells,

and further converted to LTD_4 and LTE_4[138]. The biological function of the CysLTs is principally mediated by G protein coupled CysLTs receptors named $CysLT_1$ and $CysLT_2$ receptor [138, 139] (figure 4). The CysLTs have been considered to contribute to the pathogenesis of asthma[140] and other inflammatory disorders [141, 142].

A previous study showed that functional $CysLT_1$ receptors are expressed in primary H-RS cells as well as in the HL-derived cell lines L1236 and K-MH2. Stimulation of L1236 cells with the $CysLT_1$ receptor agonist LTD_4 leads to secretion of tumor necrosis factor-alpha (TNF-α), IL-6 and IL-8 [32]. As LTD_4-producing cells, such as eosinophils, macrophages and mast cells, are part of the HL microenvironment, it is hypothesized that aberrant cytokine production by the H-RS cells is mediated via the $CysLT_1$ receptor. Thus, the $CysLT_1$ receptor signaling pathways mediating cytokine release is of great interest to study.

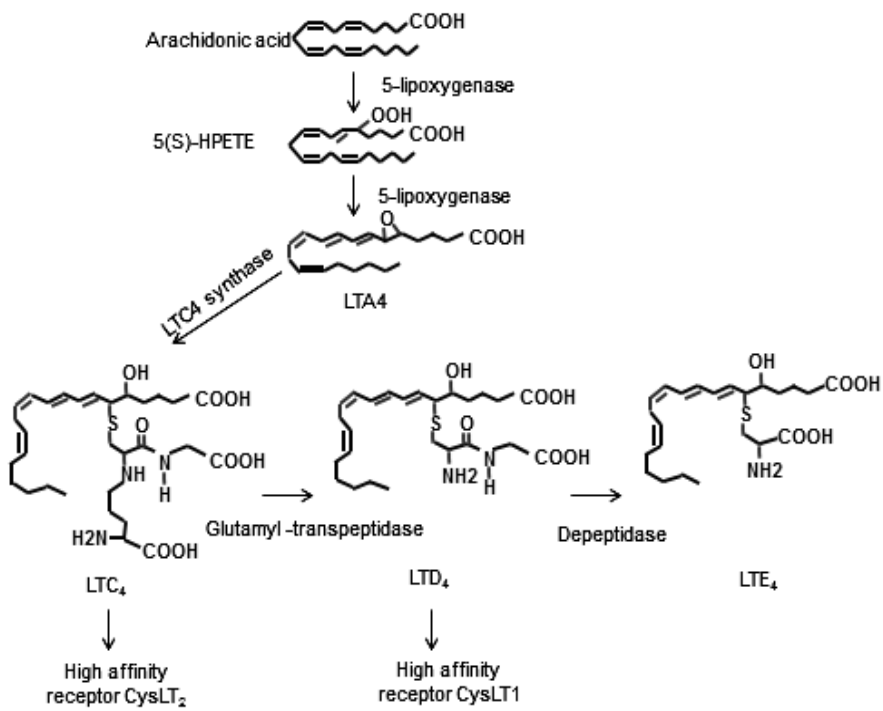

Figure 4 The 5-LOX pathway

Table 2 Transcription factors involved in HL pathogenesis

Transcription Factors involved in Hodgkin Lymphoma Pathogenesis	
STAT-6	STAT-6 is constitutively phosphorylated in primary H-RS cells (78% of analyzed cases), and HL-derived cell lines L1236, L428, KM-H2, L540 and HDLM2. Lentiviral mediated STAT-6 specific shRNA transfection significantly induces apoptosis in L1236 cells, suggesting that STAT-6 plays a critical role in H-RS cell survival [43, 44].
STAT-5	STAT-5 is phosphorylated in 38% of examined HL tumors and data suggests that the phosphorylation status of STAT-5 in H-RS cells in HL could be a prognostic marker in HL [41, 42].
STAT-3	STAT-3 is constitutively activated in 87% classical HL cases and most HL-derived cell lines including L428 and L1236. Gene knockdown experiments suggested that STAT-3 is essential for cell proliferation of these HL cells [38-40].
NF-κB	NF-κB is constitutively up-regulated in H-RS cells, and the results showed that more than 90% H-RS cells in all analyzed HL patients were positive, although HL cell lines revealed different activation mechanisms. In L428 and KM-H2 cells, the constitutive NF-κB activity correlates with defective IκBα, in L1236 and HDLM2 cells, IκBα is associated with p65 and displays an enhanced turnover. NF-κB plays a crucial role in H-RS cell survival [143-146].
GATA-2	GATA-2 was detected in 50% of tested HL patient H-RS cells, and in all tested HL-derived cell lines. The expression of GATA-2 is specific for HL cells and not seen in NHL cells or normal GC B cells, suggesting that GATA-2 is important in establishing the abnormal B-cell phenotype of H-RS cells [147, 148].
NOTCH1	Notch1 was detected in all studied H-RS cells from 25 HL patients. The data revealed that Notch is an essential upstream regulator of alternative NF-κB signaling and indicate cross talk between both the pathways in H-RS cells [149, 150].
AP-1	c-Jun and JunB overexpression is found in all tumor cells of patients with HL. AP-1 works synergistically with NF-κB and stimulates expression of the cell-cycle regulator cyclin D2, proto-oncogene c-met and the lymphocyte homing receptor CCR7, which are all strongly expressed in primary HRS cells[151]. Indicating an important role of AP-1 in lymphoma pathogenesis.

1.3.3 15-LOX-1 is highly expressed in primary H-RS cells and the L1236 cell line

15-LOX-1, also called arachidonate 15-lipoxygenase (ALOX15), is an enzyme that oxygenates polyunsaturated fatty acids and bio-membranes. In healthy subjects, this enzyme is predominantly expressed in airway epithelial cells, eosinophils, alveolar macrophages, dendritic cells and reticulocytes. Previous studies suggested that 15-LOX-1 is a pro-inflammatory enzyme that contributes to asthma pathogenesis [31, 152-

154]. In certain human cancers including colon cancer, oesophageal cancer, and pancreatic cancer, the expression of this enzyme is suppressed and has been suggested as a tumour suppressor[155-159].

In HL tumor tissue, 15-LOX-1 was found to be expressed in primary H-RS cells in 17 of 20 (85%) investigated biopsies using immunohistochemistry staining [160] (figure 5). However, this enzyme was rarely detected in Non-Hodgkin lymphoma (NHL) tissues [161], suggesting that the expression of 15-LOX-1 in H-RS cells may have important diagnostic and therapeutic implication in HL. The few numbers of H-RS cells in tumor tissue makes the research of HL difficult, therefore the *in vitro* models become important tools for the study of 15-LOX-1 biology. Among HL-derived cell lines, L1236 expresses 15-LOX-1 abundantly, while all other established HL cell lines are 15-LOX-1 negative [160]. To understand why this molecule is expressed in H-RS cells, knowledge about its transcriptional regulation is crucial.

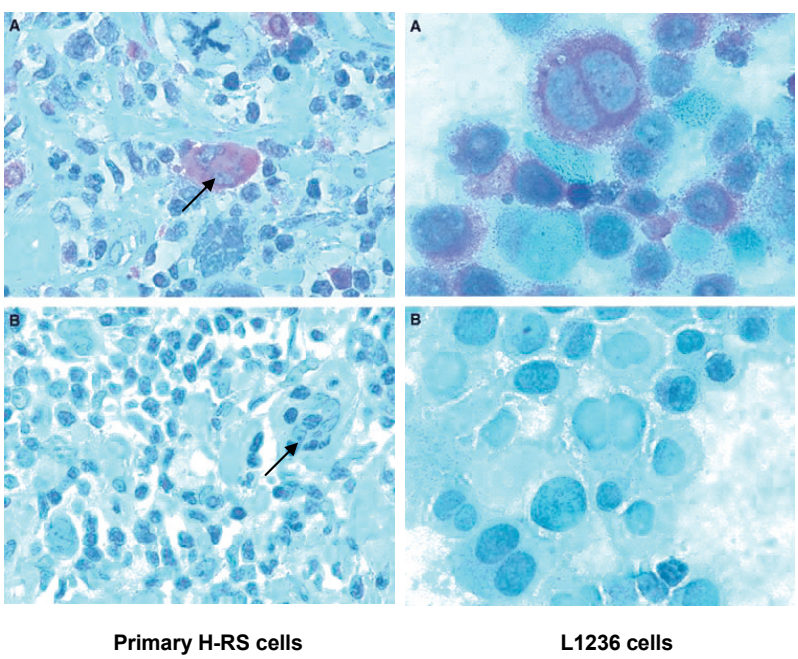

Figure 5 Primary H-RS and L1236 cells express 15-LOX-1. A, probed by 15-LOX-1 specific primary antibody, B, isotype controls. (Adapted from Claesson HE, et al. 2008, with permission)

1.4 TRANSCRIPTIONAL REGULATION

Background for understanding 15-LOX-1 regulation

Epigenetic is a definition relative to genetic, which refers to the genetic material DNA dependent inheritance that only concerns the DNA sequences information. In comparison, epigenetic is the heritable changes in gene activities which are not caused by changes in the DNA sequence. DNA decides the genotype, while epigenetic regulation controls the phenotype.

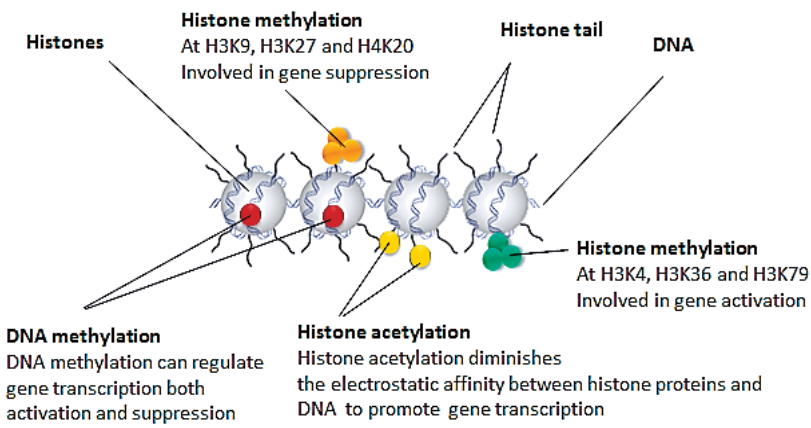

Figure 6 Schematic histone structure and modifications

The basic components of chromatin are genomic DNA and histone. Histone is a group of small proteins including H1, H2A, H2B, H3, and H4, which tightly bind to DNA and form nucleosome in eukaryotic cell nucleus, in which two copies of histone H2A, H2B, H3, and H4 assemble the core of nucleosome, and the one histone H1 locks DNA and histone as soon as the nucleosome is formed[162, 163]. The gene transcription process requires the double strand DNA to come apart temporarily, so that transcription factors and RNA polymerase are able to access to the gene promoter and the DNA template. It is therefore important for cells to have means of unwinding chromatin to permit transcription to proceed. Several mechanisms are involved in chromatin remodeling that controls gene transcription, including DNA methylation and histone modification. Histone modification is a group of events that comprises lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination

and sumoylation.

Collectively, gene transcription is regulated by multiple factors including gene activators and suppressors. In order to access to target DNA, these factors have to find their binding motifs in loosened DNA regions in the genome. Chromatin remodeling regulators such as DNA methyltransferases (DNMTs) /demethylases (DDMs), histone methyltransferases (HMTs)/demethylases (HDMs), histone acetyltransferases (HATs)/deacetylase (HDACs) are important to open or close the chromatin. Thus the chromatin remodeling regulators and gene transcription factors are working sequentially or synergistically to control gene activation.

1.4.1 DNA methylation

DNA is composed of four nucleotides comprising adenine (A), thymine (T), guanine (G) and cytosine (C), and among these nucleotides cytosine or adenine can be further modified by adding a methyl group when needed. Generally, DNA methylation typically occurs in a so called CpG Island. DNA methylation is controlled by two classes of enzymes termed DNMTs and DDMs. Although little is known about DNA DDMs so far, DNMTs are well studied. Three active DNMTs have been identified in mammals, named DNMT1, DNMT3A, and DNMT3B[164]. The CpG Island methylation displays a wide variety of biological functions in development[165], aging and cancers[166, 167]. In the present study, CpG Island methylation at the 15-LOX-1 promoter region is shown to be implicated in STAT-6 accessibility regulation, thus controlling the gene transcription in HL cell lines. More details are described in results and discussion.

1.4.2 Histone modifications

Histone modifications consist of lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation, among which lysine acetylation and lysine methylation have been widely investigated. In the following paragraphs, histone lysine acetylation and lysine methylation will be discussed.

1.4.3 Histone acetylation

Histone acetylation is achieved by introducing an acetyl group to lysine residues on histone proteins. Histone acetylation is regulated by HATs and HDACs. The introduction of acetyl groups is mediated by HATs, which consist of GNAT, MYST and p300/CBP subfamilies [168], whereas acetyl groups can be removed by HDACs that include class I, II, III and IV subgroups in mammals cells [169]. Histone acetylation diminishes the electrostatic affinity between histone proteins and DNA, thereby promoting gene transcription [170]. Several lysine residues on the histone N-terminal tails can be modified. In our current work, we found that histone H3 hyperacetylation was correlated with high 15-LOX-1 expression in L1236 cells, in which an H3 acetylation-enriched15-LOX-1 promoter is permissive for STAT-6 binding, while in L428 cells, the hypoacetylated promoter has low accessibility that inhibits gene activation[171].

1.4.4 Histone lysine methylation

Histone methylation is a critical epigenetic mechanism involved in embryonic development, immune responses, and cancer genesis. Histone methylation plays a pivotal role in the maintenance of both active and suppressed states of gene expression depending on the sites of methylation. Methylation of histone H3 at lysine (K)-4, H3K36, H3K79 is an activation sign of gene transcription, whereas methylation of histone H3K9, H3K27 and H4K20 is implicated in suppression of gene transcription[172-177]. Histone methylation is catalyzed by HMTs and HDMs, as described below (Figure 7).

1.5 TRANSCRIPTIONAL REGULATION OF THE 15-LOX-1GENE

Several mechanisms are involved

The Th2 cytokines IL-4 and IL-13 induce the expression of 15-LOX-1 in certain human cells, such as human monocytes [178, 179], human umbilical vein endothelial cells[180], orbital fibroblasts[181], SCC 1483 oral cavity cancer cells[182] and lung epithelial carcinoma A549 cells[183]. The classical IL-4/IL-13 triggered STAT-6 pathway is involved and plays an important role in 15-LOX-1 induction by directly binding to the promoter [184, 185]. However, the STAT-6 activation is not sufficient to

induce15-LOX-1 expression in dermal fibroblasts [181] and the human monocytic cell line THP-1 cells [183]. Moreover, the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) suppresses STAT-6 expression, but on the other hand stimulates the expression of 15-LOX-1[186], indicating that also other pathways are involved in the transcriptional activation of the gene.

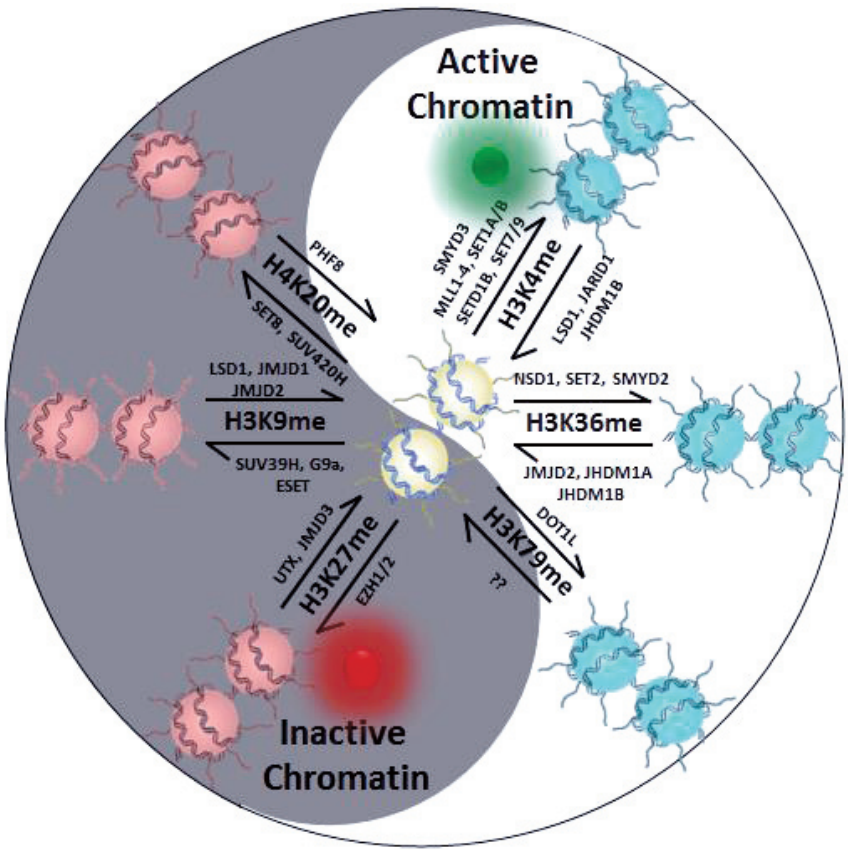

Figure 7 Histone lysine modifications and key regulators. Histone lysine methylation is a major epigenetic regulation of gene transcription both positively and negatively. Many histone modifiers have been identified. Lysine methylation has been implicated in both transcriptional activation (H3 Lys4, 36, 79) and silencing (H3 Lys9, 27, H4 Lys20) (Illustration modified from Cell Signaling Technology, Inc. www.cellsignal.com with a permission).

In human monocytes, apart from the STAT-6 pathway, an important regulatory role of ERK1/2 was demonstrated, ERK1/2 mediates IL-13-induced 15-LOX-1 expression via the transcription factors early growth response (EGR)-1 and cAMP response element-binding protein (CREB)[179]. Several other factors are suggested to be important in IL-

4 induced 15-LOX-1 expression, including activator protein 2, GATA motif-binding transcription factor (GATA)1, nuclear factor (NF)1, SP-1[180] and vimentin[187], while GATA-6 is a suppressor of 15-LOX-1 in colorectal cancer cells [188, 189]. Interestingly, the Th1 cytokine INF- γ has negative effects on 15-LOX-1 induction, indicating that this enzyme is a Th1 and Th2-counterregulated-mediator [190, 191]. The promoter methylation of DNA by DNA DNMTs in CpG-rich regions has been well described as a critical component of epigenetic silencing in human cells. DNA CpG methylation is also involved in the regulation of 15-LOX-1 transactivation. In colorectal cancer, promoter methylation of 15-LOX-1 was detected both *in vivo* and *in vitro* and has been suggested to contribute to the 15-LOX-1 expression inhibition, [192]. The association of hypermethylated promoter and 15-LOX-1 suppression was also demonstrated in HL cell lines [193]. Interestingly, another study suggested that the DNMTs can inhibit 15-LOX-1 transcription by a DNA methylation-independent mechanism [192]. Moreover, in prostate cancer cells, promoter hypermethylation has been suggested to promote 15-LOX-1 expression [194]. Thus, different mechanisms are operating in different cell types. This might be due to the fact that different types of cells express different transcription factors.

Histone modification is another type of epigenetic regulation involved in development, cancer and other diseases (see also above). Recent experimental evidence revealed that histone modification also plays a crucial role in 15-LOX-1 transcriptional regulation. H3K9me2 demethylation catalysed by lysine (K)-specific demethylase 3A (KDM3A) transcriptionally activates 15-LOX-1 in colorectal cancer cells. Moreover, histone H3 and histone H4 acetylation mediated by histone acetyltransferase lysine (K)-acetyltransferase 3B (KAT3B) is also implicated in 15-LOX-1 transcriptional activation [195]. The association of histone acetylation with 15-LOX-1 expression was also found in the HL cell line L428 cells. In these cells, the transcription of 15-LOX-1 was induced only after stimulation with both trichostatin A (TSA) and 5-Aza [193], indicating that histone acetylation and DNA methylation synergistically regulate gene transcription.

2 AIMS OF THE STUDY

HL is one of the most common malignant disorders among young adults. Although the disease is largely curable, improved and targeted treatment with less early and late side effects is urgently needed. The inflammatory microenvironment in HL is a unique feature of this disease; therefore an understanding of the molecular mechanisms involved in the inflammatory component of HL is of great interest and of potential importance for the development of future more specific treatment. Two inflammation-related molecules, $CysLT_1$ and 15-LOX-1, are highly expressed in primary H-RS cells and the HL cell line L1236.

This study specifically aims to:

1. Characterize the functional role of the $CysLT_1$ receptor in the pathogenesis of HL, focusing on the signaling pathways that bridge the LTD_4 stimulation to cytokine induction.

2. Elucidate the involvement of constitutively activated STAT-6 in the expression of 15-LOX-1 in HL cell lines, and eventually understand the mechanism of 15-LOX-1 expression in primary H-RS cells.

3. Appraise the role of epigenetic regulation in 15-LOX-1 transcription in HL-derived cells.

    a. Delineate promoter CpG methylation in 15-LOX-1 transcriptional activation
    b. Evaluate the involvement of histone acetylation in 15-LOX-1 transcription
    c. Assess the histone methylation mediated by SMYD3 and UTX in 15-LOX-1 transactivation
    d. Define the cooperation of DNA methylation, histone modification and transcription factor in 15-LOX-1 transactivation regulation.

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL MATERIALS

3.1.1 HL Biopsies and Cell lines

HL tumor tissue was collected from lymph node biopsies and the studies on patient samples were approved by the local ethics committee. The HL-derived cell lines L1236 (so far the only Hodgkin cell line formally shown to be derived from H-RS cells), L428 and KM-H2 cells were used in the present study, and all three HL cell lines were kind gifts from Professor Volker Diehl, Cologne, Germany. The human lung epithelial carcinoma A549 cell line was used as a 15-LOX-1 inducible cell model for studying epigenetic regulation. A549 cells were purchased from Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Brunswick, Germany. Another 15-LOX-1 positive cell line was used in this study, the human prostate adenocarcinoma cell LNCaP and this cell line was purchased from the American Type Culture Collection, Manassas, VA. Detailed information on the studied cell lines is listed in table 3.

Table 3 Cell lines used in the study

Cell lines	**L1236**	**L428**	**KM-H2**	**LNCaP**	**A549**
Derivation	HL (MC)	HL (NS)	HL(MC to LD)	human prostate adenocarcinoma	lung epithelial carcinoma
Specimen site	peripheral blood	pleural effusion	pleural effusion	lymph node	epithelial cells
Cell type	B-cell type	B-cell type	B-cell type	epithelial cells	epithelial cells
Patient	34-year-old male	37-year-old female	37-year-old male	50-year-old male	58-year-old male
15-LOX-1	Positive	Negative	Negative	Positive	Inducible

3.2 METHODS

3.2.1 SYBR Green-Based Quantitative Real-Time PCR

Quantitative Real-time PCR (qRT-PCR) is a technique which is a major development of the polymerase chain reaction that enables reliable detection and measurement of gene transcription levels. The material needed for this method is achieved through total RNA extraction. The basic theory of qRT-PCR is to use fluorescence to detect the threshold cycle (Ct) during PCR when the level of fluorescence gives a signal over the background and is in the linear portion of the amplified curve. This Ct value is used for the accurate quantization of qRT-PCR. The SYBR Green is an asymmetrical cyanine dye that intercalates with double-stranded DNA, which introduces fluorescence of the SYBR. The qRT-PCR machine detects the fluorescence and the software calculates Ct values from the intensity of the fluorescence. All qRT-PCR performed in this study was carried out in an ABI 7900HT real-time PCR thermo cycler using pre-designed primers (all detailed primer sequences used in this study can be found in the attached papers). The levels of gene transcription were calculated by using the 2(-Delta Delta Ct) Method [196]. The house keeping genes Beta-2 microglobulin (β2m) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls.

3.2.2 Western blot

Western blot is a protein detection method based on antibody-antigen specific recognition. In this study, whole cell lysate was prepared, followed by SDS-PAGE electrophoresis and subsequent transfer to PVDF membranes. The membranes were then probed by antibodies against the target proteins. Horseradish peroxidase conjugated secondary anti-mouse or rabbit IgG were used to detect the enrichment of the target protein by developing with the enhanced chemiluminescent method. Beta-actin was used as a loading control. In this study we applied this method to detect the target proteins including EGR-1, STAT-6, 15-LOX-1, SMYD3, SMCX, UTX and β-ACTIN (more details in the constituent papers).

3.2.3 15-LOX-1 activity assay

In order to measure the catalytic activity of 15-LOX-1, the concentration of 15(s)-hydroxy eicosatetraenoic acid (15(S)-HETE) was measured by ELISA. 15-LOX-1 catalyzes the conversion of AA to 15(S)-HETE which is the major mediator formed through this pathway. Briefly, the L1236 cells were harvested and washed by PBS

followed by centrifugation. The cell pallet was re-suspended in PBS. The cells were then incubated in a 37°C water bath with AA for 5 minutes and subsequently put on dry ice to stop the reaction. The concentration of 15(s)-HETE in the supernatant was used as a biomarker for activation of this pathway.

3.2.4 Immunofluorescence

Immunofluorescence is another immune-recognition-based technique that is commonly used for detection of the target molecule. Basically, this technique applies the specificity of antibodies to their antigen, and specifically targets fluorescent dyes to the molecules as desired. There are two main methods of immunofluorescence including direct and indirect immunofluorescence. The more frequently used method is the indirect immunofluorescence, whereby the primary antibody specifically recognizes the molecule of interest, and the primary antibody is then targeted by a second anti-immunoglobulin antibody that is tagged with the fluorochrome. Under excitation light, the fluorochrome expresses emission light that can be captured by microscope-mounted camera.

3.2.5 Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay is also an antibody-antigen based technique that is commonly used for probing protein-DNA interactions within the natural chromatin context of the cell. The ChIP assay can be used to assess the histone modification status during gene expression regulation and to determine the binding of transcription factors and other DNA interaction proteins. Briefly, the cells were fixed directly in the culturing medium by adding formaldehyde to a final concentration of 1% (fixation may not be needed when studying histone modification), which reversibly preserved the protein-DNA interaction occurring in the cells under certain conditions. The cells were then lysed and chromatin isolated and fragmented using sonication. The chromatin was then subjected to immunoprecipitation using antibodies specific to a particular protein or modified histone of interest to pull down the DNA sequences that are associated with the particular protein or histone modification. After immunoprecipitation, the protein-DNA cross-links were reversed and the DNA purified. The enrichment of a particular DNA sequence can be detected by quantitative PCR.

3.2.6 Luciferase Reporter-Based Promoter Activity Assay

The luciferase reporter based promoter activity detection system is basically constructed by the promoter of interest followed by the firefly luciferase coding region. This luciferase reporter-based system provides an intracellular tool for quantitatively studying the *cis*- or *trans*-regulating elements that putatively modulate gene expression. To determine the regulation of the promoter of interest, the reporter vector is transfected into certain cells. Simultaneously, the *Renilla* reniformis luciferase-containing vector is co-transfected as a loading control that normalizes the transfection handling error.

3.2.7 Lentiviral Transduction System for Gene Knock-down

A potent approach to transduce cells is the use of virus derived vectors, among which the lentiviral vector (derived from HIV-1) is a widely used vector that is able to transduce both dividing and non-dividing cells as the viral preintegration complex can get through the intact membrane of the nucleus in the target cell. Lentiviral vectors are usually created in a transient transfection system, in which the packaging cells (the HEK293FT cells were used in this study) are transfected with three different plasmids, including a transfer vector plasmid (pLKO.1 and pGIPZ were used in this study), a packaging plasmid (psPAX2 was used in this study) and a envelop plasmid (pMD2.G was used in the present study). The virus particles are then produced and released to the supernatant that is used to transduce the cells of interest. The transfer vector can be used both to over-express and knock-down target gene depending on the aim of the study. In the present study, the pGIPZ was used for EGR-1 knock-down and the pLKO.1 was used for UTX knock-down.

3.2.8 Microarray Gene Expression Analysis

Microarray gene expression analysis is a technique for gene profiling, which enables high-throughput gene expression quantification on a small chip. This method has been developing though the recent decades, and it is widely used for screening for candidate disease-associated transcripts, diagnostic markers and therapeutic targets. Microarray is also very useful for investigating signaling pathways. Total RNA preparation is required to initiate the microarray analysis. The high quality total cellular RNA is subsequently converted to cDNA or cRNA that hybridizes with tagged DNA probes in each DNA spot on the chip. The probe-target hybridization is usually detected and

quantified by fluorescence intensity that represents the abundance of gene transcripts. In this study the affymetrix Human Gene 1.0 ST Array was performed. The gene transcription fold change was analyzed by comparing the LTD_4 treated L1236 cells versus controls.

4 RESULTS AND DISCUSSION

4.1 H-RS CELLS EXPRESS THE CYSLT$_1$ RECEPTOR THAT MEDIATES CYTOKINE AND CHEMOKINE EXPRESSION VIA THE TRANSCRIPTION FACTOR EGR-1

Accumulating data reveal that the H-RS cells and the infiltrating cells are interdependent. The H-RS cells secret an abundance of cytokines and chemokines that recruit inflammatory cells to the tumor sites. The infiltrating cells in turn produce different growth factors to form a favorable microenvironment for the H-RS cells [11, 30, 35, 36, 74, 113, 197-201]. To understand the crosstalk between H-RS cells and the bystander cells, knowledge about the mechanisms leading to H-RS cell cytokine/chemokine production is crucial. Our previous studies have uncovered part of these mechanisms; by immunohistochemistry in formalin-fixed paraffin-embedded tissue, CysLT$_1$ receptor expression was noted in H-RS cells in 12 out of 20 HL patients and the expression of CysLT$_1$ receptors was also confirmed by microarray analysis in microdissected primary H-RS cells [32]. This finding was of great interest since the CysLT$_1$ receptor is a high affinity receptor for LTD$_4$, which presumably is released by eosinophils, macrophages and mast cells in the HL tumor microenvironment. In the same study referenced above, L1236 cells were studied as a mechanistic model of the H-RS cells since the CysLT$_1$ receptor is also expressed by these cells (Figure 8).

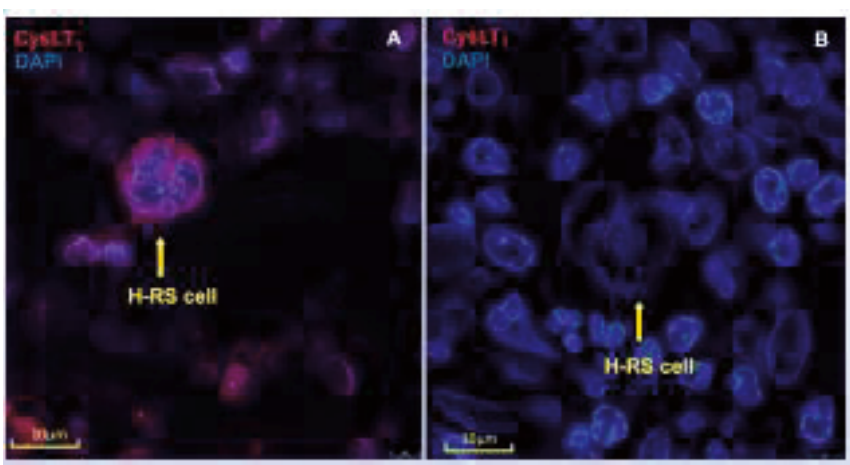

Figure 8 Primary H-RS cells express the CysLT$_1$ receptor

Interestingly, LTD_4 stimulation significantly induced the release of certain cytokines including IL-6, IL-8 and TNF-α in a dose-dependent fashion. In the present study, we have checked the transcription level of these cytokines upon LTD_4 treatment. Not surprisingly, the mRNA levels of IL-6, IL-8 and TNF-α were also dose- and time-dependently induced upon LTD_4 stimulation. The expression and release of the cytokines were mediated by the $CysLT_1$ receptor since a specific receptor antagonist against $CysLT_1$ blocked the action of LTD_4 on cytokine release. IL-8 is an important inflammatory chemokine that specifically induces chemotaxis of neutrophils. Thus, release of IL-8 by the H-RS cells may recruit neutrophils into the tumor site. TNF-α is another important inflammatory mediator, which promotes the synthesis of other proinflammatory compounds by various cell types [35]. Therefore, we demonstrated the grounds for a paracrine loop that partially may depict the interdependency between H-RS cells and infiltrating inflammatory cells. Furthermore, two other chemokines, Mip-1α and Mip-1β, were also induced upon LTD_4 stimulation. Mip-1α and Mip-1β are also named CCL3 and CCL4, both of which are chemoattractants for T cells and monocytes and were detected in HL tumor tissues [35].

The interesting finding of the LTD_4/$CysLT_1$-cytokine axis in HL provides the $CysLT_1$ receptor as a potential target in HL therapy that warrants further attention. However, the intracellular signaling pathway(s) bridging the $CysLT_1$ receptor activation to cytokine induction is (are) still unclear. In order to delineate the picture of the signaling pathways triggered by LTD_4 stimulation, microarray profiling was performed. Several transcription factors were identified and verified to be induced by LTD_4 through the $CysLT_1$ receptor, including EGR-1, NGFI-A binding (NAB) proteins member 2 (NAB-2), steroid-thyroid hormone-retinoid receptor Nuclear Receptor Subfamily 4, Group A, Member 3 (NR4A3), FOS protein B (FOSB), as well as Tribbles homolog 1 (TRIB-1), which gave us an idea about how LTD_4-$CysLT_1$ ligation leads to cytokine trans-activation in H-RS cells. Among these factors, we further studied the transcription factor EGR-1 that has been proved to be a critical mediator of LTD_4 triggered cytokine transcription [202]. The results demonstrated that EGR-1 is required for expression of IL-6, TNF-α, Mip-1α and Mip-1β, but the induction of IL-8 was not affected by EGR-1 knockdown, indicating that alternative pathways are involved in IL-8 induction upon LTD_4 stimulation. Recent studies revealed that LTD_4 initiates activities through robust induction of multiple signaling pathways in human cells. These pathways are mediated in part by activation of mitogen-activated protein kinase (MAPK), which in turn activates a large number of

transcriptional regulators including EGRs and AP-1. Thus, the MAPK pathway might be involved in LTD_4 induced EGR-1 activation. Downstream targets of EGRs and AP-1 include multiple inflammatory cytokines that might contribute to LTD_4–pathophysiology [203-206].

In addition to EGR-1, the FOSB, a member of the Fos family (c-Fos, FOSB, Fra-1, and Fra-2) was induced dramatically at the transcriptional level in L1236 cells upon LTD_4 challenge. Fos family members combine with Jun family members (c-Jun, JunB, and Jun D) [207], to form the transcriptional factor AP-1. AP-1 is a pro-inflammatory transcription factor that might be implicated in IL-8 induction in L1236 cells since IL-8 was induced through AP-1 in HEK293 cells stably transfected with $CysLT_1$ and stimulated with LTD_4 [208]. However, EGR-1 knockdown only partially inhibited the cytokine induction, reflecting either that additional pathways are involved or insufficient RNA interference efficiency. NR4A3 and TRIB-1 were also identified as early response genes upon LTD_4 stimulation. Both of NR4A3 [209-211] and TRIB-1[212] have been shown to be involved in inflammation and cancer. Identification of the target genes of AP1, NR4A3 and TRIB-1 might further depict the role of the $CysLT_1$ receptor in the pathogenesis of HL. Based on the overall results, a mechanistic model of LTD_4-induced signaling in H-RS cells is proposed (figure 9).

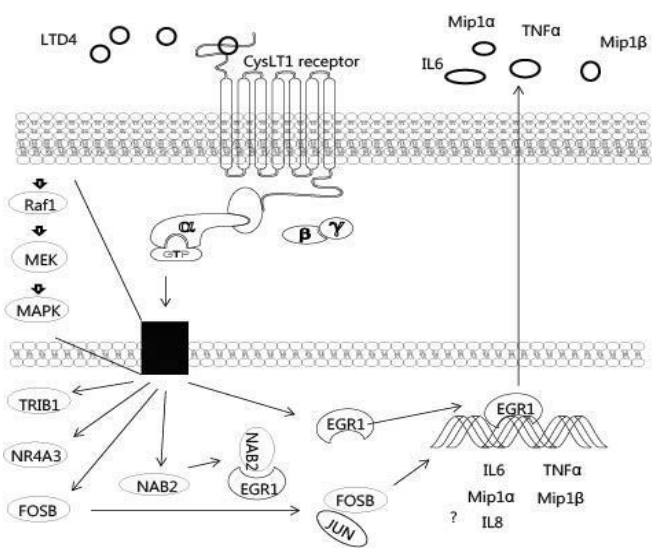

Figure 9 Schematic model of LTD_4-mediated signaling in L1236 cells

Apart from L1236 cell, the HL cell line KM-H2 was also reported in our previous study to express $CysLT_1$ [32]. The cytokine induction pattern upon LTD_4 stimulation was slightly different from L1236 cells, which might be explained by different origin of these two cell lines. A possible pathophysiological role of the $CysLT_1$ receptor in HL has been suggested (figure 10), as summarized above, the signaling pathway triggered by LTD_4 was also partially depicted (Figure 9). However, it must be considered that not all tumors exhibit H-RS cells expressing the $CysLT_1$ receptor, emphasizing the complexity of the mechanisms underlying the inflammatory feature of HL.

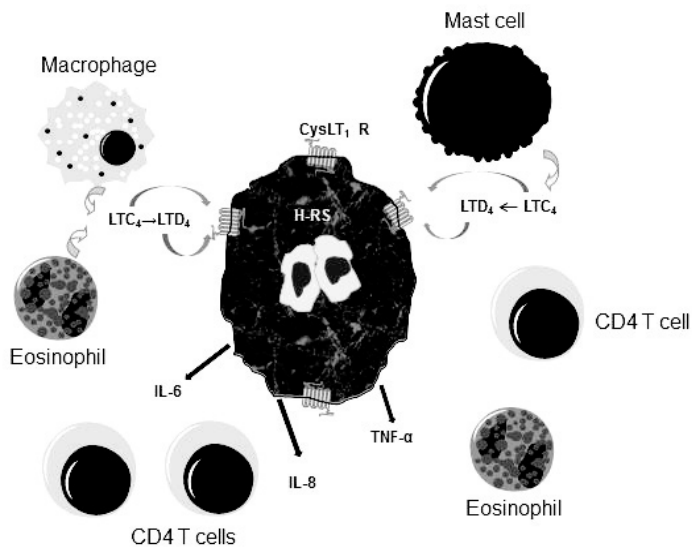

Figure 10 The $CysLT_1$ receptor mediates cytokine production that contributes to HL-microenvironment – a hypothetical model

4.2 STAT-6 IS REQUIRED FOR 15-LOX-1 ACTIVATION IN L1236 CELLS

STAT-6 is constitutively activated in primary H-RS cells as well as cultured L1236 cells. This abnormal activation attributes to IL-13/IL-13R mediated autocrine stimulation [47, 200]. As mentioned above, primary H-RS cells and L1236 cells express 15-LOX-1, of which the promoter contains three putative STAT-6 binding

motifs. Thus the question we would like to ask and to answer was: Dose constitutively activated STAT-6 stimulate 15-LOX-1 expression in H-RS cells?

The classical tool for investigating trans-activation of promoters is a luciferase-based promoter reporter. Therefore, a 1081 base pair long fragment from the 15-LOX-1 promoter was cloned into the luciferase reporter vector pGL3-basic. After transfection into L1236 cells, luciferase activity was measured and used as an indicator of transcriptional activity. In order to pinpoint transcriptionally important regions, series of 5'-deletion constructs were produced in pGL3-basic reporter vectors. The luciferase activity indicated that the region from − 1003 to – 866 including two predicted STAT-6 binding sites was found to be essential for transcription.

To specifically elucidate the contribution of STAT-6 to 15-LOX-1 transcription, three mutant plasmids were created by site-directed mutagenesis of pGL3-wt at three putative STAT-6 binding motifs. The results indicated that all three putative STAT-6 binding sites are required for full 15-LOX-1 transcription in L1236 cells. Further, we assessed the association of STAT-6 with the 15-LOX-1 promoter. The results showed a physical interaction of STAT-6 and the promoter of 15-LOX-1. The critical role of STAT-6 in 15-LOX-1 expression was verified by STAT-6 siRNA knockdown experiments, in which 15-LOX-1 mRNA transcription was remarkably reduced by STAT-6 specific siRNA transfection compared to control siRNA.

In conclusion, constitutively activated STAT-6 contributes to the highly expressed 15-LOX-1 in L1236 cells, in which STAT-6 is recruited at the 15-LOX-1 promoter to STAT-6 binding motifs. As the L1236 cell line has been proved to be derived from primary H-RS cells, it is considered likely that 15-LOX-1 expression also in primary H-RS cells is attributable to STAT-6. However, in another HL cell line, L428, 15-LOX-1 is not expressed despite STAT-6 being activated also in this cell line. Additional regulatory factors therefore likely exist in 15-LOX-1 trans-activation.

4.3 EPIGENETIC MODIFICATIONS CONTROL STAT-6 ACCESSIBILITY THAT REGULATES 15-LOX-1 EXPRESSION IN HL CELLS

To identify the factors that cause the different expression pattern of 15-LOX-1 in HL cell lines L1236 and L428 the following experimental setup was used. The promoter of

15-LOX-1 in both HL cell lines was sequenced and blasted in NCBI. No mutation was detected indicating that epigenetic regulation is likely playing a critical role in 15-LOX-1 gene activation. STAT-6 has been shown to be recruited to the 15-LOX-1 promoter and activating the gene transcription in L1236 cells. Interestingly, ChIP analysis revealed no binding of STAT-6 at the 15-LOX-1 promoter in L428 cells, suggesting that STAT-6 accessibility is suppressed in this particular cell. Notably, a previous study demonstrated the importance of DNA methylation and histone acetylation in 15-LOX-1 expression in L428 cells, in which the co-stimulation of 5'-azadeoxycytidine (DNA methyltransferase inhibitor) with either IL-4 or trichostatin (HDAC inhibitor) induces 15-LOX-1 transcription [193], meaning that DNA methylation and histone acetylation of the 15-LOX-1 promoter are involved in 15-LOX-1 expression in HL cells. Thus, the methylation status of the 15-LOX-1 promoter was determined with methylation-specific PCR (MSP). The results revealed that the 15-LOX-1 promoter region is hypomethylated in L1236 cells while hypermethylated in L428 cells. Therefore, our data suggest a strong correlation of the 15-LOX-1 promoter methylation pattern with STAT-6 accessibility and 15-LOX-1 gene activation in cultured HL cells.

We and others previously showed that histone acetylation was involved in 15-LOX-1 transcription. The acetylated H3 is a sign of gene activation; therefore, ChIP assay was employed to compare the acetylation status of histone H3 at the 15-LOX-1 promoter in both HL cell lines. Histone H3 hyperacetylation was demonstrated in the 15-LOX-1 promoter region in L1236 cells, while hardly detectable in L428 cells. Collectively, these data suggested a correlation of 15-LOX-1 promoter CpG island methylation and histone acetylation status with STAT-6 accessibility and 15-LOX-1 gene activation in cultured HL cells.

However, in prostate cancer cells, the hypermethylation of specific CpG island in the 15-LOX-1 promoter leads to the upregulation of 15-LOX-1 expression [194]. Moreover, lack of correlation between promoter methylation and expression of the 15-LOX-1 gene was reported in colorectal cancer cell line and primary tumor specimens [192]. Additional or alternative epigenetic controlling mechanisms are therefore likely involved in 15-LOX-1 gene activation.

4.4 HISTONE H3 LYSINE 4 METHYLATION/DEMETHYLATION CONTROLS 15-LOX-1 TRANSCRIPTION IN HL CELL LINES

To seek the additional epigenetic mechanism(s) involved in 15-LOX-1 transcriptional regulation, we focused on histone methylation that has dual effects on gene activation and suppression, depending on the precise residues and levels of methylation [213]. In particular, H3K4me3 and H3K4me2 associate with gene activation. We compared the methylation status of the 15-LOX-1 promoter in the two HL cell linesL1236 and L428 by using ChIP assay. The results showed remarkably higher H3K4me3 levels in L1236 cells compared to L428 cells, suggesting that the H3K4me3 level is positively correlated with 15-LOX-1 expression in HL cell lines.

Because histone methyltransferase SMYD3 and histone demethylase SMCX are major H3K4 methylation modifiers, we hypothesized that they might be responsible for H3-K4 methylation/demethylation at the 15-LOX-1 promoter, thereby regulating 15-LOX-1 gene transcription. RT-PCR and immuno-blotting demonstrated that SMYD3 was highly expressed in L1236 cells but not present in L428 cells, while SMCX was only detectable in L428 cells. Furthermore, SMYD3 inhibition reduced 15-LOX-1 expression by decreasing promoter activity in L1236 cells. SMYD3 knocking down in these cells abolished trimethylation of H3-K4, coupled with diminished occupancy by STAT-6, and histone H3 acetylation at the 15-LOX-1 promoter. In contrast, inhibition of SMCX led to upregulation of 15-LOX-1 expression through induction of H3-K4 trimethylation, histone acetylation and STAT-6 recruitment at the 15-LOX-1 promoter in L428 cells. Interestingly, a putative SMYD3 binding site was identified in the 15-LOX-1 promoter. Introduction of a point mutation in the site remarkably affected the activity of the 15-LOX-1 promoter in L1236 cells. In addition, we observed strong SMYD3 expression in the prostate cancer cell line LNCaP and its inhibition led to decreased 15-LOX-1 expression. Taken together, the status of histone methylation/demethylation at the 15-LOX-1 promoter is critical in controlling 15-LOX-1 expression.

Taken together, it seems that the chromatin remodeling is a critical “pre-cleaning” step for transcription factors finding their binding sites. In the 15-LOX-1 negative cell line L428, the 15-LOX-1 promoter region is occupied by HDM SMCX. The promoter is not accessible to STAT-6 due to the hypomethylation of H3K4 and the hypoacetylation of

H3, and thus the transcription of 15-LOX-1 is inhibited. Suppression of SMCX results in increased H3K4 methylation and leads to transcriptional activation of the 15-LOX-1 gene transcription (Figure 11, left panel). In L1236 cells, the 15-LOX-1 promoter is associated with SMYD3 coupled with hypermethylation of H3K4 and consequently activated 15-LOX-1 gene transcription (Figure 11, right panel).. Notably, these results suggested that chromatin modifiers and transcriptional factors either work sequentially or synergistically to strictly control the expression of genes important for cell differentiation and survival.

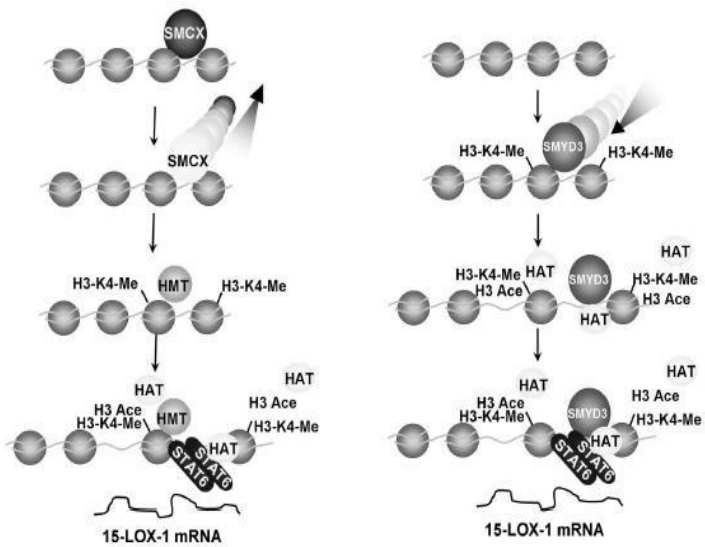

Figure 11 schematic model of 15-LOX-1 activation on HL cell lines

4.5 UTX MEDIATES IL-4/IL-13 INDUCED 15-LOX-1 EXPRESSION

IL-4 triggers mRNA transcription and protein expression of 15-LOX-1 in A549 cells and human monocytes (Figure 12) [178, 179]. Furthermore, highly expressed 15-LOX-1 in HL primary H-RS cells as well as in cultured L1236 cells [171, 185, 193] was thought to result from IL-13 autocrine stimulation. However, while binding of the cytokine to its cognate surface receptor is required for gene expression, it is not

sufficient in all cells expressing functional IL-4 receptors, indicating more complex mechanisms to be involved in the induction process. In order to address the specific mechanisms, A549 cells were established as a model for inducible 15-LOX-1 gene expression.

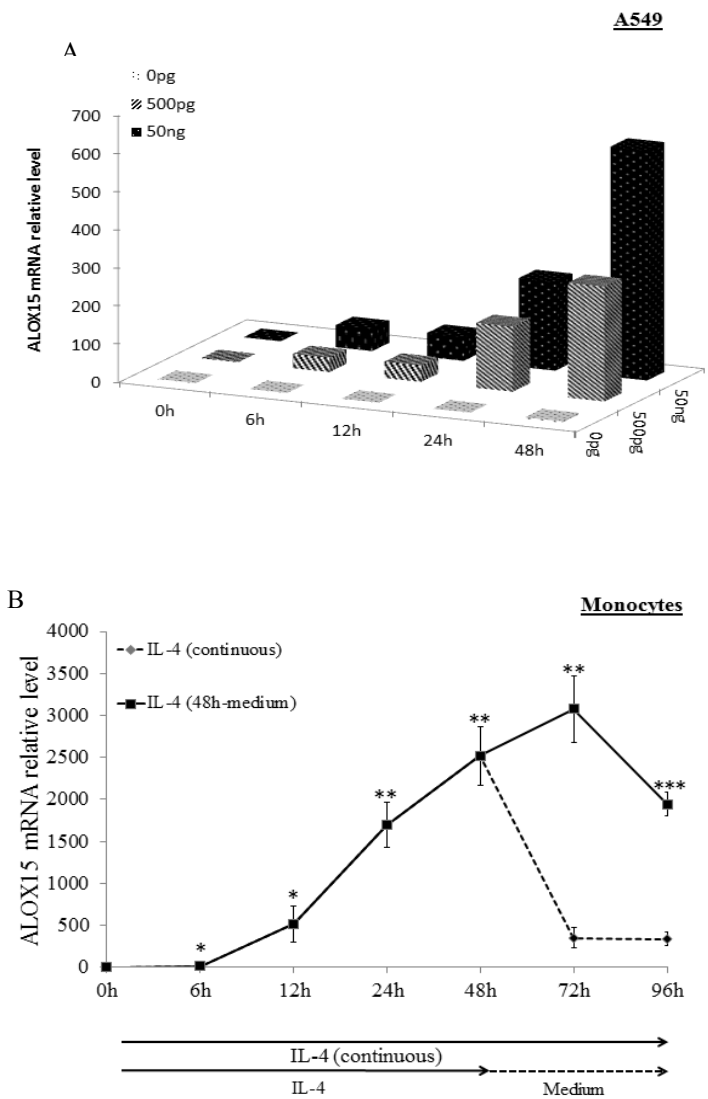

Figure 12 The transcription of 15-LOX-1is induced by IL-4.
(A)A549 cells and (B) human monocytes

To delineate the uncovered mechanisms in IL-4 induced 15-LOX-1 expression, we focused on the suppressive histone modification H3K27 methylation, which has been

implicated in IL-4 mediated macrophage differentiation. We examined the H3K27 trimethylation level of the 15-LOX-1 promoter upon IL-4 stimulation [214]. The ChIP results showed that the non-IL-4-treated A549 cells exhibit relatively high levels of H3K27me3 in the proximal promoter region of 15-LOX-1, and that IL-4 treatment substantially reduced the H3K27me3 level in these regions, indicating that histone H3K27 demethylation is correlated with 15-LOX-1 transcriptional induction. Tri-methylation of H3K27 is a repressive marker of gene transcription, which is mediated by Polycomb-Repressive Complexes (PRCs) [215]. In untreated A549 cells, 15-LOX-1 is not expressed. We hypothesized that occupancy of PRCs at the 15-LOX-1 promoter might contribute to the silencing of the gene in these cells.

Several histone modifiers are reported to be the components of the PRCs, including the H3K27 demethylase UTX [216]. Thus we evaluated the expression level of UTX and its family member JMJD3. While Jmjd3 was hardly detectable, a relatively high level of UTX was detected in untreated A549 cells, although IL-4 treatment did not increase the expression. Interestingly, enrichment of UTX at the 15-LOX-1 promoter was observed upon IL-4 stimulation by using ChIP assay. In order to investigate the importance of UTX in 15-LOX-1 expression mediated by histone modification, we transfected UTX specific siRNAs prior to IL-4 treatment, followed by qRT-PCR and ChIP assay. The results showed that inhibition of UTX not only attenuated demethylation of H3K27me3, but also reduced 15-LOX-1 induction by IL-4. This indicates that UTX is a key demethylase implicated in IL-4 mediated H3K27me3 demethylation of the 15-LOX-1 promoter in A549 cells. Upon over-expression of UTX in A549 cells by transfecting a UTX expression vector, however, 15-LOX-1 expression was not induced, which indicates that global expression of UTX alone is insufficient to activate 15-LOX-1 transcription. Taken together, these results suggest that IL-4 signaling mediates specific recruitment of UTX to the 15-LOX-1 promoter, and subsequent demethylation of H3K27me3 and 15-LOX-1 transcriptional activation.

As 15-LOX-1 is one of the top IL-4-induced genes in human peripheral monocytes, we further performed the same analyses in monocytes. To assess the role of UTX in human monocytes, we inhibited UTX by using lentivector shRNA methodology. Analyses of these cells showed that the IL-4-induced transcription of 15-LOX-1 mRNA was significantly attenuated; indicating an important role of UTX in IL-4 induced 15-LOX-

1 expression in human monocytes, as seen in A549 cells. However, IL-4 stimulation did not change the H3K27Me3 status at the 15-LOX-1 promoter, suggesting that UTX might also have an H3K27Me3-demethylase-independent regulatory function in terms of 15-LOX-1 induction in monocytes. Consistently, a histone demethylase-independent function of UTX was recently described in mice [217, 218].

Although these two cell models revealed distinct mechanisms of UTX action, the critical role of UTX was demonstrated. In addition, L1236 was considered a useful model for investigating 15-LOX-1 regulation because of the constitutively expressed 15-LOX-1. Both IL-13 and its active receptor IL-13R alpha1 are expressed in L1236 cells, and the high expression level of 15-LOX-1 in L1236 cells is believed to be a consequence of the autocrine pathway mediated by IL-13. The potential role of UTX in the 15-LOX-1 regulation in L1236 cells was investigated. UTX depletion significantly inhibited the expression of 15-LOX-1, however, UTX knockdown did not markedly influence on the methylation level of H3K27, suggesting an H3K27Me3-demethylase-independent regulatory function by UTX also in these cells.

In summary, these findings illuminate the mechanisms involved in epigenetic regulation of 15-LOX-1 transcription mediated by IL-4/IL-13 stimulation. We demonstrate the critical role of UTX through specific H3K27me3 demethylation in A549 cells, and the results also suggest an H3K27Me3-demethylase-independent function of UTX in human monocytes and L1236 cells. Further studies aiming at characterization of the mechanisms associated with UTX H3K27Me3-demethylase-independent activation of 15-LOX-1 are warranted.

Collectively, a number of epigenetic mechanisms have been identified that are implicated in 15-LOX-1 transcriptional regulation, including DNA methylation, histone acetylation and histone methylation. How UTX finds its target promoter is still unknown. We attempted to pinpoint the potential interaction between UTX and STAT-6 in 15-LOX-transactivation, however, it remains unclear if all these factors form a regulatory complex or modify the promoter sequentially. Additionally, the results showed is that different cell types seem to have distinct machineries to control gene expression.

5 SUMMARY AND CONCLUSIONS

In the present study, the crosstalk between infiltrating inflammatory cells and H-RS cells in the HL microenvironment was delineated by using *in vitro* cell models. The transcriptional regulation of a highly expressed inflammation-related enzyme, 15-LOX-1 was assessed. Several findings were demonstrated, including:

1. The transcriptional factor EGR-1 mediates cytokine expression and secretion upon LTD_4 challenge of L1236 cells, via the $CysLT_1$ receptor. The $CysLT_1$ receptor was also detected in most primary H-RS cells, suggesting that LTD_4, derived from surrounding cells, might contribute to the inflammatory microenvironment. Additionally, Mip-1α and Mip-1β were identified to be transcriptionally induced by LTD_4 stimulation via the $CysLT_1$ receptor.
2. Several mechanisms involved in 15-LOX-1 regulation were identified. STAT-6 has been proved to be an important transcription regulator of 15-LOX-1. Three putative STAT-6 binding motifs were predicted and were shown to be important for the full activation of 15-LOX-1 transcription.
3. The STAT-6 accessibility was correlated with CpG methylation and histone acetylation of the 15-LOX-1 promoter, and consequently associated with 15-LOX-1 transactivation.
4. The histone H3 lysine (K)-4 specific methyltransferase SMYD3 plays a critical role in 15-LOX-1 activation in HL L1236 cells by modifying H3K4, while H3K4 demethylase SMCX inhibits 15-LOX-1 expression in L428 cells. Furthermore, the hypermethylated H3K4 is associated with histone hyperacetylation and STAT-6 binding, which leads to high expression of 15-LOX-1.
5. UTX is involved in epigenetic regulation 15-LOX-1 transcription mediated by IL-4 stimulation.

6 FUTURE PERSPECTIVES

In the present study, the regulations of 15-LOX-1 was investigated, however, the biologic function of this enzyme in H-RS cells is still obscure. To identify the role of this enzyme, we have successfully knocked down 15-LOX-1 in L1236 cells, and established stable cell lines. Preliminary data reveal that 15-LOX-1 seems not to be required for L1236 survival and proliferation. However, due to the limitation of RNA interference, the expression of 15-LOX-1 cannot be totally shut down, thus the remaining protein might be sufficient for the cells to behave "normally", although more than 80% knockdown efficiency was achieved. One way to accomplish a total shut off could be the use of the cutting-edge technology termed RNA-guided clustered regularly interspaced shortpalindromic repeats (CRISPR), which modifies the gene at the genome level leading to complete gene silence [219, 220]. Additionally, the potential role of 15-LOX-1 in the accumulation of inflammatory cells in the HL microenvironment needs to be evaluated.

During dendritic cell (DC) differentiation, 15-LOX-1 is one of the most induced genes [127, 221] however, the function of 15-LOX-1 during this process is unknown. Human 15-LOX-1 has been suggested to play a critical role in cancer cell terminal differentiation [222-224] . Therefore, the biologic function of this enzyme in DCs, macrophages and mast cells is of great interest to depict. Our preliminary results support the hypothesis of role in differentiation. When we knocked down 15-LOX-1 in DCs, the maturation of the cells was remarkably disturbed. A future step of this project will be functional analysis to determine the role of 15-LOX-1 in DCs.

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