INNATE IMMUNE POTENTIAL OF

PRIMARY LYMPHATIC ENDOTHELIAL CELLS

by

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ABSTRACT

Lymphatic endothelial cells (LECs) are loosely overlapping cells that line the lymphatic vasculature. In tissues, LECs are often located directly beneath the mucosal epithelial layer, and therefore, are likely to be among the first cells that come in contact with incoming pathogens and/or vaccine antigens and adjuvants when there is a breakage at the epithelial barrier. We are only starting to appreciate the role of LECs in the development of host innate and adaptive immune responses during infection or vaccine administration. This is largely due to the difficulties in studying LECs in vivo and the challenges in obtaining pure LEC cultures for study ex vivo. My work focused on isolation and establishment of primary LEC cultures derived from different tissue origins of commonly used animal models – ferrets and rhesus macaques – and assessment of the potential of these LECs to respond to pathogen-associated molecular patterns (PAMPs) or a subset of microbes (SIV/HIV-1). In addition, I also partially compared the phenotype and functionality of LECs to dendritic cells (DCs), an immune cell type that acts as a "bridge" between the host innate and adaptive immunity. My findings revealed that LECs were highly heterogeneous in their gene expression profiles. They also endogenously expressed multiple known viral entry and restriction factors for SIV/HIV-1. LECs responded to several

PAMPs by producing proinflammatory cytokines/chemokines that are known to recruit immune cells to sites of inflammation. However, LECs were not highly susceptible or permissible to infection using genetically engineered, single-cycle competent SIV/HIV-1 or wild-type SIV. Interestingly, LECs expressed phenotypic markers that have been shown to be expressed by DCs and showed some functional similarities. LECs were able to take up and process model antigens, although it was not determined if these antigens were presented by MHC I and II molecules. These findings are of public health importance because they expand our knowledge of the emerging potential of LECs as key players in innate immunity during pathogen-host interactions or vaccinations. Improving our understanding of LECs will positively impact our knowledge of mucosal infections and will help in development of improved treatment and vaccination strategies. These primary cells will serve as tools to study LEC immunobiology and will also be useful in development of vaccines/therapeutics for human diseases of public health importance that target LEC and/or its crosstalk with other immune cells.

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DEDICATION

For Mom and Dad

PREFACE

In his book entitled *Out of Our Minds: Learning to be Creative*, Sir Ken Robinson wrote, "What we become as our lives evolve depends on the quality of our experiences here and now."

And I fully agree with that statement. This dissertation would not have been possible without the encouragement, support, and love that I received from so many people. I do not have words to adequately convey my deepest and sincerest gratitude to each and every one of you, and it is my hope that this body of work is yours as much as it is mine.

To my advisor and mentor, Dr. Todd A. Reinhart: Thank you for fostering in me the passion, enthusiasm, and tremendous desire to understand the beauty and complexity of immunology. I have benefited greatly from the opportunity to work and to grow as a scientist under your mentorship and guidance. You helped "flick" the switch in me that allowed me to discover and continue to explore my lifelong journey as a scientist and a scholar. It is my sincerest hope that moving forward in my career from your lab I will take with me not only the technical skills but also the qualities of a dedicated academician. Dr. Reinhart – from the bottom of my heart, thank you.

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To my lab family, especially Carissa, Beth, Nicole, and Cynthia: Thank you for your friendship. There's an anonymous quote that says, "Good friends are hard to find, harder to leave, and impossible to forget." I will always treasure our friendships and I know that I have found lifelong friends in each one of you.

To my own family, especially my dad, my late mom, and my siblings: Thank you for your love. Thank you for your understanding. And for tolerating my absence on so many important family occasions. Dad – thank you for telling me not to be afraid to try just because I might fail. Mom – thank you for telling me that not all things are good but not all things are bad either. And, I know that you are now a star that shines your light down on me, guiding me as always. Diana and Jeffery – thank you for filling the shoes of the eldest borne on so many occasions on my behalf. I could not have asked for a better sister and brother-in-law.

And finally to you, the reader: Thank you for your interest in this body of work.

1.0 INTRODUCTION

1.1 LYMPHATIC ENDOTHELIUM

1.1.1 Historical perspective

The mammalian vascular system is made of two highly related but functionally distinct and specialized vasculature networks, namely the blood vasculature and the lymphatic vasculature (1, 2). These vasculature systems both are made of similarly extensive networks of capillaries and vessels. However, due to its importance in the blood vascular (cardiovascular) system, the blood vasculature has received far more attention than its sister vasculature, the lymphatic vasculature. The blood vascular system is a closed circulatory system pumped by the heart, which main function is to deliver oxygen to cells in organs (3). In contrast, the lymphatic vascular system is a blunt, open-ended, unidirectional system, which main function is to return excess tissue fluids, cells, and macromolecules, collectively in the form of lymph, from the interstitial spaces of tissues and organs to draining lymph nodes and eventually to the blood circulation through the thoracic duct or cisterna chili that joined to the subclavian veins (Figure 1) (4).

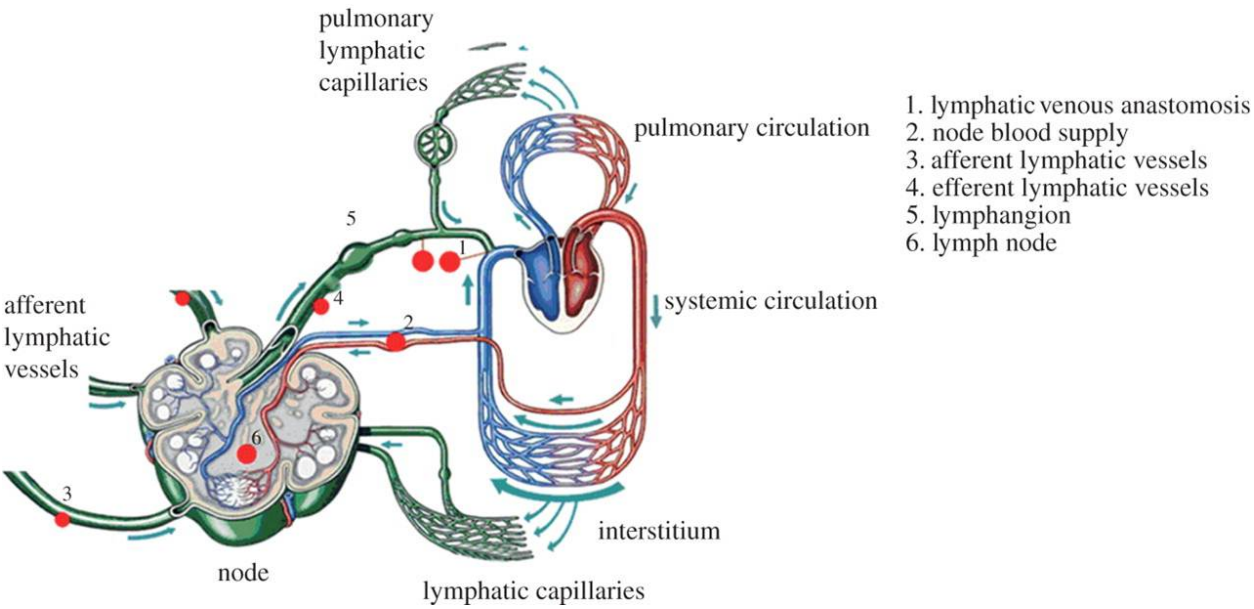


Figure 1. An overview of the blood (cardiovascular) system and the lymphatic vasculature system.

The blood vasculature network is a circular and closed network, in which the blood leaves from and returns to the heart. The lymphatic vasculature is a linear and an opened network, in which the lymphatic capillaries at the peripheral tissues drain the interstitial fluids (lymph) containing cells, proteins, and macromolecules and transport it back to the blood vasculature network through lymph node via afferent lymphatics and the lymphatic-blood junction at the end of the thoracic duct via efferent lymphatics. Figure adapted from (4) with permission.

The lymphatic system was first described by a number of astute ancient Greek physicians between 300BC to 199AD, including both Aristotle and Hippocrates (5). Aristotle in his observation described the lymphatic vessels as "fibers that take a position between blood vessels and nerves, which contain a colorless liquid", meanwhile Hippocrates described the lymphatic vessels in the axillary lymph node as "vessels containing white blood" (5, 6). In the year 1622, an Italian professor in anatomy and surgery named Gasparo Aselli published an observation describing the lymphatic structure and course of the lymphatic vessels in the canine mesentery, which he named "lacteal vessels" (5). However, Aselli's work did not describe much on the function of these vessels. It was not until the mid-18th century (between the years 1740 to 1787) that the actual function of the lymphatic vasculature and the lymphatic system was first described by the findings of three British anatomists -- William Hunter, William Hewson, and William

Cruikshank, in which they described the lymphatic vessel function was to absorb liquid waste in the body (5). A closer observation by Hewson reported that lymphatic vessels evolved to produce a substance called lymph, which contained smaller particles and were essential to body growth and health (7). Advancement in microscopy techniques and the use of in vivo vital stains, contrast substances, and radionuclide substances enabled visualization and documentation of lymphatic capillaries and vessels in the organs in the absence or presence of diseases in the next centuries (5). Identification of sentinel lymph node and other secondary lymphoid organs highlighted the importance of the lymphatic system, comprised of the lymphatics as well as the lymphoid organs, in inflammation and pathological conditions (8-11).

Despite the long list of history and medical breakthroughs, exploration of the lymphatic vasculature function at the molecular and cellular levels is still challenging. Our appreciation on how knowledge of the lymphatics has evolved over the centuries when combined with recent discoveries of lymphatic endothelium-specific markers and growth factors as well as growing availability of small animal and in vitro models in lymphatic research will hopefully provide us with tools to impact future challenges and potential breakthroughs in the field.

1.1.2 Structure and function

The lymphatic vasculature is made of highly branched networks of endothelium that penetrate most vascularized organs and tissues with the exception of the central nervous system and avascular tissues such as the cartilage, and the bone (3, 12, 13). Because of its leakiness, the blood vessels are usually located near the lymphatics, which are responsible for removal of the extravasated fluids from the interstitial tissues spaces back to the blood circulation (14).

The lymphatic endothelium consists of distinct compartments, such as initial capillaries, pre-collecting, and collecting vessels (2, 4, 14, 15). The initial lymphatic capillaries are the thinnest capillaries and are made of blind-ended structures, which lack a basement membrane and are directly anchored to the extracellular matrix by fibrillin-anchoring filaments (Figure 2C) (4, 15, 16). The luminal walls of these capillaries are lined by a single layer of loosely overlapping cells (Figure 2B) that form button-like junctions (13, 14, 17), which under high interstitial pressure conditions are pulled open by the anchoring filaments and allow lymph to flow unidirectionally from the interstitial spaces into the capillaries and then subsequently into the pre-collecting and collecting vessels (14). The pre-collecting vessels connect the lymphatic capillaries to the collecting vessels. Unlike the initial capillaries, the pre-collectors are also lined by irregular layers of smooth muscle, which contribute to their role in both lymph absorption and propulsion (4, 15, 18). The main function of the collecting lymphatic vessels is to transport lymph and thus these vessels are lined by cells that form continuous tight zipper-like junctions as well as a basement membrane and continuous muscular layer that help propel lymph flow (4, 17, 19). The collecting vessels function very much like the veins in which they contain bileaflet one-way valves that prevent retrograde flow of the lymph (3, 4, 19). The collecting vessels are connected to lymph nodes where lymph is filtered (Figure 1) and thus can be discerned into pre- (afferent) and post-nodal (efferent) lymphatic vessels (18). The lymph flows out from the lymph nodes via the efferent lymphatics and eventually drains into the thoracic duct and is returned to the bloodstream via the veins at the right jugulo-subclavian junction (Figure 2A) (2).

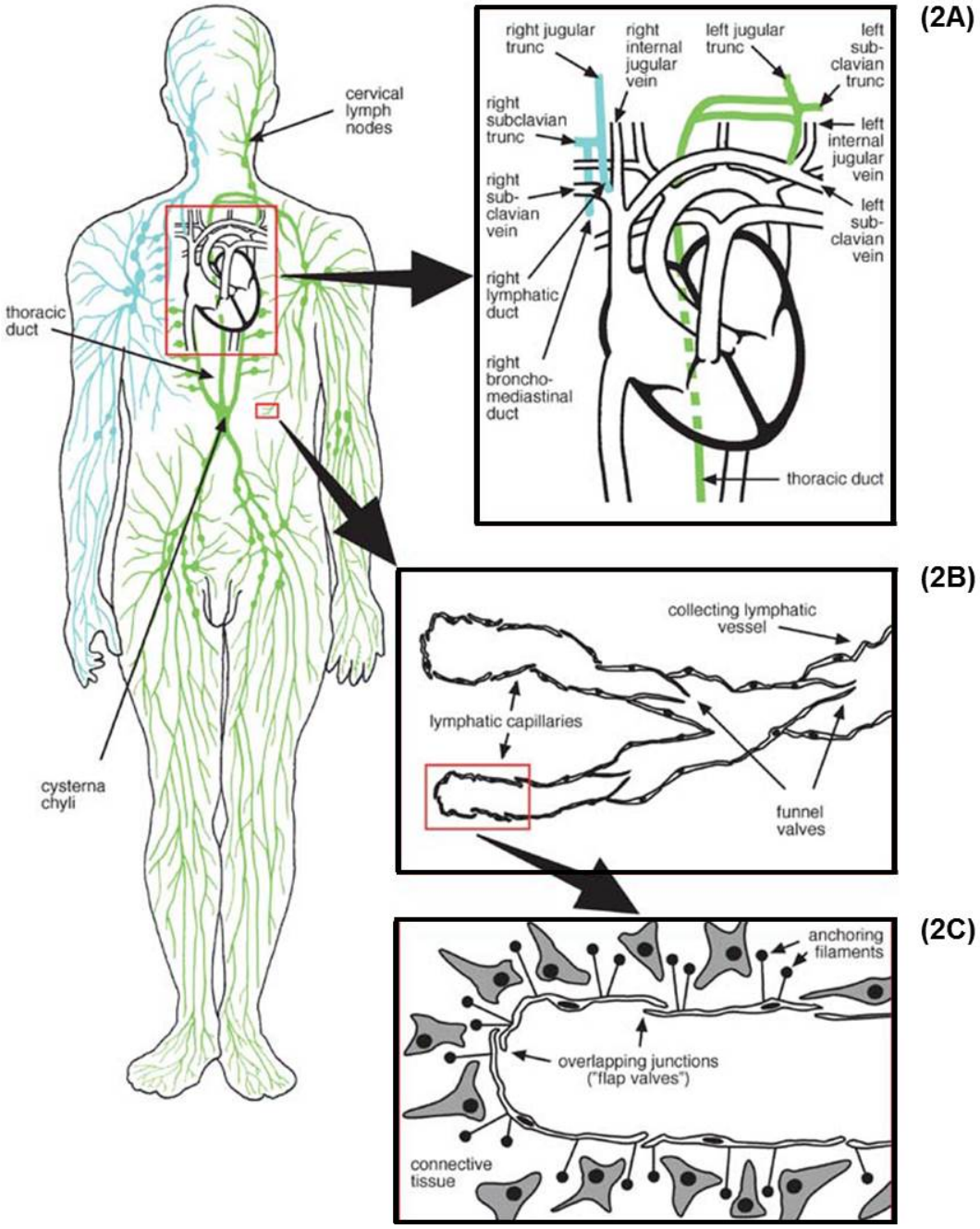


Figure 2. Schematic of the lymphatic vasculature in adult human.

The lymphatic vasculature network is made of a complex network of open-ended, thin-walled capillaries and collecting vessels. The lymphatic vasculature transports the lymph unidirectionally from the interstitium and returns it to the blood circulation via the thoracic duct at the junction of the jugular and subclavian veins at the base of the neck (2A). Lymphatic capillaries are connected to the larger connecting lymphatic vessel by the precollecting lymphatic vessel (2B). The lymphatic capillaries are blunt-ended and are made of overlapping, single layer of cells endothelial cells anchored by fibrillin filaments. The anchoring filaments attach endothelial cells to the extracellular matrices and prevent the capillaries from collapsing. Under the conditions when interstitial pressure increases due to fluid accumulation, the anchoring filaments pull the lymphatic endothelial cells and open the cell-to-cell junction of the overlapping cells to allow lymph and others (immune cells or antigens) to enter the lumen of the lymphatics (2C). Figure adapted from (2) with permission.

1.1.3 Role in health and disease

1.1.3.1 Removal of fluids

The unique structural features of the lymphatic vasculature make it highly suitable and well-adapted to function as a drainage system for removal of excess interstitial fluids in order to maintain tissues homeostasis. In healthy or steady-state conditions, the extravasation of fluids from the blood vasculature is balanced by the lymphatics through drainage and return to the blood circulation (16). The afferent lymphatics transport the interstitial fluids into the draining lymph nodes of which are filtrated and reabsorbed into the bloodstream in the lymph nodes or are transported via efferent lymphatics to be returned to the blood circulation via thoracic duct through the jugular and subclavian veins (4, 10, 16). Lymphatic malfunctions result in accumulation of tissue fluids in the interstitium and often times caused swelling and pain in the afflicted area. This condition is known as lymphedema and may occur when filtration and reabsorption of excess interstitial fluids back into the blood circulatory exceeds the lymphatic drainage for an extended period of time. The accumulation of these protein-rich fluids are also associated with inflammatory reactions, tissue fibrosis, and susceptibility to infections (10).

Lymphedema can be classified into primary (hereditary) lymphedema or secondary (acquired) lymphedema. Primary (hereditary) lymphedema is due to genetic defects that affect the lymphatic development at birth or usually start appearing around puberty (3, 10). Secondary (acquired) lymphedema is mainly caused by compromised lymphatic functions due to infections, surgery, or radiation (3). The most common form of secondary lymphedema is caused by parasitic filarial worm infections which causes blockage of lymph flow and remodeling of the infected lymphatics (20-22). Lymphatic filariasis currently affects more than 100 million people worldwide in over 80 countries, mostly in tropical Africa and Asia (3, 22).

1.1.3.2 Immune surveillance and immune cell trafficking

The extensive networks formed by the lymphatic endothelium make them an ideal conduit for antigen and immune cell transport during immune surveillance (23). In steady state conditions, naïve and semi-naïve immune cells such lymphocytes and DCs are transported along with lymph to the bloodstream for recirculation via the lymph node (4). Constant sampling of the lymph for host self-antigens by resident immune cells at the draining lymph node provides a constant update on the antigen statuses that are critical for host tolerance (Figure 3) (13, 24).

Soluble foreign antigens can either be directly transported to the draining lymph node via the lymphatics or loaded onto antigen-presenting cells (APCs) such as DCs before migration from the periphery to the draining lymph node via the afferent lymphatics (25). During an infection or an induction of inflammation, the regional lymph node provides a unique microenvironment that facilitates optimal encounters between naïve T cells and B cells with foreign antigens for mounting of cell-mediated and humoral adaptive immunity (Figure 3) (23, 26). Thus, the lymphatic vessels are critical key structural components in the microarchitecture of the lymph node for proper activation of the immune response and also the subsequent dissemination of the activated effector cells (T cells and B cells) to the blood circulation via the efferent lymphatics during inflammation (13, 27).

More recent discoveries have revealed that the lymphatic endothelium is far from just a passive conduit for immune cell trafficking. Evidence from several studies showed that the lymphatic endothelium plays an active role in mediating the entry of activated DCs and lymphocytes into the afferent lymphatics through expression of chemokines (homeostasis and proinflammatory signals) (Figure 3) (23, 28-32) and adhesion molecules (33, 34). In both steady state and inflammatory conditions, DC transmigration into the lumen of the afferent lymphatic

capillaries is driven by the interaction of chemokine receptors, such as CCR7, with CCL21 and CCL19, which are generated by the lymphatic endothelium (28, 29, 35). Lymphatic vessels in the skin produce the chemokine CXCL12 after antigen exposure and thus trigger chemotactic migration of dermal DCs (36). A more recent study showed that another chemokine, CX3CL1, is also expressed by activated lymphatics during inflammation and are responsible for recruiting DCs and leukocytes expressing the cognate receptor, CX3CR1, resulting in migration towards lymphatic endothelium (31). Adhesion molecules such as VCAM-1 and ICAM-1 facilitate the initial attachment, rolling, and transmigration of leukocytes into the luminal sides of activated lymphatics vessels (33, 34).

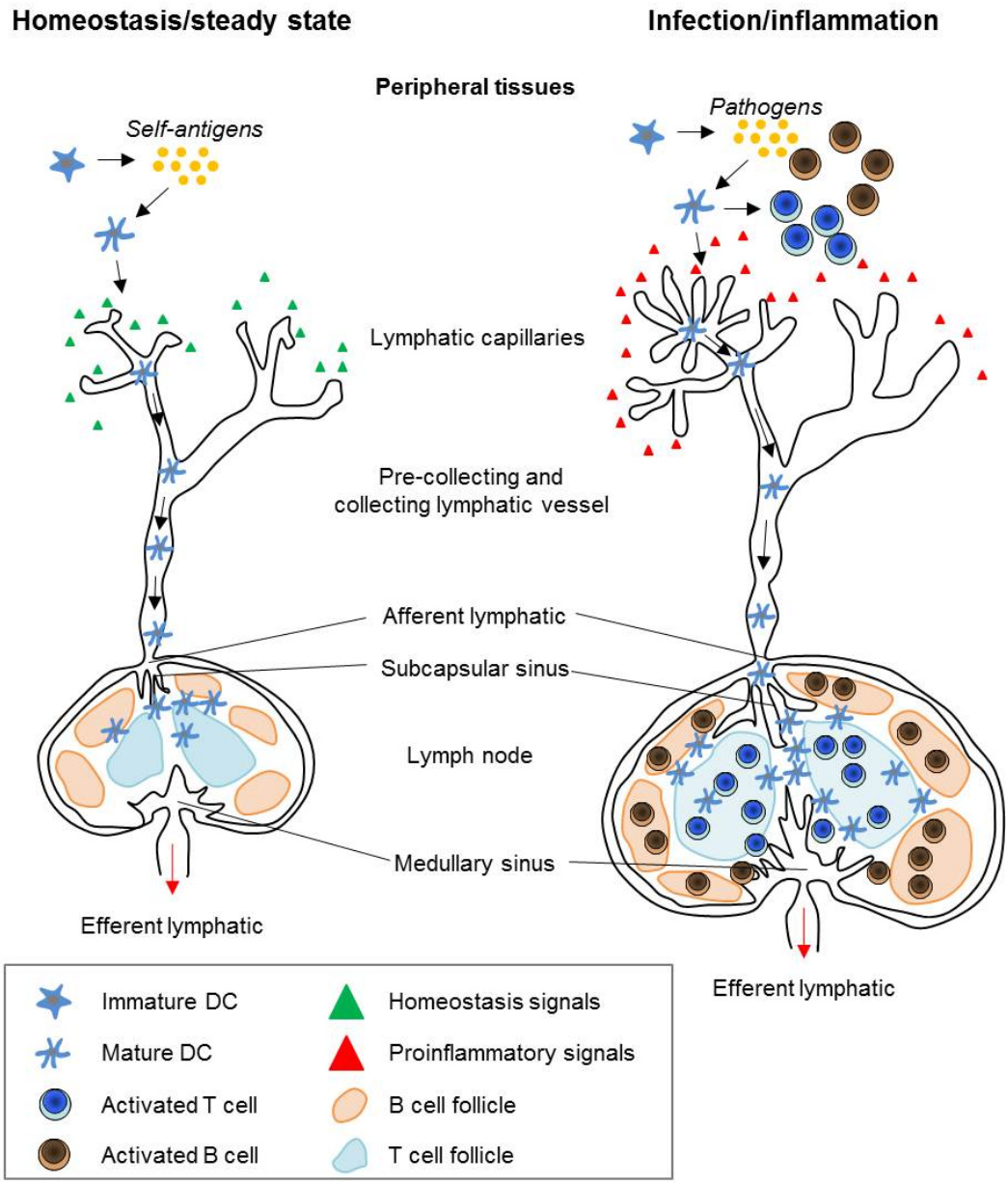


Figure 3. Lymphatic endothelium during homeostasis and proinflammatory conditions.

During a homeostasis or steady state condition, immune surveillance carried out by resident immune cells such as DCs at the peripheral tissues is orchestrated by the homeostasis signals secreted by the lymphatic endothelium. Homeostasis signals (chemokines) mediate migration of DCs towards the blunt-ended lymphatic capillaries, and into the draining lymph node via the afferent lymphatics. During an infection or inflammatory condition, proinflammatory signals (cytokines/chemokines) facilitate the migration of antigen-loaded mature DCs travel to the draining lymph node, and trigger the activation of naïve T cells and B cells. Activated T cells and B cells then exit the lymph node via the efferent lymphatics, into the blood circulation, and finally the inflammation site to begin their effector functions. Proinflammatory cytokines/chemokines secretion by the lymphatic capillaries facilitate the trafficking of these effector immune cells to the site of inflammation by creating local proinflammatory within the local peripheral tissue environment.

1.1.3.3 Lipid adsorption in the gut

Another important physiological function of the lymphatic endothelium is the adsorption and transports of intestinal nutrients, especially lipids and fat-soluble vitamins (1, 2, 9-11). High molecular weight molecules and colloids are often taken up by specialized lymphatic vessels in the villi known as lacteals (2). Unfortunately, knowledge on the mechanisms of lipid macromolecule uptake into lacteals is still rudimentary and it is still unclear whether these macromolecules are being modified in any way to facilitate their uptake into lymphatics (37). However, it is very likely that the structure of the lymphatics and lymph propulsion play a major role in facilitating the uptake processes. It is well established that the integrity of the intestinal lymphatic structure affects the survival of genetically modified mice at birth (38) and is directly responsible for the onset of obesity in adult-mice due to accumulation of adipose tissues (39). Fat deposits have been shown to accumulate surrounding the leaky lymphatics and often times lead to tissue fibrosis and chronic lymphedema in mice and humans (39, 40). Research on the mechanisms of dietary fat adsorption by intestinal lymphatics is essential in order to appreciate the role of intestinal lymphatics in diseases associated with malabsorptions and vitamin deficiencies and well as potential insight into advancement of intestinal lymphatic drug transport (41).

1.2 LYMPHATIC ENDOTHELIAL CELLS (LECS)

The lymphatic endothelial cells (LECs) line the wall of the lymphatic endothelium and share the similar apico-basal polarity and flat morphology as the blood endothelial cells (BECs) (42), making them impossible to distinguish from each other in in vitro 2D cultures. The origin of LECs remains not completely understood although increasing evidence seems to be supporting one of the popular models which proposes that peripheral lymphatics develop from the cardinal vein and gain their lymphatic competence and commitment at the embryonic level (9). However, due to their specialized biological functions, there have been tremendous efforts in identifying specific markers that allow discrimination of LECs and BECs.

1.2.1 LEC phenotypic markers

Recent discoveries of LEC markers have significantly contributed to more in-depth studies of physiological role the lymphatic endothelium and its function at the cellular and molecular levels (43). These LEC markers not only allow for discrimination of LECs and BECs at the histological level but also facilitate isolation of pure cultures of both cell populations for studies in vitro. Several phenotypic markers have been considered specific for characterization of LECs in the literature. The classical LEC markers such as the fms-like tyrosine kinase receptor VEGFR-3 (Flt4) (44-46), mucin-type transmembrane glycoprotein podoplanin (47, 48), hyaluronan receptor LYVE-1 (49), transcription factor Prox-1 (38, 50), and the CC-chemokine ligand-21 (CCL21) (51) have been used for analysis of lymphatic vasculature in histological

sections and also as targets for obtaining pure LEC populations using target-specific antibodies. Advances in molecular biology have allowed for molecular profiling of LECs and BECs at the transcriptome level and thus resulted in identification of newer markers for their discrimination. Discoveries of newly defined LEC markers such as stabilin-1 (STAB-1), reelin, and COLEC-12 (42, 52, 53), as well as other LEC-associated markers (1, 54-57) have led to better understanding of the function of the lymphatic endothelium at the cellular and molecular levels.

1.2.1.1 LEC markers

VEGFR-3

Vascular endothelial growth factor receptor-3 (VEGFR-3), also known as Flt4, is a member of a subfamily of receptor protein tyrosine kinases and is structurally related to the other members of the fms-like tyrosine kinase receptors, VEGFR-1 (Flt1) and VEGFR-2 (Flk1/KDR). However, unlike VEGFR-1 and VEGFR-2, which bind to the endothelial growth factor VEGF-A, VEGFR-3 binds to the lymphatic specific endothelial growth factors, VEGF-C and VEGF-D (43, 58). Two alternatively spliced isoforms of VEGFR-3 with different lengths of cytoplasmic domains have been reported but it is still unclear if these two isoforms differ in their signaling properties (59). Interestingly, VEGFR-3 is expressed by both blood and lymphatic vessels during the embryonic stage, evidence that corroborates the notion that lymphatic endothelium originates from sprouting of the cardinal vein (44, 45, 60). However, later in the fetal development VEGFR-3 expression is restricted to the lymphatics and plays an essential role in formation of the lymphatic vessels as well as differentiation and proliferation of LEC-lineage cells (44, 45). VEGFR-3 knock out mice display abnormalities in their blood vessel development which lead to fatalities (61) and inhibition of endogenous VEGFR-3 signaling using soluble VEGFR-3, which

competes with ligand binding with the endogenous VEGFR-3 resulted in lymphatic regression in several organs of the transgenic mice (62). Administration of a specific antibody that blocked the binding of VEGFR-3 to VEGF-C also resulted in impaired lymphatic sprouting in adult mice. However, the pre-existing lymphatic vessels did not seem to be affected (63). VEGFR-3 expression also has been detected on cells of the hematopoietic system (64), suggesting a common stem cell origin of hematopoietic and endothelial cells. Although VEGFR-3 expression is limited to the lymphatics in healthy adult tissues, it is expressed on blood capillaries in pathological conditions such as tumor neovascularization and chronic inflammatory wounds (65). Thus, the use of VEGFR-3 alone as a marker to differentiate LECs from BECs generally is not sufficient and combination with other LEC markers is needed for characterization.

Podoplanin

Podoplanin is a mucin-type membrane surface glycoprotein that is highly expressed by LECs but not BECs in vivo and in vitro (50, 51, 53, 66). Additionally, podoplanin is also expressed by other cell types including kidney podocytes, osteocytes, and lung alveolar and corneal epithelial cells (67). Podoplanin expression by the lymphatic endothelium was first discovered by immunohistochemistry as it was found to colocalize with VEGFR-3 in normal lymphatic endothelium and in vascular tumor of lymphatic origin (47, 48). Since then podoplanin expression has been associated with the lymphatic endothelium in many organs of healthy individuals, including the skin, kidney, and lung (68), as well as various human tumors of lymphatic origin (69). Podoplanin expression on LECs contributes to active recruitment of mononuclear leukocytes into the basolateral surface of the lymphatic endothelium through interaction with CCL21 (70). Podoplanin knock out mice die immediately after birth due to respiratory failure which results from defective formation of alveolar spaces and impaired

lymphatic transport (66). In vitro studies using overexpression and siRNA knock down showed that podoplanin plays an important role in LEC migration, cell adhesion, and tube formation (66). It is also possible that podoplanin plays a role in regulating transmigration of immune cells across the lymphatic endothelium (23). Podoplanin is one of the most highly expressed LEC markers in lymphatic endothelium (23), and its absence from BECs in physiological or pathological conditions makes it a strong and reliable marker for LEC and BEC discrimination.

LYVE-1

Lymphatic vessel endothelial receptor-1 (LYVE-1) is a homolog of the blood endothelial hyaluronic acid (HA) receptor CD44, and is mostly associated with the lymphatic endothelium (49). Histological analysis of LYVE-1 expression showed that it is highly expressed on both the luminal and the abluminal sides of the lymphatic capillaries (71). LYVE-1 is also expressed by other cells types including activated macrophages and sinusoidal endothelium of the liver and the spleen, where high molecular weight HA is absorbed and degraded (72). HA is known to be a key mediator of cell migration and is degraded in regional lymph nodes and liver (73). Therefore, HA interaction with LYVE-1 enables cells with surface HA such as lymphocytes and leukocytes to adhere to and migrate through the lymphatic endothelium to the draining lymph node (74). This was shown when migration of antigen-loaded DCs from the footpad of mice to the draining lymph node was abrogated by administration of antibody that binds LYVE-1 (71). However, interestingly, despite the known importance of LYVE-1 in lymphatic vessel function, mice lacking the LYVE-1 gene develop normal lymphatic vasculature and function (74, 75). Histological analysis of normal and tumor-associated lymphatics in murine and human tissues revealed that LYVE-1 expression is selectively expressed by LECs but not BECs (12, 75, 76) and analysis at the mRNA level showed no expression of LYVE-1 by BECs after birth (68).

These findings confirmed the specificity of LYVE-1 expression to that of LECs and not BECs, thus making it an important LEC marker.

Prox-1

Prospero-related homeobox gene-1 (Prox-1) is the mammalian homolog of the *Drosophila* homeobox gene *prospero,* a transcription factor that is responsible for lineage specification of LECs during embryonic lymphatic development (38, 77). Prox-1 expression localizes with VEGFR-3 in mouse embryo as early as day 8.5 in cells of lymphatic competence and is found to co-localize within the same location with LYVE-1 in the lymphatic endothelium later in development (60). In Prox-1 knock out mice the lymphatics fail to develop whereas the blood vessels appear to be unaffected (38). Prox-1 is regarded as the master regulator of LEC lineage and has the potency to reprogram BECs into LEC-like phenotype (50, 78-81). Prox-1 expression in normal adult rat tissues showed that it is exclusive to LECs (68). Prox-1 represses BEC markers (50) and Prox-1 knock out mice fail to express other LEC markers, underscoring the importance of Prox-1 in lineage commitment and differentiation of LECs (38). The expression of the lymphatic-specific transcription factor Prox-1 is mutually exclusive with that of the blood vascular-specific marker PAL-E in tissues obtained from healthy adult and lymphedema patients (82) as well as in esophageal lymphatics of the monkey (83). Thus, Prox-1 serves as an important key marker of LECs in normal and pathological conditions during disease.

CCL21

Aside from lymphatic vessels, CCL21 is expressed by T-zone stromal cells (84) as well as high endothelial venules (HEVs) (85) in mice. Nevertheless, its expression in humans was observed to be conserved to the lymphatics and not the blood vessels (86, 87). CCL21 is a homeostatic

chemokine that binds to the CC-chemokine receptor 7 (CCR7) expressed by naïve and activated leukocytes, including neutrophils, lymphocytes (T cells and B cells), and antigen presenting cells (DCs) (32, 33, 88-90). CCL21 is constitutively expressed by the lymphatics and mediates migration and entry of neutrophils (32), T cells (30, 90, 91), as well as DCs (29, 92) from the periphery to the draining lymph nodes via afferent lymphatics (30, 93). In situ hybridization analysis of CCL21 mRNA distribution in nonhuman primate lymph nodes showed that CCL21expression is higher at the afferent lymphatics compared to the efferent lymphatics (93). However, in the lymph node CCL21 is also expressed in the paracortex by stromal cells such as the fibroblastic reticular cells (FRCs) (94), thus limiting its use as lymphatic-specific marker in secondary lymphoid organs.

1.2.1.2 Newly defined LEC markers

Comprehensive analyses based on microarray data have identified new markers for characterization and differentiation of LECs and BECs (42, 50, 53). Immunohistochemical staining of widely used endothelial cell models and human tissues showed that expression of novel LEC markers such as reelin, stabilin-1 (also known as CLEVER-1), and collectin placenta 12 (COLEC12) colocalized with known LEC markers podoplanin and LYVE-1(42). Reelin is involved in NOTCH-signaling, which is important for LEC/BEC specialization and vasculature development (95). CLEVER-1 is expressed constitutively by lymphatic vessels in normal and cancerous human skin tissues (96, 97). CLEVER-1 also is involved in regulation of leukocyte migration to draining lymph nodes via lymphatics as well as leukocyte entry to sites of inflammation via inflamed vessels (96, 98). Thus, CLEVER-1 expression by lymphatic endothelium is an attractive target for development of therapeutic drugs that modulate leukocytes migration during disease conditions such as cancer metastasis via lymphatics (99).

Immunohistochemical analysis of human lymph nodes, inflamed tonsils, and malignant tissues showed that COLEC12 expression strongly co-localized with the LEC marker, LYVE-1 (42).

1.2.1.3 Other associated LEC markers

More recently, additional markers have been demonstrated to be strongly associated with LECs such as atypical chemokine receptor D6, interleukin-7 (IL-7), and spinster 2 (Spns2), and CD40. D6 is expressed on the luminal side of the lymphatic endothelium and has been shown to be expressed by lymphatics in skin and lung tissues as well as secondary lymphoid and placental tissues (100). D6 is involved in scavenging subsets of inflammatory and homeostatic CC chemokines by binding the ligands, internalizing, and degrading them (101-103). Therefore, D6 is fundamental in regulation of inflammation as well as innate and adaptive immune responses through coordination of leukocyte migration to the lymph nodes via the afferent lymphatics (54, 55, 104, 105). Analysis of sorted LECs derived from human lymph node showed substantial expression of IL-7 (57). IL-7 secretion by stromal cells, including LECs and FRCs, is critical for reconstruction and remodeling of distinct microenvironments in lymph nodes for optimal interaction between IL-7Rα+ T cells and DCs in the subcapsular sinus (57). Sphingosine-1-phosphate (S1P) is a signaling molecule required for egress of lymphocytes such as T cells and B cells in and out of the lymphatic network including the lymph node (56). S1P is made in the cytoplasm as well as the nucleus and thus needs to be transported out of the cells for it to function. Spns2 is an S1P transporter molecule and plays an important role in regulation of S1P levels in blood and lymph node (56). Spns2 knock out mice have been reported to have lower levels of plasma S1P than wild type mice and exhibit altered lymphatics that appeared collapsed (56). CD40 is expressed by many cell types such as fibroblasts, epithelial cells, and endothelial cells (106). In endothelial cells, CD40 interaction with T cells expressing CD40 ligand (or

CD154) results in activation of endothelial cells including regulation of kinases and/or transcription factors that affect immune-inflammatory responses triggered by endothelial cell-T cell crosstalk (107).

1.2.2 In vitro models for study of LECs

LECs and BECs are specialized cell types that are well adapted to their specific functions. Gene expression profiles of both types of endothelial cells will provide a basis for understanding the molecular characteristics of both LECs and BECs (51). To unravel these complex functional differences of LECs and BECs, relatively pure in vitro cultures of both cell types are crucial tools for study. Isolation and culture of bona-fide LECs has been challenging due to contamination by BECs, and vice versa, as well as contamination by other stromal cells such as fibroblasts and keratinocytes (108, 109). In addition, isolated LECs often are low in yields and have short life spans. Recent discoveries of LEC markers and growth factors have made it possible to positively or negatively select for LECs using specific antibodies to culture them in vitro and study their functions. Various techniques of cell immortalization have also been utilized to establish more stable cell lines to study LECs properties and functions.

1.2.2.1 Isolation, culture, and molecular profiling

In vitro models of LECs have been established using various isolation methods, which includes mechanical and enzymatic digestion of the tissues followed by immunoselection using antibodies against LEC markers such as podoplanin, VEGFR-3, and LYVE-, alone or in combination with antibodies against common endothelial markers, such as CD31 (PECAM-1). LECs have been isolated for culture by positive selection using antibodies specific to podoplanin (51, 108),

VEGFR-3 (110), LYVE-1 (52) as well as negative selection using antibodies specific for BECs such as CD34 and E-selectin (53). Primary human LECs were also isolated from human skin tissues from patients with lymphedema using a combination of immunoselection with anti-podoplanin and anti-LYVE-1 antibodies (111). Similarly, primary human LECs from cases of chronic tonsillitis were isolated using a combination of antibodies against CD31 and podoplanin expressed on LECs (109). Transiently expressed endothelial cell markers, upon treatment with certain stimuli such as cytokines, were also used in some cases. E-selectin specific antibody was used to obtain pure cultures of primary endothelial cells from mixed cultures containing fibroblasts and keratinocytes from human neonatal foreskin after stimulation with TNF-α (112). Establishment of in vitro LEC cultures is also made possible through identification of growth factors that enhance survival, growth, and proliferation of primary LECs, such as VEGF-C and VEGF-D (113-116). A recent study showed that cord blood derived CD34+/VEGFR-3+ cells cultured in the presence of VEGF-C in standard 2D monolayers were able to differentiate to become more LEC-like and expressed LEC markers, LYVE-1 and Prox-1 (114).

In vitro cultures of relatively pure LEC populations have enabled molecular characterization of LEC expression profiles by microarray analysis. These large datasets provided means to identify newer markers to distinguish lymphatic and blood vessels as well as their specialized functions in health and disease. Information on diseased and malignant tissues from patients also potentially identifies diagnostic markers as well as novel markers for therapeutics development. However, in vitro culture of primary cells likely alters their phenotypes to some extent. Adaptation to 2D culture has been reported to result in upregulation and/or downregulation of genes found in in vitro cultured LECs and BECs when compared to native cells isolated directly from tissues (53). Another challenge faced in in vitro culture of

primary LECs is their short lifespan. Several efforts have been made to increase the lifespans of primary LECs by transduction of oncogenic viral antigens such as SV40 T antigen (117) or infection by a retrovirus containing the coding region of the human telomerase reverse transcriptase (hTERT) (118).

In addition, in vitro studies using primary LECs from human tissues poses a lot of challenges to follow up in vivo. Therefore, in vitro cultures of LECs from commonly used animal models are attractive alternatives for study of LECs and their function in lymphatic diseases. Primary LECs have been isolated from different animal species including bovine (119), ovine (120), canine (121, 122), and murine (123-130) as well as different tissue origin including skin (129), lymphatic vessels/thoracic duct (121, 122), and lymph node (130). Conditionally immortalized primary mouse LECs were isolated from genetically modified mice and used to establish stable and long-term culture of mouse LECs in vitro (128).

1.2.3 In vivo model for study of LECs

1.2.3.1 Genetically modified mice models

Studies of knock out and transgenic mice have revealed a number of genes that are crucial for embryonic lymphatic vasculature development and growth including final remodeling of lymphatic capillaries and collecting lymphatic vessels (1, 19). Among these are genetically modified mice lacking the LEC markers Prox-1, VEGFR-3, LYVE-1, or podoplanin (Table 1). Knock out mouse models lacking the LEC marker Prox-1 (Prox-1 -/-), which is a transcription factor for controlling LEC-lineage specification, showed that these mice lacked LECs, failed to develop lymphatic vasculatures (38, 39). Mice lacking the tyrosine kinase growth factor receptor, VEGFR-3 (VEGFR-3 -/-) or with mutated VEGFR-3 (+/Chy, ENU-induced mutation)

failed to undergo lymphatic sprouting followed by cutaneous hypoplasia (61, 131). The critical role of VEGFR-3 signaling in early lymphatic development in mice was confirmed when mice lacking its ligand, VEGF-C (VEGF-C -/-) showed complete lack of lymphatic vasculature and died of prenatally due to accumulation of fluid in the tissues (115). Interestingly, knock out mice lacking the hyaluronan receptor, LYVE-1 (LYVE-1 -/-) showed no defect in lymphatic vasculature formation and function (132). However, knock out mice lacking the membrane glycoprotein, podoplanin (PDPN -/-) developed subtle defects in their lymphatic vasculature formation and were able to live until adulthood but developed severe lymphedema due to inefficient lymphatic drainage and transport (66). Transgenic mice generated using a bacterial artificial chromosome (BAC) cre-lox system revealed that interleukin-7 (IL-7) is expressed by mouse LECs and is important for remodeling of lymph node microarchitecture (57). In addition, transgenic mouse models are also used to study impaired lymphatic drainage due to gene defects. The transgenic mouse model, *K14-VEGFR-3-Ig*, expresses soluble VEGFR-3-Ig via the keratin 14 promoter, which restricts the growth of lymphatics in the skin. *K14-VEGFR-3-Ig* mouse displays defective lymphatic growth and drainage that is restricted to the skin resulting in a lymphedema-like phenotype (62) with associated impaired humoral immunity and tolerance (133). More recently, a novel transgenic mouse model, *K14-hIL8* was developed, which expressed human interleukin-8 (IL-8). The model demonstrated that human IL-8 could promote embryonic lymphangiogenesis and improved amelioration of lymphedema with lymphatic regeneration, suggestive of therapeutic potential of IL-8 in treatment of post-surgical lymphedema (134).

Table 1. Genetically modified mouse models for LEC markers and their lymphatic vascular phenotypes.

Gene/LEC marker	Mouse model	Function	Phenotype
Prox-1	Knock out (global)	Transcription factor	No LECs (-/-), chylous ascites (+/-), no lymphatic vasculature
VEGFR-3	Knock out (global), +/Chy (ENU-induced mutation)	Growth factor receptor	Defective formation and sprouting of lymphatic vasculature, hypoplastic lymphatic vessel
VEGFR-3	Transgenic (K14)	Growth factor receptor	Hypoplastic lymphatic vessel
LYVE-1	Knock out (global)	Hyaluronan receptor	No defect in lymphatic formation and interstitial lymphatic flow
Podoplanin	Knock out (global)	Membrane glycoprotein	Impaired lymphatic transport, lymphedema
VEGF-C	Knock out (global)	Ligand for VEGFR-3	No lymphatic vasculature

(adapted from (1) and (19) with permission.)

1.2.3.2 Zebrafish *(Danio rerio)*model

The zebrafish shares the same morphological, molecular, and functional characteristics of lymphatic vessels with other vertebrates (135). Immunohistochemical analysis of lymphatic vessels in adult zebrafish revealed that they expressed Prox-1 (136). However, the zebrafish model has proven to be an excellent model to study the early development of the lymphatics as the lymphatic vessels are easily observed in zebrafish compared to mouse. Lymphatic development in the zebrafish depends on VEGF-C and VEGFR-3 signaling (137). Recently, VEGF-D was shown to be able to compensate for loss of VEGF-C in lymphatic sprouting of in the zebrafish head (138). Furthermore, the zebrafish model was proven to be a useful model in screening and identification of potential lymphangiogenesis inhibitor compounds for treatment and management of tumor metastasis (139). Thus, the zebrafish model is a promising model not only for the functional study of lymphatics but also is a valuable tool for screening for potential therapeutics.

1.2.3.3 Tadpole *(Xenopus laevis)* model

The tadpole is a popular model in the study of developmental biology due to its ease of genetic manipulation and its potential as a prolific egg layer (140). The tadpole model is an attractive model for lymphatic study as the lymphatic vessels are easily distinguished by injection of dye (140). More importantly, the lymphatic vessels in this model are structurally and functionally similar to those of vertebrates and were shown to express Prox-1. Knock down of Prox-1 resulted in failure of lymphatics to develop, whereas knock down of VEGF-C resulted in lymphatic defects and development of a lymphedematous phenotype by the tadpoles (140). The mechanisms that govern the lymphatic development in the tadpole model are highly similar to the mammalians. Therefore, the tadpole model is a promising model for screening of candidate genes that are important for lymphatic development and disease.

1.3 EMERGING ROLE OF LYMPHATIC ENDOTHELIAL CELLS IN IMMUNOMODULATION

The lymphatic vasculature responds to changes in tissue microenvironments, potentially resulting in a physiological condition known as lymphangiogenesis (11, 141, 142), in which the lymphatic network undergoes proliferative expansion and remodeling due to lymphatic sprouting and enlargement of existing vessels. Lymphangiogenesis is the hallmark of many pathological conditions including infection, inflammation, wound injury, fat metabolism, hypertension, and cancer metastasis. Inflammation-induced inflammatory mediators such as growth factors and cytokines/chemokines alter the gene expression profiles of LECs and affect their function. In addition, LECs secrete subsets of chemokines and their expression is upregulated in response to

inflammatory signals. LECs also express a group of pattern recognition receptors (PRRs) known as toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) – common conserved motifs found on microbes. Increased TLRs expression has been linked to a number of autoimmune as well as chronic inflammatory diseases. Thus, LECs likely are integral players in mechanisms involved in regulation of host responses to pathogens through initiation of innate and adaptive immunity as well as host tolerance to self-antigens.

1.3.1 Role in inflammation and pathological conditions

The inflammation-induced vascular growth factors VEGF-C and VEGF-D are known for their lymphangiogenic properties. VEGF-C signaling via its receptor VEGFR-3 leads to proliferation and migration of LECs suggesting that inflammatory conditions mediate lymphatic formation (143). VEGF-C and VEGF-D are produced in inflamed tissues by stromal cells such as fibroblasts and keratinocytes as well as immune cells such as macrophages (26, 144). Inflammation-induced lymphangiogenesis in mouse cornea can be blocked by macrophage depletion (144) and CD11b+ macrophages can transdifferentiate into LECs (145). Interestingly, contradicting findings have been reported on the effects of contact hypersensitivity (CHS) induced inflammation on LECs. An earlier report using LECs isolated from inflamed murine skin induced by CHS showed that LEC markers such as LYVE-1, Prox-1, and VEGFR-3 were down-regulated, therefore making LEC markers attractive diagnostic and therapeutic targets (92). A later study using CHS-induced inflamed murine skin LECs showed that while LECs showed evidence of proliferation, they produced growth factors and chemokines that caused lymphatic vessel dilation and leakiness and thus reduction in lymphatic drainage function (146).

Inflammatory conditions increase the expression of VEGFR-3 by LECs and enhance their sensitivity to VEGF-C and VEGF-D signaling (10). Inflammatory signaling mediator such as transcription factor nuclear factor κB (NFκB) activate Prox-1 expression in LECs and in turn, increase the expression of other LEC markers, including VEGFR-3 (147). LECs also show increased expression of inter-cellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) after treatment with inflammatory mediator, tumor necrosis factor-alpha (TNF-α) (148). ICAM-1 and VCAM-1 are important molecules for transmigration of DCs and leukocytes into the lymphatics en route to the draining lymph node (33). Lymph nodes have highly dynamic and compartmentalized microarchitecture that is optimized for immune cell interaction with antigens and/or APCs. In addition, lymph node stromal cells also have microanatomic specializations and are important regulators of vascular proliferation and growth (26). Fibroblastic reticular cells (FRCs) are major producers of VEGF in lymph nodes (26). VEGF-expressing FRCs induced proliferation and migration of LECs. Lymphadenopathy or enlargement of lymph nodes is a common feature in inflammatory and autoimmune diseases (8). Inflammatory conditions created by dermal macrophage-associated cytokines induce upregulation of LEC surface marker CLEVER-1, which promotes infiltration of tumor cells into lymph nodes (97). Lymphangiogenesis also occurs in lymph nodes due to production of VEGF by follicular B cells (25). VEGF-C and VEGF-D signaling in the lymph node has been shown to facilitate tumor-associated lymphangiogenesis and promote lymph node metastasis (149). Blocking VEGF-C or VEGF-D signaling through VEGFR-3 using target-specific antibodies or siRNAs inhibits tumor-associated lymphangiogenesis and metastasis in lymph node (150).

Tumor-associated lymphangiogenesis may represent the first step in tumor dissemination to distal lymph nodes and organs for a variety of common human carcinomas and melanomas

(151, 152). In tumors associated with infection by oncogenic virus such as Kaposi's sarcoma herpesvirus (KSHV or also known as human herpesvirus-8), infected BECs are reprogramed to express LEC markers by stabilizing the LEC-lineage transcription factor, Prox-1 (153). BEC-to-LEC reprogramming also resulted in morphological transformation of LECs into spindle-like cells, the most common characteristic of KS tumor (153). Lymphatic remodeling due to structural and functional alteration of LECs is also associated with several pathophysiological conditions in chronic inflammation such as asthma, psoriasis, rheumatoid arthritis, and, inflammatory bowel disease (IBD) (9, 154). However, lymphangiogenesis in chronic inflammatory conditions has been reported to have a double-edged sword effect as it contributes to maintenance and persistence of inflammation rather than resolving it due to loss of LEC drainage function (9). Defective lymphangiogenesis in the lung during inflammation often results in bronchial lymphedema and airflow stagnation (141). Inhibition of TNF-α expression and signaling significantly reduced inflammation-mediated lymphangiogenesis in the lung (154). The role of growth factors, cytokines, and chemokines in modification of LECs during inflammation and other pathophysiological conditions remains an important research focus.

1.3.2 Emerging role of LECs in immunity and tolerance

LECs are presumably among the first stromal cell types that come in contact with invading foreign antigens that are able to cross an epithelial barrier. LEC's role in transport of immune cells for initiation of host innate as well as adaptive immune response is well established. Peripheral LECs near the mucosal epithelium constitutively express the homeostasis chemokine, CCL21, and the expression increases during inflammatory-induced immune responses to pathogens or immunization. CCL21 is essential in host immune surveillance during homeostasis

for trafficking of immune cells expressing the cognate receptor, CCR7, such as DCs, T cells, and B cells. CCL21 is also important for transmigration of antigen loaded DCs from the peripheral into the lymphatic vessels, and subsequently to the draining lymph node (34, 93). LECs also express other chemokines (31, 36, 70) and adhesion molecules (70, 89) that participate in the recruitment of naïve T cells and B cells into the lymphatic vessel from the tissue parenchyma. In addition LEC-derived chemokines and signaling molecules are crucial in maintenance of lymph node microarchitecture and compartmentalization of immune cells (57, 155). Lymph node LECs lining the subcapsular sinus may act as gatekeeper and direct antigen-loaded DCs that enter the lymph node into the T cell zone and thus allow activation of downstream adaptive immune response (57). LECs also play an important role in trafficking of activated immune cells from the lymph nodes. LEC-derived signaling molecule such as S1P facilitate the egress of T cells from lymph nodes via the efferent lymphatics (156, 157). Studies using LYVE-1 deficient mice showed absence of S1P production and retention of lymphocytes in the lymph node (158).

The active roles of LECs in innate immunity are highlighted by the expression of multiple functional TLRs, which enable them to sense incoming foreign antigens or microbes (159, 160). More recent data showed that peripheral and lymph node LECs express MHC I and II molecules as well as their known costimulatory molecules, albeit to much lower levels compared to professional APCs such as DCs. Even though MHC I expression has been reported in peripheral and lymph node LECs, the expression of MHC II was observed only in the lymph node LECs but not the peripheral tissue lymphatics, suggesting that there is a functional immunological difference between LECs in these two anatomical locations (161, 162). Cross-presentation of self-antigens on MHC I by LECs led to quiescent DCs, which suppressed activation and induced apoptosis of autoreactive CD8+ T cells (163), suggesting that antigen

presentation by LECs participates in induction of tolerance in the periphery. In addition to their role in inducing tolerance to autoreactive T cells, LECs have been reported to scavenge, endocytose, and cross-present to tumor cells expressing OVA as foreign antigen, which resulted in lymphangiogenesis and protection of tumor cells from CD8+ T cells (164). Cross-presentation of non-self antigens by LECs is responsible for rejection of grafts in organ transplantation (165). Furthermore, mouse LECs were demonstrated to capture and archive antigens derived from viral infections with influenza and vesicular stomatitis virus, and that these antigens not only persisted for prolonged time within LECs but were also able to improve effector functions of circulating memory CD8+ T cells (166).

Interaction between MHC II molecules expressed by LECs with CD4+ T cells is less well described. MHC II molecules are expressed by lymph node LECs in low levels during steady state conditions and the expression is upregulated under proinflammatory conditions (162, 167). However, MHC II expression by LECs has only been demonstrated in the lymph node and not in non-lymphoid tissue lymphatics. In addition, lymph node LECs do not express the traditional costimulatory molecules required for MHC II-antigen presentation to CD4+ T cells such as CD80 and CD86 although they do express LFA-1 (CD58), which can act as an unconventional alternative binding receptor to CD2 for activation of CD4+ T cells (162). However, data from allogeneic coculture of LECs and CD4+ T cells failed to induce CD4+ T cell proliferation or cytokine production, and coculture of IFN-γ-activated LECs with DCs and CD4+ T cells also resulted in impaired proliferation of CD4+ T cells by DCs, suggesting that potential LEC-associated mechanisms were responsible for the impaired T cell activation (162). Thus, taken together these data highlight the emerging contribution of LECs to modulation of local microenvironments as well as the functions of immune cells such as DCs and T cells.

1.4 ANIMAL MODELS FOR STUDY OF HUMAN DISEASES

Animal models of human diseases are crucial for translation of scientific discoveries into meaningful applications in studying disease pathobiology and development of new biomarkers or therapeutics (168). Animal models overcome the limitations of in vitro tissue culture models and can often serve as surrogates for study of human disease pathologies and immune responses as well as study of toxicity and efficacy prediction of newly developed therapeutics. The ideal animal model should replicate very closely the disease outcome, its underlying causality, and its mechanism of action in humans (169). In addition to that, such models preferably should have similar genetic properties to humans and also similar in response to infectious agents or therapeutics. Several animal models currently exist for many human diseases including bacterial/viral infections, cancers, neurologic, pulmonary, chronic, and metabolic diseases (170). However, the predictive value of any single animal model or a collection of animal models in fully recapitulating the human host remains a constant challenge.

1.4.1 Ferrets as animal models

Ferrets are becoming an increasingly popular small animal model for study of many human diseases, including reproductive biology (171-173), cancer (174), and infectious diseases of viral and bacterial origin (159, 175-181). The ferret model is a commonly used model for infectious diseases and is considered the “gold standard” to model human influenza infection since the 1930s (182). Ferrets are naturally susceptible to infection with various strains of human influenza viruses. In addition, ferrets show disease presentation and clinical symptoms that are similar to humans. The severity of influenza disease in ferrets depends on the specific strain of

influenza virus used (183). Seasonal influenza causes mild to moderate upper respiratory tract infections in ferrets (182, 184). In contrast, highly pathogenic influenza infection is characterized by severe pathology and rapid progression of the disease (185). Infected ferrets not only share pathological changes but also similar cytokine responses to humans when infected with influenza virus. Therefore, ferrets are often used as models to evaluate antivirals as well as vaccine efficacies for prevention of influenza infection and pathogenesis (186). Ferrets are instrumental in evaluating the efficacies of influenza vaccine candidates as well as vaccine adjuvants in inducing robust innate and adaptive host immune responses. Experimental vaccine formulations, for example, using known TLR ligands as adjuvants, have been demonstrated to enhance immune responses and prevent the spread of influenza virus to susceptible animals after vaccination (187).

Ferrets easily transmit influenza to each other thus making them ideal small animal model for influenza virus transmission (185). Transmission models for influenza have been studied by measuring the efficiency of influenza virus to spread from infected ferret to naïve ferret in the presence or absence of direct contact. The development of aerosol delivery systems using ferrets allows for more accurate recapitulation of natural transmission of influenza (183). As observed in humans, highly pathogenic influenza viruses transmit poorly when compared to seasonal influenzas. The ferret model has also been used to compare the transmissibility of antiviral resistant influenza viruses. These studies provide an important estimate of public health threat posed by antiviral drug resistant influenza viruses. The ferret model has proven to be an advantageous small animal model to study both pathogenicity and transmissibility of different influenza virus strains.

1.4.2 Nonhuman primate models

Nonhuman primate (NHP) models have been instrumental in several areas of biomedical research, including drug development such as pharmacokinetic (188, 189), toxicity and efficacy of therapeutics (190) as well as normal physiological functions (191), and chemically-induced conditions that mimic human diseases (191, 192). The close genetic similarity between rhesus macaques and humans allows for cross-reactivity of commercially available reagents as well as ex vivo assays for probing innate and adaptive immune responses of both species upon infection, vaccination, or disease progression.

Since the discovery of HIV-1 and SIV in 1989, there has been widespread use of NHP models to study pathogenesis as well as vaccine and therapeutic interventions for HIV/AIDS. Unintentional infection of SIV in non-natural hosts such as the Asian macaques resulted in a disease that resembled AIDS in human, and thus led to discovery of SIV-macaque pathogenic models of HIV-1 infections in humans (193). There are three commonly used macaque species that are susceptible to infection with SIV and developed AIDS-like disease, namely rhesus macaques (*Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*), and pigtailed macaques (*Macaca nemestrina*) (193). The rhesus macaque model is the most established and well-characterized NHP model for SIV infection. SIV infection in rhesus macaques mimics very closely HIV-1 infection in humans with respect to the cell types that are susceptible to viral infection, the development of disease, and the presentation of similar disease outcomes (194). SIV replication in rhesus macaques results in progressive CD4+ T cells depletion in the gastrointestinal mucosal sites such as the gut-associated lymphoid tissues (GALT) (195). In addition, SIV replication during primary infection leads to mucosal inflammation and epithelial injury that might enhance the pathogenicity of the virus. Ongoing local SIV replication creates

local mucosal inflammatory conditions that attract more CD4+ T cells and DCs to viral replication sites, which leads to establishment of hyper-immune activation in the gut (195). Establishment of initial infection and dissemination of the replicating virus occur through infected DCs (196). These persistent immune activation and inflammation conditions also result in epithelial cell injury in the gut of SIV-infected animals followed by microbial translocation of microbes from the lumen of the gut into the parenchyma of mucosal tissues (197). Translocation of these microbes and microbial products from the gut lumen into the gastrointestinal mucosa may stimulate innate immune cells of the gut through TLR sensing and signaling pathways, thus contributing to the proinflammatory milieu and systemic immune activation during chronic SIV infection (198).

SIV-macaque models have also been instrumental in the development of antiretroviral (ART) drugs and therapeutics as well as microbicides against HIV-1. NHP models have been used to study the toxicity, pharmacokenetics, and efficacy of ART such as Tenofovir in preclinical testing (199, 200). Tenofovir is currently one of the most widely used drugs in ART regimens for treatment of HIV-1-infected persons (201, 202). Studies in SIV-infected macaque models have also contributed significantly to different interventional aspects of ART drug administration for prophylactic prevention and therapeutic treatment of HIV-1 infections in humans. Adult and infant macaques have been used to model the effects of ART drugs combinations in preventing SIV infection during early exposure time points via the many viral inoculation routes, including oral (203-205), intravaginal (206, 207), and intrarectal (208). In addition, SIV-infected macaque models have contributed to safety testing of ART drugs usage in pregnant HIV-1-infected mothers as well as their efficacies in reducing mother-to-infant transmission of the virus (209-211).

Even though SIV and HIV-1 are closely related at the genetic levels, there are some fundamental differences between the two viruses that limit the translational value of the SIV-macaque model to HIV-1-infected patients. For example SIV is not sensitive to many drugs that are designed to inhibit HIV-1 virus enzymes, such as protease, reverse transcriptase (RT), and integrase (193, 212, 213). In addition, SIV and HIV-1 may use different co-receptors for viral entry, and this potentially complicates testing of drugs that target these molecules in macaque models (214). These limitations led to the development of SIV/HIV-1 chimeric viruses known as SHIVs, which are designed to either express HIV-1 viral enzymes (215, 216) or HIV-1 viral envelope glycoproteins (193, 217), or combinations of both (218, 219). These recombinant viruses enable evaluation of some non-nucleoside RT inhibitors (NNRTIs) that are formerly only effective against HIV-1 but not SIV (212, 220), and at the same time provide insights into disease pathogenesis in the presence of ART treatment in macaque models. Highly active antiretroviral therapy (HAART) drugs successfully suppress HIV-1 viral replication in infected individuals, thus resulting in slower disease progression and longer life spans. However, the emergence of ART- and HAART-resistant virus mutants in drug-treated macaques and humans, highlighted the relevance of NHP models in the study of HIV-1 infection in humans. Macaque models have been utilized to gain insights into the emergence as well as clinical implications of these drug resistance viruses, with regard to their replication fitness and virulence (221).

One of the main advantages of SHIVs is that these viruses are able to replicate and persist in infected macaque models and can now be used in development and evaluation of vaccines against HIV-1 (222). Passively transferred neutralizing antibodies have been evaluated in SHIV-infected macaques for their ability to prevent infection by blocking virus entry and binding (223-225). SHIV infection in macaques faithfully replicates the acute and rapid phase of HIV-1

infection in humans (226, 227), and SIV- and SHIV-mucosal challenge models, primarily via the genital or the gastrointestinal routes, are crucial for devising strategies to prevent mucosal transmission of HIV (228-230). Additionally, SHIV-infected macaque models can be utilized in studies aimed at inducing vaccine-driven neutralizing antibodies or testing of vaccine-mediated protection by candidate vaccines, evaluation of the use of molecular adjuvants in vaccine formulations, and validation of vaccine-induced protection during host immune responses to vaccinations (223, 231-233). Ongoing comparison of the SIV-infected or SHIV-infected macaque models to HIV-1-infected individuals will lead to our knowledge in development of potential cures and intervention strategies to eradicate HIV/AIDS.

Taken together, animal models such as ferrets and nonhuman primates provide us with tools for further improvement and validation of the translational value of these animal models in advancing our knowledge to prevent and eradicate important human viral pathogens such as influenza and HIV-1. Isolation of pure LEC populations from these animal models would be instrumental tools to understand the involvement and contribution of LECs during host-pathogen interactions.

1.5 SIV/HIV AND THE LYMPHATIC ENDOTHELIUM

The vast majority of HIV-1 infections occurs through mucosal transmission of the virus. Sexual mucosal transmission, either via the vaginal or rectal, remains as the major routes of viral infection in adults (234, 235). However, in pediatric HIV-1 infection, maternal-to-child transmission through the gastrointestinal portal of entry is most likely route, which could occur from ingestion of HIV-1-contaminated maternal fluids during delivery or from breast milk (236,

237). Similarly, studies of SIV and SHIV infections in nonhuman primate models demonstrated that these viruses are readily transmitted across intact mucosal surfaces such as rectal, vaginal, cervical, oral, and penile of these animals (230).

1.5.1 Mucosal transmission and early stage infection of SIV/HIV

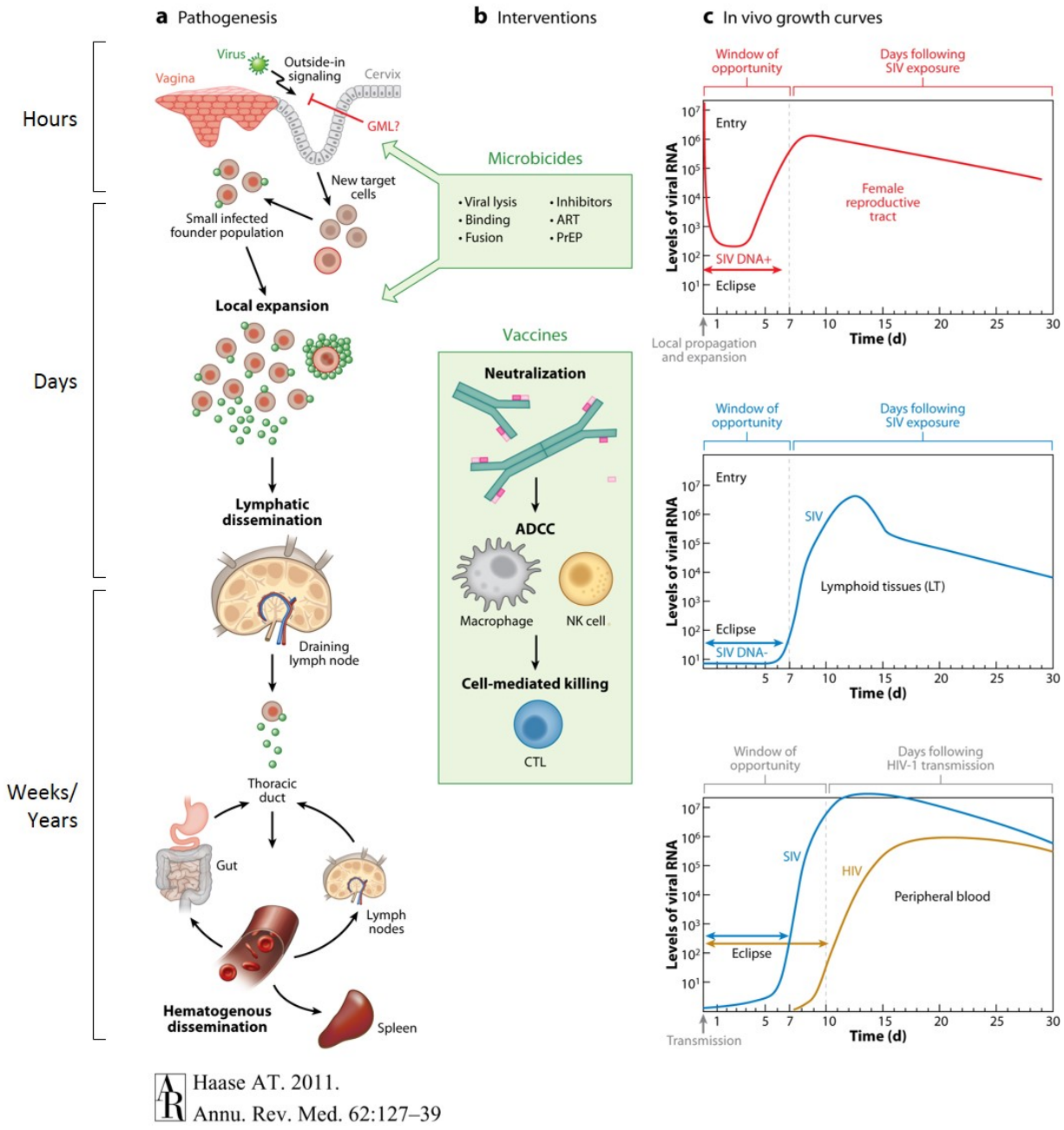
Establishment of SIV/HIV-1 infection occurs rapidly in the first hours and days after crossing the mucosal barriers (238, 239). Mucosal tissue morphology and integrity, as well as the distribution of susceptible and permissible cell types within the mucosae, greatly influence successful productive infection and local replication of the virus. In the NHP model, the critical events that determine the establishment of early stage infection of SIV following high dose mucosal exposure to SIV begin within hours after the virus crosses the mucosal barrier (Figure 4A) (238, 240). The main target cells for SIV (and also HIV-1) infection and replication within the mucosae are the resting CD4+ T cells, which then contribute to establishment of initial small founder virus populations (240). These founder virus populations then rapidly undergoes local expansion through infection of other cell types, including activated CD4 T cells, macrophages, and DCs. As a result, these infected cells not only produce more viruses but are also disseminated first to the draining lymph nodes via the lymphatics, and subsequently to other secondary lymphoid tissues and organs (systemic infection) via the bloodstream or the lymphatics (240).

The gastrointestinal (GI) tract is the largest secondary lymphoid organ in the human body (241). During the establishment of the early stages of SIV and HIV-1 acute infection, the intestinal gut-associated lymphoid tissues (GALT) serve as the major amplification site of viral replication, and remains as major site of viral persistence during chronic infection (235). The

lamina propria is an effector site of the intestinal immune system and contains the largest population of T cells, B cells, and other immune cell populations such as DCs, macrophages, eosinophils, and mast cells (241). Thus, it is not surprising that during early stage SIV infection, over 60% of resting memory CD4+ T cells in the gut are infected by the virus (239, 242). Similarly, in HIV-1-infected individuals, serial GI tract sampling during the early stages of infection demonstrated depletion of CD4+ T cells, although somewhat less dramatic than in the SIV-macaque model (243-245). Local expansion and amplification of the founder virus population could also impair the intestinal epithelial barrier and increased its permeability. The disruption of the intestinal epithelial barrier function during primary infection in HIV-1-infected individuals often coincides with massive depletion of CD4+ T cells in the GALT (245). The loss of the epithelial barrier integrity and permeability leads to microbial translocation of commensal gut microbes and microbial products from the gut lumen into the mucosal layer resulting in systemic immune activation. Translocating microbes and microbial products stimulate innate immune cells and stromal cells through PRRs and other innate immune receptors, and in turn contribute further to the proinflammatory cytokine milieu and systemic immune activation and inflammation associated with chronic SIV/HIV-1 infection (197, 245).

In the SIV-macaque model, key events in the virus-host interaction that enables establishment of systemic infection occurs within the first 7 to 10 days after vaginal exposure to virus (239). During the eclipse phase, which occurs approximately 7 days after exposure to the virus, SIV viral DNA is detected in the cervical/vaginal tissues but not in the lymphoid tissues, suggestive of low level local propagation and expansion of the virus at the portal of entry (Figure 4C). This period is known as the window of opportunity, and thus poses several potential intervention strategies to prevent early infection and subsequent systemic dissemination of the

virus (Figure 4B). During the first week of infection, the host has the upper hand as the founder virus population is still very small with very few infected cells. Intervention strategies such as the use of microbicides and pre-exposure prophylaxis with ART as well as broadly neutralizing mucosal antibodies could prevent establishment of the small founder virus population (246). A second intervention strategy that could be applied during the window of opportunity is to prevent local expansion of the small founder virus population through vaccine design that improves the effector function of the mucosal virus-specific cytotoxic T lymphocyte (CTL) or vaccine design that improves the antibody-dependent cellular cytotoxicity (ADCC) of virus-specific antibodies (240).



Haase AT. 2011.
Annu. Rev. Med. 62:127–39

Figure 4. Schematic representation of SIV/HIV-1 virus transmission, stages of infection, and their potential interventions.

The diagram shows the key events that occurs following mucosal (vaginal) transmission of SIV/HIV-1 with regards to time for interactions between virus and host as well as outline for the maximally time for effective interventions to block establishment of the virus during early stage of infection. (4A) In the first hours of infection, virus crosses the mucosal epithelial barrier to infect a small number of available target cells to establish a small population of founder virus. These founder viruses infect more naïve target cells that are recruited to the site of infection and inflammation and undergo local expansion and amplification. Once the virus is established at the point of entry, dissemination quickly takes place as infected cells including CD4+ T cells, macrophages, and DCs are transported to the draining lymph node via the lymphatics. In the lymph node, amplification of the virus occurs at exponential rate due to availability and close proximity of susceptibly infected cells, which then leads to dissemination of virus and

also infected cells to distal organs via the lymphatics or blood circulatory system. (4B) Interventions for mucosal transmission of SIV/HIV-1 ideally targets the vulnerability of the virus at the point of entry shortly after exposure. Early stage of infection offers multiple intervention opportunities to prevent establishment of the small population of founder SIV/HIV-1 viruses, and thus avoiding the systemic phase of infection by the virus. (4C) The optimal and maximal time for interventions at the portal of entry is known as the "window of opportunity", which occurs between 7 to 10 days in SIV-infected macaque models, and could be longer in HIV-1-infected humans. During the first 7 days postinfection, SIV DNA and RNA is undetectable in the lymphoid tissues, and this period is known as the "eclipse" phase of the virus. Following this phase, a number of key events take place, which leads to establishment of systemic phase of infection. SIV production grows exponentially in the lymphatic tissues (lymph node, spleen, gut), coupled by massive loss of CD4+ T cells in the gut, and establishment of latent/chronic virus infection. Figure adapted from (240) with permission.

1.5.2 Potential role of LECs in local expansion and dissemination of SIV/HIV

Dissemination of SIV/HIV-1 and SIV/HIV-1-infected cells from the portal of entry is made possible via the lymphatics. Lymphatic drainage transports the virus and virus-infected cells to the draining lymph nodes, where large numbers of susceptible cells are available in close proximity, thus providing optimal conditions for viral production (238).

Due to their direct interaction with incoming virus or virus-infected cells during lymphatic drainage, LECs are likely to have direct or indirect roles during virus-host interaction. In vitro studies of primary LECs demonstrated that not only do LECs express functional PRRs including TLRs (160, 247, 248), they also secrete functional proinflammatory cytokine and chemokines that recruit and modulate immune cells (248). More recently, LECs have been shown to capture and archive viral antigens and vaccine antigens under inflammatory conditions for a prolonged period of time and thus contribute to antigen persistence (166). These investigators also showed that the vaccine-elicited antigen persistence improved the effector function of circulating memory CD8+ T cells, which positively influenced protective immunity of the host.

The draining lymph node has been well recognized as the site of induction of host adaptive immune responses during an infection or vaccination. In a recent study, a group of researchers sought to map the distribution of LECs in the human lymph node by staining with antibodies specific for LECs as well as other immune cells (155). Interestingly, they found that there were two distinct LEC populations in the different sinuses of the human lymph node, and this observation was conserved between draining lymph nodes of different peripheral regions, namely the axillary, cervical, and mesenteric lymph nodes. In addition, the study also revealed that the paracortical and medullary sinus resident LECs co-expressed the APC marker CD209, but the resident LECs found in the subcapsular and trabecular sinuses did not, suggesting that the different phenotypes of LECs could have distinct functions depending on their locations within a specific organ.

Altogether, these results highlighted the potential involvement and function of LECs during pathogen-host interactions, not only in the context of SIV/HIV-1 but also other viral pathogens. In the context of SIV/HIV-1 infection, LECs may contribute to the local expansion of the founder viruses through PRR sensing of the virus and subsequent recruitment of naïve immune cells by LEC-induced proinflammatory cytokines/chemokines. LECs also might play an important role in regulating the transport of SIV/HIV-1-infected immune cells in the periphery as well as in the draining lymph node. LECs are important cells in establishment of structure and compartmentalization within the lymph node, and thus LECs may be responsible in directing antigen-loaded APCs and infected immune cells to populations of naïve immune cells in the lymph node. In addition to that, due to their ability to capture and store antigens LECs potentially may become a reservoir for SIV/HIV-1 during chronic infection stage, if viral antigens that are taken up are stored in the form of infectious virions.

Thus by obtaining better understanding of the different phenotypes of LECs and how they may crosstalk with virus and immune cells during host responses to infection/vaccination, this will help us better elucidate the process disease development in the context of SIV/HIV-1 and other infections, and hopefully help us design improved vaccines and therapeutics that target the different stages of the disease.

1.6 SUMMARY

LECs are key players in regulation of immune cell trafficking during immune surveillance under homeostatic and inflammatory conditions. We are only beginning to understand the contributions of LECs to the initiation of host innate and adaptive immune responses during infection or vaccination. It remains to be determined whether LEC interactions with immune cells such as DCs and T cells impact the functions of these cells in the absence or presence of disease conditions. Understanding the role of LECs in host immune responses and their crosstalk with immune cells in the context of SIV/HIV-1 and other infections can potentially lead to discovery of novel targets for therapeutics and vaccines for HIV-1 and other pathogens.

2.0 STUDY PREMISE

2.1 RATIONALE

Due to their unique location, LECs are likely to be among the first cells to come in contact with microbes that cross the epithelial barrier, with first wave, pathogen-induced host inflammatory signals, and with vaccine antigens and adjuvants after exposure. LECs line the initial lymphatic vessels, which then form larger collecting lymphatic vessels, and ultimately drain into the lymph node. In the lymph node, LECs are localized in the subcapsular, cortical, and medullary sinuses, where they regulate the processes for incoming and exiting APCs, leukocytes, and lymphocytes (249). LECs control these processes through expression of chemoattractant chemokines/cytokines (23, 36, 83), signaling molecules (56, 156, 158), and adhesion molecules (89, 148, 250) for immune cell recruitment, migration, and homing. The role of LECs in host innate immune responses is highlighted by documented expression of functional PRRs, including TLRs that recognize common conserved motifs found on microbes, PAMPs (160, 247, 248). TLRs are also expressed by professional antigen presenting cells APCs such as DCs, and LECs have also been shown to share several phenotypic characteristics with DCs (93, 155). Analysis of LECs in the lymph node showed that LECs express DC-SIGN (CD209), a known DC marker (155). LECs also have been shown to express MHC I and MHC II molecules but not their costimulatory molecules (162, 164, 251). In addition, LECs have the ability to capture and

archive antigens, which results in antigen persistence in the host (166). These findings led us to believe that LECs have more direct involvement than previously appreciated in shaping the host innate immune responses during infections or vaccinations, as well as transmission or (or resistance to) infectious agents.

Therefore, **my overall hypothesis for this project was that LECs initiate host innate immune responses to pathogens through expression of multiple functional PRRs and are involved in initial establishment or restriction of infection by viral pathogens through expression of intrinsic and cellular viral restriction factors.** I proposed that LECs were active participants in immediate host innate immune responses to pathogens and foreign antigens through sensing of PRRs and helped create inflammatory environments that attracted other immune cells to the sites of infection or vaccine administration. I also proposed that LECs also have the ability to control the spread of viral pathogens, either (1) directly through expression of intrinsic and cellular viral restriction factors, or (2) indirectly through antigen uptake and processing, and subsequently targeting these antigens to degradation or archiving them for optimal contact with APCs such as DCs or T cells.

Thus, during pathogen-host interactions the role of LECs might not be limited to development of initial host innate immunity but also to modulation of the local microenvironments within the tissues that could result in alteration of the outcome of an infection or disease progression.

2.2 SPECIFIC AIMS

To address my hypothesis, my specific aims were:

Specific Aim 1: To develop new culture models for ex vivo analysis of LECs.

Studies of LECs has been hampered by the challenges in obtaining pure in vitro cultures of LECs and the inability to mimic the quiescent physiologic state of LECs *in vivo* using the standard two-dimensional (2D) cell culture system. In chapter 3 and 4 of this dissertation, I sought to isolate primary LECs from different animals (macaque and ferret) and tissue types (skin, lung, tracheal, jejunal, mesenteric lymph node, and thoracic duct) to establish 2D in vitro cultures of homotypic primary LEC populations. These primary LEC populations were used to study the phenotypic characteristics and functionality of LECs as well as their interactions with microbes.

Specific Aim 2: To determine the expression profiles of PRRs by LECs.

PRR sensing of PAMPs rapidly triggers host innate immune responses through activation of complex signaling pathways that induce production of proinflammatory cytokines and chemokines to facilitate eradication of pathogens. PRRs can be classified into three different protein families, namely the toll-like receptors (TLRs), the NOD-like receptors (NLRs), and the RIG-I-like receptors (RLRs). Although the expression of multiple functional TLRs has been reported in LECs, the expression of NLRs and RLRs has not yet been examined. In chapter 3 and 4, I studied in more detailed and more comprehensively the expression profiles of the different PRRs express by primary ferret LECs and primary macaque LECs from different

animals and tissue types and determined the functionality of these PRRs to respond to their known ligands in comparison to their human counterparts (the model human dermal LECs).

Specific Aim 3: To compare a subset of phenotypic and functional characteristics of DCs and LECs.

Previous data from our laboratory showed that LECs and DCs responded to mycobacterial components in a similar fashion and interestingly shared a number of phenotypic characteristics (Pegu, unpublished data). In chapter 4, I examined the phenotypic expression of DC-associated markers by primary macaque LECs and also evaluated their functional ability to perform antigen uptake and processing of DQ-ovalbumin, which is a hallmark of APCs such as DCs.

Summary: Altogether, my studies contributed to establishment of accessible tools for studying endothelial cell biology of lymphatics from different species and tissue origins. In addition to that, my work on phenotypic and functional characteristics of LECs revealed aspects of LEC immunobiology that have not yet been fully explored or understood and could potentially contributed to our understanding of innate immunologic functions of LECs for improved vaccines and therapeutics design strategies.

3.0 ISOLATION, CHARACTERIZATION, AND FUNCTIONAL ANALYSIS OF PRIMARY FERRET LYMPHATIC ENDOTHELIAL CELLS

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This chapter comprises of the completed experiments and published data from studies that were performed to isolate and establish primary ferret LEC populations in vitro, as well as studies that were undertaken to characterize and evaluate the phenotypic profiles and functional functions of the primary ferret LECs in response to known TLR ligands, including poly I:C in in vitro model. All of the experiments on primary ferret LECs were performed by Stella Joan Berendam with Beth Fallert Junecko's assistance in in situ images acquisition.

(Published manuscript)

3.1 PREFACE

In order to begin to understand the potential role of LECs during pathogen-host interactions, I sought to obtain and establish in vitro models of primary LECs. I focused initially on the isolation of primary LECs from the commonly used animal model for human diseases, namely the ferret model. Ferrets are becoming increasingly popular small animal models for modelling of human respiratory diseases as well as development of vaccines and therapeutics for infectious agents of viral origins. Our group was part of a collaborative group of investigators looking at the effect of DNA vaccinations of influenza in ferrets. Vaccine antigens are likely to travel to the draining lymph node via the lymphatics. Thus, I took the opportunity to ask (1) whether we were able to culture primary ferret LECs from different animal ferrets and tissue origins, (2) whether these primary LECs maintained their phenotypic and functional characteristics ex vivo in a 2D in vitro culture system, and (3) whether the lymphatic distribution in ferret tissues was similar to that of humans. All work was performed by Stella Berendam, with the exception of in situ hybridization image acquisition, which was performed by Beth A. Fallert-Junecko.

This study was accepted for publication in Veterinary Immunology and Immunopathology in its entirety and used in this dissertation with permission from the publisher.

3.2 ABSTRACT

The lymphatic endothelium (LE) serves as a conduit for transport of immune cells and soluble antigens from peripheral tissues to draining lymph nodes (LNs), contributing to development of host immune responses and possibly dissemination of microbes. Lymphatic endothelial cells (LECs) are major constituents of the lymphatic endothelium. These specialized cells could play important roles in initiation of host innate immune responses through sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including toll-like receptors (TLRs). LECs secrete pro-inflammatory cytokines and chemokines to create local inflammatory conditions for recruitment of naïve antigen presenting cells (APCs) such as dendritic cells (DCs) to sites of infection and/or vaccine administration. In this study, we examined the innate immune potential of primary LEC populations derived from multiple tissues of an animal model for human infectious diseases -- the ferret. We generated a total of six primary LEC populations from lung, tracheal, and mesenteric LN tissues from three different ferrets. Standard RT-PCR characterization of these primary LECs showed that they varied in their expression of LEC markers. The ferret LECs were examined for their ability to respond to poly I:C (TLR3 and RIG-I ligand) and other known TLR ligands as measured by production of proinflammatory cytokine (IFN-α, IL-6, IL-10, Mx1, and TNF-α) and chemokine (CCL5, CCL20, and CXCL10) mRNAs using real time RT-PCR. Poly I:C exposure induced robust proinflammatory responses by all of the primary ferret LECs. Chemotaxis was performed to determine the functional activity of CCL20 produced by the primary lung LECs and showed that the LEC-derived CCL20 was abundant and functional. Taken together, our results continue to

reveal the innate immune potential of primary LECs during pathogen-host interactions and expand our understanding of the roles of LECs might play in health and disease in animal models.

Keywords (indexing terms)

Lymphatics, lymphatic endothelial cell, ferret, toll-like receptor, chemokine

3.3 INTRODUCTION

The lymphatic vasculature (LV) is often described as a network of unidirectional, blind-ended capillaries and larger collecting vessels made up of a single layer of loosely overlapping cells – lymphatic endothelial cells (LECs) (9, 10). Since the LV is often located within micrometers beneath mucosal surfaces, they are likely to be among the first cells to participate in early host innate immune responses upon contact with microbes, host inflammatory signals, and vaccine antigens. LECs secrete chemoattractant cytokines (chemokines), such as CCL20, which recruit immature DCs to sites of inflammation, and CCL21, which draws antigen-loaded mature DCs into the collecting lymphatic vessels and then downstream into the LN paracortices, wherein a unique environment is created to optimize activation of adaptive immune responses (28, 84).

Model human LECs express functional toll-like receptors (TLRs) that recognize multiple pathogen-associated molecular patterns (PAMPs) on microbes (160, 247). TLR activation results in signaling that triggers the production of pro-inflammatory cytokines and chemokines, including type I interferons (IFNs), that are crucial not only for pathogen clearance during innate responses, but also enhance the induction of antigen-specific responses during subsequent adaptive immunity (252, 253). Thus, the use of TLR ligands as vaccine adjuvants to increase vaccine efficacy in inducing host immune responses is an attractive strategy for development of next generation vaccines. Recent studies on the use of TLR ligand-conjugated vaccines have been promising in non-human primates (254) as well as small animal models such as mice (255, 256) and ferrets (187). However, different TLR ligand adjuvants mediate distinct cellular and molecular profiles of early innate responses in the periphery and the lymphatic organs of non-

human primates (254). Despite their potential, there is still limited understanding of the local and systemic immune responses and potential toxicities associated with their use in vivo.

Ferrets are becoming an increasingly examined small animal model for the study of human diseases, including neurobiology (171-173), cancer (174), and infectious diseases of viral and bacterial origin (176-178, 180, 181). The use of the ferret as an animal model for studying human respiratory diseases has offered a number of advantages. First, ferret airways resemble and share many anatomical and physiological similarities to humans making them useful for study of human respiratory infections (257). In addition, ferrets are highly susceptible to a number of human respiratory pathogens that often require no laboratory adaptation prior to infection (183, 184). Furthermore, ferrets are considered an accurate small animal model to study both human and avian influenza (182). In this regard, the ferret model is used to study not only seasonal and highly pathogenic avian influenza virus pathogenicity, but also viral transmission (185, 258) and the development of vaccines and antiviral therapeutics (186).

Despite the utility and increasing use of this animal model there is still a major lack of ferret-specific reagents for use in research, despite efforts that have been invested to obtain reagents to enable development of ferret-specific assays at the cellular and molecular levels (257, 259). Molecular cloning and phylogenetic analysis of ferret immune-related genes provides tools to assess the inflammatory cytokine and chemokine profiles in infected animals and determine their importance in disease progression and/or clearance of infection (260, 261). The expression of functional TLRs by human LECs (247) has highlighted that the LE could be a target for new vaccine adjuvancy strategies, alongside monocytes and DCs. In this light, we isolated, cultured, and characterized primary LECs from multiple ferret tissues, and determined their responsiveness to known TLR ligands by measuring the production of proinflammatory

cytokine and chemokine mRNAs using real time RT-PCR. In addition, we also cloned and sequenced ferret LEC marker partial cDNAs for in situ hybridization (ISH) analysis to probe the lymphatic vasculature in ferret tissues. Altogether, these findings provide insight into the function and microanatomy of ferret lymphatics and establish a foundation for examination of the roles of LECs during infection and immunization.

3.4 MATERIALS AND METHODS

Ferrets and Tissue Processing

The ferrets from which tissues for histological analysis and isolation of LECs were obtained were available from other non-infectious studies, and were 6-7 month old females that ranged in weight from 695-825g. These ferrets were vaccinated for Canine Distemper virus, descented, and single housed at the University of Pittsburgh. All animal work was approved by the University of Pittsburgh Institutional Animal Care and Use Committee, although the tissues contributing to these studies were excess tissues available at necropsy.

RT-PCR, cloning, and phylogenetic analysis of ferret LEC markers

Due to the lack of complete ferret genomic sequence information, design of ferret-specific primers for amplification of ferret cDNAs, including LEC markers (Table 2), was based on published canine sequences available in GenBank, National Center for Biotechnology Information (NCBI). Previous analyses of ferret cytokine cDNAs reported that ferret sequences were closely related to canine sequences (260). Total cellular RNAs were obtained from ferret lung, spleen, and LN tissues, both untreated and stimulated overnight with unmethylated CpG oligonucleotides (ODN), poly I:C, or lipopolysaccharide (LPS). RNA extractions were performed using Trizol (Life Technologies, Rockville, MA, USA) according to the manufacturer's recommendation. Total RNA (2 μg) was reverse transcribed using oligo-dT primer (Promega, Madison, MI, USA) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, MI, USA). The resulting cDNAs for each tissue type were pooled and

standard RT-PCR was performed using GoTaq DNA Polymerase (Promega, Madison, MI, USA) with ferret-specific primers (Table 3) using PCR conditions as follows: 94°C for 3 min, 94°C for 30 sec, 58°C for 30 sec, and 72°C 2 min for 35 cycles, followed by 72°C for an additional 10 min before holding at 4°C. Amplified products were run on a 1% agarose gel prestained with GelRed™ Nucleic Acid Stain (Invitrogen, Carlsbad, *CA, USA).* PCR products were purified from gel slices using the QIAEX II gel extraction kit (QIAGEN, USA) before subcloning into the pGEMT cloning vector (Promega, Madison, MI, USA). The inserts were sequence verified and analyzed using the Vector NTI program (Invitrogen, Life Technologies). Sequences were aligned with corresponding sequences from other species as available through the NCBI/GenBank database using the ClustalX 2.1 multiple sequence alignment program (262). Phylogenetic trees were generated by the neighbor-joining method after DNA distance calculation using the PHYLIP 3.69 program (263), and drawn with Tree View 1.6.6 (264).

Isolation and culture of primary ferret lymphatic endothelial cells from tissues

LEC isolation was performed as described (108). Briefly, ferret tissues were mechanically digested by mincing with sterile surgical scissors, followed by enzymatic digestion with 0.25% trypsin overnight at 4°C. Digested cells were passed through a 100 µM cell strainer once, and twice through 70 µM cell strainers. Primary LECs were cultured on rat tail collagen I (BD Biosciences) coated culture dishes using the EGM2 endothelial specialized medium (Lonza), supplemented with 2% FBS, and a final concentration of 100 ng/ml recombinant human VEGF-C (R&D Systems, Minneapolis, MN, USA). Cells were grown at 37°C with 5% CO_2 until they reached 80-90% confluency, and then passaged at between 1:4 to 1:8 splits. The resulting cell

populations were characterized by standard RT-PCR using primers specific for ferret LEC markers (Table 3) using PCR conditions described above.

Stimulation of ferret LECs with TLR ligands

Near confluent monolayers (80-90%) of primary ferret LECs were exposed to ligands for TLRs 1 to 9 (InvivoGen, San Diego, CA, USA) in parallel with mock treated cultures for each LEC population. Concentrations of TLR agonists were as described (247). After 24 hr of treatment, total RNA extractions were performed using Trizol (Life Technologies, Rockville, MA, USA) according to the manufacturer's recommendation. Statistical analyses were performed using the Prism software package (GraphPad, San Diego, CA). Paired *t*-tests were used to compare mock versus TLR ligand treated samples because no nonparametric method for paired data can be properly applied to sample size less than 6. A *p*-value of <0.05 was considered significant.

Real-time RT-PCR

Primers for real-time RT-PCR measurement of podoplanin, LYVE-1, and CCL20 mRNA levels were design based on the ferret sequences presented here (Table 3). Primers for GAPDH, CCL5, CXCL10, IFN-α, IL-6, IL-10, TNF-α, and Mx1 were obtained from published studies (187, 265). SYBR Green real-time RT-PCR (comparative Ct method) was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) as described (266). All samples were assayed in duplicate and with concurrent controls that lacked reverse transcriptase in the cDNA synthesis reaction.

Chemotaxis

Chemotaxis assay was performed using murine L1.2 cells engineered to express ferret CCR6 as described (261). Briefly, a total of 200,000 of L1.2.fCCR6 responder cells were loaded on top of the porous membrane (ChemoTx® Disposable Chemotaxis System, pore size 5 μm, NeuroProbe) in triplicate and incubated at 37°C/5% CO_2 for 2 hr in a humid chamber. The number of migrating cells was counted using a hemacytometer. Chemotactic index (CI) was calculated relative to control wells with no chemokine. Chemically synthesized ferret CCL20 (261) was used as a control.

In situ hybridization (ISH)

Paraffin embedded ferret tissue sections were cut at 5 μM and mounted on Superfrost Plus slides (Bio-Optica). ^{35}S-UTP-labeled riboprobes were generated (93) and used for stringent ISH on ferret tissues. Autoradiographic exposure times for ferret specific LEC markers after ISH were 14 days for LYVE-1 and CCL21 mRNA targets and 21 days for podoplanin, Prox-1, and VEGFR-3 mRNA targets. We note that for reasons not yet clear, in these studies and others, ISHs with many probes in ferret tissues have resulted in marginally acceptable signal-to-noise ratios.

3.5 RESULTS

Cloning, sequencing, and phylogenetic analysis of ferret LEC marker cDNAs

Given the paucity of ferret-specific or cross-reactive reagents available for research, particularly for lymphatic analyses, we designed primers for use in RT-PCRs to acquire ferret LEC marker partial cDNAs based on published canine sequences, a species highly related to the ferret (260, 261). These studies were driven by the goal of obtaining primary ferret LECs for ex vivo analyses (below) and it was imperative to obtain information on LEC target sequences to allow development of assays for measurement of mRNA levels in the LEC populations. We successfully obtained cDNAs containing full-length (podoplanin and CCL21) or partial (LYVE-1, Prox-1, and VEGFR-3) ORFs from total cellular RNAs prepared from ferret tissues. Sequence homology analysis of each of the ferret LEC markers with other species showed the highest similarity to the order *Carnivora*, with *Canis lupus* being the most related species (Table 2). This corroborated earlier findings with ferret immune-related genes (260, 261). Ferret LEC markers shared between 74% to 98% sequence homology to the corresponding human sequences at the nucleotide level and between 60% to 100% sequence homology at the amino acid level. Analysis of the Prox-1 partial ORF showed it is well conserved across multiple species at both the nucleotide (98%) and amino acid (99-100%) levels. Phylogenetic analysis of the podoplanin complete ORF revealed that ferret sequences clustered together with species in the order *Carnivora* (Figure 5). Phylogenetic trees constructed for the other ferret LEC markers revealed highly similar results (data not shown).

Table 2. Nucleotide sequence relatedness of ferret LEC marker cDNAs to other animal species.

	Sequence identity (%)				
	Podoplanin (complete ORF)	**LYVE-1 (partial ORF)**	**Prox-1 (partial ORF)**	**VEGFR-3 (partial ORF)**	**CCL21 (complete ORF)**
Species					
Dog	81[a] (73)	91 (85)	98 (100)	92 (96)	89 (85)
Cat	82 (65)	90 (83)	98 (100)	94 (99)	90 (88)
Pig	71 (53)	80 (71)	98 (100)	92 (95)	84 (78)
Human	74 (60)	80 (65)	98 (100)	91 (94)	86 (80)
Rhesus	73 (59)	80 (67)	98 (100)	91 (95)	82 (77)
Mouse	78 (48)	76 (56)	95 (100)	87 (94)	80 (79)
Rat	80 (48)	76 (53)	95 (100)	86 (94)	76 (70)
Guinea pig	42 (37)	75 (63)	97 (99)	89 (94)	80 (76)
Syrian hamster	82 (46)	76 (60)	94 (99)	88 (96)	80 (71)

Data shown are the percent relatedness to the corresponding stretch of nucleotide sequences in the ferret cDNAs obtained here. The numbers in parentheses represent the percent relatedness of the corresponding deduced amino acid sequences.

Table 3. Primers and sequences used for standard and real-time RT-PCR.

RT-PCR	Primer name	Primer sequence (5'→3')	Amplicon size (bp)	Source
Standard	CaPDPNF1	AGATGTGGAGGGTGCCAGT	534	This study. Designed based
	CaPDPNR1	AATTCTTCAGCTCTTTAGGGCGAG		on NM_001003220.
Standard	CaLYVE-1F1	CTGGTGGTTGTCTGCTTCCAT	761	This study. Designed based
	CaLYVE-1R2	TGCAAAGAAGAGGAGTGCGAG		on XM_003639783.
Standard	CaProx-1F1	AATAGCCTCTAAACAGTTTC	382	This study. Designed based
	CaProx-1R1	TATCCTCCTGATGTACTTCG		on XM_853042.
Standard	CaVEGFR-3F3	GAGCTCTATGACATCCAGCTGT	537	This study. Designed based
	CaVEGFR-3R3	GGCACATTCACCACCAGCTCCAG		on XM_538585.
Standard	CaCCL21F1	TCCACCTCGCGCACTACTC	414	This study. Designed based
	CaCCL21R2	CTCTAGGCTGGTCACTGGG		on NM_001005258.
Standard	CaGAPDHF1	ATGGTGAAGGTCGGAGTCAA	300	This study. Designed based
	CaGAPDHR1	GAAGACCCCAGTGGACTCCA		on NM_001003142.
Standard	FePDPNF1	ATGTGGAGGGTGCCAGTTCT	430	This study. Designed based
	FePDPNR1	CTGTCGCCAGACCATCTTTT		on obtained ferret sequence.
Standard	FeLYVE1F1	GCCAAATGCTTCAGCCTGGT	564	This study. Designed based
	FeLYVE1R1	AGCCCGAGCAGAAGTAGGAG		on obtained ferret sequence.
Standard	FeProx-1F1	ATGCCTGACCATGACAGCAC	286	This study. Designed based
	FeProx-1R1	GCTGGGAAATTATGGTTGCT		on obtained ferret sequence.
Standard	FeVEGFR3F1	TGAGCTCTATGACATCCAGC	482	This study. Designed based
	FeVEGFR3R1	CCTTTAGTACCAGGGCATGC		on obtained ferret sequence.
Standard	FeCCL21F1	ATGGCTCAGTTACTGACTCC	310 (smaller fragment)	This study. Designed based
	FeCCL21R1	TACAGCCCTGGACTTGTTTC	~500 (larger fragment)	on obtained ferret sequence.
Standard	FeGAPDHF1	CATCGTGGAGGGCCTCATGA	215	This study. Designed based
	FeGAPDHR1	ATACATTGGGGGTGGGGACAC		on obtained ferret sequence.
Real time	SYBRfPDPNF1	GAGGATGGGCCAACTCAAGA	79	This study. Designed based
	SYBEfPDPNR1	GTTGTGGTGCTCTGGCTTTCT		on obtained ferret sequence.
Real time	SYBRfLYVE1F1	GCTTCAGCCTGGTGTTGCTT	79	This study. Designed based
	SYBRfLYVE1R1	GATGTGACCAGGAGCCTTGTG		on obtained ferret sequence.
Real time	SYBRfGAPDHF	TTGCTGACAATCTTGAGGGAGTT	81	This study. Designed based
	SYBRfGAPDHR	CTGCTGATGCCCCCATGT		on obtained ferret sequence.
Real time	SYBRfCCL20F	TGCTCCTGGCTACTTTGATGTC	89	This study. Designed based
	SYBRfCCL20R	TGCTTGCTGCTTCTGACTTG		on published ferret CCL20 (261)

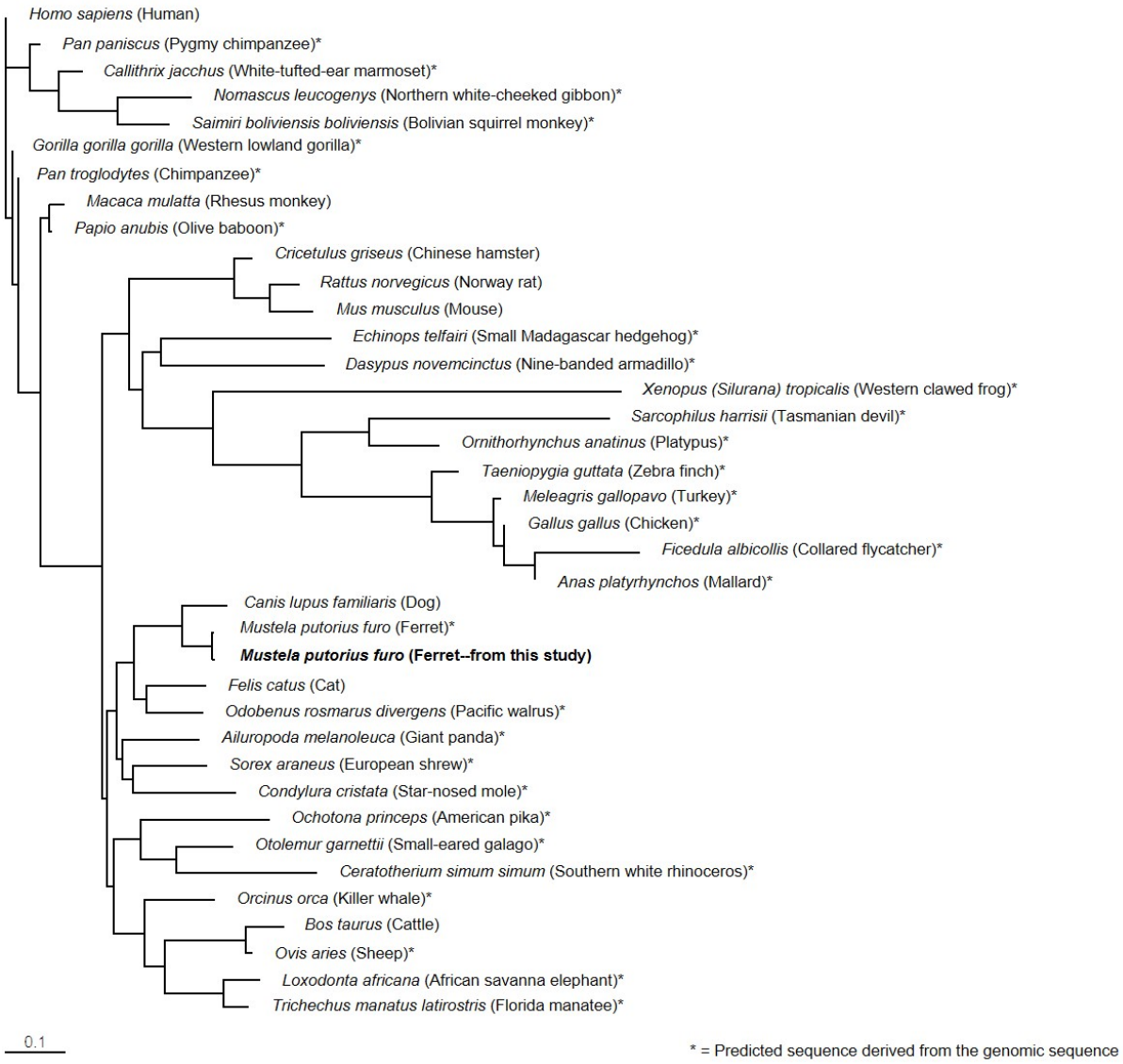


Figure 5. Phylogenetic analysis of the ferret podoplanin cDNA nucleotide sequence.

The ferret podoplanin cDNA sequence obtained here was examined for its relatedness to podoplanin sequences from other species available from the NCBI/GenBank database. Predicted complete podoplanin ORF sequences derived from genomic DNA are indicated by (*). The phylogenetic tree was generated by the neighbor-joining method using the PHYLIP free program with 1000 bootstrap analyses and was drawn with Tree View 1.6.6 (Molecular Evolution).

Isolation and characterization of primary ferret LECs

To obtain LECs from ferrets, we isolated a total of six primary ferret LEC populations from three different ferrets and four different tissue types (skin, lung, trachea, and mesenteric LN). Cultured primary ferret LECs formed confluent monolayers approximately 10 to 14 days after initial plating onto collagen-coated plastic surfaces, these cultures being designated as passage zero (P0). The primary ferret LEC monolayers showed the typical cobbled-stone morphology of endothelial cells observed in conventional 2D culture systems (Figure 6).

The primary cultures were characterized first by standard RT-PCR for expression of the LEC markers podoplanin, LYVE-1, Prox-1, VEGFR-3, and CCL21 (Figure 7). All of the primary ferret LEC populations isolated from all tissues types expressed podoplanin and Prox-1 mRNAs to high levels. In contrast, the expression of LYVE-1 and VEGFR-3 varied among the LEC populations. LYVE-1 expression was not detected in the primary ferret LECs isolated from skin (F1 skin), whereas VEGFR-3 expression was not detected in the skin (F1 skin) and trachea (F2 trachea) LEC populations. CCL21 mRNA was detected in all cultured primary ferret LECs, although the CCL21-specific primers yielded two differently sized bands. Gel extraction and sequencing of both bands revealed that the larger fragment contained all sequences present in the smaller fragment as well as what was likely an additional intron, consistent with alternative splicing of an mRNA precursor.

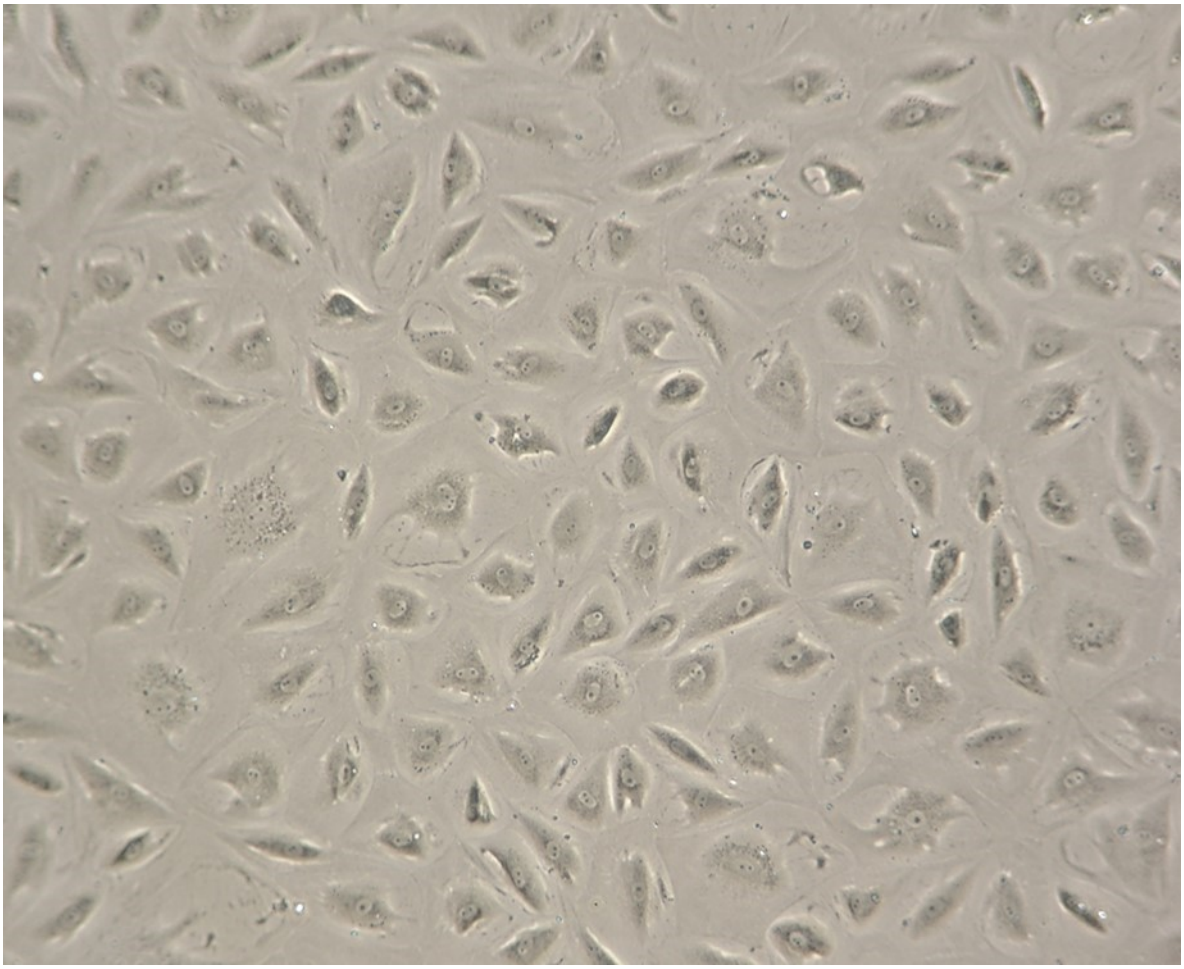


Figure 6. Ferret lung LECs grown in culture show the typical cobblestone shape.

Shown is a phase contrast micrographic image of confluent ferret lung LECs at passage 3 and at 11 days after plating. (Original magnification at 100X).

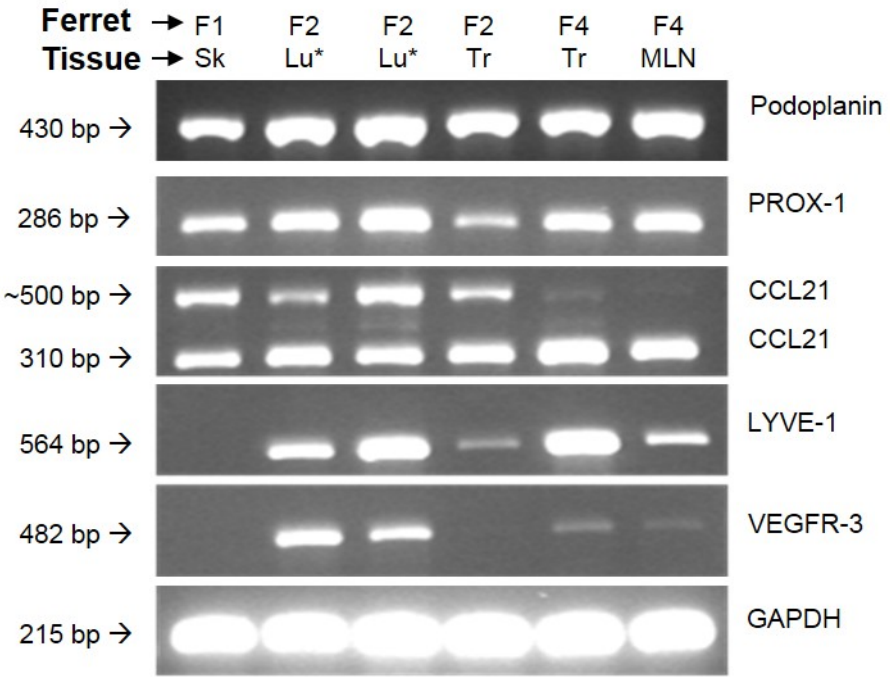


Figure 7. Ferret LECs express multiple LEC marker mRNAs.

Standard RT-PCR was used to examine the expression of podoplanin, LYVE-1, Prox-1, VEGFR-3, and CCL21 in total RNA preparations from six ferret LEC populations. The ferrets and tissue types are noted above each column of bands, whereas the target gene is noted to the right of each gel image. The two lung LEC populations, noted by the asterisks (*), were derived from the left or right lobes of lung from the same animal.

Ferret LECs respond to TLR ligand stimulation

To determine whether the ferret LECs responded to PAMPs, we first exposed all LEC populations for 24 hr to poly I:C, a double-stranded viral mimetic and a known TLR3 and RIG-I ligand,. Since two of the primary lung LEC populations were derived from the same ferret and revealed similar LEC marker expression profiles by standard RT-PCR, we included just one of the populations in these and subsequent studies. Responsiveness was assessed by measurement of proinflammatory cytokine (TNF-α, IFN-α, IL-6, and IL-10), chemokine (CCL5, CCL20, and CXCL10), and IFN-stimulated gene (ISG) Mx1 mRNA levels using newly designed or published (187, 260) ferret-specific real-time RT-PCR primers (Table 3). All primary ferret LEC populations responded to poly I:C, with increases in levels of all eight mRNAs examined ($p<0.05$ for all mRNAs; Figure 8). The levels in increase ranged from 5-fold (IL-10) to 633,031-fold (Mx1), as determined from the ratio of the geometric means for the treated and control groups.

To determine further whether LECs derived from lung tissues, which comprise a major host/pathogen/environment interface, are responsive to multiple TLR ligands, one of the primary ferret lung LEC populations (F2 lung LEC, left lobe) was exposed to different TLR ligands (TLR1-9) for 24 hr. Responsiveness was measured again by real time RT-PCR. The ferret lung LECs responded strongly to most of the TLR ligands examined (Figure 9), although this varied depending upon the TLR ligand and the mRNA measured. The gene showing the greatest overall response across TLR ligands was CCL20 (Figure 9) with a mean induction (geometric mean) of 5,041-fold across all TLR ligands. In contrast, the mean induction levels for TNF-α, IFN-α, and IL-10 were all less than 1.5-fold across all TLR ligands. Analysis of the LEC responses across all genes for the individual TLR ligands revealed that poly I:C was the most

potent TLR ligand under these conditions inducing the eight genes examined an average of 2,424-fold (geometric mean). The low molecular weight form of poly I:C induced these genes an average of 288-fold. In contrast, all of the other TLR ligands induced the same genes only 0.9-fold (LPS and flagellin) to 10.2-fold (FSL-1).

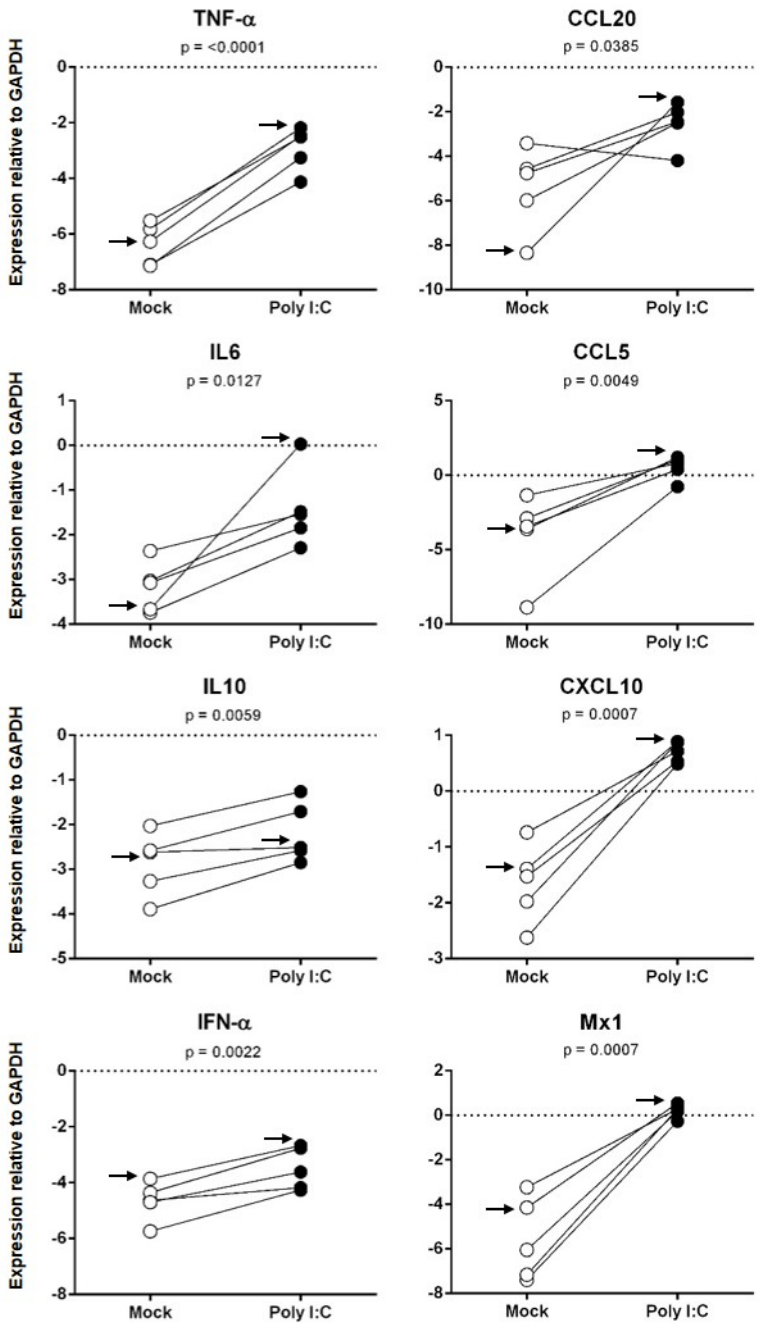


Figure 8. Primary ferret LECs respond strongly to poly I:C.

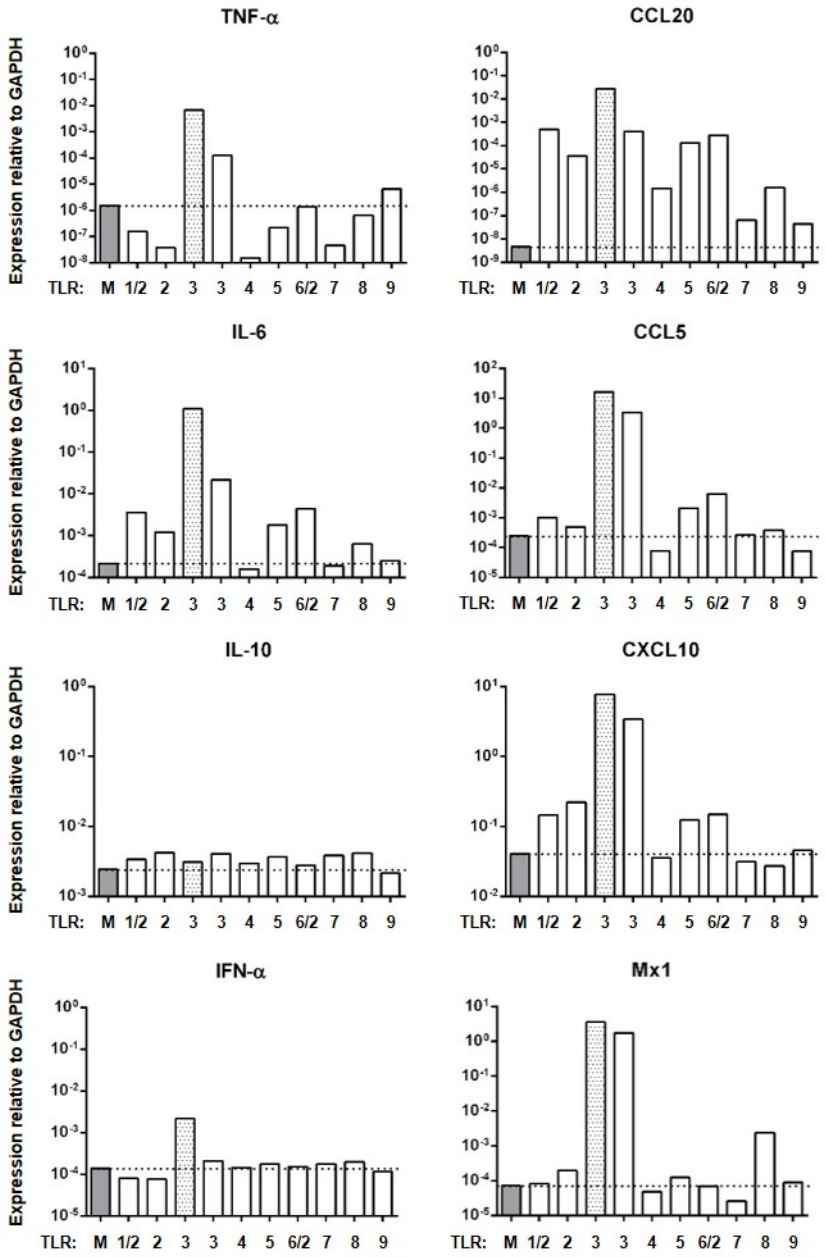
Confluent monolayers of ferret skin (1 population), lung (1 population), trachea (2 populations), and mesenteric LN (1 population) LECs were exposed to poly I:C for 24 hours and responses were measured by SYBR Green real time RT-PCR for mRNAs encoding the indicated cytokine, chemokine, or ISG. Data are presented as values normalized to the endogenous control GAPDH calculated as $2^{(-\Delta Ct)}$. The arrow denotes the data obtained from the lung LEC population used in Figure 9.

To unveil possible relations among the mRNA expression changes induced in the ferret LECs by TLR ligands, we performed Spearman correlation analyses (Table 4). Pairwise correlation analyses revealed that IL-6 mRNA levels correlated with four other mRNAs (CCL5, CCL20, CXCL10, and Mx1). Similarly CXCL10, CCL5, and CCL20 mRNA levels were all positively correlated with each other and with a total of three other mRNAs. Amongst this small set of host response genes, the levels of IFN-α, TNF-α, and IL-10 were not correlated with the levels of any other mRNAs (Table 4). The lack of correlation between IFN-α and the ISGs CXCL10 and Mx1 was surprising, but induction of other IFNs like drove the induction of these two ISGs. Additionally, these collective analyses are complicated by the analysis of this data set driven by stimulation and signaling through nine different TLRs and their respective signaling pathways.

Primary ferret lung LECs stimulated with TLR ligands produce functional CCL20

Given that the chemokine CCL20 was the most responsive of the genes examined after TLR stimulation, and that we have previously developed a chemotaxis assay for ferret CCL20 (261), we measured the CCR6-responsive activities of supernatants collected from the TLR ligand treated LECs. Chemically synthesized ferret CCL20 protein was used as a positive control for cell migration and media alone was used as a negative control. Migration of the ferret CCR6+ responder cells to serial dilutions of the synthetic peptide generated the typical bell-shaped, dose-dependent responses (Figure 10B), with maximum migration at 10nM, yielding a chemotactic index (CI) of 393 (with 20,000-30,000 cells typically migrating). Among the TLR ligands examined, the supernatant from the Pam3CSK-treated LECs induced the greatest cell migration with a CI of 169, followed by supernatant from the FSL-1-treated cells (CI of 162), poly I:C-

treated cells (CI of 111), flagellin-treated cells (CI of 27), and ssRNA40-treated cells (CI of 30) (Figure 10A). Although the real-time RT-PCR data showed that all of the TLR ligands increased the levels of CCL20 mRNA (Figure 9), although only five out of eight LEC-stimulated culture supernatants recruited ferret CCR6+ responder cells above medium alone. It is possible that the concentrations of CCL20 protein in the other LEC culture supernatants were at or higher than 1 μM, which is a concentration above which a responder cell is unable to distinguish a concentration gradient from the front and the back of the cell (Figure 10B).



TLR	Ligand
M	Mock
1/2	Pam3CSK
2	HKLM
3	Poly I:C (HMW)
3	Poly I:C (LMW)
4	LPS
5	Flagellin
6/2	FSL-1
7	Imiquimod
8	ssRNA40
9	ODN2006

Figure 9. Ferret lung LEC responses to different TLR ligands.

Ferret lung LECs (Ferret 2, left lobe lung LEC) were grown until 80 – 90% confluent and exposed to the indicated TLR ligands for 24 hr. The levels of the indicated mRNA targets were measured by real-time RT-PCR as presented in Figure 7. The dashed line notes the level of expression of each mRNA in untreated cells. The dark grey bar denotes the basal expression of the target gene without any stimulation (mock). The light grey bar denotes the poly I:C responses as a frame of reference for comparison to Figure 8.

Table 4. Spearman's correlation analyses of mRNA expression levels in TLR ligand treated ferret LECs.

	CCL5	**CCL20**	**CXCL10**	**IFN-α**	**IL-6**	**IL-10**	**Mx1**	**TNF-α**
CCL5	ID[a]	**0.95[b] (<0.0001)**	**0.76 (0.009)**	0.46 (0.155)	**0.93 (0.0001)**	0.36 (0.286)	0.56 (0.076)	0.37 (0.261)
CCL20		ID	**0.75 (0.011)**	0.37 (0.261)	**0.89 (0.0005)**	0.30 (0.371)	0.53 (0.100)	0.25 (0.468)
CXCL10			ID	0.12 (0.735)	**0.85 (0.0018)**	0.06 (0.860)	0.56 (0.082)	0.46 (0.173)
IFN-α				ID	0.41 (0.2141)	0.21 (0.539)	0.45 (0.173)	0.51 (0.114)
IL-6					ID	0.17 (0.615)	**0.67 (0.028)**	0.58 (0.066)
IL-10						ID	0.41 (0.214)	-0.33 (0.327)
Mx1							ID	0.56 (0.076)
TNF-α								ID

[a]ID, identical with r = 1.00.
[b]Shown are the r-values with the p-values presented in parentheses.

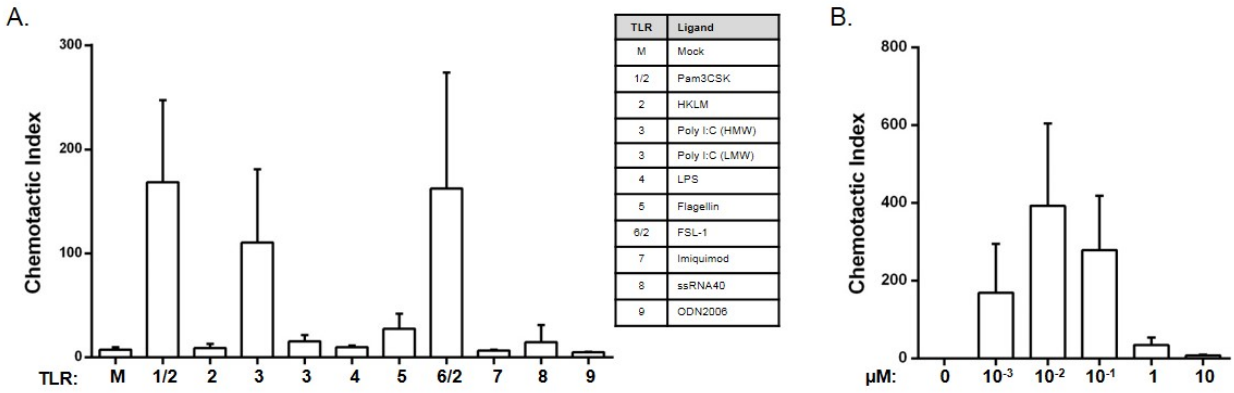


Figure 10. Chemotactic responses of ferret CCR6+ responder cells to LEC culture supernatants after TLR ligand stimulation.

(A). Chemotaxis was performed using responder cells expressing ferret CCR6 and supernatants from control and TLR ligand treated ferret lung LECs. Chemotactic index (CI) was calculated relative to the medium only control (M). (B). Chemically synthesized ferret CCL20(261) was used as a control (right). The data presented were combined from two independent experiments.

Distribution of LEC markers in ferret tissues

Given the essential role of the LE for transport of soluble antigens and immune cells for host responses and immune surveillance, we examined the distribution of the LV in multiple ferret tissues by using in situ hybridization (ISH). To do this, we generated ^{35}S-radiolabeled riboprobe for detection of ferret LE-associated mRNAs in ferret LN, lung, spleen, and jejunal tissues. These attempts to map the LV in ferret tissues using podoplanin or VEGFR-3 specific ISH probes was complicated by high background signals and low specific signals, whereas the LYVE-1 and CCL21 probes yielded signals that were reasonable and robust, respectively. In LN tissues, these ISHs revealed a distribution of LYVE-1 mRNA at the subcapsular sinus (Figure 11) with limited expression in LN parenchyma, consistent with expression by the LV. In LN, CCL21 mRNA was abundant in the cortical and paracortical regions (not shown) as we (93) and others have observed in other animals models. In jejunum, LYVE-1 (Figure 11) and CCL21 (not shown) were in the lacteals of villi, which are part of the LV immediately beneath the intestinal epithelium. We also observed networks of LV expressing high levels of CCL21 in the jejunal smooth muscle layer (not shown). In lung tissues, ferret CCL21 mRNA was expressed by widely distributed, small, thin-walled vessels in areas near (Figure 11) and distal to conducting airways. Parallel hybridization of subjacent tissue sections with a sense control riboprobe yielded no specific signal for any of the tissues examined (Figure 11). Overall these findings provide a first analysis of lymphatic structure in ferret tissues and reveal the LV to be widely distributed in ferret lung.

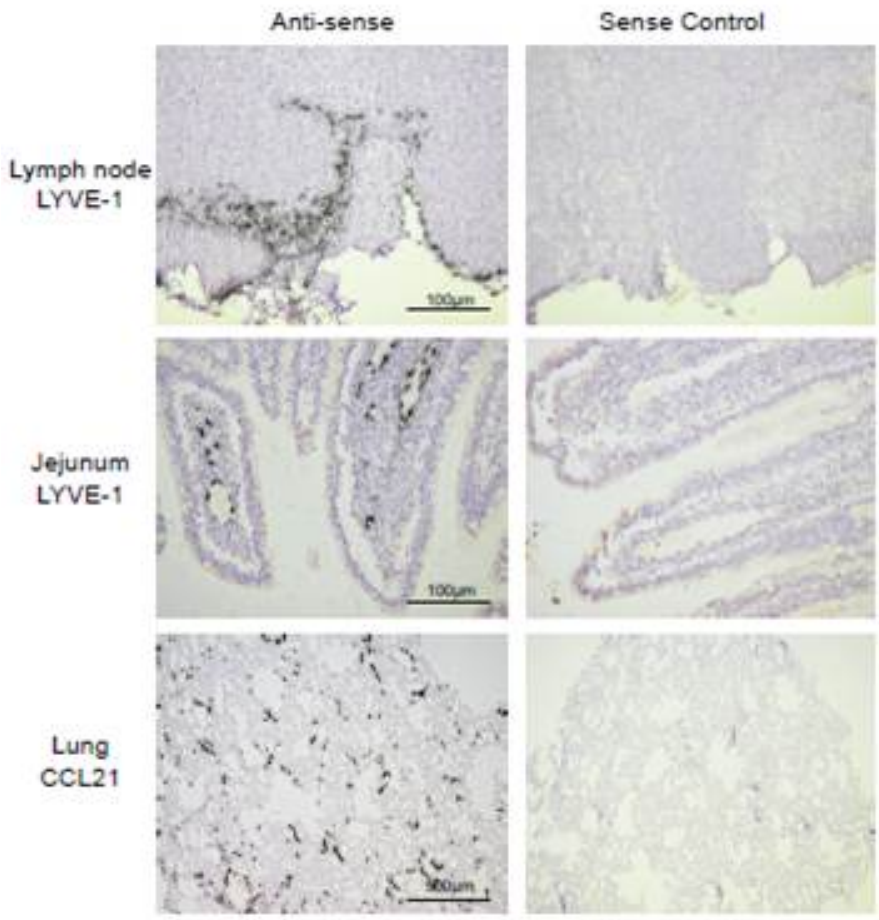


Figure 11. In situ hybridization localization of ferret CCL21 mRNA in ferret lymph node, lung, spleen, and jejunal tissues.

In situ hybridization localization of lymphatic marker mRNAs in ferret ferret lymph node, jejunal and lung tissues. ^{35}S-UTP-labeled riboprobes specific for ferret LYVE-1 or CCL21 were generated and used localize cells expressing the respective mRNAs in the indicated tissue from normal ferrets. ISH signal is visualized by collections black silver grains over cells. Parallel ISHs were performed with the cognate sense control probe (micrographs on the right). Exposure times were 21d.

3.6 DISCUSSION

The LV system is a critically important vascular system that drains interstitial fluids, particulates, and cells from peripheral tissues, disruption of which leads to lymphedema. Despite serving this important function, the LECs that serve as the major constituents of lymphatic vessels are understudied in comparison to their counterparts, blood endothelial cells (BECs), in blood vessels. Identification of LEC markers has facilitated research on LECs and helped in distinguishing them from BEC. The findings presented here describe the first isolation, culture, and functional characterization of ferret LECs, paving the way for studying LEC contributions to health and disease processes in ferret models.

An approach used for isolation and characterization of primary LECs from humans, mice, or rats involves mechanical and enzymatic dissociation of tissues followed by enrichment using LEC marker-specific antibodies (51, 109, 126, 127). Due to lack of ferret-specific or cross-reactive LEC marker-specific antibodies we were unable to enrich primary ferret LECs. Not surprisingly, though, immunosorting of LECs using marker-specific antibodies can contribute to differences observed in the molecular and functional profiles of isolated LECs (123), which could be expected to be heterogeneous in different anatomic and microanatomic compartments. Therefore, it will be important to keep in mind this potential for bias towards enrichment of subpopulations of LECs expressing moderate to high levels of surface antigens (123). On the other hand, cell types other than LECs can express the markers used for enrichment (267), such as with LYVE-1, which is expressed by heterogeneous populations of cells, including subpopulations expressing CD68, CD206, or CD208 (111), which in turn could lead to inclusion

of non-LECs in the immune-enriched populations obtained and studied. A recent study showed that LECs obtained by digesting pooled brachial, axillary, inguinal, and popliteal LNs from wild-type C57BL/6J mouse and simple bulk culture in the common DMEM medium, yielded LECs that preserved their LEC characteristics over at least 20 passages (130).

The LE is actively involved in the regulation of cellular traffic from peripheral tissues to draining LNs, which is important for the initiation and maintenance of antigen-specific immune responses during infection and vaccination. Primary human LECs express functional TLRs that sense PAMPs and secrete proinflammatory cytokines and chemokines at peripheral sites for recruitment of APCs to and through the LV (34, 247). However, little is known about primary LECs from other species commonly used as animal models for human diseases. The ferret is an attractive small animal model to study human respiratory viruses, especially influenza virus, due to the unique physiologic and anatomic similarities of its respiratory tract relative to humans (178, 182-184). The ferret model has been utilized as an animal model to study seasonal and avian influenza virus pathogenesis, as well for vaccine and therapeutic development and studies of viral transmission (185, 186, 258, 268). We describe here for the first time the isolation of primary ferret LECs and their responses to TLR ligands. A comprehensive analysis of ferret lung LEC responses to TLR1-9 ligands revealed that they respond to a large repertoire of TLR ligands. This ability to recognize and respond to a diverse set of microbes is consistent with our previous findings with commercially obtained human LECs (247). This information will be useful in extrapolating information on LEC-involved host responses to infection and mechanisms of vaccine-mediated protection in ferret animal models to humans.

Due to its primary function in gas exchange the lung is widely exposed to the external environment and to diverse microorganisms. The lung, however, has evolved a number of mechanical and physiological barriers that inhibit colonization by pathogenic microbes (269).

There is a necessarily delicate balance in the lung among the different host immune responses, including inflammation (270). Initiation of host immune responses against invading pathogens in the lung mucosal surface requires recognition of PAMPs and activation of complement and other innate and then adaptive immune responses. At the same time, exacerbation of these responses through overproduction of proinflammatory cytokines and chemokines could result in severe immunopathological damage to the lung. Recent studies have provided evidence for the direct involvement of the lymphatics in both the induction and the resolution of inflammation (55, 271, 272). Such findings can provide assistance in the development of strategies to modulate innate immune responses to limit inflammation and lung injury during infection. Chemokines are important mediators of host homeostatic processes and also of host immune responses to pathogens. However, chemokines, such as CCL20, can contribute to the pathology of a number of chronic and severe inflammatory pulmonary diseases (273, 274). We have shown here that TLR stimulation of ferret lung LECs led to high levels of production of functional CCL20. The CCL20 and overall patterns of responsiveness to TLR ligands were similar to that of human pulmonary LECs (247). Given that the receptor for CCL20, CCR6, is expressed on immature DCs and IL-17-producing Th17 cells (275), LECs could play fundamental direct and indirect roles in immune surveillance and mucosal homeostasis.

In summary, our findings represent the first isolation and functional characterization of LECs isolated from ferret, an increasingly commonly used animal model for human diseases. In addition, these cells and supporting cDNA resources provide a foundation for deeper studies of lymphatic structure and function in ferrets. Our data also report for the first time a comprehensive analysis of the TLR ligand responsiveness of ferret lung LECs. These observations provide further evidence that the LV is not simply a passive conduit, but that LECs

have more active roles in induction of host innate immune responses and in regulation of inflammation after infection. In sum, the LV presents ongoing opportunities for development of improved vaccine, infection control, and inflammation control strategies.

3.7 ACKNOWLEDGEMENTS

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4.0 FUNCTIONAL ANALYSIS OF MACAQUE LYMPHATIC ENDOTHELIAL CELLS: GENE EXPRESSION PROFILES AND INFECTIVITY STUDIES WITH SIV

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This chapter is comprised of the studies on the isolation and establishment of primary rhesus macaque LEC populations ex vivo, as well as assessment of their phenotypic gene expression profiles and functional properties response to known PRR ligands including exposure to SIV. All of the experiments generating and analyzing macaque LECs were performed by Stella Joan Berendam.

(Manuscript in preparation)

4.1 PREFACE

I extended the effort to obtain and establish primary LEC cultures from another commonly used animal model for human diseases, namely the rhesus macaque. In addition to characterization and evaluation of the primary macaque LECs at the mRNA and protein levels, I was able to determine the functionality of these LECs by measuring their ability to respond to known PRR ligands and assessing their susceptibility and permissiveness to infection by genetically engineered SIV and HIV-1 viruses as well as wild-type SIV. Studies in this chapter were also designed to dissect the potential innate immune contribution of LECs during SIV, HIV-1, or other infections by (1) sensing of the viral pathogens through multiple functional PRRs, (2) creating local proinflammatory microenvironments through secretion of cytokines/chemokines, and (3) blocking of early stages of viral infection and inter-species transmission through endogenous expression of intrinsic viral restriction factors. Studies were performed to partially compare the ability of LECs to take up and process antigens, an innate immune hallmark of DCs. These comparisons corroborated the potential innate immune function of LECs during pathogen-host interactions.

4.2 ABSTRACT

Despite their known involvement in controlling immune cell trafficking, the role of LECs during pathogen-host interactions is understudied. Due to the location of the initial lymphatic vessels, often closely beneath the mucosal epithelium, LECs are likely to come in contact with pathogens that translocate across the epithelium, including SIV/HIV-1. We hypothesized that LECs are involved in host innate immune responses via initial sensing of invading microbes (including SIV/HIV-1) through expression of functional pattern recognition receptors (PRRs) including toll-like receptors (TLRs). In this role, LECs could potentially influence the transmission and establishment of infection. To address this in a nonhuman primate model, we generated primary rhesus macaque LEC populations from multiple animals and tissue types and assessed their ability to respond to known PRR ligands. We also examined the susceptibility of these macaque LECs to SIV entry and infection. Comprehensive phenotypic characterization revealed that macaque LECs express known SIV/HIV-1 viral entry and viral restriction factors to differing extents and were resistant to infection with engineered single-cycle competent or wild-type SIV/HIV-1. They also responded to treatment with poly I:C alone or with different PAMPs to varying degrees and were capable of performing antigen capture and processing under in vitro environments that mimic physiological conditions. In summary, we have established primary macaque LEC populations that will serve as new tools to study the immunobiology of LECs during pathogen-host interactions in the context microbial transmission, pathogenesis, and vaccination.

4.3 INTRODUCTION

The human body has two major vasculature systems, namely the blood and the lymphatic vasculature. Although these two systems share many overlapping functional, structural, and physiological similarities, the lymphatic vasculature (LV) has been slightly overlooked and understudied compared to its counterpart, the blood vasculature (BV). Lymphatic endothelial cells (LECs) are major constituents of the LV. LECs contribute to immunological functions of LV by regulating the migration of immune cells such as dendritic cells (DCs) and leukocytes into LV and their transport from the peripheral tissues to the draining lymph nodes (LNs) through secretion of chemokines (29-32, 91, 276, 277) and expression of adhesion molecules (33, 89, 148, 250, 278-280). In vitro studies using primary human LECs showed that they expressed multiple functional toll-like receptors (TLRs) (281, 282) that responded to pathogen-associated molecular patterns (PAMPs) by production of proinflammatory cytokines/chemokines for recruitment of immune cells to sites of inflammation. Despite their known involvement in immunomodulation of host immune responses, LECs' contributions and involvement during pathogen-host interactions are not yet well-characterized.

The macaque model is the most commonly used nonhuman primate model in the biomedical research of human diseases. Since the discovery of SIV, the rhesus macaque model has become an indispensable animal model to study disease pathogenesis as well as disease progression of HIV-1 infection to AIDS as SIV infection in macaques resembles closely the HIV-1 infection in humans (193, 283). Rhesus macaques share more than 90% similarity to humans at the genome level (284) and thus share many orthologous genes that are responsible

for immune response to infection by HIV-1 (193, 284), making them highly relevant models to study human diseases including virus-host interactions and host immune responses. Rhesus macaques are considered the "gold standard" animal model for development and validation of therapeutics and vaccines for HIV-1 (202, 240). The rhesus macaque model has been used in evaluation of antiviral therapeutic drugs against HIV-1 (202) and testing of TLR agonists as adjuvants for improved HIV-1 vaccine formulations to induce better innate as well as adaptive host responses (231-233, 254, 285, 286). Functional TLR expression by LECs makes LEC an attractive target for development of TLR ligands as adjuvants in vaccines. Therefore, it is important to understand the involvement of LECs in host innate immune response during infection/vaccination as this could benefit the development of future vaccines and therapies.

The study of LECs is hampered due to the challenge of studying these cells in vivo and also the difficulties in obtaining pure LECs to study in cultures ex vivo. However, recent identification of gene markers to distinguish LECs from BECs has contributed to the advancement of LEC research (42, 43, 50, 52, 53). In this study, we adapted a previously published protocol for human LEC (108) isolation and established primary rhesus macaque LEC populations from multiple animals and tissue types. We proposed that LECs are involved in initiation of host innate immune response via sensing of invading microbes (including SIV/HIV-1) through expression of functional PRRs including TLRs and thus, could potentially influence the initial establishment and/or transmission of these pathogens. To address this, we derived multiple macaque LEC populations, characterized their mRNA expression profiles, and measured their responsiveness to TLR ligands and exposure to SIV.

4.4 MATERIALS AND METHODS

Animals and tissues

These studies were performed under the approval and guidance of the University of Pittsburgh Institutional Animal Care and Use Committee.

Isolation and culture of primary macaque LECs

LEC isolation was performed as described (108) and then cultured and passaged as describe (248). All primary macaque LEC populations used in this study were between passages 3 to 9.

Cell culture

293 cells and Hela cells were culture in using complete DMEM (Life Technologies, Rockville, MA, USA) supplemented with 10% FBS (Hyclone™, Thermo Scientific), L-glutamine (Life Technologies, Rockville, MA, USA), and penicillin-streptomycin (Life Technologies, Rockville, MA, USA) at 37°C, 5% CO_2. CEMx174 cells were cultured using RPMI medium (Life Technologies, Rockville, MA, USA) supplemented with 10% FBS (Hyclone™, Thermo Scientific), L-glutamine (Life Technologies, Rockville, MA, USA), and penicillin-streptomycin (Life Technologies, Rockville, MA, USA) at 37°C, 5% CO_2. All cell lines were plated at the same cell density and treated in similar manner as the macaque LECs.

Real-time RT-PCR characterization of macaque LECs

Total cellular RNA extraction was carried out using Trizol (Life Technologies, Rockville, MA, USA) followed by column purification and RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. cDNA synthesis was then performed as described (266). Detection of mRNAs of target genes of interest were performed using purchased Taqman real-time RT-PCR assays (Applied Biosystems) whenever available or in-house design primers using SYBR Green-based real-time RT-PCR assays (Table 5). Gene clustering analysis was performed using the R software for statistical computing. Detection of SIV and HIV-1 *gag* DNA by real-time PCR were performed as described (216).

Flow cytometry

Confluent monolayer of macaque LECs was detached by enzymatic reaction using Accutase® (Sigma-Aldrich, St. Louis, MO, USA). Detached cells were treated with a final concentration of 0.1 mg/ml of DNAse I (Roche, USA) for 30 minutes at 37°C, followed by live-dead exclusion with LIVE/DEAD® Fixable Aqua Dead Stain Kit (Molecular Probes, Eugene, OR, USA) for 20 minutes at RT each according to the manufacturers' recommendations before staining with Abs in ice-cold FC Receptor Blocker (Innovex, Biosciences, USA) staining buffer for 45 minutes at 4°C with anti-human podoplanin-PE (AngioBio, Del Mar, CA, cat. No. 11009-PE) and anti-human LYVE-1-APC (R&D Systems, USA, cat. no. FAB20892A) antibodies. After staining, cells were washed twice with 1XPBS and fixed in a final concentration of 0.25% paraformaldehyde (PFA) before data acquisition using BD LSRII Fortessa and analyzed using FlowJo software (Tree Star, Ashland, OR). Data acquisition was compensated using BD Compensation Beads (BD Biosciences, San Diego, CA). Unstained cells, cells stained with

isotyped- and concentration-matched Abs controls, and “fluorescent minus one” controls were used to allow appropriate acquisition parameters to be established, and to aid proper gating during data analysis.

Stimulation with PRR ligands

Primary macaque LECs were grown to approximately 80-90% confluency and were exposed to TLR1-9 ligands (Human TLR1-9 Agonists Kit, InvivoGen, San Diego, CA, USA, cat. no. tlrl-kit1hw), NLR ligand (MurNAc-L-Ala-γ-D-Glu-mDAP (M-TriDAP), InvivoGen, San Diego, CA, USA, cat. no. tlrl-mtd), CLR ligand (Zymosan, InvivoGen, San Diego, CA, USA, cat. no. tlrl-zyn), and CDS ligand (poly(deoxyadenylic-deoxythymidylic) (Poly(dA:dT)), InvivoGen, San Diego, CA, USA, cat. no. tlrl-patn). The final concentration for each ligand was as follows: Pam3CSK4 (1 µg/ml) (TLR1/2 ligand); heat killed *Listeria monocytogenes* (HKLM, 10^8 cells/ml) (TLR2 ligand); high molecular weight poly(I:C) (25 µg/ml) (TLR3 ligand); low molecular weight poly(I:C) (25 µg/ml) (TLR3 ligand); *Escherichia coli* K12 lipopolysaccharide (LPS, 100 ng/ml) (TLR4 ligand); *Salmonella typhimurium* flagellin (1 µg/ml) (TLR5 ligand); FSL1 (1 µg/ml) (TLR6/2 ligand); imiquimod (2.5 µg/ml) (TLR7ligand); ssRNA40 (2.5 µg/ml) (TLR8 ligand); ODN2006 (10 µg/ml) (TLR9 ligand); MTri-DAP (10 µg/ml) (NOD1/NOD2 ligand); Zymosan (100 µg/ml) (Dectin-1 ligand), and poly(dA:T) (5 µg/ml) (AIM2 inflammasome inducer).

NanoString mRNA expression analysis

NanoString nCounter analysis was performed using custom-synthesized CodeSet probes (N = 223) for Type I Interferon stimulated genes and housekeeping genes (287). Cells were treated as

described above to obtain total RNA, and a total of 1 µg of primary macaque jejunal LECs RNAs (equal mass volume), primary human dermal LECs (Clonetics™, Lonza), each with or without poly I:C treatment were pooled was directly hybridized to the custom designed gene expression CodeSet and analyzed on an nCounter Digital Analyzer (Nanostring Technologies). Counts were normalized to housekeeping genes. Gene clustering analysis was performed using the R software for statistical computing of NanoString data.

Antigen uptake and processing

Final concentrations of 10 µg/ml BODIPY-conjugated DQ-BSA, 10 µg/ml and 5 µg/ml BODIPY-conjugated DQ-Ovalbumin (DQ-OVA) (Molecular Probes, Life Technologies, USA) in complete EGM2 media (as described above) were added to a confluent monolayer of macaque LECs grown and incubated at 37°C or 4°C for 1 hr. After incubation, cells were washed with 1XPBS to remove unbound DQ-BSA and DQ-OVA molecules and detached from plate using Accutase® (Sigma-Aldrich, St. Louis, MO, USA). Detached cells were treated with a final concentration of 0.1 mg/ml of DNAse I (Roche, USA) for 30 minutes at 37°C, followed by live-dead exclusion with LIVE/DEAD® Fixable Aqua Dead Stain Kit (Molecular Probes, Eugene, OR, USA) for 20 minutes at RT each according to the manufacturers' recommendations, washed once in 1XPBS before fixing with final concentration of 0.25% PFA prior to analysis using flow cytometry.

Infectivity study using single-cycle competent VSV-G pseudotyped SIV/HIV-1 and wild-type SIVmac239

Single-cycle EGFP expressing VSV-G pseudotyped SIV and luciferase expressing VSV-G pseudotyped HIV-1 viruses stocks were made as described (288, 289). For the infectivity study using the VSV-G pseudotyped viruses, macaque LECs and Hela cells (control population) were seeded at the initial density of 10,000cells/cm^2 (20,000 cells per well) in a collagen coated 24-well plate and cultured as described above until 80 – 90% confluency. Viruses were added onto cultured cells at m.o.i of 0.5, with or without inhibitor (FTC, final concentration of 25 μM), and left in the culture at 37°C, 5% CO_2 for 4 hours before washing twice with 1X PBS followed by addition of fresh growth media. Upon harvest, cell pellets were analyzed by flow cytometry (eGFP-expressing virus), luminescence (luciferase activity), and real-time PCR for SIV or HIV-1 viral *gag* DNA.

For infectivity study using the WT SIVmac239, macaque LECs and CEMx174 cells (control population) were grown to 40 – 50% confluency. WT SIVmac239 virus was then added onto cultured cells at m.o.i of 0.5 and left in the culture at 37°C, 5% CO_2 for 4 hours before washing twice with 1X PBS followed by addition of fresh growth media, and further incubated at 37°C, 5% CO_2. CEMx174 cells were spun down and washed twice also with 1XPBS, before addition of fresh growth media, followed by further incubation at 37°C, 5% CO_2. Culture media were replenished by replacing half of the culture media with fresh media at day 3, 7, and 10. Supernatants from virus exposed cultures were collected at day 1, 3, 7, 10 and 14 post exposures and analyzed by real-time RT-PCR for SIV *gag* RNA as described (266). Cell pellets were also collected at day 14 by trypsinization and subjected to real-time PCR for SIV *gag* DNA as described above.

Statistical analysis

Statistical analysis was performed using paired Student t-test with Prism software (GraphPad, San Diego, CA). Results are shown as mean ± SD, and *p values* of <0.05 was considered significant.

Table 5. List of purchased Taqman assays and in-house designed primer sequences for real time RT-PCR.

Gene	Species Reactivity	Catalog number/Primers sequences
LEC Markers		
Podoplanin	Human	Hs00366766_m1
Podoplanin	Rhesus (in house designed)	F:5'-GTGACTGGTTGCCACATTTGA-3' R:5'-TCCACGCCCAAGGACAA-3'
LYVE-1	Human	Hs00272659_m1
LYVE-1	Rhesus	Rh01119302_m1
Prox-1	Human	Hs00160463_m1
Prox-1	Rhesus	Rh00896293_m1
VEGFR-3	Human	Hs00176607_m1
VEGFR-3	Rhesus	Rh02742716_m1
CCL21	Human/Rhesus	Hs00171125_m1
CD31	Human/Rhesus	Hs01065279_m1
Stabilin-1	Human/Rhesus	Hs01109068_m1
COLEC-12	Human/Rhesus	Rh02852855_m1
Reelin	Human/Rhesus	Rh02856443_m1
BEC Markers		
NRCAM	Human/Rhesus	Rh01031595_m1
MCAM	Human/Rhesus	Hs00174838_m1
CXCL1	Human	Hs00236937_m1
CXCL1	Rhesus	Rh03456656_m1
Viral Restriction Factors		
APOBEC3G	Human	Hs00222415_m1
APOBEC3G	Rhesus	Rh02788475_m1
Tetherin (BST2)	Human/Rhesus	Hs00171632_m1
Trim5-α	Human	Hs01552559_m1
Trim5-α	Rhesus	Rh02788631_m1
SAMHD1	Human/Rhesus	Hs00210019_m1
Schlafen-11	Human/Rhesus	Rh02885088_m1

Table 5 Continued

Viral Entry Factors		
CD4	Human	Hs00181217_m1
CD4	Rhesus	F:5'-CAGCAAGGCCACAATGAACC-3'
		R:5'-GCCCAGCACCACTTTCTTTC-3'
CCR5	Human	Hs00152917_m1
CCR5	Rhesus	F:5'-TCTGGGCTCCCTACAACATTG-3'
		R:5'-GGCTTGGTCCAACCTGTTAGAG-3'
CXCR4	Human	Hs00237052_m1
CXCR4	Rhesus	F:5'-AGCGCAAGGCCCTCAAGAC-3'
		R:5'-GGAGTCGATGCTGATCCCAAT-3'
D6	Human	Hs00174299_m1
D6	Rhesus	F:5'-CAGGCAGATGACTGAGGTCTATC-3'
		R:5'-GAAGGGCAGTGTCACCAGAAAC-3'
CXCR6	Human/Rhesus	Rh03043279_s1
GPR15	Human/Rhesus	Rh02915721_s1
DC-Associated Markers		
MMR (CD206)	Human/Rhesus	Hs00267207_m1
DC-SIGN (CD209)	Human	Hs01588349_m1
DC-SIGN (CD209)	Rhesus	Rh02788032_m1
DEC-205 (CD205)	Human/Rhesus	Hs001588966_m1
Siglec-1 (CD169)	Human/Rhesus	Rh00988061_m1
CD40	Human/Rhesus	Rh02621776_m1
Other Genes		
IL-7-RA	Human/Rhesus	Rh02841231_m1
IL-7	Human/Rhesus	Rh02621732_m1
Spinster-2	Human/Rhesus	Hs01390449_g1
CD26 (DPP4)	Human/Rhesus	Hs00175210_m1
MIR-21	Human/Rhesus	Hs04231424_s1
GUSB	Human/Rhesus	Hs99999908_m1
TLR3	Human	Hs01551078_m1
RIG-I	Human	Hs00204833_m1
RIG-I	Rhesus	Rh02789252_m1
CCL5	Human/Rhesus	Hs00174575_m1
CXCL10	Human	Hs00171042_m1
CXCL10	Rhesus	F:5'-TGTCCACATGTTGAGATCATTGC-3'
		R:5'-TCTTGATGGCCTTAGATTCTGGAT-3'
CCL20	Human/Rhesus	Hs00171125_m1

4.5 RESULTS

Primary rhesus macaque LECs are heterogeneous

To study the LECs involvement in host response to pathogens, we generated a total of 22 primary macaque LECs from multiple jejunal, LN, skin, lung, and thoracic duct of 10 different rhesus macaques (Table 6). These cultures were established by plating single cells obtained from an enzymatic digestion of tissues directly onto collagen-coated surface matrix using specialized endothelial and pushed using recombinant human VEGF-C for better survival of LECs. Cultured primary macaque LECs typically formed confluent monolayers 10 – 14 days after the initial plating and were designated as passage zero (P0). The established macaque LEC monolayers showed the typical cobbled-stone morphology of endothelial cells observed in conventional 2D culture systems (Figure 12).

Table 6. Animal IDs and type of tissues for derivation of primary macaque LECs.

	Type of Tissues				
Macaque	**Jejunum**	**Mesenteric LN**	**Skin**	**Lung**	**Thoracic duct**
R74	X[a]				
R507	X	X	X	X[a]	X
R59	X	X[a]	X	X[a]	
R70	X				
R564	X	X			
R24	X	X	X	X[b]	X
R58	X[a]	X[a]	X	X[a]	X
R704	X				
R69		X[a]	X	X[b]	
R701		X			

X = Denotes one derived LEC population
[a] = Denotes LEC population that was not viable after several passages
[b] = Denotes slower growth LEC population

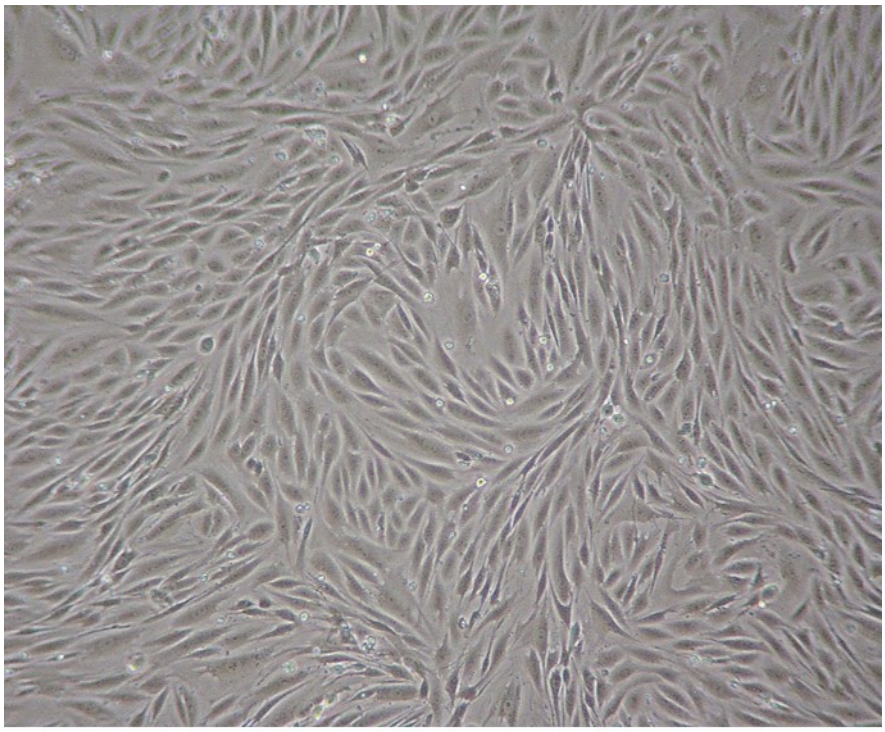


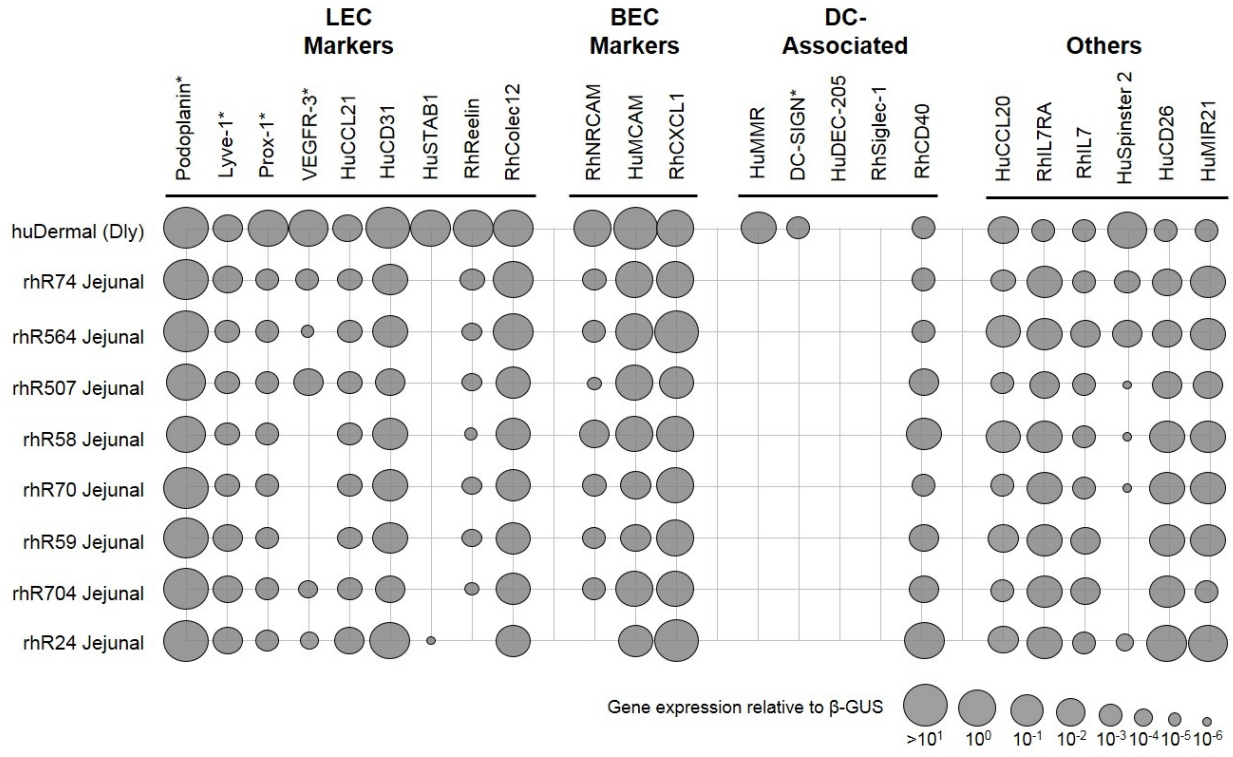
Figure 12. Primary macaque LECs show the typical cobbled-stone morphology of endothelial cells and form monolayers when cultured on collagen-coated 2D surfaces.

Cultured primary macaque LECs formed confluent monolayers approximately 10 to 14 days after initial plating onto collagen-coated plastic surfaces, these cultures being designated as passage zero (P0). The macaque LEC monolayers showed the typical cobbled-stone morphology of endothelial cells observed in conventional 2D culture systems. Showed here is one representative macaque jejunal LEC population (rhR564 Jejunal), cultured at passage 3.

To determine whether the cell populations cultured in endothelial cell growth medium with VEGF-C were LECs, we determined their expression profiles for LEC and other markers, in comparison to commercially available model human dermal LECs. We first characterized all of the macaque LEC populations using real-time RT-PCR and found that they expressed not only the pan-endothelial marker, CD31 but also most of the accepted LEC markers, including podoplanin, LYVE-1, PROX-1, and VEGFR-3 (43) and newly-assigned LEC markers COLEC12, reelin, and stabilin-1 (42, 50, 52, 53) (Fig. 13A and 13B). Podoplanin, considered to

be a marker strongly distinguishing LECs from BECs, was the most abundant LEC marker mRNA measured in macaque LECs and its expression level was comparable to that in human dermal LECs (Figure 13 A). Interestingly, there are wide variability in VEGFR-3 mRNA levels in all of the macaque LEC populations, possibly a consequence of 2D culture adaptation (290). The expression of newly identified LEC markers reelin, and stabilin-1, were mutually exclusive with only the rhR24 LECs expressing stabilin-1. It is possible such variation was due to anatomical differences in the locations of the jejunal tissues used for isolation.

(A)



(B)

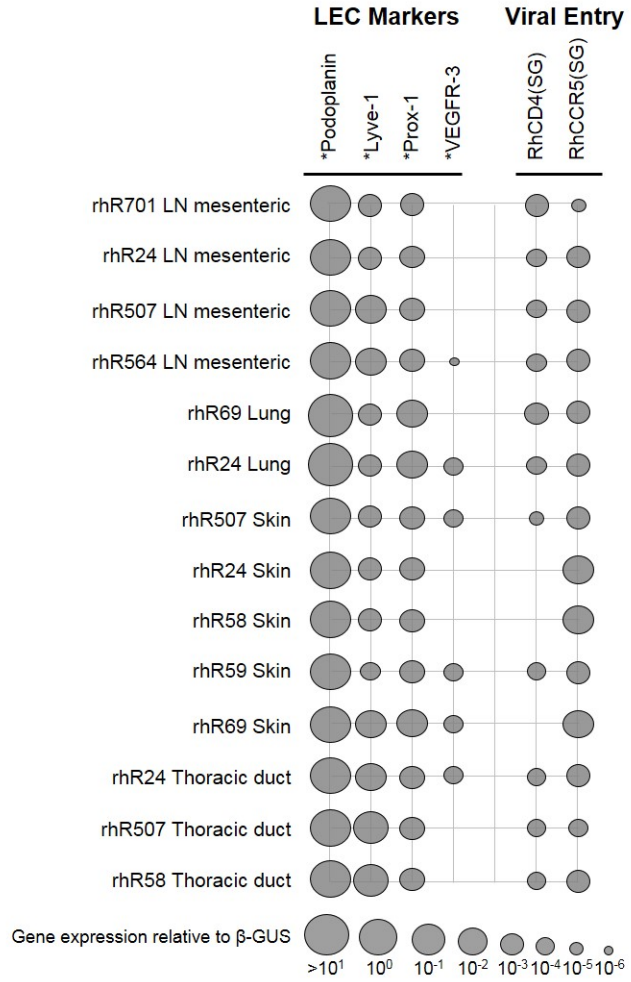


Figure 13. Primary macaque LECs are highly heterogeneous.

Comprehensive phenotypic analysis of (A) 8 macaque jejunal LEC populations and (B) 14 primary nonjejunal macaque LECs by real-time RT-PCR. Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values). * indicates species specific assays were used, Hu denotes human-specific assays, Rh denotes rhesus-specific assays, and (SG) denotes that SYBR Green based assays were used with in-house design primers.

We next examined whether these cultured macaque LECs expressed other markers known to be associated with their counterparts, the blood endothelial cells (BECs). We performed real-time RT-PCR to measure the expression levels of BEC markers and found that most of the BEC markers were expressed to relatively high levels by the macaque jejunal LECs (Figure 13A). However, these BEC markers were also expressed by the model human dermal LEC population used for comparative purposes in this study, suggesting to us that these markers are not exclusively expressed by BECs. A recent publication also showed that endothelial progenitor cells of BEC lineage (CD34+) that expressed VEGFR-3 were able to differentiate towards LECs after stimulation with VEGF-C in vitro (114).

We also investigated whether the macaque LECs expressed markers associated with DCs, which are innate immune cells that are potent APCs. Our data showed that although the human dermal LECs expressed multiple DC-associated markers (CD206, CD209, and CD40), only the expression of CD40 was observed in all of the macaque jejunal LEC populations (Figure 13A). CD40 is a known master regulator of rapid and global response of endothelial cell after activation by T cells (107). We also asked whether the macaque LECs expressed other genes that have been directly or indirectly associated with LV functions in vivo (Figure 13A). CD26 is also known as dipeptidyl peptase (DPP4) and is interesting because it has been shown to be used by virus as entry receptor and we observed abundant CD26 expression in all of the macaque jejunal LEC populations consistent with an earlier finding that CD26 in expressed strongly by LECs compared to BECs (291). CD26 was initially reported as potential coreceptor for HIV-1 virus entry (292) and more recently reported as the receptor for the Middle East respiratory syndrome coronavirus (MERS-CoV) (293). We also found that out of eight macaque jejunal LEC populations analyzed, only three of them expressed Spinster-2 mRNA. Spinster-2 is a

transporter molecule for Sphingosine-1-phosphate (S1P), a potent lipid mediator that plays an important role in lymphocytes traffic and egress from the LN in mice (56). It is possible that differences in Spinster-2 expression could be a consequence of differences in LEC function in different microanatomical compartments.

These gene expression profiles of macaque jejunal LECs were also examined by hierarchical clustering analysis (Figure 14). The resulting heat map re-analysis of the mRNA expression profiling revealed that the macaque jejunal LEC expression profiles closely resembled that of the human dermal LECs, with LEC marker podoplanin being the most abundantly expressed gene and the DC-associated markers, CD205 (DEC-205), CD169 (Siglec-1), CD209 (DC-SIGN), and CD206 (MMR) being the least abundantly expressed.

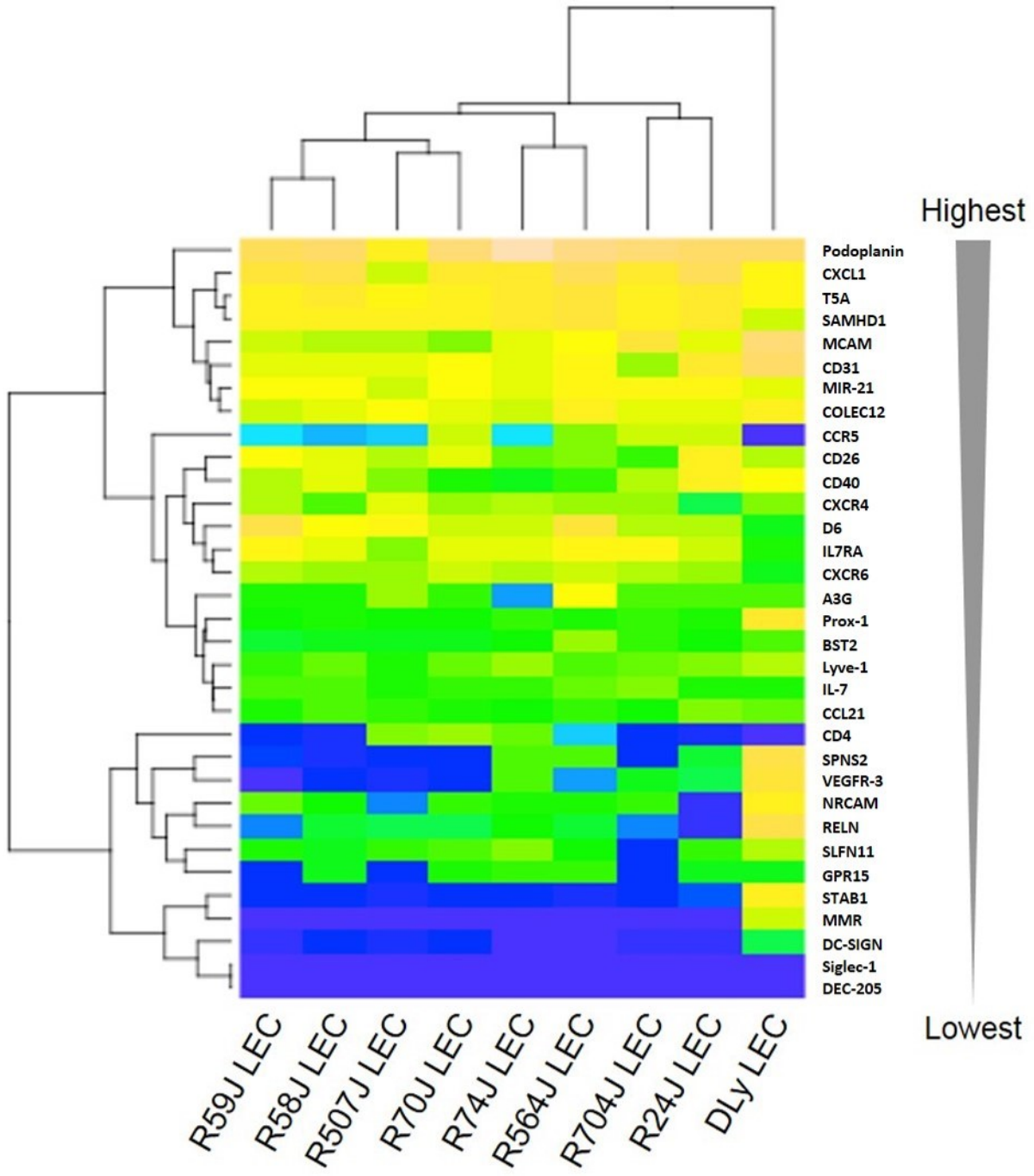


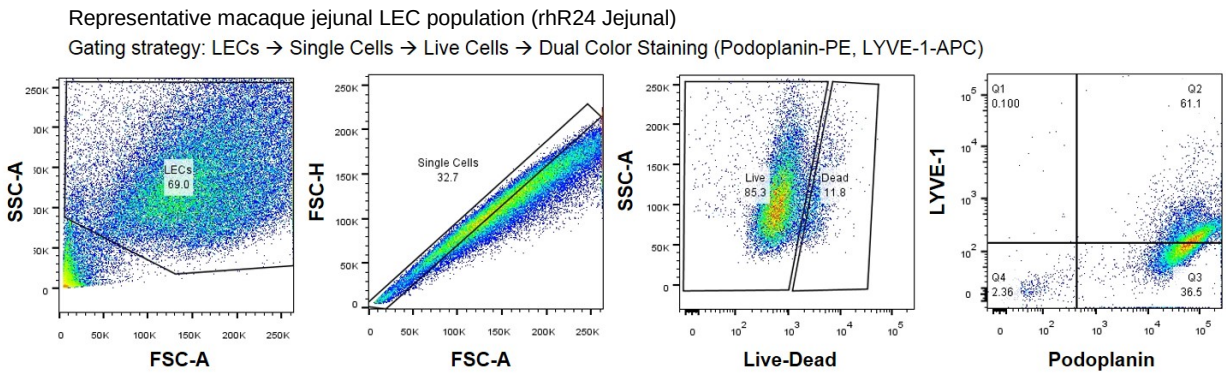
Figure 14. Macaque jejunal LECs share similar gene expression profiles with model human dermal LECs.

Heat map analysis was performed using real-time RT-PCR data (relative expression values to beta-glucoronidase, GUSB) (2-dCt values) for 33 genes endogenously expressed by the macaque jejunal LECs and model human dermal LECs. The most abundance gene expressed by both macaque and human LEC populations was podoplanin, which is a known LEC marker. CD31, which is a pan-endothelial marker was also one of the most abundant gene detected. COLEC12, which is a newly designated marker for LECs was also one the most abundant genes detected. Interestingly, both primary macaque and human LECs had low levels of expression of DC-associated markers such as, CD205 (DEC-205), CD169 (Siglec-1), CD209 (DC-SIGN), and CD206 (MMR).

Macaque jejunal LECs express LEC surface marker, podoplanin and LYVE-1

We next examined whether these macaque LECs expressed known surface markers for LECs using flow cytometry. Gating was based on flow cytometric profiles using unstained cells, cells stained with isotype- and concentration-matched control antibodies, and fluorescence minus one controls. Our data revealed that more than 90% of macaque jejunal LECs (live, singlets) expressed cell surface podoplanin, of which 19–61% co-expressed cell surface LYVE-1 (Figure 15). Altogether, these flow cytometric data and the mRNA expression profiling data support the interpretation that the macaque populations isolated by culture in endothelial growth medium with VEGF-C are indeed LECs.

The expression profiles of these macaque jejunal LECs were compared to a macaque lymph node LEC culture that was established after live cell sorting based on surface expression of podoplanin and LYVE-1. Macaque LN LECs derived by live cell sorting for podoplanin and LYVE-1 expression had remarkably a similar expression profile compared to unsorted, VEGF-C treated jejunal LECs at mRNA and protein levels (Figure 16). These data indicated that the culture conditions used to obtain LECs from jejunum (108) did not grossly effect the expression of the LEC markers examined.



Primary LECs	% Podoplanin +ve	% LYVE-1 +ve
rhR24 Jejunal	97.6	61.1
rhR564 Jejunal	98.6	21
rhR704 Jejunal	97.2	39.8
rhR70 Jejunal	99.3	34.2
rhR59 Jejunal	98	19.5
huLung (Lly)	98	68

Figure 15. Macaque jejunal LECs express the LEC surface markers podoplanin and LYVE-1.

Dual color flow cytometry staining was performed using PE-conjugated anti-human podoplanin and APC-conjugated anti-human LYVE-1 antibodies for five macaque jejunal LEC populations and one model human LEC population (Lly). Shown here is a representative figure for one population (rhR24 Jejunal) by FlowJo software (Tree Star, Ashland, OR). Gating strategy was first determined based on the forward and side scatter, followed by single cells analysis (at 45 degree angle, slope of 1), and then live-dead selection staining before dual color flow staining using PE-conjugated anti-human podoplanin and APC-conjugated anti-human LYVE-1 antibodies. Unstained cells, cells stained with isotyped- and concentration-matched Abs controls, and "fluorescent minus one" controls were used to allow appropriate acquisition parameters to be established, and to aid proper gating during data analysis. Data acquisition was compensated using BD Compensation Beads (BD Biosciences, San Diego, CA). The table showed the summary of five primary macaque jejunal LECs and one primary human lung LECs with the percentage of cells expressing the LEC surface marker, podoplanin, and the percentage of cells coexpressing the surface marker, LYVE-1.

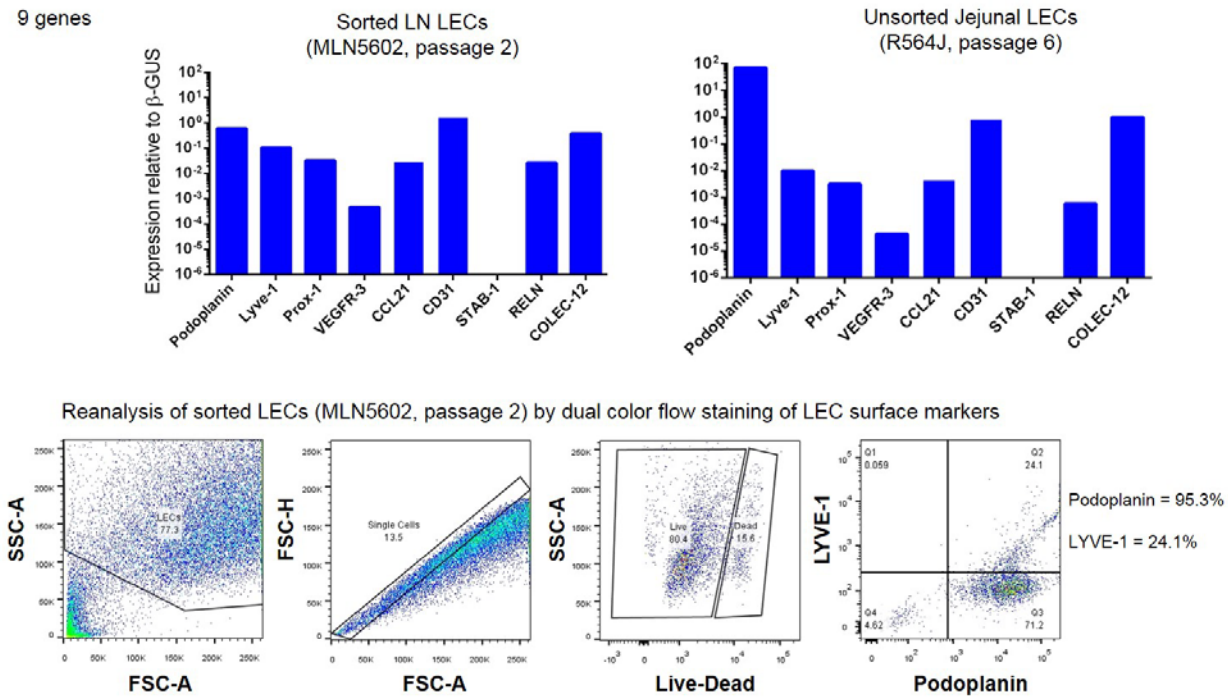


Figure 16. Primary macaque LECs sorted with antibodies against LEC surface markers (anti-human podoplanin and anti-human LYVE-1) share similar profiles as the unsorted primary macaque LEC when cultured in collagen-coated 2D surface.

One macaque LEC population was established by prior sorting from a LN tissue using PE-conjugated anti-human podoplanin and APC-conjugated anti-LYVE-1 antibodies showed similar mRNA expression profiles to the unsorted primary macaque jejunal LECs when culture at low passage number in collagen-coated 2D surfaces. Reanalysis of the sorted macaque LN LECs by dual color flow cytometry staining using PE-conjugated anti-human podoplanin and APC-conjugated anti-LYVE-1 antibodies showed similar profiles as observed earlier with the unsorted primary macaque jejunal LEC populations and the models human LECs (Figure 15), in which more than 90 percent of the LECs express podoplanin (95.3%), and a fraction coexpressed the LEC marker, LYVE-1 (24.1%).

Macaque LECs respond to poly I:C treatment

We have previously reported that primary human and ferret LECs respond to treatment with poly I:C, a known double-stranded RNA virus mimetic, by producing proinflammatory cytokines and chemokines, including CCL5, CXCL10, and CCL20 (248, 281). We asked whether the macaque LECs derived from jejunal and nonjejunal tissues were able to respond to poly I:C in the same manner as their human and ferret counterparts. To evaluate the responsiveness of the macaque LECs (N = 16) to a 24hr exposure to poly I:C, we performed real-time RT-PCR for detection of

proinflammatory chemokines, CCL5, CXCL10, and CCL20 (Fig. 17A-C). Our data showed that all of the macaque jejunal (N = 7) (Figure 17A) and nonjejunal (N = 9) (Figure 17B) LECs responded robustly to poly I:C with p values of 0.0012 and <0.0001 for CCL5, p values of 0.0065 and <0.0001 for CXCL10, and p values of 0.0412 and 0.0008 for CCL20, respectively. Interestingly, one macaque jejunal LEC population (rhR704 jejunal) and one macaque nonjejunal LEC population (rhR24 mesenteric LN) showed decreased CCL20 mRNA expression after poly I:C stimulation. These data indicated that macaque LECs were able to sense and respond to a viral RNA mimetic in similar fashion as their human and ferret counterparts.

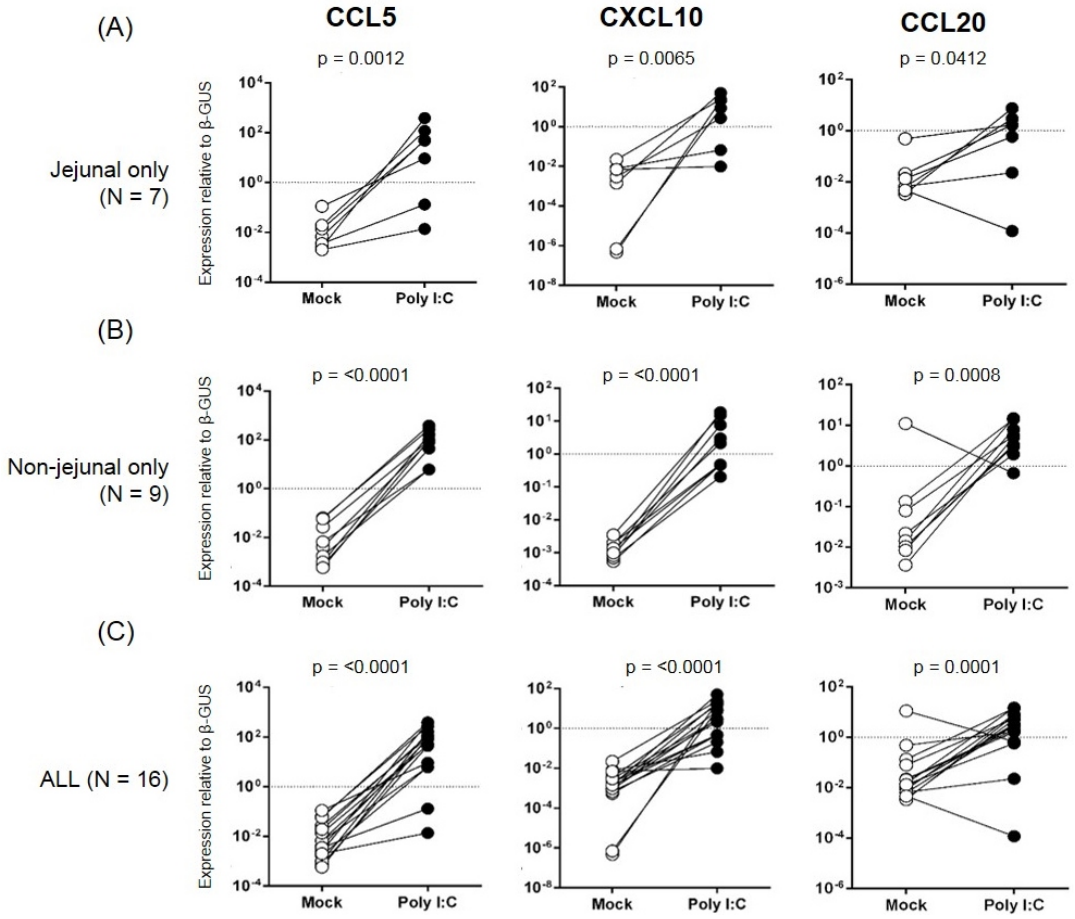


Figure 17. Poly I:C induces robust proinflammatory chemokines production by primary macaque LECs.

Confluent monolayers of primary macaque LECs were exposed to a final concentration of 25 µg/ml poly I:C for 24 hr. Response to poly I:C was measured by production of proinflammatory chemokines, CCL5, CXCL10, and

CCL20, mRNAs by real-time RT-PCR. (A) Analysis of primary macaque jejunal LECs (N = 7) showed increased production of CCL5 (p = 0.0012), CXCL10 (p = 0.0065), and CCL20 (p = 0.0412), and similarly (B) analysis of nonjejunal primary macaque LECs (N = 9) showed increase production of CCL5 (p = <0.0001), CXCL10 (p = <0.0001), and CCL20 (p = 0.0008). (C) All primary macaque LECs (N = 16) significantly induced CCL5 (p = <0.0001), CXCL10 (p = <0.0001), and CCL20 (p = 0.0001) after stimulation with poly I:C. Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values), and were transformed to log10 values.

Macaque jejunal LECs express SIV/HIV-1 viral entry receptors

We also examined whether these macaque LECs expressed known receptors and coreceptors used by SIV/HIV-1 for viral entry. We measured the expression of CD4 and CCR5 mRNAs in all of the macaque (jejunal and nonjejunal) LECs (Figure 18A and Figure 13B). Although CD4 was expressed by most of the LEC populations, there were variability in its expression. CCR5 was detected in all macaque LECs to varying degrees. We then focused on the jejunal LEC populations for expression of other known coreceptors (CXCR4, CXCR6, GPR15) and an alternative viral entry factor, and D6, which is an atypical chemokine receptor (Figure 18A). CXCR4 expression was detected in all macaque jejunal LEC populations, CXCR6 was high in all of the LEC populations, and expression of GPR15 was variable. Both CXCR6 and GPR15 have been reported to be utilized by HIV-1 virus as coreceptors albeit to low efficiency (294). D6 was highly expressed in agreement with findings in human LECs by others (55). Interestingly, the dermal human LECs did not express CD4 and CCR5 mRNAs, suggesting there are differences in their abilities to serve as targets for HIV-1 or SIV viral entry.

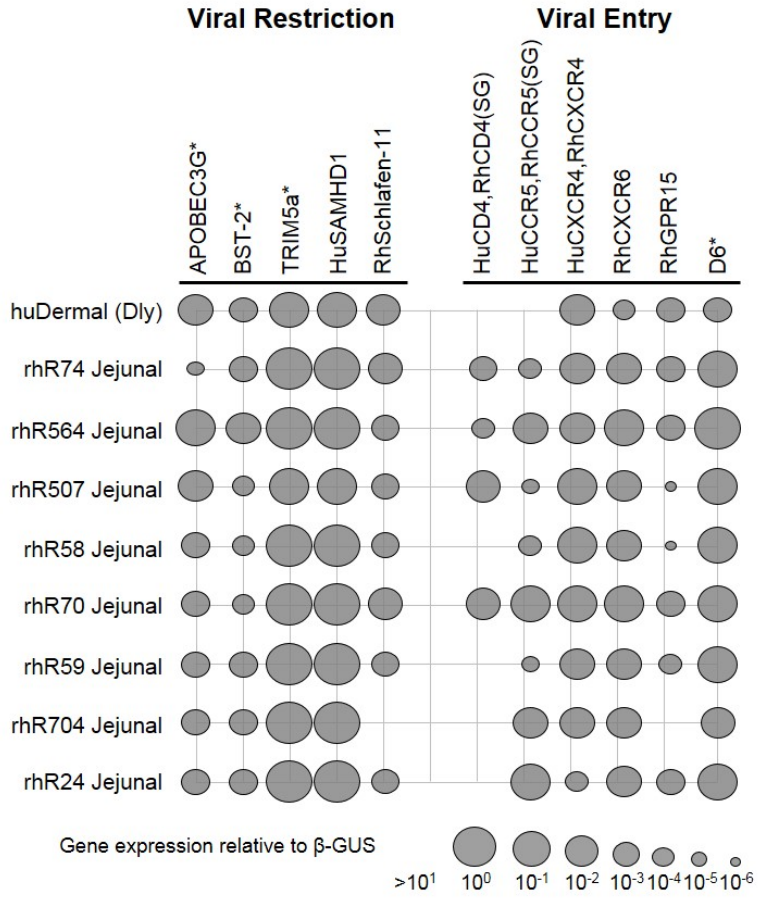
To determine the innate immune potential of LEC, we next asked whether these primary macaque LECs expressed intrinsic cellular viral restriction factors known to work against lentiviruses, such as APOBEC3G, tetherin, Trim5-α, SAMHD1, and Schlafen-11. Viral restriction factors act as frontline defenses during a viral infection as they can be constitutively expressed by host cells, are induced by IFNs, and are able to impede productive viral infection.

The expression of viral restriction factors can contribute to the permissiveness of a cell to a virus and in some cases they can contribute to limiting cross-species transmission and tropism of a virus (295, 296). Our data showed that primary human and macaque LECs constitutively express the viral restriction factors APOBEC3G, tetherin, Trim5-α, SAMHD1, and Schlafen-11, to a high level (Figure 18A).

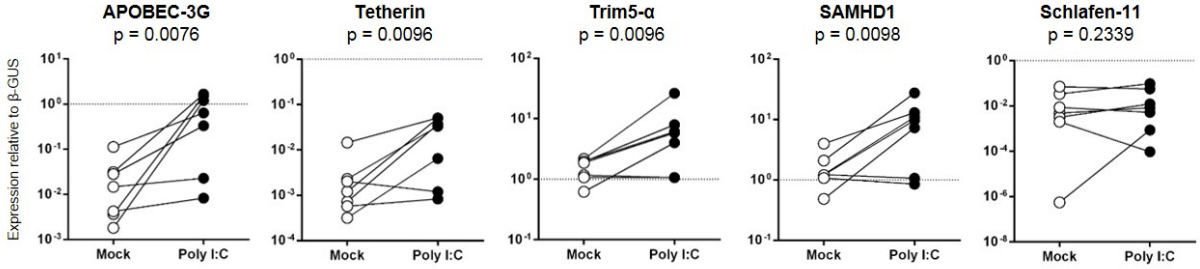
Response to poly I:C induced antiviral factors and immune receptors by rhesus macaque LECs

In addition to proinflammatory chemokines, poly I:C stimulation induced the expression of viral restriction factors and other immune receptors (Figure 18B and 18C). APOBEC3G, tetherin, Trim5-α, and SAMHD1 mRNAs were significantly increased after poly I:C treatment with p values of 0.0076, 0.0096, 0.0096, and 0.0098, respectively, but not Schlafen-11 ($p = 0.2239$) (Figure 18B). In addition, levels of mRNAs encoding TLR3 and RIG-I, both known receptors for poly I:C, were significantly upregulated upon stimulation with poly I:C suggesting that there is broad coverage in the sensing of PAMPs by both PRRs during local viral infections (Figure 18C). We also examined the effects of poly I:C exposure on the levels of known alternative virus entry factors for SIV/HIV-1 and found that the expression of D6 ($p = 0.0081$) and CD26 ($p = 0.0303$) were significantly upregulated, whereas Siglec-1 (CD169) ($p = 0.0514$) was marginally upregulated (Figure 18C).

(A)



(B) Viral Restriction Factors



(C) Immune Receptors

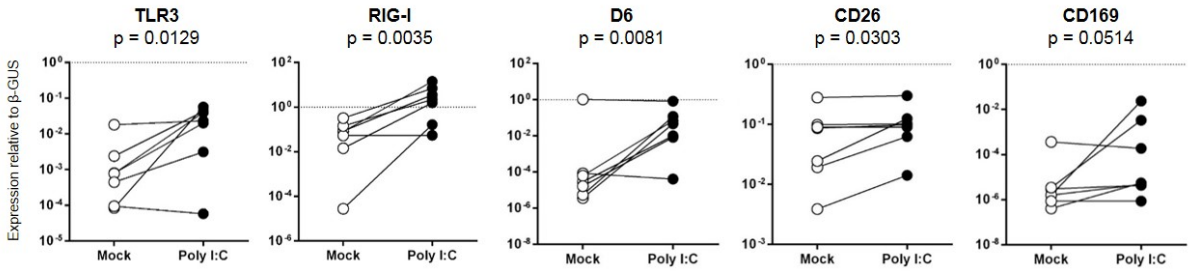
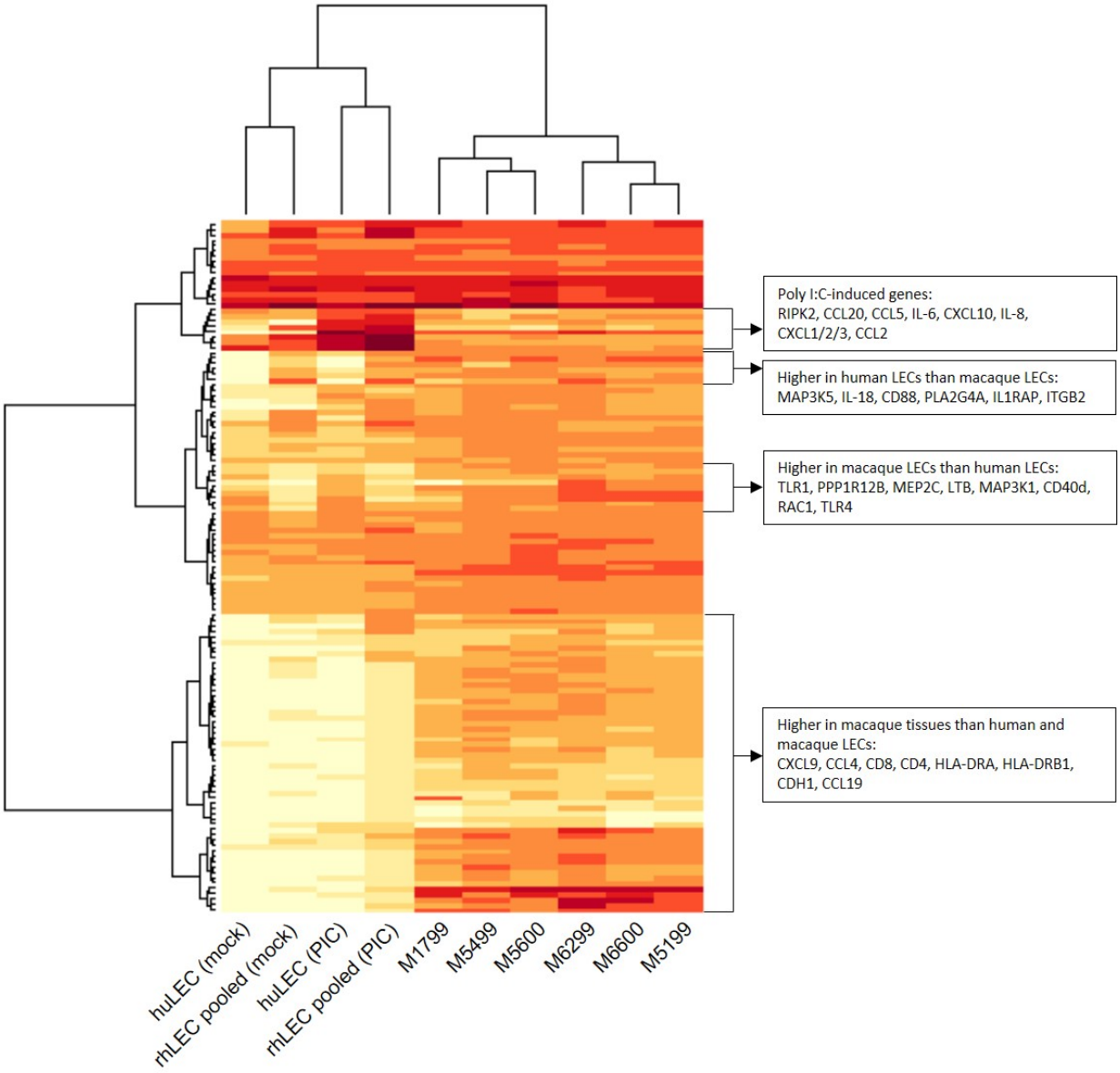


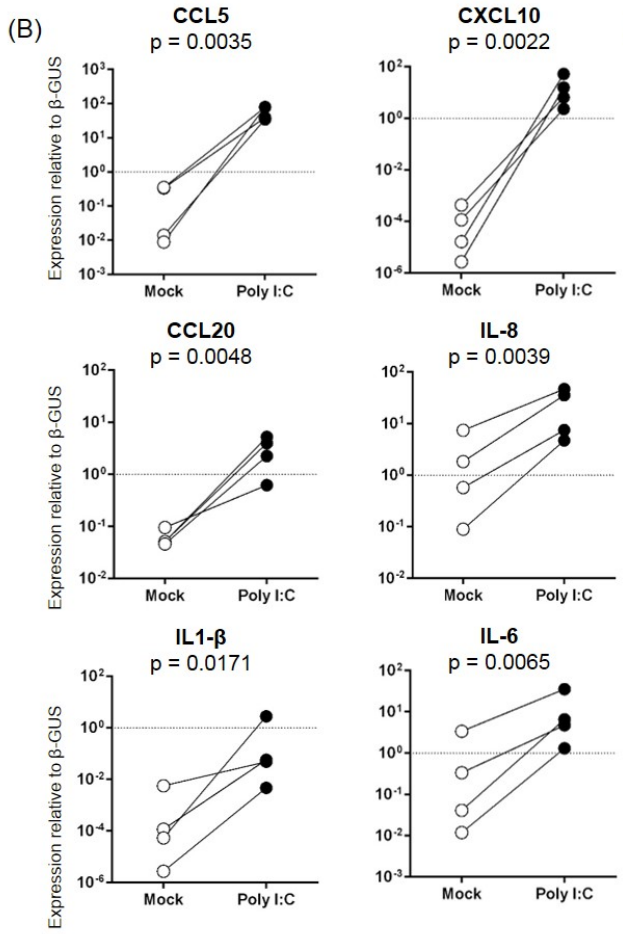
Figure 18. Primary macaque LECs express known viral restriction and entry factors for SIV and HIV-1 endogenously and their expression levels are upregulated by poly I:C treatment.

(A) Analysis by real-time RT-PCR showed that the primary macaque jejunal LECs and the model human LECs expressed multiple innate effector mechanisms that inhibit virus replication, such as APOBEC3G, BST2 (tetherin), Trim5-α, SAMHD1, and Schlafen-11, as well as known viral entry factors for SIV and HIV-1, such as CD4, CCR5, CXCR4, CXCR6, GPR15, and D6 (an atypical chemokine receptor). (B) Confluent layer of macaque LECs were exposed to a final concentration of 25 μg/ml poly I:C for 24 hr. Poly I:C treatment resulted in upregulation of viral restriction factors, APOBEC-3G ($p = 0.0076$), tetherin ($p = 0.0096$), Trim5-α ($p = 0.0093$), and SAMHD1 ($p = 0.0098$) but not Schlafen-11 ($p = 0.2339$), which in an inhibitor of viral protein synthesis. (C) Poly I:C also induced upregulation of its receptors, TLR3 ($p = 0.0129$) and RIG-I ($p = 0.0035$) and other immune receptors, D6 ($p = 0.0081$), CD26 ($p = 0.0303$), and CD169 or Siglec-1 ($p = 0.0514$). Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values), and were transformed to log10 values.

To analyze the responses of macaque jejunal LEC responses to poly I:C more comprehensively, we used custom designed probes for type I IFN upregulated genes to examine the gene expression profiles of pooled mock and poly I:C treated macaque jejunal LECs and compared them to the expression profiles of mock and poly I:C treated primary human dermal LECs by NanoString RNA profiling analysis and followed up on some target genes of interest using real-time RT-PCR (Figure 19A-C). Our data not only revealed that these two populations shared markedly similar profiles before (mock) and after poly I:C stimulation, they also shared overlapping most differentially expressed genes (13 out of 20 genes) in response to poly I:C (Figure 19C). Among the most differentially expressed genes after poly I:C treatment in both primary human dermal and macaque jejunal LECs were the proinflammatory chemokines, CCL5 ($p = 0.0035$), CXCL10 ($p = 0.0022$), CCL20 ($p = 0.0048$), as well as the proinflammatory cytokines IL-8 ($p = 0.0039$), IL1-beta ($p = 0.0171$), and IL-6 ($p = 0.0065$) (Figure 19B). We also included the analysis of RNA from uninfected and infected rhesus macaque tissues (ileum), and found that their expression profiles were similar to each other but not to the primary LECs (data not shown).

(A)


Poly I:C-induced genes:
RIPK2, CCL20, CCL5, IL-6, CXCL10, IL-8,
CXCL1/2/3, CCL2
Higher in human LECs than macaque LECs:
MAP3K5, IL-18, CD88, PLA2G4A, IL1RAP, ITGB2
Higher in macaque LECs than human LECs:
TLR1, PPP1R12B, MEP2C, LTB, MAP3K1, CD40d,
RAC1, TLR4
Higher in macaque tissues than human and
macaque LECs:
CXCL9, CCL4, CD8, CD4, HLA-DRA, HLA-DRB1,
CDH1, CCL19
huLEC (mock)
rhLEC pooled (mock)
huLEC (PIC)
rhLEC pooled (PIC)
M1799
M5499
M5600
M6299
M6600
M5199



(C) **20 most differentially expressed genes**

huLEC (Dly)	Fold-change (PIC/mock)	rhLEC (pooled)	Fold-change (PIC/mock)
CXCL10	27,523	CCL5	1,970
IL6	219	CXCL10	1,850
CCL5	196	IL-1a	280
CXCL5	91	CCL20	95
TLR2	53	IL-1b	93
IL8	31	CXCL1/2/3	45
IL-1b	26	CCL3	44
CCL20	21	CXCL5	36
MAP3K5	20	CCL2	31
TLR3	19	CCL13	27
CXCL1/2/3	19	IL23A	25
CXCL9	17	TLR2	17
STAT1	16	TNFA	17
IL1RAP	15	CCL7	16
IL12A	13	CXCL9	13
IL15	12	IL6	12
CCL3	9	CCL4	12
LTB	8	CCL19	12
PLA2G4A	7	IL8	11
TNFA	7	CD40	9

Figure 19. Primary macaque LECs share similar RNA expression profiles with primary human LECs before and after treatment of poly I:C as examined NanoString RNA profiling.

A total of 1 µg total RNA for each sample preparation was analyzed for a total of 223 host response genes code sets by NanoString RNA profiling. Counts were normalized to housekeeping genes and gene clustering analysis was performed using the R software for statistical computing. (A) Pooled macaque jejunal RNAs and primary model human dermal LECs (Dly) clustered differently from the uninfected and acute AIDS rhesus tissues RNAs, which were included as control populations. Species specific genes in both pooled primary macaque jejunal LECs and primary human dermal LECs were highlighted. (B and C) Poly I:C treatment (24 hr) induced upregulation of similar host response genes in both primary macaque jejunal LECs and primary human dermal LECs. (B) Follow-up analysis by real-time RT-PCR showed significant increase of mRNA levels of CCL5 ($p = 0.0035$), CXCL10 ($p = 0.0022$), CCL20 ($p = 0.0048$), IL-8 ($p = 0.0039$), IL1-β ($p = 0.0171$), and IL-6 ($p = 0.0065$) by paired t-test analysis. Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values), and were transformed to log10 values. (C) Fold-change analysis of 20 most differentially expressed genes in primary human and macaque LECs after poly I:C by NanoString analysis showed several overlapping genes.

Macaque jejunal LEC responsiveness to other PRR ligands

Having demonstrated the ability of the macaque jejunal LECs to respond to poly I:C, we then explored the ability of these macaque jejunal LECs to respond to other known TLR and PRR ligands by producing proinflammatory chemokines CCL5 and CXCL10 (Figure 20A and 20B). We first examine the endogenous expression of TLR1-9 by these primary rhesus jejunal LECs (Figure 21), and our data showed that TLR1-6 were expressed to high levels by these macaque LECs but not TLR7-8, which were consistent with previous studies using human LECs (281, 282). Interestingly, we did not detect TLR9 expression in these primary macaque jejunal LECs, although that could be due to the sensitivity of the real-time RT-PCR assay detection. We found that Pam3CSK (TLR1/2 ligand), high molecular weight poly I:C (TLR3 ligand), flagellin (TLR5 ligand), and MTri-DAP (NOD1/NOD2 ligand) significantly induced CCL5 and CXCL10 expression in all primary macaque jejunal LEC populations, with p values of 0.0052, 0.007, 0.0087, and 0.0086 for CCL5, and p values of 0.0015, 0.0183, 0.0193, and 0.0216 for CXCL10 (Figure 20A and 20B). Interestingly, FSL-1 (TLR6/2 ligand) only induced the expression of CCL5 ($p = 0.0403$) but not CXCL10, meanwhile Imiquimod (TLR7 ligand) only induced the expression of CXCL10 ($p = 0.0389$) but not CCL5. It is also worth noting that although the high molecular weight poly I:C significantly upregulated the expression of both proinflammatory chemokines ($p = 0.007$ for CCL5, and $p = 0.0183$ for CXCL10) by macaque jejunal LECs, their levels were only marginally induced using stimulation with low molecular weight poly I:C ($p = 0.0736$ for CCL5, and $p = 0.0813$ for CXCL10), suggesting to us that exposure to microbes with almost identical PAMPs may not induce similar host responses.

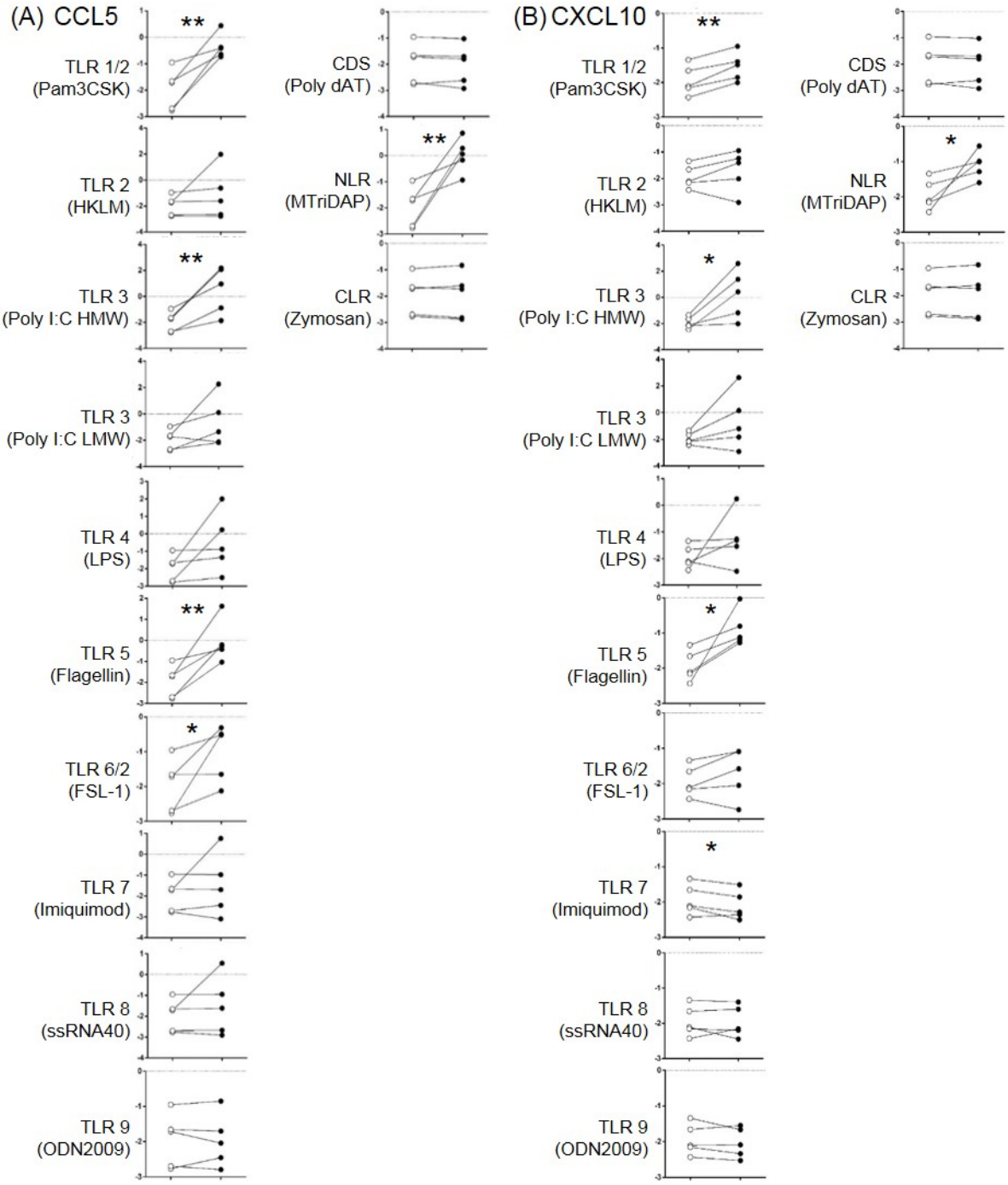


Figure 20. Primary macaque jejunal LECs respond in varying degrees to stimulation with several PRR ligands.

Confluent monolayer of primary macaque jejunal LECs were exposed to different PPR ligands (refer to methods for final concentrations used) for 24 hr. Respond to different PRR ligands were measured by production of proinflammatory chemokines, (A) CCL5 and (B) CXCL10 mRNAs by real-time RT-PCR. CCL5 and CXCL10 mRNA levels were significantly induced by treatment with Pam3CSK (TLR1/2 ligand), high molecular weight poly I:C (TLR3 and RIG-I ligand), flagellin (TLR5 ligand), and MTri-DAP (NOD-like receptor ligand) by paired t-test analysis. Interestingly, CCL5 mRNA levels was significantly induced with FSL-1 (TLR6/2 ligand) treatment but not CXCL10 mRNA levels by the primary macaque jejunal LECs. And CXCL10

mRNA levels was significantly induced imiquimod (TLR7) but not the CCL5 mRNA levels by the primary macaque jejunal LECs. ** denotes $p < 0.05$ and * denotes $p < 0.01$. Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values), and were transformed to log10 values.

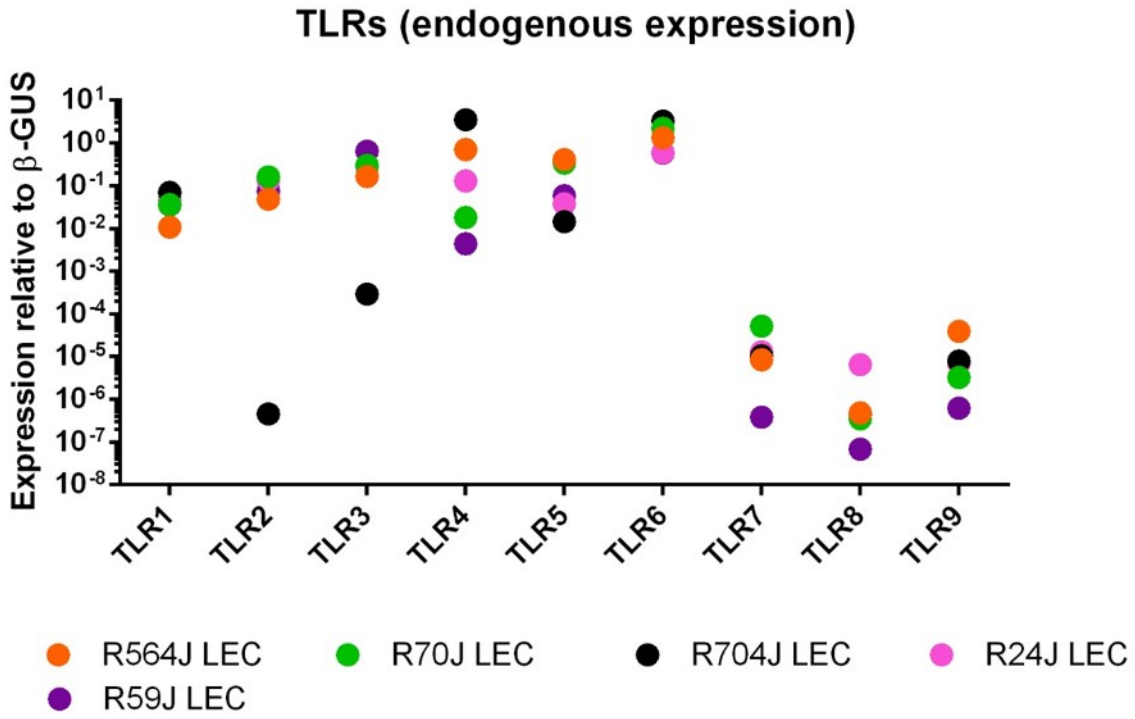


Figure 21. Primary macaque jejunal LECs express multiple endogenous TLRs.

TLR1 to 6 were abundantly expressed in all five primary macaque jejunal LEC populations with slightly lower expression of TLR2 and TLR3 in one population, rhR24 Jejunal. All five primary macaque jejunal LEC population expressed low levels of TLR7, TLR8, and TLR9. Data were normalized to relative expression of beta-glucuronidase (GUSB) (2-dCt values). Primer sequences for real-time SYBR Green RT-PCR were published (297).

Macaque jejunal LECs are able to perform antigen uptake and processing

The ability of LN-derived LECs to take up and process exogenous antigens has been reported recently in mice (163, 164, 166). We explored the ability of the macaque jejunal LECs, which were derived from different species and tissue origin, to perform antigen uptake and processing too by exposing them to BODIPY-conjugated DQ-OVA and BODIPY-conjugated DQ-BSA, known substrates for antigen uptake and processing. We found that macaque jejunal LECs were

comparable to the model human lung LECs in their ability to take up and process antigen when incubated in an environment that closely mimics the physiological condition (37°C) but were less efficiently in doing so when incubated at 4°C (Figure 22). We also found that the antigens were taken up and stored in endosomal compartments within the cytoplasm of the macaque jejunal LECs (data not shown). However, it was not determined how long these antigens were stored and whether they were subjected to antigen presentation.

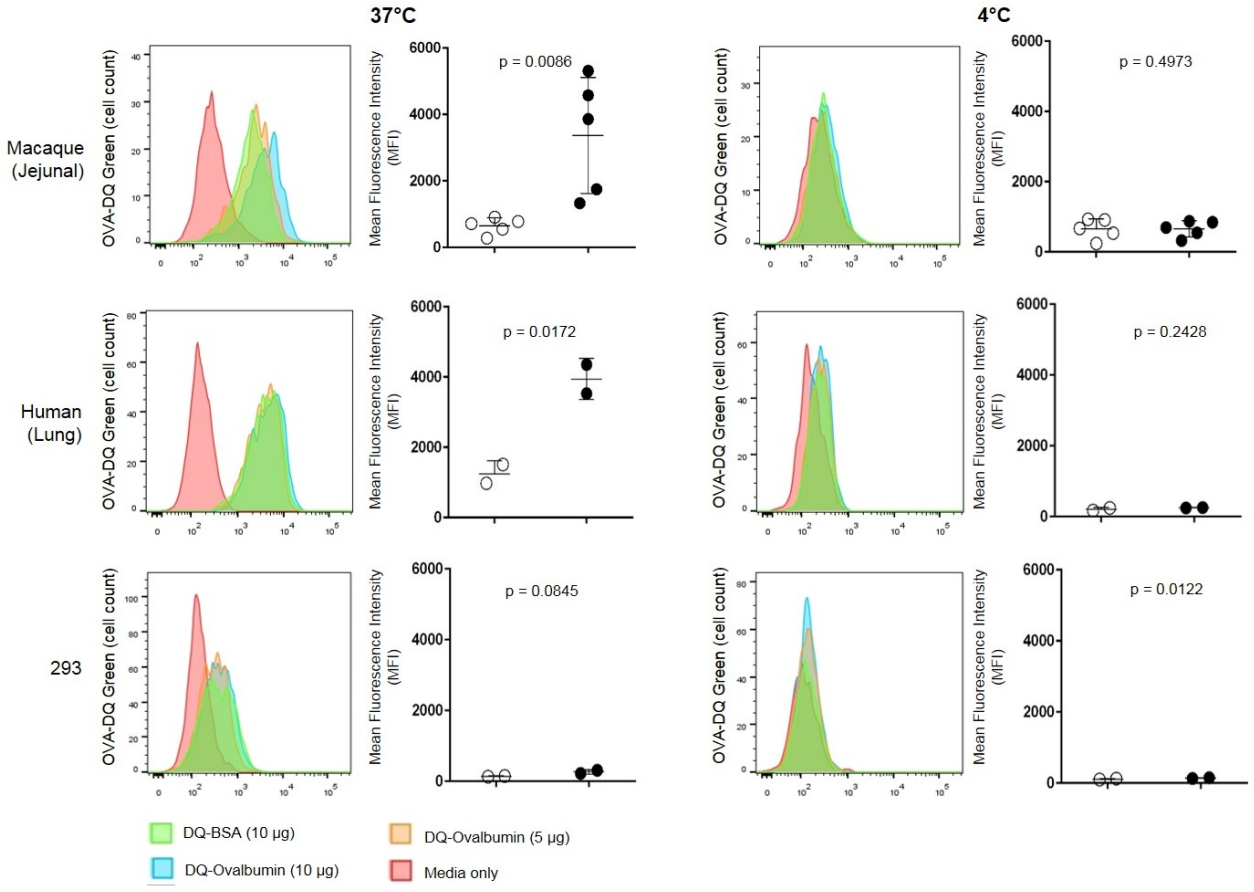


Figure 22. Primary macaque jejunal LECs are able to take up and process antigen.

Confluent monolayer of primary macaque jejunal LECs were exposed to different final concentrations of BODIPY-conjugated DQ-Ovalbumin and BODIPY-conjugated DQ-BSA for 1 hr. Cells were detached by enzymatic reaction (Accutase®), subjected to live/dead selection using cellular amines reactive dye, fixed with PFA (final concentration of 0.25%), and analyzed by flow cytometry. Model human lung LECs and macaque jejunal LECs were capable of antigen uptake and processing at 37°C but not 4°C. 293 cells were used as negative control population. Media only (o) wells were used as basal level comparison to DQ-OVA (final concentration of 5 µg/ml) (•). Geometric mean of

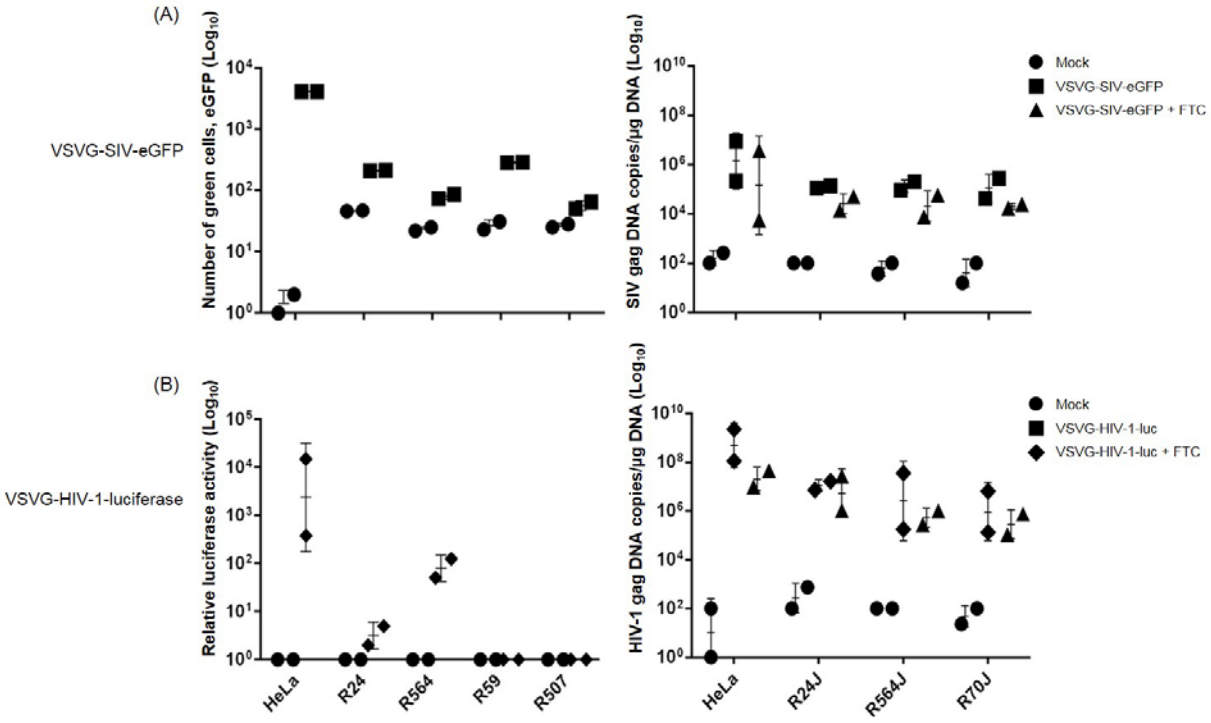
mean fluorescence index (MFI) of individual primary human lung LECs (N = 2), primary macaque jejunal LECs (N = 5), and 293 cells (N = 2) were from two independent experiments.

Macaque jejunal LECs were resistant to infection with single-cycle VSV-G pseudotyped SIV and HIV-1 viruses

We next asked whether macaque jejunal LECs were susceptible and permissible to infection with SIV/HIV-1 viruses. We investigated this by using genetically engineered VSV-G pseudotyped SIV and HIV-1 viruses that were designed to bypass receptor-mediated entry into cells and poised to perform a single-cycle reverse transcription in permissible cells upon entry (Figure 23A and 23B). Our data showed that unlike the HeLa cells (positive control population), the primary macaque jejunal LECs were not susceptible to infection by both VSV-G pseudotyped SIV (Figure 23A) and VSV-G pseudotyped HIV-1 (Figure 23B) viruses and their cellular milieus were not permissible for viral reverse transcription of both viruses to take place. Real-time PCR for detection of SIV and HIV-1 viral *gag* DNA copies showed marked decrease of SIV and HIV-1 *gag* copies after treatment with FTC, a nucleoside reverse transcriptase inhibitor (NRTI) in the HeLa cells but not the macaque jejunal LECs, suggesting to us that the blocking of these genetically engineered viruses potentially occurred before the reverse transcription step in the macaque jejunal LECs.

Our data showed that the macaque jejunal LECs expressed known viral entry factors for wild-type SIV such as CXCR6 and GPR15 (Figure 18A). Therefore, we sought to determine whether the macaque jejunal LECs were susceptible and permissible to infection with wild-type virus, SIVmac239. We used CEMx174 as control population for susceptibility and permissibility of SIVmac239 and determined the susceptibility and permissibility of these cells for viral replication through detection of SIV viral *gag* RNA in the culture supernatants and also

through detection of SIV viral *gag* DNA in the cell pellets (Figure 23C). Our data revealed that CEMx174 were readily infected by wild-type SIVmac239, and the viral SIV *gag* RNA copies per ml in the culture supernatant peaked by day 3 post-infection. Real-time PCR for detection for SIV viral *gag* DNA from the CEMx174 cell pellets by day 14 evidently showed presence of SIV *gag* DNA indicating productive replication of the wild-type SIVmac239 virus. Interestingly, although the SIV viral *gag* RNAs were detected to comparable levels to the CEMx174 in the culture supernatants of the macaque jejunal LECs at day 1, the SIV viral *gag* RNA copies were markedly decreased by day 3 post-infection. Additionally, the SIV viral *gag* RNA copies per ml in these macaque jejunal LECs were somewhat sustained at low levels up to day 14 post-infection. Real-time PCR data for SIV *gag* viral DNA showed presence of low copy number of SIV *gag* viral DNA in the macaque jejunal LEC cell pellets, suggestive of low levels (and perhaps controlled) viral replication.


(A)
VSVG-SIV-eGFP
Number of green cells, eGFP (Log10)
SIV gag DNA copies/µg DNA (Log10)
Mock
VSVG-SIV-eGFP
VSVG-SIV-eGFP + FTC
(B)
VSVG-HIV-1-luciferase
Relative luciferase activity (Log10)
HIV-1 gag DNA copies/µg DNA (Log10)
Mock
VSVG-HIV-1-luc
VSVG-HIV-1-luc + FTC
HeLa
R24
R564
R59
R507
HeLa
R24J
R564J
R70J

(C)

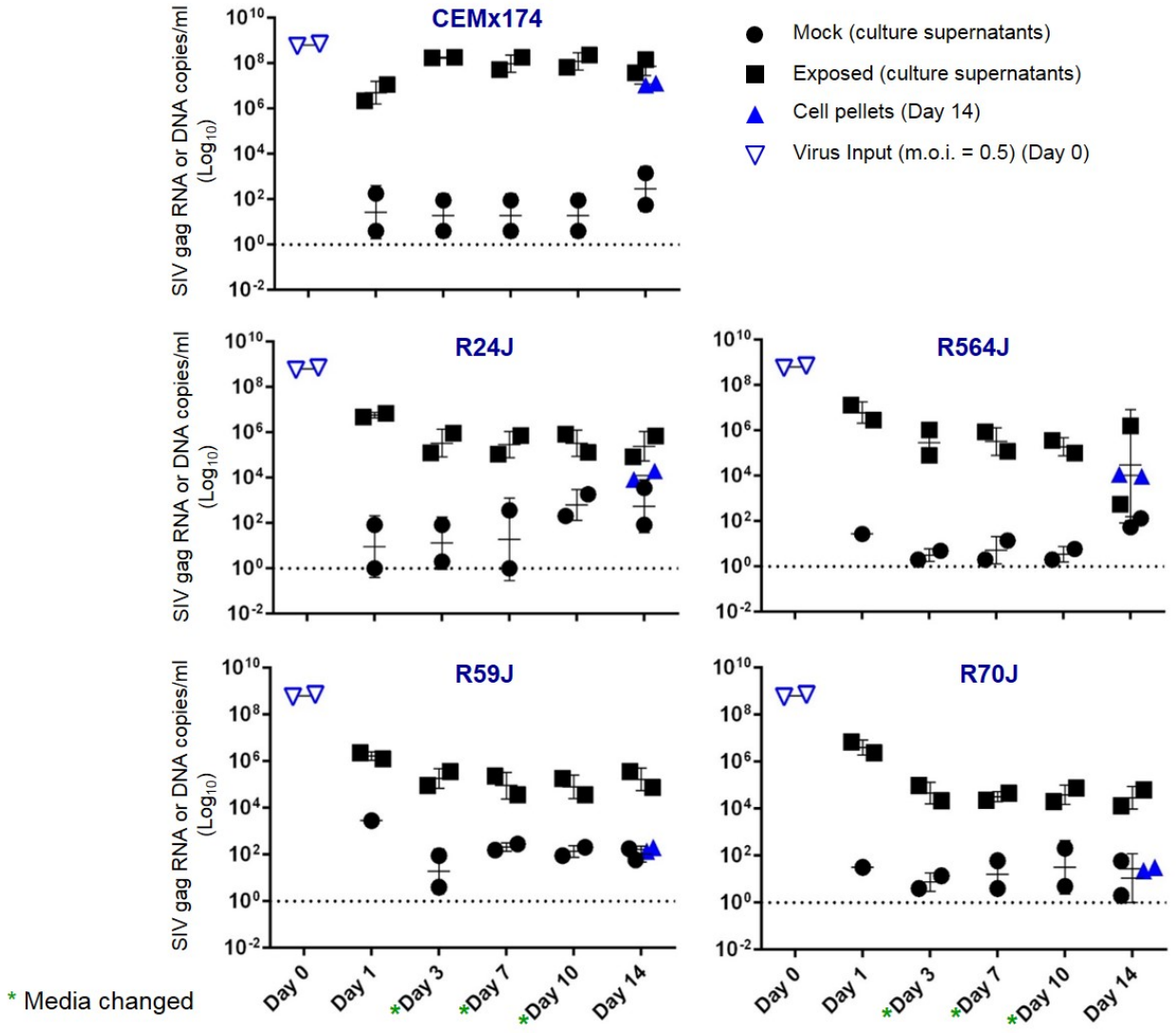


Figure 23. Macaque jejunal LECs are not susceptible to infection VSVG-pseudotyped SIV and HIV-1 virus as well as wild type SIV (SIVmac239), and their cellular milieus are not permissible for viral reverse transcription to occur.

(A and B) Confluent monolayer of HeLa cells (control population) and macaque jejunal LEC populations were exposed to genetically engineered VSV-G pseudotyped SIV expressing eGFP (m.o.i. = 0.5) and VSV-G pseudotyped HIV-1 expressing luciferase (m.o.i. = 0.5) viruses that were able to undergo single cycle reverse transcription upon entry into permissible cells after 48 hr post-infection. Evidence for viral entry and reverse transcription was measured by either flow cytometry analysis or luciferase activity for cells infected with virus expressing the eGFP or luciferase proteins and by performing real-time PCR on cell pellets of mock and virus infected cells. (A) HeLa cells were susceptible and permissible for reverse transcription of VSV-G pseudotyped SIV expressing eGFP as measure by flow cytometry and real-time PCR for SIV gag DNA copies. Detection of SIV gag DNA copies was markedly reduced with addition of FTC, a nucleoside reverse transcriptase inhibitor (NRTI). The macaque jejunal LECs were resistant to infection by the VSV-G pseudotyped SIV expressing eGFP, as shown by the flow analysis and SIV gag DNA real-time PCR data. There were no difference in the number of SIV gag DNA copies in the macaque LECs exposed to virus only and with presence of the inhibitor, suggesting that the cellular milieus of the macaque LECs were not permissible for viral reverse transcription to occur. (B) Similarly, the HeLa

cells were susceptible and permissible for reverse transcription of VSV-G pseudotyped HIV-1 expressing luciferase as measure by relative luciferase activity unit (RLU) and real-time PCR for HIV-1 gag DNA copies. Detection of HIV-1 gag DNA copies was markedly reduced with addition of FTC, a nucleoside reverse transcriptase inhibitor (NRTI). The macaque jejunal LECs remained resistant to infection by the VSV-G pseudotyped HIV-1 expressing luciferase, as shown by relative luciferase activity unit (RLU) and HIV-1 gag DNA real-time PCR. There were no difference in the number of HIV-1 gag DNA copies in the macaque LECs exposed to virus only and with presence of the inhibitor, suggesting that the cellular milieus of the macaque LECs were not permissible for viral reverse transcription to occur. (C) Confluent monolayer of macaque jejunal LECs and CEMx174 cells (control population) were exposed to wild-type SIVmac239 (m.o.i. = 0.5) and viral supernatants sampled from day 1, 3, 7, 10, and 14 were subjected to real-time RT-PCR for detection of SIV gag RNA. Culture media were replenished at day 3, 7, and 10. On day 14, cell pellets from mock and virus exposed populations were harvested and subjected to real-time PCR for detection of SIV gag DNA. CEMx174 (control population) were susceptible to infection by the wild-type SIVmac239 and virus replication peaked by day 3 post-infection. Real-time PCR of the cell pellets obtained from day 14 showed presence of SIV gag DNA in the CEMx174 cells. The primary macaque jejunal LECs showed evidence for SIV gag RNA detection in the culture supernatants of SIVmac239 exposed populations. However, the number of SIV gag RNA copies detected in all macaque jejunal LECs decreased by approximately 100-fold by day 3 and were sustained at low levels until day 14. Real-time PCR analysis of cell pellets obtained from day 14 showed low copy numbers of SIV gag DNA in the primary macaque jejunal LECs. Data showed for both VSV-G pseudotyped viruses and wild-type SIV were mean ± SD obtained from two independent experiments.

4.6 DISCUSSION

Identification of LEC markers has facilitated research on LECs by allowing them to be distinguished them from BECs (42, 50, 52, 53, 298). One approach for isolation and characterization of primary LECs from humans, mice, or rats involves mechanical and enzymatic dissociation of tissues followed by enrichment using LEC marker-specific antibodies (112, 125, 128, 298-300). Although this approach yields fairly pure populations of cells, it can result in bias through selection of subpopulations of LECs with high surface expression of the surface markers recognized by antibodies used for selection (301). Another factor is that some dissociation methods may preferentially isolate sub-populations of LECs while omitting others that are more resistant to mechanical and/or enzymatic dissociation procedure (123). Our study presents a comprehensive analysis of phenotypic and functional characteristics of primary macaque LECs obtained by direct plating onto collagen coated surface matrix and pushed with the endothelial growth factor, VEGF-C for enhanced survival. We and others (123, 302) have shown that this is an effective approach for bulk isolation of LECs. One caveat in utilizing this method is that there is a potential for contamination by other cell types. To evaluate this, we compare the phenotypic and surface markers characteristics of LECs derived by plating and provision with VEGF-C to an LEC population that was live cell sorted. Our findings revealed that these populations were remarkably similar in both their phenotype and surface marker expression profiles, suggesting that a direct plating method in the presence of VEGF-C yields pure LEC populations as representative as cell sorting and short-term culturing. In preliminary studies, the effect of exogenous recombinant human VEGF-C on macaque LEC growth appeared

to affect the overall health and appearance of the LECs, but did not seem to affect their overall growth rates (data not shown). It is conceivable that endogenous VEGF-C or VEGF-D in the culture media contributes to a nurturing environment for the LECs. A recent study using the zebrafish model showed different lymphatic growth factors can be involved in lymphatic development and that the loss of VEGF-C can be compensated by VEGF-D (138).

Intestinal epithelial mucosal barrier disruption is one of the hallmarks of progression to AIDS following HIV-1 infection (303, 304). Translocation of pathogenic bacteria or gastrointestinal microflora into the lamina propria and eventually into the systemic circulation may be responsible, at least in part, for the chronic immune activation that follows pathogenic HIV-1 and SIV infections. Although the exact mechanism of immune activation is not entirely clear, the main players in activation of host innate immunity seem to be via sensing of microbial products by TLRs and other PRRs. Studies have shown that blocking of early microbial translocation in a nonhuman primate model infected with SIV reduced virus-mediated inflammation and viral replication (198) and the use of TLR agonist as adjuvants in mucosal vaccine formulations reduced mucosal SIV translocation in the gut (286).

We present here for the first time a detailed analysis of the responsiveness of macaque jejunal LECs to PRR ligands. Our data revealed that macaque jejunal LECs express functional TLRs and other PRRs, thus unmasking them as attractive targets for development of mucosal vaccines against SIV/HIV-1 in the gut, of course alongside other innate immune cells, such as DCs and macrophages. SIV infection has been shown to cause accumulation of plasmacytoid DCs in the gut (305) and it will be interesting to study the cross-talk between LECs and other immune cells in the context of SIV/HIV-1-induced chronic inflammation in the gut.

Enhanced innate viral restriction factors following an infection with SIV has been reported as one of the contributing factors for enhanced mucosal recovery in the gut of some animals despite the lack of complete CD4+ T cell restoration (306). Our data revealed that macaque jejunal LECs highly express multiple viral restriction factors including APOBEC3G, tetherin, Trim5-α, SAMHD1, and Schlafen-11. Thus, LECs, if targets for viral entry or uptake, then have the potential to impose strong barriers to pathogens (including SIV/HIV-1) and limit the initiation of early infection or transmission by these pathogens. Through viral PAMPS, viral infection stimulates cellular factors through PRRs, which leads to increased IFN production that in turn upregulates expression of IFN stimulated genes (ISGs), including viral restriction factors (307, 308). Although the precise mechanisms are not fully clear, LECs are resistant to infection with VSV-G pseudotyped and WT SIV/HIV-1 (Figure 23A-C), and viral restriction factors likely play some role in this restriction.

The roles of LECs in innate and adaptive immunity is becoming increasingly appreciated (24). Recent studies showed that LEC plays a critical role in not only in initiation (92) but also the resolution (163) of inflammatory conditions. In addition, LECs have been reported to mediate tolerance to self-antigens via MHC-I in tissue graft and transplant rejection (161, 165, 309). LN-derived LECs in mice express MHC-II molecule as well as functional costimulatory molecules after treatment with exogenous IFN-γ although interestingly, the MHC-II expressed was unable to activate allogeneic naïve T cells and impaired their proliferation stimulated by DCs (162). In addition, LECs are able to take up tumor antigens and process them for cross-presentation to naïve T cells via MHC-I (164) and LECs are major archiving cells for storage of persisting antigens following viral challenge or vaccination in a mice model (166). Our data and others' data (166) have shown that the LECs are not only able to take up and process antigens

but they also can store the captured antigens for an extended period of time, the latter resulting in increased effector and protective function of memory CD8+ T cells. These findings highlight a potential innate immune function of LECs as the ability to perform antigen uptake and processing is a hallmark of antigen presenting cells (APCs) such as DCs. A reasonable extrapolation is that LECs are potentially actively involved in innate immunity during pathogen-host interactions, not only through sensing via functional PRRs and through expression of viral entry and/or restriction factors, but also through the ability to capture and store antigens without becoming infected themselves. This in turn provides them with a key role in controlling and halting pathogen infection and transmission, defining them as having potential barrier functions in addition to their structural role within the conduit of the lymphatic vasculature. The macaque LEC populations presented here will be beneficial in exploring further the innate immune functions of LECs and to study their cross-talk with other immune cells such as T cells and DCs, in SIV, HIV-1, and other microbial infections and resulting inflammation, as well as vaccine-elicited host responses.

4.7 ACKNOWLEDGEMENTS

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(MOSTI), Government of Malaysia and partially by the Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh.

5.0 FINAL DISCUSSION

LECs were initially thought have passive roles as conduits for transport of immune cells from the periphery to the draining lymph nodes during immune surveillance under homeostasis conditions or during initiation of host immune responses to infections or vaccinations. However, a growing number of studies have shown that LECs play an active role in regulating the kinetics of antigen presentation by APCs, and that LECs also are important contributors in shaping the cellular milieus during host immune responses to pathogens. LECs help initiate the host innate immune response through PRR sensing of microbes, and continue to modulate the local proinflammatory microenvironments through expression of multiple cytokines and chemokines, signaling molecules, and adhesion molecules to recruit immune cells to sites of infection (247). Nevertheless, LEC modulation of the local cellular milieu might potentially be a double-edged sword for the host. Whilst LEC-derived proinflammatory cytokines and chemokines, signaling molecules, and adhesion molecules are crucial for recruitment of naïve immune cells and for the migration of effector immune cells to the sites of infection, these inflammatory signals or molecules likely modulate and alter the functions of the immune cells and cause autoimmunity diseases (252). Similarly, LEC-induced inflammatory conditions are beneficial in wound healing but are a likely cause of graft rejection during organ transplantations (65, 144). During cancer metastasis, lymphangiogenesis enables the spread of cancer cells to distal organs (65).

5.1 OVERVIEW OF DISSERTATION FINDINGS

Due to their emerging roles in multiple aspects of human health and diseases, LECs and the lymphatic vascular system should no longer be neglected or regarded as a vascular system of secondary importance to the body (3). LEC function is less well understood compared to BEC function due to challenges in studying LECs. Discoveries of LEC markers as well as improved culture conditions have advanced the field substantially in the last decade (43, 108). However, one of the major challenges that still remains in establishing ex vivo cultures of LEC is obtaining pure LECs given that they are a rare cell type in tissues.

In the first aim of this study, we first sought to establish pure cultures of primary LEC populations from multiple animals and tissue origins in order to understand better the basic characteristics and functions of LECs in different organs (Chapter 3 and 4). These homotypic primary LEC cultures were first established in a 2D in vitro culture system, and can be used in the future to develop in vitro three-dimensional (3D) cell cultures of primary LECs, alone or with other cells. We have successfully established and characterized six primary LEC populations from the skin, lung, mesenteric lymph node, and trachea from four different animal ferrets (Chapter 3). We also have successfully established and characterized 22 primary LEC populations from the jejunum, skin, mesenteric lymph node, thoracic duct, and lung from 10 different animal macaques (Chapter 4).

In the second aim of this study, I sought to begin to understand the potential involvement and contributions of LECs during pathogen-host interactions (Chapter 3 and 4). I utilized primary LECs that were cultured in low passage number in 2D in vitro cultures and assessed their ability to respond to known PRRs ligands (PAMPs). I measured the ability of these primary LECs to respond to PAMPs by their ability to secrete proinflammatory cytokines and

chemokines. I found that LECs were able to secrete functional cytokines and chemokines upon stimulation with multiple PAMPs, suggesting that they were able to sense, recognize and respond to microbes. Interestingly, poly I:C, which is a double-stranded RNA viral mimetic was the most potent inducer of robust proinflammatory responses in primary human, macaque, and ferret LECs. The ability of primary LECs to respond to a viral RNA suggested that LECs may have an active contribution in shaping the initial host responses to an early stage viral infection, such as by SIV/HIV-1, and can potentially be manipulated to block the initial local establishment and/or amplification of these viruses. I then examined whether primary LECs were susceptible and permissible to infection with single-cycle, genetically engineered VSV-G-pseudotyped SIV and HIV-1, as well as wild-type SIV (Chapter 4). We found that primary LECs were not highly susceptible to infection by VSVG-pseudotyped and wild-type viruses, and that their cellular milieus were not highly permissive for reverse transcription of the viral genome of the single-cycle, genetically engineered VSV-G-pseudotyped viruses. However, prolonged culture of primary LECs post-exposure to wild-type SIVmac239 indicated that low levels of infection occured, which resulted in low level production of viral RNA and DNA.

In the third aim of this study, we evaluated the potential of LECs to act as innate immune players and barriers during pathogen-host interactions by comparing them to a subset of DC phenotypes, well-known immune cells at the interface between innate and adaptive immunity (Chapter 4). We found that LECs – human LECs in particular – shared the expression of several phenotypic markers with DCs, and that LECs and DCs shared the functional ability to take up and process model antigens. However, it was not determined if these processed antigens were subjected to terminal degradation or antigen presentation by the primary LECs.

Thus, taken together these findings highlighted the innate immune potential of LECs during pathogen-host interactions that merit further examination in order to advance our knowledge of LECs and their crosstalk with pathogens and other immune cells, therefore potentially targeting LECs for development of new and improved therapeutics or vaccines for human diseases.

5.2 IMPORTANCE OF IN VITRO MODELS FOR STUDYING LYMPHATIC ENDOTHELIAL CELLS

The main objective of an in vitro culture of mammalian cells is to provide a more uniform population of cells and a more controlled environment for investigators to study the basic physiological and biochemical properties of the different cell types (310). Typically, cells are obtained from tissues by treating them with proteolytic enzymes that digest the proteins in the extracellular matrix, and including treatment with agents that chelate Ca^{2+}, which is important for cell-to-cell adhesion. The dissociated cells are then grown as a monolayer on a plastic or glass surfaces (hence the term in vitro, which means “in glass” in Latin) with medium containing source of nutrients, and maintained at body temperature (37°C) (311). Since the first in vitro establishment of the “L cell line” by Earle and colleagues in 1940s, in vitro culture has been an instrumental tool in some of the most important scientific discoveries in human medical history (312). The first human “transformed” cell line was derived from human cervical carcinoma in the 1950s, and is still used extensively to this day in biomedical research. The ability of polio virus to grow in in vitro cultures of human kidney cell lines enabled the development of the Salk vaccine for prophylaxis against polio virus infection. Subsequently, the live-attenuated version

of the polio virus known as the Sabin vaccine, was produced also in in vitro kidney cell lines. The production of these vaccines eventually led to the near world-wide eradication of polio virus infection. Since then, more vaccines for important human diseases were produced using in vitro cell culture methods. Vaccines for measles, mumps and rubella, as well as other veterinary diseases such as foot-and-mouth disease were produced using in vitro culture of cell lines of human and other species origins (313).

The rapid advancement in establishment of 2D in vitro cultures of primary LECs from humans and other animal species has been facilitated by the discoveries of LEC specific markers, which allows for isolation and enrichment of pure LEC populations using target-specific antibodies (108). Further advancement has come through the development of specific culture conditions and growth factors that enhance the survival and growth of LECs in vitro. Our observations and those of others have shown that in vitro cultured LECs initially grow in clusters, and expand to form a confluent monolayer with the typical cobbled-stone morphology of endothelial cells (248, 314). Individual LEC cells appear to be flattened on the 2D surface, and are slightly elongated during their proliferative stage. However, the morphological characteristics of LEC change in higher subculture passages, in which LECs become slightly increased in size, have a more flattened appearance, and are slightly hexagonal in shape, suggestive of a move toward senescence (unpublished observations). In our experience as well as others, primary LECs isolated and cultured in vitro can be successfully subcultured, frozen, and thawed with minimal changes in phenotype (108). We have successfully subcultured primary macaque and ferret LECs up to nine passages, and characterization at the mRNA level by standard and real-time RT-PCR, and at the protein level by flow cytometry analysis showed stable and sustained expression of LEC markers (Chapter 3 and 4).

Establishment of in vitro cultures of primary LECs has enabled studies focused on understanding the molecular mechanisms that are involved in lineage commitment and development of the lymphatic vasculature, as well as its functions in health and disease. Primary LECs have been isolated and established from different tissues of human and other animal species of origins in 2D in vitro culture systems (51, 53, 108, 109, 117, 123-127, 129, 160, 248, 314, 315). Phenotypic and functional characterization, such as measurement of gene expression profiles and responses to cytokines and growth factors, suggested that LECs from these animal models expressed similar gene profiles and are able to response to exogenous stimuli to comparable extents as their human counterparts (248, 272). These findings suggest that data obtained using the primary LECs from other species are relevant to study human diseases. This knowledge has led to development of therapeutics for treatment and management of diseases that depend on the lymphatics drainage and transport functions, such as lymphedema, which is caused by dysfunctional lymphatics. To date, several targets for lymphedema therapies have been discovered through transcriptomal profiling of in vitro models of LECs isolated from healthy donors and comparing them to lymphedema patients (111, 290). Among the most studied therapeutic target is VEGFR-3, and its ligand VEGF-C, and their additional roles in lymphangiogenesis (113). VEGF-C signaling through VEGFR-3 has been demonstrated to induce lymphangiogenesis and improve lymphatic drainage functions in several animal models, suggesting its potential as a therapeutic target for lymphedema. Surgical lymphedema is still the most common complications for cancer survivors, in which 25–56% of breast cancer patients develop mild-to-severe cases of lymphedema after cancer treatment. In vitro cultures of LECs in inflammatory conditions have also contributed to identification of LEC-derived adhesion molecules that are utilized by cancer cells for their dissemination to distal organs during

metastasis. Additionally, transcriptional profiling of in vitro cultured LECs from healthy and diseased human tissues have enabled investigators to identify new target genes that serve as biomarkers for improved diagnosis and prognosis of diseases in patients. More recently, in vitro models of LECs have been used to study the crosstalk of LEC with immune cells in promoting tolerance or autoimmunity, and also to study the role of LEC as one of the major regulators of host immune responses (309).

Two-dimensional (2D) in vitro culture system does not faithfully recapitulate the physiologic conditions of native tissues in vivo. Cells in native tissues exist in conditions that allows for cell-to-cell and cell-to-extracellular matrix (ECM) interactions that are not modeled in 2D culture environments (316). As a result, many of the complex biological cues as well as molecular processes and functions these cells in vivo are lost in the 2D in vitro culture system. Therefore, there have been growing efforts to improve the routinely used 2D in vitro cell culture system by switching to a 3D in vitro cell culture system that mimics closer the spatial organization of ECM microenvironments, cell-to-cell adhesions, and polarity of cells in vivo (317, 318). Several studies have demonstrated that the 3D in vitro cell culture microenvironments were able to improve cell viability, proliferation, differentiation, and lifespan in vitro (319). Cells grown in 3D in vitro culture also showed improved cell-to-cell communication, cell migration, and response to stimuli (319).

To date, there are several upcoming and promising methods of 3D in vitro cell culture, depending on the types of native tissue microenvironments that individual model attempts to mimic. The multicellular spheroid (MCS) format relies on the ability of cells to aggregate and adhere to themselves. MCS can be generated through several methods including the forced-floating method (coating of culture flask with poly-2-hydroxyethyl methacrylate or poly-HEMA

to create non-adherent surface) and the hanging drop method. The MCS is becoming increasingly popular 3D in vitro culture method for high-throughput screening of efficacy and toxicity of drugs using tumor cells from patients or cancer cell lines (316). Liver cancer cells and breast cancer cells have been shown to form pathologically and physiologically relevant MCS that are "tissue-like", and thus improved the accuracy and efficacy of tested drugs. In this study, we observed that primary human and macaque LECs were able to form MCS that were intact and tightly packed with little variability in sizes using both poly-HEMA forced floating and hanging drop method (Appendix). Another group demonstrated that primary human LEC MCS cultured for extended period of time demonstrated the ability to sprout, suggestive of lymphangiogenesis (320). Another 3D in vitro culture method that is gaining popularity due to its relevance to mimic the ECM microenvironments in the tissue is the gel matrix system. In this system, cells are embedded within organic substance (collagen or matrigel), or scaffold (synthetic fiber) to mimic the ECM. Primary LECs cultured using the matrigel 3D in vitro system were able to form tubular structures that resembled the lymphatic vessels (114).

Establishment of 2D in vitro cultures of primary LEC is a step forward to advancing our knowledge of LEC biology. However, due to the limited ability of the 2D in vitro culture system to recapitulate the environment found in tissues, there is a need to develop 3D in vitro culture system of primary LECs that closely mimic their physiological and molecular functions in vivo.

5.3 HETEROGENEITY OF LYMPHATIC ENDOTHELIAL CELLS

Endothelial cells have remarkable heterogeneity and plasticity in different organs and vascular beds. LEC and BEC diversity is reflected at the molecular level during embryonic pre-

development and also post-development of the vasculature systems. LEC heterogeneity and plasticity are often associated with anatomical compartments and disease related pathological conditions in different organs or tissue types, which could be reflective of their specific functions in health and disease.

5.3.1 Plasticity and heterogeneity of lymphatic endothelial cells

Transcriptomal analysis of human dermal LECs and BECs revealed that the two types of endothelial cells shared approximately 95% of their genes (53). We found that primary LECs isolated from different animals and tissue types shared the same expression of LEC markers, podoplanin, LYVE-1, Prox-1, VEGFR-3, and CCL21, albeit to varying levels, suggestive of their heterogeneous phenotypes. However, in primary ferret and macaque LECs, Prox-1, which is a known transcription factor for LEC commitment and lineage, was expressed to an abundant degree in all primary LEC populations irrespective of the tissue and species origins. Prox-1 was highly conserved at the nucleotide and amino acid levels when compared among different species, which could potentially be mean that it has highly preserved function as LEC-lineage regulator too. Prox-1 induces the expression of other LEC markers and down regulates the expression of BEC-specific markers (50). Overexpression of Prox-1 resulted in lymphatic reprogramming of the BECs to become more LEC-like (50). In addition, inhibition of Prox-1 during embryonic developmental stage of lymphatic vasculature formation resulted in the loss of LEC-phenotype in both in vivo and in vitro experimental settings (78). Thus, Prox-1 is not only required to maintain LEC identity and phenotype but is also required to maintain the heterogeneity and also plasticity of LEC.

The LEC specific surface marker, podoplanin, was also found to be abundantly expressed by all primary LEC populations from different species and tissues types, and by far in our observation was the strongest and most robust LEC marker. Podoplanin expression by in vitro cultured primary macaque jejunal LECs were detected very strongly by flow cytometry analysis as well as real-time RT-PCR even after several subculture passages. These data suggest that combination of podoplanin and other LEC surface markers, such as LYVE-1 and VEGFR-3, are suitable for isolation and establishment of pure populations of LECs. However, heterogeneity in podoplanin expression was observed in vivo, in which two types of LECs with high and low expression of podoplanin were observed and isolated by flow cytometry (267). LECs with higher podoplanin expression showed higher ability to express CCL21, and were mostly located in the initial lymphatics, meanwhile LECs with lower expression of podoplanin demonstrated higher expression of CCL27, and were most located at the precollecting and collecting lymphatic vessels. These differences in chemokines secretion could potentially explain the anatomical specific functions of LECs in vivo. We found that VEGFR-3 was expressed to varying levels in the in vitro cultured primary LECs, and the variability was observed across different animal species and tissue types. This could be due to a number of contributing factors. Firstly, the survival rate of LECs from the different anatomical parts of the tissue. Secondly, the adaptation to the 2D in vitro culture conditions, such pushing the LECs to be more LEC-like using VEGF-C, caused some of the LEC populations to down regulate their expression of its receptor, VEGFR-3.

We also found that primary LECs shared some overlapping markers with BECs. This could be due to contaminants BECs in the culture or the fact that LECs were derived from BECs in early embryogenesis, and did not completely lose some of the BEC markers. However, it is

quite possible that the functions of these BEC markers in LECs are suppressed by Prox-1 activity. Studies by others have shown that BECs have a remarkable ability to become more LEC-like under the control of their microenvironments, both in vitro and in vivo. BECs cultured in a 3D in vitro culture system using collagen and matrigel showed altered gene expression profiles to be more LEC-like, and reverted back to BEC expression profiles when cultured in a 2D in vitro culture system (321). CD34+ VEGFR-3+ endothelial progenitor cells isolated from human cord blood and cultured in vitro in presence of VEGF-C showed the propensity to differentiate into LEC-phenotypes (322). More recently, macrophage-derived LEC progenitor cells (M-LECPs) have been reported to have the ability to integrate themselves into the peripheral lymphatic vessels in LPS-induced lymphangiogenesis in mice (145). MLECPs also were demonstrated to express LEC markers such as podoplanin, LYVE-1, and VEGFR-3 upon exposure to LPS, although it was not determined if Prox-1 expression was also induced and if this LEC-like phenotype was reversible as observed in as CD34+ VEGFR-3+ endothelial progenitor cells. Mouse DCs when cultured in different in vitro culture conditions were shown to upregulate the expression of endothelial markers as well as angiogenic factors (323). We found that primary LECs expressed several overlapping phenotype markers that are known to be associated with DCs, such as DC-SIGN, MMR, CD169, and CD40. CD40 was expressed to high levels endogenously in all of the primary macaque jejunal LECs, suggestive of the potentials of LECs as immune modulators in vivo. CD169 expression was really low and almost undetectable endogenously, but its expression was increased significantly in the primary macaque jejunal LECs when exposed to poly I:C, a double-stranded RNA virus mimetic (Figure 18C). We found that primary macaque jejunal LECs also shared the functional ability to take up and process

antigens in a similar fashion and almost as efficient as DCs (Figure 22). Although, it was not determined further if LECs were also capable of antigen presentation.

5.3.2 Lymphatic endothelial cell and tissue microenvironments

It has been well established that LEC gene expression profiles and functions are influenced by the surrounding ECM and organ compartments, as well as the types of physiological conditions they are in, whether steady-state or inflammatory (321). LEC heterogeneity in the lymph node has been shown in the defined distribution of LEC markers in uninfected, SIV infected, and *Mycobacterium tuberculosis* infected cynomolgus macaques (93). Although LEC markers podoplanin, Prox-1, and VEGFR-3 were reported to be expressed in both afferent and efferent lymphatics, CCL21 expression was only observed in the afferent lymphatics whereas LYVE-1 expression was only observed in the efferent lymphatics. These findings suggest that LECs in different anatomical compartments within an organ may have different biological functions. Similarly, genome-wide comparative studies of intestinal versus dermal LECs showed that although the two LEC populations showed similar overall gene expression profiles, however there were more than 200 genes that were differentially expressed (324). Studies of LECs isolated from different human secondary lymphoid organs such as the lymph node, spleen, thymus, palatine tonsil, and iliac lymphatic vessels displayed differential expression profiles of LEC and vascular markers (314).

Our data also revealed that primary LECs expressed other genes that were known to be important for lymph node remodeling and immune processes such as the egress of lymphocytes from the efferent lymphatics. IL-7, a homeostatic survival cytokine for naïve and memory T-cells, was shown to be important for lymph node remodeling after a viral infection as well as

lymph node reconstruction after avascular transplantation (57). Furthermore, LEC-derived IL-7 also contributed to de novo lymphangiogenesis of LYVE-1-positive vessels within the lymph node and surrounding tissues after transplantation. Our data corroborated the findings that LECs express IL-7 and its receptor IL-7R, which have been showed to work in autocrine fashion in vivo to promote lymphatic drainage as well as lymphangiogenesis. We also found that primary LECs expressed Spinster-2, an important transporter molecule for sphingosine-1-phosphate, a known mediator for lymphocytes egress from secondary lymphoid organs (156).

In addition to physiological conditions, LEC heterogeneity has been demonstrated to be associated with several pathological inflammatory conditions within tissue microenvironment, including tumor-induced lymphangiogenesis. Crosstalk between LEC and cancer cells induced the expression of LEC-mediated signals, which can promote lymphangiogenesis and proliferation of cancer cells (325). Tumor-associated LECs upregulated the expression of several adhesion molecules that have been shown to be involved in regulating the permeability of LEC tight junctions, and thus promoting dissemination of cancer cells during metastasis. Tumor-induced lymphangiogenesis upregulates the expression of LYVE-1 in both LECs and BECs, which mediates the adhesion of hyaluronan-expressing cancer cells to the lymphatic vessel and induces tumor invasion. Similarly, abnormal expression of VEGFR-3 in BECs promotes lymphangiogenesis and neovascularization in multiple tumors and granulomas. Several other LEC markers as well as adhesion molecules including Stabilin-1, mannose receptor (CD206), and Thy-1 (CD90) have been demonstrated to contribute to adhesion to and subsequent infiltration of cancer cells into the lymph node via the lymphatics. LEC-secreted chemokines such as CCL21 and CCL5 recruit cancer cells that express their respective receptors, CXCR4 or CCR7 and CCR5, and thus promoted transport of cancer cells into the draining lymph node via

the lymphatics (325). Our data revealed that primary LECs expressed CCL21 and CCL5 endogenously amongst other cytokines and chemokines, and the expression of these cytokines and chemokines were increased in proinflammatory conditions.

Recently, LEC-induced modification of the local tissue microenvironments have been associated with host immune tolerance of tumor antigens. Cross presentation of VEGF-C-expressing tumor cells antigens by lymph node LECs via their MHC I molecules resulted in tolerance of peripheral tissue antigens derived from tumor cells by tumor-specific CD8+ T cells (164). In addition, LECs have been shown to transfer exogenous antigens to DCs, which resulted in impaired antigen presentation function by DCs via MHC II molecules, and thus resulting in tolerance of to CD4+ T cells (162). In addition, inflammation-induced lymphangiogenesis during corneal transplantation resulted in rejection of the transplanted graft (144, 165). However, the graft survival was improved greatly after administration of antibody targeting the LEC marker, VEGFR-3.

5.3.3 Lymphatic endothelial cell reprogramming and viral infections

Heterogeneity and plasticity of endothelial cells in pathological conditions are best exemplified in the case of HHV8-associated Kaposi's sarcoma (KS). KS is the most common malignant tumor in AIDS patients. KS tumor is highly vascularized, suggestive of the endothelial origin of the disease pathogenesis. Initial studies of gene expression profiles of KS tumor suggested that they were of LEC origin. In addition, in vitro infection of LECs and BECs with the HHV8 virus showed greater susceptibility to infection in LECs compared to BECs (3). However, that HHV8 infection caused BECs to reprogram into LEC-like phenotypes and functions. HHV8 encodes a viral homolog of the IL-6 protein, which signals through various proinflammatory and

proangiogenic pathways that are sufficient to induce Prox-1, and subsequently other LEC markers such as podoplanin, LYVE-1, and VEGFR-3 (326). Another latent viral protein, known as Kaposin B, stabilizes the Prox-1 mRNAs in BECs upon infection, thus induced the reprogramming of BECs to LECs (327). Furthermore, additional mechanism of Prox-1 activation in BECs has been proposed. Activation of IL3-Rα signaling pathway in BECs and LECs leads to up regulation of Prox-1 and Prox-1 mediated lymphatic reprogramming, while at the same time induces the down regulation of Notch and COUP-TFII, which are essential for maintaining BEC phenotypes (328).

Overall, both LECs and BECs showed remarkable heterogeneity and plasticity in physiological and pathological conditions. Understanding of the molecular mechanisms that control the heterogeneity and plasticity in LECs is important to understand the underlying pathologic processes that could facilitate design of improved therapeutics, as well as discovery of novel therapeutic strategies that target LECs.

5.4 LYMPHATIC ENDOTHELIAL CELLS INNATE IMMUNITY ROLE IN PATHOGEN-HOST INTERACTIONS

5.4.1 Pattern recognition receptors

The concept of innate immunity was first proposed by Charles A. Janeway Jr. in the late 1980s (329). Janeway suggested that innate immune system recognition is based on germ-line encoded receptors, known as pathogen recognition receptors (PRRs) that recognizes the conserved motifs on microbes and microbial products known as pathogen-associated molecular

patterns (PAMPs) (329, 330). Since then several groups of PRR families have been discovered, including toll-like receptors (TLRs), retinoic acid-inducible gene 1-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and C-type lectin receptors (CLRs). Viral detection of PRRs through sensing of viral nucleic acids and proteins leads to initiation of type I interferon (IFN) responses in the host, which includes increase production of proinflammatory cytokines and chemokines, as well as interferon stimulating genes (ISGs) such as intrinsic cellular viral restriction factors (331).

The expression of TLRs by LECs is well established. Primary human LECs from different organs have been demonstrated to express multiple functional TLRs and responded to their known ligands to varying degrees (160, 247). We found that primary ferret and macaque LECs too expressed multiple functional TLRs that responded to known PAMPs in a similar fashion as their human counterparts. We found that poly I:C, a double-stranded RNA virus mimetic and a known ligand for TLR3 and RIG-I was the most potent inducer of proinflammatory responses in primary ferret and macaque LECs. A more comprehensive analysis of primary ferret lung LECs and primary macaque jejunal LECs demonstrated that Pam3CSK4 (TLR2 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand) were also able to induce significant induction of proinflammatory responses in in vitro cultured LECs upon treatment. Analysis of a more comprehensive list of ISGs by Nanostring analysis revealed that primary macaque jejunal LECs shared similar gene expression profiles with primary human LECs before and after treatment with poly I:C, suggesting that LECs from different species responded to PAMPs in similar manner. Therefore, in vitro cultured primary macaque and ferret LECs derived from this study are invaluable tools to study LEC-pathogen interactions of human viral pathogens, and the potential of LECs as target for improved vaccine designs that induce more

robust host innate immunity and subsequently more effective host adaptive immunity to pathogens.

Our data also revealed that poly I:C treatment of primary LECs induced production of other ISGs such as the intrinsic viral restriction factors. Viral restriction factors such as APOBEC3G, tetherin (BST2), Trim5-α, and SAMHD1 were expressed endogenously to relative high levels and were significantly increased after exposure to poly I:C by primary macaque jejunal LECs. Intrinsic viral restriction factors have been shown to counteract productive viral infection and replication in the host cells. In addition, intrinsic cellular viral restriction factors have also been demonstrated to contribute to permissiveness of the host cells to infection by viral pathogens.

Taken together, the ability of LECs to sense incoming viral pathogens and induce type I IFN responses as well as other ISGs demonstrate their potential innate immune functions. LEC sensing of PAMPs via PRRs can potentially be exploited for improved designs of vaccine formulations that incorporated the use of PRR ligands as adjuvants. Similarly, LEC-induced ISGs such as the intrinsic viral restriction factors also suggest their innate immune capability during early phase of infection in blocking different stages of viral life cycle. In addition, LECs may also act as barrier to prevent transmission of these viral pathogens.

5.4.2 LECs as targets for TLR ligand vaccine adjuvants

The use of adjuvants in vaccine formulations is first to increase the magnitude of host innate immune response to the vaccine administered, which will then contribute to enhanced host adaptive immune response (332). The second aim of the use of adjuvants is to shape the type of host innate immune response triggered by the vaccine administered, which will then lead to more

targeted activation of the host adaptive immune response. The PRRs are attractive targets for vaccine adjuvants as they are key players in priming, activation, expansion, and polarization of host innate immunity through induction of cytokine, chemokine, and costimulatory molecules that are essentials in these cellular processes (333-335). Moreover, PRRs are expressed by both hematopoietic cells and stromal cells, and are integral components of crosstalk of these cells with each other and also their local tissue microenvironments (336, 337).

PRR ligands can be used as adjuvant either alone or in combination with existing vaccine formulations (332). Currently, there are several PRR ligands undergoing preclinical as well as clinical development for both efficacy and safety for use in multiple applications related to human and veterinary diseases candidate vaccines. For example, several synthetic analogs of poly I:C (TLR3 and RIG-I ligand) have been used as adjuvants for development of soluble proteins, DC-targeting construct, and inactivated viral vaccines (338, 339). The use of poly I:C in these vaccine formulations enhanced the magnitude of innate immunity and resulted in prolonged durability of adaptive immunity in vaccinated host (339). Initial development of LPS (TLR4 ligand) as vaccine adjuvants led to the discovery of a much less toxic LPS-derivative called monophosphoryl lipid A (MPL), which is a potent inducer of TLR4 (332). MPL has been used as vaccine components in combination with alum for HBV and HPV vaccines in humans (340). It was documented that combination of MPL and alum stimulated a polarized Th1 response in contrast to mixed Th1 and Th2 response when using alum alone (340-342). Similarly, bacterial flagellin (TLR5 ligand) was reported to induce mixed Th1 and Th2 response when used as vaccine adjuvants alone (343). Therefore, the current development of flagellin as vaccine adjuvant focused on generation of recombinant fusion proteins expressing the vaccine antigens and flagellin. The production of the fusion protein required TLR5 signaling and thus

target both hematopoietic cells and stromal cells when tested in mouse model (344). Similarly, the development of subunit vaccines candidates that incorporates covalently coupled TLR7-TLR8 ligands and TLR9 ligand was able to enhance the uptake and subsequent presentation of the vaccine antigens by DCs (332).

Evidently, the use of TLR ligands as vaccine adjuvants has been proven to mediate enhanced immunity towards the vaccine antigens administered. However, there are lingering safety issues when it comes to the use of TLR ligands as vaccine adjuvants in humans, such as the increased risk of autoimmune disease. Thus, there is a need to develop in vitro models as well as animal models that faithfully predict PRRs response and immune activation in humans. In conclusion, it is possible to shape the desired immune responses through incorporation of PRR ligands as adjuvants, alone or in combination with other non-PRR adjuvants, for both prophylactic and therapeutic vaccines for infectious diseases and cancer.

5.4.3 Viral restriction factors

Intrinsic cellular viral restriction factors are important component of the host intracellular innate immune system. Unlike PRRs, intrinsic cellular viral restriction factors are able to act immediately and inhibit viral replication by binding directly to viral components (296). These viral restriction factors exist endogenously in host cells although their expression can be induced further upon contact with viral proteins or components. The expression of these viral restriction factors often determine the susceptibility and permissiveness of a cell type to virus infection as well as an important determining factor of virus tropism. Many of these restriction viral factors are by products of strong coevolutionary process between the host and the virus, thus a native viral restriction factors may not be as efficient or are only weakly efficient in blocking the viral

infection in its natural hosts. However, interspecies inhibition of viral replication is highly efficient, thus viral restriction factors have important in innate immune barrier function in preventing the spread of interspecies zoonotic diseases to humans. This interspecies barrier function of viral restriction factors is exemplified in the efficiency of the simian APOBEC3G protein to confer resistance to degradation mediated by HIV-1 Vif protein, and thus resulted in successful inhibition of the viral genome reverse transcription process by genome editing and introducing premature stop codon into the new viral genome (345-348). In addition, APOBEC3G inhibition of HIV-1 is through its function as a cytidine deaminase that induces hypermutations (G to A) in the negative strand of the viral genome, thus preventing its integration into host genome (349). Innate mechanism of APOBEC3G has also been proposed to occur prior to the reverse transcription stage of the viral RNA, thus making it an attractive target for anti-HIV agent (350). Similarly, human Trim5-α restricts murine leukemia virus (MLV) infection but not HIV-1 and SIV, and rhesus Trim5-α restricts HIV-1 but not SIV (296). Trim5-α promotes rapid uncoating of HIV-1 capsid in vitro, and also acts as ubiquitin E3 ligase in both proteasome-dependent and proteasome-independent pathways. In addition, viral restriction factor may also contributed to species specificity and shaping the evolution of virus genomes, such as in the case of primate lentivirus (351). For example, most SIV isolates do not encode the viral protein Vpu, which promotes degradation of tetherin (BST2). Tetherin (BST2) promotes degradation of newly formed HIV-1 virion by tethering the virion to the infected cell membrane and prevent viral release (352). These virions are then internalized back into the cells by endocytosis and subjected for degradation in the endosomes. SIV Nef protein is unable to antagonize the human tetherin activity due to missing amino acids for binding of the viral protein

(296, 353). SAMHD1 blocks HIV-1 virus reverse transcription process by depleting the dNTPs pool in myeloid cells (354).

Mucosal expression of viral restriction proteins along with other type I IFN molecules have been correlated with enhanced mucosal recovery in the lung and restoration of CD4+ T cells in after HIV infection in macaque model (355). Viral restriction factors are expressed by a number of different cell types including DCs, macrophages, and also other stromal cells such as LECs. Thus, viral restriction factors may have additional role in innate immune signaling during viral infection. Trim5-α also acts as sensory mechanism for viral capsid protein during infection with MLV, SIV, and HIV-1 virus, and sensing by Trim5-α triggers type I IFN responses in host (296). However, the involvement of viral restriction factors in immune signaling may not always have positive effect on the host and can be more advantageous to the virus. Tetherin binds to a membrane receptor selectively expressed by plasmacytoid DCs, known as ILT7, which leads to inhibition of TLR-mediated effector function of DCs.

Investigation of host viral restriction factors will elucidate how viruses evade the host innate immune system and use it to their advantage. At the same time, this knowledge will allow us to understand the mechanisms of evasion utilized by these viruses as targets to design better therapeutics for viral infections in humans.

5.5 LYMPHATIC ENDOTHELIAL CELLS AS POTENTIAL TARGETS FOR PATHOGENS

LECs and BECs that line the lymphatic and blood vasculature are poised to come in contact with infectious agents that are transported in the lymph to the draining lymph node or in the blood

circulation. There are several human pathogens of bacterial and viral origins that have been designated as truly endothelium-targeted (356). Most of these pathogens are able to enter and replicate within endothelial cells throughout the course of infection. In addition to that the clinical pathological hallmark of these diseases are the loss of endothelial vasculature permeability possibly as a result of infection of the endothelial cells themselves or damaging effect of inflammatory cytokines and chemokines. Most of the endothelial-targeting pathogens we know currently targets BECs and very few pathogens are known to target LECs. For example, hemorrhagic fever viruses including bunyaviruses (Hantavirus), phleboviruses (Rift Valley Fever virus), filoviruses (Ebola virus and Marburg virus), and flaviviruses (Dengue virus) target and infect BECs in both in vitro and in vivo models. In some cases, even though BECs may not be the direct target for infection by viral pathogens, production of costimulatory molecules and adhesion molecules by the BECs could contribute to pathophysiological consequences in vivo. In the case of Nipah virus infection, increase permeability of the blood brain barrier function of BECs enabled the virus to enter the brain and infected its target cells, the neurons, and thus caused viral encephalitis. Furthermore, endothelial infection by viral pathogens may be species-species such as in the case of lung endothelial infection by highly pathogenic avian influenza virus (HPAIV) (357). HPAIV targets the lung endothelial cells for infection in terrestrial poultry and wild bird species however in human infections of HPAIV the lung endothelial cells have been shown to be less susceptible to viral infection and play a more important role in controlling the local inflammatory milieu upon exposure to the virus.

5.5.1 Viral entry factors

BECs and LECs are similar in many aspect of their gene expression profiles, and thus it is highly possible that LECs are also potential targets for pathogens that infect BECs. We found that primary human dermal LECs express the macrophage mannose receptor (MMR or CD206), which has been proposed for viral entry of dengue virus into host cells (358). We also found that CD26 was highly expressed in both primary human and macaque LECs. CD26 was recently proposed to be the viral entry factor for Middle Eastern Respiratory Syndrome Coronavirus (MERS-Cov) (293, 359). A more comprehensive analysis of the expression of viral entry factors and coreceptors by LECs showed that primary macaque LECs of different tissue origins express the CD4 and CCR5 molecules, which are known receptor and coreceptor for HIV-1 virus entry. Our data also demonstrated that primary human dermal LECs and primary macaque jejunal LECs showed that these primary LECs express several other molecules that have been shown to be utilized by both HIV-1 and SIV for entry into susceptible host cells such as CXCR4, CXCR6, GPR15, and D6, suggestive of their potentials as target for SIV and HIV-1 infection. LECs involvement in the pathogenesis of HIV infection and AIDS development may not be only restricted to their potentials as target cells. Our group and others have demonstrated the ability of LECs to take up and archive exogenous antigens. We also found that primary macaque LECs were resistant to infection with genetically engineered VSVG-pseudotyped SIV and HIV-1 and also wild-type SIV when compared to control cell populations, the CEMx174 cells and the HeLa cells. However, analysis for SIV RNA and DNA from culture supernatants and cell pellets of wild type SIV-exposed primary macaque jejunal LECs suggested that low level and possibly controlled SIV virus replication had occurred.

The restricted susceptibility of BEC to different strains of HIV-1 and HIV-2 viral infection has been demonstrated (360). BECs that lined the larger blood vessels did not express the CD4 molecule but were infected through their constitutive expression of CXCR4 molecule (361). It was reported that mature virus production in infected endothelial cells was evident during the first few days post-infection but declined to undetectable levels in both supernatants and virus-exposed endothelial cells (362). HIV-infected BECs did not result in syncytia forming cytopathic effect when culture alone in in vitro model. However, coculture of HIV-infected BECs with CD4+ lymphoid and mononuclear cells resulted in formation of syncytia in these cells, suggesting that BECs might have been infected by HIV-1 although syncytia formation was not observed. Other studies have also demonstrated that HIV-1 infection in in vitro culture of microvascular BECs from brain, kidney glomeruli, hepatic sinusoid, and bone marrow potentially resulted in productive infections that were devoid of syncytia formation and cell cytolysis as well (361). HIV-1 infection of brain endothelial cells is highly studied due to its relevance in HIV-associated neurological diseases. Brain endothelial cells were reported to be able to endocytose HIV-1 virus through interaction of the virus gp120 glycoprotein with CCR5 and CXCR4 molecules expressed by the cells (363).

Taken altogether, these data suggest that LECs could potentially be infected to some extent and act as a low level viral reservoir and facilitate the dissemination as well as chronic infection of the SIV and HIV-1 virus.

5.5.2 Other immune receptors

Our data also revealed that primary LECs expressed the atypical chemokine receptor, D6, which binds to homeostatic and proinflammatory chemokines, and then internalizes them for recycling

(54, 364). Thus, D6 is an important regulator of the homeostasis as well as inflammatory processes. D6 has been demonstrated to control the interaction of inflammatory leukocytes with LECs and also discrimination of mature and immature DCs by LECs (55). In addition, D6 expression by LECs could potentially be involved in KSHV virus pathogenesis through interaction with the viral homolog of IL-6 protein (55). Although the exact mechanism is still unknown, it was proposed that the KSHV viral IL-6 homolog increased D6 expression in KSHV-infected cells, and resulted in impaired release of proinflammatory CC chemokines from the tumor microenvironment, and compromised clearance of the tumor cells by tumor-specific host immune cells. Our data also revealed that CD40 was endogenously expressed to high level in primary LECs. CD40 has been proposed to play a role in KS tumor pathogenesis. In vitro culture of KS tumor cells revealed high level of CD40 expression, and addition of IFN-γ increased the anti-apoptotic and pro-angiogenic properties of these tumor cells (365). Thus, CD40 is potential contribute to KS pathogenesis through mechanisms that stimulate tumor growth, neoangiogenesis, and anti-apoptosis processes.

Taken together, these data suggested that LECs can either directly or indirectly contribute to pathogenesis due to viral infections. LECs can be targeted for infection by pathogens, which then potentially alter or impair their physiological and immunological functions. LECs can also modulate the local microenvironment through production of proinflammatory cytokines and chemokines, which can alter or impair the effector functions of host immune cells as well as host cells processes. Understanding of the mechanisms involved in contribution of LECs in pathogen-host interactions and disease progression would offer the possibility of developing endothelium-specific therapeutics and vaccines for important human viral infections.

6.0 OVERALL SUMMARY

Due to their unique anatomical locations, LECs are poised to come in contact with incoming pathogens or vaccine antigens and are actively involved in the initial development of host innate immune responses to these foreign antigens. Based on our findings and others, I propose three different models to frame LEC innate immune involvement and contributions in pathogen-host interactions during infection or vaccine administration. Each of these models is discussed in detailed below.

6.1 MODEL ONE: PATHOGEN RECOGNITION RECEPTOR SENSING OF PATHOGENS AND PRODUCTION OF PROINFLAMMATORY CYTOKINE AND CHEMOKINES

We demonstrated that LECs express multiple functional PRRs and were able to respond to PAMPs by producing proinflammatory cytokines and chemokines. Thus, we propose that LECs are actively involved in host innate immune responses through initial sensing of incoming pathogens or vaccine antigens by PRRs and subsequent secretion of proinflammatory mediators. These LEC-derived proinflammatory signals will then recruit immune cells including immature DCs. Consequently, antigen-loaded mature DCs will carry these foreign antigens to the draining lymph node to further activate the host adaptive immune response.

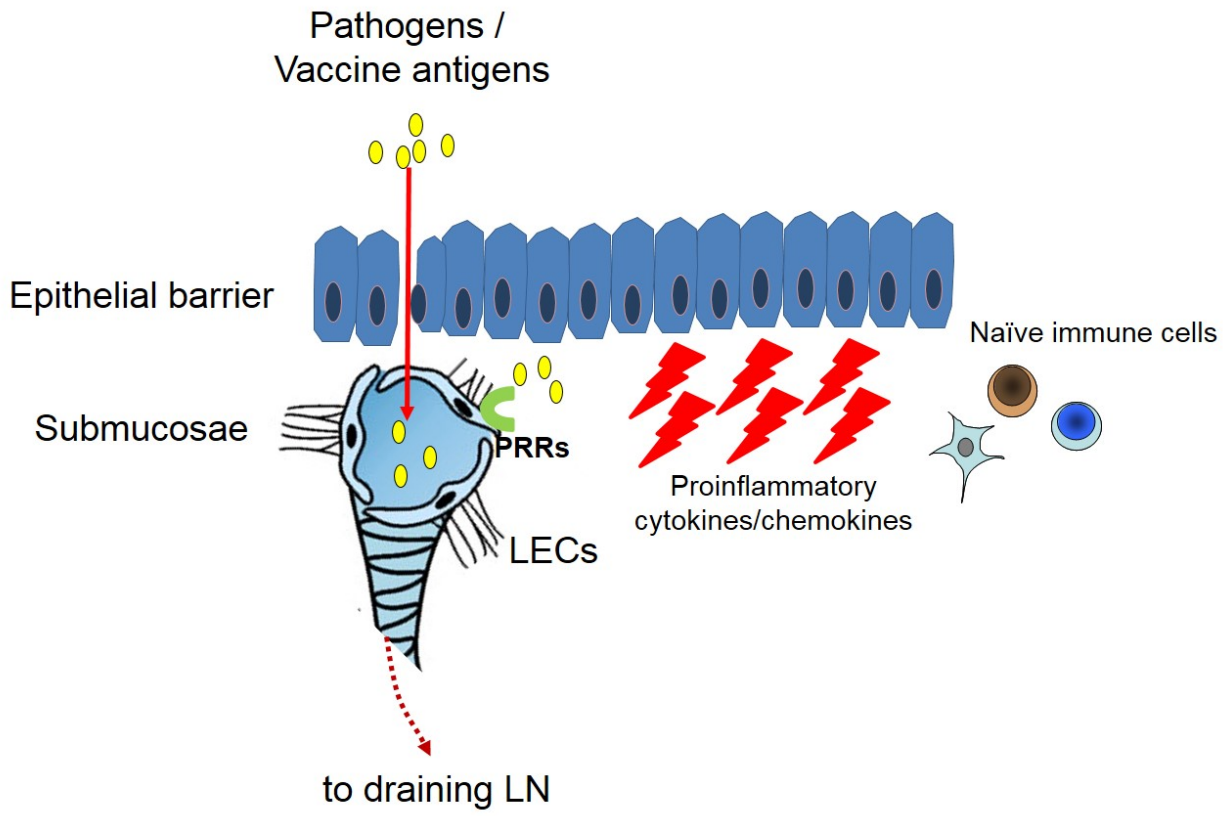


Figure 24. Innate Immune Potential of LECs - Model One

PRR sensing of pathogens results in production of proinflammatory chemokines/cytokines by LECs, which leads to recruitment of naïve immune and immune effector cells.

6.2 MODEL TWO: ANTIGEN UPTAKE, PROCESSING, AND ARCHIVING

We have also demonstrated that primary LECs are able to take up and process exogenous antigens in in vitro 2D models. Other have shown that in vivo LECs also share the same functional capability (166). Thus, we propose that LECs contribute to host innate immunity through their ability to perform antigen uptake and processing, and also archiving of these exogenous antigens for optimal contact with APCs, such as immature DCs.

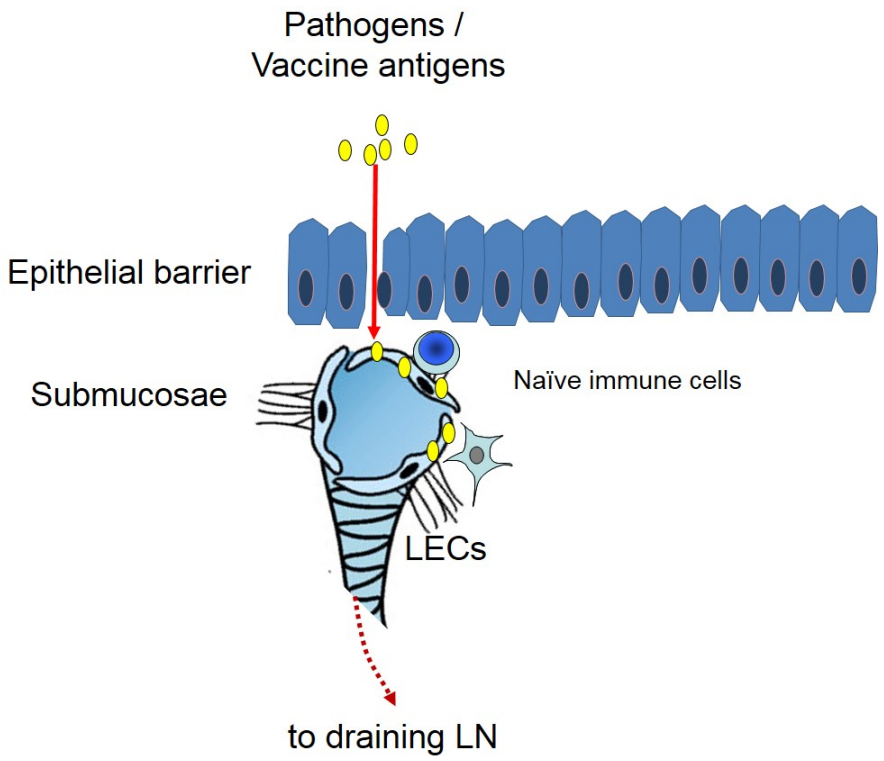


Figure 25. Innate Immune Potential of LECs - Model Two

LECs potentially capture and archive pathogens by antigen uptake/processing, and thus enable optimal contact of antigens with immune cells.

6.3 MODEL THREE: EXPRESSION OF INTRINSIC CELLULAR VIRAL RESTRICTION FACTORS

We also found that LECs endogenously expressed known intrinsic viral restriction factors against HIV-1, SIV, and other RNA viruses. It is possible that LECs also endogenously express additional as of yet unknown intrinsic viral restriction factors against other important human viral pathogens. Thus, we propose that LECs can act as an innate immune barrier in pathogen-host interactions during viral infection, and prevent initial establishment of infection, productive viral replication, and also viral transmission. We also propose that LEC-derived viral restriction factors contribute to their resistance to infection by viruses.

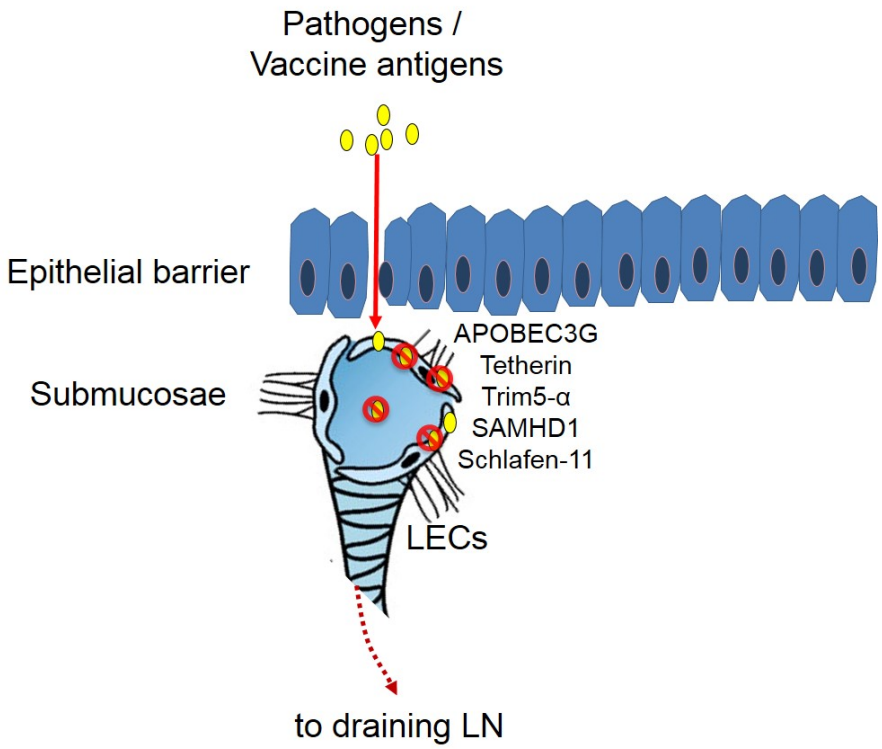


Figure 26. Innate Immune Potential of LECs - Model Three

LECs potentially act as barriers to viruses through expression of several intrinsic and viral restriction factors that presumably control and block productive infection to prevent establishment and transmission of the viral pathogens.

7.0 PUBLIC HEALTH SIGNIFICANCE

Vaccines are an effective public health measure to prevent and eliminate the spread of infectious diseases. Despite many successful vaccines, there are still no vaccines available for a vast number of important human pathogens of viral origins, such as HIV-1, Dengue, Ebola, and many others. Most successful vaccines are based on live-attenuated or inactivated (killed) forms of viruses, as well as viral antigenic subunits, such as proteins, synthetic peptides, polysaccharides, and glycoconjugates. These vaccines mimic the real pathogens and are designed to induce robust and long-term protective immunity in the host. As we understand more about the human immune system, we begin to appreciate the importance of innate immunity for generation of robust and adaptive immune responses as a result of vaccination. It is well established that the quality of adaptive immune responses is dependent on the magnitude of innate immune responses. Stromal cells such as LECs are integral players in initiation of host innate immunity during pathogen-host interactions through their ability to sense pathogens via PRRs, secrete proinflammatory molecules for recruitment of immune cells, and also crosstalk with hematopoietic cells, including antigen presenting cells such as DCs. Therefore, LECs are attractive target cells in our efforts to improve current vaccines and to develop effective vaccines strategies against emerging and chronic viral infections. In the context of HIV-1 vaccine development, LECs can potentially be manipulated to generate more robust responses for development of pre- or post-exposure prophylaxis therapeutics using PRR ligands as adjuvants,

thus preventing the initial establishment of the small founder virus population. LECs are involved in transport of vaccine antigens from the peripheral tissues to the draining lymph node during vaccination, and thus crosstalk of LECs with antigen presenting cells (DCs) and T cells can be useful in revising current methods of vaccine delivery. Improved understanding of the mechanisms involved in innate immune barrier functions of LECs through the expression of intrinsic viral restriction factors could potentially lead to discovery of new antiviral mechanisms against HIV-1 and other viruses, which can potentially be applied to development of novel antiviral therapeutics.

8.0 FUTURE DIRECTIONS

This body of work has expanded our understanding of LEC immunobiology and provided us with the tools to investigate further the important aspects of LEC crosstalk with other immune cells during pathogen-host interactions. Over the course of this study, our findings led to more exciting possibilities and questions that could not be addressed here due to time constraints. The sections below outline some of the key areas that warrant future investigations in order to expand our understanding of innate immune potential of LEC.

8.1 DEVELOPMENT OF 3D IN VITRO CULTURE OF LECS

I have isolated, characterized, and established 2D in vitro cultures of primary LECs from different animals and tissues types. These primary cells can now be used as tools to develop 3D in vitro cultures of primary LEC that mimic closer the physiological and functional conditions of these cells in vivo. Three-dimensional in vitro cultures of LEC can then be used to investigate further the crosstalk of LECs with microbes including HIV-1 and SIV, as well as cell-to-cell interactions of LECs with other immune or nonimmune cells. These future studies will hopefully give us more accurate representation of the innate immune potentials of LECs.

8.2 PROFILING OF IMMUNE RECEPTORS EXPRESSED BY PRIMARY LECS

My data showed that primary LECs expressed a set of immune receptors that potentially contribute to their innate immune ability as well as their ability to modulate the local tissue microenvironments during homeostasis or as part of pathogen-host interactions. However, there is a need to confirm these findings functionally in in vitro and in vivo models. In addition, a more comprehensive analysis of the immune receptor profiles expressed by LECs in absence or presence of an infection is needed. This knowledge will not only expand our understanding of LEC contributions and involvement in host innate and adaptive immunity but could also lead to discoveries of novel strategies to improve current therapeutics and vaccines against important human pathogens such as HIV-1.

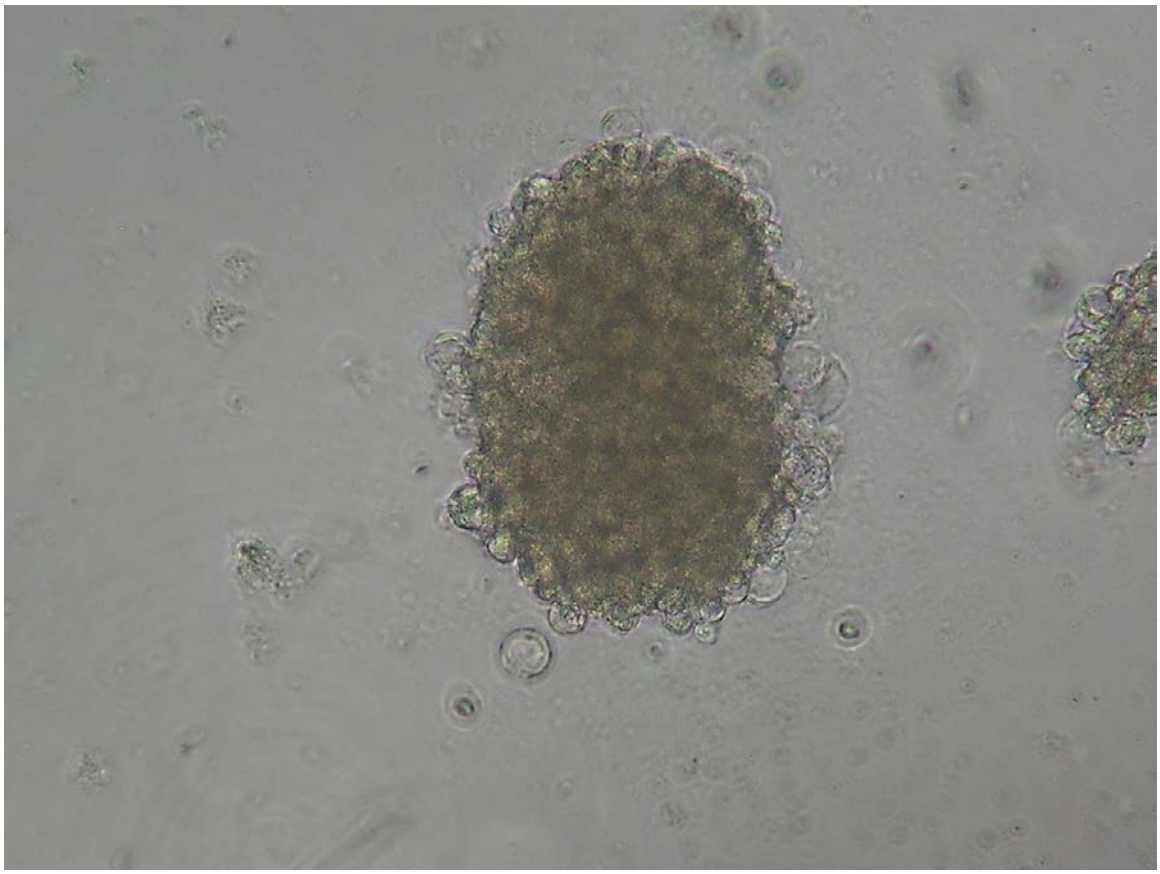
8.3 PROFILING OF MICRORNAS EXPRESSED BY PRIMARY LECS

MicroRNAs (miRNAs) are short, non-coding cellular RNAs that bind to host mRNAs based on sequence complementarity and are important regulators of genes that control the key cellular processes including cellular lineage, differentiation, and death. More recently, host miRNAs have been reported to directly target HIV-1 and influence cells permissiveness to infection. More importantly, additional evidence showed that miRNAs modulate the expression of host defense factors, including PRRs and other immune receptors, during HIV-1 infection. Comprehensive analysis of the primary LEC miRNA profiles in the absence and presence of HIV-1 infection could lead to discovery of new biomarkers for virus-induced disease progression in HIV-1-infected individuals.

8.4 ANTIGEN PRESENTATION VIA MHC I AND MHC II BY LECS

Data from this study corroborated the findings that LECs were capable of performing antigen uptake and processing, albeit less efficient than DCs. However, it was not clear whether these exogenous antigens were subjected to terminal degradation, transferred to incoming naïve DCs for antigen presentation, or were directly presented by the LECs via MHC I or MHC II molecules to T cells. In addition, it will also be interesting to examine further whether potential antigen presentation by LEC-derived MHC I and MHC II to autologous T cells results in the same outcomes as antigen presentation by DCs.

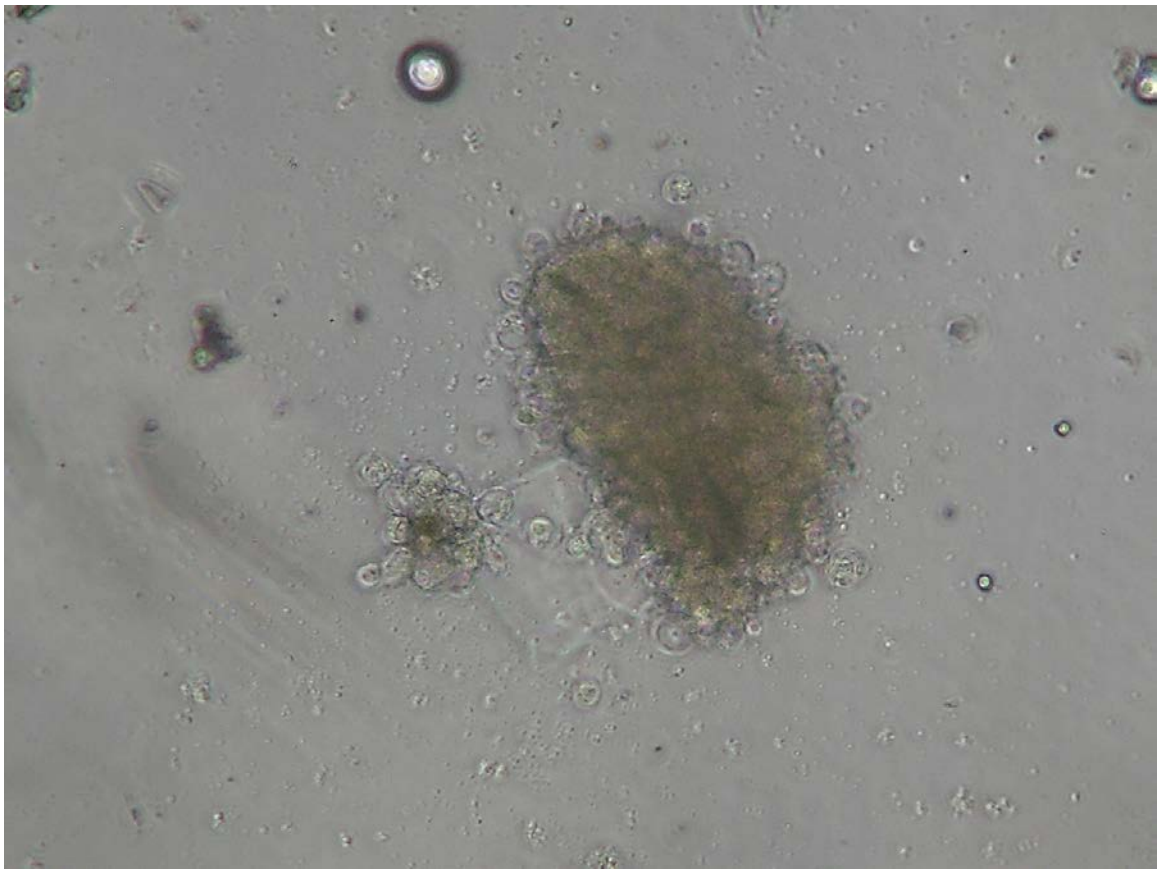
APPENDIX: 3D MODEL OF PRIMARY HUMAN AND MACAQUE LECS



(100X)

3D multicellular spheroid culture of model human dermal LECs using poly-HEMA coating method at day 3 after plating.

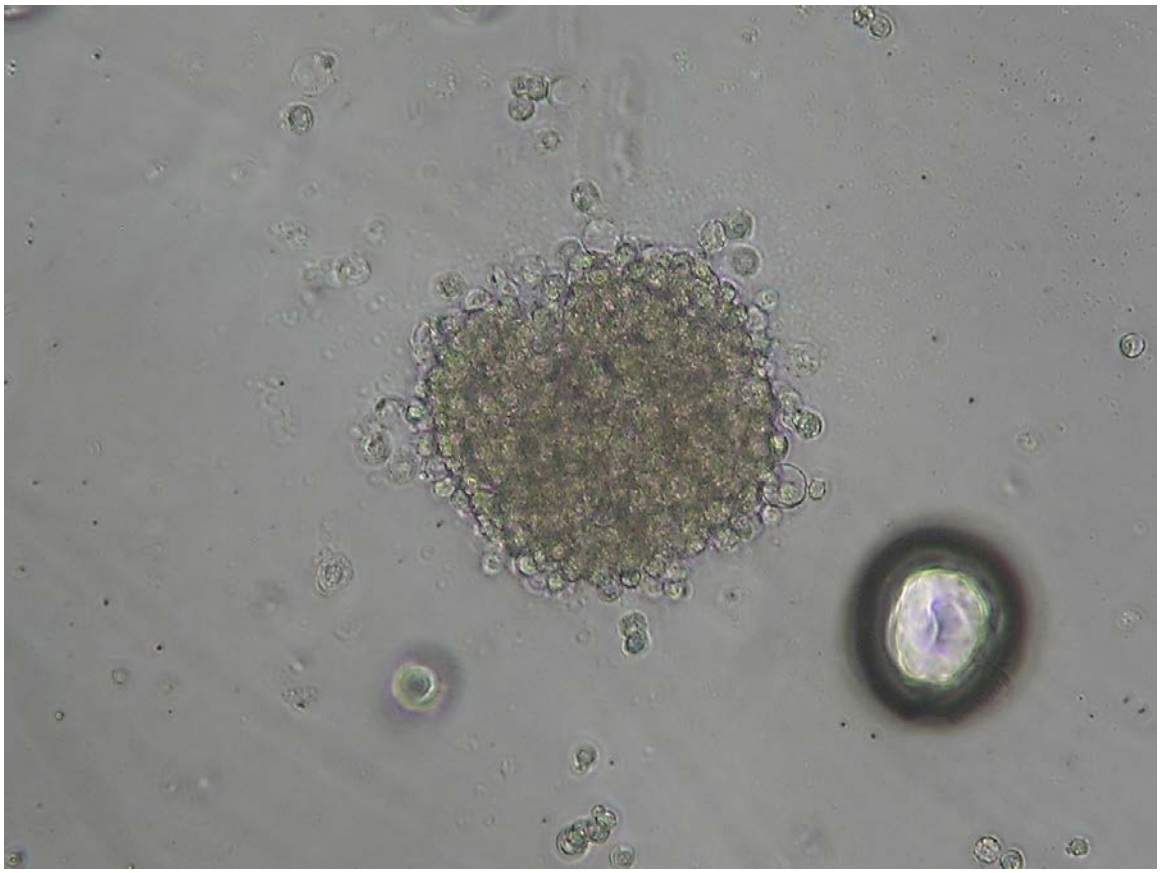
A total number of 500 live cells were plated onto each well of a round bottom 96-well plate coated prior with poly-HEMA. Cells were cultured using EGM2-MV media (Lonza) supplemented with 5% FBS at 37°C, 5% CO_2 for three days. Image was taken using a brightfield microscope using Nikon camera.



(100X)

3D multicellular spheroid culture of one primary jejunal LEC polulation (rhR564 Jejunal) using poly-HEMA coating method at day 3 after plating.

A total number of 500 live cells were plated onto each well of a round bottom 96-well plate coated prior with poly-HEMA. Cells were cultured using EGM2 media (Lonza) supplemented with 2% FBS at 37°C, 5% CO_2 for three days. Image was taken using a brightfield microscope using Nikon camera.



(100X)

3D multicellular spheroid culture of model human dermal LECs using hanging drop method at day 3 after plating.

A total number of 500 live cells were plated in a final volume of 25 µl of final concentration of 20% methyl cellulose in EGM2-MV (Lonza) supplemented with 2% FBS and cultured at 37°C, 5% CO_2 for three days. Image was taken using a brightfield microscope using Nikon camera.

BIBLIOGRAPHY

1. Cueni, L. N., and M. Detmar. 2008. The lymphatic system in health and disease. *Lymphatic Research and Biology* 6: 109-122.
2. Jeltsch, M., T. Tammela, K. Alitalo, and J. Wilting. 2003. Genesis and pathogenesis of lymphatic vessels. *Cell and Tissue Research* 314: 69-84.
3. Choi, I., S. Lee, and Y. K. Hong. 2012. The new era of the lymphatic system: no longer secondary to the blood vascular system. *Cold Spring Harbor Perspectives in Medicine* 2: a006445.
4. Margaris, K. N., and R. A. Black. 2012. Modelling the lymphatic system: challenges and opportunities. *Journal of the Royal Society, Interface / the Royal Society* 9: 601-612.
5. Loukas, M., S. S. Bellary, M. Kuklinski, J. Ferrauiola, A. Yadav, M. M. Shoja, K. Shaffer, and R. S. Tubbs. 2011. The lymphatic system: a historical perspective. *Clinical Anatomy* 24: 807-816.
6. Crivellato, E., L. Travan, and D. Ribatti. 2007. The Hippocratic treatise 'On glands': the first document on lymphoid tissue and lymph nodes. *Leukemia* 21: 591-592.
7. Miller, A. J., and A. Palmer. 1995. The three Williams--Hunter, Hewson and Cruikshank: their unique contributions to our knowledge of the lymphatics. *Lymphology* 28: 31-34.
8. Liao, S., and T. P. Padera. 2013. Lymphatic function and immune regulation in health and disease. *Lymphatic Research and Biology* 11: 136-143.
9. Jurisic, G., and M. Detmar. 2009. Lymphatic endothelium in health and disease. *Cell and Tissue Research* 335: 97-108.
10. Wang, Y., and G. Oliver. 2010. Current views on the function of the lymphatic vasculature in health and disease. *Genes & Development* 24: 2115-2126.
11. Alitalo, K. 2011. The lymphatic vasculature in disease. *Nature Medicine* 17: 1371-1380.
12. Oliver, G., and M. Detmar. 2002. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes & Development* 16: 773-783.
13. Kesler, C. T., S. Liao, L. L. Munn, and T. P. Padera. 2013. Lymphatic vessels in health and disease. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine* 5: 111-124.
14. Tammela, T., and K. Alitalo. 2010. Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* 140: 460-476.
15. Bielenberg, D. R., and P. A. D'Amore. 2013. All vessels are not created equal. *Am J Pathol* 182: 1087-1091.
16. Rovenska, E., and J. Rovensky. 2011. Lymphatic vessels: structure and function. *The Israel Medical Association Journal : IMAJ* 13: 762-768.

17. Baluk, P., J. Fuxe, H. Hashizume, T. Romano, E. Lashnits, S. Butz, D. Vestweber, M. Corada, C. Molendini, E. Dejana, and D. M. McDonald. 2007. Functionally specialized junctions between endothelial cells of lymphatic vessels. *J Exp Med* 204: 2349-2362.
18. Scavelli, C., E. Weber, M. Agliano, T. Cirulli, B. Nico, A. Vacca, and D. Ribatti. 2004. Lymphatics at the crossroads of angiogenesis and lymphangiogenesis. *Journal of Anatomy* 204: 433-449.
19. Schulte-Merker, S., A. Sabine, and T. V. Petrova. 2011. Lymphatic vascular morphogenesis in development, physiology, and disease. *The Journal of Cell Biology* 193: 607-618.
20. Bennuru, S., and T. B. Nutman. 2009. Lymphatics in human lymphatic filariasis: in vitro models of parasite-induced lymphatic remodeling. *Lymphatic Research and Biology* 7: 215-219.
21. Bennuru, S., and T. B. Nutman. 2009. Lymphangiogenesis and lymphatic remodeling induced by filarial parasites: implications for pathogenesis. *PLoS Pathogens* 5: e1000688.
22. Hosking, B., and T. Makinen. 2007. Lymphatic vasculature: a molecular perspective. *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 29: 1192-1202.
23. Johnson, L. A., and D. G. Jackson. 2008. Cell traffic and the lymphatic endothelium. *Annals of the New York Academy of Sciences* 1131: 119-133.
24. Card, C. M., S. S. Yu, and M. A. Swartz. 2014. Emerging roles of lymphatic endothelium in regulating adaptive immunity. *The Journal of Clinical Investigation* 124: 943-952.
25. Angeli, V., and G. J. Randolph. 2006. Inflammation, lymphatic function, and dendritic cell migration. *Lymphatic Research and Biology* 4: 217-228.
26. Benahmed, F., S. Ely, and T. T. Lu. 2012. Lymph node vascular-stromal growth and function as a potential target for controlling immunity. *Clinical Immunology* 144: 109-116.
27. Aebischer, D., M. Iolyeva, and C. Halin. 2014. The inflammatory response of lymphatic endothelium. *Angiogenesis* 17: 383-393.
28. Saeki, H., A. M. Moore, M. J. Brown, and S. T. Hwang. 1999. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* 162: 2472-2475.
29. Ohl, L., M. Mohaupt, N. Czeloth, G. Hintzen, Z. Kiafard, J. Zwirner, T. Blankenstein, G. Henning, and R. Forster. 2004. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 21: 279-288.
30. Bromley, S. K., S. Y. Thomas, and A. D. Luster. 2005. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nature Immunology* 6: 895-901.
31. Johnson, L. A., and D. G. Jackson. 2013. The chemokine CX3CL1 promotes trafficking of dendritic cells through inflamed lymphatics. *Journal of Cell Science* 126: 5259-5270.
32. Beauvillain, C., P. Cunin, A. Doni, M. Scotet, S. Jaillon, M. L. Loiry, G. Magistrelli, K. Masternak, A. Chevailler, Y. Delneste, and P. Jeannin. 2011. CCR7 is involved in the migration of neutrophils to lymph nodes. *Blood* 117: 1196-1204.
33. Johnson, L. A., S. Clasper, A. P. Holt, P. F. Lalor, D. Baban, and D. G. Jackson. 2006. An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. *J Exp Med* 203: 2763-2777.

34. Johnson, L. A., and D. G. Jackson. 2010. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol* 22: 839-849.
35. Russo, E., M. Nitschke, and C. Halin. 2013. Dendritic cell interactions with lymphatic endothelium. *Lymphatic Research and Biology* 11: 172-182.
36. Kabashima, K., N. Shiraishi, K. Sugita, T. Mori, A. Onoue, M. Kobayashi, J. Sakabe, R. Yoshiki, H. Tamamura, N. Fujii, K. Inaba, and Y. Tokura. 2007. CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol* 171: 1249-1257.
37. Van Dyck, F., C. V. Braem, Z. Chen, J. Declercq, R. Deckers, B. M. Kim, S. Ito, M. K. Wu, D. E. Cohen, M. Dewerchin, R. Derua, E. Waelkens, L. Fiette, A. Roebroek, F. Schuit, W. J. Van de Ven, and R. A. Shivdasani. 2007. Loss of the PlagL2 transcription factor affects lacteal uptake of chylomicrons. *Cell Metabolism* 6: 406-413.
38. Wigle, J. T., and G. Oliver. 1999. Prox1 function is required for the development of the murine lymphatic system. *Cell* 98: 769-778.
39. Harvey, N. L., R. S. Srinivasan, M. E. Dillard, N. C. Johnson, M. H. Witte, K. Boyd, M. W. Sleeman, and G. Oliver. 2005. Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. *Nature Genetics* 37: 1072-1081.
40. Brorson, H., K. Ohlin, G. Olsson, and M. Nilsson. 2006. Adipose tissue dominates chronic arm lymphedema following breast cancer: an analysis using volume rendered CT images. *Lymphatic Research and Biology* 4: 199-210.
41. Nordskog, B. K., C. T. Phan, D. F. Nutting, and P. Tso. 2001. An examination of the factors affecting intestinal lymphatic transport of dietary lipids. *Advanced Drug Delivery Reviews* 50: 21-44.
42. Keuschnigg, J., S. Karinen, K. Auvinen, H. Irjala, J. P. Mpindi, O. Kallioniemi, S. Hautaniemi, S. Jalkanen, and M. Salmi. 2013. Plasticity of blood- and lymphatic endothelial cells and marker identification. *PloS One* 8: e74293.
43. Sleeman, J. P., J. Krishnan, V. Kirkin, and P. Baumann. 2001. Markers for the lymphatic endothelium: in search of the holy grail? *Microscopy Research and Technique* 55: 61-69.
44. 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. *Proceedings of the National Academy of Sciences of the United States of America* 92: 3566-3570.
45. Kukk, E., A. Lymboussaki, S. Taira, A. Kaipainen, M. Jeltsch, V. Joukov, and K. Alitalo. 1996. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* 122: 3829-3837.
46. Jussila, L., and K. Alitalo. 2002. Vascular growth factors and lymphangiogenesis. *Physiological Reviews* 82: 673-700.
47. Breiteneder-Geleff, S., A. Soleiman, H. Kowalski, R. Horvat, G. Amann, E. Kriehuber, K. Diem, W. Weninger, E. Tschachler, K. Alitalo, and D. Kerjaschki. 1999. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 154: 385-394.
48. Weninger, W., T. A. Partanen, S. Breiteneder-Geleff, C. Mayer, H. Kowalski, M. Mildner, J. Pammer, M. Sturzl, D. Kerjaschki, K. Alitalo, and E. Tschachler. 1999. Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a

lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. *Laboratory Investigation; A Journal of Technical Methods and Pathology* 79: 243-251.

49. 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. *The Journal of Cell Biology* 144: 789-801.
50. Petrova, T. V., T. Makinen, T. P. Makela, J. Saarela, I. Virtanen, R. E. Ferrell, D. N. Finegold, D. Kerjaschki, S. Yla-Herttuala, and K. Alitalo. 2002. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *The EMBO Journal* 21: 4593-4599.
51. Kriehuber, E., S. Breiteneder-Geleff, M. Groeger, A. Soleiman, S. F. Schoppmann, G. Stingl, D. Kerjaschki, and D. Maurer. 2001. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med* 194: 797-808.
52. Podgrabinska, S., P. Braun, P. Velasco, B. Kloos, M. S. Pepper, and M. Skobe. 2002. Molecular characterization of lymphatic endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* 99: 16069-16074.
53. Hirakawa, S., Y. K. Hong, N. Harvey, V. Schacht, K. Matsuda, T. Libermann, and M. Detmar. 2003. Identification of vascular lineage-specific genes by transcriptional profiling of isolated blood vascular and lymphatic endothelial cells. *Am J Pathol* 162: 575-586.
54. Lee, K. M., C. S. McKimmie, D. S. Gilchrist, K. J. Pallas, R. J. Nibbs, P. Garside, V. McDonald, C. Jenkins, R. Ransohoff, L. Liu, S. Milling, V. Cerovic, and G. J. Graham. 2011. D6 facilitates cellular migration and fluid flow to lymph nodes by suppressing lymphatic congestion. *Blood* 118: 6220-6229.
55. McKimmie, C. S., M. D. Singh, K. Hewit, O. Lopez-Franco, M. Le Brocq, S. Rose-John, K. M. Lee, A. H. Baker, R. Wheat, D. J. Blackbourn, R. J. Nibbs, and G. J. Graham. 2013. An analysis of the function and expression of D6 on lymphatic endothelial cells. *Blood* 121: 3768-3777.
56. Nagahashi, M., E. Y. Kim, A. Yamada, S. Ramachandran, J. C. Allegood, N. C. Hait, M. Maceyka, S. Milstien, K. Takabe, and S. Spiegel. 2013. Spns2, a transporter of phosphorylated sphingoid bases, regulates their blood and lymph levels, and the lymphatic network. *FASEB Journal : official publication of the Federation of American Societies for Experimental Biology* 27: 1001-1011.
57. Onder, L., P. Narang, E. Scandella, Q. Chai, M. Iolyeva, K. Hoorweg, C. Halin, E. Richie, P. Kaye, J. Westermann, T. Cupedo, M. Coles, and B. Ludewig. 2012. IL-7-producing stromal cells are critical for lymph node remodeling. *Blood* 120: 4675-4683.
58. Hong, Y. K., J. W. Shin, and M. Detmar. 2004. Development of the lymphatic vascular system: a mystery unravels. *Dev Dyn* 231: 462-473.
59. Pajusola, K., O. Aprelikova, E. Armstrong, S. Morris, and K. Alitalo. 1993. Two human FLT4 receptor tyrosine kinase isoforms with distinct carboxy terminal tails are produced by alternative processing of primary transcripts. *Oncogene* 8: 2931-2937.
60. Wigle, J. T., N. Harvey, M. Detmar, I. Lagutina, G. Grosveld, M. D. Gunn, D. G. Jackson, and G. Oliver. 2002. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *The EMBO Journal* 21: 1505-1513.

61. Dumont, D. J., L. Jussila, J. Taipale, A. Lymboussaki, T. Mustonen, K. Pajusola, M. Breitman, and K. Alitalo. 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 282: 946-949.
62. Makinen, T., L. Jussila, T. Veikkola, T. Karpanen, M. I. Kettunen, K. J. Pulkkanen, R. Kauppinen, D. G. Jackson, H. Kubo, S. Nishikawa, S. Yla-Herttuala, and K. Alitalo. 2001. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nature Medicine* 7: 199-205.
63. Pytowski, B., J. Goldman, K. Persaud, Y. Wu, L. Witte, D. J. Hicklin, M. Skobe, K. C. Boardman, and M. A. Swartz. 2005. Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *Journal of the National Cancer Institute* 97: 14-21.
64. Fournier, E., P. Dubreuil, D. Birnbaum, and J. P. Borg. 1995. Mutation at tyrosine residue 1337 abrogates ligand-dependent transforming capacity of the FLT4 receptor. *Oncogene* 11: 921-931.
65. Martinez-Corral, I., D. Olmeda, R. Dieguez-Hurtado, T. Tammela, K. Alitalo, and S. Ortega. 2012. In vivo imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis. *Proceedings of the National Academy of Sciences of the United States of America* 109: 6223-6228.
66. Schacht, V., M. I. Ramirez, Y. K. Hong, S. Hirakawa, D. Feng, N. Harvey, M. Williams, A. M. Dvorak, H. F. Dvorak, G. Oliver, and M. Detmar. 2003. T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *The EMBO Journal* 22: 3546-3556.
67. Kabgani, N., T. Grigoleit, K. Schulte, A. Sechi, S. Sauer-Lehnen, C. Tag, P. Boor, C. Kuppe, G. Warsow, S. Schordan, J. Mostertz, R. K. Chilukoti, G. Homuth, N. Endlich, F. Tacke, R. Weiskirchen, G. Fuellen, K. Endlich, J. Floege, B. Smeets, and M. J. Moeller. 2012. Primary cultures of glomerular parietal epithelial cells or podocytes with proven origin. *PloS One* 7: e34907.
68. Kato, S., H. Shimoda, R. C. Ji, and M. Miura. 2006. Lymphangiogenesis and expression of specific molecules as lymphatic endothelial cell markers. *Anatomical Science International* 81: 71-83.
69. Martin-Villar, E., F. G. Scholl, C. Gamallo, M. M. Yurrita, M. Munoz-Guerra, J. Cruces, and M. Quintanilla. 2005. Characterization of human PA2.26 antigen (T1alpha-2, podoplanin), a small membrane mucin induced in oral squamous cell carcinomas. *International Journal of Cancer. Journal International du Cancer* 113: 899-910.
70. Kerjaschki, D., H. M. Regele, I. Moosberger, K. Nagy-Bojarski, B. Watschinger, A. Soleiman, P. Birner, S. Krieger, A. Hovorka, G. Silberhumer, P. Laakkonen, T. Petrova, B. Langer, and I. Raab. 2004. Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *Journal of the American Society of Nephrology : JASN* 15: 603-612.
71. Jackson, D. G., R. Prevo, S. Clasper, and S. Banerji. 2001. LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends in Immunology* 22: 317-321.
72. Jackson, D. G. 2003. The lymphatics revisited: new perspectives from the hyaluronan receptor LYVE-1. *Trends in Cardiovascular Medicine* 13: 1-7.
73. Jackson, D. G. 2009. Immunological functions of hyaluronan and its receptors in the lymphatics. *Immunological Reviews* 230: 216-231.

74. Jackson, D. G. 2004. Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. *APMIS : Acta Pathologica, Microbiologica, et Immunologica Scandinavica* 112: 526-538.
75. Johnson, L. A., R. Prevo, S. Clasper, and D. G. Jackson. 2007. Inflammation-induced uptake and degradation of the lymphatic endothelial hyaluronan receptor LYVE-1. *The Journal of Biological Chemistry* 282: 33671-33680.
76. Swartz, M. A., and M. Skobe. 2001. Lymphatic function, lymphangiogenesis, and cancer metastasis. *Microscopy Research and Technique* 55: 92-99.
77. Oliver, G. 2004. Lymphatic vasculature development. *Nature Reviews. Immunology* 4: 35-45.
78. Hong, Y. K., N. Harvey, Y. H. Noh, V. Schacht, S. Hirakawa, M. Detmar, and G. Oliver. 2002. Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 225: 351-357.
79. Johnson, N. C., M. E. Dillard, P. Baluk, D. M. McDonald, N. L. Harvey, S. L. Frase, and G. Oliver. 2008. Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes & Development* 22: 3282-3291.
80. Hong, Y. K., K. Foreman, J. W. Shin, S. Hirakawa, C. L. Curry, D. R. Sage, T. Libermann, B. J. Dezube, J. D. Fingeroth, and M. Detmar. 2004. Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nature Genetics* 36: 683-685.
81. Aguilar, B., I. Choi, D. Choi, H. K. Chung, S. Lee, J. Yoo, Y. S. Lee, Y. S. Maeng, H. N. Lee, E. Park, K. E. Kim, N. Y. Kim, J. M. Baik, J. U. Jung, C. J. Koh, and Y. K. Hong. 2012. Lymphatic reprogramming by Kaposi sarcoma herpes virus promotes the oncogenic activity of the virus-encoded G-protein-coupled receptor. *Cancer Research* 72: 5833-5842.
82. Wilting, J., M. Papoutsi, B. Christ, K. H. Nicolaides, C. S. von Kaisenberg, J. Borges, G. B. Stark, K. Alitalo, S. I. Tomarev, C. Niemeyer, and J. Rossler. 2002. The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. *FASEB Journal : official publication of the Federation of American Societies for Experimental Biology* 16: 1271-1273.
83. Ji, R. C. 2005. Characteristics of lymphatic endothelial cells in physiological and pathological conditions. *Histology and Histopathology* 20: 155-175.
84. Luther, S. A., H. L. Tang, P. L. Hyman, A. G. Farr, and J. G. Cyster. 2000. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proceedings of the National Academy of Sciences of the United States of America* 97: 12694-12699.
85. Gunn, M. D., K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America* 95: 258-263.
86. Carlsen, H. S., G. Haraldsen, P. Brandtzaeg, and E. S. Baekkevold. 2005. Disparate lymphoid chemokine expression in mice and men: no evidence of CCL21 synthesis by human high endothelial venules. *Blood* 106: 444-446.
87. Manzo, A., S. Bugatti, R. Caporali, R. Prevo, D. G. Jackson, M. Uguccioni, C. D. Buckley, C. Montecucco, and C. Pitzalis. 2007. CCL21 expression pattern of human

secondary lymphoid organ stroma is conserved in inflammatory lesions with lymphoid neogenesis. *Am J Pathol* 171: 1549-1562.
88. Achen, M. G., R. A. Williams, M. E. Baldwin, P. Lai, S. Roufail, K. Alitalo, and S. A. Stacker. 2002. The angiogenic and lymphangiogenic factor vascular endothelial growth factor-D exhibits a paracrine mode of action in cancer. *Growth Factors* 20: 99-107.
89. Teijeira, A., A. Rouzaut, and I. Melero. 2013. Initial afferent lymphatic vessels controlling outbound leukocyte traffic from skin to lymph nodes. *Front Immunol* 4: 433.
90. Chauhan, S. K., D. R. Saban, T. H. Dohlman, and R. Dana. 2014. CCL-21 conditioned regulatory T cells induce allotolerance through enhanced homing to lymphoid tissue. *J Immunol* 192: 817-823.
91. Debes, G. F., C. N. Arnold, A. J. Young, S. Krautwald, M. Lipp, J. B. Hay, and E. C. Butcher. 2005. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nature Immunology* 6: 889-894.
92. Vigl, B., D. Aebischer, M. Nitschke, M. Iolyeva, T. Rothlin, O. Antsiferova, and C. Halin. 2011. Tissue inflammation modulates gene expression of lymphatic endothelial cells and dendritic cell migration in a stimulus-dependent manner. *Blood* 118: 205-215.
93. Pegu, A., J. L. Flynn, and T. A. Reinhart. 2007. Afferent and efferent interfaces of lymph nodes are distinguished by expression of lymphatic endothelial markers and chemokines. *Lymphatic Research and Biology* 5: 91-103.
94. Denton, A. E., E. W. Roberts, M. A. Linterman, and D. T. Fearon. 2014. Fibroblastic reticular cells of the lymph node are required for retention of resting but not activated CD8+ T cells. *Proceedings of the National Academy of Sciences of the United States of America* 111: 12139-12144.
95. Forster, E., H. H. Bock, J. Herz, X. Chai, M. Frotscher, and S. Zhao. 2010. Emerging topics in Reelin function. *The European Journal of Neuroscience* 31: 1511-1518.
96. Salmi, M., K. Koskinen, T. Henttinen, K. Elima, and S. Jalkanen. 2004. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood* 104: 3849-3857.
97. Ammar, A., R. A. Mohammed, M. Salmi, M. Pepper, E. C. Paish, I. O. Ellis, and S. G. Martin. 2011. Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis. *Analytical Cellular Pathology* 34: 67-78.
98. Karikoski, M., H. Irjala, M. Maksimow, M. Miiluniemi, K. Granfors, S. Hernesniemi, K. Elima, G. Moldenhauer, K. Schledzewski, J. Kzhyshkowska, S. Goerdt, M. Salmi, and S. Jalkanen. 2009. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. *European Journal of Immunology* 39: 3477-3487.
99. Karikoski, M., F. Marttila-Ichihara, K. Elima, P. Rantakari, M. K. Hollmen, T. Kelkka, H. Gerke, V. Huovinen, H. Irjala, R. Holmdahl, M. Salmi, and S. Jalkanen. 2014. Clever-1/Stabilin-1 Controls Cancer Growth and Metastasis. *Clinical Cancer Research : an official journal of the American Association for Cancer Research* 20(24): 6452-6464.
100. Nibbs, R., G. Graham, and A. Rot. 2003. Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. *Seminars in Immunology* 15: 287-294.
101. Fra, A. M., M. Locati, K. Otero, M. Sironi, P. Signorelli, M. L. Massardi, M. Gobbi, A. Vecchi, S. Sozzani, and A. Mantovani. 2003. Cutting edge: scavenging of inflammatory

CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol* 170: 2279-2282.
102. Galliera, E., V. R. Jala, J. O. Trent, R. Bonecchi, P. Signorelli, R. J. Lefkowitz, A. Mantovani, M. Locati, and B. Haribabu. 2004. beta-Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *The Journal of Biological Chemistry* 279: 25590-25597.
103. Weber, M., E. Blair, C. V. Simpson, M. O'Hara, P. E. Blackburn, A. Rot, G. J. Graham, and R. J. Nibbs. 2004. The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. *Molecular Biology of the Cell* 15: 2492-2508.
104. Vetrano, S., E. M. Borroni, A. Sarukhan, B. Savino, R. Bonecchi, C. Correale, V. Arena, M. Fantini, M. Roncalli, A. Malesci, A. Mantovani, M. Locati, and S. Danese. 2010. The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6. *Gut* 59: 197-206.
105. Lee, K. M., R. J. Nibbs, and G. J. Graham. 2013. D6: the 'crowd controller' at the immune gateway. *Trends in Immunology* 34: 7-12.
106. Phipps, R. P. 2008. CD40: Lord of the endothelial cell. *Blood* 112: 3531-3532.
107. Pluvinet, R., R. Olivar, J. Krupinski, I. Herrero-Fresneda, A. Luque, J. Torras, J. M. Cruzado, J. M. Grinyo, L. Sumoy, and J. M. Aran. 2008. CD40: an upstream master switch for endothelial cell activation uncovered by RNAi-coupled transcriptional profiling. *Blood* 112: 3624-3637.
108. Nisato, R. E., R. Buser, and M. S. Pepper. 2009. Lymphatic endothelial cells: establishment of primaries and characterization of established lines. *Methods Mol Biol* 467: 113-126.
109. Garrafa, E., G. Alessandri, A. Benetti, D. Turetta, A. Corradi, A. M. Cantoni, E. Cervi, S. Bonardelli, E. Parati, S. M. Giulini, B. Ensoli, and A. Caruso. 2006. Isolation and characterization of lymphatic microvascular endothelial cells from human tonsils. *J Cell Physiol* 207: 107-113.
110. Makinen, T., T. Veikkola, S. Mustjoki, T. Karpanen, B. Catimel, E. C. Nice, L. Wise, A. Mercer, H. Kowalski, D. Kerjaschki, S. A. Stacker, M. G. Achen, and K. Alitalo. 2001. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *The EMBO Journal* 20: 4762-4773.
111. Ogunbiyi, S., G. Chinien, D. Field, J. Humphries, K. Burand, B. Sawyer, S. Jeffrey, P. Mortimer, S. Clasper, D. Jackson, and A. Smith. 2011. Molecular characterization of dermal lymphatic endothelial cells from primary lymphedema skin. *Lymphatic Research and Biology* 9: 19-30.
112. Richard, L., P. Velasco, and M. Detmar. 1998. A simple immunomagnetic protocol for the selective isolation and long-term culture of human dermal microvascular endothelial cells. *Experimental Cell Research* 240: 1-6.
113. Shin, J. W., R. Huggenberger, and M. Detmar. 2008. Transcriptional profiling of VEGF-A and VEGF-C target genes in lymphatic endothelium reveals endothelial-specific molecule-1 as a novel mediator of lymphangiogenesis. *Blood* 112: 2318-2326.
114. Tan, Y. Z., H. J. Wang, M. H. Zhang, Z. Quan, T. Li, and Q. Z. He. 2014. CD34+ VEGFR-3+ progenitor cells have a potential to differentiate towards lymphatic endothelial cells. *Journal of Cellular and Molecular Medicine* 18: 422-433.

115. Karkkainen, M. J., P. Haiko, K. Sainio, J. Partanen, J. Taipale, T. V. Petrova, M. Jeltsch, D. G. Jackson, M. Talikka, H. Rauvala, C. Betsholtz, and K. Alitalo. 2004. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nature Immunology* 5: 74-80.
116. Kesler, C. T., A. H. Kuo, H. K. Wong, D. J. Masuck, J. L. Shah, K. R. Kozak, K. D. Held, and T. P. Padera. 2014. Vascular endothelial growth factor-C enhances radiosensitivity of lymphatic endothelial cells. *Angiogenesis* 17: 419-427.
117. Nakamura, R., M. Sugano, Y. Sone, K. Sakabe, T. Itoh, and M. Kiyono. 2014. Establishment and characterization of a human lymphatic endothelial cell line. *Biological & Pharmaceutical Bulletin* 37: 683-687.
118. Nisato, R. E., J. A. Harrison, R. Buser, L. Orci, C. Rinsch, R. Montesano, P. Dupraz, and M. S. Pepper. 2004. Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. *Am J Pathol* 165: 11-24.
119. Pepper, M. S., S. Wasi, N. Ferrara, L. Orci, and R. Montesano. 1994. In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells. *Experimental Cell Research* 210: 298-305.
120. Borron, P., and J. B. Hay. 1994. Characterization of ovine lymphatic endothelial cells and their interactions with lymphocytes. *Lymphology* 27: 6-13.
121. Gnepp, D. R., and W. Chandler. 1985. Tissue culture of human and canine thoracic duct endothelium. *In vitro Cellular & Developmental Biology : Journal of the Tissue Culture Association* 21: 200-206.
122. Tsunemoto, H., F. Ikomi, and T. Ohhashi. 2003. Flow-mediated release of nitric oxide from lymphatic endothelial cells of pressurized canine thoracic duct. *The Japanese Journal of Physiology* 53: 157-163.
123. Whitehurst, B., C. Eversgerd, M. Flister, C. M. Bivens, B. Pickett, D. C. Zawieja, and S. Ran. 2006. Molecular profile and proliferative responses of rat lymphatic endothelial cells in culture. *Lymphatic Research and Biology* 4: 119-142.
124. Djoneidi, M., and P. Brodt. 1991. Isolation and characterization of rat lymphatic endothelial cells. *Microcirc Endothelium Lymphatics* 7: 161-182.
125. Mizuno, R., Y. Yokoyama, N. Ono, F. Ikomi, and T. Ohhashi. 2003. Establishment of rat lymphatic endothelial cell line. *Microcirculation* 10: 127-131.
126. Ando, T., P. Jordan, T. Joh, Y. Wang, M. H. Jennings, J. Houghton, and J. S. Alexander. 2005. Isolation and characterization of a novel mouse lymphatic endothelial cell line: SV-LEC. *Lymphatic Research and Biology* 3: 105-115.
127. Sironi, M., A. Conti, S. Bernasconi, A. M. Fra, F. Pasqualini, M. Nebuloni, E. Lauri, M. De Bortoli, A. Mantovani, E. Dejana, and A. Vecchi. 2006. Generation and characterization of a mouse lymphatic endothelial cell line. *Cell and Tissue Research* 325: 91-100.
128. Yamaguchi, T., T. Ichise, O. Iwata, A. Hori, T. Adachi, M. Nakamura, N. Yoshida, and H. Ichise. 2008. Development of a new method for isolation and long-term culture of organ-specific blood vascular and lymphatic endothelial cells of the mouse. *The FEBS Journal* 275: 1988-1998.
129. Kazenwadel, J., G. A. Secker, K. L. Betterman, and N. L. Harvey. 2012. In vitro assays using primary embryonic mouse lymphatic endothelial cells uncover key roles for FGFR1 signalling in lymphangiogenesis. *PloS One* 7: e40497.

130. Jordan-Williams, K. L., and A. Ruddell. 2014. Culturing Purifies Murine Lymph Node Lymphatic Endothelium. *Lymphatic Research and Biology* 12(3): 144-149.
131. Karkkainen, M. J., A. Saaristo, L. Jussila, K. A. Karila, E. C. Lawrence, K. Pajusola, H. Bueler, A. Eichmann, R. Kauppinen, M. I. Kettunen, S. Yla-Herttuala, D. N. Finegold, R. E. Ferrell, and K. Alitalo. 2001. A model for gene therapy of human hereditary lymphedema. *Proceedings of the National Academy of Sciences of the United States of America* 98: 12677-12682.
132. Gale, N. W., R. Prevo, J. Espinosa, D. J. Ferguson, M. G. Dominguez, G. D. Yancopoulos, G. Thurston, and D. G. Jackson. 2007. Normal lymphatic development and function in mice deficient for the lymphatic hyaluronan receptor LYVE-1. *Molecular and Cellular Biology* 27: 595-604.
133. Thomas, S. N., J. M. Rutkowski, M. Pasquier, E. L. Kuan, K. Alitalo, G. J. Randolph, and M. A. Swartz. 2012. Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol* 189: 2181-2190.
134. Choi, I., Y. S. Lee, H. K. Chung, D. Choi, T. Ecoiffier, H. N. Lee, K. E. Kim, S. Lee, E. K. Park, Y. S. Maeng, N. Y. Kim, R. D. Ladner, N. A. Petasis, C. J. Koh, L. Chen, H. J. Lenz, and Y. K. Hong. 2013. Interleukin-8 reduces post-surgical lymphedema formation by promoting lymphatic vessel regeneration. *Angiogenesis* 16: 29-44.
135. Yaniv, K., S. Isogai, D. Castranova, L. Dye, J. Hitomi, and B. M. Weinstein. 2006. Live imaging of lymphatic development in the zebrafish. *Nature Medicine* 12: 711-716.
136. Shimoda, H., and S. Isogai. 2012. Immunohistochemical demonstration of lymphatic vessels in adult zebrafish. *Acta Histochemica et Cytochemica* 45: 335-341.
137. Kuchler, A. M., E. Gjini, J. Peterson-Maduro, B. Cancilla, H. Wolburg, and S. Schulte-Merker. 2006. Development of the zebrafish lymphatic system requires VEGFC signaling. *Current Biology : CB* 16: 1244-1248.
138. Astin, J. W., M. J. Haggerty, K. S. Okuda, L. Le Guen, J. P. Misa, A. Tromp, B. M. Hogan, K. E. Crosier, and P. S. Crosier. 2014. Vegfd can compensate for loss of Vegfc in zebrafish facial lymphatic sprouting. *Development* 141: 2680-2690.
139. Astin, J. W., S. M. Jamieson, T. C. Eng, M. V. Flores, J. P. Misa, A. Chien, K. E. Crosier, and P. S. Crosier. 2014. An in vivo anti-lymphatic screen in zebrafish identifies novel inhibitors of mammalian lymphangiogenesis and lymphatic-mediated metastasis. *Molecular Cancer Therapeutics* 13(10): 2450-2462.
140. Shin, W. S., and S. G. Rockson. 2008. Animal models for the molecular and mechanistic study of lymphatic biology and disease. *Annals of the New York Academy of Sciences* 1131: 50-74.
141. Baluk, P., T. Tammela, E. Ator, N. Lyubynska, M. G. Achen, D. J. Hicklin, M. Jeltsch, T. V. Petrova, B. Pytowski, S. A. Stacker, S. Yla-Herttuala, D. G. Jackson, K. Alitalo, and D. M. McDonald. 2005. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *The Journal of Clinical Investigation* 115: 247-257.
142. Kelley, P. M., A. L. Connor, and R. M. Tempero. 2013. Lymphatic vessel memory stimulated by recurrent inflammation. *Am J Pathol* 182: 2418-2428.
143. Kerjaschki, D., N. Huttary, I. Raab, H. Regele, K. Bojarski-Nagy, G. Bartel, S. M. Krober, H. Greinix, A. Rosenmaier, F. Karlhofer, N. Wick, and P. R. Mazal. 2006. Lymphatic endothelial progenitor cells contribute to de novo lymphangiogenesis in human renal transplants. *Nature Medicine* 12: 230-234.

144. Cursiefen, C., J. Cao, L. Chen, Y. Liu, K. Maruyama, D. Jackson, F. E. Kruse, S. J. Wiegand, M. R. Dana, and J. W. Streilein. 2004. Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. *Investigative Ophthalmology & Visual Science* 45: 2666-2673.
145. Hall, K. L., L. D. Volk-Draper, M. J. Flister, and S. Ran. 2012. New model of macrophage acquisition of the lymphatic endothelial phenotype. *PloS One* 7: e31794.
146. Lachance, P. A., A. Hazen, and E. M. Sevick-Muraca. 2013. Lymphatic vascular response to acute inflammation. *PloS One* 8: e76078.
147. Flister, M. J., A. Wilber, K. L. Hall, C. Iwata, K. Miyazono, R. E. Nisato, M. S. Pepper, D. C. Zawieja, and S. Ran. 2010. Inflammation induces lymphangiogenesis through up-regulation of VEGFR-3 mediated by NF-kappaB and Prox1. *Blood* 115: 418-429.
148. Xu, H., H. Guan, G. Zu, D. Bullard, J. Hanson, M. Slater, and C. A. Elmets. 2001. The role of ICAM-1 molecule in the migration of Langerhans cells in the skin and regional lymph node. *European Journal of Immunology* 31: 3085-3093.
149. Skobe, M., T. Hawighorst, D. G. Jackson, R. Prevo, L. Janes, P. Velasco, L. Riccardi, K. Alitalo, K. Claffey, and M. Detmar. 2001. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Medicine* 7: 192-198.
150. Chen, Z., M. L. Varney, M. W. Backora, K. Cowan, J. C. Solheim, J. E. Talmadge, and R. K. Singh. 2005. Down-regulation of vascular endothelial cell growth factor-C expression using small interfering RNA vectors in mammary tumors inhibits tumor lymphangiogenesis and spontaneous metastasis and enhances survival. *Cancer Research* 65: 9004-9011.
151. Achen, M. G., B. K. McColl, and S. A. Stacker. 2005. Focus on lymphangiogenesis in tumor metastasis. *Cancer Cell* 7: 121-127.
152. Karpanen, T., and K. Alitalo. 2008. Molecular biology and pathology of lymphangiogenesis. *Annual Review of Pathology* 3: 367-397.
153. Carroll, P. A., E. Brazeau, and M. Lagunoff. 2004. Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. *Virology* 328: 7-18.
154. Baluk, P., L. C. Yao, J. Feng, T. Romano, S. S. Jung, J. L. Schreiter, L. Yan, D. J. Shealy, and D. M. McDonald. 2009. TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *The Journal of Clinical Investigation* 119: 2954-2964.
155. Park, S. M., C. E. Angel, J. D. McIntosh, C. M. Mansell, C. J. Chen, J. Cebon, and P. R. Dunbar. 2014. Mapping the distinctive populations of lymphatic endothelial cells in different zones of human lymph nodes. *PloS One* 9: e94781.
156. Cyster, J. G. 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annual Review of Immunology* 23: 127-159.
157. Lo, C. G., Y. Xu, R. L. Proia, and J. G. Cyster. 2005. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. *J Exp Med* 201: 291-301.
158. Pham, T. H., P. Baluk, Y. Xu, I. Grigorova, A. J. Bankovich, R. Pappu, S. R. Coughlin, D. M. McDonald, S. R. Schwab, and J. G. Cyster. 2010. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med* 207: 17-27.

159. Rodeheffer, C., V. von Messling, S. Milot, F. Lepine, A. R. Manges, and B. J. Ward. 2007. Disease manifestations of canine distemper virus infection in ferrets are modulated by vitamin A status. *The Journal of Nutrition* 137: 1916-1922.
160. Garrafa, E., L. Imberti, G. Tiberio, A. Prandini, S. M. Giulini, and L. Caimi. 2011. Heterogeneous expression of toll-like receptors in lymphatic endothelial cells derived from different tissues. *Immunology and Cell Biology* 89: 475-481.
161. Cohen, J. N., E. F. Tewalt, S. J. Rouhani, E. L. Buonomo, A. N. Bruce, X. Xu, S. Bekiranov, Y. X. Fu, and V. H. Engelhard. 2014. Tolerogenic properties of lymphatic endothelial cells are controlled by the lymph node microenvironment. *PloS One* 9: e87740.
162. Norder, M., M. G. Gutierrez, S. Zicari, E. Cervi, A. Caruso, and C. A. Guzman. 2012. Lymph node-derived lymphatic endothelial cells express functional costimulatory molecules and impair dendritic cell-induced allogenic T-cell proliferation. *FASEB Journal : official publication of the Federation of American Societies for Experimental Biology* 26: 2835-2846.
163. Hirosue, S., E. Vokali, V. R. Raghavan, M. Rincon-Restrepo, A. W. Lund, P. Corthesy-Henrioud, F. Capotosti, C. Halin Winter, S. Hugues, and M. A. Swartz. 2014. Steady-state antigen scavenging, cross-presentation, and CD8+ T cell priming: a new role for lymphatic endothelial cells. *J Immunol* 192: 5002-5011.
164. Lund, A. W., F. V. Duraes, S. Hirosue, V. R. Raghavan, C. Nembrini, S. N. Thomas, A. Issa, S. Hugues, and M. A. Swartz. 2012. VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics. *Cell Reports* 1: 191-199.
165. Dietrich, T., F. Bock, D. Yuen, D. Hos, B. O. Bachmann, G. Zahn, S. Wiegand, L. Chen, and C. Cursiefen. 2010. Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation. *J Immunol* 184: 535-539.
166. Tamburini, B. A., M. A. Burchill, and R. M. Kedl. 2014. Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nature Communications* 5: 3989.
167. Baptista, A. P., R. Roozendaal, R. M. Reijmers, J. J. Koning, W. W. Unger, M. Greuter, E. D. Keuning, R. Molenaar, G. Goverse, M. M. Sneeboer, J. M. den Haan, M. Boes, and R. E. Mebius. 2014. Lymph node stromal cells constrain immunity via MHC class II self-antigen presentation. *eLife* 3: e04433.
168. Wendler, A., and M. Wehling. 2010. The translatability of animal models for clinical development: biomarkers and disease models. *Current Opinion in Pharmacology* 10: 601-606.
169. McGonigle, P., and B. Ruggeri. 2014. Animal models of human disease: challenges in enabling translation. *Biochemical Pharmacology* 87: 162-171.
170. Prabhakar, S. 2012. Translational research challenges: finding the right animal models. *Journal of Investigative Medicine : the official publication of the American Federation for Clinical Research* 60: 1141-1146.
171. Atkinson, C. S., G. A. Press, P. Lyden, and B. Katz. 1989. The ferret as an animal model in cerebrovascular research. *Stroke; a Journal of Cerebral Circulation* 20: 1085-1088.
172. Bock, A. S., J. F. Olavarria, L. A. Leigland, E. N. Taber, S. N. Jespersen, and C. D. Kroenke. 2010. Diffusion tensor imaging detects early cerebral cortex abnormalities in

neuronal architecture induced by bilateral neonatal enucleation: an experimental model in the ferret. *Frontiers in Systems Neuroscience* 4: 149.
173. Medina, A. E., T. E. Krahe, and A. S. Ramoa. 2005. Early alcohol exposure induces persistent alteration of cortical columnar organization and reduced orientation selectivity in the visual cortex. *Journal of Neurophysiology* 93: 1317-1325.
174. Kim, Y., X. S. Liu, C. Liu, D. E. Smith, R. M. Russell, and X. D. Wang. 2006. Induction of pulmonary neoplasia in the smoke-exposed ferret by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK): a model for human lung cancer. *Cancer Letters* 234: 209-219.
175. Danesh, A., C. Seneviratne, C. M. Cameron, D. Banner, M. E. Devries, A. A. Kelvin, L. Xu, L. Ran, S. E. Bosinger, T. Rowe, M. Czub, C. B. Jonsson, M. J. Cameron, and D. J. Kelvin. 2008. Cloning, expression and characterization of ferret CXCL10. *Molecular Immunology* 45: 1288-1297.
176. Svitek, N., and V. von Messling. 2007. Early cytokine mRNA expression profiles predict Morbillivirus disease outcome in ferrets. *Virology* 362: 404-410.
177. Chu, Y. K., G. D. Ali, F. Jia, Q. Li, D. Kelvin, R. C. Couch, K. S. Harrod, J. A. Hutt, C. Cameron, S. R. Weiss, and C. B. Jonsson. 2008. The SARS-CoV ferret model in an infection-challenge study. *Virology* 374: 151-163.
178. Geisbert, T. W., K. M. Daddario-DiCaprio, A. C. Hickey, M. A. Smith, Y. P. Chan, L. F. Wang, J. J. Mattapallil, J. B. Geisbert, K. N. Bossart, and C. C. Broder. 2010. Development of an acute and highly pathogenic nonhuman primate model of Nipah virus infection. *PloS One* 5: e10690.
179. Fadel, H. J., D. T. Saenz, R. Guevara, V. von Messling, M. Peretz, and E. M. Poeschla. 2012. Productive replication and evolution of HIV-1 in ferret cells. *Journal of Virology* 86: 2312-2322.
180. Martina, B. E., B. L. Haagmans, T. Kuiken, R. A. Fouchier, G. F. Rimmelzwaan, G. Van Amerongen, J. S. Peiris, W. Lim, and A. D. Osterhaus. 2003. Virology: SARS virus infection of cats and ferrets. *Nature* 425: 915.
181. Woods, J. B., C. K. Schmitt, S. C. Darnell, K. C. Meysick, and A. D. O'Brien. 2002. Ferrets as a model system for renal disease secondary to intestinal infection with Escherichia coli O157:H7 and other Shiga toxin-producing E. coli. *The Journal of Infectious Diseases* 185: 550-554.
182. Bouvier, N. M., and A. C. Lowen. 2010. Animal Models for Influenza Virus Pathogenesis and Transmission. *Viruses* 2: 1530-1563.
183. O'Donnell, C. D., and K. Subbarao. 2011. The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. *Microbes and Infection / Institut Pasteur* 13: 502-515.
184. Belser, J. A., J. M. Katz, and T. M. Tumpey. 2011. The ferret as a model organism to study influenza A virus infection. *Disease Models & Mechanisms* 4: 575-579.
185. Herfst, S., E. J. Schrauwen, M. Linster, S. Chutinimitkul, E. de Wit, V. J. Munster, E. M. Sorrell, T. M. Bestebroer, D. F. Burke, D. J. Smith, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* 336: 1534-1541.
186. Banner, D., and A. A. Kelvin. 2012. The current state of H5N1 vaccines and the use of the ferret model for influenza therapeutic and prophylactic development. *Journal of Infection in Developing Countries* 6: 465-469.

187. Fang, Y., T. Rowe, A. J. Leon, D. Banner, A. Danesh, L. Xu, L. Ran, S. E. Bosinger, Y. Guan, H. Chen, C. C. Cameron, M. J. Cameron, and D. J. Kelvin. 2010. Molecular characterization of in vivo adjuvant activity in ferrets vaccinated against influenza virus. *Journal of Virology* 84: 8369-8388.
188. Kao, L. M., K. Bush, R. Barnewall, J. Estep, F. W. Thalacker, P. H. Olson, G. L. Drusano, N. Minton, S. Chien, A. Hemeryck, and M. F. Kelley. 2006. Pharmacokinetic considerations and efficacy of levofloxacin in an inhalational anthrax (postexposure) rhesus monkey model. *Antimicrobial Agents and Chemotherapy* 50: 3535-3542.
189. Glogowski, S., K. W. Ward, M. S. Lawrence, R. J. Goody, and J. W. Proksch. 2012. The use of the African green monkey as a preclinical model for ocular pharmacokinetic studies. *Journal of Ocular Pharmacology and Therapeutics : the official journal of the Association for Ocular Pharmacology and Therapeutics* 28: 290-298.
190. Jarvis, P., S. Srivastav, E. Vogelwedde, J. Stewart, T. Mitchard, and G. F. Weinbauer. 2010. The cynomolgus monkey as a model for developmental toxicity studies: variability of pregnancy losses, statistical power estimates, and group size considerations. *Birth defects research. Part B, Developmental and Reproductive Toxicology* 89: 175-187.
191. Chan, A. W. 2013. Progress and prospects for genetic modification of nonhuman primate models in biomedical research. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources* 54: 211-223.
192. Vezoli, J., K. Fifel, V. Leviel, C. Dehay, H. Kennedy, H. M. Cooper, C. Gronfier, and E. Procyk. 2011. Early presymptomatic and long-term changes of rest activity cycles and cognitive behavior in a MPTP-monkey model of Parkinson's disease. *PloS One* 6: e23952.
193. Hatziioannou, T., and D. T. Evans. 2012. Animal models for HIV/AIDS research. *Nature Reviews. Microbiology* 10: 852-867.
194. Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers. 1985. Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* 228: 1201-1204.
195. Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 280: 427-431.
196. Hu, J., M. B. Gardner, and C. J. Miller. 2000. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *Journal of Virology* 74: 6087-6095.
197. Estes, J. D., L. D. Harris, N. R. Klatt, B. Tabb, S. Pittaluga, M. Paiardini, G. R. Barclay, J. Smedley, R. Pung, K. M. Oliveira, V. M. Hirsch, G. Silvestri, D. C. Douek, C. J. Miller, A. T. Haase, J. Lifson, and J. M. Brenchley. 2010. Damaged intestinal epithelial integrity linked to microbial translocation in pathogenic simian immunodeficiency virus infections. *PLoS Pathogens* 6: e1001052.
198. Kristoff, J., G. Haret-Richter, D. Ma, R. M. Ribeiro, C. Xu, E. Cornell, J. L. Stock, T. He, A. D. Mobley, S. Ross, A. Trichel, C. Wilson, R. Tracy, A. Landay, C. Apetrei, and I. Pandrea. 2014. Early microbial translocation blockade reduces SIV-mediated inflammation and viral replication. *The Journal of Clinical Investigation* 124: 2802-2806.

199. Tsai, C. C., K. E. Follis, A. Sabo, T. W. Beck, R. F. Grant, N. Bischofberger, R. E. Benveniste, and R. Black. 1995. Prevention of SIV infection in macaques by (R)-9-(2-phosphonylmethoxypropyl)adenine. *Science* 270: 1197-1199.
200. Tsai, C. C., K. E. Follis, A. Sabo, R. Grant, and N. Bischofberger. 1995. Efficacy of 9-(2-phosphonylmethoxyethyl)adenine treatment against chronic simian immunodeficiency virus infection in macaques. *The Journal of Infectious Diseases* 171: 1338-1343.
201. Arribas, J. R., A. L. Pozniak, J. E. Gallant, E. Dejesus, B. Gazzard, R. E. Campo, S. S. Chen, D. McColl, C. B. Holmes, J. Enejosa, J. J. Toole, and A. K. Cheng. 2008. Tenofovir disoproxil fumarate, emtricitabine, and efavirenz compared with zidovudine/lamivudine and efavirenz in treatment-naive patients: 144-week analysis. *Journal of Acquired Immune Deficiency Syndromes* 47: 74-78.
202. Van Rompay, K. K. 2012. The use of nonhuman primate models of HIV infection for the evaluation of antiviral strategies. *AIDS Research and Human Retroviruses* 28: 16-35.
203. Van Rompay, K. K., C. J. Berardi, N. L. Aguirre, N. Bischofberger, P. S. Lietman, N. C. Pedersen, and M. L. Marthas. 1998. Two doses of PMPA protect newborn macaques against oral simian immunodeficiency virus infection. *Aids* 12: F79-83.
204. Van Rompay, K. K., M. B. McChesney, N. L. Aguirre, K. A. Schmidt, N. Bischofberger, and M. L. Marthas. 2001. Two low doses of tenofovir protect newborn macaques against oral simian immunodeficiency virus infection. *The Journal of Infectious Diseases* 184: 429-438.
205. Van Rompay, K. K., B. P. Kearney, J. J. Sexton, R. Colon, J. R. Lawson, E. J. Blackwood, W. A. Lee, N. Bischofberger, and M. L. Marthas. 2006. Evaluation of oral tenofovir disoproxil fumarate and topical tenofovir GS-7340 to protect infant macaques against repeated oral challenges with virulent simian immunodeficiency virus. *Journal of Acquired Immune Deficiency Syndromes* 43: 6-14.
206. Otten, R. A., D. K. Smith, D. R. Adams, J. K. Pullium, E. Jackson, C. N. Kim, H. Jaffe, R. Janssen, S. Butera, and T. M. Folks. 2000. Efficacy of postexposure prophylaxis after intravaginal exposure of pig-tailed macaques to a human-derived retrovirus (human immunodeficiency virus type 2). *Journal of Virology* 74: 9771-9775.
207. Veazey, R. S., M. S. Springer, P. A. Marx, J. Dufour, P. J. Klasse, and J. P. Moore. 2005. Protection of macaques from vaginal SHIV challenge by an orally delivered CCR5 inhibitor. *Nature Medicine* 11: 1293-1294.
208. Garcia-Lerma, J. G., R. A. Otten, S. H. Qari, E. Jackson, M. E. Cong, S. Masciotra, W. Luo, C. Kim, D. R. Adams, M. Monsour, J. Lipscomb, J. A. Johnson, D. Delinsky, R. F. Schinazi, R. Janssen, T. M. Folks, and W. Heneine. 2008. Prevention of rectal SHIV transmission in macaques by daily or intermittent prophylaxis with emtricitabine and tenofovir. *PLoS Medicine* 5: e28.
209. Tuntland, T., C. Nosbisch, W. L. Baughman, J. Massarella, and J. D. Unadkat. 1996. Mechanism and rate of placental transfer of zalcitabine (2',3'-dideoxycytidine) in Macaca nemestrina. *American Journal of Obstetrics and Gynecology* 174: 856-863.
210. Van Rompay, K. K., M. Hamilton, B. Kearney, and N. Bischofberger. 2005. Pharmacokinetics of tenofovir in breast milk of lactating rhesus macaques. *Antimicrobial Agents and Chemotherapy* 49: 2093-2094.
211. Van Rompay, K. K., L. L. Brignolo, D. J. Meyer, C. Jerome, R. Tarara, A. Spinner, M. Hamilton, L. L. Hirst, D. R. Bennett, D. R. Canfield, T. G. Dearman, W. Von Morgenland, P. C. Allen, C. Valverde, A. B. Castillo, R. B. Martin, V. F. Samii, R.

Bendele, J. Desjardins, M. L. Marthas, N. C. Pedersen, and N. Bischofberger. 2004. Biological effects of short-term or prolonged administration of 9-[2-(phosphonomethoxy)propyl]adenine (tenofovir) to newborn and infant rhesus macaques. *Antimicrobial Agents and Chemotherapy* 48: 1469-1487.

212. Witvrouw, M., C. Pannecouque, W. M. Switzer, T. M. Folks, E. De Clercq, and W. Heneine. 2004. Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. *Antiviral Therapy* 9: 57-65.
213. Ishimatsu, M., H. Suzuki, H. Akiyama, T. Miura, M. Hayami, and E. Ido. 2007. Construction of a novel SHIV having an HIV-1-derived protease gene and its infection to rhesus macaques: a useful tool for in vivo efficacy tests of protease inhibitors. *Microbes and Infection / Institut Pasteur* 9: 475-482.
214. Riddick, N. E., E. A. Hermann, L. M. Loftin, S. T. Elliott, W. C. Wey, B. Cervasi, J. Taaffe, J. C. Engram, B. Li, J. G. Else, Y. Li, B. H. Hahn, C. A. Derdeyn, D. L. Sodora, C. Apetrei, M. Paiardini, G. Silvestri, and R. G. Collman. 2010. A novel CCR5 mutation common in sooty mangabeys reveals SIVsmm infection of CCR5-null natural hosts and efficient alternative coreceptor use in vivo. *PLoS Pathogens* 6: e1001064.
215. Uberla, K., C. Stahl-Hennig, D. Bottiger, K. Matz-Rensing, F. J. Kaup, J. Li, W. A. Haseltine, B. Fleckenstein, G. Hunsmann, B. Oberg, and et al. 1995. Animal model for the therapy of acquired immunodeficiency syndrome with reverse transcriptase inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 92: 8210-8214.
216. Ambrose, Z., V. Boltz, S. Palmer, J. M. Coffin, S. H. Hughes, and V. N. Kewalramani. 2004. In vitro characterization of a simian immunodeficiency virus-human immunodeficiency virus (HIV) chimera expressing HIV type 1 reverse transcriptase to study antiviral resistance in pigtail macaques. *Journal of Virology* 78: 13553-13561.
217. Ho, S. H., S. Tasca, L. Shek, A. Li, A. Gettie, J. Blanchard, D. Boden, and C. Cheng-Mayer. 2007. Coreceptor switch in R5-tropic simian/human immunodeficiency virus-infected macaques. *Journal of Virology* 81: 8621-8633.
218. Li, J. T., M. Halloran, C. I. Lord, A. Watson, J. Ranchalis, M. Fung, N. L. Letvin, and J. G. Sodroski. 1995. Persistent infection of macaques with simian-human immunodeficiency viruses. *Journal of Virology* 69: 7061-7067.
219. Smith, J. M., A. Dauner, B. Li, P. Srinivasan, J. Mitchell, M. Hendry, D. Ellenberger, S. Butera, and R. A. Otten. 2010. Generation of a dual RT Env SHIV that is infectious in rhesus macaques. *Journal of Medical Primatology* 39: 213-223.
220. Lewis, M. G., S. Norelli, M. Collins, M. L. Barreca, N. Iraci, B. Chirullo, J. Yalley-Ogunro, J. Greenhouse, F. Titti, E. Garaci, and A. Savarino. 2010. Response of a simian immunodeficiency virus (SIVmac251) to raltegravir: a basis for a new treatment for simian AIDS and an animal model for studying lentiviral persistence during antiretroviral therapy. *Retrovirology* 7: 21.
221. Van Rompay, K. K., J. A. Johnson, E. J. Blackwood, R. P. Singh, J. Lipscomb, T. B. Matthews, M. L. Marthas, N. C. Pedersen, N. Bischofberger, W. Heneine, and T. W. North. 2007. Sequential emergence and clinical implications of viral mutants with K70E and K65R mutation in reverse transcriptase during prolonged tenofovir monotherapy in rhesus macaques with chronic RT-SHIV infection. *Retrovirology* 4: 25.

222. Reimann, K. A., J. T. Li, R. Veazey, M. Halloran, I. W. Park, G. B. Karlsson, J. Sodroski, and N. L. Letvin. 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. *Journal of Virology* 70: 6922-6928.
223. Hessell, A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. Parren, P. A. Marx, and D. R. Burton. 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nature Medicine* 15: 951-954.
224. Hessell, A. J., E. G. Rakasz, P. Poignard, L. Hangartner, G. Landucci, D. N. Forthal, W. C. Koff, D. I. Watkins, and D. R. Burton. 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. *PLoS Pathogens* 5: e1000433.
225. Lakhashe, S. K., V. Velu, G. Sciaranghella, N. B. Siddappa, J. M. Dipasquale, G. Hemashettar, J. K. Yoon, R. A. Rasmussen, F. Yang, S. J. Lee, D. C. Montefiori, F. J. Novembre, F. Villinger, R. R. Amara, M. Kahn, S. L. Hu, S. Li, Z. Li, F. R. Frankel, M. Robert-Guroff, W. E. Johnson, J. Lieberman, and R. M. Ruprecht. 2011. Prime-boost vaccination with heterologous live vectors encoding SIV gag and multimeric HIV-1 gp160 protein: efficacy against repeated mucosal R5 clade C SHIV challenges. *Vaccine* 29: 5611-5622.
226. Nishimura, Y., M. Shingai, R. Willey, R. Sadjadpour, W. R. Lee, C. R. Brown, J. M. Brenchley, A. Buckler-White, R. Petros, M. Eckhaus, V. Hoffman, T. Igarashi, and M. A. Martin. 2010. Generation of the pathogenic R5-tropic simian/human immunodeficiency virus SHIVAD8 by serial passaging in rhesus macaques. *Journal of Virology* 84: 4769-4781.
227. Sina, S. T., W. Ren, and C. Cheng-Mayer. 2011. Coreceptor use in nonhuman primate models of HIV infection. *Journal of Translational Medicine* 9 Suppl 1: S7.
228. Keele, B. F., H. Li, G. H. Learn, P. Hraber, E. E. Giorgi, T. Grayson, C. Sun, Y. Chen, W. W. Yeh, N. L. Letvin, J. R. Mascola, G. J. Nabel, B. F. Haynes, T. Bhattacharya, A. S. Perelson, B. T. Korber, B. H. Hahn, and G. M. Shaw. 2009. Low-dose rectal inoculation of rhesus macaques by SIVsmE660 or SIVmac251 recapitulates human mucosal infection by HIV-1. *J Exp Med* 206: 1117-1134.
229. Ruprecht, R. M., T. W. Baba, V. Liska, S. Ayehunie, J. Andersen, D. C. Montefiori, A. Trichel, M. Murphey-Corb, L. Martin, T. A. Rizvi, B. J. Bernacky, S. J. Buchl, and M. Keeling. 1998. Oral SIV, SHIV, and HIV type 1 infection. *AIDS Research and Human Retroviruses* 14 Suppl 1: S97-103.
230. Ma, Z. M., B. F. Keele, H. Qureshi, M. Stone, V. Desilva, L. Fritts, J. D. Lifson, and C. J. Miller. 2011. SIVmac251 is inefficiently transmitted to rhesus macaques by penile inoculation with a single SIVenv variant found in ramp-up phase plasma. *AIDS Research and Human Retroviruses* 27: 1259-1269.
231. Sui, Y., Q. Zhu, S. Gagnon, A. Dzutsev, M. Terabe, M. Vaccari, D. Venzon, D. Klinman, W. Strober, B. Kelsall, G. Franchini, I. M. Belyakov, and J. A. Berzofsky. 2010. Innate and adaptive immune correlates of vaccine and adjuvant-induced control of mucosal transmission of SIV in macaques. *Proceedings of the National Academy of Sciences of the United States of America* 107: 9843-9848.

232. Verthelyi, D., R. T. Kenney, R. A. Seder, A. A. Gam, B. Friedag, and D. M. Klinman. 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol* 168: 1659-1663.
233. Wille-Reece, U., B. J. Flynn, K. Lore, R. A. Koup, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2005. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America* 102: 15190-15194.
234. Xu, H., X. Wang, and R. S. Veazey. 2013. Mucosal immunology of HIV infection. *Immunological Reviews* 254: 10-33.
235. Cavarelli, M., and G. Scarlatti. 2014. HIV-1 infection: the role of the gastrointestinal tract. *American Journal of Reproductive Immunology* 71: 537-542.
236. Rousseau, C. M., R. W. Nduati, B. A. Richardson, M. S. Steele, G. C. John-Stewart, D. A. Mbori-Ngacha, J. K. Kreiss, and J. Overbaugh. 2003. Longitudinal analysis of human immunodeficiency virus type 1 RNA in breast milk and of its relationship to infant infection and maternal disease. *The Journal of Infectious Diseases* 187: 741-747.
237. Royce, R. A., A. Sena, W. Cates, Jr., and M. S. Cohen. 1997. Sexual transmission of HIV. *The New England Journal of Medicine* 336: 1072-1078.
238. Haase, A. T. 2005. Perils at mucosal front lines for HIV and SIV and their hosts. *Nature Reviews. Immunology* 5: 783-792.
239. Haase, A. T. 2010. Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* 464: 217-223.
240. Haase, A. T. 2011. Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annual Review of Medicine* 62: 127-139.
241. Mowat, A. M., and W. W. Agace. 2014. Regional specialization within the intestinal immune system. *Nature Reviews. Immunology* 14: 667-685.
242. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434: 1093-1097.
243. Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar. 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *Journal of Virology* 77: 11708-11717.
244. Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 200: 761-770.
245. Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 200: 749-759.
246. Veazey, R. S., P. J. Klasse, T. J. Ketas, J. D. Reeves, M. Piatak, Jr., K. Kunstman, S. E. Kuhmann, P. A. Marx, J. D. Lifson, J. Dufour, M. Mefford, I. Pandrea, S. M. Wolinsky, R. W. Doms, J. A. DeMartino, S. J. Siciliano, K. Lyons, M. S. Springer, and J. P. Moore. 2003. Use of a small molecule CCR5 inhibitor in macaques to treat simian

immunodeficiency virus infection or prevent simian-human immunodeficiency virus infection. *J Exp Med* 198: 1551-1562.
247. Pegu, A., S. Qin, B. A. Fallert Junecko, R. E. Nisato, M. S. Pepper, and T. A. Reinhart. 2008. Human lymphatic endothelial cells express multiple functional TLRs. *J Immunol* 180: 3399-3405.
248. Berendam, S. J., B. A. Fallert Junecko, M. A. Murphey-Corb, D. H. Fuller, and T. A. Reinhart. 2015. Isolation, characterization, and functional analysis of ferret lymphatic endothelial cells. *Veterinary Immunology and Immunopathology* 163: 134-145.
249. Grigorova, I. L., M. Panteleev, and J. G. Cyster. 2010. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure. *Proceedings of the National Academy of Sciences of the United States of America* 107: 20447-20452.
250. Ma, J., J. H. Wang, Y. J. Guo, M. S. Sy, and M. Bigby. 1994. In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes. *Cellular Immunology* 158: 389-399.
251. Tewalt, E. F., J. N. Cohen, S. J. Rouhani, C. J. Guidi, H. Qiao, S. P. Fahl, M. R. Conaway, T. P. Bender, K. S. Tung, A. T. Vella, A. J. Adler, L. Chen, and V. H. Engelhard. 2012. Lymphatic endothelial cells induce tolerance via PD-L1 and lack of costimulation leading to high-level PD-1 expression on CD8 T cells. *Blood* 120: 4772-4782.
252. Beutler, B. 2009. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunological Reviews* 227: 248-263.
253. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nature Reviews. Immunology* 4: 499-511.
254. Kwissa, M., H. I. Nakaya, H. Oluoch, and B. Pulendran. 2012. Distinct TLR adjuvants differentially stimulate systemic and local innate immune responses in nonhuman primates. *Blood* 119: 2044-2055.
255. Zhu, Q., C. Egelston, S. Gagnon, Y. Sui, I. M. Belyakov, D. M. Klinman, and J. A. Berzofsky. 2010. Using 3 TLR ligands as a combination adjuvant induces qualitative changes in T cell responses needed for antiviral protection in mice. *The Journal of Clinical Investigation* 120: 607-616.
256. Zhang, W. W., and G. Matlashewski. 2008. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against Leishmania major in BALB/c mice. *Infection and Immunity* 76: 3777-3783.
257. Bruder, C. E., S. Yao, F. Larson, J. V. Camp, R. Tapp, A. McBrayer, N. Powers, W. V. Granda, and C. B. Jonsson. 2010. Transcriptome sequencing and development of an expression microarray platform for the domestic ferret. *BMC Genomics* 11: 251.
258. Russell, C. A., J. M. Fonville, A. E. Brown, D. F. Burke, D. L. Smith, S. L. James, S. Herfst, S. van Boheemen, M. Linster, E. J. Schrauwen, L. Katzelnick, A. Mosterin, T. Kuiken, E. Maher, G. Neumann, A. D. Osterhaus, Y. Kawaoka, R. A. Fouchier, and D. J. Smith. 2012. The potential for respiratory droplet-transmissible A/H5N1 influenza virus to evolve in a mammalian host. *Science* 336: 1541-1547.
259. Camp, J. V., T. L. Svensson, A. McBrayer, C. B. Jonsson, P. Liljestrom, and C. E. Bruder. 2012. De-novo transcriptome sequencing of a normalized cDNA pool from influenza infected ferrets. *PloS One* 7: e37104.

260. Nakata, M., T. Itou, and T. Sakai. 2008. Molecular cloning and phylogenetic analysis of inflammatory cytokines of the ferret (Mustela putorius furo). *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science* 70: 543-550.
261. Qin, S., C. R. Klamar, B. A. Fallert Junecko, J. Craigo, D. H. Fuller, and T. A. Reinhart. 2013. Functional characterization of ferret CCL20 and CCR6 and identification of chemotactic inhibitors. *Cytokine* 61: 924-932.
262. Jeanmougin, F., J. D. Thompson, M. Gouy, D. G. Higgins, and T. J. Gibson. 1998. Multiple sequence alignment with Clustal X. *Trends in Biochemical Sciences* 23: 403-405.
263. Felsenstein, J. 1989. Mathematics vs. Evolution: Mathematical Evolutionary Theory. *Science* 246: 941-942.
264. Page, R. D. 1996. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12: 357-358.
265. Nakata, M., T. Itou, and T. Sakai. 2009. Quantitative analysis of inflammatory cytokines expression in peripheral blood mononuclear cells of the ferret (Mustela putorius furo) using real-time PCR. *Veterinary Immunology and Immunopathology* 130: 88-91.
266. Sanghavi, S. K., and T. A. Reinhart. 2005. Increased expression of TLR3 in lymph nodes during simian immunodeficiency virus infection: implications for inflammation and immunodeficiency. *J Immunol* 175: 5314-5323.
267. Wick, N., D. Haluza, E. Gurnhofer, I. Raab, M. T. Kasimir, M. Prinz, C. W. Steiner, C. Reinisch, A. Howorka, P. Giovanoli, S. Buchsbaum, S. Krieger, E. Tschachler, P. Petzelbauer, and D. Kerjaschki. 2008. Lymphatic precollectors contain a novel, specialized subpopulation of podoplanin low, CCL27-expressing lymphatic endothelial cells. *Am J Pathol* 173: 1202-1209.
268. Leon, A. J., D. Banner, L. Xu, L. Ran, Z. Peng, K. Yi, C. Chen, F. Xu, J. Huang, Z. Zhao, Z. Lin, S. H. Huang, Y. Fang, A. A. Kelvin, T. M. Ross, A. Farooqui, and D. J. Kelvin. 2013. Sequencing, annotation, and characterization of the influenza ferret infectome. *Journal of Virology* 87: 1957-1966.
269. Yoo, J. K., T. S. Kim, M. M. Hufford, and T. J. Braciale. 2013. Viral infection of the lung: Host response and sequelae. *J Allergy Clin Immunol* 132(6): 1263-1276.
270. La Gruta, N. L., K. Kedzierska, J. Stambas, and P. C. Doherty. 2007. A question of self-preservation: immunopathology in influenza virus infection. *Immunology and Cell Biology* 85: 85-92.
271. Wilting, J., J. Becker, K. Buttler, and H. A. Weich. 2009. Lymphatics and inflammation. *Current Medicinal Chemistry* 16: 4581-4592.
272. Chaitanya, G. V., S. E. Franks, W. Cromer, S. R. Wells, M. Bienkowska, M. H. Jennings, A. Ruddell, T. Ando, Y. Wang, Y. Gu, M. Sapp, J. M. Mathis, P. A. Jordan, A. Minagar, and J. S. Alexander. 2010. Differential cytokine responses in human and mouse lymphatic endothelial cells to cytokines in vitro. *Lymphatic Research and Biology* 8: 155-164.
273. Demedts, I. K., K. R. Bracke, G. Van Pottelberge, D. Testelmans, G. M. Verleden, F. E. Vermassen, G. F. Joos, and G. G. Brusselle. 2007. Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine* 175: 998-1005.

274. Lukacs, N. W., D. M. Prosser, M. Wiekowski, S. A. Lira, and D. N. Cook. 2001. Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J Exp Med* 194: 551-555.
275. Singh, S. P., H. H. Zhang, J. F. Foley, M. N. Hedrick, and J. M. Farber. 2008. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. *J Immunol* 180: 214-221.
276. Saeki, H., A. M. Moore, M. J. Brown, and S. T. Hwang. 1999. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. *J Immunol* 162: 2472-2475.
277. Pegu, A., J. L. Flynn, and T. A. Reinhart. 2007. Afferent and efferent interfaces of lymph nodes are distinguished by expression of lymphatic endothelial markers and chemokines. *Lymphatic Research Biology* 5: 91-103.
278. Johnson, L. A., and D. G. Jackson. 2010. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *International Immunology* 22: 839-849.
279. Maddaluno, L., S. E. Verbrugge, C. Martinoli, G. Matteoli, A. Chiavelli, Y. Zeng, E. D. Williams, M. Rescigno, and U. Cavallaro. 2009. The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells. *J Exp Med* 206: 623-635.
280. Rouzaut, A., S. Garasa, A. Teijeira, I. Gonzalez, I. Martinez-Forero, N. Suarez, E. Larrea, C. Alfaro, A. Palazon, J. Dubrot, S. Hervas-Stubbs, and I. Melero. 2010. Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN-alpha. *European Journal of Immunology* 40: 3054-3063.
281. Pegu, A., S. Qin, B. A. Fallert Junecko, R. E. Nisato, M. S. Pepper, and T. A. Reinhart. 2008. Human lymphatic endothelial cells express multiple functional TLRs. *J Immunol* 180: 3399-3405.
282. Garrafa, E., L. Imberti, G. Tiberio, A. Prandini, S. M. Giulini, and L. Caimi. 2011. Heterogeneous expression of toll-like receptors in lymphatic endothelial cells derived from different tissues. *Immunology Cell Biology* 89: 475-481.
283. Sui, Y., S. Gordon, G. Franchini, and J. A. Berzofsky. 2013. Nonhuman primate models for HIV/AIDS vaccine development. *Current Protocols in Immunology / edited by John E. Coligan ... [et al.]* 102: Unit 12 14.
284. Rhesus Macaque Genome, S., C. Analysis, R. A. Gibbs, J. Rogers, M. G. Katze, R. Bumgarner, G. M. Weinstock, E. R. Mardis, K. A. Remington, R. L. Strausberg, J. C. Venter, R. K. Wilson, M. A. Batzer, C. D. Bustamante, E. E. Eichler, M. W. Hahn, R. C. Hardison, K. D. Makova, W. Miller, A. Milosavljevic, R. E. Palermo, A. Siepel, J. M. Sikela, T. Attaway, S. Bell, K. E. Bernard, C. J. Buhay, M. N. Chandrabose, M. Dao, C. Davis, K. D. Delehaunty, Y. Ding, H. H. Dinh, S. Dugan-Rocha, L. A. Fulton, R. A. Gabisi, T. T. Garner, J. Godfrey, A. C. Hawes, J. Hernandez, S. Hines, M. Holder, J. Hume, S. N. Jhangiani, V. Joshi, Z. M. Khan, E. F. Kirkness, A. Cree, R. G. Fowler, S. Lee, L. R. Lewis, Z. Li, Y. S. Liu, S. M. Moore, D. Muzny, L. V. Nazareth, D. N. Ngo, G. O. Okwuonu, G. Pai, D. Parker, H. A. Paul, C. Pfannkoch, C. S. Pohl, Y. H. Rogers, S. J. Ruiz, A. Sabo, J. Santibanez, B. W. Schneider, S. M. Smith, E. Sodergren, A. F. Svatek, T. R. Utterback, S. Vattathil, W. Warren, C. S. White, A. T. Chinwalla, Y. Feng, A. L. Halpern, L. W. Hillier, X. Huang, P. Minx, J. O. Nelson, K. H. Pepin, X. Qin, G. G. Sutton, E. Venter, B. P. Walenz, J. W. Wallis, K. C. Worley, S. P. Yang, S. M. Jones, M.

A. Marra, M. Rocchi, J. E. Schein, R. Baertsch, L. Clarke, M. Csuros, J. Glasscock, R. A. Harris, P. Havlak, A. R. Jackson, H. Jiang, Y. Liu, D. N. Messina, Y. Shen, H. X. Song, T. Wylie, L. Zhang, E. Birney, K. Han, M. K. Konkel, J. Lee, A. F. Smit, B. Ullmer, H. Wang, J. Xing, R. Burhans, Z. Cheng, J. E. Karro, J. Ma, B. Raney, X. She, M. J. Cox, J. P. Demuth, L. J. Dumas, S. G. Han, J. Hopkins, A. Karimpour-Fard, Y. H. Kim, J. R. Pollack, T. Vinar, C. Addo-Quaye, J. Degenhardt, A. Denby, M. J. Hubisz, A. Indap, C. Kosiol, B. T. Lahn, H. A. Lawson, A. Marklein, R. Nielsen, E. J. Vallender, A. G. Clark, B. Ferguson, R. D. Hernandez, K. Hirani, H. Kehrer-Sawatzki, J. Kolb, S. Patil, L. L. Pu, Y. Ren, D. G. Smith, D. A. Wheeler, I. Schenck, E. V. Ball, R. Chen, D. N. Cooper, B. Giardine, F. Hsu, W. J. Kent, A. Lesk, D. L. Nelson, E. O'Brien W, K. Prufer, P. D. Stenson, J. C. Wallace, H. Ke, X. M. Liu, P. Wang, A. P. Xiang, F. Yang, G. P. Barber, D. Haussler, D. Karolchik, A. D. Kern, R. M. Kuhn, K. E. Smith, and A. S. Zwieg. 2007. Evolutionary and biomedical insights from the rhesus macaque genome. *Science* 316: 222-234.

285. Wille-Reece, U., B. J. Flynn, K. Lore, R. A. Koup, A. P. Miles, A. Saul, R. M. Kedl, J. J. Mattapallil, W. R. Weiss, M. Roederer, and R. A. Seder. 2006. Toll-like receptor agonists influence the magnitude and quality of memory T cell responses after prime-boost immunization in nonhuman primates. *J Exp Med* 203: 1249-1258.
286. Sui, Y., S. Gagnon, A. Dzutsev, Q. Zhu, H. Yu, A. Hogg, Y. Wang, Z. Xia, I. M. Belyakov, D. Venzon, D. Klinman, W. Strober, B. Kelsall, G. Franchini, and J. A. Berzofsky. 2011. TLR agonists and/or IL-15 adjuvanted mucosal SIV vaccine reduced gut CD4(+) memory T cell loss in SIVmac251-challenged rhesus macaques. *Vaccine* 30: 59-68.
287. Ambrose, Z., C. Kline, P. Polacino, and S. L. Hu. 2014. Dysregulation of multiple inflammatory molecules in lymph node and ileum of macaques during RT-SHIV infection with or without antiretroviral therapy. *Journal of Medical Primatology* 43: 298-309.
288. Lee, K., Z. Ambrose, T. D. Martin, I. Oztop, A. Mulky, J. G. Julias, N. Vandegraaff, J. G. Baumann, R. Wang, W. Yuen, T. Takemura, K. Shelton, I. Taniuchi, Y. Li, J. Sodroski, D. R. Littman, J. M. Coffin, S. H. Hughes, D. Unutmaz, A. Engelman, and V. N. KewalRamani. 2010. Flexible use of nuclear import pathways by HIV-1. *Cell Host & Microbe* 7: 221-233.
289. Kim, S. S., N. Kothari, X. J. You, W. E. Robinson, Jr., T. Schnell, K. Uberla, and H. Fan. 2001. Generation of replication-defective helper-free vectors based on simian immunodeficiency virus. *Virology* 282: 154-167.
290. Wick, N., P. Saharinen, J. Saharinen, E. Gurnhofer, C. W. Steiner, I. Raab, D. Stokic, P. Giovanoli, S. Buchsbaum, A. Burchard, S. Thurner, K. Alitalo, and D. Kerjaschki. 2007. Transcriptomal comparison of human dermal lymphatic endothelial cells ex vivo and in vitro. *Physiol Genomics* 28: 179-192.
291. Shin, J. W., G. Jurisic, and M. Detmar. 2008. Lymphatic-specific expression of dipeptidyl peptidase IV and its dual role in lymphatic endothelial function. *Experimental Cell Research* 314: 3048-3056.
292. Neubert, R., H. Helge, and D. Neubert. 1995. Thalidomide and the immune system. 4. Down-regulation of the CD26 receptor, probably involved in the binding of HIV components to T cells in primates. *Life Sciences* 56: 407-420.

293. Lu, G., Y. Hu, Q. Wang, J. Qi, F. Gao, Y. Li, Y. Zhang, W. Zhang, Y. Yuan, J. Bao, B. Zhang, Y. Shi, J. Yan, and G. F. Gao. 2013. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. *Nature* 500: 227-231.
294. Pohlmann, S., M. Krumbiegel, and F. Kirchhoff. 1999. Coreceptor usage of BOB/GPR15 and Bonzo/STRL33 by primary isolates of human immunodeficiency virus type 1. *The Journal of General Virology* 80 (Pt 5): 1241-1251.
295. Liu, L., N. M. Oliveira, K. M. Cheney, C. Pade, H. Dreja, A. M. Bergin, V. Borgdorff, D. H. Beach, C. L. Bishop, M. T. Dittmar, and A. McKnight. 2011. A whole genome screen for HIV restriction factors. *Retrovirology* 8: 94.
296. Yan, N., and Z. J. Chen. 2012. Intrinsic antiviral immunity. *Nature Immunology* 13: 214-222.
297. Sanghavi, S. K., R. Shankarappa, and T. A. Reinhart. 2004. Genetic analysis of Toll/Interleukin-1 Receptor (TIR) domain sequences from rhesus macaque Toll-like receptors (TLRs) 1-10 reveals high homology to human TLR/TIR sequences. *Immunogenetics* 56: 667-674.
298. Kriehuber, E., S. Breiteneder-Geleff, M. Groeger, A. Soleiman, S. F. Schoppmann, G. Stingl, D. Kerjaschki, and D. Maurer. 2001. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med* 194: 797-808.
299. Ando, T., P. Jordan, T. Joh, Y. Wang, M. H. Jennings, J. Houghton, and J. S. Alexander. 2005. Isolation and characterization of a novel mouse lymphatic endothelial cell line: SV-LEC. *Lymphatic Research Biology* 3: 105-115.
300. Garrafa, E., G. Alessandri, A. Benetti, D. Turetta, A. Corradi, A. M. Cantoni, E. Cervi, S. Bonardelli, E. Parati, S. M. Giulini, B. Ensoli, and A. Caruso. 2006. Isolation and characterization of lymphatic microvascular endothelial cells from human tonsils. *Journal of Cellular Physiology* 207: 107-113.
301. Wick, N., D. Haluza, E. Gurnhofer, I. Raab, M. T. Kasimir, M. Prinz, C. W. Steiner, C. Reinisch, A. Howorka, P. Giovanoli, S. Buchsbaum, S. Krieger, E. Tschachler, P. Petzelbauer, and D. Kerjaschki. 2008. Lymphatic precollectors contain a novel, specialized subpopulation of podoplanin low, CCL27-expressing lymphatic endothelial cells. *The American Journal of Pathology* 173: 1202-1209.
302. Jordan-Williams, K. L., and A. Ruddell. 2014. Culturing Purifies Murine Lymph Node Lymphatic Endothelium. *Lymphatic Research Biology* 12(3): 144-149.
303. Marchetti, G., C. Tincati, and G. Silvestri. 2013. Microbial translocation in the pathogenesis of HIV infection and AIDS. *Clinical Microbiology Reviews* 26: 2-18.
304. Moeller, A. H., M. Shilts, Y. Li, R. S. Rudicell, E. V. Lonsdorf, A. E. Pusey, M. L. Wilson, B. H. Hahn, and H. Ochman. 2013. SIV-induced instability of the chimpanzee gut microbiome. *Cell Host & Microbe* 14: 340-345.
305. Reeves, R. K., T. I. Evans, J. Gillis, F. E. Wong, G. Kang, Q. Li, and R. P. Johnson. 2012. SIV infection induces accumulation of plasmacytoid dendritic cells in the gut mucosa. *The Journal of Infectious Diseases* 206: 1462-1468.
306. Verhoeven, D., M. D. George, W. Hu, A. T. Dang, Z. Smit-McBride, E. Reay, M. Macal, A. Fenton, S. Sankaran-Walters, and S. Dandekar. 2014. Enhanced innate antiviral gene expression, IFN-alpha, and cytolytic responses are predictive of mucosal immune recovery during simian immunodeficiency virus infection. *J Immunol* 192: 3308-3318.

307. Tanaka, Y., H. Marusawa, H. Seno, Y. Matsumoto, Y. Ueda, Y. Kodama, Y. Endo, J. Yamauchi, T. Matsumoto, A. Takaori-Kondo, I. Ikai, and T. Chiba. 2006. Anti-viral protein APOBEC3G is induced by interferon-alpha stimulation in human hepatocytes. *Biochemical and Biophysical Research Communications* 341: 314-319.
308. Neil, S. J., V. Sandrin, W. I. Sundquist, and P. D. Bieniasz. 2007. An interferon-alpha-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein. *Cell Host & Microbe* 2: 193-203.
309. Cohen, J. N., C. J. Guidi, E. F. Tewalt, H. Qiao, S. J. Rouhani, A. Ruddell, A. G. Farr, K. S. Tung, and V. H. Engelhard. 2010. Lymph node-resident lymphatic endothelial cells mediate peripheral tolerance via Aire-independent direct antigen presentation. *J Exp Med* 207: 681-688.
310. Sato, G. H., J. D. Sato, T. Okamoto, W. L. McKeehan, and D. W. Barnes. 2010. Tissue culture: the unlimited potential. *In Vitro Cell Dev Biol Anim* 46: 590-594.
311. Breslin, S., and L. O'Driscoll. 2013. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today* 18: 240-249.
312. Alberts, B. 2002. *Molecular Biology of the Cell*. Garland Science, New York.
313. Betakova, T., D. Svetlikova, and M. Gocnik. 2013. Overview of measles and mumps vaccine: origin, present, and future of vaccine production. *Acta Virologica* 57: 91-96.
314. Garrafa, E., L. Trainini, A. Benetti, E. Saba, L. Fezzardi, B. Lorusso, P. Borghetti, T. Bottio, E. Ceri, N. Portolani, S. Bonardlli, S. M. Giulini, G. Annibale, A. Corradi, L. Imberti, and A. Caruso. 2005. Isolation, purification, and heterogeneity of human lymphatic endothelial cells from different tissues. *Lymphology* 38: 159-166.
315. Kraling, B. M., and J. Bischoff. 1998. A simplified method for growth of human microvascular endothelial cells results in decreased senescence and continued responsiveness to cytokines and growth factors. *In Vitro Cell Dev Biol Anim* 34: 308-315.
316. Baker, B. M., and C. S. Chen. 2012. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *Journal of Cell Science* 125: 3015-3024.
317. Haycock, J. W. 2011. 3D cell culture: a review of current approaches and techniques. *Methods Mol Biol* 695: 1-15.
318. Antoni, D., H. Burckel, E. Josset, and G. Noel. 2015. Three-Dimensional Cell Culture: A Breakthrough in Vivo. *International Journal of Molecular Sciences* 16: 5517-5527.
319. Shamir, E. R., and A. J. Ewald. 2014. Three-dimensional organotypic culture: experimental models of mammalian biology and disease. *Nature Reviews. Molecular Cell Biology* 15: 647-664.
320. Blacher, S., C. Erpicum, B. Lenoir, J. Paupert, G. Moraes, S. Ormenese, E. Bullinger, and A. Noel. 2014. Cell invasion in the spheroid sprouting assay: a spatial organisation analysis adaptable to cell behaviour. *PloS One* 9: e97019.
321. Amatschek, S., E. Kriehuber, W. Bauer, B. Reininger, P. Meraner, A. Wolpl, N. Schweifer, C. Haslinger, G. Stingl, and D. Maurer. 2007. Blood and lymphatic endothelial cell-specific differentiation programs are stringently controlled by the tissue environment. *Blood* 109: 4777-4785.
322. Salven, P., S. Mustjoki, R. Alitalo, K. Alitalo, and S. Rafii. 2003. VEGFR-3 and CD133 identify a population of CD34+ lymphatic/vascular endothelial precursor cells. *Blood* 101: 168-172.
323. Osterbur, J., L. Sprague, M. Muccioli, M. Pate, K. Mansfield, J. McGinty, Y. Li, Y. Li, V. Shirure, M. C. Courreges, and F. Benencia. 2013. Adhesion to substrates induces

dendritic cell endothelization and decreases immunological response. *Immunobiology* 218: 64-75.
324. Norrmen, C., W. Vandevelde, A. Ny, P. Saharinen, M. Gentile, G. Haraldsen, P. Puolakkainen, E. Lukanidin, M. Dewerchin, K. Alitalo, and T. V. Petrova. 2010. Liprin (beta)1 is highly expressed in lymphatic vasculature and is important for lymphatic vessel integrity. *Blood* 115: 906-909.
325. Lee, E., N. B. Pandey, and A. S. Popel. 2015. Crosstalk between cancer cells and blood endothelial and lymphatic endothelial cells in tumour and organ microenvironment. *Expert Reviews in Molecular Medicine* 17: e3.
326. Cancian, L., A. Hansen, and C. Boshoff. 2013. Cellular origin of Kaposi's sarcoma and Kaposi's sarcoma-associated herpesvirus-induced cell reprogramming. *Trends in Cell Biology* 23: 421-432.
327. Yoo, J., J. Kang, H. N. Lee, B. Aguilar, D. Kafka, S. Lee, I. Choi, J. Lee, S. Ramu, J. Haas, C. J. Koh, and Y. K. Hong. 2010. Kaposin-B enhances the PROX1 mRNA stability during lymphatic reprogramming of vascular endothelial cells by Kaposi's sarcoma herpes virus. *PLoS Pathogens* 6: e1001046.
328. Yoo, J., H. N. Lee, I. Choi, D. Choi, H. K. Chung, K. E. Kim, S. Lee, B. Aguilar, J. Kang, E. Park, Y. S. Lee, Y. S. Maeng, N. Y. Kim, C. J. Koh, and Y. K. Hong. 2012. Opposing regulation of PROX1 by interleukin-3 receptor and NOTCH directs differential host cell fate reprogramming by Kaposi sarcoma herpes virus. *PLoS Pathogens* 8: e1002770.
329. Janeway, C. A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor Symposia on Quantitative Biology* 54 Pt 1: 1-13.
330. Medzhitov, R. 2013. Pattern recognition theory and the launch of modern innate immunity. *J Immunol* 191: 4473-4474.
331. Brennan, K., and A. G. Bowie. 2010. Activation of host pattern recognition receptors by viruses. *Current Opinion in Microbiology* 13: 503-507.
332. Coffman, R. L., A. Sher, and R. A. Seder. 2010. Vaccine adjuvants: putting innate immunity to work. *Immunity* 33: 492-503.
333. Higgins, S. C., and K. H. Mills. 2010. TLR, NLR Agonists, and Other Immune Modulators as Infectious Disease Vaccine Adjuvants. *Current Infectious Disease Reports* 12: 4-12.
334. Duthie, M. S., H. P. Windish, C. B. Fox, and S. G. Reed. 2011. Use of defined TLR ligands as adjuvants within human vaccines. *Immunological Reviews* 239: 178-196.
335. Steinhagen, F., T. Kinjo, C. Bode, and D. M. Klinman. 2011. TLR-based immune adjuvants. *Vaccine* 29: 3341-3355.
336. Malhotra, D., A. L. Fletcher, and S. J. Turley. 2013. Stromal and hematopoietic cells in secondary lymphoid organs: partners in immunity. *Immunological Reviews* 251: 160-176.
337. Crozat, K., E. Vivier, and M. Dalod. 2009. Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. *Immunological Reviews* 227: 129-149.
338. Gowen, B. B., M. H. Wong, K. H. Jung, A. B. Sanders, W. M. Mitchell, L. Alexopoulou, R. A. Flavell, and R. W. Sidwell. 2007. TLR3 is essential for the induction of protective immunity against Punta Toro Virus infection by the double-stranded RNA (dsRNA), poly(I:C12U), but not Poly(I:C): differential recognition of synthetic dsRNA molecules. *J Immunol* 178: 5200-5208.

339. Stahl-Hennig, C., M. Eisenblatter, E. Jasny, T. Rzehak, K. Tenner-Racz, C. Trumpfheller, A. M. Salazar, K. Uberla, K. Nieto, J. Kleinschmidt, R. Schulte, L. Gissmann, M. Muller, A. Sacher, P. Racz, R. M. Steinman, M. Uguccioni, and R. Ignatius. 2009. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. *PLoS Pathogens* 5: e1000373.
340. Casella, C. R., and T. C. Mitchell. 2008. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cellular and Molecular Life Sciences : CMLS* 65: 3231-3240.
341. Mata-Haro, V., C. Cekic, M. Martin, P. M. Chilton, C. R. Casella, and T. C. Mitchell. 2007. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* 316: 1628-1632.
342. Didierlaurent, A. M., S. Morel, L. Lockman, S. L. Giannini, M. Bisteau, H. Carlsen, A. Kielland, O. Vosters, N. Vanderheyde, F. Schiavetti, D. Larocque, M. Van Mechelen, and N. Garcon. 2009. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol* 183: 6186-6197.
343. Huleatt, J. W., V. Nakaar, P. Desai, Y. Huang, D. Hewitt, A. Jacobs, J. Tang, W. McDonald, L. Song, R. K. Evans, S. Umlauf, L. Tussey, and T. J. Powell. 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine* 26: 201-214.
344. Sanders, C. J., D. A. Moore, 3rd, I. R. Williams, and A. T. Gewirtz. 2008. Both radioresistant and hemopoietic cells promote innate and adaptive immune responses to flagellin. *J Immunol* 180: 7184-7192.
345. Bogerd, H. P., B. P. Doehle, H. L. Wiegand, and B. R. Cullen. 2004. A single amino acid difference in the host APOBEC3G protein controls the primate species specificity of HIV type 1 virion infectivity factor. *Proceedings of the National Academy of Sciences of the United States of America* 101: 3770-3774.
346. Mangeat, B., P. Turelli, S. Liao, and D. Trono. 2004. A single amino acid determinant governs the species-specific sensitivity of APOBEC3G to Vif action. *The Journal of Biological Chemistry* 279: 14481-14483.
347. Schrofelbauer, B., D. Chen, and N. R. Landau. 2004. A single amino acid of APOBEC3G controls its species-specific interaction with virion infectivity factor (Vif). *Proceedings of the National Academy of Sciences of the United States of America* 101: 3927-3932.
348. Xu, H., E. S. Svarovskaia, R. Barr, Y. Zhang, M. A. Khan, K. Strebel, and V. K. Pathak. 2004. A single amino acid substitution in human APOBEC3G antiretroviral enzyme confers resistance to HIV-1 virion infectivity factor-induced depletion. *Proceedings of the National Academy of Sciences of the United States of America* 101: 5652-5657.
349. Chiu, Y. L., and W. C. Greene. 2008. The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. *Annual Review of Immunology* 26: 317-353.
350. Chiu, Y. L., V. B. Soros, J. F. Kreisberg, K. Stopak, W. Yonemoto, and W. C. Greene. 2005. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature* 435: 108-114.

351. Neil, S. J., S. W. Eastman, N. Jouvenet, and P. D. Bieniasz. 2006. HIV-1 Vpu promotes release and prevents endocytosis of nascent retrovirus particles from the plasma membrane. *PLoS Pathogens* 2: e39.
352. Evans, D. T., R. Serra-Moreno, R. K. Singh, and J. C. Guatelli. 2010. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends in Microbiology* 18: 388-396.
353. Wolf, D., and S. P. Goff. 2008. Host restriction factors blocking retroviral replication. *Annual Review of Genetics* 42: 143-163.
354. Lahouassa, H., W. Daddacha, H. Hofmann, D. Ayinde, E. C. Logue, L. Dragin, N. Bloch, C. Maudet, M. Bertrand, T. Gramberg, G. Pancino, S. Priet, B. Canard, N. Laguette, M. Benkirane, C. Transy, N. R. Landau, B. Kim, and F. Margottin-Goguet. 2012. SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nature Immunology* 13: 223-228.
355. Raposo, R. A., M. Abdel-Mohsen, M. Bilska, D. C. Montefiori, D. F. Nixon, and S. K. Pillai. 2013. Effects of cellular activation on anti-HIV-1 restriction factor expression profile in primary cells. *Journal of Virology* 87: 11924-11929.
356. Valbuena, G., and D. H. Walker. 2006. The endothelium as a target for infections. *Annual Review of Pathology* 1: 171-198.
357. Short, K. R., E. J. Veldhuis Kroeze, L. A. Reperant, M. Richard, and T. Kuiken. 2014. Influenza virus and endothelial cells: a species specific relationship. *Frontiers in Microbiology* 5: 653.
358. Miller, J. L., B. J. de Wet, L. Martinez-Pomares, C. M. Radcliffe, R. A. Dwek, P. M. Rudd, and S. Gordon. 2008. The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathogens* 4: e17.
359. Cai, Y., S. Q. Yu, E. N. Postnikova, S. Mazur, J. G. Bernbaum, R. Burk, T. Zhang, S. R. Radoshitzky, M. A. Muller, I. Jordan, L. Bollinger, L. E. Hensley, P. B. Jahrling, and J. H. Kuhn. 2014. CD26/DPP4 cell-surface expression in bat cells correlates with bat cell susceptibility to Middle East respiratory syndrome coronavirus (MERS-CoV) infection and evolution of persistent infection. *PloS One* 9: e112060.
360. Lafon, M. E., J. L. Gendrault, C. Royer, D. Jaeck, A. Kirn, and A. M. Steffan. 1993. Human endothelial cells isolated from the hepatic sinusoids and the umbilical vein display a different permissiveness for HIV1. *Research in Virology* 144: 99-104.
361. Bussolino, F., S. Mitola, G. Serini, G. Barillari, and B. Ensoli. 2001. Interactions between endothelial cells and HIV-1. *The International Journal of Biochemistry & Cell Biology* 33: 371-390.
362. Scheglovitova, O., M. R. Capobianchi, G. Antonelli, D. Guanmu, and F. Dianzani. 1993. CD4-positive lymphoid cells rescue HIV-1 replication from abortively infected human primary endothelial cells. *Archives of Virology* 132: 267-280.
363. Huang, M. B., M. Hunter, and V. C. Bond. 1999. Effect of extracellular human immunodeficiency virus type 1 glycoprotein 120 on primary human vascular endothelial cell cultures. *AIDS Research and Human Retroviruses* 15: 1265-1277.
364. Graham, G. J. 2009. D6 and the atypical chemokine receptor family: novel regulators of immune and inflammatory processes. *European Journal of Immunology* 39: 342-351.
365. Biancone, L., V. Cantaluppi, M. Boccellino, L. Del Sorbo, S. Russo, A. Albini, I. Stamenkovic, and G. Camussi. 1999. Activation of CD40 favors the growth and vascularization of Kaposi's sarcoma. *J Immunol* 163: 6201-6208.