LYMPHATIC ENDOTHELIAL CELLS EXPRESS VIRAL ENTRY RECEPTORS AND RESTRICTION FACTORS

by

Christopher D. Bowen

B.S. in Biology, Pennsylvania State University, 2008

Submitted to the Graduate Faculty of

Infectious Diseases and Microbiology

Graduate school of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2011

UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

This thesis was presented

by

Christopher D. Bowen

It was defended on

July 14th, 2011

and approved by

Tianyi Wang, PhD
Assistant Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

Robert E. Ferrell, PhD
Professor, Department of Human Genetics
Graduate School of Public Health, University of Pittsburgh

Thesis Advisor: Todd A. Reinhart, ScD
Professor, Department of Infectious Diseases and Microbiology
Graduate School of Public Health, University of Pittsburgh

LYMPHATIC ENDOTHELIAL CELLS EXPRESS VIRAL ENTRY RECEPTORS AND RESTRICTION FACTORS

Christopher D. Bowen, M.S.

University of Pittsburgh, 2011

Lymphatic endothelial cells (LECs) line lymphatic vessels and are present at mucosal portals of entry for many pathogens, including simian immunodeficiency virus (SIV) and human immunodeficiency virus type-1 (HIV-1). Recent studies have shown that LECs express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), capable of recognizing pathogen-associated molecular patterns (PAMPs). PAMPs are structurally similar molecules expressed by groups of pathogens. LECs have also been shown to express chemokines, a group of small molecules secreted by cells that induce chemotaxis in responsive cells, such as CCL21, which is used by $CCR7^+$ mature antigen presenting dendritic cells (DCs) to migrate to draining lymph nodes (LNs). These previous findings indicate that LECs might play an integral role in innate immune responses to a wide variety of microbes. In this study, I set out to characterize the expression of antiviral restriction factors as well as possible viral entry receptors for SIV/HIV-1 within three populations of human LECs. Real-time RT-PCR and immunofluorescent staining techniques were used to determine the relative expression of the restriction factors BST-2/Tetherin, APOBEC3G, and TRIM5-α. All of these factors have been shown to inhibit the replicative cycle of HIV-1 and have othologs present in nonhuman primates (NHPs). Expression of the viral entry receptors CD4, CXCR4, CCR5, DEC-205/CD205, D6/CCBP2, and CD209 as well as the LEC-specific markers podoplanin and LYVE-1 was also investigated. In addition, LEC populations were exposed to SIV, HIV-1, and markers

internalization to determine to what extent LECs interact with virus *in vitro*. Data from populations exposed to HIV-1 as well as other substrates for internalization of extracellular materials illustrate the ability of LECs to actively monitor the extracellular milieu. LECs exposed to SIV showed multi-spliced viral transcripts possibly due to *de novo* transcription. Taken together, this study provides evidence that LECs are equipped with tools not only to bind and internalize pathogens, but may also serve as a low-level replicative cellular substrate for virus. Further studies to characterize LECs are of great public health relevance, particularly at mucosa sites of microbial exposure, due to their potential roles during transmission/infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to sincerely thank Dr. Reinhart for his constant guidance and support to my project. In the short two years that I have been a part of his laboratory, he has helped to shape me into a professional scientist. The enthusiasm for science and learning environment in lab was palpable and has made me a better researcher. I would also like to thank my committee members, Dr. Tianyi Wang and Dr. Robert E. Ferrell for their gracious time and thoughtful suggestions to better my project.

Also, thank you to the members of the laboratory; Beth Junecko, Shulin Qin, Cynthia Klamar, Carissa Lucero, Stella Berendam, and Lauren Sciullo for project related and un-related conversation. The beginning of my scientific career has been shaped by the best of people, and I am forever grateful. Special thank you to Deena Ratner and Dr. Phalguni Gupta for supplying virus stock and work space and to Courtney Zych and Dr. Velpandi Ayyavoo for tagged virus. To the faculty, staff, and students of IDM, I am thankful for the relationships formed and the openness of this department, which has truly been enjoyable to be a part of.

Finally I would like to thank my parents for the constant support and love that has encouraged me to always challenge myself, and strive to be better. I am also appreciative of the love and support from my sister, who has always been supportive, as well as my family and friends. Thank you all.

1.0 INTRODUCTION

1.1 LYMPHATICS

The lymphatic system is a collection of vessels and organs that serves as a unidirectional conduit for lymph, a fluid from peripheral tissues containing excess water, cellular waste products, protein, lymphocytes from peripheral tissues, and other macromolecules (105). These lymphatic vessels serve to regulate the amount of fluid present in peripheral tissues and removes excess fluid that is eventually returned to the blood stream via the left and right lymphatic ducts (34). The lymphoid organs (i.e. thymus, spleen, and bone marrow) serve as the main production sites of lymphocytes that actively survey peripheral tissues for foreign pathogens (111). The lymphatic network functions in a similar manner to the blood vascular system, and as such has distinct yet parallel characteristics.

Lymphatic vessels are lined with a specialized population of epithelial cells termed lymphatic endothelial cells (LECs) which function similarly to the related blood vascular endothelial cells (BVECs) which line the interior wall of blood vasculature creating a smooth

inner surface ideal for free flowing of blood (92,111). Although similar to BVECs, LECs display unique markers and function in a markedly different manner (38). LECs line the lymphoid vessels responsible for the drainage of interstitial fluid from the periphery to secondary lymphoid tissues. Mucosal portals of entry, used by HIV-1 and other disease-causing pathogens, are ever changing environments that display a complex interaction between host and agent. Lymphatic structures are key features associated with the mucosa, such as the gut-associated lymphoid tissue (GALT), bronchus-associated LT (BALT) (102), and eye-associated LT (EALT) (104) along with many other specialized tissues, commonly referred to collectively as mucosa-associated lymphoid tissue (MALT) (103) (Figure 1). Due to the fact lymphatic vessels function as a conduit for interstitial fluid, LECs are much more permeable to allow for the uptake of fluids and molecules from the periphery. There is evidence that lymphatic vessels have a poorly developed basement membrane and are not associated with pericytes, a connective tissue cell population associated with the support of blood vessels (34). LECs also express the homeostatic chemokine CCL21, used by mature, antigen loaded $CCR7^+$ APCs during migration to secondary lymphoid tissues (32). Not only do LECs secrete immune regulating molecules, they also express functional TLRs that can recognize PAMPs implying a more active role in immune surveillance (23).

The lymphatic system is important in a number of conditions including the build-up of interstitial fluid in peripheral compartments (lymphedema) as well as metastasis of tumors (34,38). Tumor vascularization by lymphatic vessels is often a measure of prognosis. The lymphatic system can be damaged to the point where proper functioning is impaired. Decreased ability to remove excess lymph can lead to pooling of fluid in peripheral extremities. A singular

cause of primary lymphedema is still unknown, although a growing genetic relationship, including missense mutations in the vascular endothelial growth factor receptor 3 (VEGFR-3) has been observed (106). During times of increased inflammation, such as during radiation therapy, surgery, or LN dissection, secondary lymphedema can occur resulting in similar lymphatic defect. The afflicted tissues are often susceptible to infection as a result of this condition because lymphocyte migration is disrupted (107). There is also evidence that LECs themselves are involved in infection by different pathogens. Kaposi's sarcoma-associated herpesvirus, a highly vascularized tumor-causing virus, infects endothelial derived spindle cells and seems to up-regulate the expression of LEC specific markers (108). There is even evidence that shows endothelial cells express the receptor for influenza virus (109).

Until recently, it had been difficult to isolate pure populations of LECs from BVECs. With the identification of LEC specific markers, studies on homogenous populations are now capable. LECs can be isolated from mixed endothelial populations by using antibodies to surface molecules. VEGFR-3, LYVE-1, podoplanin, and Prox1 have all been shown to be effective markers for isolating LECs (35,36,37,38).

1.1.1 LYMPHATIC ENDOTHELIAL CELL MARKERS

LEC research in vivo can be very difficult as the lymphatic system is very complex. Therefore, in vitro studies have been used to characterize LECs. Until recently, it had proved difficult to adequately isolate LECs, but with the discovery of the LEC specific markers LYVE-1, podoplanin, VEGFR-3, and Prox1, *in vitro* experiments are now possible.

1.1.1.1 LYVE-1

Lymphatic endothelial hyaluronan receptor-1 (LYVE-1) is a receptor for the glycosaminoglycan hyaluronan (an integral protein in extracellular matrix). LYVE-1 is thought to play a role in lymphangiogenesis due to its ability to interact with the extracellular matrix (ECM). Hyaluronan is thought to be important both in tumor metastasis and migration of leukocytes (39). LYVE-1 is generally thought to aid in the turnover and processing of hyaluronan possibly indicating a potential mechanism by which LECs contribute to tumor metastasis and immune migration. LYVE-1 has been shown to be present on LECs while BVECs are negative (5).

1.1.1.2 PODOPLANIN

Podoplanin is expressed on LECs but not BVECs, making it an important marker (5). A mucin-type-I, 43 kDa integral membrane glycoprotein, podoplanin is thought to function as a regulator of lymphatic vascular formation as well as platelet aggregation (6). The EDxxVTPG extracellular domain of podoplanin (platelet aggregation-stimulating domain, (PLAG) has been shown to be important in platelet aggregation, particularly the threonine residue (40). Platelet aggregation is essential for separation of lymphatic vessels and blood vessels, making podoplanin an important factor critical for proper LEC function (7). Podoplanin has been shown to be expressed by glomerular podocytes and Bowman's capsule epithelial kidney cells along with LECs, rat neuronal, alveolar type I cells, mouse keratinocytes, and epithelial cells (41,42,43,98). P odoplanin has also been shown to play a critical role in tube formation, as lymphangiogenesis is inhibited by RNAi silencing of podoplanin (44). Similar inhibition of lymphangiogenesis and tube formation is seen when an inhibitor of the GTPase RhoA pathway

in the human primary lung endothelial cell population HMVEC-LLy (44). RhoA is known to regulate the cell cytoskeleton indicating a potential role in cytoskeleton regulation by podoplanin signaling.

1.1.1.3 VEGFR-3

The lymphatic vasculature plays a critical role during cancer progression, as invasion of this system can lead to metastasis of tumors. Expression of the growth factors VEGF-C and VEGF-D by tumor cells can lead to lymphangiogenesis and tumor metastasis (29,30). Vascular endothelial growth factor receptor 3 (VEGFR-3) is a member of the *fms*-like tyrosine kinase family and binds to ligands VEGF-C and VEGF-D, but not VEGF-A (4). VEGFR-3 has been shown to be expressed on both blood and lymph vessels during embryogenesis. However, VEGFR-3 expression becomes restricted to LECs in post-embryonic tissue (36). Incubation of VEGFR-3^+ cells with VEGF-C and VEGF-D has been shown to induce proliferation of an LEC-specific lineage (4,34). VEGFR-3 has been shown to be important in the induction of sprouting angiogenesis, in which endothelial cells encounter growth factors causing the dissociation of the basement membrane, allowing endothelial cells to migrate and proliferate "sprouting" newly formed lymphatic tubes (45). LECs have been shown to secrete VEGF-C, indicating possible autocrine modulation of LECs (45).

1.1.1.4 PROX1

The evolutionarily conserved transcription factor Prospero-related homeobox-1 (Prox1) has also been shown to be an effective marker of LEC populations. Prox1 has been shown to repress the characteristic BVEC-specific markers induced by Notch (arterial) and COUP-TFII (venous) on BVECs and induce the expression of lymphatic endothelial cell markers leading to the

derivation of LECs from a progenitor source (47,48). The *Drosophila melanogaster* equivalent to human Prox1 is *Prospero,* which has been shown to control neural cell development (46). Prox1 is also thought to be a tumor suppressor and has been shown to bind the liver receptor homolog-1 (LRH-1), a member of the *fushi tarazu* factor-1 sub-family of nuclear receptors, resulting in the repression of the bile acid-synthesizing enzyme Cholesterol 7-α-hydroxylase (CYP7A1) (49).

1.1.1.5 RELEVANCE

Early time points in the transmission of HIV-1 are of great interest to researchers today. Due to the fact that LECs are in close juxtaposition to mucosal barriers where transmission of many pathogens, including SIV and HIV-1 (Figure 1) occurs, studies aimed at characterizing LECs could prove to be important in understanding these early time points of infection. Determining if LECs expressed viral entry receptors capable of binding and internalizing virus, and if LECs expressed restriction factors capable of inhibiting viral replication would be important in characterizing this cellular population and discerning if LECs can recognize virus. If LECs were actively taking up virus, they could potentially elicit an immune response or could serve as a compartment of replication. Interactions between LECs and virus, in particular SIV and HIV-1, has huge implications, as the worldwide AIDS epidemic is a major public health concern, and therapeutic vaccines still elude us. LEC research in vivo can be very difficult as the lymphatic system is very complex. Therefore, *in vitro* studies, using podoplanin and LYVE-1^{+} primary dermal, lung and long-lived hTERT-transduced LEC populations could evaluate the relationship between virus lymphatics.

1.2 HIV

1.2.1 HIV/AIDS BURDEN AND DISEASE

HIV-1 is a member of the *Retroviridae* family and *Lentivirus* genus. The *Lentivirus* genus also contains SIV as well as other complex retroviruses. HIV-1 and SIV are the etiological agents of acquired immunodeficiency syndrome (AIDS) in humans and non-human primates (NHPs), respectively (25, 26). Lentiviruses infect $CD4^+$ cell subsets such as resting T-cells, macrophages, and DCs by binding to CD4 through viral glycoprotein gp120. Gp120 is a 120 kDa viral protein present on the surface of a virion as a homotrimer and is anchored to the viral membrane by the transmembrane protein gp41 (27). Binding of CD4 by gp120 leads to conformational changes in viral protein structure, revealing a chemokine binding epitope leading to interaction with the viral co-receptors CXCR4 by T-cell tropic (T-tropic) viral variants and CCR5 by macrophage tropic (M-tropic) variants (22). V irus is then internalized via receptor-mediated endocytosis where the viral envelope fuses with the host cellular membrane. Viral genome as well as reverse transcriptase are released from the viral capsid and reverse transcription takes place whereupon the reverse transcribed viral genome migrates to the nucleus. Integrase incorporates the lentiviral genome into the host genomic material, after which host machinery is use for the transcription of the single, primary viral transcript. Viral transcripts must then migrate or be chaperoned to the cytoplasm where they can be translated into viral proteins by host machinery or migrate to the plasma membrane where packaging, budding, and eventual virion maturation can occur. Initial infection takes place at mucosal portals such as the vagina or rectum where SIV and HIV-1 are

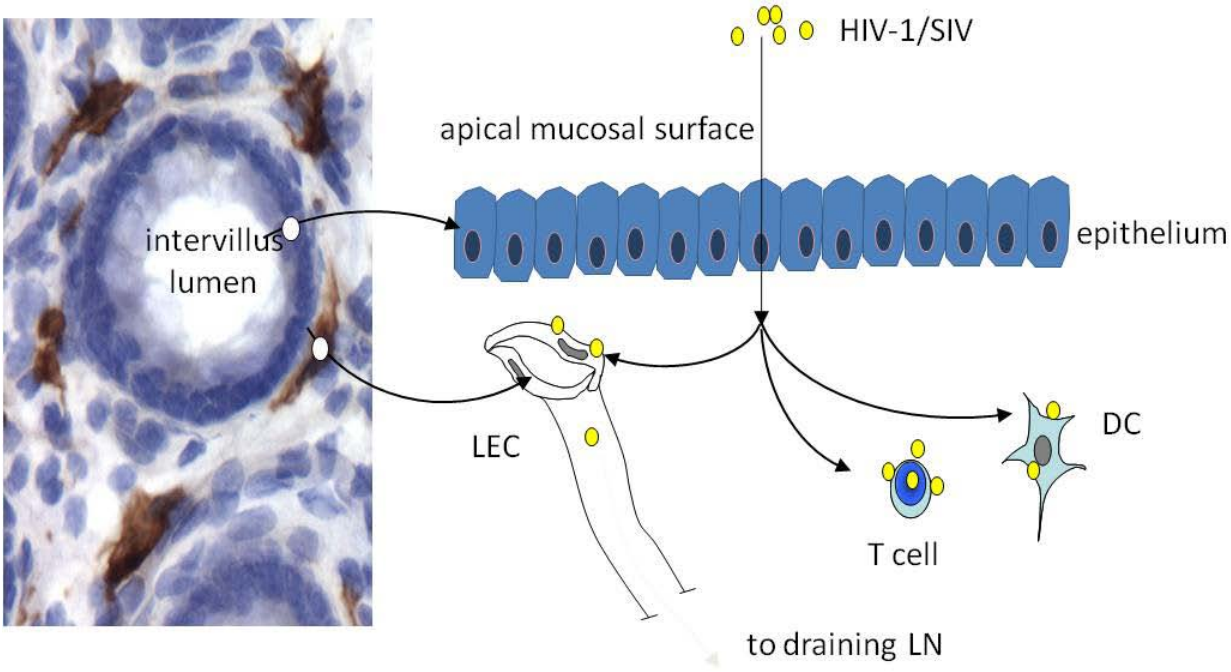


Figure 1: Schematic of LECs role in migration of virus to secondary lymphoid organs.
LECs line the inner surface of afferent lymphatic vessels which are in close juxtaposition to the mucosal surface as seen by the immunohistochemical analysis for the LEC marker LYVE-1. Virus can be loaded on permissive cell types which then migrate to secondary lymphoid organs interacting with LECs along the way, or cell free virus can freely migrate to potential target cells. LECs are in close proximity to virus at the moment of infection.

able to establish infection in activated and resting T-lymphocytes, as well as be taken up by APCs (96,97). Migration to secondary lymphoid tissues allows the virus to come into contact with a large population of target $CD4^+$ cells, leading to the dissemination of infection. Disease progression is characterized by an initial acute phase where viral load increases with an initial loss of $CD4^+$ T-cell populations. Host control of infection leads to a viral set point which is directly correlated to disease prognosis (93). Infection leads to eventual loss of $CD4^+$ T-cell populations, which concurs with increase in plasma viremia. This loss of $CD4^+$ T-cell populations leads to deficiencies in cell-mediated immunity, allowing for the occurrence of opportunistic infections (94).

HIV/AIDS has a profound effect on worldwide public health as there are approximately 35 million cases worldwide (~25 million in Sub-Saharan Africa) and 3 million deaths annually accounting for the fourth highest cause of death worldwide (28). HIV not only has an profound effects in a public health context, but also socio-economic effects as labor, productivity, and average national growth are detrimentally effected by HIV/AIDS, particularly in developing equatorial nations in Sub-Saharan Africa and southeast Asia.

1.2.2 INNATE ANTIVIRAL FACTORS

Retroviruses have been around for a long time. There is evidence that ancient retroviruses have become permanently integrated in germ-line cells and these viruses, termed human endogenous retroviruses (HERVs), have been shown to play a role in autoimmune diseases. HERVs may have even helped speed up the evolutionary rate of humans (68,69). As with any foreign microbe that has been infecting a host population for thousands of years, the host has developed a multitude of cellular factors that are efficient at inhibiting replication at a number of viral life-cycle stages (Figure 2). These families of factors can be found in primates and many other high-order mammals, supporting their evolutionary importance (Table 1). Host factors that are able to effectively inhibit HIV-1 replication have been identified and their potential to counteract retrovirus infection and the possibility of developing synthetic therapeutics based on the activities of these factors underscores their importance. These viral restriction factors include the following.

1.2.2.1 APOBEC3G

A recently described group of restriction factors is the apoplipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like (APOBEC) super-family of cytidine deaminases, and more specifically the APOBEC3 subfamily. The APOBEC superfamily contains 11 cytidine deaminase proteins, of which APOBEC3 has four different members (APOBEC3A-H) that all have been shown to have some inhibitory effect on HIV-1 replication (8,10). APOBEC3G in particular has been extensively researched and has been shown to be encapsidated within newly synthesized virions by interacting with the nucleocapsid region of the HIV-1 genome. This interaction is facilitated by an N-terminal catalytic domain present in APOBEC proteins (8, 9). Once encapsidated in immature virions, a C-terminal domain induces deamination of cytosines within the viral genome. These mutations effectively curtail the newly formed virion's ability to replicate (31). It has also been proposed that APOBEC3G inserts itself within lipid rafts in host cellular plasma membranes and that this interaction facilitates the proper encapsidation and future inhibitory effect on HIV-1 replication by APOBEC3G (9). The cytidine deaminase activity of APOBEC3G has been shown to act specifically on single stranded DNA rather than free nucleotides or RNA, in contrast to other members of the cytidine deaminase family (51). This property is essential for APOBEC3G to act as a strong viral restriction factor.

Lentiviruses have evolved to contain an accessory protein called viral infectivity factor (Vif) a 23 kDa accessory protein that is able to interact with host APOBEC3G through highly conserved motifs and acts as an adaptor protein resulting in recruitment of the Cul-5/E3 ubiquitin ligase complex comprised of ElonginB, ElonginC, Cullin5 and RING-box-1 (13). This interaction leads to the recruitment of a protein complex that directs the polyubiquitination and

eventual degradation of a substantial amount of cellular APOBEC3G via the proteasomal pathway (50). The Vif-mediated degradation of APOBEC3G contributes to the efficient replication of Vif-competent virions (11). Virions deficient in Vif are susceptible to APOBEC3G encapsidation and eventual mutational inhibition of replication (12).

1.2.2.2 TRIM5-α

Along with the APOBEC family of restriction factors, tripartite motif (TRIM) containing factors, in particular TRIM5-α, are another large family of restriction factors with many orthologs in humans and NHPs and that have been shown to induce some sort of antiviral activity (50,52). TRIM5-α is able to effectively inhibit infection of Old World monkeys with

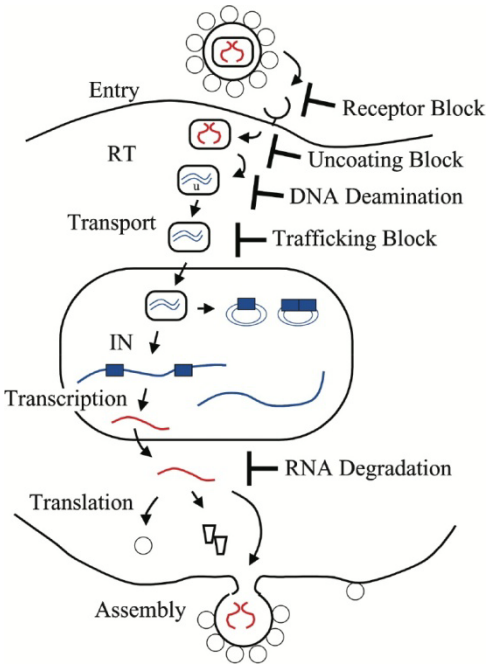


Figure 2: Host factors inhibit replication at multiple steps in the viral life-cycle.
Along with APOBEC3G, TRIM5-α, and BST-2 inhibiting replication at RT, uncoating, and budding respectively, host factors have been shown to inhibit viral replication at almost every step of the viral life-cycle indicating a long-lived battle between host and retroviruses. Reprinted from Molecular Cell, 16/6, Stephen P. Goff, Retrovirus Restriction Factors, 849-859, 2004, with permission from Elsevier.

Table 1: Properties of Dominant Retrovirus Resistance Genes.

Species	Restriction System	Time of Block	Restricted Viruses	Unrestricted Viruses
Mouse	Fv4	Entry	Ecotropic MuLV	Amphotropic MuLV lentiviruses
Human, Chimpanzee	APOBEC3G	Viral DNA formation	Vif-negative HIV-1	Vif-positive HIV-1
			SIV_{AGM}	
			EIAV	
			HBV	
African green monkey	APOBEC3G	Viral DNA formation	HIV-1	Vif-positive SIV_{AGM}
				Vif-negative SIV_{AGM}
Human	APOBEC3F	Viral DNA formation	Vif-negative HIV-1	Vif-positive HIV-1
				Vif-positive HIV-2
				MuLV
Mouse	APOBEC3	Viral DNA formation	HIV-1	MuLV
Mouse	$Fv1^n$	Viral DNA trafficking	B-MuLV	N-MuLV
				NB-MuLV
				HIV-1
Mouse	$Fv1^b$	Viral DNA trafficking	N-MuLV	B-MuLV
				NB-MuLV
Rhesus macaque	TRIM5α (Lv1)	Early postentry	HIV-1	N-MuLV
				B-MuLV
				SIVmac
African green monkey	TRIM5α (Lv1/Ref1)	Early postentry	N-MuLV	B-MuLV
			HIV-1	SIV_{AGM}
			HIV-2	
			SIVmac	
			EIAV	
Squirrel monkey	TRIM5α (Lv1)	Early postentry	SIVmac	N-MuLV
				B-MuLV
				HIV-1
Rabbit	Lv1	Early postentry	HIV-1	–
Human	TRIM5α (Ref1)	Early postentry	N-MuLV	B-MuLV
			EIAV	NB-MuLV
				HIV-1
				HIV-2
				SIVmac
				SIV_{AGM}
Chimpanzee	Ref1	Early postentry	N-MuLV	–
Cow, Pig, Bat	Ref1	Early postentry	N-MuLV	B-MuLV
Owl monkey	TRIM-Cyp	Early postentry	HIV-1	N-MuLV
				B-MuLV
				SIVmac
Rodent	ZAP	Viral RNA expression	MuLV alphaviruses	HIV-1
				VSV
				Poliovirus
				DNA viruses

Reprinted from Molecular Cell, 16/6, Stephen P. Goff, Retrovirus Restriction Factors, 849-859, 2004, with permission from Elsevier.

HIV-1. However, antiviral activity in humans is not as potent. TRIM factors contain a ring-finger zinc domain which is responsible for ubiquitination of targets for degradation (15). A coiled-coil domain allows TRIM factors to form multimers, which bind HIV-1 capsid protein through the PRY/SPRY (B30.2) motif leading to eventual degradation via the proteasomal pathway (16,53). Multiple TRIM isoforms formed by alternative splicing patterns have been described and viral restriction activity has been observed for multiple viruses (54). The isoform TRIM5-α also contains a C-terminal SPRY domain that is not conserved amongst all TRIM isoforms and may be important for the effective protection against certain lentiviruses (14). Better understanding of the inhibitory effect TRIM5-α confers upon old world monkeys could lead to a better understanding of overall SIV and HIV-1 immunity as well as eventual therapeutics.

1.2.2.3 BST-2

Tetherin, or bone marrow stromal antigen 2 (BST-2), is an interferon induced, type-II transmembrane protein with a cytoplasmic N-terminus and one membrane-spanning domain as well as a C-terminal glycosyl-phosphatidylinositol (GPI) anchor. This GPI anchor is capable of binding retroviruses at the cell surface thus inhibiting budding and maturation of infectious particles (17). There has also been evidence that BST-2 is capable of binding other pathogens, such as Ebola, at the cell surface through interactions with Ebola viral glycoprotein (Gp) (18). Complex lentiviruses as well as Ebola have adapted to avoid BST-2 inhibition. HIV-1 encodes for an accessory protein, viral protein u (Vpu) that associates with BST-2 located in membrane lipid rafts (17,99). Vpu binds cell-surface BST-2 through transmembrane interactions, leading to lysosomal degradation, partially in a β_TrCP-dependent manner (55). Ebola virus produces a secreted, soluble viral Gp that binds to BST-2, inhibiting its antiviral activity (18,99,113). The

incorporation of a BST-2 transmembrane domain into a budding enveloped virus tethers the newly synthesized virion to the surface of the infected cell, inhibiting release of virus.

1.2.3 HIV-1 AND SIV ALTERNATIVE ENTRY RECEPTORS

HIV-1 and SIV infect cells primarily in a CD4-dependent manner, through binding of HIV-1 gp120 to CD4, leading to conformational changes in gp120, which allows binding to a chemokine co-receptor leading to membrane fusion. There has been evidence however that HIV-1 and SIV can be internalized by permissive cells in a CD4-independent fashion. Certain HIV-1 isolates have been shown to be able to use the atypical scavenger chemokine receptor chemokine binding protein 2 (CCBP-2 or D6), the C-type lectin DC-SIGN (CD209) that is expressed on macrophages and dendritic cells, DEC-205 (CD205) expressed primarily by lymphoid and myeloid DCs, as well as through CD4-dependent mechanisms (1,19,20). Investigation of these alternative receptors has been extensive, describing multiple cell populations that show expression of these receptors. Characterizing the presence or absence of these receptors in LECs is important as their expression could lead to binding and subsequent internalization of virus into endothelial cells, either ending in a degradative manner or possibly leading to latent or productive replication of infectious virions.

1.2.3.1 CHEMOKINE BINDING PROTEIN 2 (CCBP-2 or D6)

Chemokine binding protein (CCBP2/ D6) is an atypical, scavenger, seven transmembrane spanning, chemokine receptor that acts as a chemokine sink (71). D6 is called a “silent” chemokine receptor, as upon binding to a chemokine ligand, D6 does not signal through a G protein pathway, but rather quickly internalizes and degrades the chemokine leading to the

theory that D6 acts to regulate chemokine levels (73,74). D6 shows incredible breadth in its ability to bind a variety of different CC chemokine including CCL3, CCL4, and CCL5 (72). D6 has been shown to be expressed in lymphatic endothelium of the skin and lungs, as well as placental tissue and secondary lymphatic tissues (74). There is evidence that D6 can bind HIV-1 isolates and act as a receptor for HIV-1 in a CD4-independent manner (1). Our laboratory has previously shown that D6 is expressed by hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy LEC populations and that expression was sensitive to treatment with TLR agonists (23).

1.2.3.2 DC-SIGN

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a C-type lectin type II transmembrane receptor with a single carbohydrate recognition domain (CRD) expressed primarily by DCs and macrophages that acts as a PRR, binding mannose type carbohydrates found on many viruses (76,77,78). DC-SIGN has been shown to internalize a wide variety of pathogens such as hepatitis C virus (HCV), Ebola, and *Mycobacterium tuberculosis* (114,115,116). There is evidence that DC-SIGN is able to bind HIV-1 through interaction with viral gp120 leading to efficient internalization but not degradation, allowing virus to traffic within DCs to secondary lymphoid tissues that are rich in $CD4^+$ targets (76). It has also been seen that DC-SIGN on activated B-cell sub-populations can bind HIV-1 and lead to infection of $CD4^+$ T-cells in *trans* (79). DC-SIGN seems to play a role in internalizing virus and in *trans* infection making cell populations that express DC-SIGN potential intermediaries of infection.

1.2.3.3 DEC-205

DEC-205 or CD205, is a C-type multi-lectin type-1 cell surface protein, similar to DC-SIGN, that is present on DCs, macrophages, T-cells, and B-cells (80,81). DEC-205 contains 10 extracellular CRDs allowing DEC-205 to play a role in antigen uptake and processing as binding of antigen results in efficient internalization within early proteasomes and eventual antigen presentation at the cellular surface via MHC class II (82). DEC-205 is efficiently shuttled into and out of DCs similar to the PRR macrophage mannose receptor (MMR) in a manner that allows the immune system to survey the environment for foreign antigen PAMPs. However, DEC-205 is associated with late-endosome MHC class II complexes while MMR is not (83). There is evidence that DEC-205 can efficiently mediate the presentation HIV-1 Gag antigen from DCs to $CD8^+$ T-cells, inducing a potent response (84). There is also evidence that DEC-205-specific internalization of HIV-1 can lead to a non-productive infection of certain cell populations, such as renal tubular cells (19).

1.3 SUMMARY

The lymphatic system regulates interstitial fluid and surveys peripheral tissue for pathogens. LECs are present at mucosal portals of entry for many pathogens, including SIV and HIV-1. LECs also express PRRs and secrete migratory chemokines, indicating an active role during an immune response to infection. Previously, it had been difficult to isolate LECs form heterogeneous populations, until LEC-specific surface markers such as LYVE-1 and podoplanin were discovered. Lentiviruses such as SIV and HIV-1 infect $CD4^+$ T-cells, but there is evidence that HIV-1 can gain access to $CD4^-$ cell populations through a variety of different entry

receptors. Over time, host populations have evolved a number of different ways to combat disease, some host factors have been shown to have antiviral activity; inhibiting viral replication at a number of different steps in the viral life-cycle. LECs possibly could express any number of these entry receptors and restriction factors, allowing them to possibly bind and internalize virus, and may serve as a low-level replication site. Researching and characterizing LECs in the context of susceptibility is important as LECs potentially come into contact with virus at sites of transmission.

2.0 STATEMENT OF THE PROBLEM

The lymphatic endothelium is a major conduit for draining lymph fluid and cells from peripheral tissues to lymph nodes (LNs) via the afferent lymphatics. LECs line the interior surface of lymphatic vessels and are localized at mucosal portals of entry for many pathogens, including the natural route of infection of HIV-1 and SIV. HIV-1 is taken up at mucosal portals by peripheral dendritic cells (DCs) as well as $CD4^+$ T-cells and macrophages. Cell free virus as well as virus carried by professional antigen presenting cells (pAPC) can be transported to draining LNs via the afferent lymphatics. Once there, virus comes into contact with susceptible cell populations leading to propagation of infection. LECs could play a critical role during infection by producing migratory chemokines like CCL21, which play a role in DC and lymphocyte migration, or possibly by actively interacting with pathogens or pathogen-loaded APCs. LECs have been shown to express PRRs such as TLRs 1-6 and TLR 9, which recognize PAMPs leading to induction of innate immune responses (23). Because LECs are proximally located to portals of infection used by many pathogens, express multiple innate immune receptors, and regulate migration of immune cells, it is possible that LECs could actively engage in eliciting innate immune responses at early time pints of infection by foreign pathogens. Therefore, LECs could participate in HIV-1 infection and disease progression, and this has not been explored thus far. **The objective of this study is to examine the expression of innate antiviral restriction**

factors and viral entry receptors in model LECs and determine if LECs can engage virus, and other markers of uptake, in a variety of ways *in vitro*.

2.1 SPECIFIC AIM 1: DEFINE THE EXPRESSION PROFILE OF VIRAL ENTRY AND RESTRICTION FACTORS IN MODEL LECS

Virus entry has been well studied for many pathogens and is often dependent on binding to a host cell receptor. The majority of viruses gain access to permissive host cell types by receptor-mediated endocytosis. HIV-1 uses the receptor CD4 as well as co-receptors CCR5 and CXCR4 to infect permissive cell populations. There is evidence that atypical viral receptors such as the chemokine scavenger receptor D6 and the c-type lectin DC-SIGN can be used to internalize HIV-1 isolates. To date, research not been conducted focusing on the specific expression of restriction factors such as TRIM5-α, BST-2, and APOBEC3G in LECs. Global expression profile data through microarray analysis (88,89,90,91) of LECs has been conducted, but specific expression profiles for entry receptors and restriction factors has not been analyzed. The expression of entry receptors and restriction factors on LECs would be intriguing as their presence could increase the potential for LECs to recognize virus. Using real-time RT-PCR, I examined the levels of mRNA for these target genes of interest present in three LEC populations and determined if primary human dermal, lung, or long lived hTERT-transduced LECs, which have a longer life span than primary dermal and lung LEC populations, expressd entry and antiviral factors associated with viral replication. Additionally, I characterized these cell populations further by determining if protein expression correlated with RNA profiles in these populations by immunofluorescent (IF) staining. Through these studies, I found that while

model LECs did not express the typical CD4 viral receptor, they did express a variety of atypical viral entry receptors along with a multitude of restriction factors.

2.2 SPECIFIC AIM 2: DETERMINE THE OUTCOME AFTER LEC EXPOSURE TO VIRUS

It is well established that HIV-1 and SIV productively infect $CD4^+$ cells that are in peripheral tissues and secondary lymphoid organs. There has been little research however, dedicated to understanding the interaction between endothelial cells, present in peripheral mucosa and lymphatic vessels, and cell free virus. SIV and HIV-1 could possibly encounter LECs during the natural route of infection; therefore, it would be beneficial to understand the outcomes of LEC exposure to virus. Virus could either not interact with LECs, be degraded in some endocytic pathway, enter LECs and terminate replication at some abortive stage, or productively infect LECs. Human LEC cultures were exposed to SIV and HIV-1, as well as other immune activating compounds, such as the mimetic dsRNA polyI:C, to determine if virus is degraded by LECs, actively taken up by LECs, or productively infected LECs.

Using markers of endocytosis and phagocytosis, I also characterized the ability of LECs to actively engage and internalize target molecules. Finally, using an HIV-eGFP construct that has eGFP tagged Vpr incorporated into virions, I examined if LEC populations were able to bind virus *in vitro.* Through these studies I have found that LECs are able to internalize different markers of endocytosis and phagocytosis *in vitro,* while LECs exposed to virus had multi-spliced

viral transcripts present in total RNA samples and seemed to actively take up virus, though future experiments will elucidate to what extent.

3.0 MATERIALS AND METHODS

3.1 CELL CULTURE

Long-lived human dermal LECs transduced with human telomerase reverse transcriptase (hTERT-HELEC) (21) between passage number 22-25, as well as commercially available primary dermal (HMVEC-DLy) and lung (HMVEC-LLy) LECs (Cambrex Bio Science) between passage number 3-5, were cultured in EGM-2MV microvascular endothelial growth medium (Clonectics) at 37^{o}C and 5% CO_2 in T-25 and T-75 vented culture flasks (Falcon) . When collecting RNA samples, T-25 flasks were seeded at a concentration of 5,000 cells/cm^2 and allowed to grow for 3 days.

3.2 RNA ISOLATION

Trizol (Invitrogen) was used to lyse cells. Samples were then isolated using phenol/chloroform extraction methods according to the Invitrogen instructions to users. Samples were treated with DNA-free DNase system (Ambion) as well as RNeasy column (Qiagen) purification. Total RNA

samples were used to make 80ng/ul working aliquots and cDNAs were synthesized using a Reverse Transcription system (Promega) with random hexamer primers for standard RT-PCR or by Applied Biosystems specifications for real-time RT-PCR (Table 2) (100).

Table 2: cDNA synthesis for real-time RT-PCR

Reagent	Volume (uL)
10X PCR Buffer (ABI)	10
25 mM $MgCl_2$ (ABI)	22
25mM dNTPs (Invitrogen)	2
125 units Superscript II RT (Invitrogen)	.625
40 units RNAse inhibitor (Promega)	1
100 uM random hexamers (ABI)	2.5
Nuclease Free Water (Ambion)	56.875
400ng cDNA template	5
Total volume	100

PCR CYCLE

25ºC for 10min.
48ºC for 30min.
95ºC for 5min.
4ºC forever

3.3 REAL-TIME RT-PCR

Synthesized cDNAs from hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy RNA preperations were mixed with TaqMan universal PCR master mix (Applied Biosystems) and ready-made commercially available TaqMan assays (Applied Biosystems) for specific targets of interest (Table 3) to manufacturers specifications(100). Samples were loaded into MicroAmp 96 well support plates (Applied Biosystems) (101). Ct values were detected by an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The level of expression for each target was measured using the comparative Ct method and $2^{\Delta Ct}$ values presented are relative to the expression of the endogenous control β-glucuronidase (101).

Table 3: TaqMan ready-made assays for real-time RT-PCR

Primer Probe	Manufacturer (Inventoried/made to order)	Catalog #
LYVE-1	Applied Biosystems (Inventoried)	Hs00272659_M1
Podoplanin	Applied Biosystems (Inventoried)	Hs00366766_M1
APOBEC3G	Applied Biosystems (Inventoried)	Hs00222415_M1
BST-2	Applied Biosystems (Inventoried)	Hs00171632_M1
TRIM5-α	Applied Biosystems (Inventoried)	Hs01552559_M1
DEC-205	Applied Biosystems (Inventoried)	Hs00158966_m1
CD4	Applied Biosystems (Inventoried)	Hs00181217_M1
CCR5	Applied Biosystems (Inventoried)	Hs00152917_M1
CXCR4	Applied Biosystems (Inventoried)	Hs00237052_M1
CD209	Applied Biosystems (Inventoried)	Hs01588349_M1
D6	Applied Biosystems (Inventoried)	Hs00174299_M1
B-GUS *	Applied Biosystems (Inventoried)	Hs99999908_M1

3.4 IMMUNOFLUORESCENT STAINING

hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy LECs were seeded at 5,000 cells/cm^2 in 4 well chamber slides (Lab-Tek) and cultured in EGM-2MV (Clonetics) microvascular endothelial growth medium at 37^{o}C and 5% CO_2. Samples were then washed 3 times in 1X PBS (Lonza) for 3 minutes. Cells were fixed in 2% paraformaldehyde (PFA) for 15 minutes and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes then blocked with 20% secondary antibody specific, donkey sera (Abcam) in 1X PBS. Cell cultures were incubated for 1 hour in a humid chamber with primary goat α-human affinity purified polyclonal antibodies (Abs) for TRIM5-α (Abcam), LYVE-1 (R&D Systems), and D6 (Abcam) as well as polyclonal affinity purified rabbit α-human antibodies for CXCR4 (Abcam) and APOBEC3G (Abcam) and finally polyclonal affinity purified mouse α-human (BST-2 (Abcam), DEC-205 (BD Pharminigen)) and monoclonal affinity purified mouse α-human (Podoplanin (Angio Bio)) (Table 4). Cultures were then washed in 1X PBS supplemented with 0.5% bovine serum albumin (BSA) from Sigma-Aldrich 3 times for 5 min. Secondary antibodies fluorescently conjugated to Alexa Fluor 488 and Alexa Fluor 647 (Invitrogen) and isotype-specific for primary antibodies were used to bind to primary antibodies for 1 hour in a humid chamber (Table 4). Slides were washed with 1X PBS supplemented with 0.5% BSA 3 times for 5 min. Slides were then immersed in 1X PBS for 5 min followed by incubation in 70% ethanol for 5 minutes. Autofluorescent Eliminator reagent (Millipore) was then added to wells for 5 minutes followed by 3 washes with 70% ethanol for 1 min each. After washing with 1X PBS 3 times for 3 min, chambers were removed and slides were mounted with Prolong Gold antifade with DAPI (Invitrogen). Images were captured using

Table 4: Antibodies used for Immunofluorescent Staining

Primary antibodies	**Species**	**Specificity**	**Dilution**	**Supplier**	**Catalog #.**
CXCR4	Rabbit	α-human	1:100	Abcam	Ab2074
APOBEC3G	Rabbit	α-human	1:250	Abcam	Ab54257
TRIM5-α	Goat	α-human	1:100	Abcam	Ab4389
LYVE-1	Goat	α-human	1:20	R&D systems	Af2089
D6	Goat	α-human	1:200	Abcam	Ab1658
DEC-205	Mouse	α-human	1:50	Abcam	Ab79458
Podoplanin	Mouse	α-human	1:20	Angio Bio	11-003
BST-2	Mouse	α-human	1:100	Abcam	Ab88523
Secondary Ab					
Alexa Fluor 647	Rabbit	α-human	1:100	Invitrogen	A31573
Alexa Fluor 488	Goat	α-human	1:100	Invitrogen	A11055
Alexa Fluor 488	Mouse	α-human	1:100	Invitrogen	A21202
Control Ab			**Stock Conc.**		
Rabbit Ig	Rabbit	α-human	15g/L	DAKO	X0936
Goat IgG	Goat	α-human	1mg/ml	R&D Systems	AB-108-C
Mouse IgG	Mouse	α-human	1mg/ml	Vector	KO225

a SPOT digital camera mounted on a Nikon E600 microscope fitted with a 20X plan Apochromat objective using METAVUE software (Molecular Devices).

3.5 PHRODO™ BIOPARTICLES® ENDOCYTOSIS/PHAGOCYTOSIS

hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy were grown to approximately 70% confluence in T-25 flasks in EGM-2MV (Clonetics) as previously described. Cultures were then trypsinized and 4-well chamber slides (Lab-tek) were seeded at a density of 5,000 cells/mm^2. Cultures were allowed to grow to confluence and then incubated with varying concentrations of either pHrodo™ BioParticles® conjugate (pH dependent dye conjugated with *E. coli*, or pHrodo™ BioParticles® dextran MW 10,000 endocytosis kit (Invitrogen)) per the supplier's recommendations. Cultures were then washed 3 times with 1XPBS (Lonza) to remove excess and unbound pHrodo conjugates. Culture wells were incubated with 1% PFA for 5 minutes and again washed 3 times with 1XPBS. Culture wells were then covered with Prolong gold anti-fade with DAPI (Invitrogen) to stain cell nuclei. Images were captured using a SPOT digital camera mounted on a Nikon E600 microscope fitted with a 20X plan Apochromat objective using METAVUE software (Molecular Devices).

3.6 EXPOSURE TO VIRUS

hTERT-HDLECs were cultured in EGM-2MV microvascular endothelial growth medium (Clonectics) at 37^{o}C and 5% CO_2 in T-25 and T-75 flasks. When collecting RNA samples, T-25

flasks were seeded at a concentration of 5,000 cells/cm^2 and allowed to grow to approximately 70% confluence. Cultures were exposed to 1ml of un-titred SIVmac251 stock, the dsRNA mimetic poly I:C, or media only. Total RNA samples were isolated at 48 and 96 h for SIV exposed cultures and 48h for poly I:C and control cultures. Forward primer PreMSGSDF1_CB 5'-TGGTCTGTTAGGACCCTTTCTGCT-3' and reverse primer PostMSGSDR1GagUnspR1_ CB 5'-ATGTTCTCGGGCTTAATGGCAGGT-3' were used for amplifying SIV region flanking the major sub-genomic splice donor site and PreMSGSDF1_CB 5'-TGGTCTGTTAG GACCCTTTCTGCT-3' and reverse primer PostRevS.A.MSR3_CB 5'-AGGACTTCTC GAATCCTCTGTAGGGT -3' (Integrated DNA Technologies) were used to identify spliced *rev* RNA in LEC total RNAs exposed to SIVmac251. LEC cultures were exposed to media only (mock), poly I:C (25ug/ml) or lab-adapted , X4 tropic, HIV-1IIIb virus (5^7 $TCID_{50}$/ml) (courtesy of Deena Ratner and Dr. Phalguni Gupta) for 3 days, at which point cultures were washed 3 times for 3 min with 1X PBS. Fresh media was added and at day 7 and 10, remaining cultures were washed, and lysed for isolation of RNA samples. Forward primer HIV.MSGSD.F_1_CB 5'-TCAAGTAGTGTGTGCCCGTCTGTT-3' and reverse primer HIV.GAG,UnSp_R_1_CB 5'-ACTTCTGGGCTGAAAGCCTTCTCT-3' (Integrated DNA Technologies) (Designed using sequences from the Los Alamos National Laboratory HIV Database, which is funded by the Division of AIDS of the National Institute of Allergy and Infectious Diseases (NIAID), a part of the National Institutes of Health (NIH)) were used to amplify HIV-1 sequences flanking the major sub genomic splice donor site from LEC RNA samples. Supernatant samples were taken at each time-point from HIV-1 exposed and mock cultures for ELISA analysis (courtesy of Dr. Phalguni Gupta and Mary White). Forward primer BGUS.control.F_1_CB 5'- ACTTCCTG AAGTTGGACGAAGCGA-3' and reverse primer BGUS.control.R_1_CB 5'- TTGGGA

CGTTAGCAAAGACGAGGT-3' (Integrated DNA Technologies) were designed for the endogenous control β-glucuronidase based on sequences obtained from NCBI/GenBank (accession number = NM_000181).

3.7 LEC EXPOSURE TO HIV-EGFP

To visualize the interaction between virus and LECs, I used an HIV-1 construct tagged with enhanced green fluorescent protein (eGFP) fused to Vpr (courtesy of Courtney Zych and Dr. Velpandi Ayyavoo). The virus stock was diluted in endothelial growth medium and incubated with cultures of hTERT-HDLECs and HMVEC-LLys, grown to ~70% confluency in 4-well chamber slides, at a Gag concentration of 100 pg/mlin hTERT-HDLECs, 200pg/ml in HMVEC-LLys, or 0pg/ml (mock control) for 3 hours. Cultures were then washed 3 times for 3 minutes in 1X PBS and fixed in 4% PFA for 15 minutes. Cultures were washed again 3 times for 3 minutes in 1X PBS and mounted with ProLong gold antifade with DAPI (Promega) for nuclear staining. Images were captured using a SPOT digital camera mounted on a Nikon E600 microscope fitted with a 20X plan Apochromat objective using METAVUE software (Molecular Devices).

3.8 CLONING AND SEQUENCING OF SIV AND HIV-1 PCR PRODUCTS

Total RNAs from populations exposed to SIV or HIV-1 were used to generate cDNAs as previously described. Viral sequences were amplified using a PTC-200 Peltier thermal cycler (MJ Research) and visualized in 2% agarose gels. Bands of corresponding size to targets were

extracted from gels using a Q IAquick gel extraction kit (Qiagen). Isolated and extracted sequences were ligated into pGEMT cloning vector (Promega) and DNA sequenced by the Pittsburgh University core sequencing facility. Comparison and analysis of sequence with known sequences available on NCBI was conducted using Vector NTI Advance software (Invitrogen).

4.0 RESULTS

4.1 LECS EXPRESS MRNAS ENCODING VIRAL UPTAKE AND RESTRICTION FACTORS

To determine if hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cell populations expressed target receptors and antiviral factors, total RNA samples were isolated from LEC cultures. Real-time RT-PCR was conducted on c DNA samples synthesized from total RNAs and levels of expression were normalized to the levels of the endogenous control β-glucuronidase (β-GUS). β-GUS proved to be an acceptable endogenous control because fluctuations in expression levels across cell populations and treatments were negligible (<1 cycle threshold (C_t) value at the most). For each target gene $2^{-\Delta Ct}$ values were calculated allowing for mRNA levels of each target to be observed relative to the endogenous control (designated by a dotted-line) (Figure 3). In hTERT-HDLECs the LEC marker LYVE-1 was expressed at levels comparable to the endogenous control. In contrast, the LEC marker podoplanin was expressed at as much as 10 times the level of the endogenous control, indicating a strong constitutive expression level. This is consistent with podoplanin and LYVE-1 being known markers of LECs. hT ERT-HDLEC populations did not express mRNAs for the typical HIV-1 receptor CD4, as well as the beta-

chemokine co-receptor CCR5, used by M-tropic variants (Figure 3) within the limits of sensitivity of the assay. However, hTERT-HDLEC populations did express mRNAs for the HIV-1 T-tropic co-receptor CXCR4. hTERT-HDLEC populations that were not stimulated in any manner also expressed the innate viral restriction factors BST-2 and TRIM5-α as well as the surface receptors D6 and DEC-205, which are capable of binding and internalizing HIV-1 (Figure 3). hTERT-HDLEC populations did not express the C-type lectin DC-SIGN, which has been shown to play a role in internalization of HIV-1 by DCs, and the antiviral factor APOBEC3G, which has been shown to induce mutations that abrogate HIV-1 replication in Vif-

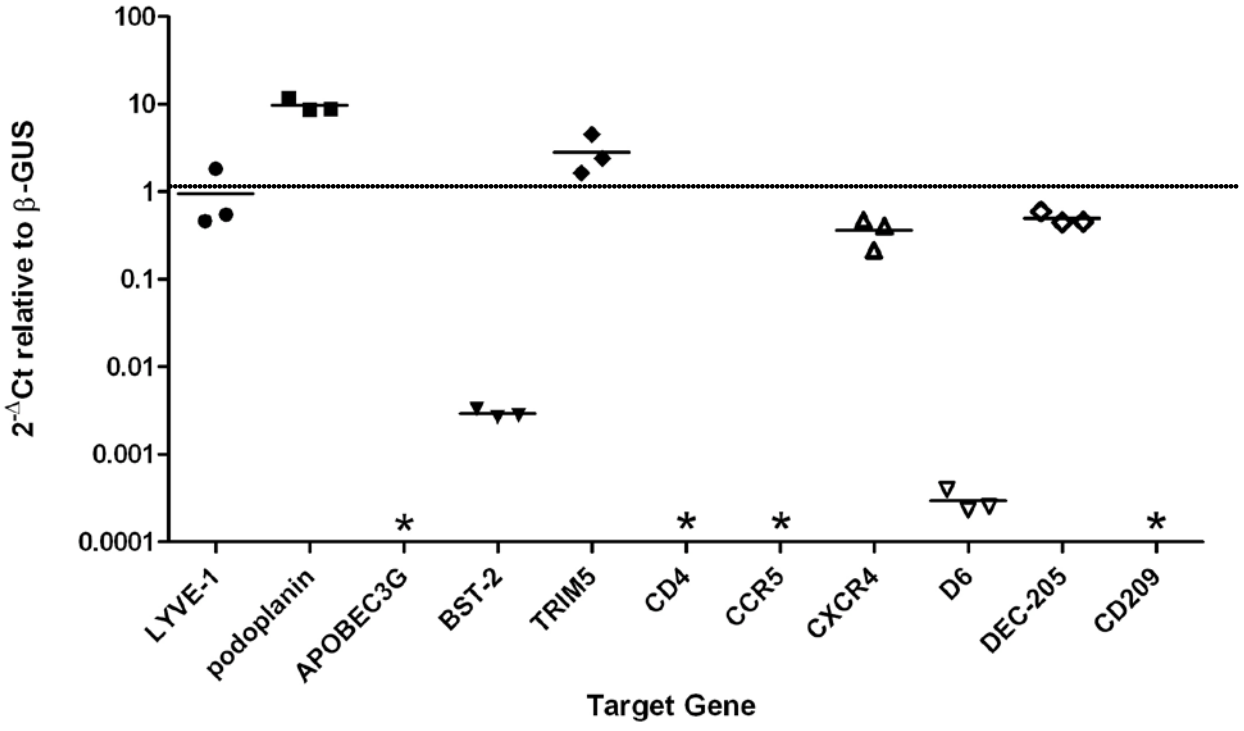


Figure 3: Real-time RT-PCR analysis of hTERT-HDLEC total RNAs.
hTERT-HDLEC RNAs were used to analyze the expression profiles for the entry and restriction factors described using the comparative Ct method. All target gene expression levels are relative to the endogenous control β-glucuronidase. Each data point represents the average of duplicate wells from 1 experiment. The dotted-line represents the expression level of the endogenous control, defined as 1.0.

deficient viral variants. The presence of antiviral factor and surface receptor mRNAs in non-stimulated cell populations indicates a constitutive expression of factors that play roles in viral uptake and replication inhibition.

When investigating the expression profile of these same target genes in the commercially available primary dermal HMVEC-DLy LEC population, there were interesting similarities and differences. HMVEC-DLy populations also expressed LYVE-1 at a comparable level to the

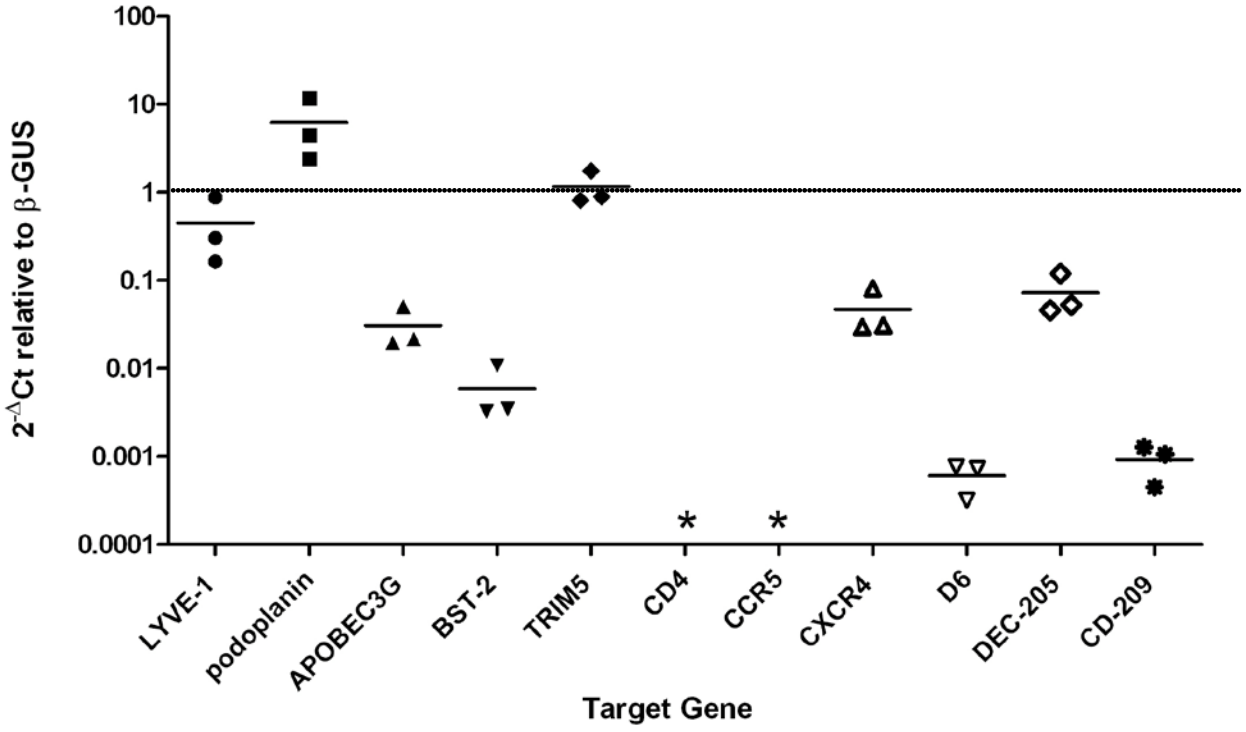


Figure 4: Real-time RT-PCR analysis of HMVEC-DLy total RNAs

HMVEC-DLy RNAs were used to analyze the expression profiles of viral entry and restriction factors using the comparative Ct method. All target gene expression levels are relative to the endogenous control β- glucuronidase. Each data point represents the average of duplicate wells from 1 experiment. Dotted-line represents the expression level of the endogenous control, defined as 1.0.

endogenous control, while the LEC marker podoplanin was present at levels higher than β-GUS (Figure 4). HMVEC-DLys also expressed the antiviral factors BST-2 and TRIM5-α.

Conversely, the primary dermal population showed expression of the antiviral factor APOBEC3G not present in hTERT-HDLEC populations. HMVEC-DLy samples were also the only populations analyzed in these studies that expressed DC-SIGN mRNA (Figure 4). The expression of APOBEC3G in primary cell populations, and its absence in transduced, long-lived

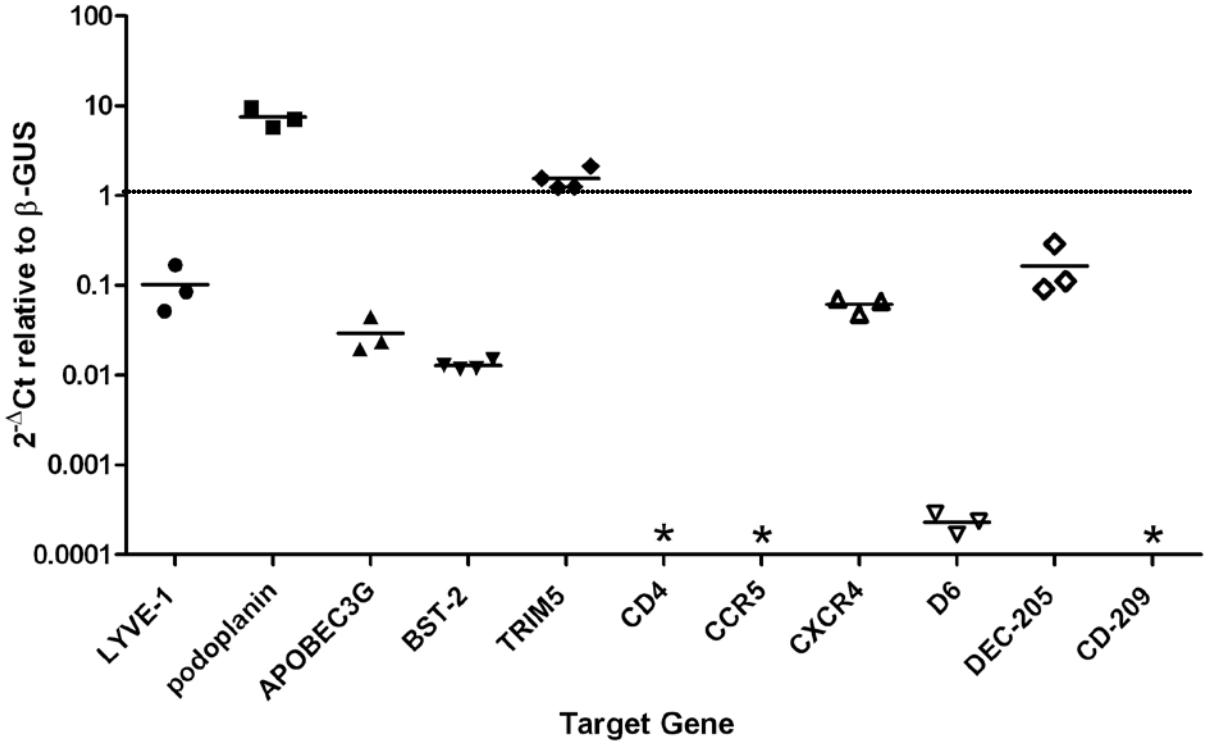


Figure 5: Real-time RT-PCR analysis of HMVEC-LLy total RNAs.
HMVEC-LLy RNAs were used to analyze the expression profiles of viral entry and restriction factors using the comparative Ct method. All target gene expression levels are relative to the endogenous control β- glucuronidase. Each data point represents the average of duplicate wells from 1 experiment. Dotted-line represents the expression level of the endogenous control, defined as 1.0.

hTERT-HDLEC populations could potentially be due to consequences of transfection and altered cell cycle characteristics. This should be taken into account when conclusions are drawn from *in vitro* studies in transfected cell populations. Expression profiles of different receptors and antiviral factors varying throughout populations is biologically plausible as different cell populations present at different areas in the body may have specialized functions specific to that tissue. Thus differences in the expression of different factors throughout even closely related cell population's occurred. HMVEC-DLy populations also expressed the atypical chemokine receptor D6, viral co-receptor CXCR4, and the antigen uptake factor DEC-205 similar to hTERT populations (Figure 4) suggesting LECs are equipped with receptors capable of binding and internalizing HIV-1 through CD4 independent mechanisms.

A primary lung LEC population, the HMVEC-LLy cells, was also analyzed for the expression of the same LEC marker, viral entry, and viral restriction target genes previously described. HMVEC-LLy cells expressed the LYVE-1 and podoplanin LEC markers. As with all populations analyzed thus far, HMVEC-LLy cells were not expressing the viral entry receptors CD4 and CCR5. HMVEC-LLy cells were expressing all three antiviral factors investigated (BST-2, APOBEC3G, and TRIM5-α) as well as the atypical entry receptors D6 and DEC-205 (Figure 5). In summary, three populations of LECs, defined as such by their expression of markers of LEC lineage, were also constitutively expressing a wide variety of viral entry and restriction factors. A lthough some differences were seen in expression levels throughout the populations, overall expression profiles were similar.

4.2 LECS EXPRESS VIRAL ENTRY AND RESTRICTION FACTORS AT THE PROTEIN LEVEL

Having determined the expression profiles of hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy LEC populations by real-time RT-PCR, analysis was then conducted to determine if corresponding proteins were expressed and what their subcellular distribution was. Using antibodies previously described, hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy populations were fluorescently labeled to determine if these cells were expressing protein at levels consistent with RNA levels previously measured (Figure 6-8). Staining for the LEC lineage markers LYVE-1 and podoplanin (Figure 6c-h) showed robust cytoplasmic signal throughout the three cell populations which correlated with the high levels of mRNA expression previously observed relative to the β-GUS (Figure 6a-b). These data suggest that for these markers, mRNA levels observed in real-time RT-PCR analysis correlate with protein expression and thus support the concept that mRNA expression levels are a good marker of the overall expression profiles for these populations (Figure 6). The expression profiles of the hTERT, DLy, and LLy cell populations for the antiviral restriction factors investigated by real-time RT-PCR were also examined by immunofluorescent staining. A ll three populations were expressing TRIM5-α which inhibits uncoating of the viral capsid, as well as the membrane associated protein BST-2, which inhibits budding virions by tethering them to the host cell surface (Figure 7). Although these data correlated with the expression levels of the corresponding mRNAs, there seemed to be distinct compartments that both BST-2 and TRIM5-α were sequestered in and the staining patterns were not similar to what was seen in the staining for the LEC markers LYVE-1 and podoplanin. There is evidence, however, that suggests both BST-2 and TRIM5-α are

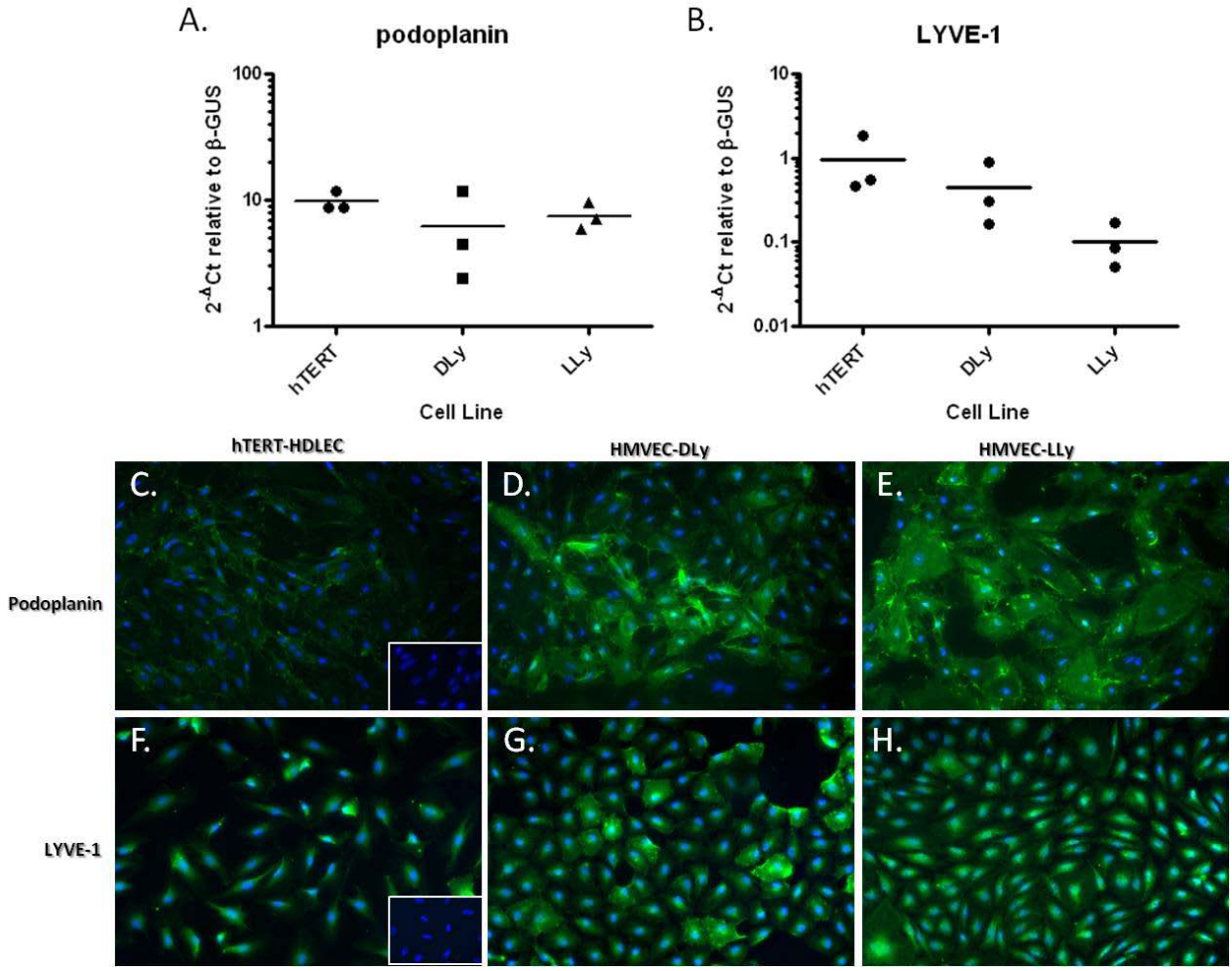


Figure 6: Expression of markers of LEC lineage.
hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy populations were positive for the LEC markers podoplanin and LYVE-1. Real-time RT-PCR data previously described was re-organized to view all populations' expression of a single target. Podopalnain (a,c-e) and LYVE-1 (b,f-h) analysis showed that not only mRNA (a,b) but protein (c-h) for these markers was present. IgG (LYVE-1) and Isotype (Podoplanin) controls were negative for signal (inset images). Original magnifications = 200X.

associated with lipid rafts in the cellular membrane, and this could be a reason as to why staining for these restriction factors are distributed unevenly like this throughout the cellular membrane (56,57,58). Lipid rafts are small microenvironments within the cell membrane that are rich in cholesterols and sphingolipids which allow for the organization and compartmentalization of many cell surface proteins that often have roles in receptor mediated endocytosis and signal

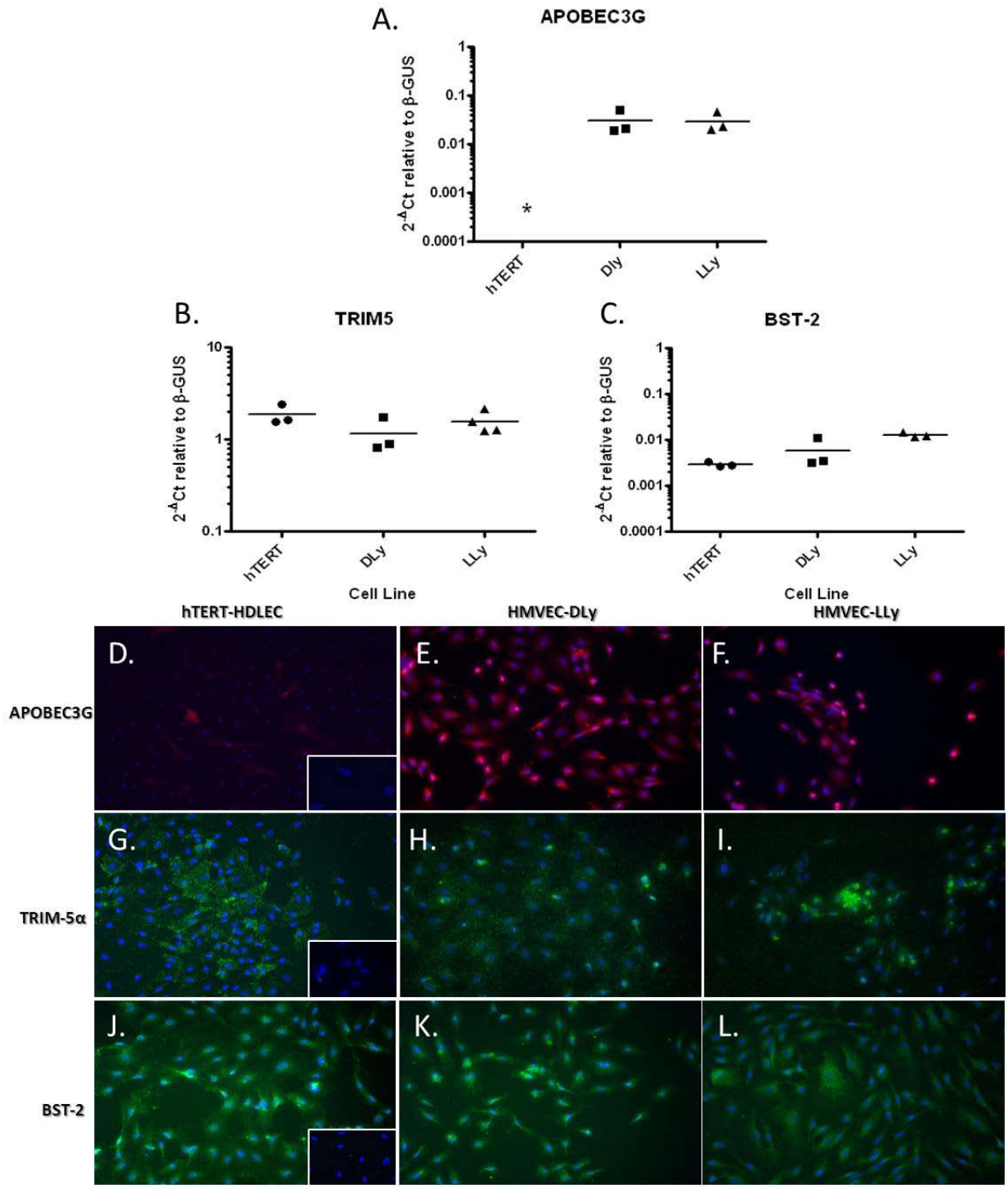


Figure 7: Expression of restriction factors by LEC populations.

Real-time RT-PCR data previously described was re-organized to view all populations expression level for a single target(a-c). Level of expression was measured relative to expression of the endogenous control β- glucuronidase using the comparative Ct method. APOBEC3G (d-f) TRIM5-α (g-i) and BST-2 (j-l) protein expression profiles, measured by immunofluorescent staining, correlated with real-time analysis. Inset images are representative IgG negative control culture chambers for each target. Original magnifications= 200X.

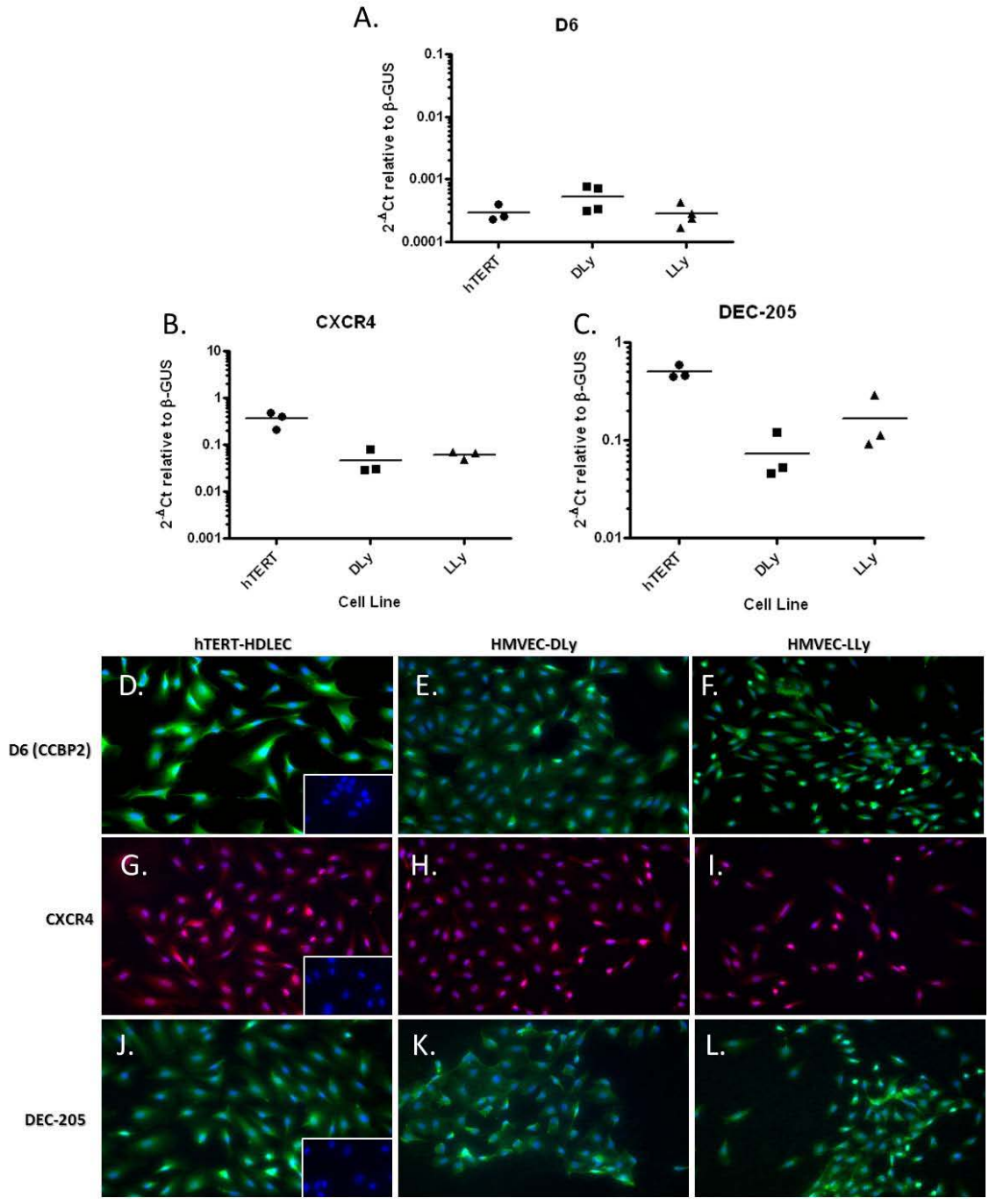


Figure 8: Expression of alternate entry factors on LEC populations.

Real-time RT-PCR data previously described was re-organized to view all populations expression level for a single target(a-c). Levels of expression were measured relative to the endogenous control β- glucuronidase using the comparative Ct method. Immunofluorescent staining of the three cell populations for D6 (d-f) CXCR4 (g-i) and DEC-205 (j-l) showed positive signal for each target indicating the presence of translated protein. IgG controls were negative for signal (inset images). Original magnifications = 200X.

transduction (59). Lipid rafts also contain microdomains where progeny virions are assembled (61,62). It is not unreasonable to expect that these lipid rafts would be a key compartment in which host antiviral restriction factors would be present to target assembling viruses.

Finally, the expression of alternative entry receptors was investigated based on the initial findings that D6, DEC-205, and CXCR4 mRNAs were present in total RNA isolations from hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cell populations (Figure 8 a-c). All three cell populations displayed staining when incubated with primary antibodies specific for the D6, CXCR4, and DEC-205 (Figure 8 d-l). This correlated with the previous real-time RT-PCR data that LEC populations were expressing alternative entry receptor mRNA (Figure 8a-c).

Taken together, the immunofluorescent staining data provide further characterization of the LEC populations. Not only are LECs actively transcribing mRNAs for LEC markers, antiviral restriction factors, and alternative entry receptors, but these populations are also translating these mRNAs to proteins, as visualized by immunofluorescent staining *in vitro*. These data suggest that as hypothesized, LECs are well equipped to actively survey the surrounding environment (i.e. lymph returning from peripheral tissues) and effectively engage microbial targets. If LECs are internalizing virus, they could have a sieve effect on the system, removing virus with tropism for these receptors. More intriguingly, LECs could offer a site of productive or latent infection not previously described

4.3 HTERT-HDLEC EXPOSURE TO POLY I:C

Previous studies in our laboratory have shown that LEC populations can respond to a number of different PAMPs, including the mimetic dsRNA poly I:C, through signaling via TLRs (23). Poly I:C binding to TLR3 induces signaling through IRF3 and NF-κB leading to the induction of an interferon response and subsequent expression of interferon stimulated genes (ISGs) (66,67) To determine if there were any differences in the expression profiles for the entry and restriction factors previously described after treatment with poly I:C, as well as after exposure to the X4 tropic lab-adapted viral strain HIV-IIIB, cultures were incubated with media only (mock control), media supplemented with poly I:C, or media with virus. Total RNAs from hTERT-HDLEC populations were isolated and the relative expression levels of the three viral restriction factors, which are also ISGs, APOBEC3G, TRIM5-α, and BST-2 were measured using real-time RT-PCR. All of these mRNAs increased in expression by at least 5-fold with APOBEC3G and BST2 expression increasing dramatically in response to poly I:C exposure (Figure 9a-c). Expression levels of these factors also increased in response to virus though not to the same extent indicating that exposing LECs to this amount of viral supernatant induced only a slight interferon response (Figure 9). It is interesting to note that APOBEC3G, which was not detectable in mock cultures of hTERT-HDLECs, was induced to express at such a high level when exposed to poly I:C (Figure 9a). There was also a significant decrease in the expression of CXCR4 from mock cultures to poly I:C treated cultures. Cultures incubated with poly I:C for 24 hours showed a significant decrease in expression level of CXCR4 than mock (media only) exposed cultures (Figure 9d).

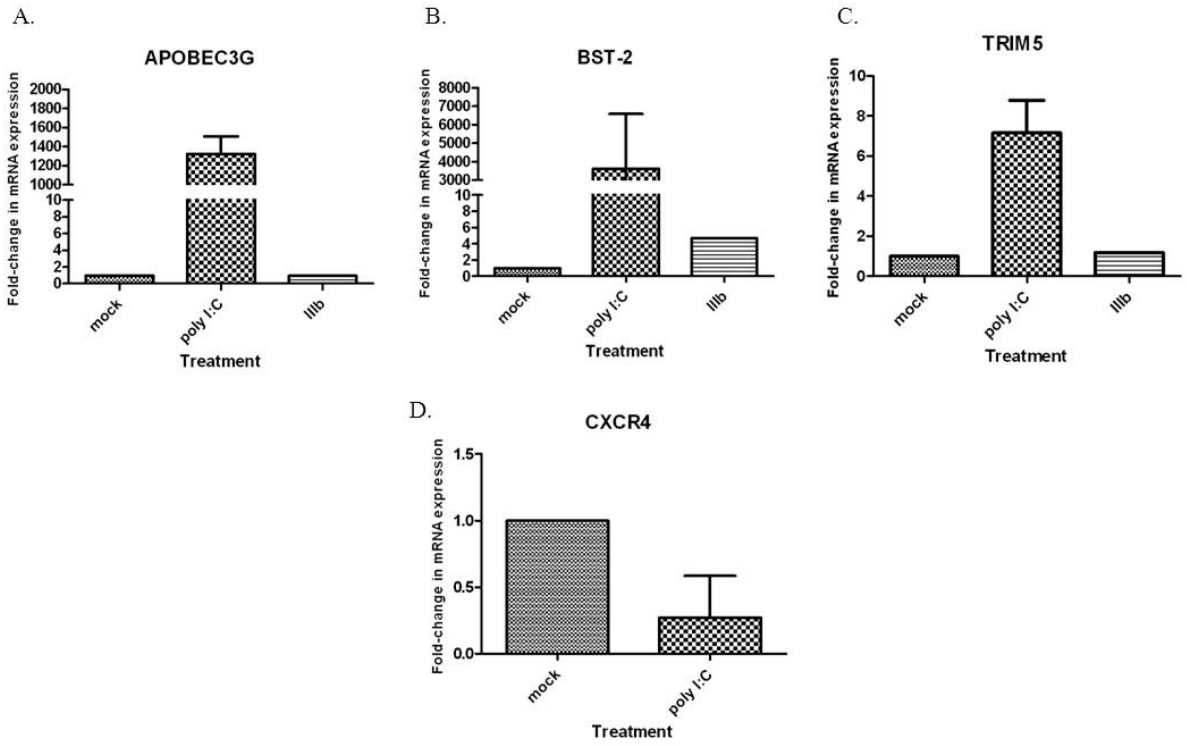


Figure 9: LECs respond to exposure to immunogenic molecules by altering expression profiles. hTERT-HDLECs exposed to media only (mock) the mimetic dsRNA poly I:C or the X4 tropic HIV-IIIb showed an induction of expression of three ISGs, APOBEC3G (a) BST-2 (b) and TRIM5-α (c). Increases in expression were also seen in cultures exposed to HIV-IIIb. Expression of the HIV-1 co-receptor CXCR4 was also significantly decreased in cultures that were incubated with poly I:C (d). Results in a-c represent averages of 2 experiments run in duplicate. Results in d are averages from 3 separate experiments run in duplicate.

Taken together, this evidence shows that LECs are not only constitutively expressing factors that can recognize foreign molecules, but the expression of these factors can be altered depending on the cells exposure to TLR ligands, including a dsRNA mimetic of viral replicative intermediates. These results strongly suggest that LECs are playing active roles in immune surveillance and response.

4.4 PHRODO BIOPARTICLE EXPOSURE

To assess whether LECs had the ability to sample the surrounding environment by both endocyctic and phagocytic mechanisms, hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cultures were exposed to dextran, a complex sugar that is internalized via early endosomes, that was directly conjugated to a pH sensitive rhodamine flurophore. Upon binding and internalization by early endosomes, the reduced pH in the endosome as it matures increases the intensity of rhodamine fluorescent signal. pHrodo dextran at normal pH has minimal emission, therefore cells that have actively taken up dextran can be identified directly using fluorescence microscopy or flow cytometry. Cells from hTERT-HDLEC and HMVEC-DLy populations were seeded on 4 well chamber slides and allowed to grow to approximately 70% confluence. Cell cultures were incubated with 30ug/ml pHrodo dextran or PBS only for 15 minutes and then visualized (Figure 10). Cultures incubated with pHrodo-labeled dextran showed robust, cell-associated, signal whereas mock control cultures were negative. These results suggest that LEC populations are actively engaging their environment in the form of internalization of dextran through endocytic pathways and that these endosomes are maturing to low pH late endosomes.

To investigate if LEC populations could internalize microbes through that would be recognized by TLRs in their environment, I also exposed cultures to pHrodo labeled K-12 *Escherichia coli* (*E. coli*) bioparticles that are internalized via phagocytosis and that also increase in red fluorescent signal as vesicles mature and the pH drops. *E. coli* is internalized by TLR4 mediated binding of LPS on the bacterial surface (63). Our laboratory has previously shown that

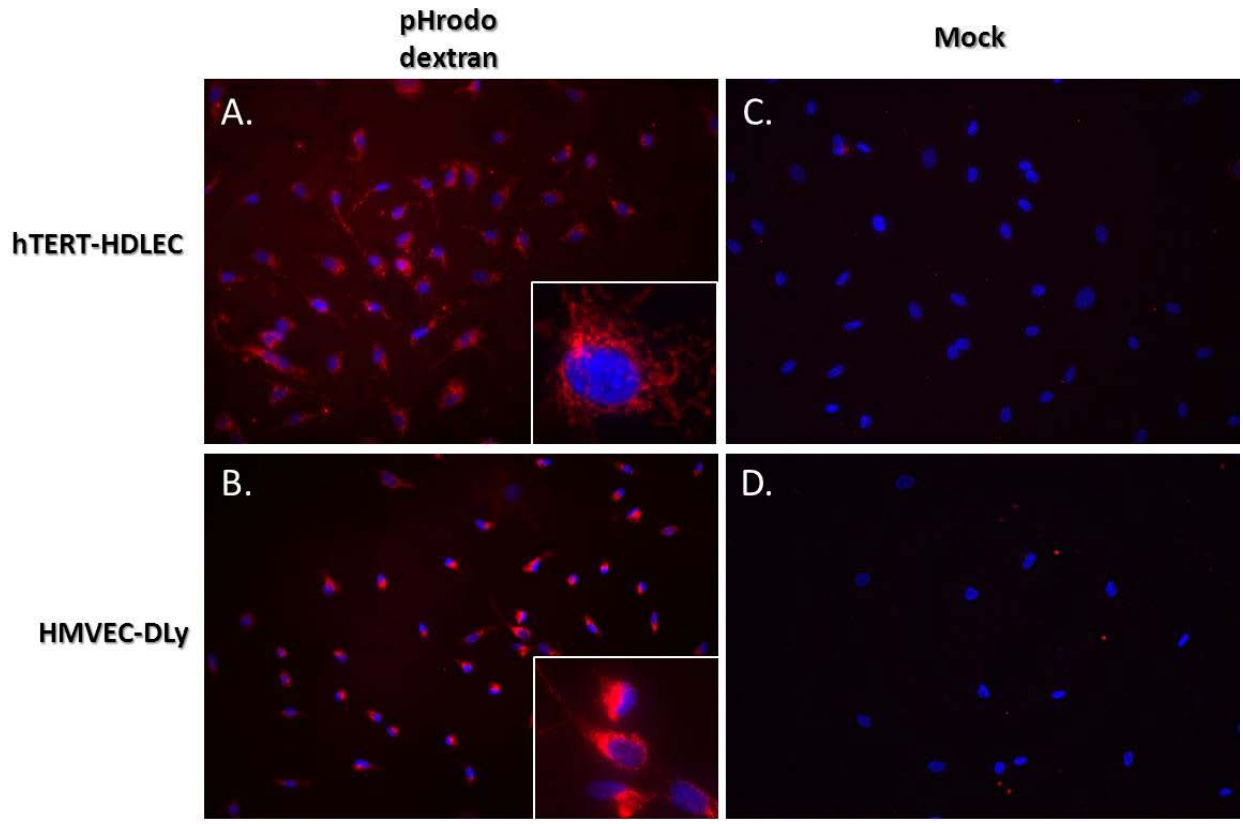


Figure 10: LEC populations exhibit endocytic activity
hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cultures were grown to approximately 70% confluence and incubated with media only (d-f) or pHrodo-labeled dextran particles diluted in media for 20 min. O riginal magnifications = 200X with inset images = 600X. Images representative of repeated experiments.

hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy populations express functional TLRs at the cell surface (23). *E. coli* conjugated particles were re-suspended in HBSS supplemented with 20nM HEPES uptake solution and diluted to appropriate concentration in endothelial growth media. Incubation with approximately 70% confluent monolayers of hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy populations occurred at 37°C for 3 hours. After nuclear staining, cultures were visualized and shown to be positive for signal, indicating the ability of all three LEC populations to actively phagocytose pHrodo labeled *E. coli* particles (Figure 11). Mock

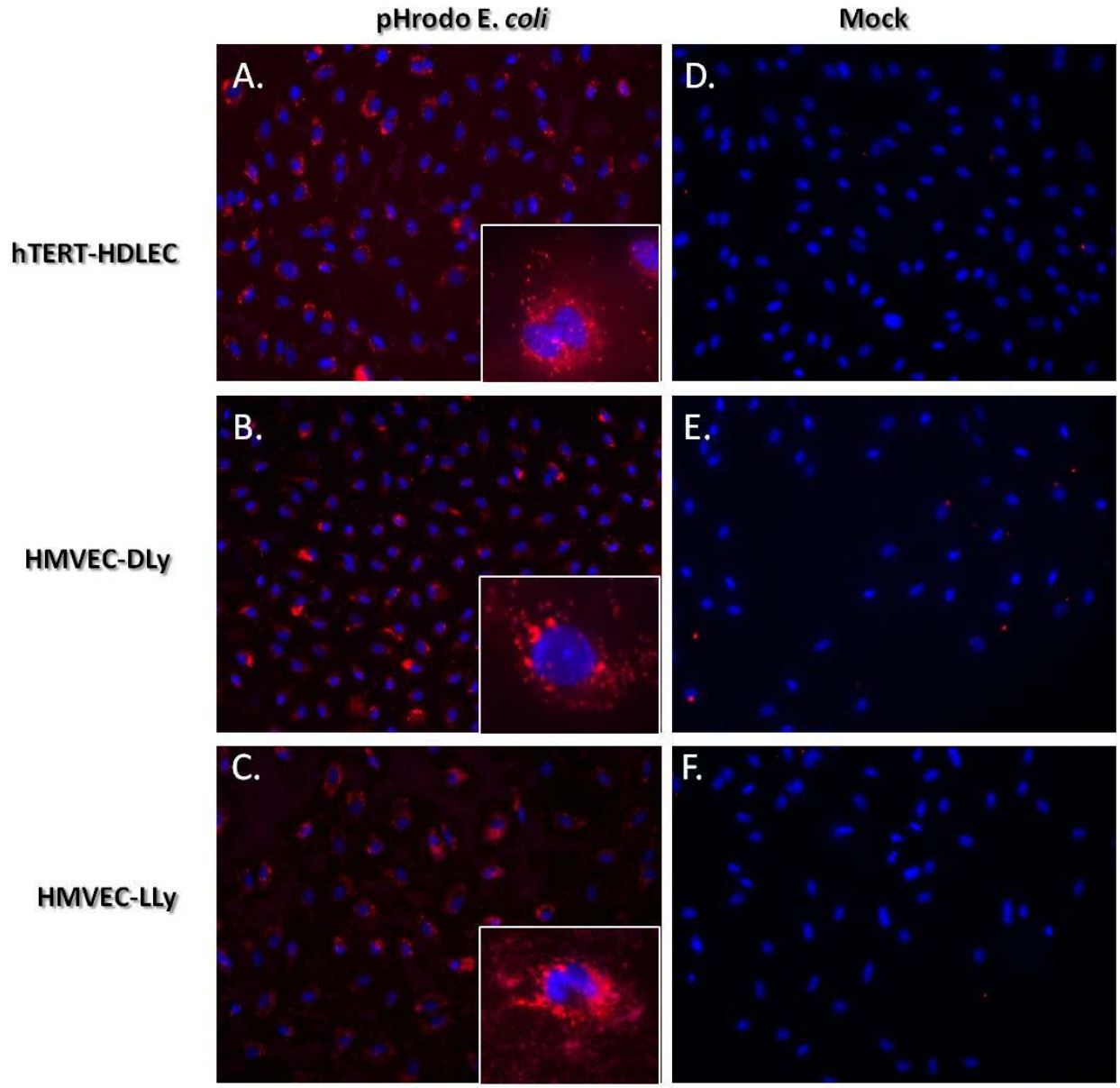


Figure 11: LEC populations exhibit phagocytic nature when exposed to pH sensitive rhodamine labeled K-12 Escherichia coli.
hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cultures were grown to 70% confluence and incubated with media only (d-f) or pHrodo labeled *E. coli* particles diluted in media for 3 h. Original magnifications = 200X with inset images = 600X. Images representative of repeated experiments.

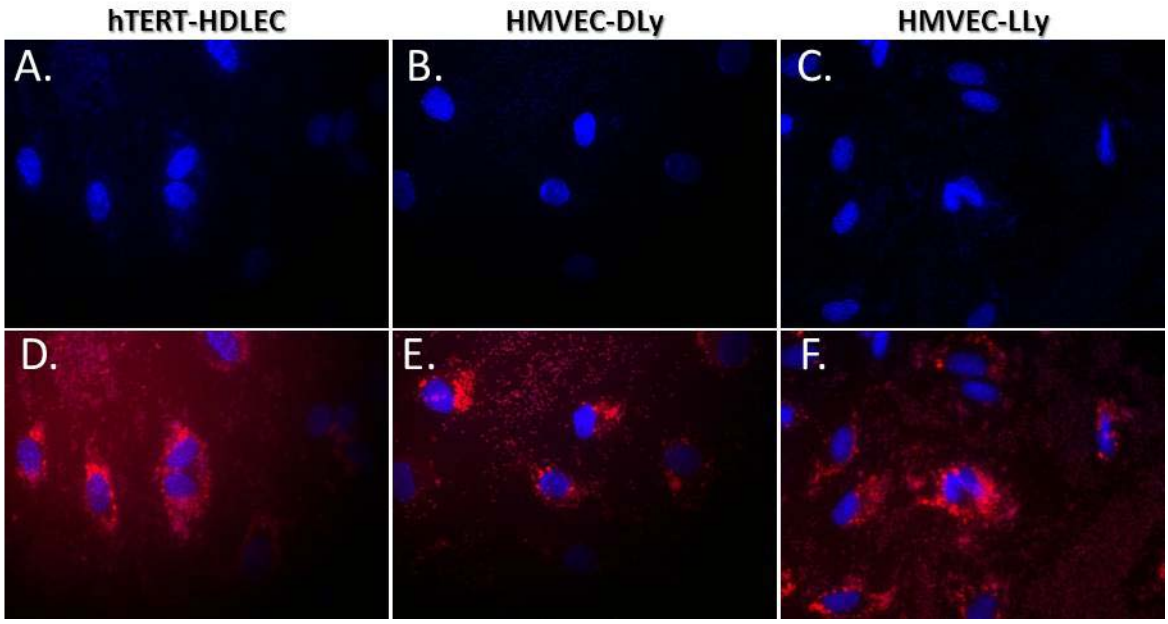


Figure 12: LECs exposed to pHrodo labeled E. coli bioparticles show extra-nuclear staining. hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cultures were grown to approximately 70% confluence and incubated with pHrodo-conjugated *E. coli* particles diluted in media for 3 h. Nuclear staining (a-c) shows positive signal outside of the defined nucleus contributed by *E. coli* genomic DNA bound by LECs. pHrodo signal (d-f) induced by acidification of vesicles containing internalized *E. coli* co localize with some of DAPI stain, but other areas where pHrodo signal is low is contributed by *E. coli* not internalized and/or bound to the slide. Original imaged= 400X. Images representative of repeat experiments.

cultures, which were exposed to media supplemented with the appropriate amount of uptake solution, did not show cell associated signal. Cultures exposed to pHrodo labeled *E. coli* particles also displayed an interesting staining pattern that further served as an internal control for the system. Cultures that were incubated with pHrodo labeled *E. coli* bioparticles were not only positive for cell-associated pH sensitive pHrodo signal, but when visualizing the nuclear staining by DAPI, there was a clear defined nucleus for each cell, as well as a diffuse granular signal that was distributed over the entire cell. The DNA staining localized with the pHrodo signal, and represents the *E. coli* genetic material (Figure 12). This signal was not present in mock exposed cultures as well as all cultures exposed to pHrodo labeled dextran particles which do not contain the genomic DNA present in the *E. coli* (Figure 10). This finding, along with the evidence of endocytosis and phagocytosis previously described, shows that LECs are actively

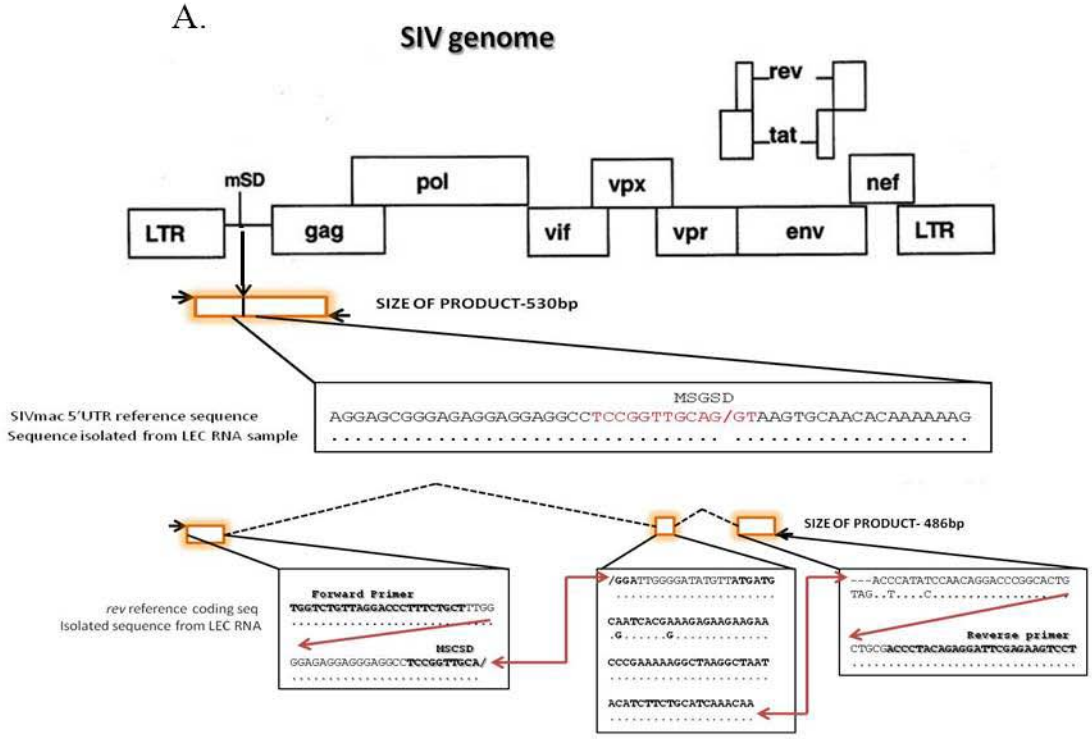
surveying the environment and are able to bind and internalize microbes, such as *E. coli,* in acidic vesicles through receptors that recognize PAMPs.

4.5 LECS EXPOSED TO SIV SHOW EVIDENCE OF VIRAL ENTRY

Characterization of LECs showed populations were actively expressing viral entry and restriction factors. LECs are also able to actively engage their surroundings and alter the expression of certain factors due to TLR signaling. To determine if LECs could bind and internalize virus, I exposed LEC cultures to SIV and examined if virus-associated PCR products could be isolated. The outcomes from exposure to virus include no interaction, virus degradation through cellular mechanisms, sequestering of virus on t he cell surface, or possible replication to some downstream endpoint (i.e. latent, abortive, productive). In order to evaluate this, SIVmac251 virus was added to T-25 flasks containing hTERT-HDLECs at approximately 70% confluence as exploratory first experiment to determine if viral transcripts could be isolated from total RNA samples from LEC cultures that had been exposed to virus. I developed a forward primer that was upstream of the major sub-genomic splice donor site (mSD), a splice site located upstream of the ψ packaging signal of the viral genome that is responsible for encapsidation (70). A reverse primer was also developed that was downstream of this site. Amplification of a PCR product of expected size was obtained when cDNAs synthesized from total RNAs of LECs exposed to SIV were used as input templates. This PCR product would be present in input viral genome as well as *de novo* synthesized viral primary transcripts. The presence of this PCR product in LEC total RNAs is indicative of SIV primary transcripts being present in LEC RNAs, potentially from virus uptake or virus attached to the cellular surface (Figure 13 a,b,d).

Although this finding was interesting, amplification of this target alone could not distinguish between input virus that had been added to the system and newly synthesized viral mRNA. To distinguish between these two possibilities, I developed a downstream reverse primer within the second coding exon of the *rev* open reading frame that when paired with the forward primer upstream of the mSD previously described, would only amplify a multi-spliced *rev* viral transcript. Since spliced *rev* transcripts are not incorporated into virions, the only way this PCR product would be present in hTERT-HDLEC total RNAs exposed to virus would be if there is some form of *de novo* replication occurring. Standard RT-PCR was conducted on hTERT-HDLEC RNAs from cultures exposed to media only (mock) or SIVmac251 for 48 or 96 hours (Figure 13d). Interestingly, products of appropriate size for both unspliced and spliced viral transcripts were amplified from RNAs obtained from cultures exposed to virus for 48 and 96 hours. Amplification was not observed when template RNAs from mock exposed cultures were examined (Figure 13d). Bands of appropriate size were extracted from the gel and purified, and then ligated into the pGEMT cloning vector prior to sequencing. Sequencing analysis showed that the target expected to be derived from the unspliced target RNA was >99% homologous to the reference sequence obtained from NCBI (Figure 13b). This finding indicated that SIV bound to hTERT-HDLECs *in vitro*, and was either staying bound to the surface of LECs, being sequestered in some cellular compartment, or possibly replicating to some endpoint, whether abortive or productive.

A.



B.

forward primer

Isolated 5′ UTR sequence (1) **TGGTCTGTTAGGACCCTTTCTGCT**TTGGGAAACCGAAGCAGGAAAATCCCTAGCAGATTGGCGCCCGAACAGG

SIVmac 5′ UTR reference seq(752) .....

GACTTGAAGGAGAGTGAGAGACTCCTGAGTACGGCTGAGTGAAGGCAGTAAGGGCGGCAGGAACCAACCACGACGGAGTGCTCCTATAAAGG

-.....

MSGSD

CGCGGGTCGGTACCAGACGGCGTGAGGAGCGGGAGAGGAGGAGGCC**TCCGGTTGCAG/GTAAG**TGCAACACAAAAAAGAAATAGCTGTCTTGT

....

TATCCAGGAAGGGATAATAAGATAGAGTGGGAGATGGGCGCGAGAAACTCCGTCTTGTCAGGGAAGAAAGCAGATGAATTAGAAAAAATTAG

.....

GCTACGACCCGGCGGAAAGAAAAAGTACATGTTGAAGCATGTAGTATGGGCAGCAAATGAATTAGATAGATTTGGATTAGCAGAAAGCCTGT

.....

TGGAGAACAAAGAAGGATGTCAAAAAATACTTTCGGTCTTAGCTCCATTAGTGCCAACAGGCTCAGAAAATTTAAAAAGCCTTTATAAT**ACT**

.....

reverse primer

GTCTGCGTCATCTGGTGCATT

.....

C.

```
                                                                                forward primer
rev reference coding seq.                                          (41) TGGTCTGTTAGGACCCTTTCTGCTTTGGGAAACCGAAGCAGGAAAATCCC
Isolated sequence from LEC RNA                                      (1) ..................................................

TAGCAGATTGGCGCCCGAACAGGGACTTGAAGGAGAGTGAGAGACTCCTGAGTACGGCTGAGTGAAGGCAGTAAGGGCGGCAGGAACCAACCACGACGGAG
..............T.....................................................................................................
                                                                                MSGSD
TGCTCCTATAAAGGCGCGGGTCGGTACCAGACGGCGTGAGGAGCGGGAGAGGAGGAGGCCTCCGGTTGCAG/GGATTGGGGATATGTTGGATTGGGGATAT
.....................................................A..............  ..........................
   start codon
GTTATGAGCAATCACGAAAGAGAAGAAGAACTCCGAAAAAGGCTAAGGCTAATACATCTTCTGCATCAAACAATAGACCCATATCCAACAGGACCCGGCCA
.........G.....G.....................................................---..T....C.................

TGCCAACCAGAGAAGGCAAAGAAAGAGACGGTGGAGAAGGCGGTGGCAACAGCTCCTGGCCTTGGCAGAATATATTCATTTCCTGATCCGCCAACTGATACG
.......................G.....................................................................................
                                                                                                reverse primer
CCTCTTGACTTGGCTATTCAGCAACTGCAGAACCTTGCTATCGAGAGTATACCAGATCCTCCAACCAATACTCCAGAGGCTCTCTGCGACCCTACAGAGGAT
.........A.................................C.....................................................................

TCGAGAAGTCCT
............
```

D.

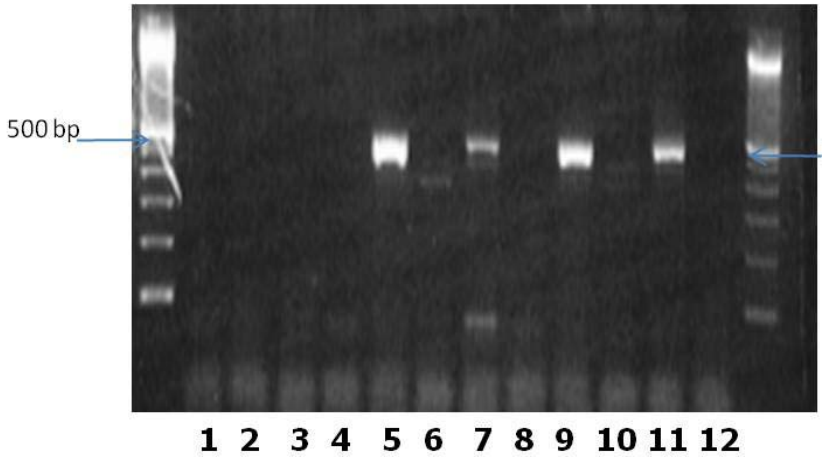


Figure 13:: LEC exposed to SIV contain viral transcripts in total RNA samples.
(a) A schematic of the SIV genome with corresponding designed primer pairs to amplify unspliced and spliced viral transcripts. (b-c) Sequencing results from amplified targets isolated via gel band extraction. Sequences for both unspliced 5'UTR (b) and multi-spliced rev transcripts (c) showed >95% homology to reference sequences from NCBI. Color coding of rev sequence correlates with 1st noncoding exon (red) 1st coding exon (green) and second coding exon (purple). Amplified targets sent for sequencing were obtained by Standard RT-PCR of hTERT-HDLEC total RNAs exposed to SIV for 48 and 96 hours (d). Lanes 1-4 correspond with mock exposed samples for each primer pair designed. Lanes 5-8 are 5'UTR, tat, and rev targets respectively in 48 hour SIV exposure samples with NRT control (8) Lanes 9-12 are 5'UTR, tat, and rev targets respectively in 96 hour SIV exposure samples with NRT control (12). Samples isolated from 48 hour lanes were ligated into a pGEMT vector and sent for sequencing which is seen in b and c.

Sequencing analysis of the target expected to be derived from multi-spliced *rev* mRNA showed >95% homology to the reference sequence with some interesting defining features (Figure 13c). The PCR product contained upstream sequences that were homologous with reference sequence expected upstream of the mSD. At the mSD however, the sequence of the PCR product was now homologous with the first coding exon of *rev* (Figure 13c). The product next contains sequences expected to be present in spliced *rev* mRNAs, containing an additional three nucleotides at the splice site between the first and second coding exons (Figure 13c). This additional three nucleotide stretch is consistent with alternative splice sites regularly seen in viral transcripts (112). Given that the coding regions of *rev* lay more than 5,000 nucleotides downstream of the mSD and that this stretch of >5kb is not present in the amplified product that was obtained, these data show that multi-spliced *rev* transcripts are present in total RNA samples from hTERT-HDLECs exposed to SIVmac251 for 48 hours. Spliced viral mRNAs are not incorporated into virions and they would not be present if virus was only bound by LECs, strongly suggesting that SIV is somehow being internalized by hTERT-HDLECs and transcription and splicing must be occurring.

4.6 LECS EXPOSED TO HIV-1 SHOWED EVIDENCE OF PRIMARY TRANSCRIPTS AND VIRAL PROTEIN SYNTHESIS

After exposing hTERT-HDLECs to SIV produced intriguing results, LECs were exposed to HIV-1 to determine if human cell populations could be infected in a more natural model. LECs were cultured and allowed to grow to approxametaly70% confluence. Cultures were incubated

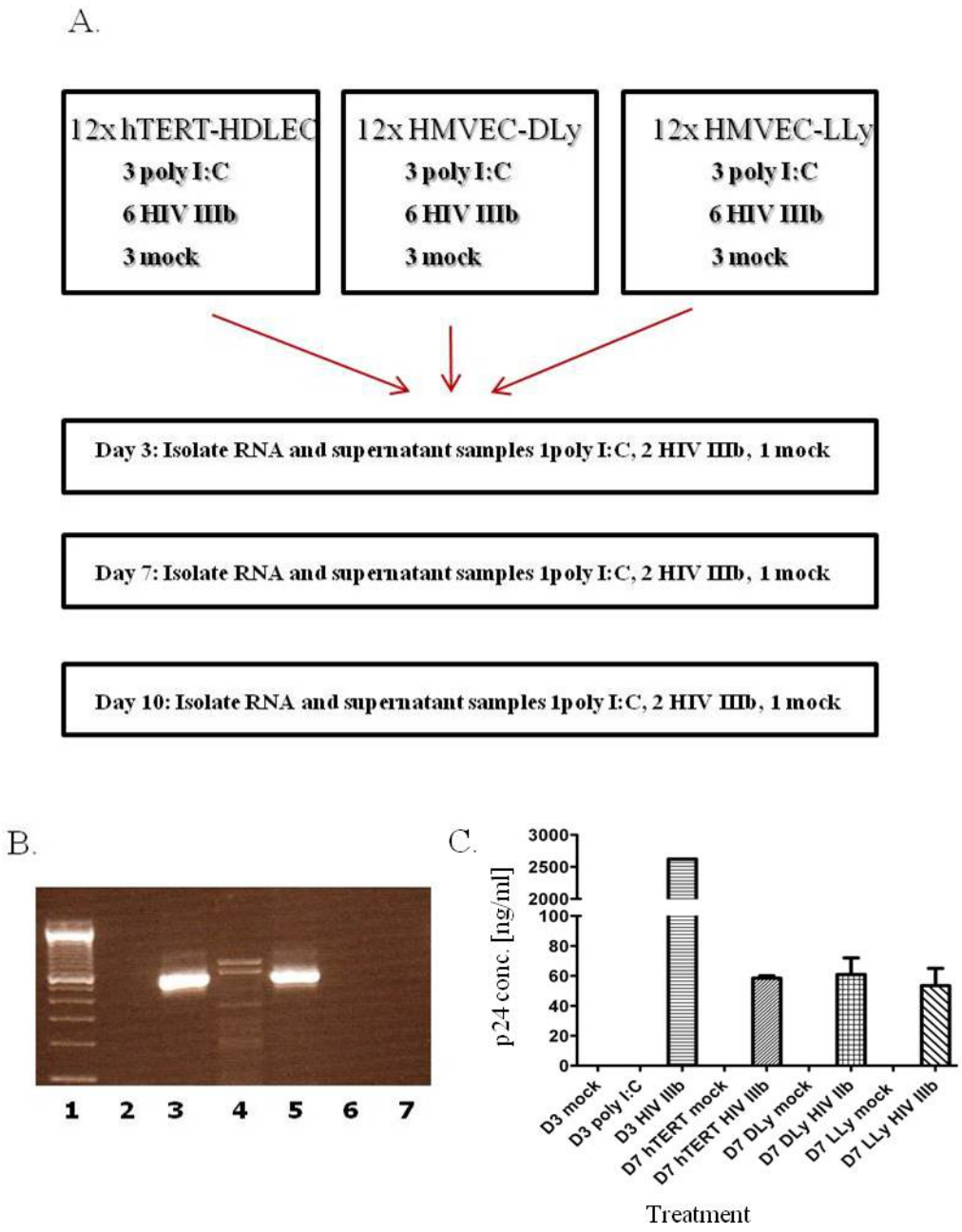


Figure 14: LECs exposed to HIV-1.

(a) Experimental design for exposure of hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy to X4 tropic HIV IIIb. Standard RT-PCR (b) of hTERT-HDLECs mock exposed (Lane 2,3) or HIV exposed (4,5) for the endogenous control β- glucuronidase (3,5) or for the mSD flanking region (2,4). NRT controls (6,7). p24 ELISA of culture supernatants from 3 cell populations (c). C ultures were washed extensively at day 4 and media was replaced. Results from 1 experiment run in duplicate.

for 3 days with either media only (mock) poly I:C (25 ug/ml) or the X4 tropic HIV IIIb (courtesy of Deena Ratner and Dr. Phalguni Gupta). HIV IIIb, a group-M, lab-adapted virus that utilizes the CXCR4 co-receptor, has also been shown to be able to use the CXCR4 receptor in the absence of CD4, making it an ideal initial strain to examine as it had been previously shown that LECs express CXCR4 but do not express CD4 (24). Cultures were designated for harvest on days 3, 7, and 10 after initial exposure with all cultures being washed extensively at day 3 and fresh media added (Figure 14a). Using the same strategy that was described for LECs exposed to SIVmac251, I developed a forward primer upstream of the mSD for HIV-1 IIIb using sequence obtained from the Los Alamos National Laboratory HIV Database. Primers were also designed for the endogenous control β- glucuronidase to serve as an internal control for the system. Standard RT-PCR was conducted on hTERT-HDLEC RNAs from cultures exposed to media only (mock) or HIV-1 IIIb (Figure 14b) at 7 days after exposure. The endogenous control β- glucuronidase mRNA was amplified from RNA samples from both mock exposed and HIV-1 IIIb exposed culture samples. However, amplification of the mSD flanking region was only seen in samples from the HIV-1 IIIb exposed cultures (Figure 14 b). These data indicate that HIV-1 IIIb can bind to hTERT-HDLECs *in vitro*, but sequence analysis has not succeeded due to poor isolation results. Future studies will focus on trying to isolate and sequence viral transcripts.

Supernatant samples were also taken from HIV-1 IIIb exposed cultures at days 3, 7, and 10 to determine if there is production of new virus particles by LEC cultures. Using a p24 ELISA kit, supernatant concentrations of viral Gag were measured in day 3 samples exposed to media only (control), poly I:C and HIV-1 IIIb exposed (before input virus was removed and

cultures were washed) as well as day 7 mock control and virus exposed cultures (Figure 14c). Levels of viral Gag were above 2000 ng/ml in day 3 samples. However, this value includes input virus as cultures had not been washed extensively since initial exposure. Therefore, day 3 samples are only serving as a positive control for the system. Mock control and poly I:C exposed cultures served as negative controls for the system as neither culture was exposed to virus. Day 7 samples however were washed extensively at day 3 to remove input virus and then incubated with fresh media. Viral Gag was present in each of the three cell population's supernatant samples, where Gag was undetectable in mock samples, indicating that hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy cultures incubated with HIV-1 IIIb are likely producing newly synthesized viral Gag (Figure 14c).

4.7 LEC EXPOSURE TO HIV-EGFP SHOWED CELL-ASSOCIATED SIGNAL

To better understand the interactions that were occurring between virus and LECs *in vitro*, I used an eGFP tagged, virus system based on the pNL4.3 viral vector construct, but with a deleterious mutation in the viral accessory protein Vpr (courtesy of Courtney Zych and Dr. Velpandi Ayyavoo). Viral vector deficient in vpr was co-transfected with a vector expressing eGFP-tagged vpr in a permissive cell line and viral supernatant contained fluorescently labeld viral particles. This virus can be used to visualize input virus bound to cells after exposure and thorough wash, as eGFP emits a fluorescent signal when exposed to blue light (85,86). Fluorescently tagged virus was incubated with hTERT-HDLEC and HMVEC-LLy LECs for 3 hours and then cultures were extensively washed. Interestingly enough, fluorescent signal not

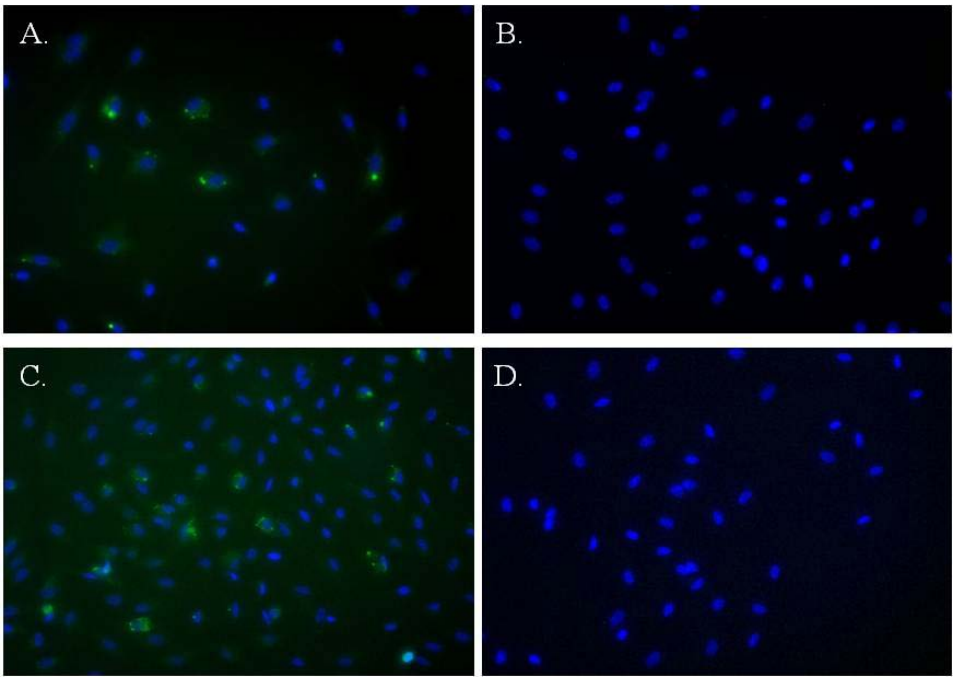


Figure 15: LECs associate with eGFP tagged HIV-1
hTERT-HDLEC(a,b) and HMVEC-LLy(c,d) were incubated with virus at a concentration of 100pg/ml(a) 200pg/ml(c) or media only (b,d). Nuclei were stained and mounted. Original magnification = 200X

seen in mock control cultures was associated with hTERT-HDLECs and HMVEC-LLys (Figure 15). Signal however was seen at relatively low magnification, where typically signal from single viral particles would not be observed. If virus was sequestered into a general sub-cellular compartment, the signal in that area would increase, possibly resulting in the images observed. The results for this experiment were from preliminary data, and future studies will be focused on definitively determining if these virus particles are indeed interacting with LECs *in vitro*. Other viral constructs which can determine to what extent virus is interacting with cells in culture such as viral luciferase constructs may be employed to further understand this complex relationship.

4.8 RESULTS CONCLUSION

LECs are expressing the LEC lineage markers LYVE-1 and podoplanin, the antiviral restriction factors APOBEC3G, TRIM5-α, and BST-2, as well as the viral entry receptors D6, DEC-205, DC-SIGN and CXCR4. Expression levels of some targets are different across cell populations and could reflect differences in tissue derivations or effects of altering cell populations via transduction with hTERT cDNA . Expression levels of these factors are also sensitive to the surrounding environment, as exposure to the dsRNA mimetic poly I:C induced changes in expression profiles. mRNA expression correlated with protein expression in these cell populations. LECs are actively engaging the environment through endocytic and phagocytic mechanisms. LECs exposed to SIV *in vitro* had viral elements present in total RNA samples that are multi-spliced, indicating *de novo* transcription is occurring to some extent. In addition, LECs exposed to HIV had viral Gag present in culture supernatants 4 days after virus was removed and cultures were washed extensively.

Taken together, it is clear that the perception of LECs as just a constituent of lymphatics that forms vessels has to change. LECs are important players in the surveillance of peripheral tissues via lymph, as well as in early innate immunity, by actively recognizing foreign pathogens through PRRs and engaging in the migration of pAPCs by secretion of chemokines. This initial evidence argues for more research focused on these specialized populations of cells and defining their role in the immune system.

5.0 DISCUSSION

The lymphatic endothelium plays a necessary role in removing residual fluid, cells, and metabolic particulates produced in tissues by peripheral lymphatic vessels (33,34). In circumstances where the lymphatic system fails, the body's ability to regulate internal fluid levels can be disrupted. Disorders of lymphatic vessels, such as lymphedema, Hodgkin's disease, and different lymphomas can disrupt the normal regulation of interstitial fluid. The lymphatic system has until now been described as a conduit for migrating DCs, lymphocytes, and interstitial fluid from peripheral tissues to the LNs. Lymph collects via the left and right lymphatic ducts and is eventually re-circulated through the blood stream (110). I believe, however, that the lymphatic endothelium has a much more active role by not only facilitating the migration of mature APCs and lymphocytes, but actively surveying interstitial fluid and engaging foreign pathogens via surface receptors and intracellular restriction factors. The objective of this study was to examine the expression of innate restriction factors and viral entry receptors on model LECs. Therefore, three cell populations (hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy) were used to assess in LECs the expression pattern of a wide variety of factors, known to play roles in a multitude of immune responses. In addition, I also exposed LECs to SIVmac251, the lab-adapted, X4 tropic, HIV-IIIb variant, and an eGFP tagged HIV-1 variant to evaluate if LECs can bind virus, and if so, to what extent virus replicates.

There is previous evidence that suggests LECs express alternative receptors for viral entry and viral restriction factors. A recent study analyzed the expression profile of human dermal LECs treated with the inflammation-inducible cytokine tumor necrosis factor-α (TNF-α) by microarray analysis (87). Expression profiles were then measured using GeneChip software and the level of expression for each gene was ranked. Genes were then placed into percentiles according to their expression levels relative to other genes. The expression levels for the specific genes of interest measured in the studies described in this thesis strongly correlated with the observed expression pattern in the microarray study (Table 5). Dermal LEC populations showed high levels of expression for the standard LEC lineage markers podoplanin and LYVE-1 as they both were ranked in the $>90^{th}$ percentile. Expression of CXCR4, DEC-205, D6 (though at low levels similar to what I observed), and DC-SIGN, was seen in microarray analysis. The restriction factors TRIM5-α, APOBEC3G, and BST-2 (87) were also expressed, supporting the data presented in this thesis. Interestingly, there was detectable expression of the HIV-1 receptor CD4 and co-receptor CCR5 in the microarray data, however at low percentile levels compared to other genes. These findings support the data in this thesis, showing that LECs are actively expressing these factors. There are other microarray studies published that show that not only do LECs express the genes examined here, but they are able to effectively regulate expression profiles in reaction to IFN-α, IFN-γ, and a wide variety of different immune regulatory compounds (88,89,90,91).

Table 5: Percentile ranks for level of gene expression in control (mock) and TNF-α exposed primary human dermal LECs

Levels of gene expression measured by microarray analysis were ranked based on overall expression in mock and TNF-α exposed dermal LEC cultures. These ranks and expression levels are similar to the data presented in this thesis.

Accession # GSE6257		
Target Gene	%ile control	%ile Treatment (TNF-α)
LYVE-1	98	81
Podoplanin	94	96
CXCR4	87	77
DEC-205	75	79
D6	17	17
DC-SIGN	60	60
TRIM5-α	92	93
APOBEC3G	81	89
BST-2	7	87
CD4	29	29
CCR5	42	43
B-GUS	95	93

LECs were expressing different restriction and entry factors. Previous work has shown that LECs effectively regulate migration and maturation of DCs, can mediate inflammation by secreting factors, and possibly control infection of microbes recognizable by PAMPs (23,95). The role LECs play during infection and subsequent immune responses is an active one (Figure 16). LECs express a wide variety of surface receptors that can bind PAMPs, efficiently take up and process antigen, and internalize an assortment of pathogens (Table 6). LECs can respond to

Table 6: Comparison of DCs, T-cells, and LECs

	DCs	T-cells	LECs
Endocytosis	Yes	Yes	Yes
Phagocytosis	Yes	No	Yes
PRRs & Restriction Factors	Yes	Yes	Yes
Chemokine/Cytokine secretion	Yes	Yes	Yes
Typical and Atypical Viral Receptors	Yes	Yes	Yes
Virus Uptake	Yes	Yes	Possibly
Antigen presentation	Yes	No	???
Immune effector function	No	Yes	???

their environment by regulating the expression of restriction factors and even express chemokines that regulate trafficking of APCs to secondary lymphoid tissues (Table 6). LECs also maintain this arsenal of restriction factors that are able to inhibit replication at different stages of the viral life cycle. This characterization of LECs highlights the fact that LECs are able to actively monitor the environment, and elicit a response if necessary, possibly in a manner similar to DCs and T-cells (Table 6). LEC cultures were able to efficiently internalize both pHrodo-labeled dextran and pHrodo-labeled *E. coli* bioparticles (Figure 10-12). T hese mechanisms highlight the ability of LECs to actively engage the environment through endocytic and phagocytic mechanisms, possibly in a manner similar to DCs.

LECs exposed to SIVmac251 had not only un-spliced, viral genomic RNA but multi-spliced viral transcripts that would only be present in LEC total RNAs if some sort of *de novo* viral replication was occurring. The presence of *rev* transcripts indicated that virus is not only

bound to the surface of LECs but internalized, possibly through some receptor I have described, and replicating (Figure 13) to either productive replication or some downstream abortive stage. Although HIV-1 PCR products obtained with LEC total RNAs as templates has not been sequenced yet, viral Gag protein was found in day 7 supernatant samples that were exposed to virus for 3 da ys and then washed, indicating that these cultures were producing newly synthesized viral protein (Figure 14). Finally, preliminary findings indicate that LEC were interacting with eGFP tagged virus, yet more experiments are required to fully understand this (Figure 15).

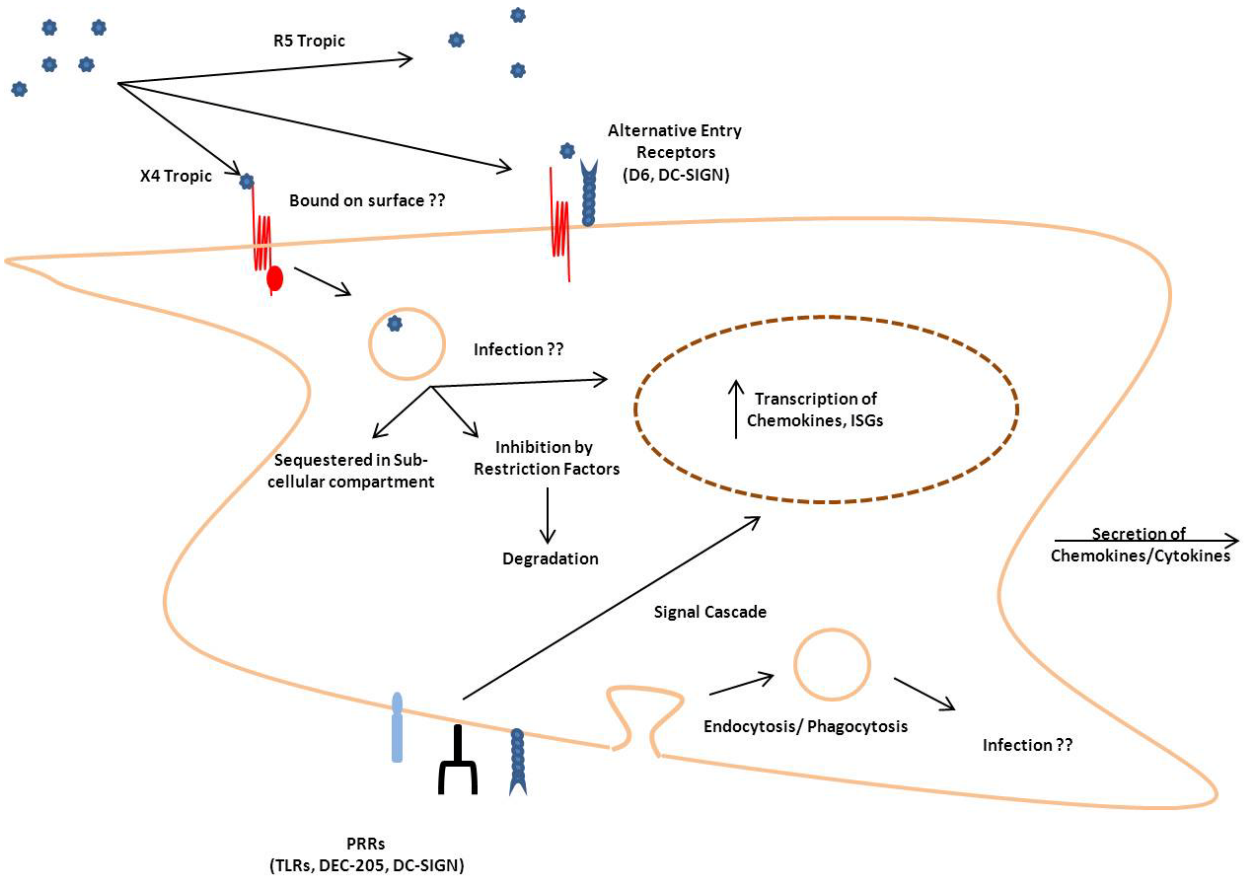


Figure 16: Model LEC and potential roles during infection

The fact that LECs express a wide variety of entry factors could have major implications in the transmission of SIV and HIV-1. It is largely acknowledged R5 tropic viral variants are responsible for the vast majority of transmission events and that co-receptor switching occurs at a later time point of infection. Viral isolates from newly infected patients are R5 predominate (118). High mutation rates lead to variances in viral envelope, allowing for co-receptor ambiguity (117). LECs express CXCR4 as well as other entry receptors and are present at areas where transmission events occur. LECs could potentially act as a viral sieve, removing X4-tropic viral variants from the environment while allowing R5-tropic variants to pass (Figure 16) whereupon they can infect other target cells (i.e., T-cells and DCs). This could have some effect on the viral heterogeneity seen in newly infected patients. If LECs are internalizing virus, they are potentially able to inhibit replication, as antiviral factors that are able to inhibit replication in a number of different ways are being expressed by LECs.

In summary, these results have shown that LECs constitutively express viral entry receptors and restriction factors. LECs have proved to be more than a subset of endothelial cells that line the inner walls of vessels, but rather an active player in the surveillance of peripheral tissues for infection. A complex relationship between APCs, peripheral migrating lymphocytes, and the afferent lymphatics (specifically LECs) is likely occurring. The preliminary data indicating active engagement and internalization of virus provides exciting possibilities. If LECs are actively engaging and internalizing virus *in vitro,* they could potentially be doing the same thing *in vivo*, serving as a viral sieve, taking up cell free virus as it migrates to LNs. More intriguingly, LECs could potentially act as a viral reservoir or auxiliary site of replication. This

possibility in itself makes researching the lymphatic endothelium during healthy and disease states very important.

6.0 FUTURE DIRECTIONS/ PUBLIC HEALTH RELAVENCE

To date, LECs have been characterized for the expression of markers of LEC lineage, antiviral restriction factors, and alternate receptors for HIV-1 on three human LEC populations. Spliced and un-spliced viral transcripts could be retrieved from LEC populations that had been exposed to SIVmac251, suggesting *de novo* transcription was occurring. Cell populations have also been exposed to a number of microbes and molecules able to visualize the endocytic and phagocytic nature of LECs. The work to date has effectively characterized LECs in a manner that highlights their ability to actively survey the environment as well as their capability of effectively engaging foreign microbes through a multitude of different receptors and mechanisms. The following experiments could be conducted to further describe LECs' roles in immune responses.

6.1 PROTEIN ANALYSIS OF LECS

To elucidate further the expression of the restriction and entry factors previously described on LECs, flow cytometry analysis of hTERT-HDLEC, HMVEC-DLy, and HMVEC-LLy could be conducted in coordination with previous immunofluorescent staining. Flow cytometry would be able to not only detect positive cells within the population, but could establish if expression is homogeneous throughout the population. Flow cytometry analysis would also be able to determine differences in expression after treatment with stimulating molecules, such as poly I:C,

to determine if protein expression changes in response to the environment. Whole cell lysate of LEC populations could be used to support the data already collected.

6.2 VIRUS EXPOSURE

Evidence from experiments conducted thus far indicates that not only are LEC populations able to bind and internalize virus *in vitro*, they are also permissive for SIV *de novo* transcription of multi-spliced viral transcripts. Data from exposure to HIV variants has been less conclusive in defining the relationship between virus and LEC. Future experiments could focus on determining exactly to what extent virus can interact with LEC populations *in vitro*. In experiments where LEC populations were exposed to eGFP tagged virus, preliminary results were obtained. Exposure to a luciferase lentiviral construct would prove valuable as presence of signal from this viral construct not only indicates entry, but productive reverse transcription and integration leading to replication. LTR-circle and Alu-PCR could be conducted to determine if virus is actively integrating into the host genome, as well as to establish to what endpoint infection of LEC cultures is occurring.

6.3 CONFOCAL MICROSCOPY

Although immunofluorescent staining done to date has provided evidence regarding expression of certain targets of interest, confocal microscopy would be able to better illustrate the cellular distribution of these targets. Confocal microscopy would also allow for co-localization

experiments to be conducted thus determining what factors expressed by LECs does virus interact with, possibly identifying a pathway not previously described in LEC populations. Finally, confocal images of the endocytic and phagocytic reactions to pHrodo bioparticles would produce images of a section of the cell, better identifying particular vesicles instead of the cumulative fluorescent signal of the cell seen in traditional immunofluorescent images.

6.4 PUBLIC HEALTH RELAVENCE

It is widely known that HIV-1 infected individuals often have a replication-competent reservoir of virus in resting, memory CD4 T-cells (65), even when plasma viral levels are undetectable. In times of immunosuppression or non-adherence to drug regimens, however, virus from latent reservoirs can induce productive replication leading to increased viremia and possible complications due to infection (i.e. drug resistant strains, opportunistic infections). It has also been established that there are alternative entry mechanisms that HIV-1 can use to gain entry to a wide variety of cell populations other than the typical CD4+ T-cell populations. To date, research has not been conducted, however, on the specific role LECs could play in both topics. LECs express many of the factors previously described as used by HIV to gain entry, and are present at portals of transmission where virus contact likely occurs. If there is some low level replication of virus in LECs as described, then LECs could potentially be another target for infection. Either way, there is evidence now that LECs are actively expressing many factors that are important in many innate immune responses to a wide array of foreign pathogens. The presence of LECs proximal to mucosal portals of transmission means that the potential for LECs to come directly into contact with virus either presented by APCs or cell free virus is high. It is

therefore biologically relevant and important in a therapeutic context to better understand the possible interactions and outcomes between virus and LECs.

7.0 CONCLUDING REMARKS

June 5th, 2011 marked the 30th anniversary of the first publication from the CDC regarding the AIDS epidemic. Great strides have been taken to transform the disease from a devastating illness, which took seemingly healthy young adults and ravaged their immune system, to a now chronic and manageable disease. The discovery of different classes of antiretroviral drugs, and the ability of HAART to control viral replication has allowed many infected patients to live active lives with very little disturbance. This has led some to believe that the battle is won. I however believe there is much still to be accomplished. The lack of a prophylactic vaccine that can effectively protect the population is still out of reach. The access to healthcare disparities between races and socioeconomic classes is still a major problem even in developed countries. The spread of new cases may have plateaued in developed countries but more people are living with AIDS now than ever before. Other complications from long-term HAART are now being observed as patients are starting to reach older ages that they previously have not survived to. Research on the lymphatic system's role in immune surveillance and its ability to effectively traffic lymphocytes to sites of infection has been extensive. The ability of LECs to contribute to such a response however has not. Recent evidence has suggested that for a therapeutic vaccine to be efficacious, it will need to be able to protect every cell at the portal of entry from infection, and be able to sustain this ability over time. For this to occur, I believe that the role of the

lymphatic system, in particularly LECs, must further be described and understood if we ever hope to develop efficacious vaccines.

BIBLIOGRAPHY

1. Neil S, Aasa-Chapman M, Clapham P, Nibbs R, McKnight A, Weiss R,.The Promiscuous CC Chemokine Receptor D6 Is a Functional Coreceptor for Primary Isolates of Human Immunodeficiency Virus Type 1 (HIV-1) and HIV-2 on A strocytes J. Virol. 2005 79: 9618-9624.
2. Timothy S. Olson, and Klaus Ley Chemokines and chemokine receptors in leukocyte trafficking *Am J Physiol Regul Integr Comp Physiol* 283: R7-R28, 2002. d oi:10.1152/ ajpregu.00738.2001.
3. Murdoch C, Monk P N, Finn A,. CXC Chemokine Receptor Expression on H uman Endothelial Cells *Cytokine* 1999. 11(9) 704-712.
4. Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A*. 1995; 92: 3566–3570.
5. Hirakawa S, Hong YK, Harvey N, et al. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol*. 2003; 162: 575–586.
6. Kaneko M, Kato Y, Kitano T, Osawa M (2006) Conservation of a platelet activating domain of Aggrus/Podoplanin as a platelet aggregation-inducing factor. *Gene* 378: 52–57.
7. Bertozzi C, Hess P, Kahn M,. P latelets: Covert Regulators of Lymphatic Development Arterioscler Thromb Vasc Biol 30: 2368-2371.
8. Chiu, Y.L. and Greene, W.C. (2008) The APOBEC3cytidine deaminases: an innate defensivenetwork opposing exogenous retroviruses andendogenous retroelements. Annual Reviewof Immunology 26, 317-353.
9. Jing Ma Xiaoyu Li* Jian Xu Quan Zhang , Zhenlong Liu , Pingping Jia , Jinming Zhou , Fei Guo , Xuefu You , Liyan Yu , Lixun Zhao , Jiandong Jiang and Shan Cen The cellular source for APOBEC3G's incorporation into HIV-1*Retrovirology* 2011, 8:2doi:10.1186/1742-4690-8-2.
10. Albin J, and Harris R,. Interactions of host APOBEC3 restriction factors with HIV-1 in vivo: implications for therapeutics. *Expert Reviews in Molecular Medicine.* 2010. DOI: 10.1017/S1462399409001343.
11. A. Mehle et al., Vif overcomes the innate antiviral activity of APOBEC3G by promoting its degradation in the ubiquitin-proteasome pathway, J. Biol. Chem. 279 (2004), pp. 7792–7798.
12. A.M. Sheehy, N.C. Gaddis, J.D. Choi and M.H. Malim, Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (6898) (2002), pp. 646–650.
13. Golia-Gaur R, Strebel K,. HIV-1 Vif, APOBEC, and intrinsic immunity. *Retrovirology*, (2008) Jun 24;5;51.
14. Reymond, A. et *al.*The tripartite motif family identifies cell compartments. *EMBO J.* 20, 2140-2151 (2001).
15. Reddy BA, Etkin LD, Freemont PS,. A novel zinc finger coiled-coil domain in a family of nuclear proteins. *Trends Biochem,* Sci. 17(9):344-5.

16. Reymond A, Meroni G, Fantozzi A, Merla G, Cairo S, Luzi L, Riganelli D, Zanaria E, Messali S, Cainarca S et al.: The tripartite motif family identifies cell compartments. EMBO J 2001,20:2140-2151.
17. S. Kupzig *et al.*, Bst-2/HM1.24 is a raft-associated apical membrane protein with an unusual topology, *Traffic* 4 (2003), pp. 694–709.
18. Lopez L, Yang S, Hauser H, Exline C, Haworth K, Oldenburg J, Cannon P,. Ebola virus glycoprotein counteracts BST-2/Tetherin restriction in a sequence-independent manner that does not require tetherin surface removal. *Journal of Virology*. 2010 Jul;84(14):7243-55.
19. Hatsukari I, Singh P, Hitosugi N, Messmer D, Valderrama E, Teichberg S, Chaung W, Gross E, Schmidtmayerova H, Singhal PC: DEC-205-Mediated internalization of HIV-1 results in the establishment of silent infection in renal tubular cells. *J Am Soc Nephrol* 2007;18:780–787.
20. Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, vanDuijnhoven GC, Middel J, Cornelissen IL, Nottet HS, Kewal RamaniVN, Littman DR, Figdor CG, van KY (2000) DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of Tcells. *Cell* 100:587–597.
21. Nisato R, Harrison J, Buser R, Orci L, Rinsch C, Montesano R, Dupraz P, Pepper M, Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. *Am J Pathol.* 2004 Jul;165(1):11-24.
22. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, et al. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 1998 393: 648–659.
23. Pegu A, Qin S, Fallert Junecko BA, Nisato RE, Pepper MS, Reinhart TA.Human Lymphatic Endothelial Cells Express Multiple Functional TLRs. *J Immunol.* 2008 Mar 1;180(5):3399-405.
24. LaBranche C, Berson J, Doms R, Hoxie J, Matthews T. Characterization of an HIV-1/IIIB that uses CXCR4 as its receptor in the absence of CD4. *Keyst Symp Mol Cell Biol Keyst Symp Mol Cell Biol.* 1998 Mar 13-19; 69.
25. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C Vezinet-Brun F, Rouzioux C, Rozenbaum W and Montagnier L. Isolation of a T -lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 20 May 1983: Vol. 220 no. 4599 pp. 868-871.
26. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, and et. al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 4 May 1984: 224 (4648), 500-503.
27. Rizzuto CD, Wyatt R, Hernandez-Ramos N, Sun Y, Kwong PD, Hendrickson WA, and Sodroski J. A Conserved HIV gp120 Glycoprotein Structure Involved in Chemokine Receptor Binding. Science 19 June 1998: Vol. 280 no. 5371 pp. 1949-1953
28. World Health Organization-HIV/AIDS Data and Statistics. http://www. who.int/hiv/data/en/. viewed 6-27-11.
29. Stacker SA, Caesar C, Baldwin ME, Thronton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells *via* the lymphatics *Nature Medicine* 7, 186 - 191 (2001).

30. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Valesco P, Riccardi L, Alitalo K, Claffey K, Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Medicine* 7, 192-198. (2001).
31. Navarro F, Bollman B, Chen H, Konig R, Yu Q, Chiles K, Landau NR. Complementary function of the two catalytic domains of APOBEC3G. *Virology.* 333.2 374-386. (2005)
32. Gunn MD, Tangemann K, Tam C, *et al.* (1998). "A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes.". *Proc. Natl. Acad. Sci. U.S.A.* 95 (1): 258–63.
33. Aukland, K., and R.K. Reed. 1993. Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol. Rev.* 73:1–78.
34. Schmid-Schönbein, G.W. 1990b. Microlymphatics and lymph flow.*Physiol. Rev.* 70:987–1028.
35. Wigle, J T., N Harvey, M Detmar, I Laqutina, and G Oliver. "An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype." *EMBO J* 21.72 Apr. (2002): 1505-13. Web. 6 June 2011.
36. Kaipainen, A., J. Korhonen, T. Mustonen, V. W. van Hinsbergh, G. H. Fang, D. Dumont, M. Breitman, and K. Alitalo. 1995. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl. Acad. Sci. U. S. A* 92:3566.
37. Banerji, S., J. Ni, S. X. Wang, S. Clasper, J. Su, R. Tammi, M. Jones, and D. G. Jackson. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol.* 144:789.
38. Podgrabinska, S., P. Braun, P. Velasco, B. Kloos, M. S. Pepper, and M. Skobe. 2002. Molecular characterization of lymphatic endothelial cells. *Proc. Natl. Acad. Sci. U. S. A* 99:16069.
39. Ouhtit A, et al. *In vivo* evidence for the role of CD44s in promoting breast cancer metastasis to the liver. Am J Path. 2007;171:2033-2039.
40. Kato Y, Fujita N, Kunita A, Sato S, Kaneko M, Osawa M and Tsuruo T: Molecular identification of Aggrus/T1α as a platelet aggregation-inducing factor expressed in colorectal tumors. JBiol Chem *278*: 51599-51605, 2003.
41. Dobbs, L G., M C. Williams, and R Gonzalez. "Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 970.230 June (1988): 146-56. Web. 6 June 2011.
42. Rishi, A K., M Joyce-Brady, J Fisher, L G. Dobbs, and M C. Williams. "Cloning, characterization, and development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives." *Dev Biol* 167.1 Jan. (1995): 294-306. Web. 6 June 2011.
43. Gandarillas, A, F G. Scholl, N Benito, C Gamallo, and M Quintanilla. "Induction of PA2.26, a cell-surface antigen expressed by active fibroblasts, in mouse epidermal keratinocytes during carcinogenesis." *Molecular Carcinogenesis* 20.17 Dec. (1998): 10-18. Web. 6 June 2011.
44. Navarro, A, R E. Perez, M Rezaiekhaligh, S M. Mabry, and I I. Ekekezie. "T1α/podoplanin is essential for capillary morphogenesis in lymphatic endothelial cells." *American Journal of Physiology Lung Cell Molecular Physiology* 29525 July (2008): 543-51. Web. 6 June 2011.

45. Tammela, T., Zarkada, G., Wallgard, E., Murtomaki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellstro¨m, M., Schomber, T., Peltonen, R. et al. (2008) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454, 656–660.
46. Chu-Lagraff, Q., Wright, D.M., McNeil, L.K., and Doe, C.Q. (1991). The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila. Dev. Suppl. 2, 79–85.
47. Amatschek S, Kriehuber E, Bauer W, et al. Blood and lymphatic endothelial cell-specific differentiation programs are stringently controlled by the tissue environment. Blood. 2007; 109:4777-4785.
48. Petrova TV, Makinen T, Makela TP, et al. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. EMBO J. 2002;21:4593-4599.
49. Qin, J, D M. Gao, Q F. Jiang, Q Zhou, and Y H. Xie. "Prospero-related homeobox (Prox1) is a corepressor of human liver receptor homolog-1 and suppresses the transcription of the cholesterol 7-alpha-hydroxylase gene." *Molecular Endocrinology* 18.10 Oct. (2004): 2424-39. Web. 6 June 2011.
50. Huthoff, H, and G T. Towers. "Restriction of retroviral replication by APOBEC3G/F and TRIM5a." *Trends in microbiology* 16.12 Dec. (2008): 612-19. Web. 6 June 2011.
51. K.M. Chen *et al.*, Structure of the DNA deaminase domain of the HIV-1 restriction factor APOBEC3G, *Nature* 452 (2008), pp. 116–119.
52. M. Stremlau *et al.*, The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys, *Nature* 427 (2004), pp. 848–853.
53. M.W. Yap *et al.*, A single amino acid change in the SPRY domain of human Trim5alpha leads to HIV-1 restriction, *Curr. Biol.* 15 (2005), pp. 73–78.
54. Nisole, S, J P. Storye, and Ali Saib. "TRIM family proteins: retroviral restriction and antiviral defence." *Nature Reviews Microbiology* 3 Oct. (2005): 799-808. Web. 6 June 2011.
55. Iwabu, Y, H Fujita, M Kinomoto, K Kaneko, and K Tokunga. "HIV-1 Accessory Protein Vpu Internalizes Cell-surface BST-2/Tetherin through Transmembrane Interactions Leading to Lysosomes." *The journal of biological Chemistry* 284.5011 Dec. (2009): 35060-72. Web. 6 June 2011.
56. Rollason, R, V Korolchuk, C Hamilton, P Schu, and G Banting. "Clathrin-mediated endocytosis of a lipid-raft-associated protein is mediated through a dual tyrosine motif." *Journal of Cell Science* 1201 Nov. (2007): 3850-58.
57. Ohmine, S, R Sakuma, T Sakuma, T Thatava, and Y Ikeda. "Cytoplasmic body component TRIM5{alpha} requires lipid-enriched microdomains for efficient HIV-1 restriction." *Journal of Biological Chemistry* 285.455 Nov. (2010): 34508-17.
58. Friedrichson, T.& Kurzchalia, T. V. “Microdomains of GPI-anchored proteins in living cells revealed by crosslinking.” *Nature* 394, 802–805 (1998).
59. Simons, K, and D Toomre. "Lipid rafts and signal transduction." *Nature reviews Molecular Cell Biology* 1 Oct. (2000): 31-39.
60. Ono, A. "Relationships between plasma membrane microdomains and HIV-1 assembly." *Biol Cell* 102.625 Mar. (2010): 335-50.
61. Schroeder, C. "Cholesterol-Binding Viral Proteins in Virus Entry and Morphogenesis." *Subcellular Biochemistry* 51 (2010): 77-108.

62. Waheed, A A., and E O. Freed. "Lipids and membrane microdomains in HIV-1 replication." *Virus research* 143.2 Aug. (2009): 162-76.
63. Blander, J M., and R Medzhitov. "Regulation of Phagosome Maturation by Signals from Toll-Like Receptors." *Science* 304.567314 May (2004): 1014-18. Print.
64. Goff, S P. "Retrovirus Restriction Factors." *Molecular Cell* 16.622 Dec. (2004): 849-59.
65. Chun, T-W. *et al*. "Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection." *Nature* 387, 183–188 (1997).
66. Takeda, K, and S Akira. "TLR signaling pathways." *Seminars in Immunology* 16 (2004): 3-9.
67. Matsumoto, M, H Oshiumi, and T Seya. "Antiviral responses induced by TLR3 pathway." *Reviews in Medical Virology* 2110 Feb. (2011): 67-77.
68. Hughes, J F., and J M. Coffin. "Evidence for genomic rearrangements mediated by human endogenous retroviruses during primate evolution." *Nature genetics* 2912 Nov. (2001): 487-89.
69. Belshaw, R, V Pereira, A Katzourakis, G Talbot, and M Tristem. "Long-term reinfection of the human genome by endogenous retroviruses." *PNAS* 101.1425 Mar. (2004): 4894-99.
70. Abbink, T E., and B Berkhout. "RNA Structure Modulates Splicing Efficiency at the Human Immunodeficiency Virus Type 1 Major Splice Donor." *Journal of Virology* 82.6 Mar. (2008): 3090-98.
71. Fra, A, M Locati, K Otero, M Sironi, and A Mantovani. "Cutting Edge: Scavenging of Inflammatory CC Chemokines by the Promiscuous Putatively Silent Chemokine Receptor D6." *Journal of Immunology* 170.51 Mar. (2003): 2279-82.
72. Nibbs, R J., S M. Wylie, J Yang, N R. Landau, and G J. Grahm. "Cloning and Characterization of a Novel Promiscuous Human β-Chemokine Receptor D6." *The Journal of Biological Chemisrty* 27219 Dec. (1997): 32078-83.
73. Nibbs RJ, Kriehuber E, Ponath PD, Parent D, Qin S, Campbell JD, Henderson A, Kerjaschki D, Maurer D., Graham GJ., Rot A. "The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors." *Am J Pathol* 2001;158:867–877.
74. Nibbs, R, G Graham, and A Rot. "Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6." *Semminars in Immunology* 15.5 Oct. (2003): 287-94.
75. Geijtenbeek, T. B. *et al*. "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances *trans*-infection of T cells." *Cell* 100, 587–597 (2000).
76. Altfeld, M, L Fadda, D Frleta, and N Bhardwaj. "DCs and NK cells: critical effectors in the immune response to HIV-1." *Nature Reviews* 11 Mar. (2011): 176-86.
77. McGreal, E P., J L. Miller, and S Gordon. "Ligand recognition by antigen-presenting cell C-type lectin receptors." *Current Opinion in Immunology* 17.1 Feb. (2005): 18-24. Print.
78. Geijtenbeek, T B., R Torensma, S J. van Vilet, G F. van Duijnhoven, and C G. Figdor. "Identification of DC-SIGN, a Novel Dendritic Cell–Specific ICAM-3 Receptor that Supports Primary Immune Responses." *Cell* 100.53 Mar. (2000): 575-85. Print.
79. Rappocciolo, G, P Piazza, C L. Fuller, T A. Reinhart, and C A. Rinaldo Jr. "DC-SIGn on B Lymphocytes is Required for Transmission of HIV-1 to T Lymphocytes." *PLOS Pathogens* 2.7 July (2006): 691-704.
80. Jiang, W, W J. Swiggard, C Heufler, M Peng, and M C. Nussenzweig. "The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing." *Nature* 37511 May (1995): 151-55.

81. Witmer-Pack, M D., W J. Swiggard, A Mizra, K Inaba, and R M. Steinman. "Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression in situ in lymphoid and nonlymphoid tissues." *Cell Immunology* 163.1 June (1995): 157-62.
82. Bonifaz, L, D Bonnyay, K Mahanke, M Rivera, and R M. Steinman. "Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8+." *Journal of Experimental Medicine* 192.1216 Dec. (2002): 1627-38.
83. Mahnke, K, M Guo, S Lee, H Sepulveda, and R M. Steinman. "The Dendritic Cell Receptor for Endocytosis, Dec-205, Can Recycle and Enhance Antigen Presentation via Major Histocompatibility Complex Class II–Positive Lysosomal Compartments." *The Jouranl of Cell Biology* 151.330 Oct. (2000): 673-84. Print.
84. Bozzacco, L, C Trumpfheller, F P. Siegal, S Mehandru, and R P. Steinman. "DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes." *PNAS* 104.423 Jan. (2007): 1289-94. Print.
85. Tsien, R Y. "THE GREEN FLUORESCENT PROTEIN." *Annual Review of Biochemistry* 67 July (1998): 509-44.
86. Adachi, A, H E. Gendelman, S Koenig, T Folks, and M A. Martin. "Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone." *Journal of Virology* 59.2 Aug. (1986): 284-91.
87. Johnson, L A., S Clasper, A P. Holt, P F. Lalor, and D G. Jackson. "An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium." *Journal of Esperimental Medicine* 203.1227 Nov. (2006): 2763-77.
88. Indraccolo, S, U Pfeffer, S Minuzzo, G Esposito, and A Amadori. "Identifciation of Genes Selectively Regulated by IFNs in Endothelial Cells." *Journal of Immunology* 17830 Mar. (2007): 1122-35.
89. Irigoyen M, Ansó E, Martínez E, Garayoa M et al. Hypoxia alters the adhesive properties of lymphatic endothelial cells. A transcriptional and functional study. *Biochim Biophys Acta* 2007 Jun;1773(6):880-90
90. Hiyama K, Otani K, Ohtaki M, Satoh K et al. Differentially expressed genes throughout the cellular immortalization processes are quite different between normal human fibroblasts and endothelial cells. *Int J Oncol* 2005 Jul;27(1):87-95.
91. Sana TR, Janatpour MJ, Sathe M, McEvoy LM et al. Microarray analysis of primary endothelial cells challenged with different inflammatory and immune cytokines. *Cytokine* 2005 Mar 21;29(6):256-69.
92. Eichten, A, W C. Hyun, and L M. Coussens. "Distinctive Features of Angiogenesis and Lymphangiogenesis Determine Their Functionality during De novo Tumor Development." *Cancer Research* 71.1215 June (2011): 5211-20.
93. Mellors, J W., C R. Rinaldo Jr., P Gupta, R M. White, and L A. Kingsley. "Prognosis in HIV-1 Infection Predicted by the Quantity of Virus in Plasma." *Science* 272.526524 May (1996): 1167-70.
94. Kaplan, J E., C Benson, K K. Holmes, J T. Brooks, and H Mausr. "Guidelines for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents Recommendations from CDC, the National Institutes of Health, and the HIV Medicine

Association." *CDC MMWR*. Department of Health and Human Services , 10 Apr. 2009. Web. 23 June 2011.
95. Pegu, A, J L. Flynn, and T A. Reinhart. "Afferent and Efferent Interfaces of Lymph Nodes are Distinguished by Expression of Lymphatic Endothelial Markers and Chemokines." *Lymphatic Research and Biology* 515 Oct. (2007): 91-103.
96. Zhang, Z Q., S W. Wiegrefe, Q Li, L Duan, and A T. Haase. "Roles of substrate availability and infection of resting and activated CD4+ T cells in transmission and acute simian immunodeficiency virus infection." *PNAS* 101.1513 Apr. (2004): 5640-45.
97. Zhang, Z, T Schuler, M Zupancic, S Wietgrefe, and A T. Haase. "Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells." *Science* 286.544317 Dec. (1999): 1353-57.
98. Matsui, M, K Nagy-Bojarsky, P Laakkonen, S Krieger, and D Kerjaschki. "Lymphatic Microvessels in the Rat Remnant Kidney Model of Renal Fibrosis: Aminopeptidase P and Podoplanin Are Discriminatory Markers for Endothelial Cells of Blood and Lymphatic Vessels." *JASN* 14 (2003): 1981-89.
99. Strebel, Klaus. "Structure and Function of HIV-1 Vpu." National Institutes of Health, n.d. Web. 24 June 2011. <http://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1996/PART-III/2.pdf>.
100. "Real-Time PCR Systems Chemistry Guide." *Applied Biosystems*. Applied Biosystems, 2005. Web. 24 J une 2011.<http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_041440.pdf>.
101. "User Bulletin # 2 ABI Prism 7700 S equence Detection System." *Applied Biosystems*. Applied Biosystems, 2001. W eb. 24 June 2011. < http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf>.
102. Bienenstock, J., Johnston, N. & Perey, D.Y. Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab. Invest*. 28, 686–692 (1973).
103. Kraehenbuhl JP, Neutra MR. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol. Rev.* 1992;72:853–879
104. Knop, E, and N Knop. "The role of eye-associated lymphoid tissue in corneal immune protection." *Journal of Anatomy* 206.3 Mar. (2005): 271-85. Print.
105. "lymphatic system." *Encyclopædia Britannica. Encyclopædia Britannica Online*. Encyclopædia Britannica, 2011. Web. 25 Jun. 2011. <http://www.britannica.com/EBchecked/topic/352770/lymphatic-system>.
106. Karkainen, M J., A Saaristo, L Jussila, K A. Karila, and K Alitalo. "A model for gene therapy of human hereditary lymphedema." *PNAS* 98.229 Oct. (2001): 12677-82.
107. Drinker, C K., M E. Field, H K. Ward, and C Lyons. "Increased susceptibility to local infection followin blockage of lymph drainage." *Am J Physiol* 112 (1935): 74-81.
108. Carroll, P A., E Brazeau, and M Lagunoff. "Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation." *Virology* 328.115 Oct. (2004): 7-18.
109. Thongratsakul, S, Y Suzuki, H Hiramatsu, T Sakpuaram, and T Songserm. "Avian and human influenza A virus receptors in trachea and lung of animals." *Asian Pac J Allergy Immunol* 28.428 Dec. (2010): 294-301.
110. Adams, R H., and K Alitalo. "Molecular Regulation of Angiogenesis and lymphangiogenesis." *Nature Reviews Molecular Cell Biology* 8 June (2007): 464-78.

111. Lee, S, I Choi, and Y K. Hong. "Heterogeneity and Plasticity of Lymphatic Endothelial Cells." *Seminars in Thrombosis and Hemostasis* 36.3 Apr. (2010): 352-61.
112. Viglianti, G A., P L. Sharma, and J I. Mullins. "Simian Immunodeficiency Virus Displays Complex Patterns of RNA Splicing." *Journal of Virology* 64.916 Mar. (1990): 4207-16.
113. Fuchs, S Y., V S. Spiegelman, and K G. Suresh Kumar. "The many faces of B-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer." *Oncogene* 23 (2004): 2028-36.
114. Ludwig, I S., A N. Lekkerkerker, E Depla, F Bosman, and H Geijtenbeek. "Hepatitis C Virus Targets DC-SIGN and L-SIGN To Escape Lysosomal Degradation." *Journal of virology* 78.15 Aug. (2004): 8322-32.
115. Alvarez, C P., F Lasala, J Carrillo, O Muniz, and R Delgado. "C-type lectins DC-SIGN and L-SIGN mediate cellular entry by Ebola virus in cis and in trans." *Journal of Virology* 76.13 July (2002): 6841-44.
116. Geijtenbeek, T B., S J. Van Vliet, E A. Koppel, M Sanchez-Hernandez, and Y Van Kooyk. "Mycobacteria target DC-SIGN to suppress dendritic cell function." *Journal of Experimental Medicine* 197.16 Jan. (2003): 7-17.
117. Coetzer, M, R Nedellec, J Salkowitz, and D E. Mosier. "Evolution of CCR5 use before and durin coreceptor switching." *Journal of Virology* 82.23 Dec. (2008): 11758-66. Print.
118. van't Wout, A B., N A. Kootstra, G A. Mulder-Kampinga, L N. Albrecht-van, and H Schuitemaker. "Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission." *Journal of Clinical Investigation* 94.5 Nov. (1994): 2060-67.