ROLE OF HODGKIN AND REED-STERNBERG CELL-DERIVED LYMPHOTOXIN-ALPHA IN T CELL RECRUITMENT INTO THE MICROENVIRONMENT OF HODGKIN LYMPHOMA LESIONS

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(B.Sc. (Hons), NUS)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2013

DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entity. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has not been submitted for any degree in any university previously.

FHU CHEE WAI

10th August 2013

ACKNOWLEDGEMENT

I express my heartfelt gratitude to my supervisor, Dr.Lim Yaw Chyn for her continuous guidance and supervision throughout the four years of my PhD study. She has taught me a lot about critical thinking and experimental design. Besides that, she has also implanted techniques of scientific presentation and poster preparation in me for my future career. Thank you very much, Dr.Lim.

I would also extend my thanks and gratitude to Dr.Anne Graham and Associate Prof. Chong Siew Meng. Dr.Anne Graham provided invaluable guidance and advice throughout this project. I am also grateful for the time spent in her laboratory in the University of Bradford, United Kingdom, where I greatly improved my Western blot techniques under her supervision. Associate Prof. Chong Siew Meng helped me a lot in my project. As the hematolymphoid pathologist in the team, he provided many insightful opinions and asked critical questions about how my data could fit into the clinical/histological features seen in classical Hodgkin Lymphoma. I am indeed grateful to both of them.

I also want to thank my fellow lab mates, Joe Thuan, Chi Kuen, Pin Yan, Kim Yee, I Fon and Lee Lee for making my lab experience one that is memorable and enjoyable. I had a great time working with them. I will always remember the time we have spent together and thank you for making a difference in my life.

Next, I also feel grateful to my girlfriend, Shan May, for being so understanding and supportive during the last two years of this study. Last but not least, I would like to thank my family members, especially my parents, for being so supportive and providing me with a home full of love.

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SUMMARY

Classical Hodgkin Lymphoma (cHL) is a lymphoid malignancy characterized by the presence of a minority of malignant Hodgkin and Reed-Sternberg cells (HRS cells) surrounded by massive inflammatory infiltrate. $CD4^+$ T helper 2 cells, regulatory T cells and $CD8^+$ cytotoxic T cells form a significant part of this cellular infiltrate. However, the mechanisms underlying T cell recruitment into the involved lymphoid lesions are still unknown. The aim of this study is to understand how HRS cells modulate endothelial cell function to facilitate T cell recruitment.

My study demonstrated that culture supernatant (C/S) derived from HRS cells (KM-H2, L1236 and L428) can stimulate the endothelial cells (ECs) to increase ICAM-1, VCAM-1 and E-selectin expression. Besides that, C/S stimulated ECs can also support naïve and memory T cell interactions under dynamic flow condition. Blocking assays revealed that ICAM-1 on endothelial cells; L-selectin, CD18b and CD44 on naïve T cells are crucial in mediating naïve T cell-EC interactions. The following experiment treating ECs with hyaluronidase suggested that hyaluronic acid (HA) synthesis was induced on C/S stimulated ECs to facilitate naïve T cell interactions through binding with CD44. Results from static transwell transmigration assays showed that C/S stimulated ECs could enhance naïve and memory T cell transmigration in response to SDF-1α.

Data from L929 cytotoxic bioassay managed to show biologically active lymphotoxin-α (LTα) in the KM-H2 cells. In combination with LTα neutralizing antibody, LTα derived from KM-H2 cells is proven to be the dominant mediator in stimulating ECs. ECs stimulated with KM-H2 C/S pre-treated with LTα neutralizing antibody also show reduced ICAM-1, VCAM-1 and E-selectin expression as compared to respective untreated control. Production of LTα by H-RS cells *in-situ* is verified by immunohistochemical staining of tissue samples from Hodgkin Lymphoma patients. NFκB, JNK and

COX enzymatic pathway are involved in LTα production in KM-H2 cells. Consistently, NFκB inhibitor (Bay 11-7085), JNK inhibitor (SP600125) and Cox enzymatic activity inhibitor (Indomethacin)-treated KM-H2 cells show reduced LTα production. ECs stimulated by C/S harvested from SP600125- and Indomethacin-treated KM-H2 cells show reduced ICAM-1, VCAM-1 and E-selectin expression as well as reduced naïve T cell interactions with stimulated ECs.

Mechanistic studies were carried out to understand the signaling pathways involved in regulating production of LTα by HRS cells. Western blot analysis showed that treatment of KM-H2 cells with Bay 11-7085 reduced expression of nuclear p65 and, unexpectedly, phosphorylated c-Fos and total c-Fos. Treatment of KM-H2 cells with SP600125 reduced both phosphorylated JNK as well as phosphorylated and total c-Jun protein but level of phosphorylated c-Fos and total c-Fos remained unchanged. Interestingly, while the levels of phosphorylated c-Fos and total c-Fos were reduced significantly in Cox inhibitor treated KM-H2 cells, phosphorylated JNK and c-Jun were up-regulated in the Indomethacin-treated KM-H2 cell. This piece of data suggested that signals from Cox and NFκB pathways might converge at c-Fos and co-operate with c-Jun in AP-1 pathway regulated LTα production.

The data suggest that in cHL, malignant H-RS cells secrete soluble LTα which can modulate ECs function. NFκB, JNK and COX pathways are involved in regulating the production of LTα from KM-H2 cells.

(500 words)

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LIST OF ABBREVIATIONS

ALCL	Anaplastic large cell lymphoma
AP-1	Activator Protein-1
ATF2	Activating protein 2
bZIP	Basic zipper leucine
CAF	Cancer associated fibroblast
cHL	classical Hodgkin lymphoma
CRE	cAMP response element
Cox-1	Cyclooxygenase-1
Cox-2	Cyclooxygenase-2
C/S	Culture supernatant
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
EMMPRIN	Extracellular matrix metalloproteinase inducer or CD174
ERK	Extracellular signal-regulated kinase
GPCR	G-protein coupled receptor
HA	Hyaluronic acid
HEV	High endothelial venule
HL	Hodgkin lymphoma
HRP	Horseradish peroxidase
HRS	Hodgkin and Reed-Sternberg
HUVEC	Human umbilical cord vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon-gamma

IHC	Immunohistochemistry
IκB	Inhibitor κB
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-12	Interleukin-12
IL-13	Interleukin-13
IP-10	Inducible protein-10
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
LFA-1	Lymphocyte function associated antigen-1
LT	Lymphotoxin
LTα	Lymphotoxin-alpha
LTβ	Lymphotoxin-beta
MCP	Monocyte chemoattractant protein-1
MDC	CCL22/ Macrophage derived chemokine
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T cell
NK	Natural killer
NLS	Nuclear localization signal
NSAIDS	Non-steroidal anti-inflammatory drugs
PD-L1	Programed cell death ligand 1
PG	Prostaglandin

PLN	Peripheral lymph node
PNAd	Peripheral node addressins
PSGL-1	P-selectin glycoprotein ligand-1
RANTES	CCL5/ Regulated on Activation, Normal T cell Expressed and Secreted
SEM	Standard error of mean
SRE	Serum response element
STAT	Signal Transducer and Activator of Transcription
TAM	Tumor associated macrophages
TARC	CCL17/ Thymus and activation-regulated chemokine
T_{EF}	Effector T cell
T_{EM}	Effector memory T cell
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor-alpha
TRE	TPA response element
TXA2	Thromboxane A2
UAE	United Arab of Emirates
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4

Chapter 1 : Introduction

1.1 Tumor microenvironment

Cancer development has been identified as a multi-step process in which a healthy somatic cell will undergo an initiating event upon exposure to external stimuli and subsequent tumor transformation steps to become a cancerous cell. This event accumulates genetic modifications. The fact that cancer cells have mutated genomes is well established (Hanahan and Weinberg, 2000). In addition, many cancers will develop as a result of chronic inflammation due to infections for example Hepatitis B and C infection in hepatocellular carcinoma and *Helicobacter pylori* in gastric cancer.

Chronic inflammation is strongly associated with cancer risk. A few examples of cancers tightly linked to inflammation include inflammatory bowel disease, colon cancer and cervical cancer (Mbeunkui and Johann, 2009). Chronic inflammation helps to establish a tumor microenvironment that is full of deregulated proliferative signaling network that are important for tumorigenesis and tumor progression. Inflammation process also supply bioactive molecules including growth factor that can sustain the proliferative signaling, survival factors that limit cell death, proangiogenic factors and extra-cellular modifying enzymes that facilitate angiogenesis, invasion and metastasis (Hanahan and Weinberg, 2011). These signals are, in part, orchestrated by inflammatory cells which are the indispensable participants in neoplastic process (Coussens and Werb, 2002).

Cells that form tumor microenvironment in different cancer types include myofibroblast, fibroblast, adipocytes, epithelial cells, glial cells, endothelial cells, macrophages and leukocytes. The tumor microenvironment is characterized by the crosstalk between tumor cells and different cell types. In the tumor periphery, macrophages (or also known as tumor associated macrophages, TAM) foster local invasion by supplying matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteinases

(Kessenbrock et al., 2010). The reciprocal interactions between TAM and cancer cells faciltate cancer cells intravasation into circulatory system and metastatic dissemination. In a metastatic breast cancer model, TAM provide epidermal growth factor (EGF) to breast cancer cells while breast cancer cells provide colony stimulating factor-1 (CSF-1) to support the growth of TAM (Wyckoff et al., 2007). Besides TAM, cancer associated fibroblast (CAF) also plays a significant role in tumor initiation, progression and metastasis. Study by Olumi et al showed co-injection of CAF with immortalized prostate epithelial cells in the mice resulted in the development of larger tumors (Olumi et al., 1999). Allinen et al and Orimo et al showed that secretion of SDF-1 (CXCL12) by CAF promotes tumor growth and angiogenesis in invasive breast carcinomas (Allinen et al., 2004; Orimo et al., 2005).

Hodgkin lymphoma (HL) is a lymphoid malignancy with a unique tumor microenvironment which features a complicated crosstalk between the cancerous Hodgkin and Reed-Sternberg (HRS) cells and the inflammatory infiltrates. HRS cells are surrounded by an enormous number of reactive infiltrates that frequently provide survival signals. In fact, once the HRS cells are removed from their microenvironment, they are unable to survive (Kuppers et al., 2012). Evidence provided from various studies highlighted the importance to cross-talk between HRS cells and surrounding immune infiltrates or stromal cells. The interaction of HRS cells with surrounding microenvironment had been studied extensively for many years and is regarded to be important for the pathogenesis of HL.

Various studies had been carried out to better understand the cell-cell signaling pathways between the HRS cells and the nonmalignant reactive and stroma cells in lymphatic tissues. Findings so far pointed out immune cells in the microenvironment that are associated with favorable or unfavorable response to HL treatment. Steidl et al showed that overexpression of macrophages signature was associated with the failure of primary treatment (Steidl et al., 2010). In contrast, expression of genes belonging to B cell

clusters including BCL11A, BANK1, STAP1, BLNK, FCER2, CD24 and CCL21 are all associated with favorable outcome in HL (Sanchez-Aguilera et al., 2006). The presence of cytotoxic T cells and regulatory T cells in the HL microenvironment also serve as the important prognostic factor for HL. Paradoxically, high cytotoxic T cells and low regulatory T cells had been reported to negatively influence event free survival and disease free survival of classical Hodgkin lymphoma (cHL) patients. Alvaro et al reported that in four cHL patients that relapse is associated with high TIA-1 positive cytotoxic T cells and low number of regulatory T cells (Alvaro et al., 2005). Another recent study by Greaves et al suggested that a combination of several immune cells markers, CD68 and FOXP3 in particular, can further improve prognostic stratification (Greaves et al., 2013).

1.2 T cells

Generally, T cells can be divided into three main classes which are naïve T cells, memory T cells and effector T cells. A more detailed classification of T cells based on their functions can divide T cells into T helper cells, cytotoxic T cells and regulatory T cells.

1.2.1 T helper (T_H) Cells

There are four basic types of T_{Helper} cells: $T_{Helper}1$, $T_{Helper}2$, $T_{Helper}17$ and T_{reg} cells (Figure 1.1) (Zhu and Paul, 2010). Each subset of T_{Helper} cells is generated by a different route of differentiation regulated by the surrounding cytokine milieu during T cell activation (O'Garra and Arai, 2000). For an optimal immune response, each subset of T_H cells has different distinct function and different characteristic cytokine production profiles.

IL-12 is the determinant cytokine that drives the differentiation of $T_{Helper}1$ cells. IL-12 is produced by macrophages and dendritic cells in the presence of microbial infection or upon CD40 ligation (Cella et al., 1996). In addition, Th1 development can be further enhanced by interferon-γ (IFN-γ) which up-regulates IL-12 receptors and inhibits the growth of $T_{Helper}2$ cells (Figure 1.1) (O'Garra, 1998). $T_{Helper}1$ cells are essential for the eradication of intracellular pathogens including bacteria, parasites, viruses and yeasts. The cytokine hallmark of $T_{Helper}1$ cells is the production of IFN-γ and lymphotoxin which can activate anti-microbial activity in macrophages and induce cytokine production. A $T_{Helper}1$ immune response is often accompanied by the production of complement fixing antibodies of IgG2a subtype as well as the activity of natural killer (NK) cells and cytotoxic T cells (Abbas et al., 1996). If $T_{Helper}1$ immune response is left uncontrolled, it could cause autoimmune disease such as Type I diabetes and multiple sclerosis (O'Garra et al., 1997).

IL-4 determines the development of $CD4^+$ precursor T cells into $T_{Helper}2$ cells (Figure 1.1). Early production of IL-4 in the immune response directs the development of $T_{Helper}2$ cells accompanied by the production of IL-4, IL-5 and IL-13. Cytokines produced by $T_{Helper}2$ cells can activate mast cells and eosinophils, thereby eradicating helminths and other extracellular parasites (O'Garra and Arai, 2000). In addition, these cells are also implicated in allergic and atopic manifestations where $T_{Helper}2$-derived cytokines can induce airway hypersensitivity as well as the production of IgE (Sher and Coffman, 1992).

$T_{Helper}1$ and $T_{Helper}2$-derived cytokines are antagonistic in nature and are able to inhibit the growth and development of each other's cell function.

1.2.2 Regulatory T (T_{reg}) cells

Regulatory T cell (Treg) can be subdivided into natural occurring T regulatory cell (nTreg) or induced T regulatory cell (iTreg). Tregs are important for the prevention of autoimmune diseases and in maintaining a balance between peripheral immune self-tolerance and the potential to generate life-long immunity to a variety of pathogenic microbes (Sakaguchi et al., 2008). nTreg cells represent 1-10% of total T cells in thymus, peripheral blood and lymphoid tissues. nTregs which are generated in the thymus (Figure1.1) express the IL-2 receptor alpha chain (CD25) constitutively. They also express CD127 and Foxp3 (forkhead winged helix family transcriptional regulator).

nTreg do not produce pro-inflammatory cytokine upon antigenic stimulation and are not pathogenic towards highly reactive self-antigens carrying cells. Instead, they potently suppress activation, proliferation and effector functions of $CD4^+$, $CD8^+$, natural killer cells, natural killer T cells, B cells and dendritic cells (Piccirillo, 2008) (Figure 1.1).

Circulating peripheral naïve T cells can also acquire regulatory functions under unique, differentiation signals *in vitro* and *in vivo* (Piccirillo, 2008) In general, most iTreg cells arise after continuous exposure to antigen presented by antigen presenting cells in the absence of co-stimulatory signal or following the activation of CD4+CD25- cells in the presence of TGFβ (Vigouroux et al., 2004). iTreg are categorized based on their phenotype and, their relative cytokine production capabilities. An example of CD4+ iTreg is the antigen-specific, IL-10 producing type 1 regulatory T cells (Tr1), which requires IL-10 as a priming factor and mediates its biological activity in an IL-10 dependent fashion.

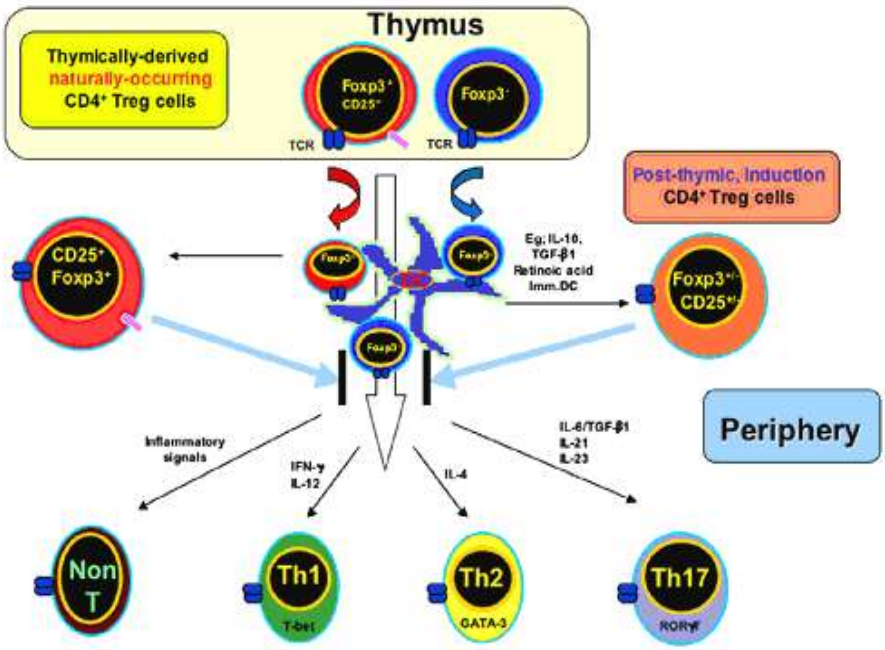


Figure 1.1: T cell differentiation. Conventional $CD4^+$ T cells (blue) exit the thymus, and upon activation by dendritic cells differentiate into effector Th1, Th2 and Th17 cells; and collectively contribute to a vast variety of peripheral immune response (Piccirillo, 2008). Thymic derived, naturally occurring (red) and peripherally induced (blue) $CD4^+$ regulatory T cell subsets can downregulate the activation, differentiation, and function of Th1, Th2 and Th17 effector cells as well as non T cells (brown). While $CD4^+CD25^+Foxp3^+$ nTreg cells differentiate in the thymus and are found in the normal, naïve T cells repertoire; multiple iTreg cell subsets, possibly expressing CD25 and Foxp3, originate from the activation and differentiation of conventional $CD4^+$ cells in the periphery under unique stimulatory conditions. Both Treg subsets conceivably synergize to assure regulation of immune responses.
(adapted with permission from Cytokine. 43:395-401 (2008))

1.2.3 Cytotoxic T cells (CTL)

CD8+CTL forms a major part of body's defense against viral infection and tumor progression by finding and eliminating viral infected and tumorigenic cells. CTL kills the target cells either by ligation of the death receptor on the target cell (Fas death receptor ligation) or by granule exocytosis, where perforin and granule specific serine proteases (granzymes) are delivered to the target cells (Waterhouse et al., 2004). The primary role of perforin is to assure the correct trafficking of granzymes into the target cells (Browne et al., 1999).

In addition, perforin also induces direct lysis of target cells (San Mateo et al., 2002).

1.3 Hodgkin Lymphoma

Hodgkin lymphoma is a lymphoid malignancy described by Thomas Hodgkin more than 150 years ago (Küppers, 2009). The cancerous cell of this disease is the mononucleated Hodgkin and multinucleated Reed-Sternberg cells (Figure 1.2) which was described by Dorothy Reed and Carl Sternberg in 1900. Hodgkin lymphoma (HL) can be divided into classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) (Cancer, 2008). NLPHL only accounts for about 5% of all the HL cases. cHL can be further subdivided into nodular sclerosis, mixed cellularity, lymphocyte rich and lymphocyte depletion Hodgkin lymphoma subtypes (Figure 1.3). Nodular sclerosis which accounts for about 60% of cases of Hodgkin lymphoma is characterized by extensive fibrotic bands separating nodules containing Hodgkin and Reed-Sternberg (HRS) cells. Mixed cellularity accounts for about 30% of cases of Hodgkin lymphoma is characterized by a prominent mixed cellular infiltration.

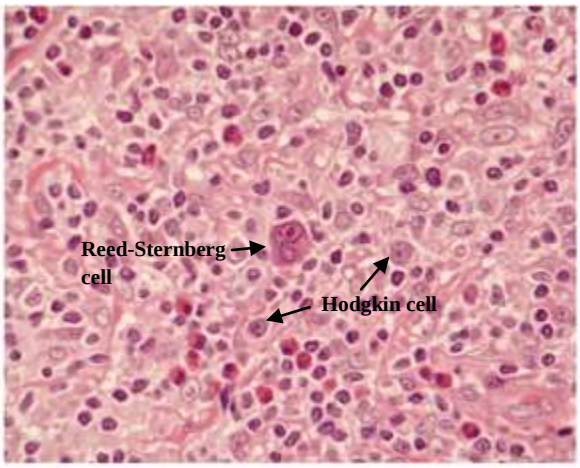


Figure 1.2: Diagram showed the morphological appearance of mononuclear Hodgkin and multinucleated Reed-Sternberg cells in the affected lymph nodes (Kuppers et al., 2012). H&E staining of mixed cellularity HL showing binucleated HRS cells is visible in the middle of the image, surrounded by histiocytes, lymphocytes and eosinophilic granulocytes. (adapted with permission from Journal of Clinical Investigation. 122:3439-3447 (2012))

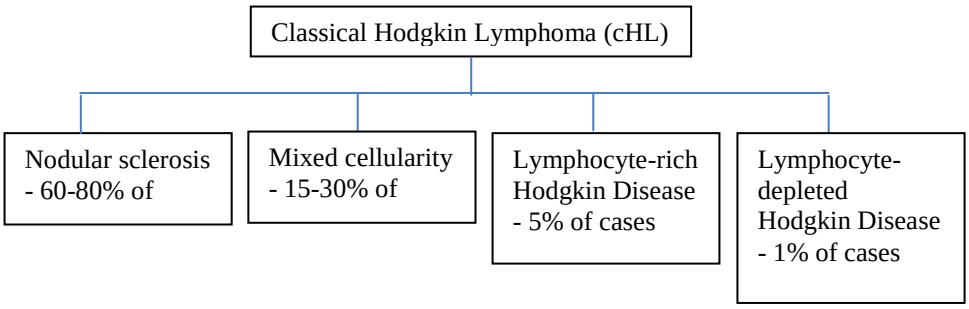


Figure 1.3: cHL can be subdivided into four subtypes, which are nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted HL (Kuppers, 2009). Nodular sclerosis and mixed cellularity cHL account for more than 95% of the total cHL cases worldwide.
(adapted with permission from Nature Reviews Cancer. 9:15-27 (2009))

In the United States, between 2006 to 2010, the median age of patients diagnosed with HL was 38 years of age. Approximately 13% was diagnosed under age 20, 31.2% was diagnosed between 20 and 34, 14.6% between 35 and 44; 12.7% between 45 and 54; 10.7% between 55 and 64; 8.8% between 65 and 74; 6.7% between 75 and 84; and 2.2% at 85+ years of age. The age-adjusted incidence rate was 2.8 per 100,000 men and women per year. The

age-adjusted mortality rate was 0.4 per 100,000 men and women per year (This data is extracted from Surveillance Epidemiology and End Result Website). In Singapore, of the 366 cases of HL reported between 1996 to 2004, nodular sclerosis constitute 66% (n=241) and mixed cellularity constitute 20% (n=73) of the total cases. Besides that, incidence rate of HL is reported to have increased significantly in adolescence and young adults to produce a second incidence peak peak in addition to the one seen for over 50 years of age (Hjalgrim et al., 2008).

Current treatment of HL involved the use of multi-agent chemotherapy and radiation agent which could achieve cure rate of about 80-90% (Diehl et al., 2004). The high cure rate of HL by a combination of chemotherapy and radiotherapy has been encouraging. However, it is also associated with high side effects, and about 20-30% of patients relapsed within 5 years after achievement of complete remission (Gaudio et al., 2011). Hence there is still need of alternative therapeutic agents to improve treatment outcome and quality of life for these patients (Klimm et al., 2005).

1.3.1 HRS cell origin

The cancerous HRS cells in cHL and the HRS cells variant in the NLPHL which is called lymphocytic and histiocytic (L&H) cells, usually account for only 1-10% of total cell population in the tumor lesions (Kuppers et al., 2012). Both HRS and L&H cells are found to originate from germinal centre B cells. However, there are slight differences in the surface marker expression profile between HRS cells and L&H cells. HRS cells are reported to co-express surface markers from several different lineages, unlike any other cells in the hematopoietic system. HRS cells can express markers of T cells (CD3, Notch 1, GATA 3), cytotoxic cells (granzyme B, perforin), B cells (Pax 5, CD20), dendritic cells (fascin, CCL17), NK cells (ID 2), myeloid cells (CSFR 1) and granulocytes (CD 15) (Kuppers et al., 2012). The B cell origin of HRS cells was demonstrated by the presence of clonal and somatically mutated

heavy and light chain gene rearrangement in these neoplastic cells (Kuppers et al., 1994). About 25% of the HRS cells in cHL cases showed loss of function immunoglobulin (Ig) gene mutations including nonsense mutation in their V region (Brauninger et al., 2003; Kanzler et al., 1996; Kuppers et al., 1994; Marafioti et al., 2000). Surprisingly, these crippled HRS cells have acquired mechanisms to survive and escape the apoptotic pathway, a fate that normally happens to germinal centre B cells that have acquired such mutations. Analysis of some cases of cHL carrying T cell markers revealed that some fraction of HRS cells express T cell receptor gene rearrangement and lack Ig gene rearrangement. Thus, it appears that HRS cells could be derived from T cells in rare cases of cHL (Aguilera et al., 2006; Muschen et al., 2000; Seitz et al., 2000; Tzankov et al., 2005).

1.3.2 Deregulated transcription factors network of HRS cells

The rarity of HRS cells has hampered the clarification of their cellular origin and identification of their genetic lesions for the longest time. Several pathogenic mechanisms have been revealed using molecular cytogenetic techniques and microdissection analysis of HRS cells. Mechanism to escape apoptosis is one of the molecular pathogenesis of cHL and signaling pathways involve in regulating apoptosis reaction had been studied thoroughly in HRS cells. TP53 mutation is a hallmark of various types of cancer which allows the cancerous cells to escape apoptosis or cell growth arrest (Greenblatt et al., 1994). Earlier analyses for TP53 mutation on primary HRS cells showed restricted mutations on selected exons. More recently, deletion of TP53 was identified on HRS cell lines (Feuerborn et al., 2006) and therefore, TP53 alterations on primary HRS cells may be more frequent than was previously anticipated (Feuerborn et al., 2006; Maggio et al., 2001; Montesinos-Rongen et al., 1999). Besides TP53 mutation, several other anti-apoptotic proteins are also up-regulated in HRS. HRS cells had been shown to up-regulate expression of CASP 8 and FADD-like apoptosis regulator (CFALR) to inhibit

FAS signaling and XIAP which suppresses caspase activation (Dutton et al., 2004; Kashkar et al., 2003; Mathas et al., 2004).

Besides TP53, activity of Janus Kinase (JAK) -STAT and NFκB pathways are commonly dysregulated in the HRS cells (Figure 1.4). Many cytokines signal through members of the Jak family which phosphorylate STAT factor on activation (Rawlings et al., 2004). The phosphorylated STATs dimerize and translocate to the nucleus and function as transcription factors. There are frequent genomic gain of JAK2 and suppressor of cytokine signaling 1 (SOCS1), which is a negative regulator of JAK-STAT signaling in HRS cells. Hence, JAK-STAT signaling is often somatically mutated and inactivated in cHL (Joos et al., 2000; Mottok et al., 2007; Weniger et al., 2006). Besides genetic mutation, constitutive activation of JAK-STAT signaling on HRS cells can also be caused by autocrine/paracrine events. Four STATs subunit are highly active in HRS cells, STAT3, STAT5A, STAT5B and STAT6 (Baus and Pfitzner, 2006; Kube et al., 2001; Scheeren et al., 2008; Skinnider et al., 2002). Expression of IL-13 and IL-13 receptor on HRS cells activates STAT6 in an autocrine manner (Kapp et al., 1999). Similarly, STAT5A, STAT5B and STAT3 on HRS cells are activated by autocrine signaling of IL-21 and IL-21 receptor on the HRS cells (Lamprecht et al., 2008; Scheeren et al., 2008).

NFκB activity is constitutively active in HRS cells. NFκB activity is affected by several types of genetic alterations. REL, a member of NFκB transcription factor family, shows genomic gain and amplification which contributes to higher REL protein expression in nearly half of the cHL cases (Barth et al., 2003; Martin-Subero et al., 2002). In rarer instances, BCL-3 which can positively up-regulated NFκB activity is also affected by genomic gain and translocation (Martin-Subero et al., 2006; Mathas et al., 2005). Besides that, IκBα which inhibits NFκB signaling by binding to NFκB in the cytoplasm and preventing their nuclear translocation is discovered to undergo mutation in about 20% of cHL cases (Cabannes et al., 1999; Emmerich et al., 1999). Signaling events contributing to the activation of NFκB go through two well-

known pathways, namely the canonical and non-canonical pathways involving the TNF receptor family. HRS cells express CD30, CD40, BCMA, TACI and RANK which are members of TNF receptor family. Interaction of these receptors with their respective ligands, often expressed on the immune infiltrate, may activate NFκB activity in HRS cells. For example, T cells expressing CD40 ligand are always found to be in close contact with HRS cells (Carbone et al., 1995). CD30 ligand is expressed by eosinophils and mast cells which are also present in the tumor microenvironment (Molin et al., 2001; Pinto et al., 1996). APRIL (TNSF13), one of the ligand for TACI and BCMA is produced by neutrophils in the HL microenvironment and BAFF (TNSF13B), the second ligand of these receptor, is expressed by HRS cells and other cells in the lesion (Chiu et al., 2007; Schwaller et al., 2007). In contrast, RANK activation is slightly different. RANK can be activated in an autocrine manner because HRS cell lines are found to express RANK ligand (Fiumara et al., 2001).

In addition to NFκB and JAK-STAT signaling, PI3K, ERK (extracellular signal-regulated kinase), AP-1 and receptor tyrosine kinase pathway are also deregulated and constitutively activated in HRS cells. The PI3K pathway in HRS cells is activated by CD30, CD40 RANK and receptor tyrosine kinase. Activity of this pathway is implicated by the presence of the phosphorylated form of AKT in HRS cells. Inhibition of AKT causes death of HRS cell lines further supports its role in regulating the survival and pathogenesis of the disease (Dutton et al., 2005; Georgakis et al., 2006). ERK pathway which regulates proliferation, apoptosis and cell differentiation may also be activated through CD30, CD40 and RANK interactions in the HRS cells. Active forms of ERK kinases, ERK1, ERK2 and ERK5, are expressed by the HRS cells. Inhibition of ERK activation in HRS cells *in vitro* causes anti-proliferative effect (Nagel et al., 2007; Zheng et al., 2003). The AP-1 transcription factor comprises of dimerized members of Jun and Fos families. HRS cells demonstrate high expression of c-Jun and JunB with especially strong nuclear localization, implying that they are highly active (Mathas et al., 2002). AP-1 induces many target genes in the HRS cells, including CD30 and galectin-1

(Juszczynski et al., 2007; Watanabe et al., 2003). These genes are involved in promoting proliferation of HRs cells and maintaining an immunosuppressive microenvironment. Interestingly, while NFκB activity can contribute to the up-regulation of JunB, the mechanisms mediating c-Jun up-regulation in HL is not as well defined (Mathas et al., 2002).

Receptor tyrosine kinases are involved in the regulation of cell proliferation, survival, growth and differentiation. HRS cells show aberrant expression of receptor of tyrosine kinases, including platelet-derived growth factor receptor-α (PDGFRA), epithelial discoidin domain containing receptor 2 (DDR2), macrophage-stimulating protein receptor (MSPR), TRKA and TRKB. Mutations of genes corresponding to these receptors have not yet been identified. This raised the possibility that they may be activated by autocrine or paracrine mechanisms. Expression of receptor tyrosine kinase is found predominantly in the nodular sclerosis subtype of HL, but is also detected at varying level of expression in other subtypes (Renne et al., 2005).

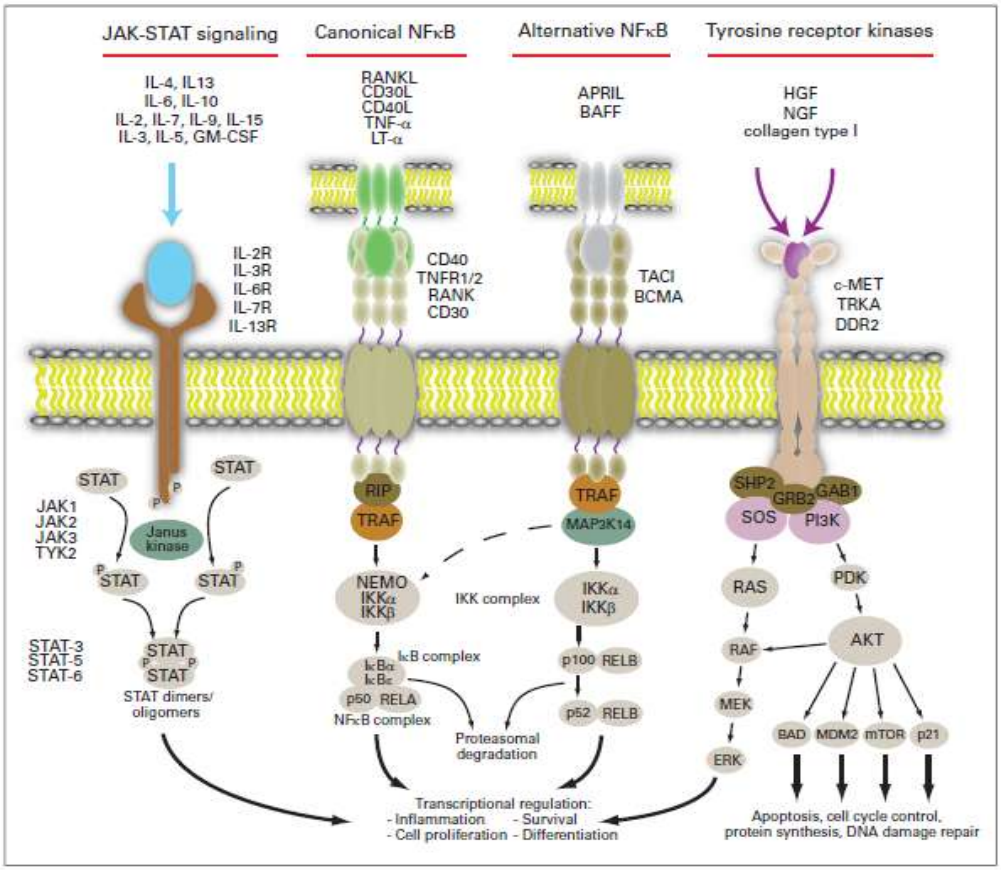


Figure 1.4: This simplified diagram shows the activation of various pathways in HRS cells by signals received from the tumor microenvironment (Steidl et al., 2011). Soluble and membrane bound signaling molecules produced by reactive cells (paracrine activation) activate JAK-STAT, canonical and non-canonical NFκB pathways and receptor tyrosine kinases. For JAK-STAT signaling pathway, the most commonly expressed interleukin and interleukin receptors are shown. For the NFκB pathway, only the principal activation pathways are shown and the activation of inhibitor of κ kinases (IκK) by other kinases is described. Downstream signaling of receptor tyrosine kinases is shown using the example of tyrosine kinase receptor A (TRKA) and illustrating the Ras and Akt pathway.
(adapted with permission from Journal of Clinical Oncology. 29:1812-1826 (2011))

1.3.3 The Hodgkin Lymphoma microenvironment

cHL is characterized by massive infiltration of immune cells into the lymphoma tissues (Kuppers et al., 2012). These immune infiltrates include T cells, particularly T_{helper} 2 (T_H2) and regulatory T (T_{reg}) cells, B cells, plasma cells, neutrophils, eosinophils, macrophages and mast cells. The malignant

HRS cells only represent 1-10% of the total cell population in the lesion (Figure 1.5). Evidence so far proved that these immune cells are actively recruited by HRS cells through chemokines and cytokines secretion (Skinnider and Mak, 2002). HRS cells secrete RANTES (CCL5, Regulated on Activation, Normal T cell expressed and secreted chemokine), TARC (CCL17, Thymus and activation-regulated chemokine) and MDC (CCL22, Macrophage-derived chemokine) to attract $T_{helper}2$ (T_H2) cells and regulatory T (T_{reg}) cells (Aldinucci et al., 2008; Skinnider and Mak, 2002). The secretion of IL-5, CCL5, CCL28 and granulocyte-macrophage-colony stimulating factor by HRS cells actively recruits eosinophils into the HL microenvironment. HRS cells also secrete IL-8 to attract neutrophils (Skinnider and Mak, 2002). Chemokines produced by HRS cells not only contribute to immune cell recruitment but can also contribute to promoting survival and proliferation of HRS cells. For example TARC produced upon CD40 ligation by HRS cell lines, including L1236, KM-H2, L428 and L540, proved to be vital in promoting clonogenic growth of HRS cells. Recombinant neutralizing antibody of CCL5 can inhibit the basal proliferation of these HL-derived cell lines (Aldinucci et al., 2008).

B cells of various maturation stages are part of the normal constituent in the normal lymph node. B cells are found mainly in the primary and secondary follicles, and marginal zones. However, in cHL the lymph node architecture is disturbed to varying degree. It remains an open question of how reactive B cells are recruited into the cHL lesions or whether they are the remnants that are yet to be displaced by the neoplastic lesion. As in other pro-inflammatory reactions, HRS cells produce TNF-α, lymphotoxin-α (LTα) which can affect B cells proliferation, differentiation and chemotaxis could play a role in intiation of germinal centre B cell reactions (Foss et al., 1993; Vu et al., 2008; Xerri et al., 1992).

Macrophages are also commonly found in cHL lesions. HRS cells secrete granulocyte colony stimulating factor (CSF1) and fractalkine (CXCL13) and

other differentiation factors that recruit and drive differentiation of monocytes (Ma et al., 2008; Truman et al., 2008). The type of macrophages present within a tumor microenvironment may exert a profound effect on tumor progression or tumor regression. M2 macrophages had been shown to be very important for the promotion of tumor progression, cell migration and suppression of anti-tumor response in various cancers including lymphoma (Qian and Pollard, 2010). A recent study reported that the number of tumor associated macrophages within the HL lesion is strongly correlated to shortened survival of cHL patients (Steidl et al., 2010).

Besides actively recruiting different subset of immune cells into the lymphoma tissues, HRS cells are also able to modulate the phenotype of specific immune cells into subset that could contribute to their survival and growth. The most obvious example is the shifting of the anti-tumor $T_{Helper}1$ response to tumor-promoting $T_{Helper}2$ response (Tan and Coussens, 2007). Recently, a HRS cell line, KM-H2, was shown to exhibit the capability of fostering a tumor privilege condition by inducing regulatory T cells differentiation of naïve T cells that were in close contact with the neoplastic cells *in vitro* (Tanijiri et al., 2007).

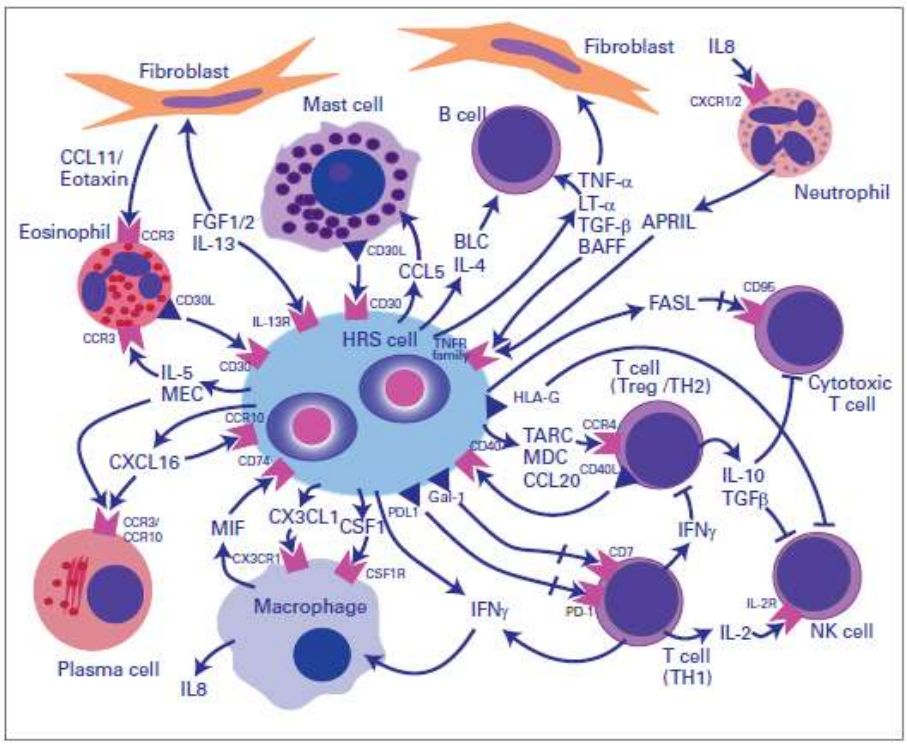


Figure 1.5: Schematic diagram showing the cross-talk between HRS cells and the tumor microenvironment in the cHL (Steidl et al., 2010). In the center, the HRS cell is shown to express various cell surface molecules as well as secreted cytokines and chemokines. Surrounding the HRS cell are cell types representative of the nonmaglinant cells attracted by these molecules. The cells in the microenvironment can, in turn, express various chemokines and cytokines that further shape the reactive infiltrate and provide signals for the HRS cell.
(adapted with permission from Journal of Clinical Oncology. 29:1812-1826 (2011))

1.3.4 Importance of T cells in cHL

The most abundant immune infiltrate around HRS cells is $CD4^+$ T cells. T cells are essential for the pathogenesis of HL disease. CD40 ligand expressed on rosetting T cells can trigger CD40 receptor on the HRS cells to provide survival signal (Carbone et al., 1995). Besides that, IL-3 receptor expressed on HRS cells could induce IL-3 secretion on activated T cells to provide more survival and growth signal (Aldinucci et al., 2002).

A considerable proportion of infiltrating $CD4^+$ T cells are T_{reg} cells which are important to provide an immunosuppressive microenvironment for the survival and growth of HRS cells. T_{reg} cells produce IL-10 and TGF-β which exert inhibitory effects on the functions of effector T cells, especially cytotoxic T lymphocytes (CTL). The presence of large population of T_{reg} in the HL microenvironment is not due solely to active recruitment induced by chemokines produced from HRS cells but also via direct modulation of naïve T cells in close contact with HRS cells (Tanijiri et al., 2007). Surprisingly, the presence of a high number of T_{reg} is linked to good prognosis in HL disease (Alvaro et al., 2005). This suggested that T_{reg} cells may have some suppressive effect on the HRS cells or on other inflammatory cells that support HRS cell survival and proliferation.

Another subset of T cells presence in the tumor microenvironment is CTL. CTL can produce granzyme B (GrB) and TIA-1 to induce apoptosis on HRS cells. Oudejans et al has found an increased number of CTL in tissue biopsies of patients and that was associated with unfavorable clinical outcome (Oudejans et al., 1997). Paradoxically, high percentage of GrB^+ cells in the tissue biopsies was associated with poor prognosis. They reported that optimal discrimination between patients with good or bad prognosis was easily differentiated when threshold was set at 15% GrB^+ cells (Oudejans et al., 1997).

1.4 Leukocyte recruitment

Leukocyte must adhere to the endothelium before they can migrate from the endothelium into tissues. Adhesion and subsequent transendothelial migration takes place preferentially at specialized sites in blood vessels called post-capillary venules in the non-lymphoid tissues and high endothelial venules in lymph nodes. The flowing leukocyte that comes into brief contact with the vessel wall will slow its movement, and rolls on the endothelium if the endothelium is activated. Exposure of the rolling leukocyte to chemokines will

trigger integrin activation allowing the cell to come to a halt. The adhered cell will flatten its shape and undergo diapedesis and transmigration across endothelium in a few minutes. There are four basic steps that regulate the extravasation of leukocytes across the blood vessel, which are tethering, triggering, firm adhesion and migration (Figure 1.6).

1.4.1 Tethering

Tethering is mediated by a family of lectin-like calcium dependent binding molecules which promotes the slow rolling of leukocytes under flow condition (Bevilacqua, 1993). The three family members, L-selectin, E-selectin and P-selectin are named according to the cell types they were first discovered in. L-selectin is found on lymphocyte, E-selectin is found on endothelial cell, and P-selectin is found on platelet and endothelial cells. L-selectin is expressed constitutively on neutrophils, monocytes and eosinophils. Majority of the B cells and naïve T cells express L-selectin while only a subpopulation of memory T cells are L-selectin positive. Optimal L-selectin function involves change of receptor affinity after cellular activation and requires an intact cytoplasmic domain (Kansas et al., 1993). Lymphocytes and neutrophils experience a reversible loss of L-selectin after cellular activation. Loss of L-selectin is always accompanied by up-regulation of other adhesion molecules.

P-selectin is constitutively found in the Weibel-Palade bodies of the endothelial cells and alpha-granules of the platelets (Hsu-Lin et al., 1984; McEver et al., 1989). P-selectin inducing agent includes thrombin, histamine complement fragments, oxygen-derived free radicals and cytokines. Expression of P-selectin is very short-lived. Within minutes of activation by inducing agents, P-selectin is mobilized to the cell surface. However, *in-vivo* studies also suggested that it might be an important regulator of leukocyte-endothelial interactions at the later time point. Level of P-selectin mRNA expression was increased in mice after treatment with lipopolysaccharide (LPS)

or cytokines with the maxima level of expression detected at 4 hours after TNF-α stimulation (Tedder et al., 1995).

E-selectin expression on endothelial cells is induced upon activation. E-selectin production is strongly and rapidly induced by IL-1β, TNF-α, interferon-γ (IFN-γ) and LPS (Bevilacqua et al., 1987). E-selectin expression on human umbilical cord vein endothelial cells (HUVEC) peaks at 4-6 hours after activation but the expression returns to basal level after 24-48 hours. However, E-selectin expression on HUVEC *in-vitro* may not reflect the temporal and spatial expression of E-selectin on microvessel endothelial cells derived from other tissues since E-selectin expression is always up-regulated at the inflammatory sites including arthritic joint, psoriasis and in heart or kidney undergoing allograft rejection (Tedder et al., 1995).

Selectins are suitable for mediating tethering of leukocytes on the endothelium because they have long molecular structure that extended above the surrounding glycocalyx and allows them to capture passing leukocytes that express the appropriate receptors (Lasky, 1992). Selectin mediated interactions are strong enough to slow down the leukocytes but not strong enough to induce firm adhesion and completely stop leukocytes on the endothelium (Lawrence and Springer, 1991). The transient nature of this entire process is crucial to allow the leukocytes to sample the local endothelium for trigger factors that can activate the integrins and allow the nest step in the cascade to proceed. Interestingly, L-selectin (Finger et al., 1996), E- and P-selectin (Lawrence et al., 1997) actually require shear stress for optimal function.

Studies of the molecular basis of selectin mediated interactions have focused on carbohydrate recognition by the lectin domains. The tetrasaccharide sialyl Lewis^x (sLe^x) has been identified as the prototype ligand for E- and P-selectin although all three selectins can bind to sialyl Lewis^x (sLe^x) or sialyl Lewis^a under appropriate conditions (Carlos and Harlan, 1994). The dependence of

the selectin functions on carbohydrate ligands and the importance of fucose metabolism to generate sLe^x and related structure had been highlighted by studied on leukocyte adhesion deficiency syndrome II (Harlan, 1993).

P-selectin glycoprotein ligand-1 (PSGL-1) has the dominant role as a ligand that binds all three selectins although it was originally being described as the ligand for P-selectin (Sako et al., 1993). Binding of PSGL-1 with L-selectin mediates leukocyte-leukocyte interaction to facilitate secondary leukocyte capture and tethering. PSGL-1 is expressed on all leukocytes and on certain types of endothelial cells (da Costa Martins et al., 2007; Rivera-Nieves et al., 2006). PSGL-1 requires specific glycosylation to become functional (Moore et al., 1995). Besides PSGL-1, ligands of L-selectin also include GlyCAM-1, CD34 and mucosal vascular addressin cell-adhesion molecule-1 (MadCAM-1). E-selectin is also found to bind to glycosylated CD44 and E-selectin ligand 1 (ESL1) (Hidalgo et al., 2007).

1.4.2 Triggering

A triggering step is needed to activate integrins and promote strong adhesion because integrins molecules on leukocytes cannot bind well to their respective receptors on the endothelial cells without activation. During inflammation, endothelial cells will be activated by inflammatory cytokines to express adhesion molecules and synthesize chemokines and lipid chemoattractants that are presented on the luminal surface. Activated endothelial cells also transport chemokines, produced by resident cells such as macrophages and mast cells, from their abluminal surface to the luminal surface (Middleton et al., 1997). Some chemokines are generated by proteolytic cleavage in activated mast cells and platelet, and delivered to endothelial cells by circulating microparticles or exocytosis of intracellular granules. RANTES, PF4 (CXCL4, platelet factor 4) and ENA-78 (CXCL5, epithelial-derived neutrophil-activating peptide 78) are examples of chemokines that are deposited by platelets on activated

endothelial cells to trigger the arrest of monocytes (von Hundelshausen et al., 2001; 2005).

Interestingly, chemokines have the ability to induce leukocyte subset specific adhesion and migration. Specificity in leukocyte arrest is conventionally attributed, in part, to the differential expression and activation state of integrin subtypes, as well as the repertoire of chemokine receptors found on the leukocyte surface. Activated lymphocytes or transformed lymphoblasts often constitutively express high affinity forms of integrins which makes them bind more readily to activated endothelium. Ligation of chemokine to its specific G-protein coupled receptor (GPCR) triggers the activation of a complex signaling network almost instantaneously or within milliseconds. This GPCR-triggered signaling network is often known as inside-out signaling (Ley et al., 2007). Chemokine triggered signaling networks can regulate the activation of distinct integrins expressed on different leukocyte subsets. The well-known examples are monocyte chemoattractant protein 1 (MCP-1) which acts on monocyte, macrophage inflammatory protein 1α (MIP-1α), MIP-1β and RANTES which act on monocytes and distinct T cell subsets (Schall, 1991).

1.4.3 Firm adhesion

Strong adhesion of leukocytes to the endothelium is mediated by integrins. Integrins are a family of heterodimeric membrane glycoproteins that consists of α and β subunits (Ley et al., 2007). They are grouped into different subfamily based on their β subunit. The most important β1 integrin for leukocyte-endothelial cell interaction is called Very Late Antigen-4 (VLA-4) which comprises of a β1 chain paired with a α4 chain ($\alpha_4\beta_1$ or CD49aCD49f). The important β2 integrin is called Lymphocyte Function Associated Antigen-1 (LFA-1) which comprises of a β2 chain pairing with a αL chain ($\alpha_L\beta_2$ or CD11aCD18). Integrins are involved in mediating leukocytes rolling and firm adhesion on endothelium (Shimizu et al., 1992). VLA-4 dependent rolling is

seen on monocytes and monocyte-like cell lines (Chan et al., 2001; Huo et al., 2000), T cells (Singbartl et al., 2001) and T cell lines (Berlin et al., 1995).

β2 integrins bind to intercellular adhesion molecule 1 (ICAM-1) and ICAM-2 which are expressed on endothelium. ICAM-2 is constitutively expressed on the endothelium. ICAM-1 expression is induced upon endothelial cell activation. ICAM-1 expression is induced by IL-1 and TNF-α (Dustin et al., 1986; Pober et al., 1986). Kadono et al showed that rolling of human lymphocytes was enhanced and slowed when ICAM-1 was co-expressed with L-selectin ligands on a human vascular endothelial cell line (Kadono et al., 2002). They proposed that LFA-1/ICAM-1 interactions can influence L-selectin-mediated leukocyte rolling; and that functional synergy between L-selectin and Ig family is essential for optimal conversion of the rolling leukocytes to the stably adhered phenotype.

The α4β1 integrin (VLA-4) binds to vascular cell adhesion molecule-1 (VCAM-1). Expression of VCAM-1, like ICAM-1, is inducible upon activation. IL-1 and TNF-α can induce VCAM-1 expression on activated endothelial cells with maxima expression level peaking at 6-12 hours (Wellicome et al., 1990). Interestingly, IL-4 also acts on endothelial cells to induce VCAM-1 but not ICAM-1 and E-selectin expression (Schleimer et al., 1992; Thornhill and Haskard, 1990).

Several signaling pathways are involved in regulating adhesion molecule expression in endothelial cells. The most commonly studied signaling pathways are MAP kinases (Keshet and Seger, 2010), including ERK and p38 as well as JNK, and NFκB pathways. Endothelial cells are sensitive to various stimuli such as TNF-α, IL-1 and IL-6 to up-regulate adhesion molecule expression. Phosphorylated forms of p38 and JNK are important in the regulation of ICAM-1 expression. AP-1, which is downstream of JNK, and p38 is also important in regulating ICAM-1 expression on the endothelial cells.

Attenuation of p38 phosphorylation by specific tyrosine phosphorylation inhibitor resulted in the inhibition of ICAM-1 expression on human pulmonary microvascular endothelial cells (Tamura et al., 1998). Similar function of p38 was also observed on human umbilical cord vein endothelial cell (HUVEC) (Yan et al., 2002). AP-1 rather than NFκB was shown to be important in oxidative stress induced ICAM-1 expression on HUVEC (Roebuck et al., 1995). This suggested that different subsets of MAP kinase are responsible for ICAM-1 expression in different situation. However, the role of ERK, p38 and JNK may be dispensable in the synthesis of ICAM-1 and VCAM-1 on TNF-α stimulated endothelial cells. Work done by Zhou et al (Zhou et al., 2007) suggested that even though TNF-α stimulated endothelial cells could up-regulate phosphorylated ERK, p38 and JNK expression, treatment with inhibitors to these three MAP kinase signaling molecules did not prevent the induction of ICAM-1 and VCAM-1 expression on the TNF-α stimulated HUVEC. Their study suggested that TNFR1-induced NFκB signaling was the main pathway for the induction of ICAM-1 and VCAM-1 in TNF-α stimulated HUVEC.

In addition to ICAM-1 and VCAM-1, CD44 also plays a role in regulating leukocyte adhesion to activated endothelial cells. CD44 is a ubiquitously expressed cell surface adhesion molecule involve in cell-cell interaction and cell-matrix interactions. The multiple protein isoforms are coded by the same gene but are generated by alternative splicing and are further modified by a range of post-translational modifications (Hofmann et al., 1991). The principal ligand of CD44 is hyaluronic acid (HA) (Aruffo et al., 1990), which is an integral component of extracellular matrix. Other CD44 ligands include fibronectin and collagen. CD44 is found on many cell types including, fibroblasts, epithelial cells, keratinocytes, neurons, erythrocytes and leukocytes. CD44 plays an important role in regulating neutrophil migration across endothelium and adhesion of activated T cells to HA on endothelial cells (Bonder et al., 2006; Khan et al., 2004). In addition, antibodies to CD44 have been reported to block lymphocyte adhesion to the high endothelial

venules of muscosal lymphoid tissues and to other activated endothelium (Jalkanen et al., 1987; Picker et al., 1989).

1.4.4 Migration

After firm adhesion, leukocytes migrate through the endothelial barrier under the influence of promigratory factor. Chemokines also act as the chemotatic factors that attract the bound leukocytes to transmigrate across the endothelium into the interstitium. For example, immobilized MIP-1α has been demonstrated to direct the migration of specific T cell subsets across the endothelium (Tanaka et al., 1993; Taub et al., 1993). In addition, junctional proteins such as endothelial junctional proteins such as platelet/endothelial-cell adhesion molecule 1 (PECAM-1) and junctional adhesion molecule (JAM), are also important for regulating leukocyte migration across the endothelial monolayer. PECAM-1, JAM-A and ICAM-2 mediate leukocyte migration in response to IL-1β but not TNF-α (Nourshargh et al., 2006). Thus, the interactions of different adhesion molecules with their cognate receptors regulate leukocyte transmigration in a cell-specific and stimulus specific manner.

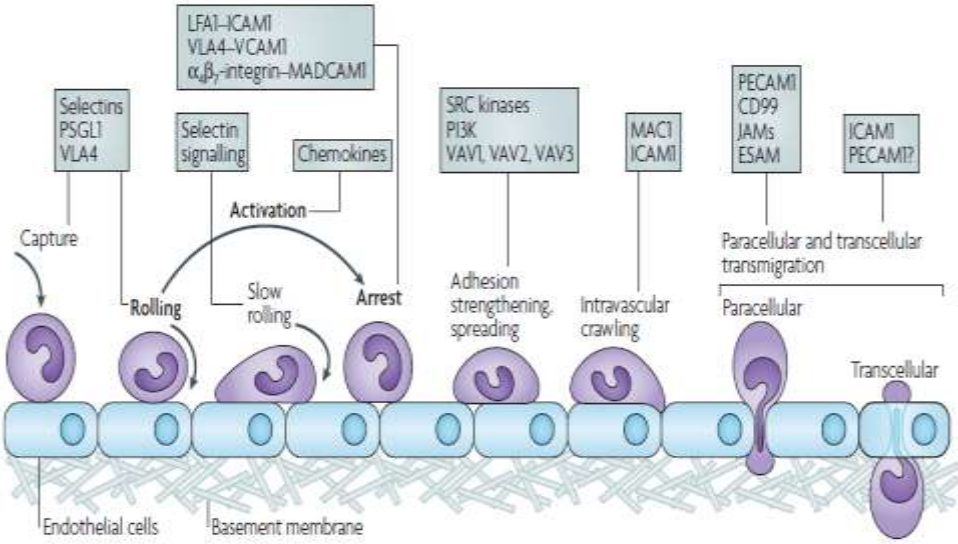


Figure 1.6: Diagram shows 4 important steps of leukocyte adhesion cascade (Ley et al., 2007). The three steps shown in bold are critical steps before leukocyte transmigration can occur. Rolling is mediated by selectins, activation is mediated by chemokines and arrest is mediated by integrins. (adapted with permission from Nature Reviews Immunology. 7:678-689 (2007))

1.4.5 Preferential migratory patterns of leukocytes

Leukocyte binding to endothelium is dependent on the site of the endothelial cells and the nature of the cytokines or stimulatory factor. For example TNF-α induced ICAM-1 and VCAM-1 expression on the activated endothelial cells therefore, allowing leukocyte-endothelial cell binding via β1 and β2 integrins whereas IL-4 only induces VCAM-1 expression hence only allows leukocyte-endothelial cell binding via β1 integrin (Schleimer et al., 1992). Leukocyte migration is important to provide immune surveillance and mount inflammatory responses against antigens. Different subset of leukocytes have different patterns of recruitment and different functional capacity. Neutrophils as early response effector cells recruited in huge numbers to the inflammatory sites to mount the first wave of immune response. T cells are recruited later and more selectively than neutrophils where they perform antigen restricted functions.

Differential expression of adhesion molecules or integrin subtypes on naïve and memory T cells gives rise to different recruitment patterns between naïve and memory T cells to inflammatory site specific and peripheral lymph nodes under physiological condition. Naïve T cells express high level of L-selectin, moderate level of CD44 and low level of α_4 integrin (Picker et al., 1990). In contrast, memory T cells have bimodal expression of L-selectin but definitely higher expression of CD44 and α_4 integrin (Lim et al., 2000; Picker et al., 1990). Initially, it was believed that memory T cells do not express L-selectin on the surface. However, various reports suggested otherwise. As mentioned by Picker et al, bimodal expression of L-selectin occurs on memory T cells and Li et al reported that L-selectin is expressed on naïve T cells and a small population of memory T cells (Li et al., 1993; Picker et al., 1990).

Naïve T cells migrate exclusively through lymph node and secondary lymphoid organ (Figure 1.7) (Marelli-Berg et al., 2008). Memory T cell acquires a distinct ensemble of adhesion molecules on the surface which allows it to migrate through the tissues more readily. In addition, lymphocyte recirculation is also regulated by the presence of other adhesion molecules preferentially expressed on specific sites of the tissues. One such molecule is PNAd (peripheral node addressins) which is expressed primarily in the peripheral lymph nodes and interact with L-selectin expressing T cells.

1.4.6 Naïve T cell recirculation

Naïve T cell recirculation through peripheral lymph node (PLN) and lymphoid tissues is largely mediated by two homing receptors or addressin pairs: first is L-selectin with PNAd; second is integrin activation induced by CCR7 engagement with chemokines, ELC (CCL19 or EBl 1 ligand chemokine) and SLC (CCL21 or secondary lymphoid-tissue chemokine), expressed on the surface of high endothelial venule (HEV) (Figure 1.7) (Miyasaka and Tanaka, 2004; von Andrian and Mempel, 2003). Studies utilizing specific gene knock-out mice had facilitated our understanding of the roles of these molecules in

mediating naïve T cell migration. L-selectin knock out mice showed less binding of lymphocytes to the PLN and markedly fewer number of lymphocytes localized in PLN (Arbones et al., 1994). Similarly, CCR7 knock out mice have fewer naïve T lymphocytes in the PLN as well as extensively disrupted PLN architecture (Baekkevold et al., 2001). Another set of adhesion molecule interaction that plays a role in naïve T cell homing to secondary lymphoid tissues is the binding of LFA-1 to ICAM-1. Lymphocytes from LFA-1 deficient mice failed to home to lymph node (Berlin-Rufenach et al., 1999). Besides that, Reichardt et al showed that LFA expression on T cells is important for prolonged PLN residence. $LFA^{-/-}$ CD4 T cells have lower PLN residence dwell time compared to $LFA^{+/+}$ CD4 T cells (Reichardt et al., 2013).

Naïve T cell migration into gut associated lymphoid tissues such as Payer's patches and mesenteric lymph nodes (MLNs) is mainly dependent on $\alpha_4\beta_7$-MAdCAM-1 pairing (Wagner et al., 1996). Besides CCR7, naïve T cells also expressed CXCR4 which is the receptor for SDF-1α (CXCL12, stromal-cell derived factor 1 alpha). The CXCR4-SDF-1α axis has been shown to promote migration of naïve T cells across HEV *in-vitro* (Campbell et al., 1998).

1.4.7 Memory T cell recirculation

Antigen-experienced T cells are more diverse than naïve T cells with respect to their migratory property and they can be subdivided into memory T cells (T_{CM}), effector T cells (T_{EF}), and effector memory T cells (T_{EM}) subsets based on their expression of specific homing receptors and functional differences (Marelli-Berg et al., 2008). T_{CM} express peripheral lymph node (PLN) homing molecules like L-selectin and CCR7 receptors. These molecules are critical for T_{CM} retention in the secondary lymphoid organ. Besides that, T_{CM} can also localize at peripheral tissues and sites of inflammation (Figure 1.7) (Sallusto et al., 1999).

In contrast, T_{EF} and T_{EM} subsets do not express CCR7 and negligible or low expression of L-selectin. T_{EF} and T_{EM} subsets do not migrate into PLN (Sallusto et al., 1999). Both T_{EF} and T_{EM} preferentially home to non-lymphoid tissues. The differential expression of different homing molecules on the different subsets of memory T cells confers different tissue specific homing properties. Memory T cells that preferentially circulate to skin express cutaneous lymphocyte antigen (CLA) (Picker et al., 1991) and the chemokine receptors, CCR4 (Campbell et al., 1999) and CCR10 (Reiss et al., 2001). CLA binds to E-selectin which is constitutively expressed on the skin post-capillary venule. CCR4 and CCR10 bind respectively to TARC and CCL27 (CTACK), leading to integrin activation. CCL17 was shown to induce integrin-dependent adhesion to ICAM-1 of skin-derived memory T cells under static and physiological flow condition *in-vitro* (Campbell et al., 1999).

Constitutive migration of effector/memory T cells into lamina propria of the small intestine requires the interaction of $\alpha_4\beta_7$ and chemokine receptor CCR9 (Berlin et al., 1993) expressed on lymphocyte surface with MadCAM-1 and CCL25 (TECK, Thymus-Expressed Chemokine) (Wagner et al., 1996) found on the endothelial cells of gut lamina propria venules. T cells deficient in β_7-integrin chain are severely impaired in their ability to home to intestinal mucosal (Lefrancois et al., 1999). Similarly, CCL25 blockade or CCR9 knock down clearly reduced $CD8^+$ T cells migration to the small intestine (Svensson et al., 2002).

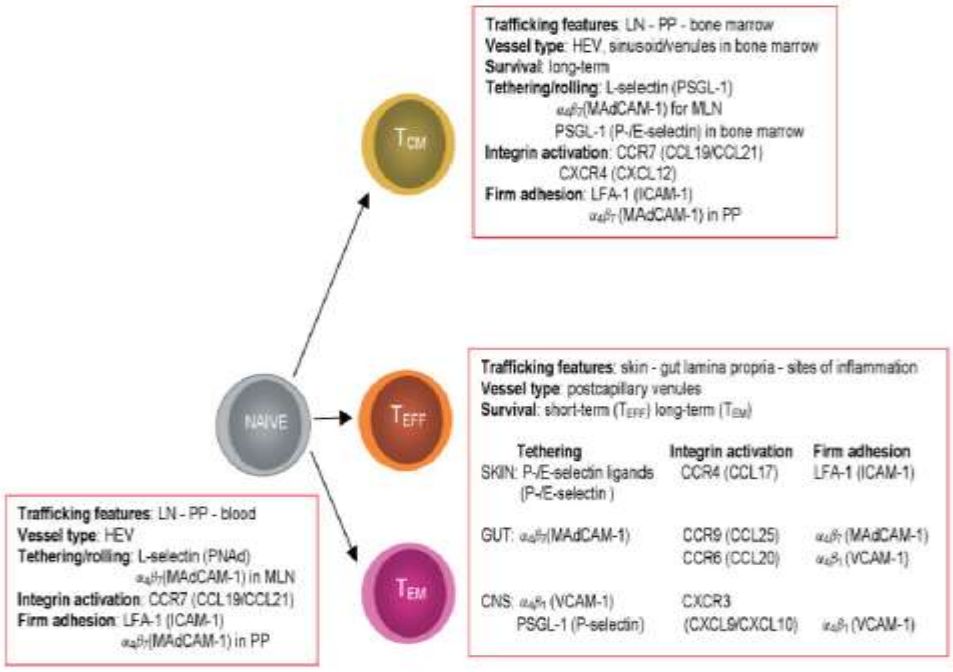


Figure 1.7: This diagram summarized the recirculation patterns and molecular interactions involved in the trafficking of naïve and memory T cells (Marelli-Berg et al., 2008).
(adapted with permission from Journal of Pathology. 214:179-189 (2008))

1.5 Lymphotoxin (LT)

LT was first described by G.Granger and co-workers in 1968 as a protein that is produced by lymphocytes that kill tumor cells. LT was the first cytotoxic chemokine to be purified from a B-lymphoblastoid cell line (Aggarwal et al., 1984) and its amino acid sequence was determined by traditional sequencing method. LT has 30% sequence homology with TNF and there are functional similarity between LT and TNF (Calmon-Hamaty et al., 2011). Human LT gene maps to chromosome 6 (Nedwin et al., 1985), within the MHC (major histocompatibility complex) gene locus (Spies et al., 1986) and is tightly linked to TNF. The human LT gene consists of 4 exons and 3 introns. The position of LT and TNF within the MHC gene locus is very unique. It has been shown that LT can exist in at least 2 different variants. It can either form the membrane bound LTαβ heterotrimers ($LT\alpha_1\beta_2$ or $LT\alpha_2\beta_1$) or soluble $LT\alpha_3$ homotrimer (Browning et al., 1993; 1995). LTα is expressed on T cells, B

cells, NK cells and lymphoid tissue inducer cells under normal physiological condition, LTβ is mainly expressed on parenchyma cells and stroma cells in secondary lymphoid organ and the thymus as well as in myeloid cells (Aggarwal, 2003; Norris and Ware, 2007). Recently, it had been shown that LTαβ heterotrimer can be shed from the cell surface by proteolytic cleavage and execute its functions on more distant cells (Young et al., 2010). LT expression can be induced by T cell mitogen, phorbol ester, other cytokines and viruses (Worm et al., 1998).

1.5.1 Function of LTα

Primary function of LTα is to exert killing effect on target cells. Cytotoxic activity of LTα was first described by Ruddle et al in 1968 as inhibitory growth of syngeneic primary rat embryo fibroblast (Ruddle and Waksman, 1968). Subsequently, malignantly transformed cells were reported to be more susceptible to LTα than the normal cells by two separate groups of researchers. Studies by Evans and Heinbaugh as well as Meltzer and Bartlett reported that rapidly proliferating chemical carcinogen transformed cells were vulnerable to LTα killing (Evans and Heinbaugh, 1981; Meltzer and Bartlett, 1972). While not all malignant cells are susceptible to LTα, LTα can work synergistically with minute amounts of IFN-γ to exert killing effect on cell lines that were originally not sensitive to LTα. This observation was reported by Williams and Bellanti who showed increased killing of Hela and WI38 cells by human LTα in the presence of human IFN-α and IFN-γ (Williams and Bellanti, 1984). Similar observation was reported by Lee et al using B16 melanoma cell line (Lee et al., 1984).

In addition to cytotoxic effect, lymphotoxin (LT) also induces expression of antigen on several different target cells that were resistant to the cytokine. In many ways, this could be due to the induction of a more highly differentiated state. Human endothelial cells (HEC) in particular are not killed by LT, but its morphology changed drastically from epithelial to fibroblastoid form. There is

also an increase in class I MHC antigen and other molecules associated with a more differentiated/activated state. These include expression of E-selectin (H4/18) and ICAM-1, which are associated with leukocyte recruitment (Pober et al., 1987).

LT also involved in the activation and differentiation of polymorphonuclear leukocytes (PMN). Shalaby et al showed that LT, TNF and IFN-γ all increased PMN's ability to ingest latex beads and enhanced PMN mediated antibody dependent cell cytotoxic (ADCC)-reactions (Shalaby et al., 1985). Synergistic effect was also seen between LT with IFN-γ or TNF. These results were confirmed by Perussia et al who showed that LT at 40 units/ml caused PMN cytotoxicity but could enhance PMN phagocytic and ADCC activity at 20 units/ml (Perussia et al., 1987). In addition, LT activity is also detected in osteolysis reaction. Osteolysis or osteoclast activation is initiated by LT stimulation. LT stimulated osteoblastic cells to release an autocrine factor that stimulates osteoclasts to reabsorb bone (Thomson et al., 1987).

1.5.2 Receptors for LT

Similar to TNF homotrimers, $LT\alpha_3$ and $LT\alpha_2\beta_1$ bind to TNFRI and TNFRII whereas $LT\alpha_1\beta_2$ signaled exclusively via LTβ receptor (Ware, 2005). Stimulation of TNFRI and LTβR causes activation of canonical and non-canonical NFκB pathway and hence play a role in cell survival, proliferation, differentiation and apoptosis. Signals transmitted through TNFRI and TNFRII, which are also activated by pro-inflammatory cytokines TNF, IL-6 and IFN-γ, will activate the canonical NFκB pathway. Engagement of $LT\alpha_3$ with TNFRI causes recruitment of a complex (which includes TRADD, TRAF2, TRAF5, receptor-interaction protein (RIP)-1 and cellular inhibitor of apoptosis (cIAP)) (Vandenabeele et al., 2010). This triggers the activation of IKKβ that leads to phosphorylation and degradation of IκBα, therefore allowing nuclear translocation of p50/RelA ($NF\kappa B_1$) heterodimer complex and the induction of proinflammatory and prosurvival factors (Remouchamps et al., 2011). Non-

canonical NFκB pathway is triggered when the canonical pathway is inhibited. Initiation of TNFRI non-canonical pathway usual results in cell death. This involves formation of a complex (which includes TRADD, FADD, caspase 8, RIPI and RIP3), leading to caspase-8 mediated apoptosis or RIP1/3 mediated necroptosis (Micheau and Tschopp, 2003; Vandenabeele et al., 2010).

In contrast, engagement of LTβR with $LT\alpha_1\beta_2$ can independently activate the non-conical pathway independent of IKKβ and NEMO (Senftleben et al., 2001). In this case, ligand engagement leads to activation of NFκB-inducing-kinase (NIK) and its downstream target IKKα2 (Ling et al., 1998). This eventually leads to p100 degradation and formation of the p52/RelB heterodimer which translocates into the nucleus to initiate gene transcription. This pathway is critical for mediating inflammation, development of thymus, secondary lymphoid organ as well as B cell survival and maturation of follicular dendritic cells (Weih and Caamano, 2003; Zhu and Fu, 2011).

1.5.3 Role of LT in lymphoid tissue development

LT plays a crucial role in lymphoid neogenesis. The studies of LT in lymphoid neogenesis and maintenance of lymphoid microarchitecture started two decades ago. Several knockout mouse model, $LT\alpha^{-/-}$, $LT\beta^{-/-}$ and $LT\beta R^{-/-}$ as well as overexpressing LT transgenic mouse were developed to study LT function extensively. In 1994, results from two different groups showed that $LT\alpha^{-/-}$ mice which cannot generate all three forms of LT ($LT\alpha_3$, $LT\alpha_2\beta_1$, $LT\alpha_1\beta_2$) lack Peyer's patches, matured follicular dendritic cells, peripheral and mesenteric lymph nodes and have a highly disrupted splenic microarchitecture (Banks et al., 1995; De Togni et al., 1994). $LT\beta^{-/-}$ mice which still retains the ability to produce $LT\alpha_3$ showed very similar but less severe effects on splenic microarchitecture with developing cervical lymph nodes and mesenteric lymph nodes (Alimzhanov et al., 1997; Koni et al., 1997). $LT\beta R^{-/-}$ mice showed a much adverse effect in lymphoid organ development. Mice without LTβR showed complete absence of lymph node and Peyer's patches

development suggesting that NFκB non-canonical pathway plays an important role in this biological process (Futterer et al., 1998; Rennert et al., 1996).

1.5.4 Pathological role of LT in cancer

LT signaling is strongly linked to cancer development. Genetic studies showed that LTA genetic polymorphism contributes differently to cancer risk. LTA gene with the allelic composition of LTα1/1 or LTα1/2 in the patients with bladder cancer showed higher risk of high-grade tumor than those patients carrying LTα2/2 allele (Nonomura et al., 2006). Single nucleotide polymorphism of LTA gene with C804A resulting in higher level of LTα expression was associated with poor prognosis in diffuse large B cell lymphoma (Chae et al., 2010) but the same LTA C804A polymorphism was associated with lower risk of lung cancer in Japanese male population (Takei et al., 2008). These differences could be explained by the multi-functionality of LTA whereby LTA can promote cell growth and adhesion and potentially favor the growth of certain tumors.

Deregulated NFκB signaling due to mutations in its regulator can be found in various forms of B-cell lymphoma. Gain of function mutation in LTβR which constitutively activates NFκB signaling can be found in multiple myeloma (Compagno et al., 2009). Keats et al showed in cell lines and patient samples that ligand-independent activation of non-canonical pathway is the rate limiting step in malignant plasma cell transformation (Keats et al., 2007). Direct evidence of transforming activity involving LTβR was provided by Fujiwara et al. Using a retroviral cDNA librabry from pancreatic ductal carcinoma cell line, Mia-PaCa-2, they showed that both NH_2-terminally trucated and full-length LTβR variants could induce growth of 3T3 cells in soft agar and promote tumor formation in nude mice (Fujiwara et al., 2005). More recently, Ammirante et al showed that LT produced by tumor-infiltrating B cells could induce IKKα activation and STAT3 phosphorylation,

leading to androgen independent survival of castration-resistant prostate cancer cells (Ammirante et al., 2010).

It was previously reported that activation of IKKα could down-regulate Maspin expression which is a fate decisive factor in primary tumor growth and metastasis of prostate and breast cancer model (Luo et al., 2007). Results from the study showed that LT induced IKKα activation was associated with RANK-mediated NFκB activation via RANKL expression on tumuor-infiltrating T cells (Luo et al., 2007). Taken together, these studies suggests that LT derived from RANKL expressing tumor infiltrating B cells (or T cells) could promote prostate and breast cancer initiation.

Lymphotoxin also plays a role in organizing tumor tissue structure during tumor development. LTβR abrogation in fibrosarcoma cells leads to tumor growth and angiogenesis inhibition (Hehlgans et al., 2002). A subsequent study showed that LTβR ligands, LT and Tumor necrosis factor ligand superfamily member 14 (LIGHT), expressed by tumor infiltrating lymphocytes induce expression of pro-angiogenic MIP-2α in fibrosarcoma cells (Daller et al., 2011).

1.5.5 Anti-tumor role of lymphotoxin (LT) in cancer

LT is important for maintaining homogeneity of the immune system. Ito et al demonstrated that NK cells with defective LT production are immature and exhibit defective homing properties leading to improper anti-tumor response and more rapid tumor growth as well as metastasis (Ito et al., 1999). However, a contradicting finding by Zhou et al showed that in prostate cancer, ablation of LT in T cells rescued the anti-tumor response by inhibiting clonal-deletion of tumor specific T cells, thus decreasing tumor incidence and inhibiting metastasis (Zhou et al., 2009).

Tumor surveillance is of great importance. LT and its related downstream signaling of NFκB pathway has been shown to influence tumor growth and metastasis in a variety of tumor models via various mechanistic pathways. However, the exact mechanism is not fully elucidated and there is still no clear picture of the role of LT plays in tumor progression and development. To address this issue, Kuprash et al completely deleted LT and TNF signaling in their $p53^{-/-}$ mouse model (Kuprash et al., 2008). Their study showed no significant difference in spontaneous tumor formation besides a slight delay in tumor-associated mortality between their LT and TNF signaling and non-signaling $p53^{-/-}$ mouse model tumor group. The author concluded that inflammatory signaling has no protective role in tumor development and has only a minor role in tumor promotion. One of the major caveats in this study was that the deletion was not performed specifically on hematopoietic cell; leaving various questions such as the intrinsic effects of TNF-α on tumor cells or LT-mediated signaling in inflammatory cells unanswered.

1.5.6 LTα and Lymphoma

Various studies had proven that LT plays a role in the development of cancers. However, there are very few studies on the role of LT in lymphoma, particularly HL. Up to date, the exact role of lymphotoxin in the pathogenesis of lymphoma still remains unclear although various studies have established the notion that LTα polymorphism serves as an important prognostic factor in various types of lymphomas disease across different populations in the world. LTA single nucleotide polymorphism serves as a poor prognostic factor in diffuse large B cell lymphoma in the Chinese and Korean population (Chae et al., 2010; Zhang et al., 2013). Besides that, polymorphism of LTA gene is also a poor prognostic factor in Burkitt lymphoma and B cell acute lymphoblastic leukemia in European population (Seidemann et al., 2005). These observations could be due to increased production of LTα which eventually leads to deregulation of NFκB pathway and uncontrolled aberrant B cells expansion.

Recently, Rehm et al demonstrated LT acts as a niche-forming cytokine in T cell lymphoma in the Eμ-Myc transgenic mice (Rehm et al., 2011). These lymphoma cells home to bone marrow by CCR7 where they disrupt stromal cellularity by activating LTβR signaling on resident stromal reticular cells. Inhibiting this interaction prevented lymphoma growth suggesting the potential of neutralizing or inhibiting LT function as a possible therapy for lymphoma.

Study of LT in HL is very limited. Although LTα can be found in the HL lesions, the exact role of LTα in HL remained unknown. Tissues from the cHL patients expressed moderate to abundant levels of LTα mRNA (Sappino et al., 1990). In contrast, TNF-α mRNA level in the tissue samples was relatively low. However, Sappino et al could not find any correlation between LTα mRNA expression and histologic pattern, histologically defined criteria for necrosis, neoangiogenesis, hyalinosis or stromal reaction. Neither could they correlate LTα mRNA expression in cHL lymph nodes with B symptoms (Sappino et al., 1990). This observation was later supported by Warzocha et al who correlated plasma levels of TNF-α and LTα with several prognostic factors of HL including B symptoms (Warzocha et al., 1998). Results from this study showed that unlike TNF-α, plasma level of LTα in cHL patients was not statistically different from that of healthy controls.

1.6 NFκB

NFκB is a family of inducible transcription factors found commonly across all cell types. It was discovered by Baltimore and Sen in 1986 (Sen and Baltimore, 1986). Since its discovery, much had been discovered about its mechanism of activation, its target genes, its' functions in a variety of human diseases including cancers, asthma, arthritis and inflammation. NFκB family has many members. They include Rel A(p65), $NF\kappa B_1$ (p50; p105), $NF\kappa B_2$ (p52; p100), c-Rel and Rel B (Ghosh et al., 1998; Verma et al., 1995). The Rel proteins contain c-terminal transactivation domains which are often not conserved at

the sequence level across all species. However, $NF\kappa B_1$ and $NF\kappa B_2$ subfamily are differentiated from Rel proteins by their long C-terminal that contains multiple copies of ankryin repeats, which acts to inhibit these proteins (Figure 1.8).

The NFκB subfamily has to undergo ubiquitination and proteolysis involving proteasome to become active DNA binding subunits (p105 to p50 and p100 to p52). These subunits then associate with members of the Rel family to become fully activated heterodimeric transcription activators (Gilmore, 2006). In addition, each member of the NFκB family except Rel B can also form homodimers. The main activator of NFκB protein is the heterodimer of p65 subunit associated with p50 or p52 subunit. p50 homodimers which lack the transactivation domain, functions as a transcription repressor with the capability of binding to NFκB consensus sites on DNA (May and Ghosh, 1997). p65 and p50 is ubiquitously expressed on all cell types whereas Rel B expression is restricted to specific regions of the thymus, lymph nodes and Peyer's patches. The expression of c-Rel is limited to hematopoietic cells and lymphocytes. Transcription of Rel B, c-Rel and p105 is regulated by NFκB (Ghosh et al., 1998; Verma et al., 1995).

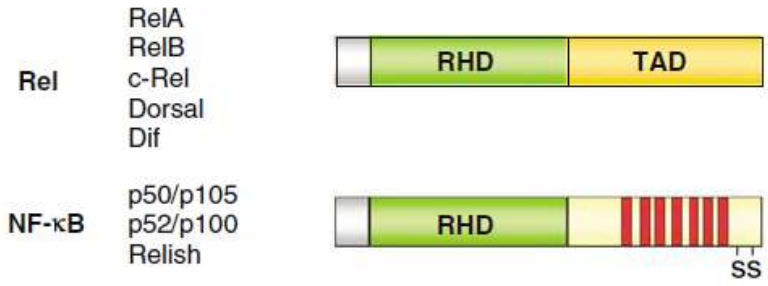


Figure 1.8: Structures of the NFκB proteins (Gilmore, 2006). The generalized structure of Rel and NFκB subfamily proteins are shown here. All the subunits contain a DNA binding domain called Rel homology domain (RHD) which also has a sequence for nuclear translocation or localization and IκB binding sites. The c-terminal of Rel proteins contain transcriptional activation domain (TAD) whereas the c-terminal of NFκB subfamily protein contain ankryin repeat-containing inhibitory domains which can be removed by proteasome-mediated proteolysis.
(adapted with permission from Oncogene. 25:6680-6684 (2006))

Collectively, NFκB transcription factor dimers bind to 9-10 base pair of NFκB consensus site on the DNA which is commonly identified as 5'-GGGACTTTCC-3'. However, it is recognized that NFκB consensus is greatly variable than originally thought and the base pairs of the DNA sites are revised as 5'-GGGRNWYYCC-3' (R represents A or G; N represents any nucleotide; W represents A or T; Y represents C or T) (Gilmore, 2006). This combinatorial diversity is important to accommodate the different NFκB homodimers and heterodimers for optimal transcriptional responses since each dimer has its own preferred DNA binding sites, specific protein interactions when bound and unique transcription signatures under specific physiological conditions (Gilmore, 2006).

1.6.1 Inhibitor κB (IκB) proteins

NFκB proteins in the cytoplasm are in the inactive form, a consequence of their association with IκB proteins. IκB proteins are expressed in three isoforms: $I\kappa B_{\alpha,}$ $I\kappa B_{\beta}$ and $I\kappa B_{\varepsilon}$ (Ghosh et al., 1998). These inhibitory proteins are identified by the presence of many ankyrin repeats. The general belief is that IκB proteins retain NFκB proteins in the cytoplasm by masking nuclear localization sequences (NLS) on NFκB subunits. However, recent studies revealed that cytoplasmic localization of inactive NFκB is achieved by constant movement of the complexes between cytoplasmic and nuclear compartments (Birbach et al., 2002; Huang et al., 2000; Huang and Miyamoto, 2001; Huxford et al., 1998; Johnson et al., 1999; Malek et al., 2001). Structural and biochemical findings demonstrated that there are two NLS sites on the NFκB dimers. However, only one of the two NLS sites is masked by the $I\kappa B_{\alpha}$ in the NFκB- $I\kappa B_{\alpha}$ complex, which allow the complex to shuttle into the nucleus. At the same time, the nucleus-exporter sequence (NES) located at the amino terminal of the $I\kappa B_{\alpha}$ protein functions to expel the NFκB- $I\kappa B_{\alpha}$ complex from the nucleus. It was shown that the export process is more efficient than the import process. Similar phenomenon is observed on NFκB-$I\kappa B_{\varepsilon}$ complex (Lee and Hannink, 2002). However, for the NFκB- $I\kappa B_{\beta}$

complex, the NFκB subunits are retained in the cytoplasm because IκB$_\beta$ mask both NLS sites (Tam and Sen, 2001). This has been well documented that IκB$_\alpha$ regulates transient NFκB activation but IκB$_\beta$ maintain persistent NFκB activation (May and Ghosh, 1997). Interestingly, IκB$_\alpha$ is degraded rapidly in response to stimuli and quickly resynthesized, due to the presence of the NFκB response element in its promoter region. The newly synthesized IκB$_\alpha$ contains NLS, therefore, it can translocate into the nucleus, binds to the active NFκB complex, displaces it from the DNA binding site and transport the NFκB-IκB$_\alpha$ complex to the cytoplasm. This is critical for post-induction repression of NFκB activation (Fenwick et al., 2000). In contrast, IκB$_\beta$ is less sensitive to stimulus-induced degradation than IκB$_\alpha$. It is believed that selective interaction between Ras- IκB$_\beta$ is crucial for inhibiting IκB$_\beta$ activation during NFκB activation (Fenwick et al., 2000). IκB$_\beta$ does not have any NES, therefore newly synthesized IκB$_\beta$ protein can only bind to NFκB complex at the promoter region without displacing them and the outcome is sustained NFκB activation.

1.6.2 Mechanism of NFκB activation

NFκB activation is regulated by two main pathways: canonical and non-canonical NFκB activation pathways. The canonical NFκB pathway applies to dimer that are comprised of Rel A, c-Rel and p50 which are prevented from translocating into nucleus by specific inhibitor which is known as inhibitor of κB (IκB) proteins. The non-canonical NFκB pathway affects primarily NFκB$_2$ which form a heterodimer with Rel B (Solan et al., 2002). In the canonical NFκB pathway, IκB proteins are phosphorylated by activated IκB kinase (IKK) complex at specific site equivalent to Ser 32 and Ser36 of IκB$_\alpha$. Phosphorylation of the NFκB-IκB complex triggers polyubiquitination at sites equivalent to Lys21 and Lys22 of IκB$_\alpha$. Degradation is carried out by 26S proteasome and hence released NFκB dimer to translocate into the nucleus for gene transcription (Karin and Ben-Neriah, 2000). IKK complex consists of IKKα, IKKβ, and the regulatory subunit NFκB essential modulator (NEMO)

which has no known intrinsic kinase activity but is crucial for protein-protein interactions (Karin and Ben-Neriah, 2000). The IKK complex is the converging point for NFκB activation by various stimuli. IKKα and IKKβ can phosphorylate all three isoforms of IκB - $IκB_{α,}$ $IκB_{β,}$ and $IκB_{ε.}$ Although IKK isoforms shared similar biological activity, they differ in the signals that they mediate (Figure 1.9).

In the alternative non-canonical pathway which preferentially affects $NFκB_2$ (p100)-Rel B dimer, the mechanism of action is slightly different. This pathway selectively activates IKKα and another protein kinase called NIK. Together, IKKα and NIK induce phosphorylation-dependent proteolytic removal of the IκB like c-terminal domain on p100. This causes the degradation of p100 to p52 and thus forms an active complex with Rel B. Active p52-Rel B complex can translocate into the nucleus to elicit its function (Senftleben et al., 2001).

The classical pathway is typically triggered by ligand binding to TNFRI or TNFRII, T-cell receptors (TCR), B-cell receptors (BCR) and Toll-like receptors (TLR) which includes IL-1 receptor superfamily members. Classical NFκB pathway primarily regulates the transcription of target genes encoding chemokines, cytokines, adhesion molecules, persistent inflammatory response and promoting cell survival. In contrast, non-canonical NFκB pathway is triggered by activation of specific subset of TNF receptor family members including LTβR and B-cell activating factor belonging to the TNF receptor family (BAFF-R). Non-canonical pathway primarily functions to regulate development of lymphoid organ and adaptive immune system.

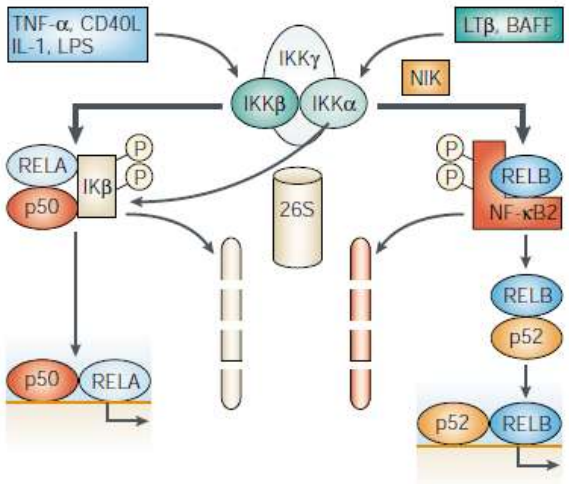


Figure 1.9: The IKK kinases comprises of IKKα, IKKβ and IKKγ (NEMO) (Karin et al., 2002). IKK complex is the converging point of various stimuli to activate two different pathways of NFκB. In response to stimuli such as TNF, CD40L, IL-1 and LPS, IKKβ subunit is activated and phosphorylates IκB at two serine residues. This phosphorylation event triggers proteasomal degradation mediated by 26S which eventually leads to nuclear translocation of p50-Rel A complex. Selective activation of IKKα by LTβ and BAFF phosphorylates $NFκB_2$-Rel B complex leads to ubiquitination of $NFκB_2$. The ubiquitinated product, p52, forms an active complex with Rel B to undergo nuclear translocation and function as active transcription factor.
(adapted with permission from Nature Reviews Cancer. 2:301-310 (2002))

1.6.3 NFκB and inflammation

NFκB is one of the important regulators of pro-inflammatory gene expression and it induces transcription of cytokines, chemokines, adhesion molecules, matrix metalloproteinase (MMP), cyclooxygenase 2 (Cox-2) and inducible nitric oxide synthase (iNOS) (Tak and Firestein, 2001). NFκB activity is high at sites of inflammation in various diseases, such as rheumatoid arthritis, asthma, inflammatory bowel disease and psoriasis. Expression of NFκB proteins as well as higher nuclear localization of NFκB was found to be higher in patient samples from these diseases than normal tissues. These changes are often related to enhanced recruitment of immune infiltrate and increased production of IL-6, IL-1, TNF-α and IL-8 at the inflammatory sites. However, it remained unclear whether increased cytokine expression is the cause or consequence of NFκB activation.

The pathogenic effects of overactivation of NFκB in inflammatory diseases are indicated by studies of p50 and c-Rel knock-out mice which do not develop airway inflammation when sensitized and challenged with allergenic ovalbumin (Yang et al., 1998). Inhibition of NFκB activity has been shown to be essential for controlling inflammatory responses in some of these models. Overexpression of IκBα has also been shown to reduce NFκB activity and inhibit both the inflammatory response and tissue destruction in rheumatoid arthritis (Bondeson et al., 1999).

Tumor microenvironment closely resembles an inflamed tissue. NFκB has been shown to play a key role in establishing a strong link between tumor development and inflammation. The one well-known example would be colitis associated colon cancer (CAC). In CAC, NFκB activity on the lamina phobia macrophages is important to produce several cytokines, including IL-6, IL-11, IL-22 which drive the proliferation of premalignant intestinal epithelial cells (IECs). IL-6 and IL-11 produced by the macrophages exert their proliferative effect through the STAT 3 signaling pathway which further synergizes with NFκB to enhance the expression of survival genes (DiDonato et al., 2012).

1.6.4 NFκB and a role in tumorigenesis

According to Hanahan and Weinberg, cancer cells acquire eight criteria during their development: self-sufficiency in growth signals; insensitive to growth inhibition; resistant to apoptosis; immortalization; tissues invasion and metastasis; angiogenesis; reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). NFκB activation had been shown to have the capability to induce several of these properties including self-sufficiency in providing growth signal, resistant to apoptosis and also stimulating angiogenesis. This had been further supported by the many different cancer types that exhibit high NFκB activity. This aberrant constitutive activation of NFκB could be the consequence of cancer-associated chromosomal translocations, deletions and mutations which disrupted genes

that encode NFκB and IκB proteins or disturbed the regulatory network of NFκB pathway. Finally, autocrine or paracrine production of pro-inflammatory cytokines, activated upstream signaling molecules and chronic infections have also been shown to constitutively activate IKK activity which leads to persistent NFκB activation (Karin et al., 2002).

NFκB regulates cellular proliferation by activating target genes IL-2, granulocyte macrophage colony stimulating factor (GMCSF), and CD40 ligand (CD40L) which encodes protein that stimulate the proliferation of lymphoid and myeloid cells (Karin et al., 2002). Constitutive expression of these factors could stimulate cellular proliferation via autocrine and paracrine manners. NFκB also functions as an apoptosis inhibitor. NFκB is known to regulate expression of several target genes which could block the apoptosis process induced by TNF-α or other pro-apoptotic factors (Van Antwerp et al., 1996). The genes involved in inhibiting apoptosis process are cIAP, c-FLIP and Bcl-X_L (Karin and Lin, 2002). Anti-apoptotic effect of NFκB is critical in the prevention of cell death in neoplastic cells that have undergone aberrant chromosomal rearrangement or other types of DNA damage.

Another important aspect of tumorigenesis is angiogenesis. Angiogenesis is strongly influenced by chemokines and MMP expression, two important classes of genes that are promoted by NFκB activation (Bond et al., 1998; Koch et al., 1992). Cells with elevated expression of NFκB were showed to have deregulated production of chemokines and increased migratory behavior. IL-8 which is one of the target genes of NFκB has been shown to promote angiogenesis (Koch et al., 1992). Interestingly, production of vascular endothelial growth factor (VEGF), which promotes angiogenesis is also regulated by NFκB (Huang et al., 2000). Cancer cells with high level of activated NFκB expression often exhibit enhanced MMP production and characteristic of extracellular matrix destruction had been detected surrounding the cancer cells (Takeshita et al., 1999; Wang et al., 1999).

1.6.5 NFκB and HL

Leukemia and lymphoma are cancers of the bone marrow and lymph nodes, characterized by the uncontrolled clonal expansion of blood cells. Knowing that NFκB is essential in regulating various cellular processes ranging from cellular proliferation to immune response, it is not surprising that NFκB is involved in the development of such cancers. Hodgkin and Reed-Sternberg (HRS) cells in classical Hodgkin Lymphoma (cHL) expressed activated NFκB dimers, p65-p50 and p50-p50. They also express various NFκB target genes including TRAF1, BIRC3, BCL21A, BCL2L1 (encoding TRAF1, c-IAP2, Bfl-1 and Bcl-X_L respectively) which confer resistance to Fas-FasL-induced apoptosis (Hinz et al., 2002). Mechanisms that contribute to the overexpression or activation of NFκB proteins in the HRS cells have been studied widely. Genetic, viral and autocrine activation may play a role or work together in activating NFκB pathway (Küppers, 2009). Activation of cell surface molecules including, CD30, CD40 and RANK via both canonical and non-canonical pathways have been observed in various studies (Thomas et al., 2004). High level of expression of CD30 on HRS cells could trigger ligand-independent activation of NFκB through recruitment and aggregation of TRAF2 and TRAF5 (Horie et al., 2002). CD40 mediated activation of canonical NFκB pathway can be induced by CD40L expressing T cells in the HL microenvironment. Co-expression of RANK and RANKL on the HRS cells is believed to regulate constitutive RANK signaling and activation of NFκB via TRAF2, 5, and 6 (Fiumara et al., 2001). Different genetic mutations that contribute to activation of NFκB in HRS cells have been identified. Mutation of IκB by deletions, insertions or nonsense mutations that caused inactivation of IκB protein have been identified in several studies and contribute up to 10% of the HL cases (Cabannes et al., 1999). Mutation of $I\kappa B_\varepsilon$ has also been described in several cases of HL (Emmerich et al., 1999). Besides that, amplification of Rel gene occurs in about 50% of cases and is associated with high level of nuclear c-Rel expression (Barth et al., 2003). EBV (Epstein-Barr) virus is associated with HL (Kuppers et al., 2012). Approximately, 50% of the HL cases in the developed countries are EBV^+ and these cases often express latent membrane protein 1 (LMP1), latent membrane protein 2A (LMP-2A), Epstein-Barr virus nuclear antigen-1 (EBVNA-1).

Expression of LMP-1 is crucial in mimicking of CD40-induced activation of NFκB pathway (Küppers and Rajewsky, 1998).

1.7 Activator protein 1 (AP-1)

Activator protein 1 (AP-1) is a sequence-specific transcriptional activator composed of members from the Jun, Fos, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma (MAF) families (Eferl and Wagner, 2003). Members of the Jun family include c-Jun, JunB and JunD. Members of the Fos family include c-Fos, FosB, Fra1 and Fra2. These proteins, which belong to the basic zipper leucine (bZIP) group of DNA binding proteins associate to form a variety of homodimers or heterodimers. They dimerize via leucine zipper motif and contain a α-helical structure in the basic domain for DNA interaction that allows the binding to a common DNA binding site (Eferl and Wagner, 2003). Combinatorial difference between AP-1 subunits contributes to a wide range of genes regulated by the AP-1 transcription factor. It was found to mediate transcription of genes at the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and therefore the site is also known as the "TPA response element (TRE)". Binding of AP-1 complex to the TRE can be induced by growth factors, cytokines, T cells activators, neurotransmitter and UV radiation (Angel et al., 1987).

1.7.1 Transcriptional regulation of AP-1 components

Components of AP-1 are immediate early response genes. Among these, regulation of c-Jun and c-Fos are most well studied (Karin, 1995). In c-Fos, there are two response elements situated on the promoter region which is Cis response element (CRE) and Serum response element (SRE). CRE mediates transcription of c-Fos in response to a wide array of stimuli. Neurotransmitters and polypeptides hormone can induce c-Fos expression using cAMP or Ca^{2+} as secondary messengers leading to activation of PKA or calmodulin-

dependent kinase and subsequently activation of the CRE (Sheng et al., 1991). SRE mediates c-Fos induction by growth factors, cytokines and other stimuli that could activate MAP kinase (Treisman, 1992). Besides that, a Sis-inducible enhancer mediates c-Fos induction by activation of JAK kinases has also been reported (Darnell et al., 1994). Given the different routes and wide range of stimuli that could induce c-Fos expression, it is no wonder that c-Fos transcription could be rapidly induced in response to almost any stimuli (Figure 1.10).

In contrast to c-Fos, c-Jun promoter is much simpler and most of its inducers function through one major cis element, TRE (Figure 1.10). This TRE differs from other consensus TRE by 1 base pair insertion (Angel et al., 1988) which makes it more readily recognized by c-Jun-ATF2 heterodimers than other AP-1 complexes (van Dam et al., 1993). Following exposure to external stimuli that activates JNK, member of MAPK, both c-Jun (Devary et al., 1992) and ATF2 (Gupta et al., 1995) are rapidly phosphorylated. The constitutive occupancy of c-Jun TRE suggested that phosphorylation process occurred while the proteins are bound to the promoter. Phosphorylation of c-Jun and ATF2 enhanced their ability to form a dimer and induce transcriptional activation thereby leads to c-Jun expression. Hence, c-Jun is positively autoregulated at the transcriptional level. The activity of c-Jun is regulated post-translationally especially via the phosphorylation and dephosphorylation of the protein. Collectively, increase in AP-1 activity is due to both the increased production of c-Jun and possibly c-Fos synthesis as enhanced phosphorylation of c-Jun.

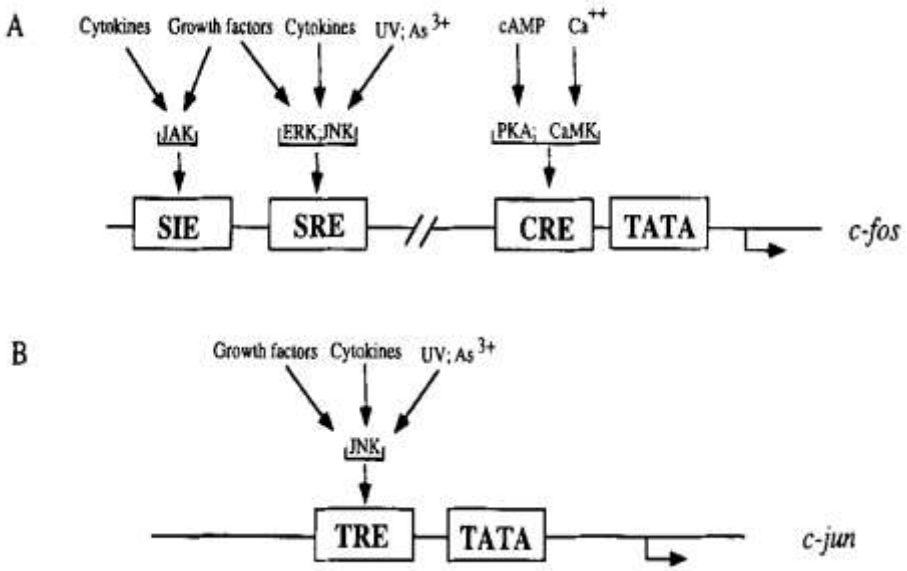


Figure 1.10: Regulation of c-Fos and c-Jun transcription in response to external stimuli (Karin, 1995). This diagram illustrates various external stimuli that could activate c-Fos and c-Jun transcription through transcriptional activation at different response element.
(adapted wih permission from Journal of Biological Chemistry. 270:16483-16486 (1995))

1.7.2 Post-translational regulation of AP-1 activity

Phosphorylation of AP-1 complexes is important to regulate their transcriptional activity. In the case of c-Jun, phosphorylation at different sites will yield totally different effects. The most commonly studied phosphorylation site is at Ser-63 and Ser-73 situated at the N-terminal region (the transactivation domain). These residues are phosphorylated by c-Jun terminal kinase (JNK). Phosphorylation of the transactivation domain of both c-Jun homodimers (Pulverer et al., 1991) and c-Fos (Deng and Karin, 1994) heterodimers will potentiate transcriptional activities. So far only JNK has the ability to phosphorylate the N-terminal site of c-Jun (Minden et al., 1994). ERK1 and ERK2 cannot phosphorylate the N-terminal of c-Jun but they phosphorylate one of the inhibitory sites located next to the c-terminal DNA binding domain (Chou et al., 1992; Minden et al., 1994). Intriguingly, c-Jun

phosphorylation at these inhibitory sites will inhibit the DNA binding activity of c-Jun homodimers but not c-Jun-c-Fos heterodimers (Boyle et al., 1991).

The sequence surrounding the N-terminal phosphoacceptors of c-Jun is conserved in the c-terminal of c-Fos (Sutherland et al., 1992). Phosphorylation of c-Fos at Thr-232, potentiates c-Fos transcriptional activity. Experimental findings showed that c-Fos phosphorylation is not carried out by JNK1 or JNK2 but by a novel 88kDa MAP kinase called FRK (Deng and Karin, 1994). Although the mechanism that regulates phosphorylation of c-Fos at Thr-232 is unclear in the context of c-Jun-c-Fos heterodimer, phosphorylation of either protein makes a similar contribution to stimulation of transcriptional activity, suggesting that both activation domains interact with the transcriptional machinery (Karin, 1995).

1.7.3 Interaction between AP-1 and MAP kinases

As described previously, three types of MAP kinases contributes to the activation of AP-1, namely ERK, JNK and FRK. It is important to note that each of these MAP kinases activates AP-1 through phosphorylation of different substrates (Figure 1.11) (Karin, 1995). In addition, MAP kinases also contribute to regulation of c-Jun and c-Fos induction

In the context of ERK, it regulates c-Fos production by phosphorylating Elk-1 and induces transcriptional activation of ternary complex factor (TCF) (Babu et al., 2000). ERK, however, could not phosphorylate c-Jun or c-Fos on sites that potentiate their transcriptional activity (Chou et al., 1992; Deng and Karin, 1994; Minden et al., 1994). Neither could ERK phosphorylate ATF2 (activating transcription factor 2) (Gupta et al., 1995). Conversely, JNK phosphorylates the stimulatory sites on c-Jun and ATF2 but does not phosphorylate c-Fos (Deng and Karin, 1994; Gupta et al., 1995; Hibi et al., 1993). JNK are also capable of phosphorylating and activating Elk-1/TCF,

suggesting that they could be involved in inducible c-Fos synthesis under certain circumstances (Bogoyevitch and Kobe, 2006). FRK, is known only for its ability to phosphorylate and regulate c-Fos activity (Deng and Karin, 1994).

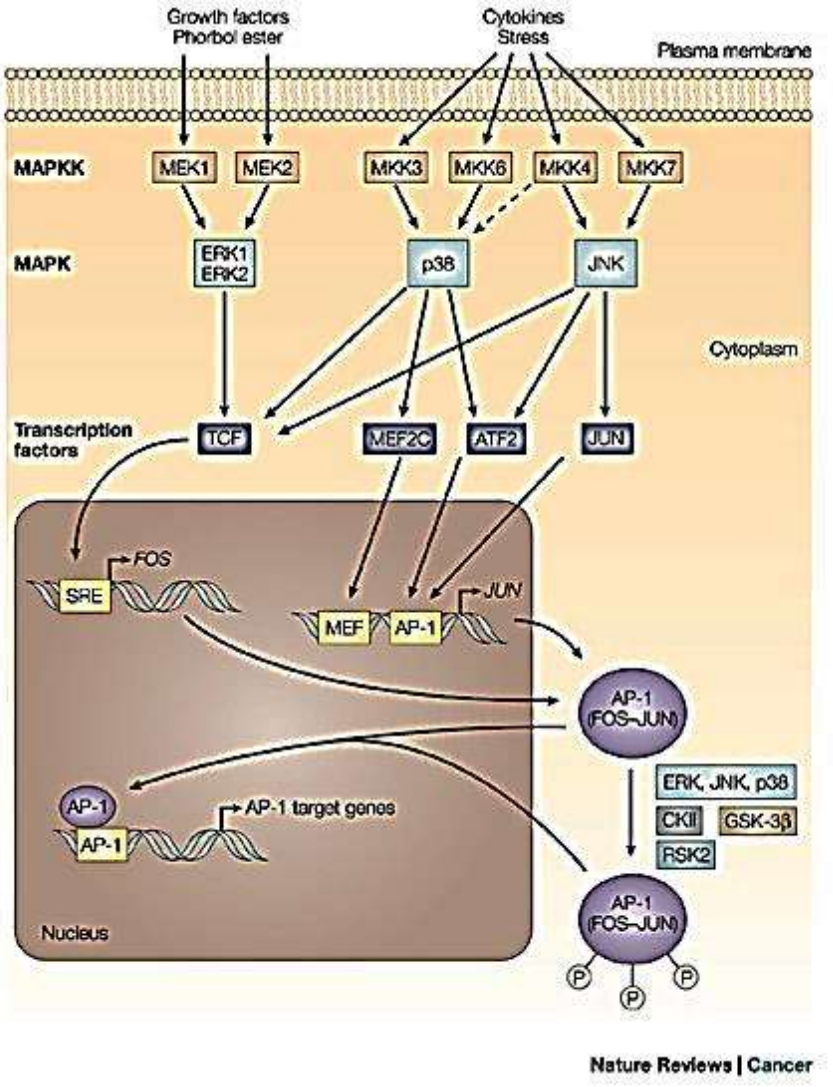


Figure 1.11: Diagram represents transcriptional and post-translational modification of AP-1. AP-1 activity is stimulated by a complex network of signaling pathway including external stimuli factor and MAPK signal (ERK, p38 and JNK) (Eferl and Wagner, 2003). The dashed arrow indicates phosphorylation of p38 by MKK4 is controversial. MAPK activates various transcription factor which leads to induced expression of c-Fos and c-Jun that can dimerize and form higher number of AP-1 complexes as well as more transcription of AP-1 target genes. Post translational phosphorylation by various other proteins enhanced AP-1 activity.
(adapted with permission from Nature Reviews Cancer. 3:859-868 (2003))

1.7.4 Interaction of AP-1 with other transcription factors

The interaction of AP-1 with other transcription factors is critically important for the complex regulatory network of gene transcription to occur at several different levels in response to different stimuli.

AP-1 complex has been shown to interact with NFAT (nuclear factor of activated T cell). AP-1-NFAT complex is important in regulating IL-2 synthesis after binding of antigen to the antigen receptor on the T cells (Jain et al., 1993). AP-1 and NFAT interact with each other to form a quaternary complex at the ARRE-2, response element on the promoter region of IL-2. The complex consists of one molecule of NFAT, and a heterodimer of c-Jun and c-Fos binding to a sequence of 15 base pairs of DNA. Results showed that the AP-1 site in ARRE-2 is a non-consensus site and binding to this site (in the absence of NFAT) is 10 fold less efficient than binding to known AP-1 consensus sites. In the presence of NFAT, AP-1 binding to this region is 3 fold greater than AP-1 binding to a consensus site (Peterson et al., 1996).

Regulation of IL-4 is thought to primarily involve NFAT, and AP-1 has been shown to interact with NFAT at a composite site within the IL-4 promoter region (Chuvpilo et al., 1993; Li-Weber et al., 1997; Rooney et al., 1995). AP-1 proteins involved include c-Fos, Far-2, Fra-1, c-Jun, JunB and JunD depending on the T cell lines investigated. For example, c-Jun dimerizes with ATF-2 in association with NFAT and bind to CRE site in the TNF-α gene in calcium stimulated T cells (Tsai et al., 1996). Besides that, c-Jun containing complex can also interact with NFκB proteins, p50/p65, to synergistically enhance the induction of TNF-α gene in lipopolysaccharide (LPS)-stimulated THP-1, a monocytic cell line (Yao et al., 1997).

Ras-raf ERK signaling pathways activate gene transcription by utilizing transcriptional control elements containing AP-1 and ets tandem DNA binding sites. Example of these genes include heparin-binding epidermal growth factor,

the urokinase-type plasminogen growth factor and type I collagenase. Function studies showed that both AP-1 and ets sites are required for gene transcription therefore, AP-1/ets element act as integrators of different signaling output (Gutman and Wasylyk, 1990; McCarthy et al., 1997).

1.7.5 AP-1 and cancer

Evidence of AP-1 transcription factor involvement in human cancers are derived from the multiple biological functions of AP-1 acting at several physiological levels. At the first level, AP-1 protein can regulate cancer cell survival and proliferation (Shaulian and Karin, 2002). AP-1 also has the ability to modulate the extracellular matrix and hence, can contribute to invasiveness and metastasis of the tumor (Eferl and Wagner, 2003b). Recently, AP-1 has also been found to support angiogenesis, thereby increasing the ability of tumor cells to cope with the resource scarce microenvironment (Folkman, 2004).

c-Fos, Fos B and c-Jun can transform cells in culture efficiently (Suzuki et al., 1994). When overexpressed in a mouse model, c-Fos caused osteosarcoma formation by transformation of osteoblasts and chondroblasts. In contrast, c-Jun is more important in the development of skin and liver tumors as reducing c-Jun/AP-1 activity using a dominant negative c-Jun in basal keratinocytes or conditional inactivation of c-Jun in the liver interfered with the development of papilloma and liver tumor (Eferl et al., 2003; Young et al., 1999). AP-1 proteins without the transactivating domain have either limited transforming activity (Fra1 and Fra2) or no transforming activity (JunB and JunD) (Eferl and Wagner, 2003a).

AP-1 components such as JunB and JunD could also function as tumor suppressor proteins (Shaulian, 2010). The c-Jun/JunB antagonistic effect in tumor formation was first described in rodent fibroblast. The anti-oncogenic

effect of JunB was confirmed *in-vivo* using JunB deficient mice carrying a JunB transgene. The transgene avoid embryonic lethality of JunB deficient fetus, but its expression was silenced in cells of the myeloid lineage. The absence of JunB resulted in progressive myeloid leukemia with increased proliferation of granulocytic progenitor cells (Passegue et al., 2001). However, role of JunD as anti-oncogenic protein is not as well described yet.

Uncontrolled tumor cell proliferation is one of the hallmarks of tumor development. AP-1 activity has been closely linked to tumor progression. c-Jun is primarily a positive regulator of cell proliferation and has been implicated to promote tumor cell proliferation. To fully promote cell proliferation, c-Jun protein needs to be activated by JNK leading to activation of AP-1 complex containing activated c-Jun and subsequently induce expression of cell cycle promoter such as cyclin D and suppress expression of cell cycle suppressor (Wisdom et al., 1999).

Interestingly, JunB and JunD are always considered as negative regulators of cell proliferation as JunB and JunD can counteract against c-Jun mediated activation or acts as repressors for regulators involved in cell cycle progression (Pfarr et al., 1994). JunB could directly regulate expression of cell cycle modulators such as INK4A (p16), independent of c-Jun. Overexpression of JunB also seems to antagonize c-Jun mediated cyclin D1 expression in fibroblast (Passegue and Wagner, 2000).

Studies have shown that AP-1 exhibit pro-apoptotic and anti-apoptotic behavior. Early studies showed that induction of AP-1 can cause apoptosis in specific cell types including human tumor cells (Eferl et al., 2003). However, AP-1 has also been implicated in promoting tumor cell survival (Park et al., 1999; Shaulian et al., 2000; Zenz et al., 2003) especially in the context of lymphoma. C-Jun and JunB are hallmarks of Hodgkin and Reed-Sternberg cells which work synergistically with NFκB to promote cell proliferation

(Mathas et al., 2002). AP-1 DNA binding activity is found to be elevated in HRS cells and in co-operation with NFκB promotes proliferation by activating cyclinD2. Similarly, a previous study also showed that oncogenic AP-1 can antagonize apoptosis in liver tumor (Shaulian and Karin, 2002).

Depending on the components and phosphorylation sites, AP-1 can act as a regulator for cell death or survival in different cell context. The dual role of AP-1 in apoptosis process is best demonstrated in neuronal cells and hepatocytes. Increased c-Jun activity promotes neuronal apoptosis in neuronal cells. When c-Jun activation is impaired, neuronal cells become less sensitive to Kainate induced cytotoxicity (Behrens et al., 1999). On the other hand, c-Jun is required for the survival of fetal hepatocytes which undergo apoptosis in c-Jun deficient mouse embryo (Eferl et al., 1999). The cell type specific pro-apoptotic or anti-apoptotic regulatory response by AP-1 is probably due to differential regulation of pro-apoptotic and anti-apoptotic genes. In neuronal cells, c-Jun regulates expression of Bim which is a pro-apoptotic protein that is crucial for neuronal apoptosis (Whitfield et al., 2001). In T cells, c-Jun and c-Fos regulate the expression of Fas ligand which could induce apoptosis in Fas dependent signaling pathway (Kasibhatla et al., 1998). Similarly, c-Jun in T cells could also provide protection from apoptosis by induction of Bcl3 (Rebollo et al., 2000). The differential regulation of pro-apoptotic and anti-apoptotic genes indicates that AP-1 can promote apoptosis in some tumor types, whereas it induces survival in others.

Some target genes regulated by AP-1 are involved in angiogenesis and metastasis of tumor cells. c-Fos and Fra1 have been identified as the main regulator of matrix metalloproteinases (MMPs) expression and the proteases of the urokinase plasminogen activator system, which promote angiogenesis and invasive growth of cancer cells (Kustikova et al., 1998). In addition to that, it has been identified that vascular endothelial growth factor D (VEGF D) is another target gene of c-Fos (Marconcini et al., 1999). Furthermore, c-Jun and JunB are also involved in the regulation of angiogenic gene products including

proliferin which promotes angiogenesis in a mouse fibrosarcoma model system (Toft et al., 2001). Moreover, JunB deficient fetus showed down-regulation of proliferin and impaired vascularization which resulted in embryonic lethality (Schorpp-Kistner et al., 1999).

Both c-Jun and c-Fos can induce epithelial-mesechymal transition (EMT) and promote tumor metastasis (Eger et al., 2000; Reichmann et al., 1992). Studies by Reichmann et al showed that overexpression of c-Fos and not c-Jun promoted invasive tumor growth in collagen gels *in-vitro* (Reichmann et al., 1992). This was further supported by *in-vivo* study where the progression of chemical induced papillomas to invasive squamous cell-carcinomas is impaired in c-Fos deficient mice (Saez et al., 1995). Besides that, c-Jun overexpression in a breast cancer cell line promotes development of an invasive phenotype (Smith et al., 1999). Together, these studies suggest that both c-Fos and c-Jun play important role in facilitating tumor cell metastasis and invasion.

A link between inflammation and cancer has been suspected for decades. Activation of regulatory kinases by pro-inflammatory signals leads to activation of AP-1. Activated AP-1, in turn, will induce expression of Cox-2, iNOS and many other pro-inflammatory genes to further enhance the inflammatory cycle. Ness at el suggested that consumption of non-steroidal anti-inflammatory drugs (NSAIDs) reduced risk of breast cancer (Ness and Modugno, 2006). Consistent with this, overexpression of Cox-2 has been reported for Her2/Neu positive breast cancer (Howe et al., 2005). Results from the study suggested that Cox-2 overexpression in these tumor cells is regulated by c-Jun, c-Fos and ATF2 binding to the CRE site of the promoter region of Cox-2 (Subbaramaiah et al., 2002).

Expression of AP-1 target genes such as Cox-2 and iNOS is induced in ulcerative colitis which could eventually progress to malignant colon cancers.

Multiple epidemiological studies correlate usage of NSAIDs with reduced risk of colon cancer (Collet et al., 1999). NSAIDs usage also associated with reduced prostate cancer risk (Nelson and Harris, 2000). Aberrant expression of IL-6 is implicated in the progression and chemoresistance characteristic of prostate cancer. IL-6 functions as a growth and differentiation factor for prostate cancer cells (Okamoto et al., 1997). The increased IL-6 expression is mediated by AP-1 activity which includes Fra1 and JunD as well as NFκB proteins (Zerbini et al., 2003). By using *in-vitro* model, the study showed that reduced AP-1 activity resulted in a reduction in IL-6 production and the prostate cancer cells become androgen sensitive again (Zerbini et al., 2003).

1.7.6 AP-1 and HL

AP-1 plays an important role in the pathogenesis of HL. AP-1 protein regulates gene expression of immunoregulatory molecules and survival proteins that contribute to the survival and growth of HRS cells.

Hodgkin and Reed-Sternberg (HRS) cells express constitutive AP-1 activity (Green et al., 2012; Juszczynski et al., 2007; Mathas et al., 2002; Rodig et al., 2008). Mathas et al showed that HRS cell lines and primary HRS cells constitutively express c-Jun and JunB. AP-1 activity on HRS cells was required to induce expression of cyclin D2, c-Met and CCR7 to promote survival of HRS cells (Mathas et al., 2002). In addition, AP-1 could also work synergistically with NFκB to regulate the expression of these genes.

In addition, AP-1 activity is also important in regulating CD30 expression on HRS cells. CD30L expressing stromal cells bind to CD30 on HRS cells to provide survival signal by activating NFκB pathway. Study by Watanabe et al showed that JunB expressed on HRS cells can relief the repressive activity of CD30 promoter microsatellite resulting in overexpression of CD30 in HRS cells (Watanabe et al., 2003).

Galectin-1 is an immunoregulatory molecule strongly expressed by HRS cell lines and primary HRS cells. Juszynski et al showed that galectin-1 expression on the HRS cells is regulated by AP-1 activity (Juszczynski et al., 2007). Subsequent study by Rodig et al showed that expression of galectin-1 is also found on anaplastic large cell lymphoma (ALCL) but not on diffuse large B-cell lymphoma, primary mediastinal large B-cell lymphoma and nodular lymphocyte-predominant cHL (Rodig et al., 2008). They further showed that expression of galectin-1 is concomitant with c-Jun expression. Galectin-1 expression in the cHL is required to induce a skewed immunosuppressive $T_{Helper}2$ microenvironment.

Besides galectin-1, AP-1 also regulates PD-L1 (programed cell death ligand 1) expression on the HRS cells. In HRS cell with constitutive AP-1 activity, PD-L1 expression is induced by AP-1 binding to a AP-1 responsive enhancer region in the PD-L1 gene (Green et al., 2012). PD-L1 functions to inhibit T-cell receptor signaling, which in turn, inhibits T-cell proliferation and IFN-γ production ability by activated T cells.

1.8 Cyclooxygenase (Cox)

Cyclooxygenase (Cox; prostaglandin G/H synthase) was purified in 1976 and cloned in 1988, is the key enzyme that catalyzes the first two steps in the biosynthesis of prostaglandins from the substrate arachidonic acid (AA) (Figure 1.12). There are two isoforms of Cox enzymes: the constitutively expressed Cox-1 and inducible Cox-2. Cox-2 was identified in 1991. These two isoforms of Cox enzymes are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their intracellular locations (Otto and Smith, 1995).

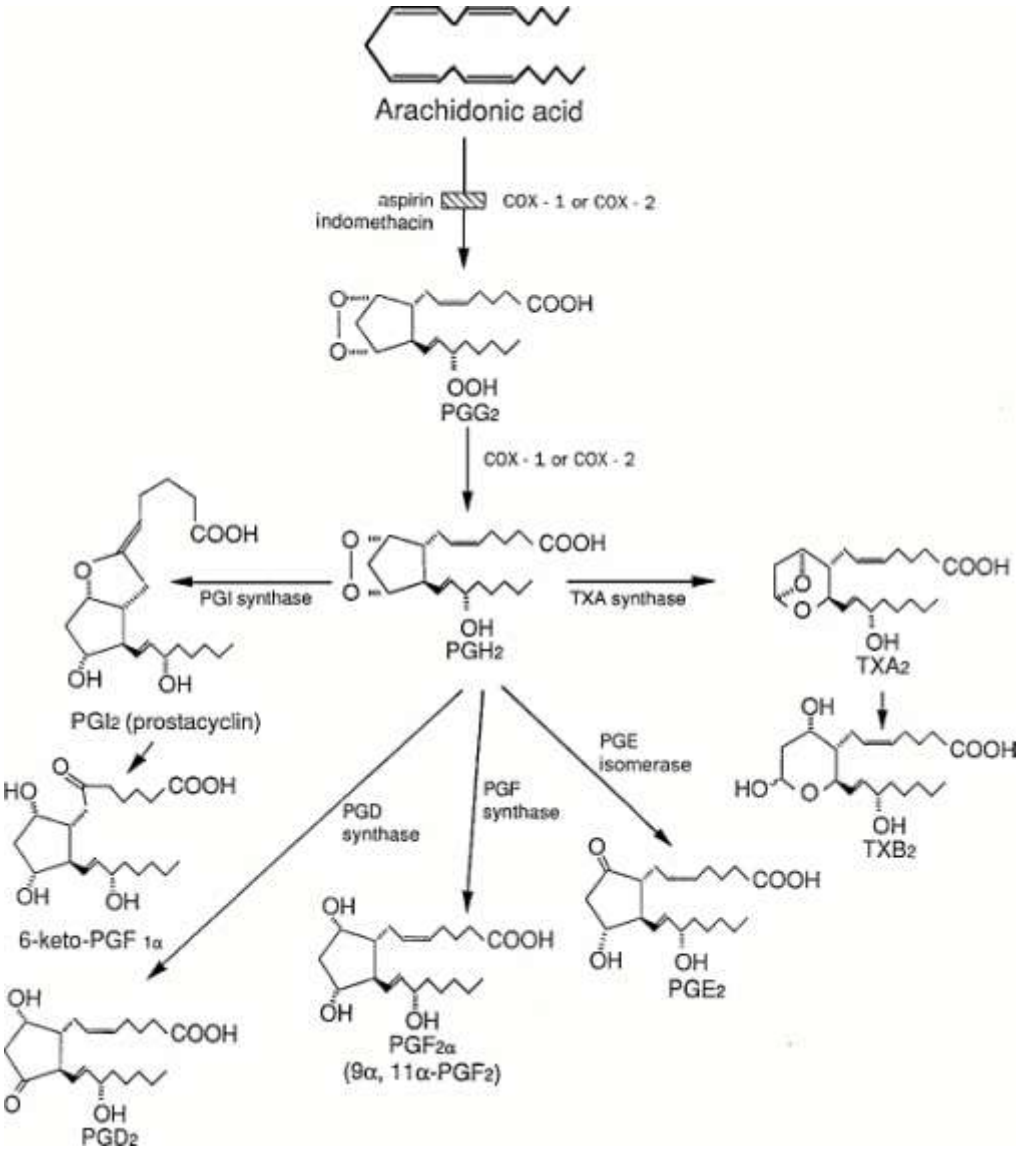


Figure 1.12: Diagram represents simplified process of prostaglandin synthesis from arachidonic acid involving Cox enzyme activity and various other prostaglandin specific enzymes (Vane et al., 1998).
(adapted with permission from Annual Review of Pharmacology and Toxicology. 38: 97-120 (1998))

1.8.1 Biochemical structure of Cox-1 And Cox-2

The inducible form of Cox-2 is very similar in structure and catalytic activity to the constitutive Cox-1. The enzymatic activity of both isoforms can be inhibited by aspirin and other NSAIDs (Vane, 1971). The inhibition of enzymatic activity by aspirin is due to the irreversible acetylation of the Cox

site of the prostaglandin H synthase, leaving the peroxidase activity of the Cox unaffected. In contrast to the irreversible action of aspirin, action of other NSAIDs such as Indomethacin or ibuprofen produces reversible or irreversible inhibition by competing with substrate AA for the active site of the enzyme.

Both isoforms have a similar molecular weight of 71kDa and are almost identical in length. Cox-1 and Cox-2 exist as homodimers of 576 and 581 amino acids, respectively but only one partner is used at a time for substrate binding (Yuan et al., 2009). Cox-1 is widely distributed and constitutively expressed in most tissues, mostly in the blood vessels, smooth muscle cells, interstitial cells, platelets and mesothelial cells (Zidar et al., 2009). The Cox-1 gene, Ptgs1, encodes a 2.8kb mRNA which is relatively stable. Ptgs2, the Cox-2 gene is an immediate early response gene that is activated by various pro-inflammatory stimuli (Chen et al., 2000; Tsuzaki et al., 2003). It codes for a 4kb mRNA with high turnover rate because it contains an unstable sequences in the 3'-untranslated region (Rouzer and Marnett, 2003; Smith et al., 2000). While Cox-2 expression is generally accepted to be highly inducible, several studies also identified constitutive Cox-2 expression in developing brain and gastric mucosa (Maslinska et al., 1999; Zimmermann et al., 1998). Differential expression of Cox-1 and Cox-2 suggesting that Cox-1 mediates prostaglandins (PGs) synthesis are required for homeostatic functions whereas, Cox-2 mediated PGs synthesis play a predominant role in inflammatory responses as well as tumorigenesis.

In the presence of oxygen molecule, the Cox pathway produces the unstable intermediate, PGG2, which is rapidly converted to PGH2 by the peroxidase activity of PGH2 synthase. Specific isomerases convert PGH2 to thromboxane A2 (TXA2) and various other forms of PGs including PGI2, PGE2 and PGD2. The physiological effects of PGs or TXA2 are regulated in part by G-protein coupled prostanoid receptors. Activation of a given receptor may elicit varying responses and some of them maybe important to inflammatory process and cancer cell biology.

1.8.2 Cox and inflammation

Cox activity or Cox products have been implicated in the inflammatory response. Cox activity within the endothelial cell the influence the endothelial/vascular function during acute and chronic inflammation. In acute or chronic inflammation, higher amount of arachidonic acid is generated by calcium-mediated activation of phospholipase A2 (cPLA2). Free arachidonic acid is metabolized by Cox enzyme within the endothelial cells to synthesize PGH2, which in turn is converted by prostacyclin synthase to PGI2, a potent vasodilator (Egan and FitzGerald, 2006). Dilation of endothelial venules leads to increased blood flow is a primary response to inflammation. Besides that, products of Cox enzyme also act on endothelial cells to induce vascular endothelial growth factor (VEGF) and adhesion molecule expression. PGE2 can stimulate endothelial cells to up-regulate VEGF expression by ERK2/JNK1 signaling pathway (Pai et al., 2001). In addition, studies by Ishizuka et al showed that TXA2 released by TNF-α stimulated endothelial cell binding to surface TXA2 receptor. Activation of TXA2 receptor activates PKC pathway and induces expression of ICAM-1, VCAM-1 and E-selectin (Ishizuka et al., 1996; 1998).

Besides acting on endothelial cells, Cox enzymatic reaction also plays an important role in modulating functions of immune cells. Cox-2 dependent production of PGE2 has been shown to induce IL-6 secretion on macrophages (Williams and Shacter, 1997). In addition, PGE2 also regulates TNF-α secretion on macrophages in a dose dependent manner (Renz et al., 1988).

Both Cox-1 and Cox-2 are co-expressed in circulating inflammatory cells in inflamed rheumatoid arthritis (RA) synovium and artherosclerotic plagues obtained from patients (Crofford et al., 1994; Schonbeck et al., 1999). A study using wildtype, Cox-1 knock-out showed that it was Cox-1 and not Cox-2 that was required for the induction of arachidonic acid-induced ear edema (Langenbach et al., 1995). In contrast, ear edema induced by tetradecanoyl phorbol acetate was not significantly different among wild-type, Cox-1 knock-

out and Cox-2 knock-out mice (Dinchuk et al., 1995; Langenbach et al., 1995; Morham et al., 1995). These studies had revealed the equal importance of Cox-1 and Cox-2 activity in mediating the inflammatory response rather than the general accepted "Cox-2 dependent inflammatory reaction". However, the involvement of either Cox-1, Cox-2 or both is dependent on the type of stimulus and the relative level of expression of each isoform in the target tissue.

1.8.3 Cox and cancer

Cox has been shown to involve in the tumorigenesis process but the relationship between Cox and cancer is focused on Cox-2. The link between Cox and cancer development was first established on colorectal cancer. The initial evidence for the involvement of Cox in colorectal cancer is based on epidemiological study. Since 1988 until now, there are more than 10 studies conducted and data to date strongly support the negative correlation between NSAIDs consumption and incidence of colorectal cancer (Giovannucci et al., 1994; 1995). In young patients with familial adenomatous polyposis (FAP), a clinical trial using sulindac (a non-selective NSAID) showed that patients treated with the drug exhibited significant improvement with drastic decrease in the number and size of polyps detected (Giardiello et al., 1993). These data strongly suggest that Cox enzymes are involved in the pathogenesis of colorectal cancer. Furthermore, increase in Cox-2 but not Cox-1 expression was reported in malignant tissues samples from patients with colorectal cancer or from polyp tissue from patients with FAP (Eberhart et al., 1994).

Human gastric and breast tumors also express higher levels of Cox-2 protein than normal tissues (Parrett et al., 1997; Ristimaki et al., 1997). Piroxicam treatment can suppress growth of human cultured breast cancer cells whereas sulindac sulfide reduces cancer incidence and the number of cancers per rat in experimental mammary carcinoma induced by 1-methyl-1-nitrosourea

(Thompson et al., 1997). Thus, these studies further support the role Cox-2 in the pathogenesis of both gastric and breast cancers.

1.8.4 Cox and HL

Cox-2 is a key enzyme in prostaglandin synthesis which has been shown to have an important role in various malignancies. While Cox-2 has been studied extensively in solid tumors, studies on the role of Cox-2 in lymphoid malignancies are limited.

Cox expression in Hodgkin Lymphoma was first discussed by Hsu et al in 1988. Hsu et al showed that Cox enzyme was present in the HRS cell lines, HDLM-1 and KM-H2 (Hsu et al., 1988). Besides that, expression of Cox enzyme was also found in the primary HRS cells from cHL of mixed cellularity and nodular sclerosis subtypes (Hsu et al., 1988). The Cox enzymes in the HRS cell lines were active since phorbol ester stimulated KM-H2 increased secretion of PGE2 while phorbol ester stimulated HDLM-1 cells increased secretion of 15-HETE (Hsu et al., 1990).

More recently, Cox enzyme expression in HL was revisited with emphasis on Cox-2 expression. Ohsawa et al showed that HRS cells in cHL expressed Cox-2. Expression of Cox-2 in the HRS cells was associated with cellular proliferation and angiogenesis in HL (Ohsawa et al., 2006). The authors reasoned that higher Cox-2 expression would contribute to more PGE2 production; which in turn, is a potent inducer of VEGF that acted on the endothelial cells. Besides that, PGE2 also contributes to the maintenance of an immunosuppressive microenvironment for the survival of HRS cells. PGE2 secreted by HRS cells could severely impair CD4+ T cells activation by inactivation of the src-kinase lck resulting in reduced phosphorylation of ZAP40 (Chemnitz et al., 2006). This entire process is dependent of T-cell receptor signaling pathway. More recently, Cox-2 expression in HRS cells was

reported to be an independent unfavorable prognostic factor in HL treated with ABVD (Mestre et al., 2012). This suggests that Cox-2 pathway is important in the pathogenesis of this disease.

1.9 Objectives of study

Numerous studies had been done to examine T cell recruitment so as to provide better understanding of the observed T cell subset profiles in cHL lymph nodes. Most of these studies were focused on the correlation of chemokine expression on HRS cells with specific T cell recruitment into the cHL lesions. In this study, I hypothesized that HRS cells secrete soluble factors to stimulate endothelial cells to facilitate T cells recruitment into the cHL lesions. Therefore, the aims of this study are to investigate how HRS cell-derived factors can modulate endothelial cell function to facilitate T cell recruitment and to identify the dominant stimulatory factor(s) involved. To this end, I will examine the effects of HRS cell-derived cytokines on endothelial cell activation in-vitro. I will also determine the ability of these activated endothelial cells to interact with memory and naïve T cells in-vitro under dynamic flow conditions. Lastly I will elucidate the signaling pathways in HRS cells that are involved in the production of the stimulatory factor(s) that can influence endothelial cell functions in cHL.

The aims and rationales for this study are as follow:

Aim 1: To determine whether HRS cell-derived soluble factors can modulate endothelial cell activation.

The vasculature in the Hodgkin Lymphoma has been shown to express various adhesion molecules. Ruco et al showed that ICAM-1, VCAM-1 and E-selectin are found in the paraffinized tissues section of HL patients. ICAM-1 expression is found on all the HL whereas VCAM-1 and E-selectin expression on HL tissues is correlated with IL-1/TNF-α production in Hodgkin disease (Ruco et al., 1992). Reports by Machado et al also showed expression of

ICAM-1, VCAM-1 and PNAd in the vasculature of HL (Machado et al., 2009). Besides that, Estrada et al showed that soluble mediator(s) derived from the HRS cell line can stimulate endothelial cells to increase E-selectin expression and support monocytic cell line, U937, adherence. This suggests that HRS cells might secrete soluble mediator(s) to modulate endothelial cells to facilitate T cells recruitment. Thus, the first objective of this study is to identify the adhesion molecule expression profile, particularly ICAM-1, VCAM-1 and E-selectin, on endothelial cells stimulated with culture supernatant (C/S) derived from HRS cell line. To achieve this, I will compare the adhesion molecule expression profile between unstimulated and C/S stimulated endothelial cells.

Aim 2: To identify the adhesive pathways that mediate T cell recruitment in cHL

To date, the roles of various addressins and adhesion molecules in T cell homing to normal lymphoid organs and sites of chronic inflammation have been well characterized. However, their role in T cell recruitment into cHL lesions is not as well characterized. The second objective of this study is to identify the adhesive pathways that mediate the T cell recruitment into cHL lesions. T.Tanijiri et al showed that naïve T cells can be differentiated by HRS cell line, KM-H2, into regulatory T cells *in-vitro* (Tanijiri et al., 2007). This suggests that naïve T cells recruitment might also contribute significantly to the pathogenesis of the disease. Thus, this part of the project will be focusing more on the interactions of naïve T cells with HRS cell-derived C/S stimulated endothelial cells *in-vitro*.

Aim 3: To identify the dominant stimulatory factor(s) secreted by HRS cells to modulate endothelial cell functions.

It is well known that HRS cells secrete many different cytokines and chemokines to modulate the composition of the cellular infiltrate and the milieu of the cHL lesions (Ohshima et al., 2003; Skinnider and Mak, 2002). In

this aim, I will determine the cytokine profiles of my cultured HRS cells and examine whether the cytokines present in the C/S, including IL-6, TNF-α and LTα, are involved in the activation of endothelial cells in the *in-vitro* system.

Aim 4: To elucidate the signaling pathways in HRS cells that regulate LTα production.

LTα is produced by activated B cells, $T_{Helper}1$ cells and macrophages. Hinz et al also presented evidence of LTα production by HRS cells (Hinz et al., 2002). However, the signaling pathway that regulates LTα synthesis is not fully elucidated yet. Hinz et al suggests that NFκB could be one of the transcription factors that regulate LTα production. As reviewed by Shebzukhov Iu and Kuprash et al, AP-1 is another transcription factor involved in the regulation of LTα production (Shebzukhov Iu and Kuprash, 2011). Thus, the fourth objective of this study is to understand the signaling pathway(s) involved in regulating LTα production by HRS cells.

Data from this study will provide more information on how HRS cells can modulate the inflammatory infiltrate and the microenvironment in the cHL lesions for survival and growth signals. This study also provides novel insights on the modulation of endothelial cell functions by HRS cells and how this can influence T cell recruitment. The knowledge gain will help us better understand the pathogenesis of cHL and help in the design of strategies to treat cHL by preventing T cell recruitment into the cHL lesions.

Chapter 2 : Materials And Methods

2.1 Common reagents and materials

Complete RPMI 1640 culture medium for culture and maintainance of Reed-Sternberg cell lines consists of 10% or 20% FCS (Gibco), 2mM of L-glutamine (Gibco), 100U/ml of Penicilin and 100µg/ml of Streptomycin (Gibco). Henceforth, RPMI 1640 with 10% FCS is referred to as R10 whereas RPMI 1640 with 20% FCS is called R20. Complete EGM-2 medium (Clonetics) was used for human umbilical cord vein endothelial cells (HUVEC) culture. Hank's Balanced Salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Sigma Aldrich, USA) was used for washing of HUVEC prior to medium change and cell detachment. The concentration of trypsin used is 0.02%. To neutralize trypsin after HUVEC cell detachment, 8ml of M199 media (Gibco) supplemented with 10% FCS (Gibco) 25mM Hepes (Sigma Aldrich, USA), 2mM of L-glutamine (Gibco), 100U/ml of Penicillin and 100µg/ml of Streptomycin (Gibco) was added.

2.2 Reed-Sternberg cell culture

Human Reed-Sternberg cell lines, KM-H2, L1236, L428 and L540, were purchased from DSMZ cell line bank (http://old.dsmz.de/human_and_animal_cell_lines/). KM-H2 was derived from plural effusion of a patient with stage IV of Hodgkin Lymphoma, mixed cellularity subtype; L1236 was established from the peripheral blood of a patient with stage IV refractory cHL. L428 was established from the pleural effusion of a Hodgkin Lymphoma patient with stage IVB refractory nodular sclerosis subtypes whereas L540 was established from the bone marrow of a stage IVB, pre-terminal stage of nodular sclerosis cHL patient. KM-H2, L1236 and L428 were cultured in R10 while L540 was maintained in R20. The cells were passaged every 2 days and maintained at optimal cell density recommended by the DSMZ cell line bank.

2.3 HUVEC Culture

2.3.1 Preparation of gelatin coated dishes

0.1% of gelatin solution was prepared from pre-warmed 0.5% geleatin solution (Sigma Aldrich, USA) with miliQ water (1 in 5 dilution). The coating of dishes was carried out weekly in a sterile tissue culture hood. 2ml of 0.1% gelatin solution was used to cover the entire growth area of the 100mm dish (Corning Costar) and left for about 2 minutes before being aspirated. Subsequently, a second coating was carried in the same manner. The dishes were then left to dry for 3-4 hours, the lids of the culture dishes were taped and the dishes stored for future use.

Similar coating steps were done on the transwell chamber and 96-well culture plate. In both cases, 50µl of 0.1% gelatin solution was added to the insert or wells and left for 10 minutes before being aspirated out. The gelatin-coated insert and 96-well culture plate were air-dried in the tissues culture hood for 3-4 hours. After drying, the lids of the culture plates were taped and the plates were stored for future use.

2.3.2 Isolation of HUVEC

Human umbilical cord vein endothelial cells (HUVEC) were isolated from human umbilical cords from consented donors (National University Hospital, Singapore). The isolation protocol was adapted and modified from original work of Lim et al (1998). The umbilical cord vein was first cannulated at both ends with two-way stopcocks. The vein was flushed with HBSS to remove blood clots using a syringe attached to one end of the stopcocks. Next, the vein was filled with 1mg/ml of collagenase, the stopcocks closed at both ends and then placed in an enclosed sterile jar. The jar was then placed in a 37°C water bath for 8 minutes. Next the umbilical cord vein was flushed with HBSS for 10-15 times using a 20ml syringe. The content of the collagenase-digested vein containing detached endothelial cells was collected and centrifuged at

350 x g for 8 minutes at 4°C. After centrifuging, the supernatant was removed. The cell pellet was resuspended in the EGM-2 (Clonetics) culture media and seeded in a 100mm gelatin-coated culture dish. On reaching 100% confluency, HUVEC monolayer was washed twice with HBSS before being detached with 0.02% trypsin. The trypsin was neutralized with M199 wash buffer and centrifuged at 350 x g for 8 minutes at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended in EGM-2 media and re-plated in new 100mm gelatin-coated culture dishes at a split ratio of 1:3. For experiments, HUVEC up to passage 6 were used.

2.3.3 Plating of HUVEC on glass coverslips

Glass coverslips were placed in six-well plates (Corning Costar) and wells were filled with 1.5ml of 70% ethanol. The glass coverslips were soaked for at least 1 minute to disinfect after which the ethanol was aspirated. The coverslips were washed with 2ml of HBSS to remove excess ethanol. After final wash, 1.5ml of HBSS containing 0.05mg/ml of Matrigel was placed in each well. The setup was incubated at 37°C for at least 3 hours. After incubation, trypsin-detached HUVEC were plated at a cell density of $0.25x10^6$ cells/ml/well and cultured for 3 days under standard condition before use.

2.4 Reagents, recombinant proteins and antibodies

2.4.1 Inhibitors used

Inhibitors were purchased from commercial sources. Each inhibitor was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each inhibitor is as listed below.

Inhibitor	Source	Final Concentration Used	Maximum solubility as stated in data sheet
NFκB inhibitor, Bay11-7085	Merck	5, 10 and 20μM	100mM in DMSO
JNK Inhibitor, SP600125	Cayman Chemical	40,60,80,100μM	90.83mM in DMSO
Cox inhibitor, Indomethacin	Sigma Aldrich	0.1 and 0.3mM	48.91mM in DMSO
Specific Cox-2 inhibitor, Celecoxib	Cayman Chemical	25, 50 and 75μM	262.21mM in ethanol

Table 2.1: List of inhibitors and their sources. The working concentrations used for each inhibitor are as stated.

2.4.2 Antibodies and recombinant proteins used

Antibodies and recombinant proteins were purchased from commercial sources. Each antibody or recombinant protein was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each reagent is as listed below.

Recombinant Protein	Source	Final Concentration Used
Neutralizing TNF-α Antibody	R&D	0.24μg/ml
Neutralizing LTα Antibody	R&D	1.0μg/ml
Neutralizing IL-6 Antibody	R&D	10μg/ml
Recombinant human TNF-α	Ebioscience	10ng/ml
Recombinant human SDF-1α	PeproTech	200ng/ml

Table 2.2: List of recombinant proteins and antibodies used in this project.

2.4.3 Recombinant proteins or antibodies used for parallel plate flow chamber assay

Antibodies were purchased from commercial sources. Each antibody was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each antibody is as listed below.

Recombinant Protein / Antibody	Source	Final Concentration Used	Origin
Anti CD11a (LFA-1, clone HI 111)	Biolegend	20ng/ml	Mouse
Anti CD18 (TS 1/18)	Biolegend	20ng/ml	Mouse
Anti L-selectin (clone Dreg 56)	Ebioscience	20ng/ml	Mouse
Mouse IgG	Invitrogen	20ng/ml	Mouse
Anti CD44 (clone IM7)	BD Pharmingen	20ng/ml	Rat
Rat IgG	Caltag	1:5000	Rat
Hyaluronidase	Sigma Aldrich	50μg/ml (37.5U/ml)	Bovine

Table 2.3: Reagents used for parallel plate flow chamber assay. This table shows the source, the clone and the final working concentration used.

2.4.4 Antibodies used for Western blot

Antibodies were purchased from commercial sources. Each new antibody was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each antibody is as listed below.

Antibody	Source	Final Concentration Used	Origin
ERK	Cell Signaling	1:1000	Rabbit
Phospho ERK	Cell Signaling	1:1000	Mouse
p38	Cell Signaling	1:1000	Rabbit
Phospho p38	Cell Signaling	1:1000	Rabbit

JNK	Cell Signaling	1:1000	Rabbit
Phospho JNK	Cell Signaling	1:1000	Rabbit
c-Jun	Cell Signaling	1:1000	Rabbit
Phospho c-Jun	Cell Signaling	1:1000	Rabbit
c-Fos	Cell Signaling	1:1000	Rabbit
Phospho c-Fos	Santa Cruz	1:450	Mouse
P65	Santa Cruz	1:450	Mouse
Beta lamin	Abcam	1:3000	Mouse
TATA Box	Abcam	1:3000	Mouse
Beta actin HRP Conjugated	Santa Cruz	1:30000	Mouse

Table 2.4: List of antibodies used for Western blot assay. The source and the dilution factor used are as shown.

2.4.5 Antibodies used for immunohistochemical (IHC) Staining

Antibodies were purchased from commercial sources. Each new antibody was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each antibody is as listed below.

Antibody	Source	Final Concentration Used	Origin
c-Jun	Cell Signaling	1:100	Rabbit
LTα	Ebioscience	1:200	Mouse
Rb sera	Dako Cytomation	1:10000	Rabbit
Biotinylated hyaluronic acid binding protein	Merck Millipore	1:500	Bovine
MouseIgG	Invitrogen	1:100	Mouse

Table 2.5: List of antibodies used for IHC staining. The source and the dilution factor used are as indicated.

2.4.6 Antibodies used for flow cytometry

Antibody was purchased from commercial source. The antibody was tittered to determine its optimal working concentration which was subsequently used

throughout the project. The source and final working concentration for the antibody is as listed below.

Antibody	Source	Dilution factor	Origin
LTα (clone LTX-21)	Ebioscience	1:50	Mouse

Table 2.6: List of antibody used for intracytopalsmic flow cytometry staining. The source and the dilution factor used are as shown.

Anti-E-selectin (H18/7), anti-ICAM-1 (Hu5/3), anti-VCAM-1 (E1/6) and anti-MHC-I (W6/32) hybridoma clones were gifts from the Vascular Research Division, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, USA. The culture supernatants were diluted 1:3 before use.

2.4.7 Secondary antibodies used

Secondary antibodies were purchased from commercial sources. Each new antibody was tittered to determine its optimal working concentration which was subsequently used throughout the project. The source and final working concentration for each antibody is as listed below.

Secondary Antibody	Source	Dilution factor	Purposes
Goat anti Mouse IgG HRP (Horseradish peroxidase)	Sigma Aldrich	1:1000	ELISA
Goat anti Mouse IgG HRP	Santa Cruz	1:5000	Western blot
Goat anti Rabbit IgG HRP	Santa Cruz	1:5000	Western blot
Goat anti Moue IgG PE (Phycoerythrin)	Caltag	1:100	Flow cytometry
Strepavidin HRP	Sigma Aldrich	1:1000	IHC

Table 2.7: List of secondary antibodies used throughout the study. The purpose, the source and the dilution factor used are as stated in the table.

2.5 Preparation of cell culture supernatant (C/S) and cell pellet

All Reed-Sternberg cell lines (L1236, L428, KM-H2, L540) were plated in R10 or R20 at a cell density of $2x10^6$ cells/ml. After 12 hours' or 24 hours' incubation, the culture supernatant containing soluble products derived from these lymphoma cells were collected after centrifugation and used immediately. All the C/S were diluted 1 in 2 or 1 in 8 with R10 for subsequent experiments.

To study the signaling pathways on the HRS cells, KM-H2, L1236 and L428 cells were treated with various doses of inhibitors for 12 hours. The doses and inhibitors used were summarized in Table 2.1. For Bay11-7085 treatment, the highest amount of DMSO used was 1µl/ 1ml of media (volume/ volume). For Indomethacin treatment, the highest amount of DMSO used was 0.6µl/ 1ml of media (volume/ volume). For SP600125 treatment, the highest amount of DMSO used was 1.1µl/ 1ml of media (volume/ volume) For Indomethacin treatment, the drug was resupplied after every 6 hours of incubation. C/S from the treated cells and respective controls were collected as described above. The cell pellets were snap frozen in liquid nitrogen and used for Western blot assay.

2.6 Preparation of T cell subsets from buffy coat

T cells were isolated from buffy coats of healthy donors (National University Hospital of Singapore). The buffy coat was first diluted with HBSS in a ratio of 1:7 and thoroughly mixed by inversion. The mixture was carefully layered over Ficoll-Pague (Ge Heatlhcare) and centrifuged at 450 x g for 30 minutes at room temperature. The PBMC (peripheral blood mononuclear cell) layer at the Ficoll-Paque/plasma interface was removed with a 1ml pipette (Gilson P1000). The cell suspension was centrifuged at 350 x g for 8 minutes. The supernatant was discarded and cell pellet was resuspended in 15ml of R10. Total number of PBMC was enumerated with trypan blue exclusion method. CD4+ T cells

were subsequently isolated from PBMC by positive selection using Whole Blood CD4+ beads (Miltenyi Biotec, Germany) kit according to manufacturer protocol. The ratio of the CD4+ beads used to cell number is 1:20 (5µl of beads to 95µl of MACS buffer for $1x10^7$ PBMC). Briefly, the PBMC were resuspended in MACS buffer (PBS + 5% BSA + 2mM EDTA) and incubated with CD4+ beads in the dark for 15 minutes at 4°C. Next, the mixture was washed once with 10-20X volume of MACS buffer by centrifuging at 350 x g for 8 minutes. Then the cell pellet was resuspended in the MACS buffer and applied to LS column (Miltenyi Biotec, Germany) attached to a magnetic stand. The LS column was washed three times with MACS buffer. Beads-bound $CD4^+$ T cells were dissociated from the LS column by removing the column from the magnetic stand and flushing the $CD4^+$ T cells out of the column with MACS buffer using a plunger. The number of $CD4^+$ T cells obtained is usually 20% of the initial number of PBMC used for the isolation process.

Isolated CD4+ T cells were further purified into naïve T cells ($CD4^+CD45RA^+CD45RO^-$) and memory T cells ($CD4^+CD45RA^-CD45RO^+$) by negative selection using CD45 RA^+ antibody- or $CDRO^+$ antibody-coated Dynal Magnetic Beads (Invitrogen, USA) as described previously (Lim et al., 2000). Briefly to isolate $CD4^+$ naïve T cells, $CD4^+$ T cells were incubated with $CD45RO^+$ antibody-coated magnetic beads; and $CD4^+$ T cells incubated with $CD45RA^+$ antibody-coated magnetic beads for isolating $CD4^+$ memory T cells. The $CD4^+$ T cells with antibody-coated magnetic beads mixture was incubated for 30 minutes at 4°C with constant rotation. After that, the beads-bound T cells were separated from the free $CD4^+$ T cells by placing the tubes on a magnetic separator for at least a minute. The unbound cells in suspension were collected into a 15ml tube and the beads washed once with 2ml of R10. The pooled cell suspension was placed on the magnetic separator again to remove any bead-bound cells that may have been dislodged when the supernatant/wash was collected. This process was repeated twice. The purity

of naïve and memory T cells were >90% as determined by flow cytometry immunofluorescence staining.

2.7 Naïve and memory T cell transmigration assay

The number of naïve and memory T cells transmigrated across a HUVEC monolayer was determined as described below. A HUVEC monolayer was grown on the inner side of the membrane of the 5μm transwell insert until it reached 100% confluency. The HUVEC were either left unstimulated, stimulated with TNF-α (10ng/ml) or KM-H2 C/S (diluted 1 in 2) for 4 hours under standard culture condition. The endothelial monolayer was washed once with HBSS before naïve or memory T cells (0.5 x 10^6cells in 200μl of R10) were placed in the upper chamber of the inserts. Either R10 only or R10 with 200ng/ml of SDF-1α (PeproTech, USA) was added to the lower chambers of the transwells. The transwell setup was then incubated at 37°C in 5% CO_2 for 4 hours (Figure 2.1). At the end of the incubation, transmigrated T cells were recovered from the lower chambers. The total number of transmigrated T cells was determined using Tryphan Blue exclusion assay. The percentage of T cells that had transmigrated was calculated by dividing the number of transmigrated T cells by total number of T cells added to the upper chamber.

To block the G-protein coupled receptor on the T cells, naïve or memory T cells were pre-treated with pertussis toxin (100μg/ml) for an hour in the incubator before using them for the transmigration experiment.

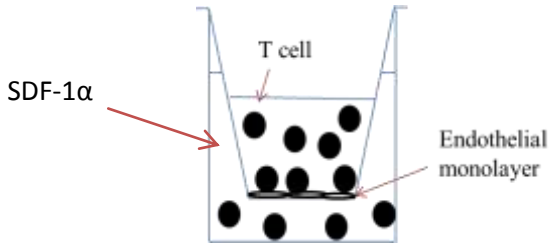


Figure 2.1: Diagram represents static transwell system used in the transmigration study.

2.8 *In-vitro* parallel plate flow chamber assay

Naïve and memory T cell interactions with endothelial cells under defined laminar flow condition were studied in the parallel plate flow chamber system as described below. A 10-cm rectangular parallel plate flow chamber containing a 5-mm wide and 0.01-inch high channel was used for the *in vitro* flow experiment. Using a syringe pump (Harvard Apparatus), the cell suspension was drawn thorough the flow chamber at three different flow rate (shear stress), 0.52ml/min (1.0 dynes/cm^2), 0.36ml/min (0.76 dynes/cm^2) and 0.26ml/min (0.5 dynes/cm^2). The wall shear stress (dynes/cm^2) which is dependent on the flow rate and viscosity of the cell suspension can be defined by the equation:

$$\text{dynes/cm}^2 = 6\ \mu Q/bh^2,$$

where μ is the viscosity of the fluid expressed in poise; Q is the flow rate of the fluid expressed in centimeters cube per second, b is the width of the chamber whereas h is the height of the chamber. Both are expressed in centimeters (Bacabac et al., 2005).

HUVEC monolayers were grown on the matrigel coated coverslip until about 100% confluency. HUVEC monolayers were either treated with TNF-α (10ng/ml), KM-H2 C/S (diluted 1:2 or 1:8) or left untreated for four hours and mounted onto a lower plate and the flow chamber assembled. The set-up was next mounted on an inverted microscope (Nikon; Eclipse TE-2000U) equipped with 20X objective lens (Nikon). Isolated naïve or memory T cells were resuspended in pre-warmed flow buffer (HBSS containing 0.1% FBS, 1mM $CaCl_2$, 20mM of HEPES, pH7.4) at 37°C at a cell density of 0.7 x 10^6cell/ml. Then, the T cells were perfused across HUVEC monolayer at decreasing flow rates. At each flow rate, T cells were allowed to interact with the HUVEC monolayer for a minute before the live time cell-cell interactions in 5 different fields were video recorded using a CCD camera and VCR (Sony; SVT-N24P). Analysis was carried out offline.

To block integrins on the T cells, naïve T cells were pre-incubated with monoclonal antibodies (20ng/ml) against various integrin subunits for 10 minutes at 37°C prior to perfusion across HUVEC monolayer. A matching mouse or rabbit IgG was used as an isotype control.

To block the adhesion molecules on HUVEC, HUVEC monolayers were pre-incubated with antibodies against ICAM-1, E-selectin and VCAM-1 (clone as listed in section 2.4.6) for 30 minutes before mounting onto the flow chamber for the experiment. Treatment with antibodies against MHC class I molecules is used as the binding non-blocking control.

To investigate effect of HA mediated naïve T cell interactions with stimulated HUVEC, HUVEC monolayer was treated with either 50μg/ml of hyaluronidase or boiled hyaluronidase for one hour in the incubator before mounting onto the flow chamber for the experiment.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Cell surface expression of ICAM-1, VCAM-1, and E-selectin on HUVEC were measured by ELISA. Briefly, HUVEC were grown until about 100% confluent in gelatin-coated 96-well flat-bottom tissue culture plate. Endothelial cells were treated with TNF-α (Ebioscience, USA) at 10ng/ml or diluted KM-H2 C/S (1:2 or 1:8 dilution) for four hours. Negative control was cells left untreated. HUVEC was washed twice with PBS+0.1% BSA before blocking with PBS+1%BSA for 20 minutes at room temperature. After the blocking step, HUVEC were incubated with optimally diluted antibodies against ICAM-1, VCAM-1, E-selectin and MHC class I molecules for one hour at 4°C. The hybridoma clones are respectively, Hu 5/3, E1/6 and H18/7 (diluted 1:3) and W6/32 (diluted 1:5 with PBS + 0.1% BSA). After 4 repeats of washing with wash buffer (PBS + 0.1%BSA); 100 μl of optimally diluted

horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (diluted 1 in 100 with PBS + 0.1% BSA) was added to each well and incubated for an hour at 4°C. After that, unbound HRP-conjugated antibody was washed off and the washes repeated 4 times. Then, 100µl of substrate buffer (0.1M citrate buffer + 0.2M phosphate buffer + Triton-X 100 + O-phenyelendiane dihydrochloride tablet + 10µl of 3% hydrogen peroxide) was added to each well and incubated for 10 minutes, after which 25µl of 3M sulphuric acid was added to stop the colorimetric reaction. Absorbance reading at 492nm of each well was determined using a Tecan plate reader.

For NFκB blocking experiment, HUVEC monolayer were pre-incubated with 20µM of Bay 11-7085 for 30 minutes in the incubator prior to stimulation with KM-H2 C/S. Fresh 20µM Bay 11-7085 inhibitor was added into the C/S during the 4 hours stimulation.

For cytokine neutralizing experiment, KM-H2 C/S was pre-incubated with neutralizing antibody for 30 minutes in the incubator prior to being used to stimulate the HUVEC monolayer.

2.10 Flow cytometry

Surface expression of ICAM-1, VCAM-1, and E-selectin on HUVEC were measured by flow cytometry. Confluent HUVEC monolayer was either treated with TNF-α (10ng/ml), KM-H2 C/S or left untreated for four hours at 37°C. After stimulation, HUVEC were detached from the culture dishes as described in section 2.3.1. After centrifugation, cells were resuspended with flow cytometry buffer (PBS + 0.1% BSA) to a cell density of 2-5x10^6 cell/ml. Subsequently, 100µl of the cell suspension was aliquoted into 5ml polystyrene round bottom tubes (BD Biosciences) and incubated with optimally diluted antibodies against ICAM-1, VCAM-1, E-selectin and MHC class I molecules.

The hybridoma clones are respectively, Hu 5/3, E1/6 and H18/7 (diluted 1:3) and W6/32 (diluted 1:5 with PBS + 0.1% BSA) for 30 minutes at 4°C. Mouse IgG was used as the isotype control. The cells were washed once with 1ml of flow cytometry buffer and pelleted by centrifuging at 350 x g for 8 minutes at 4°C. Then, the supernatant was discarded and the cells were incubated with optimally diluted PE-conjugated goat anti-mouse secondary antibody in the dark for 30 minutes at 4°C. Next, cells were washed thrice as described above; twice with flow cytometry buffer and once with PBS. Lastly, the cells were fixed with 350µl of 1% Formalin in PBS and kept in the dark at 4°C until the day of data acquisition. Cell acquisition (at least 10,000 cell per sample) was carried out on the FACS Calibur cytometer (Becton Dickinson, USA) and analysed using the CellQuest Software.

For intracytoplasmic staining, KM-H2 cells were pretreated with Golgi plug (BD Biosciences) for six hours before undergoing the intracytoplasmic staining procedure according to the manufacturer's protocol. Briefly, the Golgi-plug-treated KM-H2 cells were harvested by centrifugation. The cell pellet was resuspended with PBS to a cell density of 2-5x10^6 cell/ml. Subsequently, 100µl of cell suspension was aliquoted into 5ml polystyrene round bottom tubes. Then, the cells were pelleted down and resuspended thoroughly with 250µl of Fixation/Permeabilization solution (BD Biosciences) for 20 minutes at 4°C. Then, the cells were washed twice with BD Perm/Wash buffer (BD Biosciences) as described above. Next, supernatant was discarded and the cells were incubated with 50µl of BD Perm/Wash buffer containing optimally diluted cytokine antibody at 4°C for 30 minutes in the dark. After that, the cells were washed twice as described above followed by incubation with 50µl of BD Perm/Wash buffer containing optimally diluted PE-conjugated goat anti-mouse secondary antibody at 4°C for 30 minutes in the dark. Next, cells were washed thrice as described above; twice with BD Perm/Wash buffer and once with PBS. Lastly, the cells were fixed with 350µl of 1% Formalin in PBS and kept in the dark at 4°C until the day of data acquisition. Data acquisition and analysis was carried out as described previously.

2.11 Western blotting

Mechanistic study was carried out using Western blotting. Cell lysate was fractionated into cytoplasmic protein and nuclear protein. Cytoplasmic protein was extracted first followed by nuclear protein. HUVEC or KM-H2 cells were harvested as described in section 2.3.1 and section 2.5. Cytoplasmic protein was extracted by incubating cells with 100µl of 1x isotonic lysis buffer supplemented with 1% PMSF, 1% protease inhibitor, and 0.1mM DTT for 13 minutes on ice and followed by 6µl of 10% IGEPAL (Sigma Aldrich) for 20 seconds with vigorous vortexing. Then, the mixture was centrifuged at 10.6 x g for 30 seconds at 4°C. The supernatant was collected as cytoplasmic protein and keep for future use. The cell pellet was washed with 500µl of 1X lysis buffer by centrifuging at 10.6 x g for 30 seconds at 4°C. The supernatant was discarded and the cell pellet was incubated with 70µl of extraction buffer supplemented with 1% PMSF, 1% protease inhibitor, and 0.1mM DTT protein for 30 minutes at 4°C with gentle shaking. Next, the mixture was centrifuged at 18.8 x g for 5 minutes. The supernatant was collected as nuclear protein fraction. The cytoplasmic protein or nuclear protein was quantified using Bradford Reagent (Bio-rad, USA) according to manufacturer's protocol. A total of 25µg of proteins was loaded and separated on 10% or 12% SDS-Page gel. Then, the separated protein was transferred to a polyvinylidene fluoride (PVDF) microporous membrane (Millipore) and blocked with 5% non-fat milk (w/v) in 0.1% TBST for one hour at room temperature. The membranes were incubated with diluted primary antibodies overnight at 4°C followed by secondary antibodies for one hour at room temperature before chemiluminescent substrate detection. The working concentrations of the primary and secondary antibodies used are as listed in Table 2.4.

2.12 Immunohistochemistry (IHC) staining

4µm thick tissue sections from thirty-two cases of cHL were used for IHC staining for 2 different targets. Fourteen of the 32 cases were from the Department of Pathology, National University Hospital of Singapore whereas

18 of 32 cases were from University of Al Ain, United Arab of Emirates (UAE). LTα, and c-Jun were detected using indirect immunoperoxidase technique. Briefly, the tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Next, antigen retrieval process was performed on the rehydrated tissue sections. The antigen retrieval conditions used for the different antigens are stated in the Table 2.8 below. After that, the tissue sections were treated with 3% hydrogen peroxide for 10 minutes at room temperature. Then, the sections were washed twice with distilled water and equilibrated with TBS buffer for 5 minutes at room temperature. Next, the tissues sections were incubated with optimally diluted primary antibody (diluted with TBS diluent) overnight at room temperature. Tissue sections were washed 3 times with TBS buffer to remove excess primary antibody and incubated with HRP-conjugated secondary antibody (Envision Plus kit, Dako Cytomation) at room temperature for an hour. Excess secondary antibody was removed by 3 washings with TBS buffer. The sections were then treated with substrate reagent containing diaminobenzidine (DAB) for 5 minutes (Dako Real Envision Detection Kit). The sections were counterstained with Gill's Hematoxylin, dehydrated, cleared and mounted with Canada Balsam (Sigma Aldrich, USA). The stained tissue sections were scored for intensity of the staining and the percentage of positively stained HRS cells. The scoring was graded as 0 (undetectable), 1 (weak staining), 2 for moderate staining and 3 for intense staining. Similarly for the percentage of positively stained HRS cells, a score of 0 indicates no positively stained HRS cells, 1 for less than 30% HRS cells stained, 2 for >30%-60% HRS cells positively stained and to 3 for more than 70% of HRS cells positively stained.

Target	Antigen Retrieval Buffer	Antigen Retrieval Condition
LTα	Citrate buffer, pH 6	Boiling for 15 minutes
c-Jun	Citrate buffer, pH6	Pressure cook for 10 minutes

Table 2.8: List of antigen retrieval buffer and treatment conditions used for each molecular target.

2.13 L929 TNF cytotoxic assay

L929 cells were purchased from ATCC and cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% horse serum. L929 cells were dislodged from the culture flask with 0.02% trypsin. Cultured L929 cell number was estimated using trypan blue exclusion assay. Next, cell suspension in EMEM complete medium was plated into a 96-well plate at a cell density of 5000 cells/100µl/well. After 24 hours, EMEM complete medium was replaced with HRS cell C/S (diluted with EMEM + 2% horse serum + 0.2µg/ml of Actinomycin D) and incubated for 12 hours in the incubator. Negative controls in this study are L929 cells treated with EMEM media containing 2% horse serum and 0.2µg/ml of Actinomycin D. Positive controls are cells treated with serially diluted TNF-α (diluted with EMEM + 2% horse serum + 0.2µg/ml of Actinomycin D). At the end of the incubation, 20µl of MTS buffer (Promega) was added directly into each well and incubated in the incubator for 4 hours. The viability of L929 cells was determined by acquiring the absorbance value at 492nm using a Tecan plate reader. A standard curve of L929 cell viability against decreasing concentration of recombinant human TNF-α was included in each assay. Results are expressed as the percentage of viability relative to the untreated control.

To study the cytotoxic effect of C/S after neutralization of TNF-α or LTα, C/S of KM-H2, L1236 and L428 cells were incubated with optimally diluted antibodies against TNF-α or LTα for 30 minutes in the incubator before using them for L929 cytotoxic assay. Mouse IgG was used as the isotype control.

2.14 Cytokine antibody array

The cytokine profile of the C/S from different HRS cells (KM-H2, L1236 and L540) was determined using Quantibody Human Cytokine Array 1 from RayBiotech (Norcross, GA). In brief, antibodies against 20 different cytokines were spotted onto the cytokine array according to manufacturer's instruction.

First, the wells of the array were blocked with 100µl of sample diluent for 30 minutes. Next, the sample diluent was removed and replaced with 100µl of cytokine standards (serially diluted with sample diluent) or C/S of HRS cells (diluted 1 in 2 or 1 in 8 with sample diluent) and incubated for 2 hours at room temperature. After that, samples were decanted from each well followed by 5 minutes of washing step by using 1 x washing buffer I with gentle shaking. This washing step was repeated 4 times. Then, the array was washed with 1 x washing buffer II for twice; 5 minutes with gentle shaking each time. Wash buffer was discarded completely before incubating with 80µl of detection antibody cocktail for 1 hour at room temperature. After that, the washing steps using washing buffer I and II were carried out as described previously. Then, the well was incubated with 80µl of Cyc3 equivalent dye-conjugated streptavidin for 1 hour at room temperature in the dark. To prevent exposure to light, the array was covered by aluminium foil. At the end of incubation, the washing steps were carried out as stated above. The chip was scanned using GenePix® Professional 4200A (Synnyvale, CA) at excitation 555 nm and emission 565 nm. The image was analyzed by GenePix Pro 5.0 software program, and the amount of cytokine present in the C/S was estimated using the standard curves generated in the same array using the cytokine standards.

2.15 Lymphotoxin-α (LTα) ELISA

The secreted LTα from HRS cells (KM-H2, L1236, L428, L540) was measured using the Human TNF beta ELISA Ready-Set-Go kit (Ebioscience, USA). Briefly, 100µl of captured antibody against TNF-β (diluted with 1x coating buffer) was used to coat in the 96-well plate at 4°C for overnight. Then, the coating antibody was removed and the plate was washed three times with wash buffer (250µl/well). Next, the wells were blocked with 200µl/well with the assay diluent for an hour at room temperature. Then, the plate was washed once before incubating with 100µl of diluted C/S (diluted 1 in 2, 1 in 4 or 1 in 8) or serially diluted standard (diluted with assay diluent) at room temperature

for 2 hours. At the end of the incubation, the washing steps were carried out as described above. Next, the wells were incubated with 100µl/well of detection antibody (diluted in assay diluent) for an hour at room temperature. The detection antibody was discarded and the plate washed as stated above before incubating with 100µl/well of avidin-HRP conjugated antibody (diluted with assay diluent) for 30 minutes. At the end of incubation, avidin-HRP conjugated antibody was aspirated out and the plate was washed three times with the wash buffer. After washing, the substrate solution was added (100µl/well) and the plate was incubated for 10 minutes followed by addition of stop solution (2N sulphuric acid), at 50µl/well. The absorbance was detected at 450nm using the Tecan plate reader.

2.16 Statistical analysis

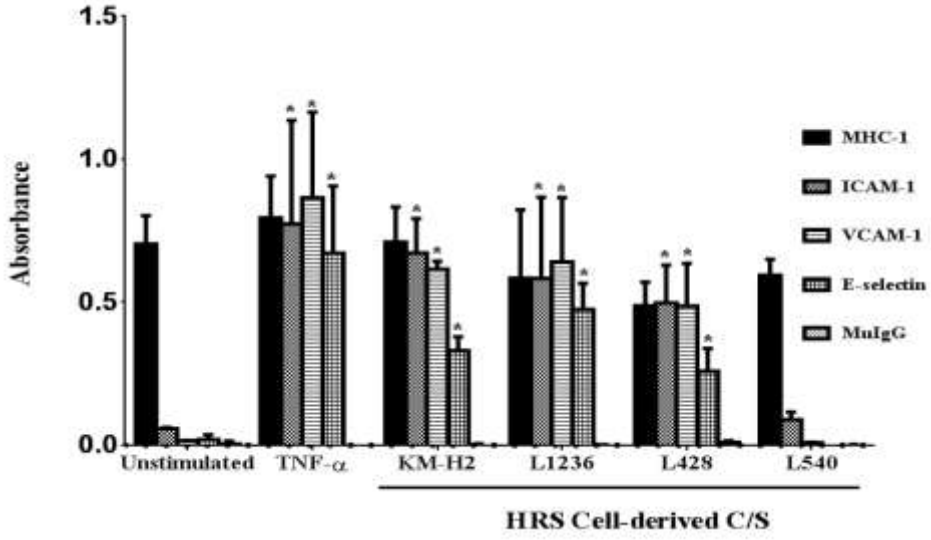
Data is expressed as mean$\pm$ standard error of mean. Statistical comparison of mean was performed by Student's T-test.

Chapter 3 : Results

3.1 HRS cell culture supernatant (C/S) can stimulate endothelial cells and induce up-regulation of adhesion molecule expression

Soluble mediators derived from human lymphomas has been shown to activate endothelial cells and this activation is analogous to that associated with leukocytes adhesion and extravasation during inflammatory response (Estrada-Bernal et al., 2003b). Knowing that HRS cells actively secrete various cytokines and chemokines to facilitate immune cells infiltration (Skinnider and Mak, 2002), I first determined the effect of HRS cell culture supernatant (C/S) on endothelial cells by cell-based ELISA. According to the ELISA results, endothelial cells stimulated with C/S harvested from three HRS cell lines, KM-H2, L1236 and L428 for 4 hours can up-regulate ICAM-1, VCAM-1 and E-selectin expression (Figure 3.1A). Endothelial cells stimulated with C/S from L540, another HRS cell line, did not show any increment in the adhesion molecule expression. The expression level of the various adhesion molecules on endothelial cells activated by C/S from KM-H2, L1236 and L428 are comparable to that induced by 10ng/ml of TNF-α. The response of endothelial cells to TNF-α was used as the positive control throughout the study. Unstimulated endothelial cells served as the negative control. Furthermore, MHC class I expression (Figure 3.1A, black bar) was used as an internal positive control for the ELISA assay. This finding was further verified by flow cytometry (Figure 3.1B). Similarly, KM-H2 C/S can stimulate endothelial cells to up-regulate ICAM-1, VCAM-1 and E-selectin expression to levels comparable to TNF-α. Thus the data confirmed that C/S from HRS cells contain soluble mediator(s) that can stimulate endothelial cells to up-regulate adhesion molecule expression.

A



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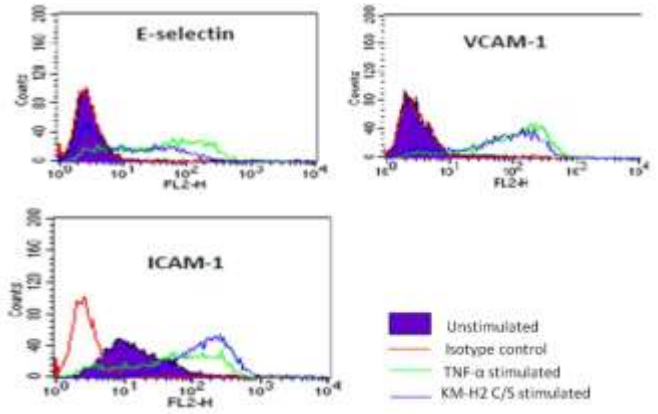


Figure 3.1: HRS cell C/S stimulates endothelial cells to up-regulate adhesion molecule expression. (A) Up-regulation of adhesion molecule expression was detected on endothelial cells stimulated with C/S from KM-H2, L1236, L428 but not L540 for four hours. Unstimulated and TNF-α stimulated endothelial cells served as the negative and positive controls, respectively. (B) KM-H2 C/S stimulated endothelial cells induced ICAM-1, VCAM-1 and E-selectin expression is comparable to that detected on TNF-α stimulated endothelial cells as assessed by flow cytometry. Values shown are the mean $\pm$ SEM (standard error of mean) from 3 independent experiments for A; and a representative experiment from 3 independent experiments for B.

3.2 HRS cell C/S stimulatory effect is not because of endotoxin contamination

To eliminate the possibility that stimulatory effect on the C/S is due to serum content or low level of endotoxin contamination, I did the following experiments. First, serum free KM-H2 C/S was used to stimulate the endothelial cells. As shown in Figure 3.2A, serum free KM-H2 C/S retained the stimulatory effect as compared to serum containing C/S. The adhesion molecule expression on the stimulated endothelial cells is similar between the two conditions. This shows that serum in the media does not contribute to the activity of soluble mediators in the C/S that activates the endothelial cells. Boiling of the C/S for 5 minutes eliminates the C/S stimulatory effect (Figure 3.2A). Treatment of C/S with polymyxin B will eliminate the possibility of endotoxin induced activation of endothelial cells. Polymyxin B will bind to the lipid A region of the lipopolysaccharide (Morrison and Jacobs, 1976) and inhibit its stimulatory effect on endothelial cells. This is important because any traces of endotoxin or lipopolysaccharide contamination in the C/S could activate endothelial cells and induce adhesion molecule expression (Lorenzon et al., 1998). According to the ELISA data shown in Figure 3.2B, C/S remains active after treatment with polymyxin B. The level of induced adhesion molecule expression on endothelial cells stimulated with polymyxin B treated C/S is comparable to the control, endothelial cells stimulated with C/S without polymyxin B treatment. Thus my data suggests that the stimulatory effect of HRS cell C/S is not due to the presence of serum or endotoxin.

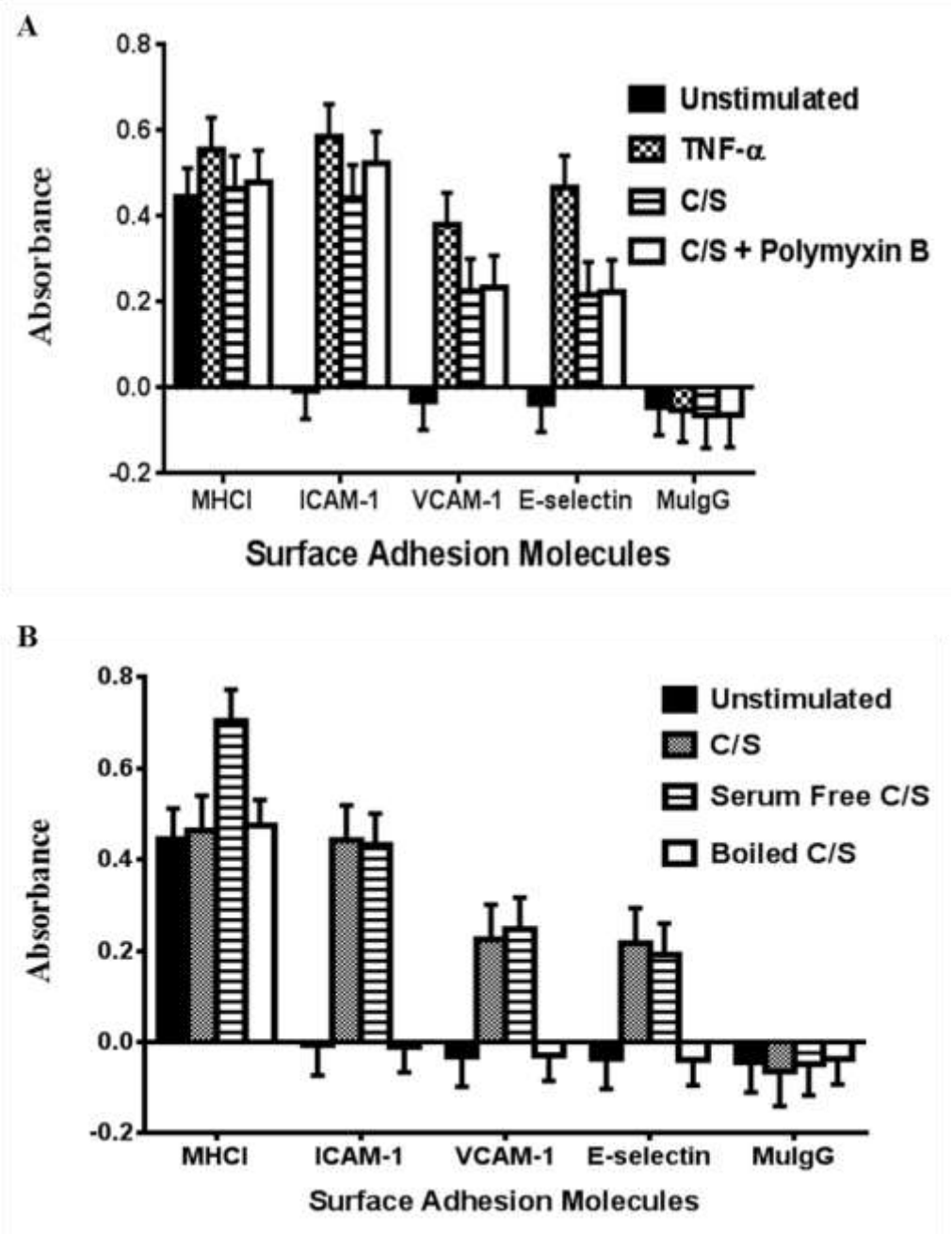


Figure 3.2: The stimulatory effect of H-RS cell C/S is not due to the presence of serum or endotoxin. (A) The expression of ICAM-1, VCAM-1 and E-selectin induced on endothelial cells activated with serum free C/S, and normal KM-H2 C/S were comparable as detected by ELISA. Boiled C/S failed to stimulate the endothelial cells. (B) 2 x 10^6 KM-H2 cells were treated with polymyxin B (10µg/ml) and cultured overnight in 1ml of culture media. C/S was harvested after 24 hours and used to stimulate endothelial cells. Stimulatory effect of polymyxin B treated KM-H2 C/S on endothelial cells was similar to that of untreated C/S as assessed by ELISA. Values shown are the mean $\pm$ standard deviation from two different experiments.

3.3 HRS cells produce highly potent soluble mediator(s) that stimulates endothelial cells

Soluble mediator(s) derived from HRS cells exhibits a very high potency for stimulating endothelial cells. The time course experiments showed that the mediators are spontaneously secreted by KM-H2 cells. C/S from KM-H2 cells conditioned for an hour already exhibits some degree of stimulatory effect on the endothelial cells. As shown in Figure 3.3A, 1 hour conditioned KM-H2 C/S can induce ICAM-1, VCAM-1 and E-selectin expression on the endothelial cells and the stimulatory efficacy increases when the conditioning time was increased from 1 hour to 24 hours. In addition, using 2-fold serially diluted KM-H2 C/S to stimulate the endothelial cells, the data revealed that soluble mediator(s) in the KM-H2 C/S is highly potent. As shown in Figure 3.3B, the stimulatory effect of C/S diluted 1 in 8 is equally potent as that of C/S diluted 1 in 2. Even at a high titre of 1 in 32, the diluted C/S could still induce ICAM-1, VCAM-1 and E-selectin expression on the endothelial cells albeit lower than the unstimulated negative control (Figure 3.3B, black bar). This result shows that the KM-H2 cells constitutively produce large amounts of highly active soluble mediator(s) in the C/S that can stimulate endothelial cells.

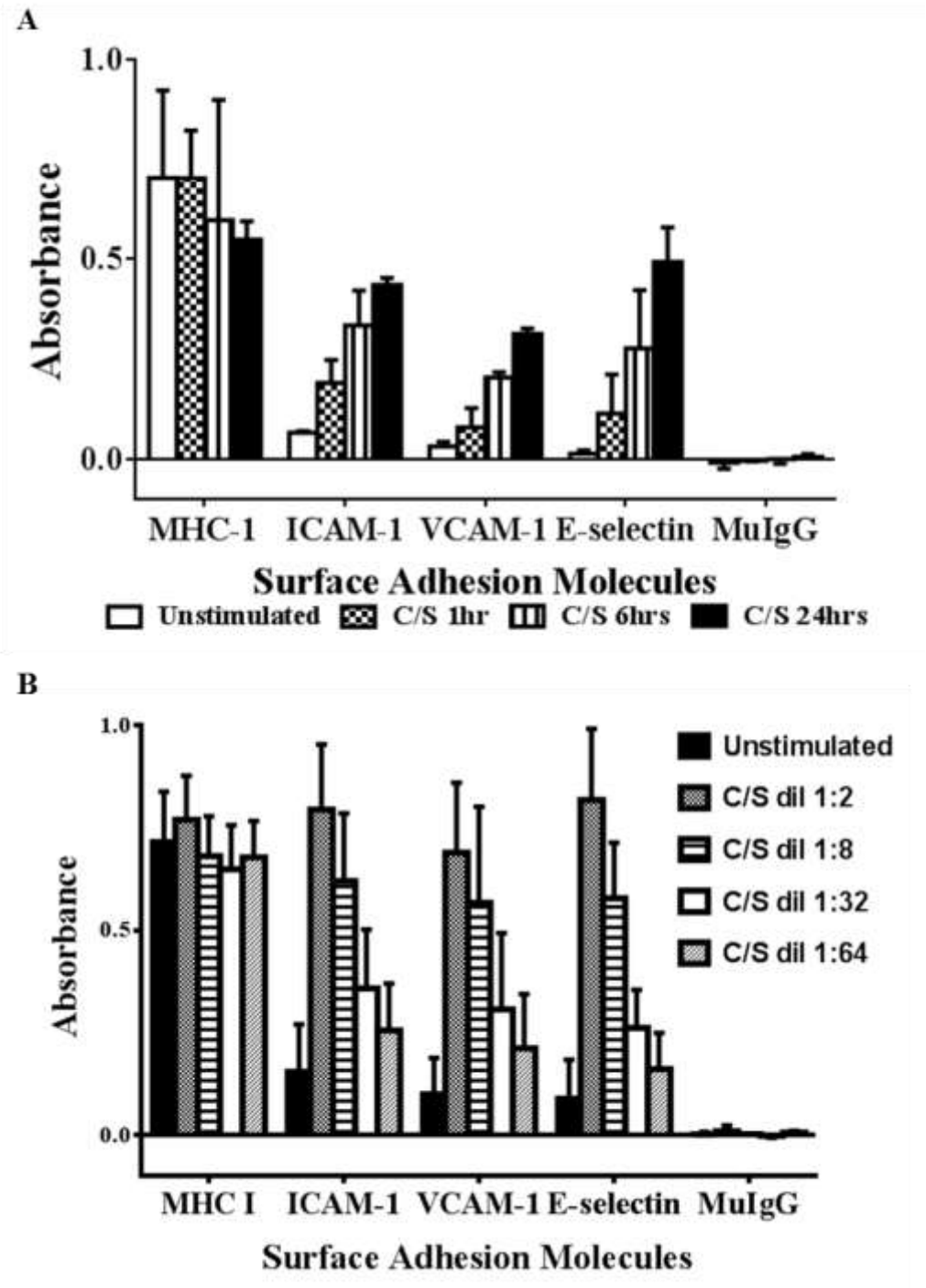


Figure 3.3: KM-H2 C/S contained very potent factor(s) that can activate endothelial cells. (A) C/S from KM-H2 cells harvested at different time points, 1hour, 6 hours and 24 hours contained soluble mediators that can up-regulate the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells as detected by ELISA. (B) 2-fold serially diluted C/S of KM-H2 cells could effectively stimulate endothelial cells at a titre of 1:32.Values shown are the mean $\pm$ standard deviation from two independent experiments.

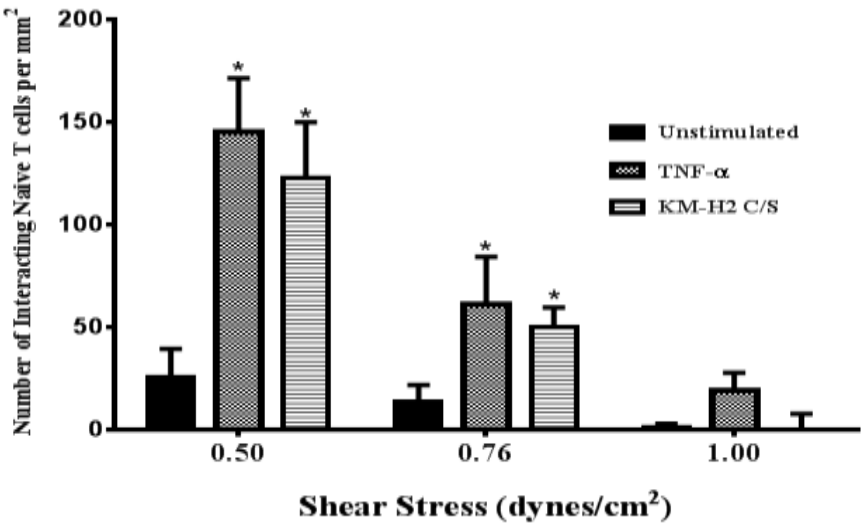
3.4 C/S activated endothelial cells exhibit enhanced interactions with T cells under dynamic flow condition

C/S stimulated endothelial cells supports higher number of naïve and memory T cell interactions (binding and rolling) under defined shear stresses as compared to unstimulated endothelial cells. As shown in Figure 3.4A and B, respectively, interactions of naïve and memory T cells with C/S stimulated endothelial cells are comparable to that of TNF-α stimulated endothelial cells. Interactions between naïve and memory T cells with C/S stimulated endothelial cells increased gradually as the shear stress was reduced from 1dynes/cm^2 to 0.5 dynes/cm^2. The interactions of memory T cell with TNF-α or C/S stimulated endothelial cells are always higher than the naïve T cells at all three shear stresses. This is not surprising since memory T cells but not naïve T cells have enhanced LFA-1, VLA-4 and associated with enhanced capacity to bind ICAM-1 and VCAM-1 on stimulated endothelial cells (Shimizu et al., 1990).

I decided to focus on examining the interactions between naïve T cells and C/S stimulated ECs in my subsequent experiments. This is because naïve T cells have a high plasticity and have been shown to be differentiated into regulatory T cells by HRS cells *in-vitro* (Tanijiri et al., 2007).

A

Naïve T cells



B

Memory T cells

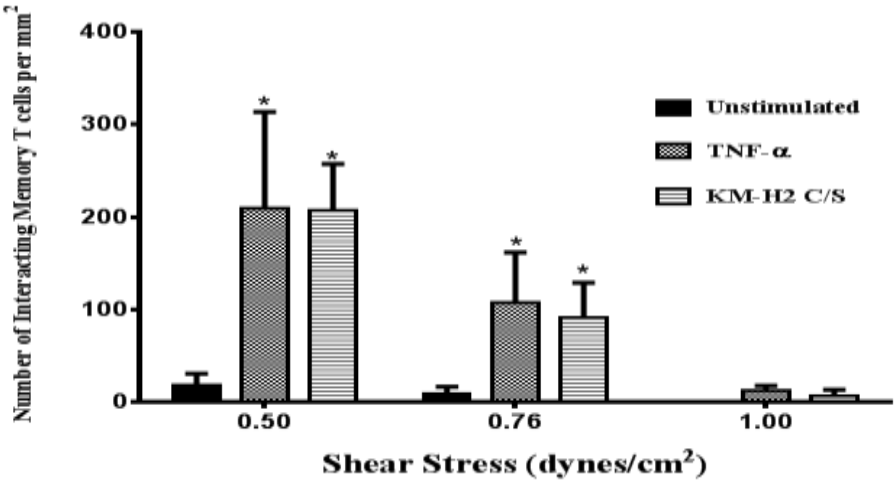


Figure 3.4: KM-H2 C/S stimulated endothelial cells mediate more interactions with naïve and memory T cells than unstimulated endothelial cells under defined shear stresses. KM-H2 C/S stimulated endothelial cells showed higher number of interactions with (A) naïve and (B) memory T cells under decreasing shear stresses as compared to unstimulated endothelial cells. Unstimulated and TNF-α stimulated endothelial cells served as negative and positive controls, respectively. Values shown are the mean $\pm$ SEM from five different experiments. * indicates statistical difference compared to unstimulated endothelial cells at $p<0.05$.

3.5 ICAM-1 and HA expressed on the C/S stimulated endothelial cells mediate naïve T cell-endothelial cells interactions

Next I carried out a series of function blocking experiments to identify the adhesion molecules that are involved in mediating the interactions between naïve T cells and C/S stimulated endothelial cells. The naïve T cells were treated with monoclonal antibody against L-selectin, CD 11a, CD18 and CD44. Antibodies against ICAM-1, VCAM-1 and E-selectin were used to block these adhesion molecules expressed on the endothelial cells. Figure 3.5A shows that blocking of ICAM-1 on the C/S stimulated endothelial cells, but not E-selectin and VCAM-1, significantly reduced naïve T cell interactions by 49±22%. Treating of endothelial cells with an antibody against MHCI, a binding non-blocking control, did not inhibit the naïve T cell interactions. Since ICAM-1 is important in regulating naïve T cell-C/S stimulated endothelial cells interactions, I first block LFA-1, the receptor of ICAM-1 on naïve T cells. LFA-1 is made up of 2 integrin subunits, CD18 (β2 integrin) and CD11a (alpha-L integrin). Interestingly, blocking data shows that blocking CD18 but not CD11a is effective in reducing the observed naïve T cell interactions (Figure 3.4B). This is probably due to the fact that integrins on naïve T cells are of low affinity and therefore the ligand binding site of LFA-1 is only partially exposed (Pribila et al., 2004).

L-selectin is the main surface molecule that mediates tethering and rolling of naïve T cells during their recirculation through lymphoid tissues (von Andrian and Mempel, 2003). Surprisingly, blocking of L-selectin on naïve T cells could only reduce naïve T cell interactions with C/S stimulated endothelial cells by 58±12%. This suggests that another molecule expressed on the naïve T cells may also be contributing to the tethering and rolling of naïve T cells to C/S stimulated endothelial cells.

Interestingly, blocking of CD44 adhesion molecules on the naïve T cells successfully inhibited the number of interacting naïve T cell by 78±14%. CD44 is a T cell activation marker and naïve T cell expresses low level of

CD44 (Mackay et al., 1990). CD44 had been reported to be involved in activated T cell migration (Baaten et al., 2010). Yet, blocking study showed that CD44 plays a major role in naïve T cell-C/S stimulated endothelial cells interactions (Figure 3.5C, black bar). This observed interaction is specific for C/S stimulated endothelial cells as blocking CD44 on naïve T cells did not affect the naïve T cell interactions with TNF-α stimulated endothelial cells (Figure 3.5C, white bars).

The binding partner of CD44 is HA (Peach et al., 1993). The data suggests that C/S stimulated endothelial cells could express HA on the surface which interacts with CD44 on the naïve T cells. To verify the presence of HA on C/S stimulated endothelial cells, the endothelial cells were treated with hyaluronidase to digest off any HA present on the surface of endothelial cells. Hyaluronidase treatment significantly reduced the number of interacting naïve T cells by 58±4% as compared to untreated negative control (Figure 3.5D). Treating the C/S stimulated endothelial cells with deactivated (boiled) hyaluronidase did not inhibit the observed naïve T cell-C/S stimulated endothelial cell interactions (Figure 3.5D, white bar). The data strongly suggests that HRS cell derived factors in the C/S could stimulate endothelial cells to upregulate HA production.

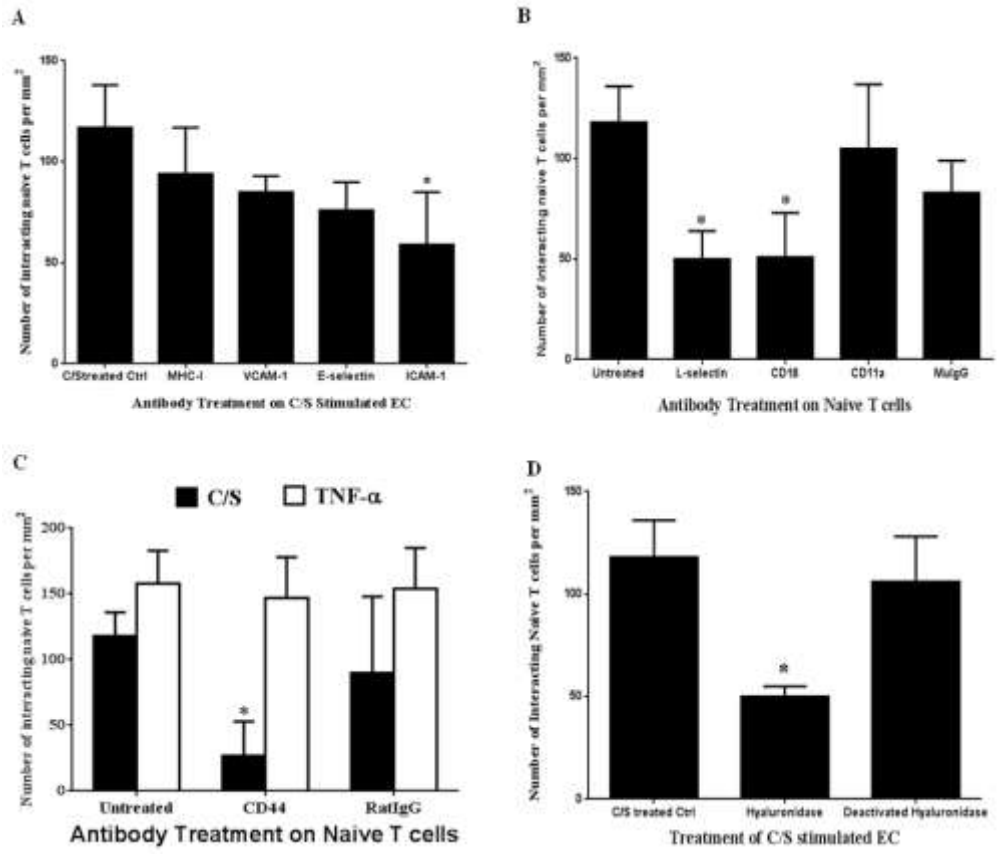


Figure 3.5: Naïve T cell interactions with KM-H2 C/S stimulated endothelial cells are mainly mediated by ICAM-1 and HA on the endothelial cells; and L-selectin, CD44 and β2-integrin on the naïve T cells. (A) Blocking of ICAM-1, but not VCAM-1 and E-selectin expressed on C/S stimulated endothelial cells inhibited naïve T cell interactions. (B) Blocking of CD18 and L-selectin on the naïve T cells significantly reduced the interaction between the T cells and C/S stimulated endothelial cells. (C) Blocking of CD44 function on the naïve T cells inhibited naïve T cell interactions with C/S stimulated endothelial cells but not TNF-α stimulated endothelial cells. (D) Treatment of C/S stimulated endothelial cells with 50μg/ml of hyaluronidase showed a drastic reduction in the number of interacting naïve T cells. Treatment with deactivated (boiled) hyaluronidase was used as the negative control. Values shown are the mean $\pm$ SEM from three different experiments. * indicates statistical difference compared to untreated endothelial cells (or untreated naïve T cells) at $p<0.05$.

3.6 C/S stimulated endothelial cells exhibit enhanced naïve and memory T cell transmigration in response to SDF-1α (CXCL12)

Transmigration of naïve and memory T cell across C/S stimulated endothelial cells in response to SDF-1α is enhanced significantly as compared to unstimulated endothelial cells (Figure 3.6A and B). Treatment of naïve T cells with G-protein coupled receptor inhibitor, pertussis toxin, partially reduced the number of transmigrated naïve T cell across C/S stimulated endothelial cells (Figure 3.6A). However, the number of transmigrated naïve T cell across TNF-α and unstimulated endothelial cells showed only a slight reduction after pertussis toxin treatment. The data suggests that transmigration of naïve T cells across C/S stimulated endothelial cells in response to SDF-1α is mediated primarily by G-protein coupled receptor. In contrast, transmigration of memory T cell in response to SDF-1α is sensitive to PTX treatment. In fact, treatment of memory T cells with pertussis toxin successfully reduced the number of transmigrated memory T cell to basal level in all the endothelial cell conditions tested (Figure 3.6B).

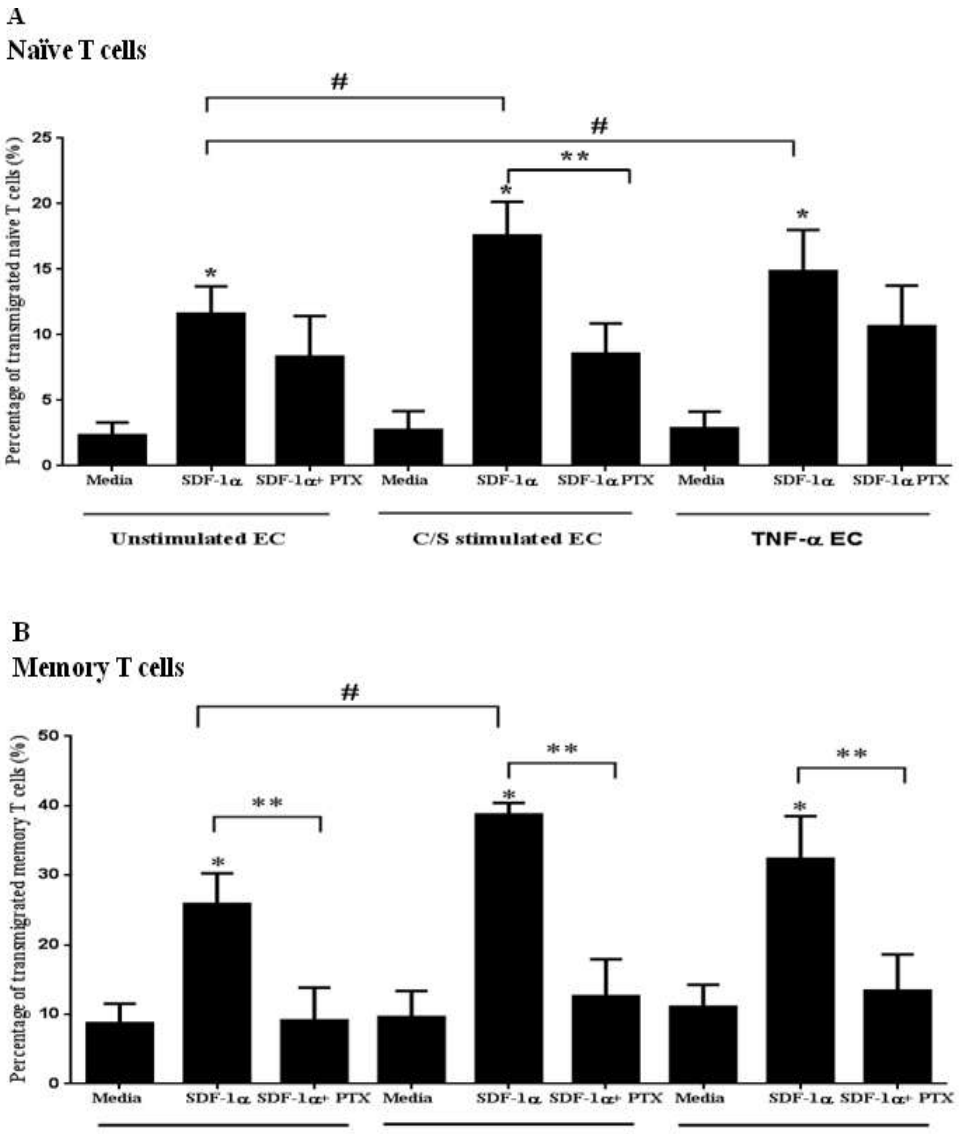


Figure 3.6: KM-H2 C/S stimulated endothelial cells show enhanced T cell transmigration in response to SDF-1 α (CXCL12). (A) Naïve T cells efficiently transmigrate across KM-H2 C/S and TNF-α stimulated endothelial cell monolayers in response to SDF-1α. Responsiveness of naïve T cells to SDF-1α is only partially pertussis toxin sensitive. (B) Memory T cells efficiently transmigrate across KM-H2 C/S stimulated and TNF-α stimulated endothelial cell monolayers in response to SDF-1α. Responsiveness of memory T cells to SDF-1α is pertusis toxin sensitive. Values shown are the mean $\pm$ SEM from four different independent experiments. * indicates statistical difference compared to untreated endothelial cells in response to SDF-1α at $p<0.05$. # indicates statistical difference compared unstimulated endothelial cells in the presence of SDF-1α at $p<0.05$. ** indicates statistical difference compared pertussin toxin treated naïve (or memory T) cells transmigrated across endothelial cells in response to SDF-1α at $p<0.05$.

3.7 C/S activation of endothelial cells is NFκB dependent

I carried out a series of protein analysis experiments to better understand the mechanisms through which KM-H2 C/S stimulated endothelial cells utilize to up-regulate adhesion molecule expression. Western blot analysis showed that stimulation of endothelial cells with C/S as early as 15 minutes can induce higher phosphorylated ERK and p38 expression. JNK activity assay was used to measure the JNK activity on the endothelial cells by detecting phosphorylated c-Jun expression. Data from JNK activity assay (Figure 3.7A, bottom panel), showed that C/S stimulated endothelial cells exhibit higher expression of phosphorylated c-Jun compared to unstimulated endothelial cells (Figure 3.7A). This implies that the KM-H2 C/S up-regulates JNK activity in the C/S stimulated endothelial cells. Besides that, KM-H2 C/S stimulated endothelial cells also expressed more nuclear p65 than unstimulated endothelial cells. The level of expression of nuclear p65 in the C/S stimulated endothelial cells was comparable to TNF-α stimulated endothelial cells (Figure 3.7B).

To verify the importance of nuclear p65 expression in regulating adhesion molecule expression on the stimulated endothelial cells, the endothelial cells were pretreated with the NFκB inhibitor, Bay 11-7085, prior to stimulating with KM-H2 C/S. Bay 11-7085 inhibits NFκB activity by inhibiting IkBa phosphorylation thus preventing the translocation of NFκB into the nucleus (Hu et al., 2001). The amount of Bay11-7085 used is twice the dose recomended in the product data sheet to inhibit 50% of TNF-α induced phosphorylation of IκBα (IC_{50}). Pretreatment of endothelial cells with the NFκB inhibitor, Bay 11-7085, prior to C/S stimulation could prevent nuclear p65 translocation. A Western blot analysis showed that nuclear p65 expression was abolished almost completely in Bay 11-7085 treated endothelial cells as compared to untreated and vehicle-treated control, DMSO (dimethyl sulfoxide), amount of DMSO used was 0.2µl / 1ml media (volume/ volume) (Figure 3.7C, upper panel). To confirm that nuclear p65 protein expression in C/S stimulated endothelial is important for the up-regulation of adhesion molecule expression, I assayed the endothelial cells for cell surface expression

of various inducible adhesion molecules. An ELISA analysis showed that pretreatment of endothelial cells with Bay11-7085 did not up-regulate ICAM-1, VCAM-1 and E-selectin expression. The expression level of ICAM-1, VCAM-1 and E-selectin on Bay 11-7085-treated C/S stimulated endothelial cells was similar to unstimulated endothelial cells (Figure 3.7C; lower panel).

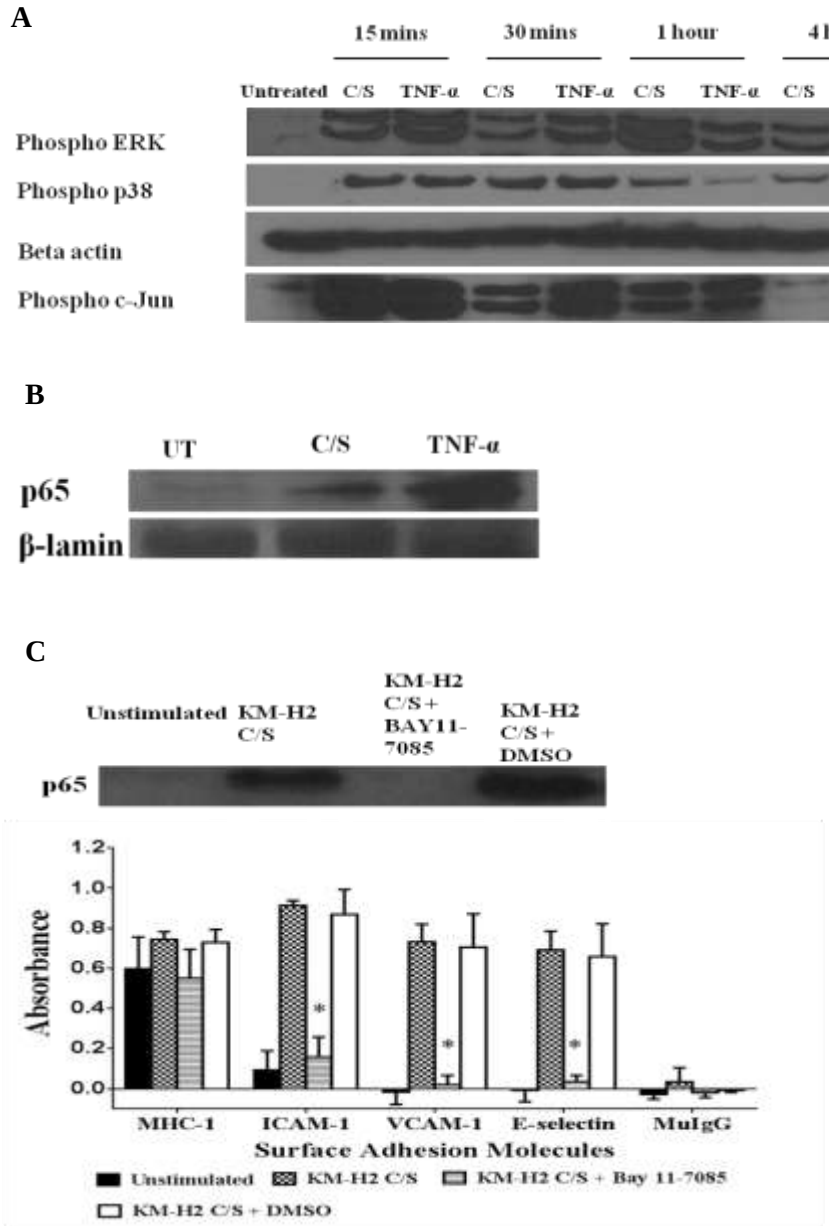


Figure 3.7: Up-regulation of adhesion molecule expression on KM-H2 C/S stimulated endothelial cells. (A) Western blot analysis showed that KM-H2 C/S stimulated endothelial cells expressed higher level of phosphorylated ERK and p38 as well as phosphorylated c-Jun as compared to unstimulated endothelial cells. Beta actin was used as loading control for cytoplasmic proteins. (B) C/S stimulated endothelial cells showed higher nuclear p65 expression as compared to unstimulated endothelial cells. TNF-α and unstimulated EC served as the positive and negative controls, respectively. B-

lamin was used as a loading control for nuclear protein. (C) Western blot analysis showed reduced nuclear p65 expression after Bay 11-7085 treatment. Inhibition of nuclear p65 translocation with 20μM of Bay 11-7085 inhibitor prevented the up-regulation of inducible ICAM-1, VCAM-1 and E-selectin expression on C/S stimulated endothelial cells. Endothelial cells were pre-treated with 20μM of Bay 117085 before stimulation with KM-H2 C/S. The Western blot analysis shown is one representative of two (for A) or three independent experiments (for B and C). Values shown are the mean $\pm$ SEM from three different experiments for C. * indicates statistical significant compared to C/S treated endothelial cells at $p<0.05$.

3.8 HRS cells actively secrete various cytokines into the C/S

To determine the soluble mediator(s) secreted by the HRS cell lines, KM-H2, L1236 and L540, I did a cytokine multiplex assay. Data showed differences in the cytokine secretion profile between KM-H2, L1236 and L540 (Table 3.1). C/S derived from KM-H2 and L1236 cells contained significantly high concentration of IL-6 compared to L540 cell C/S which contained negligible level of IL-6. The concentration of TNF-α present in all three types of HRS cell-derived C/S was low and ranged between 145-230pg/ml. The concentration of other pro-inflammatory cytokines, including IL-1α, IL-1β, IFNγ and chemokine, IL-8 were either very low or not detected in the C/S from these three HRS cell lines (Table 3.1).

Cytokines	KM-H2 (pg/ml)	L1236 (pg/ml)	L540 (pg/ml)
TNF-α	145.7	228.4	156.1
IL-6	1777.2	>2000	60.0
IL-1α	<0	<0	<0
IL-1β	<0	<0	<0
IFNγ	8.2	62.3	62.3
IL-2	<0	<0	<0
IL-4	<0	<0	<0
IL-8	<0	<0	<0
IL-10	<0	<0	<0
IL-13	111.2	104.7	107.1

Table 3.1: HRS cells secrete various cytokines into the C/S. KM-H2, L1236 but not L540 secrete high levels of IL-6. The C/S from all three HRS cell lines contain very little TNF-α.

3.9 C/S derived IL-6 does not play any role in stimulating endothelial cells

Knowing that L540 cells C/S contains negligible amount of IL-6 and that C/S derived from L540 cell line could not stimulate endothelial cells, I suspected that IL-6 is the stimulatory factor in the KM-H2 and L1236 derived C/S. To determine the role of KM-H2 derived IL-6 in stimulating endothelial cells, IL-6 neutralizing antibody (10μg/ml) was used to neutralize IL-6 in the KM-H2 C/S prior to endothelial cell stimulation. The amount of IL-6 neutralizing antibody used was able to reduce IL-6 activity derived from breast cancer cell line, MDA-MB-231, in my laboratory mate's breast cancer metastatic project (data not shown). The up-regulation of ICAM-1, VCAM-1 and E-selectin on unstimulated endothelial cells; and endothelial cells activated with C/S alone, C/S treated with IL-6 neutralizing mAb and C/S treated with mouse IgG (control) was detected by ELISA. The data show that neutralization of IL-6 in the C/S prior to endothelial cell stimulation could not inhibit the up-regulation

of ICAM-1, VCAM-1 and E-selectin expression (Figure 3.8). The expression of all three inducible adhesion molecules was comparable across all conditions. Thus, IL-6 is not the dominant stimulatory factor present in the KM-H2-derived C/S.

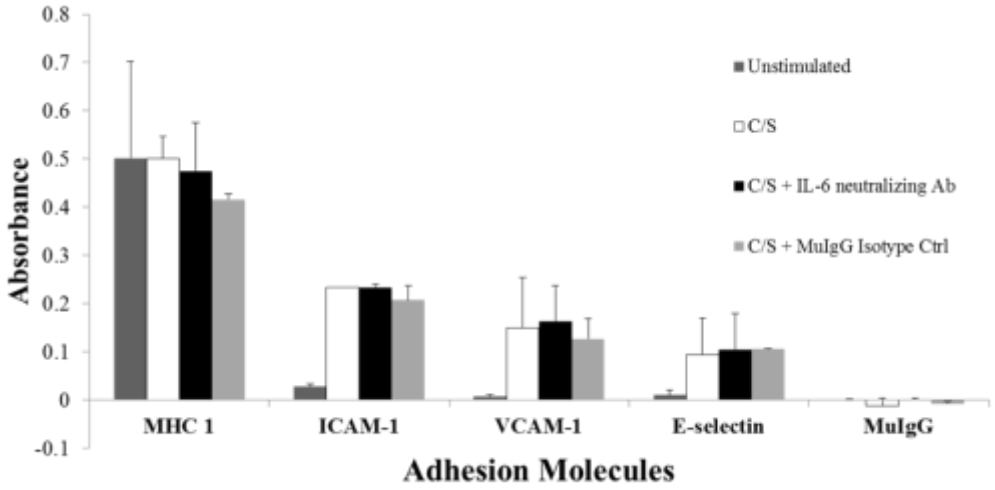


Figure 3.8: KM-H2 derived IL-6 is not involved in stimulating endothelial cells. Neutralizing IL-6 in the C/S did not have any inhibitory effect on the induction of adhesion molecule expression on the C/S stimulated endothelial cells. Values shown are the mean $\pm$ standard deviation from two different experiments.

3.10 TNF-α is not the dominant stimulating factor in the KM-H2 C/S

So far my data suggest that the C/S stimulatory effect on the endothelial cells is very much similar to the stimulatory effect of TNF-α. Hence, the next step was to determine whether the C/S contains KM-H2-derived TNF-α. To measure the bioactivity of TNF-α in the C/S, the L929 cell cytotoxic assay was used. As shown in Figure 3.9A, incubation of L929 cells with L1236, KM-H2 and L428 C/S for 12 hours induced extensive cell death of L929 cells (Figure 3.9A). However, pre-treatment of KM-H2 C/S with TNF-α neutralizing antibody did not improve the survival of L929 cells. As shown in Figure 3.8B (upper panel), incubation of L929 cells with 2-fold serially diluted C/S that were pre-treated with TNF-α neutralizing antibody did not rescue the viability of L929 cells compared to their respective untreated control condition. The concentration of the TNF-α neutralizing antibody used was sufficient to

neutralize 10ng/ml of recombinant TNF-α in control wells (Figure 3.9B, lower panel).

To further verify that TNF-α is not the stimulatory factor in KM-H2 C/S, I treated the C/S with neutralizing TNF-α antibody prior to endothelial cell stimulation. According to the ELISA result, the stimulatory effect of KM-H2 C/S on endothelial cells to induce the upregulation of ICAM-1, VCAM-1 and E-selectin was only slightly attenuated when the KM-H2 C/S was first pre-treated with TNF-α neutralizing antibody prior to use for endothelial cell stimulation. In contrast, the TNF-α-treated control wells showed significant reduction in inducing endothelial cell activation after being treated with the TNF-α neutralizing antibody (Figure 3.9C). Taken together, these data suggest that KM-H2-derived C/S contains an insignificant amount of TNF-α and TNF-α is not the dominant stimulatory factor in the C/S.

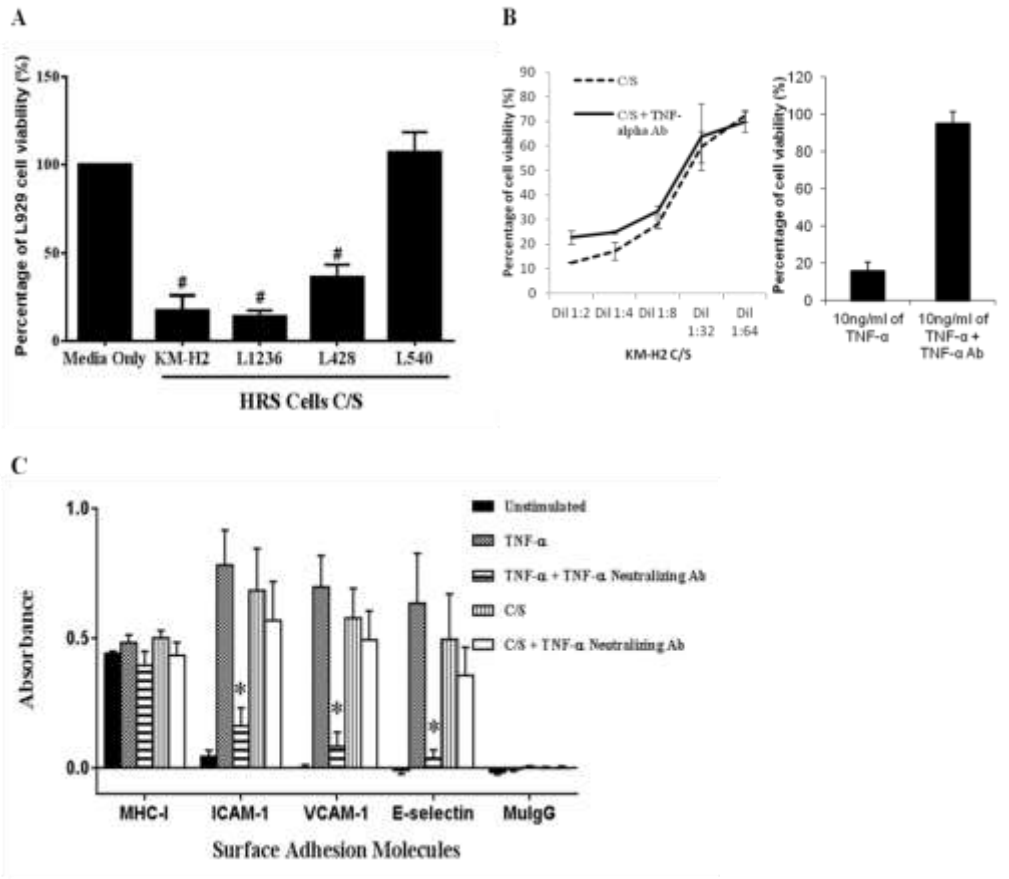


Figure 3.9: HRS cell C/S contains minimum amount of TNF-α. (A) While C/S derived from KM-H2, L1236 and L428 caused significant cytotoxicity of L929 cells, L540-derived C/S did not induce any L929 cell death. (B) Pretreatment of C/S from KM-H2 cells with the TNF-α neutralizing antibody prior to L929 cell incubation did not improved L929 cell viability. The dosage of TNF-α antibody used can effectively neutralize 10ng/ml of recombinant TNF-α *in-vitro*. (C) Neutralizing of TNF-α in the C/S did not have any profound inhibitory effect on the induction of adhesion molecule expression on C/S stimulated endothelial cells. Recombinant human TNF-α alone and recombinant human TNF-α pre-treated with TNF-α neutralizing antibody served as positive controls in this series of experiments. Values shown are the mean $\pm$ SEM from three different experiments. # indicates statistical difference compared to untreated L929 cells at $p<0.05$. * indicates significant difference compared to TNF-α stimulated endothelial cells.

3.11 HRS cells actively secrete LTα into the C/S

Data show that neutralizing TNF-α in the KM-H2 C/S did not improve L929 cell viability, thus suggesting a different mediator is killing the L929 cells. It has been previously reported that L929 cells are sensitive to both TNF-α and

lymphotoxin-α (LTα) (Cuturi et al., 1987). Furthermore, work published by Hsu et al suggested that KM-H2 and HDLM-2 cells can produce both TNF-α and LTα (Hsu and Hsu, 1989). Hence, LTα neutralizing antibody was used to determine whether biologically active LTα was present in the C/S. Pre-treatment of the KM-H2 C/S with 1µg/ml of LTα neutralizing antibody prior to incubation with L929 cells resulted in reduced cytotoxic effect of the C/S and an increase in survival of the L929 cells (Figure 3.10A, black bar). A similar finding was seen when L1236 and L428 C/S were treated with LTα neutralizing antibody prior to incubating with L929 cells (Figure 3.10A, stripped and blank bars). Treatment of the C/S with mouse IgG (non-neutralizing control) did not result in similar rescue of L929 cell survival. This suggests that HRS cells are producing LTα, a homologue of TNF-α, which is also cytotoxic to L929 cells.

Next I carried out intracytoplasmic flow cytometry taining to check for cytoplasmic LTα expression and determine the concentration of soluble LTα in the C/S by ELISA. Intracytoplasmic flow cytometry results show that LTα is present in L1236 and KM-H2 cells but not in L428 and L540 cells (Figure 3.10B). L1236 cells have the highest amount of intracytoplasmic LTα followed by KM-H2 cells. LTα ELISA result reveals a similar profile as the intracytoplasmic flow cytoplasmic staining. L1236 cells secrete the highest amount of LTα into the C/S, followed by KM-H2 cells and L428 cells (Figure 3.10C). Negligible amount of LTα was detected in the L540-derived C/S (18±20pg/ml). While intracytoplasmic expression of LTα was low (Figure 3.10B), LTα concentration in the L428-derived C/S was higher than expected (527±157pg/ml, Figure 3.10C). This suggests that LTα produced by L428 cells is rapidly secreted.

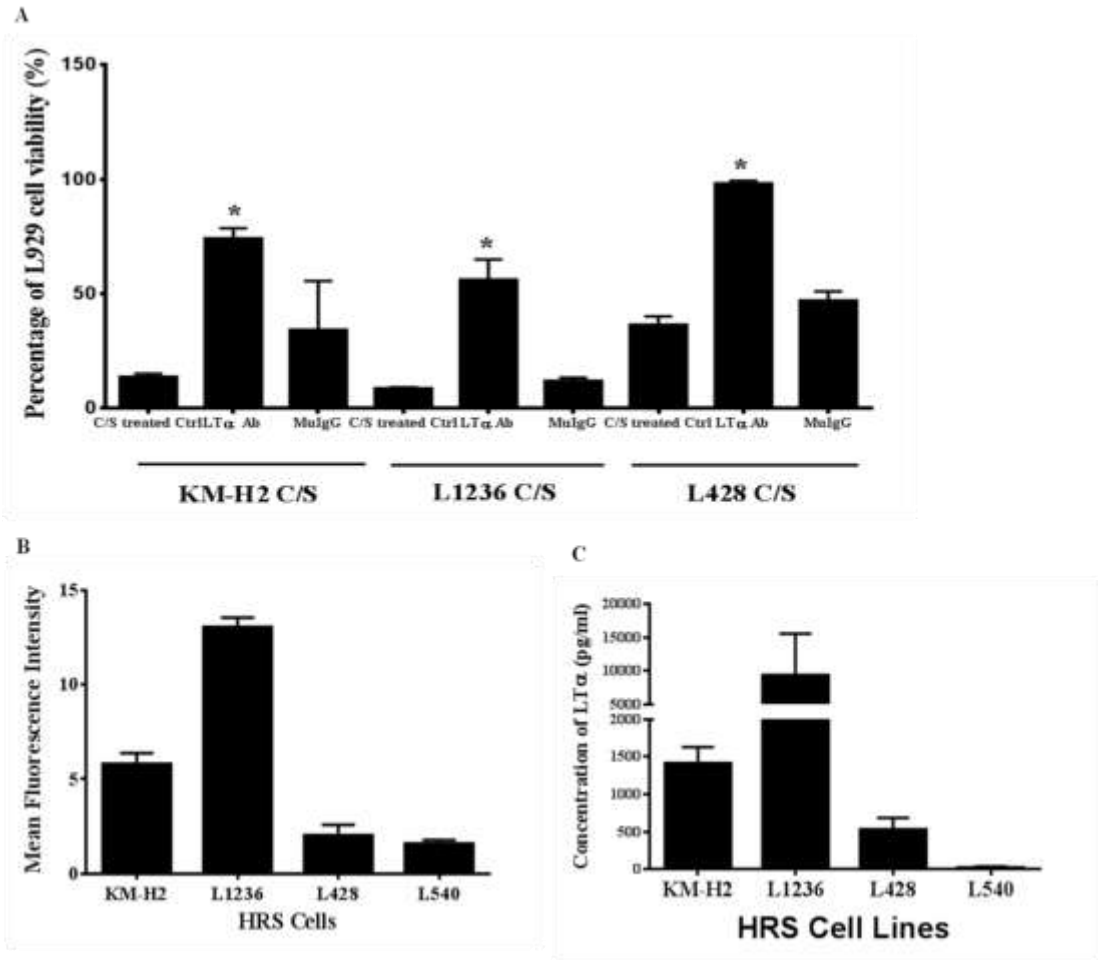


Figure 3.10: HRS cells actively produce LTα. (A) Neutralization of LTα in KM-H2, L1236 and L428 C/S improved viability of L929 cells compared to untreated and mouseIgG treated controls. (B) Intracytoplasmic flow cytometry staining showed that L1236 produced highest amount of LTα followed by KM-H2, L428 and L540. (C) LTα ELISA analysis showed that C/S derived from L1236 contained highest concentration of LTα followed by KM-H2 and L428. C/S derived from L540 cells contained negligible amount of LTα. Values shown are the mean $\pm$ SEM from three different experiments. * indicates significant difference compared to untreated control at $p<0.05$.

3.12 C/S derived LTα plays a significant role in stimulating endothelial cells

Next I proceed to confirm that LTα in KM-H2 C/S is the active soluble factor responsible for the stimulation of endothelial cells. As shown in Figure 3.11A, pre-treatment of KM-H2 C/S with LTα neutralizing antibody (1μg/ml) before use to stimulating endothelial cells successfully reduced the induction of ICAM-1, VCAM-1 and E-selectin expression by approximately 35±15% compared to their untreated and isotype control. Furthermore, endothelial cells stimulated with C/S pre-treated with LTα neutralizing antibody also show reduced naïve T cell interactions compared to untreated and mouse IgG treated control (Figure 3.11B). Taken together, my data suggest that HRS cell-derived LTα is the active factor that activates endothelial cells to facilitate T cell recruitment.

To determine whether my finding has clinical relevance, I carried out IHC staining for LTα expression on the paraffin tissue sections from two cohorts of cHL patients; from United Arab Emirates and from Singapore. Variable intensity of LTα staining was seen among the cases stained. Positive LTα expression could be detected in either in the HRS cells and /or in the stroma areas (Figure 3.11C). Collectively, strong LTα expression was detected in about 38.9% (7 out of 18 cases) and 28.5% (4 out of 14 cases), respectively, of the tissues samples from Singapore and United Arab Emirates (UAE) (Table 3.2)

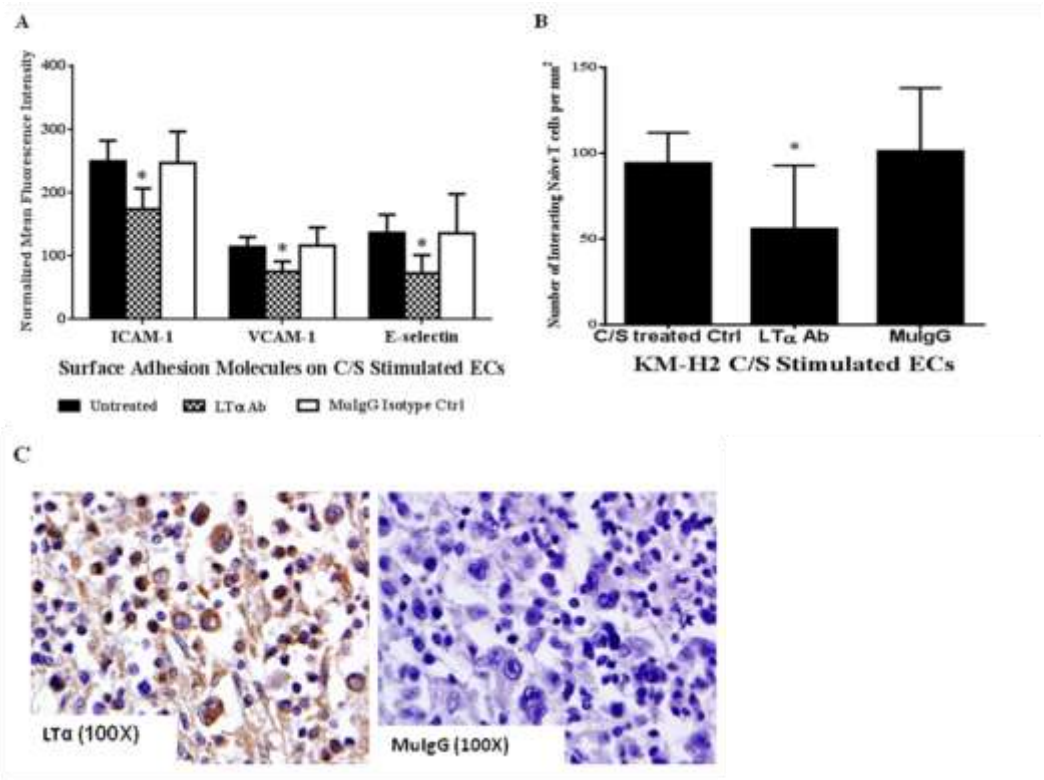


Figure 3.11: HRS cells produce biologically active LTα that stimulates endothelial cells to upregulate inducible adhesion molecules to facilitate naïve T cell interactions. (A) Neutralization of LTα in the KM-H2 C/S decreased the stimulatory effect of the C/S resulting in reduced induction of ICAM-1, VCAM-1 and E-selectin expression on endothelial cells as compared to untreated and mouse IgG isotype controls. (B) Neutralization of LTα in the C/S derived from KM-H2 cells prior to endothelial cell stimulation led to reduced naïve T cell interactions. The in-vitro flow chamber experiments were carried out at the defined shear stress of 0.36 dynes/cm^2. (C) Immunohistochemical staining of paraffinized tissue sections demonstrated LTα expression in HRS cells or in the stroma. The image shows a representative tissue sample with a LTα staining score of 3 compared to mouse IgG control. Values shown are the mean $\pm$ SEM from three different experiments for A and B. * indicates significant difference compared to untreated control at $p<0.05$. # indicates significant difference compared to untreated KM-H2 C/S stimulated endothelial cell at $p<0.05$.

cHL cases	Total cases stained	Strong	Medium	Weak
UAE	**14**	**4 (28.5%)**	**4 (28.5%)**	**6 (43.0%)**
Singapore	**18**	**7 (38.9%)**	**7 (38.9%)**	**4 (22.2%)**

Table 3.2: Summary of LTα scores for 32 cases of cHL screened. Overall, approximately 4 out of 14 cHL cases from UAE and 7 out of 18 cHL cases from Singapore show strong LTα expression.

3.13 NFκB activity in the HRS cells played a role in regulating LTα expression

There have been reports that suggested the presence of putative NFκB binding sites on the LTα promoter and enhancer region as reviewed by Shebzukhov and Kuprash (Shebzukhov Iu and Kuprash, 2011). Hinz et al showed that NFκB activity in HRS cells contributes to LTα production (Hinz et al., 2002). Hence, to confirm that the NFκB pathway also regulates LTα expression in my cells, I used the NFκB activity inhibitor, Bay 11-7085 to inhibit NFκB activity in the KM-H2 cells. As shown in Figure 3.12A, after 12 hours of inhibitor treatment, nuclear p65 expression in the Bay 11-7085-treated KM-H2 cells was inhibited in a dose dependent manner. Consistent with this, the concentration of LTα in the C/S from the inhibitor treated KM-H2 cells was also reduced in a dose-dependent manner compared to C/S from vehicle-treated (DMSO) KM-H2 cells (Figure 3.12B). Maximum reduction (approximately 39±14%) was seen when 20μM of Bay 11-7085 was used. Treatment with Bay 11-7085 and inhibition of NFκB activity could only partially reduce LTα production; thus suggesting that other signaling pathways might be involved.

To better understand the regulation of LTα production in the KM-H2 cells, I also analyzed the expression of various AP-1 complex components in the Bay 11-7085-treated cells. Western blot analysis showed that treatment of KM-H2 cells with Bay 11-7085 does not affect cytoplasmic phosphorylated JNK and total JNK expression (Figure 3.12C, upper panel). Interestingly, Bay 11-7085-treated KM-H2 cells show increased nuclear phosphorylated c-Jun expression in a dose dependent manner. Total c-Jun expression, however, remained unchanged. Furthermore, Bay 11-7085 treatment at the highest dose of 20μM, also resulted in reduced nuclear phosphorylated c-Fos and total c-Fos expression in the treated KM-H2 cells (Figure 3.12C). My findings suggest that inhibiting nuclear p65 expression on KM-H2 cells might have also affect AP-1 activity. Work done by Fujioka et al suggested that there is a correlation between NFκB activity and AP-1 activity. Abolishing NFκB activity will also

reduce AP-1 activity by reducing c-Fos protein expression (Fujioka et al., 2004).

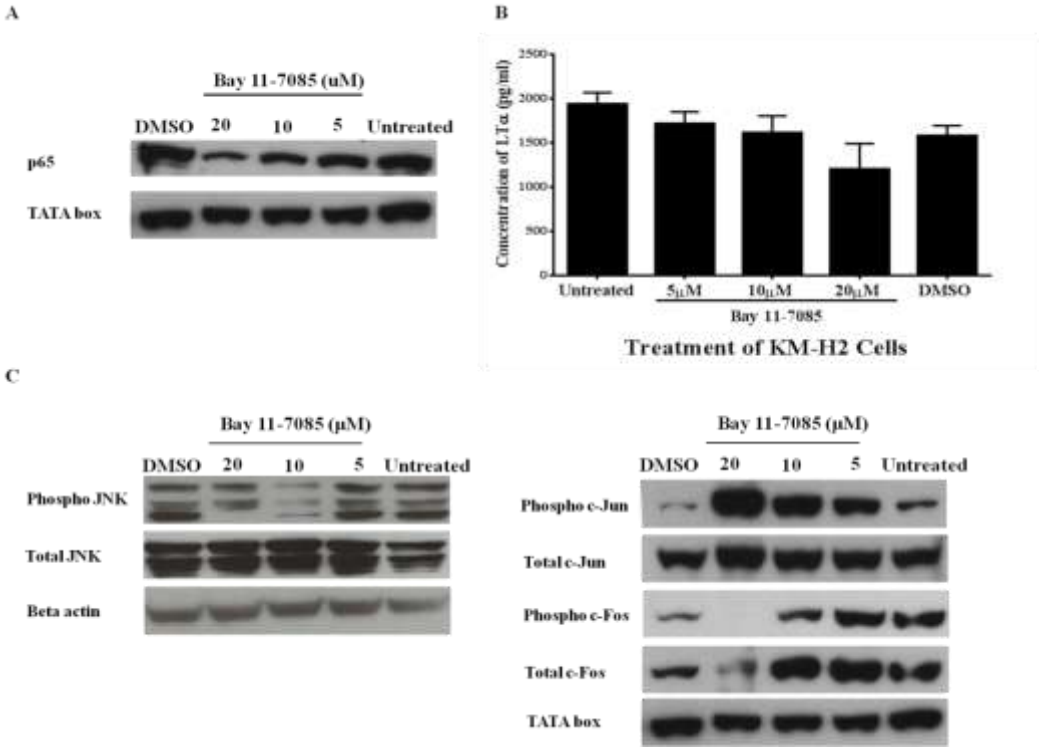


Figure 3.12: NFκB activity in HRS cells regulates LTα production. (A) Western blot analysis showed reduced nuclear p65 expression in the Bay 11-7085-treated KM-H2 cells in a dose dependent manner. TATA box served as nuclear protein loading control. (B) Treatment of KM-H2 cells with three different doses of Bay 11-7085, 5μM, 10μM and 20μM, reduced LTα production. (C) Western blot analysis showed that phosphorylated c-Jun expression increased at the 20μM inhibitor treatment. While phosphorylated and total c-Fos expression was reduced drastically at the highest concentration used, phosphorylated and total JNK expression remained unchanged. TATA box served as nuclear protein loading control. Beta actin served as cytoplasmic protein loading control. The Western blot shown is a representative picture for three independent experiments for A and C; and a representative experiment from two independent experiments for B.

3.14 The AP-1 transcription factor activity regulates LTα production in HRS cells

Subsequent literature search in combination with transcription factor database search (TFSEARCH) suggested that AP-1 could be one of the transcription factors involved in regulating LTα production. AP-1 is a transcription factor complex comprises of c-Jun and c-Fos. SP600125 is a reversible ATP-competitive JNK inhibitor which prevents phosphorylation of JNK thus concomitantly inhibiting phosphorylation of c-Jun and subsequently AP-1 transcription factor activity (Hibi et al., 1993). Treatment of KM-H2 cells with 40μM, 60μM, 80μM and 100μM of SP600125 reduced cytoplasmic phosphorylated JNK, nuclear phosphorylated c-Jun and total c-Jun expression (Figure 3.13A). C/S was harvested from KM-H2 cells treated with SP600125 for 12 hours to measure the concentration of secreted LTα. As shown in Figure 3.12B, SP600125 reduced LTα production in a dose dependent manner. The highest dose of SP600125 at100μM could reduce LTα production by 30±10% as compared to untreated KM-H2 cells.

Functional studies of C/S harvested from SP600125 treated KM-H2 cells were carried out using cell based ELISA and parallel flow chamber assay. C/S from KM-H2 cells treated with SP600125 (60μM and 100μM) exhibit reduced stimulatory effects compared to C/S from untreated KM-H2 cells. Induction of adhesion molecule expression on the endothelial cells stimulated by C/S harvested from SP600125 treated KM-H2 cells was about 30±10% lower that that induced by C/S from untreated KM-H2 cells (Figure 3.13C). The reduction is most evident with E-selectin expression followed by ICAM-1 and VCAM-1. Consistent with lower induction of inducible adhesion molecules on the endothelial cells stimulated with C/S from SP600125 treated KM-H2 cells, these endothelial cells also show reduced naïve T cell interactions at 0.76dynes/cm^2. The reduction of naïve T cells interactions is approximately 40±12% when compared to the untreated C/S stimulated endothelial cells (Figure 3.13D).

Work done by Mathas et al suggested that c-Jun expression is found in the HRS cells (Mathas et al., 2002). IHC staining data for c-Jun on the paraffin tissue sections also showed strong c-Jun expression in the HRS cells in the tissues samples from Singapore and United Arab Emirates (Figure 3.13E). Taken together, although JNK and c-Jun activity was completely abrogated, LTα production was only reduced by 30%. This suggests c-Jun and JNK activity may not be the dominant regulator of LTα production in KM-H2 cells.

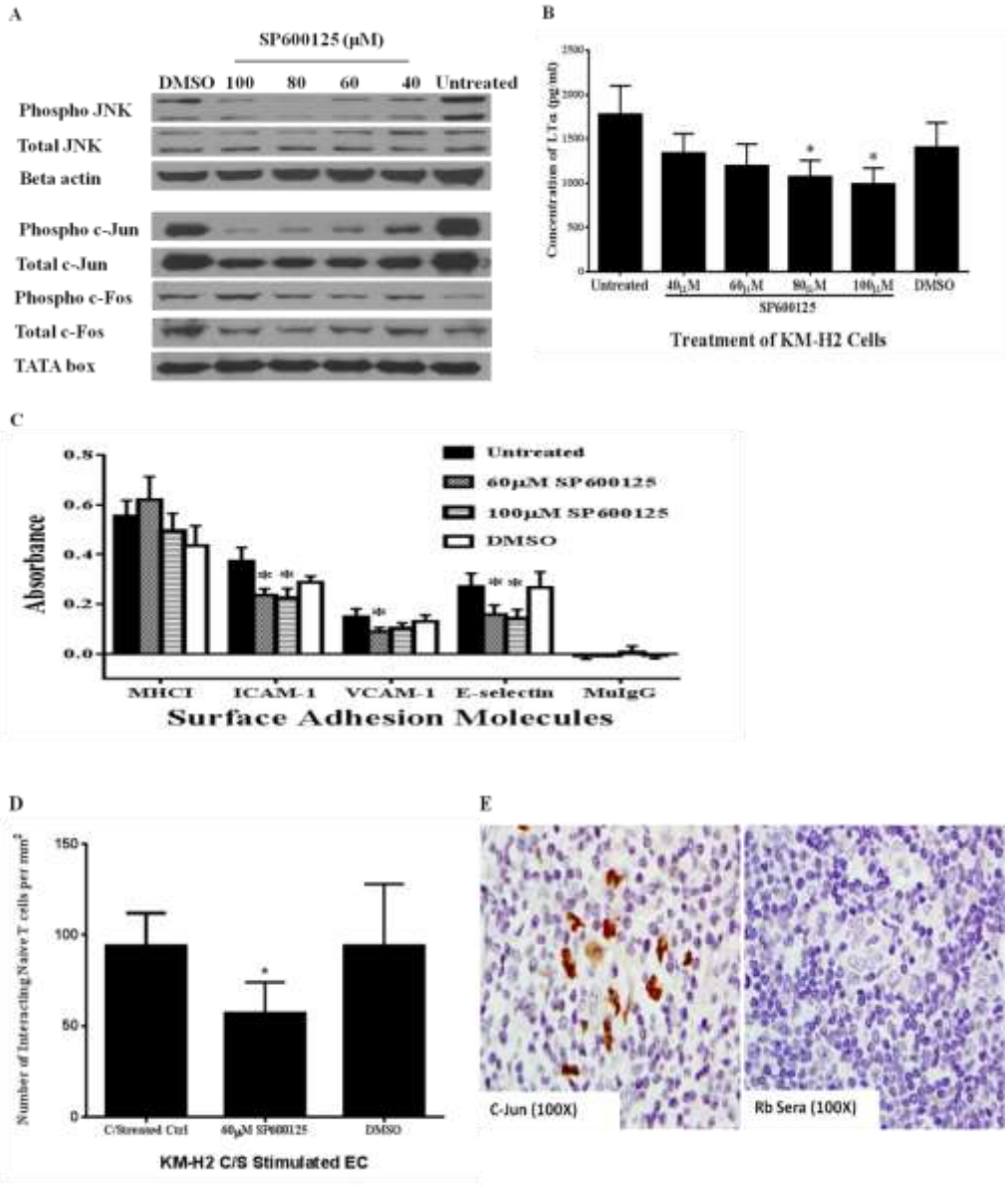


Figure 3.13: AP-1 transcription factor activity might not be the main regulator of LTα production in HRS cells. (A) A Western blot analysis showed that phosphorylated JNK and c-Jun were reduced significantly after drug treatment. In addition, total c-Jun was also reduced. However, both phosphorylated c-Fos and total c-Fos were not affected. The TATA box served as a nuclear protein loading control. Beta actin served as cytoplasmic protein loading control. (B) SP600125 treatment at 80μM and 100μM reduced LTα production by KM-H2 cells. (C) Endothelial cells stimulated with C/S from KM-H2 cells treated with 60μM or 100μM SP600125 showed lower adhesion molecule expression compared to respective control. (D) Fewer naïve T cell interactions were detected with endothelial cells activated with C/S derived from 60μM SP600125 treated KM-H2 cells at a defined shear stress of 0.76 dynes/cm^2. Endothelial cells stimulated with C/S from untreated KM-H2 cells or DMSO treated KM-H2 cells served as controls. (E) IHC staining demonstrated nuclear localization of c-Jun in the HRS cells. Rb Sera stained section served as negative control. Western blot analysis is a representative of

three independent experiments for A; values shown are the mean ± SEM from three different experiments for B, C and D; a representative case of c-Jun staining for E. * indicates significant difference compared to untreated KM-H2 cells (or untreated C/S stimulated endothelial cells) at $p<0.05$.

3.15 Cox-1 but not Cox-2 enzymatic activity regulates LTα production in HRS cells

Ferreri et al showed that PGE2 reduced LTα production by regulating its transcription on activated T cells (Ferreri et al., 1992). Besides that, PGE2 also plays a significant role in mediating homeostasis of the cHL tumor microenvironment (Kuppers et al., 1994). Cox-2 expression is found to be elevated in the HRS cells of cHL (Mestre et al., 2012). Since PGE2 is synthesized by Cox activity, I also investigated whether the Cox pathway has any role in the LTα production on HRS cells. To do this, KM-H2 cells were treated with 0.1mM, 0.3mM and 0.5mM of non-selective Cox inhibitor, Indomethacin. The LTα concentration in the C/S was measured after 12 hours of drug treatment. My data demonstrated that Indomethacin significantly reduced LTα production on the treated KM-H2 cells at the dose of 0.3mM (Figure 3.14A). However, Celecoxib, which is a Cox-2 selective inhibitor, did not have significant effect on LTα production on the treated KM-H2 cells at the ranged of dosage tested (Figure 3.14B). This data suggests that Cox-1 but not Cox-2 is the critical mediator regulating LTα production in KM-H2 cells.

Consistent with reduced LTα production, endothelial cells stimulated by C/S from the Indomethacin treated KM-H2 cells show reduced ICAM-1, VCAM-1 and E-selectin expression (Figure 3.14C). The most effective dose of Indomethacin used to reduce adhesion molecules expression is 0.3mM. Expression of adhesion molecules on the C/S stimulated endothelial cells was reduced by 67±14% on ICAM-1, 78±13% on VCAM-1 and 81±10% on E-selectin as compared to untreated control. Endothelial cells stimulated with C/S from Indomethacin treated KM-H2 cells also showed reduced naïve T cell interactions (Figure 3.14D). The number of interacting naïve T cells was

reduced by 58±16% when compared it to the untreated C/S stimulated endothelial cells.

A Western blot analysis showed that Indomethacin treatment increased cytoplasmic phosphorylated JNK and c-Jun expression at 0.3mM. Interestingly, Indomethacin treated KM-H2 cells reduced nuclear phosphorylated and total c-Fos expression. c-Fos expression diminished completely after 12 hours of Indomethacin treatment (Figure 3.14E, lower panel). However, nuclear p65 expression in Indomethacin treated KM-H2 cells remained unchanged. As shown in Figure 3.14F, combination treatment of Indomethacin (0.3mM) and SP600125 (60μM) did not improve the viability of L929 cells as compared to Indomethacin treatment alone.

Taken together, my data suggests that production of LTα in KM-H2 cells is predominantly mediated by Cox1 enzyme via the Cox pathway. Furthermore, data from my Western analysis suggests that that Cox-1 mediated LTα production might be signaling through AP-1 pathway.

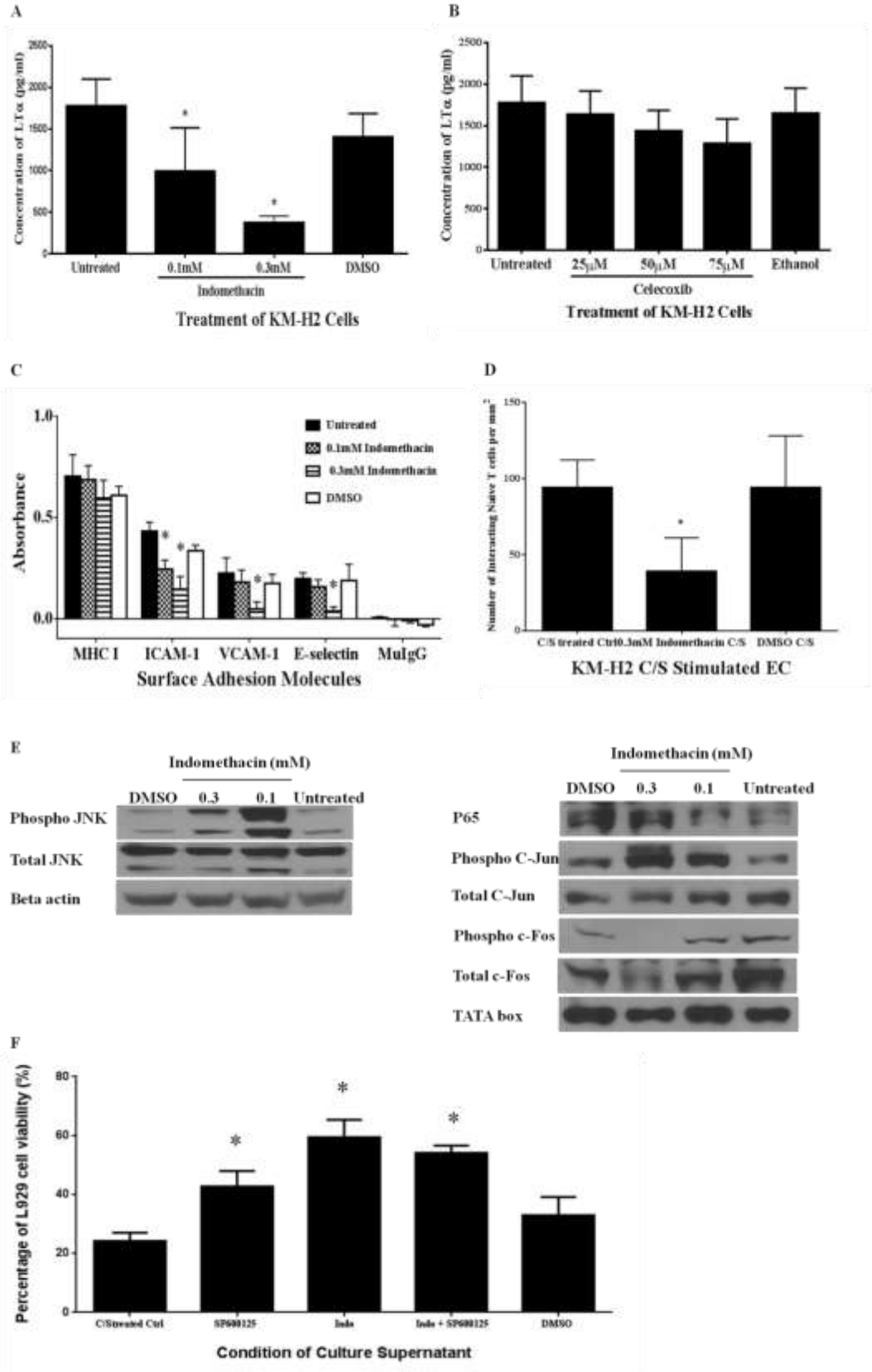


Figure 3.14: Cox-1 but not Cox-2 regulates LTα production in HRS cells. (A) Indomethacin treatment at 0.1mM and 0.3mM effectively reduced LTα production by KM-H2 cells as measure by LTα ELISA. (B) Celecoxib treatment at 25μM, 50μM and 75μM did not have any effect on LTα

production by KM-H2 cells. (C) Endothelial cells stimulated with C/S derived from KM-H2 cells treated with 0.3mM Indomethacin showed attenuated up-regulation of adhesion molecule expression. Untreated KM-H2 cells or DMSO treated KM-H2 cells served as untreated and vehicle-treated controls respectively. (D) Endothelial cells stimulated with C/S harvested from 0.3mM Indomethacin treated KM-H2 cells exhibit reduced naïve T cell interactions at a shear stress of 0.76dynes/cm^2 under defined flow condition. (E) Western blot analysis showed that increased phosphorylated JNK and c-Jun expression in 0.3mM Indomethacin treated KM-H2 cells. However, phosphorylated and total c-Fos expression in KM-H2 cells was completely abolished by the drug treatment. Nuclear p65 expression remained unchanged after drug treatment. TATA box protein served as nuclear protein loading control. Beta actin served as cytoplasmic protein loading control. (F) Combination treatment of SP600125 (60μM) and Indomethacin (0.3mM) is not more effective in reducing cytotoxicity of L929 cells compared to single treatment of Indomethacin. Values shown are the mean $\pm$ SEM from three different experiments for A, B, C, D and F; The Western blot analysis is a representative from three independent experiments for E. * indicates significant difference compared to untreated KM-H2 cells (or untreated KM-H2 C/S stimulated endothelial cells) at $p<0.05$.

Chapter 4: Discussion

cHL has a very unique tumor microenvironment (Liu et al., 2013). The cHL microenvironment resembles a typical inflammatory background. In the cHL tumor microenvironment, the minority tumor cells are surrounded by various immune infiltrates. HRS cells require a highly inflamed background to survive and grow well. To maintain a highly inflamed tumor microenvironment, HRS cells produce various cytokines and chemokines to actively recruit immune cells. Extensive studies had been done on studying cytokine production by the HRS cells and the role of HRS cell-derived chemokines on immune cell recruitment. T cell recruitment plays a significant role in the pathogenesis of the disease since HRS cells require survival signals from T cells. However, little is known about how HRS cells modulate the endothelial cells to facilitate T cell recruitment. This study describes the process by which I identify HRS cell-derived Lymphotoxin-α (LTα) as an active mediator that modulates endothelial cell function and T cell recruitment.

4.1 HRS cell-derived LTα stimulation of endothelial cells

The notion that endothelial cell function is modulated by soluble mediators derived from tumor cells is not new. Several studies have shown that soluble mediators by tumor cells can modulate endothelial cell function. Vidal-Vanalocha et al showed that B16M tumor cells can secrete products to induce hepatic sinusoidal endothelial (HSE) cells to produce IL-1, TNF-α and IL-18 that act as autocrine factors to promote VCAM-1 expression (Vidal-Vanaclocha et al., 2000). The study showed that addition of B16M conditioned medium to HSE cells resulted in increased number of B16M adherence. This was completely abrogated by neutralization of IL-18 which was responsible for the induction of VCAM-1 expression on HSE and tumour cell adhesion. Furthermore, the study also showed that IL-1β alone or together with TNF-α induced the production of IL-18 by HSE. More recently, Estrada-Bernal et al revealed that soluble products derived from Hodgkin lymphoma

cell lines have the ability to enhance E-selectin expression on stimulated endothelial cells and facilitate histiocyte cell line, U937, adherence (Estrada-Bernal et al., 2003). Finding in this study is consistent with their observation. Besides E-selectin, I also detected the up-regulation of ICAM-1 and VCAM-1, two other adhesion molecules that are critical in regulating endothelial cell-T cell interactions (Carlos and Harlan, 1994; Tedder et al., 1995). C/S derived from three out of four HRS cell lines (KM-H2, L1236 and L428 but not L540) exhibit stimulatory activity with compatible potency in activating endothelial cells. This suggests that different HRS cell lines have different cytokine expression profiles that contribute to the above observation.

The first two HRS cell lines, L428 and L540, were established in 1979 from patients with advanced stage Hodgkin lymphoma (clinical stage IVB) (Diehl et al., 1982). These cells originated from pleural effusion and bone marrow, respectively. With few exceptions, most of the established cell lines were established from bone marrow, pleural effusion and peripheral blood of advanced stage patients. So far, about thirteen cell lines had been established and described in the literature (Hoppe et al., 2007). Analysis of immunophenotype, karyotype, Immunoglobulin (Ig) or T cell receptor of these cell lines revealed heterogeneous results but evidence of their derivation from HRS cells was still lacking then (Diehl et al., 1990). In 1996, another new cell line, L1236 was established from primary peripheral blood mononuclear cells of a patient with advanced HL disease of mixed cellularity subtype. Single cell polymerase chain reaction (PCR) showed that the genomic sequence of Ig gene arrangement of the HRS cells in the bone marrow of patients was identical to that of L1236 cells (Wolf et al., 1996); thus confirming that L1236 was indeed derived from HRS cells. In this study I have carried out the experiments using four different HRS cell lines that are available commercially. As shown in my results, culture supernatants from three out of the four lines, including L1236, showed similar stimulatory effects on endothelial cells, thus suggesting that the active stimulatory factor which I have identified as LTα is made in three HRS cell lines of different origins.

LTα production by HRS cell lines remained controversial with different groups of researchers reporting differences in LTα production by the cultured HRS cells. Foss et al showed that L540 cells have comparable ability to secrete LTα compared to L428 and KM-H2 cells as determined by L929 cytotoxic assay (Foss et al., 1993). However, another report by Kretschemer et al showed that L428 cells can secrete LTα to induce high cytotoxic effect on L929 cells but L540 cells did not produce soluble LTα and cytotoxic effect was not observed. In addition, their report showed that L540 cells express LTα mRNA and immunostaining of L540 cells with LTα antibody showed fine granular cytoplasmic and pronounced Golgi reactivity. The author concluded that L540 could produce LTα which could not be secreted (Kretschmer et al., 1990). The finding is consistent with their observation. Intracytoplasmic flow cytometry staining revealed that L540 produced intracytoplasmic LTα to a level comparable to L428 cells. But the LTα ELISA revealed a very low concentration of LTα in the L540-derived culture supernatant (C/S) compared to L428-derived C/S. Similarly, Hsu and Hsu also proved that the HRS cell lines, KM-H2 and HDLM-2, can produce soluble LTα but they failed to localize LTα expression on the HRS cells in the HL tissue samples (Hsu and Hsu, 1989). Further evidence provided by Sappino et al proved the presence of LTα in the cHL lesion. Total cellular RNA was extracted from the cHL lymph node. LTα mRNA expression in the 21 out of 23 cases of cHL was found to have significantly high expression of LTα mRNA especially in the four cases of lymphocytic predominant subtype (Sappino et al., 1990). I have further confirmed my *in-vitro* findings with staining for LTα in paraffinized tissue sections from patients diagnosed with cHL. Albeit variable and weak in some cases, positive staining for LTα was detected in the HRS cells, further supporting the hypothesis that HRS cells *in-situ* can produce LTα to modulate behavior of cells in the microenvironment.

IHC staining of the tissue sections from cHL patients revealed differential expression of LTα in 32 cases investigated. In general, preliminary evidence suggests that LTα expression from Singapore cohort stained stronger than United Arab Emirates (UAE) cohort. The observed differences could be due to

technical issues such as preservation of antigen since the tissues from the two cohorts were processed differently. Alternatively, this could be due to different genetic background of the Singaporean population and UAE population. LTα polymorphism is studied extensively in the past few years and is linked strongly with various cancerous diseases. LTα polymorphisms cause different levels of LTα expression or bioactivity. The high bioactive genotype is associated with the risk of developing cancers of the lung (Shimura et al., 1994), colon or rectum (de Jong et al., 2002) and non-Hodgkin lymphoma (Wang et al., 2006). The low bioactive genotype is associated with the risk of developing cervical cancer (Niwa et al., 2005) and endometrial cancer (Niwa et al., 2007). To date, LTα polymorphism on the risk of Hodgkin lymphoma has not been reported.

However, it was previously shown that there was no correlation observed between the LTα gene expression and systemic symptoms (Sappino et al., 1990). Furthermore, Warzocha et al showed that plasma levels of LTα in HL patients with B symptoms was not different from normal healthy population (Warzocha et al., 1998). This suggests that the effect of LTα is more likely to be acting locally than systematically. Consistent with this, my data suggests that HRS cell-derived LTα can activate endothelial cells to facilitate the recruitment of T cells into the lesion.

4.2 Pathways involved in upregulation of adhesion molecules expression induced by LTα

In this study, I provided evidence that LTα derived from HRS cells can stimulate endothelial cells to up-regulate the expression of ICAM-1, VCAM-1 and E-selectin. Function of LTα in up-regulating inducible adhesion molecule expression was first described by Pober et al in 1987. LTα acts similarly as TNF-α causing a rapid and transient induction of E-selectin expression (peak 4 to 6 hours), gradual but sustained expression of ICAM-1 (plateau 24 hours)

(Pober et al., 1987). Besides that, LTα stimulatory effect on endothelial cells (human umbilical cord endothelial cells) in up-regulating adhesion molecule expression is also mentioned by Suna et al (Suna et al., 2008). LTα activates gene expression of VCAM-1 and E-selectin on the stimulated endothelial cells after two hours of stimulation as determined by DNA microarray analysis. Since LTα homotrimer only binds to TNFRI to elicit its function by the canonical NFκB pathway, this suggests that the observed activation on the endothelial cells is NFκB dependent (Aggarwal, 2003; Suna et al., 2009).

My data also strongly suggest that activation of endothelial cells by LTα is dependent on the NFκB pathway. Abrogating NFκB activity in the LTα containing C/S stimulated endothelial cells successfully prevented inducible ICAM-1, VCAM-1 and E-selectin expression. NFκB is an essential transcription factor that binds to the promoter region of ICAM-1 (Hou et al., 1994), VCAM-1 (Neish et al., 1995) and E-selectin (Schindler and Baichwal, 1994). Upon cytokine activation, p65 and p50 complexes will translocate into the nucleus and bind to the NFκB sites.

I also showed that KM-H2 derived LTα stimulated endothelial cells expressed higher level of phosphorylated ERK and p38 as well as JNK activity compared to unstimulated negative control. Work done by Suna et al suggested that expression of VCAM-1 induced by LTα is regulated primarily by NFκB and PI3K pathway. Besides that, inhibition of p38 and JNK activity by specific inhibitors could reduce LTα induced VCAM-1 expression as determined by Western blot analysis. Surprisingly, inhibition of ERK activity did not affect the expression of VCAM-1 (Suna et al., 2009). This implies that MAP kinase might play a role in regulating adhesion molecule expression on the endothelial cells induced by C/S derived LTα. The role of MAP kinase in regulating adhesion molecule expression is still controversial. Study by Zhou et al showed that ERK, p38 and JNK activity has no effect on inducible ICAM-1, VCAM-1 and E-selectin expression on the endothelial cells (Zhou et al., 2007) but other reports showed otherwise (Pietersma et al., 1997; Read et

al., 1997; Westra et al., 2005). Although, I also detected increases in the phoshorylation of ERK and p38 as well as enhanced JNK activity, the results show that NFκB is the dominant regulator of inducible adhesion molecule expression on endothelial cells.

4.3 Induction of adhesion molecules and naïve T cell recruitment

Ruco et al in 1992 showed that paraffinised lymph node sections of Hodgkin lymphoma patients expressed ICAM-1, VCAM-1 and E-selectin (Ruco et al., 1992). ICAM-1 expression was found to be present on all Hodgkin lymphoma tissues but VCAM-1 and E-selectin expression was correlated with IL-1/TNFα production in Hodgkin disease. Furthermore, they found that VCAM-1 and E-selectin expression were more pronounced in nodular sclerosis patients tissues (Ruco et al., 1992). This suggested that cytokines such as IL-1, TNFα and LTα, secreted by HRS cells can influence endothelial cell function and massive T cell infiltration observed in the Hodgkin lymphoma lymph node. Report by Machado et al further supports the above findings. They reported expression of ICAM-1, VCAM-1 and PNAd in the vasculature of HL tissues and concluded that the mechanism for T cell recruitment into HL lesion resembles that of naïve or central memory T cell migration into normal lymph node (Machado et al., 2009).

My study showed that ICAM-1, but not VCAM-1 and E-selectin, expressed on C/S stimulated endothelial cells play a role in mediating the observed naïve T cell interactions in-vitro. Function blocking of CD18 and LFA-1 on the naïve T cells using monoclonal antibodies further confirmed the importance of ICAM-1 in mediating naïve T cell binding to C/S stimulated endothelial cells. The data is expected since naïve T cells express low level of α_4 integrin or VLA4 and are therefore inefficient in binding to VCAM-1 on the stimulated endothelial cells. Even though E-selectin had been shown to interact with L-selectin, this interaction has only been reported for neutrophils (Kishimoto

et al., 1991). Kishimoto et al showed that neutrophil binding to endothelial cells was dependent on ICAM-1 and E-selectin. Blocking of L-selectin on the neutrophils or E-selectin on the endothelial cells reduced neutrophil adhesion to endothelial cells (Kishimoto et al., 1991). E-selectin-L-selectin interaction on naïve T cell has not been reported so far. This is not unexpected since E-selectin expression is usually inducible by pro-inflammatory cytokines at peripheral inflammatory sites; and naïve T cells are programmed to recirculate lymphoid tissues and not peripheral inflammatory sites. Interestingly, data from my study further suggest that up-regulation of HA expression on these HRS cell-activated endothelial cells also contributes toward naive T cell recruitment.

Numerous studies had been done to examine T cell recruitment so as to provide a better understanding of the observed T cell profiles in cHL lymph nodes. Chemokine expression profile on HRS cells had been studied extensively. Various chemokines are believed to contribute to the pathogenesis of cHL. Most of these studies were carried out using immunohistochemistry staining of cHL tissue sections or HRS cell lines. By IHC staining, HRS cells were found to secrete monokine induced by interferon gamma (MIG), interferon gamma inducible protein-10 (IP-10) and thymus and activation regulated chemokine (TARC) (Ohshima et al., 2002). It was reported that mixed cellularity cHL secretes more IP-10, MIG and TARC then the nodular sclerosis cHL. This difference in chemokine expression profile contributes to a more pronounced $T_{Helper}2$ cell infiltration observed in mixed cellularity cHL than nodular sclerosis cHL (Ohshima et al., 2002). Using *in-vitro* models, Van den Berg et al showed that HRS cell lines, L428, L1236 and L540, have high expression of TARC compared to non-Hodgkin cell lines and EBV-transformed B cell lines (van den Berg et al., 1999).

All the studies to date correlate chemokine expression in HRS cells with specific T cell subset recruitment into the cHL lesions. This is the first that examines how HRS cells could directly modulate the surrounding

microenvironment, particularly the endothelial cells, to influence T cell recruitment into the lesions. Using a combination of expression profiles and cell function assays, data show that HRS cells can, indeed, modulate endothelial cell activity to facilitate the recruitment of naïve T cells.

Until recently, all the studies had only provided evidence of activated T cell recruitment into the cHL lesions, and mostly on the memory T cell phenotype (Poppema, 1989). Recruitment of naïve T cells might also contribute to the pathogenesis of cHL. Tanijiri et al suggested that KM-H2 cell line could induce immunosuppressive regulatory T cells from naïve T cells (Tanijiri et al., 2007). Their work has offered an alternative hypothesis that regulatory T cell population observed in the cHL lesions could arise from naïve T cells recruited into the cHL lymph node. The notion that naïve T cells could be recruited into the lesions to be modulated into regulatory T cells to create the immunosuppressive environment seen in cHL is novel and fascinating.

4.4 Interaction of HA expressed on C/S stimulated endothelial cells with CD44 on naïve T cells

L-selectin is reported to be a homing receptor of lymphocytes to peripheral lymph nodes and to have an important role in initiating the adhesion of leukocytes to high endothelial venules, the first important step of the multistep leukocyte adhesion cascade. L-selectin is also important for the recruitment of leukocytes into peripheral inflammatory sites. Ligands of L-selectin include PNAd (peripheral lymph node addressin), PSGL-1 (P-selectin glycoprotein ligand-1), CD34 (Rosen, 2004) and GlyCAM-1 (Glycosylation dependent cell adhesion molecule-1) (Hwang et al., 1996). L-selectin on lymphocytes has also been reported to associate with VLA-4 and CD11/CD18 complex on TNF-α stimulated endothelial cell (Spertini et al., 1991). L-selectin ligands can be induced optimally by lipopolysaccharide and IL-1β whereas, IL-4 and

IFN-γ have been reported to down-regulate the expression of L-selectin ligands (Spertini et al., 1991).

L-selectin is critically important in regulating naïve T cell initial interactions with endothelial cells. Indeed, the data show that blocking of L-selectin function on the naïve T cells could partially reduce their interactions with both TNF-α-activated and culture supernatant (C/S)-activated endothelial cells. However my data also suggest that naïve T cells do not depend solely on L-selectin to interact with C/S stimulated endothelial cells. In fact, blocking of CD44 function on naïve T cells was more effective than blocking L-selectin in inhibiting naïve T cell interactions with C/S stimulated endothelial cells (Figure 3.4C). In contrast, blocking of CD44 did not have any inhibitory effect on naïve T cell-TNF-α stimulated endothelial cell interactions.

The function of CD44 as an adhesion molecule had been reported on activated T cells but not naïve T cells. Nacher et al showed that CD44 can associate with PSGL-1 to bind to E-selectin to induce firm arrest of activated/inflammatory T cells (Nácher et al., 2011). Using a mouse model, Nacher et al showed that activated CD4+ T cells used CD44 to interact with E-selectin expressed on the inflamed endothelial cells causes slower rolling on the T cells. Besides that, comparing the binding of the $T_{Helper}1$ cells generated *in-vitro* from CD44 deficient mice with wild-type mouse they concluded that CD44 could bind to soluble E-selectin suggesting that CD44 can serve as a ligand of E-slectin. In addition, this study also showed that both CD44 and PSGL-1 expressed on T cells can work together to support recruitment of activated T cells to the inflamed tissues *in-vivo*. Naïve T cells express low to moderate level of CD44 (Mackay et al., 1990). Upon activation, CD44 expression is rapidly increased and L-selectin is shed from activated T cells (Mackay et al., 1994). This property causes reduced homing of activated T cells to lymph node and increased probability of recirculating to inflamed sites.

The principal ligand of CD44 is HA which is a complex glycosaminoglycan that is also involved in wound healing (King et al., 1991), tumor growth (Oksala et al., 1995), adhesion and extravasation of lymphocytes (Jiang et al., 2011; Mohamadzadeh et al., 1998). Expression of HA on cultured endothelial cell lines and primary endothelial cultures is inducible by the pro-inflammatory cytokines such as TNF-α and IL-1β as well as lipopolysaccharide. However, this inducible HA expression is strictly restricted on the endothelial cells derived from microvascular but not large vessels (Mohamadzadeh et al., 1998).

Interaction of CD44 and HA is very well studied on memory T cells, activated T cell subsets and neutrophils (Butler et al., 2009). Bonder et al showed that T helper 1 and T helper 2 cells use CD44 to roll on and adhere to TNF-α stimulated microvasculature *in-vivo* (Bonder et al., 2006). Naïve T cells showed minimal interactions with HA coated coverslip *in-vitro* (Bonder et al., 2006). Siegelman et al also indicated that peripheral blood mononuclear cells increased CD44 expression upon activation and acquired enhanced capacity to bind to HA (Siegelman et al., 2000). The activated form of CD44 is associated with elevated expression of VLA-4, which is required for firm adherence of T cells following CD44-HA primary interactions on the endothelial monolayer (Siegelman et al., 2000).

However, my work showed that VCAM-1 on endothelial cells does not take part in naïve T cell-endothelial cell interactions. Degradation of HA on the endothelial cells by treatment with hyaluronidase successfully reduced the number of interacting naïve T cells with C/S stimulated endothelial cells. The data imply that soluble factor(s) in the C/S derived from HRS cells but not pro-inflammatory cytokine, TNF-α, could induce HA expression on the HUVEC, which are endothelial cells isolated from large vessels, the umbilical cord vein. Furthermore, naïve T cells can use CD44 to interact with the induced HA on the endothelial cells as an alternative to L-selectin-L-selectin

ligand interaction for tethering and rolling of naïve T cells prior to firm adhesion.

Increase in HA expression in lymphoma is not new. Hasselbalch et al observed increased serum level of HA in HL and non-Hodgkin disease (Hasselbalch et al., 1995). The level of HA was found to correlate with the tumor burden in the lymphoma patients compared to age-matched control group. They showed that serum HA levels in untreated patients with HL and non-Hodgkin disease were higher than in a healthy control group; and that the serum HA levels of relapse patients were higher than of untreated patients (Hasselbalch et al., 1995). Serum HA has also been reported to be elevated in several cancer types including lung, pancreas, breast, colorectal, ovary, sarcoma, stomach, prostate cancer and melanoma (Cooper and Forbes, 1988; Delpech et al., 1990; Manley and Warren, 1987; Yabushita et al., 2011).

The exact mechanism of how HA is synthesized in the tumor cells and the reason behind elevated serum HA in cancer patients remains unknown. One of the explanations offered was that in cancer patients the catabolism pathway of HA is disrupted and there is increased synthesis of HA in tumor tissues (Allerton et al., 1970). Another possibility is that cytokines or factors secreted by the tumour cells or by the body in response to the tumour could induce HA production. A previous study showed that LTα has the ability to induce HA synthesis on stimulated synovial fibroblasts (Butler et al., 1988). More interestingly, the synthesis of HA on the stimulated synovial fibroblasts was regulated by the endogenous cyclooxygenase product which could be inhibited by Indomethacin treatment (Butler et al., 1988). Similarly, a study published by Elias et al showed that recombinant LTα is able to induce human lung fibroblasts to up-regulate expression of HA (Elias et al., 1988). Besides that, LTα treatment could synergize with IFN-γ to further enhance the HA expression.

Here, I present new *in-vitro* evidence that LTα secreted into the C/S by HRS cells, can induce HA synthesis in the C/S stimulated endothelial cells. Furthermore, treatment of the HRS cells, KM-H2 with Indomethacin could effectively abrogate the observed LTα production and secretion. This, in turn, led to a reduction in the number of naïve T cells binding to the C/S-stimulated endothelial cells, presumably due to a decrease in HA production.

It is still unclear whether the increase in HA synthesis in many cancers is pro- or anti-tumour survival and progression. Data from this study offer a possible function for HA synthesis in HL. I postulate that HRS cells *in-situ* could induce HA expression on endothelial cells to facilitate naïve T cell recruitment into the lymphomatous lesions.

4.5 T cells transmigration across endothelial cells

Last step of lymphocytes emigration into the inflammatory sites involves transmigration across the inflamed endothelial monolayer. The data show that C/S stimulated endothelial cells have a higher capability than unstimulated endothelial cells to support naïve and memory T cell transmigrations in response to SDF-1α. SDF-1α was used in this project because SDF-1α is expressed by HRS cells (Ohshima et al., 2003) and both naïve and memory T cell were shown to respond to SDF-1α (Ding et al., 2000). This implies that C/S derived from HRS cells can stimulate endothelial cells to facilitate T cell transmigration into the cHL lesions which express a vast variety of chemokines.

In the cHL microenvironment, HRS cells are surrounded by an enormous number of T cells. The recruited T cells within the cHL lymph node lesions are predominantly $T_{Helper}2$ and regulatory T cells subtype (Kuppers et al., 2012; Ohshima et al., 2003). Chemokines are actively secreted by HRS cells to

attract the different subsets of T cells (Ma et al., 2008; Peh et al., 2001; van den Berg et al., 1999). Van den Berg et al showed that HRS cell lines, L428, L1236, L540 and L591, express different level of TARC and CCR4 receptor (receptor of TARC) in culture (van den Berg et al., 1999). However, non-Hodgkin large B cell lymphoma cell lines, ROSE and VER; anaplastic large cell lymphoma cell line, KARPAS 299, and EBV transformed cell lines, RAY and POP, did not express TARC and CCR4 expression on these cell lines were highly variable. Subsequent experiments proved that TARC expression was found in the cytoplasm of the primary HRS cells in the tissues sections of cHL of mixed cellularity and nodular sclerosis subtypes. Expression of CCR4 was not only found on the HRS cells but also on the T cells surrounding the HRS cells implied that TARC may be responsible for the attraction of CCR4 expressing $T_{Helper}2$ cells into cHL lesions (van den Berg et al., 1999).

Similarly, Ohshima et al showed that differential expression of chemokines in the cHL contributes to the recruitment of different T cell subsets into the cHL lesions. Their study demonstrated that cHL of mixed cellularity subtype expressed more chemokines than cHL of nodular sclerosis subtype (Ohshima et al., 2002). In the study, mixed cellularity subtype expressed TARC, IP-10 and MIG however, expression of these three chemokines was not consistently found in all the nodular sclerosis subtype. In addition, $T_{Helper}2$ lymphocytes were found significantly higher in cases expressing TARC, IP-10 and MIG (Ohshima et al., 2002). A subsequent study by Niens et al showed that serum level of TARC and MDC were elevated in HL patients (Niens et al., 2008). Comparison of pre- and post-treatment serum samples from nine HL patients showed that serum levels of TARC and MDC reduced drastically after treatment (Niens et al., 2008). High serum levels of TARC and MDC could be associated with infiltration of reactive lymphocytes into cHL.

Ma et al and Ishida et al showed that cultured HRS cells actively secrete chemokines into the C/S (Ishida et al., 2006; Ma et al., 2008). Ma et al showed that HRS cells lines, L1236, KM-H2, L428 and DEV, secrete RANTES,

TARC, IP-10 and MIF into the culture supernatant (Ma et al., 2008). *In-vitro* study by Ishida et al showed that HRS cells, L1236, L428, L540, KM-H2 and HDLM-2, secrete higher level of TARC as compared to Anaplastic large cell lymphoma (ALCL) cells, SUDHL-1, KARPAS (Ishida et al., 2006). However, only two HRS cell lines, HDLM-2 and L1236, produced MDC at concentrations greater than 1ng/ml. Chemotactic assay showed that C/S derived from HRS cells but not ALCL cells exhibited chemotactic effect on the $CD4^+$ T cells. The transmigrated $CD4^+$ T cells expressed $CD4^+CD25^+CCR4^+$. The transmigrated cells also exhibited regulatory T cell functions by inhibiting proliferation and IFN-γ production of normal $CD4^+$ T cells upon stimulation with TCR ligand in a co-culture system. The study concluded that transmigration of regulatory T cells in the cHL lesions is mediated by HRS cell-derived TARC or MDC.

4.6 Cytokines Profile of HRS Cell Lines

HRS cells require a highly inflamed tumor microenvironment to survive. To create and sustain this tumor microenvironment, HRS cells actively produce various cytokines and chemokines to modulate the surrounding cells to shape a highly inflamed background. Most published studies on cytokine production by HRS cells are based on products of commercially available HRS cell lines or gene array of microdissected primary HRS cells from cHL tissues section (Skinnider and Mak, 2002).

Consistent with these reports, data from my cytokine multiplex assay showed that cultured HRS cell lines produce IL-6, TNF-α, IFN-γ and IL-13 but do not express IL-1α, IL-1β, IL-2, IL-4 and IL-10. According to the study by Klein et al most of the HRS cell lines, KM-H2, L428 and L540, do not express IL-1α, IL-1β and IL-8 (Klein et al., 1992). However, Hsu et al shown IL-1 activity in the C/S derived from KM-H2 cells by using IL-1 sensitive T cell proliferation assay (Hsu et al., 1989). IL-1 was shown to be expressed by primary HRS

cells by *in-situ* hybridization (Xerri et al., 1992). My finding that IL-1α, IL-1β and IL-8 are produced by the cultured HRS cell lines is consistent with Klein et al. This difference in IL-1 secretion by KM-H2 cells from different groups, including me, once again reinforces the fact that culture conditions can influence cell behaviour. In this project, I focused on identifying the soluble mediator(s) in the C/S that contribute to the activation of the endothelial cells. The absence of IL-1β in the HRS cell-derived C/S has ruled out the possibility of IL-1β in contributing to the observed activation of endothelial cells.

Kapp et al and Skinnider et al showed that the four HRS cell lines, L1236, KM-H2, L428 and HDLM-2, could produce large amount IL-13 (Kapp et al., 1999; Skinnider et al., 2002). Besides that, Kapp et al also identified by *in-situ* hybridisation that 86% of primary HRS cells in the nodular sclerosis cHL were IL-13 positive. At the IL-13 protein level, Ohshima et al demonstrated that IL-13 was found exclusively in HRS cells and rarely in other cells within the reactive infiltrates (Ohshima et al., 2001).

C/S derived from KM-H2, L1236 and L540 cells contained low concentrations of IL-13 (about 100pg/ml) in my system. It was previously shown that IL-13, which shares a common receptor with IL-4, can induce VCAM-1 but not ICAM-1 and E-selectin expression on the endothelial cells (Bochner et al., 1995). Since all three adhesion molecules were up-regulated in the C/S-stimulated endothelial cells, this implies that IL-13 is not the active factor that activates endothelial cells in the *in-vitro* model. However, IL-13 is important for HRS cell survival. Kapp et al and Skinnider et al showed that proliferation of HDLM-2 and L1236 can be inhibited by neutralizing IL-13 in the culture (Kapp et al., 1999; Skinnider et al., 2002). This suggests IL-13 served as an autocrine growth factor for HRS cells.

I found limited quantity of TNF-α in the C/S of the four HRS cell lines used in this study. Detection of TNF-α expression in HRS cells in clinical samples by

either IHC or *in-situ* hybridisation have been reported by many different groups (Benharroch et al., 1996; Foss et al., 1993; Hsu and Hsu, 1989; Kretschmer et al., 1990; Ruco et al., 1992; Sappino et al., 1990; Xerri et al., 1992). Furthermore, high plasma levels of TNF-α, soluble TNF receptor 1 (p55) and TNF receptor 2 (p75) in the HL patients could be correlated to clinical features and clinical outcomes (Warzocha et al., 1998). They showed that high plasma level of TNF-α, p55 and p75 were associated with a lower incidence of complete responsiveness to therapy, a shorter progression free survival and overall survival of these patients (Warzocha et al., 1998).

I detected high concentrations of IL-6 in the C/S of, L1236 and KM-H2 but not L540 cells. Interestingly, the production of IL-6 in KM-H2 cells remained an unexplained puzzle. Jucker et al and Foss et al showed that IL-6 mRNA was not detectable in KM-H2 cells but high level of IL-6 was detectable in the C/S (Foss et al., 1993; Jucker et al., 1991). IL-6 gene expression was also demonstrated in microdissected primary HRS cells of both the nodular sclerosis and mixed cellularity subtypes (Karube et al., 2006). In fact, IL-6 expression in HRS cells was found to be higher than that in germinal centre cells. In addition, IL-6 expression was significantly higher in EBV positive cases of cHL compared to EBV negative cases (Herbst et al., 1997).

IL-6 was shown to exhibit stimulatory effect on endothelial cells both in-vitro and *in-vivo* (Romano et al., 1997; Watson et al., 1996). Activation of endothelial cells with recombinant IL-6 for 4 or 24 hours could up-regulate ICAM-1, VCAM-1 and E-selectin expression to enhance endothelial monolayer adhesiveness for binding of lymphocytes (under static condition) in a dose dependent manner (Watson et al., 1996). Using an *in-vivo* mouse model, Romano et al show that IL-6 required help from soluble IL-6 receptor to transduce activation signal into the endothelial cells (Romano et al., 1997; Watson et al., 1996). According to Romano et al, IL-6 with its soluble receptor could bind to gp130 to form a complex and activate endothelial cells to up-

regulate ICAM-1, MCP-1 (CCL2, monocyte chemoattractant protein-1) and IL-8 possibly through STAT3 mediated pathway (Romano et al., 1997).

IL-6 has been shown to be important clinically. Kurzrock et al reported that increased IL-6 level in the serum of Hodgkin lymphoma patients correlated with the presence of B-symptoms and significantly shortened overall survival of patients with advanced or relapsed Hodgkin disease (Kurzrock et al., 1993). Similarly, Reynolds et al also confirmed that HRS cell-derived IL-6 in cHL patients is associated with poor response to therapy and increase prevalence of 'B' symptoms (Reynolds et al., 2002). In 2004 Cozen et al showed that polymorphism of the IL-6 promoter is related to the risk of young adult HL (Cozen et al., 2004). They show that the risk decreases with increasing number of C alleles. The CC (low secreting) allele is associated with a decreased risk of young adult HL relative to GG (high secreting) allele. However, IL-6 is not the dominant factor that activates endothelial cells in my experimental set-up.

4.7 NFκB Pathway Regulated LTα Production

NFκB activity has been shown to be an important component in the immune response. NFκB activity is required to regulate cytokine production by immune cells, particularly T cells (Li and Verma, 2002). Examples of cytokines that are regulated by NFκB include IL-2, TNF-α and IFNγ (Blackwell and Christman, 1997). In addition, NFκB activity had also been proven to be highly activated at the site of inflammation in many diseases, such as inflammatory bowel disease, rheumatoid arthritis, psoriasis and asthma as reviewed by Li and Verma et al (Li and Verma, 2002).

Hodgkin disease is a lymphoid malignancy with aberrant constitutive NFκB activity. The predominant role of NFκB activity in the HL is to regulate transcription of various survival promoting proteins to enhance survival of

HRS cells. NFκB activity is required to transcribe various genes including the cell cycle regulatory protein cyclin D2, anti-apoptotic proteins Bfl-1/A1, c-IAP-2, TNFR-associated factor I, BcL-$X_{L,}$ and the cell surface receptors CD40 and CD86 which promote survival and growth of HRS cells (Hinz et al., 2001). In a following report, Hinz et al showed that NFκB activity can also induce STAT5A, IL-13 and CCR7 expression on the HRS cell lines, L1428 and HDLM-2. Together, these factors could synergize with NFκB to inhibit apoptosis and regulate cell cycle progression (Hinz et al., 2002). Besides that, NFκB expression in HRS cells can also regulate chemokine production. Liu et al showed that HRS cell line, L428, produces huge amount of CCL5 (RANTES) in the culture supernatant as compared to diffuse large B cell lymphoma cell line, Ly1 and Ly8. CCL5 derived from the L4228 cells has chemotactic effect on the CD4+ T cells. Expression of CCL5 by L428 cells was suggested to correlate with higher p65 expression in the L428 cells (Liu et al., 2011).

Interestingly, Hinz et al also suggested that NFκB could regulate LTα production in the HRS cells (Hinz et al., 2002). The study showed down-regulation of NFκB activity by adenovirus-mediated expression of IκB suppressor in L428 and HDLM-2 cells produce less LTα mRNA level as compared to untreated L428 and HDLM-2 cells. Thus, this implied NFκB is also involved in regulating LTα production. However, details of NFκB activity and LTα production at the protein level were not provided.

Further evidence on regulation of LTα production by NFκB was provided by B cells and T cells. In a study by Worm et al, CD40 ligation on B cells induced higher levels of LTα expression via NFκB activation. It was shown that CD40 responsive element on the LTα gene contains a NFκB binding sequence and mutation of the NFκB site within the region abolished CD40 induced LTα expression (Worm et al., 1998).

In 1990, Messer et al showed that NFκB can bind within the sequence at position -98 to -88 (5'-GGGGCTTCCCC-3') of the LTα promoter region (Messer et al., 1990). Upon phytohemagglutinin (PHA) or phorbol 12-myristate 13-acetate (PMA) treatment on Jurkat T cells, enhanced LTα production could be correlated with higher binding affinity of NFκB to the promoter region of LTα. Interestingly, stimulation of Jurkat cells with LTα promoted more LTα production with stronger DNA binding affinity of NFκB and transient increase in LTα mRNA suggesting that NFκB also plays a role in positive auto-regulation of LTα biosynthesis (Messer et al., 1990). Similarly, Paul et al showed that human T cell leukemia virus type I (HTLV-1) infected T cell lines have high amounts of LTα protein and mRNA expression (Paul et al., 1990). Detailed study revealed that HTLV-1 infected cells with active NFκB proteins can bind to the LTα κB binding site as shown by EMSA (electrophoretic mobility shift assay) analysis. Cells with mutant κB binding site did not bind NFκB proteins and showed reduced LTα production.

My data also show that LTα produced by HRS cell line, KM-H2, is regulated by the NFκB pathway. Blocking of NFκB activity with NFκB activity inhibitor, Bay11-7085, reduced p65 nuclear translocation in the HRS cells which correlated with reduction in soluble LTα production. This reduction of LTα production is probably due to reduced binding of NFκB to the promoter region of LTα. NFκB, however, is not the dominant signaling pathway that regulates LTα production in my model.

4.8 AP-1 regulated LTα production

The AP-1 complex is comprised of heterodimers of c-Fos and c-Jun or homodimers of Jun proteins or closely related proteins. AP-1 plays an important role in regulating cytokine production including IL-3 and IL-9, and mediating inflammatory response (Foletta et al., 1998). Besides that, AP-1 protein can also associate with other transcription factors to regulate TNF-α

and IFN-γ synthesis on activated T cells or monocytic cell lines (Foletta et al., 1998). A c-Jun/ATF2 protein combination in association with NFAT protein bind to the cAMP response element (CRE) site leading to TNF-α in the calcium and CD3 activated T cells (Tsai et al., 1996). In addition, c-Jun can co-operate with NFκB protein binding to the CRE site of TNF-α enhancer region in the LPS stimulated monocytic THP-1 cells (Yao et al., 1997). But AP-1 is not important to regulate cytokine production, IL-8, TNF-α, MIP-1α and MIP-1β, in LPS or TNF-α stimulated neutrophils (Cloutier et al., 2003). A study showed that treatment LPS- or TNF-α stimulated neutrophils with SP600125 did not inhibit inflammatory cytokine gene expression. This suggests that AP-1 regulated cytokine expression is cell line dependent.

HRS cells have constitutive AP-1 activity (Green et al., 2012; Juszczynski et al., 2007; Mathas et al., 2002). The AP-1 activity in HRS cells contributes to the modulation of an immunosuppressive environment and the regulation of cell cycle protein expression to promote the growth of HRS cells.

A report by Mathas et al showed that c-Jun and JunB were strongly expressed in the HRS cell lines and primary HRS cells from the tissues sections (Mathas et al., 2002). Constitutive AP-1 activity in the HRS cells regulated expression of cyclin D2, c-Met and CCR7. Transiently transfected HRS cells, L428, with a dominant negative AP-1 plasmid showed noticeable reduction of the production of these genes. In addition, using NFκB repressor plasmid transfected L428 cells, the authors revealed that AP-1 can work synergistically with NFκB to regulate cyclin D2 expression to enhance survival of HRS cells. Similarly, CCR7 and c-Met expression were also regulated by synergistic effect of AP-1 with NFκB (Mathas et al., 2002).

The AP-1 activity is also required to regulate galectin-1 production by HRS cells. Secretion of galectin-1 by HRS cells fostered a skewed T helper2 immunosuppressive microenvironment for the growth of HRS cells

(Juszczynski et al., 2007). The presence of galectin-1 in the co-culture system with T cells promoted the production of Th2 cytokines, IL-4, IL-5, IL-10 and IL-13. Besides that, galectin-1 also induced differentiation of PMA activated T cells into regulatory T cells. Recently, the AP-1 activity was also shown to regulate PD-L1 (programed cell death ligand 1) expression on the HRS cells (Green et al., 2012). Green et al showed that PD-L1 expression in HRS cells was augmented by the binding of AP-1 to the AP-1 responsive enhancer element in the PD-L1. In the study, transfection of L428 and L540 cells with PD-L1 luciferase plasmid containing AP-1 enhancer sites showed higher luciferase activity as compared to untreated and plasmid without the AP-1 enhancer site control cells. Furthermore, transfected L428 and L540 cells expressing a dominant negative c-Jun mutant significantly reduced PD-L1 expression. Thus, these studies show that AP-1/c-Jun activity contributes to many of the molecular features that we see in cHL.

As reviewed by Shebzukhov et al there have been reports that an AP-1 binding site is present on the promoter region and enhancer region of LTα gene (Shebzukhov Iu and Kuprash, 2011). This suggests that constitutive AP-1 activity in HRS cells might also be involved in regulating transcription of LTα. The data showed that cultured HRS cell line, KM-H2 exhibit high constitutive AP-1 activity and treatment of the KM-H2 cells with specific JNK inhibitor, SP600125 rapidly down-regulated phosphorylated JNK, c-Jun and total c-Jun expression to minimal levels. There was also a concomitant 30% reduction of LTα production. SP600125 is specifically designed to inhibit JNK activity and is very effective in reducing phosphorylation of c-Jun. The fact that an absolute loss of phosphorylated and total c-Jun only resulted in a 30% reduction of LTα production strongly suggest that AP-1 may be exerting its effects synergistically with another signaling pathway. This is not unexpected since the AP-1 protein can also associate with other transcription factors to regulate the production of various cytokines as reviewed earlier in my Introduction.

4.9 Cox Pathway Mediated LTα Production

The Cox enzymatic pathway plays a role in inflammatory responses and in the development of colorectal cancer, gastric cancer and breast cancer (Vane et al., 1998). Even though Cox activity on cancer development has been well established, the study of Cox activity in Hodgkin lymphoma is very limited. In 2006, Ohsawa et al showed that Cox-2 was detected in the HRS cells in HL tissues samples (Ohsawa et al., 2006). This expression of Cox-2 was not associated with p53 or bcl-2 expression. It was, however, associated with a higher cell proliferation rate and angiogenesis in HL. Recently, Mestre et al showed that Cox-2 was overexpressed on the HRS cells in about 30% of the HL patients (Mestre et al., 2012). The study proposed Cox-2 as an independent unfavourable prognostic factor in HL patients treated with ABVD. This suggested that increased Cox activity might contribute to the pathogenesis of HL.

Even though Cox-1 and Cox-2 enzymes produce the same products, their roles in inflammation appear to be segregated. Williams and Shacter et al showed that IL-6 synthesis on macrophages is linked to activation of Cox-2 but not Cox-1 (Williams and Shacter, 1997). Macrophages stimulated with serum albumin produced IL-6 upon up-regulation of Cox-2. Inhibiting Cox-2 activity with selective Cox-2 inhibitor, NS-398, reduced production of IL-6 but IL-6 production was unaffected by inhibiting Cox-1 activity. Results from a different study suggested that suppression of LPS-induced TNF-α secretion by murine peritoneal macrophages was mediated mainly by Cox-1-dependent prostaglandins and not by Cox-2-derived products (Rouzer et al., 2004). This suggests that Cox-1 and Cox-2 plays differential role in regulating or modulating cytokine production.

An involvement of Cox product in regulating LTα production has been previously reported. Ferreri et al showed that PGE2 negatively regulate LTα production by activated murine T cells (Ferreri et al., 1992). Furthermore, they showed that this decrease in LTα production was PGE2 specific as other Cox

products and intermediates including PGD2, PGF2 alpha, 5-hydroxyeicosatetraenoic acid and leukotriene C4 did not exert the same inhibitory effect on the activated T cells.

Interestingly, my data suggest that Cox-1 is more important than Cox-2 in mediating LTα production by HRS cells. KM-H2 cells treated with Indomethacin, a non-selective Cox inhibitor, and not those treated with Celecoxib, a specific Cox-2 inhibitor, showed a significant decrease in LTα production. My finding is consistent with a previous study by Butler et al who reported that LTα-induced HA synthesis by synovial fibroblast was regulated by the endogenous cyclooxygenase product which could be inhibited by Indomethacin treatment (Butler et al., 1988). The HRS cell-derived LTα could also induce HA expression on endothelial cells.

4.10 c-Fos, the possible dominant regulatory factor in LTα production

After knowing that inhibition of Cox-1 activity reduced LTα production, I investigated the various signaling pathway that could be activated in the KM-H2 cells following drug treatment. The first pathway that I investigated was the activation of NFκB in KM-H2 cells after Indomethacin treatment. However, I did not observe any changes in nuclear p65 expression in the Indomethacin treated KM-H2 cells. Next I examined the AP-1 pathway and found that phosphorylated and total c-Fos expression were down-regulated in Indomethacin treated KM-H2 cells. However, levels of phosphorylated c-Jun but not total c-Jun in KM-H2 cells were increased minimally. This suggests that Cox-1-mediated LTα production might involve c-Fos protein.

Cox products, particularly PGE2, had previously been shown to modulate AP-1 protein expression. DNA binding and transcriptional activity of AP-1 complex was significantly enhanced in PGE2 stimulated murine macrophages

as detected by luciferase assay (Iwahashi et al., 2000). Fitzgerald et al showed that PGE2 could induce c-Fos mRNA expression on osteoblasts via cAMP pathway (Fitzgerald et al., 2000). Besides osteoblast, PGE2 also induced c-Fos expression on the fibroblast cell line, Swiss 3T3, via the PKC pathway (Danesch et al., 1994). Consistent with these studies, the data in this study suggest that product(s) generated by constitutive Cox-1 activity is involved in some way in the regulation of c-Fos production, which in turn is essential for LTα production in KM-H2 cells. However, I have yet to identify the precise prostaglandin or intermediate that is responsible for regulating LTα production in HRS cells.

Interestingly, c-Fos protein expression on KM-H2 cells was also reduced after the NFκB activity was inhibited by Bay11-7085 treatment. This suggests that NFκB activity on KM-H2 cells might play a role in regulating c-Fos protein expression.

It was previously shown that AP-1 can work synergistically with NFκB to enhance the transcriptional activity. AP-1 and NFκB were shown to interact physically to enhance the binding capability of the complex to the NFκB or AP-1 response element within the 5' long terminal repeat of the human inmunodeficiency virus type 1 (Stein et al., 1993). Their data showed that the bZIP region of c-Fos and c-Jun could interact with NFκB/p65 through the Rel homology domain. Furthermore, their data showed that c-Fos and c-Jun only interact with the nuclear form of NFκB or more specifically p65 in the NFκB complex. The c-Fos and c-Jun interaction with p65 synergized with transcription of an AP-1 dependent promoter with a specific AP-1 tandem repeats binding site. The synergistic effect was not observed between JunB, JunD and p50 (Stein et al., 1993).

My data strongly suggest that LTα production in KM-H2 cells is regulated by c-Fos, Cox-1 enzyme and the NFκB pathway. However, it is currently unclear how the three signaling pathway come together to regulate LTα gene transcription.

4.11 HRS cells can modulate endothelial cell function to shape the microenvironment

This study showed that HRS cells could secrete soluble mediator(s) to modulate endothelial cell functions, focusing primarily on activation of endothelial cells and their interactions with T cells. However, the activity of endothelial cells and immune cell recruitment are probably only the many endothelial functions that are being modulated by HRS cells. For example, to sustain the growth and demands of the multiple cell types within the cHL lesions, it is also very likely that HRS cells can secrete factors to induce endothelial cell proliferation and promote angiogenesis.

There is little information to date regarding angiogenesis in HL. HL tissues were shown to express high level of EMMPRIN (extracellular matrix metalloproteinase inducer or CD174) (Thorns et al., 2002). In their study, EMMPRIN was expressed in about two-thirds of the 60 cases of Hodgkin lymphoma investigated. EMMPRIN is required to induce expression of VEGF and therefore plays an important role in angiogenesis (Tang et al., 2005). Hence, the presence of EMMPRIN in HL supports the notion that angiogenesis occurs in cHL lesions.

The presence of VEGF expression in the cHL microenvironment has also been reported. A study by Mainou-Fowler et al also showed that angiogenesis or micro-vessel density was associated with progression in HL disease (Mainou-Fowler et al., 2006). The study showed that VEGF expression was found on

endothelial cells of some micro-vessels and in follicular dendritic cells. VEGF expression, however, was not found on the HRS cells but they observed expression of platelet-derived endothelial growth factor (PdEGF, also known as thymidine phosphorylase), another angiogenic factor, in the cytoplasmic or nuclear of HRS cells. Conversely, Doussis-Anagnostopoulou et al reported cytoplasmic or perinuclear expression of VEGF protein in the malignant HRS cells in 25/32 cases of nodular sclerosis cHL investigated (Doussis-Anagnostopoulou et al., 2002).

VEGF enhances vascularization, is mitogenic for endothelial cells and increases micro-vessel permeability and plasma protein leakage (Senger et al., 1983). PdEGF promotes endothelial cell proliferation and chemotaxis *in vitro* and has angiogenic activity *in vivo* (Moghaddam et al., 1995). Angiogenic activity of PdEGF was demonstrated by Moghaddam et al using rat sponge model. In addition, endothelial cells stimulated with PdEGF showed higher migration ability in response to serum as compared to unstimulated endothelial cells. Expression of VEGF and PdEGF by HRS cells implies that HRS cells can produce this pro-angiogenic factor to modulate endothelial cell function to promote angiogenesis.

4. 12 Conclusion

It is well known that the malignant HRS cells in cHL directly or indirectly induce the production of a multitude of pro-inflammatory factors that act both locally and systematically. Yet, the cells could simultaneously create an abnormal immunosuppressive and tumour permissive environment within the affected lymph node. The mechanisms by which the HRS cells induce these changes are not well understood.

The cHL microenvironment consists of various inflammatory cells consisting of neutrophils, eosinophils, B cells, T cells and macrophages (Kuppers et al., 2012). How HRS cells regulate the trafficking of these cells into the lesion has been largely unexplored. In this project, I focused on how the HRS cells can modulate endothelial cell functions to facilitate T cell recruitment *in-vitro*.

I showed that HRS cell-derived LTα can induce ICAM-1, VCAM-1, E-selectin and HA expression on the endothelial cells and facilitate T cell binding and transmigration. I have also shown that while the activation of endothelial cells is dependent on the NFκB pathway, three pathways: NFκB, Cox-1 and AP-1 are involved in regulating LTα production in KM-H2 cells, the HRS cell line used in this study. My data further suggest that c-Fos protein regulated by NFκB and Cox pathway could work together with c-Jun to form an AP-1 complex to regulate LTα production. The proposed mechanism is shown Figure 4.1:

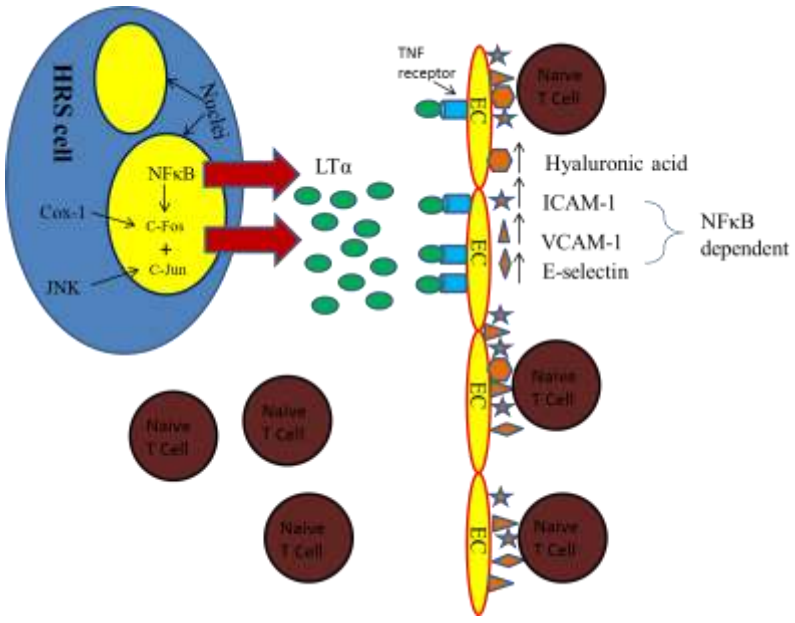


Figure 4.1: Diagram represents the proposed mechanisms of HRS cell-derived LTα in modulating endothelial cell function.

I believe that the data generated have provided insight as to how HRS cells *in-situ* may be modulating the microenvironment and the dynamic cell-cell interactions in the cHL lesion. An understanding of the mediators produced by the HRS cells and the mechanisms involved will allow us to design new therapeutics that will be helpful to alleviate inflammation, both locally and systemically. Similarly, knowing the signaling pathways involved and how HRS cells work will also allow us to design novel inhibitors that are more specific and with less long-term toxicity than current chemotherapeutic agents.

4. 13 Caveats of this study

In my system, I used endothelial cells isolated from human umbilical cord vein (HUVEC) to simulate endothelial venules in the cHL lymph node. The post capillary endothelial cells in the lymph node are termed High Endothelial Venule (HEV) which have slightly different adhesion molecule expression profile compared to normal microvasculature endothelial cells. HEV play an important role in regulating leukocyte transmigration into the lymph node by expressing organ-specific adhesion molecules, such as Peripheral node addressin (PNAd) which is not found on large vessels or microvasculature endothelial cells. PNAd is essential for the emigration of naïve T cells into the lymph node (Streeter et al., 1988). However, work done by Drayton et al suggested that PNAd could be induced on the endothelial cells by LTαβ complex signaling pathway (Drayton et al., 2003).

I had used HUVEC instead of HEV for this study because there was no reliable tissues source or protocol to generate primary HEV cultures for this study. Protocols for mouse HEV are available but these cells appear more fibroblastic endothelial cell like. Secondly, due to the plasticity of the endothelial cells, isolated HEV would have the tendency to dedifferentiate outside the lymphoid tissue microenvironment. Lacorre et al showed that freshly isolated HEV from the tonsils rapidly lost their specialized

characteristics after 2 days of culture (Lacorre et al., 2004). Striking changes occurred as early as 48 hours in culture with complete loss of the postcapillary venule-specific Duffy antigen receptor for chemokines (DARCs) and the HEV-specific fucosyltransferase Fuc-TVII. It is unclear at this juncture what soluble factors could induce the HEV characteristics in-vitro.

Using an *in-vitro* model, I investigated the mechanism(s) of C/S derived from HRS cells in modulating the endothelial cell function on T cell recruitment. I carried out immunohistochemical staining to demonstrate the presence of LTα in cHL lymph nodes, thus verifying the clinical relevance of my findings. Most studies on cHL to date had been carried out using HRS cell lines in in-vitro models and immunohistochemistry or *in-situ* hybridization of tissues samples. To date, there is still no satisfactory xenograft mouse model for the study of HRS cells and Hodgkin lymphoma *in-vivo*. Neither unmanipulated HRS cell lines nor cells isolated from biopsy tissues could be grown in nude mice or other immunodeficient mice (Kapp et al., 1992; von Kalle et al., 1992). One possible reason for this is that HRS cells require T cell signals for survival and both nude and SCID mice could not provide the correct environment because they do not have endogenous T cells.

4.14 Future Work

Experimental results suggest that LTα derived from the HRS cells contributes to the activation of endothelial cells and facilitates T cell recruitment to the lymphatic lesions. Data show that the regulatory pathways of LTα expression include the NFκB, AP-1 and Cox-1 dependent pathways. To have a better understanding of the functions of LTα stimulated endothelial cells and regulation of LTα production in the HRS cells, I have identified some interesting areas where more knowledge and information would help to give better insights into cell-cell interactions in cHL.

My data show that CD44 on naïve T cells mediates interaction with C/S stimulated endothelial cells. Studies to date suggest that CD44 only functions as an adhesion molecule when it reaches a certain threshold level and after conversion to the active form in response to appropriate stimuli such as TNF-α (Maiti et al., 1998). In line with this, most studies showed that only activated T cells exhibit CD44-HA binding to facilitate lymphocyte recruitment. Surprisingly, the CD44 expressed on naïve T cell was able to bind effectively to HA induced on the C/S stimulated endothelial cells. It would be interesting to identify the CD44 variant expressed on naïve T cells or the modification of HA on the endothelial cells induced by C/S derived from HRS cells that mediated the observed CD44-HA interactions.

My results suggest that the possible mediator that can induce HA synthesis on stimulated endothelial cells is LTα. It had been shown previously that HA synthesis could not be induced on human umbilical cord vein endothelial cells (HUVEC) via stimulation with TNF-α and IL-1β (Mohamadzadeh et al., 1998). However, Butler et al showed that LTα can also induce HA production on the synovial fibroblast like-cells (Butler et al., 1988). To clarify that, endothelial cells can be stimulated with recombinant human LTα instead of KM-H2 C/S to induce HUVEC activation and examine their interactions with naïve T cells in absence and presence of hyaluronidase treatment. Employing silencing RNA (siRNA) techniques to knock-down LTα production in the HRS cell lines would provide more solid confirmation. By using a commercially available LTα siRNA plasmid, LTα deficient HRS cells can be generated. C/S derived from this cell line will be harvested and used to stimulate endothelial cells.

According to the results, Cox-1 is more important than Cox-2 in regulating LTα production by HRS cells. Comparing the activity of Cox-1 and Cox-2 in the HRS cells might help us determine whether constitutive Cox-1 activity is higher than Cox-2 activity in HRS cells. Employing silencing RNA techniques to knock-down Cox-1 or Cox-2 would allow me to measure the activity of

each Cox enzyme. Cox enzyme activity can be correlated with LTα concentration secreted into the C/S. In addition, my data suggest that the Cox-1 pathway probably acts on the AP-1 pathway to mediate LTα production. An assay to measure AP-1 activity after Indomethacin treatment on KM-H2 cells would provide more confirmative data. Comparing the AP-1 activity between the SP600125 treated, Indomethacin treated, Cox-1 silenced cells and untreated KM-H2 cells would help to determine whether the AP-1 activity is indeed attenuated in Indomethacin treated KM-H2 cells; and whether AP-1 is acting synergistically with or downstream from the Cox-1 pathway.

The data suggest that c-Fos might play a role in mediating LTα production. To validate c-Fos involvement in regulating LTα production, c-Fos expression can be knocked down in the HRS cells. Generation of c-Fos deficient HRS cells and measuring LTα content in the C/S derived from these cells would provide direct evidence on the role of c-Fos in LTα production. Besides that, to find out whether other transcription factors is also regulated by Cox pathway to mediate LTα production, Chip Sequencing technique could be performed to analyze the transcription factors that interact with the LTα gene. Transcription factors identified on the LTα gene in the Indomethacin treated KM-H2 cells compared with untreated HRS cells would provide us a better insight of the transcription regulatory network of LTα production.

BIBLIOGRAPHY

Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787-793.

Aggarwal, B.B. (2003). Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol *3*, 745-756.

Aggarwal, B.B., Moffat, B., and Harkins, R.N. (1984). Human lymphotoxin. Production by a lymphoblastoid cell line, purification, and initial characterization. J Biol Chem *259*, 686-691.

Aguilera, N.S., Chen, J., Bijwaard, K.E., Director-Myska, A.E., Barekman, C.L., Millward, C., Lichy, J., and Abbondanzo, S.L. (2006). Gene rearrangement and comparative genomic hybridization studies of classic Hodgkin lymphoma expressing T-cell antigens. Arch Pathol Lab Med *130*, 1772-1779.

Aldinucci, D., Lorenzon, D., Cattaruzza, L., Pinto, A., Gloghini, A., Carbone, A., and Colombatti, A. (2008). Expression of CCR5 receptors on Reed-Sternberg cells and Hodgkin lymphoma cell lines: involvement of CCL5/Rantes in tumor cell growth and microenvironmental interactions. Int J Cancer *122*, 769-776.

Aldinucci, D., Poletto, D., Gloghini, A., Nanni, P., Degan, M., Perin, T., Ceolin, P., Rossi, F.M., Gattei, V., Carbone, A. Pinto, A. (2002). Expression of functional interleukin-3 receptors on Hodgkin and Reed-Sternberg cells. Am J Pathol *160*, 585-596.

Alimzhanov, M.B., Kuprash, D.V., Kosco-Vilbois, M.H., Luz, A., Turetskaya, R.L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S.A., and Pfeffer, K. (1997). Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. Proc Natl Acad Sci U S A *94*, 9302-9307.

Allerton, S.E., Beierle, J.W., Powars, D.R., and Bavetta, L.A. (1970). Abnormal extracellular components in Wilms' tumor. Cancer Res *30*, 679-683.

Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lathi-Domenici, J.,, Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A., Schnitt, S., Sellers, W.R., Polyak, K. (2004). Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell *6*, 17-32.

Alvaro, T., Lejeune, M., Salvadó, M.T., Bosch, R., García, J.F., Jaén, J., Banham, A.H., Roncador, G., Montalbán, C., and Piris, M.A. (2005). Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. Clin Cancer Res *11*, 1467-1473.

Ammirante, M., Luo, J.L., Grivennikov, S., Nedospasov, S., and Karin, M. (2010). B-cell-derived lymphotoxin promotes castration-resistant prostate cancer. Nature *464*, 302-305.

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell *55*, 875-885.

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987). Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell *49*, 729-739.

Arbones, M.L., Ord, D.C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D.J., and Tedder, T.F. (1994). Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity *1*, 247-260.

Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B., and Seed, B. (1990). CD44 is the principal cell surface receptor for hyaluronate. Cell *61*, 1303-1313.

Baaten, B.J., Li, C.R., and Bradley, L.M. (2010). Multifaceted regulation of T cells by CD44. Commun Integr Biol *3*, 508-512.

Babu, G.J., Lalli, M.J., Sussman, M.A., Sadoshima, J., and Periasamy, M. (2000). Phosphorylation of elk-1 by MEK/ERK pathway is necessary for c-fos gene activation during cardiac myocyte hypertrophy. J Mol Cell Cardiol *32*, 1447-1457.

Bacabac, R.G., Smit, T.H., Cowin, S.C., Van Loon, J.J., Nieuwstadt, F.T., Heethaar, R., and Klein-Nulend, J. (2005). Dynamic shear stress in parallel-plate flow chambers. J Biomech *38*, 159-167.

Baekkevold, E.S., Yamanaka, T., Palframan, R.T., Carlsen, H.S., Reinholt, F.P., von Andrian, U.H., Brandtzaeg, P., and Haraldsen, G. (2001). The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. J Exp Med *193*, 1105-1112.

Banks, T.A., Rouse, B.T., Kerley, M.K., Blair, P.J., Godfrey, V.L., Kuklin, N.A., Bouley, D.M., Thomas, J., Kanangat, S., and Mucenski, M.L. (1995). Lymphotoxin-alpha-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. J Immunol *155*, 1685-1693.

Barth, T.F., Martin-Subero, J.I., Joos, S., Menz, C.K., Hasel, C., Mechtersheimer, G., Parwaresch, R.M., Lichter, P., Siebert, R., and Mooller, P. (2003). Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma. Blood *101*, 3681-3686.

Baus, D., and Pfitzner, E. (2006). Specific function of STAT3, SOCS1, and SOCS3 in the regulation of proliferation and survival of classical Hodgkin lymphoma cells. Int J Cancer *118*, 1404-1413.

Behrens, A., Sibilia, M., and Wagner, E.F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet *21*, 326-329.

Benharroch, D., Prinsloo, I., Apte, R.N., Yermiahu, T., Geffen, D.B., Yanai-Inbar, I., and Gopas, J. (1996). Interleukin-1 and tumor necrosis factor-alpha in the Reed-Sternberg cells of Hodgkin's disease. Correlation with clinical and morphological "inflammatory" features. Eur Cytokine Netw *7*, 51-57.

Berlin, C., Bargatze, R.F., Campbell, J.J., von Andrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., and Butcher, E.C. (1995). alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell *80*, 413-422.

Berlin, C., Berg, E.L., Briskin, M.J., Andrew, D.P., Kilshaw, P.J., Holzmann, B., Weissman, I.L., Hamann, A., and Butcher, E.C. (1993). Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell *74*, 185-195.

Berlin-Rufenach, C., Otto, F., Mathies, M., Westermann, J., Owen, M.J., Hamann, A., and Hogg, N. (1999). Lymphocyte migration in lymphocyte function-associated antigen (LFA)-1-deficient mice. J Exp Med *189*, 1467-1478.

Bevilacqua, M.P. (1993). Endothelial-leukocyte adhesion molecules. Annu Rev Immunol *11*, 767-804.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S., and Gimbrone, M.A. (1987). Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci U S A *84*, 9238-9242.

Birbach, A., Gold, P., Binder, B.R., Hofer, E., de Martin, R., and Schmid, J.A. (2002). Signaling molecules of the NF-kappa B pathway shuttle constitutively between cytoplasm and nucleus. J Biol Chem *277*, 10842-10851.

Blackwell, T.S., and Christman, J.W. (1997). The role of nuclear factor-kappa B in cytokine gene regulation. Am J Respir Cell Mol Biol *17*, 3-9.

Bochner, B.S., Klunk, D.A., Sterbinsky, S.A., Coffman, R.L., and Schleimer, R.P. (1995). IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. J Immunol *154*, 799-803.

Bogoyevitch, M.A., and Kobe, B. (2006). Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev *70*, 1061-1095.

Bond, M., Fabunmi, R.P., Baker, A.H., and Newby, A.C. (1998). Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B. FEBS Lett *435*, 29-34.

Bonder, C.S., Clark, S.R., Norman, M.U., Johnson, P., and Kubes, P. (2006). Use of CD44 by CD4+ Th1 and Th2 lymphocytes to roll and adhere. Blood *107*, 4798-4806.

Bondeson, J., Foxwell, B., Brennan, F., and Feldmann, M. (1999). Defining therapeutic targets by using adenovirus: blocking NF-kappaB inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. Proc Natl Acad Sci U S A *96*, 5668-5673.

Boyle, W.J., Smeal, T., Defize, L.H., Angel, P., Woodgett, J.R., Karin, M., and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell *64*, 573-584.

Brauninger, A., Wacker, H.H., Rajewsky, K., Kuppers, R., and Hansmann, M.L. (2003). Typing the histogenetic origin of the tumor cells of lymphocyte-rich classical Hodgkin's lymphoma in relation to tumor cells of classical and lymphocyte-predominance Hodgkin's lymphoma. Cancer Res *63*, 1644-1651.

Browne, K.A., Blink, E., Sutton, V.R., Froelich, C.J., Jans, D.A., and Trapani, J.A. (1999). Cytosolic delivery of granzyme B by bacterial toxins: evidence that endosomal disruption, in addition to transmembrane pore formation, is an important function of perforin. Mol Cell Biol *19*, 8604-8615.

Browning, J.L., Dougas, I., Ngam-ek, A., Bourdon, P.R., Ehrenfels, B.N., Miatkowski, K., Zafari, M., Yampaglia, A.M., Lawton, P., and Meier, W. (1995). Characterization of surface lymphotoxin forms. Use of specific monoclonal antibodies and soluble receptors. J Immunol *154*, 33-46.

Browning, J.L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E.P., Hession, C., O'Brine-Greco, B., Foley, S.F., and Ware, C.F. (1993). Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface. Cell *72*, 847-856.

Butler, D.M., Vitti, G.F., Leizer, T., and Hamilton, J.A. (1988). Stimulation of the hyaluronic acid levels of human synovial fibroblasts by recombinant human tumor necrosis factor alpha, tumor necrosis factor beta (lymphotoxin), interleukin-1 alpha, and interleukin-1 beta. Arthritis Rheum *31*, 1281-1289.

Butler, L.M., Rainger, G.E., and Nash, G.B. (2009). A role for the endothelial glycosaminoglycan hyaluronan in neutrophil recruitment by endothelial cells cultured for prolonged periods. Exp Cell Res *315*, 3433-3441.

Cabannes, E., Khan, G., Aillet, F., Jarrett, R.F., and Hay, R.T. (1999). Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IkappaBalpha. Oncogene *18*, 3063-3070.

Calmon-Hamaty, F., Combe, B., Hahne, M., and Morel, J. (2011). Lymphotoxin alpha revisited: general features and implications in rheumatoid arthritis. Arthritis Res Ther *13*, 232.

Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N., Wu, L., Butcher, E., C. (1999). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. Nature *400*, 776-780.

Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C. (1998). Chemokines and the arrest of lymphocytes rolling under flow conditions. Science *279*, 381-384.

Cancer, T.I.A.f.R.o. (2008). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue, 4 edn.

Carbone, A., Gloghini, A., Gruss, H.J., and Pinto, A. (1995). CD40 ligand is constitutively expressed in a subset of T cell lymphomas and on the microenvironmental reactive T cells of follicular lymphomas and Hodgkin's disease. Am J Pathol *147*, 912-922.

Carlos, T.M., and Harlan, J.M. (1994). Leukocyte-endothelial adhesion molecules. Blood *84*, 2068-2101.

Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., and Alber, G. (1996). Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med *184*, 747-752.

Chae, Y.S., Kim, J.G., Sohn, S.K., Moon, J.H., Kim, S.N., Lee, S.J., Park, T.I., and Lee, M.H. (2010). Lymphotoxin alfa and receptor-interacting protein kinase 1 gene polymorphisms may correlate with prognosis in patients with diffuse large B cell lymphoma treated with R-CHOP. Cancer Chemother Pharmacol *65*, 571-577.

Chan, J.R., Hyduk, S.J., and Cybulsky, M.I. (2001). Chemoattractants induce a rapid and transient upregulation of monocyte alpha4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. J Exp Med *193*, 1149-1158.

Chemnitz, J.M., Driesen, J., Classen, S., Riley, J.L., Debey, S., Beyer, M., Popov, A., Zander, T., and Schultze, J.L. (2006). Prostaglandin E2 impairs CD4+ T cell activation by inhibition of lck: implications in Hodgkin's lymphoma. Cancer Res *66*, 1114-1122.

Chen, C.C., Sun, Y.T., Chen, J.J., and Chiu, K.T. (2000). TNF-alpha-induced cyclooxygenase-2 expression in human lung epithelial cells: involvement of the phospholipase C-gamma 2, protein kinase C-alpha, tyrosine kinase, NF-kappa B-inducing kinase, and I-kappa B kinase 1/2 pathway. J Immunol *165*, 2719-2728.

Chiu, A., Xu, W., He, B., Dillon, S.R., Gross, J.A., Sievers, E., Qiao, X., Santini, P., Hyjek, E., Lee, J.W., Cesarman E., Chadburn A., Knowles D.M., Cerutti A. (2007). Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL. Blood *109*, 729-739.

Chou, S.Y., Baichwal, V., and Ferrell, J.E. (1992). Inhibition of c-Jun DNA binding by mitogen-activated protein kinase. Mol Biol Cell *3*, 1117-1130.

Chuvpilo, S., Schomberg, C., Gerwig, R., Heinfling, A., Reeves, R., Grummt, F., and Serfling, E. (1993). Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. Nucleic Acids Res *21*, 5694-5704.

Cloutier, A., Ear, T., Borissevitch, O., Larivée, P., and McDonald, P.P. (2003). Inflammatory cytokine expression is independent of the c-Jun N-terminal kinase/AP-1 signaling cascade in human neutrophils. J Immunol *171*, 3751-3761.

Collet, J.P., Sharpe, C., Belzile, E., Boivin, J.F., Hanley, J., and Abenhaim, L. (1999). Colorectal cancer prevention by non-steroidal anti-inflammatory drugs: effects of dosage and timing. Br J Cancer *81*, 62-68.

Compagno, M., Lim, W.K., Grunn, A., Nandula, S.V., Brahmachary, M., Shen, Q., Bertoni, F., Ponzoni, M., Scandurra, M., Califano, A., Bhagat, G., Chadburn, A., Dalla-Favera, R., Pasqualucci, L. (2009). Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. Nature *459*, 717-721.

Cooper, E.H., and Forbes, M.A. (1988). Serum hyaluronic acid levels in cancer. Br J Cancer *58*, 668-669.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Cozen, W., Gill, P.S., Ingles, S.A., Masood, R., Martinez-Maza, O., Cockburn, M.G., Gauderman, W.J., Pike, M.C., Bernstein, L., Nathwani, B.N., Salam, M.T., Danley, K.L., Wang, W., Gage, J., Gundell-Miller, S., Mack, T.M. (2004). IL-6 levels and genotype are associated with risk of young adult Hodgkin lymphoma. Blood *103*, 3216-3221.

Crofford, L.J., Wilder, R.L., Ristimaki, A.P., Sano, H., Remmers, E.F., Epps, H.R., and Hla, T. (1994). Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. J Clin Invest *93*, 1095-1101.

Cuturi, M.C., Murphy, M., Costa-Giomi, M.P., Weinmann, R., Perussia, B., and Trinchieri, G. (1987). Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med *165*, 1581-1594.

da Costa Martins, P., Garcia-Vallejo, J.J., van Thienen, J.V., Fernandez-Borja, M., van Gils, J.M., Beckers, C., Horrevoets, A.J., Hordijk, P.L., and Zwaginga, J.J. (2007). P-selectin glycoprotein ligand-1 is expressed on endothelial cells and mediates monocyte adhesion to activated endothelium. Arterioscler Thromb Vasc Biol *27*, 1023-1029.

Daller, B., Musch, W., Rohrl, J., Tumanov, A.V., Nedospasov, S.A., Mannel, D.N., Schneider-Brachert, W., and Hehlgans, T. (2011). Lymphotoxin-beta receptor activation by lymphotoxin-alpha(1)beta(2) and LIGHT promotes

tumor growth in an NFkappaB-dependent manner. Int J Cancer *128*, 1363-1370.

Danesch, U., Weber, P.C., and Sellmayer, A. (1994). Arachidonic acid increases c-fos and Egr-1 mRNA in 3T3 fibroblasts by formation of prostaglandin E2 and activation of protein kinase C. J Biol Chem *269*, 27258-27263.

Darnell, J.E., Jr., Kerr, I.M., and Stark, G.R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science *264*, 1415-1421.

de Jong, M.M., Nolte, I.M., te Meerman, G.J., van der Graaf, W.T., de Vries, E.G., Sijmons, R.H., Hofstra, R.M., and Kleibeuker, J.H. (2002). Low-penetrance genes and their involvement in colorectal cancer susceptibility. Cancer Epidemiol Biomarkers Prev *11*, 1332-1352.

De Togni, P., Goellner, J., Ruddle, N.H., Streeter, P.R., Fick, A., Mariathasan, S., Smith, S.C., Carlson, R., Shornick, L.P., Strauss-Schoenberger, J., John, H. Russel., Robert, Karr, David, D. Chaplin. (1994). Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science *264*, 703-707.

Delpech, B., Chevallier, B., Reinhardt, N., Julien, J.P., Duval, C., Maingonnat, C., Bastit, P., and Asselain, B. (1990). Serum hyaluronan (hyaluronic acid) in breast cancer patients. Int J Cancer *46*, 388-390.

Deng, T., and Karin, M. (1994). c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK. Nature *371*, 171-175.

Devary, Y., Gottlieb, R.A., Smeal, T., and Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell *71*, 1081-1091.

DiDonato, J.A., Mercurio, F., and Karin, M. (2012). NF-κB and the link between inflammation and cancer. Immunol Rev *246*, 379-400.

Diehl, V., Kirchner, H.H., Burrichter, H., Stein, H., Fonatsch, C., Gerdes, J., Schaadt, M., Heit, W., Uchanska-Ziegler, B., Ziegler, A., Heintz, F., Sueno, K. (1982). Characteristics of Hodgkin's disease-derived cell lines. Cancer Treat Rep *66*, 615-632.

Diehl, V., Thomas, R.K., and Re, D. (2004). Part II: Hodgkin's lymphoma--diagnosis and treatment. Lancet Oncol *5*, 19-26.

Diehl, V., von Kalle, C., Fonatsch, C., Tesch, H., Juecker, M., and Schaadt, M. (1990). The cell of origin in Hodgkin's disease. Semin Oncol *17*, 660-672.

Dinchuk, J.E., Car, B.D., Focht, R.J., Johnston, J.J., Jaffee, B.D., Covington, M.B., Contel, N.R., Eng, V.M., Collins, R.J., Czerniak, P.M., Gorry, S.A., Trzaskos, J.M. (1995). Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature *378*, 406-409.

Ding, Z., Xiong, K., and Issekutz, T.B. (2000). Regulation of chemokine-induced transendothelial migration of T lymphocytes by endothelial activation: differential effects on naive and memory T cells. J Leukoc Biol *67*, 825-833.

Doussis-Anagnostopoulou, I.A., Talks, K.L., Turley, H., Debnam, P., Tan, D.C., Mariatos, G., Gorgoulis, V., Kittas, C., and Gatter, K.C. (2002). Vascular endothelial growth factor (VEGF) is expressed by neoplastic Hodgkin-Reed-Sternberg cells in Hodgkin's disease. J Pathol *197*, 677-683.

Drayton, D.L., Ying, X., Lee, J., Lesslauer, W., and Ruddle, N.H. (2003). Ectopic LT alpha beta directs lymphoid organ neogenesis with concomitant expression of peripheral node addressin and a HEV-restricted sulfotransferase. J Exp Med *197*, 1153-1163.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., and Springer, T.A. (1986). Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J Immunol *137*, 245-254.

Dutton, A., O'Neil, J.D., Milner, A.E., Reynolds, G.M., Starczynski, J., Crocker, J., Young, L.S., and Murray, P.G. (2004). Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. Proc Natl Acad Sci U S A *101*, 6611-6616.

Dutton, A., Reynolds, G.M., Dawson, C.W., Young, L.S., and Murray, P.G. (2005). Constitutive activation of phosphatidyl-inositide 3 kinase contributes to the survival of Hodgkin's lymphoma cells through a mechanism involving Akt kinase and mTOR. J Pathol *205*, 498-506.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S., and DuBois, R.N. (1994). Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology *107*, 1183-1188.

Eferl, R., Ricci, R., Kenner, L., Zenz, R., David, J.P., Rath, M., and Wagner, E.F. (2003). Liver tumor development. c-Jun antagonizes the proapoptotic activity of p53. Cell *112*, 181-192.

Eferl, R., Sibilia, M., Hilberg, F., Fuchsbichler, A., Kufferath, I., Guertl, B., Zenz, R., Wagner, E.F., and Zatloukal, K. (1999). Functions of c-Jun in liver and heart development. J Cell Biol *145*, 1049-1061.

Eferl, R., and Wagner, E.F. (2003). AP-1: a double-edged sword in tumorigenesis. Nat Rev Cancer *3*, 859-868.

Egan, K., and FitzGerald, G.A. (2006). Eicosanoids and the vascular endothelium. Handb Exp Pharmacol, 189-211.

Eger, A., Stockinger, A., Schaffhauser, B., Beug, H., and Foisner, R. (2000). Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of beta-catenin and upregulation of beta-

catenin/lymphoid enhancer binding factor-1 transcriptional activity. J Cell Biol *148*, 173-188.

Elias, J.A., Krol, R.C., Freundlich, B., and Sampson, P.M. (1988). Regulation of human lung fibroblast glycosaminoglycan production by recombinant interferons, tumor necrosis factor, and lymphotoxin. J Clin Invest *81*, 325-333.

Emmerich, F., Meiser, M., Hummel, M., Demel, G., Foss, H.D., Jundt, F., Mathas, S., Krappmann, D., Scheidereit, C., Stein, H., Dorken, B. (1999). Overexpression of I kappa B alpha without inhibition of NF-kappaB activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. Blood *94*, 3129-3134.

Estrada-Bernal, A., Mendoza-Milla, C., Ventura-Gallegos, J.L., López-Bojórquez, L.N., Miranda-Peralta, E., Arechavaleta-Velasco, F., Vadillo-Ortega, F., Sánchez-Sánchez, L., and Zentella-Dehesa, A. (2003). NF-kappaB dependent activation of human endothelial cells treated with soluble products derived from human lymphomas. Cancer Lett *191*, 239-248.

Evans, C.H., and Heinbaugh, J.A. (1981). Lymphotoxin cytotoxicity, a combination of cytolytic and cytostatic cellular responses. Immunopharmacology *3*, 347-359.

Fenwick, C., Na, S.Y., Voll, R.E., Zhong, H., Im, S.Y., Lee, J.W., and Ghosh, S. (2000). A subclass of Ras proteins that regulate the degradation of IkappaB. Science *287*, 869-873.

Ferreri, N.R., Sarr, T., Askenase, P.W., and Ruddle, N.H. (1992). Molecular regulation of tumor necrosis factor-alpha and lymphotoxin production in T cells. Inhibition by prostaglandin E2. J Biol Chem *267*, 9443-9449.

Feuerborn, A., Moritz, C., Von Bonin, F., Dobbelstein, M., Trumper, L., Sturzenhofecker, B., and Kube, D. (2006). Dysfunctional p53 deletion mutants in cell lines derived from Hodgkin's lymphoma. Leuk Lymphoma *47*, 1932-1940.

Finger, E.B., Puri, K.D., Alon, R., Lawrence, M.B., von Andrian, U.H., and Springer, T.A. (1996). Adhesion through L-selectin requires a threshold hydrodynamic shear. Nature *379*, 266-269.

Fitzgerald, J., Dietz, T.J., and Hughes-Fulford, M. (2000). Prostaglandin E2-induced up-regulation of c-fos messenger ribonucleic acid is primarily mediated by 3',5'-cyclic adenosine monophosphate in MC3T3-E1 osteoblasts. Endocrinology *141*, 291-298.

Fiumara, P., Snell, V., Li, Y., Mukhopadhyay, A., Younes, M., Gillenwater, A.M., Cabanillas, F., Aggarwal, B.B., and Younes, A. (2001). Functional expression of receptor activator of nuclear factor kappaB in Hodgkin disease cell lines. Blood *98*, 2784-2790.

Foletta, V.C., Segal, D.H., and Cohen, D.R. (1998). Transcriptional regulation in the immune system: all roads lead to AP-1. J Leukoc Biol *63*, 139-152.

Folkman, J. (2004). Angiogenesis and c-Jun. J Natl Cancer Inst *96*, 644.

Foss, H.D., Herbst, H., Oelmann, E., Samol, J., Grebe, M., Blankenstein, T., Matthes, J., Qin, Z.H., Falini, B., Pileri, S., Diamantstein, T., Stein, H. (1993). Lymphotoxin, tumour necrosis factor and interleukin-6 gene transcripts are present in Hodgkin and Reed-Sternberg cells of most Hodgkin's disease cases. Br J Haematol *84*, 627-635.

Fujioka, S., Niu, J., Schmidt, C., Sclabas, G.M., Peng, B., Uwagawa, T., Li, Z., Evans, D.B., Abbruzzese, J.L., and Chiao, P.J. (2004). NF-kappaB and AP-1 connection: mechanism of NF-kappaB-dependent regulation of AP-1 activity. Mol Cell Biol *24*, 7806-7819.

Fujiwara, S., Yamashita, Y., Choi, Y.L., Wada, T., Kaneda, R., Takada, S., Maruyama, Y., Ozawa, K., and Mano, H. (2005). Transforming activity of the lymphotoxin-beta receptor revealed by expression screening. Biochem Biophys Res Commun *338*, 1256-1262.

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity *9*, 59-70.

Gaudio, F., Giordano, A., Pavone, V., Perrone, T., Curci, P., Pastore, D., Delia, M., De' Risi, C., Spina, A., Liso, V., Specchia, G. (2011). Outcome of Very Late Relapse in Patients with Hodgkin's Lymphomas. Adv Hematol *2011*, 707542.

Georgakis, G.V., Li, Y., Rassidakis, G.Z., Medeiros, L.J., Mills, G.B., and Younes, A. (2006). Inhibition of the phosphatidylinositol-3 kinase/Akt promotes G1 cell cycle arrest and apoptosis in Hodgkin lymphoma. Br J Haematol *132*, 503-511.

Ghosh, S., May, M.J., and Kopp, E.B. (1998). NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol *16*, 225-260.

Giardiello, F.M., Hamilton, S.R., Krush, A.J., Piantadosi, S., Hylind, L.M., Celano, P., Booker, S.V., Robinson, C.R., and Offerhaus, G.J. (1993). Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med *328*, 1313-1316.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680-6684.

Giovannucci, E., Egan, K.M., Hunter, D.J., Stampfer, M.J., Colditz, G.A., Willett, W.C., and Speizer, F.E. (1995). Aspirin and the risk of colorectal cancer in women. N Engl J Med *333*, 609-614.

Giovannucci, E., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Ascherio, A., and Willett, W.C. (1994). Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. Ann Intern Med *121*, 241-246.

Greaves, P., Clear, A., Coutinho, R., Wilson, A., Matthews, J., Owen, A., Shanyinde, M., Lister, T.A., Calaminici, M., and Gribben, J.G. (2013). Expression of FOXP3, CD68, and CD20 at diagnosis in the microenvironment of classical Hodgkin lymphoma is predictive of outcome. J Clin Oncol *31*, 256-262.

Green, M.R., Rodig, S., Juszczynski, P., Ouyang, J., Sinha, P., O'Donnell, E., Neuberg, D., and Shipp, M.A. (2012). Constitutive AP-1 activity and EBV infection induce PD-L1 in Hodgkin lymphomas and posttransplant lymphoproliferative disorders: implications for targeted therapy. Clin Cancer Res *18*, 1611-1618.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res *54*, 4855-4878.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science *267*, 389-393.

Gutman, A., and Wasylyk, B. (1990). The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J *9*, 2241-2246.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Harlan, J.M. (1993). Leukocyte adhesion deficiency syndrome: insights into the molecular basis of leukocyte emigration. Clin Immunol Immunopathol *67*, S16-24.

Hasselbalch, H., Hovgaard, D., Nissen, N., and Junker, P. (1995). Serum hyaluronan is increased in malignant lymphoma. Am J Hematol *50*, 231-233.

Hehlgans, T., Stoelcker, B., Stopfer, P., Muller, P., Cernaianu, G., Guba, M., Steinbauer, M., Nedospasov, S.A., Pfeffer, K., and Mannel, D.N. (2002). Lymphotoxin-beta receptor immune interaction promotes tumor growth by inducing angiogenesis. Cancer Res *62*, 4034-4040.

Herbst, H., Samol, J., Foss, H.D., Raff, T., and Niedobitek, G. (1997). Modulation of interleukin-6 expression in Hodgkin and Reed-Sternberg cells by Epstein-Barr virus. J Pathol *182*, 299-306.

Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev *7*, 2135-2148.

Hidalgo, A., Peired, A.J., Wild, M.K., Vestweber, D., and Frenette, P.S. (2007). Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. Immunity *26*, 477-489.

Hinz, M., Lemke, P., Anagnostopoulos, I., Hacker, C., Krappmann, D., Mathas, S., Dörken, B., Zenke, M., Stein, H., and Scheidereit, C. (2002). Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. J Exp Med *196*, 605-617.

Hinz, M., Loser, P., Mathas, S., Krappmann, D., Dorken, B., and Scheidereit, C. (2001). Constitutive NF-kappaB maintains high expression of a characteristic gene network, including CD40, CD86, and a set of antiapoptotic genes in Hodgkin/Reed-Sternberg cells. Blood *97*, 2798-2807.

Hjalgrim, H., Seow, A., Rostgaard, K., and Friborg, J. (2008). Changing patterns of Hodgkin lymphoma incidence in Singapore. Int J Cancer *123*, 716-719.

Hofmann, M., Rudy, W., Zöller, M., Tölg, C., Ponta, H., Herrlich, P., and Günthert, U. (1991). CD44 splice variants confer metastatic behavior in rats: homologous sequences are expressed in human tumor cell lines. Cancer Res *51*, 5292-5297.

Hoppe, R.T., Mauch, P.M., Armitage, J.O., Diehl, V., and Weiss, L.M. (2007). Hodgkin Lymphoma.

Horie, R., Watanabe, T., Ito, K., Morisita, Y., Watanabe, M., Ishida, T., Higashihara, M., and Kadin, M. (2002). Cytoplasmic aggregation of TRAF2 and TRAF5 proteins in the Hodgkin-Reed-Sternberg cells. Am J Pathol *160*, 1647-1654.

Hou, J., Baichwal, V., and Cao, Z. (1994). Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding intercellular adhesion molecule 1. Proc Natl Acad Sci U S A *91*, 11641-11645.

Howe, L.R., Chang, S.H., Tolle, K.C., Dillon, R., Young, L.J., Cardiff, R.D., Newman, R.A., Yang, P., Thaler, H.T., Muller, W.J., Hudis, C., Brown, A.M., Hla, T., Subbaramaiah, K., Dannenberg, A.J. (2005). HER2/neu-induced mammary tumorigenesis and angiogenesis are reduced in cyclooxygenase-2 knockout mice. Cancer Res *65*, 10113-10119.

Hsu, P.L., and Hsu, S.M. (1989). Production of tumor necrosis factor-alpha and lymphotoxin by cells of Hodgkin's neoplastic cell lines HDLM-1 and KM-H2. Am J Pathol *135*, 735-745.

Hsu, S.M., Hsu, P.L., Lo, S.S., and Wu, K.K. (1988). Expression of prostaglandin H synthase (cyclooxygenase) in Hodgkin's mononuclear and Reed-Sternberg cells. Functional resemblance between H-RS cells and histiocytes or interdigitating reticulum cells. Am J Pathol *133*, 5-12.

Hsu, S.M., Krupen, K., and Lachman, L.B. (1989). Heterogeneity of interleukin 1 production in cultured Reed-Sternberg cell lines HDLM-1, HDLM-1d, and KM-H2. Am J Pathol *135*, 33-38.

Hsu, S.M., Xie, S.S., and Hsu, P.L. (1990). Cultured Reed-Sternberg cells HDLM-1 and KM-H2 can be induced to become histiocytelike cells. H-RS cells are not derived from lymphocytes. Am J Pathol *137*, 353-367.

Hsu-Lin, S., Berman, C.L., Furie, B.C., August, D., and Furie, B. (1984). A platelet membrane protein expressed during platelet activation and secretion. Studies using a monoclonal antibody specific for thrombin-activated platelets. J Biol Chem *259*, 9121-9126.

Hu, X., Janssen, W.E., Moscinski, L.C., Bryington, M., Dangsupa, A., Rezai-Zadeh, N., Babbin, B.A., and Zuckerman, K.S. (2001). An IkappaBalpha inhibitor causes leukemia cell death through a p38 MAP kinase-dependent, NF-kappaB-independent mechanism. Cancer Res *61*, 6290-6296.

Huang, T.T., Kudo, N., Yoshida, M., and Miyamoto, S. (2000). A nuclear export signal in the N-terminal regulatory domain of IkappaBalpha controls cytoplasmic localization of inactive NF-kappaB/IkappaBalpha complexes. Proc Natl Acad Sci U S A *97*, 1014-1019.

Huang, S., Robinson, J.B., Deguzman, A., Bucana, C.D., and Fidler, I.J. (2000). Blockade of nuclear factor-kappa B signaling inhibits angiogenesis and tumorigenecity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Res *60*, 5334-5339

Huang, T.T., and Miyamoto, S. (2001). Postrepression activation of NF-kappaB requires the amino-terminal nuclear export signal specific to IkappaBalpha. Mol Cell Biol *21*, 4737-4747.

Huo, Y., Hafezi-Moghadam, A., and Ley, K. (2000). Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions. Circ Res *87*, 153-159.

Huxford, T., Huang, D.B., Malek, S., and Ghosh, G. (1998). The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. Cell *95*, 759-770.

Hwang, S.T., Singer, M.S., Giblin, P.A., Yednock, T.A., Bacon, K.B., Simon, S.I., and Rosen, S.D. (1996). GlyCAM-1, a physiologic ligand for L-selectin, activates beta 2 integrins on naive peripheral lymphocytes. J Exp Med *184*, 1343-1348.

Ishida, T., Ishii, T., Inagaki, A., Yano, H., Komatsu, H., Iida, S., Inagaki, H., and Ueda, R. (2006). Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. Cancer Res *66*, 5716-5722.

Ishizuka, T., Kawakami, M., Hidaka, T., Matsuki, Y., Takamizawa, M., Suzuki, K., Kurita, A., and Nakamura, H. (1998). Stimulation with thromboxane A2 (TXA2) receptor agonist enhances ICAM-1, VCAM-1 or ELAM-1 expression by human vascular endothelial cells. Clin Exp Immunol *112*, 464-470.

Ishizuka, T., Suzuki, K., Kawakami, M., Hidaka, T., Matsuki, Y., and Nakamura, H. (1996). Thromboxane A2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells. Eur J Pharmacol *312*, 367-377.

Ito, D., Back, T.C., Shakhov, A.N., Wiltrout, R.H., and Nedospasov, S.A. (1999). Mice with a targeted mutation in lymphotoxin-alpha exhibit enhanced tumor growth and metastasis: impaired NK cell development and recruitment. J Immunol *163*, 2809-2815.

Jain, J., Miner, Z., and Rao, A. (1993). Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. J Immunol *151*, 837-848.

Jalkanen, S., Bargatze, R.F., de los Toyos, J., and Butcher, E.C. (1987). Lymphocyte recognition of high endothelium: antibodies to distinct epitopes of an 85-95-kD glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. J Cell Biol *105*, 983-990.

Jiang, D., Liang, J., and Noble, P.W. (2011). Hyaluronan as an immune regulator in human diseases. Physiol Rev *91*, 221-264.

Johnson, C., Van Antwerp, D., and Hope, T.J. (1999). An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha. Embo J *18*, 6682-6693.

Joos, S., Kupper, M., Ohl, S., von Bonin, F., Mechtersheimer, G., Bentz, M., Marynen, P., Moller, P., Pfreundschuh, M., Trumper, L., Lichter, P. (2000). Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res *60*, 549-552.

Jucker, M., Abts, H., Li, W., Schindler, R., Merz, H., Gunther, A., von Kalle, C., Schaadt, M., Diamantstein, T., Feller, A.C. (1991). Expression of interleukin-6 and interleukin-6 receptor in Hodgkin's disease. Blood *77*, 2413-2418.

Juszczynski, P., Ouyang, J., Monti, S., Rodig, S.J., Takeyama, K., Abramson, J., Chen, W., Kutok, J.L., Rabinovich, G.A., and Shipp, M.A. (2007). The AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune privilege in classical Hodgkin lymphoma. Proc Natl Acad Sci U S A *104*, 13134-13139.

Kadono, T., Venturi, G.M., Steeber, D.A., and Tedder, T.F. (2002). Leukocyte rolling velocities and migration are optimized by cooperative L-selectin and intercellular adhesion molecule-1 functions. J Immunol *169*, 4542-4550.

Kansas, G.S., Ley, K., Munro, J.M., and Tedder, T.F. (1993). Regulation of leukocyte rolling and adhesion to high endothelial venules through the cytoplasmic domain of L-selectin. J Exp Med *177*, 833-838.

Kanzler, H., Kuppers, R., Hansmann, M.L., and Rajewsky, K. (1996). Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. J Exp Med *184*, 1495-1505.

Kapp, U., Wolf, J., von Kalle, C., Tawadros, S., Röttgen, A., Engert, A., Fonatsch, C., Stein, H., and Diehl, V. (1992). Preliminary report: growth of Hodgkin's lymphoma derived cells in immune compromised mice. Ann Oncol *3 Suppl 4*, 21-23.

Kapp, U., Yeh, W.C., Patterson, B., Elia, A.J., Kägi, D., Ho, A., Hessel, A., Tipsword, M., Williams, A., Mirtsos, C. Itie, A., Moyle, M., Mak, T.W. (1999). Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. J Exp Med *189*, 1939-1946.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem *270*, 16483-16486.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol *18*, 621-663.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer *2*, 301-310.

Karin, M., and Lin, A. (2002). NF-kappaB at the crossroads of life and death. Nat Immunol *3*, 221-227.

Karube, K., Ohshima, K., Suzumiya, J., Kawano, R., Kikuchi, M., and Harada, M. (2006). Gene expression profile of cytokines and chemokines in microdissected primary Hodgkin and Reed-Sternberg (HRS) cells: high expression of interleukin-11 receptor alpha. Ann Oncol *17*, 110-116.

Kashkar, H., Haefs, C., Shin, H., Hamilton-Dutoit, S.J., Salvesen, G.S., Kronke, M., and Jurgensmeier, J.M. (2003). XIAP-mediated caspase inhibition in Hodgkin's lymphoma-derived B cells. J Exp Med *198*, 341-347.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A., and Green, D.R. (1998). DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. Mol Cell *1*, 543-551.

Keats, J.J., Fonseca, R., Chesi, M., Schop, R., Baker, A., Chng, W.J., Van Wier, S., Tiedemann, R., Shi, C.X., Sebag, M., Braggio, E., Henry, T., Zhu, Y.X, Fogle, H., Price-Toska, T., Ahmann, G., Mancini, C., Brents, L.A., Kumar, S., Greipp, P., Dispenzieri, A., Bryant, B., Mulligan, M., Bruhn, L., Barrett, M., Valdez, R., Trent, J., Stewart, A.K., Carpten, J., Bergsagel, P.L . (2007). Promiscuous mutations activate the noncanonical NF-kappaB pathway in multiple myeloma. Cancer Cell *12*, 131-144.

Keshet, Y., and Seger, R. (2010). The MAP kinase signaling cascades: a system of hundreds of components regulates a diverse array of physiological functions. Methods Mol Biol *661*, 3-38.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the tumor microenvironment. Cell *141*, 52-67

Khan, A.I., Kerfoot, S.M., Heit, B., Liu, L., Andonegui, G., Ruffell, B., Johnson, P., and Kubes, P. (2004). Role of CD44 and hyaluronan in neutrophil recruitment. J Immunol *173*, 7594-7601.

King, S.R., Hickerson, W.L., and Proctor, K.G. (1991). Beneficial actions of exogenous hyaluronic acid on wound healing. Surgery *109*, 76-84.

Kishimoto, T.K., Warnock, R.A., Jutila, M.A., Butcher, E.C., Lane, C., Anderson, D.C., and Smith, C.W. (1991). Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/DREG-56 antigen) and endothelial cell ELAM-1 inhibit a common CD18-independent adhesion pathway in vitro. Blood *78*, 805-811.

Klein, S., Jucker, M., Diehl, V., and Tesch, H. (1992). Production of multiple cytokines by Hodgkin's disease derived cell lines. Hematol Oncol *10*, 319-329.

Klimm, B., Schnell, R., Diehl, V., and Engert, A. (2005). Current treatment and immunotherapy of Hodgkin's lymphoma. Haematologica *90*, 1680-1692.

Koch, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S.G., and Strieter, R.M. (1992). Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science *258*, 1798-1801.

Koni, P.A., Sacca, R., Lawton, P., Browning, J.L., Ruddle, N.H., and Flavell, R.A. (1997). Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. Immunity *6*, 491-500.

Kretschmer, C., Jones, D.B., Morrison, K., Schluter, C., Feist, W., Ulmer, A.J., Arnoldi, J., Matthes, J., Diamantstein, T., Flad, H.D., Gerdes, J. (1990). Tumor necrosis factor alpha and lymphotoxin production in Hodgkin's disease. Am J Pathol *137*, 341-351.

Kube, D., Holtick, U., Vockerodt, M., Ahmadi, T., Haier, B., Behrmann, I., Heinrich, P.C., Diehl, V., and Tesch, H. (2001). STAT3 is constitutively activated in Hodgkin cell lines. Blood *98*, 762-770.

Kuppers, R. (2009). The biology of Hodgkin's lymphoma. Nat Rev Cancer *9*, 15-27.

Kuppers, R., Engert, A., and Hansmann, M.L. (2012). Hodgkin lymphoma. J Clin Invest *122*, 3439-3447.

Kuppers, R., Rajewsky, K., Zhao, M., Simons, G., Laumann, R., Fischer, R., and Hansmann, M.L. (1994). Hodgkin disease: Hodgkin and Reed-Sternberg cells picked from histological sections show clonal immunoglobulin gene

rearrangements and appear to be derived from B cells at various stages of development. Proc Natl Acad Sci U S A *91*, 10962-10966.

Kuprash, D.V., Qin, Z., Ito, D., Grivennikov, S.I., Abe, K., Drutskaya, L.N., Blankenstein, T., and Nedospasov, S.A. (2008). Ablation of TNF or lymphotoxin signaling and the frequency of spontaneous tumors in p53-deficient mice. Cancer Lett *268*, 70-75.

Kurzrock, R., Redman, J., Cabanillas, F., Jones, D., Rothberg, J., and Talpaz, M. (1993). Serum interleukin 6 levels are elevated in lymphoma patients and correlate with survival in advanced Hodgkin's disease and with B symptoms. Cancer Res *53*, 2118-2122.

Kustikova, O., Kramerov, D., Grigorian, M., Berezin, V., Bock, E., Lukanidin, E., and Tulchinsky, E. (1998). Fra-1 induces morphological transformation and increases in vitro invasiveness and motility of epithelioid adenocarcinoma cells. Mol Cell Biol *18*, 7095-7105.

Küppers, R., and Rajewsky, K. (1998). The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease. Annu Rev Immunol *16*, 471-493.

Lacorre, D.A., Baekkevold, E.S., Garrido, I., Brandtzaeg, P., Haraldsen, G., Amalric, F., and Girard, J.P. (2004). Plasticity of endothelial cells: rapid dedifferentiation of freshly isolated high endothelial venule endothelial cells outside the lymphoid tissue microenvironment. Blood *103*, 4164-4172.

Lamprecht, B., Kreher, S., Anagnostopoulos, I., Johrens, K., Monteleone, G., Jundt, F., Stein, H., Janz, M., Dorken, B., and Mathas, S. (2008). Aberrant expression of the Th2 cytokine IL-21 in Hodgkin lymphoma cells regulates STAT3 signaling and attracts Treg cells via regulation of MIP-3alpha. Blood *112*, 3339-3347.

Langenbach, R., Morham, S.G., Tiano, H.F., Loftin, C.D., Ghanayem, B.I., Chulada, P.C., Mahler, J.F., Lee, C.A., Goulding, E.H., Kluckman, K.D., Kim, H.S, Smithies, O. (1995). Prostaglandin synthase 1 gene disruption in mice reduces arachidonic acid-induced inflammation and indomethacin-induced gastric ulceration. Cell *83*, 483-492.

Lasky, L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science *258*, 964-969.

Lawrence, M.B., Kansas, G.S., Kunkel, E.J., and Ley, K. (1997). Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). J Cell Biol *136*, 717-727.

Lawrence, M.B., and Springer, T.A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859-873.

Lee, S.H., Aggarwal, B.B., Rinderknecht, E., Assisi, F., and Chiu, H. (1984). The synergistic anti-proliferative effect of gamma-interferon and human lymphotoxin. J Immunol *133*, 1083-1086.

Lee, S.H., and Hannink, M. (2002). Characterization of the nuclear import and export functions of Ikappa B(epsilon). J Biol Chem *277*, 23358-23366.

Lefrancois, L., Parker, C.M., Olson, S., Muller, W., Wagner, N., Schon, M.P., and Puddington, L. (1999). The role of beta7 integrins in CD8 T cell trafficking during an antiviral immune response. J Exp Med *189*, 1631-1638.

Ley, K., Laudanna, C., Cybulsky, M.I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol *7*, 678-689.

Li, Q., and Verma, I.M. (2002). NF-kappaB regulation in the immune system. Nat Rev Immunol *2*, 725-734.

Li, X.Y., Matsuzaki, G., Yoshikai, Y., Muramori, K., and Nomoto, K. (1993). T cells expressing both L-selectin and CD44 molecules increase in number in peritoneal exudate cells and in vitro-stimulated spleen cells from mice immunized intraperitoneally with Listeria monocytogenes. Immunology *78*, 28-34.

Li-Weber, M., Salgame, P., Hu, C., and Krammer, P.H. (1997). Characterization of constitutive and inducible transcription factors binding to the P2 NF-AT site in the human interleukin-4 promoter. Gene *188*, 253-260.

Lim, Y.C., Snapp, K., Kansas, G.S., Camphausen, R., Ding, H., and Luscinskas, F.W. (1998). Important contributions of P-selectin glycoprotein ligand-1-mediated secondary capture to human monocyte adhesion to P-selectin, E-selectin, and TNF-alpha-activated endothelium under flow in vitro. J Immunol *161*, 2501-2508.

Lim, Y.C., Wakelin, M.W., Henault, L., Goetz, D.J., Yednock, T., Cabanas, C., Sanchez-Madrid, F., Lichtman, A.H., and Luscinskas, F.W. (2000). Alpha4beta1-integrin activation is necessary for high-efficiency T-cell subset interactions with VCAM-1 under flow. Microcirculation *7*, 201-214.

Ling, L., Cao, Z., and Goeddel, D.V. (1998). NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176. Proc Natl Acad Sci U S A *95*, 3792-3797.

Liu, F., Zhang, Y., Wu, Z.Q., and Zhao, T. (2011). Analysis of CCL5 expression in classical Hodgkin's lymphoma L428 cell line. Mol Med Rep *4*, 837-841.

Liu, Y., Sattarzadeh, A., Diepstra, A., Visser, L., and van den Berg, A. (2013). The microenvironment in classical Hodgkin lymphoma: An actively shaped and essential tumor component. Semin Cancer Biol *24*, 15-22.

Lorenzon, P., Vecile, E., Nardon, E., Ferrero, E., Harlan, J.M., Tedesco, F., and Dobrina, A. (1998). Endothelial cell E- and P-selectin and vascular cell adhesion molecule-1 function as signaling receptors. J Cell Biol *142*, 1381-1391.

Luo, J.L., Tan, W., Ricono, J.M., Korchynskyi, O., Zhang, M., Gonias, S.L., Cheresh, D.A., and Karin, M. (2007). Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. Nature *446*, 690-694.

Ma, Y., Visser, L., Roelofsen, H., de Vries, M., Diepstra, A., van Imhoff, G., van der Wal, T., Luinge, M., Alvarez-Llamas, G., Vos, H., Poppema, S., Vonk, R., van dern Berg, A. (2008). Proteomics analysis of Hodgkin lymphoma: identification of new players involved in the cross-talk between HRS cells and infiltrating lymphocytes. Blood *111*, 2339-2346.

Machado, L., Jarrett, R., Morgan, S., Murray, P., Hunter, B., Hamilton, E., Crocker, J., Thomas, W., Steven, N., Ismail, T., Chapman, A., Adams, D.H, Lee, S.P. (2009). Expression and function of T cell homing molecules in Hodgkin's lymphoma. Cancer Immunol Immunother *58*, 85-94.

Mackay, C.R., Marston, W.L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. J Exp Med *171*, 801-817.

Mackay, C.R., Terpe, H.J., Stauder, R., Marston, W.L., Stark, H., and Gunthert, U. (1994). Expression and modulation of CD44 variant isoforms in humans. J Cell Biol *124*, 71-82.

Maggio, E.M., Stekelenburg, E., Van den Berg, A., and Poppema, S. (2001). TP53 gene mutations in Hodgkin lymphoma are infrequent and not associated with absence of Epstein-Barr virus. Int J Cancer *94*, 60-66.

Mainou-Fowler, T., Angus, B., Miller, S., Proctor, S.J., Taylor, P.R., and Wood, K.M. (2006). Micro-vessel density and the expression of vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PdEGF) in classical Hodgkin lymphoma (HL). Leuk Lymphoma *47*, 223-230.

Maiti, A., Maki, G., and Johnson, P. (1998). TNF-alpha induction of CD44-mediated leukocyte adhesion by sulfation. Science *282*, 941-943.

Malek, S., Chen, Y., Huxford, T., and Ghosh, G. (2001). IkappaBbeta, but not IkappaBalpha, functions as a classical cytoplasmic inhibitor of NF-kappaB dimers by masking both NF-kappaB nuclear localization sequences in resting cells. J Biol Chem *276*, 45225-45235.

Manley, G., and Warren, C. (1987). Serum hyaluronic acid in patients with disseminated neoplasm. J Clin Pathol *40*, 626-630.

Marafioti, T., Hummel, M., Foss, H.D., Laumen, H., Korbjuhn, P., Anagnostopoulos, I., Lammert, H., Demel, G., Theil, J., Wirth, T., Stein, H. (2000). Hodgkin and reed-sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. Blood *95*, 1443-1450.

Marconcini, L., Marchio, S., Morbidelli, L., Cartocci, E., Albini, A., Ziche, M., Bussolino, F., and Oliviero, S. (1999). c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro. Proc Natl Acad Sci U S A *96*, 9671-9676.

Marelli-Berg, F.M., Cannella, L., Dazzi, F., and Mirenda, V. (2008). The highway code of T cell trafficking. J Pathol *214*, 179-189.

Martin-Subero, J.I., Gesk, S., Harder, L., Sonoki, T., Tucker, P.W., Schlegelberger, B., Grote, W., Novo, F.J., Calasanz, M.J., Hansmann, M.L., Dyer, M.J, Siebert, R. (2002). Recurrent involvement of the REL and BCL11A loci in classical Hodgkin lymphoma. Blood *99*, 1474-1477.

Martin-Subero, J.I., Wlodarska, I., Bastard, C., Picquenot, J.M., Hoppner, J., Giefing, M., Klapper, W., and Siebert, R. (2006). Chromosomal rearrangements involving the BCL3 locus are recurrent in classical Hodgkin and peripheral T-cell lymphoma. Blood *108*, 401-402; author reply 402-403.

Maslinska, D., Kaliszek, A., Opertowska, J., Toborowicz, J., Deregowski, K., and Szukiewicz, D. (1999). Constitutive expression of cyclooxygenase-2 (COX-2) in developing brain. A. Choroid plexus in human fetuses. Folia Neuropathol *37*, 287-291.

Mathas, S., Hinz, M., Anagnostopoulos, I., Krappmann, D., Lietz, A., Jundt, F., Bommert, K., Mechta-Grigoriou, F., Stein, H., Dorken, B., Scheidereit, C. (2002). Aberrantly expressed c-Jun and JunB are a hallmark of Hodgkin lymphoma cells, stimulate proliferation and synergize with NF-kappa B. Embo J *21*, 4104-4113.

Mathas, S., Johrens, K., Joos, S., Lietz, A., Hummel, F., Janz, M., Jundt, F., Anagnostopoulos, I., Bommert, K., Lichter, P., Stein, H., Scheidereit, C., Dorken, B. (2005). Elevated NF-kappaB p50 complex formation and Bcl-3 expression in classical Hodgkin, anaplastic large-cell, and other peripheral T-cell lymphomas. Blood *106*, 4287-4293.

Mathas, S., Lietz, A., Anagnostopoulos, I., Hummel, F., Wiesner, B., Janz, M., Jundt, F., Hirsch, B., Johrens-Leder, K., Vornlocher, H.P., Bommert, K., Stein, H., Dorken, B. (2004). c-FLIP mediates resistance of Hodgkin/Reed-Sternberg cells to death receptor-induced apoptosis. J Exp Med *199*, 1041-1052.

May, M.J., and Ghosh, S. (1997). Rel/NF-kappa B and I kappa B proteins: an overview. Semin Cancer Biol *8*, 63-73.

Mbeunkui, F., and Johann, D.J., Jr. (2009). Cancer and the tumor microenvironment: a review of an essential relationship. Cancer Chemother Pharmacol *63*, 571-582.

McCarthy, S.A., Chen, D., Yang, B.S., Garcia Ramirez, J.J., Cherwinski, H., Chen, X.R., Klagsbrun, M., Hauser, C.A., Ostrowski, M.C., and McMahon, M. (1997). Rapid phosphorylation of Ets-2 accompanies mitogen-activated protein kinase activation and the induction of heparin-binding epidermal

growth factor gene expression by oncogenic Raf-1. Mol Cell Biol *17*, 2401-2412.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L., and Bainton, D.F. (1989). GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. J Clin Invest *84*, 92-99.

Meltzer, M.S., and Bartlett, G.L. (1972). Cytotoxicity in vitro by products of specifically stimulated spleen cells: susceptibility of tumor cells and normal cells. J Natl Cancer Inst *49*, 1439-1443.

Messer, G., Weiss, E.H., and Baeuerle, P.A. (1990). Tumor necrosis factor beta (TNF-beta) induces binding of the NF-kappa B transcription factor to a high-affinity kappa B element in the TNF-beta promoter. Cytokine *2*, 389-397.

Mestre, F., Gutierrez, A., Ramos, R., Martinez-Serra, J., Sanchez, L., Matheu, G., Ros, T., Garcia, J.F., and Rodriguez, J. (2012). Expression of COX-2 on Reed-Sternberg cells is an independent unfavorable prognostic factor in Hodgkin lymphoma treated with ABVD. Blood *119*, 6072-6079.

Micheau, O., and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. Cell *114*, 181-190.

Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., and Rot, A. (1997). Transcytosis and surface presentation of IL-8 by venular endothelial cells. Cell *91*, 385-395.

Minden, A., Lin, A., Smeal, T., Dérijard, B., Cobb, M., Davis, R., and Karin, M. (1994). c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol Cell Biol *14*, 6683-6688.

Miyasaka, M., and Tanaka, T. (2004). Lymphocyte trafficking across high endothelial venules: dogmas and enigmas. Nat Rev Immunol *4*, 360-370.

Moghaddam, A., Zhang, H.T., Fan, T.P., Hu, D.E., Lees, V.C., Turley, H., Fox, S.B., Gatter, K.C., Harris, A.L., and Bicknell, R. (1995). Thymidine phosphorylase is angiogenic and promotes tumor growth. Proc Natl Acad Sci U S A *92*, 998-1002.

Mohamadzadeh, M., DeGrendele, H., Arizpe, H., Estess, P., and Siegelman, M. (1998). Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. J Clin Invest *101*, 97-108.

Molin, D., Fischer, M., Xiang, Z., Larsson, U., Harvima, I., Venge, P., Nilsson, K., Sundstrom, C., Enblad, G., and Nilsson, G. (2001). Mast cells express functional CD30 ligand and are the predominant CD30L-positive cells in Hodgkin's disease. Br J Haematol *114*, 616-623.

Montesinos-Rongen, M., Roers, A., Kuppers, R., Rajewsky, K., and Hansmann, M.L. (1999). Mutation of the p53 gene is not a typical feature of Hodgkin and Reed-Sternberg cells in Hodgkin's disease. Blood *94*, 1755-1760.

Moore, K.L., Patel, K.D., Bruehl, R.E., Li, F., Johnson, D.A., Lichenstein, H.S., Cummings, R.D., Bainton, D.F., and McEver, R.P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J Cell Biol *128*, 661-671.

Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A., Smithies, O. (1995). Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell *83*, 473-482.

Morrison, D.C., and Jacobs, D.M. (1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry *13*, 813-818.

Mottok, A., Renne, C., Willenbrock, K., Hansmann, M.L., and Brauninger, A. (2007). Somatic hypermutation of SOCS1 in lymphocyte-predominant Hodgkin lymphoma is accompanied by high JAK2 expression and activation of STAT6. Blood *110*, 3387-3390.

Muschen, M., Rajewsky, K., Brauninger, A., Baur, A.S., Oudejans, J.J., Roers, A., Hansmann, M.L., and Kuppers, R. (2000). Rare occurrence of classical Hodgkin's disease as a T cell lymphoma. J Exp Med *191*, 387-394.

Nagel, S., Burek, C., Venturini, L., Scherr, M., Quentmeier, H., Meyer, C., Rosenwald, A., Drexler, H.G., and MacLeod, R.A. (2007). Comprehensive analysis of homeobox genes in Hodgkin lymphoma cell lines identifies dysregulated expression of HOXB9 mediated via ERK5 signaling and BMI1. Blood *109*, 3015-3023.

Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D., Jarrett-Nedwin, J., Pennica, D., Goeddel, D.V., and Gray, P.W. (1985). Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids Res *13*, 6361-6373.

Neish, A.S., Read, M.A., Thanos, D., Pine, R., Maniatis, T., and Collins, T. (1995). Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. Mol Cell Biol *15*, 2558-2569.

Nelson, J.E., and Harris, R.E. (2000). Inverse association of prostate cancer and non-steroidal anti-inflammatory drugs (NSAIDs): results of a case-control study. Oncol Rep *7*, 169-170.

Ness, R.B., and Modugno, F. (2006). Endometriosis as a model for inflammation-hormone interactions in ovarian and breast cancers. Eur J Cancer *42*, 691-703.

Niens, M., Visser, L., Nolte, I.M., van der Steege, G., Diepstra, A., Cordano, P., Jarrett, R.F., Te Meerman, G.J., Poppema, S., and van den Berg, A. (2008).

Serum chemokine levels in Hodgkin lymphoma patients: highly increased levels of CCL17 and CCL22. Br J Haematol *140*, 527-536.

Niwa, Y., Hirose, K., Matsuo, K., Tajima, K., Ikoma, Y., Nakanishi, T., Nawa, A., Kuzuya, K., Tamakoshi, A., and Hamajima, N. (2005). Lymphotoxin-alpha polymorphism and the risk of cervical cancer in Japanese subjects. Cancer Lett *218*, 63-68.

Niwa, Y., Ito, H., Matsuo, K., Hirose, K., Ito, N., Mizuno, M., Hamajima, N., Tajima, K., and Nakanishi, T. (2007). Lymphotoxin-alpha polymorphisms and the risk of endometrial cancer in Japanese subjects. Gynecol Oncol *104*, 586-590.

Nonomura, N., Tokizane, T., Nakayama, M., Inoue, H., Nishimura, K., Muramatsu, M., and Okuyama, A. (2006). Possible correlation between polymorphism in the tumor necrosis factor-beta gene and the clinicopathological features of bladder cancer in Japanese patients. Int J Urol *13*, 971-976.

Norris, P.S., and Ware, C.F. (2007). The LT beta R signaling pathway. Adv Exp Med Biol *597*, 160-172.

Nourshargh, S., Krombach, F., and Dejana, E. (2006). The role of JAM-A and PECAM-1 in modulating leukocyte infiltration in inflamed and ischemic tissues. J Leukoc Biol *80*, 714-718.

Nácher, M., Blázquez, A.B., Shao, B., Matesanz, A., Prophete, C., Berin, M.C., Frenette, P.S., and Hidalgo, A. (2011). Physiological contribution of CD44 as a ligand for E-Selectin during inflammatory T-cell recruitment. Am J Pathol *178*, 2437-2446.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogeneous T helper cell subsets. Immunity *8*, 275-283.

O'Garra, A., and Arai, N. (2000). The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell Biol *10*, 542-550.

O'Garra, A., Steinman, L., and Gijbels, K. (1997). CD4+ T-cell subsets in autoimmunity. Curr Opin Immunol *9*, 872-883.

Ohsawa, M., Fukushima, H., Ikura, Y., Inoue, T., Shirai, N., Sugama, Y., Suekane, T., Kitabayashi, C., Nakamae, H., Hino, M. Ueda, M. (2006). Expression of cyclooxygenase-2 in Hodgkin's lymphoma: its role in cell proliferation and angiogenesis. Leuk Lymphoma *47*, 1863-1871.

Ohshima, K., Akaiwa, M., Umeshita, R., Suzumiya, J., Izuhara, K., and Kikuchi, M. (2001). Interleukin-13 and interleukin-13 receptor in Hodgkin's disease: possible autocrine mechanism and involvement in fibrosis. Histopathology *38*, 368-375.

Ohshima, K., Karube, K., Hamasaki, M., Suefuji, H., Tutiya, T., Yamaguchi, T., Suzumiya, J., and Kikuchi, M. (2003). Imbalances of chemokines,

chemokine receptors and cytokines in Hodgkin lymphoma: classical Hodgkin lymphoma vs. Hodgkin-like ATLL. Int J Cancer *106*, 706-712.

Ohshima, K., Tutiya, T., Yamaguchi, T., Suzuki, K., Suzumiya, J., Kawasaki, C., Haraoka, S., and Kikuchi, M. (2002). Infiltration of Th1 and Th2 lymphocytes around Hodgkin and Reed-Sternberg (H&RS) cells in Hodgkin disease: Relation with expression of CXC and CC chemokines on H&RS cells. Int J Cancer *98*, 567-572.

Okamoto, M., Lee, C., and Oyasu, R. (1997). Interleukin-6 as a paracrine and autocrine growth factor in human prostatic carcinoma cells in vitro. Cancer Res *57*, 141-146.

Oksala, O., Salo, T., Tammi, R., Häkkinen, L., Jalkanen, M., Inki, P., and Larjava, H. (1995). Expression of proteoglycans and hyaluronan during wound healing. J Histochem Cytochem *43*, 125-135.

Olumi, A.F., Grossfeld, G.D., Hayward, S.W., Carroll, P.R., Tlsty, T.D., and Cunha, G.R. (1999). Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res *59*, 5002-5011.

Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seidedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., and Weinberg, R.A. (2007). Stromal fibroblast present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell *121*, 335-348.

Otto, J.C., and Smith, W.L. (1995). Prostaglandin endoperoxide synthases-1 and -2. J Lipid Mediat Cell Signal *12*, 139-156.

Oudejans, J.J., Jiwa, N.M., Kummer, J.A., Ossenkoppele, G.J., van Heerde, P., Baars, J.W., Kluin, P.M., Kluin-Nelemans, J.C., van Diest, P.J., Middeldorp, J.M., Meijer, C.J. (1997). Activated cytotoxic T cells as prognostic marker in Hodgkin's disease. Blood *89*, 1376-1382.

Pai, R., Szabo, I.L., Soreghan, B.A., Atay, S., Kawanaka, H., and Tarnawski, A.S. (2001). PGE(2) stimulates VEGF expression in endothelial cells via ERK2/JNK1 signaling pathways. Biochem Biophys Res Commun *286*, 923-928.

Park, J.M., Adam, R.M., Peters, C.A., Guthrie, P.D., Sun, Z., Klagsbrun, M., and Freeman, M.R. (1999). AP-1 mediates stretch-induced expression of HB-EGF in bladder smooth muscle cells. Am J Physiol *277*, C294-301.

Parrett, M., Harris, R., Joarder, F., Ross, M., Clausen, K., and Robertson, F. (1997). Cyclooxygenase-2 gene expression in human breast cancer. Int J Oncol *10*, 503-507.

Passegue, E., Jochum, W., Schorpp-Kistner, M., Mohle-Steinlein, U., and Wagner, E.F. (2001). Chronic myeloid leukemia with increased granulocyte

progenitors in mice lacking junB expression in the myeloid lineage. Cell *104*, 21-32.

Passegue, E., and Wagner, E.F. (2000). JunB suppresses cell proliferation by transcriptional activation of p16(INK4a) expression. Embo J *19*, 2969-2979.

Paul, N.L., Lenardo, M.J., Novak, K.D., Sarr, T., Tang, W.L., and Ruddle, N.H. (1990). Lymphotoxin activation by human T-cell leukemia virus type I-infected cell lines: role for NF-kappa B. J Virol *64*, 5412-5419.

Peach, R.J., Hollenbaugh, D., Stamenkovic, I., and Aruffo, A. (1993). Identification of hyaluronic acid binding sites in the extracellular domain of CD44. J Cell Biol *122*, 257-264.

Peh, S.C., Kim, L.H., and Poppema, S. (2001). TARC, a CC chemokine, is frequently expressed in classic Hodgkin's lymphoma but not in NLP Hodgkin's lymphoma, T-cell-rich B-cell lymphoma, and most cases of anaplastic large cell lymphoma. Am J Surg Pathol *25*, 925-929.

Perussia, B., Kobayashi, M., Rossi, M.E., Anegon, I., and Trinchieri, G. (1987). Immune interferon enhances functional properties of human granulocytes: role of Fc receptors and effect of lymphotoxin, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor. J Immunol *138*, 765-774.

Peterson, B.R., Sun, L.J., and Verdine, G.L. (1996). A critical arginine residue mediates cooperativity in the contact interface between transcription factors NFAT and AP-1. Proc Natl Acad Sci U S A *93*, 13671-13676.

Pfarr, C.M., Mechta, F., Spyrou, G., Lallemand, D., Carillo, S., and Yaniv, M. (1994). Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. Cell *76*, 747-760.

Piccirillo, C.A. (2008). Regulatory T cells in health and disease. Cytokine *43*, 395-401.

Picker, L.J., Kishimoto, T.K., Smith, C.W., Warnock, R.A., and Butcher, E.C. (1991). ELAM-1 is an adhesion molecule for skin-homing T cells. Nature *349*, 796-799.

Picker, L.J., Nakache, M., and Butcher, E.C. (1989). Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. J Cell Biol *109*, 927-937.

Picker, L.J., Terstappen, L.W., Rott, L.S., Streeter, P.R., Stein, H., and Butcher, E.C. (1990). Differential expression of homing-associated adhesion molecules by T cell subsets in man. J Immunol *145*, 3247-3255.

Pietersma, A., Tilly, B.C., Gaestel, M., de Jong, N., Lee, J.C., Koster, J.F., and Sluiter, W. (1997). p38 mitogen activated protein kinase regulates endothelial VCAM-1 expression at the post-transcriptional level. Biochem Biophys Res Commun *230*, 44-48.

Pinto, A., Aldinucci, D., Gloghini, A., Zagonel, V., Degan, M., Improta, S., Juzbasic, S., Todesco, M., Perin, V., Gattei, V., Hermann, F., Gruss, H.J., Carbone, A. (1996). Human eosinophils express functional CD30 ligand and stimulate proliferation of a Hodgkin's disease cell line. Blood *88*, 3299-3305.

Pober, J.S., Gimbrone, M.A., Jr., Lapierre, L.A., Mendrick, D.L., Fiers, W., Rothlein, R., and Springer, T.A. (1986). Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. J Immunol *137*, 1893-1896.

Pober, J.S., Lapierre, L.A., Stolpen, A.H., Brock, T.A., Springer, T.A., Fiers, W., Bevilacqua, M.P., Mendrick, D.L., and Gimbrone, M.A., Jr. (1987). Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. J Immunol *138*, 3319-3324.

Poppema, S. (1989). The nature of the lymphocytes surrounding Reed-Sternberg cells in nodular lymphocyte predominance and in other types of Hodgkin's disease. Am J Pathol *135*, 351-357.

Pribila, J.T., Quale, A.C., Mueller, K.L., and Shimizu, Y. (2004). Integrins and T cell-mediated immunity. Annu Rev Immunol *22*, 157-180.

Pulverer, B.J., Kyriakis, J.M., Avruch, J., Nikolakaki, E., and Woodgett, J.R. (1991). Phosphorylation of c-jun mediated by MAP kinases. Nature *353*, 670-674.

Qian, B.Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell *141*, 39-51.

Rawlings, J.S., Rosler, K.M., and Harrison, D.A. (2004). The JAK/STAT signaling pathway. J Cell Sci *117*, 1281-1283.

Read, M.A., Whitley, M.Z., Gupta, S., Pierce, J.W., Best, J., Davis, R.J., and Collins, T. (1997). Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J Biol Chem *272*, 2753-2761.

Rebollo, A., Dumoutier, L., Renauld, J.C., Zaballos, A., Ayllon, V., and Martinez, A.C. (2000). Bcl-3 expression promotes cell survival following interleukin-4 deprivation and is controlled by AP1 and AP1-like transcription factors. Mol Cell Biol *20*, 3407-3416.

Rehm, A., Mensen, A., Schradi, K., Gerlach, K., Wittstock, S., Winter, S., Buchner, G., Dorken, B., Lipp, M., and Hopken, U.E. (2011). Cooperative function of CCR7 and lymphotoxin in the formation of a lymphoma-permissive niche within murine secondary lymphoid organs. Blood *118*, 1020-1033.

Reichardt, P., Patzak, I., Jones, K., Etemire, E., Gunzer, M., and Hogg, N. (2013). A role for LFA-1 in delaying T-lymphocyte egress from lymph nodes. EMBO J *32*, 829-843.

Reichmann, E., Schwarz, H., Deiner, E.M., Leitner, I., Eilers, M., Berger, J., Busslinger, M., and Beug, H. (1992). Activation of an inducible c-FosER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. Cell *71*, 1103-1116.

Reiss, Y., Proudfoot, A.E., Power, C.A., Campbell, J.J., and Butcher, E.C. (2001). CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. J Exp Med *194*, 1541-1547.

Remouchamps, C., Boutaffala, L., Ganeff, C., and Dejardin, E. (2011). Biology and signal transduction pathways of the Lymphotoxin-alphabeta/LTbetaR system. Cytokine Growth Factor Rev *22*, 301-310.

Renne, C., Willenbrock, K., Kuppers, R., Hansmann, M.L., and Brauninger, A. (2005). Autocrine- and paracrine-activated receptor tyrosine kinases in classic Hodgkin lymphoma. Blood *105*, 4051-4059.

Rennert, P.D., Browning, J.L., Mebius, R., Mackay, F., and Hochman, P.S. (1996). Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J Exp Med *184*, 1999-2006.

Renz, H., Gong, J.H., Schmidt, A., Nain, M., and Gemsa, D. (1988). Release of tumor necrosis factor-alpha from macrophages. Enhancement and suppression are dose-dependently regulated by prostaglandin E2 and cyclic nucleotides. J Immunol *141*, 2388-2393.

Reynolds, G.M., Billingham, L.J., Gray, L.J., Flavell, J.R., Najafipour, S., Crocker, J., Nelson, P., Young, L.S., and Murray, P.G. (2002). Interleukin 6 expression by Hodgkin/Reed-Sternberg cells is associated with the presence of 'B' symptoms and failure to achieve complete remission in patients with advanced Hodgkin's disease. Br J Haematol *118*, 195-201.

Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1997). Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res *57*, 1276-1280.

Rivera-Nieves, J., Burcin, T.L., Olson, T.S., Morris, M.A., McDuffie, M., Cominelli, F., and Ley, K. (2006). Critical role of endothelial P-selectin glycoprotein ligand 1 in chronic murine ileitis. J Exp Med *203*, 907-917.

Rodig, S.J., Ouyang, J., Juszczynski, P., Currie, T., Law, K., Neuberg, D.S., Rabinovich, G.A., Shipp, M.A., and Kutok, J.L. (2008). AP1-dependent galectin-1 expression delineates classical hodgkin and anaplastic large cell lymphomas from other lymphoid malignancies with shared molecular features. Clin Cancer Res *14*, 3338-3344.

Roebuck, K.A., Rahman, A., Lakshminarayanan, V., Janakidevi, K., and Malik, A.B. (1995). H2O2 and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through

distinct cis-regulatory elements within the ICAM-1 promoter. J Biol Chem *270*, 18966-18974.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., van Hinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., Mantovani, A. (1997). Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity *6*, 315-325.

Rooney, J.W., Hoey, T., and Glimcher, L.H. (1995). Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. Immunity *2*, 473-483.

Rosen, S.D. (2004). Ligands for L-selectin: homing, inflammation, and beyond. Annu Rev Immunol *22*, 129-156.

Rouzer, C.A., and Marnett, L.J. (2003). Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. Chem Rev *103*, 2239-2304.

Ruco, L.P., Pomponi, D., Pigott, R., Gearing, A.J., Baiocchini, A., and Baroni, C.D. (1992). Expression and cell distribution of the intercellular adhesion molecule, vascular cell adhesion molecule, endothelial leukocyte adhesion molecule, and endothelial cell adhesion molecule (CD31) in reactive human lymph nodes and in Hodgkin's disease. Am J Pathol *140*, 1337-1344.

Ruddle, N.H., and Waksman, B.H. (1968). Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. I. Characterization of the phenomenon. J Exp Med *128*, 1237-1254.

Saez, E., Rutberg, S.E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S.H., and Spiegelman, B.M. (1995). c-fos is required for malignant progression of skin tumors. Cell *82*, 721-732.

Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775-787.

Sako, D., Chang, X.J., Barone, K.M., Vachino, G., White, H.M., Shaw, G., Veldman, G.M., Bean, K.M., Ahern, T.J., and Furie, B. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. Cell *75*, 1179-1186.

Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature *401*, 708-712.

San Mateo, L.R., Chua, M.M., Weiss, S.R., and Shen, H. (2002). Perforin-mediated CTL cytolysis counteracts direct cell-cell spread of Listeria monocytogenes. J Immunol *169*, 5202-5208.

Sanchez-Aguilera, A., Montalban, C., de la Cueva, P., Sanchez-Verde, L., Morente, M.M., Garcia-Cosio, M., Garcia-Larana, J., Bellas, C., Provencio, M., Romagosa, V., de Sevilla A.F., Menarquez, J., Sabin, P., Mestre, M.J., Mendez, M., Fresno, M.F., Nicolas, C., Piris, M.A., Garcia, J.F., Spanish

Hodgkin Lymphoma Study Group. (2006). Tumor microenvironment and mitotic checkpoint are key factors in the outcome of classic Hodgkin lymphoma. Blood *108*, 662-668.

Sappino, A.P., Seelentag, W., Pelte, M.F., Alberto, P., and Vassalli, P. (1990). Tumor necrosis factor/cachectin and lymphotoxin gene expression in lymph nodes from lymphoma patients. Blood *75*, 958-962.

Schall, T.J. (1991). Biology of the RANTES/SIS cytokine family. Cytokine *3*, 165-183.

Scheeren, F.A., Diehl, S.A., Smit, L.A., Beaumont, T., Naspetti, M., Bende, R.J., Blom, B., Karube, K., Ohshima, K., van Noesel, C.J., Spits, H. (2008). IL-21 is expressed in Hodgkin lymphoma and activates STAT5: evidence that activated STAT5 is required for Hodgkin lymphomagenesis. Blood *111*, 4706-4715.

Schindler, U., and Baichwal, V.R. (1994). Three NF-kappa B binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol Cell Biol *14*, 5820-5831.

Schleimer, R.P., Sterbinsky, S.A., Kaiser, J., Bickel, C.A., Klunk, D.A., Tomioka, K., Newman, W., Luscinskas, F.W., Gimbrone, M.A., Jr., McIntyre, B.W., Bochner, B.S. (1992). IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J Immunol *148*, 1086-1092.

Schonbeck, U., Sukhova, G.K., Graber, P., Coulter, S., and Libby, P. (1999). Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol *155*, 1281-1291.

Schorpp-Kistner, M., Wang, Z.Q., Angel, P., and Wagner, E.F. (1999). JunB is essential for mammalian placentation. Embo J *18*, 934-948.

Schwaller, J., Went, P., Matthes, T., Dirnhofer, S., Donze, O., Mhawech-Fauceglia, P., Myit, S., and Huard, B. (2007). Paracrine promotion of tumor development by the TNF ligand APRIL in Hodgkin's Disease. Leukemia *21*, 1324-1327.

Seidemann, K., Zimmermann, M., Book, M., Meyer, U., Burkhardt, B., Welte, K., Reiter, A., and Stanulla, M. (2005). Tumor necrosis factor and lymphotoxin alfa genetic polymorphisms and outcome in pediatric patients with non-Hodgkin's lymphoma: results from Berlin-Frankfurt-Munster Trial NHL-BFM 95. J Clin Oncol *23*, 8414-8421.

Seitz, V., Hummel, M., Marafioti, T., Anagnostopoulos, I., Assaf, C., and Stein, H. (2000). Detection of clonal T-cell receptor gamma-chain gene rearrangements in Reed-Sternberg cells of classic Hodgkin disease. Blood *95*, 3020-3024.

Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell *46*, 705-716.

Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.C., Karin, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science *293*, 1495-1499.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., and Dvorak, H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science *219*, 983-985.

Shalaby, M.R., Aggarwal, B.B., Rinderknecht, E., Svedersky, L.P., Finkle, B.S., and Palladino, M.A., Jr. (1985). Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J Immunol *135*, 2069-2073.

Shaulian, E. (2010). AP-1--The Jun proteins: Oncogenes or tumor suppressors in disguise? Cell Signal *22*, 894-899.

Shaulian, E., and Karin, M. (2002). AP-1 as a regulator of cell life and death. Nat Cell Biol *4*, E131-136.

Shaulian, E., Schreiber, M., Piu, F., Beeche, M., Wagner, E.F., and Karin, M. (2000). The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. Cell *103*, 897-907.

Shebzukhov Iu, V., and Kuprash, D.V. (2011). Transcriptional regulation of TNF/LT locus in immune cells. Mol Biol *45*, 47-57.

Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.

Sher, A., and Coffman, R.L. (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. Annu Rev Immunol *10*, 385-409.

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. Immunol Today *13*, 106-112.

Shimizu, Y., van Seventer, G.A., Horgan, K.J., and Shaw, S. (1990). Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding, and costimulation. Immunol Rev *114*, 109-143.

Shimura, T., Hagihara, M., Takebe, K., Munkhbat, B., Odaka, T., Kato, H., Nagamachi, Y., and Tsuji, K. (1994). The study of tumor necrosis factor beta gene polymorphism in lung cancer patients. Cancer *73*, 1184-1188.

Siegelman, M.H., Stanescu, D., and Estess, P. (2000). The CD44-initiated pathway of T-cell extravasation uses VLA-4 but not LFA-1 for firm adhesion. J Clin Invest *105*, 683-691.

Singbartl, K., Thatte, J., Smith, M.L., Wethmar, K., Day, K., and Ley, K. (2001). A CD2-green fluorescence protein-transgenic mouse reveals very late

antigen-4-dependent CD8+ lymphocyte rolling in inflamed venules. J Immunol *166*, 7520-7526.

Skinnider, B.F., Elia, A.J., Gascoyne, R.D., Patterson, B., Trumper, L., Kapp, U., and Mak, T.W. (2002). Signal transducer and activator of transcription 6 is frequently activated in Hodgkin and Reed-Sternberg cells of Hodgkin lymphoma. Blood *99*, 618-626.

Skinnider, B.F., and Mak, T.W. (2002). The role of cytokines in classical Hodgkin lymphoma. Blood *99*, 4283-4297.

Smith, L.M., Wise, S.C., Hendricks, D.T., Sabichi, A.L., Bos, T., Reddy, P., Brown, P.H., and Birrer, M.J. (1999). cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. Oncogene *18*, 6063-6070.

Smith, W.L., DeWitt, D.L., and Garavito, R.M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem *69*, 145-182.

Solan, N.J., Miyoshi, H., Carmona, E.M., Bren, G.D., and Paya, C.V. (2002). RelB cellular regulation and transcriptional activity are regulated by p100. J Biol Chem *277*, 1405-1418.

Spertini, O., Luscinskas, F.W., Kansas, G.S., Munro, J.M., Griffin, J.D., Gimbrone, M.A., Jr., and Tedder, T.F. (1991). Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J Immunol *147*, 2565-2573.

Spies, T., Morton, C.C., Nedospasov, S.A., Fiers, W., Pious, D., and Strominger, J.L. (1986). Genes for the tumor necrosis factors alpha and beta are linked to the human major histocompatibility complex. Proc Natl Acad Sci U S A *83*, 8699-8702.

Steidl, C., Connors, J.M., and Gascoyne, R.D. (2011). Molecular pathogenesis of Hodgkin's lymphoma: increasing evidence of the importance of the microenvironment. J Clin Oncol *29*, 1812-1826.

Steidl, C., Lee, T., Shah, S.P., Farinha, P., Han, G., Nayar, T., Delaney, A., Jones, S.J., Iqbal, J., Weisenburger, D.D., Bass, M.A., Rosenwald, A., Muller-Hermelink, H.K., Rimsza, L.M., Campo, E., Delabie, J., Braziel, R.M., Cook, J.R., Tubbs, R.R., Jaffe, E.S., Lenz, G., Connors, J.M., Staudt, L.M., Chan, W.C., Gascoyne, R.D. (2010). Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. N Engl J Med *362*, 875-885.

Stein, B., Baldwin, A.S., Ballard, D.W., Greene, W.C., Angel, P., and Herrlich, P. (1993). Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J *12*, 3879-3891.

Streeter, P.R., Rouse, B.T., and Butcher, E.C. (1988). Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. J Cell Biol *107*, 1853-1862.

Subbaramaiah, K., Norton, L., Gerald, W., and Dannenberg, A.J. (2002). Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. J Biol Chem *277*, 18649-18657.

Suna, S., Sakata, Y., Sato, H., Mizuno, H., Nakatani, D., Shimizu, M., Usami, M., Takashima, S., Takeda, H., and Hori, M. (2008). Up-regulation of cell adhesion molecule genes in human endothelial cells stimulated by lymphotoxin alpha: DNA microarray analysis. J Atheroscler Thromb *15*, 160-165.

Suna, S., Sakata, Y., Shimizu, M., Nakatani, D., Usami, M., Matsumoto, S., Mizuno, H., Ozaki, K., Takashima, S., Takeda, H., Tanaka, T., Hori, M., Sato, H. (2009). Lymphotoxin-alpha3 mediates monocyte-endothelial interaction by TNFR I/NF-kappaB signaling. Biochem Biophys Res Commun *379*, 374-378.

Sutherland, J.A., Cook, A., Bannister, A.J., and Kouzarides, T. (1992). Conserved motifs in Fos and Jun define a new class of activation domain. Genes Dev *6*, 1810-1819.

Suzuki, T., Murakami, M., Onai, N., Fukuda, E., Hashimoto, Y., Sonobe, M.H., Kameda, T., Ichinose, M., Miki, K., and Iba, H. (1994). Analysis of AP-1 function in cellular transformation pathways. J Virol *68*, 3527-3535.

Svensson, M., Marsal, J., Ericsson, A., Carramolino, L., Broden, T., Marquez, G., and Agace, W.W. (2002). CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. J Clin Invest *110*, 1113-1121.

Tak, P.P., and Firestein, G.S. (2001). NF-kappaB: a key role in inflammatory diseases. J Clin Invest *107*, 7-11.

Takei, K., Ikeda, S., Arai, T., Tanaka, N., Muramatsu, M., and Sawabe, M. (2008). Lymphotoxin-alpha polymorphisms and presence of cancer in 1,536 consecutive autopsy cases. BMC Cancer *8*, 235-240.

Takeshita, H., Yoshizaki, T., Miller, W.E., Sato, H., Furukawa, M., Pagano, J.S., and Raab-Traub, N. (1999). Matrix metalloproteinase 9 expression is induced by Epstein-Barr virus latent membrane protein 1 C-terminal activation regions 1 and 2. J Virol *73*, 5548-5555.

Tam, W.F., and Sen, R. (2001). IkappaB family members function by different mechanisms. J Biol Chem *276*, 7701-7704.

Tamura, D.Y., Moore, E.E., Johnson, J.L., Zallen, G., Aiboshi, J., and Silliman, C.C. (1998). p38 mitogen-activated protein kinase inhibition attenuates intercellular adhesion molecule-1 up-regulation on human pulmonary microvascular endothelial cells. Surgery *124*, 403-407; discussion 408.

Tan, T.T., and Coussens, L.M. (2007). Humoral immunity, inflammation and cancer. Curr Opin Immunol *19*, 209-216.

Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S. (1993). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. Nature *361*, 79-82.

Tang, Y., Nakada, M.T., Kesavan, P., McCabe, F., Millar, H., Rafferty, P., Bugelski, P., and Yan, L. (2005). Extracellular matrix metalloproteinase inducer stimulates tumor angiogenesis by elevating vascular endothelial cell growth factor and matrix metalloproteinases. Cancer Res *65*, 3193-3199.

Tanijiri, T., Shimizu, T., Uehira, K., Yokoi, T., Amuro, H., Sugimoto, H., Torii, Y., Tajima, K., Ito, T., Amakawa, R., Fukuhara, S. (2007). Hodgkin's reed-sternberg cell line (KM-H2) promotes a bidirectional differentiation of CD4+CD25+Foxp3+ T cells and CD4+ cytotoxic T lymphocytes from CD4+ naive T cells. J Leukoc Biol *82*, 576-584.

Taub, D.D., Conlon, K., Lloyd, A.R., Oppenheim, J.J., and Kelvin, D.J. (1993). Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. Science *260*, 355-358.

Tedder, T.F., Steeber, D.A., Chen, A., and Engel, P. (1995). The selectins: vascular adhesion molecules. FASEB J *9*, 866-873.

Thomas, R.K., Re, D., Wolf, J., and Diehl, V. (2004). Part I: Hodgkin's lymphoma--molecular biology of Hodgkin and Reed-Sternberg cells. Lancet Oncol *5*, 11-18.

Thompson, H.J., Jiang, C., Lu, J., Mehta, R.G., Piazza, G.A., Paranka, N.S., Pamukcu, R., and Ahnen, D.J. (1997). Sulfone metabolite of sulindac inhibits mammary carcinogenesis. Cancer Res *57*, 267-271.

Thomson, B.M., Mundy, G.R., and Chambers, T.J. (1987). Tumor necrosis factors alpha and beta induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol *138*, 775-779.

Thornhill, M.H., and Haskard, D.O. (1990). IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN-gamma. J Immunol *145*, 865-872.

Thorns, C., Feller, A.C., and Merz, H. (2002). EMMPRIN (CD 174) is expressed in Hodgkin's lymphoma and anaplastic large cell lymphoma. An immunohistochemical study of 60 cases. Anticancer Res *22*, 1983-1986.

Toft, D.J., Rosenberg, S.B., Bergers, G., Volpert, O., and Linzer, D.I. (2001). Reactivation of proliferin gene expression is associated with increased angiogenesis in a cell culture model of fibrosarcoma tumor progression. Proc Natl Acad Sci U S A *98*, 13055-13059.

Treisman, R. (1992). The serum response element. Trends Biochem Sci *17*, 423-426.

Truman, L.A., Ford, C.A., Pasikowska, M., Pound, J.D., Wilkinson, S.J., Dumitriu, I.E., Melville, L., Melrose, L.A., Ogden, C.A., Nibbs, R., Graham,

G., Combadiere, C., Gregory, C.D. (2008). CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood *112*, 5026-5036.

Tsai, E.Y., Jain, J., Pesavento, P.A., Rao, A., and Goldfeld, A.E. (1996). Tumor necrosis factor alpha gene regulation in activated T cells involves ATF-2/Jun and NFATp. Mol Cell Biol *16*, 459-467.

Tsuzaki, M., Guyton, G., Garrett, W., Archambault, J.M., Herzog, W., Almekinders, L., Bynum, D., Yang, X., and Banes, A.J. (2003). IL-1 beta induces COX2, MMP-1, -3 and -13, ADAMTS-4, IL-1 beta and IL-6 in human tendon cells. J Orthop Res *21*, 256-264.

Tzankov, A., Bourgau, C., Kaiser, A., Zimpfer, A., Maurer, R., Pileri, S.A., Went, P., and Dirnhofer, S. (2005). Rare expression of T-cell markers in classical Hodgkin's lymphoma. Mod Pathol *18*, 1542-1549.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., and Verma, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science *274*, 787-789.

van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P., Zantema, A., Angel, P., and van der Eb, A.J. (1993). Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. Embo J *12*, 479-487.

van den Berg, A., Visser, L., and Poppema, S. (1999). High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltratein Hodgkin's lymphoma. Am J Pathol *154*, 1685-1691.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol *11*, 700-714.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nat New Biol *231*, 232-235.

Vane, J.R., Bakhle, Y.S., and Botting, R.M. (1998). Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol *38*, 97-120.

Verma, I.M., Stevenson, J.K., Schwarz, E.M., Van Antwerp, D., and Miyamoto, S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. Genes Dev *9*, 2723-2735.

Vidal-Vanaclocha, F., Fantuzzi, G., Mendoza, L., Fuentes, A.M., Anasagasti, M.J., Martin, J., Carrascal, T., Walsh, P., Reznikov, L.L., Kim, S.H., Novick, D., Rubinstein, M., Dinarello, C.A. (2000). IL-18 regulates IL-1beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. Proc Natl Acad Sci U S A *97*, 734-739.

Vigouroux, S., Yvon, E., Biagi, E., and Brenner, M.K. (2004). Antigen-induced regulatory T cells. Blood *104*, 26-33.

von Andrian, U.H., and Mempel, T.R. (2003). Homing and cellular traffic in lymph nodes. Nat Rev Immunol *3*, 867-878.

von Hundelshausen, P., Koenen, R.R., Sack, M., Mause, S.F., Adriaens, W., Proudfoot, A.E., Hackeng, T.M., and Weber, C. (2005). Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. Blood *105*, 924-930.

von Hundelshausen, P., Weber, K.S., Huo, Y., Proudfoot, A.E., Nelson, P.J., Ley, K., and Weber, C. (2001). RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation *103*, 1772-1777.

von Kalle, C., Wolf, J., Becker, A., Sckaer, A., Munck, M., Engert, A., Kapp, U., Fonatsch, C., Komitowski, D., Feaux de Lacroix, W., Diehl, V. (1992). Growth of Hodgkin cell lines in severely combined immunodeficient mice. Int J Cancer *52*, 887-891.

Vu, F., Dianzani, U., Ware, C.F., Mak, T., and Gommerman, J.L. (2008). ICOS, CD40, and lymphotoxin beta receptors signal sequentially and interdependently to initiate a germinal center reaction. J Immunol *180*, 2284-2293.

Wagner, N., Lohler, J., Kunkel, E.J., Ley, K., Leung, E., Krissansen, G., Rajewsky, K., and Muller, W. (1996). Critical role for beta7 integrins in formation of the gut-associated lymphoid tissue. Nature *382*, 366-370.

Wang, S.S., Cerhan, J.R., Hartge, P., Davis, S., Cozen, W., Severson, R.K., Chatterjee, N., Yeager, M., Chanock, S.J., and Rothman, N. (2006). Common genetic variants in proinflammatory and other immunoregulatory genes and risk for non-Hodgkin lymphoma. Cancer Res *66*, 9771-9780.

Wang, W., Abbruzzese, J.L., Evans, D.B., and Chiao, P.J. (1999). Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. Oncogene *18*, 4554-4563.

Ware, C.F. (2005). Network communications: lymphotoxins, LIGHT, and TNF. Annu Rev Immunol *23*, 787-819.

Warzocha, K., Bienvenu, J., Ribeiro, P., Moullet, I., Dumontet, C., Neidhardt-Berard, E.M., Coiffier, B., and Salles, G. (1998). Plasma levels of tumour necrosis factor and its soluble receptors correlate with clinical features and outcome of Hodgkin's disease patients. Br J Cancer *77*, 2357-2362.

Watanabe, M., Ogawa, Y., Ito, K., Higashihara, M., Kadin, M.E., Abraham, L.J., Watanabe, T., and Horie, R. (2003). AP-1 mediated relief of repressive activity of the CD30 promoter microsatellite in Hodgkin and Reed-Sternberg cells. Am J Pathol *163*, 633-641.

Waterhouse, N.J., Clarke, C.J., Sedelies, K.A., Teng, M.W., and Trapani, J.A. (2004). Cytotoxic lymphocytes; instigators of dramatic target cell death. Biochem Pharmacol *68*, 1033-1040.

Watson, C., Whittaker, S., Smith, N., Vora, A.J., Dumonde, D.C., and Brown, K.A. (1996). IL-6 acts on endothelial cells to preferentially increase their adherence for lymphocytes. Clin Exp Immunol *105*, 112-119.

Weih, F., and Caamano, J. (2003). Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. Immunol Rev *195*, 91-105.

Wellicome, S.M., Thornhill, M.H., Pitzalis, C., Thomas, D.S., Lanchbury, J.S., Panayi, G.S., and Haskard, D.O. (1990). A monoclonal antibody that detects a novel antigen on endothelial cells that is induced by tumor necrosis factor, IL-1, or lipopolysaccharide. J Immunol *144*, 2558-2565.

Weniger, M.A., Melzner, I., Menz, C.K., Wegener, S., Bucur, A.J., Dorsch, K., Mattfeldt, T., Barth, T.F., and Moller, P. (2006). Mutations of the tumor suppressor gene SOCS-1 in classical Hodgkin lymphoma are frequent and associated with nuclear phospho-STAT5 accumulation. Oncogene *25*, 2679-2684.

Westra, J., Kuldo, J.M., van Rijswijk, M.H., Molema, G., and Limburg, P.C. (2005). Chemokine production and E-selectin expression in activated endothelial cells are inhibited by p38 MAPK (mitogen activated protein kinase) inhibitor RWJ 67657. Int Immunopharmacol *5*, 1259-1269.

Whitfield, J., Neame, S.J., Paquet, L., Bernard, O., and Ham, J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron *29*, 629-643.

Williams, J.A., and Shacter, E. (1997). Regulation of macrophage cytokine production by prostaglandin E2. Distinct roles of cyclooxygenase-1 and -2. J Biol Chem *272*, 25693-25699.

Williams, T.W., and Bellanti, J.A. (1984). In vitro protective and enhancing effects of interferons from several species on human lymphotoxin-induced target cell killing. Cell Immunol *83*, 255-261.

Wisdom, R., Johnson, R.S., and Moore, C. (1999). c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. Embo J *18*, 188-197.

Wolf, J., Kapp, U., Bohlen, H., Kornacker, M., Schoch, C., Stahl, B., Mucke, S., von Kalle, C., Fonatsch, C., Schaefer, H.E., Hansmann, M.L., Diehl, V. (1996). Peripheral blood mononuclear cells of a patient with advanced Hodgkin's lymphoma give rise to permanently growing Hodgkin-Reed Sternberg cells. Blood *87*, 3418-3428.

Worm, M.M., Tsytsykova, A., and Geha, R.S. (1998). CD40 ligation and IL-4 use different mechanisms of transcriptional activation of the human lymphotoxin alpha promoter in B cells. Eur J Immunol *28*, 901-906.

Wyckoff, J.B., Wang, Y., Lin, E.Y., Gowswami, S., Stanley, E.R., Segall, J.E., Pollard, J.W., and Condeelis, J. (2007). Direct visualization of macrophage-associated tumor cell intravasation in mammary tumors. Cancer Res *67*, 2649-2656.

Xerri, L., Birg, F., Guigou, V., Bouabdallah, R., Poizot-Martin, I., and Hassoun, J. (1992). In situ expression of the IL-1-alpha and TNF-alpha genes by Reed-Sternberg cells in Hodgkin's disease. Int J Cancer *50*, 689-693.

Yabushita, H., Iwasaki, K., Kanyama, K., Obayashi, Y., Zhuo, L., Itano, N., Kimata, K., and Wakatsuki, A. (2011). Clinicopathological Role of Serum-Derived Hyaluronan-Associated Protein (SHAP)-Hyaluronan Complex in Endometrial Cancer. Obstet Gynecol Int *2011*, 739150.

Yan, W., Zhao, K., Jiang, Y., Huang, Q., Wang, J., Kan, W., and Wang, S. (2002). Role of p38 MAPK in ICAM-1 expression of vascular endothelial cells induced by lipopolysaccharide. Shock *17*, 433-438.

Yang, L., Cohn, L., Zhang, D.H., Homer, R., Ray, A., and Ray, P. (1998). Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. J Exp Med *188*, 1739-1750.

Yao, J., Mackman, N., Edgington, T.S., and Fan, S.T. (1997). Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. J Biol Chem *272*, 17795-17801.

Young, J., Yu, X., Wolslegel, K., Nguyen, A., Kung, C., Chiang, E., Kolumam, G., Wei, N., Wong, W.L., DeForge, L., Townsend, M.J., Grogan, J.L. (2010). Lymphotoxin-alphabeta heterotrimers are cleaved by metalloproteinases and contribute to synovitis in rheumatoid arthritis. Cytokine *51*, 78-86.

Young, M.R., Li, J.J., Rincon, M., Flavell, R.A., Sathyanarayana, B.K., Hunziker, R., and Colburn, N. (1999). Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc Natl Acad Sci U S A *96*, 9827-9832.

Yuan, C., Sidhu, R.S., Kuklev, D.V., Kado, Y., Wada, M., Song, I., and Smith, W.L. (2009). Cyclooxygenase Allosterism, Fatty Acid-mediated Cross-talk between Monomers of Cyclooxygenase Homodimers. J Biol Chem *284*, 10046-10055.

Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., Sibilia, M., and Wagner, E.F. (2003). c-Jun regulates eyelid closure and skin tumor development through EGFR signaling. Dev Cell *4*, 879-889.

Zerbini, L.F., Wang, Y., Cho, J.Y., and Libermann, T.A. (2003). Constitutive activation of nuclear factor kappaB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer. Cancer Res *63*, 2206-2215.

Zhang, Y., Chen, M.B., Zhou, X.Y., and Hong, X.N. (2013). Lymphotoxin Alpha (LTA) Polymorphism Is Associated with Prognosis of Non-Hodgkin's Lymphoma in a Chinese Population. PLoS One *8*, e66411 (1-6).

Zheng, B., Fiumara, P., Li, Y.V., Georgakis, G., Snell, V., Younes, M., Vauthey, J.N., Carbone, A., and Younes, A. (2003). MEK/ERK pathway is aberrantly active in Hodgkin disease: a signaling pathway shared by CD30, CD40, and RANK that regulates cell proliferation and survival. Blood *102*, 1019-1027.

Zhou, P., Fang, X., McNally, B.A., Yu, P., Zhu, M., Fu, Y.X., Wang, L., Liu, Y., and Zheng, P. (2009). Targeting lymphotoxin-mediated negative selection to prevent prostate cancer in mice with genetic predisposition. Proc Natl Acad Sci U S A *106*, 17134-17139.

Zhou, Z., Connell, M.C., and MacEwan, D.J. (2007). TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. Cell Signal *19*, 1238-1248.

Zhu, J., and Paul, W.E. (2010). Heterogeneity and plasticity of T helper cells. Cell Res *20*, 4-12.

Zhu, M., and Fu, Y.X. (2011). The role of core TNF/LIGHT family members in lymph node homeostasis and remodeling. Immunol Rev *244*, 75-84.

Zidar, N., Odar, K., Glavac, D., Jerse, M., Zupanc, T., and Stajer, D. (2009). Cyclooxygenase in normal human tissues--is COX-1 really a constitutive isoform, and COX-2 an inducible isoform? J Cell Mol Med *13*, 3753-3763.

Zimmermann, K.C., Sarbia, M., Schror, K., and Weber, A.A. (1998). Constitutive cyclooxygenase-2 expression in healthy human and rabbit gastric mucosa. Mol Pharmacol *54*, 536-540.

APPENDIX I

Preparation of solution:

Western blot

Isotonic lysis buffer (5X concentrated, pH 7.5)

0.05M Tris HCl, 10mM $MgCl_2$, 15mM $CaCl_2$, 1.5M Sucrose

Extraction buffer (pH 7.9)

20mM HEPES, 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA, 25% Glycerol

TBS buffer (10X,pH 7.6)

24.23g Tris, 80.06g NaCl, 1000ml of MiliQ water

TBST buffer (1000ml)

100ml of 10X TBS, 900ml MiliQ water, 1ml Tween-20

Running buffer (1000ml)

6.06g of Tris, 28.8g of glycine, 1000ml of MiliQ water

Transfer buffer (1000ml)

6.06g of Tris, 28.8g of glycine, 200ml of methanol, 800ml of MiliQ water

Resolving gel for SDS-PAGE

40% bis-acrylamide, 1.5M Tris HCl (pH 8.8), 10% SDS, MiliQ water, 10% APS (add fresh), TEMED (add fresh)

4% Stacking gel for SDS-PAGE

40% bis-acrylamide, 1M Tris HCl (pH 6.8), 10% SDS, MiliQ water, 10% APS (add fresh), TEMED (add fresh)

IHC Staining

Citrate buffer (10mM, pH6)

2.94g of $C_6H_5Na_3O_7$, 1000ml of MiliQ water

TBS buffer (10 litre, pH7.4)

500ml of 1M Tris, 90g of NaCl, 10 litre of deionized water

TBS diluent

0.1% of BSA, 0.01% of Sodium azide, TBS buffer

APPENDIX II

Poster Presentation

1. FHU, C W, S M CHONG, S M T Yap, AM Graham and Y C Lim, "Hodgkin and Reed-Sternberg cells secrete soluble factors to modulate endothelial cell-T cell interactions in classical Hodgkin lymphoma." Cancer Research, 71, no. 18, part. Supplement (2011): A4. United States. (Second AACR International Conference on Frontiers in Basic Cancer Research, 14 - 18 Sep 2011, InterContinental San Francisco, San Francisco, United States).

2. FHU, C W, S M CHONG, S M T Yap, AM Graham and Y C Lim, "Soluble factors by Hodgkin and Reed-Sternberg cells that modulate endothelial cell - T cell interactions in classical Hodgkin Lymphoma". (AACR Annual Meeting 2013, 6 – 10 April 2013, Washington DC Convention Centre, Washington DC, United States).

Oral Presentation

1. Lim, Y C, C W FHU, S M T Yap, AM Graham and S M CHONG, "Hodgkin and Reed-Sternberg cells modulate endothelial cell function in classical Hodgkin lymphoma." Journal of Immunology, 188 (Meeting Abstract Supplement) (2012): 61.9. United States. (Immunology 2012, 4 - 8 May 2012, Hynes Convention Centre, Boston, United States).