HIV-1 TAT AND VPU MEDIATE B LYMPHOMA CELL GROWTH AND ADHESION TO ENDOTHELIAL CELLS: AN *IN VITRO* MODEL FOR AIDS-ASSOCIATED NON-HODGKIN'S LYMPHOMA

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LIST OF ABBREVIATIONS

Ad/ = adenovirus expressing

Ad/trans = adenovirus expressing tet off transactivator

AIDS = acquired immunodeficiency syndrome

AIDS-BL = acquired immunodeficiency syndrome-associated Burkitt's lymphoma

AIDS-DLCL = acquired immunodeficiency syndrome-associated diffused large cell lymphoma

AIDS-NHL = acquired immunodeficiency syndrome-associated non-Hodgkin's lymphoma

BL = B lymphoma

CA = HIV capsid protein

CAM = camptothecin

CD40L = CD40 ligand

CMV = human cytomegalovirus

CNS = central nervous system

CTL = cytotoxic T lymphocyte

CCR = chemokine receptor with CC motif

CXCR = chemokine receptor with CXC motif

DLCL = diffused large cell lymphoma

DMVEC = dermal microvascular endothelial cells

DNA = deoxyribonucleic acid

EBV = Epstein-Barr virus

EBNA-1 = Epstein-Barr virus nuclear antigen-1

EBNA-2 = Epstein-Barr virus nuclear antigen-2

EC = endothelial cells

EGFP = enhanced green fluorescent protein

EF1-EGFP = enhanced green fluorescent protein driven by elongation factor-1 promotor

Env = HIV envelope glycoprotein

FACS = fluorescence activated cell sorter = flow cytometry analysis

FN = fibronectin

Gag = HIV group specific antigens

GFP = green fluorescent protein

GM-CSF = granulocyte-macrophage colony-stimulatory factor

gp = HIV glycoprotein

HAART = highly active anti-retroviral therapy

HCMV=human cytomegalovirus

HHV8 = human herpes virus 8 = KSHV

HIV = human immunodeficiency virus type-1

HIV/VSV-G = human immunodeficiency type-1 pseudotyped
with vesicular stomatitis virus glycoprotein

HPV = human papilloma virus

HUVEC = human umbilical vein endothelial cells

IBLP = immunoblastic lymphoma plasmacytoid

ICAM-1 = intercellular adhesion molecule - 1

ICCS = intracellular cytokine staining

IFA = immunofluorescence analysis

IFN = interferon

IgSF = immunoglobulin superfamily

IL- = interleukin-

IN = integrase

KS = Kaposi's sarcoma

KSHV = Kaposi's sarcoma-associated herpes virus = HHV8

LFA-1 = leukocyte function associated antigen -1

LIF = leukemia inhibitory factor

LMP-1 = Epstein-Barr virus latent membrane protein - 1

LNCCL = large non-cleaved cell lymphoma

LTR = HIV long terminal repeat

MA = HIV matrix protein

MAdCAM-1 = mucosal addessin cell adhesion molecule -1

MCP-1 = monocyte chemoattractant protein - 1

MHC = major histocompatibility antigen

MIPα = macrophage inflammatory protein alpha

MMP = matrix metalloproteinases

MOI = multiplicity of infection

mRNA = messenger RNA = messenger ribonucleic acid

MVEC = microvascular endothelial cells

NC = HIV nucleocapsid protein

Nef = HIV negative regulator factor

NHL = non-Hodgkin's lymphoma

NK cells = natural killer cells

ORF = open reading frame

PCNSL = primary central nervous system lymphoma

PCR = polymerase chain reaction

PECAM-1 = platelet endothelial cell adhesion molecule -1

PEL/BCBL = primary effusion lymphoma/body cavity based lymphoma

PKC = protein kinase C

Pol = HIV polymerase

PR = HIV protease

PSGL-1 = P-selectin glycoprotein ligand -1

Rev = HIV nuclear RNA export factor

RNA = ribonucleic acid

RNase = ribonuclease H

RNP = ribonucleoprotein

RRE = Rev responsive element

RT = reverse transcriptase

SNCCL = small non-cleaved cell lymphoma

TAR = Tat activating region = Tat responsive element

Tat = HIV transactivator protein

tet = tetracycline

tetO = tetracycline operator DNA sequence

TetR = tetracycline repressor

TGFβ = transforming growth factor beta

Th 1 = T helper cell type 1

Th 2 = T helper cell type 2

TK poly (A) = herpes simplex virus type-1 thymidine kinase poly (A)

TNF = tumor necrosis factor

TNFR = tumor necrosis factor receptor

TRAF = tumor necrosis factor receptor-associated factor

TRE = tetracycline response element

tRNA = transfer RNA = transfer ribonucleic acid

V1-V5 = hypervariable regions 1-5 in HIV envelope gp120

VCAM-1 = vascular cell adhesion molecule -1

VLA-4 = very late activation antigen - 4

Vif = HIV virion infectivity factor

Vpu = HIV viral protein U

Vpr = HIV viral protein R

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ABSTRACT

AIDS-associated lymphoma continues to be a significant clinical problem among individuals infected with human immunodeficiency virus type-1 (HIV), but the mechanisms by which HIV mediates B cell lymphomagenesis remain unresolved. In the absence of direct HIV infection of B lymphoma (BL) cells, infection of non-malignant microvascular endothelial cells (MVEC) is thought to play a role in driving lymphomagenesis. These cells interact closely with B cells at the blood-tissue interface and may thus contribute to homing and aggressive growth of AIDS-associated non-Hodgkin's lymphoma (AIDS-NHL) at extranodal tissue sites.

Moses *et al.* (1997) have shown that brain and bone marrow MVEC develop an enhanced capacity to support BL cell adhesion and growth following HIV infection. Furthermore, HIV-mediated upregulation of CD40 and enhanced expression of VCAM-1 following CD40 ligation were found to be key events driving the lymphoma support phenotype. The goal of this thesis project is to identify the HIV gene(s) responsible for inducing this phenotype in endothelial cells (EC). Using adenovirus vectors to individually express HIV genes in EC, Vpu was found to induce CD40 surface expression on EC. Importantly, this protein also rendered EC susceptible to VCAM-1 induction following CD40 ligation with anti-CD40 antibodies; an event that may be triggered *in vivo* by cross-linking of CD40 with the CD40 ligand (CD40L) expressed on T cells, macrophages or the BL cells themselves. Vpu was a strong inducer of CD40 and VCAM-1 expression, and Vpu-expressing EC were able to support the tight adhesion of CD40L-positive BL cells added in co-culture. In addition, while EC infected with a vesicular stomatitis virus glycoprotein

(VSV-G) pseudotyped HIV supported BL cell adhesion, this property was lost when a *vpu*-deletion pseudotype was used. These data collectively suggest that Vpu is the principle HIV gene mediating BL cell adhesion to HIV-infected EC.

While non-adherent BL cells typically grew as a single cell suspension in EC co-culture, prominent BL cell aggregation and a significantly enhanced growth rate were observed upon co-culture with EC that expressed Tat. These effects may be due to Tat-induced factors being secreted into the culture medium. Indeed, neutralization experiments and intracellular cytokine staining of Tat-expressing EC revealed that B cell growth factors IL-6 and IL-10 contribute to enhance B lymphoma cell growth.

In summary, HIV Vpu and Tat both induce elements of the lymphoma support phenotype in EC. *In vivo*, these genes may act co-operatively to facilitate EC-lymphoma cell adhesion and enhanced lymphoma cell proliferation and survival, thus allowing the formation of a multi-layered lymphoma aggregate attached to the vessel wall. On the basis of our findings with Vpu and Tat, we have proposed a model for the mechanism by which HIV-1 infection of endothelial cells contributes to the development of B cell lymphomas.

Chapter 1

1.1 INTRODUCTION

Many viruses cause human malignancies by directly transforming virally infected cells. Examples include Epstein-Barr virus (EBV) in B cell lymphoma, human papilloma virus (HPV) in squamous cell carcinoma, and human herpes virus 8 (HHV8) in Kaposi's sarcoma and primary effusion lymphoma (PEL). In other viruses, exemplified by human immunodeficiency virus type-1 (HIV), infection is associated with cancer development without direct infection of the malignant cell. In the spectrum of AIDS-associated malignancies, the incidence of B cell non-Hodgkin's lymphoma (NHL) is second only to Kaposi's sarcoma (KS) [Beral *et al.*, 1991; Schulz *et al.*, 1996]. The AIDS-associated NHL (AIDS-NHL) encompass a heterogeneous group of high-grade lymphomas that are characterized by aggressive growth at extranodal tissue sites. Section 1.2 of this chapter provides a more detailed description of the subtypes of AIDS-NHL. Because HIV does not directly infect and transform B lymphoma cells in AIDS-NHL, mechanisms including HIV-mediated immunologic imbalance [Gaidano *et al.,* 1992] and HIV infection of endothelial cell that interact with malignant B cells at extranodal sites [Moses *et al.*, 1997] have been proposed to explain the abnormal incidence of NHL in AIDS patients.

HIV infection of microvascular endothelial cells (MVEC) from the brain, liver and bone marrow has previously been demonstrated *in vivo* [An, *et al.*, 1999, An,

et al., 1999, Bagasra, *et al.*, 1996, Sun, *et al.*, 1989, Valle, *et al.*, 2000, Wiley, *et al.*, 1986] and *in vitro* [Didier, *et al.*, 2002, Moses, *et al.*, 1993, Moses, *et al.*, 1994, Moses, *et al.*, 1996, Mukhtar, *et al.*, 2000, Steffan, *et al.*, 1992]. Moreover, Moses *et al.* (1997) showed that *ex vivo* HIV infection of stromal MVEC isolated from the bone marrow of HIV-seronegative patients with B cell NHL led to the rapid expansion of residual B lymphoma cells that were not able to survive in control stromal cultures. An *in vitro* B cell lymphomagenesis system (Figure 1.1) containing co-cultures of HIV-infected brain microvessel-derived MVEC and uninfected malignant B lymphoma (BL) cells was used to recapitulate and further investigate the *in vivo* microenvironment of HIV infection in AIDS-NHL. With this system, HIV-infected endothelial cells (EC) were shown to support significant adhesion and proliferation of malignant NHL cells, therefore mimicking the aggressive extranodal growth pattern of AIDS-NHL *in vivo*. In the context of this *in vitro* co-culture system, HIV-induced EC-BL cell adhesion is mediated by the interaction of two receptor/ligand pairs, CD40/CD40 ligand (CD40L) and vascular cell adhesion molecule-1 (VCAM-1)/very late activation antigen-4 (VLA-4), because blocking of either interaction inhibits growth and adhesion of B lymphoma cells. HIV infection was found to induce CD40 surface expression on MVEC but did not influence the basal levels of VCAM-1 without CD40 ligation. However, cross-linking of CD40 on HIV-infected MVEC by co-culture with CD40L-positive BL cells led to increased VCAM-1 expression on MVEC, with subsequent VCAM-1/VLA-4-mediated EC-BL cell adhesion [Moses, *et al.*, 1997].

Although our *in vitro* co-culture system has provided evidence of an important role for HIV-infected EC in AIDS-NHL, the mechanisms by which HIV infection supports this malignancy remain unclear. Therefore, the experiments described in this thesis were designed to elucidate such mechanisms. An *in vitro* B lymphomagenesis system involving EC and B lymphoma cells was designed to systematically explore the ability of HIV-encoded proteins to promote lymphomagenesis in AIDS-NHL. Specifically, EC infected with HIV deletion-mutant viruses pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSVG) were used to determine if a specific HIV protein was necessary for supporting B lymphoma cells. Furthermore, EC infected with adenoviruses expressing individual HIV regulatory or accessory genes were used to evaluate the potential role of these genes in promoting a B lymphoma support phenotype. These experiments have suggested that HIV-induced EC-B lymphoma cell interactions may play an important role in AIDS-NHL. This thesis encompasses studies that define the mechanisms by which HIV mediates lymphomagenesis of AIDS-NHL. With this project, I have answered the following questions:

(1) Which HIV proteins are responsible for the lymphoma support phenotype?

(2) How do these viral proteins contribute to the lymphoma support phenotype?

1.2 AIDS-ASSOCIATED NON-HODGKIN'S LYMPHOMA (AIDS-NHL)

1.2.1 Clinical Significance of AIDS-NHL:

AIDS-NHL is the second most common malignancy in HIV-infected adults [Beral, *et al.*, 1991, Schulz, *et al.*, 1996] and the most frequent neoplasm in HIV-infected children [McClain, *et al.*, 1996]. Along with Kaposi's sarcoma, NHL has been classified as an AIDS-defining malignancy [CDC, 1985].

In an AIDS-Cancer Match Registry, the incidence of NHL for individuals with AIDS was significantly increased when compared to the general population [Goedert, *et al.*, 1998]. This study reported a 113-fold increase for NHL (1793/98,336) in patients with AIDS. The incidence of Kaposi's sarcoma (7028/98,336) was 310-fold higher when compared to an uninfected control group [Goedert, *et al.*, 1998]. Only a few other malignant disorders were increased in HIV-infected individuals including angiosarcoma (37-fold), Hodgkin's disease (8-fold), multiple myeloma (4.5-fold) and brain cancer (3.5-fold) [Goedert, *et al.*, 1998].

AIDS-NHL occurs in as many as 10% of HIV-infected individuals with moderate to severe immunodeficiency [Biggar, *et al.*, 1996]. With the introduction in late 1995 of highly active antiretroviral therapy (HAART) to improve immune status and bone marrow function, opportunistic infections and AIDS-related deaths have been significantly reduced [Tirelli, *et al.*, 2002]. HAART includes a combination of drugs that target various viral enzymes important for HIV replication;

therefore, HAART has resulted in reduction of HIV viral load and increase in CD4 T cell counts in HIV-infected individuals. While the rate of Kaposi's sarcoma and primary central nervous system lymphoma has declined [Cattelan, *et al.*, 1999, Lebbe, *et al.*, 1998], the prevalence of systemic NHL has remained relatively stable [Buchbinder, *et al.*, 1999, Rabkin, *et al.*, 1999]. Two additional recent clinical studies [Tirelli, *et al.*, 2002, Vilchez, *et al.*, 2002] have confirmed that AIDS-NHL-related mortality continues in patients on HAART. Furthermore, the incidence of AIDS-NHL is likely to increase as HAART increases longevity. On-going B cell stimulation and dysregulation of the immune system are likely to contribute to the development of B cell lymphomas in these patients [Biggar, *et al.*, 1996, Cote, *et al.*, 1997, Levine, *et al.*, 2001]. Current treatments for NHL involving chemotherapy have a very low success rate. Therefore, AIDS-NHL remains a significant clinical problem and a therapeutic challenge. The mechanisms of AIDS lymphomagenesis must be elucidated before we can develop effective alternate therapeutic strategies for this clinically challenging disease.

1.2.2 Classification and characteristics of AIDS-NHL

The term AIDS-NHL encompasses a heterogeneous group of lymphomas with variable histopathologies and genetic alterations. AIDS-NHL are broadly classified into systemic lymphomas and primary central nervous system lymphomas (PCNSL). Systemic lymphomas are found in 80% of AIDS-NHL while PCNSL are less common. Systemic AIDS-NHL are categorized into four

histological subtypes. The intermediate grade small non-cleaved cell lymphoma (SNCCL) is prevalent in approximately 30% of systemic disease cases and is associated with high CD4 T cell counts. Diffuse large cell lymphoma (DLCL) is a more common and aggressive subtype, with poor prognosis. DLCL comprise approximately 70% of systemic lymphomas and are generally associated with low CD4 T cell counts. DLCL are further divided into large non-cleaved cell lymphoma (LNCCL) and immunoblastic lymphoma plasmacytoid (IBLP). The remaining two subtypes, anaplastic large cell lymphoma and primary effusion lymphoma (PEL/BCBL) are rare. In CNS disease, the primary central nervous system lymphoma (PCNSL) constitutes an immunoblastic subtype of DLCL.

The histological subtypes mentioned above are associated with specific oncogenic lesions or viral involvements. Small non-cleaved cell lymphomas (including Burkitt's lymphomas) are derived from germinal center B cells, often with activating c-*Myc* translocations and p53 mutations [Ballerini, *et al.*, 1993, Chaganti, *et al.*, 1983, Shibata, *et al.*, 1991, Subar, *et al.*, 1988]. Occasionally, EBV nuclear antigen-1 (EBNA-1) is detected in these tumor cells, correlating with latent EBV infection. Interestingly, EBV infection is found in only 30% of SNCCL neoplasms. Among the two DLCL sub-types, DLCL-LNCCL are also derived from germinal center B cells, with dysregulated bcl-6 proto-oncogene expression and occasional EBV infection [Gaidano, *et al.*, 1994, Gaidano, *et al.*, 1997, Ye, *et al.*, 1993]. DLCL-IBLP differs in that the malignant cells are derived from post germinal center B cells with an overexpression of the tcl-1 oncogene

[Teitell, *et al.*, 1999]. These lymphomas are invariably positive for EBNA-2 & LMP-1, indicating universal EBV infection [Hamilton-Dutoit, *et al.*, 1993, Liebowitz, 1998]. The fact that HHV8 plays an important role in the pathogenesis of PEL/BCBL is now widely accepted [Nador, *et al.*, 1996].

Despite the histologic and genetic diversity of AIDS-NHL, common features include a B cell origin, an aggressive growth profile and the propensity for growth at extranodal sites such as gastrointestinal tract, liver, bone marrow and meninges [Kaplan, *et al.*, 1989, Levine, 1992, Ziegler, *et al.*, 1984]. Clinically, NHL in AIDS patients often presents as a high-grade, rapidly progressive B cell tumor at extranodal sites. Systemic lymphoma and brain involvement with perivascular cuffing within the brain parenchyma are also common. Peripheral and central lymphadenopathy as well as hepatosplenomegaly and thrombocytopenia are often common findings [Levine, *et al.*, 2000].

1.2.3 B cell ontogeny and neoplastic transformation of B cell lymphoma

Normal B cell ontogeny can be divided into antigen-independent and -dependent differentiation. Antigen-independent differentiation of pre-B cells in the bone marrow involves immunoglobulin gene (VDJ and VJ) rearrangements giving rise to clones that are released in G_0 phase to the periphery. These bone marrow pre-germinal center B cells enter the blood stream and are distributed into lymphoid tissues where immature B cells are sequestered from encountering antigens in the paracortical regions of the lymph node. Within the germinal center follicles of the

lymph node, antigen-dependent B-cell differentiation takes place by somatic hypermutation, affinity maturation, and isotype (class) switching. Antigen-driven somatic hypermutations commonly result in mutations within the complementary determining regions (CDR) of the antibody V gene. The expression of variable region mutations in B cell tumors is the hallmark for tumors derived from the germinal center and can be used to evaluate the neoplastic transformation of a particular B cell tumor [Lossos, *et al.* 2000]. Isotype class switching of immature B cells surface IgD and IgM to IgG1, IgG2, IgG3, IgE or IgA takes place in the germinal center. After exiting the germinal center, post germinal center B cells (plasma cells and memory B cells) are capable of secreting various immunoglobulins to mediate humoral responses.

AIDS-NHL pathogenesis is a complex process. Aberrant B cell immunoregulations, chronic antigen hyperstimulation leading to polyclonal B cell activation and immune deficiency are all forces that drive clonal expansion and acquisition of mutations in genes (*c-Myc* and *bcl-6*) that confer growth advantages on these B cell lymphomas [Jain *et al.* 1994; Diebold *et al.* 1997]. Lymphomas derived from immunosuppressed individual such as AIDS patients or post-transplantation patients are believed to have a more disordered differentiation pattern from those with an intact immune system [Hollingsworth *et al.* 1994]. Since 95% of AIDS-NHL are of B cell origin, all of these NHL express B-cell differentiation antigens such as IgM and CD20 [Ng and McGrath 1998] and the

majority of these are derived from the germinal center where antigen-driven somatic hypermutation, affinity maturation, and isotype switching take place.

Location and types of mutations in a malignant clone have been evaluated by many techniques. FACS analysis can be used to determine immunoglobulin expression while Southern blot analysis is used for evaluating c-*Myc* or immunoglobulin gene rearrangement to determine the origin of the tumor cells from different sites in the body. The occurrence of an isotype switch in tumors from different sites is suggestive of an ongoing role for antigen stimulation and selection in the evolution of a malignant clone [Jain *et al.,* 1994].

B cell immortalization is believed to result from accumulation of proto-oncogene and tumor suppressor gene mutations that confer a growth advantage to the malignant clone [Pelicci *et al.,* 1986; Ng and McGrath 1998]. Pelicci *et al.* demonstrated a high frequency of *c-Myc* oncogene rearrangements in AIDS-related lymphoproliferative disorders and AIDS-NHL [Pelicci *et al.,* 1986]. *c-Myc* translocation takes place along with VDJ rearrangements in pre-B cells making the bone marrow the initial site of neoplastic transformation. Subsequent *bcl-6* and *p53* mutations are proposed to occur at the germinal center. Therefore, the mutation profile of *c-Myc, bcl-6, and p53* through physiologic B cell maturation can be used as clonal marker for clinical monitoring and histologic classification of AIDS-NHL. For example, *bcl-6* expression is downregulated in post germinal center B effector cells so *bcl-6* can serve as a marker for B cell transition through

the germinal center [Gaidano *et al.,* 1998].Other markers such as CD10 and CD138 are also used in the evaluation of B cell NHL because CD10 is found on germinal center derived B cells while CD138 is present only on post germinal center B cells [Carbone *et al.,* 1999; Huang *et al.,* 2002]. Therefore, *bcl-6*-positive/CD138-negative tumor cells represent germinal center derived lymphomas such as AIDS-BL, AIDS-LNCCL, AIDS-SNCCL, while *bcl-6*-negative/CD138-positive cells are characteristic of post germinal center derived lymphomas such as AIDS-IBLP [Carbone *et al.,* 1999]. Campomenosi *et al.* proposed that *p53* mutations take place late in the differentiation process following *c-Myc* translocation and VDJ rearrangements. This idea was based on the observation that B cells with a wild-type *p53* gene differentiate more robustly than those carrying mutated *p53* [Campomenosi *et al.,* 1997].

1.2.4 AIDS-NHL and HIV-infected microvascular endothelial cells

Since all B-cell derived AIDS-NHL are not infected with HIV [Knowles, 1997], HIV is believed to play an indirect role in the pathogenesis of this disease [Biggar, *et al.*, 1996]. Defects in immune regulation, loss of CD4+ T cells, altered cytokine profiles, changes in the architecture of germinal center and lymphoid tissues, chronic antigen stimulation and loss of immune surveillance may all contribute to a unique microenvironment that contributes to the development of AIDS-NHL. [Gaidano, *et al.*, 1998, Hamilton-Dutoit, *et al.*, 1991, Knowles, 1997, Raphael, *et al.*, 1994]. Furthermore, immunosuppression and sustained B cell stimulation in polyclonal hypergammaglobulinemia are common in AIDS

patients. These two factors are believed to be important in driving the development of B cell malignancies [Grulich, *et al.*, 2000]. Polyclonal B cell proliferation is considered to predispose B cells to accumulate genetic errors, thereby leading to the development of monoclonal B-cell lymphoma [Levine, *et al.*, 2001, Przybylski, *et al.*, 1996].

HIV has been reported by several groups to infect microvascular endothelial cells (MVEC) of the brain, bone marrow and liver [An, *et al.*, 1999, An, *et al.*, 1999, Bagasra, *et al.*, 1996, Sun, *et al.*, 1989, Valle, *et al.*, 2000, Wiley, *et al.*, 1986] and *in vitro* [Didier, *et al.*, 2002, Moses, *et al.*, 1993, Moses, *et al.*, 1994, Moses, *et al.*, 1996, Mukhtar, *et al.*, 2000, Steffan, *et al.*, 1992]. Notably, these organs are all common sites for extranodal AIDS-NHL involvement. Infection of non-malignant endothelial cells at extranodal sites may promote a malignant support phenotype in a manner analogous to the hematopoietic support function of bone marrow stroma [Moses, *et al.*, 1997]. Similarly, the role of tumor-endothelial cell interactions in another B cell malignancy, multiple myeloma (MM), is well established and involves both cell-cell contact and cytokines [Bataille, *et al.*, 1997]. Furthermore, while angiogenesis and endothelial cell physiology have long been considered important factors in solid tumor growth, a similar role in the pathophysiology of hematologic malignancies has recently been established. For example, an increase in microvessel density in lymph node and bone marrow correlates with the severity of disease in acute myelogenous leukemia (AML), MM and NHL [Hussong, *et al.*, 2000, Ribatti, *et al.*, 1996, Vacca, *et al.*, 1994]

and the antiangiogenic drug endostatin stabilizes tumor growth in a NOD/SCID mouse model of high-grade NHL [Bertolini, *et al.*, 2000]. Since MVEC of the brain, bone marrow and liver are all susceptible to HIV infection, HIV-mediated changes in EC phenotype and function could conceivably contribute to the development of lymphomagenesis. Working with the hypothesis that HIV plays a role in the development of AIDS-NHL in part through infection of EC, I have demonstrated in studies reported in this thesis that certain HIV proteins as well as HIV-induced endothelial proteins, can significantly influence malignant B cell adhesion and growth by inducing an altered microenvironment that is favorable for B cell lymphomagenesis. These findings strongly implicate a role for HIV in the lymphomagenesis of AID-NHL.

1.3 HUMAN IMMUNODEFICIENCY VIRUS TYPE-1

1.3.1 Viral life cycle:

Human immunodeficiency virus type-1 (HIV) is a positive-sense retrovirus with a non-segmented RNA genome of approximately 10 kb [Freed, 2001]. HIV belongs to the genus *Lentivirinae* and is a member of the *Retroviridae* family. Besides the standard structural genes (*gag*, *pol* and *env*) encoded by prototypic retroviruses, HIV has a complex genome with six additional genes: two regulatory genes (*rev* and *tat*) and four accessory genes (*vpu*, *nef*, *vpr* and *rev*) (Figure 1.2). In negatively stained electron micrographs, HIV virions appear as mature retroviral particles with an envelope, a cone-shaped central core, and visible glycoprotein spikes on a lipoprotein surface (Figure 1.3). The interior of an HIV virion consists

of matrix proteins (p17MA) and a cone-shaped core consisting of capsid proteins (CA), nucleocapsid proteins (NC), reverse transcriptase (RT), protease (PR), integrase (IN) and viral genomic RNA.

Adsorption of HIV virions to cell targets takes place when an envelope glycoprotein, gp120, interacts with a cellular receptor, CD4, on the surface of the host cell (Figure 1.4). CD4+ T cells are the preferential targets for HIV replication, but HIV also infects other CD4+ cells such as macrophages, and other cell type specific replication patterns also exist [Levy, *et al.*, 1985]. . Following the initial Env-CD4 interaction, binding to a coreceptor is essential for HIV fusion and entry into cells targeted for infection [Berger, *et al.*, 1999]. The primary HIV co-receptors were recently identified as the chemokine receptors CCR5 and CXCR4. Others, including CCR2b, CCR3, CCR8, APJ, Bonzo, BOB and US28, are used to a limited extent in a cell-type specific manner [Edinger, *et al.*, 1999, Simmons, *et al.*, 2000]. The formation of a ternary complex of Env, CD4 and chemokine receptor induces a conformational changes in the Env glycoproteins, in particular a change in gp41from a non-fusogenic state to a fusion-active state by exposing a fusion peptide at the amino-terminus of gp41. Fusion of the viral envelope with the cellular membrane takes place by insertion of this fusion peptide into the target cellular membrane [Doranz, *et al.*, 1999a, Doranz, *et al.*, 1999b, Salzwedel, *et al.*, 2000]. The six-helix bundle present adjacent to the gp41 fusion peptide facilitates viral/cellular membrane fusion by bringing the two membranes into close proximity [Chan, *et al.*, 1997, Weissenhorn, *et al.*, 1997].

Following membrane fusion, viral uncoating takes place when viral core enters the cytoplasm of the target cell.

In addition to being essential for virus entry, chemokine receptors play an important role in determining HIV tropism. For example, CCR5 is used by macrophage-tropic (R5) HIV to enter macrophages [Cocchi, *et al.*, 1995, Dragic, *et al.*, 1998] while CXCR4 is used by T cell-tropic (X4) HIV to enter CD4+ T cells [Feng, *et al.*, 1996] and some dual-tropic (R5/X4) HIV strains can infect either macrophages or T cells.

While the CD4 molecule allows for an efficient initial virus-host cell interaction, HIV may infect cells via CD4-independent, chemokine-receptor mediated pathways, suggesting a wider host cell range for this virus [Edinger, *et al.*, 1998, Endres, *et al.*, 1996]. Endothelial cells are a primary CD4-negative target for HIV infection, and positioned at the blood-tissue interface, are likely an important portal of entry for HIV to multiple tissue sites. In humans, HIV infection of endothelial cells from the brain, bone marrow, placenta and retina has been detected and has been implicated in blood brain barrier breakdown and dementia, hematopoietic dysfunction and ocular microangiopathy [Bagasra, *et al.*, 1996, Martin, *et al.*, 1992, Skolnik, *et al.*, 1989, Wiley, *et al.*, 1986]. Syndromes such as HIV-associated cardiomyopathy suggest additional endothelial targets. Endothelial cell infection has been reported for other retroviruses such as the murine leukemia viruses [Ruscetti, *et al.*, 1992] and human T cell leukemia virus

1 [Hoxie, *et al.*, 1984], as well as by simian [Mankowski, *et al.*, 1994, Strelow, *et al.*, 1998] and feline immunodeficiency viruses [Steffan, *et al.*, 1996]. Many have documented HIV infection of *in vivo* targets such as EC from adipose tissue, umbilical vein, kidney and liver [Chi, *et al.*, 2000, and references therein]. *In vitro*, HIV infection of EC is non-cytopathic and persistent, raising the possibility that HIV may persist in endothelium *in vivo* and cause functional and phenotypic deficits that contribute to viral pathogenesis.

A distinguishing feature of retroviruses from other RNA viruses is the expression of a unique viral enzyme, reverse transcriptase (RT) that allows the virus to form double-stranded DNA from viral RNA. The incoming positive-sense RNA in the virus particle does not serve as mRNA following infection. Instead, these positive-sense RNAs are used as template for the synthesis of the minus DNA strands by RT using a host tRNA as the primer. This DNA strand is then released when the RNA/DNA hybrid is digested by ribonuclease H (RNase H). The nascent DNA strand is transferred to the 3′ end of the DNA for synthesis of the second DNA strand. As a result of the reverse transcription reaction, three forms of viral DNA structures are formed: a linear DNA containing two LTRs integrate in the host genome as well as two unintegrated circular double-stranded DNAs. The two unintegrated circular double-stranded DNAs can be a circle with one LTR or a circle with two tandem LTRs. The formation of the double-stranded DNA occurs in a ribonucleoprotein (RNP) complex or preintegration complex in the cytoplasm. This RNP complex consisting of RT, IN, NC and the linear viral

DNA migrates to the nucleus through the nuclear pore, which is facilitated by p17MA (matrix protein) and Vpr (an accessory protein) [Freed, 2001]. After transport to the nucleus, the double-stranded viral DNA is called provirus. Some viral DNAs are integrated into host chromosomal DNA by viral integrase (IN). Once integrated, the viral genome becomes part of the host cell genome in the infected cells. Similar to the cellular DNA, integrated proviral DNA is transcribed by cellular DNA-dependent RNA polymerase II to produce viral RNAs[Freed, 2001]., Three kinds of RNAs are produced; full-length unspliced progeny RNA (which is also the mRNA for *gag* and *pol*), partially spliced mRNAs (encoding for *vif*, *vpr*, *vpu* and *env*) and completely spliced mRNA (encoding for *tat*, *rev*, and *nef*) [Freed, 2001]. Unspliced and spliced mRNAs are exported to the cytoplasm for translation into viral proteins. Virion proteins (Env, Vif, Vpr, Gag and Gag/Pol), progeny RNA and tRNA molecules all assemble close to the cell surface during genome packaging. The immature viral particle buds through the membrane and is released. Maturation occurs when precursors of Gag-Pol are cleaved into their respective functional products by the viral protease. During maturation, structural rearrangements take place within the virion forming a mature infectious virus with a cone-shaped condensed core[Freed, 2001].,

1.3.2 Structural genes:

Gag (group specific antigens) encodes a precursor Pr55Gag for all the internal core proteins. A Rev-dependent unspliced mRNA produces a Gag-Pol fusion

protein by ribosomal frameshifting. This fusion protein is eventually cleaved by protease (PR) into Gag and viral polymerase (Pol), IN, PR and RT. During virus budding, activated viral protease (PR) cleaves Pr55gag into mature Gag proteins p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC) and p6gag. These Gag proteins form the virus structure with MA inside the lipid bilayer, CA forming the cone-shaped core structure, and NC complexed with the viral RNA genome to form the ribonucleoprotein. In addition, Gag protein oligomerization plays an important role in targeting virus particles to the plasma membrane, and facilitates close association between the cell membrane lipid bilayer and the viral Env protein before virus particles are released from the cell [Freed, 2001].

Matrix protein (MA) possesses a membrane-binding signal that may be responsible for directing precursor Pr55Gag to the plasma membrane [Zhou *et al.*, 1994]. As a trimer, MA facilitates incorporation of Env into the newly formed viral particles during budding. Since HIV has the ability to infect non-dividing cells, viral mechanisms have evolved that mediate viral genome entry into the nucleus of non-dividing cells such as monocytes and lymphocytes [Nottet *et al.*, 1996]. Along with Vpr and IN, MA, possibly through a potential nuclear localization signal, may facilitate transport of the preintegration complex into the nucleus [Bukrinsky, *et al.*, 1993, Heinzinger, *et al.*, 1994, von Schwedler, *et al.*, 1994].

Capsid (CA) is composed of an amino-terminal core domain and a carboxyl-terminal dimerization domain. The dimerization domain promotes multimerization of Gag proteins while the core domain is important for core condensation [Freed, 2001]. Unique to HIV-1, but absent in HIV-2 or SIV, is the discovery that cellular protein cyclophilin A iis incorporated into the virion. Although the mechanism remains unclear, CA protein interaction with cyclophilin A is considered to increase viral infectivity [Freed, 2001].

Nucleocapsid (NC) functions to encapsidate full-length HIV genomic RNA into the virus particles by interacting with a packaging signal or encapsidation element between the 5′ LTR and the Gag initiation site [Berkowitz, *et al.*, 1996]. The zinc finger motifs found in the p7 NC have previously been shown to be important for RNA packaging during encapsidation and infectivity [Miller, *et al.*, 2000]. The amino-terminal basic domain may be critical for multimerization of Gag proteins, leading to tight packing of Gag proteins in the virion [Bennett, *et al.*, 1993]. The last cleavage product of Gag proteins is a proline-rich protein of 6 kD termed p6Gag that directs the incorporation of Vpr into the virions and plays a role in virus production and release.

Pol (polymerase) encodes the precursor for viral enzymes (IN, PR, and RT), which are essential for replication. Enzymatic cleavage of the Gag-Pol fusion protein (PR160 Gag-Pol) gives rise to Gag and Pol proteins as described above.

Integrase (IN) is a 32-kDa protein produced from the carboxyl-terminal of the Gag-Pol polyprotein. Enzymatic activities of IN include removal of two to three nucleotides from the 3′ end of the proviral DNA, random cleavage of the cellular DNA and processing/joining of the 3′ end of the viral DNA to the host chromosome following reverse transcription (see viral life cycle section). Gaps in the DNAs are then filled in by IN and the cellular repair machinery to form an intact proviral DNA.

Active HIV protease (PR) belongs to the family of aspartic proteases that use two aspartate residues at the active site to coordinate catalysis of peptide bond hydrolysis [Freed, 2001]. This viral enzyme is tightly controlled and is critical for virus infectivity. Proteolytic digestion of Gag and Gag-Pol polyprotein precursors by PR during budding and immediately after virion release is essential for virus maturation.

Reverse transcriptase (RT) consists of two p66 and p51 subunits from the cleavage product of Pr160Gag-Pol that form a heterodimer. RT has three enzymatic activities; an RNA-dependent DNA polymerase activity for minus-strand DNA synthesis, a DNA-dependent DNA polymerase activity for second or positive-strand synthesis, and ribonuclease H (RNase H) activity that degrades tRNA primer and the RNA strand in the RNA/DNA hybrid in the reverse transcriptase reaction, thus releasing the DNA strand for double strand synthesis. The description of the sequence of actions by RT can be found in the viral life

cycle section of this thesis. The lack of exonucleolytic proofreading activity in RT is the key factor contributing to the high mutation rates of HIV (1:1000 bases).

Inhibitors of protease and RT have been shown to be effective in controlling HIV replication and are included as part of a triple therapy regime for treating HIV-infected individuals. Also known as highly active anti-retroviral therapy (HAART), this regime usually includes one protease inhibitor and two RT inhibitors. PR inhibitors are developed based on the knowledge of the structure and active sites of PR. Inhibitors of RT, including the nucleoside analogs AZT, 3TC, ddI, and ddC, act as chain terminators and nonnucleoside inhibitors that bind to the active site of RT to induce allosteric change. HAART has been shown to block production of infectious virus and lower viral load in HIV-infected patients, although some viral escape mutants are not sensitive to treatments with these drugs.

Env (envelope) is produced as a second open reading frame by a biscistronic mRNA encoding for Vpu and Env. The *env* mRNA codes for an integral membrane precursor protein gp160 that is cleaved intracellularly at the Golgi into surface glycoprotein SU (gp120) and transmembrane TM regions (gp41) by a cellular furin-like protease[Hallenberger, *et al.*, 1992]. gp120 and gp41associate non-covalently in a complex and are transported to the cell surface. Functional Env glycoproteins that consist of several conserved disulfide bonds that are

heavily glycosylated multimerize to form spikes on the virion (Figure 1.3). Env glycoproteins are immunogenic and can elicit immune responses in the host. As described above, specific sequences in gp120 interact with the CD4 receptor and the chemokines receptor (HIV coreceptor) for virus binding and entry to the host cell. Considerable sequence diversity can be found in the five-hypervariable regions (V1-V5) of gp120. The V3 loop and regions of gp120 that interact with the coreceptor have been shown to be responsible for coreceptor usage and cell tropism [Gu, *et al.*, 1993, Hoffman, *et al.*, 1999, Hwang, *et al.*, 1991, Moses, *et al.*, 1996, Ross, *et al.*, 1998]. In addition, V1/V2 regions also play a role in influencing coreceptor usage.

1.3.3 Regulatory genes:

Tat (transactivator) is a 16-kDa protein that is encoded by two exons. Tat is the transactivator of HIV gene expression and is absolutely essential for viral replication *in vivo* [Rice, *et al.*, 1988, Rosen, *et al.*, 1990]. Tat is transcribed as a spliced mRNA early in the viral replication cycle. The major effect of Tat is mediated through binding to an RNA target called the Tat-responsive element or Tat activating region (TAR), located immediately downstream from the potent viral promoter 5′ long terminal repeat (LTR) resulting in the increase of RNA polymerase II processivity and elongation. Mechanistically this process is mediated through Tat interaction with cellular factors cyclin T (a subunit of the elongation complex) and Tat-associated kinase (TAK) [Cullen, 1998, Herrmann, *et al.*, 1995, Miller, *et al.*, 2000]. Binding of Tat to cyclin T increases the affinity

of the complex for the TAR element on mRNA transcripts. The cyclin T/Tat/TAR complex recruits TAK which hyperphosphorylates the carboxyl-terminal domain of RNA polymerase II. Tat interacts cooperatively with cellular transcriptional transactivators such as NFκB and Sp1 on the HIV LTR to produce high levels of viral RNA [Moses *et al.*, 1994].

Tat is localized to the nucleus and the nucleolus where Tat activates a number of viral and cellular promoters (e.g., JV virus late promoter, TNFα, TGFβ, collagen and fibronectin) [Biswas, *et al.*, 1995, Cupp, *et al.*, 1993, Sawaya, *et al.*, 1998, Tada, *et al.*, 1990, Taylor, *et al.*, 1992, Zauli, *et al.*, 1992]. The ability of Tat to function as a transcriptional activator has lead to the observation that this vrial protein can regulate expression of a number of cellular genes such as cytokines, extracellular matrix proteins, collagenases, adhesion molecules, enzymes and proto-oncogenes possibly by interacting with cellular transcriptional transactivator [Biswas, *et al.*, 1995, Chang, *et al.*, 1995, Cupp, *et al.*, 1993, Sawaya, *et al.*, 1998, Tada, *et al.*, 1990, Taube, *et al.*, 1999, Taylor, *et al.*, 1992, Zauli, *et al.*, 1992]. For example, Tat was shown to induce interleukin-6 (IL-6) production and activate endothelial cells by upregulating expression of E-selectin, VCAM-1 and ICAM-1 [Jeang, *et al.*, 1999, Taube, *et al.*, 1999]. Tat was also shown to promote collagenase production and increase vascular permeability [Maruo, *et al.*, 1992] as well as to upregulate IL-4 receptors, increase IL-6 and TNFα expression [Buonaguro, *et al.*, 1992, Husain, *et al.*, 1996, Iwamoto, *et al.*, 1994], and augment the motility of two AIDS-NHL B cell lines (derived from

Burkitt's lymphoma and PEL) [Chirivi, *et al.*, 1999].

Tat may be secreted from infected cells and actively absorbed by adjacent uninfected cells via the protein transduction domain [Chang, *et al.*, 1995, Schwarze, *et al.*, 1999]. Thus Tat may enter uninfected cells and activate host cellular genes. The basic domain of Tat has also been shown to bind $\alpha v\beta 5$ integrin on host cells to trigger signal transduction [Barillari, *et al.*, 1993, Vogel, *et al.*, 1993].

Rev (regulator of expression of viral proteins or nuclear RNA export factor) is a 19-kDa small positively charged protein with phosphoserine that transports viral RNA transcripts between the nucleolus and the cytoplasm. Like Tat, Rev is translated from a fully spliced transcript encoding two exons early during the HIV life cycle. In eukaryotic cells, partially or unspliced RNAs are retained within the nucleus, thereby allowing only completely spliced RNAs to leave the nucleus for translation in the cytoplasm. Thus, Rev plays an important role in efficient expression of structural and accessory proteins post-transcriptionally by exporting these partially spliced and unspliced viral mRNAs. *gag, pol, env, vif, vpr* and *vpu* mRNA contain a Rev responsive element (RRE) and are dependent on the Rev protein for efficient expression, while *tat, rev* and *nef* are fully spliced mRNAs that are not retained in the nucleus. By binding to RRE on the unspliced and partially spliced transcripts, Rev facilitates transport, stability and translation of these mRNAs. Rev contains two functional domains: (1) An arginine-rich RNA

binding and nuclear localization domain with a nuclear localization signal (NLS); (2) Nuclear export domain with nuclear export signal (NES). Both of these domains of Rev (RNA binding motif and NES sequence) mediate nuclear export of mRNAs. The NLS sequence of Rev allows Rev to shuttle back to the nucleus following unloading of mRNA cargo in the cytoplasm. The NLS of newly made Rev protein is believed to bind to the cellular import receptor Importin β (IMPβ) [Cullen, 1998], thereby recruiting Rev to the nuclear pore. IMPβ interacts with nuclear pore gatekeeper proteins known as nucleoporins to mediate translocation of Rev/IMPβ complex into the nucleus. Once inside the nucleus, GTP-bound G protein Ran (GTP-Ran) interacts with IMPβ, releasing Rev. Rev is then free to bind the RRE of transcripts and export receptor CRM1 (chromosome region maintenance 1 or exportin1) via the NES [Cullen, 1998, Miller, *et al.*, 2000] and GTP-Ran. CRM1 interacts with nucleoporins and transports the cargo mRNA/Rev/CRM1/GTP-Ran complex to the cytoplasm. In the cytoplasm, GTP-Ran is eventually hydrolyzed to GDP-Ran resulting in the dissociation of CRM1 from Rev and mRNA transcripts [Cullen, 1998]. Translation of transcripts takes place in the cytoplasm while Rev returns to the nucleus to transport more cargo mRNAs.

In addition to the nucleocytoplasmic transport function of Rev, the various amounts of spliced to unspliced mRNA are temporally regulated by Rev as well as Tat. Early during infection, activation of Tat results in the production of mostly spliced HIV mRNAs. However, at the late stage of infection, Rev regulation

ensures that predominantly unspliced mRNAs remain for maximal expression of viral proteins and full length mRNA for packaging [Malim, *et al.*, 1988]. Rev mutants are not replication competent since Rev function is essential for viral expression.

1.3.4 Accessory genes:

Nef (negative regulator factor; previously known as negative regulatory element NRE, F protein, 3′ORF, or B-ORF) is expressed in high levels from a multiply spliced mRNA and is therefore considered an early gene product during HIV replication. Nef is a 27-kDa-myristylated protein that is anchored to the cytoplasmic side of the plasma membrane but has also been found in the nucleus and in association with the cytoskeleton. Nef was first considered to be a negative regulator of HIV replication but was later shown to have distinct activities important in viral replication and pathogenicity [Simmons *et al.*, 2001]. Nef downregulates surface CD4 by direct binding to the di-leucine motif in cytoplasmic tail of CD4 with subsequent interaction with adapter proteins (AP-2)[Piguet et al.,1999]. The interaction of CD4 with AP-2 facilitates CD4 endocytosis from the cell surface by targeting CD4 into the clathrin-coated pits. Following internalization of CD4 into endosomes, Nef mediates interaction of β-COP coatomers to CD4 and targeting CD4 to lysosomes for degradation [Garcia, *et al.*, 1991, Janvier, *et al.*, 2001, Mangasarian, *et al.*, 1999, Piguet, *et al.*, 1998, Piguet, *et al.*, 1999]. The Nef-mediated loss of CD4 on the cell surface reduces the chance of viral superinfection and can therefore lower the levels of

unintegrated viral DNA. In addition, Nef has been reported to enhance virion infectivity due to the inability of *nef*-deleted virus to synthesize proviral DNA through a defect in reverse transcription. The mechanism for this function is not clear. Another function Nef is the ability of this HIV protein to downregulate MHC class I [Greenberg, *et al.*, 1998, Le Gall, *et al.*, 1998, Piguet, *et al.*, 2000] and thus inhibit cytotoxic T lymphocyte-mediated lysis of HIV- infected cells [Collins, *et al.*, 1998]. The mechanism of Nef-mediated MHC class I downregulation requires a tyrosine-based motif in MHC class I molecules. Lastly, Nef may affect T cell activation via interacting with cellular kinases to alter signal transduction to the plasma membrane and increase NFkB activation [Richard, *et al.*, 1997].

The importance of Nef in HIV pathogenesis is underscored by the existence of a group of long time survivors with no overt clinical symptoms who are infected with HIV with a deletion in *nef* [Freed, 2001]. In addition, macques infected with a pathogenic clone of SIV strain with an inactivation of the *nef* gene have undetectable viral titer and are more resistance to pathogenic sequelae [Edinger *et al.*, 1998]. Therefore, Nef may play a role in maintaining high viral load *in vivo*[Freed, 2001]. Nef may also have a role in differentiation of B-lymphocytes into immunoglobulin secreting cells primarily by upregulation of IL-6 production from monocytes [Chirmule, *et al.*, 1994].

Vif (virus infectivity factor) Vif is a 23-kDa protein found predominantly in

soluble form in the cytoplasm, but some protein can be found anchored to the inner cell membrane via the C-terminus and was shown to co-localize with Gag protein. Vif is made in high levels from partially spliced mRNA during the late phase of HIV infection, and low levels of Vif are incorporated into viral particle. Phosphorylation of Vif by p44/42 mitogen-activated protein kinase (MAPK) allows cellular and viral proteins to interact with Vif for targeting to various compartments within the cell [Yang, *et al.*, 1998, Yang, *et al.*, 1999]. The function of Vif is unclear, but Vif appears to affect HIV infectivity but not virus particle production. Virus particles lacking Vif can bind and enter into target cells to initiate but not complete reverse transcription, thus provirus integration into the host chromosome does not occur [Cullen, 1998, Miller, *et al.*, 2000]. This effect of Vif appears to be cell-type specific, and Vif may suppress the activity of a naturally occurring antiviral factor in nonpermissive cells. The expression of Vif in these nonpermissive cells renders them permissive in *in vitro* culture and vif is believed to suppress an antiviral protein named CEM15 in T cells [Madani, *et al.*, 1998, Simon, *et al.*, 1998; Sheehy *et al.*, 2002]. Furthermore, *vif* mutation was reported to result in a defect in Gag protein processing. Therefore, Vif is believed to influence the late stages of virion assembly possibly by temporally controlling protease (PR)-mediated Gag processing resulting in an abundance of Gag-derived peptides (capsid protein, matrix protein, and nucleocapsid proteins) available for viral assembly with Env and viral RNA at the plasma membrane [Kotler, *et al.*, 1997].

Vpr (viral protein R) is a 14-kDa protein expressed from a singly spliced Rev-dependent mRNA at the late phase of the viral life cycle. Vpr is incorporated at high levels into the nucleocapsid via interactions with p6gag protein. Following virus entry and uncoating, Vpr along with matrix proteins functions to facilitate the nuclear import of preintegration complexes [Paxton, *et al.*, 1993] into the nucleus of non-dividing cells such as primary macrophages [Cullen, 1998]. Vpr does not appear to affect dividing cells such as PBMC and T cells. The nuclear import of preintegration complexes may be the result of direct interaction of Vpr with nucleoporins and cellular import receptors [Popov, *et al.*, 1998, Vodicka, *et al.*, 1998]. In infected cells, Vpr can be found in the nucleus and promotes G2/M cell cycle arrest by inactivating the cell cycle phosphatase cdc25C that normally functions to activate cyclin B-associated kinase activity in infected non-dividing cells such as macrophages [He, *et al.*, 1995, Re, *et al.*, 1995]. Since cyclin B kinase activity is essential for transition from G2 to mitosis, cells expressing Vpr are arrested at the G2 phase of the cell cycle. Interestingly, HIV replication may be enhanced during the G2 phase of the cell cycle because of the ability of Vpr to induce LTR promoter activity [Goh, *et al.*, 1998]. In addition to the above mentioned functions, Vpr has been shown to stimulate LTR dependent viral gene expression [Cohen, *et al.*, 1990]. For example, Vpr can increase in *vitro* activity of viral promoters such as HIV LTR, CMV and SV40 [Roux, *et al.*, 2000]. Vpr has also been shown to induce cellular differentiation [Heinzinger, *et al.*, 1994, Levy, *et al.*, 1993, Vodicka, *et al.*, 1998] as well as apoptosis [Stewart, *et al.*, 1997]. Similar to other HIV viral proteins, Vpr has been shown to affect cellular

gene expression [Kino, *et al.*, 1999, Sawaya, *et al.*, 1998, Wang, *et al.*, 1995] and induce cytokine production, in particular, IL-6 and IL-10 in macrophages, TNFα in T cells and IL-8 in primary T cells, macrophages and epithelial cells [Roux, *et al.*, 2000].

Vpu (viral protein U) is a 16-kDa oligomeric type 1 integral membrane protein that is unique to HIV-1 but is not present in HIV-2 or SIV [Cohen, *et al.*, 1988, Strebel, *et al.*, 1988, Strebel, *et al.*, 1989]. Similar to Vif and Vpr, Vpu is also a late product formed from Rev-dependent mRNA. However, Vpu is not incorporated into virions. Vpu is phosphorylated by casein kinase II at position serine 52 and serine 56 at the carboxyl-terminal cytoplasmic tail. Vpu is produced by a bicistronic vpu/env mRNA. The two known biological functions of Vpu are: (1) Degradation of nascent CD4 in the ER by binding to the cytoplasmic tail of CD4, which reduces or prevents CD4 transport to the plasma membrane [Raja, *et al.*, 1994, Vincent, *et al.*, 1993]. In addition to binding of CD4 to Vpu, Vpu recruits cellular factor h-βTrCP that interacts with Skp1, a factor involved in ubiquitin-mediated proteolysis. Therefore, Vpu-mediated CD4 degradation by the ubiquitin pathway indirectly promotes Env trafficking from the Golgi apparatus to the plasma membrane preventing newly made Env from interacting with CD4 intracellularly; (2) Augmentation of virion release from the plasma membrane of HIV-infected cells by promoting budding of the virions. Increased plasma membrane permeability as a result of the ability of Vpu to multimerize and form ion channels from the amino-terminal transmembrane domain has been proposed

to be responsible for Vpu-mediated virion release. Vpu has been shown to have structural homology to influenza virus M2 membrane protein that also forms ion channels that are important for virion uncoating in influenza virus-infected cells [Shimbo, *et al.*, 1996]. Vpu-mediated viral particle release requires the expression of HIV matrix protein [Lee, *et al.*, 1997]. Additional functions of Vpu include interference with synthesis and surface expression of MHC Class 1 molecules [Kerkau, *et al.*, 1997] and enhanced susceptibility of Jurkat cells to Fas-mediated killing induced by anti-Fas antibodies [Casella, *et al.*, 1999]. Subcellular localization studies indicate that Vpu can be found in the perinuclear region as well as dispersed throughout the cytoplasm in CD4+ cells [Fujita, *et al.*, 1996, Klimkait, *et al.*, 1990, Tiganos, *et al.*, 1998]. However, Vpu is targeted to the Golgi apparatus when CD4 and gp160 are absent [Kimura, *et al.*, 1994]. Moreover, Vpu appears to be a potential regulator of proteolysis and protein transport between the ER and the plasma membrane with undefined function [Vincent, *et al.*, 1995].

1.3.5 HIV pathogenesis:

Since 1981, over 22 million people have died from Acquired Immunodeficiency Syndrome (AIDS) worldwide, and more than 36 million are infected with HIV [Freed, 2001]. Undoubtedly, the HIV epidemic will remain a major health concern worldwide without the development of an effective vaccine.

HIV pathogenesis is complex and involves dysfunction of many different arms of the immune system. During early HIV infection, viral dissemination in the lymphoid system is evident and the lymphoid tissues remain an important reservoir of HIV. HIV often infects immune cells such as T cells and macrophages, but many other non-immune cell types such as endothelial cells are also susceptible to HIV infection [An, *et al.*, 1999, An, *et al.*, 1999, Bagasra, *et al.*, 1996, Sun, *et al.*, 1989, Valle, *et al.*, 2000, Wiley, *et al.*, 1986] and *in vitro* [Didier, *et al.*, 2002, Moses, *et al.*, 1993, Moses, *et al.*, 1994, Moses, *et al.*, 1996, Mukhtar, *et al.*, 2000, Steffan, *et al.*, 1992]. HIV infection is characterized by an early immune system dysfunction and subsequent destruction of lymphoid architecture, CD4+ T cells loss, and clonal exhaustion of CD8+ T cells. *In vitro*, cytopathic effects caused by HIV include syncytium formation and cell death by many different mechanisms, which eventually cause a profound host immunodeficiency resulting in increased incidence of opportunistic infections and neoplasms. In addition to the loss of CD4 T cells and T-cell dysfunction, a number of HIV proteins (gp120 and Tat) or HIV-induced cytokines such TGFβ and IL-10 are immunosuppressive [Shearer, *et al.*, 1993]. The HIV-induced dysregulation of the immune system as a result of early immune hyperactivation and subsequent the loss of CD4 T helper cells may contribute to the inability of the immune system to eliminate HIV. Furthermore, the genetic variants generated by HIV replication due to the infidelity of RT to generate mutations during reverse transcription of the viral RNA genome may also contribute to the inability of the immune system to elicit an anti-HIV response. Increasing evidence

suggests that the cellular reservoirs of HIV may include resting T cells and EC where the HIV LTR has minimal activity [Belmonte *et al.,* 2003; Kulkosky *et al.*, 2002 ; Blankson *et al.*, 2002; Pomerantz *et al.*, 2002; Eckstein *et al*, 2001; Lum *et al.*, 2001; Siliciano *et al.*, 1999; Bagasra *et al.*, 1996; Corbeil *et al.*, 1995]. In these relatively quiescent cells, viral release likely corresponds to cellular activation. The ability of HIV to infect non-dividing cells and modulate cellular gene expression by HIV regulatory and accessory genes may be another reason for the inability of the immune system to eliminate the infection, resulting in persistent infection and long disease course.

A number of HIV proteins such as Tat, Vpr, and Nef have effects on the pathogenesis of other comorbidities (opportunistic infections, malignancies, encephalitis and dementia) that exist with HIV infection. These same proteins have been shown to interact with the cellular machinery to promote virus propagation (Tat, Vpr, Nef, Vpu) [Freed, 2001]. In addition, the viral promoter is highly inducible and has sites for the binding of cellular transcription factors such as Sp1 and NFκB [Moses, *et al.*, 1994]. Therefore, activation of infected cells, which leads to NFκB translocation to the nucleus, may simultaneously stimulate viral transcription. The relationship between HIV proteins and AIDS-associated diseases has been discovered in some disease processes, but others remain to be identified. In this thesis, I have provided strong evidence that HIV Tat and Vpu play a crucial role in the development of B cell non-Hodgkin's lymphoma, the second most common malignancy afflicting HIV-infected individuals.

1.4 Cytokines: B cell growth factors

1.4.1 Interleukin-6 and HIV infection

The production of interleukin-6 (IL-6) can be induced in monocytes, macrophages, lymphocytes, fibroblasts, and endothelial cells [Akira, *et al.*, 1993, Kishimoto, 1989, May, *et al.*, 1989, Podor, *et al.*, 1989, Shalaby, *et al.*, 1989, Sironi, *et al.*, 1989]. As a paracrine growth factor, IL-6 can stimulate proliferation and differentiation of T- and B-cells as well as smooth muscle cells [Van Snick, 1990]. IL-6 is essential for the terminal differentiation of B cells into plasma cells and polyclonal B cell proliferation [Kishimoto, *et al.*, 1988, Poupart, *et al.*, 1987, Watson, *et al.*, 1995]. In addition, IL-6 is a systemic mediator of the acute-phase response [Heinrich, *et al.*, 1990, Van Snick, 1990]. As part of the acute-phase response, IL-6 can activate EC and increase endothelial cell permeability, and potentiate the paracrine production of additional IL-6 by the same cells [Maruo, *et al.*, 1992]. This B-cell differentiation factor can also act as an autocrine growth factor for B cells and B cell tumors such as PEL and DLCL that express IL-6R. Therefore, IL-6 has been implicated in B lymphoma cell pathogenesis [Yee, *et al.*, 1989].

The serum IL-6 level is elevated in patients suffering from AIDS-NHL, suggesting a role for IL-6 in this disease [Pluda, *et al.*, 1993]. In these patients, CD4 T cell counts are usually low, indicating significant viral burden as well as an immunocompromised state. HIV induces IL-6 in monocytes and macrophages

in vitro [Nakajima, *et al.*, 1989]. Many groups have speculated that HIV-induced IL-6 production from T cells or macrophages affects B lymphoma cells in a paracrine fashion[Scala *et al.*, 1994; Buonaguro *et al*, 1992; Sharma *et al*, 1995; Iwamoto *et al*, 1994; Nath *et al*, 1999]. While a number of roles for IL-6 in AIDS lymphomagenesis have been described, the spectrum of mechanisms has yet to be fully defined. Two HIV proteins have been shown to modulate IL-6 expression. Nef was shown to induce IL-6 in monocytes, with subsequent activation and differentiation of B cells into plasma cells [Chirmule, *et al.*, 1994], while Tat was shown to interact with a transcription factor, CAAT enhancer-binding protein β (NF-IL6) for IL-6 induction [Ambrosino, *et al.*, 1997, Scala, *et al.*, 1994]. In addition, Hofman *et al.* (1993) showed that HIV Tat activates human endothelial cells to express IL-6, IL-8, and E-selectin. Tat has been proposed to induce IL-6 mRNA in human brain endothelial cells by activating the protein kinase C and cyclic AMP protein kinase pathways [Zidovetzki, *et al.*, 1998]. Exogenous recombinant Tat can also activate endothelial cells, leading to IL-6 production [Hofman, *et al.*, 1993]. Therefore, Tat expression in HIV-infected endothelial cells, T cells and macrophages, may play a major role in inducing the IL-6 that induces proliferation of B lymphoma cells in a paracrine manner.

Indeed, I have shown in this thesis that Tat-induced endothelial IL-6 can contribute to the proliferation of B lymphoma cells *in vitro*, in the absence of T cells or macrophages. As described in Chapter 2 of this thesis, EC-derived IL-6 and IL-10 are important Tat-induced cytokines driving B lymphoma cell growth.

1.4.2 Interleukin-10 and HIV infection

HIV infection induces production of the immunoregulatory cytokine interleukin 10 (IL-10), from target cells [Badou, *et al.*, 2000, Blazevic, *et al.*, 1996, Kundu, *et al.*, 1999, Masood, *et al.*, 1994, Sharma, *et al.*, 1995]. Importantly, serum IL-10 is significantly elevated in HIV-infected patients, and IL-10 levels have been implicated in disease progression to AIDS. IL-10 was originally identified as a cytokine produced by Th2 cells that inhibits the proliferation and function of Th1 cells, and IL-10 was thus regarded as an anti-inflammatory cytokine with immunosuppressive activity. In particular, IL-10 inhibits production of pro-inflammatory cytokines (IFNγ, IL-1α, TNFα, IL-6, IL-2, IL-12 and GM-CSF) from monocytes and T cells [Chadban, *et al.*, 1998, Ding, *et al.*, 1993, Mielcarek, *et al.*, 1997, Song, *et al.*, 1997], and downregulates MHC class I and II molecules. These events result in a switch from a Th1 to a Th2 T cell response and altered monocyte function [de Waal Malefyt, *et al.*, 1991, de Waal Malefyt, *et al.*, 1991, Moore, *et al.*, 1993]. IL-10 has also been implicated in the loss of T cells and macrophages in HIV infection, and in disruption of the autocrine loop of TNFα and IL-6 production in HIV-infected macrophages and T cells. IL-10 also appears to have an inhibitory effect on IL-2 and IFNγ production and can thereby regulate CD8+ T cell, natural killer cell, endothelial cell and B cell function [Song, *et al.*, 1997]. In endothelial cells, IL-10 directly downregulates antigen presentation by a reduction of MHC class II, and modulates angiogenesis [Pugin, *et al.*, 1993, Vora, *et al.*, 1994].

In addition to mediating immunosuppression, IL-10 is an autocrine as well as paracrine B cell growth factor [Masood, *et al.*, 1995] and is produced at high levels by certain lymphomas including Burkitt's lymphomas [Benjamin, 1995]. Interestingly, the serum levels of both IL-6 and IL-10 are elevated in HIV patients, as well as in patients suffering from B cell lymphoma, leading many groups to speculate that HIV-induced IL-6 and IL-10 produced by circulating monocytes, macrophages and T cells [Emilie, *et al.*, 1992, Marsh, *et al.*, 1995, Masood, *et al.*, 1994, Masood, *et al.*, 1995, Nakajima, *et al.*, 1989, Tohyama, *et al.*, 1990] may contribute to the development of AIDS-related lymphoma. Blazevic and colleagues [Blazevic, *et al.*, 1996] demonstrated that IL-10 mRNA levels in Th1 and Th2 T cells from HIV-infected individuals can be enhanced by treatment with a Tat peptide. In addition, monocytes treated with soluble Tat produce high levels of IL-10 via a process involving the activation of protein kinase C, phosphorylation of MAP Kinases ERK1 and ERK2 and NFκB activation [Badou, *et al.*, 2000]. Furthermore, Kundu and colleagues [Kundu, *et al.*, 1999] demonstrated that Tat expression upregulated IL-6 and IL-10 mRNA in the lymphoid tissue in transgenic mice, with some animals developing splenic B cell lymphoma.

1.4.3 Additional B cell growth factors

IL-1α is a proinflammatory cytokine secreted by endothelial cells, epithelial cells, monocytes, macrophages, B cells, and dendritic cells. IL-1α has different

biological activities on different target cells. For example, IL-1α promotes B cell maturation and clonal expansion, activates T cells and macrophages, enhances NK cell activity, increases adhesion molecules on vascular endothelial cells, and induces synthesis of acute phase proteins in hepatocytes [Dinarello, 1996, Mrak, *et al.*, 2001, Rosenwasser, 1998]. Additionally, IL-1α is an important chemotactic cytokine for macrophages and neutrophils, and is an important mediator of inflammation. Along with IL-6, IL-1α is an endogenous pyogen that induces fever in the hypothalamus and therefore plays a critical role in thermoregulation [Leon, 2002].

IL-3 is produced by activated T cells, NK cells, mast cells and thymic epithelial cells. IL-3 supports the growth and differentiation of hematopoietic cells of neutrophil, eosinophil, basophil, megakaryocyte and erythroid lineages [Aglietta, *et al.*, 1993, Ihle, 1992, Suda, *et al.*, 1985]. In addition, IL-3 has been shown to induce production of human B lymphoid progenitors from uncommitted progenitor stem cells, suggesting a crucial role in B lymphopoiesis [Crooks, *et al.*, 2000]. However, another study suggests that IL-3 may also have an inhibitory effect on early development of B lymphopoiesis [Miyamoto, *et al.*, 2001]. Therefore, the role of IL-3 in B cell development remains unclear.

IL-4 (also known as BCGF-1 and BCSF-1) is derived from T cells, mast cells and NK cells and has multiple effects on B cells. IL-4 co-stimulates antigen-primed B cells, stimulates proliferation as well as differentiation of activated B cells in a

paracrine manner, and directs the differentiation of germinal center B cell into memory B cells [Zhang, *et al.*, 2001]. Furthermore, IL-4 can induce class switching to IgG_1 and IgE in activated B cells, and upregulates MHC class II expression on resting B cells. In addition to influencing B cells, IL-4 induces proliferation of thymocytes and T cells, and increases MHC class II expression and phagocytic activity in macrophages. As a Th2-response mediator, IL-4 downregulates monocyte function by inhibiting IL-6, IL-12, and TNFα production. IL-4 is also a mast cell growth factor. The effects of IL-4 on endothelial cells include changes in cell morphology [Klein, *et al.*, 1993] and increased expression of VCAM-1 on vascular endothelial cells to induce lymphocyte and monocyte adhesion [Bochner, *et al.*, 1995, Kotowicz, *et al.*, 1996, Thornhill, *et al.*, 1990]. Additionally, several reports have shown that IL-4 has angiogenic activities [Fukushi, *et al.*, 1998, Fukushi, *et al.*, 2000].

IL-7 is expressed by bone marrow and thymic stromal cells, as well as by follicular dendritic cells. IL-7 was originally known as a pre-B cell growth factor that stimulated the differentiation and growth of lymphoid stem cells into progenitor B cells during early B cell development. IL-7 was later found to have similar effect on T cell development [Appasamy, 1999]. In addition to the effect of IL-7 on B cell development, tonsillar B cells proliferate when exposed to IL-7 [Kroncke, *et al.*, 1996]. Furthermore, IL-7 acts as an important regulator of T cell homeostasis by inducing T cell expansion in T cell depleted individuals [Fry, *et al.*, 2001]. IL-7 can induce the generation of cytotoxic T cells and NK cells and

activate monocytes [Kroncke, *et al.*, 1996, Or, *et al.*, 1998]. IL-7 also induces tumor rejection in mice *in vivo* [Hock, *et al.*, 1991]. One study reported that IL-7 upregulates HIV replication in PBMC from infected individuals [Smithgall, *et al.*, 1996].

IL-8 (also known as neutrophil activating peptide 1) is a proinflammatory cytokine secreted by endothelial cells, macrophages, monocytes, neutrophils, fibroblasts and T cells [Mahieux, *et al.*, 2001]. IL-8 was first identified as an endothelial cell-derived chemotactic cytokine for neutrophils that also induced release of lysosomal enzymes from neutrophils [Matsushima, *et al.*, 1988, Walz, *et al.*, 1987]. IL-8 induces adherence of neutrophils to vascular endothelium and facilitates extravasation into tissues [Huber, *et al.*, 1991]. Further, IL-8 mediates firm adhesion of monocytes to vascular endothelium under dynamic flow [Gerszten, *et al.*, 1999]. Additional studies have shown that IL-8 also stimulates chemotaxis in basophils and T cells [Gorman, *et al.*, 1982, White, *et al.*, 1989]. Expression of IL-8 can be induced by IL-1, TNFα, and phorbol myristate acetate (PMA), and is transcriptionally controlled by NFκB in endothelial cells where IL-8 is stored in Weibel-Palade bodies [Utgaard, *et al.*, 1998, Wolff, *et al.*, 1998]. Serum IL-8 levels are increased in HIV-infected individuals [Roux, *et al.*, 2000] and HIV Tat protein has been shown to induce IL-8 production in T cells [Ott, *et al.*, 1998], astrocytes [Kutsch, *et al.*, 2000] and brain endothelial cells [Hofman, *et al.*, 1999]. Therefore, IL-8 is believed to contribute to the cytokine dysregulation in HIV-infected individuals.

MCP-1 (monocyte chemotactic protein-1 or MCAF) is a β-chemokine (C-C chemokine) produced by macrophages that functions as a potent chemoattractant to recruit monocytes and T cells to sites of inflammation [Muller, 2001]. MCP-1 plays an important role in leukocyte transmigration by promoting adhesion of leukocytes to and transmigration across activated endothelium. Like IL-8, MCP-1 also mediates firm adhesion of monocytes to vascular endothelium under dynamic flow [Gerszten, *et al.*, 1999]. Mechanical shear stress of cultured endothelial cells has been shown to increase MCP-1 expression [Shyy, *et al.*, 1994, Wang, *et al.*, 1995]. In addition to mechanical stress, endogenous HIV Tat protein also increased secretion of MCP-1 by microvascular endothelial cells and increased monocyte migration [Park, *et al.*, 2001]. Tat-induced MCP-1 production by endothelial cells is believed to take place via activation of the protein kinase C (PKC) pathway [Park, *et al.*, 2001]. In addition, Tat protein is a potent inducer of MCP-1 in astrocytes [Kutsch, *et al.*, 2000], and increased MCP-1 mRNA expression in HIV-infected macrophages has been reported. [Boven, *et al.*, 2000].

In summary, a variety of cytokines and chemokines can regulate the trafficking, proliferation, and survival of normal as well as malignant B cells. These factors act via autocrine and paracrine pathways, and likely exert their influence in co-ordination with other growth factors. Previous reports, as well as data presented in this thesis, indicate that HIV Tat expression can influence cytokine production

and responsiveness, suggesting a mechanism through which HIV can promote a microenvironment that favors B cell lymphoma development.

1.5 CD40 and CD40 ligand

1.5.1 CD40

In our *in vitro* B lymphomagenesis system, HIV-induced MVEC-B cell adhesion is mediated by the interaction of two receptor/ligand pairs, CD40/CD40 ligand (CD40L) and vascular adhesion molecule-1 (VCAM-1)/very late activation antigen-4 (VLA-4). The subsequent sections in this chapter will address in detail each of these receptor/ligand pairs and the physiological significance of their interactions.

CD40 (also known as Bp50, p50, TNFRSF5) is a 45-50-kDa type-1 integral membrane glycoprotein belonging to the Tumor Necrosis Factor Receptor (TNFR) superfamily. CD40 is a phosphoprotein and may be expressed as homodimer. This cell surface antigen was originally discovered in 1985 as a B cell antigen important in B cell development and maturation [van Kooten, *et al.*, 2000]. In B cells, CD40 has a number of inducible functions, which include isotype switching and IgE production, homotypic adhesion, adhesion molecule and integrin (LFA-1 and ICAM-1) expression, costimulatory molecule (CD23 and B7) upregulation, Fas expression, IL-6 production, memory B cell generation, clonal expansion, and protection of germinal center and neoplastic B cells from apoptosis [Banchereau, *et al.*, 1994, Clark, *et al.*, 1986]. Following ligand binding

on the B cell surface, CD40 receptor transduces downstream signals by interacting with a number of adaptor molecules called TNFR-associated factors (TRAFs), resulting in NFκB activation.

Subsequent studies have found that CD40 is expressed by many other cell types including monocytes, dendritic cells, follicular dendritic cells, thymic epithelial cells, fibroblasts and keratinocytes [Alderson, *et al.*, 1993, Clark, *et al.*, 1986, Freudenthal, *et al.*, 1990, Galy, *et al.*, 1992]. In addition, CD40 is constitutively expressed at low levels in vascular EC [Hollenbaugh, *et al.*, 1995, Karmann, *et al.*, 1995] but is inducible, acting as an important activation marker to recruit leukocyte adhesion and provide T cell costimulation. These studies implicate CD40 in inflammation and autoimmune disease.

Inflammatory cytokines such as TNFα, interferon γ and IL-1 can induce CD40 expression three-fold on endothelial cells after treatment for 24 hrs [Karmann, *et al.*, 1995]. Furthermore, in endothelial cells, CD40 activation leads to the upregulation of adhesion molecules (ICAM-1, VCAM-1, E-selectin), induction of cytokines and chemokines (IL-1α, IL-6, IL-8, LIF, GM-CSF), increased production of matrix metalloproteinases (MMP), and stimulation of procoagulant activity [Dechanet, *et al.*, 1997, van Kooten, *et al.*, 2000]. In addition, endothelial activation induced by viral infection has also been linked to CD40 induction. For example, Human cytomegalovirus (HCMV) was recently shown to upregulate CD40 expression on EC, with a subsequent increase in E-selectin levels [Maisch,

et al., 2002]. Lastly, HIV infection of microvascular endothelial cells has been shown to upregulate CD40 expression, providing a mechanism for VCAM-1 induction and adhesion of B lymphoma cells to infected EC [Moses, *et al.*, 1997].

1.5.2 CD40 ligand

CD40 ligand (also known as CD40L, CD154, Gp39, TRAP, T-BAM, TNFSF5) is a 30-33 kDa type II (extracellular C-terminus) transmembrane trimeric glycoprotein [Pietravalle, *et al.*, 1996] belonging to the TNF cytokine superfamily. CD40L was originally identified as a CD4+ T cell activation antigen [Armitage, *et al.*, 1992, Hollenbaugh, *et al.*, 1992, Spriggs, *et al.*, 1992]. CD40L is expressed by CD4+ and CD8+ T cells, mast cells, NK cells, monocytes, dendritic cells, primary B cells and B cell lines including lymphoma cells [Cocks, *et al.*, 1993, Desai-Mehta, *et al.*, 1996, Gauchat, *et al.*, 1993, Gauchat, *et al.*, 1995]. CD40L can exist as either a membrane bound or soluble form.

The importance of the role of CD40L in immune regulation was recognized in patients with X-linked hyper-IgM syndrome with a mutation in the CD40L gene, and in CD40L knockout mice [van Kooten, *et al.*, 2000]. The absence of CD40L in these individuals contributes to a breakdown in immune surveillance; therefore, the susceptibility to intracellular pathogens and the incidence of carcinoma in these patients is increased [van Kooten, *et al.*, 2000]. CD40L activates and upregulates co-stimulatory molecules on antigen presenting cells, which are in turn important in activating T cells. In addition, CD40L induces production of IL-

12 by macrophages, which is important for CD4+ T cells to differentiate into type 1 helper T cells (Th1 response). The Th1 response is required for the generation of cytotoxic T lymphocytes (CTL) against tumors or virally infected cells.

CD40L, through the interaction with CD40, also plays an important role in B cell biology. CD40, triggered by CD40L, promotes differentiation of activated B cells and class switching from IgM to IgG production [Kornbluth, 2000]. Interestingly, some B cells and B lymphoma cells express CD40L as well as CD40. Examples of constitutive expression of CD40L on B cells are malignant B cells (Burkitt's and CLL) and B cells in Systemic Lupus Erythematosus patients, which spontaneously express high levels of CD40L [Devi, *et al.*, 1998, Higuchi, *et al.*, 2002, Schattner, 2000].

CD40L is believed to play multiple roles in HIV infection [Kornbluth, 2000]. However, the significance of these roles remains obscure, since in the context of antigen presentation and activation of T cell and macrophages, CD40L appears to facilitate HIV infection of these activated cells. CD40L can also inhibit HIV replication with the induction of immunosuppressive β-chemokines [Cotter, *et al.*, 2001, Kornbluth, *et al.*, 1998, McDyer, *et al.*, 1999].

1.5.3 Functional consequence of CD40/CD40 ligand interaction

CD40 cross-linking by either CD40L or anti-CD40 monoclonal antibody leads to signal transduction and gene activation. In particular, cross-linking of CD40 on monocytes leads to tumoricidal activity by an increase of TNFα and IL-6 production [Alderson, *et al.*, 1993]. In epithelial cells, CD40 cross-linking leads to GM-CSF production [Galy, *et al.*, 1992]. The role of CD40/CD40L interactions has been established in the activation of vascular endothelial cells. Karmann *et al.* (1995) have shown that CD40 expression and ligation leads to the subsequent upregulation of adhesion molecules such as E-selectin (CD62E), ICAM-1 (CD54) and VCAM-1 (CD106) on EC. Additionally, TNFα appears to be a potent inducer of CD40 as well as VCAM-1. CD40 receptor engagement with CD40L results in CD40 multimerization and transduction of an intracellular signal cascade via the long (62 amino acid) cytoplasmic tail, which lacks intrinsic enzymatic activity. Downstream mediators known as tumor necrosis factor receptor-associated factors (TRAFs) facilitate signal transduction to the nucleus. To date, TRAFs 1 through 6 have been identified and different TRAFs can associate with distinct regions of CD40 cytoplasmic tail [Ni, *et al.*, 2000]. Signaling cascade pathways and TRAF usage may be cell type dependent. In B cells, CD40-mediated p38 activation proceeds uniquely via TRAF-3. Vascular endothelial CD40 may likely interact with a different set of TRAF molecules. However, common features of CD40 signal transduction include activation of src family tyrosine kinases, JAK and STAT proteins, activation of mitogen-activated protein kinases (MKK1, ERK, JNK, p38) and nuclear translocation of transcription factors (NFkB, AP-1,

NFAT) [Calderhead, *et al.*, 2000, Tewari, *et al.*, 1996], leading to subsequent activation of NFkB and JNK/SAPK pathways and eventual induction of gene expression.

The CD40/CD40L interaction is central to adaptive immunity [Foy, *et al.*, 1996, Grewal, *et al.*, 1997, Noelle, 1996, van Kooten, *et al.*, 2000]. Vascular CD40 and CD40L play an important role in leukocyte adhesion, atherogenesis and activation of endothelial cells, leading to the induction of adhesion molecules [Hollenbaugh, *et al.*, 1995, Karmann, *et al.*, 1995, Karmann, *et al.*, 1996, Schmitz, *et al.*, 1998, van Kooten, *et al.*, 1997, van Kooten, *et al.*, 1997, Yellin, *et al.*, 1995]. Consistent with other studies, Moses *et al* 1997 demonstrated that HIV infection increased expression of CD40 on microvascular endothelial cells (MVEC). Further, the triggering of MVEC CD40 by CD40L expressed on the B lymphoma cells led to a subsequent upregulation of VCAM-1 on MVEC. A primary goal of this thesis is to identify the HIV genes responsible for the increased expression of CD40 and subsequent upregulation of VCAM-1 on MVEC.

1.6 Vascular adhesion molecules

1.6.1 VCAM-1

Cellular interactions mediated by cellular adhesion molecules form the basis for many diverse biological processes, including embryogenesis, hematopoiesis, cellular migration and inflammation [Cavenagh *et al.,* 1998; van Dinther-Janssen *et al,* 1991]. Adhesion molecules facilitate interactions between cells of the same

(homotypic) or distinct (heterotypic) lineages and function as receptors for extracellular matrix components and infectious agents. Accordingly, adhesion molecules play a central role in normal physiologic functioning as well as in many pathologic states [Cavenagh *et al.,* 1998]. Specifically, vascular adhesion molecules expressed on the surface of vascular endothelium are central to immune reactions, inflammation and a host of pathological conditions involving adhesive interactions between endothelial cells and leukocytes or cells of non-leukocyte lineage. Cellular adhesion molecules have been structurally and functionally divided into at least five families: selectins, integrins, immunoglobulin superfamily (IgSf), cadherins and sialomucins [Henderson and Moses, 2002]. This thesis will focus on members of the immunoglobulin superfamily and integrins, in particular, vascular cell adhesion molecule-1 (VCAM-1) and the integrin ligand or binding partner, the very late activation antigen-4 (VLA-4).

Members of the immunoglobulin superfamily all possess unpaired extracellular domains of immunoglobulin-like anti-parallel β strands stabilized by disulfide bonds, a transmembrane helix and a cytoplasmic domain [Fukushi *et al.,* 2000]. IgSf molecules are important in organ development, particularly within the nervous system, and play a central function in the complex process of immune system regulation, which requires both adhesion and signaling functions. VCAM-1 (CD106, INCAM-110) is found on activated endothelial cells. Receptive ligands to VCAM-1 include VLA-4, α4β7 and αDβ2. Diverse functions include

leukocyte adhesion and transmigration, T- and B-cell maturation, embryogenesis and hematopoietic cell homing [Bochner, *et al.*, 1991, Carlos, *et al.*, 1991, Imhof, *et al.*, 1995, Kwee, *et al.*, 1995, Osborn, *et al.*, 1989, Papayannopoulou, *et al.*, 1997]. VCAM-1, expressed on activated endothelial cells, has an important role in localization of B- and T-cells and regulates adhesion of monocytes, lymphocytes, basophils and eosinophils to activated endothelium [Yusuf-Makagiansar, *et al.*, 2002].

VCAM-1 is constitutively expressed at low levels on MVEC [Swerlick *et al.*, 1992]. The expression of this adhesion molecule was shown to be inducible by cytokine stimulation [Bevilacqua, *et al.*, 1987, Gerlach, *et al.*, 1989, Swerlick, *et al.*, 1992], which allows lymphocytes and tumor cells such as Ramos cells to adhere to the activated, VCAM-1-positive endothelial cells [Carlos, *et al.*, 1991, Carlos, *et al.*, 1990, Swerlick, *et al.*, 1992]. TNFα treatment leads to a persistent and dose dependent induction of VCAM-1 [Bevilacqua, *et al.*, 1993, Swerlick, *et al.*, 1992, Thornhill, *et al.*, 1991]. This potent induction of VCAM-1 by TNFα appears to involve activation of NFκB [Collins, 1993, Heller, *et al.*, 1994, Marui, *et al.*, 1993, Weber, *et al.*, 1994], protein tyrosine kinase and protein tyrosine phosphatase [Dhawan, *et al.*, 1997, Weber, 1996]. As mentioned in the previous section, CD40 signal transduction also induces strong expression of VCAM-1 on MVEC. CD40L is believed to induce adhesion molecule expression by triggering CD40 with signaling pathways similar to those of TNFα and IL-1 [Karmann, *et al.*, 1996].

1.6.2 VLA-4

VLA-4 (α4β1, CD49d, CD29) is a member of the integrin-type family of cell adhesion molecules. VLA-4 is involved in leukocyte trafficking and extravasation [Carlos, *et al.*, 1994, Dunon, *et al.*, 1996, Imhof, *et al.*, 1995, Imhof, *et al.*, 1997, Panes, *et al.*, 1999, Springer, 1995, Tan, *et al.*, 1999]. This integrin is found on B- and T-cells, monocytes and macrophages, basophils, and eosinophils, but not neutrophils, and plays an important role in the recruitment of these cells to sites of inflammation.

Integrins are heterodimeric transmembrane glycoproteins consisting of two non-covalently linked α- and β- subunits. Various α- and β- subunits have been identified and differential pairing leads to considerable diversity within this family. Generally, integrin activation that induces conformational change is a prerequisite for ligand binding. In addition to the adhesive function, integrins mediate signal transduction following ligand binding. VLA-4 belongs to the β1 integrins (VLA subfamily) and has a 155kDa α4 and a 150 kDa β1 chain. The interacting partners of VLA-4 are VCAM-1, extracellular matrix protein (ECM) and fibronectin (FN) [Masumoto *et al.,* 1993; Johnson *et al.,* 1999, Stephens, *et al.*, 2000]. Circulating leukocytes constitutively express VLA-4 with low ligand binding capacity. In the case of leukocytes extravasation, high affinity binding of VLA-4 to VCAM-1 is preceded by activation of leukocyte integrins by local cytokines/chemokines, leading to conformational change. Conformational change

promotes a high affinity and irreversible interaction between activated integrin VLA-4 and endothelial VCAM-1. CD40-CD40L interactions may trigger a similar conformational change in B lymphoma cell VLA-4 to high affinity state, facilitating a firm attachment between B lymphoma cell VLA-4 and endothelial cell VCAM-1.

1.6.3 Physiological roles of VCAM-1 and VLA-4

Since the initial proposal of a multi-step model for leukocyte transmigration, many of the adhesion molecules involved in the leukocyte-endothelium interaction have been identified [Butcher, 1991, Butcher, *et al.*, 1996]. During an immune response, leukocytes are recruited from the bloodstream to the site of inflammation. Pro-inflammatory cytokines and chemokines secreted by the leukocytes activate the endothelium and regulate expression of the adhesion molecules required for extravasation. The extravasation process can be divided into several sequential steps, including margination, rolling/transient attachment, triggering/integrin activation, stable adhesion and diapedesis/transmigration (Figure 1.5). Margination (step 1) is a random, adhesion-independent process that allows the leukocytes to flow near the endothelium at the margin of post-capillary venules. Margination facilitates leukocyte rolling, whereby the marginated leukocytes undergo a series of reversible and low affinity attachments to the vessel wall (step 2). These transient attachments involve interaction between leukocyte L-selectin and counter-ligands on the activated endothelium such as E-selectin, P-selectin and various sialomucins. The successive expression and

shedding of L-selectin allows the leukocytes to loosely adhere to and roll across the endothelium. Rolling continues until local cytokine/chemokine signaling activates leukocyte integrins and triggers a conformational change (step 3). Conformational change promotes a high affinity and irreversible interaction between activated integrins (e.g. LFA-1 and VLA-4) and endothelial IgSf molecules (e.g. VCAM-1, MAdCAM-1, ICAM-1 and ICAM-2), which stably attach the leukocytes to the endothelium (step 4). Leukocyte migration or diapedesis across the vessel wall follows and is mediated by both homotypic (e.g. PECAM-1) and heterotypic (e.g. LFA-1/ICAM-1, VLA-4/VCAM-1) interactions (step 5). An interstitial chemokine gradient between the sites of diapedesis and inflammation facilitates directed leukocyte trafficking.

Other than the major role in leukocyte extravasation, vascular adhesion molecules (VLA-4 /VCAM-1; PSGL-1/P-selectin) also play a role in the homing of circulating hematopoietic progenitor cells to bone marrow endothelium. Furthermore, chronic inflammatory disease and transplant rejection are associated with vascular adhesion molecule dysregulation. The pathogenic mechanisms underlying many disease states involve aberrant cell adhesion and transmigration events, which are mediated in turn by vascular adhesion molecules. Many of these pathological processes share features with a normal inflammatory response.

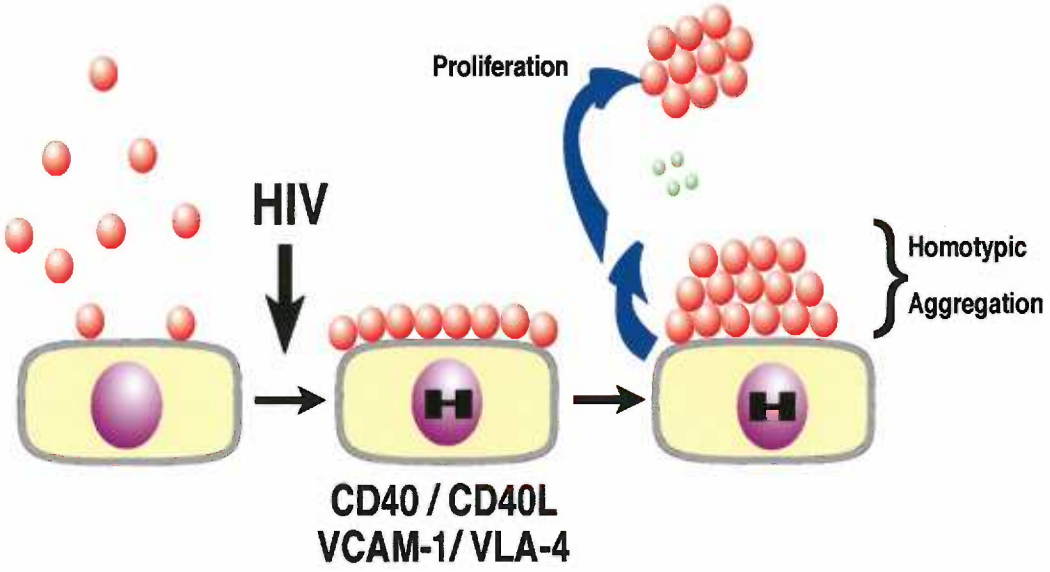
Adhesion also plays a vital role in malignant tumor cell homing and metastasis. Changes in expression profiles of cell adhesion molecules has been correlated

with tumor progression [Yoong, *et al.*, 1998], and increased expression of α4 integrin (VLA-4) has been shown to suppress metastasis [Gosslar, *et al.*, 1996]. Adhesion of cells is necessary to anchor and target tumor cells to specific sites such as the endothelium. Malignant cells enter the bloodstream, attach to the endothelium at a distal site and transmigrate across the vessel wall to form a secondary tumor.

Tumor cell adhesion to the endothelium at the distal site utilizes many of the adhesion molecules involved in normal inflammatory processes, while subsequent invasion through endothelial junctions resembles leukocyte diapedesis [Imhof, *et al.*, 1996, Lafrenie, *et al.*, 1992]. Inflammatory cytokines also regulate the dynamic interaction between the malignant cells and the endothelium [Kaji, *et al.*, 1995, Lafrenie, *et al.*, 1992].

Because of the importance of adhesion molecules in diseases, adhesion-based therapeutics using peptidase and small molecules have been used experimentally to inhibit VCAM-1/VLA-4 in inflammation and autoimmune diseases [Yusuf-Makagiansar, *et al.*, 2002]. Since adhesion of the B lymphoma cells involves the interaction of VCAM-1/VLA-4, these same treatments may be beneficial in treating B cell lymphomas. In addition, identification of additional molecules driving the EC-B lymphoma interaction may suggest general as well as more tumor or disease-type-specific therapeutic alternatives. The work presented in this thesis lays the groundwork for identification of novel treatments for AIDS-NHL.

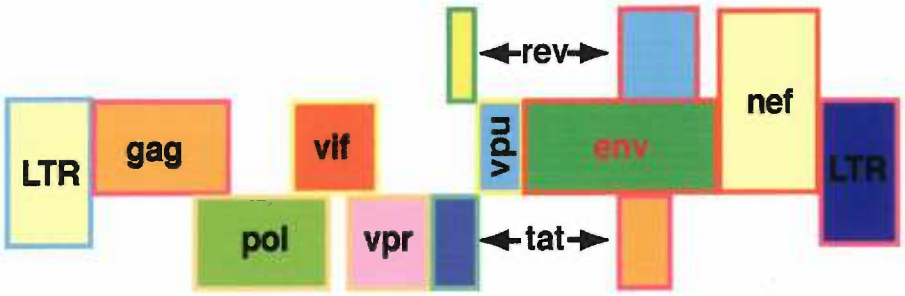
Figure 1.1 Model: A Role for HIV in Lymphomagenesis



Schematic depiction of events:

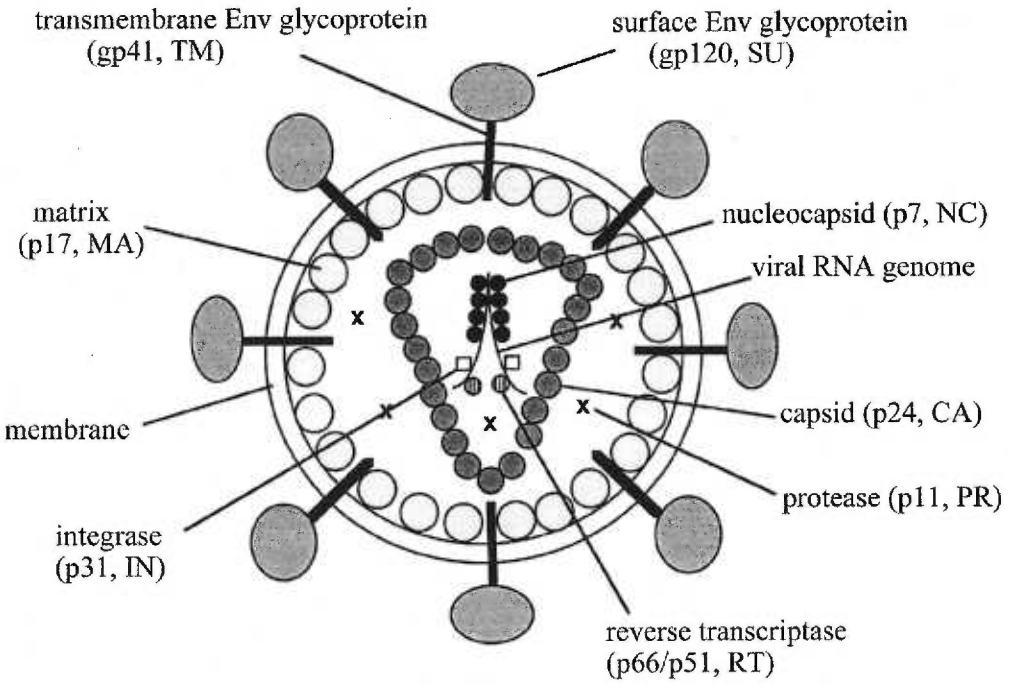
HIV-1 infection of endothelial cells (MVEC) induces MVEC-B lymphoma cell adhesion, which is mediated by the interaction of two receptor/ligand pairs, CD40/CD40L and VCAM-1/ VLA-4. The initial interaction between CD40-positive HIV-infected MVEC and CD40L-positive B lymphoma cells promotes VCAM-1 expression on MVEC and subsequent VCAM-1/VLA-4-mediated heterotypic adhesion. HIV-1 infection also induces lymphoma cell proliferation and lymphoma cell aggregation leading to the eventual formation of B lymphoma cell foci adherent to, or in close association with, infected endothelium.

Figure 1.2 Genome organization of HIV-1



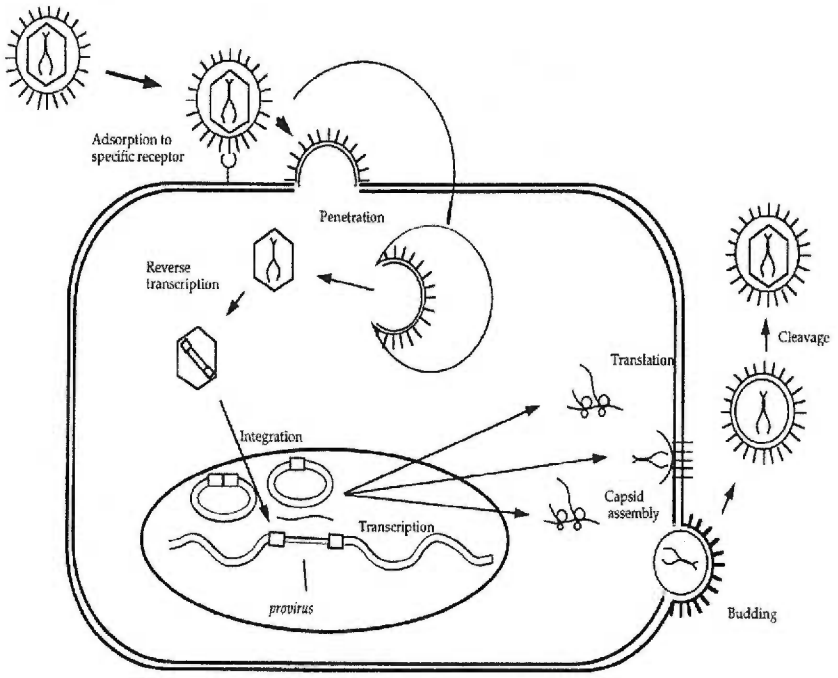
HIV-1 genome is comprised of three structural genes (*gag, pol* and *env)*, two regulatory genes (*rev* and *tat*), four accessory genes (*vpu, nef, vpr* and *vif)*, a 5' long terminal repeat (LTR) and a 3' LTR. (Adapted from *Fields Virology*, Fourth Edition, 2001)

Figure 1.3 HIV-1 viral particle



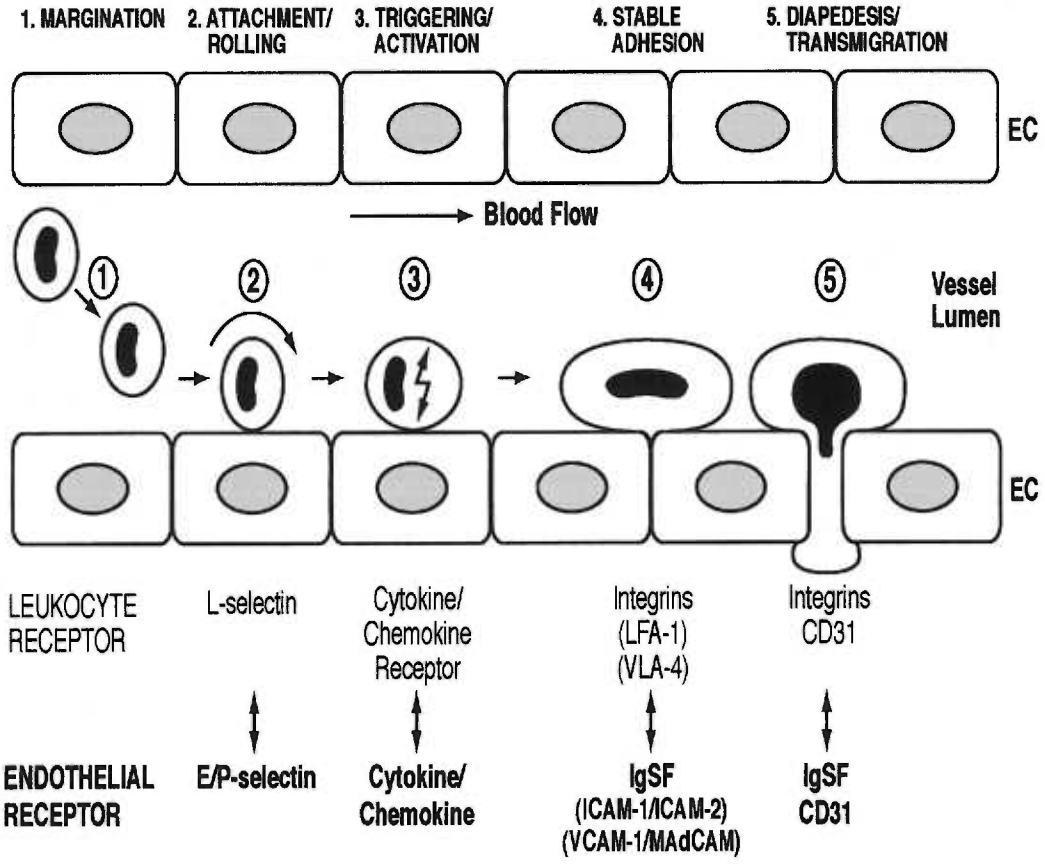
HIV-1 virion structure.
See text for the function of each virion component.
(Adapted from *Fields Virology*, Fourth Edition, 2001)

Figure 1.4 HIV-1 replication cycle



HIV-1 viral life cycle.
See text for detail description of HIV-1 replication cycle.
(Adapted from *Fields Virology*, Fourth Edition, 2001)

Figure 1.5 Leukocyte-endothelial cell interaction



The sequential steps of leukocyte-endothelial cell interaction are numbered 1-5 as described in the text. The major adhesion molecules expressed on leukocytes (normal font) and endothelial cells (bold font) at each step are shown.

Chapter 2

Endothelial cells expressing human immunodeficiency virus-1 Tat support B lymphoma cell proliferation.

ABSTRACT

AIDS-associated non-Hodgkin's B cell lymphoma (AIDS-NHL) is the second most prevalent AIDS-associated malignancy arising in individuals infected with human immunodeficiency virus type-1 (HIV). An infectious agent has not been universally identified to account for the increase in frequency of malignant B cells in AIDS-NHL. Therefore, attention has focused on indirect factors such as HIV-mediated immunologic imbalance and infection of non-malignant cells that interact with the lymphoma cells. Microvascular endothelial cells (MVEC) interact closely with B cells at the blood-tissue interface and are susceptible to HIV infection. HIV-infected endothelial cells are thus ideally situated to influence homing and aggressive growth of AIDS-NHL at extranodal tissue sites. We have previously demonstrated that brain and bone marrow MVEC develop an enhanced capacity to support the attachment and growth of B cell lymphomas following HIV infection *in vitro* [Moses, *et al.*, 1997]. The present study was designed to examine the role of HIV proteins in influencing the growth support phenotype of infected endothelial cells (EC). Recombinant adenovirus vectors were used to express HIV proteins in EC, which facilitated us in examining the ability of these particular EC to influence B lymphoma (BL) cell growth. When BL cells were co-cultured with HIV Tat-expressing EC, a significantly enhanced BL cell growth rate was observed relative to BL

cells co-cultured with control EC or EC expressing other HIV genes. HIV Tat is a potent transactivator of viral gene expression and is also capable of modulating the expression and function of a variety of cellular genes. Intracellular cytokine staining of Tat-expressing EC revealed induction of the B cell growth factors interleukin-6 (IL-6) and interleukin-10 (IL-10) which played a primary role in the enhanced B lymphoma cell growth as suggested by cytokine neutralization experiments. Taken together, these data support an important role for Tat-induced cytokines in regulating B lymphoma cell proliferation and survival. In the context of AIDS, such a mechanism could eventually lead to the aggressive growth profile of AIDS-NHL at extranodal tissue sites.

INTRODUCTION

AIDS-associated non-Hodgkin's lymphoma (AIDS-NHL) remains a significant clinical problem in individuals infected with human immunodeficiency virus type-1 (HIV). The AIDS-NHL are primarily of B cell origin and are not HIV-infected. While different histological subtypes exist, common properties include aggressive growth at extranodal sites and lack of a universal infectious agent. The unique microenvironment created by HIV infection is thus believed to play a role in the extranodal homing and growth of malignant B cells. Immunosuppression and the sustained B cell stimulation in polyclonal hypergammaglobulinemia experienced by AIDS patients are two major factors implicated in the development of B cell malignancies [Grulich, *et al.*, 2000]. Some investigators have speculated that polyclonal B cell proliferation predisposes B cells to accumulate genetic errors, thereby leading to the development of monoclonal B cell lymphoma [Levine, *et al.*, 2001, Przybylski, *et al.*, 1996]. However, the distinct mechanism(s) by which HIV influences the establishment of NHL remain unclear.

Our group has previously reported that HIV-infected microvascular endothelial cells (MVEC) support the attachment and growth of AIDS-NHL B cell lines in culture [Moses, *et al.*, 1997]. Initially, B lymphoma (BL) cells adhered tightly to the HIV-infected monolayer via a tight VCAM-1/VLA-4-mediated interaction. With continued co-culture, dividing BL cells seeded from the MVEC-adherent layer and continued to proliferate in the overlaying MVEC-conditioned supernatant. We thus hypothesized that HIV contributes both an adhesive and a growth-promoting phenotype to the endothelium, and

that the growth promoting phenotype may in part operate via a contact-independent mechanism such as the production of soluble B cell growth factors.

The aim of the present study was to identify the HIV protein(s) responsible for the B cell growth support as well as the nature of this support. Specifically, endothelial cells from large vessel and the microvasculature were transduced with adenovirus vectors expressing individual HIV proteins, and evaluated for their capacity to support the growth of the AIDS-NHL B cell line, JB. JB is an EBV-negative, HHV8-negative Burkitt's lymphoma derived from the bone marrow of an AIDS patient following *ex vivo* culture of bone marrow under conditions that promoted enrichment of supportive HIV-infected stromal MVEC. The subsequent outgrowth of malignant B cells from this *ex vivo* culture matched the Burkitt's lymphoma arising independently within the patient's bone marrow *in situ*. We report here for the first time that Tat-expressing EC support proliferation of BL cells, and that the active factors in the growth-promoting medium include IL-6 and IL-10. The BL cell proliferation phenomenon was Tat-specific since other HIV accessory and regulatory genes tested in parallel did not induce a growth support phenotype in EC.

Tat, the transactivator of HIV gene expression, also modulates expression of diverse cellular genes including cytokines, B cell growth factors, anti-apoptotic mediators and extracellular matrix (ECM) proteins, and has a stimulatory effect on various cell types, including endothelial cells [Biswas, *et al.*, 1995, Chang, *et al.*, 1995, Cupp, *et al.*, 1993, Sawaya, *et al.*, 1998, Tada, *et al.*, 1990, Taube, *et al.*, 1999, Taylor, *et al.*, 1992, Zauli, *et*

al., 1992]. For example, Tat activates endothelial cells by upregulating IL-6 production, promoting expression of adhesion molecules (e.g., E-selectin, VCAM-1 and ICAM-1), inducing collagenase production, and altering vascular permeability [Dhawan, *et al.*, 1997, Ensoli, *et al.*, 1990, Hofman, *et al.*, 1993, Maruo, *et al.*, 1992].

Our studies suggest an additional role for Tat in regulation of endothelial function; namely, induction of a phenotype that favors B lymphoma cell growth. From a broader perspective these results imply an active role for Tat in driving AIDS lymphomagenesis.

MATERIALS AND METHODS

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker, Inc. (San Diego, CA). HUVEC monolayers were cultured in an endothelial growth medium (EGM; BioWhittaker, Inc) supplemented with 10% human AB serum (Sigma, St Louis, MO), and PSG (penicillin at 100U/ ml, streptomycin at 100mg/ml and 2mM glutamine) on 35mm- or 60mm-Primaria™ (Becton-Dickinson, Bedford, MA) tissue culture dishes and used for experiments at 4^{th}-6^{th} passage. Dermal MVEC (DMVEC) were derived by immortalization of primary cells with HPV type 16 E6/E7 as previously described [Moses, *et al.*, 1999] and cultured as described for HUVEC. DMVEC retained the essential features of primary cells but were not restricted by tissue culture passage. B lymphoma (BL) cells were an EBV-negative Burkitt's lymphoma line derived from an HIV-positive patient as previously described [Moses, *et al.*, 1997]. BL cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone, St. Louis,

MO), and PSG. Human MAGI cells (H-MAGI cells) are HeLa-CD4 cells expressing β-galactosidase under the HIV-1 LTR. H-MAGI cells were obtained through the NIH AIDS Research & Reference Reagent Program (Rockville, MD) and grown on 24 well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and PSG.

Adenoviral construction and infection:

Recombinant adenovirus vectors were constructed as previously described [Hitt, 1994, Streblow, *et al.*, 1999]. HIV-1 *vpu*, *nef*, *vif, vpr, rev*, and *tat* were amplified by polymerase chain reaction (PCR) from the HIV proviral plasmid pNL4-3 (obtained from the NIH AIDS Research & Reference Reagent Program) with the gene specific forward and reverse primers. Each PCR product was cloned into the adenovirus shuttle vector pΔE1sp1Btet/EF1EGFPTKpolyA. This vector was derived from plasmid pΔE1sp1Btet as described previously [Streblow, *et al.*, 1999] with the addition of EF1-EGFP and herpes simplex virus TK poly(A). The recombinant adenoviruses were produced by co-transfection of 293 cells with a shuttle plasmid containing the individual HIV genes and pJM17 (Microbix, Toronto, Ontario, Canada), which has an E1A-deleted adenovirus genome. Recombinant adenovirus stocks were titered on 293 cells by limiting dilution. All adenovirus infections were performed with co-infection of an adenovirus transactivator-expressing virus (Ad/trans) at an equivalent, pre-optimized multiplicity of infection (MOI). Monolayers of HUVEC or DMVEC were infected with the various adenoviruses at an MOI 100 of Ad/trans and /or Ad/vpu, Ad/tat, Ad/vpr, Ad/vif, Ad/rev,

or Ad/nef in the presence of polybrene (2μg/ml hexadimethrine bromide; Sigma) for 6 hrs and incubated with fresh media for an additional 24 to 48 hrs.

HIV/VSV-G pseudotype virus infection

Recombinant HIV/VSV-G pseudotype virus was constructed as described previously [Bartz, *et al.*, 1997]. Detail of the virus construction can be found in Materials and Methods section in Chapter 3 of this thesis. Pseudotype viral infection was performed by exposing HUVEC to the titered viral inoculae with polybrene (2μg/ml; Sigma), for 2 hours in a minimal volume of medium to promote adsorption, followed by an overnight incubation under normal culture conditions. The following day, monolayers were rinsed in HBSS and recultured in EGM.

Western blot analysis

HUVEC were grown in EGM on 60 mm Primaria™ tissue culture dishes. Monolayers were infected with the tat recombinant adenovirus, Ad/tat, along with Ad/trans, or with the HIV/VSV-G pseudotype as described above. Cells infected with 2X Ad/trans or mock-infected cells were used as controls. Cells were harvested and lysed in 200-500 μl 2X SDS (2X SDS loading dye, 1M DTT and 2% bromophenol blue) with boiling for 5 minutes. 30 μl of total cell lysate from each sample was electrophoresed by SDS-PAGE on a 12% polyacrylamide gel and blotted onto nitrocellulose membranes (Schleicher and Schuell, Keen, NH). The nitrocellulose membranes were blocked with 1% nonfat milk in TBS-Tween 20 (100mM Tris-Cl [pH 7.5], 150mM NaCl and 0.1% Tween 20) for 1 hr. This was followed by incubation with a mouse anti-HIV Tat monoclonal antibody (1:100

dilution in TTBS with 1% nonfat milk; Advanced Biotechnologies Inc., Columbia , MD) for 2 hrs at room temperature. A mouse anti-paxillin monoclonal antibody (1:1000; Upstate Biotechnology, Lake Placid, NY) was used as a loading control. After incubation, membranes were washed and treated with a secondary peroxidase-labeled anti-mouse IgG (1:1500 in TTBS with 1% nonfat milk; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr. The ECL system was used to visualize the protein of interest, and bands were developed by chemiluminescence and autoradiography.

Immunofluorescence

Immunofluorescence analysis was performed as described previously [Moses, *et al.*, 1997]. For the detection of HIV Tat, HUVEC monolayers were rinsed with PBS with Ca^{2+}/Mg^{2+}, fixed in 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 20% normal goat serum (NGS) and stained with mouse anti-HIV Tat antibody (1:100; Advanced Biotechnologies Incorporated), followed by a rhodamine-conjugated anti-mouse IgG antibody (1:100; BioSource International, Camarillo, CA). Microscopic images were captured with a Nikon fluorescence microscope and camera. An absence of non-specific staining was assessed by irrelevant isotype-matched antibodies and secondary antibodies alone.

H-MAGI assay

Human MAGI (H-MAGI) assays were performed as described previously [Bartz, *et al.*, 1997, Vodicka, *et al.*, 1997]. Briefly, H-MAGI cells grown on 24-wells plate were infected with adenoviruses expressing Tat (Ad/tat) and transactivator (Ad/trans) at an

MOI of 10 and 50 for 24 hrs followed by fixing and staining for β-galactosidase. In this assay, cells expressing HIV Tat protein transactivate the expression of β-galactosidase following interaction with the HIV-1 LTR, and the blue color in cells is scored via light microscopy.

Quantitative proliferation assay

BL cells ($5x10^3$ and $1x10^4$ cells/well) were incubated for 24 and 48 hrs in 96 well trays in 150 µl conditioned supernatant harvested from DMVEC that were mock infected or infected with Ad/trans or Ad/tat for 48 hrs. The ability of these different supernatants to support BL cell proliferation was tested in an XTT proliferation assay. Control wells contained BL cells in endothelial growth medium alone. After 48 hrs, 50 µl XTT (Roche Diagnostics, Indianapolis, IN) was added to each well and 4-6 hours later, the spectrophotometrical absorbance of each sample was recorded by an ELISA reader between 450 and 500nm. XTT is metabolized to a water-soluble formazan dye by viable cells and therefore gives a quantitative determination of proliferation. The same assay protocol was also used to measure BL cell proliferation in response to recombinant cytokines. These assays were performed as above, but BL cells were incubated in 150 µl culture medium supplemented with rIL-6, rIL-7, rIL-8 or rIL-10 separately or in combination (R& D Systems, Minneapolis, MN) at doses from 5-20 ng/ml. Cytokines were reconstituted in sterile PBS with 0.1% BSA prior to use. BL cells incubated in Ad/tat supernatant was used as a positive control.

The XTT assay also serves as a measure of viable cell number, and was used for this purpose to evaluate BL cell growth following a period of EC-BL cell co-culture. For these experiments, equal numbers of BL cells ($5x10^4$ cells/35mm dish) were co-cultured with mock, Ad/trans-infected or Ad/tat-infected DMVEC monolayers at 24 hrs post adenovirus infection. 48 hours later, BL cells were rinsed from the monolayer and resuspended in equal volumes (1 ml/sample) of complete RPMI. 150 µl aliquots of cell suspension were added to quadruplicate wells of 96 well trays followed by addition of XTT. 4-6 hrs later, the OD was determined as described above.

Antibody neutralization studies

DMVEC and HUVEC were infected with Ad/tat, Ad/vpu or Ad/trans, and conditioned medium was harvested at 48 hrs post infection. The ability of these EC supernatants to support BL cell proliferation was tested in XTT assays as described above, but cytokine neutralizing antibodies were used to neutralize selected cytokines in the conditioned medium. Specifically, antibodies against IL-6, IL-7, IL-8 and IL-10 (all from R&D Systems) were added to conditioned supernatants at a concentration of 10µg/ml. Isotype-matched antibodies were also obtained from R&D systems and used as controls; an IgG_1 for anti IL-6, 7 and 8 and an IgG_{2b} for anti-IL-10. Additional control wells contained BL cells exposed to non-neutralized conditioned medium and BL cells exposed to EGM containing antibodies alone. After a 48 hr proliferation in the presence of MVEC conditioned medium containing the different neutralizing antibodies, XTT was added and BL cell proliferation evaluated spectrophotometrically.

Assay for cytokine production

HUVEC monolayers at 70-80% confluence were infected with Ad/tat or Ad/vpu for 24 hrs, followed by treatment with Brefeldin A (BD Pharmingen, San Diego, CA) for 3 hrs to collapse the Golgi apparatus in order to retain secreted cytokines intracellularly. Immunofluorescent staining for individual cytokines was performed with R-PE-conjugated rat anti-human IL-3 (0.5 μg /10^6 cells), R-PE-conjugated rat anti-human IL-4 (0.2 μg /10^6 cells), R-PE-conjugated rat anti-human IL-6 (pretitrated at 20 μl /10^6 cells), R-PE-conjugated mouse anti-human IL-8 (pretitrated at 20 μl /10^6 cells), R-PE-conjugated rat anti-human IL-10 (pretitrated at 20 μl /10^6 cells), R-PE-conjugated mouse anti-human MCP-1 (pretitrated at 20 μl /10^6 cells), R-PE-conjugated mouse anti-human TNFα (pretitrated at 20 μl /10^6 cells) or R-PE conjugated species-specific isotype-matched antibodies (all purchased from BD Pharmingen). Briefly, cells were washed following Brefeldin A treatment and treated with Cytofix/Cytoperm Plus solution (BD Pharmingen) for 20 minutes to fix and permeabilize the cells. This was followed by washing twice with Perm/Wash solution (BD Pharmingen) containing sodium azide and saponin and blocking with 20% NGS in Perm/Wash solution for 30 minutes. Incubation with PE-conjugated monoclonal antibodies against various cytokines or corresponding isotype control monoclonal antibodies was performed in Perm/Wash solution. The cells were detached non-enzymatically, and fluorescence was analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with CellQuest software (Becton-Dickinson). A minimum of 10,000 cells was analyzed for each sample.

RESULTS

HIV Tat-expressing endothelial cells support B lymphoma cell proliferation.

To elucidate the role of HIV proteins in supporting lymphoma cell growth in an *in vitro* lymphomagenesis model, HIV proteins Nef, Tat, Rev, Vif, Vpr and Vpu were individually expressed in endothelial cells using an adenovirus expression system (Appendix A).

When B lymphoma (BL) cells were co-cultured with adenovirus-infected DMVEC monolayers, an increased BL cell density was observed by light microscopy in co-culture with Ad/tat-infected EC as compared to mock-infected EC or EC infected with the Ad/transactivator (Ad/trans) alone (Figure 2.1A). A similar observation was made with HUVEC (data not shown), suggesting that Tat expression enhanced the capacity of EC to support BL cell proliferation and/or survival. Furthermore, when BL cells were exposed to cell-free supernatants harvested from DMVEC or HUVEC infected with Ad/tat or Ad/trans alone, a similar increase in cell density was observed by light microscopy only in response to conditioned medium from Tat-expressing EC (Figure 2.1B). When similar experiments were performed with EC infected with recombinant adenovirus vectors for the other HIV proteins, cell densities were similar to those observed in mock-infected or Ad/trans-infected EC (data not shown). Collectively, these data suggest that HIV Tat expression in endothelial cells uniquely promotes BL cell growth, a phenomenon that could contribute to the aggressive growth profile of AIDS-NHL *in vivo*.

The expression of HIV Tat (Ad/tat) using the recombinant adenovirus system was verified by western blot analysis (Figure 2.2A) and immunofluorescence (Figure 2.2B). Additionally, adenovirus-expressed Tat was able to transactivate the HIV-1 LTR in H-MAGI cells (Figure 2.2C), thereby confirming the functionality of the vector-expressed protein.

HIV Tat or Tat-induced factors promote B lymphoma cell growth.

As illustrated in Figure 2.1, visual examination of EC-BL cell co-cultures via light microscopy revealed that Tat expression enhanced the ability of EC to support BL cell growth. To quantitate this observation, BL cells were removed from co-culture with Ad/tat-infected DMVEC and the viable cell density evaluated using a tetrazolium-based (XTT) colorimetric cellular quantitation assay. As controls, BL cells removed from co-culture with mock-infected or Ad/trans-infected DMVEC were tested in an identical manner. As graphically illustrated in Figure 2.3A, the viable BL cell density in Ad/tat-infected DMVEC co-culture was significantly greater than under control conditions. These assays suggested that Tat-expressing EC may produce soluble factors stimulatory to BL cell growth. To address this hypothesis, cell-free supernatants were harvested from mock infected, Ad/tat-infected or Ad/trans-infected DMVEC and the ability of these conditioned supernatants to promote BL cell proliferation was tested. In this instance, constant numbers of BL cells were exposed to conditioned media and proliferation over a 48 hr period measured via the XTT assay. Tat-conditioned medium stimulated a significantly greater level of BL cell proliferation (at least two-fold) as compared to supernatants from EC infected with controls; Ad/trans alone or mock-infected EC (Figure

2.3B). Thus, these data provide quantitative proof that HIV Tat expression in EC induces a phenotype that is supportive of BL cell growth.

HIV-1 Tat induces the production of B cell growth factors by HUVEC

The support phenotype conferred on EC supernatants by expression of HIV Tat suggested that Tat induced the EC production of B cell growth factors. To directly evaluate the production of such cytokines by Ad/tat-infected EC, we performed intracellular cytokine staining (ICCS) to measure the levels of IL-3, IL-4, IL-6, IL-8, IL-10, MCP-1 and TNFα (Figures 2.4 and 2.5). ICCS is a quantitative assay for measuring the total cytokine level produced by each cell, since secretion of soluble protein is experimentally inhibited by treatment with Brefeldin A. As illustrated in Figure 2.4, increased expression of IL-6, IL-8, and IL-10 was observed in Ad/tat-infected EC as compared to Ad/vpu infected or mock-infected cells. As IL-6, IL-8 and IL-10 are all B cell growth factors, these cytokines are likely contributing to the enhanced proliferation of BL cells exposed to conditioned medium from, or in co-culture with, Ad/tat-infected EC. The effect of Tat on the production of IL-6, IL-8, and IL-10 is specific in that Tat did not have any effect on the production of other cytokines (Figure 2.5). The data revealed by the ICCS assay strongly suggests a role for Tat-induced cytokines IL-6, IL-8, and IL-10 in driving B lymphoma cell proliferation at extranodal sites of EC-B cell interaction.

Tat-induced EC- derived IL-6 and IL-10 contribute to B lymphoma cell proliferation.

To examine the hypothesis that Tat-induced cytokines play a role in lymphomagenesis, BL cells were initially exposed to different doses of recombinant IL-6 (rIL-6), IL-7 (rIL-

7), IL-8 (rIL-8) or IL-10 (rIL-10) separately or in combination and proliferation measured 24 and 48 hrs later by the XTT cellular proliferation assay. Interestingly, none of these cytokines added alone (data not shown) or in combination (Figure 2.6) as recombinant proteins induced significant BL cell proliferation, even at doses as high as 20 ng/ml while BL cells were stimulated to grow significantly by Tat complete conditioned medium alone (Figure 2.6). The fact that recombinant IL-6 or IL-10 and in combination could not substitute for Tat complete conditioned medium was not surprising since these vital growth factors may work optimally in concert with multiple additional factors present in the complex biological milieu. In addition, we hypothesize that extracellular Tat secreted by Ad/tat-infected EC (Figure 2.2A) may influence the BL cell response to growth factors present in the conditioned medium. Such an influence was not reproduced in the recombinant cytokine treatment experiments described above. Note that attempts to assay the BL cell response to recombinant tat (rTat) were unsuccessful due to the biological inactivity of commercially available rTat (as reported by our group and others; 2002 Grantees Meeting for the HIV RFA Program on Endothelial Dysfunction in HIV Infection). Thus, to assess the potential role of these cytokines in a more physiologically relevant setting, BL cell proliferation assays were repeated using conditioned media from Tat-expressing EC in the presence of neutralizing antibodies to the cytokines of interest. A neutralizing antibody to IL-7 was similarly tested, since IL-7 is thought to be an important B cell growth factor.

For these experiments, cell-free supernatants were harvested from Ad/tat-infected HUVEC (Figure 2.7A) and DMVEC (Figure 2.7B) at 48 hrs post adenovirus infection

and used directly for BL cell proliferation experiments without a freeze-thaw step. BL cell proliferation in response to these supernatants, in the presence or absence of neutralizing antibodies, was measured over a 48 hr period by the XTT assay. As controls, BL cells were exposed to supernatants similarly derived from Ad/vpu- (Figure 2.7 C and D) or Ad/trans-infected EC. In the results presented in Figure 2.7 A-D, BL cell proliferation is depicted as a percentage of proliferation measured in response to Ad/trans conditioned supernatant in the absence of any antibodies (adjusted to 100%). Note that addition of neutralizing antibodies to Ad/trans-conditioned supernatants did not significantly affect proliferation (data not shown). As depicted in Figure 2.7A and B, neutralization of IL-6 and IL-10 in test supernatants harvested from Ad/tat-infected EC significantly impaired the growth of BL cells. In fact, in the presence of the anti-IL-10 antibody, BL cell proliferation was similar to that observed in the presence of Ad/trans alone. Treatment with anti-IL-7 and anti-IL-8 antibodies, or with isotype matched antibody controls, did not influence the stimulatory capacity of conditioned supernatants from Tat-expressing EC. Further, in confirmation of previous results, conditioned supernatants from Ad/vpu-expressing DMVEC or HUVEC (Figure 2.7 C & D) did not induce BL cell proliferation and were not influenced by cytokine neutralization. Collectively, these data suggest that IL-10 and IL-6 are Tat-induced EC cytokines that play a central role in stimulating BL cell growth.

DISCUSSION

The development of tumors of B cell origin often involves inappropriate activation, inhibition of apoptosis and growth stimulation of pre-malignant and malignant cells, thus

increasing the risk of accumulation of genetic errors and promoting survival of neoplastic clones. Inappropriate stimulation is frequently provided by non-malignant support cells, and viral infection is a common feature of these cells. In our *in vitro* lymphomagenesis model, the inappropriate stimulation is provided by non-malignant endothelial cells that are infected with HIV. *In vivo*, the aggressive growth of AIDS-NHL at extranodal sites where HIV infection of the endothelium is observed, for example the brain, bone marrow, liver and kidney, provides a clear relevance for our model. To unravel the viral and cellular mechanisms responsible for the ability of HIV-infected endothelial cells to support the growth of AIDS-NHL, we examined the role of HIV proteins in supporting B lymphoma (BL) proliferation.

Our results demonstrate that when HIV proteins are individually expressed in EC, expression of Tat alone recapitulates the previously reported capacity of HIV-infected EC to promote BL cell growth [Moses, *et al.*, 1997]. Tat is known to modulate host gene expression [Jeang, *et al.*, 1999, Taube, *et al.*, 1999] and cytokines including TNFα and β, IL-2, IL-6, IL-8, MCP-1, and TGFβ are subject to Tat-mediated dysregulation [Chen, *et al.*, 1997, Ehret, *et al.*, 2001, Hofman, *et al.*, 1999, Lim, *et al.*, 2000, Mahieux, *et al.*, 2001, Nath, *et al.*, 1999, Ott, *et al.*, 1998, Park, *et al.*, 2001, Sastry, *et al.*, 1990, Weiss, *et al.*, 1999, Westendorp, *et al.*, 1995]. Given the role of Tat in transactivating a variety of cellular proteins, a central role for Tat in our system is suspected. This observation is however the first direct evidence that Tat can influence B cell lymphomagenesis via endothelial cells, and the mechanism was thus deemed worthy of further study.

ICCS studies revealed that expression of Tat in EC induced the production of a variety of cytokines, some of which have a known function as B cell growth factors. Notably, proliferation assays performed with conditioned supernatants from Tat-expressing EC in the presence of neutralizing antibodies confirmed a role for two of these cytokines, IL-6 and IL-10, in promoting BL cell proliferation. *In vivo*, secretion of these cytokines from HIV-infected EC could conceivably facilitate the development of B cell tumors.

B cell differentiation and proliferation can be induced by a variety of factors, including IL-1, IL-2, IL-4, -IL6, IL-7, IL-10, IFN-γ, and TNFα and β [Hirano, *et al.*, 1986, Jelinek, *et al.*, 1986, Jelinek, *et al.*, 1987, Paul, 1987, Saeland, *et al.*, 1991 174, Sharma, *et al.*, 1987, Zlotnik, *et al.*, 1991]. Elevated levels of these B cell growth factors have been found in the serum of HIV-infected individuals, and some, including IL-6 and IL-10, are thought to play a causative role in the clinical pathology of various AIDS-NHL. For example, EBV-positive AIDS-associated Burkitt's lymphoma (AIDS-BL) and AIDS-associated diffused large cell lymphoma (AIDS-DLCL) produce large amounts of IL-6, IL-7, IL-10 and IL-12 [Fassone, *et al.*, 2000], and AIDS-BL growth inhibition by IL-10 antisense oligonucleotides as well as strong expression of IL-6 receptors on AIDS-DLCL support a role for IL-6 and IL-10 in lymphomagenesis [Fassone, *et al.*, 2000, Masood, *et al.*, 1995]. Further, HHV8-positive primary effusion lymphoma (PEL) cells produce and respond to IL-6 and IL-10 [Fassone, *et al.*, 2000, Marsh, *et al.*, 1995]. The Burkitt's lymphoma cells used in our studies are not infected with EBV or HHV8, which reflects the situation in at least 50% (EBV) and 100% (HHV8) of the AIDS-associated Burkitt's lymphomas, but these cells maintained their responsiveness to both IL-6 and IL-10. The

proliferative ability of other types of AIDS-NHL may be similarly affected by HIV infection or Tat expression in endothelial cells. Indeed, our previously published *in vitro* studies with replicating HIV demonstrate that infected MVEC support the growth of EBV-positive and EBV-negative BL cells, as well as AIDS-DLCL [Moses, *et al.*, 1997].

In addition to directly inducing B cell proliferation in the EC-B cell microenvironment, Tat-induced IL-10 secretion could also affect lymphomagenesis in a more indirect way via its immunosuppressive properties. Early in HIV infection when immune function is relatively high, the Th-1 T cell response predominates, but disease progression is associated with a switch to the Th-2 response, with increased production of IL-4, IL-6 and IL-10. This switch contributes to the ineffective control of HIV replication with an increase in viral load and the development of AIDS [Clerici, *et al.*, 1993, Clerici, *et al.*, 1993, Maggi, *et al.*, 1994]. Tat can induce IL-10 (this manuscript and others [Badou, *et al.*, 2000, Blazevic, *et al.*, 1996, Kundu, *et al.*, 1999, Masood, *et al.*, 1994, Sharma, *et al.*, 1995]) and serum levels of IL-10 are elevated in HIV-infected patients. Tat has also been implicated in suppression of macrophage function in HIV infection [Ito, *et al.*, 1998], thus contributing to suppression of Th-1-driven anti-viral and anti-tumor responses. An immunosuppressive role for Tat is further implied by reports that Tat suppresses the expression of IL-2 in Jurkat T cells [Purvis, *et al.*, 1992] and can inhibit natural killer (NK) cell cytotoxicity and degranulation [Zocchi, *et al.*, 1998]. Tat-induced IL-10 may thus suppress the development of anti-viral as well as anti-tumor cytotoxic T lymphocytes (CTL) thereby exacerbating virus-associated B cell stimulation and preventing tumor surveillance. Our finding that IL-10 secreted by Tat-expressing EC

stimulates malignant B cell growth suggests an additional way in which IL-10 promotes a pro-tumor environment in the context of AIDS.

In vivo, HIV infects T cells and macrophages in addition to EC. In the *in vitro* system described here, macrophages and T cells were not present, allowing us to examine the exclusive contribution of Tat-expressing EC. *In vivo*, cytokines produced by other HIV-infected cells may contribute to the high level of IL-6 and IL-10 detected in patients with AIDS-NHL, and such cells may also participate in the local microenvironment where extranodal tumors prosper. Indeed, HIV-infected EC may play an active role in recruitment of other cell types to these sites. Finally, while this study has focused almost exclusively on the role of Tat in influencing the EC lymphoma support phenotype, while in the context of an HIV-infected cell, other HIV proteins could contribute to EC support. As will be discussed in detail in Chapter 3 of this thesis, HIV Vpu was found to be necessary and sufficient to induce adhesion of lymphoma cells to HIV-infected EC. Adhesion of lymphoma cells to EC would provide an important initial step for cell homing as well as bringing these cells into local proximity for exposure to effective concentrations of growth stimulatory cytokines.

In summary, this study was designed to examine the mechanisms by which HIV-infected EC support the robust proliferation of B lymphoma cells. To our knowledge, this is the first report to show that HIV can promote lymphoma growth by expression of Tat in EC, resulting in the production of B cell growth factors. This work provides compelling

evidence that Tat plays a major role in regulating B lymphoma growth, and identifies Tat and/or Tat-induced proteins as targets for lymphoma therapy.

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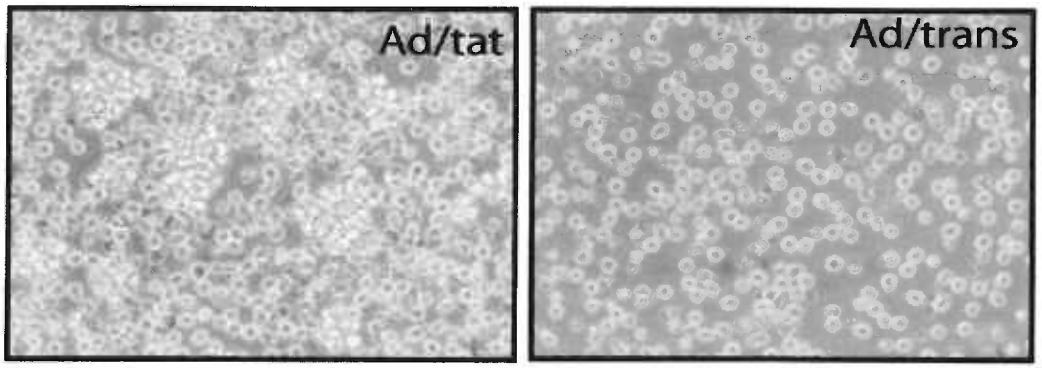
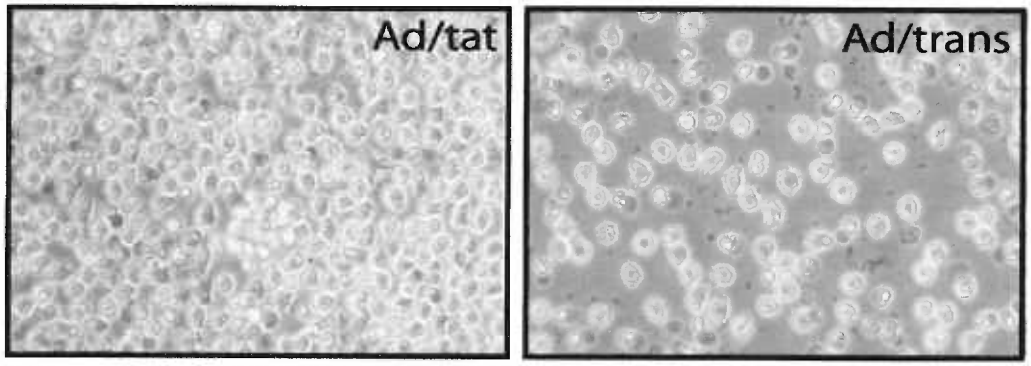
Figure 2.1 HIV-1 Tat-expressing dermal microvascular endothelial cells (DMVEC) induce B lymphoma (BL) cell proliferation.

(A) MVEC-B lymphoma cell co-culture



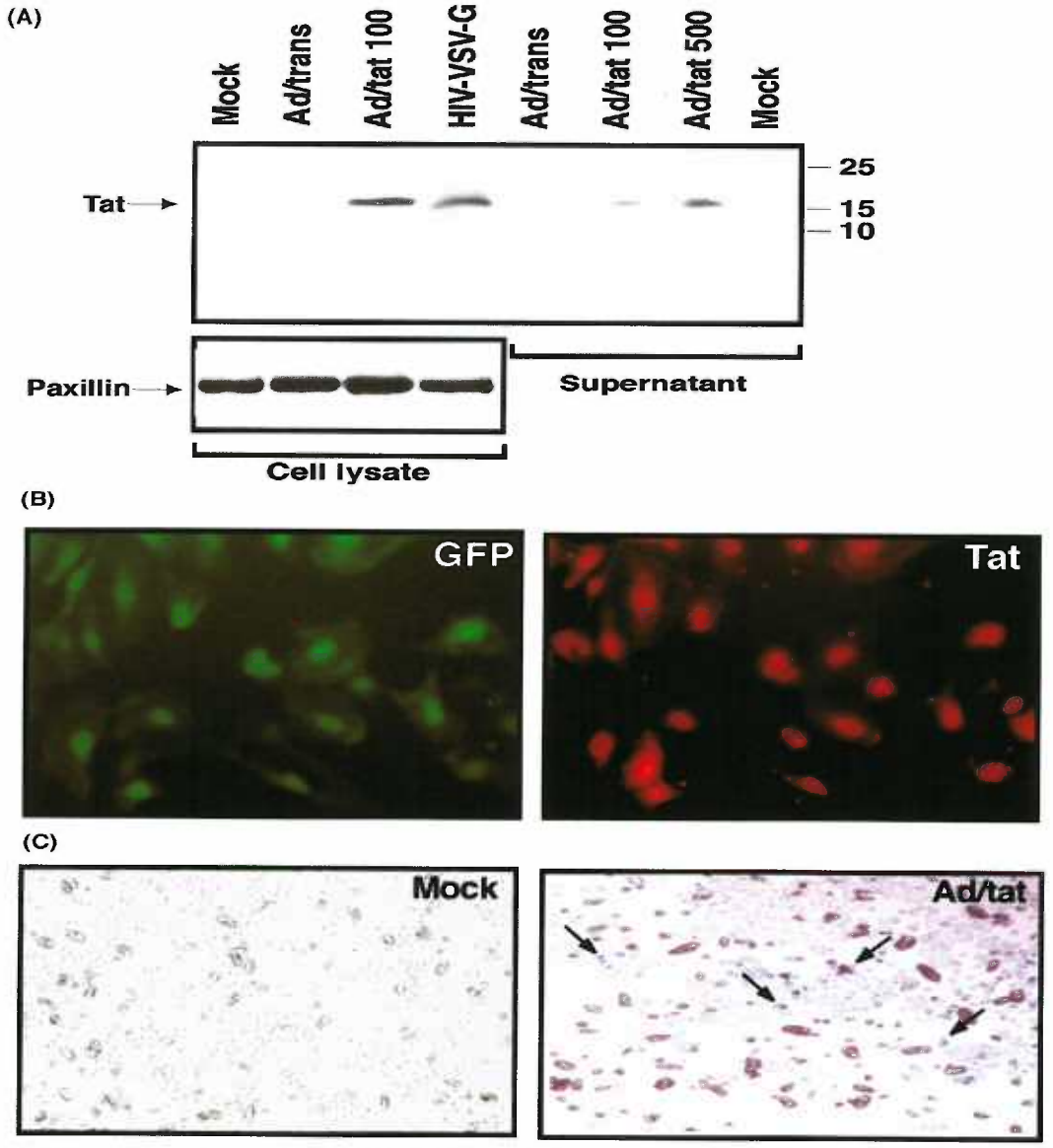
(B) MVEC-conditioned supernatant



(A) When DMVEC-BL cell co-culture experiments were performed following adenovirus infection, increased BL cell density and homotypic BL cell-BL cell aggregates were observed in co-culture with Ad/tat-infected DMVEC (Ad/tat) as compared to Ad/trans-infected DMVEC (Ad/trans). Representative fields of co-cultures observed by light microscopy are shown.

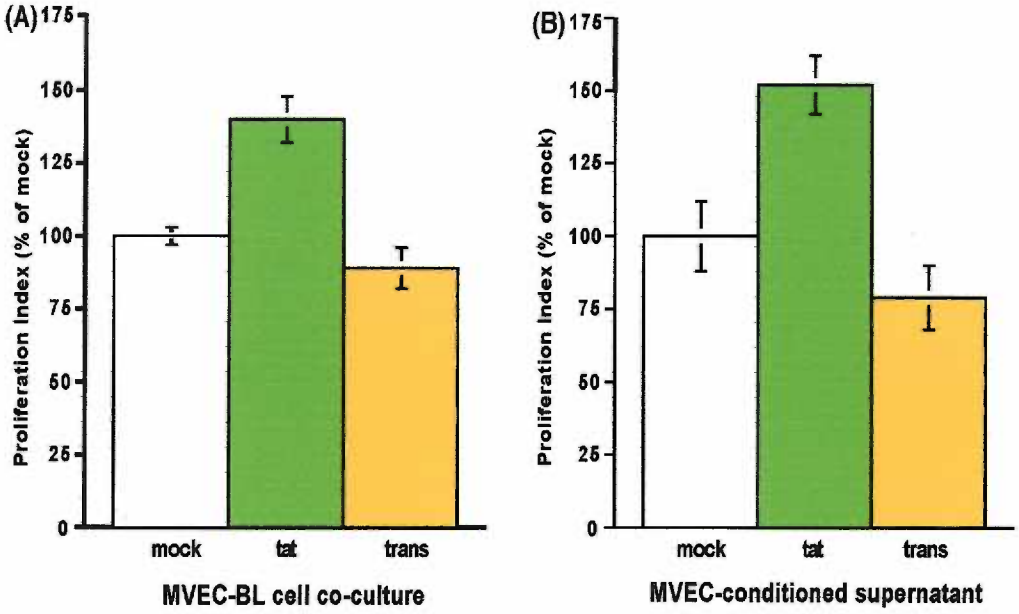
(B) When BL cells were exposed to cell-free supernatants harvested from DMVEC infected with Ad/tat or Ad/trans, supernatant from Ad/tat-infected DMVEC supported increased BL cell proliferation. Proliferation is reflected in the increased cell density observed by light microscopy exclusively in the Tat-conditioned medium (Ad/tat). In addition, some cell aggregation were seen also exclusively in Tat-conditioned medium.

Figure 2.2 Functional HIV-1 Tat is expressed with an adenovirus expression system.



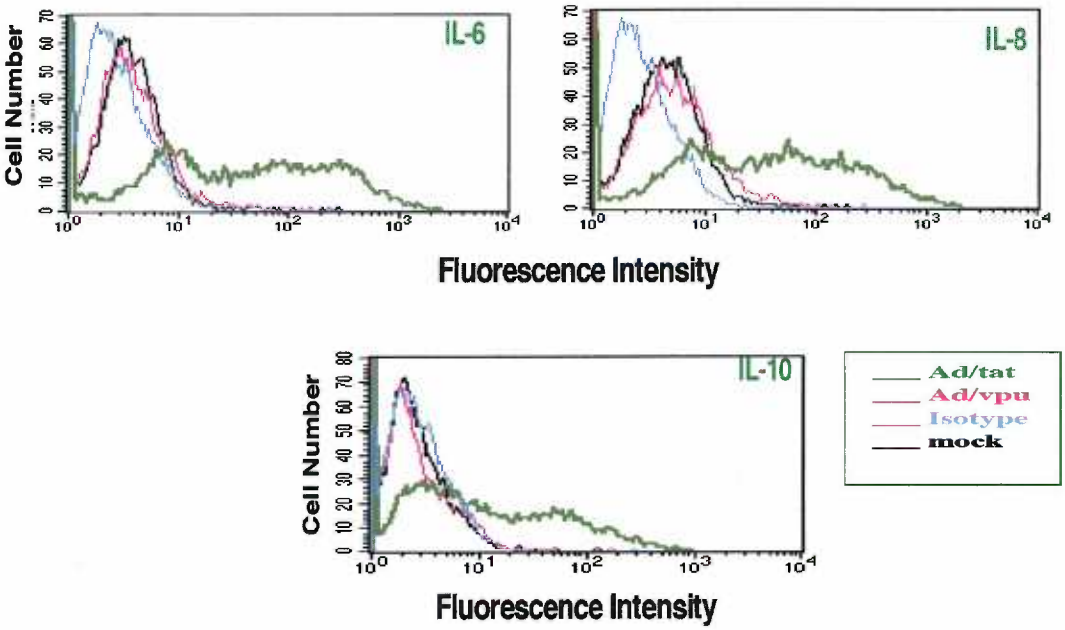
The expression of the HIV Tat protein was established by western blot analysis (A) and by immunofluorescence (B). Molecular weights were calculated using full range rainbow markers. Cellular paxillin levels are shown to confirm that an equal amount of cell lysate was loaded in each lane. HIV Tat expressed by adenovirus (Ad/tat) transactivates the HIV-1 LTR in H-MAGI cells (C), thereby confirming the functionality of the adenovirus-expressed Tat. Mock-infected H-MAGI cells are shown as controls. H-MAGI cells were infected with Ad/tat at low multiplicity of infection. Black arrows indicate four Tat-expressing, β-gal-positive cells.

Figure 2.3 HIV-1 Tat or Tat-induced factors promote B lymphoma cell proliferation



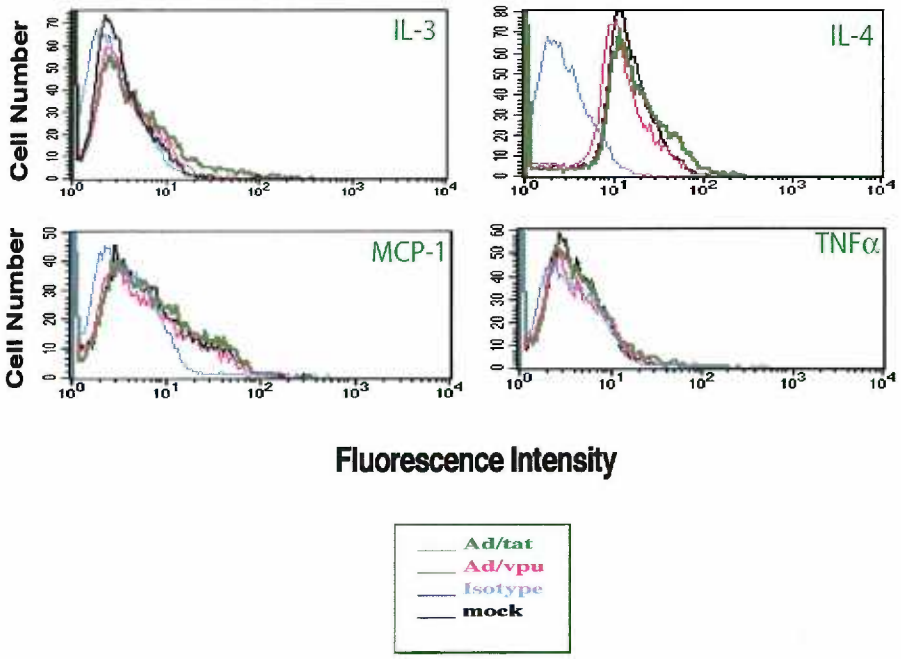
Quantitative B lymphoma cell proliferation assay. (A) BL cells co-cultured with Ad/tat-infected DMVEC (green) were removed from co-culture with EC after 48 hours, and cell density in co-culture supernatants measured with a tetrazolium-based cellular quantitation assay (XTT assay) as described in Materials and Methods. As controls, BL cells in supernatants removed from mock-infected (white) or Ad/trans-infected (orange) DMVEC were counted in an identical manner. As graphically illustrated, the BL cell growth in Ad/tat-infected DMVEC co-culture was significantly greater than growth in controls. (B) BL cell proliferation induced by equal amount of cell-free supernatants conditioned by Ad/tat-infected DMVEC was measured by XTT assay. Supernatants from Tat-expressing DMVEC (green) stimulated a significantly greater level of B cell proliferation than control supernatants. Columns represent the mean ± SD of quadruplicate wells. The experiments shown are representative of three similar experiments.

Figure 2.4 HIV-1 Tat induces the production of B cell growth factors by HUVEC



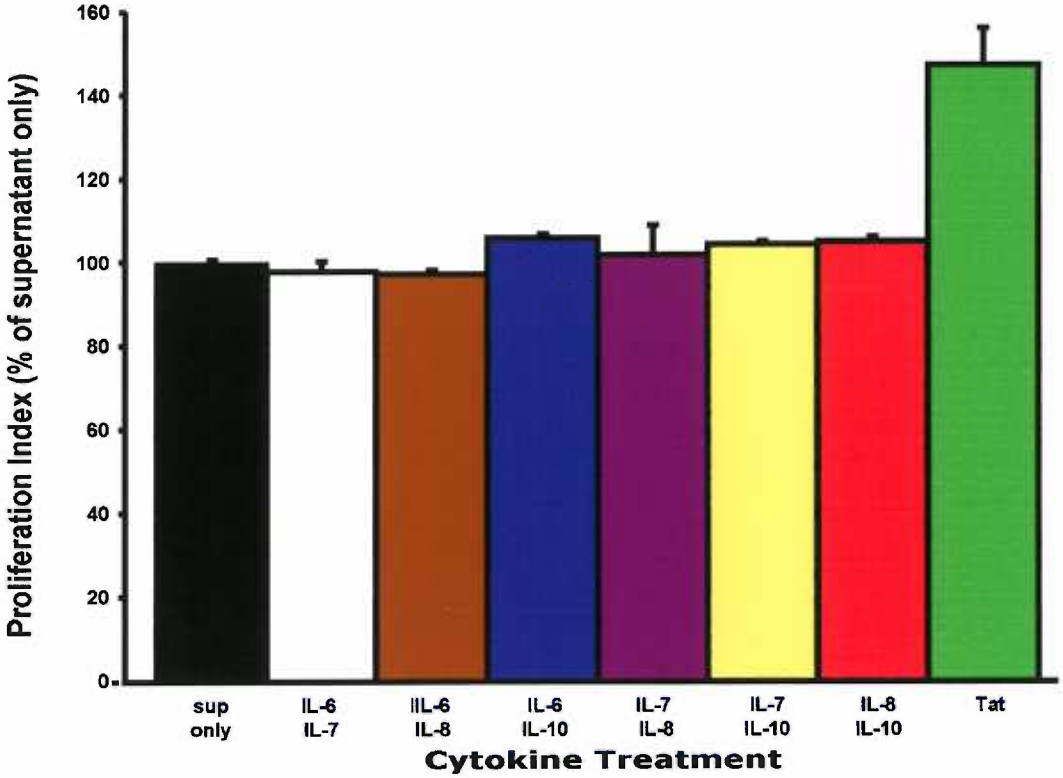
Intracellular cytokine staining (ICCS) for the B cell growth factors Interleukin (IL)-6, IL-8 and IL-10. Briefly, HUVEC were infected with Ad/tat or Ad/vpu for 42 hrs, followed by treatment with Brefeldin A for 3 hours to collapse the Golgi apparatus in order to retain all secreted cytokines within the cells. This was followed by immunofluorescence staining for individual cytokines and FACS analysis. Infection with Ad/tat (green) significantly induced expression of all three cytokines, IL-6, IL-8 and IL-10. In contrast, minimal induction of these cytokines was measured in Ad/vpu-infected cells (pink) or mock-infected cells (black). The effect of HIV-1 Tat on the production of IL-6, IL-8, and IL-10 was specific in that Tat did not have any effect on the production of other cytokines (see Figure 2.5). Isotype-matched controls (blue) were included.

Figure 2.5 HIV-1 Tat does not induce IL-3, IL-4, MCP-1 or TNFα by HUVEC



Intracellular cytokine staining (ICCS) for various cytokines IL-3, IL-4, MCP-1 and TNFα. Intracellular cytokine staining protocol was performed as described for Figure 2.4. Although HIV-1 Tat induced IL-6, IL-8, and IL-10 to a significant level (Figure 2.4), Tat (green) failed to induce other cytokines such as IL-3, IL-4, MCP-1 and TNFα.

Figure 2.6 Recombinant cytokines in combination have no effect on B lymphoma proliferation



B lymphoma (BL) cells were cultured in supernatants harvested from DMVEC with or without various cytokines treatment. Supernatants were untreated (black) or treated with 10ng/ml each of rIL-6+rIL-7 (white), rIL-6+rIL8 (brown), rIL-6+rIL-10 (blue), rIL-7+rIL-8 (purple), rIL-7+rIL-10 (yellow), rIL-8+rIL-10 (red). BL cell proliferation was measured after 48 hours by the XTT assay. Supernatant from Ad/tat-infected DMVEC (green) as described in Figure 2.3 is included as a positive control. Proliferation of BL cells cultured in medium with various recombinant cytokines is depicted as a percentage of the proliferation of BL cells exposed to control medium harvested from DMVEC without cytokine treatment (sup only), which was adjusted to 100%. Note that recombinant cytokines in combination have no effect on B lymphoma proliferation. Each column represents the mean ± SD of quadruplicate wells of a 96 well plate. The experiment shown is representative of three similar experiments.

Figure 2.7 Neutralization of IL-6 and IL-10 in conditioned supernatants abolishes the proliferative capacity of supernatants from Ad/tat-infected endothelial cells (EC).

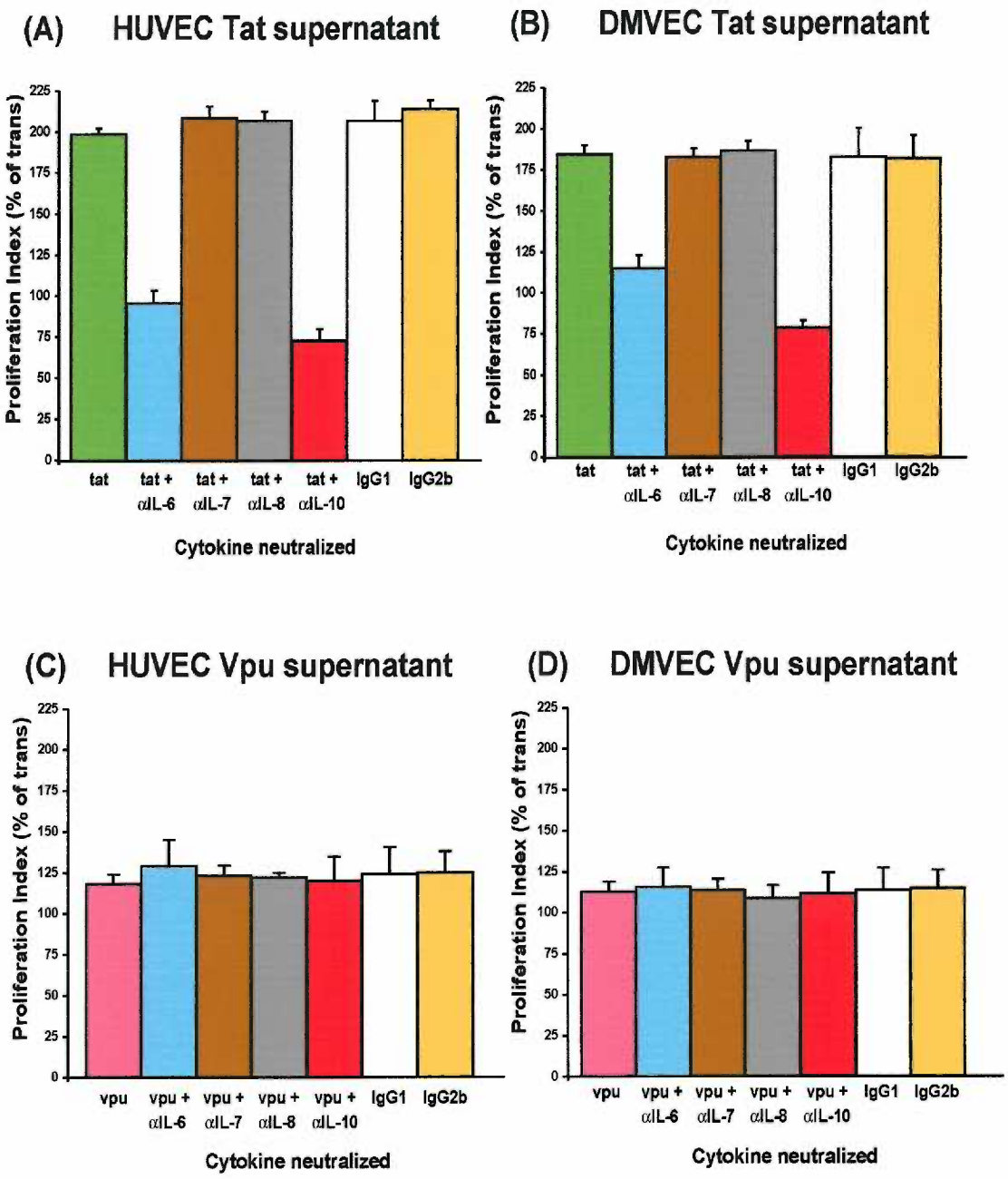


Figure 2.7 Neutralization of IL-6 and IL-10 in conditioned supernatants abolishes the proliferative capacity of supernatants from Ad/tat-infected endothelial cells (EC).

B lymphoma (BL) cells were cultured in supernatants harvested from HUVEC (A & C) and DMVEC (B & D) 48 hrs after infection with Ad/tat (A & B), Ad/vpu (C & D) or Ad/trans (A-D; proliferation index reference value). Supernatants were untreated (tat = green; vpu = pink) or treated with neutralizing antibody (10μg/ml) to IL-6 (blue), IL-7 (brown), IL-8 (gray), IL-10 (red) or isotype-matched control antibodies (IgG1 for IL-6, -7 and -8 = white; IgG2b for IL-10 = orange). BL cell proliferation was measured after 48 hours by the XTT assay.

(A & B) Proliferation of BL cells cultured in medium conditioned by Ad/tat-infected EC with or without cytokine neutralization is depicted as a percentage of the proliferation of BL cells exposed to control medium harvested from EC infected with Ad/trans alone, which was adjusted to 100%. Note that Ad/tat infection doubles the proliferative support capacity of the EC-conditioned medium (green bar), but that neutralization of either IL-6 (blue bar) or IL-10 (red bar) eliminates this proliferative advantage.

(C & D) Proliferation of BL cells cultured in medium conditioned by Ad/vpu-infected EC with or without cytokine neutralization is depicted as a percentage of the proliferation of BL cells exposed to control medium harvested from EC infected with Ad/trans alone, which was adjusted to 100%. Note that Ad/vpu infection has no effect on the proliferative support capacity of the EC-conditioned medium (pink bar) as compared to Ad/trans-infected EC supernatants, and that cytokine neutralization similarly has no effect.

Each column represents the mean ± SD of quadruplicate wells of a 96 well plate.
The experiment shown is representative of three similar experiments.

Chapter 3

Endothelial cells expressing HIV-1 Vpu support adhesion of B lymphoma cells through induction of endothelial cell surface CD40 and subsequent upregulation of VCAM-1.

ABSTRACT

AIDS-associated non-Hodgkin's B cell lymphoma (AIDS-NHL) is a significant cause of morbidity and mortality among in individuals infected with human immunodeficiency virus type-1 (HIV). The AIDS-NHL are heterogeneous, but common features of AIDS including loss of immune function, chronic B cell hyperactivation and cytokine dysregulation are thought to contribute to lymphomagenesis. HIV does not infect the NHL, but infection of endothelial cells (EC) that interact with B cells at the blood-tissue interface suggests a second mechanism for the increased lymphoma incidence in AIDS. We have previously demonstrated that HIV-infected brain and bone marrow EC develop an enhanced capacity to support the attachment and growth of B cell lymphomas, and that this is in part due to enhanced expression of VCAM-1 via a CD40-mediated pathway [Moses, *et al.*, 1997]. The present study was designed to examine the role of HIV proteins in influencing the adhesive properties of EC for B lymphoma (BL) cells. Recombinant adenovirus vectors were used to express HIV proteins in EC, and the ability of these EC to influence BL cell adhesion as well as the expression of EC surface CD40 and VCAM-1 was examined. Expression of Vpu in EC induced an adhesive phenotype, upregulation of CD40, and an enhanced response to CD40-triggered VCAM-1 expression

following antibody-induced CD40 ligation. Deletion of the *vpu* gene from an HIV pseudotype genome revealed that Vpu was absolutely necessary for effective induction of the adhesive phenotype. *In vivo*, CD40 ligation on EC may occur via the CD40 ligand (CD40L) expressed on T cells, macrophages or the BL cells themselves. HIV-infected endothelium would present high levels of CD40 for ligation and signal transduction, with increased VCAM-1 expression and subsequent BL cell adhesion being the ultimate pathologic consequence.

INTRODUCTION

Patients infected with human immunodeficiency virus-1 (HIV) are at significantly greater risk (a 113-fold increase) for developing AIDS-associated non-Hodgkin's lymphoma (AIDS-NHL) than the general population [Goedert, *et al.*, 1998]. Polyclonal B cell stimulation, reduced immune surveillance and dysregulated cytokine profiles are all indirect measures that likely contribute to this clinical phenomenon [Biggar, *et al.*, 1996, Gaidano, *et al.*, 1992, Knowles, 1997]. While malignant B cells are not themselves HIV infected, HIV could possibly contribute directly to the incidence and features of the AIDS-NHL via infection of non-malignant cells interacting with lymphoma cells. One of the distinct features of the AIDS-NHL is aggressive growth at extranodal tissue sites that include brain, bone marrow, liver and kidney [Kaplan, *et al.*, 1989, Levine, 1992, Ziegler, *et al.*, 1984]. Interestingly, these are all sites where HIV infection of the endothelium is observed [Chi, *et al.*, 2000, and references therein]. HIV infection of the endothelium thus could conceivably induce phenotypic changes that foster lymphoma attachment and growth. In support of this hypothesis, bone marrow EC derived from HIV-positive lymphoma patients were shown to support the natural outgrowth of autologous lymphoma cells *ex vivo* [Moses, *et al.*, 1997]. Similarly, EC infected *in vitro* develop a phenotype supportive of lymphoma cell attachment and growth that has been mechanistically linked to a virus-induced increase in expression of the TNF-receptor family molecule CD40 on the surface of HIV-infected cells [Moses, *et al.*, 1997]. Stimulation of EC with the cytokine CD40 ligand or an anti-CD40 antibody leads to preferential induction of vascular adhesion molecule-1 (VCAM-1) on the HIV+/CD40+ cells, which in turn promotes B lymphoma cell binding via a VCAM-1/VLA-4-mediated

interaction. *In vivo*, the initial induction of CD40, as well as post CD40-ligation signals, may both contribute to VCAM-1 induction and increased lymphoma cell attachment to the infected endothelium. Attachment signals as well as close proximity to EC-produced cytokines could in turn promote B lymphoma cell proliferation and the outgrowth of malignant foci.

The accessory (Vif, Nef, Vpr and Vpu) and regulatory (Rev and Tat) proteins of HIV have all been shown to modulate aspects of cellular function. Tat, the transactivator of HIV gene expression, is also able to transactivate many cellular proteins [Biswas, *et al.*, 1995, Chang, *et al.*, 1995, Cupp, *et al.*, 1993, Sawaya, *et al.*, 1998, Tada, *et al.*, 1990, Taube, *et al.*, 1999, Taylor, *et al.*, 1992, Zauli, *et al.*, 1992]. Vpu has been shown to degrade nascent CD4 in the endoplasmic reticulum and augment virion release from the plasma membrane likely through an ion channel activity (see chapter 1 section 1.3.4 for detailed description of Vpu function). In addition, Vpu interferes with the synthesis and surface expression of MHC Class 1 molecules [Kerkau, *et al.*, 1997] and protein transport to the plasma membrane [Vincent, *et al.*, 1995].

To identify the HIV gene(s) that promote B lymphoma cell-endothelial cell interactions, we have expressed the individual accessory and regulatory genes in EC using an adenovirus expression system and have screened the transduced EC for their ability to support adhesion of co-cultured B lymphoma (BL) cells. Using this *in vitro* co-culture system, we observed significant adhesion of BL cells to Vpu-expressing EC. Lymphoma cell adhesion was not observed in EC expressing Vif, Nef, Vpr, Rev or Tat alone. In

agreement with enhanced adhesive properties, increased CD40 expression and CD40-triggered VCAM-1 expression were evident on Vpu-expressing EC. Interestingly, expression of Tat allowed induction of VCAM-1, but was not sufficient to support significant B cell adhesion. As discussed in Chapter 2 of this thesis, Tat expression in EC is also able to promote the proliferation of BL cells. Collectively, these results suggest that in HIV-infected EC, Vpu and Tat co-operate to promote lymphoma cell attachment and growth.

MATERIALS AND METHODS

Cell lines and cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker, Inc. (San Diego, CA). HUVEC monolayers were cultured in an endothelial growth medium (EGM; BioWhittaker, Inc) supplemented with 10% human AB serum (Sigma, St Louis, MO), and PSG (penicillin at 100U/ml, streptomycin at 100 mg/ml and 2mM glutamine) on 35mm- or 60mm-Primaria™ (Becton-Dickinson, Bedford, MA) tissue culture dishes and used for experiments at 4^{th}-6^{th} passage. Dermal MVEC (DMVEC) were derived by immortalization of primary cells with HPV type 16 E6/E7as previously described [Moses, *et al.*, 1999] and cultured as described for HUVEC. DMVEC retained the essential features of primary cells but were not restricted by tissue culture passage. B lymphoma (BL) cells were an EBV-negative Burkitt's lymphoma line derived from an HIV-positive patient as previously described [Moses, *et al.*, 1997]. BL cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone, St. Louis, MO), and PSG. 293T human embryonic kidney cells (American Type Culture Collection,

Manassas, VA) and H-MAGI cells (NIH AIDS Research & Reference Reagent Program, Rockville MD) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and PSG.

Reagents

Recombinant human tumor necrosis factor-α (TNFα) was purchased from R&D System Inc. (Minneapolis, MN) and was used at 10 μg/μl on HUVEC or DMVEC monolayers.

Adenoviral construction and infection

Recombinant adenovirus vectors were constructed as previously described [Hitt, 1994, Streblow, *et al.*, 1999]. Each of the accessory (*vpu*, *vpr*, *vif* and *nef*) and regulatory (*tat* and *rev*) genes of HIV was amplified by polymerase chain reaction (PCR) from proviral plasmid pNL4-3 (NIH AIDS Research & Reference Reagent Program) with the gene-specific forward and reverse primers. Each PCR product was cloned into the adenovirus shuttle vector pΔE1sp1Btet/EF1EGFPTKpolyA. This vector was derived from plasmid pΔE1sp1Btet as described previously by Streblow *et al.* (1999) with the addition of EF1-EGFP and herpes simplex virus thymidine kinase poly (A). The recombinant adenoviruses were produced by co-transfection of 293 cells with the shuttle plasmid containing the various HIV genes and pJM17 (Microbix, Toronto, Ontario, Canada), which has an E1A-deleted adenovirus genome. The recombinant adenoviruses were titered on 293 cells by limiting dilution. All adenovirus infections were performed with co-infection of an adenovirus transactivator-expressing virus (Ad/trans) at an equivalent, pre-optimized multiplicity of infection (MOI). Monolayers of HUVEC or DMVEC were

infected with the different adenoviruses at an MOI 100 of Ad/trans and Ad/vpu, Ad/tat, Ad/vpr, Ad/vif, Ad/rev or Ad/nef in the presence of polybrene (2 μg/ml hexadimethrine bromide; Sigma) for 6 hrs and incubated with fresh media for 24 to 48 hrs. Control monolayers were mock-infected or infected with Ad/trans alone at an MOI of 200.

HIV/VSV-G pseudotype virus construction and infection

Recombinant HIV/VSV-G pseudotype viruses were constructed as described previously [Bartz, *et al.*, 1997]. Proviral plasmids (pBru3ori^*env*, pME351Δ*vpu*, pME341Δ*vpr*, pME342Δ*vif*, pMAVIIΔ*rev* and pSFiev^{+}Δ*nef*) used to construct HIV/VSV-G and deletion viruses were obtained through the AIDS Research & Reference Reagent Program. Briefly, cell free viral stock was obtained from co-transfection of 293T cells with various proviral plasmids, pL-VSV-G plasmid and EGFP plasmid (transfection control). The virus stocks were harvested, concentrated, and titered by H-MAGI assay as described previously [Vodicka, *et al.*, 1997]. Pseudotype viral infection was performed by exposing HUVEC to the titered viral inoculae at an MOI of 1 with polybrene (2μg/ml; Sigma), for 2 hours in a minimal volume of medium to promote adsorption, followed by an overnight incubation under normal culture conditions. The following day, monolayers were rinsed in HBSS and re-cultured in EGM. HIV/VSV-G infection was confirmed by the detection of p24 gag protein expression by immunofluorescent staining with an anti-p24 antibody (Dako, Carpinteria, CA).

H-MAGI assay

Human MAGI (H-MAGI) assays were performed as described previously [Bartz, *et al.*, 1997, Vodicka, *et al.*, 1997]. H-MAGIs are HeLa-CD4 cells expressing β-galactosidase under the HIV-1 LTR. Recombinant HIV-VSV-G pseudotype viruses were titered on H-MAGI cells grown on 24-wells plate by limiting dilution. Four days post infection, infected H-MAGI cells were fixed and stained for β-galactosidase. In this assay, pseudotype virus-infected cells expressing HIV Tat proteins which transactivate the expression of β-galactosidase following interaction with the HIV-1 LTR, and the blue color in cells is scored via light microscopy to determine the titer of the various pseudotype viruses.

Western blot analysis

HUVEC were grown in EGM on 60 mm Primaria™ tissue culture dishes. The cells were infected with the appropriate adenovirus (Ad/vpu, Ad/trans or mock) as described above. Monolayers were harvested, lysed in 200-500μl of 2X-SDS lysis buffer (2X SDS loading dye, 1M DTT and 2% bromophenol blue), removed and boiled for 5 minutes. 30μl of total cell lysate from each sample was electrophoresed by SDS-PAGE on 12% polyacrylamide gels and blotted onto nitrocellulose membranes (Schleicher and Schuell, Keen, NH). Membranes were blocked with 1% nonfat milk in TBS-Tween 20-(100mM Tris-Cl [pH 7.5], 150mM NaCl and 0.1% Tween 20) for 1 hr, followed by incubation with rabbit anti-Vpu antiserum (1:100 dilution in TTBS with 1% nonfat milk; AIDS Research & Reference Reagent Program) for 2 hrs at room temperature. After incubation, membranes were washed and treated with a secondary peroxidase-labeled anti-rabbit IgG (1:1500 in TTBS with 1% milk; Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr.

An ECL system was used to visualize the protein of interest and was developed by chemiluminescence and autoradiography. For determination of total CD40 levels, a western blot protocol was performed in a similar manner, but membranes were developed with a rabbit anti-human CD40 antibody (1:250; Research Diagnostics Inc, Flanders, NJ). The total CD40 levels of each lane were quantified by densitometry. A mouse anti-paxillin monoclonal antibody (1:1000; Upstate Biotechnology, Lake Placid, NY) was used as a loading control.

Flow cytometric analysis

HUVEC were infected with adenoviruses or HIV/VSV-G pseudotype viruses, or were mock-infected, and analyzed for cell surface expression of CD40 using flow cytometry as previously described [Moses, *et al.*, 1997]. Cells were washed twice with Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS) and stained indirectly using a mouse anti-human CD40 antibody (1:100; BD Pharmingen, San Diego, CA) or an isotype-matched mouse IgG antibody (1:100; BD Pharmingen), followed by incubation with Cy5-conjugated goat anti-mouse immunoglobulin secondary antibody (Amersham Pharmacia Biotech), and fixed with 2% paraformaldehyde. Cells were detached non-enzymatically and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with CellQuest software (Becton-Dickinson). A minimum of 10,000 cells was analyzed for each sample.

CD40 ligation

HUVEC monolayers were mock-infected or infected with adenoviruses expressing Vpu or Tat for 6 hrs, and re-cultured with EGM for an additional 24 hrs. To cross-link surface CD40, HUVEC were incubated with a low-endotoxin, sodium azide free anti-CD40 monoclonal antibody (5μg/μl; BD Pharmingen) for another 24 hrs. This was followed by flow cytometric analysis of VCAM-1 expression with a mouse anti-CD106 (VCAM-1) antibody (1:100; BD Pharmingen) and a Cy5-conjugated species-specific secondary antibody (1:300; Amersham Pharmacia Biotech).

B lymphoma cell adhesion assay

HUVEC or DMVEC were plated on 35 mm Primaria™ dishes and allowed to grow to 60% confluence. Monolayers were infected with various adenoviruses for 6 hrs or with HIV/VSV-G deletion mutant viruses overnight. For adhesion experiments with HUVEC, CD40L+ B lymphoma (BL) cells were pre-labeled with DAPI/Hoechst stain (1:200, Molecular Probes, Eugene, OR) and incubated with the infected or mock-infected EC at day 2 post infection (PI). After a 24 hr period of co-culture, non-adherent BL cells were removed by extensive washes with HBSS. After washing, cultured cells were re-cultured in normal medium and immediately examined under a UV microscope to assess the number of BL cells remaining tightly adherent to the EC monolayer. Representative cell fields were photographed to record the extent of BL cell adhesion. For adhesion experiments with DMVEC, a cell permeant fluorescent dye Calcein-AM (5 μl/μl cell suspension; Molecular Probes) was used to label BL cells. For these experiments, a CD40

monoclonal antibody (MAB) was used as an initial VCAM-1-induction stimulus and was added at day 2 PI for an additional 24 hr period. Calcein-AM-loaded BL cells were incubated with CD40-crosslinked MVEC for a 3 hr period followed by stringent rinsing to remove loosely adherent and non-adherent cells. Adherent fluorescent BL cells were visualized under a fluorescent microscope with a 10X objective and random fields across the monolayer recorded using a digital camera. Fluorescent Calcein-loaded B cells were readily distinguishable from GFP-expressing MVEC on the basis of morphology and the intensity of the vital dye. Numbers of fluorescent BL cells adhering to mock-infected DMVEC or DMVEC infected with Ad/vpu, Ad/tat or Ad/trans alone were counted off each digital image and marked once scored.

Immunofluorescence analysis

For the detection of MHC class I and CD40, HUVEC or DMVEC monolayers were rinsed with PBS with Ca^{2+} and Mg^{2+}, fixed in 2% paraformaldehyde, blocked with 20% normal goat serum (NGS) and stained directly with allophycocyanin (APC)-conjugated mouse anti-human HLA-A, B, C (MHC class I) (pre-titered at 20µl/10^6 cells; BD Pharmingen) or indirectly with mouse anti-human CD40 (1:100; BD Pharmingen), followed by TRITC-conjugate goat anti-mouse secondary antibody (1:100; Sigma). For detection of p24 gag protein, goat anti-mouse or goat anti-rabbit p24 antibody (Dako) followed by the appropriate specie-specific secondary antibodies was used. Microscopic images were captured with a Nikon fluorescence microscope and camera. An absence of non-specific staining was demonstrated with irrelevant isotype-matched antibodies and secondary antibodies alone.

RESULTS

HIV Vpu upregulates CD40 surface expression on HUVEC

HIV upregulates CD40 surface expression on endothelial cells, and signals transduced following CD40 ligation subsequently induce VCAM-1, thus promoting B cell-endothelial cell adhesion via VLA-4/VCAM-1 interactions [Moses, *et al.*, 1997]. To determine whether any of the HIV accessory or regulatory genes play a role in CD40 expression, individual HIV regulatory and accessory genes were expressed in HUVEC using an adenovirus vector system (Appendix A), and monolayers were evaluated for levels of CD40 surface expression by immunofluorescence analysis (IFA) (Figure 3.1A). TNFα-stimulated EC were analyzed in parallel as a positive control for CD40 expression, since this inflammatory cytokine is known to be a potent inducer of CD40 *in vitro* [Bevilacqua, *et al.*, 1993, Karmann, *et al.*, 1995]. HIV Vpu and Tat both induced CD40 surface expression on HUVEC, although at a lesser intensity than that induced by TNFα. by IFA (Figure 3.1). CD40 was not induced by infection of HUVEC with Ad/trans alone (Figure 3.1A) or any of the other HIV genes tested (Table 3.1). To confirm and more quantitatively evaluate CD40 induction, FACS analysis was performed (Figure 3.1B). In agreement with IFA, an increase in CD40 expression was detected on Vpu-expressing cells as compared to cells infected with Ad/trans alone. Ad/tat-infected cells, however, demonstrated only a minimal effect on surface CD40 expression (mean fluorescence intensity 53.50) as compared with cells infected with Ad/trans alone (MFI 37.64), but Vpu (MFI 66.50) was clearly the more effective inducer of CD40. The effect of *vpu* on

CD40 expression was specific to *vpu* since other HIV genes such as *nef, vpr*, *rev* and *vif* similarly tested did not induce CD40 surface expression (Figure 3.1B and Table 3.1).

Western blot analysis was used to demonstrate that Vpu and Tat were expressed in the recombinant adenovirus-infected HUVEC (Figure 3.2A and Figure 2.2A). In Figure 3.2A, Vpu was represented by the 16kD molecular weight band while high molecular weight background bands were observed previously with the antiserum to HIV-1 Vpu provided by the NIH AIDS research and reference reagent program. Biological assays designed to verify functionality of these proteins were also performed. Adenovirus-expressed Vpu retained the ability to downregulate MHC Class I molecules (Figure 3.2B), while adenovirus-expressed Tat was able to transactivate the HIV-1 LTR in H-MAGI cells (Figure 2.2C).

HIV Vpu is required for the upregulation of CD40 surface expression on HUVEC

To confirm an essential role for Vpu in CD40 induction, and by extension, in HIV-induced lymphoma cell adhesion, a *vpu*-deleted HIV/VSV-G pseudotype virus (Appendix B), was used to infect HUVEC, and CD40 surface expression was determined by FACS analysis. Figure 3.3 illustrates that HIV/VSV-G Δ*vpu*-infected HUVEC have significantly reduced levels of surface CD40 relative to HUVEC infected with HIV/VSV-G wild type or other deletion viruses that retain expression of Vpu. This observation suggested an essential role for Vpu in the HIV-mediated upregulation of surface CD40 expression. The expression of Tat in HIV/VSV-G Δ*vpu*-infected HUVEC did not significantly alter the CD40 levels on these infected cells suggesting that Tat is likely not

the primary protein responsible for surface CD40 upregulation. In this series of experiments, mock-infected cells were used as a control for basal CD40 expression, and TNFα-stimulated cells were included as positive controls for CD40 induction.

Total CD40 level is increased in Ad/vpu-infected HUVEC

Because Vpu was a strong inducer of CD40 surface expression, we next sought to determine whether surface CD40 induction reflected an increase in the total amount of cellular CD40 protein. Total CD40 protein levels were determined by western blot analysis of whole cell lysates. CD40 levels in HUVEC induced by infection with Ad/vpu were compared to basal levels seen by infection with Ad/trans alone. Lysates from TNFα-treated HUVEC and mock-infected HUVEC were used as controls for induced and basal CD40 levels respectively. As illustrated in Figure 3.4, total levels of CD40 as measured by densitometry in Vpu-expressing cells (244.6) were increased two-fold relative to mock-infected cells (108.8) or cells expressing transactivator alone (134). HIV Vpu may increase total CD40 protein levels by increasing protein translation and/or increasing the half-life of the synthesized product.

HIV-1 Vpu induces VCAM-1 expression on EC following CD40 ligation

HIV accessory genes were systematically evaluated for their capacity to induce VCAM-1 expression on HUVEC, both constitutively and following CD40 ligation, using FACS analysis. VCAM-1 expression on EC in the absence of CD40 cross-linking was low and was not affected by any of the HIV genes tested (data not shown). This result corroborates our prior observation that HIV infection of EC does not alter constitutive

VCAM-1 expression levels [Moses, *et al.*, 1994, Moses, *et al.*, 1997]. To evaluate CD40-mediated VCAM-1 expression on EC that expressed enhanced CD40 in response to Tat or Vpu, an anti-CD40 antibody was used to cross-link surface CD40 on Tat- or Vpu-expressing EC (Figure 3.5). Following CD40 ligation, VCAM-1 expression was significantly elevated on EC infected with Ad/vpu (Figure 3.5A) or Ad/tat (Figure 3.5B) as compared to mock-infected control EC. In the absence of CD40 ligation, VCAM-1 levels were similar to those seen on mock-infected cells. TNFα-treated cells were used as positive control for VCAM-1 expression. The above data demonstrate that CD40 stimulated by Vpu or Tat retains the capacity to deliver VCAM-1 induction signals following ligation, a capability that we previously demonstrated with CD40 stimulated by a natural HIV infection. Thus, these viral proteins could conceivably promote BL cell attachment to EC via adhesive interactions between endothelial VCAM-1 and the cognate integrin VLA-4 expressed on BL cells.

HIV-1 Vpu mediates B lymphoma cell adhesion to HUVEC and microvascular EC

Previous studies by our group have shown that BL cells adhere to EC primarily via VCAM-1/VLA-4 interactions [Moses, *et al.*, 1997]. The BL cell line utilized in our EC-BL cell adhesion studies is CD40L-positive and can thus cross-link endothelial CD40 without a requirement for exogenous soluble CD40L, creating a positive feedback loop for VCAM-1/VLA-4-mediated adhesion. To test the responsiveness of Tat- or Vpu-expressing EC to CD40L+ BL cells, HUVEC infected with Ad/vpu, Ad/tat, Ad/trans were co-cultured with CD40L+ BL cells and tested for BL cell adhesion as described in the Methods section. TNFα-stimulated HUVEC were used as a positive control. As

shown in Figure 3.6A, BL cells adhered to Ad/vpu-infected HUVEC but not to Ad/tat-infected cells or the control Ad/trans-infected cells. The adhesion was resistant to even stringent saline washes, indicating a firm adhesion molecule-ligand interaction. This result confirms our hypothesis that HIV Vpu plays a central role not only in CD40 induction, but also in the enhanced attachment of lymphoma cells to HIV-infected EC. Expression of Tat (Figure 3.6A, bottom right panel) as well as Vpr, Vif and Rev (data not shown) did not have any effect on EC-BL cell interactions. Lack of Tat-induced adhesion was not surprising, given that Tat modulates CD40 expression minimally and VCAM-1 induction slightly in response to an artificial cross-linking stimulus (anti-CD40 MAB). Thus, Tat appears to be insufficient to facilitate adhesive events induced by natural EC-BL cell cross-talk, at least when expressed in isolation.

To quantify BL cell adhesion to EC, BL cells were preloaded with a fluorescent dye (Calcein-AM) before incubation with EC, and fluorescent cells remaining adherent to the monolayer after stringent washing were scored under a fluorescence microscope. For these experiments, DMVEC were infected with Ad/vpu, Ad/tat or Ad/trans alone, or were mock-infected as a control for basal adherence properties. The results of two independent experiments are shown in Table 3.2, where the values represent the number of adherent BL cells in a 10X field. The average – standard deviation (SD) of four randomly selected fields is shown. Foci of green BL cells could easily be seen under the fluorescent microscope in Vpu-expressing EC, and substantially more fluorescence was detected in the culture with Vpu-expressing cells as compared to transactivator-expressing or Tat-expressing cells. Cell fields shown in Figure 3.6B are representative of those counted,

clearly demonstrate the increased adhesive properties seen in Vpu-expressing DMVEC. Figure 3.6C demonstrates that DMVEC supporting BL cell adhesion were limited to those expressing Vpu (identified as GFP+ cells), and only Vpu-positive cells expressed high levels of CD40 (visualized via a TRITC conjugate). These results with DMVEC are also important because they demonstrate that data obtained with HUVEC can be recapitulated in microvascular EC. While HUVEC are the most widely used cells for endothelial cell studies *in vitro*, large vessel and microvessel EC are known to possess certain phenotypic and functional differences (Swerlick et al., 1991. Swerlick et al., 1992) and HIV commonly infects EC of the microvasculature. Thus, the results obtained in HUVEC can be extended to microvascular EC, which may be more relevant in an *in vivo* setting.

B lymphoma cells adhere selectively to HIV/VSV-G-infected endothelial cells, and Vpu is required for B lymphoma cell adhesion.

To verify that Vpu is absolutely required for the EC adhesive phenotype, HUVEC were infected with HIV/VSV-G pseudotype viruses that allowed deletion of individual viral genes. HUVEC were initially infected with a pseudotype virus containing the full complement of HIV genes, except for *env,* to ensure that this system induced an adhesive phenotype similar to that seen with wild type HIV. For these studies, HUVEC were infected with HIV/VSV-G at a low multiplicity of infection (MOI of 1) to allow direct comparison of adjacent infected and uninfected cells, and partially-infected monolayers were subsequently co-cultured with BL cells. BL cells adhered exclusively to p24-positive HIV/VSV-G-infected HUVEC, indicating that the pseudotype virus was relevant

to use as an investigative tool in our system (Figure 3.7A). The pseudotype virus system was thus utilized to directly test our hypothesis that Vpu is essential for the adhesion of B lymphoma cells to HIV-infected EC. To test this prediction, HUVEC were infected with a VSV-G pseudotyped virus with a deletion in *vpu* (Δ*vpu*) (Figure 3.7A; 10x magnification) and co-cultured with BL cells. As controls, Δ*nef* (Figure 3.7A), Δ*vpr* and Δ*vif* mutants (data not shown) were tested in parallel. BL cells adhered to HIV/VSV-G-infected HUVEC that expressed Vpu, regardless of whether other accessory genes were deleted. Importantly, loss of Vpu abolished the adhesive properties of pseudotype HIV-infected cells. Infected EC monolayers were also co-cultured with BL cells pre-stained with DAPI/Hoechst prior to stringent rinsing (Figure 3.7B). As illustrated in Figure 3.7B, the adhesive phenotype abolished in HIV-VSVGΔ*vpu* —infected cells was rescued by co-infection of these cells with Ad/vpu. These findings further substantiate an essential role for Vpu in lymphoma cell adhesion to HIV-infected endothelial cells.

All of the results presented above are consistent with a primary role for HIV Vpu in mediating EC-BL cell adhesion via induction of CD40 and subsequent CD40-mediated VCAM-1 expression. *In vivo*, CD40 ligation and triggering in endothelial cells may be mediated by CD40 ligand expressed by T cells, macrophages or by the malignant B cells themselves. Following EC-BL cell adhesion, Tat may function to additionally promote BL cell growth (Chapter 2), facilitating the formation of a multi-layered lymphoma aggregate attached to the vessel wall.

DISCUSSION

To identify the HIV genes that confer a lymphoma support phenotype on endothelial cells, individual HIV regulatory and accessory genes were expressed using an adenovirus vector system. The expression of HIV genes in EC was followed by the evaluation of three characteristic features of the lymphoma support phenotype; namely, CD40 expression, CD40-mediated VCAM-1 expression and B lymphoma cell adhesion. To the best of our knowledge, this manuscript is the first report showing that Vpu induces endothelial cell CD40 expression, and functions to promote heterotypic adhesion between EC and BL cells. While the ability of HIV accessory or regulatory genes to modulate host cell phenotype is well established, a role for specific HIV genes in conferring a lymphoma support phenotype on endothelial cells is a novel finding that may have important implications for understanding the unique features of AIDS-NHL.

Our group has reported previously that upregulation of VCAM-1 on HIV-infected MVEC occurs via a CD40-mediated pathway [Moses, *et al.*, 1997]. In this report, we have identified *vpu* as the gene responsible for the CD40-induced VCAM-1 upregulation. Interactions between the cytokine CD40L and its cognate receptor, CD40, play an important role in immune activation processes. For example, CD40 triggered induction of VCAM-1 in vascular endothelial cells is believed to play a crucial role in inflammation. Specifically, during inflammation, activated endothelial cells interact with T lymphocytes and macrophages via CD40/CD40L to produce inflammatory cytokines, which further increase endothelial CD40 expression and subsequent VCAM-1 expression resulting in the recruitment of these cells to the site of inflammation [Noelle, 1996]. Like many other

disease processes, the development of AIDS-NHL is likely the result of dysregulation of the normal functions of these molecules.

Similar to our reports of HIV-induced CD40 expression on infected EC, a recent study by Maisch and colleagues [Maisch, *et al.*, 2002] reports that human cytomegalovirus (HCMV) infection of HUVEC leads to increased expression of surface CD40 and preferential expression of the adhesion molecule E-selection on the CD40-positive HCMV-infected HUVEC when co-cultured with CD40L-positive T cells. Therefore, our study and that of others have demonstrated the importance of CD40 in diseases where abnormal interactions between endothelial cells and leukocytes underlie the pathogenic process.

The adhesive properties of activated EC for B cells, T cells and macrophages increase due to induction of adhesion molecules such as ICAM-1 and VCAM-1 at the EC surface. For example, both interleukin-4 (IL-4) and interleukin-6 (IL-6) have been shown to upregulate VCAM-1, leading to T and B lymphocyte adhesion [Swerlick, *et al.*, 1992, Watson, *et al.*, 1995]. In our study, Vpu does not induce these cytokines (data shown in Figures 2.4 and 2.5), but instead renders CD40-positive EC more responsive to activation via the TNF-related cytokine CD40L, which is expressed by BL cells. In addition, HIV Tat does induce IL-6. Thus, in the context of an HIV infection, such cytokines and cytokine receptors likely both play a role in modulating EC adhesiveness.

During an active inflammation process, a CD40-transduced signal may be globally redundant, because soluble TNFα and IFNγ can by themselves induce VCAM-1 upregulation. The ability to signal through CD40 however provides an opportunity for CD40 L-positive tumor cells to home and attach specifically to a focally responsive endothelium. We hypothesize that HIV infection of the endothelium induces localized responsiveness to CD40L and promotes AIDS-NHL attachment and growth at extranodal tissue sites. CD40 is expressed constitutively at a low level on EC, and CD40 triggering by CD40L can lead to a moderate induction of VCAM-1. In HIV-infected MVEC however, VCAM-1 induced by CD40 cross-linking is significant, either as a direct consequence of increased surface CD40, activation of post-ligation signaling pathways, or a combination of both mechanisms. Regardless, CD40-mediated VCAM-1 expression may be upregulated to a level similar to that achieved in non-specific inflammation, but in regions restricted to sites of HIV infection, B lymphoma cells expressing the CD40L may then induce VCAM-1 expression by binding to the upregulated CD40 on HIV-infected EC. Importantly, CD40 ligand-expressing T cells or monocytes in the microenvironment may also supply the CD40 triggering stimulus.

Since Tat induces CD40 minimally as measured by FACS analysis when compared to Vpu (Figure 3.1B) and the presence of Tat alone in HIV/VSV-G deletion mutant of vpu did not induce surface CD40 expression on infected cells, we conclude that Tat-induced CD40 seen in Figure 3.1A may be the result of overexpression of Tat in a small number of infected cells in the adenovirus expression system. Although adenovirus expressing Vpu may lead to overexpression of Vpu, we believe that Vpu is indeed responsible for

upregulation of CD40 because the lack of *vpu* resulted in reduced level of CD40 expression (Figure 3.3) and abolished the ability of infected cells to support BL cell adhesion (Figure 3.7). Our data clearly demonstrates a relationship between Vpu expression and CD40 induction/BL cell adhesion. Note that while Vpu may be overexpressed in this recombinant system, experiments in cells infected with wild type HIV have shown that the amount of Vpu expressed in a natural infection is sufficient for effective CD40 induction [Moses, *et al.*, 1997].

The VCAM-1 induction by Tat-expressing EC (Figure 3.5B) observed in this study is interesting since co-culture of BL cells with Tat-expressing EC led primarily to BL cell-BL cell interaction (Figure 2.1) rather than BL cell-EC adhesion (Figure 3.6). The exogenous soluble CD40L or anti-CD40 antibody may efficiently crosslink surface CD40 on Tat expressing EC resulting in an artificial induction of VCAM-1 on the EC cell surface. In addition, the triggering via CD40L-positive BL cells may be insufficient to induce VCAM-1 to the same extent as exogenous soluble CD40L or anti-CD40 antibody because the threshold of CD40 level on the cell surface is not attained in Tat expressing EC. Furthermore, when exposed to the effect of Tat alone, BL cell-BL cell interactions may take preference over EC-BL cell interactions, thus leading to the formation of BL cell aggregates (Chapter 4). However, since both Tat and Vpu are expressed during an HIV infection *in vivo*, both EC-BL cell interactions and BL cell-BL cell interactions would occur, thus facilitating the formation of a multi-layered lymphoma aggregate attached to the vessel wall.

In summary, the results presented here demonstrate that HIV Vpu plays a functional role in promoting adhesion of B lymphoma cells to HIV-infected endothelial cells. Thus, HIV Vpu may play an important role in malignant B cell homing and adhesion to extranodal tissue sites.

Acknowledgments

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Figure 3.1 HIV Vpu upregulates CD40 surface expression on HUVEC

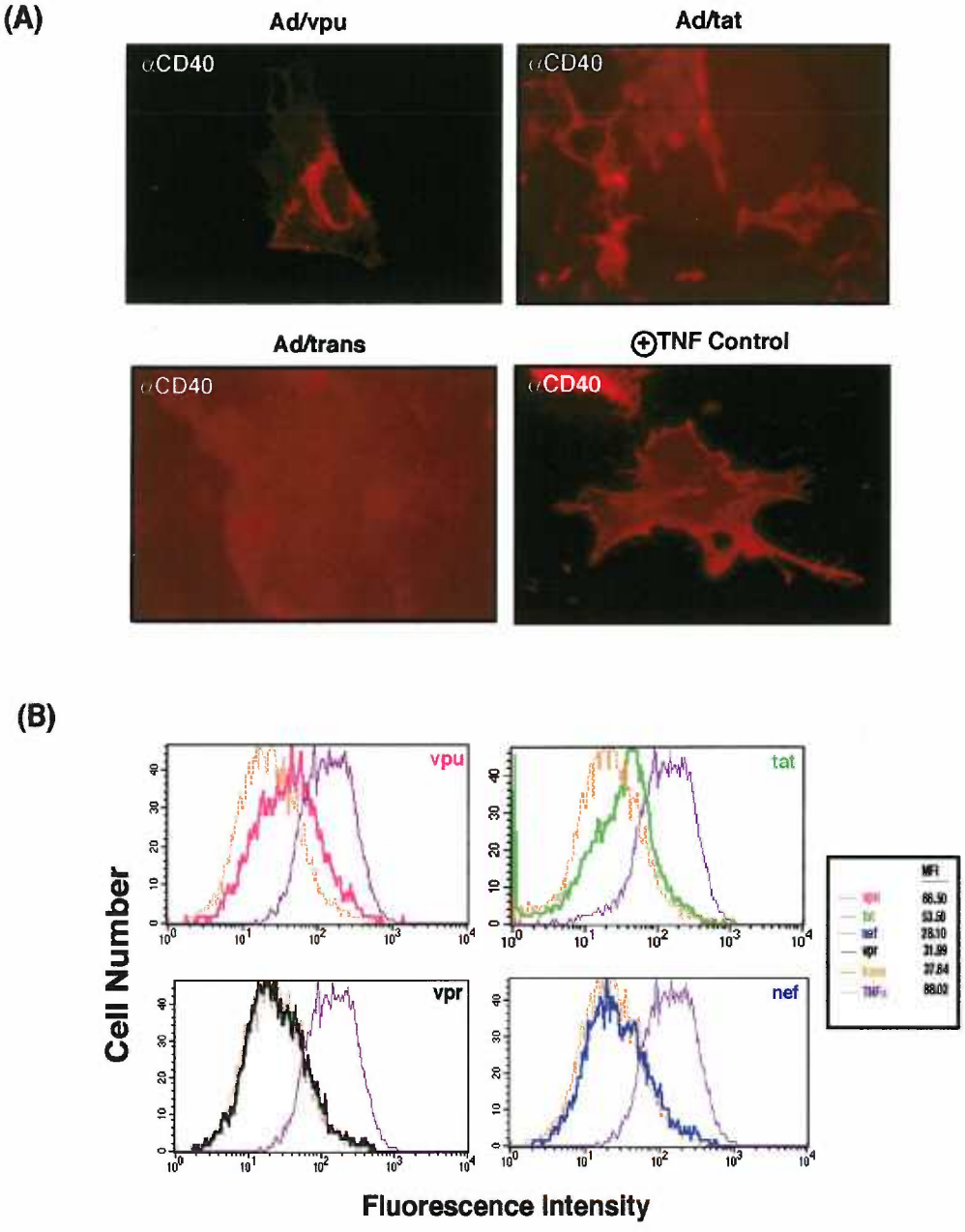
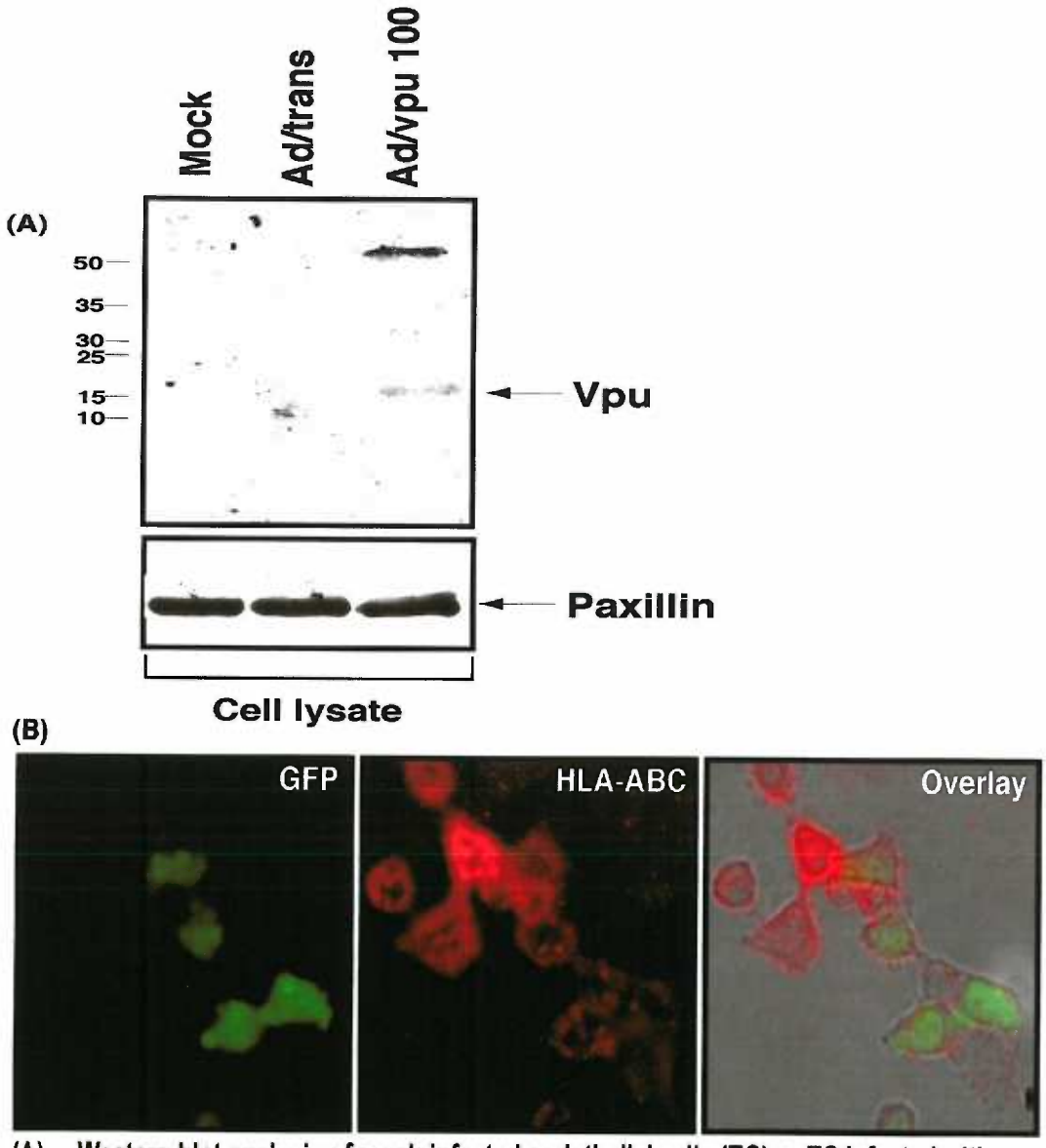


Figure 3.1 HIV Vpu upregulates CD40 surface expression on HUVEC

(A) CD40 surface expression by HUVEC infected with adenoviruses expressing vpu or tat. HUVEC were infected with recombinant adenoviruses for 48 hours, followed by evaluation of CD40 surface expression by immunofluorescence analysis. TNFα-treated cells were used as positive controls for CD40 staining. CD40 surface expression was upregulated by both Vpu and Tat as determined by immunofluorescence.

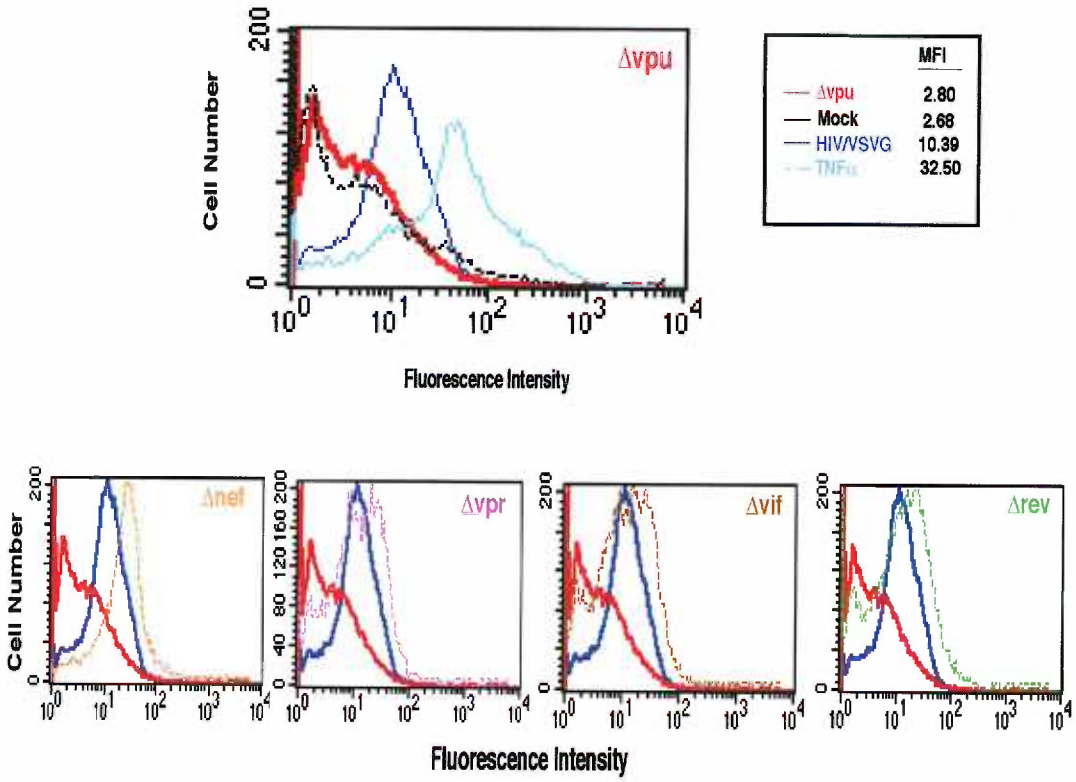
(B) Quantitative CD40 surface expression determined by FACS analysis. An increase in CD40 expression was detected on Vpu-expressing cells (pink) but not on cells expressing transactivator only (orange), cells expressing Vpr (black) or cells expressing Nef (blue). Tat-expressing cells (green) demonstrated a minimal effect on surface CD40 expression. TNFα-treated HUVEC (purple) were used as positive controls.

Figure 3.2 Functional HIV-1 Vpu is expressed with an adenovirus expression system.



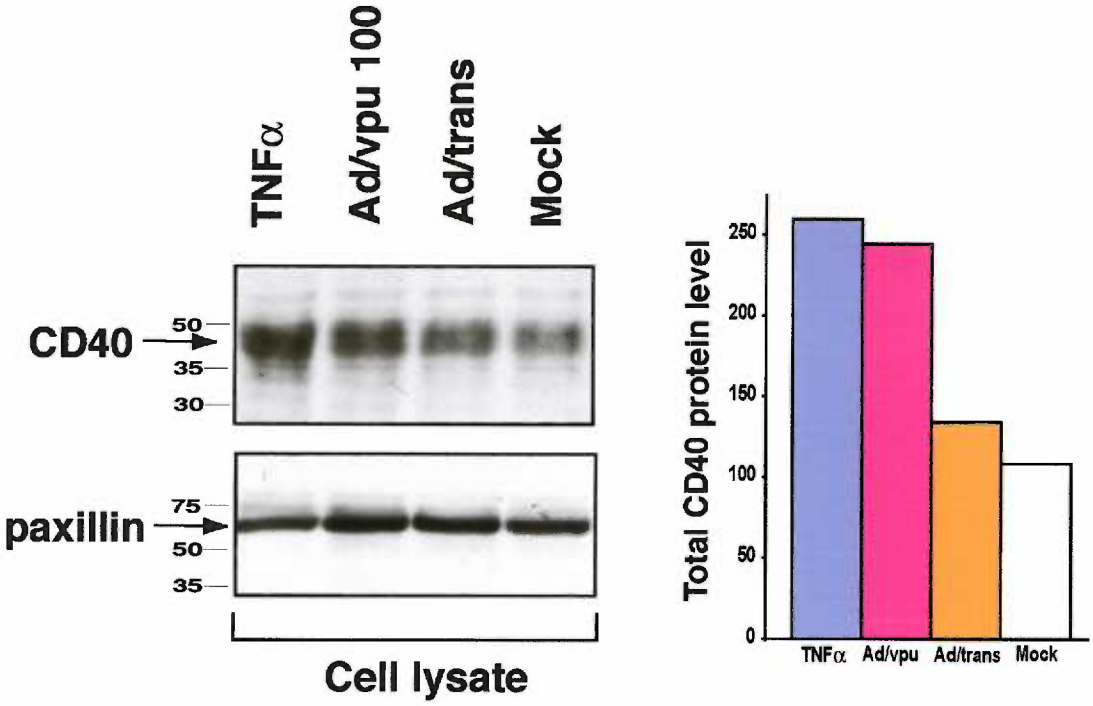
(A) Western blot analysis of mock-infected endothelial cells (EC) or EC infected with an adenovirus expressing vpu (Ad/vpu) or adenovirus expressing transactivator (Ad/trans). Molecular weights were calculated using full range rainbow markers. Cellular paxillin level are shown to confirm that an equal amount of cell lysate was loaded in each lane.
Note that higher molecular weight background bands were noted in data sheet for antiserum to HIV-1 Vpu obtained from NIH AIDS research and reference regent program.
(B) Functionality of adenovirus-expressed HIV-1 Vpu was demonstrated by the ability of Vpu to downregulate MHC Class I molecules. The GFP positive, Vpu expressing cells stained substantially lower for surface MHC Class I (HLA-ABC) than GFP negative, mock-infected cells, suggesting a Vpu-driven downregulation.

Figure 3.3 Vpu is necessary for upregulation of CD40 surface expression on HUVEC



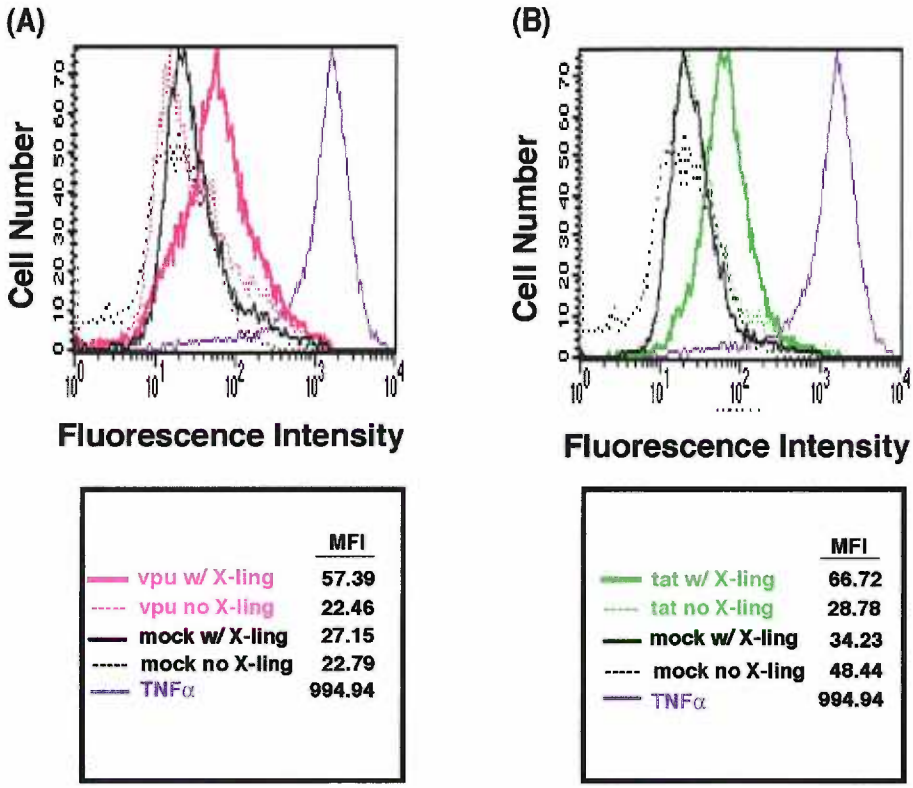
CD40 surface expression by endothelial cells (HUVEC) infected with HIV-VSV-G and HIV-VSV-G gene deletion mutants. As determined by FACS analysis, HIV-VSV-G Δ*vpu*-infected HUVEC (red) have substantially lower CD40 surface expression than cells infected with HIV-VSV-G (blue) or other deletion viruses, Δ*nef* (orange), Δ*vpr* (purple), Δ*vif* (brown) and Δ*rev* (green), suggesting that Vpu plays a direct role in induction of CD40 surface expression. TNFα-treated cells (Teal) were included as positive controls. Mock infected cells (black) were included to show baseline levels of CD40 surface expression.

Figure 3.4 Ad/vpu-infected HUVEC have increased total CD40 level.



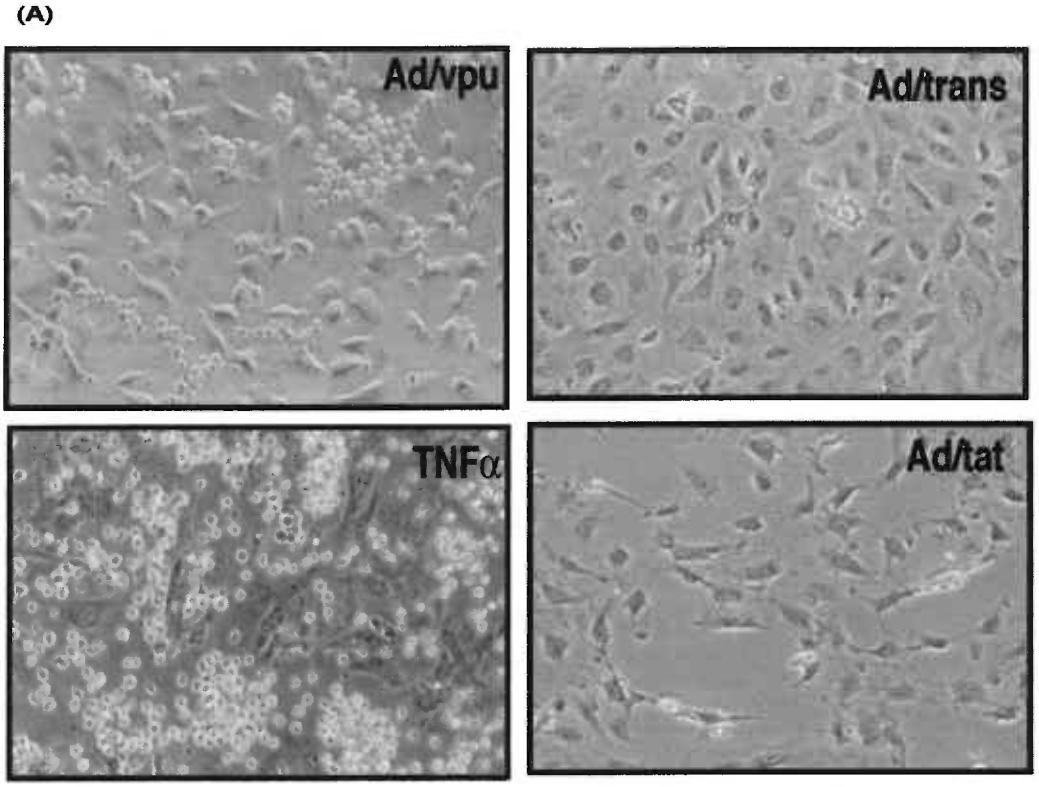
Total CD40 protein levels in whole cell lysates as determined by western blot analysis. The total levels of CD40 in Ad/vpu-infected HUVEC were increased by two-fold relative to mock-infected HUVEC or Ad/trans-infected HUVEC. TNFα-treated cells were included as positive controls. The total CD40 levels in each lane were compared quantitatively by densitometry: TNFα-treated cells (259.7), Ad/vpu 100 (244.6), Ad/trans (134.1), and Mock (108.8). Cellular paxillin levels are shown to confirm that an equal amount of cell lysate was loaded in each lane.

Figure 3.5 HIV-1 Vpu and Tat induce VCAM-1 expression on HUVEC following CD40 ligation.



CD40-mediated VCAM-1 expression on endothelial cells (HUVEC) expressing Vpu (A) or Tat (B) by FACS analysis. (A) Following CD40 ligation with anti-CD40 antibody (5 μg/ml) *in vitro*, HUVEC expressing Vpu (solid pink) displayed increased levels of VCAM-1 as compared to mock-infected cells (solid black). Consistent with previous observations made with HIV infection of EC, neither Vpu (dotted pink) nor Tat (dotted green) was sufficient to directly upregulate surface VCAM-1 expression on HUVEC. In the absence of CD40 ligation, VCAM-1 levels were similar to those seen on mock-infected cells (dotted black). TNFα-treated cells were used as positive controls for VCAM-1 expression. (B) HUVEC expressing Tat were treated with anti-CD40 antibody (solid green) as described in (A). Again, the level of VCAM-1 expression was compared to HUVEC expressing Tat without CD40 crosslinking (dotted green) and mock-infected cells with (solid black) or without CD40 triggering (dotted black).

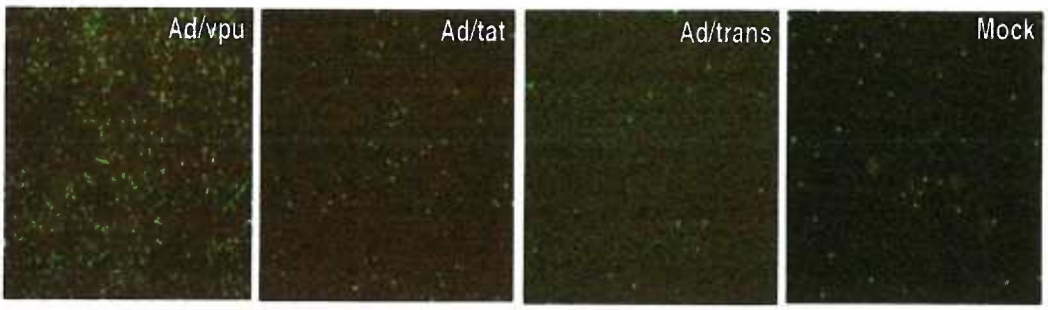
Figure 3.6 HIV-1 Vpu mediates B lymphoma cell adhesion to HUVEC and microvascular EC (MVEC).



(A) Light microscopy demonstrating B lymphoma (BL) cell adhesion to HUVEC expressing Vpu (Ad/vpu). HUVEC were infected with Ad/vpu, adenovirus expressing Tat (Ad/tat) or transactivator Ad/trans for 48 hours. Infected cells were co-cultured with BL cells for 48 hours. BL cells adhered to Ad/vpu-infected HUVEC but not to Ad/tat- or Ad/trans-infected cells. Cell fields were photographed after washing monolayers with PBS. Because TNFα treatment markedly upregulates VCAM-1 expression, TNFα-stimulated HUVEC were used as positive controls for BL cell adhesion.

Figure 3.6 HIV-1 Vpu mediates B lymphoma cell adhesion to HUVEC and microvascular EC (MVEC).

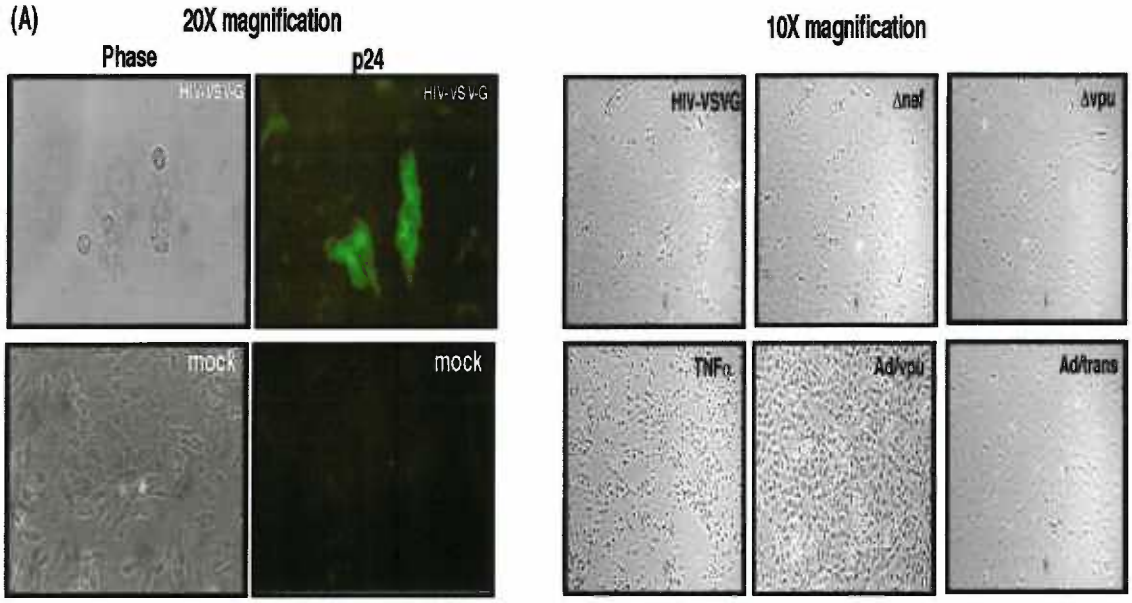
(B)



(C)

(B) Quantitation of BL cell adhesion to EC. EC were infected with various adenoviruses for 48hrs, followed by the addition of a crosslinking anti-CD40 monoclonal antibody to induce VCAM-1 expression for 18 hrs. BL cells preloaded with a fluorescent dye (Calcein-AM) were co-cultured with infected EC for 3 hrs. Representative 10X fields illustrating BL cells adherent to mock-infected MVEC or MVEC infected with Ad/vpu, Ad/tat or Ad/trans alone are shown. A minimum of four such fields was counted to quantitate adhesion (Table 3.2). (C) Adhesion assays as described in (A) were performed on MVEC. Accordingly, dermal MVEC were infected with Ad/vpu for 48 hours and co-cultured with BL cells. Following a 2-day co-culture period, non-adherent BL cells were removed by rinsing and fixed cells were stained with mouse anti-CD40 antibody and TRITC-conjugate goat anti-mouse secondary antibody and then evaluated for both CD40 surface expression and B cell adhesion. Vpu-expressing cells were detected by GFP expression. As shown, MVEC that supported B cell adhesion (phase) were those that expressed Vpu (GFP), and only Vpu-positive cells expressed high levels of CD40 (red).

Figure 3.7 B lymphoma cells adhere selectively to HIV-VSV-G-infected endothelial cells, and Vpu is required for B lymphoma cell adhesion.



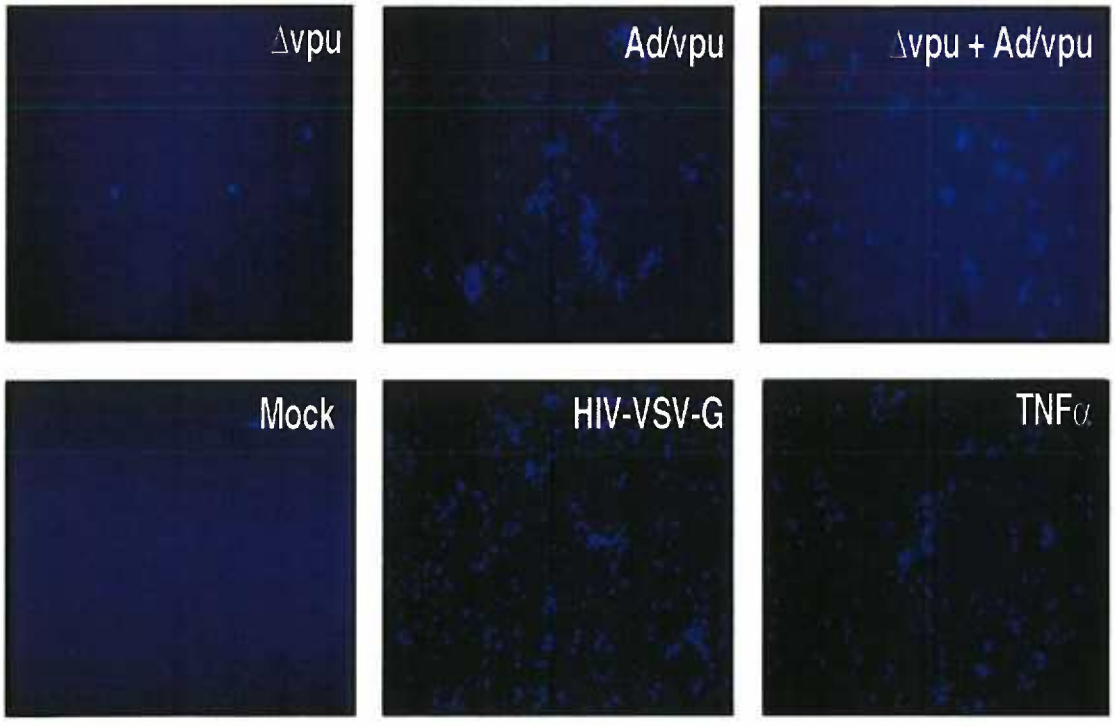


Figure 3.7 B lymphoma cells adhere selectively to HIV-VSV-G-infected endothelial cells, and Vpu is required for B lymphoma cell adhesion.

(A) Microscopy illustrating B lymphoma cell adhesion to HIV-VSV-G-infected HUVEC and the lack of B lymphoma cell adhesion to mock-infected or HIV-VSV-G Δ*vpu*-infected cells. Infected monolayers were co-cultured with BL cells for 48 hours prior to stringent rinsing. BL cells adhered exclusively to HIV-VSV-G-infected HUVEC expressing Vpu (HIV-VSV-G and Δ*nef*), which were p24 positive (green), but did not adhere to cells lacking Vpu (HIV-VSV-G Δ*vpu* or mock). TNFα–treated cells, Ad/vpu- and Ad/trans-infected cells were included as controls.

(B) Direct immunofluorescence microscopy to illustrate that DAPI-stained BL cells (blue) preferentially adhere to EC expressing Vpu (Ad/vpu, HIV-VSV-G, and Δ*vpu*+Ad/vpu) but not to EC infected with HIV-VSV-G Δ*vpu* or mock-infected EC. TNFα -treated cells were included as positive controls for B lymhoma cell adhesion.

Table 3.1 B lymphoma cell adhesion and CD40 surface expression on endothelial cells expressing various HIV-1 regulatory and accessory genes.

HIV-1 Proteins	B Lymphoma Cell Adhesion	↑CD40
HIV	+	+
Vpu	+	+
Tat	-	-/+
Nef	-	-
Vpr	-	-
Vif	-	-
Rev	-	-

Table 3.2 B lymphoma cell adherence to MVEC expressing HIV proteins

	Experiment 1#	*Experiment 2*
Mock-infected MVEC*	**38 ± 6**	**56 ± 8**
Ad/vpu-infected MVEC	**722 ± 33**	**884 ± 100**
Ad/tat-infected MVEC	**78 ± 11**	**144 ± 16**
Ad/trans-infected MVEC	**42 ± 7**	**96 ± 32**

 # Results from 2 independent experiments are shown. Counts are the mean ± SD of 4 randomly selected 10X fields.

*** MVEC were uninfected or infected with Ad/vpu, Ad/tat or Ad/trans for 48 hrs prior to addition of CD40 MAB for 24 hrs and co-culture with Calcein-AM loaded BL cells for 3 hrs. BL cells were washed stringently prior to counting to remove non-adherent and loosely adherent cells.**

Chapter 4

SUMMARY AND DISCUSSION

The development of malignant tumors in individuals infected with human immunodeficiency virus-1 (HIV) represents one of the biggest medical challenges for Acquired Immune Deficiency Syndrome (AIDS) patients and their caretakers. Indeed, 30-40% of HIV patients will develop cancer during the course of their disease [Spina, *et al.*, 1999]. The most common cancers associated with HIV infection are Kaposi's sarcoma (KS) and non-Hodgkin's lymphoma (NHL) [Goedert, *et al.*, 1998], and NHL is the most lethal malignant complication of AIDS in industrialized countries [Petruckevitch, *et al.*, 1998]. The AIDS-associated NHL (AIDS-NHL) are characteristically high-grade B cell tumors of two main histological categories, diffuse large cell lymphomas (DLCL), and small non-cleaved cell (Burkitt's and Burkitt's-like) lymphomas (SNCCL), that frequently involve extranodal primary sites such as the central nervous system (CNS), bone marrow and liver [Raphael, *et al.*, 1991]. The introduction of highly active antiretroviral therapy (HAART) has dramatically reduced the progression of HIV-infection to AIDS and mortality, but the impact of HAART on HIV-associated cancers remains uncertain. While current therapeutic strategies have significantly reduced KS incidence, they have had a relatively minor effect on NHL risk [Ledergerber, *et al.*, 1999, Matthews, *et al.*, 2000, Rabkin, 2001]. This is particularly true for the systemic Burkitt's-like lymphomas, probably because

these tumors are not as closely linked to immunosuppression as the primary CNS lymphomas [Gabarre, *et al.*, 2001]. While failure to see an improving trend for AIDS-associated NHL may reflect inadequate time after widespread initiation of therapy, and/or a need to treat earlier in the course of infection, AIDS-NHL continues to be a significant clinical problem that will become increasingly common as an AIDS-defining illness and a cause of mortality in HIV-infected population [Tirelli, *et al.*, 2002, Vilchez, *et al.*, 2002].

Heterologous genetic lesions are seen in the different AIDS-NHL subtypes, and importantly, Epstein-Barr virus (EBV) infection is not universally present in these lymphomas. A common feature however is the lack of infection by HIV, indicating an indirect role for the virus in pathogenesis. While chronic B cell stimulation and dysregulated cytokine networks are likely to facilitate B cell expansion and acquisition of genetic lesions, I hypothesize that HIV infection of endothelial cells that interact with B lymphoma cells plays an additional role in driving lymphomagenesis. Since microvascular endothelial cells (MVEC) of the brain, bone marrow and liver (major sites for extranodal AIDS-NHL involvement) are all susceptible to HIV infection, HIV-mediated changes in MVEC phenotype could conceivably contribute to homing and aggressive growth of NHL at extranodal tissue sites.

Our group has previously shown that brain and bone marrow MVEC develop an enhanced capacity to support the adhesion and growth of B cell NHL following HIV infection [Moses, *et al.*, 1997]. Specifically, we have shown that HIV-mediated upregulation of the cytokine receptor CD40, and enhanced expression of the adhesion molecule VCAM-1 following CD40 ligation, are key events facilitating MVEC-B cell interactions.

The experimental results described in this thesis have identified a group of viral and cellular genes expressed in HIV-infected endothelial cells (EC) that promote the adhesion and growth of non-Hodgkin's lymphoma-derived B cells (BL cells). Using an *in vitro* system to express HIV genes individually in EC, followed by EC phenotypic analysis and examination of EC-BL cell co-culture systems, I have demonstrated that BL cell adhesion is mediated primarily by HIV Vpu as a consequence of enhanced expression of CD40 and VCAM-1 on the surface of EC, while BL cell growth is driven by HIV Tat-induced EC cytokines. The *in vitro* lymphomagenesis system described in this thesis utilizes only two cell types, an endothelial cell monolayer that is subject to experimental manipulation, and malignant B lymphoma cells added in co-culture or exposed to EC-conditioned supernatants. While this system allows us to clearly define mechanisms of EC support, we should remember that *in vivo*, interactions with other cell types such as T cells, macrophages and dendritic cells may amplify or modulate the effects

reported in this thesis. The results of this study and their broader implications are discussed in sections 4.1 and 4.2 below.

4.1 HIV-1 Tat supports B lymphoma cell proliferation

4.1.1 HIV-1 Tat mediates B lymphoma cell proliferation by inducing production of interleukin-6 and interleukin-10 in endothelial cells:

In chapter 2 of this thesis, I have presented clear evidence that the expression of the HIV-1 regulatory gene *tat* in EC using a recombinant adenovirus expression system promotes the growth of lymphoma cells by inducing expression of B cell growth factors, in particular IL-6 and IL-10, in the tat-expressing EC.

Serum IL-6 and IL-10 levels have been shown to be elevated in patients suffering from AIDS-NHL, implicating a role for these cytokines in the disease process [Pluda, *et al.*, 1993]. Tat is synthesized early during HIV infection to transactivate viral genes, and is also able to induce the transcription of several cellular genes, including cytokines. Thus, while HIV (unlike some herpesviruses) does not encode cytokine homologs, the virus can achieve a similar biological outcome by transactivation of the cellular protein.

Tat-mediated induction of cytokines and chemokines is well documented, and cell type specific profiles have been reported (reviewed in Tat section, Chapter 1). In this thesis, I report a Tat-regulated endothelial cell cytokine profile characterized

by induction of IL-6, IL-8 and IL-10 (Figure 2.4) but not IL-1, IL-3, IL-4 or TNFα (Figure 2.5). Further, I hypothesize that IL-6 and IL-10 are defining factors in the Tat-induced EC support phenotype, since neutralization of these cytokines in EC-conditioned medium from Tat-expressing EC nullified the growth advantage of the medium, making the supernatant unsupportive than conditioned supernatant from control EC (Figure 2.7).

The Tat-induced production of IL-6 in EC reported here is consistent with other reports of Tat-induction of IL-6 from EC (reviewed in IL-6 section, Chapter 1). The manner of IL-6 induction by adenovirus-vector expressed Tat in EC was not explored in more detail in this thesis, but direct promotor transactivation and/or stimulation of secondary IL-6-induction pathways are likely mechanisms which have been described in other systems.

Tat-induced production of IL-10 has also been documented in the literature, both using *in vitro* expression systems [Badou, *et al.*, 2000] and cells from HIV-infected individuals [Blazevic, *et al.*, 1996]. Since IL-10 has both immunosuppressive and B cell stimulatory properties, IL-10 induced via Tat may facilitate evasion of malignant B cells from immune surveillance, as well as promoting B cell growth. Indeed, Kebelmann-Betzing has proposed a role for IL-10 in tumor-associated anergy [Kebelmann-Betzing, *et al.*, 2001].

In vivo, Tat may play multiple roles in regulating lymphoma growth. In addition to inducing cytokines that promote B cell proliferation (IL-6 and IL-10) and fostering an environment that retards tumor surveillance and elimination (IL-10), Tat may also contribute to lymphoma cell accumulation by protecting pre-malignant and malignant cells from apoptotic death. In the following sub-section, I will present compelling preliminary evidence that Tat-mediated anti-apoptosis of BL cells during HIV infection may contribute further to the EC-derived support.

4.1.2 HIV-1 Tat or Tat-induced factors protect B lymphoma cells from apoptosis:

To evaluate the survival of BL cells in the presence of Tat-expressing EC, the effect of EC-BL cell co-culture on BL cell apoptosis was examined. This assay was considered relevant since visual examination of EC-BL cell co-cultures suggested a decreased incidence of apoptotic cells (Figure 2.1). In addition, several lines of evidence indicate that Tat plays a dual role in cell survival as well as cell growth [Bonavia, *et al.*, 2001, Ensoli, *et al.*, 1990, Gibellini, *et al.*, 2001, Lotz, *et al.*, 1994, Park, *et al.*, 2001, Purvis, *et al.*, 1995, Zauli, *et al.*, 1993].

In the *in vitro* lymphomagenesis system utilized here, Tat appeared to deliver a survival or anti-apoptotic signal to BL cells when BL cells were co-cultured with Tat-expressing EC. Notably, these BL cells exhibited a substantially lower (13%-30%) propensity to undergo apoptotic death when treated with the pro-apoptotic

agent camptothecin (CAM) as compared to BL cells co-cultured with Nef-expressing or mock-infected EC (Figure 4.1). CAM induces apoptosis as a result of its activity against topoisomerase-1, an important enzyme in DNA replication [Han, *et al.*, 2002, Li, *et al.*, 1995]. These data suggest an additional role for Tat in promoting lymphomagenesis that involves protecting malignant B cells from apoptotic death. Additional experiments will be needed to elucidate the nature of this protection.

Our finding that Tat plays a role in protecting BL cells from apoptosis (Figure 4.1), thereby supporting B lymphoma survival, is consistent with the anti-apoptotic function of Tat reported by other researchers. For example, Tat protects epithelial cells, Kaposi's sarcoma cells and CD4-positive Jurkat T cells from apoptosis [Ensoli, *et al.*, 1990, Gibellini, *et al.*, 1995, Zauli, *et al.*, 1993]. Gibellini and colleagues [Gibellini, *et al.*, 2001] hypothesized that Tat mediates a protective effect by interfering with the TRAIL-mediated apoptosis pathway. Some contradictory data about the role of Tat in apoptosis can be resolved by the recognition that dose-dependent effects occur; Tat has an anti-apoptotic effect at nanomolar concentrations, but an apoptotic effect at micromolar concentrations [Li, *et al.*, 1995]. Tat protects T cells from apoptosis by activating ERK/MAPK pathways [Mischiati, *et al.*, 1999] while Tat-mediated apoptosis utilizes the activation of JNK pathway [Mischiati, *et al.*, 1999].

4.1.3 HIV-1 Tat-expressing endothelial cells induce B lymphoma cell aggregation:

In addition to inducing BL cell proliferation, visual observation of BL cells co-cultured with EC expressing HIV proteins revealed an additional phenomenon unique to the Tat expressing co-cultures. Specifically, a homotypic BL cell-BL cell aggregation was observed in co-cultures with Ad/tat-infected EC, but not mock-infected, or Ad/trans-infected EC (Figure 2.1A). In addition, no BL cell aggregation was observed in Vpu-, Nef-, Vpr-, Vif- or Rev-expressing EC co-cultures (data not shown). Tat expressed in EC via adenovirus vectors is found in the supernatant at levels that are readily detected via Western analysis (Figure 2.2A). When BL cells were exposed to cell-free supernatants harvested from MVEC infected with Ad/tat or Ad/trans some BL cell aggregation was also seen exclusively in the Tat-containing medium (Figure 2.1B).

This homotypic BL cell aggregation observed in the presence of Tat-containing medium is reminiscent of what is seen in activated B cells that express adhesion molecules and or/ their cognate ligands. Adhesion molecules and integrins such as VLA-4, $\alpha 4\beta 7$, LFA-1, CD23, CD45, CD48, and CD53 have all been implicated in aggregation of activated B cells [Bjorck, *et al.*, 1993, Cao, *et al.*, 1997, Garnett, *et al.*, 1994, Postigo, *et al.*, 1993, van de Wiel-van Kemenade, *et al.*, 1992, Zapata, *et al.*, 1995]. While LFA-1 can mediate this type of interaction, this molecule is likely not responsible for the aggregation in our system because the

BL cells used in our study lack LFA-1 expression (unpublished data). Other molecules, especially VLA-4, and CD40/CD40L, are more likely to be involved in the formation of B cell aggregates. Future neutralization studies will allow us to define which adhesion molecules play a role in Tat-mediated B cell aggregation.

Interestingly, in the presence of Tat-expressing EC, the BL cell aggregation was observed with minimal adhesion to the underlying infected monolayer, while another HIV protein, Vpu, induced EC-BL cell heterotypic adhesion (Chapter 3). Collectively, these data suggest that HIV infection of the endothelium could induce BL cell-BL cell as well as EC-BL cell adhesion events that would allow for the generation of multi-layered lymphoma aggregates *in vivo*.

The observation that Tat derived from HIV infected-endothelial cells can significantly influence the proliferation and survival of B lymphoma cells supports the hypothesis that Tat plays a role in the development of HIV-associated malignancies. Others have reported that expression of Tat in transgenic mice induces lymphoid proliferative disorders and tumors [Corallini, *et al.*, 1993, Kundu, *et al.*, 1999, Vellutini, *et al.*, 1995, Vogel, *et al.*, 1988]. While most groups have focused on Tat expression in macrophages and lymphocytes, as well as malignant tumor cells, the data presented in this thesis is the first to demonstrate that Tat expression in endothelial cells, a cell type that is susceptible to HIV infection and may serve as a reservoir for HIV, supports significant

growth of B lymphoma cells. Since transgenic mice express Tat in many organs and cell types, the mechanism by which Tat induces tumors in the murine system has not been resolved. I propose that the human cell-based *in vitro* system described in this thesis has allowed unique and relevant insight into the mechanism whereby Tat supports lymphoma growth.

4.2 HIV-1 Vpu expression in endothelial cells supports the attachment of B lymphomas through induction of endothelial cell surface CD40 and subsequent upregulation of VCAM-1.

HIV/VSV-G and adenovirus expression systems have been utilized in this thesis for loss of function and gain of function studies designed to identify the HIV gene(s) that play a role in promoting BL cell-EC adhesion. For loss of function studies, BL cells were shown to adhere preferentially to HIV/VSV-G-infected, but not uninfected HUVEC. This observation suggested that HIV-1 *env* is not essential for B cell adhesion to infected EC, and that other HIV gene(s) act to confer the adhesive phenotype. Since accessory and regulatory genes of HIV are well conserved among viral isolates, have a critical role in gene expression and replication, and are causally involved in the pathogenesis in AIDS, I hypothesized that some or all of these genes could potentially contribute to the HIV-induced EC-BL cell interactions. To systematically explore this hypothesis, I expressed these HIV genes individually in EC using an adenovirus expression system in a series of gain-of-function studies in order to identify the gene(s) that confer B

cell adhesive properties on EC. Results presented in Chapter 3 of this thesis clearly show that Vpu is the only gene that confers significant adhesive properties on EC. This is the first report to describe a function for Vpu in mediating cell-cell adhesion. In addition, because the model used recapitulates a disease scenario (AIDS-NHL), the study ascribes a unique role to Vpu in AIDS pathogenesis.

A characteristic feature of activated endothelium is enhanced expression of co-stimulatory and adhesion molecules, leading to increased adhesiveness for leukocytes. Cytokines produced by EC or the leukocytes themselves activate EC by binding to EC receptors. Thus, the type and density of these receptors play a central role in regulating endothelial activation [Henderson and Moses 2002, and references therein]. In activated endothelial cells, CD40 activation often leads to the upregulation of adhesion molecules (ICAM-1, VCAM-1, E-selectin). In our system, Vpu-mediated BL cell adhesion is likely the result of CD40-mediated VCAM-1 upregulation (Chapter 3). However, other cytokine/cytokine receptor pairs may also play a role. Interestingly, Vpu-expressing EC demonstrated an increase in IL-1α production, but did not show induction of other cytokines tested such as IL-3, IL-4, IL-6, IL-8, IL-10, MCP-1 and TNFα (Figures 4.2, 2.4 and 2.5). Thus, Vpu may upregulate surface CD40 expression indirectly by inducing the production of IL-1 which subsequently upregulates CD40 in an autocrine fashion. This hypothesis is consistent with the idea that IL-1α, similar to TNFα and interferonγ, can induce CD40 expression three-fold on endothelial cells at

peak level at 8-24 hours after treatment [Karmann, *et al.,* 1995]. The upregulated CD40 molecules will then be cross-linked by CD40 ligand to induce VCAM-1 expression. In addition, Karmann *et al.* showed that IL-1α is more potent than CD40 ligand in VCAM-1 induction in EC. Therefore, possibility exists that Vpu may affect CD40 expression by both an autocrine effect of IL-1α and a direct effect on CD40 expression. Future experiments are needed to evaluate the significance of IL-1α in our system.

In a natural HIV infection, virus-induced expression of the co-stimulatory and adhesion molecules, CD40 and VCAM-1 has been shown to mediate EC-BL cell adhesion [Moses, *et al.*, 1997]. CD40, a member of the TNF-R family of receptors, delivers a VCAM-1 induction stimulus following cross-linking by its ligand, the cytokine CD40L. Thus, in this thesis I have also evaluated the effect of HIV genes on expression of CD40 and VCAM-1. As presented in detail in Chapter 3, only Vpu and Tat were found to affect the surface expression of these two proteins. The significance of these findings is discussed in more detail below.

Quantitative FACS analysis of surface CD40 levels on EC indicated that Vpu was a stronger inducer of CD40 expression than Tat (Figure 3.1B). Although the mechanism by which Vpu-mediated CD40 upregulation has yet to be resolved, other studies have shown that Vpu interferes with cellular protein transport pathways. Indeed, one of the two established functions of Vpu is to indirectly

assist transport of viral Env from the endoplasmic reticulum (ER) to the plasma membrane by binding to nascent CD4. Since Vpu has been shown to interact with cellular proteins, Vpu may similarly bind to CD40 and facilitate efficient transport of CD40 to the cell surface. In addition, since Western blot analysis demonstrated that Ad/vpu-infected cells contained increased total CD40 levels (Figure 3.4), Vpu may also affect CD40 transcriptionally or post-transcriptionally. Future experiments will define the mechanism of Vpu-mediated CD40 upregulation and will address whether Vpu upregulates CD40 at the mRNA level and whether Vpu can physically associate with CD40.

A recent study by Maisch and colleagues [Maisch, *et al.*, 2002] reports that infection of EC with human cytomegalovirus (HCMV) leads to increased expression of surface CD40 that was evident by 24 hr post infection (PI) and peaked at 48-72 hrs PI. Interestingly, CD40 mRNA and total protein levels protein were unchanged at 24 hrs but significantly increased (approximately 1.5 fold and 3 fold for mRNA and total protein respectively) by 72 hrs PI. In a manner analogous to our reports of CD40L-mediated VCAM-1 induction in HIV-infected EC, Maisch and colleagues report that co-culture of EC with CD40L-positive T cells leads to preferential expression of the adhesion molecule E-selection on the CD40-positive HCMV-infected HUVEC. While HCMV infection is not associated to abnormal NHL incidence, it has been linked to other endothelial cell-associated diseases such as transplant-associated vascular

restenosis and atherosclerosis [Streblow, *et al.*, 2001]. A pathologic feature of both of these diseases is recruitment of activated T cells with local production of pro-inflammatory cytokines. CD40-CD40L interactions have previously been linked to atherosclerosis [Lutgens, *et al.*, 2002] so the link between HCMV infection, CD40 induction and development of inflammatory vascular lesions may have important implications for understanding and treating HCMV-associated vascular disease. These studies and ours highlight CD40 as an important molecule in diseases where abnormal interactions between endothelial cells and leukocytes underlie the pathogenic process.

Constitutive levels of VCAM-1 on unstimulated EC were not affected by expression of Vpu and/or Tat in these cells (Figure 3.5). This result is consistent with previous observations that HIV-1 infection does not directly upregulate VCAM-1 expression unless a CD40 activation stimulus is applied [Moses, *et al.*, 1994, Moses, *et al.*, 1997]. In keeping with observations in HIV-infected cells, both Vpu- and Tat-expressing EC displayed enhanced VCAM-1 expression in response to CD40 ligation with an anti-CD40 antibody (Figure 3.5). The VCAM-1 induction observed in Tat-expressing EC is interesting since this would imply establishment of a B cell adhesive phenotype. However, co-culture of CD40L-positive BL cells with Tat-expressing EC (an event that could potentially activate CD40 and induce VCAM-1 expression) led primarily to BL cell-BL cell interaction rather than BL cell-EC adhesion. I hypothesize that triggering via

CD40L-positive BL cells may be insufficient to induce VCAM-1 on Tat-expressing EC to the same extent as exogenous soluble CD40L or anti-CD40 antibody; thus, the threshold for EC-BL cell adhesion is not attained. Vpu on the other hand may influence CD40 expression in a manner that renders this receptor responsive to ligand delivered on the surface of BL cells. Note that *in vivo*, CD40L functions primarily as a membrane-bound cytokine. While CD40/CD40 ligand mediated VCAM-1 expression is part of the normal physiological response of activated endothelial cells, HIV Vpu expression appears to upregulate the key molecule CD40 for this interaction, thereby enhancing the subsequent VCAM-1 upregulation. Furthermore, VCAM-1/VLA-4 interactions are also a part of the normal inflammatory process leading to leukocyte extravasation. B lymphoma cells may utilize these important molecules to adhere to activated EC to initiate the formation of an extranodal tumor focus. Once the initial attachment layer is formed, the activated B cells may adhere to each other in a homotypic manner to form the multilayered lymphoma. Results presented in this thesis suggest that the coordinated effects of HIV-1 Vpu and Tat promote the development of B lymphoma foci.

4.3 A model for functional roles of HIV proteins in B cell lymphomagenesis.

Collectively, the findings presented in this thesis identify an important role for the HIV proteins Tat and Vpu in AIDS-associated B cell lymphomagenesis. On the basis of my experimental findings, I propose the model schematically illustrated

in Figure 4.3. The expression of the viral proteins Tat and then Vpu in HIV-infected endothelial cells leads to an upregulation of the cytokine receptor and co-stimulatory molecule CD40. CD40 is activated following cross-linking by the CD40 ligand, which is expressed by B lymphoma cells, or adjacent T cells and macrophages, or possibly even as a soluble cytokine. The BL cells used for the studies in this thesis were CD40L-positive, and this is the situation represented in Figure 4.3. Note however that since CD40L can be delivered from multiple sources, the mechanism illustrated may be equally relevant for the establishment of CD40 ligand-negative B lymphomas *in vivo*. As in normal physiological processes, CD40 ligation mediates a subsequent upregulation of VCAM-1 on infected endothelial cells. Importantly, the effect of HIV on CD40 is likely the key to allowing preferential induction of VCAM-1 on HIV-infected cells. Preferential induction may be due to increased levels of CD40 on the cell surface as well as to regulation of post-ligation signal transduction events leading to VCAM-1 promoter activation. Future studies are aimed at elucidating the molecular pathways involved in VCAM-1 induction. Similar to events mediating normal leukocyte binding to EC, VCAM-1 interacts with its cognate receptor VLA-4 on the B lymphoma cell surface, forming a tight intercellular bond. It is possible that in addition to activating VCAM-1 expression, the initial CD40/CD40 ligand interaction may trigger an activation signal in the B lymphoma cells, thereby changing the VLA-4 integrin from a low-affinity to a high affinity conformation. In addition, HIV-1 Tat may act as a further activation

stimulus to B lymphoma cells, resulting in homotypic BL cell aggregation as well as lymphoma cell proliferation. Ultimately, the coordinated action of viral and cellular proteins leads to the formation of B lymphoma cell foci within the unique microenvironment created by HIV-infected endothelial cells.

Like many other viral proteins, HIV Tat and Vpu appear to have manipulated the normal physiological and inflammatory responses to allow in this case for the development of B cell NHL. Like many other disease processes, the development of AIDS-NHL is likely an accidental side product of the normal functions of these molecules. The evolution of HIV and the manipulation of the host immune system are likely responsible for the etiology of this aggressive malignancy.

4.4 Physiological and clinical significance.

A significant number of HIV-positive patients with lymphoadenopathy will eventually develop AIDS-NHL [Pelicci *et al.,* 1986]. In addition, multiple clonal B cell expansion can be found in 20% of patient with AIDS-associated lymphadenopathy syndrome [Pelicci *et al.,* 1986]. Therefore, the development of AIDS-NHL appears to progress from multiclonal B cell expansion to monoclonal B cell NHL. Clonality of malignant tumors can be evaluated by immunophenotypic and genotypic analysis [Raphael *et al.,* 1991; Raphael *et al.,* 1994]. Histopathologic studies have suggested that AIDS-NHL initially develops as multiclonal B-cell expansion and transform into high-grade monoclonal B-cell

lymphoma in two to three months [Lippman *et al.,* 1988; Raphael *et al.,* 1991]. In addition, Ng *et al.* studied the molecular analyses of immunoglobulin heavy chain variable gene of B cells in AIDS-NHL and found that clonal evolution of the malignant clone is the result of antigen-mediated somatic hypermutations in a multistep process [Ng *et al.,* 1997]. Therefore a multistep lymphomagenesis model was proposed to describe the progression of AIDS-NHL from a polyclonal lymphoma during earlier stage to a dominant malignant clone [Pelicci *et al.,* 1986; Ng *et al.* 1997].

The initial steps of malignant transformation of B cell NHL, *c-Myc* and VDJ rearrangement, take place in the bone marrow. In our lymphomagenesis model, HIV-infected bone marrow MVEC provide a local microenvironment that is supportive of BL cell growth. *In vivo*, these cells may have a role in facilitating these initial steps of transformation by providing cytokines and adhesive interactions in order to sustain polyclonal B cell differentiation. Furthermore, we hypothesize that endothelial derived IL-10 may contribute to further immunosuppression *in vivo* and thereby allowing for additional accumulation of genetic error in clonally expanding B cells that are predisposed to become malignant cells.

Molecular techniques such as fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR) analysis of light and heavy chain rearrangement

have been used to distinguish between dissemination and simultaneous proliferation of different malignant clones. The majority of AIDS-NHL are monoclonal malignant B cell clones, however, polyclonal and monoclonal lymphomas have also been reported to co-exist in the same individual [Raab-Traub and Flynn 1986; Barriga *et al.,* 1988; de Mascarel *et al.,* 1989; Knowles *et al.,* 1989; McGrath *et al.,* 1991; Ballerini *et al.,* 1993; Shibata *et al.,* 1993]. Dubus *et al.* reported the identification of two different monotypic B cell tumors at the lungs and CNS in an AIDS patient [Dubus *et al.,* 1995]. These authors speculated that the immortalization of multiple B cell clones may precede *c-Myc, bcl-6* and *p53* mutations which are mutations that normally contribute to the expansion of a monotypic malignant clone.

Regardless of whether different malignant clones can be found in different sites, lymphomatous infiltrations have been found in 87% of AID-NHL especially CNS, bone marrow, GI tract, and liver [Potter 1990]. We believed that infiltrating B lymphoma cells are able home to extranodal sites as a result of the adhesive interaction between the BL cells and HIV-infected MVEC at the extranodal site. In addition, endothelial derived B cell growth factors including IL-6 and IL-10 would further support the proliferation of these infiltrating cells in the local microenvironment, leading to formation of lymphoma foci. However, the possibility exists for de novo tumor development at the extranodal sites where antigen driven mutations are likely the main driving force. Like macrophages,

infected endothelial cells can present antigens and produce cytokines to facilitate the various stages of malignant transformation of B lymphoma clones. Since chronic stimulation can result in polyclonal lymphomas, sites such as CNS during inflammation and bone marrow may represent special microenvironments for the de novo neoplastic transformation of B cells.

Proliferation of mature B cells is influenced by exogenous factors derived from T cells and macrophages and possibly by endothelial cells in the environment; therefore tumor progression will also require growth factor signals from the microenvironment. I have shown in this thesis that HIV-infected endothelial cells provided a supportive microenvironment for BL cell growth and possibly for tumor cell progression. Abnormalities outside of the B-cell compartment may also play important roles in the development of NHL in AIDS. By inducing the proliferation of other macrophages and increased production of growth factors, HIV-infected macrophages were reported to result in secondary proliferation of surrounding B cells that may be the prerequisite events leading to transformation [McGrath et al. 1991]. Therefore, the high level of B cell growth factors produced from HIV-infected endothelial cells in our model is analogous to the growth factors produced by the HIV-infected macrophages in inducing B cell proliferation.

Co-infection experiments with recombinant adenoviruses demonstrated that both B lymphoma cell aggregation and adhesion to EC were enabled when these cells expressed Vpu and Tat simultaneously (Figure 4.5). On the basis of the studies presented in this thesis, I hypothesize that Vpu and Tat act co-operatively *in vivo* to facilitate B lymphoma cell homing and adhesion to HIV-infected endothelial cells, as well as lymphoma cell survival and proliferation at the vessel wall, thus allowing the formation of multi-layered lymphoma aggregates at extranodal sites of AIDS-NHL involvement, including brain, liver and bone marrow.

The studies presented in this thesis are important from a clinical perspective since AIDS-associated lymphoma continues to be a significant medical and social problem. An emerging paradigm for cancer therapy is the identification of selective targets for therapeutic intervention. A better understanding of the role of HIV in the etiology of the AIDS-NHL will facilitate the design of rational targeted therapies for this aggressive tumor. Indeed, an experimental treatment for NHL using anti-IL-6 monoclonal antibody (BE-8) was attempted [Emilie, *et al.*, 1994]. However, most patients did not achieve remission. Since IL-10 appears to play a significant role in the *in vitro* lymphomagenesis model, I anticipate that anti-IL-10 monoclonal antibody in conjunction with anti-IL-6 therapy may be a beneficial treatment for AIDS-NHL because both cytokines are responsible for significant B lymphoma proliferation. This proposed combination therapy may

curb the proliferation of B lymphoma cells, increase Th1 anti-tumor response and may facilitate full remission of lymphoma in patients.

From a virological perspective, my study on the functions of individual HIV genes has suggested novel roles for Vpu and Tat in AIDS-associated cancer, but may also have wider implications for HIV disease. This systematic evaluation of the influence of HIV genes on endothelial cell - B lymphoma cell interactions has provided insights on the mechanisms that promote B lymphoma cell homing and growth at extranodal sites in the context of AIDS.

4.5 Conclusion

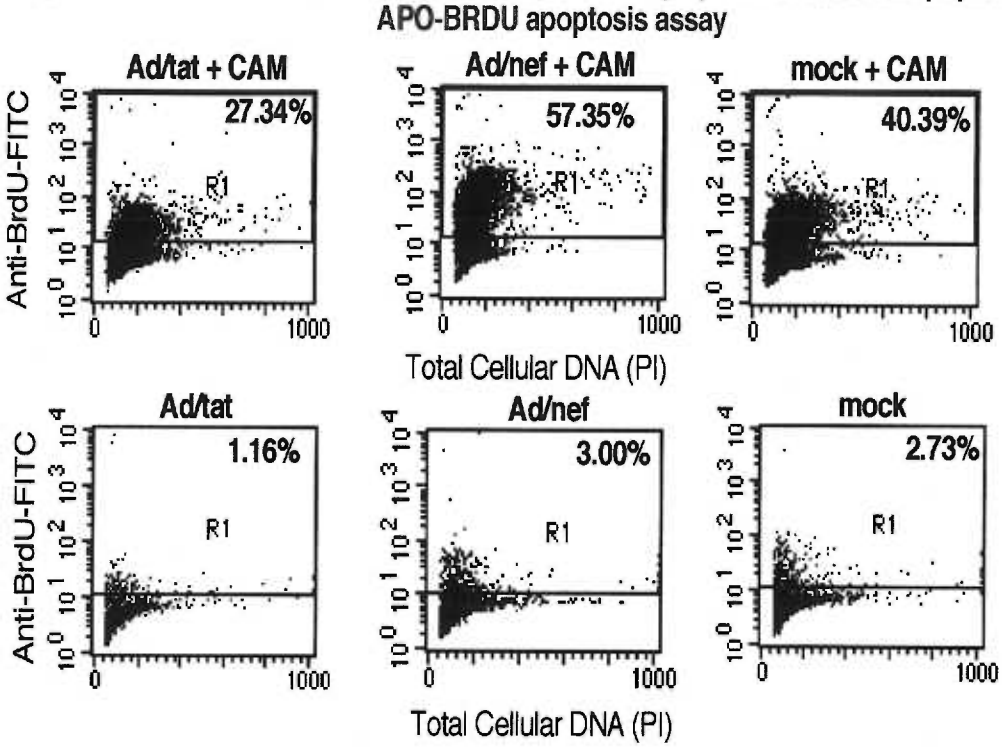
In conclusion, this dissertation has demonstrated the following:

1) HIV-1 Tat expression in endothelial cells supports the development of B cell lymphoma by promoting B lymphoma cell proliferation via Tat-induced cytokines IL-6 and IL-10.
2) HIV-1 Vpu expression in endothelial cells supports the firm adhesion of B lymphoma cells to endothelial cells.
3) HIV-1 Vpu mediates upregulation of surface CD40 on endothelial cells, while Tat induces a slight enhancement of CD40 expression on endothelial cells.
4) Surface VCAM-1 expression is not upregulated on endothelial cells expressing Vpu and/or Tat without CD40 ligation.

5) Following CD40 ligation, surface VCAM-1 is upregulated on endothelial cells expressing either Vpu or Tat.

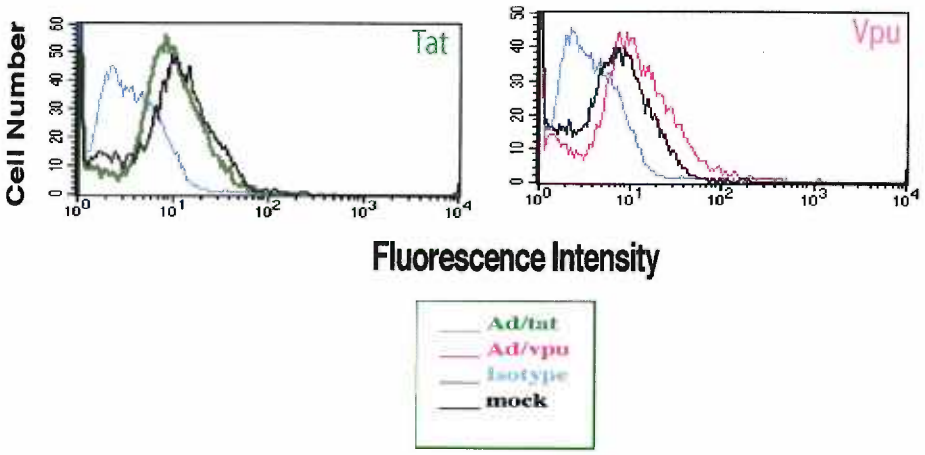
When taken together, the data presented in this dissertation identified for the first time a unique role for HIV-1 proteins Tat and Vpu expressed in HIV-infected endothelial cells, in the development of B cell AIDS-NHL.

Figure 4.1 HIV-1 Tat or Tat-induced factors protect B lymphoma cells from apopotosis.



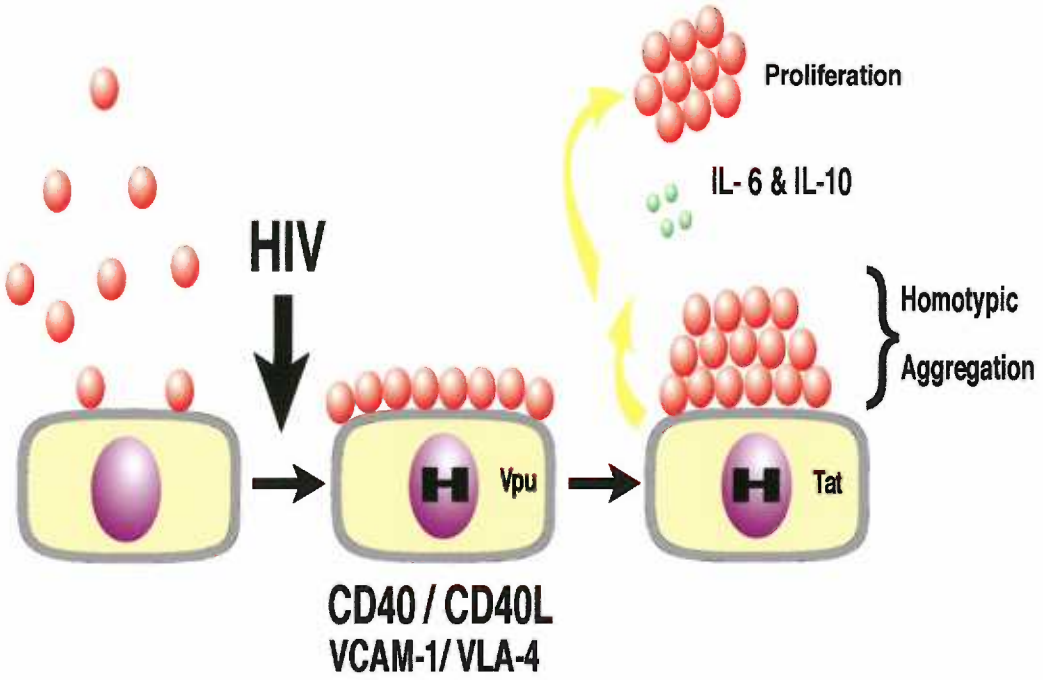
Apoptosis assays were performed on BL cells co-cultured with mock-infected EC and EC infected with Ad/tat or Ad/nef for 24 hrs. These cells were treated with camptothecin (4ug/ml CAM; Sigma) for 4 hrs prior to staining for apoptotic cells with an APO-BRDU apoptosis assay kit (Sigma). Briefly, cells were fixed in 1% paraformaldehyde incubated in 70% ethanol at -20°C. The cells were resuspended in a DNA labeling solution containing terminal deoxynucleotide transferase (TdT) enzyme and bromodeoxyuridine triphosphate (Br-dUTP) and stained with fluorescein-conjugated anti-BrdU antibody. Incorporation of Br-dUTP into the nicked chromosomal DNA was determined by FACS analysis. A minimum of 10,000 cells was analyzed for each sample.The percentage of apoptotic cells gated in R1 was compared: Ad/tat + CAM (27.34%), Ad/nef + CAM (57.35%), mock + CAM (40.39%), Ad/tat (1.16%), Ad/nef (3.00%) and mock (2.73%).

Figure 4.2 HIV-1 Vpu induces IL-1α production in HUVEC



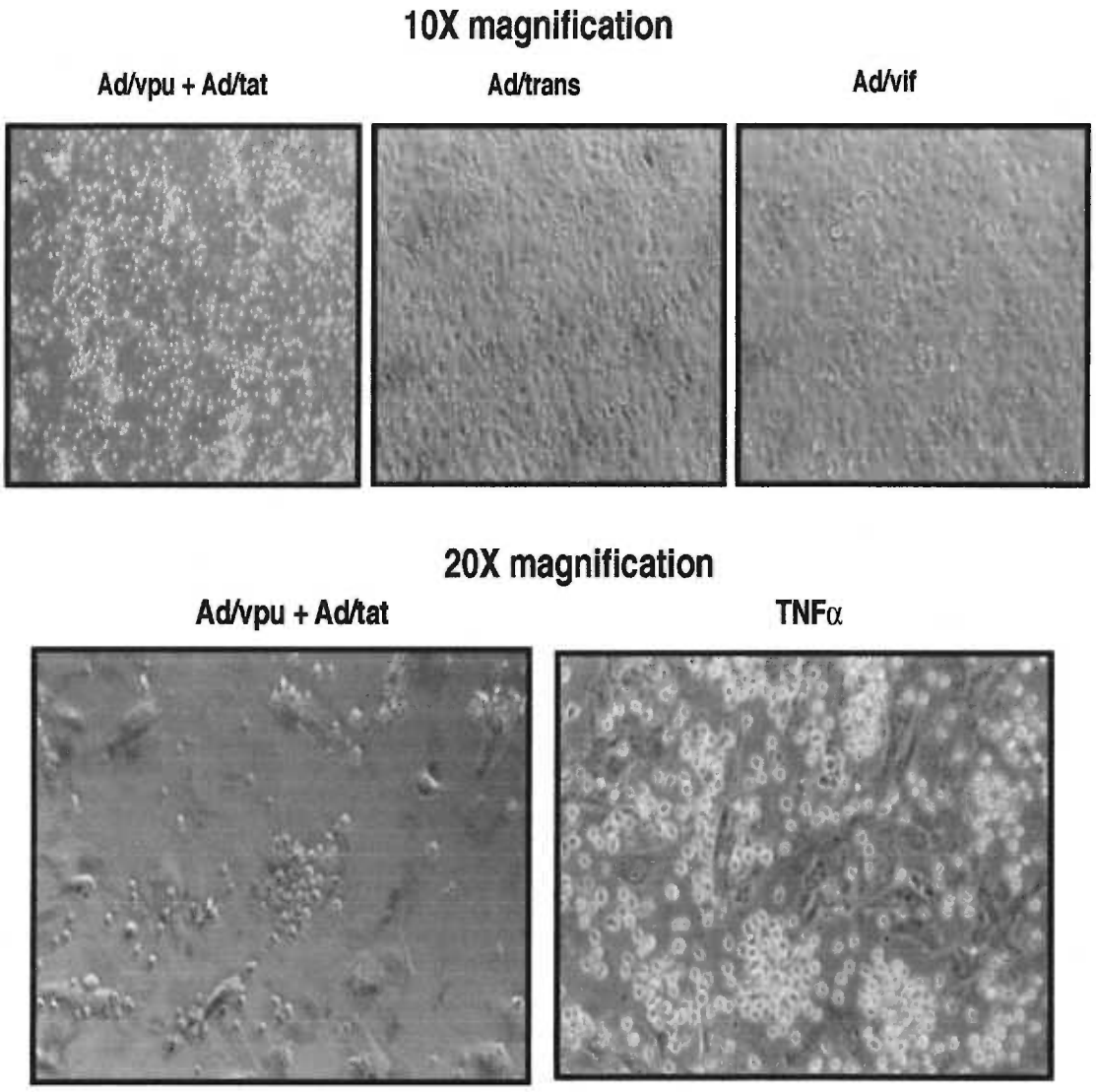
Intracellular cytokine staining (ICCS) for cytokine IL-1α. Intracellular cytokine staining protocol was performed as described for Figures 2.4 & 2.5. IL-1α was induced slightly by the expression of Vpu (pink) as compared to mock-infected cells (black). This Vpu-mediated IL-1α induction is specific for Vpu in that Tat (green) did not induce IL-1α production in HUVEC. Isotype-matched controls (blue) was included.

Figure 4.3 A model for functional roles of HIV proteins in B cell lymphomagenesis.



HIV-1 Vpu and Tat play an important role in B cell lymphomagenesis. In HIV-1 infection, the expression of Vpu leads to upregulation of CD40 on infected EC. Surface CD40 is available for ligation via the NGF/TNF-related cytokine CD40 ligand, which may be expressed by B lymphoma cells themselves, by adjacent macrophages and/or T cells, or may be present in soluble form. CD40 ligation mediates a subsequent upregulation of VCAM-1 on infected EC. VCAM-1 interacts with VLA-4 on the B lymphoma cell surface forming a tight endothelial cell to B cell adhesive interaction. HIV-1 Tat mediates lymphoma cell aggregation as well as lymphoma cell proliferation, with the eventual formation of B lymphoma cell foci in close association with the HIV-infected endothelium. Mechanisms regulating both EC-BL cell interactions and BL–BL cell interactions facilitate the formation of a multi-layered lymphoma aggregate attached to the vessel wall.

Figure 4.4 B lymphoma cells adhere to HUVEC expressing both Vpu and Tat.



Adhesion assays were performed as described previously. BL cells adhered to HUVEC expressing both HIV-1 vpu and tat (Ad/vpu + Ad/tat) but not to cells expressing transactivator (Ad/trans) or vif (Ad/vif). As seen previously, Ad/vpu-infected cells supported BL cells adhesion (Figure 3.6) while Ad/tat-infected cells facilitated BL cell aggregation (Figure 2.1), TNFα-treated cells were used as positive controls.

APPENDIX A: Adenovirus expression system

To identify the HIV gene(s) that confer the lymphoma support phenotype on endothelial cells, individual HIV regulatory and accessory genes were expressed using an adenovirus vector system. We have constructed adenoviruses expressing all of the individual accessory genes (*vpu, vif, nef, vpr*) and regulatory genes (*rev* and *tat*) for our gain of function studies.

Recombinant adenovirus vectors (see illustration 1 below) were constructed as previously described [Hitt, 1994, Streblow, *et al.*, 1999]. Each of the accessory or regulatory gene of HIV was amplified by polymerase chain reaction (PCR) from proviral plasmid pNL4-3 (AIDS Research and Reference Reagent Program) with the gene-specific forward primers with 5′ EcoRI site and reverse primers with 3′ XbaI site. Each PCR product was analyzed on agarose gel, purified and digested with restriction enzymes EcoRI and XbaI. The digested fragment was cloned into 5′ EcoRI and 3′ XbaI sites within the adenovirus shuttle vector pΔE1sp1Btet/EF1EGFPTKpolyA. This vector was derived from plasmid pΔE1sp1Btet as described previously by Streblow *et al.* (1999) with the addition of EF1-EGFP and herpes simplex virus thymidine kinase (HSV TK) poly (A). Plasmid pΔE1sp1Btet was originally cloned from pΔE1sp1B [Microbix Inc., Toronto, Ottawa, Canada] containing the tetracycline (tet)-responsive sequences, cytomegalovirus (CMV) minimal promoter and SV40 late poly (A) signal.

Using the calcium phosphate transfection method, the recombinant adenoviruses were produced by cotransfection of 293 cells with the shuttle plasmid, which contains the

various HIV genes and pJM17 (Microbix) that has an E1A-deleted adenovirus genome. The viruses were plaque purified and bulk virus stocks were titered on 293 cells by limiting dilution as well as plaque assay.

All adenovirus infection was performed with co-infection with an adenovirus Ad/trans expressing the tet off transactivator at equivalent, pre-optimized multiplicity of infection (MOI 100). Monolayers of HUVEC or MVEC were infected with different adenoviruses at an MOI 100 of Ad/trans and/or Ad/vpu, Ad/tat, Ad/vpr, Ad/vif, Ad/rev or Ad/nef in the presence of polybrene (2μg/ml hexadimethrine bromide; Sigma) for 6 hrs and incubated with fresh media for 24 to 48 hours. Adenovirus infection was confirmed by the detection of GFP in infected cells. This is followed by the determination of expression level and the functionality of the specific protein of interest.

The adenovirus expression system used in our experiments is a tet-off system (see illustration 2 below). The shuttle plasmid has the tetracycline responsive element (TRE), which is comprised of 7 copies of tetO (tet operator DNA sequence) upstream of a CMV minimal promoter. The Ad/trans virus is a tetracycline-controlled transactivator, which makes a hybrid protein (composed of a fusion between the Tet repressor [TetR] and VP16 activation domain of herpes simplex virus). TetR-VP16 fusion protein from the transactivator binds to tetO, allowing the transcription of the gene of interest, which is driven by a CMV minimal promoter. Therefore, in all the experiments involving adenovirus expressing HIV genes, adenovirus expressing tet off transactivator was used as control for the basal phenotype induced by adenovirus infection.

Illustration 1: Construction of adenovirus expressing HIV gene(s)

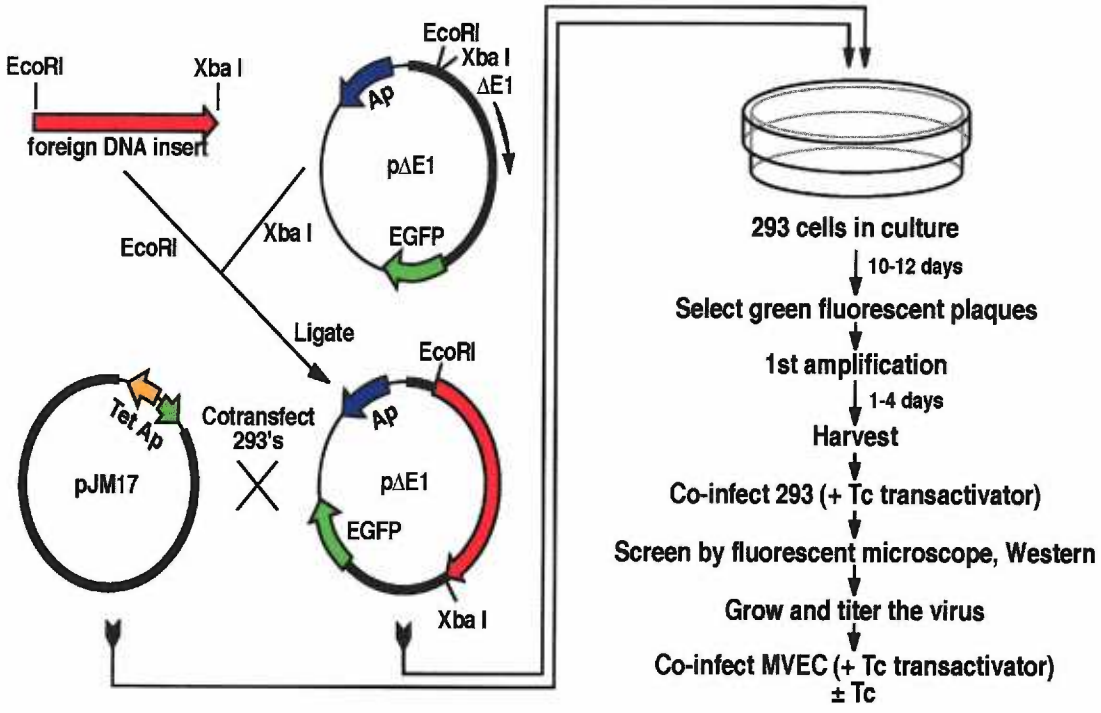
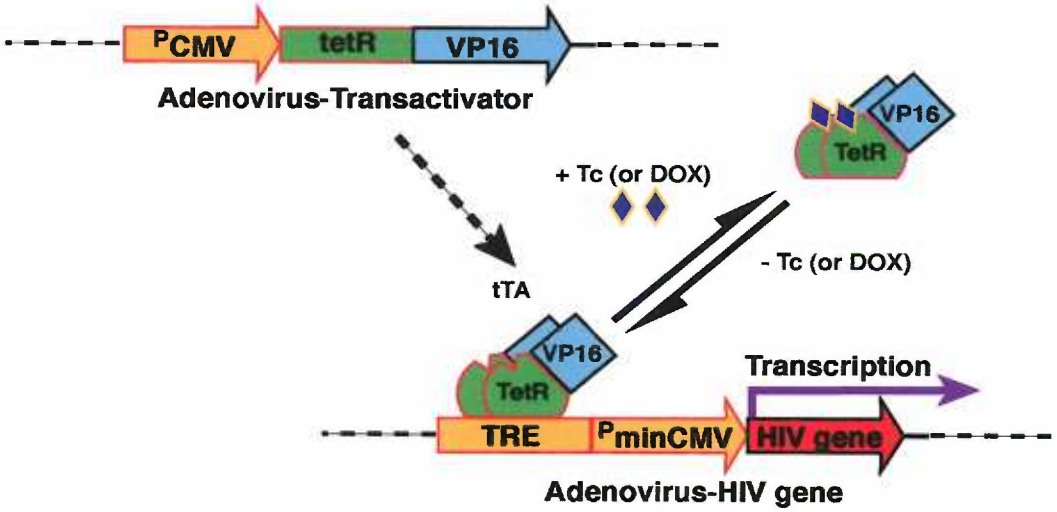


Illustration 2: "Tet off" adenovirus expression system



APPENDIX B: Construction of HIV/VSV-G pseudotype or gene-deleted HIV/VSV-G pseudotype viruses

The requirement for HIV-1 Vpu in mediating endothelial cell-B lymphoma cell adhesion was confirmed by infecting endothelial cells with a *vpu*-deleted HIV/VSV-G pseudotype virus. For this loss of function study, we used vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV constructs with deletions in the individual accessory genes (*vpu*, *vif*, *vpr*, *nef*) and one regulatory gene (*rev*). Since Tat is absolutely essential for viral gene expression and HIV replication, a *tat*-deletion mutant was not tested. The pseudotyped viruses are made by transfecting a pL-VSV-G plasmid and an HIV proviral plasmid with deletions in *env* plus additional HIV genes into mammalian cells. With this protocol, VSV-G proteins (transcribed from pL-VSV-G) are provided *in trans* and are incorporated into the virion envelope during viral packaging along with HIV proteins (transcribed from the HIV proviral plasmid). VSV-G has a wide host range, and can be subjected to ultracentrifugation and freeze-thaw cycles. Thus, HIV/VSV-G pseudotyped viruses allow efficient infection of endothelial cells with stable, high-titer viral stocks. Note that *rev*- and *vif*- deletion mutants are inefficient in viral replication and commonly have lower titers than other deletion mutants.

For the studies described in this thesis, recombinant HIV/VSV-G pseudotype viruses were constructed as described previously [Bartz, *et al.*, 1997]. Proviral plasmids with deletions in *env* and *vpu, vpr, vif, rev or nef* (pBru3ori^*env*, pME351Δ*vpu*, pME341Δ*vpr*, pME342Δ*vif*, pMAVIIΔ*rev* and pSFiΔev^{+}Δ*nef*) were obtained through the AIDS Research and Reference Reagent Program. Briefly, cell free viral stocks were obtained

from co-transfection of the 293T human embryonic kidney cell line (American Type Culture Collection) with proviral plasmids, pL-VSV-G plasmid and EGFP plasmid (as a transfection control) by the calcium phosphate transfection method. 293T cells were refed 12 hours after transfection. Virus stocks were harvested after 36 hours, concentrated by ultracentrifugation and titered by H-MAGI assay as described previously [Bartz, *et al.*, 1997, Vodicka, *et al.*, 1997]. Human MAGIs (H-MAGI) are HeLa-CD4 cells expressing β-galactosidase under the HIV-1 long terminal repeat (LTR). Briefly, H-MAGI cells (AIDS Research and Reference Reagent Program) grown on 24 well plates were infected with various dilutions of virus stock for 3 days, followed by fixing and staining for β-galactosidase. Cells that express HIV-Tat protein appear blue as a result of LTR-driven transactivation of β-galactosidase expression by Tat.

Pseudotyped-virus infection was performed by exposing HUVEC or MVEC to the individual titered viral inoculae at a multiplicity of infection (MOI) of 1 in the presence of polybrene (2μg/ml; Sigma) for 2 hours, followed by rinsing in HBSS and reculturing in endothelial growth medium. Pseudotyped-virus infection was confirmed by detection of p24 gag protein by immunofluorescence staining as previously described [Moses, *et al.*, 1993, Moses, *et al.*, 1997].

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