PRIMARY EFFUSION LYMPHOMA:

Disruption of the B cell transcriptional program and overexpression of inflammatory molecules

By

Meztli ARGUELLO

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Microbiology and Immunology

McGill University, Montreal

August, 2006

Your file Votre référence
ISBN: 978-0-494-32291-8
Our file Notre référence
ISBN: 978-0-494-32291-8

To my son, Dylan Alexander. Your birth surely was the loveliest delay in the completion of this thesis.

À mon fils, Dylan Alexander. Ta naissance a été le plus joli pépin dans la finition de cette thèse.

To my husband, Sean, for his constant love, encouragement and support were instrumental for finishing this thesis.

À mon époux, Sean, car son amour, son appui et son encouragement ont été essentiels pour compléter cette thèse.

A mi familia, que siempre creyó en mi. Especialmente a mi abuelo, el profesor.

À ma famille, pour avoir toujours cru en moi. En spécial à mon grand-père, le professeur.

ABSTRACT

Primary Effusion Lymphoma (PEL) is a lymphoproliferative disease of B cell origin associated with HHV-8 infection and characterized by migration of tumor cells to serous body cavities. PEL cells originate from post-germinal center B cells yet harbor a non-B, non-T phenotype, a characteristic that has not been fully explained. In the present study we demonstrate that PEL cells have an impaired expression of B cell-specific transcription factors and this results in a decreased activity of promoters regulating essential B cell genes. PEL cells lack PU.1 expression, although its transcription partner IRF-4 is highly upregulated, leading to decreased activity of the immunoglobulin λ and κ light chain ETS-IRF enhancers. Expression of the B cell specific transcription factor Oct-2 and the B cell specific co-activator of octamer factors (Bob-1), which are known to regulate PU.1 expression, was also impaired. Ectopic expression of Oct-2 was able to fully restore PU.1 promoter activity in the PEL cell line BCBL-1, while PU.1 expression also reconstituted the activation of the λB Ets-IRF site. In addition, protein levels of BSAP/Pax-5 and IRF-8/ICSBP were undetectable in PEL cells. The pattern of transcription factor ablation observed in PEL was found to be comparable to that observed in classical Hodgkin's disease-derived cell lines, which also lack B cell specific surface markers. Comparative analysis of gene expression by cDNA microarray of BCBL-1 cells (PEL), L-428 (cHD) and BJAB cells revealed a subset of genes that were differentially expressed in PEL cells. Among these, four genes involved in cell migration and chemotaxis were strongly upregulated in PEL cells: LTA_4H, IL-16, TSP-1, and selectin-P ligand. Upregulation of LTA_4H was investigated at the

transcriptional level. The *LTA4H* promoter exhibited 50% higher activity in BCBL-1 cells than in BJAB or L-428 cells. Deletion analysis of the *LTA4H* promoter revealed a positive *cis* regulatory element active only in BCBL-1 cells in the promoter proximal region located between –76 to –40 bp. Formation of a specific DNA-protein complex in this region was confirmed by Electromobility Shift Assay (EMSA). Co-culture of BCBL-1 cells with ionophore-stimulated primary neutrophils lead to an increased production of LTB_4 by transcellular biosynthesis compared to L-428 cells, demonstrating the functional significance of LTA_4H upregulation. BCBL-1 cells also demonstrated increased migration even in the absence of chemotactic stimulus compared to L-428 cells. These observations indicate that 1) disruption of the B-cell specific transcriptional program is likely to contribute to the incomplete B cell phenotype characteristic of PEL cells and 2) upregulation of factors involved in cell migration and chemotaxis constitute a unique characteristic of PEL cells that may contribute to the localization of this lymphoma to serous body cavities.

RÉSUMÉ

PEL est un désordre lymphoprolifératif qui dérive des cellules B et est associé avec l'infection avec le virus HHV-8. Le PEL est caractérisé par la migration des cellules cancéreuses dans les cavités séreuses. Les cellules du PEL dérivent des cellules B à un stade de développement postérieur au centre germinal (CG) mais elles présentent un phénotype non-B, non-T, une caractéristique qui n'a pas encore été expliqué. Dans cette étude, nous démontrons que les cellules du PEL n'expriment pas certains facteurs de transcription essentiels à la differentiation lymphocytes B. Les cellules PEL n'expriment pas le facteur PU.1, malgré une surexpression de son partenaire de transcription IRF-4. Ceci entraine un manque d'activité des éléments Ets-IRF des activateurs à distance dans les promoteurs des chaînes légères des immunoglobulines. L'expression du facteur de transcription Oct-2 et du co-activateur des facteurs Oct (Bob-1), qui régulent l'expression de PU.1, est aussi déficiente dans les cellules PEL. La surexpression de Oct-2 est capable de rétablir l'activité du promoteur du gène *PU.1* tandis que la surexpression de PU.1 dans les cellules PEL est suffisante pour activer l'élément Ets-IRF qui se trouve dans la chaîne légére λ. En plus, nous avons décelé un défaut d'expression des facteurs de transcription BSAP/Pax-5 et IRF-8/ICSBP dans les cellules PEL. Dans l'ensemble, le manque d'expression de facteurs de transcription observé dans le PEL ressemble à celui qui a été décrit pour le lymphome de Hodgkin's classique (cHD), pour lequel on observe également une ablation de l'expression des marqueurs de surface. L'analyse comparative de l'expression génétique par puces à ADN de la lignée cellulaire BCBL-1 (PEL) et des lignées L-428 (cHD) et BJAB a démontré qu'un nombre

réduit de gènes était exprimé de façon différentielle dans les cellules PEL. En particulier, l'expression de quatre gènes impliqués dans la migration cellulaire et le chimiotactisme est fortement augmentée dans les cellules PEL : hydrolase du leukotriène A_4 (LTA_4H), l'interleukine 16 (IL-16), la thrombospondine 1 (TSP-1) et le ligand de la selectine-P (PSGL-1). La surexpression de LTA4H a été étudié au niveau transcriptionel. L'activité du promoteur du gène *LTA4H* était 50% plus élevée dans les cellules BCBL-1 que dans les cellules L-428 ou BJAB. L'analyse des fragments de délétion du promoteur *LTA4H* ont révélé la présence d'un élément de régulation *cis* dans la région proximale du promoteur (entre -76 et -40 pb) activé uniquement dans les cellules BCBL-1. La formation d'un complexe spécifique protéine-ADN dans ce fragment a été confirmé par retard de migration sur gel. La co-culture des cellules BCBL-1 avec des neutrophils primaires stimulés avec ionophore a conduit à une production accrue de leukotriène B_4 par synthèse transcellulaire, par rapport aux cellules L-428 ou BJAB. Ces résultats suggèrent que la surexpression de LTA_4H dans les célulles PEL pourrait avoir des implications physiologiques. La migration des cellules BCBL-1 – même en absence de stimulus chimiotactic – s'est montrée supérieure à celle des cellules L-428 ou BJAB. Dans l'ensemble, nos résultats indiquent que le manque d'expression des facteurs de transcription spécifiques aux lymphocytes B contribue au phénotype ambigü des cellules du PEL tandis que la surexpression de facteurs impliqués dans la migration et le chimiotactisme est une characteristique unique au PEL qui peut contribuer à la localisation de ce lymphome dans les cavités sereuses.

ACKNOWLEDGMENTS

I would like to thank my supervisors Dr. John Hiscott and Dr. Rongtuan Lin for giving me the opportunity to work on this project. Many, many thanks to Dr. Hiscott for allowing my initial HHV-8 project to take off on a tangent far away from IRFs, which lead me to very interesting and exciting results. I absolutely have to thank you for allowing me to participate in great conferences in awesome places ("The weather was nice, the conference was great..."). Thank you for your advice, support and, most importantly, believing that I could bring the ship to safe haven.

Thank you Dr. Lin for all your helpful advice and magical cloning technique. Thank you also for your support and zen attitude in the face of adverse results (Orf45 and the such).

Special thanks to Dr. Marc Servant, who took me under his wing when I arrived and taught me everything about everything (or almost). I still quote you on a daily basis.

Thank you to all present and past lab members of the Hiscott lab. You all made my Ph.D. experience a wonderful one. I have to specially thank Maria Ricci and Delphine Dugay, who made my scientific and social life so much more happening! I wish you all the best. Marco, thank you for being the other 50% of the HHV-8 team. Eduardo, thank you for believing in microarrays. Thanks to Yael Mamane and Sonia Sharma, the IRF-4 girls, for their support, help and overall good heartedness. Thank you

to Suzanne for always being there in case of need, and always with a smile. Special thanks to Thibault for proofreading my thesis and to Nahn and Vanessa for much-needed last-minute help. Other members and ex-members of the lab (Ben T., Catherine, Ben P., Tudor, Vanessa, Delphine, Mayra, Stephanie, Jennifer, Peyman, Sun and the rest of the crew), sorry if there is not space to thank you all individually but please receive a big collective

Thank you

PREFACE

In accordance to the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of her research in classical form. A general introduction is presented in chapter I. The materials and methods are presented in chapter II. The results are described in chapters III to VII and appear in the following journal articles:

1. **Arguello, M., M. Sgarbanti, E. Hernandez, Y. Mamane, S. Sharma, M. Servant, R. Lin, and J. Hiscott.** 2003. Disruption of the B-cell specific transcriptional program in HHV-8 associated primary effusion lymphoma cell lines. Oncogene **22:**964-73.

2. **Arguello, M., S. Paz, E. Hernandez, C. Corriveau-Bourque, L. M. Fawaz, J. Hiscott, and R. Lin.** 2006. Leukotriene A_4 hydrolase expression in PEL cells is regulated at the transcriptional level and leads to increased leukotriene B_4 production. J. Immunol. **176:** 7051-7061.

3. **Sgarbanti, M., M. Arguello, B. tenOever, A. Battistini, R. Lin, and J. Hiscott.** 2004. A requirement for NF-κB induction in the production of replication-competent HHV-8 virions. Oncogene **23:** 5770-5780.

Specific contributions to the work described in Chapter III and V are as follows:

Marco Sgarbanti performed the *de novo* HHV-8 infections and subsequent analysis of NF-κB activation and viral replication in Ea.hy926 cells.

Eduardo Hernandez carried out the microarray hybridizations and raw data analysis.

Suzanne Paz contributed to the work on NF-κB and AP-1 inhibition by pharmacological compounds in PEL cells by carrying out EMSA analysis for each transcription factor.

The candidate was also involved in collaboration with other researchers in the laboratory, which resulted in the following publication:

4. Lin, R., L. Yang, M. Arguello, C. Penafuerte, and J. Hiscott. 2005. A CRM1-dependent nuclear export pathway is involved in the regulation of IRF-5 subcellular localization. J. Biol. Chem. **280:** 3088-95.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

List of Abbreviations

5-LO	5-lipoxygenase
AA	Arachidonic acid
AID	Activation-induced cytidine deaminase
AIDS	Acquired Immunodefficiency syndrome
AML	Acute myelogenous leukemia
AP-1	Activator protein-1
ATF	Activating transcription factor
AZT	Azydothymidine
BCBL/PEL	Body cavity-based lymphoma/Primary effusion lymphoma
Bcl-1/-2/-6	B-cell CLL/lymphoma-1/-2/-6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein
BLT	B-leukotriene receptor
Bob-1	B-cell Oct-binding protein-1
BSAP/Pax5	B cell lineage-specific protein/paired box gene-5
CAT	Chloroamphenicol acetyl-transferase
CBP	CREB-binding protein
CD	Cluster of differentiation
CD40L	CD40 ligand
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitors

cHD	Classical Hodgkin's disease
CLP	Common lymphoid progenitor
COX	Cyclo-oxygenase
cPLA2	Cytosolic phospholypase A2
Cyc	Cyclin
DBD	DNA-binding domain
DED	Death effector domain
DISC	Death-inducing signaling complex
EAC	Esophageal adenocarcinoma
EBF	Early B cell factor
EBV	Epstein Barr virus
EMA	Epithelial membrane antigen
EMSA	Electromobility shift assay
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FADD	FAS-associated via death domain
FLAP	5-LO activating protein
FLICE	FADD-like ICE (Caspase 8)
FLIP	FLICE inhibitory protein
GAS	IFN-γ activated sequence
GAS	Gamma stimulated sequence
GC	Germinal center
gH	Glycoprotein H
H&E	Hematoxilin and eosin

HAT	Histone acetyl-transferase
HSA	Human serum albumin
IAD	IRF association domain
ICSBP	Interferon consensus sequence binding protein
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IgV	Immunoglobulin variable region
IκB-α	Inhibitor of NF-κB-α
IKK	IκB kinase
IKKγ/NEMO	IkB kinase g/NF-kappaB essential modulator
IL	Interleukin
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IL-7R	IL-7 receptor
IRF	Interferon regulatory factor
ISRE	Interferon-stimulated response element
JAK	Janus kinase
JNK	Jun N-terminal kinase
KS	Kaposi's sarcoma
KSHV/HHV-8	Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8
LANA-1	Latent nuclear antigen-1

LCA	Leukocyte common antigen
LMP	Latent membrane protein
LPS	Lipo-polysaccharide
LT	Leukotriene
LTA_4H	Leukotriene A_4 hydrolase
LTB_4	Leukotriene B_4
LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinase
MCP	Major capsid protein
MEK	MAP/ERK kinase
MHC	Major histocompatibility complex
NF-κB	Nuclear factor κB
NHL	Non-Hodgkin's lymphoma
NIK	NF-κB inducing kinase
NSAIDs	non-steroidal anti-inflammatory drugs
Oct	Octamer binding factor
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PEST	Proline-Glutamate-Serine-Threonine
PG	Prostaglandin
PI3K	Phosphatidyl-inositol-3 kinase
PMNL	Polymorphonuclear leukocytes
PSGL-1	P-selectin glycoprotein ligand-1

PU.1	PU-box binding protein.1
Rb	Retinoblastoma
RS	Reed-Sternberg
RT-PCR	Reverse transcription-polymerase chain reaction
SCID	Severe combined immunodeficiency
STAT	Signal transducer and activator of transcription
Tat	Transcription activator
TCR	T cell receptor
TLR-4	Toll-like receptor-4
TNF	Tumor necrosis factor
TNFR	TNF-receptor
TPA	Tetradecanoyl-phorbol acetate
TR	Terminal repeat
TRAF2	TNF receptor-associated factor-2
TRAIL	TNF-related apoptosis-inducing ligand
TRE	TPA-response element
TSP-1	Thrombospondin-1
vCyc	Viral cyclin
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLIP
vGPCR	Viral G-protein coupled receptor
vIL-6	Viral interleukin-6
vMIP	Viral macrophage inflammatory protein
WCE	Whole cell extract

WHO	World Health Organization
XBP-1	X box-binding protein

CHAPTER I

General Introduction

1. Primary Effusion Lymphoma

Kaposi's sarcoma (KS) and non-Hodgkin's lymphoma (NHL) are the two most common neoplasms occurring in people with AIDS (198). In 1989, Knowles *et al.* first described an AIDS-associated neoplasm characterized by a lymphomatous effusion localized to serous body cavities in the absence of a solid tumor mass (149). The malignant cells lacked B-cell, T-cell, myeloid or monocyte lineage-restricted antigen expression pattern, but exhibited immunoglobulin light and heavy chain rearrangement in all cases, thus allowing the classification of this neoplasm as a B-cell lymphoma. Several reports with similar findings followed (39, 96, 297), but it was not until 1996 that Nador et al. proposed the classification of body cavity-based lymphoma (BCBL), also named Primary Effusion Lymphoma (PEL), as a distinct clinico-pathologic and biological entity (198). The conclusion was based on morphologic and immunophenotypic characteristic of the cellular infiltrate as well as on the strong association of KSHV/HHV-8 infection with BCBL/PEL but not with other AIDS-related or non-AIDS-associated lymphoid neoplasms. Numerous studies have increased our understanding of PEL, both at the level of its cellular origin and the involvement of HHV-8 in disease development. In turn, the use of established PEL cell lines has been an invaluable tool in the study of HHV-8 at the molecular level, particularly because KS spindle cells have proven difficult to grow in the *in-vitro* setting.

1.1. Clinical presentation – epidemiology

PEL presents itself as a lymphomatous effusion spreading along serous membranes in the pleural, peritoneal, or pericardial cavities. In general, PEL develops without obvious lymphadenopathy, tumor masses or bone marrow involvement. All PEL cases are associated with HHV-8 infection of the tumor cells. Co-infection with EBV is common (70% of cases) and - although not required for PEL development - EBV co-infection is thought to help transformation by HHV-8 by a yet undefined mechanism. PEL usually develops in male, advanced-stage AIDS patients (265), although a few reports of PEL in the absence of HIV-1 infection have been documented (23, 244). In AIDS-related PEL cases the median patient age is 40 years, whereas in HIV-negative cases, PEL develops much later, with a median patient age around 70 years. In all cases survival is poor, with a median survival rate of 5 months that remains unaffected by chemotherapy. Immunodepletion appears to be a factor in PEL development, because CD4+ cell count in HIV patients with PEL are significantly lower than that of patients with other AIDS-related non-Hodgkin's lymphomas (265).

1.2. Cellular morphology - cellular origin

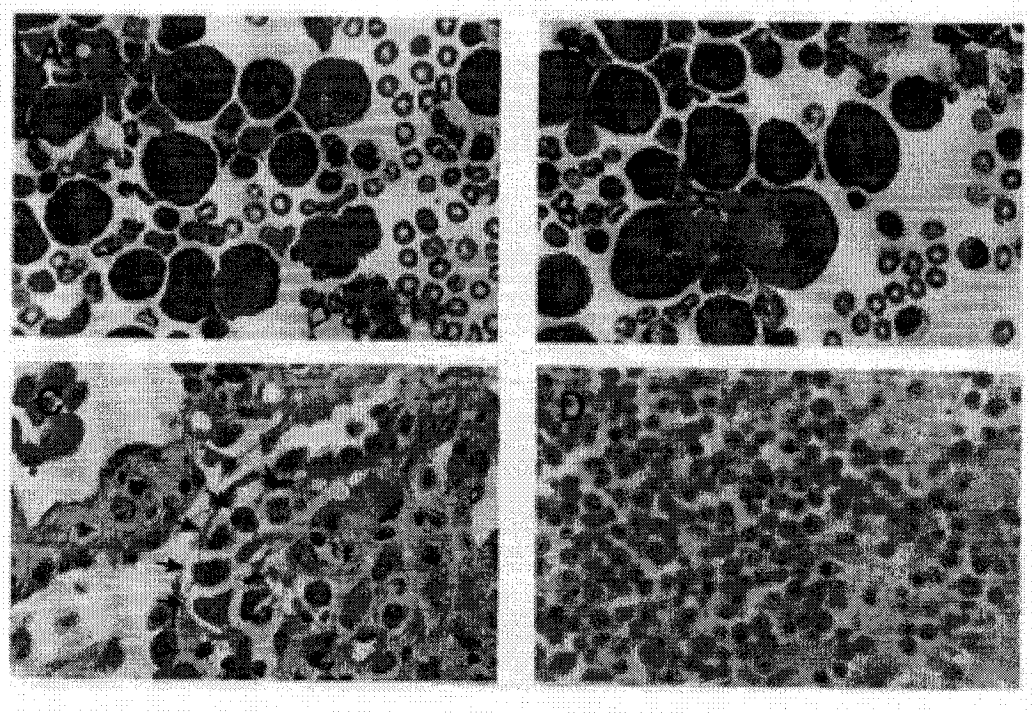
PEL cells are pleomorphic and heterogeneous in size, bridging large-cell immunoblastic and anaplastic features (198). The majority of cells is significantly larger than normal lymphocytes and possess moderate to abundant cytoplasm, basophilic to deeply basophilic, and large round or ovoid nuclei with one or more large nucleoli. A

prominent perinuclear Golgi zone is also present. A variable number of large pleomorphic cells containing markedly irregular, lobated or pleomorphic nuclei and cells with immunoblastic features are also present. Finally, some bi-nucleated cells resembling the Reed-Sternberg cells of Hodgkin's disease are also found in a majority of cases (reviewed in (32, 198)) (**FIGURE 1**).

PEL cells lack expression of B-cell, T-cell, myeloid and monocyte lineage-restricted antigens, which initially lead to its classification as a hematopoietic neoplasm of indeterminate origin (149)(96). Expression of leukocyte common antigen (LCA, CD45) confirmed that PEL cells belong to the hematopoietic lineage (149). Most cases lack expression of B-cell markers such as surface immunoglobulin (sIg), CD19, CD20, and CD22 and all cases lack expression of T-cell markers such as CD2, CD3, CD5 (reviewed in (250)). Cytoplasmic immunoglobulins have been detected in about 20% of patients. PEL cells do express some activation-associated markers such as EMA, CD30 (also expressed by 95% of anaplastic large-cell lymphoma and 70% of Hodgkin's lymphoma), CD38 and CD71, and the plasma cell marker CD138 (syndecan-1).

Final classification of PEL as a B-cell lymphoma was based on antigen receptor rearrangement analysis that demonstrated light and heavy chain gene rearrangement in all cases (198). This also demonstrated that PEL originated from a mature B cell, and the presence of CD138 suggested that PEL cells had some degree of plasma cell differentiation. Moreover, PEL cells lack expression of Bcl-6 protein while exhibiting high levels of IRF-4 expression. These characteristics are consistent with a post-germinal center (post-GC) state of differentiation (reviewed in (35, 36)). However, study of the somatic hypermutation of the immunoglobulin loci in PEL cells demonstrated that

FIGURE 1. Biopsy samples of Primary Effusion Lymphoma. A and B) Wright-Giemsa-stained preparations of HHV-8-positive PEL. The cells are considerably larger than normal benign lymphocytes and exhibit cytomorphologic features that appear to bridge large cell immunoblastic lymphoma and anaplastic large cell lymphoma. The cells display variable polymorphism and generally possess moderately abundant amphophilic to deeply basophilic cytoplasm. A prominent clear perinuclear Golgi zone is frequently present. Small cytoplasmic vacuoles are occasionally present. The nuclei vary from large and round to highly irregular, multilobated and pleomorphic (Original magnification X 630). C) Hematoxilin and eosin-stained section of a PEL case involving the pulmonary lymphatics (Original magnification X 630). D) Hematoxilin and eosin-stained pleural fluid cell block of a KSHV-positive case of PEL (Original magnification X 400). The neoplastic cells are large but appear more uniform than in other preparations. Courtesy of Blood Journal (Nador *et al.*, 1996)


A
B
C
D

the hypervariable region could be found either in germline state, actively undergoing mutations or mutated and clonally expanded, corresponding to pre-GC, GC, and post-GC states of differentiation (109, 179). In light of this, it is possible that HHV-8 infects B-cells at different stages of differentiation but transformation leads to a phenotype of post-GC, pre-plasma B cell. This is comparable to the activation state induced by EBV when it infects naïve B cells (reviewed in (310)).

PEL is not associated with rearrangements in B-Cell CLL/lymphoma-1 (Bcl-1), Bcl-2, Bcl-6 and cMyc genes, which are common in other aggressive B-cell lymphomas. PEL cells do not exhibit p53 inactivation. However, mutations in the Bcl-6 5'-non-coding region are common in PEL as it is in most AIDS-related lymphomas derived from mature B cells (reviewed in (250)).

1.3. Classification

Over the years, different classification systems have been used to differentiate lymphomas based on morphological characteristics but also on information gathered as additional techniques become available. The Working Formulation classification was introduced in the 1980s to unify previously developed classification systems. The Working Formulation was based on the morphology of H&E stained sections and did not take into account the cellular origin of the lymphoma but rather classified it based on architectural and cytological features. The advent of immunologic, cytogenetic and molecular techniques has broadened the amount of information that can be gathered from biopsy samples and used to classify lymphomas. In 2001, the World Health

Organization (WHO) classification was published and it is to date the authoritative standard of lymphoma classification (reviewed in (40)). The WHO classification groups lymphomas based on cellular origin and cytogenetic, morphologic and clinical characteristics. Unlike previous methods, the WHO classification does not classify lymphomas according to behavior or prognostic categories but rather each lymphoma is seen as a separate disease that can be more or less aggressive in individual patients. The WHO classification recognizes Primary Effusion Lymphoma as a new type of non-Hodgkin's lymphoma (NHL) mature B cell neoplasm whereas previous classification systems included PEL in the Diffuse Large B-cell Lymphoma category (52). NHL mature B cell neoplasms in the WHO classification include, among others, chronic lymphocytic leukemia, hairy cell leukemia, plasma cell neoplasms, follicular lymphoma and Burkitt's lymphoma.

Classical Hodgkin's disease (cHD) is also derived from mature B cells at a similar stage of development than PEL cells yet it falls under a different arm of the WHO classification system, i.e. Hodgkin's lymphoma (40). PEL and cHD share numerous similarities at the molecular level, such as lack expression of B-cell surface markers leading to what has been described as a "null" phenotype (11, 114, 233, 288). Investigation of transcription factor expression and activity in cHD led to the identification of a constitutive activation of NF- κB and AP-1 in the Reed-Sternberg (RS) cells that constitute the tumor cells of cHD (reviewed in (284)). PEL cells also exhibit constitutive NF-κB and AP-1 activation, owing to the action of HHV-8 viral proteins (see below). However, the similarities at the molecular level between PEL and cHD do not translate to their clinical presentation. PEL is a 'liquid' lymphoma that localizes to the serous body cavities, with >90% of cells being of malignant origin. PEL

has extremely poor prognosis and generally does not respond to conventional chemotherapy. Median survival rates hover around 6 months (reviewed in (4)). Classical Hodgkin's disease, on the other hand, is a lymphoma that localizes exclusively to the lymph nodes. Reed-Sternberg cells –the tumor cells of cHD – constitute less than 5% of the tumor load, the rest being a mixture of inflammatory cells such as neutrophils, macrophages and T cells. Conventional chemotherapy is the treatment of choice for cHD, with over 80% survival rates (reviewed in (58)). It is currently unknown which features of PEL at the molecular level lead to its particular clinical presentation, such as its exclusive localization to body cavities and its resistance to chemotherapy.

1.4. Treatment of PEL

Because PEL responds poorly to classical chemotherapy treatment, the focus of current research has been the identification of novel therapeutic venues. The use of highly active anti-retroviral therapy (HAART) to control HIV-1 infection has reduced dramatically the incidence of both KS and PEL although it is not clear if this is due to its effects on HIV or on HHV-8 infection. One study of five HIV+ PEL patients demonstrated that chemotherapy and HAART combination treatment lead to a decrease in HHV-8 load in four patients, which correlated with an improvement of CD4+ T cell counts but not with decreased HIV-1 viral load (266).

Initial studies on PEL cell lines demonstrated that treatment with NF-κB inhibitors induced apoptosis of the tumor cells (144). Treatment with Interferon-α (IFN-α), on the other hand, was able to prevent HHV-8 reactivation in PEL cells and reduced HHV-8 load in cultured peripheral blood mononuclear cells (PBMCs) (193). A breakthrough

study by Lee *et al.* demonstrated that treatment of the PEL cell line BCBL-1 with azydothymidine (AZT) and IFN-α induced apoptosis of 70% of cells compared with 10-20% of apoptosis observed with either treatment alone (158). The synergistic effect of AZT and IFN-α in PEL cell apoptosis depended on the induction of TRAIL as transfection of the decoy receptor DcR2 abrogated the synergy between IFN-α and AZT. Treatment of a 35-year old HIV-1 positive PEL patient with a combination of parenteral, twice-daily AZT 1.5 g and IFN-α 5 million units resulted in a complete resolution of the pleural effusion after 5 days (90). Six months later, the patient remained symptom-free. However, in spite of this initial report of successful treatment of PEL by AZT and IFN-α, no further studies have been published on the subject.

Recently, treatment of a PEL case with intrapleural administration of the anti-viral agent cidofovir (3-5 mg/kg) combined with radiation therapy was reported (108). The treatment efficiently halted further accumulation of pleural fluid and the patient's condition improved substantially. However, 15 months after treatment, the patient still had a pleural effusion (albeit minimal), and pleural thickening. A pilot clinical trial at the National Institutes of Health will investigate the use of a combination of the anticancer agent bortezomib, with zidovudine (AZT) and the anti-viral agent valganciclovir in the treatment of PEL (trial number NCI-05-C-0203, http://bethesdatrials.cancer.gov). Due to the inherent toxicity of each of the components of this treatment, it is likely that such a regime will result in considerable side effects. A treatment of PEL that can be routinely used with high efficiency and relatively low toxicity is still needed.

1.5. Role of HHV-8 in PEL development

Although HHV-8 infection is recognized to be associated with PEL development, the exact mechanism by which HHV-8 transforms B cells remains unknown, partially owing to the lack of a good *de novo* infection model. In PEL cells, the pattern on HHV-8 gene expression is very restricted, with virtually all cells (>95%) expressing only the latent genes Latent Nuclear Antigen-1 (LANA-1), viral Cyclin (vCyc), and viral FLIP (vFLIP), and to a lesser extent kaposin/K12 and viral Interferon Regulatory Factor 10.5 (vIRF 10.5)/Latent Nuclear Antigen-2 (LANA-2). vFLIP, vCyc and LANA-1 genes are encoded by open reading frames K13, ORF72 and ORF73, respectively, located in a DNA locus with latency-associated gene expression (reviewed in (247) and (61)). Analysis of PEL biopsy samples by immunohistochemistry demonstrated that, additionally to LANA-1 expression, a limited number of cells (≤2-5%) express viral Interleukin-6 (vIL-6), and a minority of cells (<1%) expresses lytic proteins (223).

Microarray analysis of HHV-8 gene transcription in PEL cell lines has allowed a detailed study of HHV-8 viral gene expression (132). HHV-8 positive PEL cell lines treated with TPA to induce HHV-8 lytic replication demonstrated that HHV-8 genes are expressed following a discrete pattern and that they can be broadly classified in three categories (246). Class I genes are expressed in the absence of TPA induction and their expression does not change after treatment, constituting "true" latent genes. The latent transcripts LT1 and LT2, encoding for LANA-1, vCyc and vFLIP, correspond to this category. A third 4.5 kb transcript, termed LT3, is also a class I but it has not been characterized. Class II genes include mRNAs that are detected at different levels without

TPA induction and are induced to high levels upon lytic replication. Kaposin (ORF K12), nuclear transcript 1 (nut-1), vIL-6 (ORF K2), vMIP II (ORF K4) and vIRF (ORF K9) are examples of Class II genes. Class III genes are detected only after TPA induction. These genes encode for lytic genes that are required for efficient viral replication and virion particle production. Examples of class III genes include major capsid protein (MCP, ORF 25), DNA polymerase (ORF 6) and glycoprotein H (gH, ORF21).

It should be noted that some differences in gene expression have been observed between PEL biopsy samples and cell lines, particularly pertaining to vIRF expression during latency, which was not detected in biopsy samples (223). It is possible that *in-vitro* culture conditions somewhat alter HHV-8 gene expression, a phenomenon observed also in EBV-infected cell lines. The expression of vIL-6 and vIRF 10.5/LANA-2 has been suggested to correspond to an alternative latent program established in B cells, since the endothelial cells of KS do not express these viral proteins (250, 272). The potential role of the major latent proteins LANA-1, vCyc and vFLIP as well as vIL-6 in cellular transformation is presented below.

1.5.1 Latent Nuclear Antigen-1 (LANA-1/ ORF 73)

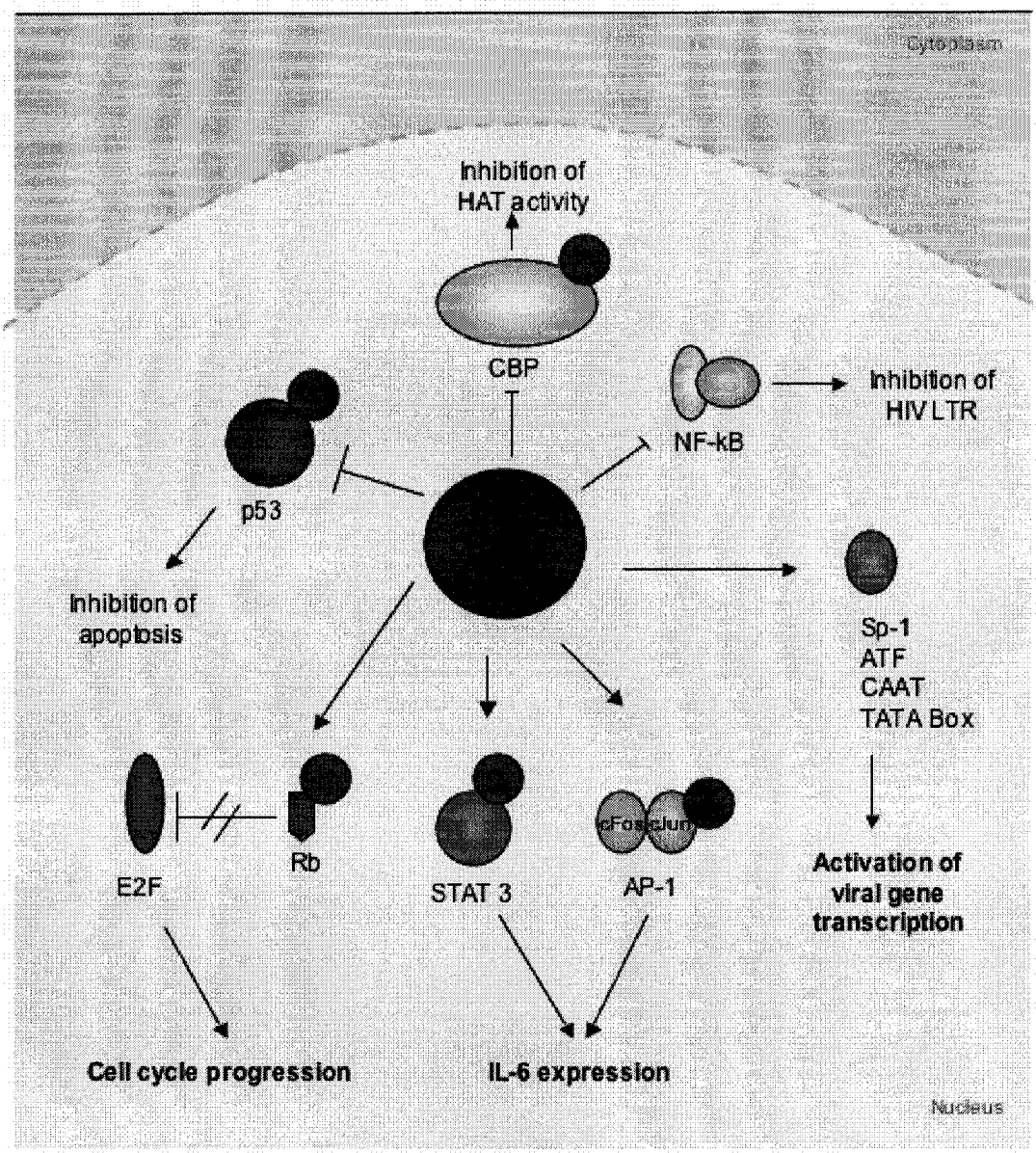
HHV-8 gene ORF 73 encodes for a 132 kDa protein, 1 162 amino acids long, known as the Latency-associated Nuclear Antigen (LANA, LANA-1, LNA-1). LANA is expressed during latency in the nucleus of HHV-8 infected cells where it accumulates in speckles referred to as LANA bodies (143). LANA protein tethers the viral chromosome

to the host's genome, mediating episome replication and persistence as well as efficient segregation of episomes to daughter nuclei. LANA oligomerizes and binds to the terminal repeat (TR) region of the circularized HHV-8 DNA via its C-terminal domain (150) and simultaneously interacts with cellular histone H1 (54). Furthermore, LANA plays an important role in lymphomagenesis, as demonstrated in LANA transgenic mice, which show expanded IgM^+ IgD^+ B cell populations, increased GC formation and spontaneous development of lymphomas (76).

LANA is a transcriptional activator and repressor of both viral and cellular promoters (**FIGURE 2**). Various DNA tumor viruses encode specific proteins that target the p53 and Retinoblastoma (Rb)/E2F regulatory pathways, such as the simian virus (SV) 40 large T antigen, adenovirus E1A protein and human papilloma virus (HPV) E7 protein. Similarly, HHV-8 LANA physically interacts with p53, inhibiting its transcriptional activity and leading to a decrease in p53-mediated apoptosis (85). However, the mechanism of inhibition of p53 by LANA is still unclear and it does not involve inhibition of DNA binding by p53 or induction of p53 degradation. LANA transactivates E2F-dependent promoters by binding to the retinoblastoma protein (pRb) when in its hypophosphorylated/active state. This effectively prevents the formation of the inhibitory Rb/E2F complex and thus promotes E2F-mediated transcription (229).

LANA is a coactivator of cJun, as demonstrated in 293 cells and BCBL-1 primary effusion lymphoma cell line. LANA physically associates to cJun and induces binding of cFos-cJun heterodimers to the AP-1 response element without physically interacting with the DNA sequence (7). This transactivation of the AP-1 response element by LANA induces the activity of the IL-6 promoter, leading to an increase in IL-6

FIGURE 2. LANA-1 can act as a transcriptional transactivator or repressor. LANA-1 promotes cell cycle progression by binding to the inhibitor Rb protein, preventing interactions with E2F. LANA-1 physically interacts with cJun and STAT3, enhancing transcription of the *IL-6* gene. LANA-1 also increases the transcription of viral promoters containing Sp-1, ATF, CAAT and TATA box elements. As an inhibitor, LANA-1 represses transcription mediated by p53, CBP and NF-κB. Interaction with p53 inhibits the induction of pro-apoptotic genes, whereas NF-κB inhibition represses the HIV-1 LTR.


Cytoplasm
Inhibition of
HAT activity
CBP
Inhibition of
HIV LTR
NF-kB
p53
Inhibition of
apoptosis
Sp-1
ATF
CAAT
TATA Box
Rb
E2F
STAT 3
AP-1
Activation of
viral gene
transcription
Cell cycle progression
IL-6 expression
Nucleus

production, as demonstrated *in-vitro* in 293 and bone marrow stromal cell lines (6). LANA physically associates with STAT3 in PEL cells to enhance its transcriptional activity, possibly leading to a further upregulation of IL-6 expression (197). LANA can also physically interact with the ubiquitous transcription factor Sp-1 and synergistically activate Sp-1 mediated transcription, as demonstrated for the human telomerase reverse transcriptase gene (295).

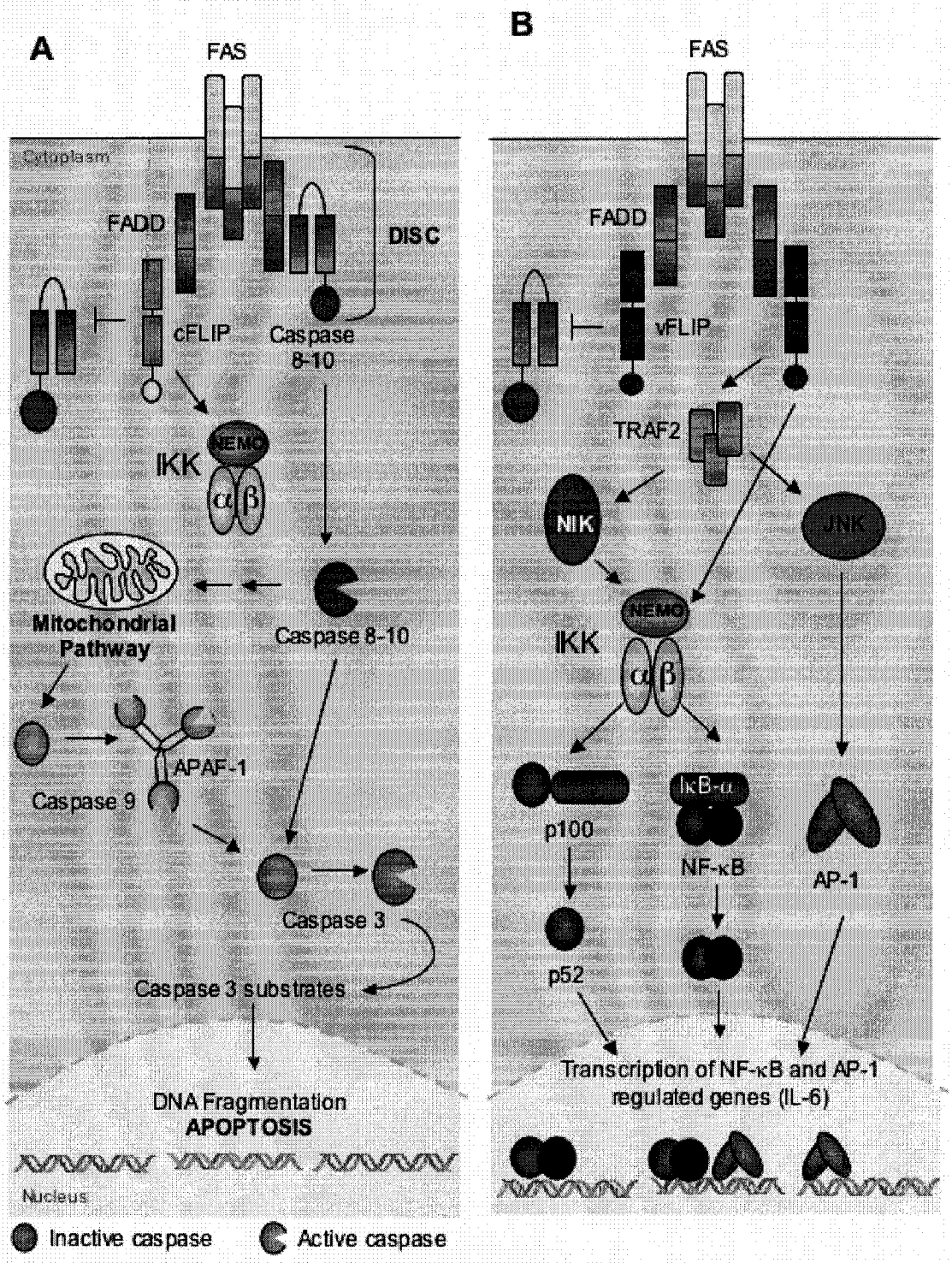
LANA can modulate viral gene expression via interactions with the basal transcription machinery or additional transcription factors, thus LANA can positively regulate viral promoters containing ATF, AP-1, CAAT, and Sp-1 binding sites or only a TATA box and negatively regulate promoters containing κB sites (235). LANA positively regulates its own expression, increasing mRNA levels up to 5.5-fold (134), whereas it inhibits the expression of Rta (ORF50) and induction of the lytic cycle (156, 165). In contrast, LANA inhibits Epstein-Barr virus (EBV) gene expression by interacting with the mSin3 correpressor complex (152). The action of LANA on HIV-1 gene expression is less clear: LANA was shown to inhibit LTR-mediated transcription by repressing NF-κB transactivation (235) yet it was shown to interact with the transactivator protein Tat to stimulate LTR transcription (125). Surprisingly, LANA has been reported to interact with CREB-binding protein (CBP) and inhibit its histone acetyl-transferase activity (162), although the physiological relevance of such interaction remains unknown.

1.5.2 Viral FLICE Inhibitory Protein (vFLIP/ ORF K13)

The open reading frame ORF71/K13 of HHV-8 encodes for vFLIP, a viral protein homologous to the cellular inhibitor of human caspase-8/Fas-associated death domain-like IL-1β-converting enzyme (FLICE) known as cFLIP (also known as MRIT, Casper, I-FLICE, FLAME, CASH, CLARP). Survival of PEL lymphoma cells and spindle cells from late stage KS lesions is associated with vFLIP expression (103, 273). Downregulation of vFLIP expression by RNA interference in PEL cells leads to abrogation of NF-κB activity and enhanced spontaneous cellular apoptosis as well as sensitization to the induction of apoptosis by extrinsic stimuli (103). Viral FLIP thus appears as a pivotal protein for the development of HHV-8 associated malignancies.

Activation of the apoptotic program by ligation of death receptors - such as the tumor necrosis factor (TNF) receptor 1 (TNFR-1) or FAS/CD95 - follows a specific cascade of signaling events (reviewed in (27)) (**FIGURE 3A**). Ligand binding to the death receptor leads to receptor multimerization and recruitment of adaptor molecules such as FADD (Fas-associated Death Domain protein) and TRADD (TNFR-associated Death Domain protein). The adaptor molecules then recruit Caspase 8/FLICE (also known as MACH, Mch5), the apical caspase of the caspase cascade, via interaction with their Death Effector Domains (DED) to form the Death Inducing Signaling Complex (DISC). Recruitment signals Caspase 8 to undergo autoactivation by proteolytic cleavage and relay the apoptotic signal to the effector caspase, Caspase 3. During the assembly of the DISC complex, cFLIP can also be recruited via association with FADD. cFLIP contains tandem DED domains but only partial or no activation domain. Recruitment of cFLIP

FIGURE 3. vFLIP is a viral homolog of cFLIP that inhibits apoptosis and activates NF-κB and AP-1. A) Death receptor-mediated apoptosis involves the recruitment of the apical caspase Caspase 8 to the receptor complex *via* interaction with adaptor molecules such as FADD, leading to auto-activation by proteolytic cleavage. Active Caspase 8 can induce the mitochondrial apoptotic pathway as well as directly activate the effector caspase Caspase 3. Cellular FLIP inhibits recruitment of caspase 8 by FADD to the DISC complex. cFLIP can also activate the NF-κB pathway and promote cell survival. B) vFLIP prevents caspase-8-mediated apoptosis and activates NF-κB either by recruiting TRAF2 and NIK or by direct interaction with the IKK complex, resulting in activation of both the classical and canonical NF-κB pathways. vFLIP also activates JNK *via* TRAF2 leading to activation of AP-1 transcription factors. vFLIP thus enhances transcription of NF-κB and AP-1 regulated genes such as *IL-6*.


A
FAS
Cytoplasm
FADD
DISC
cFLIP
Caspase 8-10
NEMO
IKK
α β
Mitochondrial Pathway
Caspase 8-10
APAF-1
Caspase 9
Caspase 3
Caspase 3 substrates
DNA Fragmentation
APOPTOSIS
Nucleus
Inactive caspase
Active caspase
B
FAS
FADD
vFLIP
TRAF2
NIK
JNK
NEMO
IKK
α β
p100
IκB-α
NF-κB
AP-1
p52
Transcription of NF-κB and AP-1 regulated genes (IL-6)

does not prevent Caspase-8 association with the DISC complex, but it prevents Caspase-8 from undergoing a productive proteolytic cleavage. Thus, cFLIP effectively blocks caspase 8-mediated apoptosis. Moreover, Chaudhary *et al.* demonstrated that cFLIP also activates the NF-κB pathway by means of its DED domain (45).

Viral FLIP efficiently inhibits death receptor-mediated apoptosis of HHV-8 infected cells by inhibiting the recruitment of pro-caspase 8 to the DISC complex (19). vFLIP activates the classical and alternative NF-κB pathways, effectively triggering the transcription of pro-survival and proliferation genes. Indeed, vFLIP physically associates with TRAF2, which binds and activates the NF-κB Inducing Kinase (NIK). NIK subsequently activates the IκB Kinase (IKK) complex leading to persistent IKK activation and phosphorylation of IκB-α, which frees NF-κB dimers to translocate into the nucleus and activate transcription of target genes (46) (**FIGURE 3B**). Additionally, vFLIP has been found to physically associate with the IKK complex presumably through interactions requiring IKKγ/NEMO, the regulatory subunit of the IKK complex, to activate NF-κB (164). vFLIP also activates the alternative NF-κB pathway, leading to increased p100 processing into p52 via IKKα kinase activity (181).

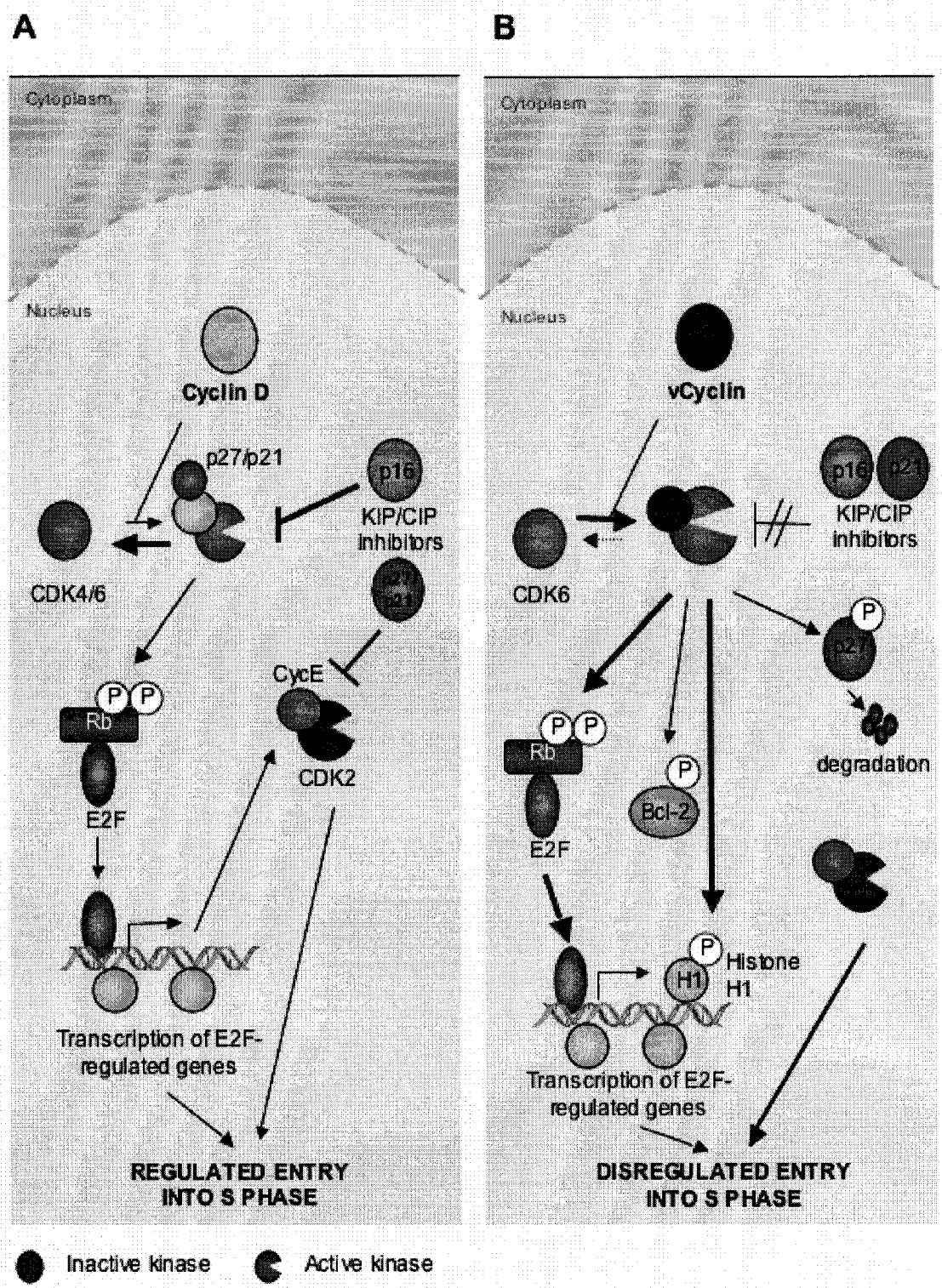
Additionally, vFLIP can activate the activation protein 1 (AP-1) pathway. Association of vFLIP with TRAF2 leads to the activation of cJun N-terminal kinase (JNK), which phosphorylates cJun leading to AP-1 translocation into the nucleus and transcription of target genes (8). Activation of NF-κB and AP-1 by vFLIP leads to increased IL-6 production, a growth factor found in all HHV-8-associated neoplasms that contributes to tumor cell survival and establishment of an inflammatory milieu (8).

1.5.3. Viral Cyclin (vCyc/ ORF72)

Cellular cyclins control progression through the cell division cycle by associating with members of the Ser/Thr kinase family of CDKs to form active holoenzymes that phosphorylate particular substrates to allow checkpoint clearance and progression through the cell cycle (reviewed in (260)). Entry from G0 to G1 and progression to S phase requires the sequential action of Cyclin D (CycD)-CDK4/6, Cyclin E (CycE)-CDK2 and cyclin A (CycA)-CDK2. When G0 cells are stimulated with mitogen to re-enter the cell cycle, active Cyclin D-CDK4/6 complexes start accumulating during G1 and phosphorylate the tumor-suppressor Retinoblastoma protein family (Rb, p107, and p130). Hypophosphorylated Rb is bound to and inhibits the E2F transcription factor, which controls the expression of a battery of genes involved in DNA metabolism and replication. Phosphorylation of pRb by CycD-CDK4/6 leads to its degradation and release of E2F, which can then transactivate target genes. Inhibitory proteins known as Cyclin-Dependent Kinase Inhibitors (CDKI) control the activation of Cyc-CDK kinase complexes. The KIP/CIP family of CDKI inhibits CycD- and CycE-activated kinases. The KIP family includes $p21^{Cip}$, $p27^{Kip}$, and $p57^{Kip2}$, which inhibit CycE/CDK2. CycD/CDK4/6 requires binding of $p21^{Cip}$ or $p27^{Kip}$ to stabilize the complex, leading to a titration of the inhibitors that regulates the activation of CycE/CDK2. The INK family – comprised of $p16^{Ink4a}$, $p15^{Ink4b}$ and $p18^{Ink4c}$ – specifically inhibits the kinase activity of CycD-CDK4/6 (**FIGURE 4A**).

HHV-8 vCyc has 32% identity and 54% similarity with mammalian cyclin D2 (CycD2) (43, 161). Association of vCyc with CDK6, but not CDK4, leads to activation

FIGURE 4. vCyclin interacts with CDK6 and promotes disregulated cell cycle progression. A) Cellular cyclin D interacts with CDK 4 and 6 to promote progression through G1/S. The active CycD/CDK kinase complex exclusively phosphorylates the Rb protein family to induce transcription of genes necessary for progression through G1/S, such as CycE. Cyclin/CDK complexes are tightly regulated by the family of KIP/CIP inhibitors: $p27^{Kip1}$ and $p21^{Cip1}$ inhibit CycE/CDK2, whereas $p16^{Ink4a}$ inhibit CycD/CDK4/6. Additionally, $p27^{Kip1}$ and $p21^{Cip1}$ bind to CycD/CDK4/6 complexes and stabilize them. B) vCyclin preferentially interacts with CDK6 to form a holloenzyme that does not require $p27^{Kip1}$/$p21^{Cip1}$ for stabilization and is resistant to the inhibitory effects of the KIP/CIP proteins $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$. vCyc/CDK6 promiscuously phosphorylates proteins involved in the G1/S and G2/M transitions, including Rb proteins, cdc25A, Id-2, the apoptosis inhibitor Bcl-2 and $p27^{Kip1}$, among others. Phosphorylation of Bcl-2 and $p27^{Kip1}$ triggers the degradation of these inhibitors of the cell cycle. vCycD thus triggers disregulated cell cycle progression.


A
B
Cytoplasm
Nucleus
Cyclin D
p27/p21
p16
KIP/CIP
inhibitors
CDK4/6
P
Rb
E2F
CycE
CDK2
Transcription of E2F-
regulated genes
REGULATED ENTRY
INTO S PHASE
vCyclin
CDK6
degradation
Bcl-2
Histone
H1
DISREGULATED ENTRY
INTO S PHASE
Inactive kinase
Active kinase

of the kinase activity and efficient phosphorylation of Rb and other substrates such as cdc25A –normally a target of CycE/CDK2 -, cdc6, ORC1 and physiological substrates of CyclinA/CDK2 (173). This is in striking contrast to the cellular CycD3-CDK4/6 complex that exclusively phosphorylates pRb (92, 161). vCyc is resistant to inhibition by $p16^{Ink4a}$, $p21^{Cip}$ and $p27^{Kip}$ and does not require binding of $p21^{Cip}$ or $p27^{Kip}$ to form stable complexes with CDK6 (276). In addition, vCyc-CDK6 can phosphorylate $p27^{Kip}$, leading to protein destabilization and degradation (70). Thus, vCyc can further promote cell cycle progression by relieving CDK2 of the inhibition by $p27^{Kip}$ (**FIGURE 4B**). In this manner, vCyc can mimic the effect of both G1-S and G2-M cyclins (reviewed in (155), (296)).

vCyc contributes to HHV-8 infection by promoting cellular entry into S phase, a phenomenon that might be required for virus reactivation from latency (111). More importantly, vCyc contributes to cellular transformation by overcoming normal cell cycle control mechanisms. In a study by Carbone *et al.*, PEL biopsy samples and cell lines as well as non-HHV-8 associated lymphomatous effusions were analyzed for vCyc, the proliferation marker Ki-67, and $p27^{Kip}$ protein expression (34). PEL cells consistently stained positive for all three proteins, suggesting that vCyc drives PEL cell proliferation regardless of the presence of the inhibitor $p27^{Kip}$. This was in contrast with non-HHV-8 associated neoplasms, were proliferation and $p27^{Kip}$ expression were generally inversely correlated.

1.5.4. Viral Interleukin-6 (vIL-6/ ORFK2)

HHV-8 viral IL-6 shares 24.8% amino acid sequence identity and 49.7% similarity to human IL-6. It is encoded by ORF K2 and it can be detected as a 1.0 kb mRNA in latent and lytically infected PEL cells (201). The abundance of vIL-6 mRNA is moderate in PEL cells but it increases to high levels upon induction of the lytic cycle by TPA treatment. Thus, vIL-6 is a class II gene, according to the classification proposed by Sarid *et al.* (246).

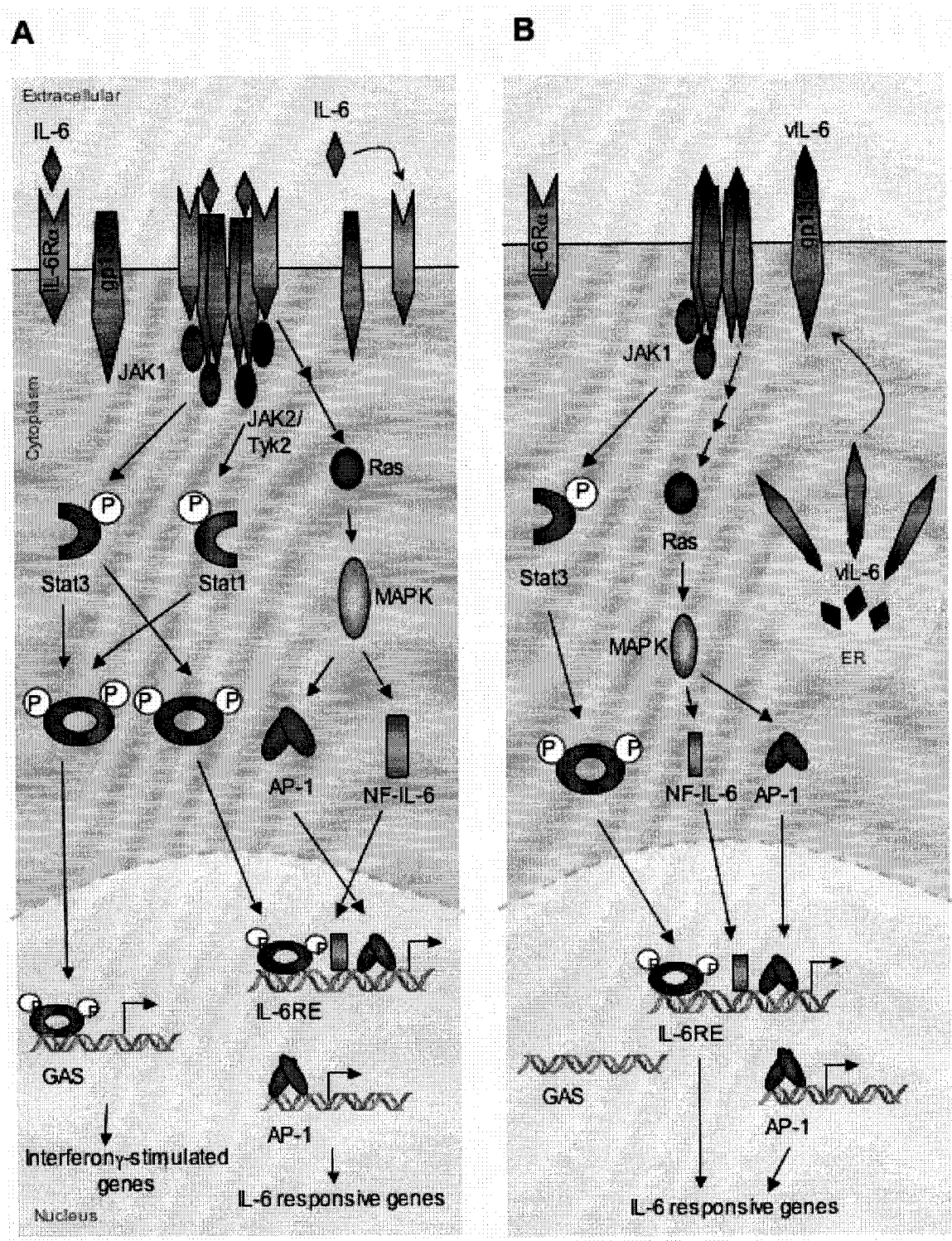
Human interleukin-6 (hIL-6 or IL-6) is a multifunctional enzyme that promotes normal B cell proliferation and terminal differentiation into plasma cells (283, 289) but has been implicated in the development of a variety of diseases, including rheumatoid arthritis and multiple myeloma (38). IL-6 is implicated in the pathogenesis of HHV-8 associated diseases: IL-6 can be detected in KS lesions, where it triggers proliferation of the spindle cells (73, 188). In multicentric Castelman's disease, IL-6 acts as a B cell proliferation factor, and IL-6 levels closely correlate with severity of the disease (210); however, the role of IL-6 in PEL pathogenesis remains controversial (63, 83, 135).

Human IL-6 signals through the IL-6 receptor, which is composed by the common chain gp130 and the specific α subunit IL-6R (gp80). IL-6 binds to IL-6R and this complex then associates with gp130, allowing it to homodimerize (160). A hexameric complex composed of two molecules of IL-6, two IL-6R and four gp130 then signals through recruitment of Janus Kinases (JAK) JAK1, JAK2 and/or Tyk2, which phosphorylate gp130 (180, 270). Signal Transducers and Activators of Transcription (STATs) are recruited to the receptor and are phosphorylated by the JAK kinases,

leading to their activation. Phosphorylation and homodimerization of STAT3 leads to its activation and binding to hIL-6 response element (IL-6RE) found in IL-6-induced genes (106, 243). Heterodimers of STAT1 and STAT3 bind to the GAS (IFNγ-activated sequence) to activate transcription of additional genes (106). IL-6 signaling also activates the Ras-dependent MAPK pathway, ultimately leading to the activation of transcription factors AP-1 and NF-IL-6 (154, 200) (**FIGURE 5A**). Binding of human IL-6 to IL-6Rα subunit is required for the recruitment of gp130 and activation of the downstream signaling cascade. This provides a regulatory mechanism for the control of IL-6 signaling by stimuli such as interferon-α (IFN-α), which can trigger downregulation of IL-6Rα expression and shut down IL-6-induced proliferation and survival (44).

HHV-8 encoded vIL-6 can bind to gp130 in the absence of IL6Rα and persistently activate transcription *via* the JAK/STAT and MAPK pathway. Viral IL-6 induces recruitment of JAK1 to gp130, leading to its phosphorylation and activation. However, unlike hIL-6, JAK2 and Tyk2 are not activated by vIL-6. Thus, as a downstream effect, only STAT3 is phosphorylated but not STAT1 (116), meaning that only genes containing the IL-6RE but not GAS sequences can be activated by vIL-6. Viral IL-6 triggers activation of the Ras-dependent MAPK pathway - as demonstrated by the phosphorylation of ERK2 and activation of MEK1 (116) - leading to activation of AP-1 transcription factors (**FIGURE 5B**). Because vIL-6 does not require the IL-6Rα chain, it can escape negative regulation by IFN-α and induce cell proliferation and survival even in the presence of this cytokine; moreover, vIL-6 disrupts IFN receptor phosphorylation of Tyk2 kinase, leading to decoupling from the Jak/STAT pathway.

FIGURE 5. Viral IL-6 signals through gp130 to activate the STAT and Ras signaling pathways. A) Cellular IL-6 binds to IL-6Rα (gp80) and recruits gp130 to form the IL-6 receptor. IL-6 activates Ras, leading to activation of the MAPK pathway and AP-1 and NF-IL6 transcription factors. Activation of the STAT pathway by IL-6 proceeds *via* recruitment of JAK1 and JAK2/Tyk2 to gp130, which phosphorylate STAT3 and STAT1, respectively. STAT3/STAT1 heterodimers bind to GAS sequences and activate transcription of IFN-γ-stimulated genes. STAT3 homodimers bind in conjunction with AP-1 and NF-IL6 to the IL-6 response element (IL-6RE) to activate transcription of IL-6 inducible genes. B) Viral IL-6 (vIL-6) binds to gp130 in the ER and signals independently of IL-6Rα. Similar to IL-6, vIL-6 activates the Ras/MAPK pathway. vIL-6 signaling recruits JAK1 but not JAK2/Tyk2 to the receptor, resulting in the activation of STAT3 but not STAT1. STAT3 homodimers together with NF-IL6 and AP-1 activate transcription of IL-6 and AP-1 responsive genes but there is no activation of IFN-γ inducible genes, owing to the lack of STAT1/STAT3 heterodimers.


A
Extracellular
IL-6
IL-6
IL-6Rα
gp130
JAK1
JAK2/
Tyk2
Cytoplasm
Ras
P
Stat3
Stat1
MAPK
AP-1
NF-IL-6
IL-6RE
GAS
Interferonγ-stimulated
genes
AP-1
IL-6 responsive genes
Nucleus
B
vIL-6
IL-6Rα
gp130
JAK1
P
Ras
Stat3
vIL-6
MAPK
ER
NF-IL-6
AP-1
IL-6RE
GAS
AP-1
IL-6 responsive genes

Viral IL-6 protects PEL cells against growth arrest and apoptosis induced by IFN-α. PEL cells rapidly upregulate vIL-6 production following exposure to IFN-α directly through two Interferon-Stimulated Response Element (ISRE) sequences found in the vIL-6 promoter, thus creating a negative feedback loop that allows HHV-8 to respond to interferon challenge (44).

PEL cells are dependent on vIL-6 autocrine signaling for spontaneous growth (135). However, exogenous recombinant vIL-6 is required at levels up to 10^3 times higher – around 1 μg/mL - than hIL-6 to induce proliferation of IL-6 dependent B cell lines (28, 163) yet physiological vIL-6 levels found in PEL samples are much lower than this value. This discrepancy led to the investigation by Meads and Medveczky of vIL-6 postranslational modifications and transport (186). Their results show that vIL-6 binds to gp130 in the ER and is transported to the surface along with the receptor (**Figure 5B**). This interaction is required for vIL-6 transport because vIL-6 is not secreted in cells lacking gp130. Interestingly, vIL-6 can stimulate signaling through gp130 and trigger STAT3 phosphorylation without need for surface expression of the receptor. This is in contrast with human IL-6, which is secreted independently of gp130 and cannot signal intracellularly. Viral IL-6 can thus efficiently establish an autocrine signaling mechanism to promote cell proliferation and survival.

1.6. Role of EBV co-infection

Epstein-Barr Virus (EBV) is a γ-herpesvirus closely related to HHV-8. EBV primarily infects nasopharyngeal epithelia and B lymphocytes. Acute infection with EBV is the

cause of infectious mononucleosis, whereas persistent infection with EBV is associated with a number of human malignancies, such as nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, immunoblastic lymphomas, etc (reviewed in (147)). In most PEL cases, the lymphoma cells are co-infected with HHV-8 and EBV (reviewed in (1)) and the quasi-ubiquitous presence of EBV in PEL cells first lead to the suggestion of an etiological role for EBV in PEL pathogenesis. Although it has now become clear that it is HHV-8 infection that is associated with PEL development and that this lymphoma can arise in the absence of EBV but not HHV-8, it is also clear that EBV and HHV-8 can interact in the infected B lymphocyte.

PEL cells co-infected with EBV usually exhibit a type II latency pattern of gene expression (31, 121), although some reports have found a latency I type of expression (152, 277). *In vivo* experiments in severe combined immunodeficiency (SCID) mice demonstrated that EBV co-infected HHV-8^+ PEL cell lines were able to induce tumor formation much more efficiently than the EBV^- HHV-8^+ cell lines (290). Indeed, the PEL cell lines BC-3 and CRO-AP/6 infected with recombinant EBV were highly tumorigenic in SCID mice when inoculated at 10 X 10^6 cells and 1 X 10^6 cells respectively. Both doses failed to induce any tumor formation when the parental cell lines were used. The dually infected cells expressed EBER-1, EBNA-1 and LMP2A but lacked EBNA 2-6, corresponding to a latency type II gene expression profile. Some cells also expressed variable levels of LMP1. It remains to be determined which EBV-encoded proteins help HHV-8 to induce transformation.

At the molecular level, somewhat conflicting reports exist of interactions between the HHV-8 latent protein LANA-1 and the EBV genome. One study (100) found that

transiently transfected LANA-1 activated transcription from the EBV major C promoter (Cp) in BJAB and 293 cells to the same level as EBNA2. In the same way, LANA-1 dramatically activated the LMP1 promoter, with up to 45-fold induction. Deletion studies of the LMP1 promoter showed that LANA-1 transactivation was mediated by the proximal region of the promoter (-204 bp to +40 bp), which contains PU.1, ATF/CRE and Sp-1 binding sites. LANA-1 has been shown to bind and activate Sp-1 to induce transcription of target genes, so it is possible that activation of the LMP-1 promoter occurs via Sp-1 and LANA-1 interactions. On the other hand, a study by Krithivas et al. (152) found that LANA-1 repressed both Cp and Qp promoter activity. LANA-1 blocked JAK-1 induced upregulation of Qp and EBNA2 induced upregulation of Cp, although LANA-1 had no effect on the basal activity of these two promoters. The negative effects of LANA-1 were at least partially mediated through its association with proteins of the mSin3 corepressor complex.

1.7. Role of HIV-1 co-infection

Long before the discovery of HHV-8, the association between Kaposi's sarcoma and HIV-1 infection had been established in the clinical setting. In the 1980s, the onset of the AIDS epidemic was accompanied by a dramatic increase in KS cases in homosexual men. The appearance of KS lesions was one of the hallmarks of the onset of AIDS and was used as a clinical marker of infection with HIV-1 (91). Similarly, PEL cases are more common in AIDS patients than in the rest of the population, suggesting some level of co-operation between HHV-8 and HIV-1 in promoting PEL. Clearly, the immunosuppression induced by HIV infection plays an indirect role in PEL and KS

pathogenesis, as other immunosuppressed states –such as in transplant patients – also correlate with increased PEL/KS incidence (reviewed in (1)).

The Tat protein of HIV-1 has been put forth as a viral protein that promotes KS development due to its pro-angiogenic properties (17). Tat can also directly promote growth of KS cells (71). Tat and basic fibroblast growth factor (bFGF) synergistically induce the proliferation of endothelial cells (72) and Tat can increase activated endothelial cell adhesion (16). Because inflammatory cytokines play such an essential role in KS development, it has been suggested that immune modulation, rather than immune suppression, by HIV-1 is a factor in KS development.

In the case of PEL, although association between disease development and late stage AIDS is striking, few studies exist on the role of HIV-1 in PEL development. HIV-1 can induce HHV-8 reactivation in PEL cell lines by inducing Rta expression and Tat can promote increased migration of PEL cells (50, 294). Thus, a certain level of interaction between viral proteins exists but the full extent of co-operation between HIV-1 and HHV-8 in PEL development still remains to be investigated.

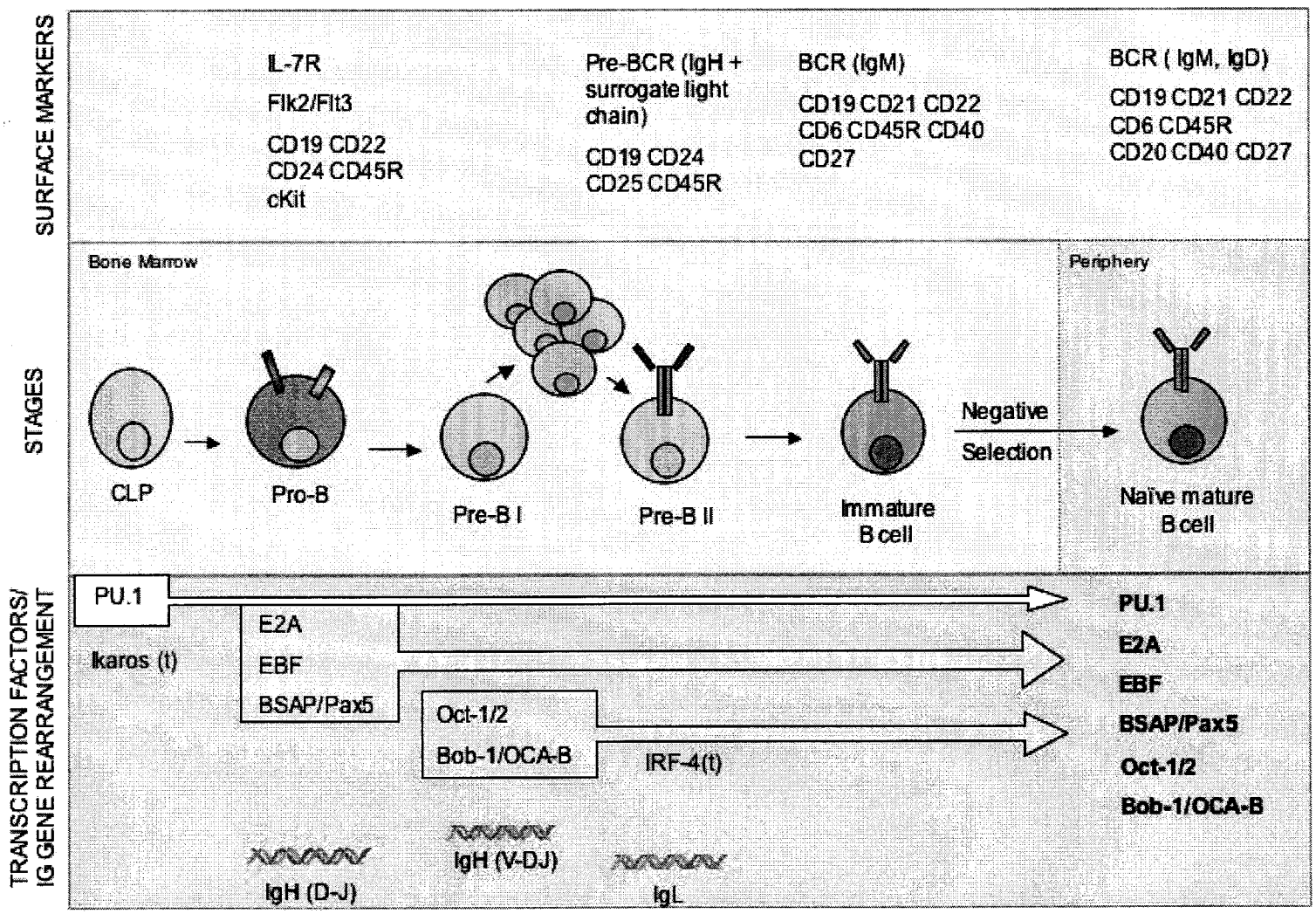
2. PEL cells and the B cell transcriptional program

Lymphomagenesis is a complex process involving multiple molecular pathways that culminate in the transformation of mature lymphoid cells. As we have seen in the previous section, infection of B lymphocytes with HHV-8 is associated with the development of PEL; however, the study of HHV-8 viral products is insufficient to explain the development and clinical presentation of PEL. The study of PEL lymphoma histogenesis is necessary to identify the normal B lymphocyte counterpart of the PEL cell and the molecular changes associated with cancer development. As seen above, PEL has been characterized as being of late-GC or post-GC origin, owing to the expression of IRF-4 and CD138/syndecan-1 and the lack of expression of Bcl-6. To put these findings into context, we give an overview of B lymphocyte development and of specific transcription factors that play an essential role in this process.

2.1. The Development of B Lymphocytes

The development of B lymphocytes proceeds through a series of tightly regulated stages that can be described based on a unique constellation of surface markers as well as Ig gene rearrangement (267) (**FIGURE 6**). For an in-depth review in lymphocyte development, please refer to (94). B cell development starts in the bone marrow, with the common lymphoid progenitor (CLP) giving rise to the pro-B cell by expressing the IL-7 and Flk2/Flt3 cytokine receptors at its surface via the combined action of PU.1 and

FIGURE 6. Schematic representation of surface marker and transcription factor expression during B cell development. The development of B lymphocytes can be described as a series of stages characterized by the rearrangement of immunoglobulin genes (bottom) and the stepwise expression of surface markers (top). This process is orchestrated by specific transcription factors that positively or negatively regulate gene expression (bottom). The transcription factors PU.1 and Ikaros (transiently expressed) regulate the expression of the IL-7 receptor, required for the emergence of the Pro-B cell from the common lymphoid progenitor (CLP). PU.1, E2A, EBF and BSAP/Pax-5 are the master regulators of the earliest stages of B cell development and are joined at the Pre-B I stage by octamer factors (Oct-1/-2) and the co-activator Bob-1. Transient expression of IRF-4 during the Pre-B II stage is required for the rearrangement of the immunoglobulin light chains. The naïve mature B cell can be distinguished by the expression of a fully mature B cell receptor (BCR) - composed of an IgM or IgD molecule, Igα and Igβ chains - and a constellation of surface markers (CD19, CD21, etc.) as well as by the expression of PU.1, E2A, EBF, BSAP/Pax-5, Oct-1/2 and Bob-1 transcription factors.



(t): transient expression

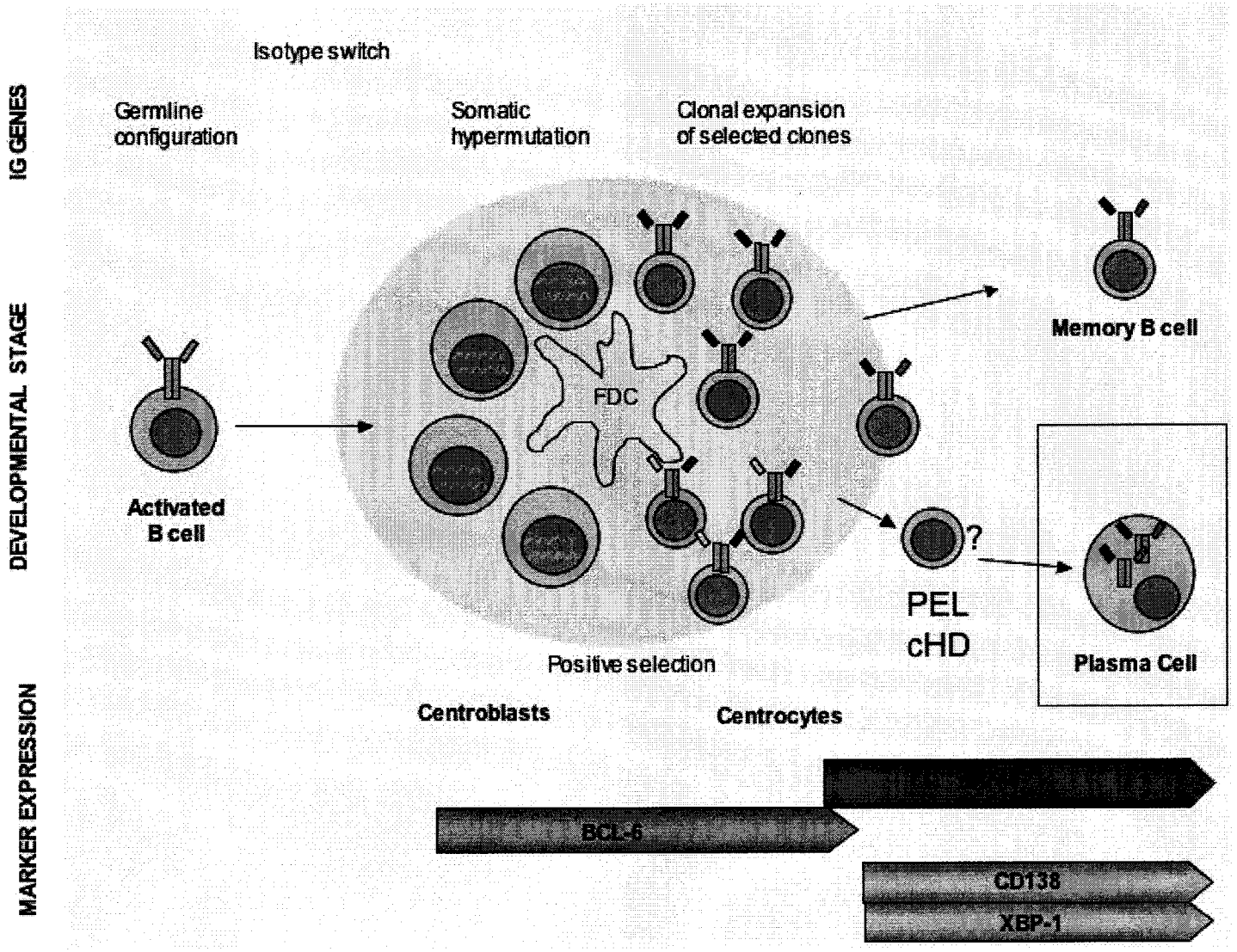
Ikaros transcription factors. During the pro-B cell stage, rearrangement of the immunoglobulin heavy chain takes place. Expression of genes essential to B cell development such as *mb-1, λ5, VpreB* and *Rag1/2* is driven by the transcription factors E2A and EBF. The presence of the pre-B cell receptor, composed of the newly rearranged heavy chain and the surrogate light chain λ5 or VpreB, marks passage to the pre-B cell stage. The transcription factor Pax-5/BSAP controls the expression of *CD19*, *Blnk* and *mb-1* genes, which encode for essential components of the B cell receptor (BCR) and co-receptor complexes. At the pre-B cell stage, rearrangement of the immunoglobulin light chain takes place and successful rearrangement leads to the expression of a complete immunoglobulin molecule at the cell surface. This signals the end of the pre-B cell stage and passage to the immature B cell stage. Therefore, an immature B cell is characterized by surface expression of the mature BCR - composed of one IgM molecule, two Igα and two Igβ chains -, the B cell co-receptor complex - composed of CD19, CD21 and CD81 -, and CD45, a tyrosine phosphatase that helps in the amplification of downstream signaling. At this point, self-reacting B lymphocytes are eliminated or inactivated by negative selection. B cells that successfully complete negative selection are termed mature B cells. The naïve mature B cells exit the bone marrow and circulate in the periphery, where pro-survival signals are further delivered to the pool of naïve B cells that successfully enter the lymphoid follicles.

The circulating naive mature B lymphocyte can encounter antigens from invading pathogens. If the antigen encountered is specifically recognized by the BCR, the B lymphocyte can become activated. Antigens can be of two sorts: thymus-dependent (proteins) or thymus-independent (LPS, lipids). The first sort requires the interaction of

the B lymphocyte with a T helper cell to fully activate the antibody response whereas the latter type can activate the B lymphocyte without T cell help. In the thymus-dependent response, cross-linking of surface Ig molecules by specific antigen triggers signaling by the Igα and Igβ components of the BCR. This results in the entry to G1 phase of the cell cycle and up-regulation of co-stimulatory molecules such as B7-1 and B7-2. Additionally, the B cell endocytoses and processes antigen to present it on its surface bound to MHC class II molecules. The activated B cell migrates to the lymph nodes where it can find its cognate antigen-primed T helper cell. Cognate T cell-B cell recognition involves 1) specific recognition of MHC class II-bound antigen at the B cell surface by the T cell receptor, 2) expression of CD40 ligand by the T cell which binds to CD40 on the B lymphocyte and 3) secretion of IL-4, IL-6 and IL-5 by the helper T cell. Together CD40L and IL-4 stimulate clonal expansion of the activated B cell. At this stage, the activated B cell also undergoes isotype switching.

The activated B cell then migrates to the primary follicles where it forms a germinal center (**FIGURE 7**). The rapidly dividing B cells known as centroblasts form the dark zone of the germinal center. These cells are large, have an expanded cytoplasm that stains intensely for RNA and have diffuse chromatin in the nucleus. During this stage, the cells undergo somatic hypermutation of the Ig hypervariable region, so that the progeny cells, the small centrocytes express slightly different Ig molecules. Centrocytes are then selected for high affinity recognition of antigen displayed by $CD4^+$ T lymphocytes (positive selection). B cells that fail to recognize antigen die by apoptosis, whereas B cells that successfully bind to their specific epitope undergo a new round of clonal expansion before differentiating into plasma or memory B cells. Thus, B

FIGURE 7. PEL cells are derived from B lymphocytes at a post-GC stage of differentiation with a partial plasma cell phenotype. During the GC reaction, B lymphocytes can be differentiated based on the configuration of the hypervariable region of the Ig genes (top) and on expression of specific molecular markers (bottom). Previous to GC formation, the activated B cell has a hypervariable region in germline configuration, whereas centroblast that are actively undergoing somatic hypermutation will exhibit numerous unique sequences containing point mutations. Centrocytes and post-GC B cells represent a clonal expansion of such mutated clones. During the GC reaction, B cells express Bcl-6 but only a few express IRF-4, whereas cells that successfully exit the GC lose Bcl-6 expression and upregulate IRF-4. B cells that undergo plasma cell differentiation also express the surface marker CD138/syndecan-1 and upregulate the transcription factor XBP-1. Both PEL and cHD share the expression of IRF-4 and CD-138 whereas they lack Bcl-6 expression. FDC= follicular dendritic cell. Adapted from (Carbone *et al.*, 2001 (34)).


IG GENES
DEVELOPMENTAL STAGE
MARKER EXPRESSION
Isotype switch
Germline configuration
Somatic hypermutation
Clonal expansion of selected clones
Activated B cell
FDC
Memory B cell
?
PEL
cHD
Plasma Cell
Positive selection
Centroblasts
Centrocytes
BCL-6
CD138
XBP-1

lymphocytes in the germinal center can be distinguished based on the sequence of their BCR: pre-GC B cells have an identical germline sequence, centroblasts and early centrocytes have somatic hypermutations in the IgV region so that each cell has slightly different BCR sequences and late centrocytes and post-GC B cells present a clonal expansion of a mutated BCR. In addition, transcription factor expression tightly regulates germinal center progression. Expression of the transcription factor Bcl-6, in particular, is a hallmark of GC B cells and it is expressed at high levels by centroblasts. Centrocytes downregulate Bcl-6 expression and activate the expression of Blimp-1 and IRF-4.

Cells that successfully exit the germinal center differentiate into long-lived plasma cells that reside in the bone marrow or into memory cells. Plasma cells express low levels of surface Ig and no MHC class II but are rather poised to produce and secrete large amounts of antibody. Plasma cells have abundant cytoplasm with multi-layered rough endoplasmic reticulum, prominent Golgi apparatus and a nucleus with peripheral chromatin condensation. Ten to 20% of all protein produced corresponds to immunoglobulin molecules. Alternatively, the B cell can become a memory B cell that can be rapidly reactivated upon subsequent antigen challenge. Plasma cells are characterized by the expression of Oct-2, Blimp-1, XBP-1 and IRF-4 transcription factors, whereas Pax-5 and Bcl-6 - which can repress transcription of XBP-1 and Blimp-1 respectively - are down-regulated in plasma cells (36, 293).

2.2. The B cell transcriptional Program

2.2.1. PU.1

The Ets-family transcription factor PU.1 plays an essential role in the development of both lymphoid and myeloid lineages (185, 252). High expression of PU.1 favors the development of macrophages whereas low levels of PU.1 induce B cell generation from hematopietic progenitors (57). PU.1 knockout mice die of severe septicemia 48 hours after birth and reveal a lack of mature macrophages, neutrophils, B cells and T cells. The lack of PU.1 was not essential for lymphocytic and myeloid commitment, since the K. O. mice could develop limited numbers of T cells and neutrophils that could be detected in antibiotic-treated animals, but generation of B cells and macrophages was completely impaired (185). In humans, heterozygous mutations of the PU.1 gene are associated with acute myeloid leukemia (AML). AML blast cells are arrested at a very early stage of myeloid development, reminiscent of myeloid cells from the PU.1$^{-/-}$ mouse (196).

Low-level expression of PU.1 favors the expression of the IL-7 receptor (IL-7R) and the generation of common lymphoid precursors (CLP) from hematopoietic stem cells (HSC) (56). During B cell development, PU.1 regulates the expression of the Ig μ heavy chain (202), Ig light chain (λ and κ) (68, 227), mb-1 (Igα) (107), B29 (Igβ) (211), CD20 (117), Btk (118), and Ig J chain (261), among other genes. Moreover, murine knockout studies have demonstrated that regulation of c-rel promoter activity by PU.1 and Spi-B is essential for mature B cell survival (122), possibly due to the anti-apoptotic properties of c-rel. PU.1 expression is also important during the GC reaction and high levels can be detected in centroblasts, where it regulates the expression of Bcl-6 (287).

PU.1 expression is regulated by a highly conserved region that surrounds the transcriptional start site (47). The 0.5 kb mouse promoter is overall 88% identical to the

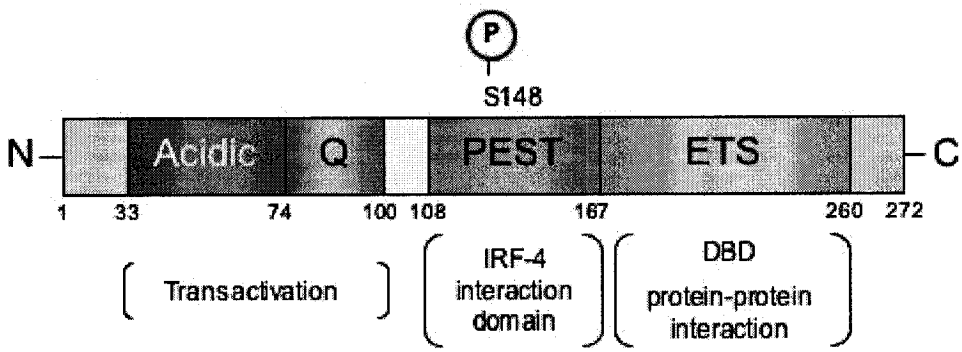
same region in the human PU.1 promoter whereas the region between -70 bp to +134 bp is even more highly conserved (95% similarity). Both murine and human promoters demonstrated specific activity in myeloid and B cells. The region located between –61 and +34 bp, contains single functional binding sites for PU.1, Octamer binding factors, GATA and Sp-1 (**FIGURE 8B**). *In vivo* studies in murine myeloid cells demonstrated that the PU.1 binding site is occupied in these cells, leading to the establishment of an auto-regulatory loop, whereas the octamer and Sp-1 sites appear vacant (47). In B cells, both the PU.1 and the octamer site are occupied, demonstrating that octamer binding (Oct) factors along with the B cell specific co-activator Bob-1 regulate the expression of PU.1 in B lymphocytes (48).

PU.1 protein is 272 aa long and it is composed of an acidic domain, a glutamine-rich domain, a PEST (proline, glutamate, serine, threonine rich) domain and a C-terminal Ets domain that confers DNA binding specificity (**FIGURE 8A**). The PEST domain contains regulatory Ser148 that is required for protein-protein interactions (228) whereas Ser41 in the N-terminal transactivation domain is phosphorylated in response to activation of the Ras-PI3 kinase-PKB pathway (236) leading to increased PU.1 transcriptional activity. The protein kinase CK2 has been shown to specifically phosphorylate PU.1 at serine residues 41, 45, 132, 133, and 148 (228).

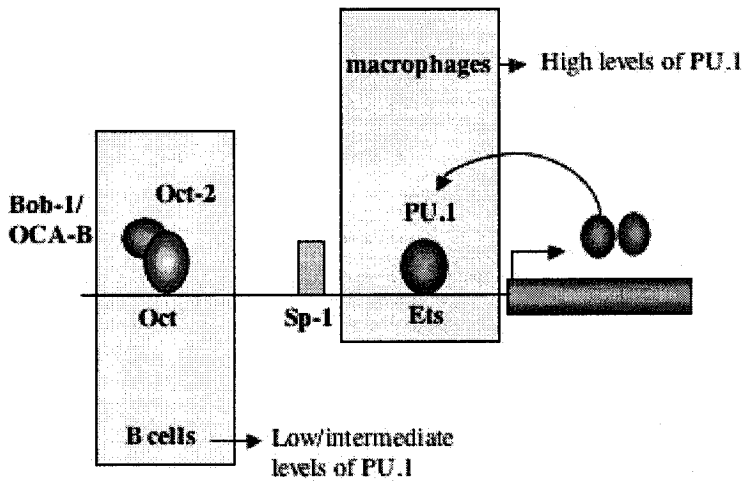
Interactions between PU.1 and Interferon regulatory factor-4 (IRF-4/Pip/ICSAT/NF-EM5) to activate transcription were first described by Pongubala *et al.* (227, 228). This group identified a novel transcription factor, that they named NF-EM5, that co-operatively bound with PU.1 to the immunoglobulin κE3' enhancer (**FIGURE 8C**). The composite Ets-IRF binding site had the sequence 5'-GAGGAANNGAAAAC-3' on the

FIGURE 8. Structure, regulation and function of the transcription factor PU.1. A) Schematic representation of PU.1 protein. The acidic domain and the glutamine (Q)-rich domain located at the N-terminus as well as the central proline- glutamate- serine- threonine-rich (PEST) domain and the C-terminal ETS domain are indicated. Numbers indicate amino acid residues at domain boundaries. Domain functions are indicated in brackets. The regulatory serine S148 – required for PU.1/IRF-4 interactions – is indicated. Ⓟ denotes phosphorylation. B) Regulation of the PU.1 promoter. In macrophages, PU.1 expression is regulated mainly through an auto-regulatory loop, leading to high levels of protein expression. In B lymphocytes, both the Octamer binding site (Oct) and the PU.1 site are essential for PU.1 transcription but only lead to low levels of protein expression. C) PU.1 interacts with IRF-4 to activate transcription via cooperative binding to the Ets-IRF composite element (EICE). Binding of IRF-4 to PU.1 leads to a conformational change in IRF-4 that unmasks the DNA-binding domain and allows interaction with the partial IRF site of the EICE element.

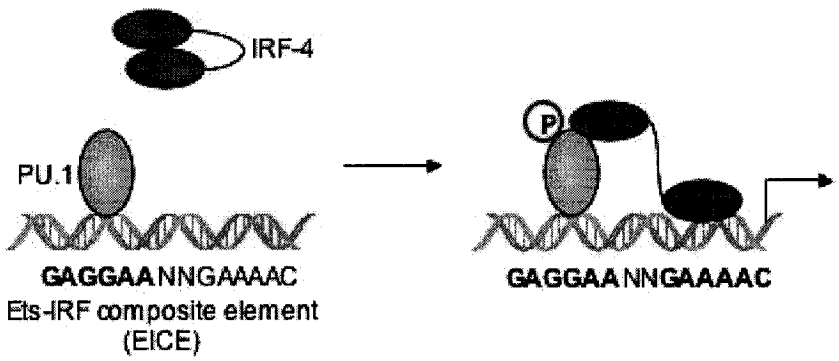
A


P
S148
N
Acidic
Q
PEST
ETS
C
1
33
74
100
108
167
260
272
Transactivation
IRF-4 interaction domain
DBD protein-protein interaction

B


macrophages
High levels of PU.1
Bob-1/ OCA-B
Oct-2
PU.1
Oct
Sp-1
Ets
B cells
Low/intermediate levels of PU.1

C


IRF-4
PU.1
P
GAGGAANNGAAAAC
Ets-IRF composite element (EICE)
GAGGAANNGAAAAC

κE3' enhancer. Interaction with IRF-4 was mediated by the PEST domain of PU.1 (227) and required phosphorylation of Ser148. Later, similar co-operation between PU.1 and IRF-4 was found to exist on the λ2-4 light chain enhancer (68) and on the CD20 promoter (117). Binding to the composite ETS-IRF site by PU.1 and IRF-4 is co-operative: IRF-4 requires the presence of PU.1 to be able to bind DNA and activate transcription (68). Interaction between the PEST domain of PU.1 and the C-terminal domain of IRF-4 induces a conformational change in IRF-4 that exposes the DNA-binding domain, allowing it to recognize its target sequence. Alternatively, in the composite Ets-IRF site found in the CD20 promoter, it is PU.1 that requires the presence of IRF-4 to form a stable protein-DNA complex (117). Co-operation between PU.1 and Interferon Regulatory Factor-8 (IRF-8/ICSBP) has been also characterized and it is established in much the same way as with IRF-4. PU.1 and IRF-8 interactions are important for the expression of $gp91^{phox}$ (69) and TLR-4 (234). More extensive co-operation between PU.1 and IRFs has been identified in the IL-1β gene, where PU.1 interacts with IRF-4 to bind to a composite ETS-IRF site found in the IL-1β enhancer (177). The PU.1/IRF-4 complex also interacts with IRF-1 and IRF-2 to form an enhanceosome that allows maximal expression of IL-1β following LPS or cytokine stimulation.

PU.1 plays an essential role in B-lymphocyte and macrophage development by regulating the expression of multiple genes essential for lineage differentiation. In B cells, PU.1 positively regulates the expression of fundamental genes such as IL-7 and the immunoglobulin chains, constituting a pivotal transcription factor in B lymphoid

development. As such, PU.1 expression can be detected as early as the CLP and its expression is not extinguished until the plasma cell stage (reviewed in (267),(199)).

2.2.2. IRF-4 and IRF-8

Interferon Regulatory Factors (IRFs) constitute a family of transcription factors that regulate the transcription of IFN genes as well as other target genes in response to IFN-α/β or IFNγ stimulation. IRFs are characterized by a common amino-terminal DNA-binding domain containing characteristic tryptophan repeats that allows them to interact with the Interferon Stimulated Response Element (IRSE, consensus: 5'- GAANT/C-3') or the Gamma-Activated Sequence (GAS, consensus: 5'- TTTNCNNNAA-3') found in the promoter of target genes. IRFs may also contain an IRF Association Domain (IAD) that allows protein-protein interactions. Extensive reviews on the IRF family have been published (119, 168) and the reader is directed to these for a more thorough overview on IRFs.

IRF-4 and IRF-8 are the two members of the IRF family with the less homology to other IRF family members – within the DBD - but the highest homology (70%) to each other (reviewed in (168),(176)). IRF-4 and IRF-8 also share a restricted pattern of expression: IRF-4 is expressed in T and B lymphocytes, where its expression is inducible by PMA treatment, CD3 or Ig crosslinking. IRF-4 can also be expressed in macrophages, although the stimuli leading to its upregulation is unknown. IRF-8 expression is largely restricted to the myeloid and lymphoid compartment. IRF-8 expression can be rapidly induced in macrophages following IFNγ stimulation. IRF-4 and IRF-8 can bind alone to ISRE sequences and act as transcriptional repressors of

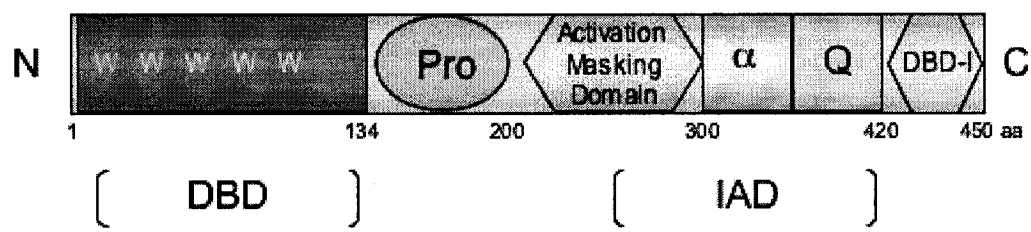
IFN-inducible genes such as MHC-I and 2'-5'-oligo-adenylate synthetase. However, it appears that the main function of IRF-4 and IRF-8 in lymphocytes and myeloid cells is to act as transcriptional activators in partnership with the Ets proteins PU.1 and Spi-B (176). In this fashion, IRF-4 and IRF-8 regulate many genes essential for lymphoid and myeloid development. We take a closer look at each of these IRFs in the sections below.

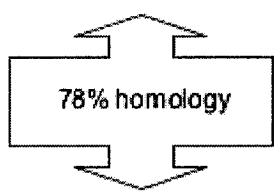
IRF-4. IRF-4 was first described as a transcriptional activator that acted in partnership with PU.1 to increase transcription of the Ig light chain genes (67, 68, 227) but can also act as a repressor when it binds to ISRE sequences (24). Thus, IRF-4 may favor lymphocyte activation while inhibiting the anti-proliferative effects of IFNα/β. IRF-4 knockout mice are unable to form germinal centers (GCs), lack plasma cells and show reduced circulating Ig levels (190). They are also unable to generate T cell-dependent cytotoxic or anti-tumor responses (190). Thus IRF-4 is essential for the function of mature B and T cells.

Structurally, IRF-4 is a 450 aa-long protein that contains the N-terminal DBD characteristic to all IRFs, followed by a proline-rich domain, an activation-masking domain, a glutamine-rich region, and a DBD-inhibitory domain (**FIGURE 9**). In resting cells, IRF-4 is present in a closed conformation, with the N-terminal DBD masked by the C-terminal inhibitory domain. Upon stimulation, DNA-bound, phosphorylated PU.1 recruits IRF-4 by interacting with the α-helical inhibitory domain (aa 399-413) (25). This induces a conformational change in IRF-4 that unveils the DBD and allows IRF-4 to bind with higher affinity to its target sequence. Phosphorylation of PU.1 at serine 148 is essential for interactions with IRF-4. In turn, IRF-4 transcriptional activity is modulated by the immunophilin FKBP52, which induces a *cis-trans* propyl

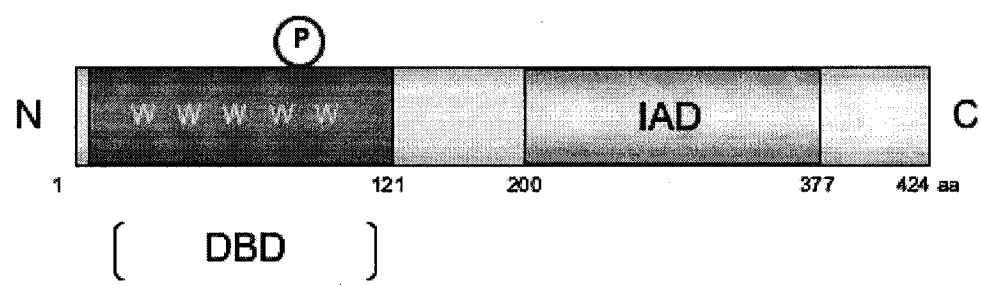
FIGURE 9. Schematic representation of IRF-4 and IRF-8. The DNA-binding domain (DBD) containing the characteristic tryptophan repeats is located at the N-terminus whereas the IRF-association homology domain (IAD) is located at the C-terminus. IRF-4 (top) also contains a proline-rich domain (Pro) followed by the IAD and the DBD-inhibitory region (DBD-I) at the C-terminus. Numbers indicate amino acid residues at domain boundaries. The IAD of IRF-4 contains an activation masking domain, an alpha-helical region (α) and a glutamine-rich (Q) domain. The activation-masking domain and the C-terminal DBD-I fold over the DBD to prevent interactions with DNA. The inhibition is relieved by binding to PU.1 as well as by the action of the immunophilin FKBP52. The α-helix is required for interaction with PU.1 whereas FKBP52 induces proline isomerization in the Pro-rich region. IRF-8 (bottom) is 78% homologous to IRF-4 in the DBD. Phosphorylation has been described for IRF-8 within the DBD.

IRF-4


N
Pro
Activation
Masking
Domain
α
Q
DBD-I
C
1
134
200
300
420
450 aa
DBD
IAD


78% homology

IRF-8


P
N
IAD
C
1
121
200
377
424 aa
DBD

isomerization in the proline-rich domain, resulting in the inhibition of IRF-4 interactions with PU.1 and transcriptional activity (171).

In B lymphocytes, IRF-4 expression can be strongly upregulated by stimuli that induce B cell activation such as LPS or IgM crosslinking. In addition, co-stimulation with CD40 and IL-4 induces a strong expression of IRF-4 (105), linking IRF-4 to the IL-4 signaling pathway. Indeed, inducible expression of CD23 – a target of IL-4 and CD40 signaling – was shown to depend on IRF-4 interactions with STAT6 at a GAS element present in the CD23 promoter. Constitutive expression of IRF-4 in B cells depends on a GC-rich element found on the –51 to –28 bp promoter region which is bound by a novel 60 kDa protein (205), whereas inducible expression in lymphocytes is regulated by cRel (101).

Expression of IRF-4 in B cells is bi-phasic: immature B cells express IRF-4, then IRF-4 expression is lost when B cells enter the GC and recovered at the end of the GC reaction to finally be expressed at a high level by plasma cells (77). Immunohistochemical analysis of healthy lymphoid tissue thus shows that GC B cells are mostly negative for IRF-4, but 3-10% expresses high levels of IRF-4. The IRF-4^{+} cells locate in the light zone of the GC and have varied morphology: some have irregular nuclei whereas others show immunoblast-like or plasmablast-like characteristics. Thus, the beginning IRF-4 expression correlates with the centrocyte state of B cell differentiation and it is sustained during the maturation as a plasma cell (88). Klein *et al.* generated a conditional *IRF-4* knockout mouse in which *IRF-4* is deleted in GC B cells (148). The conditional knockout demonstrated that IRF-4 expression is required for class-switch recombination and plasma cell development, although not for memory cell

development. IRF-4 is required during class-switch recombination to upregulate the expression of activation-induced cytidine deaminase (AID). During plasma cell development, IRF-4 and Blimp-1 were found to act non-redundantly upstream of XBP-1. Indeed, expression of XBP-1 in LPS-stimulated mature IRF-$4^{-/-}$ B cells was not sufficient for the generation of plasma cells in spite of the presence of normal levels of Blimp-1.

Because IRF-4 expression correlates with B cell exit from the GC reaction and it is associated with loss of expression of B-cell Lymphoma 6 protein (Bcl-6) – a marker of GC B cells -, the expression of IRF-4, Bcl-6 and the surface marker CD138 (Syndecan-1) can be used as histogenetic markers of B cell development (88). Activated B cells that have just entered the lymph nodes are IRF-4- Bcl-6- CD138- and they lack somatic hypermutations in their Ig genes; during the GC reaction, B cells are Bcl-6+ IRF-4- CD138- and they have multiple IgV region sequences – denoting active somatic hypermutation. Cells that are exiting the GC gain expression of IRF-4 and CD138 and lose expression of Bcl-6. Sequencing of the IgV region sequences of post-GC B reveals the clonal expansion of mutant sequences, corresponding to positively selected cells. Lymphomas can be classified in much the same manner, as the cancer cells retain most histogenetic markers from their normal cellular counterpart. Based on molecular marker analysis, PEL cells have been classified as being of late-GC or post-GC origin since most PEL cells are IRF-4^{+} Bcl-6^{-} $CD138^{+}$. However, as stated above, immunoglobulin hypervariable regions mutational analysis has led to somewhat discrepant results (179).

IRF-8. IRF-8, also known as Interferon Consensus Sequence Binding Protein (ICSBP), is a ~50 kDa protein first described as binding to IFN Response Sequences in the major histocompatibility complex class I (MHC I) genes (64). IRF-8 is a 424 aa long protein that has the conserved N-terminal DNA binding domain and the C-terminal IRF-interaction domain (IAD) common to IRF family members (**FIGURE 9**) (reviewed in (281)). IRF-8 is expressed in B lymphocytes, activated T cells and monocytes/macrophages, where expression of IRF-8 can be strongly induced by IFN-γ and to a lesser extent by LPS. IRF-8 is constitutively phosphorylated on tyrosine residues *in vivo*, leading to a downregulation of its DNA binding activity and an increased association with IRF-1/2. Thus, phosphorylated IRF-8 can only bind DNA through interaction with IRF-1/2 (256).

The IRF-8 knockout mouse is susceptible to infection with pathogens that depend on IFN-γ for clearance. These include bacteria such as *Listeria monocytogenes* and *Yersinia enterocolitica* and parasites such as *Leishmania major* and *Toxoplasma gondii*. However, the IRF-$8^{-/-}$ mouse survives infection with Vesicular Stomatitis Virus (VSV) or influenza A, controlled primarily through the IFN-α/β response (120). Mounting of a Th1-mediated response is greatly impaired in the IRF-8 K.O. mouse. This defect was ascribed primarily to the inability of IRF-$8^{-/-}$ macrophages/dendritic cells to produce IL-12 - more specifically, the IL-12p40 subunit - whose expression is directly controlled by IRF-8 (281). Lack of IL-12 leads to impaired production of IFN-γ in natural killer (NK) and $CD4^+$ T cells, resulting in an inhibition of the expression of co-stimulatory molecules necessary for proper Th1 response. Further study of the knockout mouse revealed additional defects in dendritic cell (DC) generation and maturation. IRF-$8^{-/-}$

mice were unable to sustain plasmacytoid DC (pDCs) generation or DC maturation in response to Toll-like receptor (TLR) stimulation (292). Ectopic expression of IRF-8 in pDCs restored their ability to produce IFN-α, suggesting a role for IRF-8 in type I IFN production in pDCs (291).

In contrast to the rapidly expanding knowledge of the role of IRF-8 in myeloid development, very little is known about its role in lymphocyte development. IRF-8-/- mice have increased numbers of splenic and lymph node B cells and IRF-8/IRF-4 double knockout have a block in the pre-B to B cell transition, owing to impaired downregulation of surrogate light chain expression and a profound block in rearrangement and upregulation of Ig light chain expression (166). Thus, IRF-4 and IRF-8 play additional roles in B cell development besides enhancement of light chain expression in combination with PU.1. A recent study by Lee *et al.* (157) demonstrated that IRF-8 expression is modulated during the human GC reaction. IRF-8 is expressed by all B cells in the GC, but expression is highest during the dark-zone centroblast stages and lowest in the Ig-secreting population of plasma cells. IRF-8 expression co-localized with Bcl-6 nuclear staining, in sharp contrast with IRF-4 expression (see above). IRF-8 positively regulates the expression of Bcl-6 and activation-induced cytidine deaminase (AID), two proteins essential to the GC reaction. It is not entirely clear how IRF-8 and IRF-4 can play such contrasting roles during the GC reaction, given that up to now they have been shown to be practically interchangeable in their transactivation capacity of target genes. It is possible that stage-specific expression of transcription partners might help explain this astonishing observation.

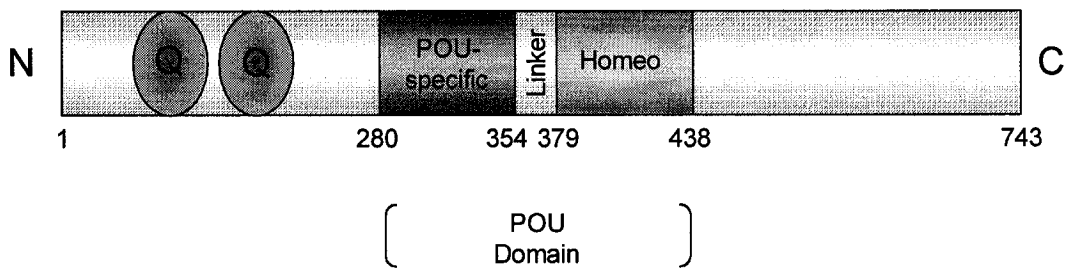
2.2.3. Octamer Binding Factors

The octamer motif (5'-TNATTTGCAT-3') is found in the promoter of numerous genes essential for B cell function such as Ig, CD20, CD21 and CD36 as well as in other ubiquitously expressed genes such as small nuclear RNAs (snRNA) and histone H2B (reviewed in (182)). The octamer motif is recognized by octamer binding proteins (Oct) that belong to the POU protein family. POU proteins contain a 150-160 bipartite DNA-binding domain consisting of a conserved N-terminal POU-specific domain and a C-terminal POU homeodomain. Two Oct transcription factors interact with the octamer motif in B lymphocytes: Oct-1 and Oct-2 (**FIGURE 10**). Oct-1 is a ubiquitously expressed 90-100 kDa protein whereas Oct-2 (60-75 kDa) is expressed exclusively in the lymphoid compartment - predominantly in B cells - and central nervous system. An auxiliary factor termed B-cell Oct-binding protein-1 (Bob-1, also known as OBF-1 and OCA-B) expressed exclusively in lymphocytes binds to Oct-1/-2 via DNA and POU domain interactions and acts as a transcriptional co-activator (102). Bob-1 is largely responsible for the specific activity of octamer sites in B lymphocytes and its expression is required at multiple stages of B cell development (115), particularly for GC formation (248).

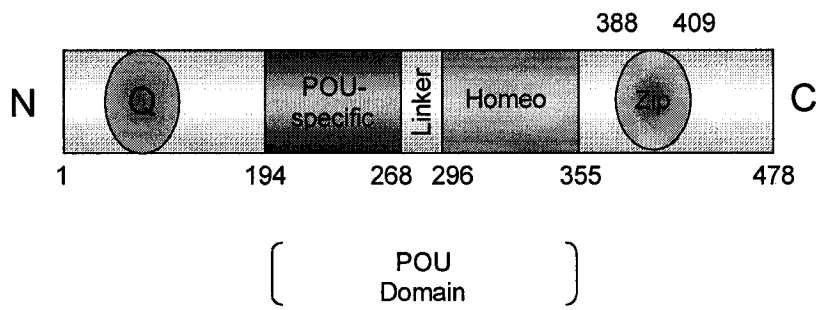
The generation of Oct-$2^{-/-}$ and Bob-$1^{-/-}$ mice has identified important roles played by these two factors in B cell function. The Oct-2 knockout mouse dies at birth of unknown causes (53). B cell lymphopoiesis is normal up to the IgM^{+} stage, thus Oct-2 is dispensable during early B cell development. However, there is a maturation defect in Oct-$2^{-/-}$ B cells because they fail to form GCs and secrete immunoglobulin when challenged *in vitro* with LPS (248). Furthermore, when Oct-2 deficient fetal liver cells

FIGURE 10. Schematic representation of the octamer binding factors Oct-1 and Oct-2. Oct-1 and Oct-2 belong to the POU-domain family of transcription factors, which contain a bi-partite DNA-binding domain composed of a POU-specific domain and a homeo-domain separated by a linker. Interactions with the co-activator Bob-1 are mediated by the POU domain. Both Oct-1 and Oct-2 contain Glutamine (Q)-rich domains at the N-terminus but Oct-2 also contains a leucine zipper (Zip) domain at the C-terminus. Numbers indicate amino acid residues at domain boundaries.

Oct-1


N
Q
Q
POU-specific
Linker
Homeo
C
1
280
354
379
438
743
POU Domain

Oct-2


388
409
N
Q
POU-specific
Linker
Homeo
Zip
C
1
194
268
296
355
478
POU Domain

were transferred into irradiated recipients, the peripheral B cell pool was decreased, the spleen showed predominantly B cells of an immature phenotype and peritoneal B1 cells were absent. Mice lacking Bob-1 are viable and show normal early B cell development although they have reduced B cell numbers in the spleen and reduced IgG serum levels (146). The humoral immune response in the Bob-$1^{-/-}$ mouse is dramatically impaired and germinal centers do not form. In the Oct-2/Bob-1 double knockout, B cells develop normally but the mature peripheral B cell pool is strongly reduced. Immunoglobulin gene transcription appears normal yet serum IgG levels are dramatically reduced (248). The double knockout animals failed to develop GCs in response to antigen challenge. Thus, Oct-2 and Bob-1 regulate essential aspects of B cell maturation that are independent of immunoglobulin gene transcription.

Although earlier studies had suggested that Oct-1 and Oct-2 bound to the octamer sequence interchangeably and that specificity was conferred by the presence or absence of the co-activator Bob-1, the knockout mouse suggested a distinct role for Oct-2. CD36 was identified by subtractive cDNA cloning as the first gene dependent on Oct-2 transcriptional activity (151), although the function of CD36 remains unknown. Analysis of the CD36 promoter revealed the presence of the octamer sequence 5'-ATGCTAAT-3', which required the expression of Oct-2 to activate transcription in B cells but did not support recruitment of Bob-1 (263). Thus, Oct-2 can regulate transcription in B cells both dependently and independently of Bob-1. The differential transcriptional activity of Oct-2 compared to Oct-1 has been mapped to the C-terminus of Oct-2 (282), which is also a site for protein phosphorylation, although the relationship between phosphorylation and transcriptional activity remains to be elucidated. Further

confirmation of the essential role played by Oct-2 in the expression of the late-stage B cell transcriptional program came from studies of myeloma cell hybrids (137, 231). When the Ig-producing myeloma cell line MPC 11 is fused to a T cell, expression of B cell-specific gene products (Ig, J chain, etc.) ceases, and this correlates with a lack of expression of Oct-2. The same phenomenon is observed when fusing an Ig-producing B cell to a fibroblast (136). In this last study, Junken et al. observed a rapid depletion in nuclear transcription factor expression - including Oct-2 and PU.1 - that preceded Ig gene silencing. Constitutive expression of Oct-2 in the B/non-B cell hybrids is enough to alleviate gene silencing (136, 137, 231). Thus, expression of Oct-2 is essential to maintain the B cell transcriptional program during the late stages of B cell development.

2.2.4. Nuclear Factor κB (NF-κB)

The family of NF-κB transcription factors is involved in such diverse cellular functions as apoptosis, inflammation and cell differentiation. It is a common target in various cancer types and its constitutive activation generally results in an inhibition of apoptosis and expression of pro-inflammatory cytokines which results in a chronic inflammatory state favorable to cancer development (for reviews refer to (98, 141)). The NF-κB transcription factor family consists of p50 (NF-κB1), p52 (NF-κB2), Rel (cRel), p65 (Rel-A), and Rel-B. Each family member contains a Rel-homology domain (RHD), which includes a DNA-binding motif, a dimerization motif and a nuclear localization sequence (NLS). Rel, p65 and Rel-B also contain a C-terminal transactivation domain. All members of the NF-κB family associate to form homo- and hetero-dimers able to

modulate transcription. NF-κB dimers are kept inactive in the cytoplasm by binding to the Inhibitors of NF-κB (IκB), of which the prototypical member is IκB-α (89).

The classical pathway of NF-κB activation is triggered following stimulation with a myriad of compounds such as TNF-α, IL-6 or CD40L that funnel into the IκB Kinase (IKK) complex (138, 220). The IKK complex is composed of two catalytic subunits, IKKα and IKKβ, and one regulatory subunit called NF-κB Essential Modulator (NEMO/IKKγ). As part of the activated IKK complex, IKKβ phosphorylates IκBα at two serine residues (Ser34/36), targeting it for proteasomal-mediated degradation. The p65/p50 NF-κB dimer, now free of the inhibitor, translocates into the nucleus and activates transcription of target genes. In the non-canonical pathway, the NF-κB Inducing Kinase (NIK) activates IKKα, which phosphorylates p100 triggering its cleavage into p52. Target genes for NF-κB include genes that promote cell growth, prevent cell death and regulate the immune response. In B lymphocytes, B cell receptor (BCR) stimulation and CD40 signaling both lead to NF-κB activation, promoting cell survival and proliferation (191, 192, 298).

In PEL, constitutive NF-κB activation, specifically of p65/p50 heterodimers, has been observed, resulting in the inhibition of apoptotic pathways and expression of pro-inflammatory cytokines such as IL-6 (present work and (144)). PEL is not associated with chromosomal translocations that constitutively activate NF-κB, as it has been demonstrated with other cancer types and in particular in Hodgkin's lymphoma (18, 302). Rather, NF-κB activation seems to be mediated by the action of HHV-8 viral genes. Activation of NF-κB during latent HHV-8 infection is mediated by vFLIP, which directly interacts with NEMO/IKKγ to activate the IKK complex (103). During the lytic

cycle, NF-κB can be further activated by vGPCR and mediate IL-6 expression (251). NF-κB activation during the lytic cycle is essential to establish a productive infectious cycle (255). Inhibition of NF-κB with pharmacological inhibitors in PEL cells results in apoptosis, highlighting the importance of NF-κB activation in driving survival of the malignant cells. It is noteworthy that PEL cells do not exhibit activation of the NF-κB subunits cRel or RelB, which are predominantly associated with lymphocyte development and function but rather of p65/p50 heterodimers, which regulate genes involved in cell survival and proliferation.

2.2.5 Activator Protein-1 (AP-1)

AP-1 is a transcriptional activator composed of members of the Fos (cFos, FosB, Fra-1, and Fra2), Jun (cJun, JunB, JunD), Maf (cMaf, MafB, MafA, MafG/F/K, Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) protein families (reviewed in (259)). The transforming potential of AP-1 is demonstrated by the fact that transforming viruses such as the osteogenic sarcoma viruses FBJ and FBR and avian sarcoma virus encode for viral homologues of Fos (*v-fos*) and Jun (*v-jun*) (187, 204). These viral oncogenes are essential for transformation of target cells by sarcoma viruses. Jun and Fos proteins associate *via* their leucine zipper domain to form a variety of homo- and heterodimers and bind to their target sequence (5'-TGACTCA-3'), the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE) (81). AP-1 activity can be induced following a variety of stimuli, including growth factors, cytokines, B and T cell receptor cross-linking and UV irradiation. Stimulation of AP-1 activity can be achieved at three levels:

1) transcriptional upregulation of AP-1 components, 2) stabilization of AP-1 proteins and 3) stimulation of AP-1 activity by post-translational modification. The mitogen-activated protein kinase (MAPK) pathway is the classical and best-studied activator of AP-1 transcription factors (reviewed in (42, 140, 259)).

Activation of the extracellular regulating kinases (ERK-1 and ERK-2) leads to increased transcription of cFos, which can bind to cJun protein present in the nucleus to form AP-1 dimers. In contrast, cJun is expressed constitutively in most cell types but its expression can be further induced through the *cjun* TRE, which is activated by cJun-ATF2 heterodimers. Activation of cJun occurs via phosphorylation of key serine residues located in the transactivation domain by the Jun kinases (JNKs). JNKs are activated primarily in response to inflammatory stimuli such as TNF-α. They can also be activated in response to UV irradiation, cytokines, growth factor deprivation, DNA damaging agents, etc.

Activation of lymphocytes requires two signaling events: signal 1 is delivered by cross-linking of the BCR or TCR whereas signal 2 is delivered by co-stimulatory molecules such as CD28 (T cells) or CD40 (B cells) (reviewed in (81)). Cross-linking of the T and B cell receptors signals through protein tyrosine kinases and activates PKC, $p21^{Ras}$ and PLC-γ. Ras activation leads to activation of ERK kinases, phosphorylation of Elk-1 and induction of c-fos expression. The $p21^{Ras}$ pathway can also activate JNK kinase *via* MEKK-1. PKC directly activates the MAPK Fos kinase, whereas it activates Erk, p38 and JNK by signaling through the $p21^{Ras}$ pathway. Finally, PLC-γ, through production of diacyl-glycerol (DAG) can signal through PKC and activate the MAPK pathway. Binding of the T cell co-receptor CD28 leads to the activation of JNK through

the guanine-nucleotide exchange factor (GEF) protein Vav pathway, which activates MEKK-1 and leads to JNK activation. In B lymphocytes, co-stimulation via CD40-CD40L interactions also leads to AP-1 activation, both by PKC-dependent and PKC-independent pathways. Activation of transcription mediated by c-fos/c-jun heterodimers, in combination with NFAT and NF-κB transcription factors, leads to the upregulation of target genes important for lymphocyte survival, differentiation and function such as IL-2, IL-3, IL-4, IL-5, IL-6 and GM-CSF. In B lymphocytes, AP-1 activity – and particularly of c-fos/c-jun heterodimers – is required for Blimp-1 expression and terminal differentiation into plasma cells (209).

PEL cells harbor constitutive activation of AP-1 transcription factors, as demonstrated by an increase in DNA binding of c-fos/c-jun heterodimers and increased transactivation of target sequences. The activation of AP-1 is mediated by two HHV-8 latent gene products: LANA-1 and vFLIP. LANA-1 physically interacts with and activates cJun (7). In addition, LANA-1 induces DNA binding of c-fos/c-jun heterodimers, although it is not itself part of the DNA-binding complex. Viral FLIP activates JNK via association with Tumor Necrosis Factor Receptor-Associated Factors (TRAFs), possibly TRAF2, leading to increased transactivation of TRE-regulated promoters. Expression of vFLIP leads to increased JNK phosphorylation and increased DNA binding of c-fos/c-jun heterodimers (8). Activation of AP-1 by LANA-1 and vFLIP – which also activates NF-κB - leads to upregulation of cellular IL-6 expression in PEL cells (7, 8). Thus, AP-1 activation contributes to the survival and proliferation of PEL cells.

3. Leukotriene B_4 and cancer

3.1. Inflammation and Cancer

The link between inflammation and cancer development has long been suspected. Already in the 19th century, Virchow had suggested that tumors might arise from areas of chronic inflammation. Recently, compelling scientific evidence linking the establishment of a chronic inflammatory state and cancer development has been put forth by several groups (14, 15, 97, 139, 269). The inflammatory process involves the complex interplay of different cell types that express biological pro-inflammatory agents such as cytokines, chemokines, and lipid mediators. The production of these agents in the tumor microenvironment can potentiate transformation and drive proliferation and survival of cancerous cells (269). Reciprocally, activation of oncogenes in cancer cells leads to the production of signaling molecules that recruit inflammatory cells and modulate their activity to drive cancer growth (reviewed in (98, 153)).

Further evidence for the involvement of chronic inflammation and cancer development came from clinical evidence that indicated that prolonged use of non-steroidal anti-infammatory drugs (NSAIDs) such as aspirin lead to a 40-50% reduction in the incidence of colon cancer (reviewed in (104)). NSAIDs are inhibitors of the cyclooxygenase enzymes COX-1 and COX-2, implicated in the synthesis of prostaglandins (PGs) from arachidonic acid. PGs mediate pain and inflammation - among other physiological functions - and are synthesized in a broad range of tissue types. Studies of familial adenomatous polyposis (FAP) in which patients were treated with NSAIDs – either non-specific or COX-2 specific – lead to a reduction of size and

number of polyps. In the $APC^{\Delta 716}$ mouse model of FAP, treatment with the COX-2 specific inhibitor Rofecoxib or with the non-specific inhibitor Sulindac lead to a decrease in polyp number and size correlating with a decrease in membrane-bound VEGF (215). Elevated levels of COX-2 have been detected in esophageal, head and neck, breast, lung, prostate and other cancers, suggesting that the use of NSAIDs may have benefits in other malignancies besides colon cancer.

3.2. Leukotrienes

Leukotrienes (LT) are a family of lipid mediators involved in inflammation and allergy (reviewed in (245)). Leukotrienes are part of the eicosainoids class of paracrine hormones, which derive from the oxidative metabolism of arachidonic acid (AA) and include prostaglandins, leukotrienes and lipoxins. Leukotrienes can be found at high levels in most inflammatory lesions and contribute to the physiological changes observed during inflammation. LTB_4 is the classic chemoattractant of neutrophils and thus regulates PMNL infiltration to the inflammation site whereas cysteinyl leukotrienes are involved in vascular permeability and smooth muscle tone.

3.3. Leukotriene Synthesis

Leukotriene synthesis is initiated by the release of arachidonic acid (AA) from the cell membrane by cytosolic phospholipase A_2 ($cPLA_2$), making this substrate available to 5-lipoxygenase (5-LO). In resting leukocytes, 5-LO is a soluble enzyme, but elevated

intracellular calcium levels (26, 225) or phosphorylation by MAPK-activated protein (MAPKAP) on Ser-271 (299-301) lead to its translocation to nuclear and perinuclear membranes where 5-LO is in close proximity to its substrate AA and to the 5-lipoxigenase-activating protein (FLAP) (59, 189). 5-LO catalyzes the conversion of AA into LTA_4 in a two-step enzymatic process: I) conversion of AA into 5-hydroperoxyeicosatetraenoic acid, which can be degraded to 5-hydroxyeicosatetraenoic acid (5-HETE), and II) subsequent formation of LTA_4 (239). LTA_4 can then meet two fates: it can be conjugated with glutathione by LTC_4 synthase to yield LTC_4, the parent compound of cysteinyl-containing LTs (LTC_4, LTD_4, LTE_4), or it can be hydrolyzed by LTA_4H to yield LTB_4 (**FIGURE 11A**).

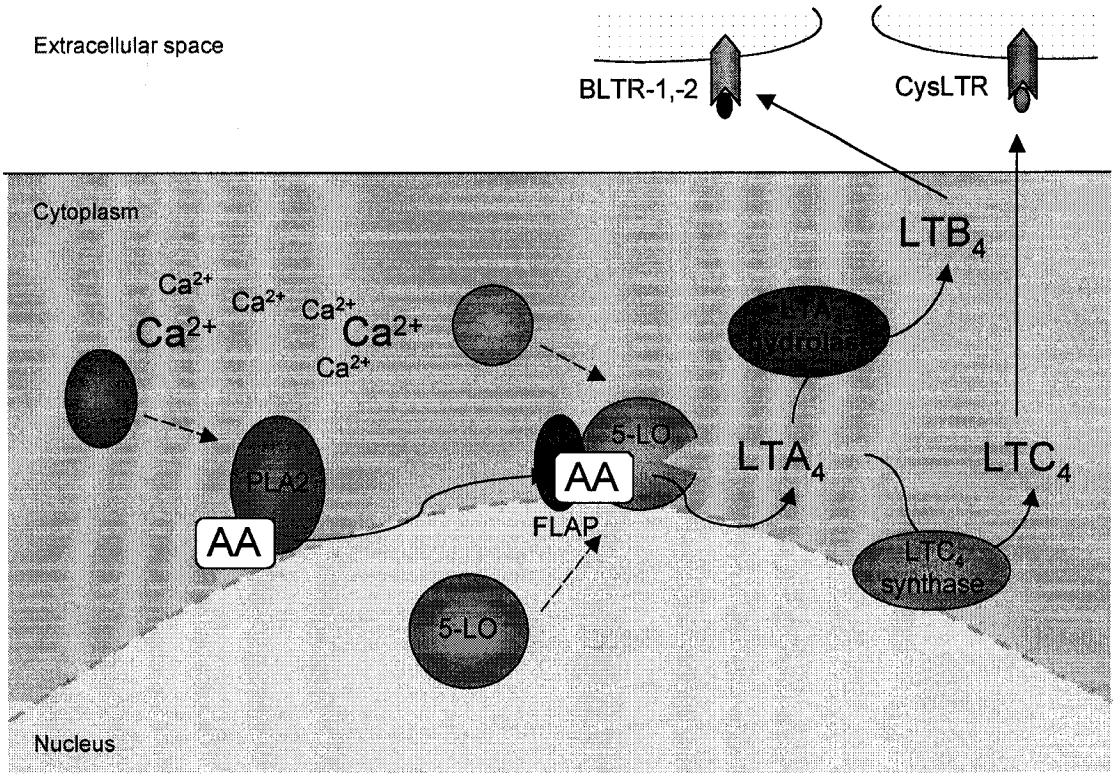
3.4. Transcellular Biosynthesis

Enzymatic cooperation between different cell types in the synthesis of certain prostaglandins and leukotrienes is a concept that originated from the observation that endothelial cells could produce prostaglandin I_2 (PGI_2) from platelet-derived prostaglandin endoperoxide H_2 (175). This process was termed transcellular biosynthesis and it involves the concerted action of an "acceptor" cell, with a constitutive enzymatic activity for the formation of a biologically active LT/PG but a deficient capacity for substrate generation, and a "donor" cell that can provide such substrate.

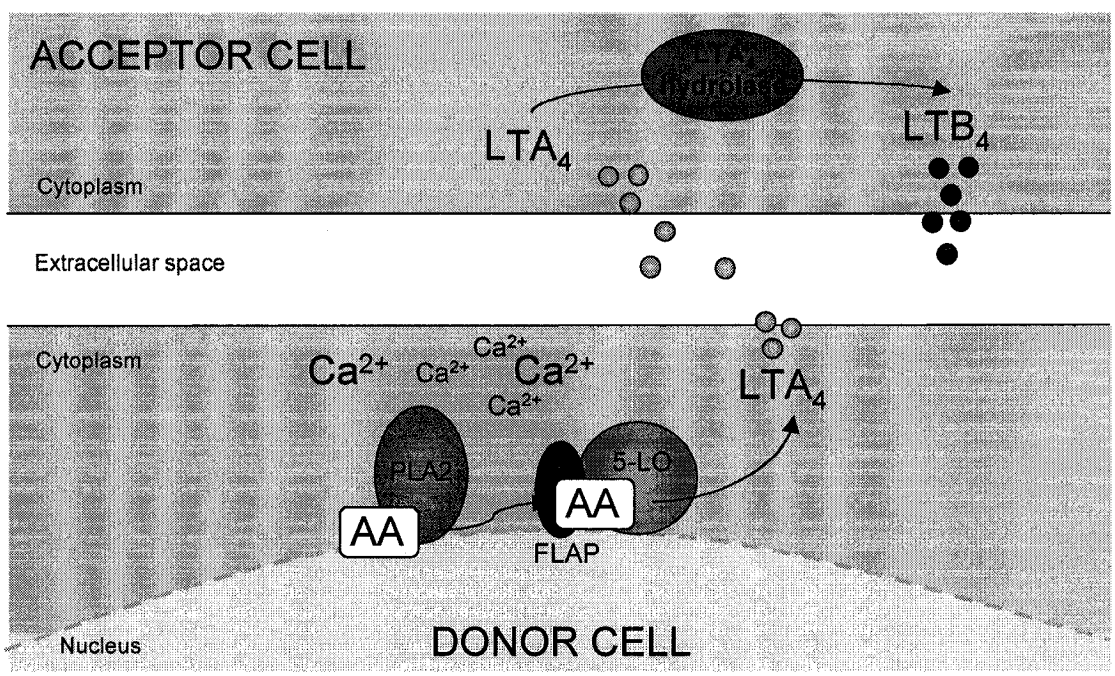
The generation of leukotrienes by transcellular biosynthesis has been observed in a variety of settings. It involves the participation of myeloid cells - in particular neutrophils - as "donors", since these cells possess 5-LO activity, and a variety of other

FIGURE 11. Schematic representation of leukotriene B_4 synthesis. A) Synthesis of leukotrienes is induced by elevated intracellular calcium levels, which stimulate phospholipase A2 (PLA2) to cleave arachidonic acid (AA) from the cell membranes (cytoplasmic or nucleic membranes), making this substrate available for 5-lipoxigenase (5-LO). Intracellular calcium also stimulates 5-LO enzymatic activity to transform AA into the unstable epoxide leukotriene A_4 (LTA_4). This process requires the presence of the co-factor molecule 5-LO activating protein (FLAP). Two fates can occur to LTA_4: conversion to LTB_4 by LTA_4 hydrolase (LTA_4H) or conversion to LTC_4 and derived cysteinyl-leukotrienes by LTC_4 synthase. LTB_4 and LTC_4 are then released into the extracellular space, where they bind their cognate receptors found mainly in granulocytes and smooth muscle cells, respectively. B) Trancellular biosynthesis of leukotrienes involves the concerted action of two cells: the "donor" cell, which possesses 5-LO activity, and the "acceptor" cell, which has LTA4H activity. Transcellular biosynthesis allows the magnification of leukotriene production, mainly in the case of LTB_4 as LTA_4H undergoes suicide inactivation.

A


Extracellular space
BLTR-1,-2
CysLTR
Cytoplasm
LTB_4
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
LTA_4 hydrolase
5-LO
PLA2
AA
AA
FLAP
LTA_4
LTC_4
LTC_4 synthase
5-LO
Nucleus

B

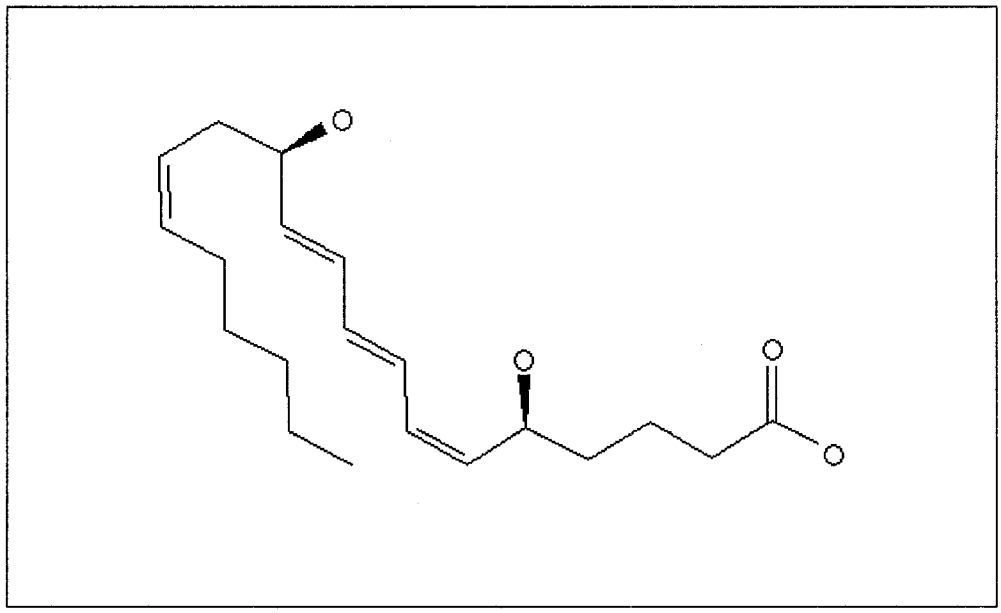

ACCEPTOR CELL
LTA_4 hydrolase
LTA_4
LTB_4
Cytoplasm
Extracellular space
Cytoplasm
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
LTA_4
PLA2
5-LO
AA
AA
FLAP
Nucleus
DONOR CELL

cell types with LTA_4H or LTC_4 synthase activity as "acceptors" (**FIGURE 11B**). Examples of acceptors cells include erythrocytes (80, 184), keratinocytes (128), alveolar macrophages (99), platelets (167), endothelial cells (78), and B and T cells (129, 130). The use of $LTA4H^{-/-}$ and $5\text{-}LO^{-/-}$ mice in transcellular biosynthesis experiments have demonstrated that this process can occur *in vivo* and that the LTs produced can contribute to the inflammatory response (75).

3.5. Leukotriene B_4

Leukotriene B_4 was initially discovered by Borgeat and Samuelsson (22) and it was soon identified as a potent chemoattractant of neutrophils, with activity detectable at subnanomolar concentrations (80, (93, 159) (**FIGURE 12**). LTB_4 contributes to the inflammatory response primarily by recruiting leukocytes to the injury site and augmenting vascular permeability (21) LTB_4 is synthesized by myeloid-derived cells such as PMNL (in particular neutrophils), dendritic cells, monocytes and macrophages and plasma levels of this leukotriene increase from less than 100 pg/ml to over 100 ng/ml following leukocyte stimulation (262, 275). In the airway passages, bronchial epithelial cells are a source of LTB_4 (9) as are keratinocytes in the epidermis (131, 238). It is now clear that LTB_4 is a potent mediator of inflammation that affects a larger spectrum of cell types including monocytes, T and B cells, with diverse biological outcomes.

FIGURE 12. Chemical structure of Leukotriene B_4.



Leukotriene B_4

3.6. Leukotriene B_4 Receptors

The activity of LTB_4 is mediated by two G protein-coupled seven transmembrane domain receptors (GPCR): BLT1 and BLT-2 (reviewed in (280)). BLT1 is a high affinity receptor initially discovered in 1997 by Yokomizo *et al.* (305). BLT1 had been previously identified as an orphan member of the GPCR superfamily (232) and chemoattractant receptor-like 1 (CMKRL1) (217) before further experiments confirmed its identity as a receptor for LTB_4 (218). Expression of BLT1 is highest in peripheral blood leukocytes but it is also detectable in the spleen, thymus, bone marrow, lymph nodes, heart, skeletal muscle, brain and liver (216). BLT1 expression is largely restricted to the leukocyte compartment, with high expression in granulocytes (neutrophils and eosinophils) and monocytes (226), low expression in B lymphocytes, and inducible expression in macrophages (124), activated T cells (95, 278) and vascular smooth muscle cells (12).

BLT2 is a low affinity receptor (307) with more ubiquitous expression. High BLT2 levels are detected in human spleen, liver, ovary and peripheral blood leukocytes (307); murine BLT2 is highly expressed in small intestine and skin (127). BLT2 binds LTB_4 with less specificity than BLT1 and several other eicosainoids – including 12(S)-HETE, 12(S)-HPETE and 15(S)-HETE - can bind to BLT2 and trigger calcium mobilization (306).

Both BLT1 and BLT2 are G protein-coupled receptors, but the identity of the G-protein that transduces intracellular signals following LTB_4 stimulation remains unknown. In fact, different cellular events may require different G proteins as

demonstrated by *Bordella pertussis* toxin treatment of BLT1- and BLT2-transfected CHO cells, which completely abolished chemotaxis but only partially diminished calcium mobilization (305, 307). Thus chemotaxis is mediated exclusively through $G\alpha_{i/o}$ subunit signaling but calcium responses involve additional G proteins. LTB_4 signaling through BLT1 leads to the activation of the NF-κB pathway(12, 123, 183). In human monocytes, BLT1 signalling activates ERK1/2 and JNK MAP kinases (MAPK), but not p38 MAPK, inducing overexpression of monocyte chemoattractant protein-1 (MCP-1)(123). Information on BLT2 signaling is limited. Stimulation of mBLT2 with LTB_4 lead to calcium mobilization, phosphorylation of ERK1/2 and inhibition of adenylyl cyclase (127).

3.7. Biological Effects of LTB_4

As part of the host's immune response, LTB_4 stimulation triggers leukocyte chemotaxis, adhesion and activation, contributing to the recruitment of effector leukocytes at the sites of pathogen invasion. At subnanomolar concentrations, LTB_4 causes neutrophil chemotaxis and chemokinesis (222). At higher concentrations, (100 nM), LTB_4 stimulation leads to neutrophil aggregation and degranulation(82) as well as superoxide anion production. LTB_4 also mediates monocyte, macrophage and eosinophil chemotaxis and adhesion (268, 279). Additional biological functions of LTB_4 in other cell types continue to emerge and it is now clear that it mediates a plethora of effects in both health and disease.

LTB_4 participates in immune modulation by activating B cells, T cells and NK-cells (reviewed in (51)). LTB_4 mediates T cell recruitment to inflammatory sites (278). $CD4^+$ T helper (T_H) cells upregulate BLT1 upon activation and LTB_4 stimulation of both T_H1- and T_H2-polarized cells induces chemotaxis and firm arrest of these cells. Two murine models of asthma – an inflammatory disease of the lung – demonstrated the importance of LTB_4 stimulation in early T_H cell recruitment (278). $CD8^+$ Cytotoxic T cells also upregulate BLT1 upon activation and LTB_4 induces chemotaxis and adherence of these cells, mediating recruitment of cytotoxic T cells to inflammation sites at both early and late time points (95). Thus, LTB_4 possibly elicits a rapid activation of integrins at the T cell surface that allows for efficient rolling and diapedesis.

In B lymphocytes, LTB_4 induces proliferation, activation (130) and differentiation (65, 303). LTB_4 induces expression of the activation marker CD23 in high-density, resting B lymphocytes when used in combination with sub-optimal concentrations of IL-2 or IL-4 (65, 130, 303). Concurrently, LTB_4 induces DNA synthesis and enhances the production of IgG and IgM (65, 303).

In monocytes, LTB_4 can trigger adhesion to the vascular endothelium via rapid changes in integrin affinity and/or avidity for its receptor (86). LTB_4 stimulation of monocytes also increases the production of monocyte chemoattractant protein-1 (MCP-1) via activation of ERK1/2 or JNK and activation of the NF-κB pathway (123).

LTB_4 can block apoptosis of neutrophils (112, 271), increasing cell survival. Indeed, glucocorticoids such as dexamethasone promote neutrophil survival via upregulation of BLT1 (271). LTB_4 also inhibited apoptosis of carcinoma cells both *in-vitro* and *in-vivo* (286).

LTB_4 activates endothelial cells, vascular smooth muscle cells, keratinocytes and osteoclasts (12, 127). LTB_4 acts on endothelial cells to increase their adhesiveness – to promote neutrophil binding and endothelial transmigration - by a yet undetermined mechanism independent of PMN activation (221). In vascular smooth muscle cells, LTB_4 induced proliferation and migration, a phenomenon related to intimal hyperplasia development (12).

3.8. LTB_4 and Inflammatory Diseases

LTB_4-mediated recruitment and activation of leukocytes participates in the pathogenesis of a number of inflammatory diseases. Inflammatory bowel disease (258), asthma (37, 55, cystic fibrosis {Konstan, 1993 #1933, 262), multiple sclerosis (203), atherosclerosis (312), rheumatoid arthritis (3) and other diseases are associated with significantly elevated levels of LTB_4 in involved tissues. Multiple animal models have demonstrated a causative role for LTB_4 in these diseases and treatment with LTB_4 receptor inhibitors usually leads to decreased leukocyte recruitment and improvement or remission of the disease (reviewed in (280)).

3.9. LTB_4 and Cancer

A clear link between AA metabolites, inflammation and cancer is starting to emerge. Non-steroidal anti-inflammatory drugs (NSAIDs) are proving effective in the chemoprevention of certain types of cancer (104, 215). LTB_4 is a potent mediator of

leukocyte recruitment and transcellular biosynthesis of this leukotriene can greatly amplify the inflammatory response. The potential role of LTB_4 in cancer development and the use of pharmacological inhibitors of the LTB_4 receptors or LTA_4H are actively being investigated.

Increased LTA_4H levels were detected in the squamous endothelium of human esophageal adenocarcinoma (EAC), colon and lung cancer samples (49). Pleural effusions associated with lung cancer showed increased levels of LTB_4 and neutrophil infiltration, comparable to those found in exudates from tuberculosis patients (219). In a rat model of EAC, treatment with the non-specific LTA_4H inhibitor Bestatin decreased LTB_4 levels and tumor incidence and severity (49). Bestatin has also been shown to inhibit squamous-cell lung carcinoma relapse (126) and induce apoptosis of human leukemic cell lines (253), although the specific involvement of LTA_4H was not demonstrated. Human pancreatic cancer cell lines expressed both BLT1 and BLT2 receptors and LTB_4 stimulation led to an increase in proliferation. Treatment of these cells with an inhibitor of the BLT receptors (LY293111) lead to decreased proliferation and apoptosis (286). LTB_4 plays a pivotal role in the activation and proliferation of chronic B lymphocytic leukemia cells (B-CLL) in response to CD40 ligation. Expression of activation markers in B-CLL cells upon CD40L stimulation was inhibited by MK-886 (a FLAP inhibitor) but could be re-established upon LTB_4 treatment (242).

LTB_4 has been implicated in Ras-induced transformation as part of the Rac signaling pathway. Rac activation of $cPLA_2$ is required for c-fos serum response element (SRE) activation and cellular transformation (145, 308). Ras-transformed Rat2 cells (Rat2-HO6) produce more LTB_4 and have higher levels of BLT2 (308). Use of the BLT

antagonist leukotriene B4-3-aminopropylamine (LTB_4-APA) determined BLT2 to be required for Ras-induced transformation: *in vitro*, LTB_4-APA reduced Ras-induced morphological changes and anchorage independence; *in vivo*, it could block tumor formation by Rat2-HO6 in athymic mice (308).

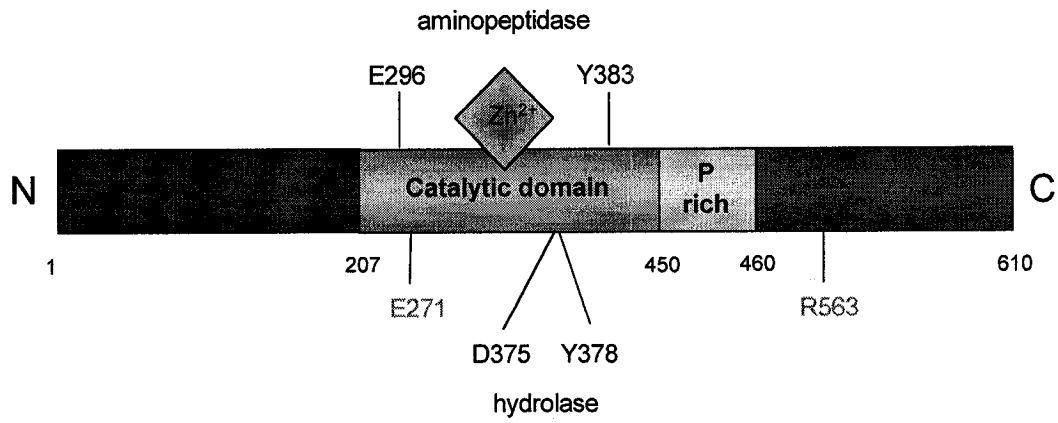
The body of evidence linking chronic inflammation, disregulated production of LTB_4 and cancer is increasing. Many questions remain as to the downstream signaling events following BLT stimulation and the transcriptional control of synthetic enzymes. Further investigation of transgenic models and the use of pharmacological inhibitors can help to shed light on these questions and propose novel therapeutic targets.

3.10. Leukotriene A_4 Hydrolase

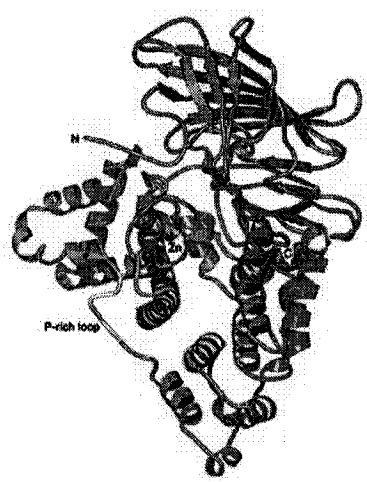
LTA_4H is a 610 amino acid-long cytosolic enzyme with a molecular mass of 69 kDa (87, 230) (**FIGURE 13**). Based on its zinc signature and aminopeptidase activity, LTA4H is classified as part of the M1 family of zinc metallopeptidases, which includes Aminopeptidase A, Aminopeptidase B, and Aminopeptidase N. LTA_4H catalyzes the final step in the synthesis of LTB_4 via its highly specific hydrolase activity. Epoxide hydrolysis of LTA_4 by LTA_4H is the rate-limiting step in the production of LTB_4, a reaction that also results in covalent modification of LTA_4H leading to suicide inactivation (74, 207).

FIGURE 13. Leukotriene A_4 hydrolase (LTA_4H). A) Schematic representation of LTA_4H, highlighting the Zn2+-containing catalytic domain. Residues important for the aminopeptidase and hydrolase activity are indicated. Residues important for both enzymatic activities are highlighted in red. P-rich indicates proline-rich domain. Numbers correspond to amino-acid residues. B) Crystal structure of LTA_4H. The zinc-containing catalytic domain is shown in green and the proline-rich loop is indicated (courtesy of Nat. Struct. Biol. (Thunnissen, M. *et al.*, 2001 (280)).

A


aminopeptidase
E296
Y383
Zn^{2+}
N
Catalytic domain
P rich
C
1
207
450
460
610
E271
R563
D375
Y378
hydrolase

B


N
P-rich loop

3.10.1. Tissue Distribution

Tissue distribution of LTA_4H is very broad, with high expression in spleen, lung and small intestine (208). LTA_4H is expressed in a variety of hematopoietic and non-hematopoietic cells, including airway epithelial cells, erythrocytes, neutrophils, and B-lymphocytes (20).

3.10.2. Gene Organization

Human LTA_4H gene exists as a single copy gene of over 35 kbp on chromosome 12q22 with a coding sequence divided in 19 exons (172). The putative *LTA4H* promoter is located in the 5'upstream region of the gene. It lacks a definitive TATA box and it contains a phorbol-ester response element (AP-2) and two xenobiotic response elements (XRE) (172). These elements were never functionally characterized and their significance remains unknown. A study by Zaitsu *et al.* (311) demonstrated the inducibility of the *LTA4H* gene by IL-4 and IL-13 treatment in polymorphonuclear leukocytes (PMN), however specific regulatory element within the promoter or transcriptions factors implicated in *LTA4H* gene regulation were not identified.

3.10.3. Protein Structure and Catalytic Activity

The crystal structure of LTA_4H in complex with the competitive aminopeptidase inhibitor Bestatin was resolved to 1.95 Å resolution to an R-factor of 18.5% (285)

(**Figure 13B**). The enzyme is globular and composed of three domains: N-terminal, catalytic and C-terminal. LTA_4H possesses two catalytic activities – hydrolase and aminopeptidase – that are exerted in distinct yet overlapping active sites. A hydrophobic pocket in the catalytic domain binds LTA_4, this pocket is lined by 21 residues that compose the catalytic peptide K21. The stereoselective introduction of water to the backbone of LTA_4 occurs several methylene groups away from the epoxide moiety, presumably through the induction of a carbocation intermediate (240, 241). The epoxide hydrolase activity is abolished by suicide inactivation that involves binding of LTA_4 to Tyr-378, which is located within the K21 peptide. The aminopeptidase activity of LTA4H proceeds via a zinc-assisted general base mechanism involving Glu-296 and Tyr-383 as general base and proton donor, respectively. LTA_4H accepts a variety of substrates for its aminopeptidase activity, including arginyl di- and tri- peptides (212) and opioid peptides (206).

3.10.4. *LTA4H* Knockout Mouse

LTA4H knockout mice were generated by homologous recombination in a 129/SvEv background (29). $LTA4H^{-/-}$ mice were obtained in the expected Mendelian ratios, thus disruption of LTA_4H expression had no effect in fetal development or perinatal survival. $LTA4H^{-/-}$ animals could not be distinguished from $LTA4H^{+/-}$ or wild-type littermates by simple observation or histological analysis. Hematopoiesis and lymphopoiesis of $LTA4H^{-/-}$ mice was normal.

LTA4H$^{-/-}$ mice challenged with intra-peritoneal administration of zymosan A did not produce LTB_4, whereas it was easily detected in wild-type animals. Thus, the *LTA4H* knockouts challenged with Zymosan A did not recruit PMNs to the site of inflammation. *LTA4H*$^{-/-}$ mice also showed impaired inflammatory response triggered by topical application of arachidonic acid (AA). *LTA4H*$^{-/-}$ mice were protected against systemic shock induced by platelet-activating factor treatment (92.3% survival compared to 50% in control group) but not by LPS. Study of the $LTA_4H^{-/-}$ mouse as well as the 5-LO and FLAP knockouts has unveiled the complexity of the inflammatory response to various stimuli and how LT production is carefully and differentially orchestrated in each case.

3.10.5. LTA_4H Pharmacological Inhibitors

LTA_4H was identified as a potential target for the development of anti-inflammatory drugs based on the specific roles of LTB_4 in acute and chronic inflammation and the identification of increased levels of LTA_4H in esophageal cancer and certain types of graft-versus-host disease. The general aminopeptidase inhibitor bestatin and the angiotensin-converting enzyme inhibitor captopril inhibit LTA_4H in a potent and reversible manner (IC_{50} 4 X 10^{-6} M and 11 X 10^{-6} M respectively)(213), consistent with a molecular resemblance of LTA_4H and other metallohydrolases at their Zn^{+2} binding sites. Several other more specific inhibitors have been developed in academic and industrial laboratories. Notably, the competitive inhibitor SC-57461A (3-[methyl[3-[4-(phenylmethyl)phenoxy]propyl] amino] propanoic acid HCl) demonstrated potent *in-vitro* activity, good cell penetration and oral activity in a mouse model (224). The use of

specific LTA_4H inhibitors alone or in combination with other existing drugs for the treatment of diseases such as atherosclerosis, rheumatoid arthritis and possibly certain types of cancer constitutes an interesting therapeutic approach to cure or improve the condition of patients afflicted with these diseases.

RESEARCH OBJECTIVE AND SPECIFIC AIMS

Detailed characterization of the molecular features of lymphomas is essential to their accurate classification, choice of appropriate treatment and targeted development of novel therapeutic agents. The advent of new technologies –such as microarray – has allowed the characterization of complex transcriptional profiles of cancerous as well as normal lymphocytes. In parallel, the study of the expression and activity of key transcription factors in normal and cancerous lymphocytes is essential as they orchestrate the cellular transcriptional program that results in the phenotypical features observed for each lymphoma. Notable examples of the transcriptional alterations observed in mature B cell lymphomas include the constitutive activation of cMyc and the inactivation of p53. These common defects however are not observed in PEL but rather development of the disease is intimately linked to infection by HHV-8. Our hypothesis is that PEL cells possess altered transcription factor expression and/or activity and that this contributes to lymphomagenesis by a) favoring HHV-8 replication and 2) inducing or suppressing the expression of target genes, which together constitute the transcriptional signature of PEL.

We undertook an investigation of the transcriptional program of PEL cells to identify alterations that 1) potentially contribute to lymphomagenesis by favoring HHV-8 infection and 2) provide a mechanistic explanation for the lack of surface marker expression observed in this lymphoma. Next, we used microarray technology to identify genes that were differentially expressed in PEL compared to cHD cells. PEL and cHD

are derived from B lymphocytes at a similar stage of development - late or post-GC with a partial plasma cell phenotype (34) and share numerous characteristics at the molecular level, yet are vastly dissimilar at the clinical level. We investigated if expression of a discrete set of genes could set apart PEL from cHD cells and help explain the clinical features and biological outcomes observed in PEL. The identification of a transcriptional signature particular to PEL – composed of both downregulated and upregulated factors – is crucial to better understand the molecular changes that contribute to the development of this lymphoma.

In order to prove our working hypothesis, we identified the following specific aims:

1. To investigate if the NF-κB pathway is activated in PEL cells and if activation of this pathway contributes to the spread of the HHV-8 virus.

2. To identify alterations in the B cell-specific transcriptional program in PEL cells that contribute to the lack of surface marker expression observed in these cells.

3. To identify specific genes expressed in PEL that can differentiate at the molecular level this lymphoma form other mature B cell malignancies, in particular cHD.

4. Following completion of objective 3, we identified leukotriene A_4 hydrolase (LTA_4H) as being upregulated in PEL, therefore we sought to investigate the

transcriptional regulation of the leukotriene A_4 hydrolase (LTA_4H) gene and identify promoter elements responsible for the overexpression of LTA_4H in PEL cells, further confirming that transcriptional regulation is altered in this lymphoma.

CHAPTER II

Materials and Methods

1. Cell lines

BCBL-1, a primary effusion lymphoma cell line infected with HHV-8 but negative for EBV or HIV-1, and BJAB, an EBV-negative Burkitt's lymphoma cell line, were a kind gift from Dr. Jae U. Jung (Harvard Medical School, MA). Both cell lines were cultured in RPMI 1640 (Wisent Inc., St. Bruno, QC) supplemented with 10% FBS, 10^{-5} M β-mercaptoethanol, and 8 μg/mL gentamicin. BC-3 (ATCC no. CRL-2277) is a primary effusion lymphoma cell line infected with HHV-8 but negative for both HIV and EBV. BCP-1 (ATCC product number CRL-2294) is a HHV-8 positive, HIV and EBV negative, clonal lymphoma cell line isolated from peripheral blood. Both PEL cell lines were maintained in RPMI 1640 supplemented with 20% FBS and 8 μg/mL gentamicin. CRO-AP6 is a HIV positive, EBV negative PEL cell line established from the pleural effusion of a 26-year-old, HIV^+ patient (described in (33), a kind gift from Dr. A. Carbone, Centro di Referimento Oncologico, IRCCS, Italy). KM-H2 and L-428 are two well characterised Hodgkin's lymphoma cell lines of B cell origin (described in (62, 114)), BL-2, BL-30, and BL-41 are Burkitt's lymphoma cell lines previously described (66), all a kind gift from Dr. Sigrun Smola. Namalwa cells are a EBV positive Burkitt's lymphoma cell line obtained from the ATCC (product number CRL-1432). They were cultured in RPMI 1640 with 10% FBS and 1% sodium pyruvate.

2. Plasmid constructions

The B4-TKCAT reporter construct was a kind gift from Dr. H. Singh (University of Chicago, Chicago, IL (68)). B4-TKCAT contains 4 copies of the ETS-IRF site found in

the immunoglobulin λB enhancer subcloned upstream of the HSV thymidine kinase promoter in the TK-CAT vector. The PU.1 expression vector PU.1/pECE was a kind gift from Dr. M. Fenton (Boston University School of Medicine, Boston, MA). The PU.1 cDNA is placed under the transcriptional control of the SV40 early promoter. The luciferase reporters under the control of the murine full length PU.1 promoter (mPU-334) and deletions (mPU-86, mPU-39 and mPU+34) as well as the Oct-2 and Oct-1 expression vectors (pCGOct-1 and pCGOct-2) were a kind gift from Dr. D. Tenen (Harvard University, Cambridge, MA) and are described in (48).

The LTA_4H promoter region was isolated from BJAB genomic DNA using the primers: 5'- CTT TCT CAA TGC TGC ATT CCT C – 3' (forward), and 5'- TAC CAG ACT CGT CGA TAG AG – 3' (reverse). The amplified 2.2 kb fragment was purified and subcloned by blunt-end ligation into the SmaI site of pGL3basic-modified (new NcoI site introduced upstream of MCS, missing original fragment spanning from Hind III to NcoI restriction sites) to create LTA4HPRO. The presence of the insert in the correct orientation was verified by analytical digestion with Hind III/XhoI, which excised a 1.3 kb fragment. Deletion fragments S1 (-1702 to +105), S2 (-1196 to +105) and S4 (-123 to +105) were amplified using the following forward primers: 5'- ATT CTG GTGTTC TCT CAG C – 3', 5'- CCT ACC TGG AAG CAT ACT GG- 3', 5'- TCA GCT CCA GGA GCA CGC TTG G - 3' respectively and blunt end cloned into SmaI site of pGL3basic to create S1/pGL3, S2/pGL3, and S4/pGL3. S6/pGL3 (-40 to +105) was constructed by digesting LTA4HPRO with NheI and NcoI followed by Kleenow treatment and blunt-end re- ligation of the plasmid.

3. Whole cell extract (WCE) preparation

Whole cell extracts (WCE) and immunoblot analysis were performed using standard protocols as described (254). Briefly, 10^7 cells were harvested and washed twice in PBS. The cells were lysed in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 30 mM β-glycerophosphate pH 7.45, 1% NP-40, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM Phenylmethyl sulfonyl fluoride (PMSF), 5 μg/mL aprotinine, 5 μg/ mL leupeptine, 5 μg/mL pepstatin A) for 45 min on ice. Cell debris was removed by centrifugation at 16 000 g for 15 min at 4°C. Protein content was quantified by Bradford assay (BioRad Laboratories Inc., Hercules, CA) as per manufacturer's instructions. WCE were stored at –80°C.

4. Immunoblot analysis

WCE (50-100 μg) prepared using Nonidet P-40 lysis buffer were subjected to electrophoresis on 7.5-12% SDS-PAGE gels, as appropriate for best resolution of protein of interest. Proteins transferred to Trans-Blot® nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA) were blocked in PBS containing 5% nonfat dry milk and 0.05% Tween 20, then incubated with 1 μg/mL primary antibody dilutions. After washes, the blots were incubated with a secondary antibody coupled to horseradish peroxidase (HRP). Blots were visualized using the NEN electrochemilumonescence (ECL) detection system (Perkin Elmer Life an Analytical Sciences Inc., Wellesley, MA). The following antibodies were used at 1 μg/mL for the study of B cell transcription factor ablation in PEL: Anti-IRF-4 goat polyclonal antibody, anti-ICSBP goat polyclonal antibody, anti-PU.1 rabbit polyclonal antibody, anti-Oct-2 rabbit polyclonal antibody,

anti-Bob-1 rabbit polyclonal antibody, anti-Spi-B goat polyclonal antibody, anti-Pax5 goat polyclonal antibody, all from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-α-actin mouse monoclonal antibody was purchased from Sigma-Aldrich Co. (St-Louis, MO) and used at a 1:10000 dilution.

The following primary antibodies were used in the study of upregulation of pro-inflammatory factors in PEL: anti- LTA_4H (rabbit polyclonal, a kind gift from Dr. Jilly Evans, Merck & Co., NJ, USA), was used at a 1:5000 dilution, anti-human Il-16 (goat polyclonal, R&D systems inc., cat # AF-316-PB) was used at 0.2 μg/mL, anti-thrombospondin-1 (mouse monoclonal, Medicorp Inc., Montreal, QC) was used at 1 μg/mL, anti-PSGL-1 (mouse monoclonal, Santa Cruz Biotechnology Inc.) was used at 1μg/mL. All antibody dilutions were prepared in blocking solution. Anti-cleaved caspase 3 antibody (rabbit polyclonal, Cell Signaling Inc., Danvers, MA) was diluted 1:2000 in 5% BSA/0.05% Tween/Tris buffered saline (TBS). Anti-β-actin mouse monoclonal antibody was purchased from Sigma-Aldrich Co. and used at a 1: 10 000 dilution.

5. Transient transfection and reporter assays

Electroporation or BJAB, BCBL-1 and L-428 cells was performed at 950 μF and 250 V using a Gene Pulser II™ apparatus (BioRad Inc.). Luciferase assays were performed following a transient transfection of 10 μg of the reporter gene (mPU-334, mPU-86, mPU-39 or mPU+34) and 1.0 μg of the *renilla* internal control (pRL-TK) into 10 X 10^6 cells. The dose response for Oct-2 was performed in a similar manner, but co-transfecting 0, 1, 2 or 4 μg of the pCGOct-2 expression vector (in which the octamer

factor cDNA is under the control of a CMV promoter) along with the reporter gene construct. The luciferase assay was performed 48 h after transfection using the Promega Dual Luciferase Reporter Assay System (Promega Corporation, Madison, WI) as per manufacturer's instructions. Transfection efficiency was normalized using the renilla activity counts.

Luciferase assays using full length LTA4HPRO and deletion constructs were performed following transient transfection of 10 μg of the reporter gene and 1.0 μg of the *renilla* internal control (pRL-null) into 10 X 10^6 cells. The luciferase assay was performed 48 h post-transfection using the Dual Luciferase Reporter Assay System as per manufacturer's instructions. Transfection efficiency was normalized using the *renilla* activity counts. Inhibition of NF-κB and AP-1 with the chemical inhibitors aspirin (acetylsalicylic acid, 10 mM) or ibuprofen (1 mM) were carried out as follows: 10 X 10^6 BCBL-1 cells were electroporated with 10 μg of LTA4HPRO and incubated for 42 h, then the appropriate inhibitor or control vehicle (100 mM Tris, pH 7.5) was added and incubated for an additional 6 h before harvesting samples and assaying for luciferase activity.

CAT assays were performed after transient transfection of 10 μg of the reporter constructs B4-TKCAT, TK-CAT, or pEGFP-C1 into 10 X 10^6 cells. PU.1 dose response was performed by co-transfecting 0, 10, 50, 150, or 300 ng of the PU.1/pECE expression vector. The CAT assay was performed 48 h after transfection as follows: cell pellet was resuspended in 0.25 M Tris pH 7.8 and lysed by three rounds of freeze-thaw. Equal volumes of protein extract (50 μL) were placed in a 7 mL glass scintillation vial and heated at 60°C for 10 min to deactivate deacetylases, then cooled on ice. To the extracts,

1 mM chloroamphenicol (Sigma-Aldrich Co., St-Louis, MO) and 0.4 nCi of ^{3}H-acetyl-coenzyme A (200.00 mCi/mmol, Perkin Elmer Life an Analytical Sciences Inc., Wellesley, MA) were added in a total volume of 250 µL buffered with 100 mM Tris pH 7.8. The mixture was overlayed with Beta Max water immiscible scintillation fluid (ICN Radiochemicals, Costa Mesa, CA) and incubated at 37°C for 6 h. The samples were then analyzed in a scintillation counter for ^{3}H measurement. Transfection efficiency was normalized by %GFP and microgram of protein.

6. Nuclear extract preparation

Nuclear extracts were prepared as described elsewhere (214). Briefly, 10^{7} cells were harvested and washed twice in PBS. The cell pellet was washed in 1 mL of buffer A (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF) then the cell membrane was lysed by incubating in 60 µL of buffer A/ 0.1% NP-40 for 10 min on ice. The intact nuclei were centrifuged down at 16 000 g for 10 min at 4°C and the cytoplasmic fraction was discarded. The isolated nuclei were lysed in 45 µL of cold buffer B (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 5 µg/mL Leupeptin, 5 µg/mL Pepstatin, 0.5 mM Spermidine, 0.15 mM Spermine, 5 µg/mL Aprotinin) for 15 min on ice. Nuclear membranes and debris were spun down for 10 min at 16 000 g at 4°C. Supernatants were collected and quantified for protein content by Bradford assay (BioRad Laboratories Inc.), aliquoted and quick frozen in liquid nitrogen. Nuclear extracts were stored at -80°C.

7. Electrophoretic Mobility Shift Assay (EMSA)

EMSA analysis using the octamer consensus sequence was performed as described in Chen *et al.* (48). Briefly, 2 X 10^5 cpm of the [γ-^{32}P] ATP labelled consensus Octamer probe (5'- TGT CGA **ATG CAA ATC** ACT AGA A – 3', Santa Cruz Biotechnology Inc.) were incubated with 5-10 μg of nuclear extracts in binding buffer (10 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1mM DTT, 0.1 mg/mL poly(dI/dC), 0.1 mg/mL BSA, and 5% glycerol) on ice for 30 min. For supershift experiments, 2 μg of anti-Oct-2 antibody (Santa Cruz Biotechnology) or anti-Oct-1 antibody (Santa Cruz Biotechnology Inc.) was added to the reaction. Competition with mutant probe was performed using 25-times molar excess of a mutated octamer probe (5' – TGT CGA **ATG CAA GCC** ACT AGA A – 3', Santa Cruz Biotechnology Inc.). Supershift with an unrelated antibody was performed by incubation with 1 μL of normal goat serum. Binding reactions were subjected to electrophoresis on a 5% polyacrylamide gel in 0.25 X TBE at 160 V for 3 hours.

Electromobility shift assays using the κB1 (5'-CTC TGC AAA GCG AAG TCC CCT TCG CAC-3'), λB (5'-AAA TAA AAG GAA GTG AAA CCA AG-3') and κE3' (5'-CCC TTT GAG GAA CTG AAA ACA GA-3') probes were performed as described above. For supershift experiments, 1-2 μg of antibodies directed against each NF-κB subunit or anti-IRF-4, anti-PU.1, anti-ICSBP, or anti-Spi-B (Santa Cruz Biotechnology Inc.) were added to the reaction.

EMSA analysis using probes derived from the *LTA4H* promoter was performed as follows. S4 and S6 EMSA probes were generated by restriction digestion of S4/pGL3 and S6/pGL3 plasmids with Kpn I and Bgl II. The remaining EMSA probes were

generated by annealing of complementary primers and had the following sequences: S8, 5'- ATC ACG CGT CGG CAC CAT GGA ACT TGT AGT TCC TTC ACC CAT CCC CCA ACG CTC GTC TGA AAG CTT ATC C -3', S9, 5'- ATC ACG CGT TCC CAG GTA GCC AAG CGC CCG CTT GCC GCG CGG CAC CAT GGA ACT TGT AGT TCC TTC ACC CAT CCC CCA ACG CTC GTC TGA AAG CTT ATC C -3', S91, 5'- TCC CAG GTA GCC AAG CGC CCG CTT GCC -3", S92, 5'- GCC GCG CGG CAC CAT GGA ACT TGT AGT -3', S93, 5' – CCA AGC GCC CGC TTG CCG CGC GGC ACC -3', S94, 5'- CAT GGA ACT TGT AGT TCC TTC -3'. EMSA analysis was performed as follows: 2 X 10^5 cpm of [γ-^{32}P]-ATP of labeled probe was incubated with 5 μg of nuclear extracts in binding buffer (10mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/mL poly (dI/dC), 0.1 mg/mL BSA, and 5% glycerol) at room temperature for 30 minutes. Competition with cold probe was performed using 25-50 fold molar excess unlabelled probe. Binding reactions were subjected to electrophoresis on a 5% polyacrylamide gel in 0.25X TBE at 160 V for 3 hours.

8. B cell purification

Fresh B lymphocytes were isolated from human tonsils discarded following surgery. The tonsils were thoroughly minced, resuspended in wash medium consisting of RPMI 1640 (Gibco by Invitrogen, Corp., Carlsbad, CA) supplemented with 2% FCS (Hyclone Laboratories Inc., Logan, UT), and 50 U/ml penicillin, 50 μg/ml streptomycin, and amphotericin B (1/500 w/v) from Life Technologies (Burlington, ON, Canada); and then layered onto a Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient. Tonsil

lymphocytes were separated by rosetting with neuraminidase-treated sheep red blood cells and Ficoll-Paque density centrifugation. Monocytes were removed from the E-rosette-negative fraction by adherence depletion; the remaining B cells were routinely demonstrated to be > 98% pure on flow cytometry by CD19 staining, with <1% CD14+ and <1% CD3+.

9. RNA isolation and microarray analysis

Total RNA was extracted from BCBL-1, L-428 and BJAB cells using the TRIZOL® method as per manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Concentrations were calculated using the OD_{260} for each sample and RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). RNA from BCBL-1 cells was compared against that of L-428 or BJAB cells. Ten micrograms of RNA from each cell line were reverse transcribed, labeled with the appropriate fluorochrome (Cy3 or Cy5, Perkin Elmer Co.) and hybridized to a human 1.7K chip (versions 4 and 8, University Health Network Microarray Center, Toronto, Canada), a double-spotted array containing 1,718 well-characterized human ESTs. Two independent experiments comprising three hybridization with corresponding reverse labeling were carried out. Data acquisition was performed using GenePix Pro (4000B, Axon Instruments, Inc.) at PTM between 600 and 700. Flagging parameters were set to reject spots with (F532 mean – B532 AND F635 – B635) intensity values lower than 200. Data analysis was performed using Iobion Gene Traffic software (V 3.0, Iobion Informatics LLC, La Joya, CA.), for a two-class experiment. Data was normalized with Lowess (sub-grid) method with background subtraction. Statistical Analysis for

Microarray (SAM) was applied on spot tables with a p-value cut-off = 0.05 and a differential expression cut-off of 1.5 fold and significant, differentially expressed genes in each class were selected.

10. Reverse transcription and Polymerase Chain Reaction (RT-PCR) analysis

Five micrograms of total RNA isolated using TRIZOL® method from the PEL cell lines BCBL-1, BC-3, BCP-1 and CRO-AP6, the cHD cell lines L-428 and KM-H2 as well as BJAB cells was subjected to reverse transcription using SuperScript II, RNAseH$^-$ reverse transcriptase (Invitrogen Corp., Carlsbad, CA) according to manufacturer's instructions. Five microliters of the obtained cDNA was then amplified by PCR using Taq polymerase (GE HealthCare Co., Fairfield, CT) as per manufacturer's instructions. The following primers were used for amplification: LTA4H (product size: 204 bp), fwd: 5'-CCC TAA AGA ACT GGT GGC ACT –3', rev: 5'- GAC TTT TCC ACC TGC TCT TTC –3', IL-16 (product size: 333 bp), fwd: 5'- AAG GGG CAT CTC CAA CAT CAT CAT –3', rev: 5'- CTC CTG CCA AGC TGA ACC CAA GAC –3', TSP-1 (product size: 493 bp), fwd: 5'- ACC GCA TTC CAG AGT CTG GC –3', rev: 5'- ATG GGG ACG TCC AAC TCA GC –3', BLT-1 (product size: 239 bp), fwd: 5'-CAC TGC TCC CTT TTT CCT TCA –3', rev: 5'- CCA GCA GAA AGG ACA ACA CC –3', BLT-2 (product size: 173 bp), fwd: 5'- ATC ACC CTG CCA GTC TTT TG –3', rev: 5'- TAA GGG CTT GGG TAC AGG TG –5', GAPDH (product size 376 bp), fwd: 5'- CCA TGG AGA AGG CTG GGG –3', rev: 5'- CAA AGT TGT CAT GGA TGA CC –3'. PCR conditions were as follows for all primers except TSP-1: 94°C for 2

min, cycles: 94°C for 45 sec, 56°C for 45 sec, 72°C for 45 sec, repeat 20-30 times depending on optimal product detection, 72°C for 10 min. For TSP-1 (110), conditions were: 94°C for 2 min, cycles: 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, 28 times, 72°C for 10 min. PCR samples were then resolved in a 2% agarose gel and visualized by Ethidium Bromide staining under UV light.

11. Neutrophil isolation

Primary human neutrophils were isolated from whole blood as follows: heparinized whole blood was fractionated using Ficoll™ (GE Healthcare Corp., Fairfield, CT) as per manufacturer's instructions and the red blood cell (RBC)/polymorphonuclear cell (PMN) fraction was collected. The pellet was resuspended in Hank's balanced salt solution (HBSS) to twice the original volume and 6% dextran 500 (GE Healthcare Corp.) in 0.9% (w/v) NaCl was added to each sample to a final concentration of 1% dextran. The RBCs were allowed to settle for 1-1.5 hours at room temperature. The PMN-rich supernatant was carefully collected and diluted 1:2 in HBSS and collected by centrifugation at 600 g for 10 minutes. Remaining RBCs were lysed in ammonium chloride lysis buffer (55 mmol/L ammonium chloride; 10 mmol/L sodium bicarbonate; 0.1 mmol/L EDTA). The pure PMN fraction was resuspended in PBS and analyzed by FACS for CD16, CD19, CD3 and CD14 expression, which showed that >98% of the isolated fraction was neutrophils ($CD16^+$) and that there was <1% contamination by B cells, T cells and monocytes.

12. Immunofluorescence staining and analysis

Primary cells were stained with phycoerytrin (PE)-labelled anti-CD16, anti-CD19, anti-CD3 or anti-CD14 antibodies (BD Pharmingen) for neutrophil characterization ($CD16^+$) or with anti-CD19, anti-CD14 and anti-CD3 (BD Pharmingen) for B cell characterization ($CD19^+$). After washing twice with PBS, 1 X 10^6 cells were labelled with the appropriate antibody for 30 min in PBS/1% FBS. After a final wash with ice-cold PBS, cells were resuspended in 400 µL FACS® buffer (PBS/CytoFix (BD Pharmingen). Flow cytometric analyses (1 X 10^4 cell/measurement) were performed using a FACScalibour™ flow cyometer with CELLQuest™ software (Becton Dickinson).

13. LTB_4 transcellular biosynthesis assay

Immediately after isolation, neutrophils were used for transcellular biosynthesis assay. A total of 0.2 X 10^6 neutrophils were seeded into 6-well plates in a volume of 1 mL of PBS. Subsequently, 10^7 B cells (BJAB, BCBL-1 or L-428) in PBS were added to the appropriate wells for a final volume of 2 mL. Leukotriene production was stimulated by addition of 2 mM $CaCl_2$ and 5 nM calcium ionophore A23187 (Sigma-Aldrich Co., St-Louis, MO). The reaction was allowed to proceed for 5 minutes at room temperature and then the supernatants were collected and diluted 1/100 to 1/200 in fresh PBS. A total of 10^7 neutrophils were assayed as a positive control as well as 10^7 B cells alone as a negative control. All samples were prepared in triplicate. Detection of LTB_4 was

performed by enzyme-linked immunoassay (Leukotriene B_4 EIA Kit, Cayman Chemical Co., Ann Arbor, MI) as per manufacturer's instructions.

13. Trans-well migration assay

Migration assay was performed using a 96-well disposable migration chamber (ChemoTx, Neuro Probe, Gaithersburg, MD) as described (84). Briefly, BCBL-1 or BJAB cells were labelled with the fluorescent dye Calcein AM (Invitrogen Corp., Carlsbad, CA) as per manufacturer's instructions and resuspended at a density of 3 X 10^6 cells/mL in RPMI 1640/10% FBS. The lower chamber of the migration chamber was filled with 29 µL of chemoattractant solution (either LTB_4, 10^{-10} M, or a 1/100 dilution of supernatant from transcellular biosynthesis assay) or vehicle alone (PBS/0.1% HSA). A total of 25 µL of the cell suspension (75 000 cells) was placed on top of a polycarbonate filter (PVP-free, 8 µm pore size, 3.2 mm diameter). The chamber was incubated for 1 hour at 37°C in a 5% CO_2 atmosphere. Non-migrated cells were removed with a cell scraper from the top of the filter. Migrated cells were counted on the bottom side of the membrane using a fluorescence microscope. The experiment was performed at least three times and every sample was prepared in triplicate.

CHAPTER III
Constitutive NF-κB activation in PEL cells

Constitutive activation of the NF-κB pathway by oncogenic viruses such as EBV, human Papilloma virus (HPV) and human T-cell Leukemia virus-1 (HTLV-1) is a common mechanism that promotes transformation (reviewed in (2)). Previous studies of the HHV-8 virus demonstrated that the lytic HHV-8 protein vGPCR could induce persistent NF-κB activation and promote tumor formation in transgenic mice by inducing VEGF expression, leading to disregulated endothelial cell proliferation (251, 304). These results clearly showed a role for NF-κB activity in Kaposi's sarcoma development but did not adress this question in PEL. Because constitutive activation of NF-κB has been associated with some mature B cell lymphomas, including cHD, we undertook an investigation of the status of NF-κB activation in PEL cells as well as the effect of NF-κB activity on HHV-8 lytic replication.

1. PEL cells have constitutive activation of p65/p50 NF-κB heterodimers

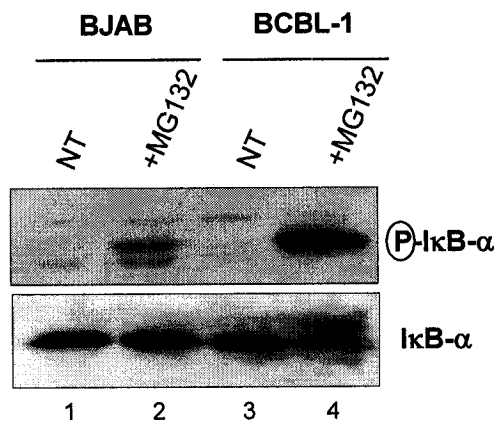
The NF-κB family of transcription factors regulates the expression of genes involved in such diverse cellular processes as proliferation, survival, inflammation and migration. These powerful transcription factors are often hijacked by viruses to drive viral gene expression and dampen the innate and adaptive immune responses. Constitutive NF-κB activation was detected in PEL cells as demonstrated by IκB-α phosphorylation and NF-κB binding and transactivation of target sequences.

Activation of NF-κB requires phosphorylation of IκB-α by the IKK complex, which triggers its proteasome-mediated degradation. Treatment of the PEL cell line BCBL-1 with the proteasome inhibitor MG132 for 6 hours lead to the accumulation of phosphorylated IκB-α, as detected by immunoblot using a phosphospecific antibody that recognizes IκB-α when phosphorylated at Ser 32 (**FIGURE 14A,** upper panel). Blotting for total IκB-α demonstrated the presence of a slower migrating form that corresponded to the phosphorylated protein (**FIGURE 14A**, lower panel). No such accumulation was seen in control BJAB cells treated with MG132. These results indicate that there is a constitutive activation of NF-κB in BCBL-1 cells but not in control BJAB cells.

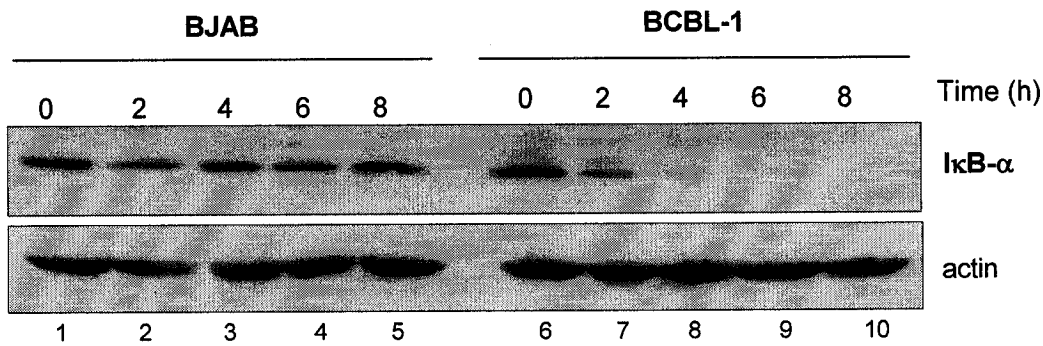
IκB-α levels are maintained through an auto-regulatory loop due to the presence of κB binding sites in its promoter region. Thus, activation of NF-κB is rapidly shut down by *de novo* synthesis of IκB-α. Inhibition of protein synthesis by chemical inhibitors such as cycloheximide allows the study of IκB-α protein degradation kinetics without the

FIGURE 14. Constitutive activation of NF-κB in PEL cells. A) MG-132 treatment to inhibit proteasome-mediated protein degradation leads to the accumulation of phospho-IκB-α in BCBL-1 but not in BJAB cells (top panel). The appearance of a slower migrating band can be seen when blotting for total IκB-α (lower panel). B) The kinetics of IκB-α degradation in BCBL-1 cells (lanes 6 to 10) compared to control BJAB cells (lanes 1 to 5) was analyzed using cycloheximide to inhibit *de novo* protein synthesis. After 6 hours of cycloheximide treatment, there is complete degradation of IκB-α in BCBL-1 cells but not in BJAB cells (compare lanes 4 and 9). Immunoblot for β-actin (lower panel) demonstrates equal loading.

A


BJAB
BCBL-1
NT
+MG132
NT
+MG132
P-IκB-α
IκB-α
1
2
3
4

B


BJAB
BCBL-1
0
2
4
6
8
0
2
4
6
8
Time (h)
IκB-α
actin
1
2
3
4
5
6
7
8
9
10

masking effects of *de novo* protein synthesis. Protein synthesis was inhibited in BCBL-1 and control BJAB cells using the chemical inhibitor cycloheximide and the disappearance of IκB-α with time was followed by immunoblot (**FIGURE 14B**). IκB-α levels remained constant in BJAB cells, whereas a marked decrease was seen in BCBL-1 cells after 30 minutes and it was barely detectable after 1 hour. The half-life of IκB-α in BCBL-1 cells was calculated to be about 12 minutes.

Binding to NF-κB dimers to target DNA sequences was analyzed by EMSA using the κB3 binding probe, corresponding to a functional κB site found in the IRF-4 promoter (5'-CTCTGCAAAGCGAAGTCCCCTTCGCAC-3'). Formation of two specific complexes could be seen in the PEL cell lines BCBL-1 and BC-3 but not in control BJAB cells (**FIGURE 15**). The upper complex could be supershifted by antibodies against p65 and p50 whereas the lower complex was supershifted by anti-p50 antibodies only. Thus, there is constitutive DNA binding of p65/p50 heterodimers and p50/p50 homodimers in PEL cells. There was a lower specific complex formed with BJAB nuclear extracts but not PEL cells extracts, however the identity of this complex was not determined. The ability of NF-κB to activate transcription of κB-containing promoters was demonstrated by reporter gene assay using a construct containing two copies of the κB site found in the RANTES promoter cloned in front of the CAT gene (p2(2)CAT). The activity of the reporter gene was 4.5 times higher in BCBL-1 cells than in control BJAB cells (**FIGURE 16**). Taken together, these results show that PEL cells exhibit constitutive activation of the NF-κB classical pathway. This was confirmed by studies published while this work was underway, which showed that the classical NF-κB pathway was activated by the latent HHV-8 protein vFLIP (8, 79, 103).

FIGURE 15. Constitutive NF-κB DNA binding in PEL cells. The activation of NF-κB in PEL cells was analyzed by EMSA using a probe corresponding to the κB1 site found in the IRF-4 promoter (5'-CTCTGCAAAGCGAAGTCCCCTTCGCAC-3'). In the PEL cell lines BCBL-1 and BC-3, constitutive binding to κB1 could be detected as two complexes (lanes 2 and 8). The upper complex could be supershifted with anti-p65 antibody (lanes 3 and 9) whereas both complexes were supershifted with anti-p50 antibody (lanes 4 and 10). Supershifted complexes are indicated by arrows. The activated NF-κB thus corresponds to p65/p50 heterodimers and p50/p50 homodimers. In control BJAB cells, the corresponding complexes could not be detected (lane 5). CTL indicates the positive control MT4 cells (lane 1). "Cold probe" corresponds to competition with 100-times excess of unlabelled probe (lane 11). Free probe is shown at the bottom.

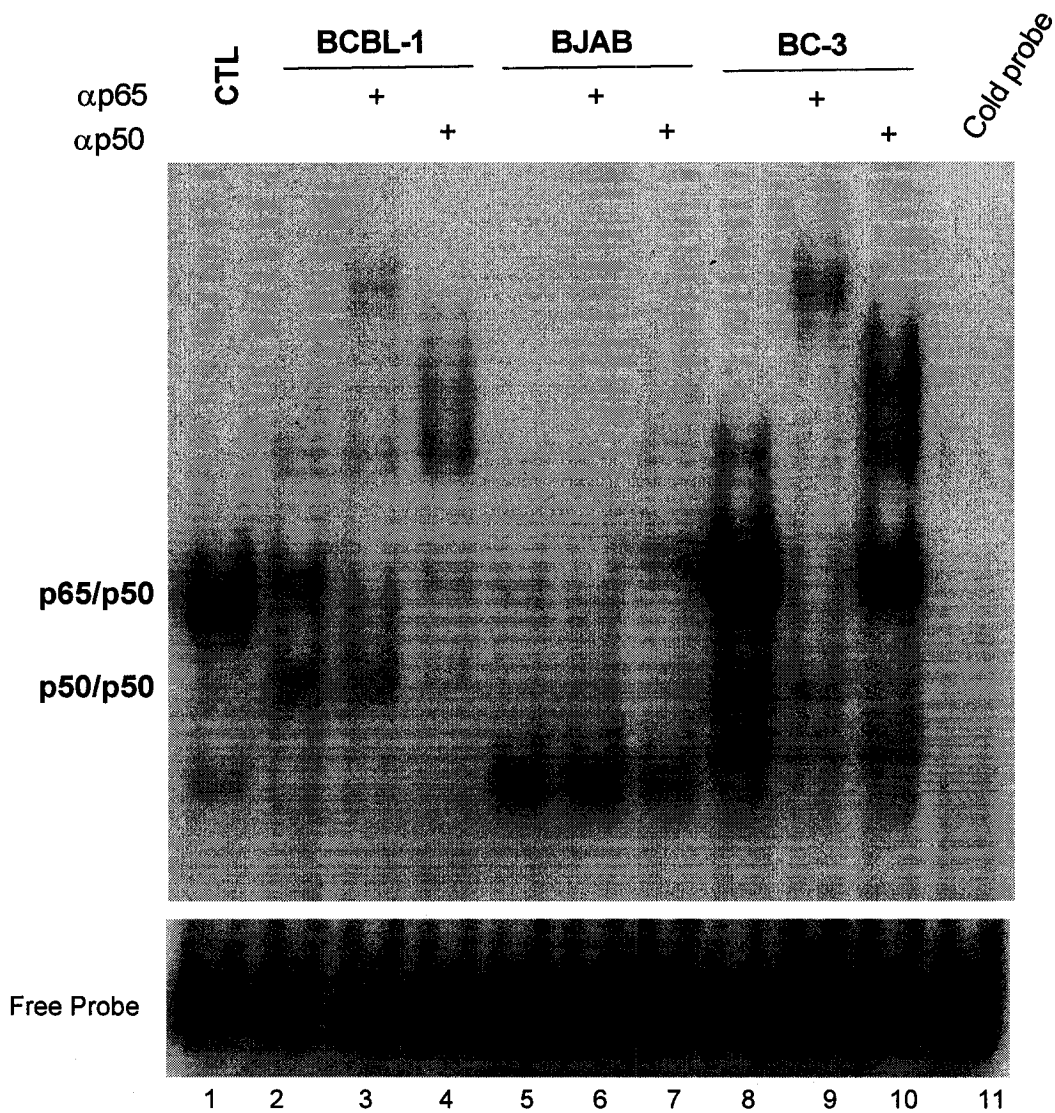
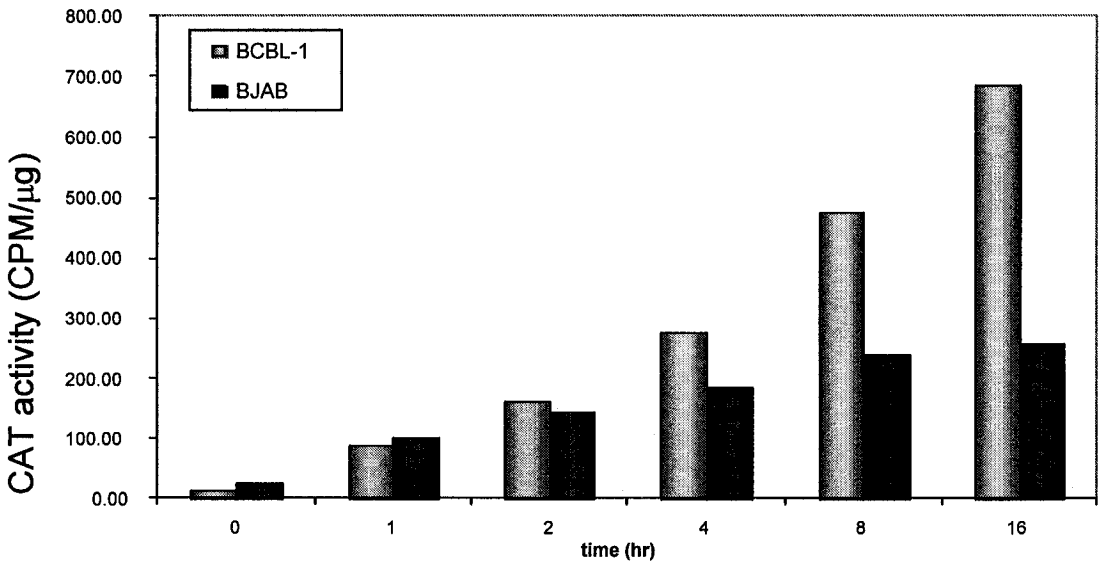

CTL
BCBL-1
BJAB
BC-3
Cold probe
αp65
αp50
+
+
+
+
+
+
p65/p50
p50/p50
Free Probe
1
2
3
4
5
6
7
8
9
10
11

FIGURE 16. The transcriptional activity of an NF-κB dependent construct is increased in BCBL-1 cells. A CAT reporter gene assay was performed in BCBL-1 (yellow bars) and BJAB (blue bars) cells using a plasmid encoding for the CAT gene under the control of two kB sites (P2(2)-CAT). CAT activity (CPM normalized to μg of protein) was followed over time (hrs). BCBL-1 cells demonstrated roughly twice as much CAT activity than control BJAB cells after 8-16 hours of measurement.

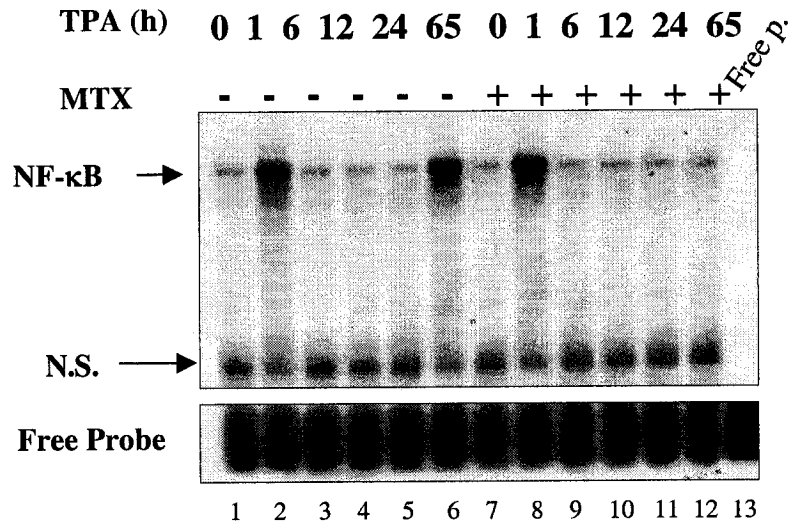

800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
CAT activity (CPM/µg)
BCBL-1
BJAB
0
1
2
4
8
16
time (hr)

2. NF-κB activation during the HHV-8 lytic cycle is required for *de novo* infection

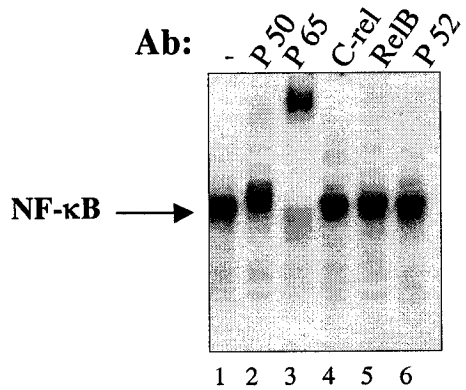
NF-κB is also activated during the HHV-8 lytic cycle, as demonstrated by electromobility shift assay of whole cell extracts from BCBL-1 cells induced with TPA to stimulate HHV-8 lytic replication (**FIGURE 17A,** lane 6). In extracts from TPA-induced cells, there is a late activation of NF-κB 65 hours post-stimulation, corresponding to p65/p50 heterodimers (**FIGURE 17B**). Activation of NF-κB coincides with late stages of viral replication, as demonstrated by the presence of the lytic viral protein encoded by ORF26 (**FIGURE 17C**). This activation can be abrogated by using the inhibitor of lytic gene transcription methotrexate (MTX) (**FIGURE 17A,** lane 12), demonstrating the need for active HHV-8 replication. In contrast, the early NF-κB activity induced by TPA treatment after one hour cannot be inhibited by MTX and it is therefore independent of HHV-8 replication (**FIGURE 17A**, lanes 2 and 8). The activation of NF-κB during the HHV-8 replicative cycle in BCBL-1 cells was essential to produce virions capable of infecting endothelial cells *de novo*. Virus produced by TPA-treated BCBL-1 cells was capable of infecting the endothelial cell line EA.hy926, establishing a lytic infection with spontaneous production of the lytic genes ORF26 and ORF K8.1 (**FIGURE 18**). However, virus produced in BCBL-1 cells encoding for the NF-κB super-repressor IκB-α 2NΔ4 were incapable of productive *de novo* infection, as demonstrated by the lack of ORF26 and ORF K8.1 expression.

FIGURE 17. Late NF-κB activation in BCBL-1 cells correlates with HHV-8 lytic replication. A) The kinetics of NF-κB activation in BCBL-1 cells following TPA treatment were analyzed by electromobility shift assay (EMSA). Nuclear extracts from BCBL-1 cells treated with TPA or with a combination of TPA and Methotrexate (MTX, an inhibitor of lytic protein production) for various time points were analyzed for NF-κB binding to the PRDII probe. The positions of the NF-κB complex as well as a non-specific band are indicated. Free probe is shown at the bottom. NF-κB binding was detected after 65 hours of TPA treatment alone (lane 6) but not in the presence of TPA plus methotrexate (lane 12). B) Nuclear extract from BCBL-1 cells after 65 h of TPA stimulation was further analyzed by EMSA supershift with specific antibodies against the NF-κB subunits p50, p65, c-Rel, RelB and p52, as indicated. C) Nuclear extracts from (A) were analyzed for lytic protein production by immunoblotting with an antibody against the product of ORF-26 (minor capsid protein). Immunoblotting with an antibody against TFIID was used as a control for equal protein loading.

A


TPA (h) 0 1 6 12 24 65 0 1 6 12 24 65
MTX - - - - - - + + + + + +
Free p.
NF-κB
N.S.
Free Probe
1 2 3 4 5 6 7 8 9 10 11 12 13

B


Ab: - P 50 P 65 C-rel RelB P 52
NF-κB
1 2 3 4 5 6

C

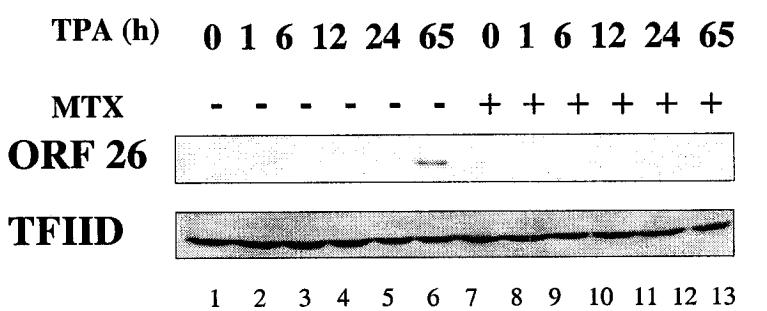
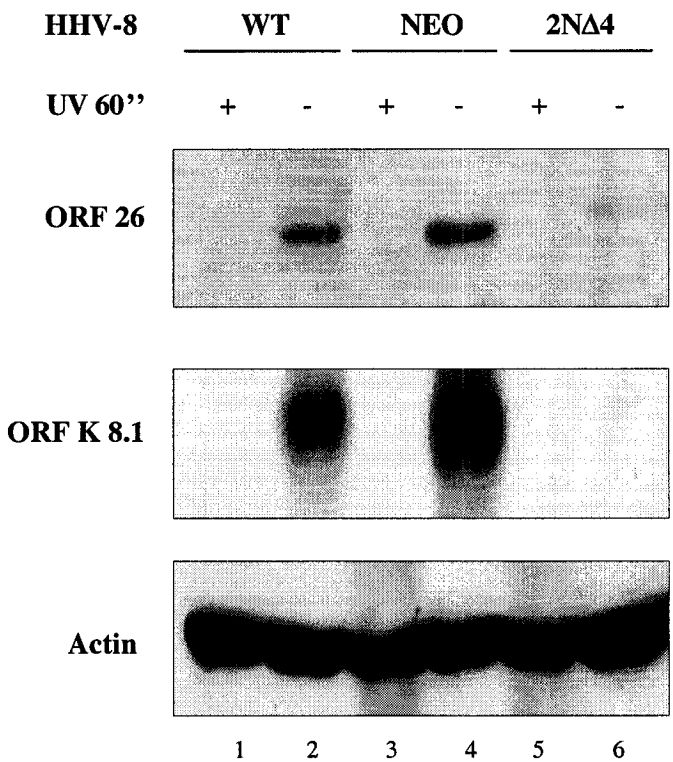

TPA (h) 0 1 6 12 24 65 0 1 6 12 24 65
MTX - - - - - - + + + + + +
ORF 26
TFIID
1 2 3 4 5 6 7 8 9 10 11 12 13

FIGURE 18. De-novo HHV-8 infection of endothelial Ea.hy926 cells. Wild-type (WT), Neo and 2NΔ4 BCBL-1 cells were stimulated with TPA and Ionomycin for 24 h to induce the HHV-8 lytic cycle and supernatants were collected 65 h post-induction. After ultracentrifugation, the virus was used to *de-novo* infect Ea.hy926 cells with a calculated MOI of 5 400. Whole cell extracts from Ea.hy926 cells at 72 h post-infection with HHV-8 derived from WT (lanes 1 and 2), Neo (lanes 3 and 4) and 2NΔ4 (lanes 5 and 6) BCBL-1 cells were analyzed by immunoblot using antibodies against ORF 26 (top panel) and ORF K8.1 (middle panel) viral products. Lanes 2, 4 and 6 show viral protein levels after *de-novo* infection. Lanes 1, 3 and 5 show viral protein levels when the viral preparations are treated by 60 sec of UV crosslinking. A blot against β-actin (bottom panel) was performed to ensure equal loading.


HHV-8
WT
NEO
2NΔ4
UV 60''
+
-
+
-
+
-
ORF 26
ORF K 8.1
Actin
1
2
3
4
5
6

Based on this and other studies, NF-κB activity seems to be required both during latent and lytic HHV-8 infection. In PEL cells, constitutive activation of p65/p50 NF-κB dimers is important for IL-6 production and survival of the tumor cells (8, 83), whereas further activation during HHV-8 lytic replication allows the production of viral progeny capable of spreading infection to neighboring cells.

CHAPTER IV

Analysis of B cell-specific transcription factor expression and activity in PEL cells

Primary Effusion Lymphoma is characterized by a lack of B cell-specific surface marker expression. In particular, PEL cells do not express the B cell receptor (BCR) in spite of productive immunoglobulin gene rearrangement in most cases. In order to elucidate if transcriptional defects are at heart of this lack of BCR expression, we undertook an investigation of the expression and activity of key transcription factors known to govern BCR expression and the B cell program.

1. Constitutive expression of IRF-4 in PEL is concomitant with a downregulation of PU.1.

The transcription factor IRF-4 requires physical association with PU.1 in order to efficiently bind and transactivate target Ets-IRF sequences found in the light chain gene enhancers (67). Analysis of IRF-4 protein levels by immunoblot demonstrated constitutive IRF-4 levels in all PEL cell lines examined (BCBL-1, BC-3, and BCP-1), compared to the control Burkitt's lymphoma BJAB cells which showed no IRF-4 expression (**FIGURE 19A**). In contrast, PU.1 protein could not be detected in any of the PEL cell lines (**FIGURE 19B**). Surprisingly, the expression pattern of IRF-8, the IRF family member most closely related to IRF-4, was inversely related to IRF-4; i.e. IRF-8 was expressed in BJAB but not in PEL cells (**FIGURE 19C**). The levels of Spi-B, another Ets family member that binds similar DNA elements and activates many of the same target genes as PU.1, remained constant in two of three PEL cell lines compared to BJAB (**FIGURE 19D**).

To determine if the abrogation of PU.1 expression in PEL cells disrupted the regulation of B cell specific genes, EMSA analysis was performed using the composite Ets-IRF elements found in the λB and κE3' light chain enhancers (**FIGURE 20**). In control BJAB cells, a protein-DNA complex was easily observed using the λB or the κE3' probe (FIGURE 20, lane 1) and the protein-DNA complex was supershifted with anti-PU.1 antisera (FIGURE 20, lane 2) but not with anti-SpiB antisera (FIGURE 20, lane 5). Anti-IRF-8/ICSBP antibody supershifted both the lower PU.1-containing

FIGURE 19. Expression of IRF-4, PU.1, IRF-8 and Spi-B in PEL cells. Immunoblot analysis was performed on whole cell extracts (WCE) from PEL cell lines BCBL-1, BC-3 and BCP-1 as well as control Cos7 and BJAB cell lines. Immunoblots were probed with anti-IRF-4 (A), anti-ICSBP/IRF-8 (B), anti-PU.1 (C), and anti-Spi-B (D) antisera. In A, C, and D the membranes were re-blotted with anti-β-actin antibody to verify equal protein loading. These immunoblots are representative of at least 3 independent experiments.

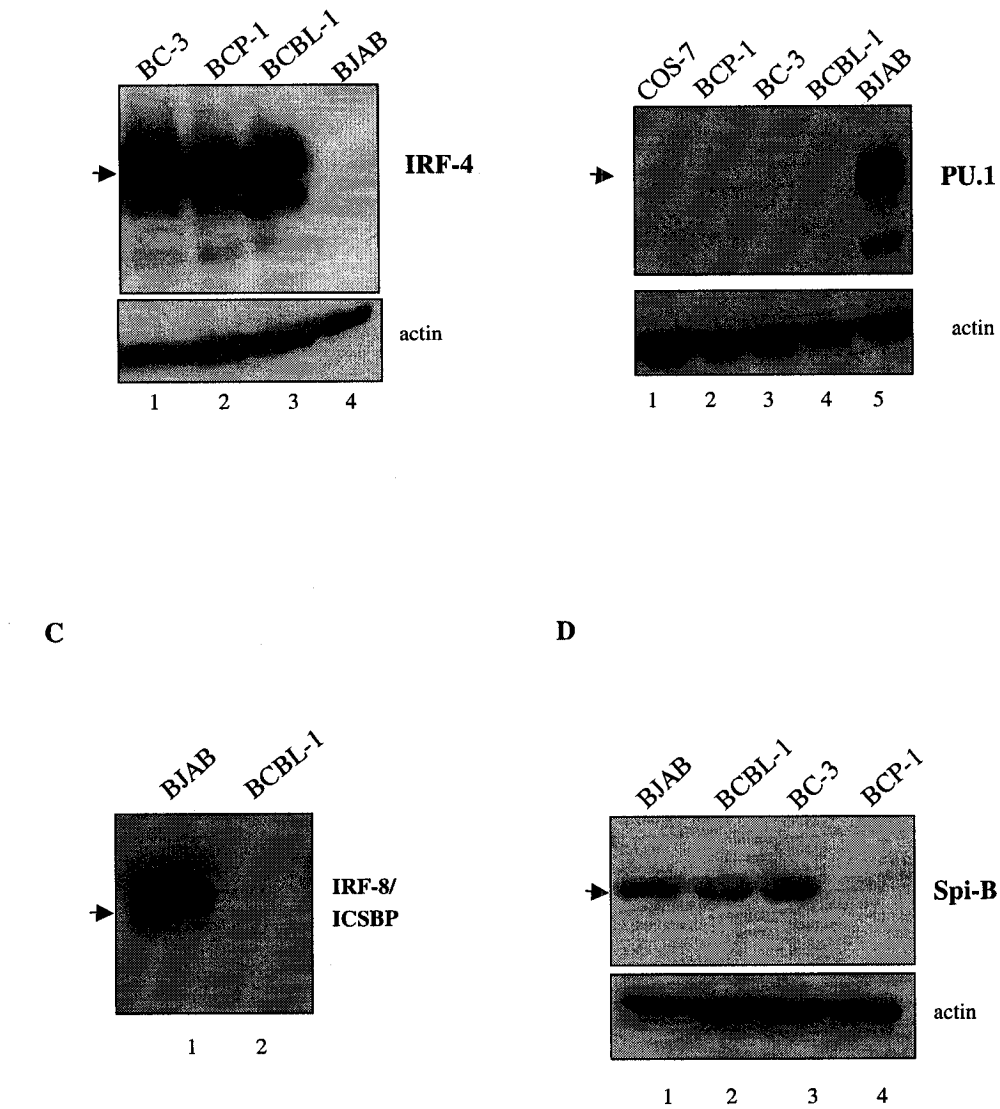
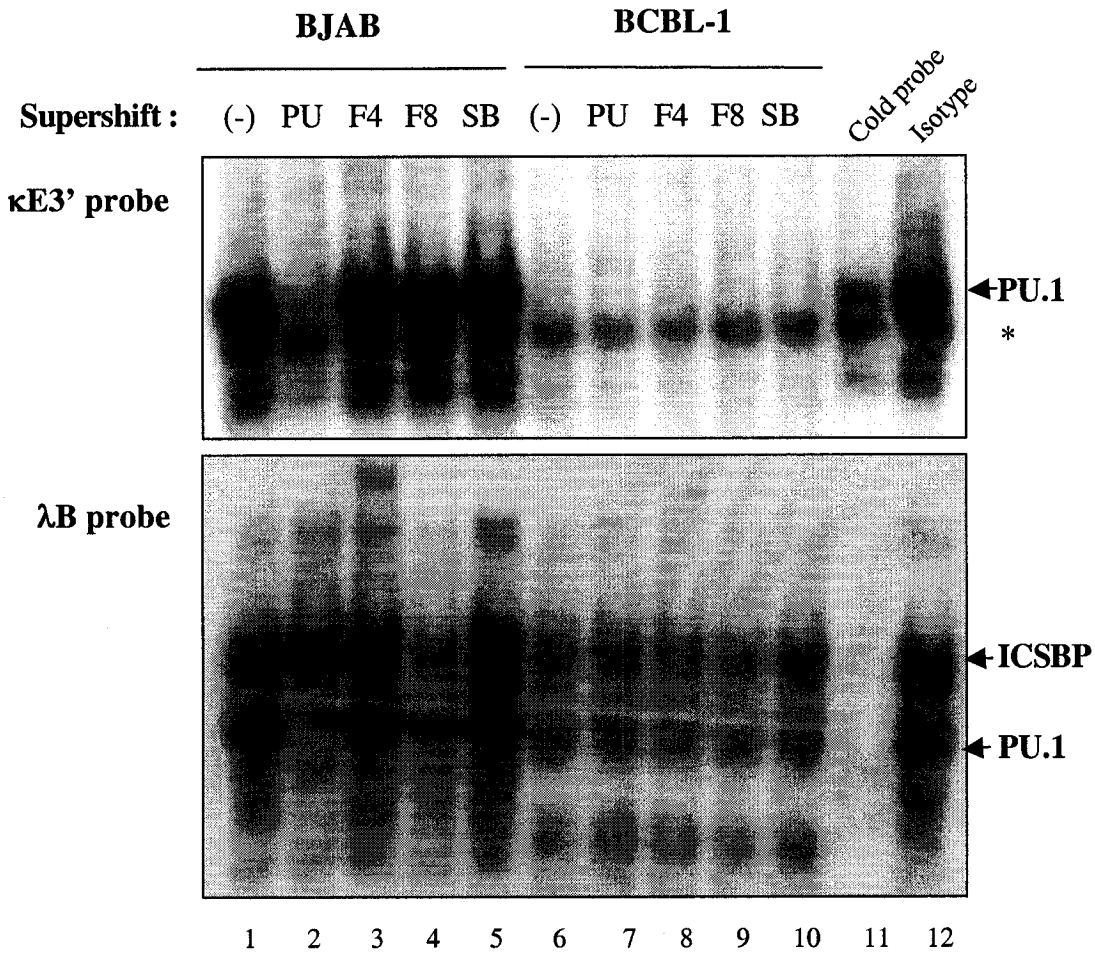

A
BC-3
BCP-1
BCBL-1
BJAB
IRF-4
actin
1 2 3 4
B
COS-7
BCP-1
BC-3
BCBL-1
BJAB
PU.1
actin
1 2 3 4 5
C
BJAB
BCBL-1
IRF-8/
ICSBP
1 2
D
BJAB
BCBL-1
BC-3
BCP-1
Spi-B
actin
1 2 3 4

FIGURE 20. PU.1 does not bind to the Ets-IRF composite element in the λ and κ light chain enhancers in PEL cells. Nuclear extracts from the PEL cell line BCBL-1 were analyzed by EMSA for binding of PU.1, IRF-4, ICSBP/IRF-8, and Spi-B to the Ets-IRF composite element found in the λ (5'-AAA TAA AAG GAA GTG AAA CCA AG-3') and κ (5'-CCC TTT GAG GAA CTG AAA ACA GA-3') immunoglobulin light chain enhancers. Nuclear extracts from BJAB cells were used as a positive control. Lanes 1-5 and 6-10 are BJAB and BCBL-1 nuclear extracts, respectively. Lanes 1 and 6 represent binding of BJAB and BCBL-1 nuclear extracts to the γ-32P-ATP-labeled EMSA probes. Supershift reactions were performed by adding the following antibodies: lanes 2 and 7, supershift with anti-PU.1 antibody (PU); lanes 3 and 8, supershift with anti-IRF-4 antibody (F4); lanes 4 and 9, supershift with anti-IRF-8/ICSBP antibody (F8), lanes 5 and 10, supershift with anti-Spi-B antibody (SB). Lane 11 represents competition with 25-fold excess cold probe. Lane 12 represents supershift with normal goat serum (isotype). Asterix indicates a non-specific complex.


BJAB
BCBL-1
Supershift :
(-) PU F4 F8 SB (-) PU F4 F8 SB
Cold probe
Isotype
κE3' probe
PU.1
*
λB probe
ICSBP
PU.1
1 2 3 4 5 6 7 8 9 10 11 12

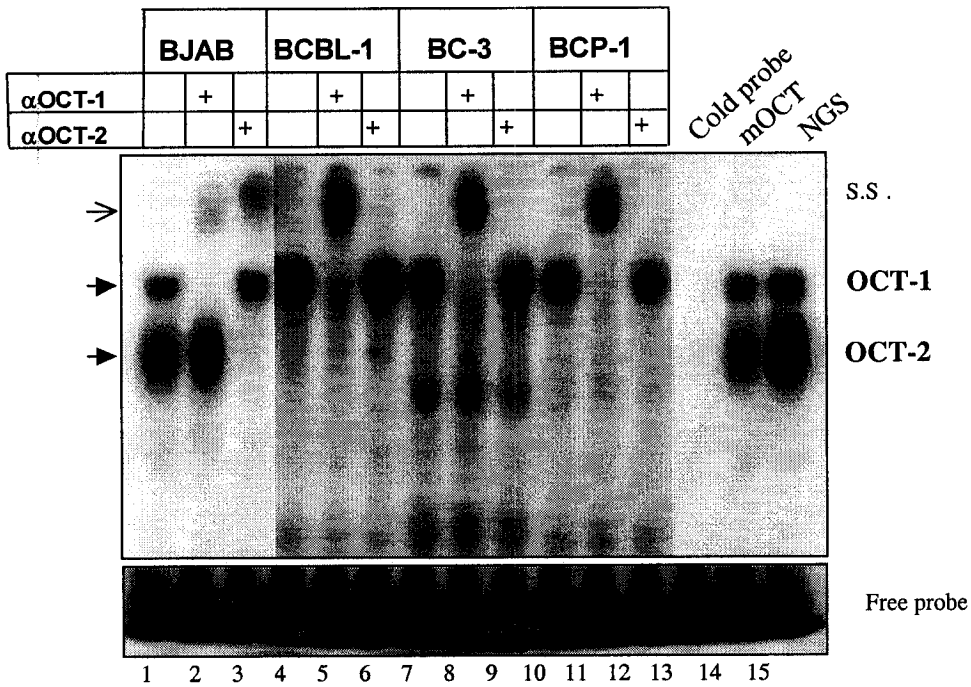
complex as well as the upper complex formed with the κE3' probe (FIGURE 20 lane 4, lower panel), thus demonstrating that in BJAB cells, PU.1 can bind alone or in partnership with IRF-8 to the Ets-IRF elements present in both light chain enhancers. As expected, anti-IRF-4 antibody did not supershift any of the complexes, consistent with the lack of IRF-4 expression in BJAB cells (FIGURE 20, lane 3). In contrast, nuclear extracts of PEL cells failed to form complexes containing either PU.1 or IRF-4 (FIGURE 20, lane 6), thus indicating that the heterodimer composed by PU.1 and IRF-4 –which is required for the normal function of composite Ets-IRF elements found in IgL chain enhancers (24, 25) – does not form in PEL cells.

2. The B cell-specific transcription factor Oct-2 and the co-activator Bob-1/OCA-B are not expressed in PEL cells

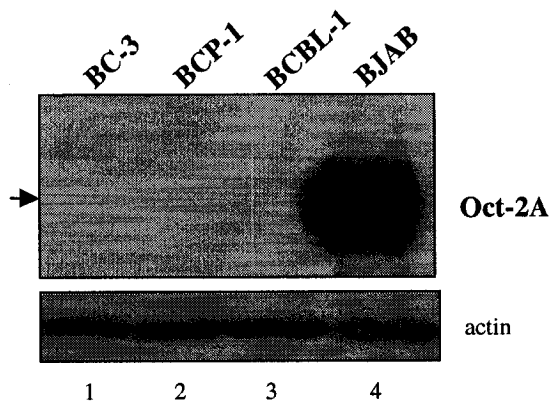
In B cells, the PU.1 promoter is regulated mainly through an octamer motif located at position -57 to -51 upstream of the PU.1 mRNA start site (48). The octamer site can be occupied by either member of the Octamer Binding Factor (Oct) family: the ubiquitous Oct-1 or the B cell-specific factor Oct-2, with the latter displaying the highest transactivation capacity in B cells. To examine the status of Oct-1 and Oct-2 binding and its role in transcriptional activation, EMSA analysis was performed with a consensus octamer sequence (**FIGURE 21 A**). A dramatic reduction in Oct-2 binding was detected specifically in PEL cells. In control BJAB cells, two complexes were observed (FIGURE 21A, lane 1), the upper complex containing Oct-1 (FIGURE 21A, lane 2) and the lower complex containing Oct-2 (FIGURE 21A, lane 3), as detected by supershift

FIGURE 21. The B cell-specific factor Oct-2 is not expressed in PEL cells. A) Nuclear extracts from PEL cell lines BCBL-1 (lanes 4-6), BC-3 (lanes 7-9) and BCP-1 (lanes 10-12) were analyzed by EMSA for binding of octamer factors to a consensus octamer binding site (5'- TGT CGA **ATG CAA ATC** ACT AGA A – 3'). Nuclear extracts from BJAB cell line were used as a positive control (lanes 1-3). The antibodies used for supershift (either anti-Oct-1 or anti-Oct-2 polyclonal antibodies) are indicated above the lanes. In lanes 13-15, *mOct* indicates competition with a mutated octamer unlabeled probe (5'- TGT CGA **ATG CAA GCC** ACT AGA A – 3'**),** *NGS* indicates supershift with an unrelated antiserum (goat serum), and *Cold Probe* indicates competition with 25-fold excess of unlabeled octamer probe. Oct-1 and Oct-2 DNA-protein complexes as well as supershift complexes (SS) are indicated by arrows. B) Whole cell extracts (100 ug) from BCBL-1, BC-3 and BCP-1 were analyzed by immunoblot. BJAB whole cell extract was used as a control. Blot was probed with anti-Oct-2 polyclonal antibody, and re-blotted with anti-β-actin antibody to demonstrate equal sample loading. C) Immunoblot analysis of whole cell extracts from BCBL-1, BC-3 and BPC-1. BJAB whole cell extract was used as a control. Blot was probed with anti-Bob-1/OCA-B antibody, and re-blotted with anti-β-actin antibody to demonstrate equal sample loading. Blots B and C are representative of at least 3 independent experiments.

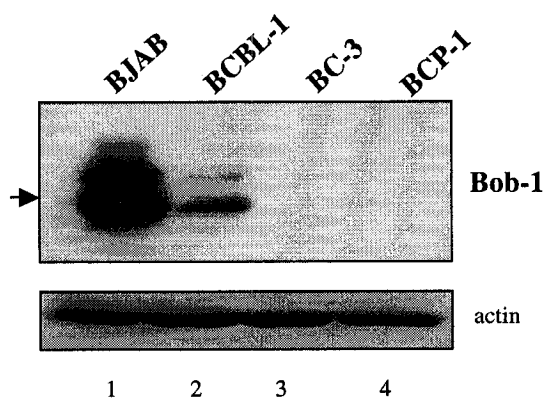
A


BJAB
BCBL-1
BC-3
BCP-1
αOCT-1
αOCT-2
Cold probe
mOCT
NGS
S.S .
OCT-1
OCT-2
Free probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B


BC-3
BCP-1
BCBL-1
BJAB
Oct-2A
actin
1 2 3 4

C


BJAB
BCBL-1
BC-3
BCP-1
Bob-1
actin
1 2 3 4

with Oct-1 and Oct-2 specific antibodies, whereas in the PEL cells only the upper, Oct-1-containing complex was observed (FIGURE 21 A, lanes 4-12). Immunoblot analysis revealed that Oct-2 protein expression was completely absent in all three PEL cell lines (**FIGURE 21 B**).

Since the transactivation potential of the Oct factors is enhanced by binding of the co-activator Bob-1 (48), the expression of the cofactor was also examined. Protein expression of the co-activator Bob-1/Oca-B was downregulated in PEL cells to different extents depending on the cell line (**FIGURE 21 C**); Bob-1 was highly expressed in BJAB (FIGURE 21 C, lane 1), whereas Bob-1 levels were about 75% lower in BCBL-1 cells compared to control BJAB (FIGURE 21 C, lane 2); in BC-3 and BCP-1 expression of Bob-1 was below detection limits (FIGURE 21 C, lanes 3 and 4).

3. PU.1, OCT-2, Bob-1/OCA-B and BSAP/Pax5 expression in other mature-B cell lymphomas

Recent studies have indicated that disruption of B cell specific transcription factor activity may also occur in other forms of B cell lymphoma, particularly in classical Hodgkin's disease (reviewed in (41, 60)). To directly compare transcription factor levels in PEL with other mature B cell-derived lymphomas, protein levels were assessed in a panel of cell lines derived from Burkitt's Lymphoma, a disease originating from pre-germinal center B cells and characterized by *c-myc* gene rearrangement (reviewed in (113)), cHD, a clonal disease of B cell origin that arises from late germinal center B lymphocytes (reviewed in (41)), and PEL, also of late/post GC origin (32, 36) (**FIGURE**

22). Immunoblot analysis demonstrated that PU.1 and Oct-2 expression was consistently found in Burkitt's lymphoma derived cell lines (BJAB, Namalwa, BL-2, BL-30, and BL-41; Fig. 6, lanes 1, 8, 9, 10 and 11). As previously reported (233, 288), PU.1 and Oct-2 expression was strongly downregulated in the cHD cell lines L-428 (FIGURE 22, lane 6) and KM-H2 (FIGURE 22, lane 7), mirroring the results obtained in PEL cell lines (FIGURE 22, lanes 2 to 5). Additionally, expression of BSAP/Pax-5 and IRF-8/ICSBP was assessed in both PEL and cHD cell lines, since it has been reported that BSAP/Pax-5 expression is downregulated in cHD cells (114) and we have found a downregulation of IRF-8 levels in PEL cells. The levels of both BSAP/Pax5 and IRF-8 transcription factors were dramatically decreased and, furthermore, the expression patterns were once again comparable between cHD and PEL. However the decrease in protein expression was more profound in PEL cell lines. These results demonstrate that transcription factor silencing is not exclusive of PEL cells but rather shared among certain types of mature B cell lymphomas.

4. Ectopic expression of Oct-2 restores the activity of the PU.1 promoter in PEL cells

To examine the functional role of Oct-2 expression on the PU.1 promoter, a luciferase assay was performed with the full-length murine PU.1 promoter as well as truncated promoter constructs lacking the Oct and Ets sites (**FIGURE 23**). In control BJAB cells, full-length PU.1 promoter activity was approximately 200-fold higher than the activity of the truncated PU.1 +34 promoter (FIGURE 23, compare lanes 1 and 4). The

FIGURE 22. Expression of B lymphocyte-specific transcription factors in mature B cell lymphoma cell lines. Immunoblot analysis were performed on WCE from the Burkitt's lymphoma cell lines BJAB (lane 1), Namalwa, BL-2, BL-30, and BL-41 (lanes 8-11), Hodgkin's lymphoma cell lines L-428 and KM-H2 (lanes 6 and 7), and the PEL cell lines BCBL-1, BC-3, BCP-1 and CRO-AP6 (lanes 2-5). Immunoblots were probed with anti-sera raised against IRF-4, IRF-8, Oct-2, Pax5, PU.1 and Spi-B, as indicated. Immunoblot with an anti-actin antibody showed equal loading of the samples. The immunoblots shown are representative of at least three independent experiments.

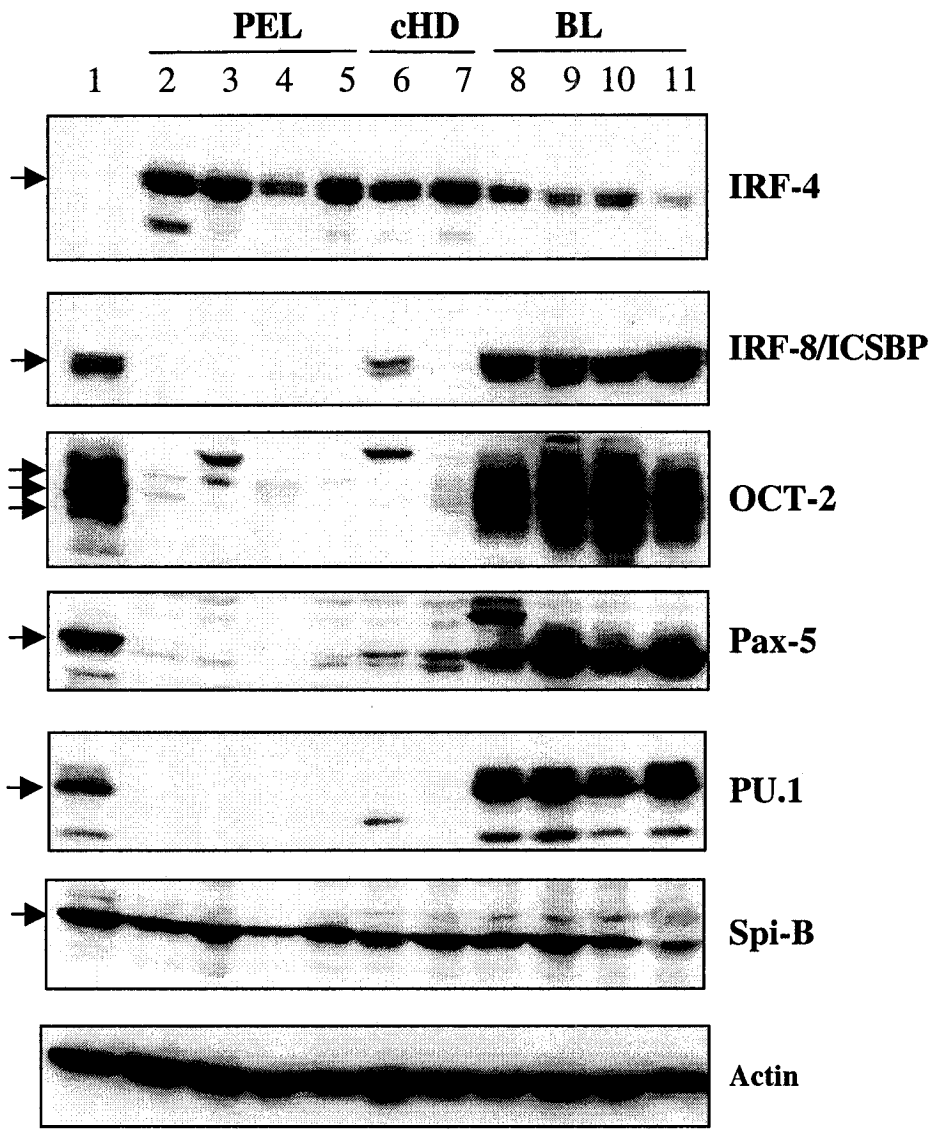
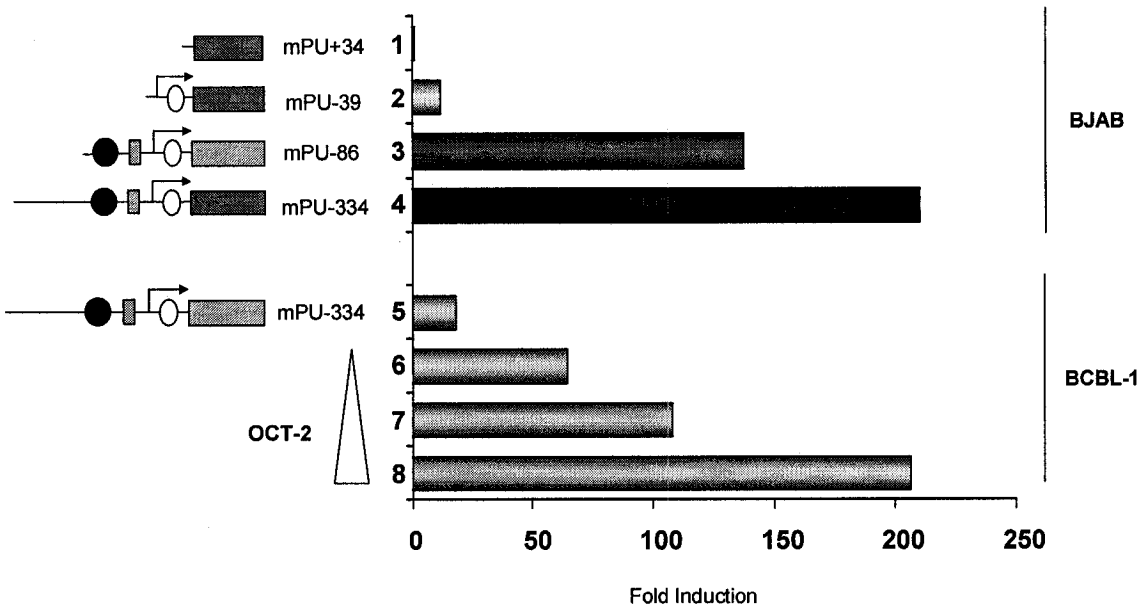

PEL
cHD
BL
1 2 3 4 5 6 7 8 9 10 11
IRF-4
IRF-8/ICSBP
OCT-2
Pax-5
PU.1
Spi-B
Actin

FIGURE 23. The activity of the PU.1 promoter is abrogated in PEL cells but can be recovered by ectopic expression of Oct-2. The luciferase reporter gene was placed under the control of the full-length murine PU.1 promoter (mPU-334, yellow bar) or truncated promoter constructs lacking the distal 5' region (mPU-86, violet bar), the Oct and Sp1 sites (mPU-39, light blue bar), or the Oct, Sp1 and PU.1 sites (mPU+34, white bar). The schematic representation of the mPU-Luc plasmids includes the location of the Oct site (●), the Sp1 site (▩) and the PU.1 site (○). For the transactivation assay, each construct was electroporated into BCBL-1 (lanes 5 to 9) or BJAB (lanes 1 to 4) cells. An Oct-2 dose response was performed in BCBL-1 cells by co-transfecting increasing amounts of pCGOct-2 expression vector along with the mPU-334 reporter construct (lanes 6 to 9). Transfection efficiency was determined by co-transfection of pRL-TK *renilla* reporter gene. Fold induction was calculated by comparing the activity of the full-length promoter (mPU-334) to that of the truncated promoter (mPU+34). Data represents a typical experiment performed in duplicate.


mPU+34
mPU-39
mPU-86
mPU-334
mPU-334
OCT-2
1
2
3
4
5
6
7
8
BJAB
BCBL-1
0
50
100
150
200
250
Fold Induction

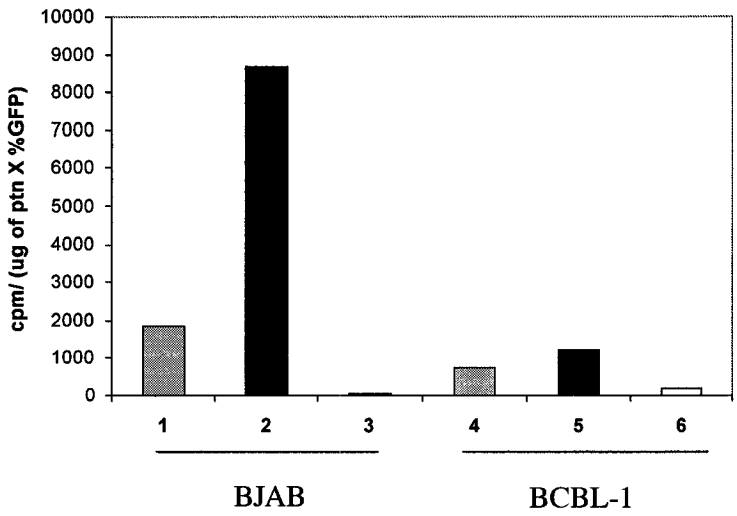
importance of the octamer site in regulating PU.1 expression in B cells was substantiated by the low activity of the mPU-39 construct, which contains the promoter proximal PU.1 binding site but lacks the Oct motif (FIGURE 23, lane 2). The 5' distal region (from –334 to –86), does not contain important regulatory sites for promoter activity in B cells, since the mPU-86 construct has equivalent transactivation potential as the full-length promoter in BJAB cells (FIGURE 23, compare lanes 3 and 4). In contrast, in BCBL-1 cells, only a basal level of PU.1 promoter activity was observed with the full-length promoter (FIGURE 23, compare lanes 1 and 5). Significantly, co-expression of increasing amounts of an Oct-2 expression plasmid in PEL cells resulted in a dose-dependent increase in promoter activity, comparable to that observed in BJAB cells (FIGURE 23, lanes 6 to 8). Thus, the absence of PU.1 protein in PEL cells appears to be directly related to the transcription block imposed by the complete lack of Oct-2 expression in PEL cells.

5. The activity of a composite Ets-IRF site is restored by ectopic PU.1 expression

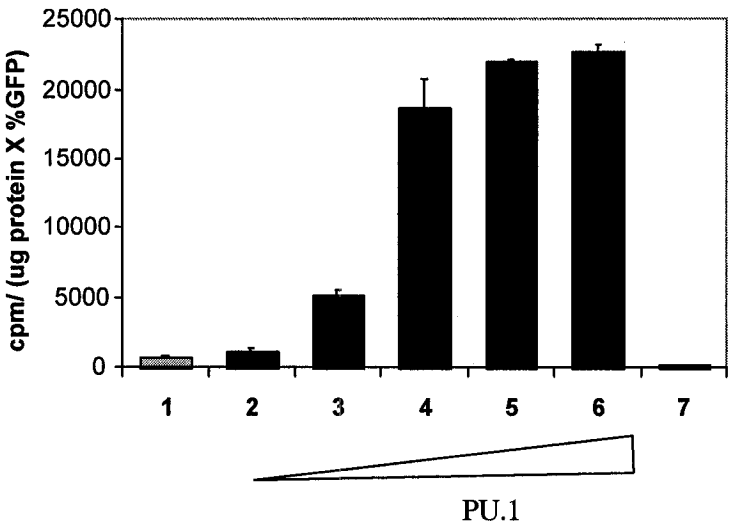
Activity of the λB and κE3'enhancer elements requires the synergistic activity of PU.1 and IRF-4 on the composite Ets-IRF site found in both enhancers. To test the activity of the λB site in PEL cells, the construct B4-TKCAT, which contains four copies of the Ets-IRF site found in the λB enhancer element was transfected into control BJAB or PEL BCBL-1 cells. In control BJAB cells, B4-TKCAT displayed a 6-fold increase in promoter activity compared to the empty vector TK-CAT (**FIGURE 24 A**,

FIGURE 24. A composite Ets/IRF site is inactive in PEL cells but its activity can be recovered by ectopic expression of PU.1. A) The activity of the Ets/IRF site was assessed in BCBL-1 cells (lanes 1-3) and BJAB cells (lanes 4-6) using a CAT reporter gene assay. The reporter gene B4-TKCAT (green bars) or the empty vector TK-CAT (yellow bars) were electroporated into each cell line and the CAT activity was measured at 48h post-transfection. A GFP expression vector was used as a negative control (white bars). B) A PU.1 dose response curve was performed by co-transfection of increasing amounts of PU.1 expression vector along with the B4-TKCAT construct in BCBL-1 cells. Lane 1, TK-CAT; lane 2-6, B4-TKCAT; lanes 3 to 6, increasing amounts of pECE-PU.1 expression plasmid; lane 7, GFP negative control. Normalization of CPM values was performed based on percent GFP positive cells and micrograms of protein. Data represents the mean and the standard error of the mean of four independent experiments.

A


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
cpm/ (ug of ptn X %GFP)
1
2
3
4
5
6
BJAB
BCBL-1

B


25000
20000
15000
10000
5000
0
cpm/ (ug protein X %GFP)
1
2
3
4
5
6
7
PU.1

columns 1 and 2). However, in BCBL-1 cells, the activity of the composite Ets-IRF construct was approximately equivalent to that of the empty vector (FIGURE 24 A, columns 4 and 5). Increasing amounts of PU.1 transfected together with B4-TKCAT restored the activity of the λB-derived construct to levels comparable to those observed in BJAB cells (**FIGURE 24 B**, columns 2-6), thus indicating that functional activity of the Ets-IRF sites in PEL cells could be restored by PU.1 expression.

CHAPTER V

Differential gene expression analysis of the PEL cell line BCBL-1 *versus* the cHD cell line L-428

Primary Effusion Lymphoma cells and the Reed-Sternberg cells of classical Hodgkin's disease originate from mature B lymphocytes at a similar stage of development: post-GC with partial plasma cell differentiation. PEL and cHD cells share numerous characteristics at the molecular level, including lack of expression of the transcription factors Oct-2, Bob-1/OCA-B, PU.1, IRF-8 and Pax-5 leading to a "null" phenotype as well as constitutive activation of NF-κB and AP-1 transcription factors. However, these two lymphomas differ widely in their clinical presentation and response to chemotherapy. In order to identify genes whose expression can differentiate PEL from cHD and provide a PEL "transcriptional signature", we undertook a microarray analysis of the PEL cell line BCBL-1 compared to the cHD cell line L-428 and the Burkitt's lymphoma cell line BJAB.

1. Microarray analysis of the PEL cell line BCBL-1 compared to the cHD cell line L-428.

In order to elucidate the signature genes that distinguish PEL from cHD at the molecular level, microarray analysis was performed using total RNA isolated from the PEL cell line BCBL-1 and compared it to the cHD cell line L-428 as well as the Burkitt's lymphoma (BL) cell line BJAB (**FIGURE 25**). A subset of about 40 genes out of 1700 was differentially regulated exclusively in BCBL-1 cells (**TABLE I**); from this list, four genes involved in inflammation at the level of chemotaxis and/or cell motility were significantly upregulated in BCBL-1. Given that the localization of PEL to serous body cavities involves migration of the tumor B cells from the lymph nodes to the peritoneal, pleural or pericardial space, it was therefore of interest to further characterize the expression of leukotriene A_4 hydrolase (LTA_4H, >5 fold induction), thrombospondin-1 (TSP-1, >6 fold induction), interleukin-16 (IL-16, >4 fold induction), and P-selectin glycoprotein ligand (PSGL-1, 4-5 fold induction).

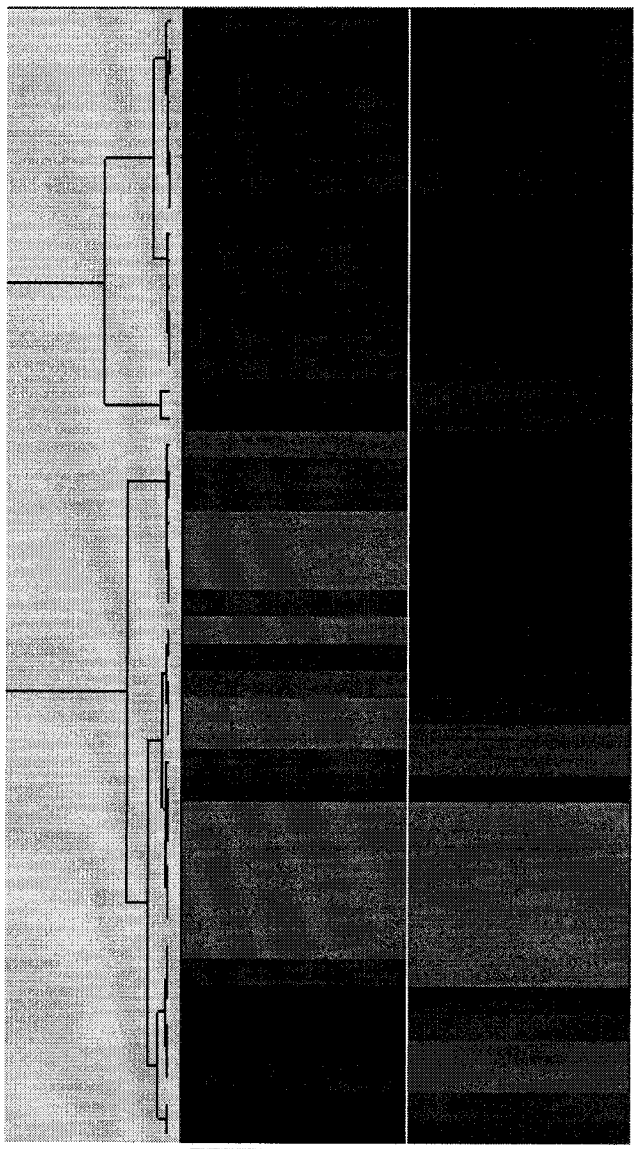
2. Leukotriene A_4 hydrolase, thrombospondin-1, interleukin-16 and P-selectin glycoprotein ligand-1 are overexpressed in PEL cells.

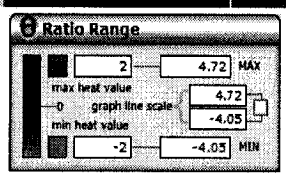
To confirm the results obtained by microarray, the expression of LTA_4H, IL-16, PSGL-1 and TSP-1 was examined at the mRNA (**FIGURE 26 A**, left panel) and/or protein (FIGURE 26 A, right panel) level by using three additional PEL cell lines (BC-3,

FIGURE 25. Gene expression profile of BCBL-1 cells compared to BJAB and L-428 cells. Gene expression is shown as a color representation of ratio values, with red being above and green being below the row/column median level of expression as shown by the scale. Each column represents the average for BCBL-1 cells compared to either BJAB or L-428 cells and each row represents one gene. The dendogram comprises a partial gene list after SAM statistical analysis with a p-value cut-off = 0.05 and a differential expression cut-off of 1.5 fold. Genes in bold were confirmed by RT-PCR or immunoblot.

BCBL-1 *vs.*

BJAB **L-428**





thrombospondin 1
endothelin receptor type B
CD48 antigen (B-cell membrane protein)
2'-5'-oligoadenylate synthetase 2, 69/71kDa
leukotriene A4 hydrolase
interleukin 16 (lymphocyte chemoattractant factor)
selectin P ligand
B-cell receptor-associated protein 31
proteoglycan 1, secretory granule
S100 calcium binding protein A6 (calcyclin)
ornithine decarboxylase antizyme inhibitor
X-box binding protein 1
lectin, galactoside-binding, soluble, 1 (galectin 1)
interferon regulatory factor 4
Rho GDP dissociation inhibitor (GDI) beta
beta-2-microglobulin
bleomycin hydrolase
phosphorylase kinase, alpha 2 (liver)
chaperonin containing TCP1, subunit 5 (epsilon)
CDW52 antigen (CAMPATH-1 antigen)
CD79B antigen (immunoglobulin-associated beta)
CD79A antigen (immunoglobulin-associated alpha)
heat shock 70kDa protein 9B (mortalin-2)
tumor necrosis factor receptor superfamily, member 5
BCL2-like 1
E74-like factor 1 (ets domain transcription factor)
autocrine motility factor receptor
immunoglobulin J polypeptide
major histocompatibility complex, class II, DQ beta 1
major histocompatibility complex, class II, DP alpha 1
glutathione S-transferase pi
CD74 antigen
major histocompatibility complex, class II, DQ alpha 1
major histocompatibility complex, class II, DR beta 3
transcription factor 4
dual specificity phosphatase 6
lipase A, lysosomal acid, cholesterol esterase
ARP1 actin-related protein 1 homolog A
dihydrolipoamide dehydrogenase
cyclin G1
thymosin, beta 4, X-linked
baculoviral IAP repeat-containing 3
chromosome 14 open reading frame 147

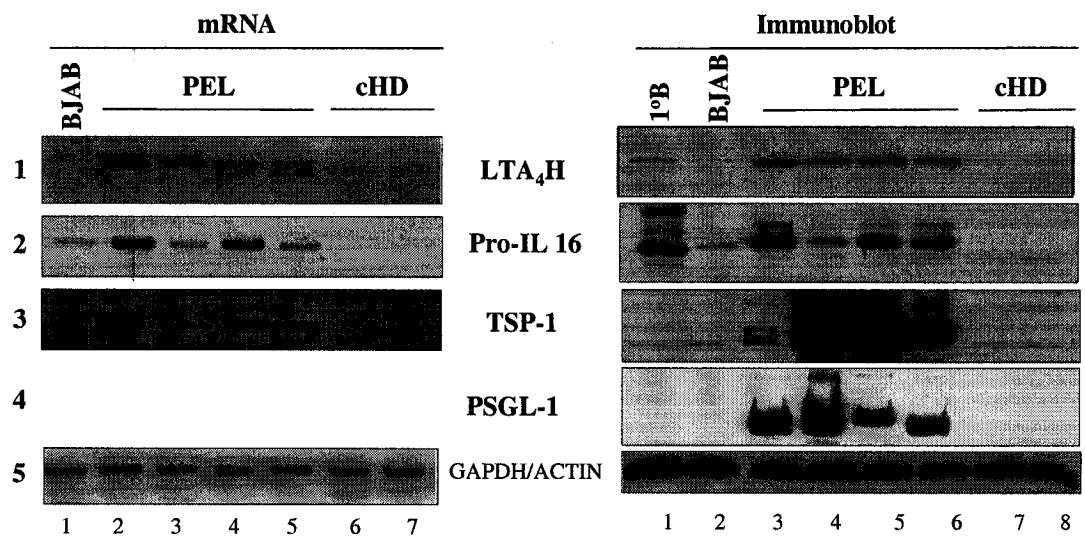
Table I. List of genes specifically expressed in BCBL-1 cells compared with L-428 and BJAB cells. The list was generated based on significant genes identified by microarray analysis. Gene names, fold change and function are indicated. Fold change value represents the average of two independent experiments carried out in triplicate. Functions are based on GO annotations and/or published data.

GENE NAME	FUNCTION	BJAB FOLD CHANGE	L-428 FOLD CHANGE	
leukotriene A4 hydrolase	INFLAMMATION, CHEMOTAXIS	-18.96	-18.22	
selectin P ligand	CELL ADHESION, MIGRATION	-4.38	-5.37	
thrombospondin 1	CELL ADHESION, CHEMOTAXIS	-6.35	-4.12	
interleukin 16	CHEMOTAXIS	-5.18	-6.05	GENES
lectin, galactoside-binding, soluble, 1	CELL ADHESION	-4.99	-1.72	UPREGULATED
CD48 antigen	B-lymphocyte activation marker	-3.06	-5.51	in BCBL-1
Selectin L	CELL ADHESION	-3.61	-2.93	
S100 calcium binding protein A6	Calcium binding, cell cycle progression	-5.49	-2.31	
endothelin receptor type B	G-protein coupled receptor	-2.89	-6.25	
2'-5'-oligoadenylate synthetase 2, 69/71kDa	Interferon response gene	-16.79	-11.53	
interferon regulatory factor 4	transcription factor	-27.74	-2.79	
B-cell receptor-associated protein 31	Transport	-1.90	-2.12	
MHC complex, class II, DP alpha 1	antigen presentation	2.21	1.87	
thymosin, beta 4, X-linked	cytoskeleton organization	1.91	8.27	
MHC complex, class II, DQ beta 1	antigen presentation	2.67	3.43	GENES
transcription factor 4	transcription factor	6.34	6.12	DOWNREGULATED
MHC complex, class II, DR beta 3	antigen presentation	5.54	5.48	IN BCBL-1
MHC complex, class II, DQ alpha 1	antigen presentation	7.79	7.18	
glutathione S-transferase pi	xenobiotic metabolism	34.42	20.21	
CD74 antigen	antigen presentation	15.54	6.74	
X-box binding protein 1	transcription factor	-3.60	-1.53	GENES
ornithine decarboxylase antizyme inhibitor	polyamine biosynthesis	-3.57	-1.65	UPREGULATED
proteoglycan 1, secretory granule	granule component	-3.18	-1.15	IN BCBL-1 AND L-428

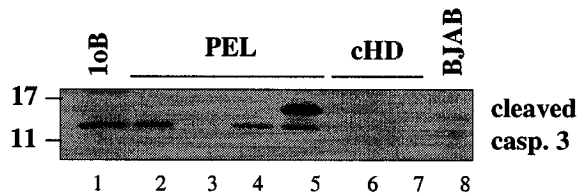
GENE NAME	FUNCTION	BJAB FOLD CHANGE	L-428 FOLD CHANGE	
E74-like factor 1	transcription factor	3.51		
BCL2-like 1	apoptosis	2.16	1.36	
phosphorylase kinase, alpha 2 (liver)	glycogen metabolism	2.48	1.11	
immunoglobulin J polypeptide	immune response, Ig production	11.63		
autocrine motility factor receptor	tumor motility	4.69		GENES
CD79B antigen	B-cell receptor	5.71	-1.03	DOWNREGULATED
CD79A antigen	B-cell receptor	18.32	-1.12	IN BCBL-1 AND
CD40 antigen	B-cell activation	8.14	1.84	L-428
bleomycin hydrolase	unknown. Inactivation of bleomycin	3.65	0.35	
heat shock 70kDa protein 9B (mortalin-2)	cell proliferation, chaperone	2.62	-0.03	
chaperonin containing TCP1, subunit 5	chaperone	2.50	1.05	
CDW52 antigen (CAMPATH-1 antigen)	unknown	17.08	-1.13	
baculoviral IAP repeat-containing 3	apoptosis	-1.06	2.56	
dihydrolipoamide dehydrogenase	mitochondria	1.32	2.78	GENES
ARP1 actin-related protein 1 homolog A	cytoskeleton organization	1.51	2.26	UPREGULATED
lipase A, lysosomal acid, cholesterol esterase	lipid metabolism		5.69	IN L-428
dual specificity phosphatase 6	MAP kinase pathway		38.80	
cyclin G1	cell cycle	1.49	3.81	
Rho GDP dissociation inhibitor (GDI) beta	Cell adhesion. Cytoskeleton organization	1.76	-14.71	DOWNREGULATED
beta-2-microglobulin	antigen presentation	-1.22	-3.37	IN L-428

FIGURE 26. Four cellular factors involved in chemotaxis and cell migration are upregulated in PEL cells. A) LTA_4H, IL-16, TSP-1 and PSGL-1 mRNA and/or protein levels in PEL cells vs. cHD cells. Left panel: Total RNA isolated from 4 PEL cell lines and 2 cHD cell lines as well as BJAB cells was analyzed for LTA_4H, IL-16 and TSP-1 mRNA levels by RT-PCR. Lane 1, BJAB, Lane 2, BCBL-1, lane 3, BC-3, lane 4, BCP-1, lane 5, CRO-AP6, lane 6, L-428, lane 7, KM-H2. GAPDH amplification was used to ensure equal RNA levels. Right panel: Protein expression of LTA_4H, pro-IL16, TSP-1 and PSGL-1 in PEL cells compared to primary tonsilar B cells and cHD cells was assessed by immunoblot with specific antibodies as indicated. Lane 1, primary tonsilar B cells, lane 2, control BJAB cells, lanes 3-6, PEL cell lines (BCBL-1, BC-3, BCP-1, CRO-AP6), lanes 7-8, cHD cell lines (L-428, KM-H2). An antibody against β-actin was used to ensure equal loading. B) Immunoblot for cleaved caspase 3 allowed the detection of the active 17 kDa form when blotted with an antibody against active caspase 3. Lane 1, primary tonsilar B cells, lanes 2-5, PEL cell lines (BCBL-1, BC-3, BCP-1, CRO-AP6), lanes 6-7, cHD cell lines (L-428, KM-H2), lane 8, control BJAB cells. C) Immunoblot against secreted TSP-1 present in growth media after 24h incubation. Cells were seeded into 6-well plates and left for 24 h at 37°C, with 5% CO_2. After the incubation period, the supernatants were harvested and blotted with a specific antibody against TSP-1. Lanes 1-3, BJAB cells (negative control), lanes 4-6, BCBL-1 cells, lane 7, media alone. Experiment was performed in triplicates.

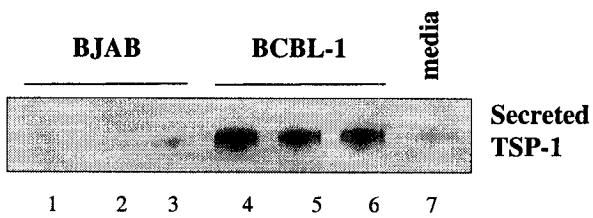
A


mRNA
Immunoblot
BJAB
PEL
cHD
1°B
BJAB
PEL
cHD
1
2
3
4
5
LTA4H
Pro-IL 16
TSP-1
PSGL-1
GAPDH/ACTIN
1 2 3 4 5 6 7
1 2 3 4 5 6 7 8

B


1oB
PEL
cHD
BJAB
17
11
cleaved
casp. 3
1 2 3 4 5 6 7 8

C


BJAB
BCBL-1
media
Secreted
TSP-1
1 2 3 4 5 6 7

BCP-1, CRO-AP6), and comparing these cells to the cHD cell lines L-428 and KM-H2 as well as the BL cell line BJAB and primary tonsilar B cells. Analysis of the expression of LTA_4H mRNA by RT-PCR identified a single 204 bp amplification product, while immunoblot analysis revealed the presence of a 69 kDa protein corresponding to LTA_4H (Figure 26 A, top row). The mRNA induction level in PEL cells as measured by RT-PCR was greater than 10-fold whereas the protein level averaged a 5-fold increase compared to cHD and BJAB cells.

RT-PCR amplification of IL-16 mRNA detected the expected 333 bp product in all PEL and BJAB cells but not in cHD cells (Figure 26 A, second row). Induction levels in PEL cells ranged from 2.7- to 21-fold increase compared to the EBV negative BJAB cells. Other EBV+ BL cell lines analyzed by RT-PCR also exhibited high IL-16 levels (data not shown), indicating that both EBV and HHV-8 can induce expression of this chemokine. IL-16 is synthesized as a large 80 kDa precursor molecule - Pro-IL16 - that is cleaved to the bioactive 20 kDa IL-16 molecule by Caspase 3 (13). Immunoblot analysis of intracellular precursor protein levels closely mirrored the results obtained at the mRNA level, with high yet variable protein levels in PEL cell lines (Figure 2A, second row). No band was detected at 20 kDa, as expected for the secreted active IL-16. Immunoblot for activated Caspase 3 detected low levels of active, 17 kDa caspase 3 protein in all PEL cell lines but not in cHD and BJAB cells (**FIGURE 26 B**), thus suggesting that bioactive IL-16 is produced and secreted by PEL cells.

TSP-1 levels were also increased in PEL cells compared to cHD and BJAB cells, both at the mRNA and protein levels (FIGURE 26 A, third row). RT-PCR analysis of TSP-1 mRNA levels identified a single 493 bp product in PEL cells; this transcript was

undetectable in BJAB and cHD cells. Immunoblot analysis under reducing conditions revealed the intracellular presence of the 145-185 kDa protein in PEL cells only; furthermore, TSP-1 protein was detected in supernatants from BCBL-1 cells, demonstrating that this extracellular matrix protein is produced and secreted by PEL cells (**FIGURE 26 C**). Similarly, intracellular PSGL-1 protein level was elevated in PEL cells but not detectable in cHD or BJAB cells (FIGURE 26 A, fourth row).

Protein expression of LTA_4H, TSP-1, PSGL-1 and IL-16 in PEL cells was also compared to expression in primary tonsilar B cells (Figure 26 A right panel, compare lane 1 to PEL samples). LTA_4H, TSP-1 and PSGL-1 were 5- to 10-fold higher in PEL compared to primary B cells; however IL-16 levels as well as caspase 3 activation were comparable in PEL and primary tonsilar B lymphocytes, suggesting that during PEL development, malignant B cells have acquired expression of LTA_4H, TSP-1 and PSGL-1, while retaining the capacity to produce IL-16.

CHAPTER VI

Analysis of the transcriptional control of the Leukotriene A_4 Hydrolase Promoter

The results from our microarray analysis demonstrated a strong upregulation of the enzyme Leukotriene A_4 hydrolase (LTA_4H) in PEL cells compared to cHD and BL cells. To date, no reports exist on the regulation of LTA_4H at the transcriptional level. In order to elucidate if regulatory elements in the promoter of the *LTA4H* gene were at the origin of the overexpression of this enzyme in PEL, we isolated the putative promoter region and assayed its activity in BCBL-1, L-428 and BJAB cells using a reporter gene assay.

1. Isolation of the *LTA4H* gene promoter region

Altered transcriptional regulation has been shown to play an important role in aberrant gene expression in PEL, both in the case of expressed (IL-6, IL-8) and silenced (BCR) genes (8, 11, 274). For this reason, we sought to investigate if transcriptional regulatory elements were involved in LTA_4H overexpression in PEL. The *LTA4H* promoter was initially described by Mancini and Evans in 1995 (172) but to date has not been functionally characterized. The promoter region of the *LTA4H* gene was located using NCBI software tools (National Center for Biotechnology Information, Bethesda, MA). A 2.1 kb fragment upstream of the transcriptional start site, as well as 105 bp of the 5' UTR was amplified from genomic DNA isolated from BJAB cells. After PCR amplification, the fragment was cloned upstream of the luciferase gene by blunt-end ligation into the pGL3basic/modified plasmid (LTA4HPRO, see schematic in **FIGURE 29 A**). The presence of the insert in the correct orientation was confirmed by restriction digest. Deletion constructs were created from LTA4HPRO by amplifying the desired fragment by PCR and blunt-end cloning into pGL3basic.

2. *LTA4H* promoter activity in PEL cells.

The activity of the *LTA4H* promoter was assessed in the PEL cell line BCBL-1 and compared to promoter activity in the Burkitt's lymphoma cell line BJAB and the cHD cell line L-428. A 130-fold induction in reporter gene activity was observed with the

LTA4H promoter relative to the empty vector (pGL3basic) in BCBL-1 cells, compared to 40- and 60-fold in BJAB and L-428 cells, respectively (**FIGURE 27**).

Expression of the HHV-8 latent gene vFLIP in PEL cells has been shown to modulate the activity of important transcription factors such as NF-κB and AP-1, which are constitutively active in PEL cells (present work, (7, 30, 144, 178). In order to assess if NF-κB or AP-1 transcription factors mediated the observed upregulation of LTA_4H in PEL cells, we transfected a vFLIP expression plasmid or a p65 expression plasmid along with the LTA4HPRO construct into BJAB cells. Activity of the *LTA4H* promoter was not further stimulated in BJAB cells by overexpression of vFLIP or p65 (**FIGURE 28 A**). To confirm that transcriptional modulation by NF-κB or AP-1 is not involved in LTA_4H overexpression in PEL cells, we used pharmacological inhibitors of these transcription factors in BCBL-1 cells and observed their effects on *LTA4H* promoter activity. Neither ibuprofen, acetylsalicylic acid nor the specific AP-1 inhibitor SP600125 had a significant influence in promoter activity in BCBL-1 (**FIGURE 28 B**). Therefore, neither NF-kB nor AP-1 appears to be involved in LTA_4H expression in PEL.

3. Identification of a LTA_4H promoter element active only in PEL cells

Four deletion fragments of the *LTA4H* promoter were constructed to identify potential regulatory elements active in PEL cells (**FIGURE 29 A**). In both BJAB and BCBL-1, fragment S1, in which the distal 319 bp were deleted, exhibited roughly the same activity as the full-length promoter. Fragment S2, from −1196 to +105, exhibited a 1.5 fold increase in activity compared to S1, demonstrating the existence of a negative *cis*

FIGURE 27. Activity of the *LTA4H* promoter in BCBL-1 cells vs. L-428 and BJAB cells. The putative promoter region and partial 5' UTR of the *LTA4H* gene (-2021 to +105 bp) was subcloned in front of the luciferase gene to create LTA4HPRO. LTA4HPRO (yellow bars) or the empty vector (pGL3basic, white bars) were electroporated into BCBL-1, L-428 or control BJAB cells along with *Renilla* (pRLnull) as internal control. Fold activity of the promoter was calculated as the ratio of LTA4HPRO activity to empty vector. Values were normalized according to *Renilla* activity. The experiment shown is representative of at least 3 independent experiments carried in duplicate.

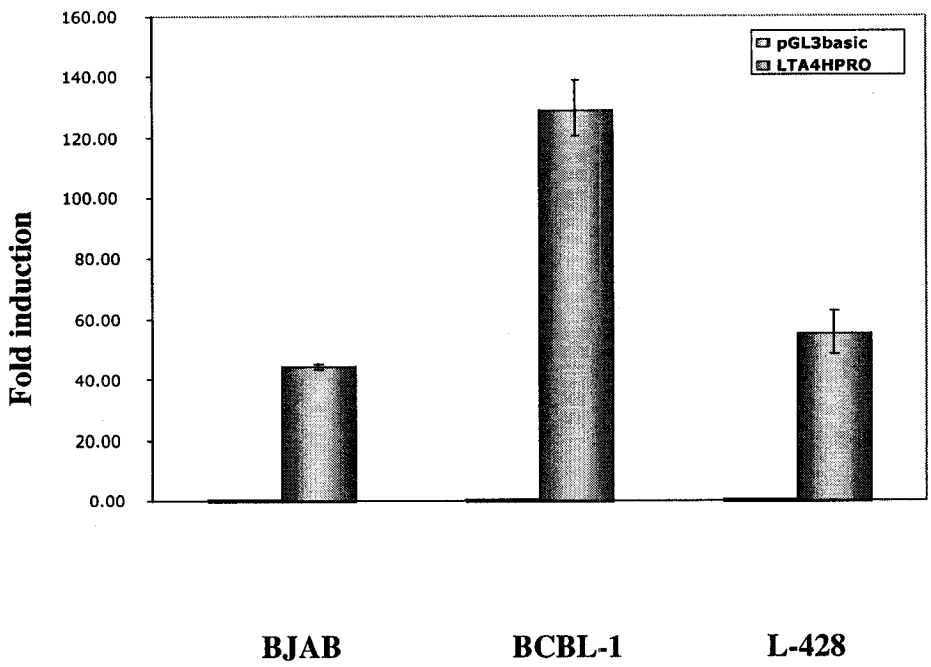
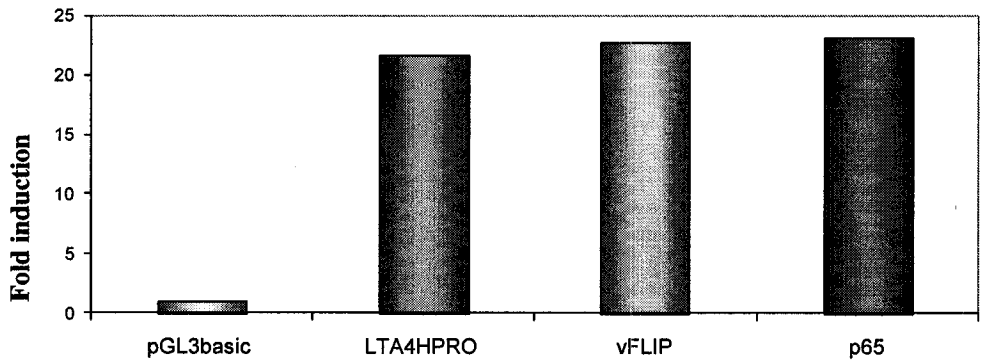

160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Fold induction
pGL3basic
LTA4HPRO
BJAB
BCBL-1
L-428

FIGURE 28. NF-κB is not involved in the regulation of the *LTA4H* promoter. A) Activation of NF-κB by transfection of vFLIP or p65 did not affect the activity of LTA4HPRO. BJAB cells were electroporated with LTA4HPRO plus the appropriate expression plasmid and then incubated for 42 hrs before assaying for luciferase activity. B) Inhibition of NF-κB and AP-1 activity in BCBL-1 cells with the chemical inhibitors aspirin (acetylsalicylic acid), ibuprofen (NF-κB inhibitor only) or the JNK inhibitor SP600125 did not affect the activity of LTA4HPRO. The appropriate inhibitor was added to transfected BCBL-1 cells 6 hrs prior to harvesting samples and assaying for LTA4HPRO luciferase activity. Fold induction was calculated as the ratio between LTA4HPRO activity to empty vector (pGL3basic) after normalization using *renilla* as an internal control (pRLnull). Representative experiments performed in duplicate are shown. At least three independent experiments were performed.

A


Fold induction
25
20
15
10
5
0
pGL3basic
LTA4HPRO
vFLIP
p65

B

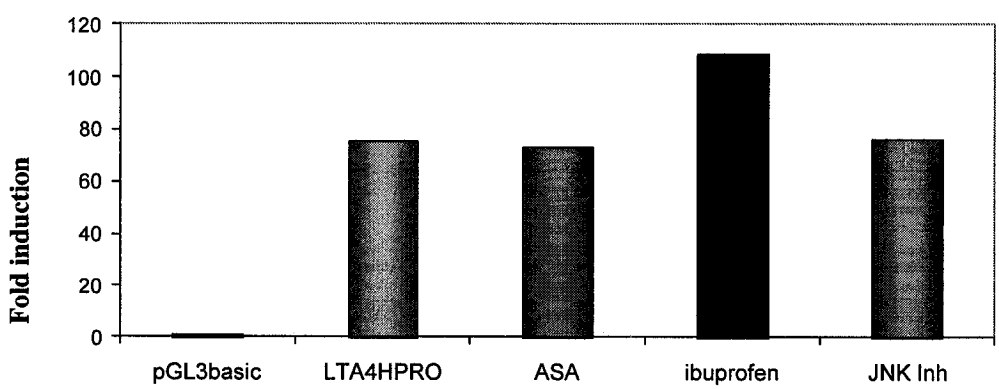
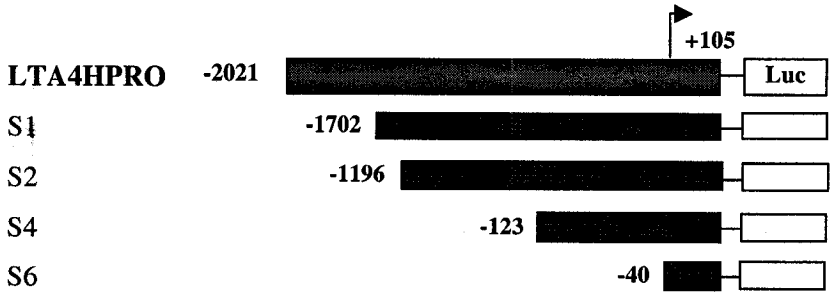
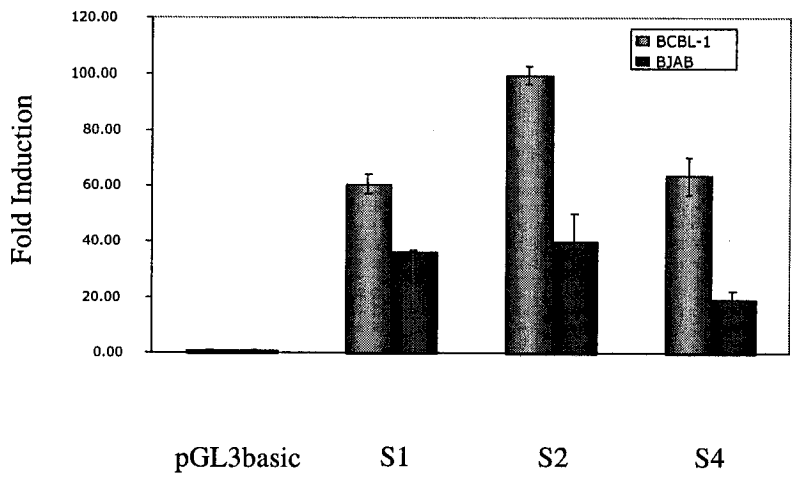

Fold induction
120
100
80
60
40
20
0
pGL3basic
LTA4HPRO
ASA
ibuprofen
JNK Inh

FIGURE 29. Identification of a positive regulatory region between –123 and –40 bp in the LTA_4H promoter active only in BCBL-1 cells but not in BJAB or L-428 cells. A) Schematic representation of LTA4HPRO and deletion constructs. B) Activity of deletion constructs S1, S2 and S4. BCBL-1 or BJAB cells were electroporated with each deletion construct or empty vector (pGL3basic), and assayed for luciferase activity. Fold induction was calculated as the ratio of luciferase activity of the deletion construct to empty vector. Values were normalized using *renilla* activity (pRLnull) as an internal control. C) Activity of S4 and S6 deletion constructs. BCBL-1, L-428 or BJAB cells were electroporated with S4 or S6 construct and assayed as in B. A representative experiment carried out in duplicate is shown. At least 3 independent experiments were performed.

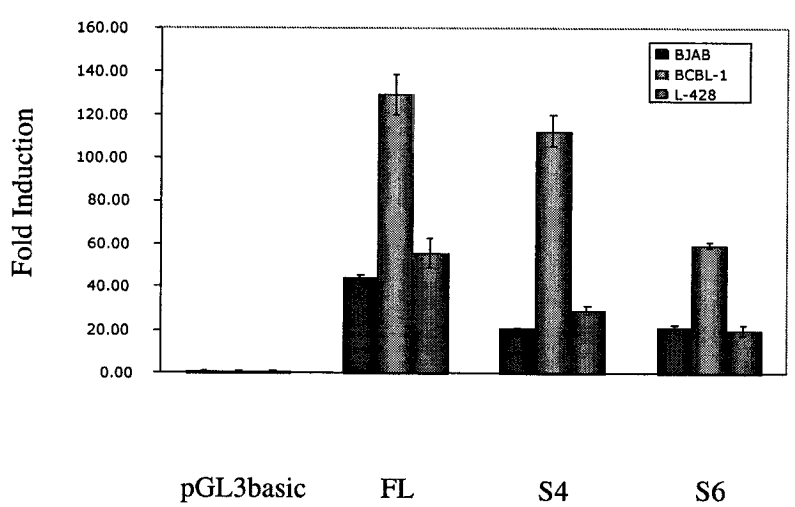
A


+105
LTA4HPRO
-2021
Luc
S1
-1702
S2
-1196
S4
-123
S6
-40

B


120.00
100.00
80.00
60.00
40.00
20.00
0.00
Fold Induction
BCBL-1
BJAB
pGL3basic
S1
S2
S4

C


160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Fold Induction
BJAB
BCBL-1
L-428
pGL3basic
FL
S4
S6

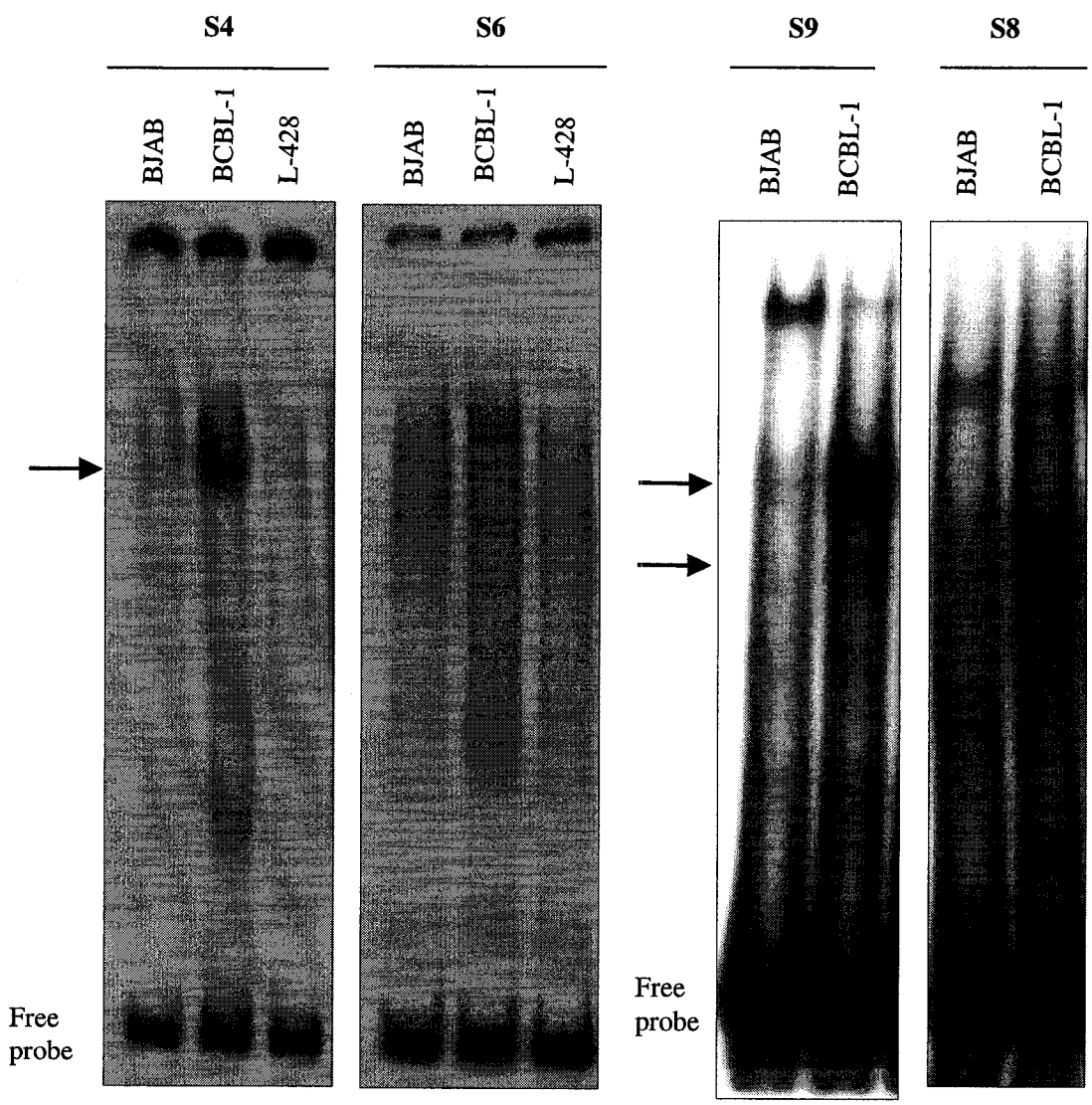
regulatory element between –1702 and -1196. Between S2 and S4 there was a 1.5 to 2-fold loss in activity, indicating the presence of one or more positive element(s) between –1196 and –123 (**FIGURE 29 B**). Interestingly, between S4 and S6 a 50% loss of activity was observed exclusively in BCBL-1 cells, whereas no significant change in promoter activity was seen in BJAB or in L-428 cells (**FIGURE 29 C**). Thus, at least one positive *cis* regulatory element is present in the *LTA4H* promoter that is active only in BCBL-1 cells; moreover, this element is located in the promoter proximal region, between –123 to –40 bp from the transcriptional start site.

4. Two protein-DNA complexes form in the –76 to -40 region of the *LTA4H* promoter in BCBL-1 cells.

Further investigation of the transcriptional control of the *LTA4H* promoter was performed by electrophoretic mobility shift assay (EMSA). Nuclear extracts from BCBL-1, BJAB and L-428 cells were incubated with probes corresponding to the S4 and S6 regions of the LTA4H promoter (**FIGURE 30**, left panels). With probe S4, the presence of a protein-DNA complex was observed in BCBL-1 cells but not in BJAB or L-428 cells. This complex disappeared when S6 was used as probe, thus demonstrating that binding occurred in the –123 to –40 bp region. This observation is in agreement with results from the reporter gene assay, where S4 contained a transactivating element that produced 2-fold higher activity than S6 in BCBL-1 cells.

To further delineate the factor(s) binding region, smaller probes were designed to fine-tune the position of the binding site. Probe S9 spanned nucleotides –76 to +5, and probe

FIGURE 30. Formation of a specific protein-DNA complex in the –76 to +5 bp region of the *LTA4H* promoter in BCBL-1 cells. Nuclear extracts from BCBL-1, BJAB or L-428 cells were analyzed by EMSA with different probes spanning the *LTA4H* promoter proximal region, as indicated, to identify specific complexes formed exclusively in BCBL-1. Probe S4 spans from –123 to +105 bp, probe S6 from –40 to +105 bp, probe S9 from –76 to +5 bp, probe S8 from –46 to +5 bp. Specific complexes are indicated by an arrow.


S4
S6
S9
S8
BJAB
BCBL-1
L-428
BJAB
BCBL-1
L-428
BJAB
BCBL-1
BJAB
BCBL-1
Free probe
Free probe

S8 from –46 to +5. Using these two probes in EMSA analysis, formation of a complex could be clearly seen in BCBL-1 cells but not BJAB nor L-428 with both probes, although S9 probe yielded a sharper band than S8 (FIGURE 30, right panel). Four additional probes: S91 (-76 to –49) and S92 (-51 to –25), S93 (-66 to –40) and S94 (-40 to –19) were used in EMSA analysis (**FIGURE 31**), to refine the mapping of the binding site(s). S91 showed formation of a faint, diffuse complex in BCBL-1 cells and a stronger higher complex present in all cell lines. S92 lead to the formation of two specific complexes in BCBL-1 cells: complex A was stronger and had lower motility, whereas complex B was very faint and migrated faster than complex A. S93 showed the presence of one strong band that roughly co-migrated with complex B. The S94 probe showed no protein binding in any cell line. The specificity of the binding was demonstrated by cold probe competition, which disrupted the formation of the higher specific complexes but not of a lower non-specific band. Therefore, there are at least two distinct binding sites in this region: binding site A is approximatively centered at position -41/42 and truncations beyond –40 bp abrogate binding to this site. Binding site B is upstream of A, approximatively centered at position –46/48 (see **FIGURE 32** for schematic). Identification of the binding protein(s) is still under investigation.

FIGURE 31. Fine mapping of the protein-DNA complex formed in BCBL-1 cells. Shorter probes spanning the region from –76 to +5 were assayed for specific complex formation by EMSA analysis. Probe S91 contains the –76 to –49 bp region, probe S92, -51 to –25 bp, probe S93, -66 to –40 bp, probe S94, -40 to –19 bp. Black circles (lanes 1, 4, 7, and 10): BJAB nuclear extract, white circles (lanes 2, 5, 8, 11, 13-15): BCBL-1 nuclear extracts, gray circles (lanes 3, 6, 9, and 12): L-428 nuclear extracts. Lanes 13-15: competition with S91, S92 and S93 cold probe at 25-fold molar excess, respectively. Specific complexes are indicated by arrows. A non-specific complex is indicated by an asterix.

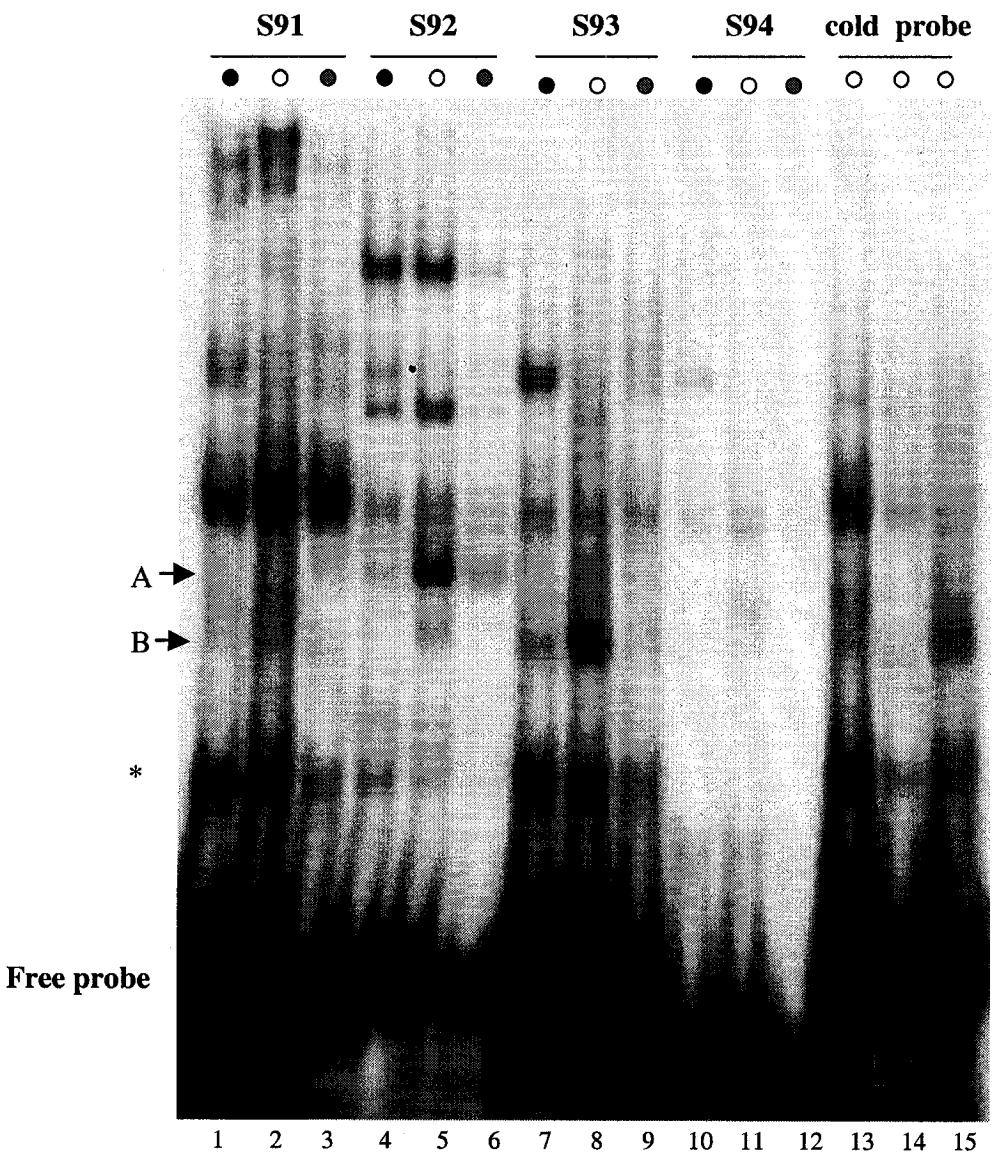
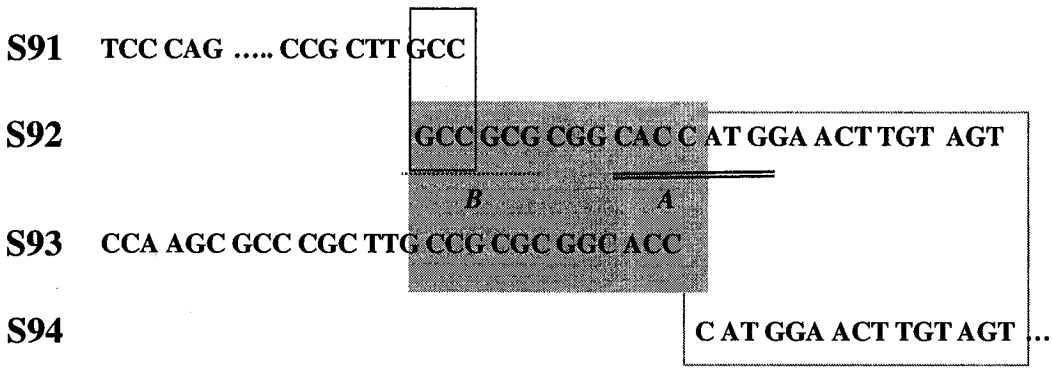

S91
S92
S93
S94
cold probe
A
B
*
Free probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 32. Schematic representation of the putative binding sites for complexes A and B formed exclusively in BCBL-1 cells.

The overlapping regions between S91, S92, S93 and S94 are indicated by boxes. The putative binding sites A and B are underlined.


S91
TCC CAG ..... CCG CTT GCC
S92
GCC GCG CGG CAC C AT GGA ACT TGT AGT
B
A
S93
CCA AGC GCC CGC TTG CCG CGC GGC ACC
S94
C AT GGA ACT TGT AGT ...

CHAPTER VII

Functional significance of LTA_4H upregulation in PEL cells

Leukotriene A_4 hydrolase is a key enzyme in the production of the potent pro-inflammatory mediator leukotriene B_4 (LTB_4). LTA_4H is the limiting reagent in the production of LTB_4, as the enzyme undergoes suicide inactivation during the reaction. Recently, it has been realized that two cells can co-operate to increase LTB_4 production by a mechanism known as transcellular biosynthesis. In order to establish a biological significance to the overexpression of LTA_4H in PEL cells, we investigated if this overexpression led to an increased production of LTB_4 by transcellular biosynthesis.

1. Transcellular biosynthesis of LTB_4 is increased in BCBL-1 cells

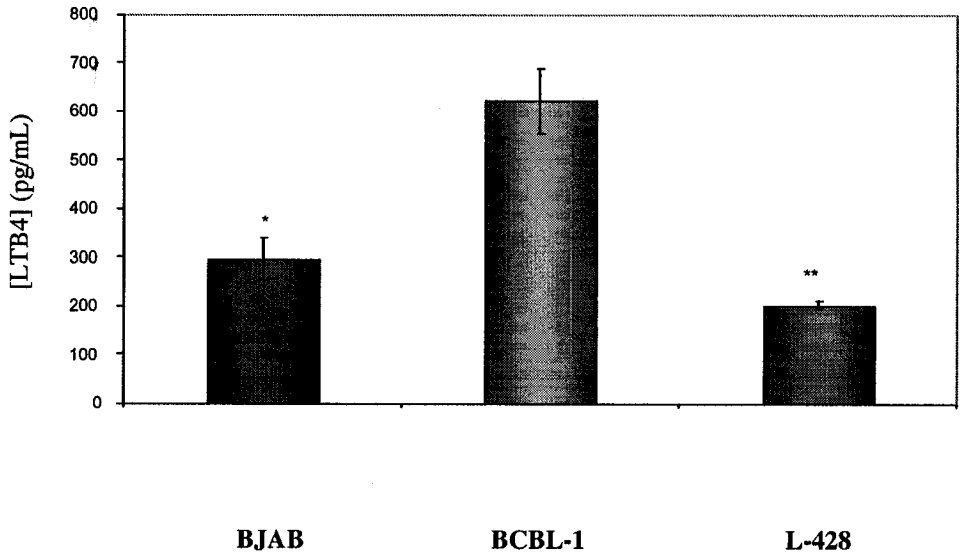
LTB_4 is a potent lipid mediator that acts as a potent chemoattractant of neutrophils, monocytes, macrophages and T cells. LTB_4 can also induce B cell proliferation and antibody secretion. Because B cells normally lack 5-LO activity, we postulated that upregulation of LTA_4H in PEL cells may contribute to increased production of LTB_4 by transcellular biosynthesis. To examine this possibility, BCBL-1 cells were co-cultured at a 1:50 ratio with primary neutrophils stimulated with calcium ionophore to induce 5-LO activity. In parallel, control BJAB and L-428 cells were assayed. Supernatants were collected and analyzed by enzyme-linked immunoassay (EIA) for LTB_4. Co-culture with BCBL-1 cells resulted in LTB_4 levels of 620 pg/mL (+/- 65 pg/mL), whereas coculture with BJAB or L-428 produced 295 pg/mL (+/- 44 pg/mL) or 201 pg/mL (+/- 7 pg/mL), respectively (**FIGURE 33 A**). Therefore, BCBL-1 cells have the capacity to produce 2-3 fold more LTB_4 than control B cells in the presence of stimulated neutrophils.

2. LTB_4 receptors are expressed in PEL cells

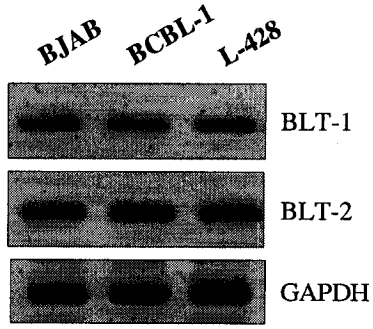
B lymphocytes have been shown to express both LTB_4 receptors, BLT-1 and BLT-2, and therefore also have the capacity to respond to exogenous LTB_4. All PEL cell lines had strong expression of BLT-1 and BLT-2 mRNA as demonstrated by RT-PCR, and these levels were comparable to those of BJAB and L-428 cells (**FIGURE 33 B**). This suggests that PEL cells have retained the potential to respond to LTB_4 stimulation.

FIGURE 33. Formation of LTB_4 by transcellular biosynthesis is increased in BCBL-1 cells. A) Transcellular biosynthesis of LTB_4 measured by EIA. BCBL-1, BJAB or L-428 cells were incubated with calcium ionophore-stimulated primary neutrophils at a ratio of 50:1. Supernatants were diluted 100-fold and assayed for secreted LTB_4 by EIA. Experiment performed in triplicate. Data significance was assessed using paired Student's t test, * P= 0.010, ** P= 0.004. B) RNA levels of the LTB_4 receptors BLT1 and BLT2 in BJAB, BCBL-1 and L-428 cells. Total RNA from each cell line was subjected to RT-PCR using specific primers for BLT-1 and BLT-2. GAPDH primers were used as a control for equal RNA content. Representative results are shown. At least 3 independent experiments performed.

A


800
700
600
500
400
300
200
100
0
[LTB4] (pg/mL)
*
**
BJAB
BCBL-1
L-428

B


BJAB
BCBL-1
L-428
BLT-1
BLT-2
GAPDH

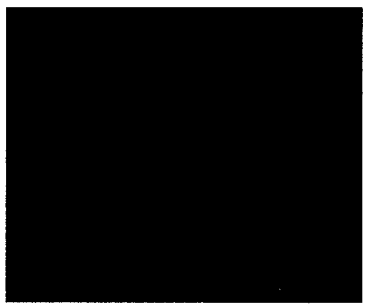
3. BCBL-1 cells do not migrate or proliferate in response to LTB_4

Migration experiments demonstrated that BCBL-1 cells migrate 50 to 100 times more readily across a porous membrane than control BJAB cells (**FIGURE 34**, compare panels A and B), even in the absence of chemotactic stimulus. Although the underlying mechanism for such an increase in motility has not been studied, it is possible that the overexpression of adhesion molecules such as L selectin and PSGL-1 might be involved in this process. LTB_4 stimulation consistently failed to induce a further increase in migration of BCBL-1 cells (**FIGURE 34**, panels B, C and D). It is possible that LTB_4 produced by PEL cells acts in a paracrine way to induce activation of endothelial cells and promote transmigration in this way, a hypothesis that requires further investigation.

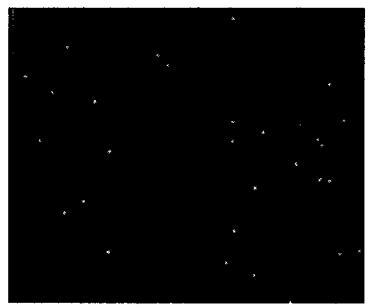
LTB_4 failed to induce BCBL-1 cell proliferation (**FIGURE 35**) when used alone or in combination with IL-4 or IL-6. Indeed, LTB_4 appeared to have a slight inhibitory effect on BCBL-1 cell proliferation when used in combination with IL-4 and high dose IL-6. The reasons for such inhibition remain unclear. Failure of LTB_4 to induce BCBL-1 cell proliferation comes as no surprise, as addition of exogenous stimuli has consistently failed to induce further PEL cell proliferation because cell growth is almost exclusively driven by autocrine mechanisms involving vIL-6 and IL-10 (135). LTB_4 seems to be no exception, and although this leukotriene can increase proliferation and maturation of normal B cells, its effects – if any - on PEL cells remain unknown.

FIGURE 34. BCBL-1 cells migrate readily across a porous membrane. A-C, Representative fields of Calcein-AM labeled cells migrated to the bottom side of a polycarbonate membrane in a transwell migration experiment. A) Control BJAB cells plus PBS/0.1% HSA. B) BCBL-1 cells plus PBS/0.1% HSA. C) BCBL-1 cells plus LTB_4 (10^{-10}M). D) Quantitation of migration experiment. The total number of migrated BCBL-1 cells on the bottom side of the polycarbonate filter were counted under the fluorescent microscope. Error bars correspond to SD on triplicate readings.

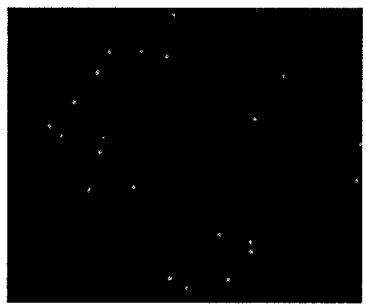
A



B



C



D

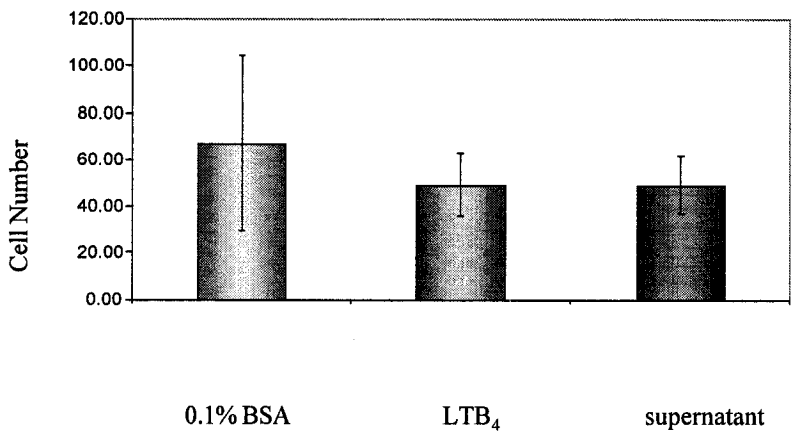
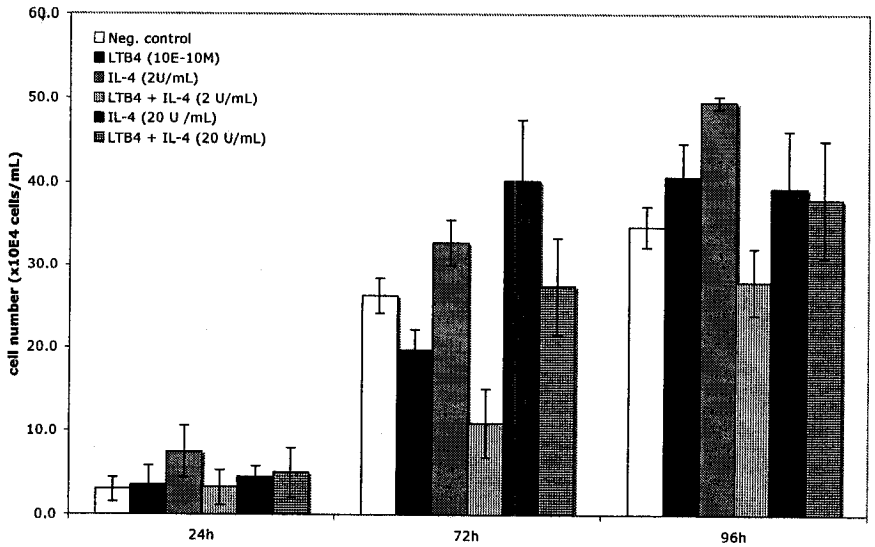
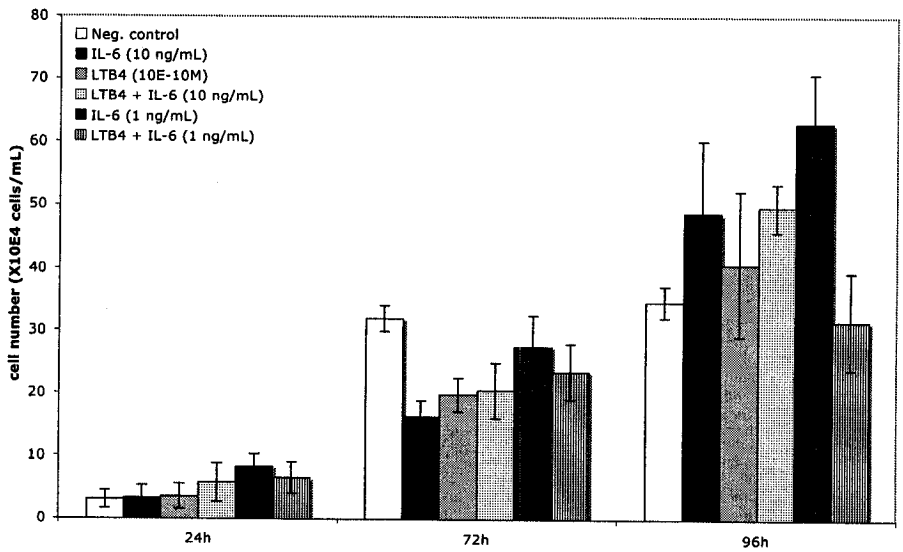

120.00
100.00
80.00
60.00
40.00
20.00
0.00
Cell Number
0.1% BSA
LTB4
supernatant

FIGURE 35. LTB_4 does not induce proliferation of BCBL-1 cells. A) BCBL-1 cells were incubated with LTB_4 alone (black bars) or in combination with IL-4 (2 U/mL, vertical hatched bars; 20 U/ mL, horizontal hatched bars) and cell number was monitored by trypan blue staining. Untreated cells (white bars) and cells treated with IL-4 alone (2 U/mL) were included as controls. B) Same as A, but BCBL-1 cells were treated with LTB_4 alone or in combination with IL-6 (1 or 10 ng/mL).

A


Neg. control
LTB4 (10E-10M)
IL-4 (2U/mL)
LTB4 + IL-4 (2 U/mL)
IL-4 (20 U /mL)
LTB4 + IL-4 (20 U/mL)
cell number (x10E4 cells/mL)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
24h
72h
96h

B


Neg. control
IL-6 (10 ng/mL)
LTB4 (10E-10M)
LTB4 + IL-6 (10 ng/mL)
IL-6 (1 ng/mL)
LTB4 + IL-6 (1 ng/mL)
cell number (X10E4 cells/mL)
80
70
60
50
40
30
20
10
0
24h
72h
96h

CHAPTER VIII

Discussion

1. Disruption of the B cell-specific transcriptional program in Primary Effusion Lymphoma

The current study demonstrates that PEL cells have undergone a severe disruption in the expression of B cell specific transcription factors - specifically PU.1, Oct-2, BOB-1, BSAP/Pax5 and IRF-8 concomitant with an overexpression of IRF-4 – a marker of a post-GC stage of differentiation. The absence of Oct-2 and the co-activator BOB-1 is noteworthy as these transcription factors are essential for the generation of germinal centers and are expressed by B-lymphocytes until the plasma cell stage. The downregulation of Oct-2 and BOB-1 plays a role in the ablation of PU.1 expression in PEL cells as the activity of the PU.1 promoter in mature B cells is largely dependent on the presence of an octamer binding site at position -57 to -51 bp from the transcriptional start site (our work and (48)). In the PEL cell line BCBL-1 - where Oct-2 expression is completely absent – the activity of the full-length PU.1 promoter was negligible. Transient expression of Oct-2 restored the activity of the PU.1 promoter to levels comparable to the control cell line BJAB. Interestingly, the endogenously expressed Oct-1 failed to activate the PU.1 promoter in BCBL-1 cells and thus could not functionally replace Oct-2, in spite of its high level of expression. Our studies thus suggest that loss of Oct-2 expression contributes to the transcriptional shut-off of PU.1 in PEL cells and thus might be at the heart of the lack of expression of B lymphocyte-specific molecules in PEL. Indeed, studies by Radomska *et al.* (231) and Junker et al. (137) using a B and non-B cell fusion model demonstrated that extinction of the B cell

transcriptional program correlates with the rapid downregulation of the expression of B cell specific transcription factors including Oct-2 and PU.1. The expression of B cell immunoglobulin genes could be recovered by ectopic expression of Oct-2.

Protein expression of the Ets transcription factor PU.1 is completely absent in PEL cells. The ablation of this important transcription factor leads to the impaired activity of PU.1-responsive elements such as the λB and κE3' enhancers that regulate the expression of the immunoglobulin light chains. These enhancer elements contain composite Ets-IRF sites that are activated by the co-operative action of PU.1 and IRF-4/IRF-8. In control BJAB cells, electromobility shift assay using the λB or the κE3' Ets-IRF site sequence as probe demonstrated that this site could be bound by PU.1 independently or co-operatively with IRF-8. Co-operative binding with IRF-4 could not be shown as BJAB cells lack IRF-4. In PEL cells, no binding to these Ets-IRF sites was observed. The activity of the λB Ets-IRF site in reporter gene assay is negligible in PEL cells - in spite of the overexpression of IRF-4 in these cells – correlating with the results obtained by EMSA analysis. Ectopic expression of PU.1 in BCBL-1 cells could restore the activity of the Ets-IRF site in a dose-dependent manner, demonstrating the specificity of this site for PU.1. Remarkably, although the Ets factor Spi-B – which has been reported to have redundant activity with PU.1 – was expressed to normal levels in PEL cells, it could not replace PU.1 in the transactivation of the λB element. The reasons for such discrepancy with previous reports (68, 227) could lie in differences in experimental models as, to our knowledge, we are the first ones to assess the activity of a composite Ets-IRF site in PEL cell lines.

The role of IRF-4 in the context of PEL cells that lack expression of PU.1 has not been studied. However, IRF-4 has been shown to regulate important events in the last stages of mature B cell differentiation such as class-switch recombination and plasma cell development. IRF-4 regulates the expression of activation-induced cytidine deaminase, most likely at the transcriptional level, and controls as yet undetermined events upstream of XBP-1 in the path towards plasma cell differentiation (148). Whether IRF-4 acts in partnership with PU.1 or with additional transcription factors in controlling these events remains to be determined. IRF-4 overexpression has been shown to occur in HTLV-1 infected leukemia T cells (257), in which IRF-4 acts as a global transcriptional repressor. IRF-4 was shown to downregulate the expression of genes involved in cell cycle control, apoptosis, DNA repair and cell adhesion (170). IRF-4 overexpression in T cells led to the downregulation of critical DNA repair genes, leading to increased sensitization to genotoxic stress and increased risk of DNA mutations (169). IRF-4 can also directly inhibit IRF-1 transactivation of interferon-responsive genes by competitively binding to target sequences (309) and it can inhibit cytokine production following TLR-4 stimulation. It will be interesting to determine if IRF-4 can act as a global repressor of B-cell specific genes in PEL cells and if IRF-4 overexpression combined with PU.1 downregulation plays a role in the transformation of B lymphocytes.

2. PEL is characterized by the overexpression of pro-inflammatory molecules

The detailed characterization of B cell lymphomas contributes to its accurate classification as well as the design of new treatments, specifically targeted towards molecular features known to contribute to the development of a particular lymphoma. PEL and classical HD derive from B lymphocytes at a similar developmental stage – late GC or post-GC with a partial plasma cell phenotype – and they share a peculiar lack of surface marker expression leading to an indeterminate phenotype. This lack of surface marker expression, and in particular of surface immunoglobulin, has been attributed to an ablation in B cell-specific transcription factor expression. Thus, both PEL and cHD cells lack PU.1, IRF-8, Oct-2, Bob-1 and Pax-5 expression yet they normally express Spi-B and IRF-4. PEL and cHD also share a constitutive activation of the transcription factors NF-κB and AP-1, although the molecular mechanisms leading to this activation are quite different. The similarities at the molecular level between PEL and cHD are not translated to the clinical level, where the presentation and response to treatment of each lymphoma are diametrically opposed. In this study, we addressed the question of identifying a set of genes that could set apart PEL from cHD and other mature B cell lymphomas by using microarray technology. Amongst a set of 1 700 ESTs, we identified a small group (approx. 40) of genes differentially expressed in PEL (**TABLE I**). Four genes involved in cell migration, chemotaxis and inflammation – *LTA4H*, *IL-16*, *TSP-1* and *PSGL-1* - were highly expressed in PEL cells but not in cHD cells. These genes are particularly interesting because PEL cells aberrantly localize to serous body cavities – pointing to cell migration defects - and because chronic inflammation is a major player in the development of Kaposi's sarcoma - a disease that like PEL is caused by the HHV-8 virus.

LTA_4H. LTA_4H catalyzes the last step in the synthesis of LTB_4, a potent chemoattractant of neutrophils with numerous pro-inflammatory properties. LTA_4H also possesses an aminopeptidase activity thought to play a role in extracellular matrix remodelling, although no physiological substrates have been identified to date. LTB_4 stimulates leukocyte generation of reactive oxygen species, degranulation and phagocytosis. LTB_4 augments human peripheral blood monocyte production of IL-6 as well as IL-2 production by CD4+ T cells (195, 237). LTB_4 can also stimulate production of IFN-γ, IL-4 and IL-10 in a model of murine splenocytes (10). In B cells, LTB_4 enhances activation, proliferation and Ig secretion. LTB_4 also acts on endothelial cells to increase their adhesiveness, thus promoting transendothelial migration. Finally, LTB_4 can inhibit apoptosis in several cancer cell models.

In the case of PEL, increased LTB_4 production by transcellular biosynthesis could contribute in numerous ways to the development of this lymphoma. The first obvious effect of LTB_4 would be the enhancement of B cell proliferation and inhibition of apoptosis. LTB_4 could also drive the early HHV-8 infected B cells towards the plasma cell phenotype – in as much as LTB_4 seems to promote B cell activation. Indeed, a study of B-cell chronic lymphocytic leukemia (B-CLL) cells showed that LTB_4 plays a pivotal role in the activation of these cells by CD40 ligation and suggests that the use of leukotriene biosynthesis inhibitors has therapeutic potential for this lymphoma (242). Secondly, LTB_4 could increase the production of IL-6 and IL-10 - two cytokines involved in PEL cell survival and proliferation – either by PEL cells or by bystander cells. Enhancement of IFN-γ production by LTB_4 could lead to induction of the HHV-8 lytic cycle in infected B cells, promoting further rounds of infection of healthy B-

lymphocytes. Endothelial activation by LTB_4 – leading to increased adhesiveness – could promote aberrant transvasation of PEL cells from the circulation into body cavities.

Increased expression of genes involved in leukotriene synthesis has been observed in several cancer types. Overexpression of 5-LO has been observed in B-CLL and human pancreatic cancer cells, whereas LTA_4H overexpression has been described for human esophageal carcinoma and increased LTB_4 levels have been detected in oral squamous cancer cells, suggesting a role for LTB_4 in head and neck cancer pathogenesis. We have now identified an overexpression of LTA_4H in PEL cells - both at the mRNA and at the protein level that leads to an increased LTB_4 production. The implications of LTA_4H overexpression in the pathogenesis of PEL are multiple and our results grant further investigation of the role of LTA_4H overespression in PEL development using *in vivo* models of this disease.

IL-16. IL-16 is synthesized as an 80 kDa precursor that is cleaved by caspase 3 into a 20 kDa active peptide. IL-16 acts on CD4+ cells such as T helper cells, dendritic cells, monocytes/macrophages, eosinophils and neuronal cells. IL-16 multimerizes and binds to the CD4 receptor, triggering multiple responses including chemoattraction of CD4+ cells. IL-16 enhances IL-2- or IL-15-induced proliferation of CD4+ T cells. Bioactive IL-16 secreted by B lymphocytes is a major player in the chemoattraction of CD4+ T and dendritic cells to sites of T-B cell interaction in the lymph nodes (142) and IL-16 can be detected in the mantle zone and GC regions of lymphatic tissue colocalized with CD20.

Some inflammatory diseases such as asthma and inflammatory bowel disease are associated with increased IL-16 levels. Similarly, increased IL-16 levels were found in the serum of multiple myeloma patients and higher levels of this cytokine correlated with decreased survival time (5). We have identified an upregulation of IL-16 precursor protein in PEL cells as well as low levels of caspase 3 activation, suggesting the production of bioactive IL-16 by PEL cells. The production of IL-16 by PEL cells could lead to the recruitment of CD4+ cells to serous body cavities and the establishment of a chronic inflammatory state favorable to disease progression. Furthermore, during the early stages of the lymphoma, the recruitment of T helper cells could provide pro-survival signals that maintain aberrant B cells alive and promote the emergence of a transformed PEL clone.

TSP-1. TSP-1 is a large trimeric protein found in the extracellular matrix that can bind to multiple receptors such as CD36 (the classical TSP-1 receptor), intergrins $\alpha v\beta 3$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha IIb\beta 3$, $\alpha 2\beta 1$, CD47 and heparin sulfate proteoglycans such as syndecans 1, 2, 3 and 4 (reviewed in (264)). Through its multiple domains, TSP-1 is involved in processes such as chemotaxis, cell adhesion and neo-vascularization with sometimes conflicting and even opposite effects. TSP-1 can support cell attachment, adhesion and spreading. TSP-1 has been associated with T cell adhesion and a synergistic effect on the activation of the MAPK and Ras pathways by CD3. TSP-1 induced RhoA inactivation via activation of Focal Adhesion Kinase (FAK), leading to focal adhesion disassembly, a process required for cell migration. No studies up to date have looked into the effects of TSP-1 in B lymphocytes.

Predicting the effects of TSP-1 upregulation in PEL can be a daunting task. Because PEL cells express CD138/syndecan-1, TSP-1 could mediate cell adhesion and migration. Similarly, interactions between TSP-1 and integrins on PEL cells could activate the p38 MAPK and JNK pathways and mediate cell adhesion. The anti-angiogenic properties of TSP-1 are well documented, and they are usually associated with decreased tumor growth. Nonetheless, in some instances, such as gastric cancer, elevated TSP-1 expression is associated with increased angiogenesis and cancer progression. It would be extremely interesting to study the effect of TSP-1 antagonists in PEL cell migration or in disease progression in *in vivo* models of this lymphoma.

PSGL-1. PSGL-1 is the sialo-mucin ligand for P-selectin, an adhesion molecule important for leukocyte rolling and adhesion (194). P-selectin expression is rapidly induced at the surface of endothelium or activated platelets in response to histamines or thrombin whereas E-selectin is expressed on endothelium following activation with inflammatory cytokines. P-selectin and E-selectin mediate the adhesion of leukocytes to the vascular endothelium. P-selectin glycoprotein ligand-1 (PSGL-1) is a heterodimer that binds P-selectin with high affinity and L-selectin at significant lower levels. PSGL-1 interactions with P-selectin mediate neutrophil rolling on endothelial cells, a process essential in for extravasation to sites of inflammation.

Upregulation of PSGL-1 in PEL cells could be a factor contributing to the extravasation of the malignant cells from the circulation into the body cavities. Increased expression of adhesion molecules such as L-selectin and several integrins has been identified by our study and others (133). It is very likely that the combined expression of these molecules increases the migration capacity of PEL cells. Indeed, an *in vitro*

migration experiment using BCBL-1 cells demonstrated that the PEL cell line migrates 50- to 100-fold more readily across a porous membrane than control BJAB cells, even in the absence of chemotactic stimulus.

Considering that PEL's exclusive localization to body cavities requires the migration of the B cell from the lymph node to the periphery and its extravasation from the circulation into serous body cavities, it is likely that the overexpression of chemotactic, adhesion and migration factors play an important role in PEL formation. HHV-8 has been shown to require the establishment of an inflammatory milieu to induce KS onset and progression, suggesting that factors involved in inflammation may play a role in the development of PEL as well. Unfortunately, detection of PEL usually occurs at the late stages of the disease and therefore the sequential progression of this lymphoma has not been studied.

3. Other genes identified as differentially expressed in PEL

*B cell receptor components: CD79A and B.*Microarray analysis of the PEL cell line BCBL-1 and the cHD cell line L-428 identified components of the B cell receptor (BCR) or B cell co-stimulatory molecules to be downregulated in these cells. Among them, CD79A and CD79B (Igα and Igβ chains) - which constitute the signaling components of the BCR - were downregulated in PEL and cHD cells. This is in agreement with previous reports that have found a lack of expression of B cell-specific surface molecules in both PEL and cHD.

Phospholipase Cγ-2 (PLC-γ2). Crosslinking of the BCR by antigen triggers a complex signaling cascade that culminates in the activation of multiple transcription factors such as NF-κB, NFAT, Elk, cFos and cJun. Although multiple reports exist on the lack of BCR components and co-stimulatory molecules in PEL, no reports to date have looked into the downstream signaling pathways. Initial microarray analysis demonstrated a downregulation in PLCγ2 levels in BCBL-1 cells and this result was confirmed by immunoblot in PEL and cHD cell lines. A study published by Marafioti et al. (174) consistently found a lack of PLCγ2, Syk and BLNK expression in biopsy samples and cell lines derived from cHD, further confirming the parallel between PEL and cHD. Thus, lack of expression of PLC-γ2 is another common trait between PEL and cHD cells, although the expression of Syk and BLNK in PEL has not been investigated.

Major Histocompatibility Complex class II (MHC II). Microarray analysis showed a decreased expression of all MHC II chains in BCBL-1 cells compared to BJAB and L-428 cells. Analysis of HLA-DR expression in a panel of PEL cell lines by RT-PCR demonstrated a significant decrease in MHC II expression, although this was not consistent accross our panel of cell line nor was it demonstrated for all MHC II alleles. This was in contrast to cHD cell lines, which demonstrated normal levels of MHC II mRNA.

Other. Microarray analysis revealed altered expression of additional molecules essential for B cell function in PEL cells: the expression of CD40 and CD74 was found to be downregulated in BCBL-1 cells whereas expression of Rho GDP dissociation inhibitor (GDI) β was upregulated (TABLE I). However, these results await confirmation by RT-PCR.

Genes such as Immunoglobulin J polypeptide (downregulated), L selectin (upregulated), CD48 (upregulated), immunoglobulin light chains λ and κ (downregulated), have already been described in the literature as altered in PEL cells and the agreement of the microarray results with previous findings confers an additional level of confidence to this study.

4. LTA_4H transcriptional regulation in PEL and functional significance of its overexpression

The involvement of lipid mediators in inflammation and cancer has been established both at the clinical and basic level. Long-term treatment with non-steroidal anti-inflammatory drugs, compounds that inhibit the synthesis of eicosainoids, reduces the incidence of colorectal cancer in large population-based studies as well as in rodent models (reviewed in (104)). The leukotriene LTB_4, which induces a vigorous inflammatory response, has been equally implicated in cancer development and the use of LTB_4 antagonists or LTA_4H inhibitors reduced cancer incidence in animal and *in vitro* models (49, 242). LTA_4H upregulation in PEL cells was initially identified by microarray analysis and further confirmed by RT-PCR and immunoblot. Our results showed that LTA_4H expression was at least 5-fold higher in PEL cells than in other B cell lines or in primary tonsilar B cells.

The present work focused on the transcriptional elements responsible for LTA_4H upregulation in PEL as well as establishing a physiological role such as increased LTB_4 synthesis by PEL cells. Isolation of the *LTA4H* promoter demonstrated that expression

of this enzyme is controlled at the transcriptional level and that promoter activity was over 3-fold higher in the PEL cell line BCBL-1 than in the control cell line BJAB. The use of truncated promoter construct in reporter gene assays identified potential regulatory sites in the *LTA4H* promoter: the distal -1996 to -1702 bp region contained negative regulatory sites whereas the -1196 to -123 bp region contained positive regulatory elements active in all cell lines. Interestingly, the promoter proximal -123 to -40 bp region contained a positive *cis*-acting element active only in BCBL-1 cells. Deletion of this region lead to a 50% decrease in promoter activity in BCBL-1 cells but had no effect in control BJAB or L-428 cells. EMSA analysis demonstrated the presence of two protein-DNA complexes in the -51 to -40 bp region in BCBL-1 cells but not control cells. Thus, we identified a positive regulatory site that controls LTA4H expression in PEL cells and leads to its overexpression. Although candidate transcription factors that could potentially bind this element were identified by computer modeling (TESS, (249)), EMSA analysis using the corresponding consensus probes and/or antibodies (C/EBP, E2F-1, Oct, Myc, Myb and others) were unsuccessful. We speculate that this site might be regulated by a novel GC-rich element binding protein.

There is increasing evidence that leukotriene synthesis *in vitro* as well as *in vivo* can be achieved by transcellular biosynthesis. This is particularly interesting in the case of LTB_4 for two reasons: 1) 5-LO expression is restricted to myeloid-derived cells and 2) LTA_4H undergoes suicide inactivation after synthesis of one molecule of LTB_4. LTA_4H expression is largely ubiquitous and therefore transcellular synthesis of LTB_4 would play an important role in the amplification of the inflammatory response by increasing the overall amount of LTA_4H available to catalyze the last step of the synthesis. The

increased ability of PEL cells to produce LTB_4 by transcellular biosynthesis was demonstrated by co-culturing BCBL-1 cells with calcium ionophore-stimulated primary neutrophils at a 1:10 and 1:50 ratio. Measurement of LTB_4 production by enzyme-linked immunosorbent assay (ELISA) showed that BCBL-1 cells were able to produce up to 50% more LTB_4 than control BJAB or L-428 cells, thus demonstrating the ability of PEL cells to magnify LTB_4 production even under limited neutrophil infiltration.

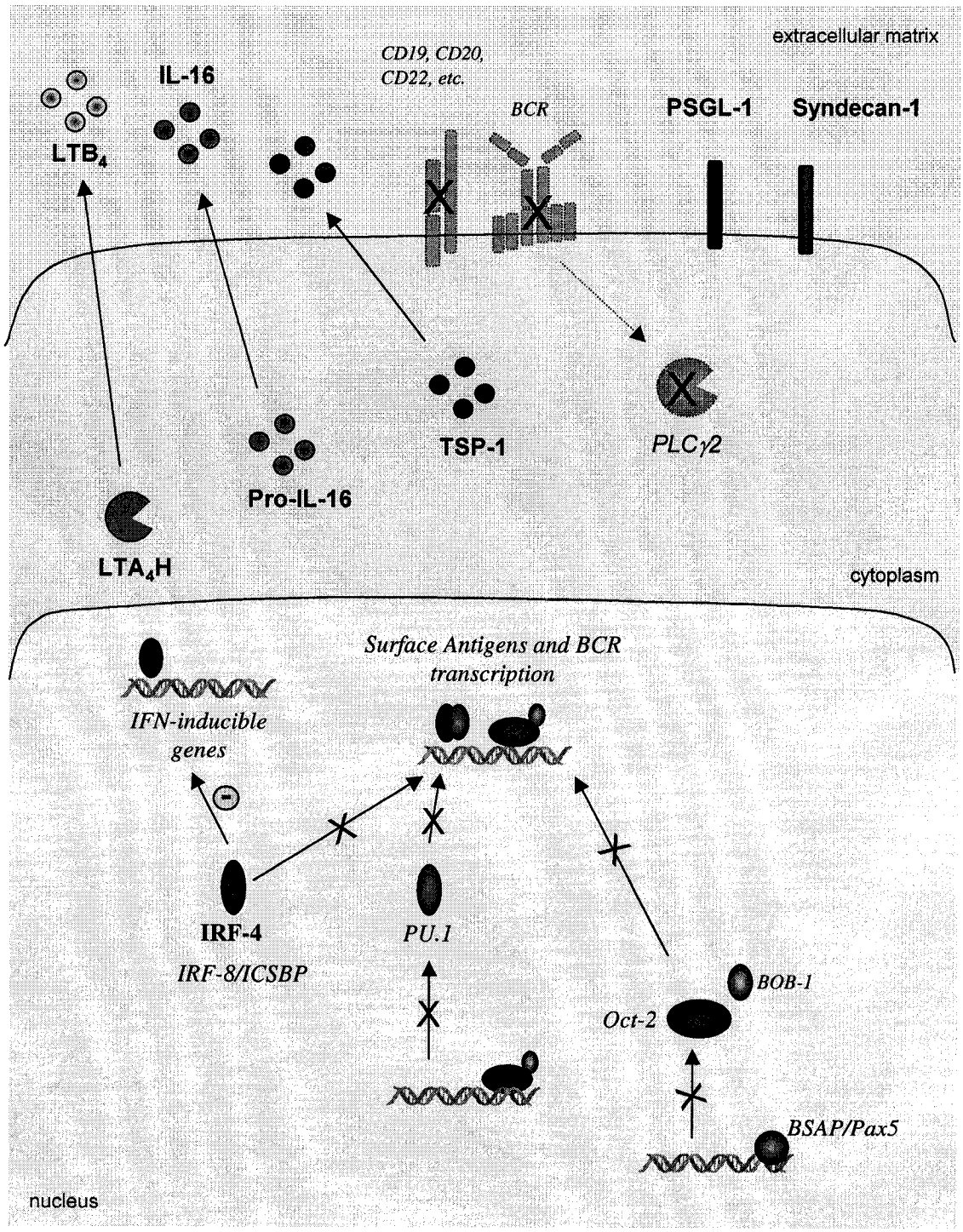
PEL cells appear to have retained the capability to respond to LTB_4, as suggested by the strong mRNA expression of both LTB_4 receptors (BLT-1 and BLT-2). However, the effect of this leukotriene on PEL cells remains to be determined. LTB_4 did not trigger BCBL-1 cell proliferation or chemotaxis at physiological concentrations. Proliferation was not induced by LTB_4 even when used in combination with IL-6 or IL-4. This is not totally surprising, since exogenous addition of most B- cell growth factors – including the potent cytokine IL-6 – have no proliferative effect in PEL cell lines. This is attributable to the fact that PEL cell lines depend mostly on autocrine signals such as vIL-6 to promote growth. However, during the early stages of PEL, before the malignant cells are growth-factor independent, it is not unlikely that LTB_4 can play a role in cell proliferation and survival, as demonstrated with normal mature B cells. Moreover, LTA_4H possesses an aminopeptidase activity that is thought to participate in extracellular matrix remodeling. It cannot be ruled out that this activity of LTA_4H may be involved in PEL establishment in serous body cavities, a possibility that remains to be explored.

In conclusion, we have found that PEL cells are characterized at the molecular level by an ablated expression of B-cell specific transcription factors such as PU.1, Oct-2, IRF-8

and BSAP/Pax-5 while exhibiting a concomitant upregulation of mediators of inflammation and cell migration such as LTA_4H, IL-16, TSP-1 and PSGL-1 (see schematic representation in **FIGURE 36**). Whereas transcripton factor ablation provides a plausible cause for the lack of expression of B cell-specific surface markers in PEL, the identification LTA_4H, IL-16, PSGL-1 and TSP-1 as molecules exclusively upregulated in PEL cells suggests that overexpression of factors involved in cell motility, inflammation and/or chemotaxis are involved in the aberrant localization of this lymphoma to the serous body cavities. Further experiments that delineate the role of each of these factors in the increased migration potential observed for PEL cells are required to understand the mechanisms governing the establishment of these cells in body cavities.

Identifying new molecules that are specifically overexpressed in PEL provides an interesting framework for the development of novel therapeutic approaches for this lymphoma. For example, the effect of the metalloproteinase inhibitor Bestatin - an anti-cancer agent that inhibits LTA_4H – could be assayed as a potential inhibitor of PEL, either by blocking LTB_4 formation or the metalloproteinase activity of LTA_4H. As well, the effect of anti-inflammatory drugs on PEL cell survival and migration should be explored. However, the development of an appropriate *in-vivo* model for PEL is essential to clearly demonstrate the role of cell migration and inflammation in the development of this lymphoma.

FIGURE 36. Schematic representation of the molecular alterations identified in PEL cells. PEL cells have ablated expression of the transcription factors PU.1, Oct-2, Bob-1, BSAP/Pax-5 and IRF-8, resulting in decreased expression immunoglobulin genes and other surface and signaling molecules. Concomitantly, PEL cells overexpress factors involved in cell migration and inflammation such as LTA_4H, IL-16, TSP-1 and PSGL-1. This may contribute to the localization of PEL to serous body cavities.


extracellular matrix
CD19, CD20,
CD22, etc.
IL-16
BCR
PSGL-1
Syndecan-1
LTB4
TSP-1
PLCγ2
Pro-IL-16
LTA4H
cytoplasm
Surface Antigens and BCR
transcription
IFN-inducible
genes
IRF-4
IRF-8/ICSBP
PU.1
BOB-1
Oct-2
BSAP/Pax5
nucleus

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Identified a lack of B cell-specific transcription factor expression in PEL cells, which contributes to the null phenotype characteristic of this lymphoma. Furthermore, this study delineated the functional significance of this ablation by confirming its effect on the activity of B cell specific promoter elements such as the PU.1 promoter and the λB and κE3' light chain enhancers.

2. Identified by microarray analysis a cluster of genes that are differentially expressed in PEL cells and therefore can be of practical relevance in the classification and diagnosis of this lymphoma. Four molecules involved in the inflammatory processes of chemotaxis, cell adhesion and migration were identified as upregulated at the mRNA and protein level in PEL cells: LTA_4H, IL-16, PSGL-1 and TSP-1. PEL cells also expressed – at least at the mRNA level – both LTB_4 receptors: BLT-1 and BLT-2

3. Functionally characterized the *LTA4H* promoter, demonstrating that LTA_4H expression is regulated at the transcriptional level by *cis*-regulatory elements that are active only in PEL cells and determine LTA_4H overexpression in this lymphoma. The regulatory site was recognized by a yet unidentified transcription factor active only in PEL cells.

4. Established that overexpression of LTA_4H in PEL cells results in an increased capability of synthesizing LTB_4 by transcellular biosynthesis. Considering that LTA_4H

and LTB_4 are implicated in the development of certain types of cancers, LTA_4H potentially represents a novel target for PEL treatment.

REFERENCES

1. **Ablashi, D. V., L. G. Chatlynne, J. E. Whitman, Jr., and E. Cesarman.** 2002. Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. Clin Microbiol Rev **15:**439-64.

2. **Aggarwal, B. B., S. Shishodia, S. K. Sandur, M. K. Pandey, and G. Sethi.** 2006. Inflammation and cancer: how hot is the link? Biochem Pharmacol **72:**1605-21.

3. **Ahmadzadeh, N., M. Shingu, M. Nobunaga, and T. Tawara.** 1991. Relationship between leukotriene B4 and immunological parameters in rheumatoid synovial fluids. Inflammation **15:**497-503.

4. **Alexandrakis, M. G., F. H. Passam, D. S. Kyriakou, and D. Bouros.** 2004. Pleural effusions in hematologic malignancies. Chest **125:**1546-55.

5. **Alexandrakis, M. G., F. H. Passam, D. S. Kyriakou, A. V. Christophoridou, K. Perisinakis, A. Hatzivasili, A. Foudoulakis, and E. Castanas.** 2004. Serum level of interleukin-16 in multiple myeloma patients and its relationship to disease activity. Am J Hematol **75:**101-6.

6. **An, J., A. K. Lichtenstein, G. Brent, and M. B. Rettig.** 2002. The Kaposi sarcoma-associated herpesvirus (KSHV) induces cellular interleukin 6 expression: role of the KSHV latency-associated nuclear antigen and the AP1 response element. Blood **99:**649-54.

7. **An, J., Y. Sun, and M. B. Rettig.** 2004. Transcriptional coactivation of c-Jun by the KSHV-encoded LANA. Blood **103:**222-8.

8. **An, J., Y. Sun, R. Sun, and M. B. Rettig.** 2003. Kaposi's sarcoma-associated herpesvirus encoded vFLIP induces cellular IL-6 expression: the role of the NF-kappaB and JNK/AP1 pathways. Oncogene **22:**3371-85.

9. **Aoki, Y., D. Qiu, G. H. Zhao, and P. N. Kao.** 1998. Leukotriene B4 mediates histamine induction of NF-kappaB and IL-8 in human bronchial epithelial cells. Am J Physiol **274:**L1030-9.

10. **Arcoleo, F., S. Milano, P. D'Agostino, and E. Cillari.** 1995. Effect of exogenous leukotriene B4 (LTB4) on BALB/c mice splenocyte production of Th1 and Th2 lymphokines. Int J Immunopharmacol **17:**457-63.

11. **Arguello, M., M. Sgarbanti, E. Hernandez, Y. Mamane, S. Sharma, M. Servant, R. Lin, and J. Hiscott.** 2003. Disruption of the B-cell specific transcriptional program in HHV-8 associated primary effusion lymphoma cell lines. Oncogene **22:**964-73.

12. **Back, M., D. X. Bu, R. Branstrom, Y. Sheikine, Z. Q. Yan, and G. K. Hansson.** 2005. Leukotriene B4 signaling through NF-kappaB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia. Proc Natl Acad Sci U S A **102:**17501-6.

13. **Baier, M., N. Bannert, A. Werner, K. Lang, and R. Kurth.** 1997. Molecular cloning, sequence, expression, and processing of the interleukin 16 precursor. Proc Natl Acad Sci U S A **94:**5273-7.

14. **Balkwill, F.** 2004. Cancer and the chemokine network. Nat Rev Cancer **4:**540-50.

15. **Balkwill, F., and L. M. Coussens.** 2004. Cancer: an inflammatory link. Nature **431:**405-6.

16. **Barillari, G., R. Gendelman, R. C. Gallo, and B. Ensoli.** 1993. The Tat protein of human immunodeficiency virus type 1, a growth factor for AIDS Kaposi sarcoma and cytokine-activated vascular cells, induces adhesion of the same cell types by using integrin receptors recognizing the RGD amino acid sequence. Proc Natl Acad Sci U S A **90:**7941-5.

17. **Barillari, G., C. Sgadari, V. Fiorelli, F. Samaniego, S. Colombini, V. Manzari, A. Modesti, B. C. Nair, A. Cafaro, M. Sturzl, and B. Ensoli.** 1999. The Tat protein of human immunodeficiency virus type-1 promotes vascular cell growth and locomotion by engaging the alpha5beta1 and alphavbeta3 integrins and by mobilizing sequestered basic fibroblast growth factor. Blood **94:**663-72.

18. **Barth, T. F., J. I. Martin-Subero, S. Joos, C. K. Menz, C. Hasel, G. Mechtersheimer, R. M. Parwaresch, P. Lichter, R. Siebert, and P. Mooller.** 2003. Gains of 2p involving the REL locus correlate with nuclear c-Rel protein accumulation in neoplastic cells of classical Hodgkin lymphoma. Blood **101:**3681-6.

19. **Belanger, C., A. Gravel, A. Tomoiu, M. E. Janelle, J. Gosselin, M. J. Tremblay, and L. Flamand.** 2001. Human herpesvirus 8 viral FLICE-inhibitory protein inhibits Fas-mediated apoptosis through binding and prevention of procaspase-8 maturation. J Hum Virol **4:**62-73.

20. **Bigby, T. D., D. M. Lee, N. Meslier, and D. C. Gruenert.** 1989. Leukotriene A4 hydrolase activity of human airway epithelial cells. Biochem Biophys Res Commun **164:**1-7.

21. **Bjork, J., P. Hedqvist, and K. E. Arfors.** 1982. Increase in vascular permeability induced by leukotriene B4 and the role of polymorphonuclear leukocytes. Inflammation **6:**189-200.

22. **Borgeat, P., and B. Samuelsson.** 1979. Transformation of arachidonic acid by rabbit polymorphonuclear leukocytes. Formation of a novel dihydroxyeicosatetraenoic acid. J Biol Chem **254:**2643-6.

23. **Boulanger, E., O. Hermine, J. P. Fermand, I. Radford-Weiss, N. Brousse, V. Meignin, and A. Gessain.** 2004. Human herpesvirus 8 (HHV-8)-associated peritoneal primary effusion lymphoma (PEL) in two HIV-negative elderly patients. Am J Hematol **76:**88-91.

24. **Brass, A. L., E. Kehrli, C. F. Eisenbeis, U. Storb, and H. Singh.** 1996. Pip, a lymphoid-restricted IRF, contains a regulatory domain that is important for autoinhibition and ternary complex formation with the Ets factor PU.1. Genes Dev **10:**2335-47.

25. **Brass, A. L., A. Q. Zhu, and H. Singh.** 1999. Assembly requirements of PU.1-Pip (IRF-4) activator complexes: inhibiting function in vivo using fused dimers. Embo J **18:**977-91.

26. **Brock, T. G., R. W. McNish, and M. Peters-Golden.** 1995. Translocation and leukotriene synthetic capacity of nuclear 5-lipoxygenase in rat basophilic leukemia cells and alveolar macrophages. J Biol Chem **270:**21652-8.

27. **Budihardjo, I., H. Oliver, M. Lutter, X. Luo, and X. Wang.** 1999. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol **15:**269-90.

28. **Burger, R., F. Neipel, B. Fleckenstein, R. Savino, G. Ciliberto, J. R. Kalden, and M. Gramatzki.** 1998. Human herpesvirus type 8 interleukin-6 homologue is functionally active on human myeloma cells. Blood **91:**1858-63.

29. **Byrum, R. S., J. L. Goulet, J. N. Snouwaert, R. J. Griffiths, and B. H. Koller.** 1999. Determination of the contribution of cysteinyl leukotrienes and leukotriene B4 in acute inflammatory responses using 5-lipoxygenase- and leukotriene A4 hydrolase-deficient mice. J Immunol **163:**6810-9.

30. **Cabannes, E., G. Khan, F. Aillet, R. F. Jarrett, and R. T. Hay.** 1999. Mutations in the IkBa gene in Hodgkin's disease suggest a tumour suppressor role for IkappaBalpha. Oncogene **18:**3063-70.

31. **Callahan, J., S. Pai, M. Cotter, and E. S. Robertson.** 1999. Distinct patterns of viral antigen expression in Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus coinfected body-cavity-based lymphoma cell lines: potential switches in latent gene expression due to coinfection. Virology **262:**18-30.

32. **Carbone, A.** 2003. Emerging pathways in the development of AIDS-related lymphomas. Lancet Oncol **4:**22-9.

33. **Carbone, A., A. M. Cilia, A. Gloghini, D. Capello, T. Perin, D. Bontempo, V. Canzonieri, U. Tirelli, R. Volpe, and G. Gaidano.** 2000. Primary effusion lymphoma cell lines harbouring human herpesvirus type-8. Leuk Lymphoma **36:**447-56.

34. **Carbone, A., A. Gloghini, D. Bontempo, P. Monini, U. Tirelli, R. Volpe, P. J. Browning, and G. Gaidano.** 2000. Proliferation in HHV-8-positive primary effusion lymphomas is associated with expression of HHV-8 cyclin but independent of p27(kip1). Am J Pathol **156:**1209-15.

35. **Carbone, A., A. Gloghini, M. R. Cozzi, D. Capello, A. Steffan, P. Monini, L. De Marco, and G. Gaidano.** 2000. Expression of MUM1/IRF4 selectively clusters with primary effusion lymphoma among lymphomatous effusions: implications for disease histogenesis and pathogenesis. Br J Haematol **111:**247-57.

36. **Carbone, A., A. Gloghini, L. M. Larocca, D. Capello, F. Pierconti, V. Canzonieri, U. Tirelli, R. Dalla-Favera, and G. Gaidano.** 2001. Expression profile of MUM1/IRF4, BCL-6, and CD138/syndecan-1 defines novel histogenetic subsets of human immunodeficiency virus-related lymphomas. Blood **97:**744-51.

37. **Carpagnano, G. E., P. J. Barnes, D. M. Geddes, M. E. Hodson, and S. A. Kharitonov.** 2003. Increased leukotriene B4 and interleukin-6 in exhaled breath condensate in cystic fibrosis. Am J Respir Crit Care Med **167:**1109-12.

38. **Catlett-Falcone, R., T. H. Landowski, M. M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J. L. Fernandez-Luna, G. Nunez, W. S. Dalton, and R. Jove.** 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity **10:**105-15.

39. **Chadburn, A., E. Cesarman, J. Jagirdar, M. Subar, R. N. Mir, and D. M. Knowles.** 1993. CD30 (Ki-1) positive anaplastic large cell lymphomas in individuals infected with the human immunodeficiency virus. Cancer **72:**3078-90.

40. **Chan, J. K.** 2001. The new World Health Organization classification of lymphomas: the past, the present and the future. Hematol Oncol **19:**129-50.

41. **Chan, W. C.** 2001. The Reed-Sternberg cell in classical Hodgkin's disease. Hematol Oncol **19:**1-17.

42. **Chang, L., and M. Karin.** 2001. Mammalian MAP kinase signalling cascades. Nature **410:**37-40.

43. **Chang, Y., P. S. Moore, S. J. Talbot, C. H. Boshoff, T. Zarkowska, K. Godden, H. Paterson, R. A. Weiss, and S. Mittnacht.** 1996. Cyclin encoded by KS herpesvirus. Nature **382:**410.

44. **Chatterjee, M., J. Osborne, G. Bestetti, Y. Chang, and P. S. Moore.** 2002. Viral IL-6-induced cell proliferation and immune evasion of interferon activity. Science **298:**1432-5.

45. **Chaudhary, P. M., M. T. Eby, A. Jasmin, A. Kumar, L. Liu, and L. Hood.** 2000. Activation of the NF-kappaB pathway by caspase 8 and its homologs. Oncogene **19:**4451-60.

46. **Chaudhary, P. M., A. Jasmin, M. T. Eby, and L. Hood.** 1999. Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene **18:**5738-46.

47. **Chen, H., D. Ray-Gallet, P. Zhang, C. J. Hetherington, D. A. Gonzalez, D. E. Zhang, F. Moreau-Gachelin, and D. G. Tenen.** 1995. PU.1 (Spi-1) autoregulates its expression in myeloid cells. Oncogene **11:**1549-60.

48. **Chen, H., P. Zhang, H. S. Radomska, C. J. Hetherington, D. E. Zhang, and D. G. Tenen.** 1996. Octamer binding factors and their coactivator can activate the murine PU.1 (spi-1) promoter. J Biol Chem **271:**15743-52.

49. **Chen, X., N. Li, S. Wang, N. Wu, J. Hong, X. Jiao, M. J. Krasna, D. G. Beer, and C. S. Yang.** 2003. Leukotriene A4 hydrolase in rat and human esophageal adenocarcinomas and inhibitory effects of bestatin. J Natl Cancer Inst **95:**1053-61.

50. **Chirivi, R. G., G. Taraboletti, M. R. Bani, L. Barra, G. Piccinini, M. Giacca, F. Bussolino, and R. Giavazzi.** 1999. Human immunodeficiency virus-1 (HIV-1)-Tat protein promotes migration of acquired immunodeficiency syndrome-related lymphoma cells and enhances their adhesion to endothelial cells. Blood **94:**1747-54.

51. **Claesson, H. E., B. Odlander, and P. J. Jakobsson.** 1992. Leukotriene B4 in the immune system. Int J Immunopharmacol **14:**441-9.

52. **Cogliatti, S. B., and U. Schmid.** 2002. Who is WHO and what was REAL? Swiss Med Wkly **132:**607-17.

53. **Corcoran, L. M., M. Karvelas, G. J. Nossal, Z. S. Ye, T. Jacks, and D. Baltimore.** 1993. Oct-2, although not required for early B-cell development, is critical for later B-cell maturation and for postnatal survival. Genes Dev **7:**570-82.

54. **Cotter, M. A., 2nd, and E. S. Robertson.** 1999. The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. Virology **264:**254-64.

55. **Crooks, S. W., D. L. Bayley, S. L. Hill, and R. A. Stockley.** 2000. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. Eur Respir J **15:**274-80.

56. **DeKoter, R. P., H. J. Lee, and H. Singh.** 2002. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. Immunity **16:**297-309.

57. **DeKoter, R. P., and H. Singh.** 2000. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science **288:**1439-41.

58. **Diehl, V., M. Sextro, J. Franklin, M. L. Hansmann, N. Harris, E. Jaffe, S. Poppema, M. Harris, K. Franssila, J. van Krieken, T. Marafioti, I. Anagnostopoulos, and H. Stein.** 1999. Clinical presentation, course, and prognostic factors in lymphocyte-predominant Hodgkin's disease and lymphocyte-rich classical Hodgkin's disease: report from the European Task Force on Lymphoma Project on Lymphocyte-Predominant Hodgkin's Disease. J Clin Oncol **17:**776-83.

59. **Dixon, R. A., R. E. Diehl, E. Opas, E. Rands, P. J. Vickers, J. F. Evans, J. W. Gillard, and D. K. Miller.** 1990. Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature **343:**282-4.

60. **Dolcetti, R., M. Boiocchi, A. Gloghini, and A. Carbone.** 2001. Pathogenetic and histogenetic features of HIV-associated Hodgkin's disease. Eur J Cancer **37:**1276-87.

61. **Dourmishev, L. A., A. L. Dourmishev, D. Palmeri, R. A. Schwartz, and D. M. Lukac.** 2003. Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. Microbiol Mol Biol Rev **67:**175-212, table of contents.

62. **Drexler, H. G.** 1993. Recent results on the biology of Hodgkin and Reed-Sternberg cells. II. Continuous cell lines. Leuk Lymphoma **9:**1-25.

63. **Drexler, H. G., C. Meyer, G. Gaidano, and A. Carbone.** 1999. Constitutive cytokine production by primary effusion (body cavity-based) lymphoma-derived cell lines. Leukemia **13:**634-40.

64. **Driggers, P. H., D. L. Ennist, S. L. Gleason, W. H. Mak, M. S. Marks, B. Z. Levi, J. R. Flanagan, E. Appella, and K. Ozato.** 1990. An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. Proc Natl Acad Sci U S A **87:**3743-7.

65. **Dugas, B., N. Paul-Eugene, J. Cairns, J. Gordon, A. Calenda, J. M. Mencia-Huerta, and P. Braquet.** 1990. Leukotriene B4 potentiates the expression and release of Fc epsilon RII/CD23, and proliferation and differentiation of human B lymphocytes induced by IL-4. J Immunol **145:**3406-11.

66. **Ehlin-Henriksson, B., A. Manneborg-Sandlund, and G. Klein.** 1987. Expression of B-cell-specific markers in different Burkitt lymphoma subgroups. Int J Cancer **39:**211-8.

67. **Eisenbeis, C. F., H. Singh, and U. Storb.** 1995. Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. Genes Dev **9:**1377-87.

68. **Eisenbeis, C. F., H. Singh, and U. Storb.** 1993. PU.1 is a component of a multiprotein complex which binds an essential site in the murine immunoglobulin lambda 2-4 enhancer. Mol Cell Biol **13:**6452-61.

69. **Eklund, E. A., A. Jalava, and R. Kakar.** 1998. PU.1, interferon regulatory factor 1, and interferon consensus sequence-binding protein cooperate to increase gp91(phox) expression. J Biol Chem **273:**13957-65.

70. **Ellis, M., Y. P. Chew, L. Fallis, S. Freddersdorf, C. Boshoff, R. A. Weiss, X. Lu, and S. Mittnacht.** 1999. Degradation of p27(Kip) cdk inhibitor triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. Embo J **18:**644-53.

71. **Ensoli, B., G. Barillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal.** 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature **345:**84-6.

72. **Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H. K. Chang, J. N. Brady, and R. C. Gallo.** 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature **371:**674-80.

73. **Ensoli, B., S. Nakamura, S. Z. Salahuddin, P. Biberfeld, L. Larsson, B. Beaver, F. Wong-Staal, and R. C. Gallo.** 1989. AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. Science **243:**223-6.

74. **Evans, J. F., P. Dupuis, and A. W. Ford-Hutchinson.** 1985. Purification and characterisation of leukotriene A4 hydrolase from rat neutrophils. Biochim Biophys Acta **840:**43-50.

75. **Fabre, J. E., J. L. Goulet, E. Riche, M. Nguyen, K. Coggins, S. Offenbacher, and B. H. Koller.** 2002. Transcellular biosynthesis contributes to the production

of leukotrienes during inflammatory responses in vivo. J Clin Invest **109:**1373-80.

76. **Fakhari, F. D., J. H. Jeong, Y. Kanan, and D. P. Dittmer.** 2006. The latency-associated nuclear antigen of Kaposi sarcoma-associated herpesvirus induces B cell hyperplasia and lymphoma. J Clin Invest **116:**735-42.

77. **Falini, B., M. Fizzotti, A. Pucciarini, B. Bigerna, T. Marafioti, M. Gambacorta, R. Pacini, C. Alunni, L. Natali-Tanci, B. Ugolini, C. Sebastiani, G. Cattoretti, S. Pileri, R. Dalla-Favera, and H. Stein.** 2000. A monoclonal antibody (MUM1p) detects expression of the MUM1/IRF4 protein in a subset of germinal center B cells, plasma cells, and activated T cells. Blood **95:**2084-92.

78. **Feinmark, S. J., and P. J. Cannon.** 1986. Endothelial cell leukotriene C4 synthesis results from intercellular transfer of leukotriene A4 synthesized by polymorphonuclear leukocytes. J Biol Chem **261:**16466-72.

79. **Field, N., W. Low, M. Daniels, S. Howell, L. Daviet, C. Boshoff, and M. Collins.** 2003. KSHV vFLIP binds to IKK-gamma to activate IKK. J Cell Sci **116:**3721-8.

80. **Fitzpatrick, F., W. Liggett, J. McGee, S. Bunting, D. Morton, and B. Samuelsson.** 1984. Metabolism of leukotriene A4 by human erythrocytes. A novel cellular source of leukotriene B4. J Biol Chem **259:**11403-7.

81. **Foletta, V. C., D. H. Segal, and D. R. Cohen.** 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. J Leukoc Biol **63:**139-52.

82. **Ford-Hutchinson, A. W., M. A. Bray, M. V. Doig, M. E. Shipley, and M. J. Smith.** 1980. Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature **286:**264-5.

83. **Foussat, A., J. Wijdenes, L. Bouchet, G. Gaidano, F. Neipel, K. Balabanian, P. Galanaud, J. Couderc, and D. Emilie.** 1999. Human interleukin-6 is in vivo an autocrine growth factor for human herpesvirus-8-infected malignant B lymphocytes. Eur Cytokine Netw **10:**501-8.

84. **Frevert, C. W., V. A. Wong, R. B. Goodman, R. Goodwin, and T. R. Martin.** 1998. Rapid fluorescence-based measurement of neutrophil migration in vitro. J Immunol Methods **213:**41-52.

85. **Friborg, J., Jr., W. Kong, M. O. Hottiger, and G. J. Nabel.** 1999. p53 inhibition by the LANA protein of KSHV protects against cell death. Nature **402:**889-94.

86. **Friedrich, E. B., A. M. Tager, E. Liu, A. Pettersson, C. Owman, L. Munn, A. D. Luster, and R. E. Gerszten.** 2003. Mechanisms of leukotriene B4--triggered monocyte adhesion. Arterioscler Thromb Vasc Biol **23:**1761-7.

87. **Funk, C. D., O. Radmark, J. Y. Fu, T. Matsumoto, H. Jornvall, T. Shimizu, and B. Samuelsson.** 1987. Molecular cloning and amino acid sequence of leukotriene A4 hydrolase. Proc Natl Acad Sci U S A **84:**6677-81.

88. **Gaidano, G., and A. Carbone.** 2000. MUM1: a step ahead toward the understanding of lymphoma histogenesis. Leukemia **14:**563-6.

89. **Ghosh, S., M. J. May, and E. B. Kopp.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol **16:**225-60.

90. **Ghosh, S. K., C. Wood, L. H. Boise, A. M. Mian, V. V. Deyev, G. Feuer, N. L. Toomey, N. C. Shank, L. Cabral, G. N. Barber, and W. J. Harrington, Jr.** 2003. Potentiation of TRAIL-induced apoptosis in primary effusion lymphoma through azidothymidine-mediated inhibition of NF-kappa B. Blood **101:**2321-7.

91. **Gnann, J. W., Jr., P. E. Pellett, and H. W. Jaffe.** 2000. Human herpesvirus 8 and Kaposi's sarcoma in persons infected with human immunodeficiency virus. Clin Infect Dis **30 Suppl 1:**S72-6.

92. **Godden-Kent, D., S. J. Talbot, C. Boshoff, Y. Chang, P. Moore, R. A. Weiss, and S. Mittnacht.** 1997. The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J Virol **71:**4193-8.

93. **Goetzl, E. J., and W. C. Pickett.** 1981. Novel structural determinants of the human neutrophil chemotactic activity of leukotriene B. J Exp Med **153:**482-7.

94. **Goldsby, R. A., T. J. Kindt, and B. A. Osborne.** 2000. Kuby Immunology, 4th ed. W. H. Freeman and Company.

95. **Goodarzi, K., M. Goodarzi, A. M. Tager, A. D. Luster, and U. H. von Andrian.** 2003. Leukotriene B4 and BLT1 control cytotoxic effector T cell recruitment to inflamed tissues. Nat Immunol **4:**965-73.

96. **Green, I., E. Espiritu, M. Ladanyi, R. Chaponda, R. Wieczorek, L. Gallo, and H. Feiner.** 1995. Primary lymphomatous effusions in AIDS: a morphological, immunophenotypic, and molecular study. Mod Pathol **8:**39-45.

97. **Greten, F. R., L. Eckmann, T. F. Greten, J. M. Park, Z. W. Li, L. J. Egan, M. F. Kagnoff, and M. Karin.** 2004. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell **118:**285-96.

98. **Greten, F. R., and M. Karin.** 2004. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett **206:**193-9.

99. **Grimminger, F., U. Sibelius, and W. Seeger.** 1991. Amplification of LTB4 generation in AM-PMN cocultures: transcellular 5-lipoxygenase metabolism. Am J Physiol **261:**L195-203.

100. **Groves, A. K., M. A. Cotter, C. Subramanian, and E. S. Robertson.** 2001. The latency-associated nuclear antigen encoded by Kaposi's sarcoma-associated herpesvirus activates two major essential Epstein-Barr virus latent promoters. J Virol **75:**9446-57.

101. **Grumont, R. J., and S. Gerondakis.** 2000. Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. J Exp Med **191:**1281-92.

102. **Gstaiger, M., L. Knoepfel, O. Georgiev, W. Schaffner, and C. M. Hovens.** 1995. A B-cell coactivator of octamer-binding transcription factors. Nature **373:**360-2.

103. **Guasparri, I., S. A. Keller, and E. Cesarman.** 2004. KSHV vFLIP is essential for the survival of infected lymphoma cells. J Exp Med **199:**993-1003.

104. **Gupta, R. A., and R. N. Dubois.** 2001. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer **1:**11-21.

105. **Gupta, S., M. Jiang, A. Anthony, and A. B. Pernis.** 1999. Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. J Exp Med **190:**1837-48.

106. **Guschin, D., N. Rogers, J. Briscoe, B. Witthuhn, D. Watling, F. Horn, S. Pellegrini, K. Yasukawa, P. Heinrich, G. R. Stark, and et al.** 1995. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. Embo J **14:**1421-9.

107. **Hagman, J., and R. Grosschedl.** 1992. An inhibitory carboxyl-terminal domain in Ets-1 and Ets-2 mediates differential binding of ETS family factors to promoter sequences of the mb-1 gene. Proc Natl Acad Sci U S A **89:**8889-93.

108. **Halfdanarson, T. R., S. N. Markovic, U. Kalokhe, and M. Luppi.** 2006. A non-chemotherapy treatment of a primary effusion lymphoma: durable remission after intracavitary cidofovir in HIV negative PEL refractory to chemotherapy. Ann Oncol.

109. **Hamoudi, R., T. C. Diss, E. Oksenhendler, L. Pan, A. Carbone, V. Ascoli, C. Boshoff, P. Isaacson, and M. Q. Du.** 2004. Distinct cellular origins of primary effusion lymphoma with and without EBV infection. Leuk Res **28:**333-8.

110. **Harada, H., K. Nakagawa, M. Saito, S. Kohno, S. Nagato, K. Furukawa, Y. Kumon, K. Hamada, and T. Ohnishi.** 2003. Introduction of wild-type p53 enhances thrombospondin-1 expression in human glioma cells. Cancer Lett **191:**109-19.

111. **Hardwick, J. M.** 2000. Cyclin' on the viral path to destruction. Nat Cell Biol **2:**E203-4.

112. **Hebert, M. J., T. Takano, H. Holthofer, and H. R. Brady.** 1996. Sequential morphologic events during apoptosis of human neutrophils. Modulation by lipoxygenase-derived eicosanoids. J Immunol **157:**3105-15.

113. **Hecht, J. L., and J. C. Aster.** 2000. Molecular biology of Burkitt's lymphoma. J Clin Oncol **18:**3707-21.

114. **Hertel, C. B., X. G. Zhou, S. J. Hamilton-Dutoit, and S. Junker.** 2002. Loss of B cell identity correlates with loss of B cell-specific transcription factors in Hodgkin/Reed-Sternberg cells of classical Hodgkin lymphoma. Oncogene **21:**4908-20.

115. **Hess, J., P. J. Nielsen, K. D. Fischer, H. Bujard, and T. Wirth.** 2001. The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. Mol Cell Biol **21:**1531-9.

116. **Hideshima, T., D. Chauhan, G. Teoh, N. Raje, S. P. Treon, Y. T. Tai, Y. Shima, and K. C. Anderson.** 2000. Characterization of signaling cascades triggered by human interleukin-6 versus Kaposi's sarcoma-associated herpes virus-encoded viral interleukin 6. Clin Cancer Res **6:**1180-9.

117. **Himmelmann, A., A. Riva, G. L. Wilson, B. P. Lucas, C. Thevenin, and J. H. Kehrl.** 1997. PU.1/Pip and basic helix loop helix zipper transcription factors interact with binding sites in the CD20 promoter to help confer lineage- and stage-specific expression of CD20 in B lymphocytes. Blood **90:**3984-95.

118. **Himmelmann, A., C. Thevenin, K. Harrison, and J. H. Kehrl.** 1996. Analysis of the Bruton's tyrosine kinase gene promoter reveals critical PU.1 and SP1 sites. Blood **87:**1036-44.

119. **Hiscott, J., P. Pitha, P. Genin, H. Nguyen, C. Heylbroeck, Y. Mamane, M. Algarte, and R. Lin.** 1999. Triggering the interferon response: the role of IRF-3 transcription factor. J Interferon Cytokine Res **19:**1-13.

120. **Holtschke, T., J. Lohler, Y. Kanno, T. Fehr, N. Giese, F. Rosenbauer, J. Lou, K. P. Knobeloch, L. Gabriele, J. F. Waring, M. F. Bachmann, R. M. Zinkernagel, H. C. Morse, 3rd, K. Ozato, and I. Horak.** 1996. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. Cell **87:**307-17.

121. **Horenstein, M. G., R. G. Nador, A. Chadburn, E. M. Hyjek, G. Inghirami, D. M. Knowles, and E. Cesarman.** 1997. Epstein-Barr virus latent gene expression in primary effusion lymphomas containing Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8. Blood **90:**1186-91.

122. **Hu, C. J., S. Rao, D. L. Ramirez-Bergeron, L. A. Garrett-Sinha, S. Gerondakis, M. R. Clark, and M. C. Simon.** 2001. PU.1/Spi-B regulation of c-rel is essential for mature B cell survival. Immunity **15:**545-55.

123. **Huang, L., A. Zhao, F. Wong, J. M. Ayala, M. Struthers, F. Ujjainwalla, S. D. Wright, M. S. Springer, J. Evans, and J. Cui.** 2004. Leukotriene B4 strongly increases monocyte chemoattractant protein-1 in human monocytes. Arterioscler Thromb Vasc Biol **24:**1783-8.

124. **Huang, W. W., E. A. Garcia-Zepeda, A. Sauty, H. C. Oettgen, M. E. Rothenberg, and A. D. Luster.** 1998. Molecular and biological characterization of the murine leukotriene B4 receptor expressed on eosinophils. J Exp Med **188:**1063-74.

125. **Hyun, T. S., C. Subramanian, M. A. Cotter, 2nd, R. A. Thomas, and E. S. Robertson.** 2001. Latency-associated nuclear antigen encoded by Kaposi's sarcoma-associated herpesvirus interacts with Tat and activates the long terminal repeat of human immunodeficiency virus type 1 in human cells. J Virol **75:**8761-71.

126. **Ichinose, Y., K. Genka, T. Koike, H. Kato, Y. Watanabe, T. Mori, S. Iioka, A. Sakuma, and M. Ohta.** 2003. Randomized double-blind placebo-controlled trial of bestatin in patients with resected stage I squamous-cell lung carcinoma. J Natl Cancer Inst **95:**605-10.

127. **Iizuka, Y., T. Yokomizo, K. Terawaki, M. Komine, K. Tamaki, and T. Shimizu.** 2005. Characterization of a mouse second leukotriene B4 receptor, mBLT2: BLT2-dependent ERK activation and cell migration of primary mouse keratinocytes. J Biol Chem **280:**24816-23.

128. **Iversen, L., V. A. Ziboh, T. Shimizu, N. Ohishi, O. Radmark, A. Wetterholm, and K. Kragballe.** 1994. Identification and subcellular localization of leukotriene A4-hydrolase activity in human epidermis. J Dermatol Sci **7:**191-201.

129. **Jakobsson, P. J., B. Odlander, and H. E. Claesson.** 1991. Effects of monocyte-lymphocyte interaction on the synthesis of leukotriene B4. Eur J Biochem **196:**395-400.

130. **Jakobsson, P. J., Odlander, B., Yamaoka, K. A., Rosen A., and Claesson H. E.** 1990. Biosynthesis and Biological Effects of Leukotriene B_4 in B lymphocytes. Advances in Prostaglandin, Thromboxane, and Leukotriene Research **21:**1005 - 1012.

131. **Janssen-Timmen, U., P. Vickers, U. Beilecke, W. D. Lehmann, H. J. Stark, N. E. Fusenig, T. Rosenbach, M. Goerig, O. Radmark, B. Samuelsson, and et al.** 1995. 5-lipoxygenase expression in cultured human keratinocytes. Adv Prostaglandin Thromboxane Leukot Res **23:**329-31.

132. **Jenner, R. G., M. M. Alba, C. Boshoff, and P. Kellam.** 2001. Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J Virol **75:**891-902.

133. **Jenner, R. G., K. Maillard, N. Cattini, R. A. Weiss, C. Boshoff, R. Wooster, and P. Kellam.** 2003. Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. Proc Natl Acad Sci U S A **100:**10399-404.

134. **Jeong, J. H., J. Orvis, J. W. Kim, C. P. McMurtrey, R. Renne, and D. P. Dittmer.** 2004. Regulation and autoregulation of the promoter for the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J Biol Chem **279:**16822-31.

135. **Jones, K. D., Y. Aoki, Y. Chang, P. S. Moore, R. Yarchoan, and G. Tosato.** 1999. Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. Blood **94:**2871-9.

136. **Junker, S., M. Lamm, V. Nielsen, and P. Matthias.** 1997. Extinction of immunoglobulin gene expression in B cells upon fusion with HeLa cells is preceded by rapid nuclear depletion of essential transcription factors and is accompanied by widespread inactivation of genes expressed in a B cell-specific manner. J Cell Sci **110 (Pt 20):**2579-87.

137. **Junker, S., S. Pedersen, E. Schreiber, and P. Matthias.** 1990. Extinction of an immunoglobulin kappa promoter in cell hybrids is mediated by the octamer motif and correlates with suppression of Oct-2 expression. Cell **61:**467-74.

138. **Karin, M.** 1999. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene **18:**6867-74.

139. **Karin, M.** 2005. Inflammation and cancer: the long reach of Ras. Nat Med **11:**20-1.

140. **Karin, M.** 2004. Mitogen activated protein kinases as targets for development of novel anti-inflammatory drugs. Ann Rheum Dis **63 Suppl 2:**ii62-ii64.

141. **Karin, M., Y. Cao, F. R. Greten, and Z. W. Li.** 2002. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer **2:**301-10.

142. **Kaser, A., S. Dunzendorfer, F. A. Offner, O. Ludwiczek, B. Enrich, R. O. Koch, W. W. Cruikshank, C. J. Wiedermann, and H. Tilg.** 2000. B lymphocyte-derived IL-16 attracts dendritic cells and Th cells. J Immunol **165:**2474-80.

143. **Kellam, P., C. Boshoff, D. Whitby, S. Matthews, R. A. Weiss, and S. J. Talbot.** 1997. Identification of a major latent nuclear antigen, LNA-1, in the human herpesvirus 8 genome. J Hum Virol **1:**19-29.

144. **Keller, S. A., E. J. Schattner, and E. Cesarman.** 2000. Inhibition of NF-kappaB induces apoptosis of KSHV-infected primary effusion lymphoma cells. Blood **96:**2537-42.

145. **Kim, B. C., and J. H. Kim.** 1997. Nuclear signalling by rac GTPase: essential role of phospholipase A2. Biochem J **326 (Pt 2):**333-7.

146. **Kim, U., X. F. Qin, S. Gong, S. Stevens, Y. Luo, M. Nussenzweig, and R. G. Roeder.** 1996. The B-cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. Nature **383:**542-7.

147. **Kis, L. L., M. Takahara, N. Nagy, G. Klein, and E. Klein.** 2006. Cytokine mediated induction of the major Epstein-Barr virus (EBV)-encoded transforming protein, LMP-1. Immunol Lett **104:**83-8.

148. **Klein, U., S. Casola, G. Cattoretti, Q. Shen, M. Lia, T. Mo, T. Ludwig, K. Rajewsky, and R. Dalla-Favera.** 2006. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. Nat Immunol **7:**773-82.

149. **Knowles, D. M., G. Inghirami, A. Ubriaco, and R. Dalla-Favera.** 1989. Molecular genetic analysis of three AIDS-associated neoplasms of uncertain lineage demonstrates their B-cell derivation and the possible pathogenetic role of the Epstein-Barr virus. Blood **73:**792-9.

150. **Komatsu, T., M. E. Ballestas, A. J. Barbera, B. Kelley-Clarke, and K. M. Kaye.** 2004. KSHV LANA1 binds DNA as an oligomer and residues N-terminal

to the oligomerization domain are essential for DNA binding, replication, and episome persistence. Virology **319**:225-36.

151. **Konig, H., P. Pfisterer, L. M. Corcoran, and T. Wirth.** 1995. Identification of CD36 as the first gene dependent on the B-cell differentiation factor Oct-2. Genes Dev **9**:1598-607.

152. **Krithivas, A., D. B. Young, G. Liao, D. Greene, and S. D. Hayward.** 2000. Human herpesvirus 8 LANA interacts with proteins of the mSin3 corepressor complex and negatively regulates Epstein-Barr virus gene expression in dually infected PEL cells. J Virol **74**:9637-45.

153. **Kulbe, H., N. R. Levinson, F. Balkwill, and J. L. Wilson.** 2004. The chemokine network in cancer--much more than directing cell movement. Int J Dev Biol **48**:489-96.

154. **Kumar, G., S. Gupta, S. Wang, and A. E. Nel.** 1994. Involvement of Janus kinases, p52shc, Raf-1, and MEK-1 in the IL-6-induced mitogen-activated protein kinase cascade of a growth-responsive B cell line. J Immunol **153**:4436-47.

155. **Laman, H., D. J. Mann, and N. C. Jones.** 2000. Viral-encoded cyclins. Curr Opin Genet Dev **10**:70-4.

156. **Lan, K., D. A. Kuppers, and E. S. Robertson.** 2005. Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein Jkappa, the major downstream effector of the Notch signaling pathway. J Virol **79**:3468-78.

157. **Lee, C. H., M. Melchers, H. Wang, T. A. Torrey, R. Slota, C. F. Qi, J. Y. Kim, P. Lugar, H. J. Kong, L. Farrington, B. van der Zouwen, J. X. Zhou, V. Lougaris, P. E. Lipsky, A. C. Grammer, and H. C. Morse, 3rd.** 2006. Regulation of the germinal center gene program by interferon (IFN) regulatory factor 8/IFN consensus sequence-binding protein. J Exp Med **203**:63-72.

158. **Lee, R. K., J. P. Cai, V. Deyev, P. S. Gill, L. Cabral, C. Wood, R. P. Agarwal, W. Xia, L. H. Boise, E. Podack, and W. J. Harrington, Jr.** 1999. Azidothymidine and interferon-alpha induce apoptosis in herpesvirus-associated lymphomas. Cancer Res **59**:5514-20.

159. **Lewis, R. A., E. J. Goetzl, J. M. Drazen, N. A. Soter, K. F. Austen, and E. J. Corey.** 1981. Functional characterization of synthetic leukotriene B and its stereochemical isomers. J Exp Med **154**:1243-8.

160. **Li, H., H. Wang, and J. Nicholas.** 2001. Detection of direct binding of human herpesvirus 8-encoded interleukin-6 (vIL-6) to both gp130 and IL-6 receptor (IL-6R) and identification of amino acid residues of vIL-6 important for IL-6R-dependent and -independent signaling. J Virol **75:**3325-34.

161. **Li, M., H. Lee, D. W. Yoon, J. C. Albrecht, B. Fleckenstein, F. Neipel, and J. U. Jung.** 1997. Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. J Virol **71:**1984-91.

162. **Lim, C., Y. Gwack, S. Hwang, S. Kim, and J. Choe.** 2001. The transcriptional activity of cAMP response element-binding protein-binding protein is modulated by the latency associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J Biol Chem **276:**31016-22.

163. **Liu, C., Y. Okruzhnov, H. Li, and J. Nicholas.** 2001. Human herpesvirus 8 (HHV-8)-encoded cytokines induce expression of and autocrine signaling by vascular endothelial growth factor (VEGF) in HHV-8-infected primary-effusion lymphoma cell lines and mediate VEGF-independent antiapoptotic effects. J Virol **75:**10933-40.

164. **Liu, L., M. T. Eby, N. Rathore, S. K. Sinha, A. Kumar, and P. M. Chaudhary.** 2002. The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the Ikappa B kinase complex. J Biol Chem **277:**13745-51.

165. **Lu, F., L. Day, S. J. Gao, and P. M. Lieberman.** 2006. Acetylation of the latency-associated nuclear antigen regulates repression of Kaposi's sarcoma-associated herpesvirus lytic transcription. J Virol **80:**5273-82.

166. **Lu, R., K. L. Medina, D. W. Lancki, and H. Singh.** 2003. IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development. Genes Dev **17:**1703-8.

167. **Maclouf, J. A., and R. C. Murphy.** 1988. Transcellular metabolism of neutrophil-derived leukotriene A4 by human platelets. A potential cellular source of leukotriene C4. J Biol Chem **263:**174-81.

168. **Mamane, Y., C. Heylbroeck, P. Genin, M. Algarte, M. J. Servant, C. LePage, C. DeLuca, H. Kwon, R. Lin, and J. Hiscott.** 1999. Interferon regulatory factors: the next generation. Gene **237:**1-14.

169. **Mamane, Y., M. Loignon, J. Palmer, E. Hernandez, R. Cesaire, M. Alaoui-Jamali, and J. Hiscott.** 2005. Repression of DNA repair mechanisms in IRF-4-expressing and HTLV-I-infected T lymphocytes. J Interferon Cytokine Res **25:**43-51.

170. **Mamane, Y., S. Sharma, N. Grandvaux, E. Hernandez, and J. Hiscott.** 2002. IRF-4 activities in HTLV-I-induced T cell leukemogenesis. J Interferon Cytokine Res **22:**135-43.

171. **Mamane, Y., S. Sharma, L. Petropoulos, R. Lin, and J. Hiscott.** 2000. Posttranslational regulation of IRF-4 activity by the immunophilin FKBP52. Immunity **12:**129-40.

172. **Mancini, J. A., and J. F. Evans.** 1995. Cloning and characterization of the human leukotriene A4 hydrolase gene. Eur J Biochem **231:**65-71.

173. **Mann, D. J., E. S. Child, C. Swanton, H. Laman, and N. Jones.** 1999. Modulation of p27(Kip1) levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. Embo J **18:**654-63.

174. **Marafioti, T., M. Pozzobon, M. L. Hansmann, G. Delsol, S. A. Pileri, and D. Y. Mason.** 2004. Expression of intracellular signaling molecules in classical and lymphocyte predominance Hodgkin disease. Blood **103:**188-93.

175. **Marcus, A. J., B. B. Weksler, E. A. Jaffe, and M. J. Broekman.** 1980. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest **66:**979-86.

176. **Marecki, S., and M. J. Fenton.** 2000. PU.1/Interferon Regulatory Factor interactions: mechanisms of transcriptional regulation. Cell Biochem Biophys **33:**127-48.

177. **Marecki, S., C. J. Riendeau, M. D. Liang, and M. J. Fenton.** 2001. PU.1 and multiple IFN regulatory factor proteins synergize to mediate transcriptional activation of the human IL-1 beta gene. J Immunol **166:**6829-38.

178. **Mathas, S., M. Hinz, I. Anagnostopoulos, D. Krappmann, A. Lietz, F. Jundt, K. Bommert, F. Mechta-Grigoriou, H. Stein, B. Dorken, and C. Scheidereit.** 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:**4104-13.

179. **Matolcsy, A., R. G. Nador, E. Cesarman, and D. M. Knowles.** 1998. Immunoglobulin VH gene mutational analysis suggests that primary effusion lymphomas derive from different stages of B cell maturation. Am J Pathol **153:**1609-14.

180. **Matsuda, T., Y. Yamanaka, and T. Hirano.** 1994. Interleukin-6-induced tyrosine phosphorylation of multiple proteins in murine hematopoietic lineage cells. Biochem Biophys Res Commun **200:**821-8.

181. **Matta, H., and P. M. Chaudhary.** 2004. Activation of alternative NF-kappa B pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 beta-converting enzyme inhibitory protein (vFLIP). Proc Natl Acad Sci U S A **101:**9399-404.

182. **Matthias, P.** 1998. Lymphoid-specific transcription mediated by the conserved octamer site: who is doing what? Semin Immunol **10:**155-63.

183. **McDonald, P. P., A. Bald, and M. A. Cassatella.** 1997. Activation of the NF-kappaB pathway by inflammatory stimuli in human neutrophils. Blood **89:**3421-33.

184. **McGee, J. E., and F. A. Fitzpatrick.** 1986. Erythrocyte-neutrophil interactions: formation of leukotriene B4 by transcellular biosynthesis. Proc Natl Acad Sci U S A **83:**1349-53.

185. **McKercher, S. R., B. E. Torbett, K. L. Anderson, G. W. Henkel, D. J. Vestal, H. Baribault, M. Klemsz, A. J. Feeney, G. E. Wu, C. J. Paige, and R. A. Maki.** 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. Embo J **15:**5647-58.

186. **Meads, M. B., and P. G. Medveczky.** 2004. Kaposi's sarcoma-associated herpesvirus-encoded viral interleukin-6 is secreted and modified differently than human interleukin-6: evidence for a unique autocrine signaling mechanism. J Biol Chem **279:**51793-803.

187. **Michiels, L., and J. Merregaert.** 1993. Retroviruses and oncogenes associated with osteosarcomas. Cancer Treat Res **62:**7-18.

188. **Miles, S. A., A. R. Rezai, J. F. Salazar-Gonzalez, M. Vander Meyden, R. H. Stevens, D. M. Logan, R. T. Mitsuyasu, T. Taga, T. Hirano, T. Kishimoto, and et al.** 1990. AIDS Kaposi sarcoma-derived cells produce and respond to interleukin 6. Proc Natl Acad Sci U S A **87:**4068-72.

189. **Miller, D. K., J. W. Gillard, P. J. Vickers, S. Sadowski, C. Leveille, J. A. Mancini, P. Charleson, R. A. Dixon, A. W. Ford-Hutchinson, R. Fortin, and et al.** 1990. Identification and isolation of a membrane protein necessary for leukotriene production. Nature **343:**278-81.

190. **Mittrucker, H. W., T. Matsuyama, A. Grossman, T. M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P. S. Ohashi, and T. W. Mak.** 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science **275:**540-3.

191. **Mizuno, T., and T. L. Rothstein.** 2005. B cell receptor (BCR) cross-talk: CD40 engagement creates an alternate pathway for BCR signaling that activates I

kappa B kinase/I kappa B alpha/NF-kappa B without the need for PI3K and phospholipase C gamma. J Immunol **174:**6062-70.

192. **Mizuno, T., and T. L. Rothstein.** 2005. B cell receptor (BCR) cross-talk: CD40 engagement enhances BCR-induced ERK activation. J Immunol **174:**3369-76.

193. **Monini, P., F. Carlini, M. Sturzl, P. Rimessi, F. Superti, M. Franco, G. Melucci-Vigo, A. Cafaro, D. Goletti, C. Sgadari, S. Butto, P. Leone, C. Chiozzini, C. Barresi, A. Tinari, A. Bonaccorsi, M. R. Capobianchi, M. Giuliani, A. di Carlo, M. Andreoni, G. Rezza, and B. Ensoli.** 1999. Alpha interferon inhibits human herpesvirus 8 (HHV-8) reactivation in primary effusion lymphoma cells and reduces HHV-8 load in cultured peripheral blood mononuclear cells. J Virol **73:**4029-41.

194. **Moore, K. L., K. D. Patel, R. E. Bruehl, F. Li, D. A. Johnson, H. S. Lichenstein, R. D. Cummings, D. F. Bainton, and R. P. McEver.** 1995. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J Cell Biol **128:**661-71.

195. **Morita, H., K. Takeda, H. Yagita, and K. Okumura.** 1999. Immunosuppressive effect of leukotriene B(4) receptor antagonist in vitro. Biochem Biophys Res Commun **264:**321-6.

196. **Mueller, B. U., T. Pabst, M. Osato, N. Asou, L. M. Johansen, M. D. Minden, G. Behre, W. Hiddemann, Y. Ito, and D. G. Tenen.** 2002. Heterozygous PU.1 mutations are associated with acute myeloid leukemia. Blood **100:**998-1007.

197. **Muromoto, R., K. Okabe, M. Fujimuro, K. Sugiyama, H. Yokosawa, T. Seya, and T. Matsuda.** 2006. Physical and functional interactions between STAT3 and Kaposi's sarcoma-associated herpesvirus-encoded LANA. FEBS Lett **580:**93-8.

198. **Nador, R. G., E. Cesarman, A. Chadburn, D. B. Dawson, M. Q. Ansari, J. Sald, and D. M. Knowles.** 1996. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. Blood **88:**645-56.

199. **Nagy, M., B. Chapuis, and T. Matthes.** 2002. Expression of transcription factors Pu.1, Spi-B, Blimp-1, BSAP and oct-2 in normal human plasma cells and in multiple myeloma cells. Br J Haematol **116:**429-35.

200. **Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. Proc Natl Acad Sci U S A **90:**2207-11.

201. **Neipel, F., J. C. Albrecht, A. Ensser, Y. Q. Huang, J. J. Li, A. E. Friedman-Kien, and B. Fleckenstein.** 1997. Human herpesvirus 8 encodes a homolog of interleukin-6. J Virol **71:**839-42.

202. **Nelsen, B., G. Tian, B. Erman, J. Gregoire, R. Maki, B. Graves, and R. Sen.** 1993. Regulation of lymphoid-specific immunoglobulin mu heavy chain gene enhancer by ETS-domain proteins. Science **261:**82-6.

203. **Neu, I., J. Mallinger, A. Wildfeuer, and L. Mehlber.** 1992. Leukotrienes in the cerebrospinal fluid of multiple sclerosis patients. Acta Neurol Scand **86:**586-7.

204. **Nishimura, T., and P. K. Vogt.** 1988. The avian cellular homolog of the oncogene jun. Oncogene **3:**659-63.

205. **Nishiya, N., K. Yamamoto, Y. Imaizumi, T. Kohno, and T. Matsuyama.** 2004. Identification of a novel GC-rich binding protein that binds to an indispensable element for constitutive IRF-4 promoter activity in B cells. Mol Immunol **41:**855-61.

206. **Nissen, J. B., L. Iversen, and K. Kragballe.** 1995. Characterization of the aminopeptidase activity of epidermal leukotriene A4 hydrolase against the opioid dynorphin fragment 1-7. Br J Dermatol **133:**742-9.

207. **Ohishi, N., T. Izumi, M. Minami, S. Kitamura, Y. Seyama, S. Ohkawa, S. Terao, H. Yotsumoto, F. Takaku, and T. Shimizu.** 1987. Leukotriene A4 hydrolase in the human lung. Inactivation of the enzyme with leukotriene A4 isomers. J Biol Chem **262:**10200-5.

208. **Ohishi, N., M. Minami, J. Kobayashi, Y. Seyama, J. Hata, H. Yotsumoto, F. Takaku, and T. Shimizu.** 1990. Immunological quantitation and immunohistochemical localization of leukotriene A4 hydrolase in guinea pig tissues. J Biol Chem **265:**7520-5.

209. **Ohkubo, Y., M. Arima, E. Arguni, S. Okada, K. Yamashita, S. Asari, S. Obata, A. Sakamoto, M. Hatano, O. W. J, M. Ebara, H. Saisho, and T. Tokuhisa.** 2005. A role for c-fos/activator protein 1 in B lymphocyte terminal differentiation. J Immunol **174:**7703-10.

210. **Oksenhendler, E., G. Carcelain, Y. Aoki, E. Boulanger, A. Maillard, J. P. Clauvel, and F. Agbalika.** 2000. High levels of human herpesvirus 8 viral load, human interleukin-6, interleukin-10, and C reactive protein correlate with exacerbation of multicentric castleman disease in HIV-infected patients. Blood **96:**2069-73.

211. **Omori, S. A., and R. Wall.** 1993. Multiple motifs regulate the B-cell-specific promoter of the B29 gene. Proc Natl Acad Sci U S A **90:**11723-7.

212. **Orning, L., J. K. Gierse, and F. A. Fitzpatrick.** 1994. The bifunctional enzyme leukotriene-A4 hydrolase is an arginine aminopeptidase of high efficiency and specificity. J Biol Chem **269:**11269-73.

213. **Orning, L., G. Krivi, G. Bild, J. Gierse, S. Aykent, and F. A. Fitzpatrick.** 1991. Inhibition of leukotriene A4 hydrolase/aminopeptidase by captopril. J Biol Chem **266:**16507-11.

214. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci U S A **86:**2336-40.

215. **Oshima, M., N. Murai, S. Kargman, M. Arguello, P. Luk, E. Kwong, M. M. Taketo, and J. F. Evans.** 2001. Chemoprevention of intestinal polyposis in the Apcdelta716 mouse by rofecoxib, a specific cyclooxygenase-2 inhibitor. Cancer Res **61:**1733-40.

216. **Owman, C., S. J. Lolait, S. Santen, and B. Olde.** 1997. Molecular cloning and tissue distribution of cDNA encoding a novel chemoattractant-like receptor. Biochem Biophys Res Commun **241:**390-4.

217. **Owman, C., C. Nilsson, and S. J. Lolait.** 1996. Cloning of cDNA encoding a putative chemoattractant receptor. Genomics **37:**187-94.

218. **Owman, C., A. Sabirsh, A. Boketoft, and B. Olde.** 1997. Leukotriene B4 is the functional ligand binding to and activating the cloned chemoattractant receptor, CMKRL1. Biochem Biophys Res Commun **240:**162-6.

219. **Pace, E., M. Profita, M. Melis, A. Bonanno, A. Paterno, C. H. Mody, M. Spatafora, M. Ferraro, L. Siena, A. M. Vignola, G. Bonsignore, and M. Gjomarkaj.** 2004. LTB4 is present in exudative pleural effusions and contributes actively to neutrophil recruitment in the inflamed pleural space. Clin Exp Immunol **135:**519-27.

220. **Pahl, H. L.** 1999. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene **18:**6853-66.

221. **Palmblad, J., R. Lerner, and S. H. Larsson.** 1994. Signal transduction mechanisms for leukotriene B4 induced hyperadhesiveness of endothelial cells for neutrophils. J Immunol **152:**262-9.

222. **Palmblad, J., C. L. Malmsten, A. M. Uden, O. Radmark, L. Engstedt, and B. Samuelsson.** 1981. Leukotriene B4 is a potent and stereospecific stimulator of neutrophil chemotaxis and adherence. Blood **58:**658-61.

223. **Parravicini, C., B. Chandran, M. Corbellino, E. Berti, M. Paulli, P. S. Moore, and Y. Chang.** 2000. Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Am J Pathol **156:**743-9.

224. **Penning, T. D., M. A. Russell, B. B. Chen, H. Y. Chen, C. D. Liang, M. W. Mahoney, J. W. Malecha, J. M. Miyashiro, S. S. Yu, L. J. Askonas, J. K. Gierse, E. I. Harding, M. K. Highkin, J. F. Kachur, S. H. Kim, D. Villani-Price, E. Y. Pyla, N. S. Ghoreishi-Haack, and W. G. Smith.** 2002. Synthesis of potent leukotriene A(4) hydrolase inhibitors. Identification of 3-[methyl[3-[4-(phenylmethyl)phenoxy]propyl]amino]propanoic acid. J Med Chem **45:**3482-90.

225. **Peters-Golden, M., and R. W. McNish.** 1993. Redistribution of 5-lipoxygenase and cytosolic phospholipase A2 to the nuclear fraction upon macrophage activation. Biochem Biophys Res Commun **196:**147-53.

226. **Pettersson, A., J. Richter, and C. Owman.** 2003. Flow cytometric mapping of the leukotriene B4 receptor, BLT1, in human bone marrow and peripheral blood using specific monoclonal antibodies. Int Immunopharmacol **3:**1467-75.

227. **Pongubala, J. M., S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison.** 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity. Mol Cell Biol **12:**368-78.

228. **Pongubala, J. M., C. Van Beveren, S. Nagulapalli, M. J. Klemsz, S. R. McKercher, R. A. Maki, and M. L. Atchison.** 1993. Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. Science **259:**1622-5.

229. **Radkov, S. A., P. Kellam, and C. Boshoff.** 2000. The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. Nat Med **6:**1121-7.

230. **Radmark, O., T. Shimizu, H. Jornvall, and B. Samuelsson.** 1984. Leukotriene A4 hydrolase in human leukocytes. Purification and properties. J Biol Chem **259:**12339-45.

231. **Radomska, H. S., C. P. Shen, T. Kadesch, and L. A. Eckhardt.** 1994. Constitutively expressed Oct-2 prevents immunoglobulin gene silencing in myeloma x T cell hybrids. Immunity **1:**623-34.

232. **Raport, C. J., V. L. Schweickart, D. Chantry, R. L. Eddy, Jr., T. B. Shows, R. Godiska, and P. W. Gray.** 1996. New members of the chemokine receptor gene family. J Leukoc Biol **59:**18-23.

233. **Re, D., M. Muschen, T. Ahmadi, C. Wickenhauser, A. Staratschek-Jox, U. Holtick, V. Diehl, and J. Wolf.** 2001. Oct-2 and Bob-1 deficiency in Hodgkin and Reed Sternberg cells. Cancer Res **61:**2080-4.

234. **Rehli, M., A. Poltorak, L. Schwarzfischer, S. W. Krause, R. Andreesen, and B. Beutler.** 2000. PU.1 and interferon consensus sequence-binding protein regulate the myeloid expression of the human Toll-like receptor 4 gene. J Biol Chem **275:**9773-81.

235. **Renne, R., C. Barry, D. Dittmer, N. Compitello, P. O. Brown, and D. Ganem.** 2001. Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. J Virol **75:**458-68.

236. **Rieske, P., and J. M. Pongubala.** 2001. AKT induces transcriptional activity of PU.1 through phosphorylation-mediated modifications within its transactivation domain. J Biol Chem **276:**8460-8.

237. **Rola-Pleszczynski, M., and J. Stankova.** 1992. Leukotriene B4 enhances interleukin-6 (IL-6) production and IL-6 messenger RNA accumulation in human monocytes in vitro: transcriptional and posttranscriptional mechanisms. Blood **80:**1004-11.

238. **Rosenbach, T., J. Grabbe, A. Moller, H. J. Schwanitz, and B. M. Czarnetzki.** 1985. Generation of leukotrienes from normal epidermis and their demonstration in cutaneous disease. Br J Dermatol **113 Suppl 28:**157-67.

239. **Rouzer, C. A., T. Matsumoto, and B. Samuelsson.** 1986. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A4 synthase activities. Proc Natl Acad Sci U S A **83:**857-61.

240. **Rudberg, P. C., F. Tholander, M. M. Thunnissen, and J. Z. Haeggstrom.** 2002. Leukotriene A4 hydrolase/aminopeptidase. Glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms. J Biol Chem **277:**1398-404.

241. **Rudberg, P. C., F. Tholander, M. M. Thunnissen, B. Samuelsson, and J. Z. Haeggstrom.** 2002. Leukotriene A4 hydrolase: selective abrogation of leukotriene B4 formation by mutation of aspartic acid 375. Proc Natl Acad Sci U S A **99:**4215-20.

242. **Runarsson, G., A. Liu, Y. Mahshid, S. Feltenmark, A. Pettersson, E. Klein, M. Bjorkholm, and H. E. Claesson.** 2005. Leukotriene B4 plays a pivotal role in CD40-dependent activation of chronic B lymphocytic leukemia cells. Blood **105:**1274-9.

243. **Sadowski, H. B., K. Shuai, J. E. Darnell, Jr., and M. Z. Gilman.** 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. Science **261:**1739-44.

244. **Said, J. W., T. Tasaka, S. Takeuchi, H. Asou, S. de Vos, E. Cesarman, D. M. Knowles, and H. P. Koeffler.** 1996. Primary effusion lymphoma in women: report of two cases of Kaposi's sarcoma herpes virus-associated effusion-based lymphoma in human immunodeficiency virus-negative women. Blood **88:**3124-8.

245. **Samuelsson, B., S. E. Dahlen, J. A. Lindgren, C. A. Rouzer, and C. N. Serhan.** 1987. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science **237:**1171-6.

246. **Sarid, R., O. Flore, R. A. Bohenzky, Y. Chang, and P. S. Moore.** 1998. Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J Virol **72:**1005-12.

247. **Sarid, R., S. J. Olsen, and P. S. Moore.** 1999. Kaposi's sarcoma-associated herpesvirus: epidemiology, virology, and molecular biology. Adv Virus Res **52:**139-232.

248. **Schubart, K., S. Massa, D. Schubart, L. M. Corcoran, A. G. Rolink, and P. Matthias.** 2001. B cell development and immunoglobulin gene transcription in the absence of Oct-2 and OBF-1. Nat Immunol **2:**69-74.

249. **Schug, J., and G. C. Overton.** 1997. TESS: Transcription Element Search Software on the WWW .Computational Biology and Informatics Laboratory School of Medicine University of Pennsylvania.

250. **Schulz, T. F.** 2001. KSHV/HHV8-associated lymphoproliferations in the AIDS setting. Eur J Cancer **37:**1217-26.

251. **Schwarz, M., and P. M. Murphy.** 2001. Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant. J Immunol **167:**505-13.

252. **Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh.** 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science **265:**1573-7.

253. **Sekine, K., H. Fujii, and F. Abe.** 1999. Induction of apoptosis by bestatin (ubenimex) in human leukemic cell lines. Leukemia **13:**729-34.

254. **Servant, M. J., B. ten Oever, C. LePage, L. Conti, S. Gessani, I. Julkunen, R. Lin, and J. Hiscott.** 2001. Identification of distinct signaling pathways leading to the phosphorylation of interferon regulatory factor 3. J Biol Chem **276:**355-63.

255. **Sgarbanti, M., M. Arguello, B. R. tenOever, A. Battistini, R. Lin, and J. Hiscott.** 2004. A requirement for NF-kappaB induction in the production of replication-competent HHV-8 virions. Oncogene **23:**5770-80.

256. **Sharf, R., D. Meraro, A. Azriel, A. M. Thornton, K. Ozato, E. F. Petricoin, A. C. Larner, F. Schaper, H. Hauser, and B. Z. Levi.** 1997. Phosphorylation events modulate the ability of interferon consensus sequence binding protein to interact with interferon regulatory factors and to bind DNA. J Biol Chem **272:**9785-92.

257. **Sharma, S., N. Grandvaux, Y. Mamane, P. Genin, N. Azimi, T. Waldmann, and J. Hiscott.** 2002. Regulation of IFN regulatory factor 4 expression in human T cell leukemia virus-I-transformed T cells. J Immunol **169:**3120-30.

258. **Sharon, P., and W. F. Stenson.** 1984. Enhanced synthesis of leukotriene B4 by colonic mucosa in inflammatory bowel disease. Gastroenterology **86:**453-60.

259. **Shaulian, E., and M. Karin.** 2002. AP-1 as a regulator of cell life and death. Nat Cell Biol **4:**E131-6.

260. **Sherr, C. J., and J. M. Roberts.** 2004. Living with or without cyclins and cyclin-dependent kinases. Genes Dev **18:**2699-711.

261. **Shin, M. K., and M. E. Koshland.** 1993. Ets-related protein PU.1 regulates expression of the immunoglobulin J-chain gene through a novel Ets-binding element. Genes Dev **7:**2006-15.

262. **Shindo, K., M. Fukumura, and K. Miyakawa.** 1995. Leukotriene B4 levels in the arterial blood of asthmatic patients and the effects of prednisolone. Eur Respir J **8:**605-10.

263. **Shore, P., W. Dietrich, and L. M. Corcoran.** 2002. Oct-2 regulates CD36 gene expression via a consensus octamer, which excludes the co-activator OBF-1. Nucleic Acids Res **30:**1767-73.

264. **Sid, B., H. Sartelet, G. Bellon, H. El Btaouri, G. Rath, N. Delorme, B. Haye, and L. Martiny.** 2004. Thrombospondin 1: a multifunctional protein implicated in the regulation of tumor growth. Crit Rev Oncol Hematol **49:**245-58.

265. **Simonelli, C., M. Spina, R. Cinelli, R. Talamini, R. Tedeschi, A. Gloghini, E. Vaccher, A. Carbone, and U. Tirelli.** 2003. Clinical features and outcome of primary effusion lymphoma in HIV-infected patients: a single-institution study. J Clin Oncol **21:**3948-54.

266. **Simonelli, C., R. Tedeschi, A. Gloghini, M. T. Bortolin, M. Spina, E. Bidoli, R. Cinelli, P. De Paoli, A. Carbone, and U. Tirelli.** 2005. Characterization of immunologic and virological parameters in HIV-infected patients with primary effusion lymphoma during antiblastic therapy and highly active antiretroviral therapy. Clin Infect Dis **40:**1022-7.

267. **Singh, H., K. L. Medina, and J. M. Pongubala.** 2005. Contingent gene regulatory networks and B cell fate specification. Proc Natl Acad Sci U S A **102:**4949-53.

268. **Smith, M. J., A. W. Ford-Hutchinson, and M. A. Bray.** 1980. Leukotriene B: a potential mediator of inflammation. J Pharm Pharmacol **32:**517-8.

269. **Sparmann, A., and D. Bar-Sagi.** 2004. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell **6:**447-58.

270. **Stahl, N., T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, B. A. Witthuhn, F. W. Quelle, O. Silvennoinen, G. Barbieri, S. Pellegrini, and et al.** 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science **263:**92-5.

271. **Stankova, J., S. Turcotte, J. Harris, and M. Rola-Pleszczynski.** 2002. Modulation of leukotriene B4 receptor-1 expression by dexamethasone: potential mechanism for enhanced neutrophil survival. J Immunol **168:**3570-6.

272. **Staskus, K. A., R. Sun, G. Miller, P. Racz, A. Jaslowski, C. Metroka, H. Brett-Smith, and A. T. Haase.** 1999. Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. J Virol **73:**4181-7.

273. **Sturzl, M., C. Hohenadl, C. Zietz, E. Castanos-Velez, A. Wunderlich, G. Ascherl, P. Biberfeld, P. Monini, P. J. Browning, and B. Ensoli.** 1999. Expression of K13/v-FLIP gene of human herpesvirus 8 and apoptosis in Kaposi's sarcoma spindle cells. J Natl Cancer Inst **91:**1725-33.

274. **Sun, Q., H. Matta, G. Lu, and P. M. Chaudhary.** 2006. Induction of IL-8 expression by human herpesvirus 8 encoded vFLIP K13 via NF-kappaB activation. Oncogene **25:**2717-26.

275. **Surette, M. E., A. Odeimat, R. Palmantier, S. Marleau, P. E. Poubelle, and P. Borgeat.** 1994. Reverse-phase high-performance liquid chromatography analysis of arachidonic acid metabolites in plasma after stimulation of whole blood ex vivo. Anal Biochem **216:**392-400.

276. **Swanton, C., D. J. Mann, B. Fleckenstein, F. Neipel, G. Peters, and N. Jones.** 1997. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. Nature **390:**184-7.

277. **Szekely, L., F. Chen, N. Teramoto, B. Ehlin-Henriksson, K. Pokrovskaja, A. Szeles, A. Manneborg-Sandlund, M. Lowbeer, E. T. Lennette, and G. Klein.** 1998. Restricted expression of Epstein-Barr virus (EBV)-encoded, growth transformation-associated antigens in an EBV- and human herpesvirus type 8-carrying body cavity lymphoma line. J Gen Virol **79 (Pt 6):**1445-52.

278. **Tager, A. M., S. K. Bromley, B. D. Medoff, S. A. Islam, S. D. Bercury, E. B. Friedrich, A. D. Carafone, R. E. Gerszten, and A. D. Luster.** 2003. Leukotriene B4 receptor BLT1 mediates early effector T cell recruitment. Nat Immunol **4:**982-90.

279. **Tager, A. M., J. H. Dufour, K. Goodarzi, S. D. Bercury, U. H. von Andrian, and A. D. Luster.** 2000. BLTR mediates leukotriene B(4)-induced chemotaxis and adhesion and plays a dominant role in eosinophil accumulation in a murine model of peritonitis. J Exp Med **192:**439-46.

280. **Tager, A. M., and A. D. Luster.** 2003. BLT1 and BLT2: the leukotriene B(4) receptors. Prostaglandins Leukot Essent Fatty Acids **69:**123-34.

281. **Tamura, T., and K. Ozato.** 2002. ICSBP/IRF-8: its regulatory roles in the development of myeloid cells. J Interferon Cytokine Res **22:**145-52.

282. **Tanaka, M., and W. Herr.** 1990. Differential transcriptional activation by Oct-1 and Oct-2: interdependent activation domains induce Oct-2 phosphorylation. Cell **60:**375-86.

283. **Tanner, J. E., and G. Tosato.** 1992. Regulation of B-cell growth and immunoglobulin gene transcription by interleukin-6. Blood **79:**452-9.

284. **Thomas, R. K., D. Re, J. Wolf, and V. Diehl.** 2004. Part I: Hodgkin's lymphoma--molecular biology of Hodgkin and Reed-Sternberg cells. Lancet Oncol **5:**11-8.

285. **Thunnissen, M. M., P. Nordlund, and J. Z. Haeggstrom.** 2001. Crystal structure of human leukotriene A(4) hydrolase, a bifunctional enzyme in inflammation. Nat Struct Biol **8:**131-5.

286. **Tong, W. G., X. Z. Ding, R. Hennig, R. C. Witt, J. Standop, P. M. Pour, and T. E. Adrian.** 2002. Leukotriene B4 receptor antagonist LY293111 inhibits proliferation and induces apoptosis in human pancreatic cancer cells. Clin Cancer Res **8:**3232-42.

287. **Torlakovic, E., A. Malecka, J. H. Myklebust, A. Tierens, H. C. Aasheim, J. M. Nesland, E. Smeland, S. Kvaloy, and J. Delabie.** 2005. PU.1 protein expression has a positive linear association with protein expression of germinal centre B cell genes including BCL-6, CD10, CD20 and CD22: identification of PU.1 putative binding sites in the BCL-6 promotor. J Pathol **206:**312-9.

288. **Torlakovic, E., A. Tierens, H. D. Dang, and J. Delabie.** 2001. The transcription factor PU.1, necessary for B-cell development is expressed in lymphocyte predominance, but not classical Hodgkin's disease. Am J Pathol **159:**1807-14.

289. **Tosato, G., K. B. Seamon, N. D. Goldman, P. B. Sehgal, L. T. May, G. C. Washington, K. D. Jones, and S. E. Pike.** 1988. Monocyte-derived human B-cell growth factor identified as interferon-beta 2 (BSF-2, IL-6). Science **239:**502-4.

290. **Trivedi, P., K. Takazawa, C. Zompetta, L. Cuomo, E. Anastasiadou, A. Carbone, S. Uccini, F. Belardelli, K. Takada, L. Frati, and A. Faggioni.** 2004. Infection of HHV-8+ primary effusion lymphoma cells with a recombinant Epstein-Barr virus leads to restricted EBV latency, altered phenotype, and increased tumorigenicity without affecting TCL1 expression. Blood **103:**313-6.

291. **Tsujimura, H., T. Tamura, C. Gongora, J. Aliberti, C. Reis e Sousa, A. Sher, and K. Ozato.** 2003. ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro. Blood **101:**961-9.

292. **Tsujimura, H., T. Tamura, and K. Ozato.** 2003. Cutting edge: IFN consensus sequence binding protein/IFN regulatory factor 8 drives the development of type I IFN-producing plasmacytoid dendritic cells. J Immunol **170:**1131-5.

293. **Underhill, G. H., D. George, E. G. Bremer, and G. S. Kansas.** 2003. Gene expression profiling reveals a highly specialized genetic program of plasma cells. Blood **101:**4013-21.

294. **Varthakavi, V., R. M. Smith, H. Deng, R. Sun, and P. Spearman.** 2002. Human immunodeficiency virus type-1 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus through induction of KSHV Rta. Virology **297:**270-80.

295. **Verma, S. C., S. Borah, and E. S. Robertson.** 2004. Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus up-regulates transcription of

human telomerase reverse transcriptase promoter through interaction with transcription factor Sp1. J Virol **78:**10348-59.

296. **Verschuren, E. W., N. Jones, and G. I. Evan.** 2004. The cell cycle and how it is steered by Kaposi's sarcoma-associated herpesvirus cyclin. J Gen Virol **85:**1347-61.

297. **Walts, A. E., I. P. Shintaku, and J. W. Said.** 1990. Diagnosis of malignant lymphoma in effusions from patients with AIDS by gene rearrangement. Am J Clin Pathol **94:**170-5.

298. **Weil, R., and A. Israel.** 2004. T-cell-receptor- and B-cell-receptor-mediated activation of NF-kappaB in lymphocytes. Curr Opin Immunol **16:**374-81.

299. **Werz, O., E. Burkert, B. Samuelsson, O. Radmark, and D. Steinhilber.** 2002. Activation of 5-lipoxygenase by cell stress is calcium independent in human polymorphonuclear leukocytes. Blood **99:**1044-52.

300. **Werz, O., J. Klemm, B. Samuelsson, and O. Radmark.** 2000. 5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases. Proc Natl Acad Sci U S A **97:**5261-6.

301. **Werz, O., D. Szellas, D. Steinhilber, and O. Radmark.** 2002. Arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MAPK-activated protein kinase 2 (MK2). J Biol Chem **277:**14793-800.

302. **Wood, K. M., M. Roff, and R. T. Hay.** 1998. Defective IkappaBalpha in Hodgkin cell lines with constitutively active NF-kappaB. Oncogene **16:**2131-9.

303. **Yamaoka, K. A., H. E. Claesson, and A. Rosen.** 1989. Leukotriene B4 enhances activation, proliferation, and differentiation of human B lymphocytes. J Immunol **143:**1996-2000.

304. **Yang, T. Y., S. C. Chen, M. W. Leach, D. Manfra, B. Homey, M. Wiekowski, L. Sullivan, C. H. Jenh, S. K. Narula, S. W. Chensue, and S. A. Lira.** 2000. Transgenic expression of the chemokine receptor encoded by human herpesvirus 8 induces an angioproliferative disease resembling Kaposi's sarcoma. J Exp Med **191:**445-54.

305. **Yokomizo, T., T. Izumi, K. Chang, Y. Takuwa, and T. Shimizu.** 1997. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature **387:**620-4.

306. **Yokomizo, T., K. Kato, H. Hagiya, T. Izumi, and T. Shimizu.** 2001. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. J Biol Chem **276:**12454-9.

307. **Yokomizo, T., K. Kato, K. Terawaki, T. Izumi, and T. Shimizu.** 2000. A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. J Exp Med **192:**421-32.

308. **Yoo, M. H., H. Song, C. H. Woo, H. Kim, and J. H. Kim.** 2004. Role of the BLT2, a leukotriene B(4) receptor, in Ras transformation. Oncogene.

309. **Yoshida, K., K. Yamamoto, T. Kohno, N. Hironaka, K. Yasui, C. Kojima, H. Mukae, J. Kadota, S. Suzuki, K. Honma, S. Kohno, and T. Matsuyama.** 2005. Active repression of IFN regulatory factor-1-mediated transactivation by IFN regulatory factor-4. Int Immunol **17:**1463-71.

310. **Young, L. S., and P. G. Murray.** 2003. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene **22:**5108-21.

311. **Zaitsu, M., Y. Hamasaki, M. Matsuo, A. Kukita, K. Tsuji, M. Miyazaki, R. Hayasaki, E. Muro, S. Yamamoto, I. Kobayashi, T. Ichimaru, O. Kohashi, and S. Miyazaki.** 2000. New induction of leukotriene A(4) hydrolase by interleukin-4 and interleukin-13 in human polymorphonuclear leukocytes. Blood **96:**601-9.

312. **Zhao, L., M. P. Moos, R. Grabner, F. Pedrono, J. Fan, B. Kaiser, N. John, S. Schmidt, R. Spanbroek, K. Lotzer, L. Huang, J. Cui, D. J. Rader, J. F. Evans, A. J. Habenicht, and C. D. Funk.** 2004. The 5-lipoxygenase pathway promotes pathogenesis of hyperlipidemia-dependent aortic aneurysm. Nat Med **10:**966-73.

APPENDIX

Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

04094-7-07.0

NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT | Licence Number / Numéro de permis

I) LICENSEE

Pursuant to section 24(2) of the Nuclear Safety and Control Act, this licence is issued to:

Hôpital Général Juif/
Jewish General Hospital
Sir Mortimer B. Davis
3755 Côte Ste-Catherine Road
Montréal, QC
H3T 1E2
Canada

hereinafter «the licensee».

II) PERIOD

This licence is valid from: August 1 2002 to July 31 2007.

III) LICENSED ACTIVITIES

This licence authorizes the licensee to possess, transfer, import, export, use and store the nuclear substances and the prescribed equipment listed in section IV) of this licence.

This licence is issued for: laboratory studies: 10 or more laboratories where radioisotopes are used or handled (836)

IV) NUCLEAR SUBSTANCES AND PRESCRIBED EQUIPMENT

ITEM	NUCLEAR SUBSTANCE	UNSEALED SOURCE MAXIMUM QUANTITY	SEALED SOURCE MAXIMUM QUANTITY	EQUIPMENT MAKE AND MODEL
1	Carbon 14	400 MBq	n/a	n/a
2	Calcium 45	40 MBq	n/a	n/a
3	Cerium 141	100 MBq	n/a	n/a
4	Chromium 51	100 MBq	n/a	n/a
5	Iron 55	100 MBq	n/a	n/a
6	Iron 59	1 GBq	n/a	n/a
7	Hydrogen 3	2 GBq	n/a	n/a
8	Iodine 125	1 GBq	n/a	n/a
9	Phosphorus 32	5 GBq	n/a	n/a
10	Phosphorus 33	100 MBq	n/a	n/a
11	Sulfur 35	3 GBq	n/a	n/a
12	Scandium 46	100 MBq	n/a	n/a
13	Strontium 85	20 MBq	n/a	n/a
14	Cesium 137	n/a	40 kBq	n/a
15	Cesium 137	n/a	1480 kBq	Beckman LS (series)
16	Radium 226	n/a	370 kBq	PerkinElmer Wallac 1200 series LS Counters
17	Europium 152	n/a	740 kBq	PerkinElmer Wallac 1400 series LS Counters

The total quantity of an unsealed nuclear substance in possession shall not exceed the corresponding listed unsealed source maximum quantity. The total quantity of nuclear substance per sealed source shall not exceed its corresponding listed sealed source maximum quantity. Sealed sources shall only be used in the corresponding listed equipment.

V) LOCATION(S) OF LICENSED ACTIVITIES

used or stored at:

Lady Davis Institute for Medical
Research
3755 Côte Ste-Catherine Road
Montréal, QC

VI) CONDITIONS

1. Prohibition of Human Use
This licence does not authorize the use of nuclear substances in or on human beings.
(2696-0)

2. Area Classification
The licensee shall classify each room, area or enclosure where more than one exemption quantity of an unsealed nuclear substance is used at a single time as:

(a) basic-level if the quantity does not exceed 5 ALI,
(b) intermediate-level if the quantity used does not exceed 50 ALI,
(c) high-level if the quantity does not exceed 500 ALI,
(d) containment-level if the quantity exceeds 500 ALI; or
(e) special purpose if approved in writing by the Commission or a person authorized by the Commission.

Except for the basic-level classification, the licensee shall not use unsealed nuclear substances in these rooms, areas or enclosures without written approval of the Commission or a person authorized by the Commission.
(2108-1)

3. Laboratory Lists
The licensee shall maintain a list of all areas, rooms and enclosures in which more than one exemption quantity of a nuclear substance is used or stored.
(2569-1)

4. Laboratory Procedures
The licensee shall post and keep posted, in a readily visible location in areas, rooms or enclosures where nuclear substances are handled, a radioisotope safety poster approved by the Commission or a person authorized by the Commission, which corresponds to the classification of the area, room or enclosure.
(2570-1)

5. Thyroid Monitoring
Every person who
(a) uses at a single time a quantity of volatile iodine-125 or iodine-131 exceeding;
(i) 5 MBq in an open room;
(ii) 50 MBq in a fume hood;
(iii) 500 MBq in a glove box;
(iv) any other quantity in other containment approved in writing by the Commission or a person authorized by the Commission; or
(b) is involved in a spill of greater than 5 MBq of volatile iodine-125 or iodine-131;
(c) or on whom iodine-125 or iodine-131 external contamination is detected; and shall, undergo thyroid screening within five days following the exposure to iodine-125 or iodine-131.
(2046-7)

6. Thyroid Screening
Screening for internal iodine-125 and iodine-131 shall be performed using:
(a) a direct measurement of the thyroid with an instrument that can detect 1 kBq of iodine-125 or iodine-131; or
(b) a bioassay procedure approved by the Commission or a person authorized by the Commission.
(2600-1)

7. Thyroid Bioassay
If thyroid screening detects more than 10 kBq of iodine-125 or iodine-131 in the thyroid, the licensee shall immediately make a preliminary report to the Commission or a person authorized by the Commission and have bioassay performed within 24 hours by a person licensed by the Commission to provide internal dosimetry.

(2601-4)

8. Extremity Dosimetry
The licensee shall ensure that any person who handles a container which contains more than 50 MBq of phosphorus 32, strontium 89, yttrium 90, samarium 153 or rhenium 186 wears a ring dosimeter. The dosimeters must be supplied and read by a dosimetry service licensed by the Commission.
(2578-0)

9. Contamination Criteria
The licensee shall ensure that for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides";
(a) non-fixed contamination in all areas, rooms or enclosures where unsealed nuclear substances are used or stored does not exceed:
(i) 3 becquerels per square centimetre for all Class A radionuclides;
(ii) 30 becquerels per square centimetre for all Class B radionuclides; or
(iii) 300 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres; and
(b) non-fixed contamination in all other areas does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; or
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres.
(2642-2)

10. Decommissioning
The licensee shall ensure that prior to decommissioning any area, room or enclosure where the licensed activity has been conducted;
(a) the non-fixed contamination for nuclear substances listed in the licence application guide table titled "Classification of Radionuclides" does not exceed:
(i) 0.3 becquerels per square centimetre for all Class A radionuclides;
(ii) 3 becquerels per square centimetre for all Class B radionuclides; and
(iii) 30 becquerels per square centimetre for all Class C radionuclides;
averaged over an area not exceeding 100 square centimetres;
(b) the release of any area, room or enclosure containing fixed contamination, is approved in writing by the Commission or person authorized by the Commission;
(c) all nuclear substances and radiation devices have been transferred in accordance with the conditions of this licence; and
(d) all radiation warning signs have been removed or defaced.
(2571-2)

11. Storage
The licensee shall:
(a) ensure that when in storage radioactive nuclear substances or radiation devices are accessible only to persons authorized by the licensee;
(b) ensure that the dose rate at any occupied location outside the storage area, room or enclosure resulting from the substances or devices in storage does not exceed 2.5 microSv/h; and
(c) have measures in place to ensure that the dose limits in the Radiation Protection Regulations are not exceeded as a result of the substances or devices in storage.
(2575-0)

12. Disposal (Laboratories)
When disposing of unsealed nuclear substances to municipal garbage or sewer systems, the licensee shall ensure that the following limits are not exceeded:

COLUMN 1	COLUMN 2(a)	COLUMN 3(b)

Material will be included in the following new publication:

Title of publication: Ablation of the B-cell specific transcriptional program and upregulation of pro-inflammatory molecules in Primary Effusion Lymphoma

Type of publication (check one): _____ Journal _____ Book _____ CD-ROM
_____ Newsletter _X_ Other (specify): Doctoral Thesis

Author/Editor: Meztli Arguello, Ph.D. candidate

Publisher: _____ Check here if *publisher* is not-for-profit: _X_

Expected date of publication: August 31st, 2006

PART B

ASH grants permission to reproduce the material as noted above provided a full citation of the *Blood* article is included with the reproduced material.

Fee: $ 0.00 Request No. **198**

Approved by:

Julia Sydnor 8/31/06
ASH Representative Date

To pay with a credit card, please complete the following and fax to (202) 292-6010 or mail. **PLEASE DO NOT DO BOTH.**

VISA/MC/AmEx Number: __ __ __ __/__ __ __ __/__ __ __ __/__ __ __ __ Expiration: __ __/__ __

Cardholder Name: _____ Signature: _____

Amount Authorized to Charge: _____

Email Address (in case of questions): _____