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Interactions of HIV with Cellular Processes and with Epstein-Barr Virus as Distinct Causes of HIV Pathogenesis

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Abstract of the Dissertation

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Antiretroviral therapy (ART) has transformed human immunodeficiency virus type I (HIV-1) infection from a rapidly terminal illness to a chronic yet manageable condition. Nevertheless, significant morbidities persist, such as metabolic abnormalities and herpesvirus-associated malignancies. The presented work explores features of these complications with the goal of enhancing the understanding of HIV pathogenesis.

HIV-1 infection is associated with metabolic abnormalities that include insulin resistance and Type II Diabetes Mellitus (DM). The HIV protein, Nef, downregulates cell surface expression of several proteins, alters signal transduction pathways, and interacts directly with the cytoskeleton and actin-polymerizing proteins. These cell functions are required for successful insulin signaling and the ensuing redistribution of Glucose Transporter 4 (GLUT4) from intracellular compartments to the adipocyte plasma membrane. We found that when adipocytes are exposed to Nef, insulin-stimulated glucose uptake is reduced. This reduction resulted from Nef-dependent inhibition of GLUT4 vesicle fusion with the plasma membrane, with a lesser effect on GLUT4 trafficking to the membrane. Nef treatment caused a decrease in Akt phosphorylation and dramatic alterations in cortical actin organization. We identify HIV Nef, which is detectable in sera of HIV-infected people, as a novel contributor to insulin resistance.

Though ART has minimized most opportunistic infections, herpesvirus infection, particularly Epstein-Barr Virus (EBV), is an important exception. EBV establishes a life-long reservoir in its human host and has growth-transforming properties. Owing to this, infection with EBV can result in malignancies that are particularly aggressive in HIV-infected people. We found significantly elevated EBV DNA levels in HIV-positive people. Immunodeficiency could not account for the higher EBV load as the cellular immune system in HIV-positive people responded robustly when challenged with EBV-encoded peptide antigens. The response was specific to EBV as tetanus toxoid elicited a cellular response that was similar to that of HIV-negative people. EBV replication may be up-regulated by the HIV accessory protein, Tat. Expression of EBV genes BZLF and gp350 was elevated after transfection of Tat in EBV-infected B-cells. There is an important relationship between HIV and EBV that likely impacts the development of EBV-associated cancers and progression of HIV disease.

Dedication

To all who are infected and affected by HIV
and
To all who have persevered and remained dedicated to ending this scourge

"The flower that follows the sun does so even on cloudy days"
~Robert Leighton (1611-1684)

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
BAFF	B-cell Activating Factor of the TNF-Family
BL	Burkitt's Lymphoma
CD	Cluster of Differentiation
CMV	Cytomegalovirus
DM	Diabetes Mellitus
EBNA	EBV Nuclear Antigen
EBV	Epstein-Barr Virus
GLUT4	Glucose Transporter 4
HD	Hodgkin's Disease
HDL	High-density Lipoprotein
HHV-8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LDL	Low-density Lipoprotein
LMP	Latent Membrane Protein
MHCI	Major Histocompatibility Complex class I
mTOR	Mammalian Target of Rapamycin
N-WASP	Neural Wiskott-Aldrich Syndrome Protein
NaByr	Sodium Butyrate
NHL	Non-Hodgkin's Lymphoma
PAK	p21-Associated Kinase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCNSL	Primary Central Nervous System Lymphoma
PCR	Polymerase Chain Reaction
PDK1	PI3K-dependent Kinase 1
PI3K	Phosphoinositide-3-Kinase
PPAR-γ	Peroxisome Proliferator-Activated Receptor-γ
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription PCR
SFU	Spot-Forming Unit
RICTOR	Rapamycin-Insensitive Companion of mTOR
SIV	Simian Immunodeficiency Virus
$T_H1/2$	Type 1/2 Helper T-cell
TNF	Tumor Necrosis Factor
vIL	viral Interleukin

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General Introduction

There are approximately 37 million people worldwide infected with Human Immunodeficiency Virus type 1 (HIV-1). Since the beginning of the epidemic almost 30 years ago, there have been an estimated 28 million AIDS-related deaths. In the most heavily affected countries, HIV infection has reduced life expectancy by more than 20 years (3). A striking 45% of all new HIV infections occur in young people aged 15-24 (4), and nearly a quarter of all infected people are unaware they are infected. HIV infection remains among the most devastating pandemics recorded in human history, not only because of the substantial human health impact, but also for the social, economical and political impacts as well.

Many years of meticulous research have provided a vast amount of knowledge regarding the virology and pathogenesis of HIV disease, and substantial progress has been made in the treatment of HIV with the development of and continued improvements on effective HIV therapies. Despite this progress, there is more to learn as neither a preventative nor therapeutic vaccine seems within close reach.

HIV Structure and Life Cycle

The human immunodeficiency virion contains two positive-sense single-stranded RNA molecules that each encode nine genes flanked by two Long Terminal repeat (LTR) segments (Fig. 1A). *Gag* encodes four structural proteins that comprise the viral capsid: p6, p7, p17, and p24. *Pol* encodes the three viral enzymes: Protease; Reverse Transcriptase; and Integrase. *Env* encodes two envelope proteins, gp120 and gp41, that act in concert to initiate infection of naïve cells. Six other regulatory genes—*vif, vpr, rev, vpu*, *nef*, and *tat*—play various roles in viral replication, protein translation and trafficking, and budding from the surface of the infected cell. The diminutive genome is enclosed within a capsid which is in turn wrapped in a lipid bilayer called the viral envelope (Fig. 1B). The envelope is studded with the viral transmembrane glycoprotein gp120 and its partner gp41. As HIV buds from its host cell, the viral envelope also acquires over 100 different host-cell proteins. This fact likely contributes to the difficulty in developing an effective vaccine.

For infection, gp120 binds to CD4 (Fig. 2), its primary receptor, but HIV also utilizes co-receptors in order to fuse with the cell membrane. These co-receptors are CCR5

and CXCR4, chemokine receptors that mainly function to promote lymphocyte extravasation from circulation to sites of inflammation. Once inside the cytoplasm of the newly infected cell, the RNA genome is reverse transcribed by Reverse Transcriptase (RT) into linear double-stranded DNA which is then imported into the nucleus where it integrates into host chromosomal DNA by the action of Integrase (Fig. 2). Cellular machinery transcribes HIV proviral DNA into single-stranded RNA which is exported out of the nucleus into the cytoplasm for translation. Here, viral assembly begins with the assistance of Protease, which cleaves the viral polyproteins into individual, functional proteins. Viral proteins are packaged and newly assembled virions bud from the cell membrane, incorporating the lipid bilayer of the host cell to form the virus's envelope (Fig. 2). Immune cells bearing the necessary receptors and co-receptors are then targeted for infection and the virus renews its life cycle.

HIV and the Immune System

The immune system is intimately and intricately linked to HIV pathogenesis. HIV productively infects immune cells, specifically cells that bear the cell-surface marker known as Cluster of Differentiation 4 (CD4). Cells that express CD4 on their surface include cells of the monocyte lineage (monocytes, macrophages, dendritic cells, Langerhan's cells and microglia) and T Helper lymphocytes (here after referred to as CD4 T-cells). HIV does not infect CD8-bearing T lymphocytes (also known as CD8+ T effector cells), nor does it productively infect B cells, but both cell types are affected by HIV, as discussed below.

Fundamentally, HIV infection leads to immunodeficiency by slow but substantial loss of CD4+ T lymphocytes, cells that are essential for coordinating immune responses to invading pathogens on many levels. This loss occurs by several mechanisms but the three major causes are apoptosis, the subversion of cell machinery needed for homeostasis, and cytotoxicity of HIV cDNA and proteins. A minimum number of circulating CD4+ cells are required to maintain immune integrity and when CD4+ T cells are depleted below this number (200 cells/μl blood), the host becomes susceptible to opportunistic pathogens including other viruses, fungi, parasites and bacteria.

Much of the notoriety of HIV infection is due to the immunodeficiency it causes, but HIV also paradoxically causes generalized chronic immune activation. During the natural course of most infections, including with HIV, immune activation is necessary to limit and clear the infection. Immune activation by viruses in general is typically short-term, but during infection with HIV-1, immune activation persists through most of the course of the disease. Immune activation and inflammation manifest in several different ways. For example, peripheral blood mononuclear cells (PBMC) isolated from HIV-positive individuals display elevated markers of activation and/or apoptosis, and produce excess amounts of cytokines such as IL-2, IL-4, IL-10, and interferon-γ (IFN-γ). Similarly, macrophages and B lymphocytes produce excess tumor necrosis factor-α (TNF-α), and B-cells are known to produce excess interleukin-6 (IL-6) (17, 19, 43, 121, 122). B-cells also produce excess immunoglobulins of all classes, but in particular IgG. This hypergammaglobulinemia occurs irrespective of whether HIV is detectable in accessible tissue (blood, lymph nodes) or not. Interestingly, the excess antibody produced after the initial stage of infection is largely HIV non-specific and also non-neutralizing (100, 117).

Another facet of abnormal immune activation concerns deregulation of distinct populations of CD4+ T-helper cells. The CD4+ T helper 1 (T_H1) subset of cells coordinates a cellular immune response to viruses and other intracellular pathogens and involves expansion and proliferation of CD8+ T cells and production of the cytokines IL-2, TNF-α and IFN-γ. The CD4+ T helper 2 (T_H2) subset coordinates the humoral immune response and involves B cell activation, antibody production and production of the cytokines IL-4, IL-5, and IL-10. The T_H1 and T_H2 responses each have components that counter the other so that when one system is activated the other is repressed. For example, during a T_H2 response, IL-10 production is increased to mediate B cell activation but also to suppress T_H1 activity. The abnormal immune activation occurring in HIV-positive people is skewed towards the T_H2 response at the expense of a suppressed but protective T_H1 response (167, 190). This can create a functional loss of effector T cells, thereby hampering viral clearance (18, 22).

Chronic immune activation affects the virus and immune system homeostasis. Hyperactivation enhances infectivity and up-regulates viral replication. For instance, immune activation results in the upregulation of CD4 as well as the chemokine receptor

CCR5, the very receptors HIV requires to infect naïve cells. HIV propagation is therefore facilitated by immune activation. Also, inflammatory cytokines such as TNF-α and IL-6 increase viral replication by various mechanisms (89, 90, 131, 139). A malevolent cycle is therefore established whereby HIV promotes immune activation and immune activation spurs HIV replication.

The body receives no benefit from chronic hyperactivation. Indeed, it is increasingly acknowledged that abnormal immune activation is a significant pathogenic process of HIV infection (10, 101, 157). The activation and deregulation debilitate host defense resources, resulting in a decreased ability to respond to HIV as well as compromising defense against a broad spectrum of other pathogens. In addition, chronic abnormal activation exhausts the regenerative capacity of the immune system, contributing to the loss of CD4+ cells and the eventual development of immunodeficiency. In fact, T cell activation is predictive of poor patient prognosis (55, 56) and the extent of activation directly correlates with progression to Acquired Immunodeficiency Syndrome (AIDS) (69).

Antiretroviral Therapy (ART)

At the beginning of the recognized HIV epidemic—1981 in the U.S.—there was a simple treatment goal: prevent opportunistic infections and prolong life. Therapy directly targeting the virus was urgently sought. Relief first came in 1987 when AZT, a nucleoside reverse transcriptase inhibitor, was approved. However, HIV replicates very rapidly: upwards of $10x10^9$ virions are released into extracellular fluid a day. In addition, reverse transcriptase has an incredibly high error rate as it lacks the editing function of mammalian DNA polymerase. RT misincorporates nucleotides at a frequency between 1/1700 and 1/4000. This is equivalent to approximately five to ten errors per HIV genome per round of replication (142, 149). Owing to its error-prone replicative capacity, the virus quickly generates quasi-subspecies that will undergo selective pressure to become resistant to ART. Those treated with AZT were afforded only short postponements from inevitable immune system collapse and death. Major success in HIV treatment was not realized until approximately 10 years later when Highly Active Antiretroviral Therapy (HAART) regimens became standard of care. HAART regimens relied on a combination of drugs that were active at different stages of the virus's life cycle simultaneously, a strategy employed

to combat the aptitude of HIV to generate drug-resistant mutants. HAART regimens were potent enough to drive HIV replication to undetectable levels in the blood for substantial amounts of time, thereby significantly improving and extending patients' lives. Nevertheless, early treatment courses were fairly toxic with significant side effects, a high pill burden and complex dosing schedules; patient compliance remained a significant issue. Since, considerable treatment advances have led to the development of more than 20 drugs falling into 5 classes (Table 1), each class targeting a different stage of the virus's life cycle (Fig. 2). Current ART regimens are effective, side effects are minimized and the number of pills required and their dosing schedules are streamlined. In developed societies where treatment is readily available, infection with HIV has been transformed from a rapidly terminal illness to a chronic yet manageable disease.

Metabolic Abnormalities

A significant number of HIV-infected people develop metabolic abnormalities. This heterogeneous collection of abnormalities is morphological and biochemical in nature:

- Generalized muscle wasting and increased resting energy expenditure.
- Lipodystrophy syndrome characterized by redistribution of adipose tissue due to adipose hypertrophy and atrophy.
- Dyslipidemia with increased triglycerides and low density lipoprotein (LDL) and decreased high density lipoprotein (HDL).
- Cardiovascular complications including atherosclerosis and coronary artery disease, likely linked to dyslipidemia.
- Bone density abnormalities including osteopenia, osteoporosis and osteonecrosis.
- Mitochondrial toxicities that can lead to anemia, myopathy, neuropathy, pancreatitis and hepatic steatosis.
- Failure to maintain glucose homeostasis with development of insulin resistance and Type II (non-insulin dependent) diabetes mellitus (DM).

Prior to successful treatment regimens, HIV-infected persons either died before metabolic disturbances developed or the problems that did arise were secondary to the primary issue of immunodeficiency. With the extensive use of ART and the extended life span it affords, previously neglected or even unrecognized metabolic derangements have

now assumed increasing importance in patient care. This is especially true for cardiovascular health, as cardiovascular events underlie greater than 10% of all deaths in the HIV-infected population, and cardiovascular disease consistently ranks within the top five leading causes of death in persons infected with HIV (156).

The clinical picture of metabolic aberrations is complex. Fittingly, the mechanisms underlying development of these issues are complex and multi-faceted. The role of ART in the development of HIV-associated metabolic alterations has been the subject of intense research (47, 72, 112, 124). Protease Inhibitors (PIs) and Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are the main culprits, each causing derangements in several different ways (76, 99). It is increasingly apparent, however, that HIV itself drives the development of metabolic abnormalities as well. In the pre-ART era, changes in resting energy expenditure, lipid levels, lipogenesis and triglyceride clearance were well-documented (61, 71, 74). More recent *in vivo* work has demonstrated altered gene expression in primary subcutaneous adipose tissue isolated from ART-naïve HIV-1-infected patients (57). An HIV-1 infection model of T cells has shown that expression of proteins involved in lipid synthesis, transport and metabolism is altered (146). Certain viral accessory proteins have also been implicated in the development of insulin resistance as well as with changes in cholesterol synthesis and storage (95, 125, 181).

HIV and other Chronic Viral Infections

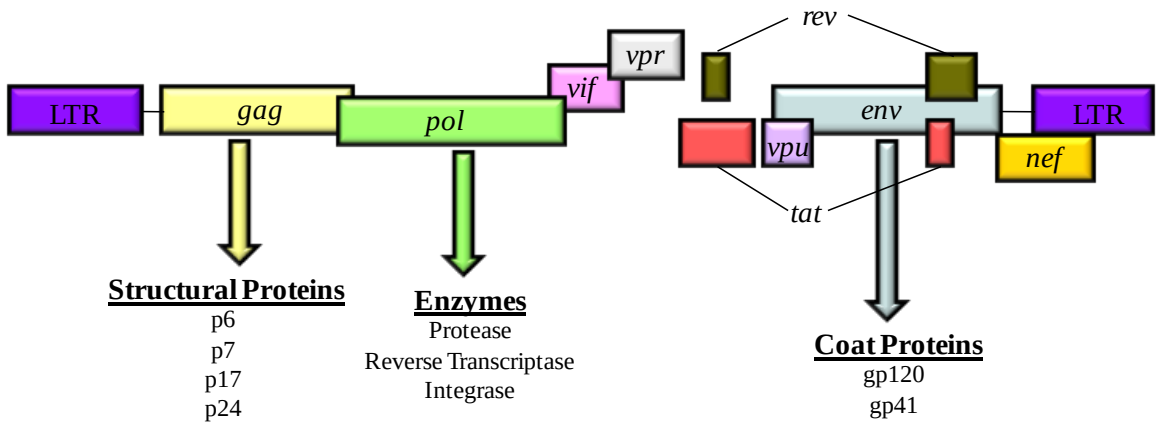
A persistent concern with HIV-infected patients is for infection with other pathogens, especially in the setting of immune decline. ART has minimized the incidence of most opportunistic infections, but there are still significant issues related to infection with members of the herpesvirus family. The herpesviruses comprise a large family of DNA viruses that infect both vertebrates and invertabrates. They are unique for their ability to establish life-long infections in their hosts. They possess a sophisticated genetic program that cloaks infected cells such that the cells remain undetected by the immune system, thereby allowing the virus to exist in a dormant state for the life of the host, called latency. There are eight recognized human herpesviruses. Their identities, target cells, and other characteristics including diseases and mode of transmission can be found in Table 2.

There are four herpesviruses that are of particular concern with regard to HIV infection (Table 3). Herpes simplex virus-2 increases the risk of acquiring HIV (48, 189), while cytomegalovirus (CMV), human herpesvirus-8 (HHV-8, or Kaposi's Sarcoma-associated virus), and Epstein-Barr virus (EBV) play an important role in HIV pathogenesis. For example, HIV-infected people with low CD4 levels have a substantially increased risk for developing serious CMV disease, and CMV is the most common viral opportunistic infection in patients with AIDS; before the ART era, up to 44% of people with AIDS suffered from CMV disease (113). CMV most commonly causes retinitis, but can also cause other central nervous system diseases as well as gastrointestinal diseases (Table 3), and is a particular problem in those who have been multiply transfused e.g. with clotting factor concentrates for hemophilia. HHV-8 causes Kaposi's Sarcoma (KS), a highly vascularized neoplasm of endothelial origin, typically involving the skin. KS occurs with up to 300-fold more frequency in the HIV-infected population (58) and is much more aggressive with involvement of the gastrointestinal and respiratory tracts (113). EBV is a ubiquitous pathogen with 95% seroprevalence in the United States. EBV is the causative agent of heterophile antibody-positive infectious mononucleosis (IM) and is associated with endemic Burkitt's lymphoma and nasopharyngeal carcinoma. Additionally, in HIV-infected people, immunoblastic and extranodal lymphomas are virtually always associated with EBV (7, 116). Not only do these malignancies occur with greater frequency among the HIV-positive population, they are also highly aggressive. These malignancies are comparatively insensitive to antiretroviral therapy (33).

General Discussion

Though ART has dramatically changed the course of HIV disease, both patients and clinicians must be alert to non-immune deficiency related issues associated with HIV infection, such as metabolic complications, abnormal immune activation and complications associated with infectious agents like the herpesviruses. These issues underscore the need for continued research into HIV pathogenesis and the effects the virus has on biological processes beyond immunodeficiency. The work presented here explores features of both metabolic abnormalities and herpesvirus infection to enhance the understanding of HIV morbidity and promote development of new and effective treatment modalities.

A.



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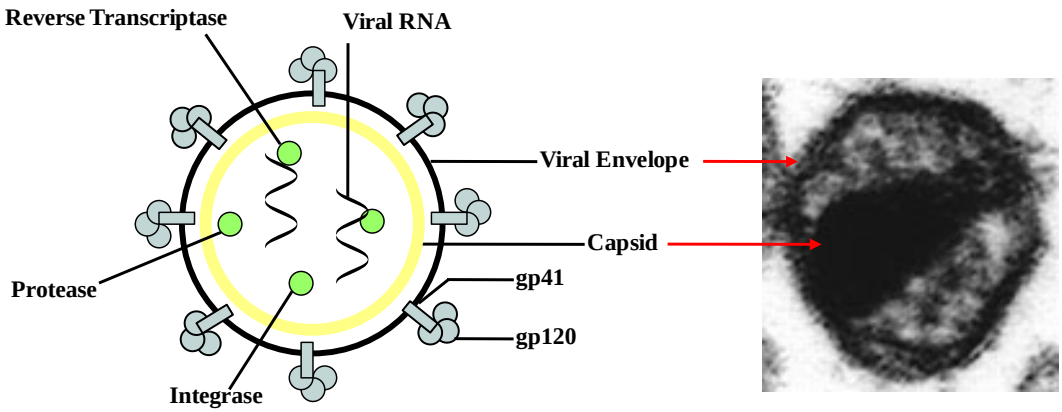


Figure 1. Schematic of HIV genome and virion. (A) The HIV genome is comprised of nine genes flanked by two long terminal repeat (LTR) regions. *Gag* encodes four structural proteins, *pol* encodes three enzymes, and *env* encodes two coat proteins. The six other accessory genes encode for proteins involved in the HIV life cycle. (B) The genome is enclosed by the viral capsid which is in turn wrapped in the viral envelope. The viral envelope is composed of a lipid bilayer, studded with glycoproteins gp41 and gp120. The electron micrograph of a mature HIV virion in (B) was adapted by permission from Macmillan Publishers Ltd: The EMBO Journal (188), © 1998.

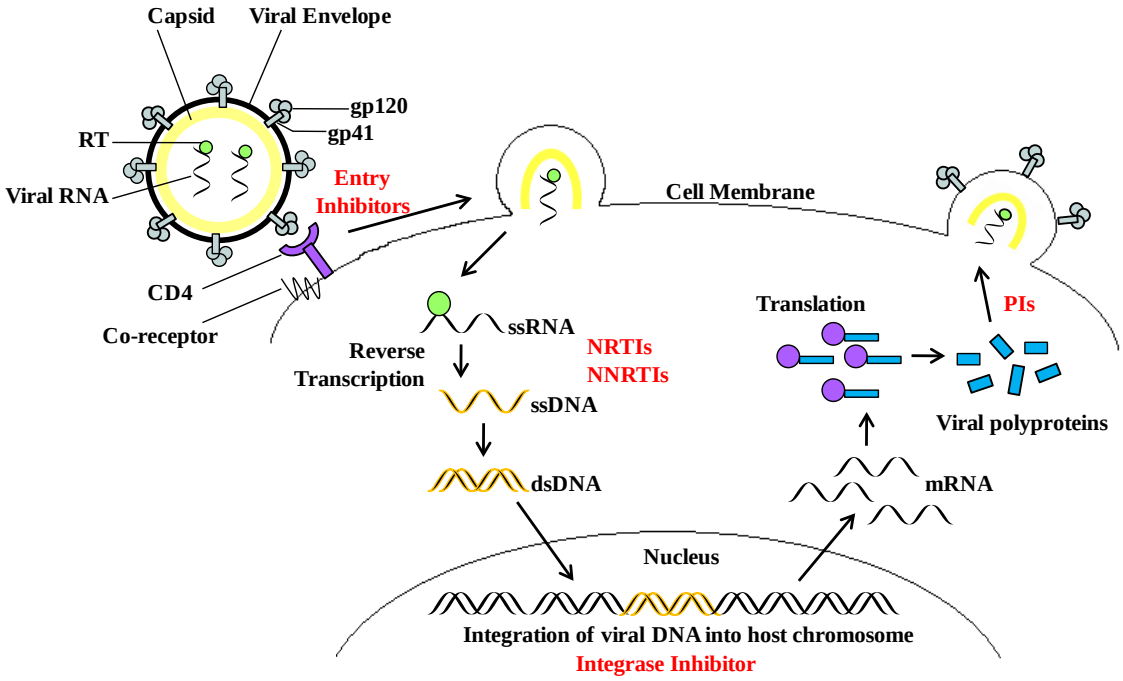


Figure 2. Schematic of HIV life cycle. Gp120 binds to the cell surface receptor CD4 and a co-receptor. The virion fuses with the membrane and releases its RNA genome into the cytoplasm for reverse transcription. Double-stranded viral DNA enters the nucleus where it integrates into host chromosomes. Cellular machinery then makes viral mRNA and in turn viral polyproteins. HIV Protease processes the polyproteins into distinct protein units. Viral proteins are then packaged and new virions bud from the cell surface. Antiretroviral drugs (shown in red) target different aspects of the viral life cycle including entry into the cell (Entry Inhibitors), reverse transcription (NRTIs and NNRTIs), integration (Integrase Inhibitor) and processing of viral proteins (PIs).

Table 1. Currently available antiretroviral drugs listed according to class.

NRTIs	NNRTIs	PIs	Entry Inhibitors	Integrase Inhibitor
Abacavir	Delavirdine	Atazanavir	Enfuvirtide	Raltegravir
Didanosine	Efavirenz	Darunavir	Maraviroc	
Emtricitabine	Etraviripine	Fosamprenavir		
Lamivudine	Nevirapine	Indinavir		
Stavudine		Lopinavir		
Tenofovir		Nelfinavir		
Zidovudine		Ritonavir		
		Saquinavir		
		Tipranavir		

NRTIs: Nucleoside Reverse Transcriptase Inhibitors; terminate the growing DNA strand
NNRTIs: Non-nucleoside Reverse Transcriptase Inhibitors; block the RT active site
PIs: Protease Inhibitors

Table 2. Features of recognized human herpesviruses.

Virus	Synonym	Primary Target Cell	Pathophysiology	Site of Latency	Mode of Transmission
HHV-1	Herpes simplex virus-1 (HSV-1)	Mucoepithelial	Oral/ genital herpes (predominantly oral) Encephalitis	Sensory neurons	Close contact*
HHV-2	Herpes simplex virus-2 (HSV-2)	Mucoepithelial	Oral/ genital herpes (predominantly genital)	Sensory neurons	Close contact
HHV-3	Varicella Zoster virus (VZV)	Mucoepithelial	Chickenpox and shingles	Sensory neurons	Close contact
HHV-4	Epstein-Barr Virus (EBV)	B cells	Infectious Mononucleosis, Burkitt's lymphoma$^{\alpha}$, Nasopharyngeal carcinoma, Post-transplantation lymphoproliferative disease$^{\alpha}$	B cells	Close contact, transfusion and transplantation
HHV-5	Cytomegalovirus (CMV)	Monocytes? Neutrophils?	CMV mononucleosis Retinitis Low birth weight, microcephaly, seizures, hearing loss, visual impairments, mental retardation$^{\beta}$	Monocytes? Neutrophils?	Close contact, transfusion and transplantation
HHV-6	Genus Roseolovirus	T cells?	Roseola Infantum	T cells?	Close contact
HHV-7	Genus Roseolovirus?	T cells?	Roseola Infantum?	T cells?	Close contact
HHV-8	Kaposi's Sarcoma-Associated herpesvirus (KSHV)	Lymphocytes and others	Kaposi's Sarcoma$^{\alpha}$, Multicentric Castleman's Disease	B cells	Close contact, transfusion and transplantation

*Close contact includes saliva, semen, breast milk, and other bodily fluids
$^{\alpha}$, Associated with immune suppression
$^{\beta}$, These complications result when infection occurs pre- or peri-natally
?, Inadequate data

Table 3. Pathophysiology of Herpesvirus infections in HIV-infected people.

HSV-2	EBV	CMV	HHV-8
Increases risk of acquiring HIV May also enhance infection of naïve cells	Increased incidence and severity of EBV-associated malignancies including BL[α], PTLD[β], HD[γ], and PCNSL[δ] Oral Hairy Leukoplakia	Retinitis Encephalitis Ventriculitis Myelitis Polyradiculopathy Esophageal ulcers CMV colitis Pancreatitis	Highly aggressive and potentially fatal Kaposi's Sarcoma (KS) Primary Effusion Lymphoma (PEL)

[α], BL, Burkitt's Lymphoma
[β]; PTLD, Post-transplantation Lymphoproliferative Disease
[γ]; HD, Hodgkin's disease
[δ]; PCNSL, Primary Central Nervous System (CNS) Lymphoma

Chapter 1: Nef Inhibits Glucose Uptake in Adipocytes and Can Account for the Insulin Resistance Caused by Human Immunodeficiency Virus Type-I

Introduction

Substantial strides have been made in the treatment of HIV-1 disease with the advancements of antiretroviral therapy (ART); both the quality and longevity of life for those infected with HIV-1 have significantly improved. However, a considerable number of patients on ART develop metabolic abnormalities including insulin resistance and Type II (non-insulin-dependent) diabetes mellitus (DM). The correlation between ART and metabolic abnormalities has been the subject of intense research (72, 76, 112).

Antiretroviral drugs cannot explain the complete clinical picture of metabolic derangements as alterations in metabolism were documented before the ART era when patients were not on therapy. For example, resting energy expenditure as well as fat-oxidation rates were higher in a group of asymptomatic HIV-infected patients relative to HIV-negative controls matched for age and body composition (74). Hellerstein *et al.* found that de novo hepatic lipogenesis was increased 3- to 4-fold in asymptomatic HIV-infected patients compared to HIV-negative controls (71). Serum triglyceride concentrations were also elevated in the HIV-positive cohort and corresponded in magnitude to degree of lipogenesis (71). Another study found that plasma triglyceride levels were significantly higher in HIV-positive patients and that rates of triglyceride clearance were slower in the patients when compared to HIV-negative controls (61). In addition, serum High-density Lipoprotein (HDL) cholesterol levels were significantly lower in HIV-positive patients (61). That these studies were conducted with ART-naïve patients suggests that HIV-1 itself drives the development of these abnormalities as well. Indeed, El-Sadr *et al.* uncovered various direct associations between markers of HIV disease and lipid profiles in HIV-positive patients who were naïve to antiretroviral therapy. For example, lower CD4 lymphocyte counts and higher HIV RNA (viral load) were each independently associated with higher serum cholesterol (42). Giralt *et al.* found that gene expression in primary subcutaneous adipose tissue is altered in ART-naïve HIV-1-infected patients; the amounts of mRNA for adipocyte metabolic markers PPAR-γ, GLUT4, and lipoprotein lipase were all significantly decreased below those of HIV-negative controls (57). The mRNA levels of markers denoting mitochondrial

function were also significantly altered in HIV-infected people (57). Rasheed *et al.* recently demonstrated that HIV replication has a striking influence on protein expression along pathways involved in lipid metabolism *in vitro*. The authors were able to study genome-wide changes in the proteome without confounding variables (i.e. in the absence of antiretroviral drugs or human genetic and epigenetic factors) because they utilized a human T cell line and a laboratory clone of HIV. A few examples of genes whose expression was altered post- HIV-infection are LDL receptor 1 and apolipoprotein-B100 —both dramatically upregulated—and apolipoprotein-A1 which was synthesized *de novo* only after infection (146).

Though much of the research into the effects of HIV on metabolism has focused on lipids, HIV also contributes to derangements in glucose homeostasis. HIV infection is associated with increases of at least 2-fold in the relative risk of developing Type II DM (23), and the incidence of disorders of glucose metabolism in the HIV-infected population is estimated to be between 2 and 25% (159). In addition, higher CD4 cell counts in ART-naïve HIV-positive people are associated with lower blood insulin concentrations, suggesting that as HIV disease advances and CD4 lymphocyte counts decline, glucose homeostasis will not be maintained (42).

Regulation of blood glucose begins with the release of insulin from pancreatic beta cells when blood glucose is elevated post-prandially. Insulin stimulates muscle and adipose tissue to take in glucose for storage and also blocks the liver from releasing glucose into circulation. Glucose uptake in response to insulin is crucial for maintaining blood glucose and, ultimately, metabolic homeostasis. Simply put, insulin resistance occurs when insulin fails to stimulate sufficient glucose uptake by muscle and adipose tissue, and fails to fully prevent glucose release from the liver. Hyperglycemia results with a compensatory increase in insulin production by the pancreatic beta cells. Chronically elevated insulin can compensate for the unresponsiveness of the tissue for only a short period of time, however, before the balance between beta cell function and peripheral insulin resistance gives out, finally resulting in the development of Type II DM.

The molecular mechanisms underlying insulin resistance are complex. Normally, insulin binds to the insulin receptor expressed on the surface of myocytes and adipocytes

(Fig. 3). This induces a complex signal transduction cascade that involves, among many proteins, phosphoinositol-3-kinase (PI3K), PI3K-dependent kinase 1 (PDK1), and mammalian target of rapamycin/ rapamycin-insensitive companion of mTOR (mTOR/RICTOR) (Fig. 3). This cascade results in activation of downstream protein kinase B, also known as Akt. Activated Akt then spurs rapid translocation of vesicles containing glucose transporter 4 (GLUT4), the major insulin-responsive glucose transporter, from intracellular storage compartments to the plasma membrane (Fig. 3). Dynamic changes of the adipocyte cortical actin meshwork, induced by insulin-stimulated recruitment of Neural Wiskott-Aldrich Syndrome Protein (N-WASP), causes fusion of the GLUT4 vesicle with the plasma membrane (Fig. 3). Glucose influx into the cell then occurs. Insulin resistance is, in part, due to reduced expression of the insulin receptor on the cell surface, as well as to reduced activation of the signaling cascade needed to mobilize GLUT4 to the cell membrane.

The contributions HIV makes to metabolic abnormalities, including insulin resistance, are poorly elucidated. Some studies have implicated viral accessory proteins. For example, Vpr was shown to counteract the insulin-induced negative regulation of a transcription factor, FoxO3a, thereby potentially contributing to insulin resistance (95). In a model of HIV infection consisting of transformed T-lymphocytes, the viral accessory protein Nef was shown to induce expression of genes involved in cholesterol biosynthesis and uptake (181), and Nef increases the biosynthesis of cholesterol as well (197). In addition, Mujawar *et al.* demonstrated that Nef impairs cholesterol efflux from macrophages such that the lipid-laden macrophages resemble foam cells characteristic of atherosclerotic lesions (125). The authors speculated that HIV, via Nef and its effects on cholesterol transport, contributes to the development of atherosclerosis. In support of their hypothesis, HIV-infected cholesterol-loaded macrophages were identified within biopsies of atherosclerotic plaques (125). Nef has also been implicated in AIDS-associated wasting syndrome. Otake *et al.* showed that Nef within the nucleus of T-cells and macrophages suppressed expression of PPAR-γ, a transcription factor required for adipocyte differentiation and glucose metabolism (133).

HIV-1 Nef (Fig. 4) is a 27 kDa non-structural protein, encoded by a small gene overlapping the 3’ long-terminal repeat region (Fig. 1A). Nef plays a critical role in viral

replication. Using mitogen-stimulated primary CD4 T-cells, Spina *et el.* demonstrated that, relative to wild-type virus, a Nef-deleted strain of HIV had a severely reduced capacity to replicate (169). More recently, it was shown in primary dendritic cell/T-cell co-cultures that replication of any of three different HIV clones, each with deletions within the Nef gene, was significantly impaired relative to wild-type virus (137). Nef-mutant viruses were also shown to have a 7- to 14-fold reduction in infectivity toward a T-lymphoid cell line (35). One way Nef enhances infectivity is by altering the composition of lipid rafts, microdomains in the cell membrane from which HIV virions bud (24, 197). Nef also significantly contributes to the pathogenesis of HIV infection. For example, some people infected with HIV progress to AIDS much more slowly than most others. These so-called long-term non-progressors—who maintain low viral loads as well as normal and stable CD4 lymphocyte counts for more than 10 years—often harbor Nef-defective virus strains (40, 96, 158). Conversely, Nef was shown to be required for the maintenance of high viral loads and progression to AIDS in rhesus monkeys (92, 174). These effects may be due to the vital role Nef plays in viral replication and infectivity. Nef also contributes to pathogenesis by dampening the immune response towards the virus. As will be discussed below, Nef down-regulates the cell-surface expression of CD4 and Major Histocompatibility Complex class I (MHCI), both of which are needed for T-cell activation. Nef also interferes with production of antibodies by B-cells (143, 192), thereby perturbing humoral immunity.

Interestingly, Nef has no known enzymatic activity, but modulates a striking variety of cellular processes through protein-protein interactions (Fig. 4; Table 4). Its suppression of the cell surface expression of CD4 and MHCI molecules (71, 151) is accomplished by interaction with proteins that regulate protein trafficking, though by different mechanisms. Nef primarily prohibits MHCI from exiting to the cell surface by rerouting MHCI, via interaction with AP-1, from the Trans-Golgi Network to lysosomes. It induces rapid internalization of CD4 *from* the surface by inducing AP-2/ clathrin-mediated endocytosis (26, 32, 87, 152). Nef also alters signal-transduction pathways by altering phosphorylation patterns and localization of key signal transducing proteins (147). For example, Haller *et al.* demonstrated that Nef decreases N-WASP phosphorylation (and therefore N-WASP activity) as well as recruitment of N-WASP to

the cortical actin of stimulated T-cells (63). Nef interacts directly with components of the actin cytoskeleton (28, 44) as well as proteins involved in actin polymerization, such as p21-associated kinase (PAK) (12, 63). Though Nef mainly localizes to the cytoplasmic side of the plasma membrane of an infected cell, it can be detected in the extracellular environment (49, 111) and is measurable in the sera of HIV-infected people (49). Extracellular Nef remains functional. For example, it was shown to induce HIV replication in infected T-cells (50), and also induce migration of monocytes in a concentration-dependent manner in an *in vitro* migration assay (107). Moreover, Nef has been shown to enter dendritic cells and B cells, and in both cases influences cell function once inside (143, 144, 192).

Protein trafficking, signal transduction, cytoskeletal structure and actin polymerization are all essential components of insulin-stimulated glucose uptake in adipocytes. Given that HIV-1 Nef can interact with proteins within each of these components, we hypothesized that HIV-1, via Nef, alters adipocyte function with regard to insulin action, glucose uptake and glucose homeostasis, thereby making a direct contribution to the development of insulin resistance in HIV-infected people.

Experimental Procedures

Cell Culture Conditions

3T3L1 pre-adipocytes (ATCC) were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 25mM glucose,10% bovine calf serum (BCS), 1% penicillin/streptomycin and 1% L-glutamine at 37 °C with 5% CO_2. Two days past confluence, cells were differentiated into adipocytes with DMEM containing 10% fetal bovine serum (FBS) and the above supplements, plus 1µg/ml insulin (Sigma-Aldrich), 1µM dexamethasone (Sigma-Aldrich), and 0.5mM isobutyl-1-methylxanthine (Sigma-Aldrich). Medium was replaced with DMEM containing 10% FBS and 1µg/ml insulin on the 4^{th} day of differentiation. Before experiments, cells were treated overnight with 0.01µg/ml or 0.1µg/ml recombinant SIV Nef, or with 0.1µg/ml recombinant HIV-1 Nef. Cells were then serum-starved in DMEM for 2-3 hours before stimulating with insulin and continuing.

Glucose Uptake Assay

Differentiated adipocytes were plated onto 24-well culture plates, and treated as described above. Cells were washed twice with Krebs-Ringer bicarbonate buffer supplemented with 30mM HEPES, pH 7.4, and 0.1% Bovine Serum Albumin (KRBH/BSA). The cells were then incubated in KRBH/BSA for 30 minutes. Two wells per treatment were incubated in KRBH/BSA/0.4mM cytochalasin B (Sigma-Aldrich) to determine non-specific glucose uptake. Cells were left unstimulated or stimulated with 10nM insulin for 20 min. The assay was initiated by the addition of 2-deoxyglucose-D-[1-^{3}H] glucose (1µCi per well; Amersham Biosciences) and 0.1mM 2-deoxy glucose. The assay was terminated after 10 minutes by adding PBS supplemented with 10mM glucose and 0.2mM cytochalasin B. Cells were washed three times with ice-cold PBS/10mM glucose. Cell lysates were collected in 0.05N NaOH/1% Triton X-100 and the protein concentration in each was determined using the BCA protein assay (Pierce). Cell-associated radioactivity (expressed as dpm) was normalized to the corresponding protein concentration of each sample, and the rate of glucose uptake in nmol/min/pg protein was calculated.

Transient GLUT4 Transfection and Immunofluorescence

Adipocytes were transfected with a pcDNA3-Myc-GLUT4-GFP plasmid. Briefly, this construct was prepared as follows: the DNA sequence encoding the myc epitope was inserted into GLUT4 cDNA by site-directed mutagenesis (82). GLUT4 cDNA was cloned into a pEGFP vector to generate a carboxyl-terminal green fluorescent protein (GFP) fusion protein. This was subsequently cloned into a pcDNA3 vector (178). Cells were electroporated using the Gene Pulser II (Bio-Rad) with settings of 0.16 kV and 950 microfarads. Following electroporation, cells were plated on glass cover slips and allowed to recover for 12-16 hours. Cells were treated with Nef as described, then left unstimulated or stimulated with 1nM, 10nM or 100nM insulin for 20 min. Adipocytes were fixed for 10 min in 4% paraformaldehyde and blocked in 5% donkey serum (Sigma-Aldrich) containing 1% BSA (Sigma-Aldrich) for 1 hour at room temperature. Cells were probed with c-Myc epitope 9E10 monoclonal antibody (Santa Cruz Biotechnology, Inc.) and stained with a Texas Red-conjugated donkey anti-mouse IgG antibody (Jackson Immunoresearch Laboratories) prior to mounting with Vectashield (Vector Labs). Fluorescence images were acquired by confocal fluorescence microscopy and fluorescence intensity measurements were performed using Zeiss LSM510 software. The degree of GLUT4 fusion with the plasma membrane was quantified by measuring Texas Red fluorescence at the plasma membrane and dividing by the GFP signal of the whole cell (Fig. 5). The degree of GLUT4 translocation to the plasma membrane was quantified by measuring the GFP signal at the plasma membrane and dividing by the total GFP signal of the cell (Fig. 5). At least 10 cells per condition were analyzed from each of three independent experiments.

Actin Polymerization Assay

Adipocytes were treated as described under *Cell Culture Conditions*, except for one control group in which 20µM Latrunculin B (LatB; Sigma-Aldrich) was added during serum starvation. Cells were then stimulated with 10nM insulin for 20 min, or left unstimulated. Cells were fixed as above and stained with rhodamine-conjugated phalloidin (Invitrogen). All samples were mounted on glass slides with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs). Fluorescent images

were obtained by confocal fluorescence microscopy. Fifty rhodamine-stained cells per condition were scored positive or negative based on the detection of the circumferential filamentous (F)-actin ring about the plasma membrane, called the cortical actin ring. Cells with a smooth rim around the membrane, indicating an intact cortical actin ring, were scored negative. Cells were scored positive if the fluorescence around the plasma membrane was discontinuous (patchy) indicating disruption of the actin ring. Three independent experiments were performed.

Immunoblotting

Adipocytes were stimulated with 0nM, 10nM, or 100nM insulin for 7 min. Cells were washed with ice-cold PBS and collected in ice-cold RIPA buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich) and Phosphatase Inhibitor Cocktail I (Sigma-Aldrich). After rotation at 4° for 10 minutes, lysates were clarified at 15,000 rpm for 15 min at 4°C. The protein concentration of each lysate was quantified using the BCA protein assay. Equal amounts of protein from each sample were resolved by Sodium Dodecyl Sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluordide (PVDF) membranes. Membranes were then subjected to immunoblotting with one of the following antibodies, where indicated: monoclonal phospho-Akt (Thr-308), monoclonal phospho-Akt (Ser-473) (Cell Signal Technology), polyclonal phospho-N-WASP (Tyr-256), or polyclonal phospho-N-WASP (Ser-484, Ser-485) (ECM Biosciences). Membranes were visualized with SuperSignal West Chemiluminescent Substrate (Pierce). Where indicated, membranes were stripped and re-probed with polyclonal rabbit antibodies to total Akt (Cell Signaling Technology) or to total N-WASP (ECM Biosciences), where indicated. Each immunoblot was performed independently at least two times. Densitometric analysis was performed using Gel-Pro Analyzer software version 4.0. Maximum optical density (OD) for phosho-Akt bands was divided by the maximum OD for the corresponding total Akt bands.

Data Analysis

Significance values of all experiments were calculated by the two-tailed Paired-Samples T-Test, unless otherwise noted. Statistical tests were performed using SPSS version 12.

Results

Nef inhibits insulin-stimulated glucose uptake in 3T3L1 cultured adipocytes. Insulin-stimulated glucose uptake in skeletal muscle and adipocytes is required for maintaining post-prandial blood glucose homeostasis. We measured glucose uptake in response to 10nM insulin in differentiated 3T3L1 adipocytes treated with 0.1µg/ml HIV Nef. In the absence of insulin stimulation, there was no difference in the mean basal glucose uptake between control cells and HIV Nef-treated cells (Fig.7A; $p= 0.2$) indicating that exposure to Nef did not affect basal metabolic processes. Upon stimulation with 10nM insulin, the mean rate of glucose uptake in HIV Nef-treated cells was significantly reduced relative to control cells (Fig. 7A; $p<0.02$). Control adipocytes displayed an approximate 6-fold increase in glucose uptake after insulin stimulation compared to unstimulated cells (Fig. 7B). In contrast, following HIV Nef-treatment, insulin increased glucose uptake by only 3-fold relative to basal uptake (Fig. 7B). Thus, treatment of adipocytes with HIV Nef resulted in a significant reduction of insulin-stimulated glucose uptake of approximately 50% relative to control cells (Fig. 7B; $p< 0.02$). Exposure to HIV Nef did not affect the viability of the adipocytes, as the proportions of control cells and Nef-treated cells that were viable was the same (80%) as determined by Trypan Blue exclusion.

Insulin-stimulated glucose uptake in adipocytes treated with 0.1µg/ml SIV Nef was also evaluated. In the absence of insulin stimulation, the mean basal glucose uptake between control cells and SIV Nef-treated cells was similar (3.9nmol glucose/min/mg protein vs. 4.8nmol glucose/min/mg protein, respectively), indicating that as for HIV Nef, SIV Nef did not interfere with basal metabolic processes. Upon stimulation with 10nM insulin, the mean rate of glucose uptake in SIV Nef-treated cells was reduced relative to control cells (28.1 nmol glucose/min/mg protein vs. 31.3nmol glucose/min/mg protein, respectively). While control adipocytes displayed an approximate 8-fold increase in glucose uptake after insulin stimulation, SIV Nef-treated cells increased glucose uptake by only 5.8-fold. Thus, treatment of adipocytes with SIV Nef reduced insulin-stimulated glucose uptake by approximately 30% relative to control cells (Fig. 7C).

SIV Nef inhibits GLUT4 fusion with the plasma membrane in insulin-stimulated adipocytes. Glucose Transporter 4 (GLUT4) is the primary insulin-responsive glucose transporter expressed in adipocytes. In resting cells, GLUT4 accumulates in intracellular compartments, but is rapidly translocated from these sites to the plasma membrane upon insulin stimulation. Subsequent integration with the plasma membrane allows glucose influx to occur. Since Nef has been associated with alterations in cell-surface expression of other proteins, we sought to determine whether the decrease in glucose uptake by Nef-treated cells was due to a decrease in GLUT4 surface expression upon insulin stimulation. Differentiated adipocytes were transiently transfected with a Myc-GLUT4-GFP plasmid and then stimulated with insulin as described in Methods. Staining the exofacial myc tag of unpermeabilized cells permits a sensitive and quantitative measurement of the amount of GLUT4 integration into the plasma membrane after insulin stimulation, as only integrated GLUT4 will be detected (Fig. 6).

Little or no GLUT4 was inserted into the plasma membranes of unstimulated control cells as evidenced by the lack of red fluorescence at the plasma membrane (Fig. 8A, left panel). After stimulation of control adipocytes with 1nM, 10nM or 100nM insulin, there was integration of GLUT4 with the plasma membrane, as evidenced by the bright red fluorescent signal at the cell membrane (Fig. 8C, panels 1, 7 and 13). The signal is dose-responsive with respect to insulin, appearing smoother and brighter with each increase (Fig. 8C, panel 1 vs. 7 vs. 13), and upon quantification, indicating more GLUT4 integration with the membrane.

Unstimulated adipocytes treated with either 0.01µg/ml or 0.1µg/ml SIV Nef appeared similar to control cells, with very little surface expression of GLUT4 (Fig. 8A, middle and right panel, respectively). Without insulin stimulation, there was no significant difference in the mean red-fluorescence intensity between control cells and cells treated with either 0.01µg/ml or 0.1µg/ml SIV Nef (Fig. 8B and 8D; Table 5).

Adipocytes treated with 0.01µg/ml SIV Nef and stimulated with 1nM, 10nM or 100nM insulin did not differ from control cells treated similarly (Fig. 8B). Thus, with 0.01µg/ml SIV Nef, there was no significant difference between the mean red-fluorescence intensity at the plasma membranes of control cells and the mean membrane fluorescence of cells stimulated with any of the 3 insulin concentrations tested (Fig. 8C;

Table 5). Nonetheless, the mean fluorescence intensity of Nef-treated cells after insulin stimulation is consistently less than that of control cells (Fig. 8B). This suggests that Nef begins to inhibit insulin-stimulated GLUT4 integration with the plasma membrane even at 0.01 µg/ml SIV Nef.

Adipocytes treated with SIV Nef at 0.1µg/ml and stimulated with 1nM insulin were also similar in appearance to control cells (Fig. 8C, panel 1 vs. 4). Quantitatively, there was no significant difference in the mean fluorescence intensity between control cells and cells treated with 0.1µg/ml SIV Nef stimulated with 1nM insulin (Fig. 8D; Table #). But when stimulated with 10nM or with 100nM insulin, adipocytes looked markedly different from control cells. GLUT4 integration was substantially less and appeared more punctate (Fig. 8C, panel 7 vs. 10, and panel 13 vs. 16). At higher insulin concentrations there was a significant reduction in the mean fluorescence intensity (Table 5) at the plasma membranes of cells stimulated with 10nM insulin (Fig. 8D; 32% reduction in mean florescence intensity) and with 100nM insulin (Fig. 8D; 34% reduction). These experiments indicate that 0.1µg/ml SIV Nef inhibits insulin-induced GLUT4 fusion with the plasma membrane.

HIV Nef inhibits GLUT4 fusion with the plasma membrane in insulin-stimulated adipocytes. We performed the same analysis with HIV Nef at 0.1µg/ml and 10nM or 100nM insulin, as these concentrations were affected by SIV Nef relative to control cells. Without insulin stimulation, little GLUT4 was inserted into the plasma membranes (Fig. 9A), and there was no significant difference in the mean red-fluorescence intensity between control and HIV Nef-treated cells (Fig. 9C; $p= 0.2$). After stimulation with 10nM insulin or 100nM insulin (Fig. 9B, panels 1 and 7, respectively), there was substantial integration of GLUT4 with the plasma membranes of control cells. In contrast, the insertion of GLUT4 into the plasma membranes of HIV Nef-treated cells was markedly less and appeared more punctate than in control cells at the corresponding insulin concentration (Fig. 9B, panels 4 and 10, respectively). There was a significant reduction in the mean red-fluorescence intensity at the plasma membranes of HIV Nef-treated cells upon stimulation with 10nM (Fig. 9C; 32% relative to control cells), or 100nM insulin (64% reduction, Fig. 9C). These data are comparable to those for SIV Nef

and indicate that HIV Nef inhibits insulin-induced GLUT4 fusion with the plasma membrane.

As Nef was added directly to adipocyte culture media, we wanted to ensure that Nef did not interfere with the binding of the myc antibody to its target, generating a false reduction in fluorescence intensity. To address this concern, cells were serum-starved, stimulated with 10nM insulin and fixed as described, without overnight incubation with the Nef protein. The myc antibody was then mixed with 0.1µg/ml HIV Nef prior to cell labeling. There was no significant difference in the fluorescence intensity between control cells and cells probed with the myc antibody/ Nef mixture ($p= 0.27$, by Wilcoxon Signed Ranks test), indicating that exogenous HIV Nef did not interfere with antibody recognition of the myc epitope.

Nef does not substantially inhibit insulin-stimulated GLUT4 translocation in adipocytes. A decrease in the insertion of GLUT4 into the plasma membrane when Nef is present may be due to a decrease in the amount of GLUT4 reaching the plasma membrane from intracellular storage sites after insulin stimulation. We took advantage of the GFP tag on the GLUT4 molecule to quantify GLUT4 translocation to the plasma membrane in control and Nef-treated cells after insulin stimulation.

As expected, there was little or no GLUT4 translocation to the plasma membranes of control cells without insulin stimulation, with most GLUT4 occupying a perinuclear space (Fig. 8A, left panel). Unstimulated adipocytes treated with 0.01µg/ml or 0.1µg/ml SIV Nef appeared similar, with significant intracellular accumulation of GLUT4 but little to no translocation to the periphery (Fig. 8A, middle and right panels, respectively). There was no significant difference in the mean intensity of green fluorescence at the plasma membrane between unstimulated control cells and SIV Nef-treated cells at either Nef concentration ($p=0.08$ for 0.01µg/ml and $p= 0.1$ for 0.1µg/ml).

In the absence of Nef, there was translocation of GLUT4 to the plasma membrane after 1nM, 10nM or 100nM insulin stimulation, as evidenced by the circumferential bright green fluorescent signal (Fig. 8C, panels 2, 8 and 14). The signal appears smoother and brighter with each successive rise in insulin concentration (Fig. 8C, panel 2 vs. 8 vs. 14), confirming that a greater degree of GLUT4 translocation to plasma membrane

occurred with higher amounts of insulin. This verifies that GLUT4 translocation to the plasma membrane is dose-dependent with respect to insulin concentration.

Adipocytes treated with 0.01µg/ml SIV Nef and stimulated with 1nM, 10nM or 100nM insulin appeared similar to control cells stimulated with the same concentrations of insulin. There was no significant difference in the mean green fluorescence intensity at the plasma membranes of control cells and that of cells treated with 0.01µg/ml SIV Nef at any of the insulin concentrations tested (1nM insulin, $p=0.6$; 10nM insulin, $p= 0.7$; and 100nM insulin, $p= 0.3$).

Adipocytes treated with 0.1µg/ml SIV Nef and stimulated with 1nM also appeared similar to control cells (Fig. 8C, panel 2 vs. 5). Moreover, there does not appear to be any difference in GLUT4 translocation between control cells and cells treated with 0.1µg/ml SIV Nef stimulated with 10nM insulin and with 100nM insulin (Fig. 8C, panel 8 vs. 11, and panel 14 vs. 17). The yellow fluorescence in panels 3, 6, 9, 12, 15 and 18 of Figs. 8C confirms that the red and green fluorescence represents the same, insulin-responsive protein. Quantitative analysis confirmed the qualitative observations. There was no significant difference in the mean fluorescence intensity between control cells and cells treated with 0.1µg/ml SIV Nef stimulated with 1nM insulin ($p=0.6$), 10nM insulin ($p=0.2$), or 100nM insulin ($p=0.2$).

GLUT4 translocation in insulin stimulated adipocytes after treatment with 0.1µg/ml HIV Nef was also analyzed. There was no difference in GLUT4 translocation between control cells and cells treated with HIV Nef without insulin stimulation ($p=0.44$). After 10nM insulin stimulation, there was a slight difference in the degree of GLUT4 translocation between control and HIV Nef-treated cells (Fig. 9B, panels 2 and 5, respectively). There was also a subtle difference between control and HIV Nef-treated cells after 100nM insulin stimulation (Fig. 9B, panels 8 and 11, respectively). The yellow fluorescence in panels 3, 6, 9 & 12 of Fig. 9B confirms that both red and green fluorescence signals represent the same, insulin-responsive protein. Quantitative analysis performed as described in Methods confirmed a significant difference in mean fluorescence intensity between HIV Nef-treated and control cells after 10nM insulin stimulation ($p<0.03$), but only borderline significance after 100nM insulin stimulation ($p= 0.06$). These data suggest that in contrast to the dramatic decrease in GLUT4 fusion

with the plasma membrane, HIV Nef affects the trafficking of GLUT4 to the plasma membrane only slightly.

Nef alters the proximal signal-transduction pathway of insulin. Activation of Akt via phosphorylation of Thr-308 and Ser-473 is required for insulin-stimulated GLUT4 mobilization (59, 93). Akt is phosphorylated at these residues by different kinases. The mTOR/RICTOR complex phosphorylates Akt at Ser-473 (5, 160, 163) whereas PDK1 [activated by phosphoinositide-3-kinase (PI3K)], phosphorylates Akt at Thr-308 (6). To determine if the decrease in GLUT4 fusion observed in HIV Nef-treated cells was due to disruption of the proximal insulin signal-transduction cascade, we performed immunoblot analysis of phosphorylation sites of Akt. Without insulin stimulation, cell lysates revealed minimal phosphorylation of Akt at Thr-308 and Ser-473 in either control or Nef-treated cells (Fig. 10A). In control cells, the degree of Thr-308 and Ser-473 phosphorylation increased dose-dependently with insulin concentration. HIV Nef-treated cells revealed a similar insulin dose-dependence at Thr-308 and Ser-473, but there was a considerable reduction in the phosphorylation of Akt at Thr-308 at either 10nM or 100nM insulin (Fig. 10A). In marked contrast, there was no appreciable difference in the degree of Akt phosphorylation at Ser-473 between Nef-treated and control cells at either insulin concentration tested (Fig. 10A). Densitometric analysis of the immunoblots confirmed these observations: phosphorylation of Thr-308 was significantly reduced in Nef-treated cells compared to control cells at both insulin concentrations tested (Fig. 10B) while there was no difference between control and Nef-treated cells at Ser-473 (Fig. 10C). Similarly, insulin-stimulated adipocytes treated with 0.1µg/ml SIV Nef revealed no difference in Akt phosphorylation at Ser-473 when compared to that of control cells (Fig. 10D). Taken together, these data show that Nef affects the activation of Akt by influencing the PI3K signaling cascade, but not the cascade upstream of mTOR/RICTOR.

Nef disrupts F-actin at the cortical actin ring. Dynamic changes of the adipocyte cortical actin meshwork are required for GLUT4 translocation and fusion with the plasma membrane (82, 86, 179). To determine if the decrease in GLUT4 fusion in cells treated with Nef was attributable to disruption of actin polymerization dynamics, we examined

actin organization in control, HIV Nef-treated, and Latrunculin B (LatB)-treated cells. As expected, control cells had very smooth, intact cortical actin rings both without and with 10nM insulin stimulation (Fig. 11A, panels 1 and 4, respectively). By comparison and expectedly, LatB-treated cells demonstrated significant perturbation of the cortical actin rings, whether without or with insulin stimulation (Fig. 11A, panels 3 and 6, respectively). HIV Nef-treated cells were morphologically intermediate, with perturbation of the cortical actin rings under basal and insulin-stimulated conditions, though not to the degree seen in LatB-treated cells (Fig. 11A, panels 2 and 5, respectively). This qualitative assessment was confirmed quantitatively as described in Methods. Only a small number of control cells were positive for disrupted F-actin (8.7%; Fig. 11B), whereas a high proportion of LatB-treated cells had disrupted cortical actin polymers (89.3%; Fig. 11B). HIV Nef-treated cells fell between control and LatB-treated cells with 56.7% showing perturbed cortical F-actin (Fig. 11B). Compared to control cells, there was a significant difference in the proportion of HIV Nef-treated cells showing disrupted cortical actin, both in the basal and insulin-stimulated states ($p < 0.05$). These data indicate that actin dynamics are disrupted in adipocytes exposed to HIV Nef.

Nef does not alter N-WASP phosphorylation. Insulin causes cortical ring localization of the Neural Wiskott-Aldrich Syndrome Protein (N-WASP) protein, a requirement for actin filament rearrangement in adipocytes (82) and other cells. N-WASP is a key regulator of actin dynamics by virtue of its relationship with the actin nucleating complex Arp2/3. Phosphorylation of N-WASP at tyrosine 256 stabilizes the “open” conformation of N-WASP, enhancing its activity toward Arp2/3. To determine if the cortical actin ring disruptions caused by Nef were due to alterations in N-WASP phosphorylation, we performed immunoblot analysis of N-WASP phosphorylation at Tyr-256. As expected, Tyr-256 was phosphorylated in control cells and was unaffected by insulin treatment at either 10nM or 100nM (Fig. 12). In addition, HIV Nef treatment had no significant effect on N-WASP Tyr-256 phosphorylation in adipocytes (Fig. 12). While N-WASP is also phosphorylated constitutively at Ser-484 and Ser-485, we detected no significant difference between control and HIV Nef-exposed adipocytes in N-WASP phosphorylation at these residues (Fig. 12).

Discussion

An increased prevalence of insulin resistance, glucose intolerance and frank diabetes has been reported in patients with HIV infection. Though substantial research has shown important links between antiretroviral therapy (ART) and the development of insulin resistance and diabetes, it has also been shown that HIV contributes directly to metabolic disturbances as well. We provide new insights into the effects of HIV-1 on adipocyte function related to insulin-stimulated glucose uptake. We show that recombinant Nef from either HIV-1 or SIV significantly reduces glucose uptake in insulin-stimulated adipocytes by inhibiting GLUT4 surface expression. Nef also inhibits insulin-induced Akt phosphorylation which may explain the GLUT4 translocation deficit we observed. In addition, Nef inhibited insulin-stimulated cortical actin dynamics which we take to be the reason GLUT4 cannot fuse with the plasma membrane. Disruption of cortical actin dynamics is not attributable to an inhibition of phosphorylation in N-WASP.

Pancreatic beta cells release insulin when blood glucose levels rise. Insulin then binds its receptor on adipocytes, inducing a sequence of events culminating in glucose uptake. Vesicles containing GLUT4 and, in the resting state, residing in the insulin-responsive compartment are mobilized and rapidly translocated to the plasma membrane. There, fusion with the membrane initiates glucose influx. We found that after exposure to Nef from HIV or SIV, adipocytes stimulated with insulin took in significantly less glucose than control adipocytes (Fig. 7). No detectable non-specific cytotoxicity was caused by HIV Nef, as both basal glucose uptake and viability in culture were similar between control and Nef-treated cells.

The decrease in glucose uptake is attributable to a Nef-dependent reduction in the surface expression of GLUT4 molecules. Nef down-regulates the cell surface expression of several proteins, including CD4 (9, 52, 114) and MHCI molecules (36, 164). The decrease in surface expression of GLUT4 upon insulin stimulation (Fig. 9) is therefore consistent with this effect of Nef. Nef mediates CD4 and MHCI down-regulation within the secretory trafficking pathway (26, 32, 87, 151, 152), and GLUT4-containing vesicles traverse this pathway upon insulin stimulation. Our finding that GLUT4 translocation to the plasma membrane was only slightly affected by Nef (Fig. 9) suggests the mechanisms

by which CD4 and MHCI proteins are down-regulated in immune cells are not the same as mechanisms by which Nef down-regulates surface GLUT4 in adipocytes.

Successful GLUT4 translocation to and fusion with the plasma membrane is the result of several converging cellular processes, including multiple signaling cascades. Activation of Akt is one major product of insulin signaling. Full activation of Akt is dependent initially on phosphorylation of Ser-473 by mTOR/RICTOR containing complex (5, 160, 163), and subsequently PI3K-stimulated phosphorylation of Thr-308. In Nef-exposed adipocytes stimulated with insulin, there was a considerable difference in the extent of Akt phosphorylation at Thr-308 relative to control cells (Fig. 10). This finding is consistent with the known effect of Nef on phosphorylation patterns in stimulated T-cells (14, 62, 63, 78, 147, 177). In adipocytes, insulin stimulates GLUT4 translocation through co-existent Akt-dependent and independent pathways. Consequently, inhibition of Akt blocks GLUT4 translocation, but not entirely (59, 83). In our hands, Nef treatment modestly reduced GLUT4 translocation (Fig. 9), partially mimicking the effect of Akt inhibition on GLUT4. We hypothesize that Nef reduces Akt activity by decreasing Thr-308 phosphorylation and inhibiting Akt-dependent GLUT4 translocation, but having minimal effect on Akt-independent GLUT4 translocation. The mechanism by which Nef reduces insulin-stimulated Akt-phosphorylation at Thr-308 in adipocytes remains unknown, but may reflect interference with PI3K activity (60). It is interesting that there was no discernible difference in Akt phosphorylation at Ser-473 between insulin-stimulated control and Nef-treated cells (Fig. 10), suggesting that Nef affects neither mTOR/RICTOR activity nor signaling proteins upstream of the mTOR/RICTOR complex.

Once GLUT4 has docked at the plasma membrane, insulin-stimulated rearrangements within the cortical actin meshwork induce GLUT4 vesicle fusion (86). It is well established that Nef alters actin cytoskeletal structure and disrupts actin-remodeling processes (28, 45, 62, 63, 110). For example, Nef induces actin cytoskeletal rearrangements in podocytes (110), and also prevents circumferential actin ring formation in stimulated T-cells (63). Our data are consistent with these observations, as we saw abnormal cortical actin structures in adipocytes exposed to Nef (Fig. 11). Thus, we propose that decreased GLUT4 fusion in insulin-stimulated adipocytes is due to Nef-

dependent alterations of the cortical actin ring. N-WASP regulates actin dynamics by interacting with the Arp2/3 complex, and phosphorylation of N-WASP enhances its activity towards actin/Arp2/3. Haller *et al*. found that phosphorylation of N-WASP tyrosine was decreased in Nef-expressing T-cells stimulated through the T-cell receptor (63), likely contributing to the defect the authors observed in formation of the circumferential actin ring. In adipocytes, by contrast, Nef did not decrease phosphorylation of N-WASP tyrosine either before or after insulin stimulation (Fig. 12), suggesting the cortical actin ring defect we observed is not due to decreased N-WASP activity *per se*. Haller *et al*. also found that recruitment of N-WASP to signaling microclusters was decreased in Nef-expressing stimulated T-cells (63). Since insulin causes recruitment of N-WASP to the cortical actin rings of adipocytes (82), it is possible that Nef inhibits N-WASP recruitment to the adipocyte plasma membrane.

Another important component of insulin signaling is PI3K as it plays multiple roles in provoking GLUT4 mobilization. PI3K activity is responsible for Akt activation leading to GLUT4 translocation. Acting through Akt-independent channels, PI3K also mediates GLUT4 vesicle fusion (59), and has been implicated in actin remodeling processes (115). Nef is known to inhibit PI3K activity (60) and, in our experiments, Nef reduced Akt phosphorylation, decreased GLUT4 vesicle fusion, and prohibited cortical actin dynamics. Nef may inhibit just PI3K (Fig. 13A) to produce the multiple phenotypes we observed in insulin-stimulated adipocytes. This would be consistent with the known roles of PI3K in GLUT4 trafficking. Alternatively, Nef may act at several points along the insulin-stimulated GLUT4 trafficking pathway in addition to PI3K (Fig. 13B), a scenario consistent with its ability to bind a plethora of different proteins and influence numerous different cellular processes. These two scenarios remain as fruitful areas for further investigation. More specifically, future research will focus on identifying the proteins Nef interacts with to mediate the adipocyte perturbations observed here. Because Nef has an impressive array of functional amino acid motifs, additional studies will map the determinants of Nef that mediate each effect documented here.

It is not surprising that similar aberrations were observed in insulin-stimulated adipocytes treated with either HIV-1 or SIV Nef, as the proteins bear striking sequence homology (Fig. 14; Table 6). Nef has a flexible N-terminal anchor domain, a globular core

domain, a flexible loop region, and a C-terminal domain. The core domains of HIV-1 and SIV Nef share the greatest amount of sequence identity while the N- and C-terminals are less similar (Table 6). SIV Nef has an additional C-terminal tail of 10–30 amino acids. HIV and SIV Nef are also functionally homologous; virtually all of the functions ascribed to HIV-1 Nef are also attributed to SIV Nef (Table 7) (97). It is interesting to note, however, that HIV-1 and SIV Nef utilize divergent strategies to mediate similar functions. For example, the PxxP and RR motifs within HIV-1 Nef are required for its interaction with the zeta chain of the TCR, but were dispensable for SIV Nef to interact with the same protein (75). Also, HIV-1 Nef depends on a leucine-based motif to down-regulate CD4, while SIV Nef depends on a tyrosine-based motif (77). Functional differences also exist between the proteins. SIV Nef more potently dampens T-cell activation in response to presented antigens than HIV-1 Nef. SIV Nef accomplishes this by down-regulating the T-cell surface markers CD3 and CD28, important co-stimulatory proteins. HIV-1 Nef does not down-regulate CD28 as efficiently as SIV Nef, and completely fails to down-regulate CD3 (97). That SIV Nef did not reduce insulin-stimulated glucose uptake and GLUT4 trafficking to the same extent as HIV-1 Nef may reflect differences in their primary sequences and, by extension, their functionality. Further exploration into the similarities and differences between HIV-1 and SIV Nef with respect to glucose homeostasis in this model system are warranted.

It should be noted that native Nef is co-translationally myristoylated. This modification is thought to be critical for many activities of Nef. For example, down-regulation of CD4 and MHCI (136), as well as interaction with PAK2 (161) are dependent on myristoylation. However, myristoylation is not an absolute requirement for full Nef activity. For example, Briggs *et al.* report that Nef binding to and activation of the Src kinase Hck is myristoylation-independent (20). Also, intracellular localization of Nef changes in a manner dependent on myristoylation: non-myristoylated Nef is diffuse within the cytosol and nucleus, while myristoylated Nef localizes to the plasma membrane (46). Dennis, *et al.* showed that myristoylation alters the structure and the oligomeric state of Nef, concluding that Nef can carry out various functions, in different locations of the cell, depending on the presence or absence of the myristoyl group (41). Several reports have documented an attenuation, rather than complete abolition, of Nef

activity when non-myristoylated (35, 46, 66, 143). Fackler *et al.* showed that incorporation of Nef into newly-forming virions—an event considered important for viral infectivity—does not require myristoylation, but that infectivity of a myristoylation-defective Nef mutant virus was 40-60% as efficient as wild-type virus (46). The preparation of Nef used in the experiments described here was non-myristoylated. Therefore, our measurements may underestimate the degree to which native Nef can alter insulin-stimulated glucose uptake and GLUT4 trafficking in adipocytes. Also, defects in addition to those observed here may result when the myristoyl moiety is present. Further studies will concentrate on the effects native Nef may have on adipocytes with respect to glucose uptake and GLUT4 trafficking.

An important consideration of the work presented here is whether adipocytes are exposed to Nef *in vivo*. Nef protein lacks the canonical secretory signal sequence, yet several reports document its presence extracellularly. It was detected in the culture media of *S. cerevisiae* infected with HIV-1 plasmid constructs (111), and of MOLT-4 cells transfected with a Nef-producing baculovirus (49). Most pertinent is that Nef has been found in the sera of HIV-infected people. Fuji *et al.* detected Nef in the sera of HIV-infected people in amounts up to 20ng/ml (49), and in collaboration with Immunodiagnostics®, using a proprietary ELISA kit, we have detected Nef in sera of viremic patients in amounts up to 50ng/ml. In addition to the extracellular environment, Nef has been detected within HIV-uninfected primary lymphoid B cells, and recombinant Nef can collect within B cells as well as dendritic cells *in vitro* (143, 144, 192). It is to be expected that Nef concentration in local tissue is higher than in serum, especially in adipose tissue which can harbor a significant number of macrophages. Macrophages are resistant to the cytopathic effects of HIV and thus serve as a reservoir for persistent HIV infection. In addition, the capacity of monocytes and macrophages to migrate into and survive in organs and tissues makes them potential conveyors of HIV-1 and viral components to other cells. Indeed, one mechanism by which Nef penetrates B cells has recently been elucidated, and macrophages take central stage in this phenomenon. Xu *et al.* showed that Nef induces thin cytoskeletal protrusions from macrophages that then contact neighboring B cells. Nef is shuttled through these cytoskeletal "conduits" both as soluble protein and within trafficking vesicles, consequently accumulating within B cells

(192). The authors also showed that Nef-containing structures budding from the macrophage navigate through the extracellular space to dock on neighboring B cells (192). Lastly, adipose cells can support viral entry as they do express all the necessary HIV receptors, and replication can occur at least to a small degree in adipocytes (68). It seems likely, therefore, that adipocytes are exposed to and may incorporate Nef in one or more ways: in the extracellular environment via serum, by Nef-producing macrophages, by conduit-forming macrophages, by Nef-containing vesicles budded from macrophages, and by direct infection. Regardless of the route, we hypothesize that adipocytes chronically exposed to Nef *in vivo* are impaired sufficiently with regard to glucose uptake, that the threshold for development of insulin resistance in HIV-infected people will be lower. The focus of future work will be to determine whether Nef can accumulate in adipocytes *in vitro* and *in vivo*, and to elucidate the roles HIV-infected macrophages, in particular adipose tissue macrophages, may play in these phenomena.

Our findings implicate Nef in the development of HIV-associated insulin resistance and Type II Diabetes. This is an important finding in light of the prolonged life expectancy HIV-infected people are afforded by ever-improving ART. As the HIV-infected population ages, they will be subject to the forces that drive insulin resistance in the general population but with the added risks of HIV-associated factors such as Nef. Hence, further exploration of the roles that HIV itself plays in the development of metabolic diseases, in particular insulin resistance, is vital for enhancing the clinical management of HIV disease.

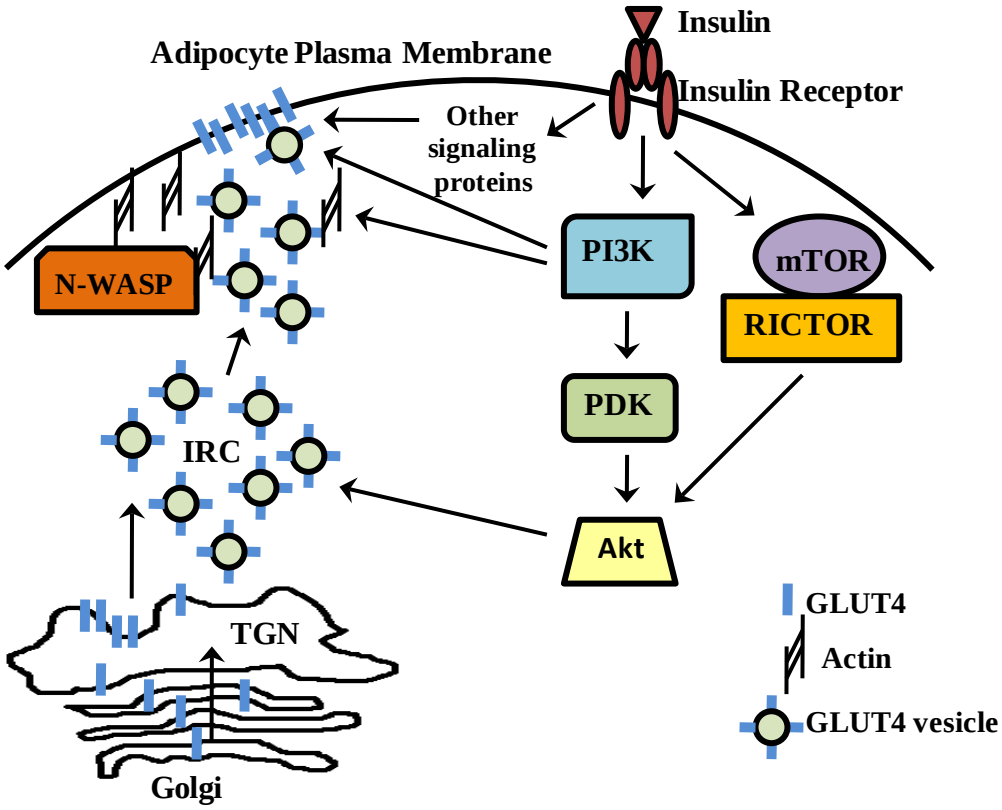


Figure 3. Molecular mechanism of insulin-induced GLUT4 translocation and glucose uptake in adipocytes. In unstimulated cells, vesicles containing GLUT4 reside in the so-called insulin-responsive compartment (IRC). When insulin binds its receptor on the cell surface, a signal transduction cascade is initiated. PI3K and mTOR/RICTOR become activated. Both the mTOR/RICTOR complex and PDK phosphorylate Akt to activate it. Activated Akt then rapidly mobilizes GLUT4 within the IRC to translocate to the plasma membrane. Insulin also induces recruitment of N-WASP to the membrane and mediates rearrangements of the cortical actin ring that are required for GLUT4 to fuse with the plasma membrane. Subsequent integration with the membrane allows glucose influx to occur.

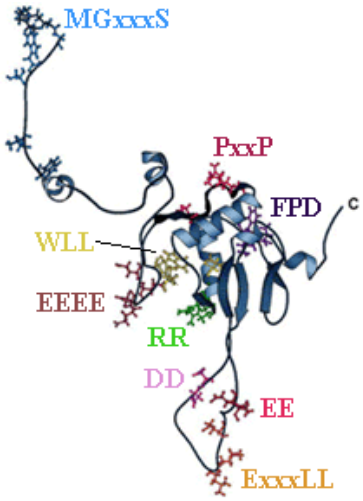


Figure 4. Structure of full-length HIV-1 Nef with color-coded functional amino acid motifs. The N-terminal domain is structurally flexible while the core domain of Nef is the only portion that adopts a stable tertiary fold. The flexibility of Nef facilitates conformational changes, sometimes dictated by its binding partner. In addition, the less-structured regions of Nef provide an extensive accessible surface that could be used to connect to many proteins simultaneously (11). Adapted by permission from Macmillan Publishers Ltd: EMBO reports (54) © 2001.

Table 4. Amino acid sequence motifs of HIV-1 Nef, their functions, and examples of proteins interacting with each motif. Adapted by permission from Macmillan Publishers Ltd: EMBO reports (54) © 2001.

Motif	Function	Interacting Proteins
MGxxxS	Protein Modification	N-myristoyl transferase
WLL	Protein Trafficking	Cytoplasmic tail of CD4
EEEE	Protein Trafficking	PACS-1[α]
RR	Signaling	PAK 1/2[β]
DD	Protein Trafficking	V1H[γ]
ExxxLL	Protein Trafficking	AP-1/2/3[δ] Clathrin
EE	Protein Trafficking	β-COP[ε]
FPD	Protein Trafficking	Thioesterase
PxxP	Signaling Cytoskeleton remodeling	SH3 domains of Hck, Lck Vav[ζ] T-cell Receptor chain ζ

[α], PACS-1, phosphofurin acidic cluster sorting protein-1; a coat protein that enables the formation of vesicles travelling from the endosome to the golgi. Nef binds to PACS-1 to re-route vesicles containing MHCI molecules.
[β], PAK, p21-associated kinase 1/2; kinases involved in cytoskeletal regulation.
[γ], V1H, subunit H of the vacuolar membrane ATPase; a component of the proton pump required for the acidification of endosomes and lysosomes, and also binds AP-2.
[δ], AP-1/2/3, adapter protein 1/2/3; clathrin-based vesicle coat proteins required for transport of proteins between membrane-bound organelles along the exocytic and endocytic pathways.
[ε], β-COP, β Coat protein; a non-clathrin-based vesicle coat protein component of COP-1 needed for retrograde vesicle transport through the trans-golgi network.
[ζ], Vav; guanine-nucleotide exchange factor for the Rho-like small GTPases that regulate cytoskeletal reorganization.

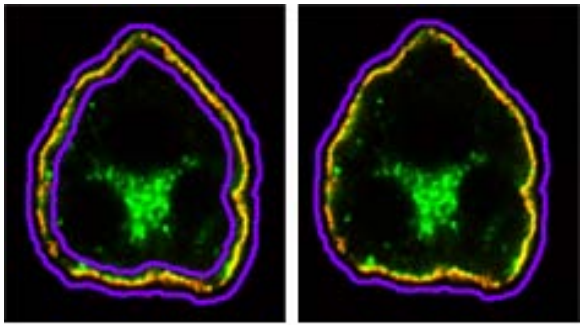


Figure 5. Quantification of GLUT4 integration and translocation to the plasma membrane of insulin-stimulated adipocytes. The fluorescent signal at the cell surface, whether red or green, was quantified by measuring the signal at the membrane (left panel, area inside the purple lines) and normalizing to the total green signal of the whole cell (right panel, area inside the single purple line).

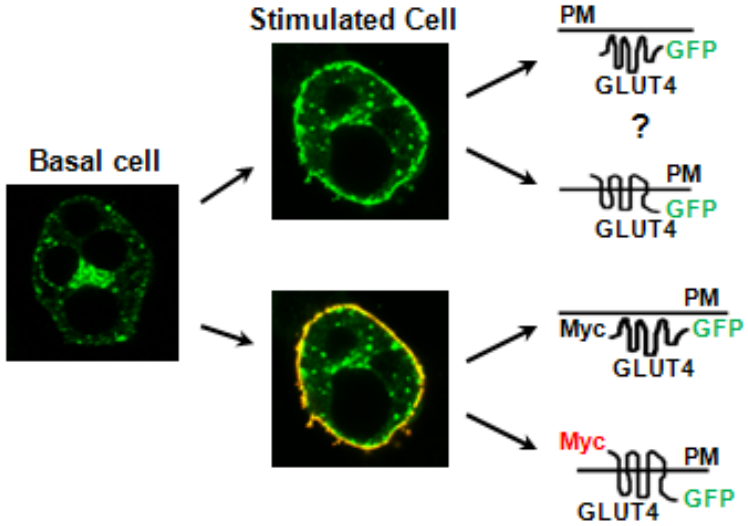


Figure 6. Insulin-stimulated translocation and insertion of double-tagged GLUT4 into the adipocyte plasma membrane (PM). GLUT4 accumulates intracellularly under basal (unstimulated) conditions, but is rapidly translocated to the membrane upon insulin stimulation. The GFP tracks intracellular GLUT4 translocation to the membrane, but does not differentiate between GLUT4 docked *on* the plasma membrane and GLUT4 inserted *in* the membrane (top path). The exofacial myc tag permits differentiation of these two states (bottom path).

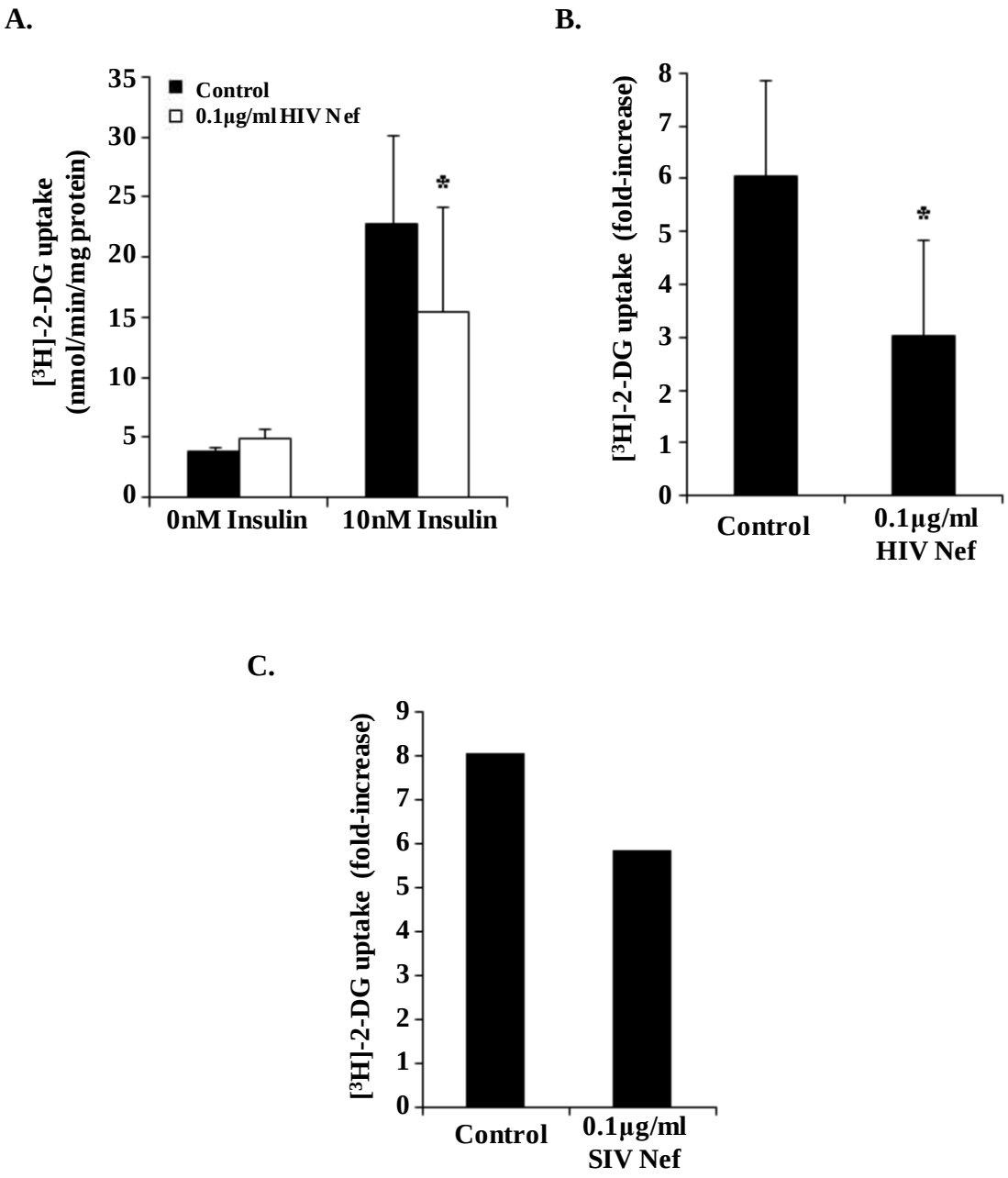


Figure 7. Glucose uptake in control and Nef-treated adipocytes. (A-B) Insulin-stimulated glucose uptake in HIV Nef-treated cells. (C) Insulin-stimulated glucose uptake in SIV Nef-treated cells. (A) Results are expressed as the mean rate of [^{3}H]-2-deoxyglucose (2-DG) uptake ± SD of triplicate determinations (*, $p<0.02$ by paired-samples T-test). (B) Results are expressed as the fold insulin-stimulated increase in glucose uptake relative to basal uptake, ± SD of triplicate determinations (*, $p<0.02$ by paired-samples T-test). (C) Results are expressed as the fold insulin-stimulated increase in glucose uptake relative to basal uptake.

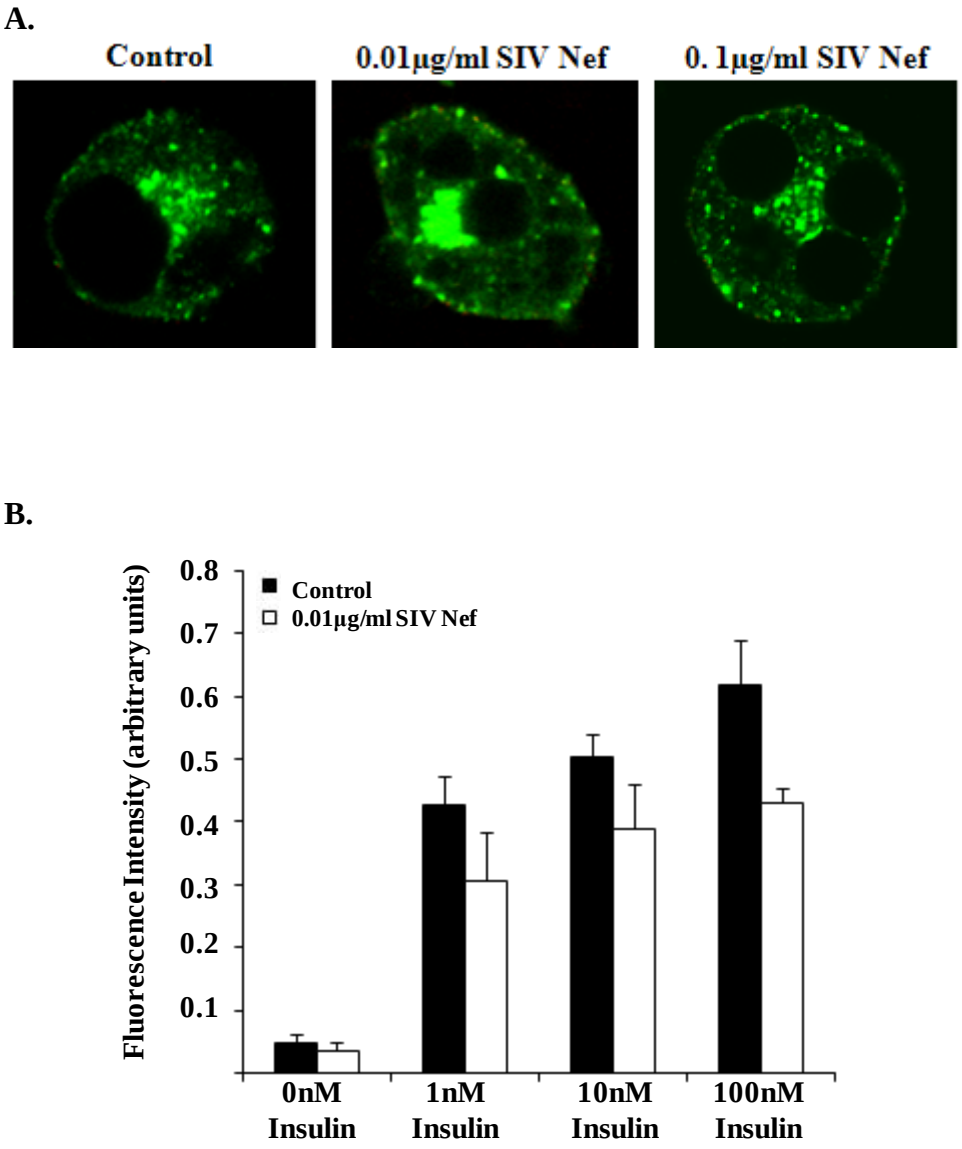
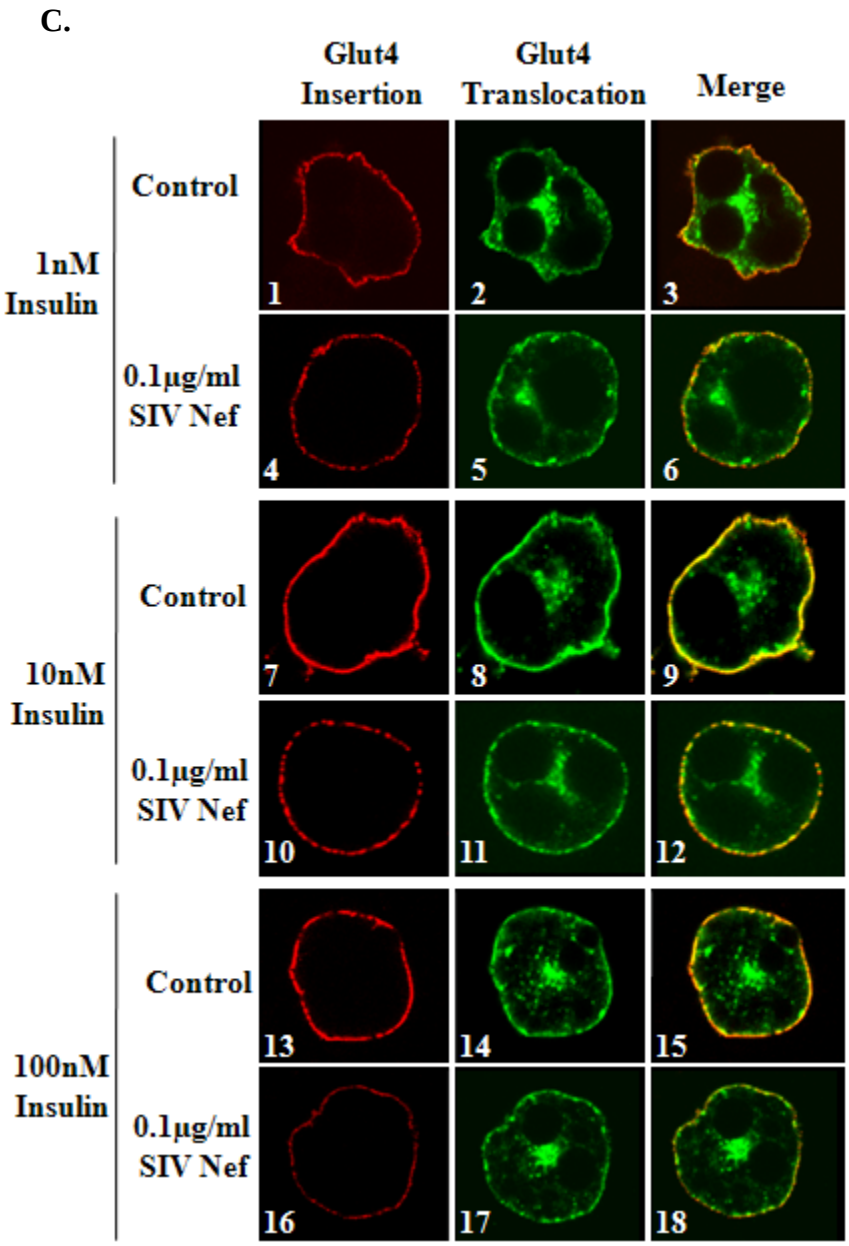


Figure 8. Glut4 integration and translocation in insulin-stimulated control and SIV Nef-treated adipocytes. (A,C) Immunofluorescence analysis of Myc-Glut4-GFP-transfected control and Nef-treated adipocytes with no insulin stimulation (A), and 1nM, 10nM or 100nM insulin (C), stained with mouse monoclonal antibody to c-Myc followed by Texas Red-conjugated rabbit anti-mouse IgG. Original magnification, x63. The yellow fluorescence in panels 3, 6, 9, 12, 15 and 18 confirms that the red and green fluorescence represents the same, insulin-responsive protein visualized two ways. (B, D) The red-fluorescence intensity of Myc-Glut4-GFP transfected control and Nef-treated cells with no insulin, 1nM, 10nM or 100nM insulin stimulation. Values represent the mean ± SD of 3 independent experiments (*, $p < 0.04$; **, $p < 0.002$, by paired-samples T-Test).



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D.

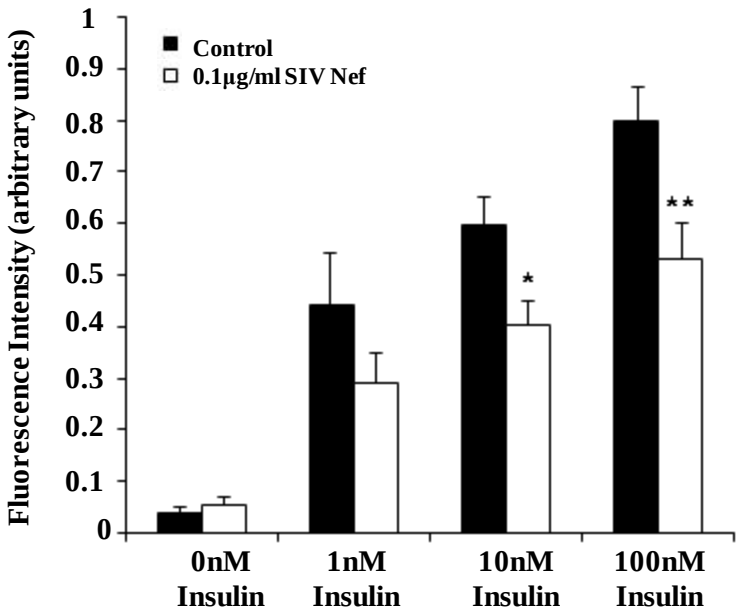

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Table 5. P values derived from paired-samples T-tests of mean red-fluorescence intensity of Nef-treated adipocytes compared to control cells stimulated with the same insulin concentrations. Significant values are italicized.

Insulin	0.01µg/ml SIV Nef	0.1µg/ml SIV Nef
0nM Insulin (basal)	p= 0.3	p= 0.2
1nM Insulin	p=0.1	p=0.058
10nM Insulin	p=0.2	*p< 0.04*
100nM Insulin	p=0.2	*p<0.002*

A.

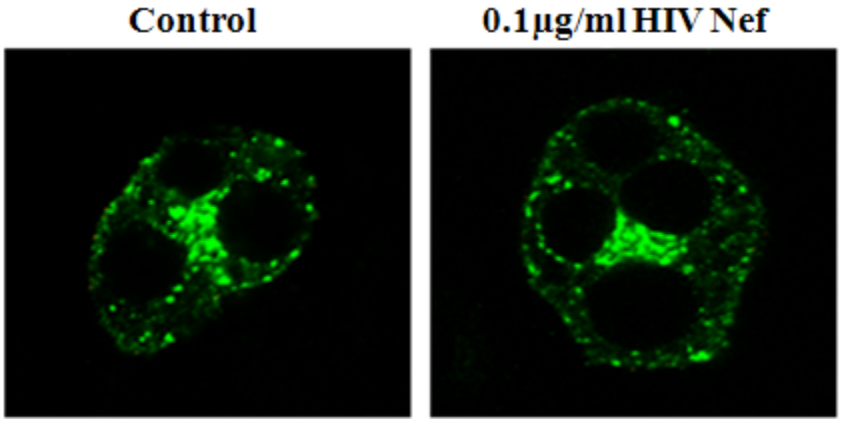

Figure 9. Glut4 fusion and translocation in insulin-stimulated control and HIV Nef-treated adipocytes. (A-B) Immunofluorescence analysis of Myc-Glut4-GFP-transfected control and Nef-treated adipocytes with no insulin stimulation (A), and 10nM or 100nM insulin (B), stained with mouse monoclonal antibody to c-Myc followed by Texas Red-conjugated rabbit anti-mouse IgG. Original magnification, x63. (B) The yellow fluorescence in panels 3, 6, 9 and 12 confirms that the red and green fluorescence represents the same, insulin-responsive protein visualized two ways. (C) The red-fluorescence intensity of Myc-Glut4-GFP transfected control and Nef-treated cells with no insulin, 10nM or 100nM insulin stimulation. Values represent the mean ± SD of 3 independent experiments (*, $p < 0.05$; **, $p< 0.03$, by paired-samples T-Test).

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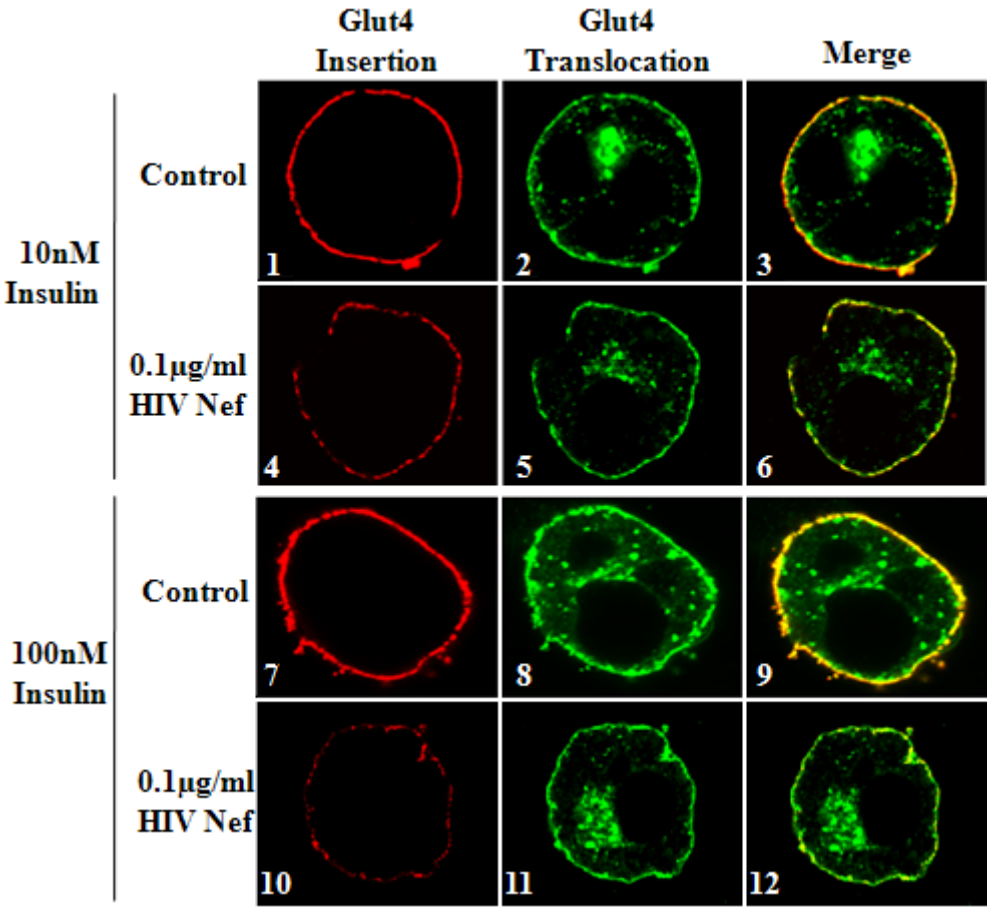
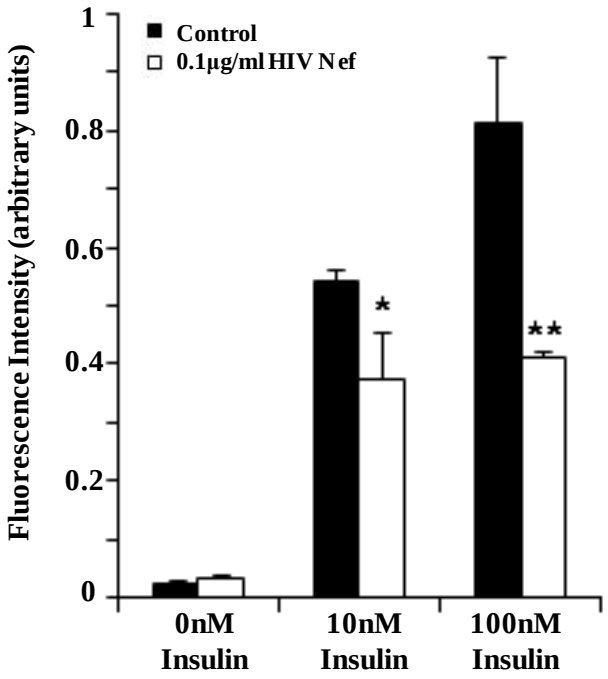


Figure 9. Glut4 fusion and translocation in insulin-stimulated control and HIV Nef-treated adipocytes. (A-B) Immunofluorescence analysis of Myc-Glut4-GFP-transfected control and Nef-treated adipocytes with no insulin stimulation (A), and 10nM or 100nM insulin (B), stained with mouse monoclonal antibody to c-Myc followed by Texas Red-conjugated rabbit anti-mouse IgG. Original magnification, x63. (B) The yellow fluorescence in panels 3, 6, 9 and 12 confirms that the red and green fluorescence represents the same, insulin-responsive protein visualized two ways. (C) The red-fluorescence intensity of Myc-Glut4-GFP transfected control and Nef-treated cells with no insulin, 10nM or 100nM insulin stimulation. Values represent the mean ± SD of 3 independent experiments (*, $p < 0.05$; **, $p < 0.03$, by paired-samples T-Test).

C.



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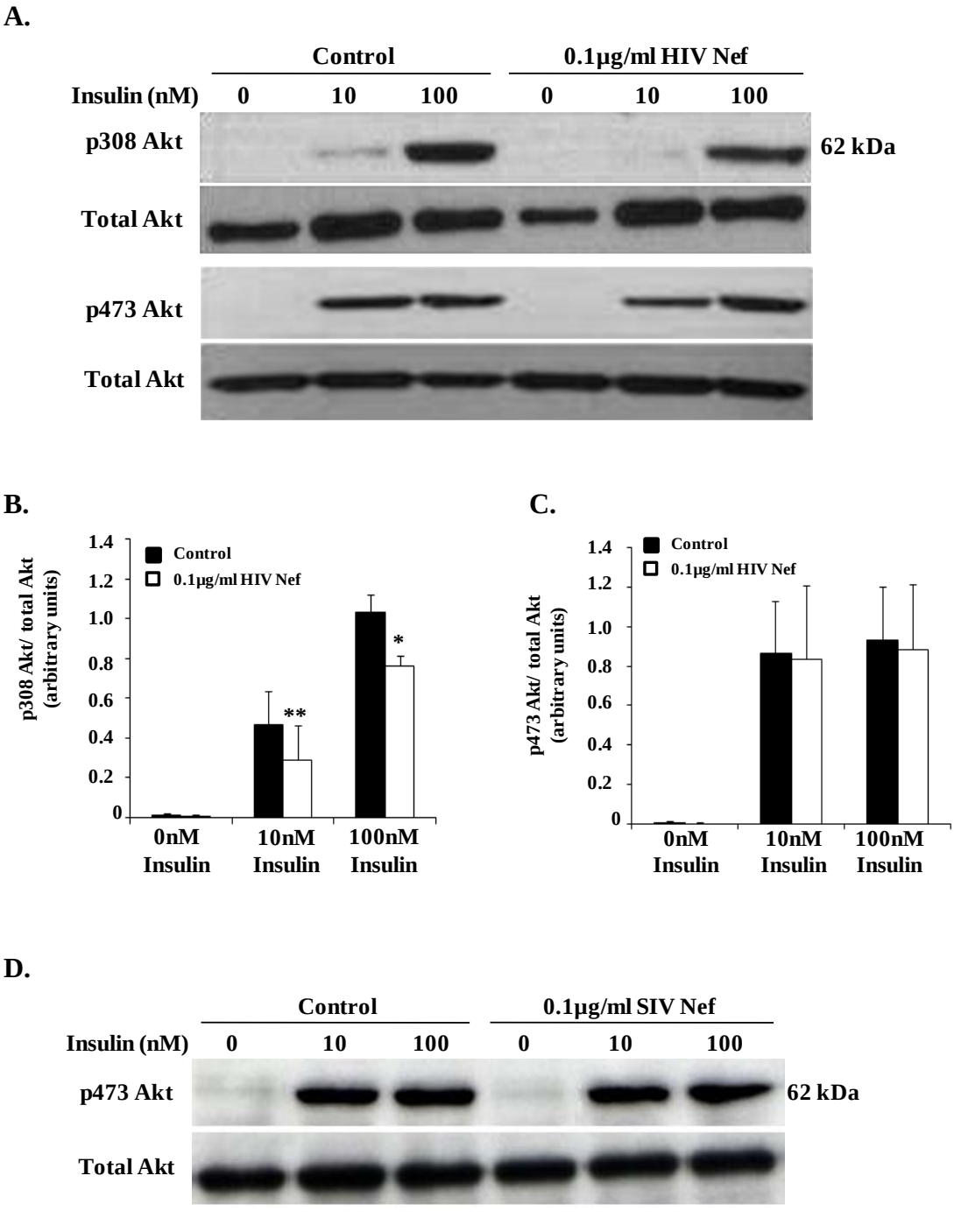

Figure 10. Akt phosphorylation in insulin-stimulated control and Nef-treated adipocytes. (A,D) Immunoblot of lysates collected from control and HIV Nef-treated adipocytes (A), and SIV Nef-treated adipocytes (D) unstimulated or stimulated with 10nM or 100nM insulin. (B,C) Densitometric analysis of phospho-308 Akt (B) and phospho-473 Akt (C) immunoblots. Values represent the mean optical density of phospho-Akt bands divided by the mean O.D. of total Akt bands from at least 2 independent experiments, ± SD (*, $p<0.05$; **, $p<0.01$).

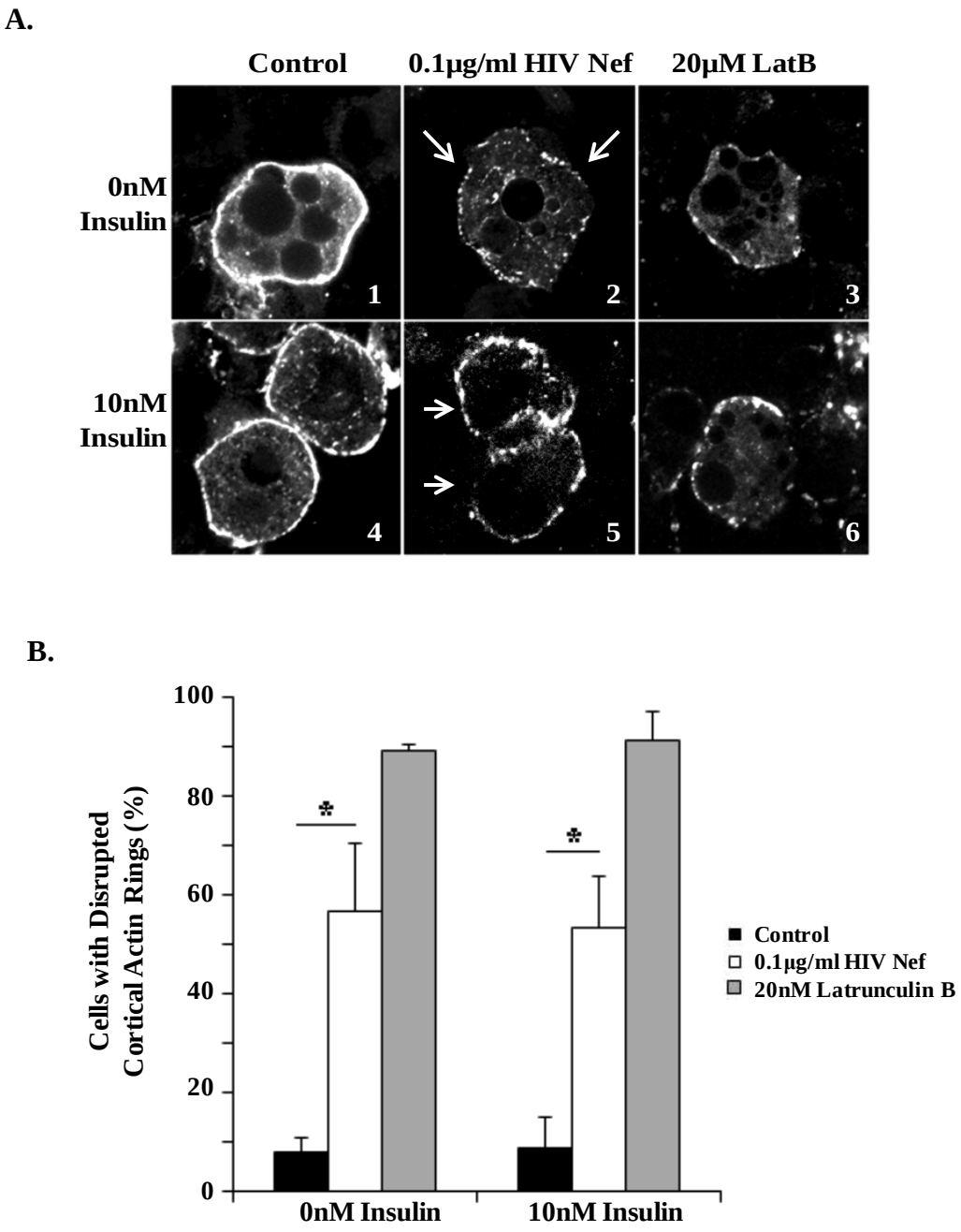

Figure 11. Cortical actin rings of control, Nef-treated and Latrunculin B-treated adipocytes. (A) Fluorescence microscopy of control, Nef-treated and Latrunculin B-treated adipocytes without and with 10nM insulin stimulation. Cells were stained with rhodamine-conjugated phalloidin. The arrows (panels 2 and 5) designate areas of disrupted f-actin within the cortical rings. (B) The percent of control, Nef-treated and Latrunculin B-treated cells with perturbed cortical actin rings, without and with 10nM insulin stimulation. Values are the mean percent of cells positive for disrupted cortical actin ± the SD from 3 independent experiments (*, $p < 0.05$ by paired-samples T-Test).

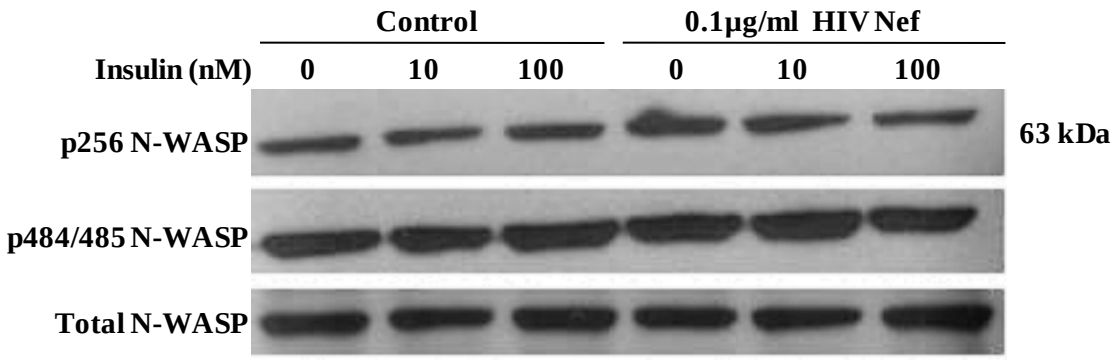

Figure 12. N-WASP phosphorylation in insulin-stimulated control and Nef-treated adipocytes. Immunoblot of lysates collected from control and Nef-treated adipocytes unstimulated or stimulated with 10nM or 100nM insulin.

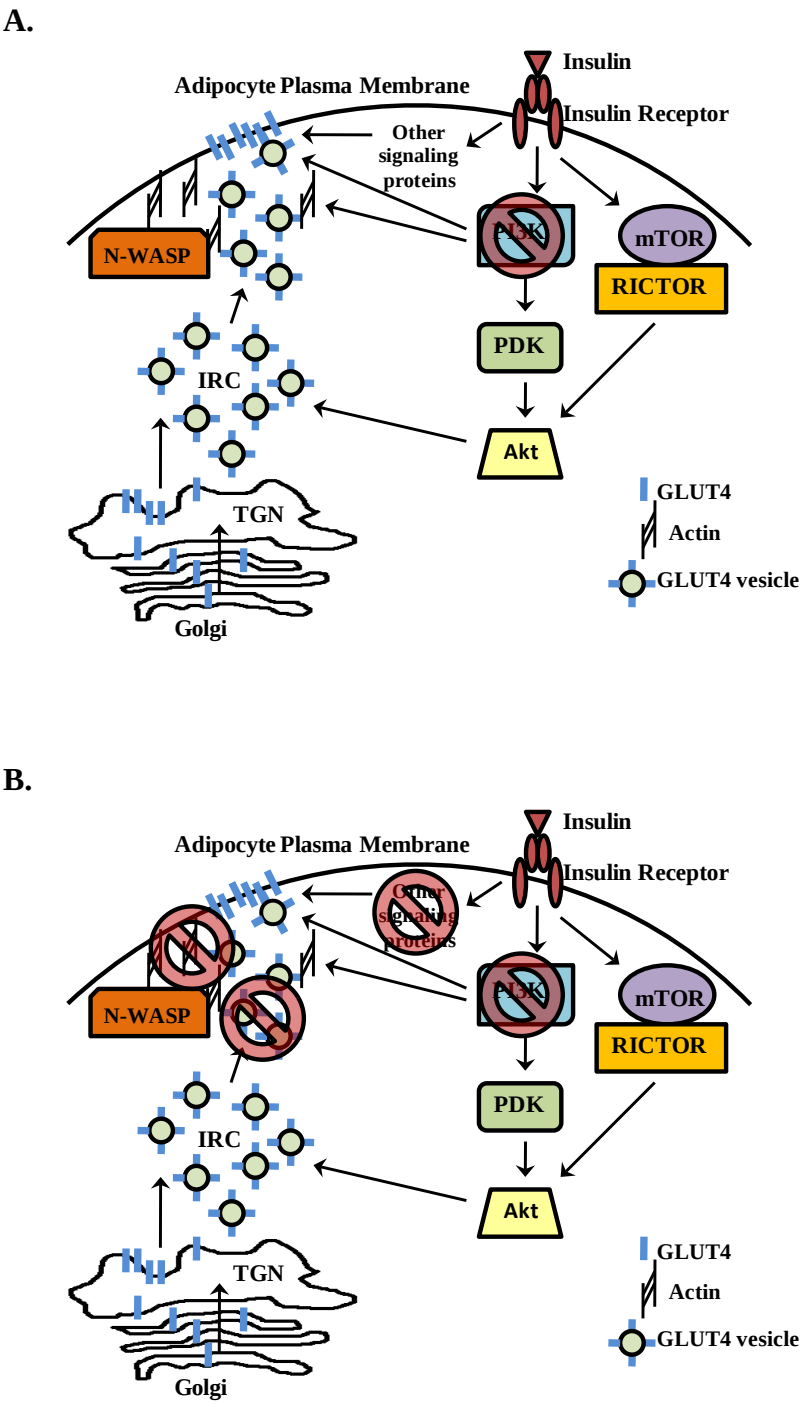

Figure 13. Nef affects insulin-induced GLUT4 translocation and glucose uptake in adipocytes. (A) Nef may inhibit just PI3K such that Akt is not efficiently activated, actin rearrangements are inhibited, and GLUT4 fusion is impeded. (B) Alternatively, Nef may interfere with proteins at several points along the insulin-stimulated GLUT4 trafficking pathway.

Figure 14. Sequence alignment of HIV-1 and SIV Nef. Nef has a flexible N-terminal anchor domain (green underline), a globular core domain (blue underline), a flexible loop region (pink underline), and a C-terminal domain (yellow underline). The core domains of HIV-1 and SIV Nef share the greatest amount of sequence identity. The N- and C-terminals are less similar. SIV Nef also has an additional C-terminal tail of 10–30 amino acids (151).

Table 6. Percentage of sequence homology between regions of HIV-1 and SIV Nef (151).

Region	Identical (% amino acids)	Similar (% amino acids)
N-terminus	17	21
Globular Core	57	69
Flexible Loop	32	39
C-terminus	20	24

Table 7. Similarities and differences of HIV-1 and SIV Nef functionality (97).

Nef Clone	CD4	MHCI	CD3	CD28	Replication	Infectivity
HIV-1	+++	++	-	(+)	++	++
SIV	+++	++	++	++	++	+++

+++, high activity; ++ moderate activity; (+), marginal activity; -, no activity

Chapter 2: HIV and EBV Act in Concert to Promote EBV-associated Malignancies

Introduction

Infection with opportunistic pathogens is a persistent concern for those infected with HIV, especially as the immune system declines. Though ART has to a great extent minimized the incidence and complications of most opportunistic infections, controlling infection with herpesviruses remains a significant issue. There are four herpesviruses that contribute substantially to HIV morbidity and mortality (Table 3), but it is HHV-8 and EBV that perhaps have the most mysterious and interesting roles in the pathogenesis and progression of HIV disease.

Kaposi's Sarcoma (KS; Fig. 15) was first described in 1872 in older Mediterranean males (113), but it was not until 1981 when 50 young men in the U.S. were reported to have aggressive KS involving the skin, lymph nodes, mucosa and viscera (1) that interest in this disease was sparked. In 1994, a PCR-based technique was used to look for DNA, such as viral DNA, that was present in KS tissue but not healthy tissue, and found herpesvirus-like DNA sequences (31), subsequently given the designation Human Herpesvirus 8 (HHV-8). Most herpesviruses cause ubiquitous infections worldwide, but HHV-8 does not. HHV-8 is most prevalent in sub-Saharan Africa with approximately 50% of people infected, but seroprevalence ranges from 10% in the Mediterranean region and 5% in the U.S. to as low as 0.2% in Japan. A syndrome associated with primary infection in immune-competent hosts has not been described. However, immune suppression can contribute to the development of pathologies associated with HHV-8, such as KS, Primary Effusion Lymphoma (PEL), and multicentric Castleman's disease.

Epstein-Barr Virus (EBV; also called Human Herpesvirus 4 (HHV-4); Fig. 16) is a ubiquitous pathogen with a worldwide distribution. Seroprevalence in adults in the U.S. and Great Britain is 95%, meaning that infection in a population co-infected with HIV is virtually assured. At least 50% of adults in these and most other countries were infected before 5 years of age (113), as a result of crowded living conditions, siblings, daycare, etc. Infection usually occurs after exposure to oral secretions of a seropositive individual, and when occurring within the first decade of life, is typically subclinical if not clinically silent. Primary infection in the second decade of life or later manifests as Infectious

Mononucleosis (IM). IM encompasses a broad spectrum of illness typically characterized by a triad of sore throat with palatal exudates, fever, and lymphadenopathy, but other symptoms may be present, such as abdominal pain, hepatosplenomegaly, malaise, chills, and myalgias. IM is generally self-limiting with a 2- to 3- week course, but complications such as autoimmune hemolytic anemia, thrombocytopenia, encephalitis and renal abnormalities can occur. Lymphocytic infiltrations of the spleen coupled with rapid splenic enlargement predispose the spleen to rupture during IM, and though this is a dramatic complication, it is rare (113). As described for HHV-8, EBV is also associated with certain malignancies. These include lymphoproliferative disease (typically occurring post-transplantation; PTLD), endemic Burkitt's lymphoma (BL), Hodgkin's disease (HD), and nasopharyngeal carcinoma.

The HIV-infected population, along with other immune-suppressed people, suffers uniquely from HHV-8 and EBV-associated pathologies, particularly the malignancies. KS occurs with an astoundingly greater frequency in those infected with HIV (58). In fact, in 1982, the U.S. Centers for Disease Control and Prevention (CDC) included KS within its initial case definition of AIDS (2). Most pertinently, KS is much more aggressive and potentially fatal in HIV-infected persons with AIDS (113). PEL also occurs with greater frequency in the HIV-infected population (113). Interestingly, malignant cells of PEL are typically infected with both HHV-8 and EBV. EBV-associated malignancies also occur with greater frequency in HIV-infected people. Indeed, the incidence of primary central nervous system lymphoma (PCNSL) was once 3600-fold greater than in the general population (37), and was also included in the CDC's initial case definition of AIDS (2). EBV-infected B-cells are found in virtually all cases of HD (7). Other EBV-associated malignancies occurring with greater incidence in the HIV setting are the aggressive diffuse large B-cell lymphoma and BL. Whether HHV-8- or EBV-associated, these malignancies follow a more aggressive course in HIV-infected people. Despite a decline in the incidence of HIV-associated cancers, attributable, at least in part, to ART (33), malignancies with a viral etiology have not diminished as much, so are becoming a major cause of death in AIDS.

To better understand the role of herpesviruses in the development of cancer, specifically in the HIV setting, a better understanding of both the virology and

immunology is necessary. Herpesviruses are unique for their ability to escape immune clearance and establish life-long infections within the hosts. This may explain, in part, why they remain problematic for a population that we recognize as suffering from a complex combination of immune suppression and activation. Initial infection of B cells by EBV incites the growth program, which induces naïve B cells to differentiate into memory B cells where EBV will lay dormant, or into plasma cells in which EBV will lytically replicate. Lytic replication follows a program of expression of nearly all the genes in its large DNA repertoire. Though most infected cells are destroyed by an activated T_H1 immune response, this viral class is defined by the ability to remain in a very small number (as few as a million) of infected memory cells. Their genomes circularize and persist extra-chromosomally as episomes within the nucleus of infected cells. The episomes are replicated in concert with host chromosomes by cellular enzymes. For EBV in latency (Table 8), almost all of the roughly 80 viral promoters are silenced, encompassing all immediate-early, early and late-lytic cycle promoters, the origins of lytic replication, and also most latency promoters. Since only a small subset of latency promoters is active, neither lytic viral replication nor EBV-mediated activation of B-cell proliferation takes place. Depending on the EBV latency program being enacted, no viral genes or only a few genes are expressed (Table 8). This retreat renders EBV essentially invisible to the immune system.

Though considered "dormant," the few gene products remaining are active within the infected cells, where they serve to promote cell survival and proliferation so that the viral genome is maintained. For example, HHV-8 produces a homologue of human cell cyclin D, called ORF72, which stimulates the G1-to-S-phase transition within the cell cycle. Viral cyclin D is resistant to natural inhibitors of cellular cyclin D, and so cell division can occur unchecked (113). T-cells bind to the coreceptor CD40 on the surface of B cells to deliver survival and proliferation signals by direct contact. The EBV gene product, latent membrane protein 1 (LMP1), acts as a constitutively active, ligand-independent coreceptor in B cells, homologous to CD40 in function (176). Given the potent cell-survival effects of these genes, it is not surprising that these viruses drive malignant transformation so well. Indeed, B cells infected with EBV *in vitro* are driven to continuously proliferate, and can be propagated indefinitely (140). The ability of EBV to

immortalize B-cells has been exploited in many genetic studies to preserve samples from human donors (171).

Immune competence is a large factor in determining whether a herpesvirus-associated cancer will develop. During primary infection with EBV, 20-50% of circulating B cells can be infected (73, 150). With B-cells acting as antigen presenting cells, the immune response towards EBV is necessarily aggressive, complex and overwhelming. The cellular response is manifest primarily by massive oligoclonal expansion of the cytotoxic CD8 T-cell compartment. One study showed that 40% of activated CD8 T-cells isolated during IM were reactive towards a single EBV antigen (27). CD4 T-cells and Natural Killer cells as well as humoral immunity are also integral to the immune response towards EBV. Though this sizable response is what contributes most to the symptoms of IM, it must be this order of magnitude to keep proliferating cells in check and help clear the virus. Indeed, Piriou *et al.* speculate that it is the progressive loss of EBV-specific CD4 and CD8 T-cells that contributes to development of non-Hodgkin's Lymphoma (NHL) in HIV-positive people (138). Naturally, EBV has evolved mechanisms to elude this extensive immune response. For example, EBV Nuclear Antigen 1 (EBNA1) is the only viral protein needed for genome maintenance and replication of the virus during the cell cycle, and therefore must be expressed in order to ensure that the genome is passed on to daughter B-cells. To subvert the immune response against replicating cells expressing this vital protein, EBNA1 contains a long stretch of glycine-alanine repeats that prevent proteosomal degradation. Without proteosomal processing, EBNA1 antigens cannot be presented on MHCI molecules. Consequently, cells expressing full-length EBNA1 evade immune surveillance (128). Only occasionally are EBNA1-specific T-cell responses detected *ex vivo* and this is thought to be the result of presentation of EBNA-1 defective ribosomal products (DRiPs) that are not dependant on proteosomal degradation for presentation on MHCI molecules (175). Additionally, the EBV protein BCRF1 is highly homologous to human IL-10 (109). Human IL-10 shifts the CD4 T-cell response towards T_H2, which will stimulate humoral immunity but dampen T_H1, the response more effective against viruses. Without an intact immune system, the virus is free to drive cellular proliferation, thwart "feeble" efforts of a disabled immune system, and thus cause malignant transformation of infected cells.

One would anticipate that with reconstitution of the immune system as afforded by sophisticated ART regimens, HIV-associated malignancies would decline. Though there has been a reduction in the incidence of PCNSL and KS, BL incidence does not appear to have declined substantially (33). There are likely multiple other elements besides immune competence contributing to herpesvirus-associated pathologies in HIV infection. This is evidenced by the relationship HIV shares with HHV-8; a relationship more complex than simply residing within the same host. Casselli *et al.* noted increased HIV replication in both macrophages and endothelial cells co-infected with HHV-8 *in vitro*, as well as after infection with HHV-8 of PBMC from asymptomatic HIV-positive people *ex vivo* (29). The same group also showed that the HHV-8 gene product, ORF50, could induce HIV replication in a T-cell as well as a B-cell line (30). HHV-8 can also induce HIV replication via action of its homologue of human IL-6 called viral IL-6 (vIL-6) (51, 126). This is by no means a one-sided relationship, as HIV can induce HHV-8 replication (184, 185, 195). The HIV transcription factor Tat was shown by experiments *in vitro* and *ex vivo* to mediate HHV-8 reactivation (65, 118). Since HHV-8 replication correlates with KS disease progression (172), these data directly implicate an HIV protein in the development of KS. Thus, it is easy to understand why concomitant infection with HIV and HHV-8 poses significant problems for the immune system and control of HIV disease.

We may suppose EBV has an equally complex relationship with HIV, although far less studied. EBV up-regulates HIV replication *in vitro*. Scala *et al.* stably transfected a B-cell line with the EBV gene EBNA-2 which is responsible for transactivating most viral genes and for B-cell immortalization (128). After transfection of these cells with a plasmid encoding the HIV LTR fused to the chloramphenicol acetyltransferase (CAT) gene, there was high LTR activation, as measured by CAT activity. Their data indicate that EBV activates the HIV promoter (162). Zhang *et al.* reported similar findings: an HIV-infected T-cell line also infected with EBV upregulated expression of HIV reverse transcriptase (196). HIV may also induce EBV replication. Ling *et al.* and Miller *et al.* both report significantly elevated levels of EBV DNA in the blood and saliva of HIV-infected people relative to HIV-negative people (108, 119). It was noted in HIV-infected children that EBV DNA load was comparable to that observed during IM in HIV-negative people, and that the elevated EBV DNA load persisted through ART (15). In addition to increasing the risk of

developing EBV-associated malignancies, detectable EBV DNA in peripheral blood leukocytes was also shown to delay immune reconstitution in HIV-infected adults beginning antiretroviral therapy (134).

Despite the evidence that an important relationship exists between HIV and EBV, there are no reports that describe specific molecular interplays. Given that immune competence plays such an important role in suppressing EBV, we hypothesized that immune dysfunction in the setting of HIV infection contributes to elevated EBV DNA documented in patients. In addition, since HIV directly alters the life cycle of HHV-8, we hypothesized that HIV acts similarly towards EBV. In summary, we propose an important interchange between HIV and EBV that contributes to the development of EBV-associated pathologies.

Experimental Procedures

Study Participants

Our laboratory has followed, with Stony Brook University Internal Review Board (IRB) approval, cohorts of HIV-seropositive patients for up to seven years. All patients, regardless of their clinical state and HIV disease received antiretroviral therapy (ART), but may have been ART-naïve or ART-experienced at recruitment, depending upon their respective studies. The people enrolled ranged from healthy individuals with CD4 lymphocyte counts within normal limits, to patients with AIDS-defining illnesses, persistent viral loads (HIV RNA copies/ml blood) of $\log_{10} = 4$ or above, and very low (sometimes unmeasurable) blood CD4 cells. At pre-determined time-points during the course of the studies, whole blood was collected from study participants in Vacutainer® tubes containing EDTA or acid citrate dextrose. Peripheral Blood Mononuclear Cells (PBMC) were isolated using Ficoll-Hypaque density gradient centrifugation. PBMC were also collected from healthy, HIV-negative donors with IRB approval. PBMC were cryopreserved at -80° or below in RPMI-1640 supplemented with 20% FBS and 5% dimethyl sulfoxide (DMSO), until used in experiments described below. Before experiments, PBMC were thawed and washed at least once with phosphate buffered saline (PBS).

For analyses, the HIV-positive group was divided into two subgroups: aviremic and viremic. Aviremia was defined as having an HIV viral load below the limit of detection (less than 400 HIV RNA copies/ml blood) in a standard Amplicor® (Roche) assay for at least 2 months before and after the collection date. There were 37 aviremic patients with CD4 lymphocyte counts ranging from 154 to 1423 per µl blood with a mean of 533. Viremia was defined as having detectable HIV RNA (>400 copies/ml) at the time of blood collection as well as within 2 months before and after the blood collection. There were 33 viremic patients with a CD4 lymphocyte count ranging from 29 to 999/ µl blood, mean 337. The range of viral loads among viremic patients was 508 to 700,000 HIV RNA copies/ml blood with a mean of just over 52,000.

Quantitative Polymerase Chain Reaction (qPCR)

Genomic DNA was isolated using DNeasy Blood and Tissue kit (Qiagen) from washed PBMC of a total of 70 HIV-positive and 25 HIV-negative people. The DNA concentration was measured by spectrophotometry, and equal amounts of DNA from each patient were added to a PCR reaction containing primers specific for the EBV gene Epstein-Barr Nuclear Antigen 3 (EBNA3; Table10) and Sybr Green (Quantitect Sybr Green; Qiagen) for quantitation purposes. The primers for EBNA3 were designed using the online program Primer-BLAST from NCBI (National Center for Biotechnology Information). Thermocycling conditions for amplification of EBV DNA were optimized, after much trial and error, using the condition for stringency of a single band upon electrophoresis, representing a single, specific PCR product, and were 95° for 5 min, followed by 45 cycles at 95° for 45 sec, 60.8° for 45 sec, and 72° for 30 sec. For absolute quantification of EBNA DNA, a standard curve was generated using DNA isolated from IB4 cells, a B-cell line containing precisely two EBV genome copies per cell. The standard curve was included in all experiments as well as a no-template (water) control. DNA isolated from Ramos cells, a B-cell lymphoblastoma line lacking EBV, was included in each experiment as a negative control. The DNA from each patient was amplified in triplicate. Quantification based on the standard curve was performed using 7300 System SDS Software (Applied Biosystems). To ensure qPCR specificity, all samples were electrophoresed in 2% agarose with 0.5µg/ml ethidium bromide for visual confirmation of the 277 base-pair (bp) product.

Interferon-gamma (IFN-γ) ELISpot

PBMC from the 70 HIV-positive and 25 HIV-negative people used in the qPCR experiment were analyzed in parallel for immune competence by ELISpot. Washed cells were resuspended in RPMI-1640 supplemented with 10% FBS, 1% penicillin/ streptomycin and 1% L-glutamine at a concentration of $3x10^6$ cells/ml. To each well of a 96-well plate coated with antibody towards IFN-γ, 100µl ($3x10^5$) cells were added. Also added were 40µl aliquots from a pool of EBV peptides at a concentration of 10µg of each peptide per ml, 5ng/ml PMA or 0.06% DMSO. The EBV-encoded lytic and latent peptide antigens (Table 9) used in the assay are highly immunogenic and associated with the more common HLA subtypes. Fifteen were selected from published lists of peptide sequences (148, 191)

and custom-synthesized by American Peptide Company (Sunnyvale, CA). Phorbol-12-myristate-13-acetate (PMA; Sigma-Aldrich) was used as a positive control (maximum IFN-γ production), and DMSO; the peptide diluent, was used as a negative control (to quantify basal IFN-γ production). Tetanus toxoid (Massachusetts Department of Health), represented a second ubiquitous antigen used in the assay as a control for the specificity of an anticipated response to EBV. Tetanus, like EBV, will induce a T_H1 immune response (135, 155). The ELISpot assay was performed according to manufacturer's instructions (BD Biosciences). Each PBMC sample was run in triplicate. ELISpot plates were analyzed by Cellular Technologies Ltd (Cleveland, OH). For representative data-spots of an ELISpot assay, see Figure 17.

Transient Tat expression and EBV Reactivation

HH514-16 cells, a B-cell line latently infected with EBV (a kind gift from Dr. George Miller), were transiently transfected with a HIV-1 Tat-expressing vector. The vector was sequenced prior to transfection to confirm correct Tat sequence. HH514-16 cells were transfected with 50-75µg DNA by electroporation with settings of 0.25kV and 950µFarads. Additional cells were transfected with empty vector (pSVdhfr; ATCC), or mock-transfected with TE buffer. Mock-transfected cells as a positive control were treated with 4mM sodium butyrate (NaByr) to reactivate latent EBV. Sodium butyrate is a histone deacetylase inhibitor that induces latent EBV to undergo lytic replication (120). Untreated cells were included as a negative control. All cells were cultured for 12, 24 and 36h, judged sufficient time to allow expression of Tat. They were then collected and snap-frozen in microcentrifuge tubes until used in experiments described below.

Quantitative Reverse-Transcription PCR (qRT-PCR)

RNA was isolated from HH514-16 cells using the RNeasy kit (Qiagen), then reverse-transcribed to cDNA using Quantitect Reverse Transcription kit (Qiagen). Each cDNA was subject to qRT-PCR with primers and probes specific for two EBV genes, *BZLF1* and *gp350*, and two cellular genes, *GAPDH* and DNA polymerase β (*Polβ*) (Table 10). BZLF1 is a protein encoded by an immediate-early gene and essential for EBV replication to kick-start from latency. By contrast, *gp350* is a late-transcribed gene needed for virion assembly,

thus giving a measure of whether mature viral particles were produced. *GAPDH* is a ubiquitous cellular house-keeping gene commonly used for normalization. *Polβ* is a cellular gene upregulated in B-cells expressing HIV Tat (170), and used here as the positive control for Tat protein expression. Primers and probes were designed by Applied Biosystems (Table 10). RNA from each condition at each time point was analyzed in duplicate for each of the four genes described. The comparative C_t method ($2^{-\Delta\Delta Ct}$) was used to analyze the fold-changes in gene expression. The equation is $\Delta\Delta C_t = \Delta C_{t\text{-sample}} - \Delta C_{t\ \text{reference}}$ where $\Delta C_{t\text{-sample}}$ is the C_t value for a sample normalized to GAPDH, and $\Delta C_{t\ \text{control}}$ is the C_t value for the control, also normalized to GAPDH. For an example calculation, see Table 11.

Immunoblot Analysis

Transfected HH514-16 cells were washed with ice-cold PBS and collected in ice-cold RIPA buffer containing Protease Inhibitor Cocktail (Sigma). After 10 min rotation at 4°, lysates were clarified at 15,000 rpm for 15 min at 4°C. The protein concentration of each lysate was quantified using the BCA protein assay (Pierce). Equal amounts of protein from each sample were resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to PVDF, and subjected to immunoblotting with a monoclonal anti-BZLF1 antibody (Argene), or a polyclonal anti-Tat antibody (Santa Cruz Biotechnology) where indicated, and visualized with the SuperSignal West® Chemiluminescent Substrate kit (Pierce). Membranes were stripped and reprobed with a polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology) to evaluate loading consistency.

Data Analysis

Statistical tests were performed using SPSS version 12.

Results

HIV-positive people have more EBV DNA than HIV-negative people. The presence (or absence) of EBV DNA and the quantity were determined in PBMC of 70 HIV-positive and 25 HIV-negative people by qPCR specific for the EBV gene EBNA3. We first compared the number of HIV-positive people who were positive for EBV DNA by PCR to the number of HIV-negative people who were positive for EBV. A subject was considered positive for EBV DNA if greater than 1pg of EBNA3 was measured by qPCR. This value was above the lowest point on the standard curve and just at the limit of visual detection with ethidium bromide after agarose gel electrophoresis. Of the HIV-negative group, 28% were positive for EBV DNA. In stark contrast, 84% of HIV-positive people were positive for EBV DNA; a difference that is statistically significant (Table 12). Viremic patients tested positive for EBV DNA with greater frequency than aviremic patients (Table 12), and both viremic and aviremic groups separately tested positive with greater frequency than HIV-negative people (Table 12).

We then compared the mean amount of EBV DNA detected between each group. The mean amount of EBV DNA was greater in the HIV-positive group relative to the HIV-negative group (Fig. 18A). When comparing aviremic or viremic patients to HIV-negative donors, the amount of EBV DNA detected was consistently greater (Fig. 18B). There was little difference between aviremic and viremic patients in the amount of EBV DNA detected (Fig. 18B; $p= 0.07$ by Mann-Whitney U-Test).

Gel electrophoresis of all samples confirmed the specificity of the primers and generation of the correct product as judged by size (Fig. 19). There was no concern with regard to contamination as there are no non-specific bands in any samples tested, and the negative controls were consistently band-free (Fig. 19). A few patient samples did not have bands of consistent signal-strength (triplicates of lane 5, Fig. 20). We do not feel this is contamination because of the scarce nature of the target: when as little as 1 B-cell in a million are infected with EBV, the possibility that EBV DNA was left out of the small sample aliquoted into the PCR reaction tube is great. These data show that HIV-positive people have more EBV DNA, regardless of the amount of HIV RNA.

The immune systems of HIV-positive people recognize EBV as robustly as HIV-negative people. It is well known e.g. from the nature of post-transplant lymphoproliferative disease, that immune integrity is an important factor in continuing, lifelong surveillance and control of EBV replication. To determine if the elevated EBV DNA observed in HIV-positive people was due to immune dysfunction, we evaluated the ability of HIV-infected individuals to mount an immune response to EBV antigens, using an IFN-γ ELISpot assay. IFN-γ is secreted by memory CD4 and CD8 T-cells in response to viral peptide antigens and so this assay allowed us to assess immune integrity specific for EBV. The ELISpot data were analyzed in a similar manner to the EBV DNA results. First, the number of HIV-positive people who were positive for recognition of EBV antigens by ELISpot was compared to the number of HIV-uninfected people who were positive. A subject was considered positive for EBV recognition if the spot count was greater than twice the standard deviation of the mean spot count of the DMSO-treated cells across all subjects. Anyone negative for a response to PMA was excluded from analysis as PBMC were assumed dead if unable to produce IFN-γ to the strong stimulator. Only three people were excluded: all were HIV-negative.

There was no difference in the number of HIV-positive people responding to EBV antigens relative to HIV-negative people (Table 13). Also, there was no difference when comparing aviremic to viremic patients, as well as these two HIV-positive groups separately to HIV-negative donors (Table 13). The mean number of EBV-specific IFN-γ secreting CD4/CD8 T-cells responding to a pool of EBV antigens was also assessed. There was a large difference in the mean spot count between HIV-positive and HIV-negative people (Fig. 20A), and surprisingly, HIV-positive people as a whole had a greater number of EBV-specific T-cells among both lineages. This large difference is contributed mostly by viremic patients, as the difference between them and HIV-negative people is significantly larger (Fig. 20B; $p < 0.02$) than a borderline-significant difference between aviremic and HIV-negative people (Fig. 20B; $p = 0.054$). These data indicate that the cellular immune system in HIV infection, even when plasma HIV RNA is detected, is capable of recognizing and mounting a strong response to EBV.

If the immune response directed against EBV were skewed towards latent antigens as opposed to lytic antigens, then the immune response may be ineffectual

against lytically replicating EBV. We analyzed the number of HIV-positive and HIV-negative people with positive responses towards EBV lytic antigens in comparison to EBV latent antigens. There was no difference in the number of HIV-positive and HIV-negative people who responded to EBV lytic antigens (Table 14), as well as to latent antigens (Table 15). When comparing the aviremic group to the viremic group as well as each separately to the HIV-negative group, there was also no difference for either antigen class (Tables 14 and 15). In fact, the spot-counts produced in response to lytic antigens positively correlate with the spot-counts generated towards latent antigens whether aviremic, viremic, or HIV-negative (Fig. 21). Neither HIV-positive nor HIV-negative donors responded with any preference towards lytic versus latent antigens. In other words, the immune response towards EBV was not skewed towards lytic or latent antigens, but was robust in both instances, showing that the cellular immune response is to both classes.

As mentioned, HIV-positive people experience abnormal immune activation. This is long-term, and increasingly recognized as central to HIV pathogenesis, even in those being successfully treated with ART. To determine if the elevated response to EBV is a manifestation of generalized immune activation, we also tested the patients' ability to respond to another ubiquitous (though bacterial) antigen, tetanus toxoid, employing an analogous ELISpot assay. Not one HIV-negative person responded positively to the tetanus toxoid (0/5), and only 3 of 22 HIV-positive people responded positively. There was no difference in the mean spot count between HIV-positive and HIV-negative people, though the mean was higher for HIV-positive people (Fig. 22; $p= 0.2$ by Mann Whitney U-Test). It is important to note that the standard deviation of the mean for HIV-positive people is large, owing to one outlier who demonstrated a rather robust response to the tetanus toxoid (mean SFU of 112). Even with this outlier removed from analysis, there was no significant difference between HIV-positive and HIV-negative people ($p= 0.13$ by Mann Whitney U-Test). These data indicate that the immune response generated towards EBV is not simply a manifestation of generalized abnormal immune activation. They imply that the heightened response of CD4/CD8 lymphocytes towards EBV antigens was driven by and specific for EBV. Importantly, these data also show that the elevated EBV DNA observed in HIV-

positive people cannot be explained by EBV gaining ground in the absence of immune surveillance.

HIV Tat reactivates latent EBV. HIV Tat is a promiscuous transcription factor that penetrates uninfected bystander cells and has been shown to reactivate latent HHV-8 (184) by a mechanism(s) that remains unclear, but may involve JAK/STAT signaling (195). To determine if the increased EBV DNA load is due to increased EBV replication spurred by the action of HIV Tat, we transfected HH514-16 cells with a *tat*-expressing plasmid and measured induction of EBV gene expression by qRT-PCR. HH514-16 is a cell line derived from Burkitt's lymphoma, in which EBV manifests very low background levels of spontaneous reactivation (120), and in which EBV replication is easily and vigorously induced with sodium butyrate (NaByr). After 12h of NaByr-treatment, cells expressed 6-fold more BZLF relative to untreated cells. After 24h, BZLF expression substantially increased to almost 240-fold more, and increased to just over 280-fold more than control cells after 36h (Fig. 23A). BZLF expression was increased 15-fold in cells transfected with Tat after 24h. Unlike NaByr, Tat-induced BZLF mRNA expression declined between 24 and 36h to near the level expressed in cells transfected with empty vector (Fig. 23A). Transfection with the empty vector did not substantially increase BZLF expression relative to control cells at any time point (Fig. 23A). NaByr treatment caused a dramatic upregulation of mRNA expression of gp350 after 24- and 36h of NaByr treatment; the time course for gp350 induction appeared slower than for BZLF induction, as there was not a significant increase in gp350 mRNA after 12hrs of treatment (Fig. 23B). Transfection with Tat induced a modest increase of gp350 mRNA of approximately 3-fold after 24h relative to control cells, and similarly to BZLF mRNA expression, gp350 expression also declined between 24- and 36h (Fig. 23B). The empty vector did not induce any significant change in gp350 gene expression at any time point. Expression of the DNA Polymerase β (Polβ) gene increases in B-cells expressing Tat (170) so, as a positive control for Tat expression, induction of expression of this gene was also analyzed. Tat induced a considerable increase in Polβ mRNA expression, with nearly a 9-fold increase relative to control cells after 24h (Fig. 23C). Pertinently, expression of this gene was not elevated in either NaByr-treated cells or cells transfected with an empty vector (Fig. 23C). These data show that Tat can

induce EBV replication in a B-cell model by up-regulating BZLF mRNA expression. Though modest, the Tat-induced increase in gp350 mRNA expression confirms that EBV replication was not simply stalled at BZLF expression, but that it was firmly on the way to produce virions.

We performed western blot analysis to confirm expression of BZLF and Tat protein in the cells. There was no detectable BZLF protein after 12h NaByr treatment, but it was detectable after 24h, and even more so after 36h, (Fig. 24A), concomitant with the mRNA increase. Though Tat-transfection induced a 15-fold increase in BZLF gene expression, this was not reflected in any increase in BZLF protein (Fig. 24A). Lysates from either control cells or cells transfected with an empty vector showed no BZLF protein in agreement with the qRT-PCR data (Fig. 24A). In addition, we did not detect expression of Tat protein in cell lysates (Fig. 24B), despite there being an increase in Polβ mRNA.

Discussion

Herpesviruses, particularly HHV-8 and EBV, and their propensity to cause aggressive malignancies still remain a significant source of morbidity and mortality for HIV-infected people, even in the ART-era. It is widely believed that EBV-associated cancers occur in the HIV setting because of HIV-associated immune compromise, a situation that allows unrestrained proliferation of EBV-infected lymphocytes. The development of EBV-associated malignancies in HIV-infected people appears more complex, however, and there are likely multiple factors involved. We show that EBV DNA in the blood of HIV-positive people is significantly elevated relative to HIV-negative controls. Immunodeficiency cannot account for the higher EBV load as the cellular immune system in HIV-positive people responded robustly when challenged with EBV-encoded peptide antigens, *ex vivo*. Moreover, the response was specific as tetanus toxoid elicited a far lesser cellular response comparable to that of HIV-negative people. Therefore, we suggest that EBV replication may be up-regulated by the HIV accessory protein Tat as expression of EBV early and late genes (*BZLF* and *gp350*, respectively) was elevated after transfection of Tat in EBV-infected B-cells.

Using a stringent quantitative PCR assay maximized for a scarce target, we found that EBV DNA is detectable in HIV-infected people with almost a 3-fold greater frequency than HIV-negative people (Table 12). In addition, there is on average, almost 6-times more EBV DNA detected in HIV-infected people (Fig. 18). These data are consistent with a previous finding in our lab, that HIV-infected people have a greater number of EBV-infected circulating B cells (Ocampo, unpublished results). They are also consistent with previous findings of elevated EBV DNA load in patients' blood, PBMC, and saliva, (15, 108, 119, 134). We amplified EBV DNA from PBMC and so the amount measured reflects infection of circulating blood cells rather than freely circulating virus, viral debris, or virus harbored in solid organs and tissues. Additional work is needed to determine the cell-free EBV DNA load in the sera of HIV-infected people as well as that in lymph nodes, where the majority of B- cells, the primary target of EBV, reside.

There are several possible mechanisms that may drive the increased EBV DNA in HIV-infected people, with active EBV replication being the largest contributing factor.

EBV reactivation from latency back to lytic replication may result from the loss of immune control. Immune compromise can manifest as a physical loss of immune cells, such as in the progression of HIV disease. We did not find any correlation between CD4 T-cell counts and EBV DNA load in our patient population. In fact, the HIV-positive patients with undetectable HIV RNA (aviremic) had a mean CD4 T-cell count of 533 cells/μl blood, within normal limits. Therefore, the elevated EBV DNA cannot be explained by an absolute loss of immune cells, at least in aviremic patients. The average CD4 T-cell count among HIV-positive patients with detectable HIV RNA (viremia) was significantly lower than the aviremic patients (mean of 337 cells/μl blood; $p<0.006$ by independent samples T-Test), and while significantly more viremic patients had detectable EBV DNA, the mean DNA load was not significantly higher than in aviremic patients. This is in agreement with Ling *et al.* who also noted no significant differences in EBV detection and EBV load between HIV-infected people with high versus low CD4 T-cell counts (108). These data suggest a physical loss of immune control may play a small role in increasing EBV DNA load, but with other factors making additional contributions.

Immune compromise can also manifest as a qualitative loss of immune cell function. We examined EBV-specific immunity mediated by CD4/ CD8 T-cells in 70 HIV infected people. There was no difference in the number of HIV-positive versus HIV-negative people positively responding to the EBV antigens (Table 13). In addition, there was a significant difference between HIV-positive and HIV-negative people in the mean number of EBV-specific CD4/ CD8 T-cells, with HIV-positive people having the greater number of cells (Fig. 20). By contrast, the HIV-infected group responded to a different ubiquitous pathogen, *Clostridium tetani* (as Tetanus Toxoid) in the same manner as HIV-negative controls. That is, neither group contained a significant number of people responding measurably to tetanus antigens (Fig. 22). These data indicate that the response to EBV was not a demonstration of generalized abnormal immune activation, and that EBV-specific immunity is as robust in our cohort of HIV-infected people as for HIV-negative people. Additionally, there was no correlation between EBV DNA load and the number of EBV-specific CD4/ CD8 T-cells, again indicating that the elevated EBV DNA loads in HIV-positive people are not caused by physical or functional loss of EBV-specific immunity. It is important to note that the ELISpot assay tested EBV-specific *memory* CD4

and CD8 T-cells. Though immune memory to EBV remains intact in the HIV-positive patients, we cannot determine whether effector immune cells would respond appropriately to the signals provided by activated memory T-cells. This remains an interesting area for further investigation.

That immunity to EBV in our HIV-positive patients remains intact is in contrast to previous reports. Gasser *et al.* found that HIV-infected people lacked EBV-specific CD4 T-cell function as determined by ELISpot analysis, despite prolonged ART-induced normalization of the patients' absolute CD4 lymphocyte count (53). In another report, the number of EBV-specific CD8 T-cells was comparable between HIV-infected people and HIV-negative people, but the functional capacity of those cells was impaired in the HIV-positive cohort (182). Legoff *et al.* found that EBV DNA load correlated inversely with EBV-specific immunity as measured by the same assay employed here (106). van Baarle and colleagues observed in a group of HIV-infected people i) that most EBV-specific CD8 T-cells were directed against a lytic antigen, with much fewer directed towards a latent EBV epitope; and ii) that CD8 T-cell responses to both antigen classes declined with time. The authors concluded that loss of both lytic and latent antigen-specific CD8 T-cells could lead to an increase in EBV DNA (182). We noted no preference in the response towards lytic antigens relative to latent antigens (Fig. 21) and therefore cannot conclude that EBV DNA is elevated owing to a skewed immune response favoring poor viral control. One noteworthy difference between other reports and the findings presented here is that the HIV-infected patients who lost EBV-specific immunity had all progressed to non-Hodgkin's lymphoma or PCNSL, whereas we know of no EBV-associated malignancies in the present study to date. Speculatively, loss of EBV immunity coupled with an increase in EBV DNA load may serve as a marker for eventual development of EBV-associated malignancies in HIV-infected people.

Others have reported a reconstitution of herpesvirus-specific immunity in HIV-positive people taking ART. HIV-infected people treated for 55 months with ART experienced an increase in HIV-, CMV- and EBV-specific CD4+ T-cell proliferative capacity (80), and herpes simplex virus 2-specific T-cell immunity also improved with ART (145). All of the HIV-infected patients in our study were taking ART, a fact that may account for the robust EBV-specific immune response observed. Notably, however,

ART-induced recovery of herpesvirus-specific immunity is slow and incomplete (25, 141, 145). Though we chose EBV epitopes that are highly immunogenic, we only evaluated immunity towards a few epitopes and so we may have failed to detect immune abnormalities that may have appeared if additional antigens were utilized. Besides, ART (and by extension immune reconstitution) may not have a great influence on EBV loads, as one study showed that the ranges of EBV copies in patients who were or were not on ART did not differ significantly (130). Data from various cohorts show no significant decrease in the incidence of systemic non-Hodgkin's lymphoma (NHL) and Hodgkin's disease (HD) in those taking ART (79, 84, 103, 104, 186, 187). These varied findings highlight the complexity of EBV immunity as well as the mechanisms driving EBV loads and development of EBV-associated cancer in the HIV setting.

In addition to the role the immune system plays in EBV reactivation, HIV-associated factors, like Tat, may also drive EBV replication. We found that after transfection of HIV Tat into an EBV-infected B-cell line, EBV replication was induced as reflected in both BZLF and gp350 mRNA expression relative to control cells (Fig 23). The effect was specific to Tat as empty vector did not induce BZLF or gp350. Tat was not as efficient as NaByr in reactivating EBV, but there are multiple reasons that may explain this. First, NaByr causes global hyperacetylation of the histone-bound chromatin allowing very potent stimulation of gene transcription, including *BZLF*. As a transcription factor with specific gene targets, Tat would be restricted in its ability to induce EBV replication. Also, transfection efficiency, which was not directly evaluated, may have been low; HH514-16 cells are difficult to transfect (G. Miller, personal communication). In addition, EBV replication occurs most vigorously during the S-phase of the cell cycle. Zeng *et al.* achieved maximum HHV-8 replication after transfecting Tat into cells going through S-phase in synchrony (195). EBV replication may be stimulated to a greater degree if cells were synchronized during S-phase, although this would not reflect their natural *in vivo* state. Nonetheless, Merat *et al.* saw a consistent 6-fold induction of HHV-8 replication after Tat transfection (118). Therefore, the level of reactivation observed here likely accurately reflects the capability of Tat to provoke EBV replication.

A preliminary western blot analysis did not detect Tat protein production in the transfected cells (Fig. 24). Technical factors which may have played a role include poor

transfection efficiency, loss of Tat into culture supernatant, or loss of Tat to the insoluble fraction of cell lysates during clarification. Nonetheless, the other marker for Tat protein expression, mRNA of Polβ was heartily increased (Fig. 23), and pertinently, augmented only in Tat-transfected cells, indicating Tat protein was expressed. Western blot analysis did not confirm BZLF protein production in Tat-transfected cells either (Fig. 24) despite clearly increased expression of BZLF mRNA. In NaByr-treated cells, BZLF mRNA expression increased after 12h, but was also not detected by western blot at this time point (Fig. 24). BZLF protein was not detected until 24h after NaByr treatment, when the mRNA expression was approximately 230-fold greater than control cells (Fig. 23 and 24). We could not detect BZLF protein in Tat-transfected cells, similarly to NaByr-treated cells, likely because the amount produced was below the limit of detection of the assay.

Presumably, patients with higher HIV RNA loads, and therefore greater amounts of Tat, would have more EBV DNA than patients with less HIV RNA. We show that HIV-viremic patients had more detectable EBV DNA relative to HIV aviremic patients, but we did not find any correlation between HIV and EBV viral loads in the HIV-positive patients. Almost certainly, the amount of virus circulating is not reflective of the total viral burden as lymphoid tissue is a harbor for the bulk of both HIV- and EBV-infected cells. It is to be expected, therefore, that Tat within lymphoid tissue greatly exceeds the amount in serum, and so EBV replication may be the same. This scenario would explain the lack of correlation between HIV and EBV viral loads in the blood. Further investigation will focus on the specific mechanism by which Tat induces EBV replication *in vitro*, and determine the role Tat plays in EBV reactivation *in vivo*, including determining amounts of Tat in serum and lymph node biopsy tissue.

An important consideration is whether B cells encounter HIV Tat *in vivo*. First, though not typically thought of as permissive for HIV infection, HIV can productively infect B-cells, including both EBV-infected and EBV-naïve B-cell lines (39, 123). Intriguingly, EBV-infected B-cell lines produce higher amounts of HIV RNA than their EBV-negative counterparts (123). Tat is also released by infected cells and readily taken up by bystander cells; Tat has been detected in AIDS-related NHL B-cells (102). Tat is targeted to the nucleus, and from there it modulates a plethora of cellular functions. For example, Tat was shown to enhance the proliferation of germinal center B-cells (105), a

phenomenon that may augment EBV replication and development of lymphomas. Though B-cells represent the primary target of EBV infection, EBV can also infect other lymphocytes, such as CD4 and CD8 T-cells. This has been noted in such conditions as Chronic Active EBV (94), EBV-associated hemophagocytic lymphohistiocytosis (88, 173), and even in HIV infection in children (15). EBV would undoubtedly be exposed to products of HIV were it to infect CD4 cells that are co-infected. Future work will determine to what degree primary B-cells of HIV-infected donors contain HIV and express Tat. Which lineages in adults are co-infected with EBV and HIV; whether co-infection spurs either or both viruses to replicate; and the specific mechanisms by which this occurs; are all issues that deserve additional investigation.

There are also indirect mechanisms by which HIV may reactivate EBV. BAFF (B cell activating factor of the TNF family) was found in this laboratory to be elevated in sera from a small group of HIV-infected aviremic patients (Ocampo, unpublished results). This cytokine which promotes peripheral B-cell survival is produced by innate immune cells such as neutrophils, macrophages and dendritic cells. Excess BAFF may prevent the clearance of EBV-infected B cells, thereby elevating EBV DNA loads in the six patients tested for BAFF. We found a strong positive correlation between EBV DNA load and the amount of circulating BAFF ($R= 0.943$; $p<0.005$ by Spearman's rho), suggesting BAFF is associated with the accumulation of EBV-infected B cells in HIV-infected people, thus spurring development of EBV-associated lymphomas. Indeed, BAFF plays an important role in the accumulation of malignant B-cells in non-Hodgkin's lymphoma (NHL). He *et al*. showed that macrophages from patients with NHL express more BAFF, and that NHL B-cells express both BAFF and BAFF receptor. NHL B-cells thus receive paracrine and autocrine pro-survival signals that would promote massing of malignant NHL cells (70). It would also maintain EBV genomes within abnormal B-cells, giving EBV the foothold to replicate and contribute to persistence of malignant B-cells. Indeed, EBV contributes some selective advantage to Burkitt's lymphoma cells as well as Hodgkin's lymphoma cells (118). Also, normal B cells up-regulate BAFF expression when stimulated through CD40 (70), and as mentioned, the EBV gene product LMP1 behaves as a constitutively active mimic of CD40. A vicious cycle may then ensue, whereby reactivated EBV will induce BAFF production via LMP1, and BAFF will then

stimulate B-cell growth and survival, which in turn preserves EBV. Additional studies in HIV-infected patients will focus on BAFF, specifically evaluating serum and tissue BAFF levels, and determining the role BAFF may play in EBV reactivation and survival of EBV-infected B-cells in HIV-infected patients.

In addition to expression of BAFF, HIV infection is also accompanied by elevated interleukin-10 (IL-10). Interleukin-10 (IL-10) is produced by macrophages and activated T- and B-cells. It is up-regulated in HIV infection by several mechanisms, including extracellular Nef (21) and the viral protein gp41 (13). IL-10 does not up-regulate HIV replication, but it does assist HIV in evading immune attack. IL-10 potently induces a T_H2 immune response which, as discussed, is not as effective against viral pathogens as a T-cell based T_H1 response. Human IL-10 bears striking homology to an EBV gene product called BCRF1 (or viral IL-10, vIL-10) in both sequence and function (67, 166). Viral IL-10 binds the IL-10 receptor, can efficiently block macrophage activation, and stimulates B cell survival and proliferation (109, 127). Viral IL-10 is expressed during EBV lytic cycle replication, and is detectable in the blood during IM, just as human IL-10 is significantly upregulated during IM as well as in CAEBV and even in certain EBV-associated T-cell lymphomas (85, 132, 193). Therefore, those concomitantly infected with HIV and EBV are likely receiving "triple doses" of IL-10. Through IL-10, both HIV and EBV receive the benefit of suppressed antiviral responses, and EBV enjoys the advantage of enhanced B-cell survival and proliferation. Additionally, there is evidence that vIL-10 per se promotes B-cell transformation (127). Further investigation into the role IL-10 plays in HIV pathogenesis, including EBV-associated malignancies is warranted.

IL-10 is far from the only cytokine produced in abnormal amounts in HIV-infection. IL-6 is a pro-inflammatory cytokine produced by T-cells, B-cells and macrophages which tilts immune responses towards a T_H2 phenotype with stimulation of B cells, antibody synthesis, and humoral immunity. Up-regulation of IL-6 occurs by several mechanisms, including action of HIV as well as EBV. HIV drives production of IL-6 via collaboration of Tat with transcriptional co-activators acting on the IL-6 promoter (8). EBV induces IL-6 production via binding of gp350 to its receptor, CD21 on B cells, which activates multiple signaling pathways resulting in transcriptional up-

regulation of IL-6 (38). EBV-immortalized B cells express the IL-6 receptor and use this cytokine as a paracrine and autocrine growth factor (180). A similar feedback pattern to that for IL-10 emerges with IL-6: HIV and EBV collaborate to induce IL-6, i) contributing to abnormal immune activation and a reduced T_H1 response against the viruses; and ii) maintaining EBV-infected B cells with the potential for eventual malignant transformation. Elucidation of more specific roles for IL-6 in this phenomenon will be undertaken in additional studies.

In this report, we confirm previous observations of increased EBV DNA load in HIV-infected people. Our data suggest that immune control is but one cog within the host-cell machinery that should suppress EBV, and that HIV Tat undermines those efforts. There are a dizzying number of other factors in HIV infection likely involved in the induction of EBV replication and malignant transformation, including though not limited to other HIV proteins; EBV proteins such as LMP1 and vIL-10; and human BAFF, IL-6 and IL-10 (Fig. 25). Further exploration of the roles these proteins and others play in the development of EBV-associated malignancies is critical for enhancing the medical management of HIV disease.

A. **B.**

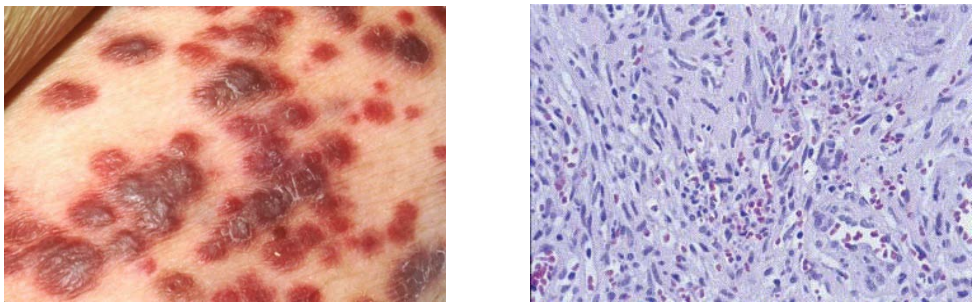

Figure 15. Kaposi's Sarcoma. (A) Plaques and nodules characteristic of KS, found on the skin of an AIDS patient. The lesions appear red-to-purple due to the highly vascular and angiogenic nature of the neoplasm. Image provided with permission by the National Cancer Institute, AV# 8500-3620. (B) Microscopic view of a KS lesion biopsied from the mandible of an HIV-infected person. Spindle cells are the characteristic cell type of KS. Poorly formed vascular spaces can be seen with entrapped erythrocytes. Image adapted from (129) © (2007) with permission from Elsevier.

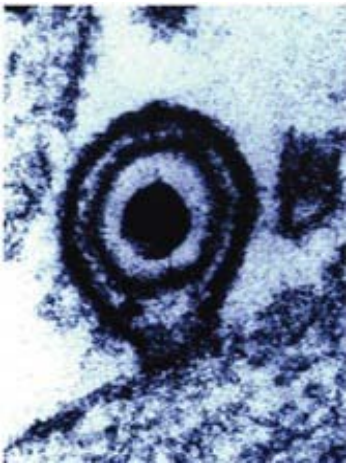

Figure 16. Electron micrograph of the Epstein-Barr virion. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (194) © 2004.

Table 8. EBV latency programs (128, 176).

Latency Program	Genes Expressed	Function
Latency I (Latency)	None (LMP2A[α] EBNA1[β])	Allows persistence of the virus in memory B cells in a manner that is non-pathogenic and invisible to the immune system
Latency II (Default)	EBNA1[β] LMP1[γ] LMP2A	Provides survival signals for infected B cells to differentiate into memory B cells Maintains persistently infected memory B cells
Latency III (Growth)	EBERs[δ] BARTs[ε] EBNA 1-6[β] LMP1 LMP2A	Activates a resting B cell to proliferate

[α], LMP2A; Latent Membrane protein 2A, 2B; constitutively active mimic of the B cell receptor.
[β], EBNA; EBV Nuclear Antigen -1, -2, -3A, -3B, -3C, -LP; transcription factors that maintain the viral episome, prevent immune recognition of infected cells, and induce cellular immortalization.
[γ], LMP1; Latent Membrane Protein 1; constitutively active mimic of CD40.
[δ], EBERs; EBV small RNAs; block interferon signaling to prevent apoptosis of infected cell.
[ε], BARTs; BamHI A rightward transcripts; modulate EBNA2 expression and modify cell signaling.

Table 9. Amino acid sequences and the proteins from which they are derived (italicized and underlined) of highly immunogenic EBV-encoded lytic and latent antigens used in the ELISpot assay.

Lytic Peptides	Latent Peptides
BRLF1 RALIKTLPRASYSSH	*LMP2* RRRWRRLTV PYLFWLAAI FLYALALLL
BZLF1 RAKFKQL	*EBNA 3A* QAKWRLQTL AYSSWMYSY RYSIFFDY FLRGRAYGL
BMLF1 GLCTLVAML	*EBNA 3B* AVLLHEESM IVTDFSVIK
BMRF1 FRNLAYGRTCVLGK	*EBNA 3C* EGGVGWRHW LLDFVRFMGV

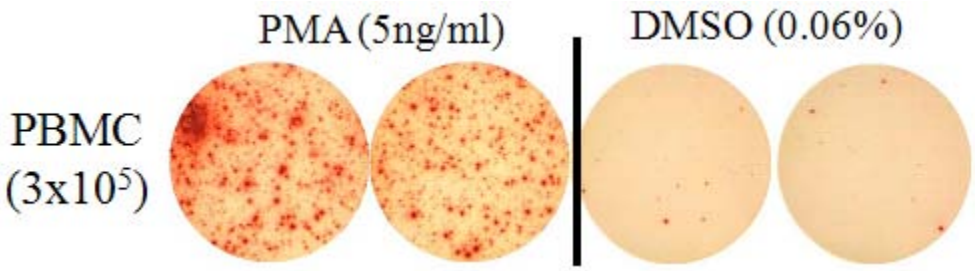

Figure 17. Example ELISpot results. $3x10^5$ PBMC were incubated overnight with either 5ng/ml PMA (positive control) or 0.06% DMSO (negative control). An ELISpot assay is similar to an ELISA, except the readout is spots, which are IFN-γ secreting CD4 and CD8 T-cells.

Table 10. Primer sequences used to detect genes in qPCR and qRT-PCR.

Gene		Primer Sequence
EBNA3	For:	TTT GCA GCC CAT ACA GGT TAT
	Rev:	TTC CTG TTT AGC AGT TCC TCC GC
BZLF1	For:	TGC AAT GTT TAG TGA GTT ACC TGT CT
	Rev:	TCT GTG ATG TCA TGG TTT GGG AC
	Probe:	AAA GCC AAG GCA CCA G
gp350	For:	CAG GTC TAC CCA GCT CTA CTC A
	Rev:	GGT GTT GGG CTG GTG ACA T
	Probe:	CCT CAC CGC ACC TGC
GAPDH	For:	proprietary
	Rev:	proprietary
	Probe:	ATT GGG CGC CTG GTC ACC AGG GCT G
Polβ	For:	proprietary
	Rev:	proprietary
	Probe:	TTG GGA GTC ACT GGA GTT GCA GGA G

Table 11. Example calculation of the comparative C_t method of determining fold-change in gene expression. In this example, expression of BZLF by cells treated with sodium butyrate (NaByr) for 36 hours was compared to expression of the same gene by control cells at the same time point. Control and NaByr-treated samples were normalized to GAPDH expression at the corresponding time point. Here, BZLF expression by NaByr-treated cells is approximately 200-fold greater than control cells after 36h of treatment.

Sample	**C_t (duplicates)**	**Mean C_t**	**ΔC_t (normalized C_t)**	**$\Delta\Delta C_t$**	**$2^{-\Delta\Delta Ct}$**
NaByr-treated 36 hrs GAPDH	17.62 16.82	17.22	(20.63-17.22) 3.41	(3.41-11.07) -7.66	$2^{-(-7.66)}$ 201.6
NaByr-treated 36 hrs BZLF	20.69 20.57	20.63			
Control 36 hrs GAPDH	17.68 15.23	16.46	(27.52-16.46) 11.07		
Control 36 hrs BZLF	27.08 27.96	27.52			

Table 12. Frequency of EBV DNA positivity in HIV-positive and HIV-negative people. P values were derived by the chi-squared test.

Comparison Groups	Percent EBV Positive (N)	P value
HIV+ vs. HIV-	84% vs. 28% (59/70 vs. 7/25)	<0.0001
Aviremic vs. Viremic	76% vs. 94% (28/37 vs. 31/33)	<0.05
Aviremic vs. HIV-	76% vs. 28% (28/37 vs. 7/25)	<0.0001
Viremic vs. HIV-	94% vs. 28% (31/33 vs. 7/25)	<0.0001

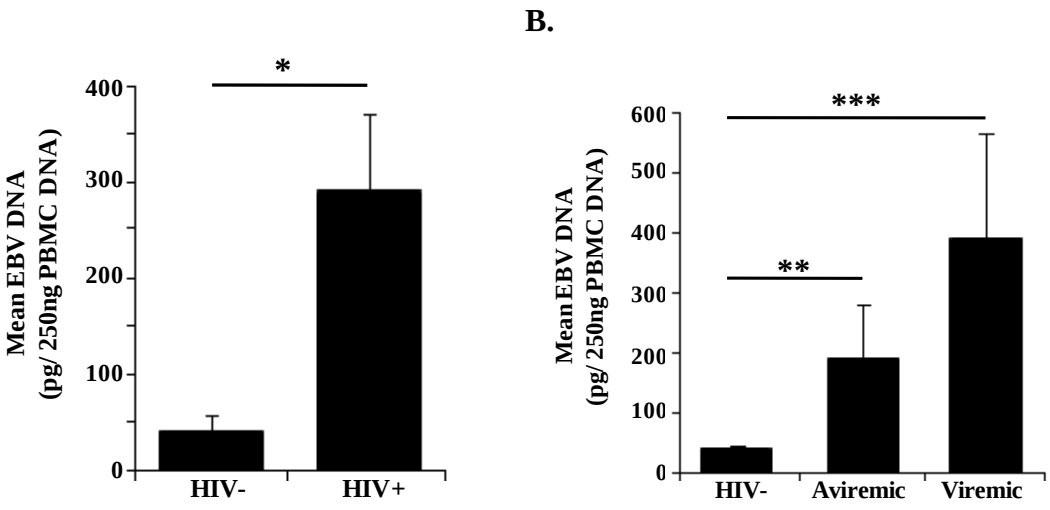

Figure 18. Mean EBV DNA detected by PCR in HIV-positive vs. HIV-negative people. (A,B) Values represent the mean ± SD of each group (*, $p < 0.02$ by independent samples T-Test; **, $p < 0.004$ and ***, $p < 0.0001$ by Mann-Whitney U test).

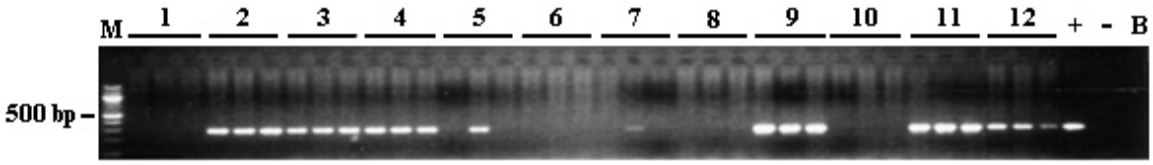

Figure 19. Quality control of qPCR samples. All DNA samples analyzed by qPCR were electrophoresed in 2% agarose to confirm primer specificity, correct product size (277bp) and lack of contamination. M, Marker (DNA ladder); 1-12, DNA samples from patients analyzed in triplicate; +, 1ng IB4 DNA, representing one point on the standard curve, also a positive control; -, 250ng Ramos DNA (negative control); B, Blank (no template PCR control).

Table 13. Frequency of ELISpot positivity in HIV-positive and HIV-negative people. P values were derived by the chi-squared test.

Comparison Groups	Percent ELISpot Positive (N)	p value
HIV+ vs. HIV-	59% vs. 44% (41/70 vs. 11/25)	= 0.25
Aviremic vs. Viremic	54% vs. 64% (20/37 vs. 21/33)	= 0.47
Aviremic vs. HIV-	54% vs. 44% (20/37 vs. 11/25)	= 0.61
Viremic vs. HIV-	64% vs. 44% (21/33 vs. 11/25)	= 0.19

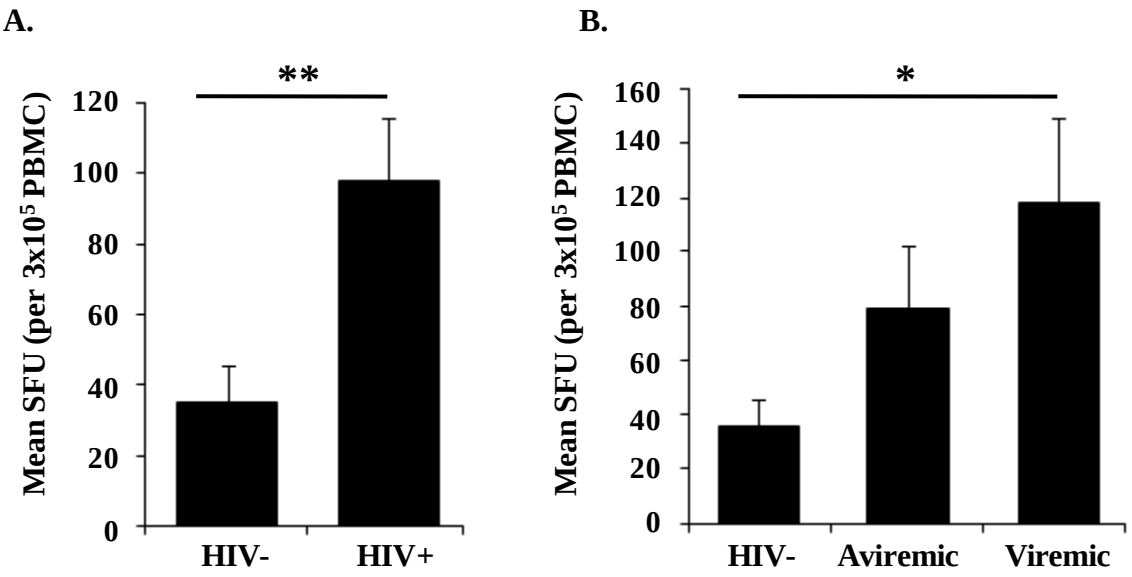

Figure 20. Mean EBV-specific IFN-γ secreting CD4/ CD8 T-cells in HIV-positive and HIV-negative people. (A,B) Values represent the mean spot-forming-unit (SFU) ± SD of each group (*, $p < 0.02$; **, $p < 0.004$ by independent samples T-Test).

Table 14. Comparison between HIV-positive and HIV-negative people of the frequency of ELISpot positivity for EBV *lytic* peptide antigens. P values were derived by the chi-squared test.

Comparison Groups	Percent ELISpot Positive for Lytic EBV Peptides (N)	p value
HIV+ vs. HIV-	22% vs. 26% (2/9 vs. 10/39)	= 0.99
Aviremic vs. Viremic	20% vs. 29% (3/15) vs. (7/24)	= 0.71
Aviremic vs. HIV-	20% vs. 22% (3/15 vs. 2/9)	= 0.99
Viremic vs. HIV-	29% vs. 22% (7/24 vs. 2/9)	= 0.99

Table 15. Comparison between HIV-positive vs. HIV-negative people of the frequency of ELISpot positivity for EBV *latent* peptide antigens. P values were derived by the chi-squared test.

Comparison Groups	Percent ELISpot Positive for Latent EBV Peptides (N)	p value
HIV+ vs. HIV-	22% vs. 21% (2/9 vs. 8/39)	= 0.99
Aviremic vs. Viremic	13% vs. 25% (2/15 vs. 6/24)	= 0.45
Aviremic vs. HIV-	13% vs. 22% (2/15 vs. 2/9)	= 0.62
Viremic vs. HIV-	25% vs. 22% (6/24 vs. 2/9)	= 0.99

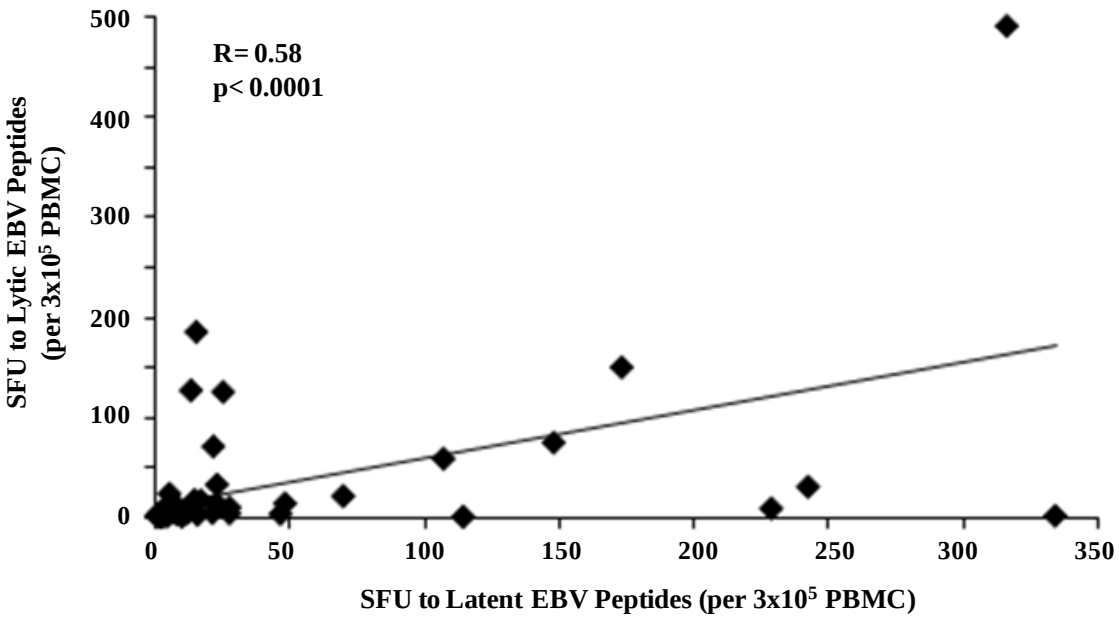

Figure 21. IFN-γ secreting CD4/ CD8 T-cells specific for lytic and latent EBV peptide antigens. Values represent the spot-forming unit (SFU) of all individual subjects ($p < 0.0001$ by Spearman's rho).

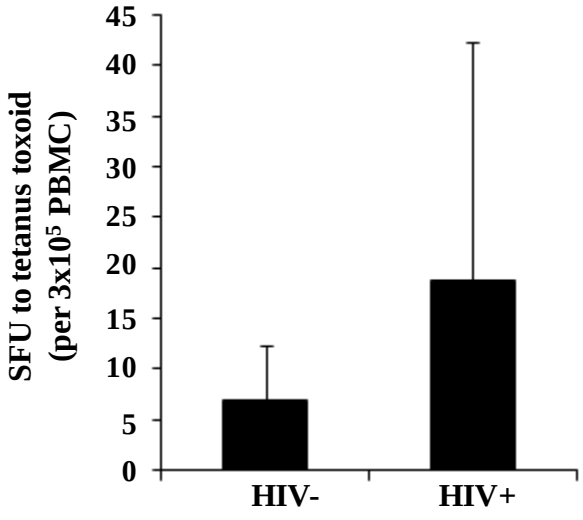

Figure 22. Tetanus-specific IFN-γ secreting CD4/ CD8 T-cells in HIV-positive and HIV-negative people. Values represent the mean spot-forming-unit (SFU) ± SD of each group.

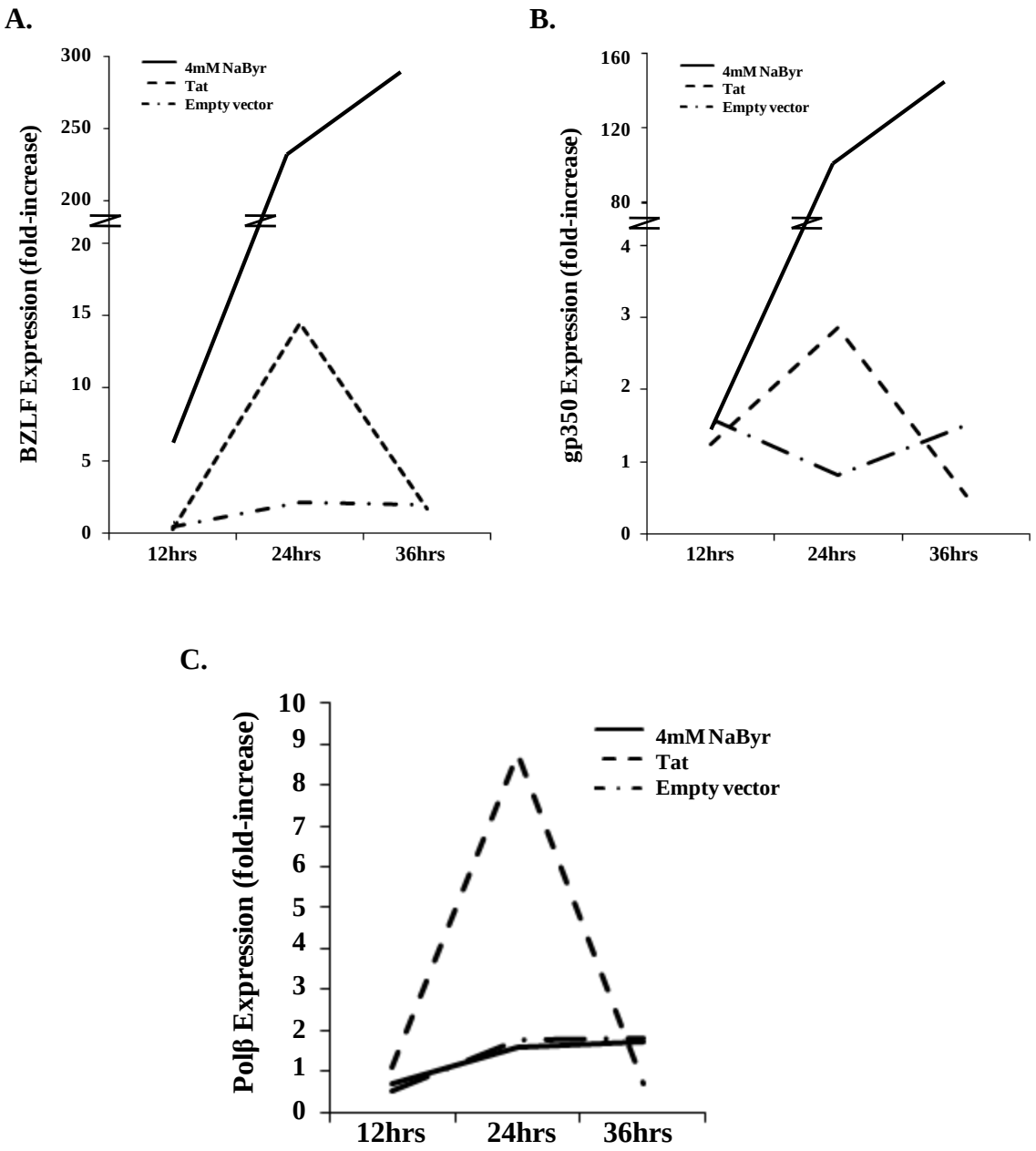

Figure 23. EBV gene expression in NaByr-treated and Tat-transfected HH514-16 cells. (A-C) Fold increase in BZLF (A), gp350 (B), and Polβ (C) gene expression. Samples were normalized to GAPDH, and the fold-increase is relative to control cells at each corresponding time point, calculated by the comparative C_t method.

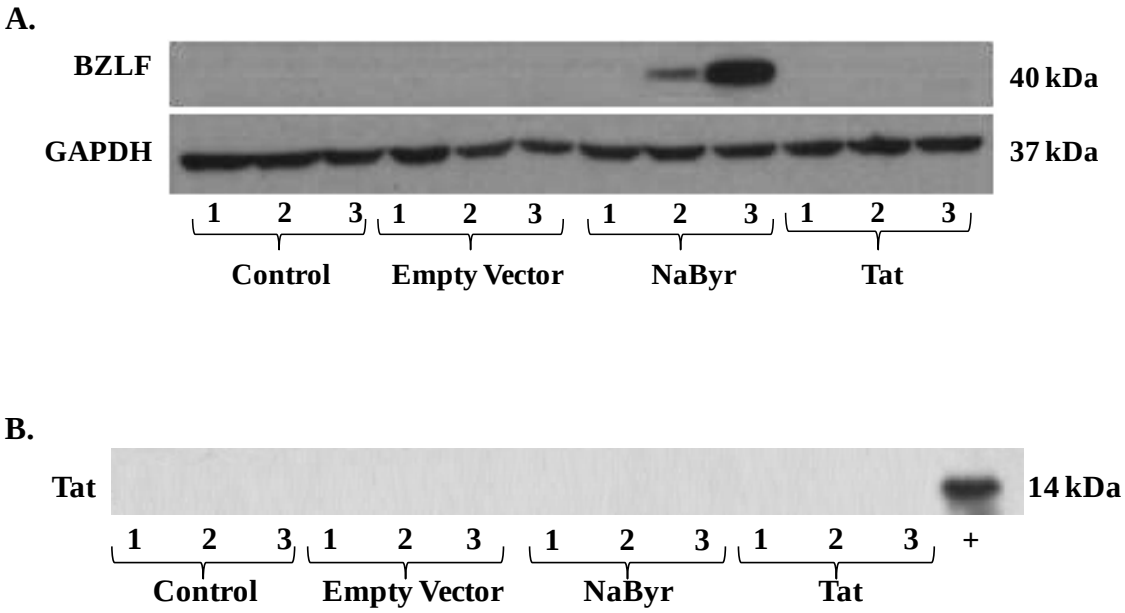

Figure 24. BZLF and Tat expression in HH514-16 cells. (A-B) Immunoblot of lysates collected from control cells, cells treated with 4mM Sodium Butyrate (NaByr) and cells transfected with HIV Tat or an empty vector. (A) BZLF expression. (B) Tat expression. (1, 12h; 2, 24h; 3, 36h).

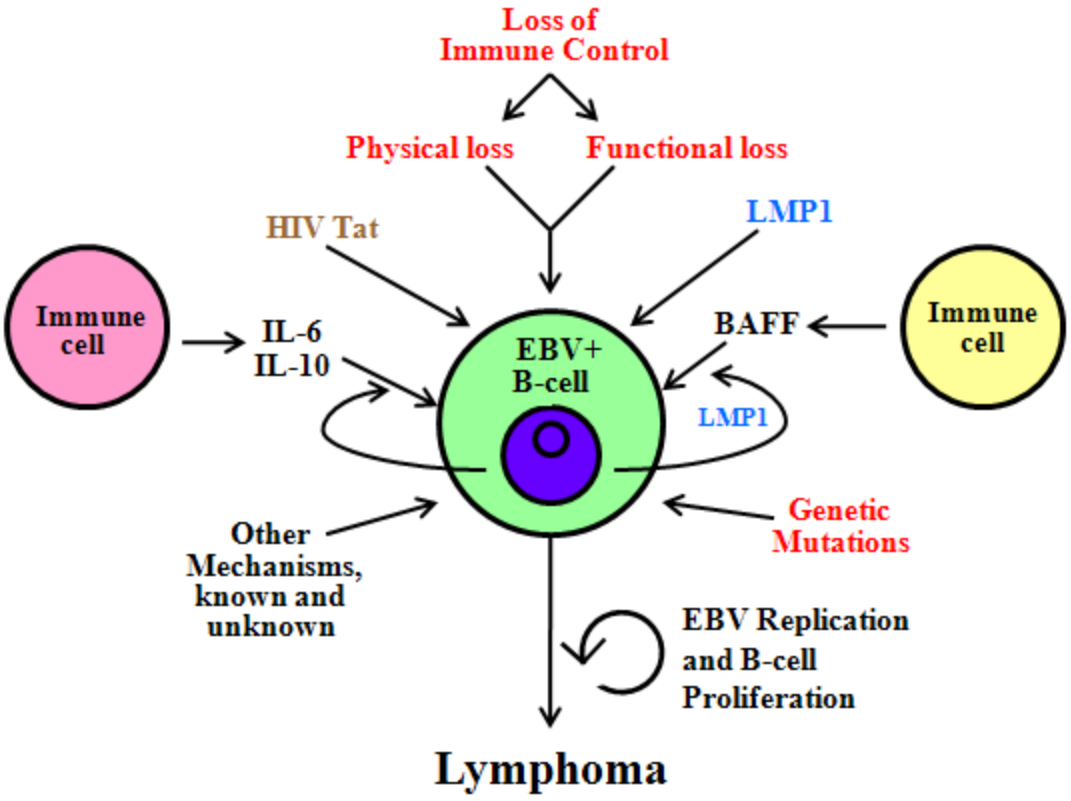

Figure 25. Potential molecular mechanisms for development of EBV-associated malignancies in HIV infection. Host factors are red, HIV-associated factors are brown, EBV-associated factors are blue, and factors associated with multiple sources are black. Loss of immune control, by physical and/or functional loss of immune cells allows uninhibited EBV replication. HIV Tat can reactivate EBV directly and may also have indirect roles. LMP1 has multiple effects on EBV-infected B-cells, including inducing production of BAFF, which serves to activate B-cell, and in turn, EBV replication. Cytokines like IL-6 and IL-10 also stimulate B-cells, and therefore EBV replication as well. Genetic mutations, as well as other mechanisms, both cellular and viral in nature, spur B-cell and EBV propagation, eventually leading to development of lymphoma.

General Discussion

Nearly 40 million people worldwide are infected with HIV and the assault on humanity has been distressingly difficult to counter. No disease has provoked a similar enlistment of political, financial, and human resources (3) and the best results at blunting the epidemic have been the development of antiretrovirals. Though ART has dramatically changed the course of HIV disease, significant complications remain. These experiments sough to better understand two aspects of HIV pathogenesis: HIV-driven insulin resistance and Epstein-Barr virus-associated malignancies. These are different important considerations in the clinical management of HIV disease that have not proved as responsive to therapy as others. We have shown that HIV Nef disrupts adipocyte-mediated glucose homeostasis by altering insulin-stimulated uptake of glucose and trafficking of GLUT4. We have also shown an increased EBV DNA load in HIV-infected people which cannot be explained by loss of immune control, but may be explained by action of HIV Tat. We have identified a multitude of issues relating both to insulin resistance and to EBV replication in the HIV setting that deserve additional attention:

- Elucidate the specific mechanisms by which Nef alters insulin-stimulated signaling cascades, cortical actin organization, GLUT4 trafficking and glucose uptake, and identify proteins interacting with Nef that mediate these changes.
- Define the amino acid sequences that mediate each effect of Nef, including the role myristoylation may play in adipocyte dysfunction.
- Establish clinical relevance *in vivo* of Nef-induced derangement of glucose homeostasis.
- Determine whether and how Nef accumulates within adipocytes, including any role the macrophages residing in adipose tissue may play.
- Better define the EBV DNA load in HIV-infected patients, particularly within B-cells harbored in lymphoid tissue.
- Characterize in more detail how and when EBV-specific immunity declines to allow EBV reactivation during HIV infection.
- Determine whether and how EBV may be exposed to Tat *in vivo*, and the specific mechanism by which Tat induces EBV replication.

- Elucidate the roles that cellular and viral proteins play in enhancing both HIV and EBV replication and infectivity.

This list is by no means exhaustive. In enhancing the knowledge pertaining to HIV-associated insulin resistance and EBV-associated malignancies, more efficacious therapies against HIV and EBV can be developed.

Nef as a Drug Target

Owing to its central role in HIV pathogenesis, Nef appears as an attractive therapeutic target. The initial focus for a Nef-based therapeutic intervention was a *nef*-deleted attenuated virus vaccine. Interest for this type of therapy evolved from the observation that many long-term non-progressors are infected with viruses bearing mutations and deletions of the *nef* gene. However, there are others defined as slow-progressors who harbor *nef*-deleted mutants yet do gradually advance to AIDS. This renders a *nef*-deleted attenuated vaccine too dangerous.

The foremost problem with targeting Nef is the overwhelming complexity of its multiple functions. That Nef mediates most of what it does by employing separate and distinct modalities, it is likely that any drug will only debilitate one or two functions of Nef, rather than Nef in general. Therefore, the most effective drug would need to disable the function of Nef that contributes most to pathogenesis, either down-regulation of CD4 or enhancement of viral infectivity being the best candidates. Another obstacle in targeting Nef is that it has no known enzymatic activity, mediating its many effects through protein–protein interactions (PPI). Though interest in PPI inhibitors has recently sparked with improved mapping techniques of molecular binding surfaces, it is a newly developing field. Recently, Betzi *et al.* identified a small library of drug-like compounds that bound Nef (in the micromolar range) within its SH3 domain and also competed for Nef–SH3 interactions in cell-based assays (16). Unfortunately, the molecules only weakly prevented down-regulation of MHCI, with limited ability to prevent CD4 down-regulation.

All potential targets for countering HIV, including Nef, should be considered, no matter the challenges. In light of the recently failed Merck vaccine trial and the dim

prospect of developing any effective vaccine in the near future (165), there isn't any other current option.

Targeting Epstein-Barr Virus-associated Tumor Cells

Actively replicating EBV can be efficiently controlled by a competent immune system. Indeed, the only treatment for IM is palliative care. Acyclovir, which is used in the treatment of herpes simplex 1 and 2 infections, can modestly inhibit EBV replication and reduce viral shedding, but has no significant effect on the symptoms of IM (183), and is therefore not typically utilized. At any rate, acyclovir is only effective against replicating EBV as it serves as a nucleoside analogue that targets DNA polymerase to terminate viral DNA elongation. It is the fact that EBV lives predominantly in a dormant state that makes it such a formidable challenge to control pharmacologically.

One approach for cancer therapy is to induce EBV replication, either chemically or by delivery of EBV lytic cycle genes, so that acyclovir will be effective. Additionally, the EBV- infected B-cell is usually killed in the process of viral reactivation. Lytic reactivation may, therefore, be used as a strategy to destroy EBV-positive tumor cells. One difficulty to this approach is that lytic replication must only be induced in tumor cells, as EBV-infected non-tumor cells could also reactivate.

Alternatively, hydroxyurea has been shown to induce the loss of episomal EBV *in vitro* and has shown clinical promise in treating AIDS-related PCNSL (34, 168). Hydroxyurea is an anti-neoplastic drug that selectively inhibits ribonucleoside diphosphate reductase, an enzyme required to convert ribonucleoside diphosphate into deoxyribonucleoside diphosphate. This prevents cells from leaving the S phase of the cell cycle. The mechanism by which hydroxyurea causes the loss of EBV episomes is not well understood, but may be related to alterations in the timing of EBV replication during the S phase of the cell cycle. Zhou *et al.* recently showed that EBV replicates in mid- to late S phase and that hydroxyurea accelerated the replication timing of EBV, which reduced viral genome stability (198). Regrettably, resistance to hydroxyurea has been documented *in vitro*, by upregulation of ribonucleoside diphosphate reductase (81).

Another approach is to target individual EBV proteins, such as LMP1. Antisense RNA targeted to LMP-1 was shown to suppress LMP1 expression in EBV-infected

lymphoblastoid cell lines which correlated with the down-regulation of LMP-induced anti-apoptotic genes and stimulation of apoptosis, cell proliferation inhibition, and improvement in the sensitivity to etoposide, a chemotherapeutic agent (91). EBNA1 is also an appealing target as it is the one viral protein expressed in all EBV-positive tumors. Antisense RNA against EBNA1 partially suppressed EBNA-1 protein expression and inhibited proliferation of EBV-immortalized lymphoblastoid cells (154). Dominant-negative mutants of EBNA1 have also been developed that can substantially inhibit wild-type EBNA-1 function (98). The clinical relevance of antisense EBNA1 and dominant-negative EBNA1 has yet to be established.

Immunotherapy directed against EBV-positive tumors is an attractive approach as the immune system plays such as important role in controlling EBV. Indeed, bone-marrow transplant patients infused with autologous EBV-specific CD8 T-cells stimulated and expanded *in vitro* experienced significant success in treating and preventing PTLD (64, 153). However, the majority of these stimulated effector cell preparations are primarily specific for immunodominant epitopes of EBV (194), which are not necessarily expressed in all EBV-associated tumor cells. Another problem with this approach is that T-cell-mediated immune attack could still be thwarted by immunosuppressive cytokines, like IL-6 and IL-10.

EBV has gained substantial notoriety for its leading role in diverse and particularly aggressive malignancies in HIV-infected people. Many mechanistic insights concerning the biology of EBV and its ability for malignant transformation have been elucidated. The challenge now is to exploit these insights to develop novel therapies for treating virus-associated disease.

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