THE RESPONSE OF THE LYMPHATIC ENDOTHELIUM TO INFLAMMATION AND INFECTION IN *IN VITRO* AND *IN VIVO* SYSTEMS

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The lymphatic endothelium is involved in the drainage of interstitial fluid and in the migration of immune cells like dendritic cells (DCs) from the periphery to draining lymph nodes (LNs). Tuberculosis has been declared a pandemic infectious disease accounting for more than 2 million deaths annually and is caused by the intracellular bacteria, *Mycobacterium tuberculosis*. The chronic inflammatory response to *M. tuberculosis* infection is characterized by the formation of granulomatous structures in the pulmonary compartments of infected individuals. These structures contain excess interstitial fluid and are enriched with immune cells including DCs. Therefore, the lymphatic vessels might play important roles in regulating drainage of fluid and migration of immune cells from granulomas to the draining LNs. My hypothesis was that there is an increased concentration of lymphatic vessels in these granulomatous structures and that the inflammatory environment including mycobacterial components present in granulomas and at other sites of infection elicit an inflammatory response from these lymphatic vessels which contribute to the overall immune response to *M. tuberculosis* infection. To address this hypothesis I have examined the distribution of lymphatic vessels in granulomatous and LN tissues obtained from nonhuman primates infected with *M. tuberculosis* and analyzed their expression of multiple chemokines and lymphatic markers. In addition, I evaluated the response of LECs to inflammatory mediators that included multiple TLR ligands, *M. tuberculosis* components and cytokines. I observed an association of lymphatic vessels with granulomas, and

found that there was heterogeneity in the expression of chemokines and lymphatic markers by LECs in tissues. I also found that primary human LECs expressed multiple TLR molecules and responded to TLR ligands, cytokines and *M. tuberculosis* components by increasing expression of inflammatory chemokines, cytokines and adhesion molecules. These LECs also demonstrated phenotypic similarities with DCs. Overall my findings support the involvement of the lymphatic endothelium in the inflammatory immune response to pathogens like *M. tuberculosis*. From the perspective of public health relevance, these studies provide direction in the development of new therapeutic targets against *M. tuberculosis* infections and aid in the development of better adjuvants for vaccines for infectious diseases and cancers.

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FOREWORD

A step into the ever-expanding world of science, I have taken
For the past twenty-seven years been working towards this goal
After many a sacrifice and things that I have forsaken
Family, friends and everyone I have met, all have played a role
In this pursuit of answers to questions that arise
Out of the thirst for an understanding of life's mysteries
In the quest for a better life, the ultimate prize
To provide tools and means in the fight against disease
It's been a journey, long, hard and tiresome
With endless days, sleepless nights and many a mistake
And inspite of all the obstacles and pitfalls that have come
It's been fun on the bench without a break
Hopefully it's not the end just a beginning
On this road of life, long and winding
With many more twists and turns coming
Wish till the end, this song, I'll still be singing.

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LIST OF ABBREVIATIONS

5’-Nase	5’-Nucleotidase
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen presenting cell
BEC	Blood endothelial cell
CD	Crohn’s disease
COX	Cyclooxygenase
DAB	Diaminobenzidine
DC	Dendritic cell
DC-LAMP	Dendritic cell lysosome-associated membrane glycoprotein
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
DLAR	Division of laboratory animal resources
DQ-Ova	DQ-ovalbumin
ELISA	Enzyme-linked immunosorbent assay
FGF-2	Fibroblast growth factor 2
FIND	IL-4 inducible protein
FITC	Fluorescein isothiocynate
HEV	High endothelial venule
HGF	Hepatocyte growth factor
HHV8	Human herpesvirus 8
HIV-1	Human immunodeficiency virus type 1
HLN	Hilar lymph node
HMVEC	Human microvascular endothelial cell
HMVEC-LLy	Lung lymphatic microvascular endothelial cell
HMVEC-dLy	Dermal lymphatic microvascular endothelial cell
hTERT	Human telomerase reverse transcriptase
IACUC	Institutional animal care and use committee
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
iDC	Immature dendritic cell
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
ISH	*In situ* hybridization
KS	Kaposi’s sarcoma
KSHV	Kaposi’s sarcoma associated herpesvirus

LE	Lymphatic endothelium
LEC	Lymphatic endothelial cell
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LYVE-1	Lymphatic endothelial hyaluronan receptor 1
mDC	Mature dendritic cell
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MLR	Mixed leukocyte reaction
nt	Nucleotide
ODN	Oligodeoxynucleotide
OSM	Oncostatin M
PAMP	Pathogen-associated molecular pattern
PE	Phycoerythrin
PRR	Pattern recognition receptor
Prox-1	Prospero-related homeobox 1
RT-PCR	Reverse transcriptase polymerase chain reaction
SIV	Simian immunodeficiency virus
TB	Tuberculosis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
UI	Uninfected
VCAM-1	Vascular cell adhesion molecule 1
VEGF-C	Vascular endothelial growth factor C
VEGF-D	Vascular endothelial growth factor D
VEGFR-3	Vascular endothelial growth factor receptor 3
VLA-4	Very late antigen 4
WHO	World health organization

1.0 INTRODUCTION

The mammalian vascular system is made up of two distinct but interrelated endothelial networks – the blood endothelial network and the lymphatic endothelial network. The blood endothelial network forms a closed loop that is involved in the circulation of blood pumped by the heart, whereas the lymphatic endothelial network is a unidirectional, open-ended network comprised of lymphatic vessels that collect lymph, a protein-rich exudate from blood vessels, in tissues and drain it back into the venous circulation via larger collecting lymphatic vessels and the thoracic duct (1,2). The lymphatic endothelial network is also involved in immune surveillance since immune cells that include lymphocytes and antigen-presenting dendritic cells (DCs) travel from peripheral tissues through lymphatic vessels to draining lymph nodes (LNs) to initiate specific immune responses (3). Despite the importance of the lymphatic endothelial network in initiation of immune responses, there has been a dearth of studies on the involvement of the lymphatic endothelial network in the immune system as compared to the extensive research focused on the blood endothelial network. The lymphatic endothelial network can be actively involved in the regulation of cellular traffic from the periphery to the LNs, which would be important during pathogenic infections for the initiation and maintenance of antigen specific immune responses.

1.1 LYMPHATIC ENDOTHELIUM: CELLULAR STRUCTURE AND MARKERS

The lymphatic endothelial network consists of the terminal lymphatic vessels and the larger collecting lymphatic vessels, collectively referred to as the lymphatic endothelium (LE) and lymphoid organs that include LNs, tonsils, Peyer's patches, spleen and thymus, which are involved in the generation and maintenance of immune responses (2). Lymphatic vessels are present throughout the body in almost all tissues with the exception of the central nervous system, bone marrow and avascular tissues such as cornea, cartilage and epidermis (1,2). The lymphatic vessels share phenotypic similarities with blood vessels although there are key differences between them due to their distinct functional roles. The terminal lymphatic vessels are thin-walled, relatively large vessels, composed of a single, non-fenestrated layer of overlapping endothelial cells that lack a continuous basement membrane as well as pericyte or smooth muscle cell coverage unlike blood vessels. The lymphatic endothelial cells (LECs) are anchored to the extracellular matrix by specialized fibrillin-containing anchoring filaments (4), which in the case of fluid accumulation exerts pressure on the LECs to open the overlapping cell junctions leading to enhanced uptake of interstitial fluid, macromolecules, and cells. The larger collecting lymphatic vessels have a smooth muscle layer, basement membrane and luminal valves that prevent backflow of fluid.

Lymphatic vessels were first described in the seventeenth century, although not until at the start of the twentieth century was the developmental origin of lymphatic vessels known. Only in the past decade were the first growth factors and molecular markers specific for lymphatic vessels identified. Due to the close structural and functional relationship of blood and lymphatic vessels, it has been a challenge to differentiate between the two vessel types. The identification of several lymphatic-specific markers has aided in the isolation and study of

relatively pure LECs and blood endothelial cells (BECs) from tissues (5-8). These studies have focused on the molecular characteristics of LECs and BECs, and have revealed that both cell types share a similar gene expression profile indicative of their close genetic relationship. However, these studies have also led to the identification of several new and previously unknown markers specific for LECs that will aid in the further study of lymphatic vessel development and function.

1.1.1 VEGFR-3

Vascular endothelial growth factor receptor-3 (VEGFR-3), also known as Flt4, was one of the first lymphatic-specific markers to be identified (9). VEGFR-3 is a member of the *fms*-like tyrosine kinase family and specifically binds the lymphatic growth factors, vascular endothelial growth factor (VEGF)-C and VEGF-D. During early embryonic development,VEGFR-3 is expressed both in developing venous and in presumptive LE, whereas in normal adult tissues, its expression is largely restricted to the LE (9,10). Although the inactivation of V*egfr3* in mice led to cardiovascular failure and death of the embryo before the emergence of lymphatic vessels (11), the association of mutations in *Vegfr3* gene with hereditary lymphedema in patients has provided support for an important role of this gene in lymphatic development (12,13). VEGFR-3 expression has also been observed on some blood capillaries associated with tumor neovascularization or wound granulation tissue (10,14,15), and on antigen presenting cells in corneal tissue (16,17). Therefore, depending on the tissue and the developmental stage, VEGFR-3 alone may not be sufficient to serve as a specific marker for lymphatic vessels.

1.1.2 Prox1

The transcription factor Prospero-related homeobox-1 (Prox1) is a homolog of the *Drosophila* homeobox gene *prospero* (18) and is considered to be the most specific lineage marker in endothelial cells for LECs. Inactivation of Prox1 in mice leads to embryonic lethality and a complete absence of lymphatic vasculature in these mice (19). It was also observed that in Prox1 null mice, the endothelial cells failed to express LE markers (20) indicating the importance of Prox1 in the early stages of LEC differentiation from progenitor cells. The ectopic expression of Prox1 in differentiated BECs was shown to reprogram these cells to adopt a lymphatic phenotype (21,22) further illustrating the importance of Prox1 for lymphatic cell differentiation. Amongst endothelial cells, Prox1 expression is observed exclusively in lymphatic vessels of adult tissues and tumors (23).

1.1.3 LYVE-1

The lymphatic endothelial hyaluronan receptor-1 (LYVE-1) is a transmembrane receptor that binds to the glycosaminoglycan hyaluronan and is a homolog of the BEC-specific hyaluronan receptor CD44 (24,25). It was identified as a specific cell surface protein of LECs and macrophages (24,26,27), although LYVE-1 expression has also been detected in liver sinusoidal endothelial cells (28). Interestingly, LYVE-1 deficient mice have normal development of the lymphatic vasculature and exhibit minimal functional abnormalities (29,30). LYVE-1 is expressed on all embryonic LECs, whereas in adult tissues its expression becomes restricted to the terminal lymphatic vessels (31).

1.1.4 Podoplanin

Podoplanin is a mucin-type transmembrane glycoprotein that controls podocyte shape and platelet aggregation (32). Podoplanin expression has been observed in osteoblastic cells, kidney podocytes, and lung alveolar type I cells (33), with strong expression in LECs both *in vivo* and *in vitro* (5,6,22,33,34). Podoplanin deficient mice die perinatally and have malformed lymphatic vessels and diminished tissue drainage (35,36) suggesting the importance of podoplanin in lymphatic development. Podoplanin promotes LEC adhesion, migration and tubulogenesis formation *in vitro* (36). Podoplanin expression also has been observed in several non-endothelial cell types particularly in tumors suggesting involvement in tumor progression (37).

1.1.5 CCL21

The CC-chemokine ligand-21 (CCL21, also known as 6Ckine, Exodus or SLC) is secreted by LECs and not BECs (6) and binds to the CC-chemokine receptor 7 (CCR7). It has been shown to mediate the homing of lymphocytes and migration of antigen-loaded DCs into lymphatic vessels and LNs (3,38,39), thus playing an important role in the regulation and activation of immune responses. Besides LECs, CCL21 has also been shown to be highly expressed by cells other than LECs in the LN paracortex and therefore limiting its use as a lymphatic marker in lymphoid tissues (40).

Additional markers that have been used to identify lymphatic vessels include the chemokine decoy receptor D6 (41,42), 5'-nucleotidase (5'-Nase) activity (43-45), and desmoplakin (46). A large number of previously unknown LEC-specific genes have also been identified using microarray analysis which include macrophage mannose receptor 1, reelin,

plakoglobin, and integrin α9 (5,22), although their exact functions in LECs is still under investigation.

1.2 CELLULAR TRAFFIC THROUGH LYMPHATIC VESSELS

Lymphatic vessels not only play an essential role in the drainage of interstitial fluid and maintenance of normal fluid balance, but also are important for cellular trafficking and antigen delivery that are required for the regulation and initiation of immune responses. Several different immune cell types have been shown to traffic through the lymphatic vessels to the draining LNs, which supports the important role of the lymphatic system in immune surveillance.

The cellular traffic moving through peripheral lymphatic vessels was first described as containing 'veiled' cells of mononuclear lineage and lymphocytes (47-50). Since then these veiled cells were identified as mature DCs, although immature DCs, macrophages and monocytes have also been shown to be present within lymphatic vessels (51).

DCs are professional antigen-presenting cells (APCs) that are regarded as the sentinels of the immune system and they act as key regulators of immunity (52). Immature DCs present in the peripheral tissues are capable of phagocytosis and express the chemokine receptor CCR6, whereas after activation the mature DCs lose their phagocytic capacity, and express the chemokine receptor CCR7 (53-56). These CCR7-expressing mature DCs are then recruited to the lymphatic vessels, which in current models constitutively secrete the CCR7 ligand CCL21, leading to the migration of the DCs to the draining LNs (57-59). The trafficking of DCs through the lymphatic vessels occurs at a constitutive level under homeostatic conditions and this traffic

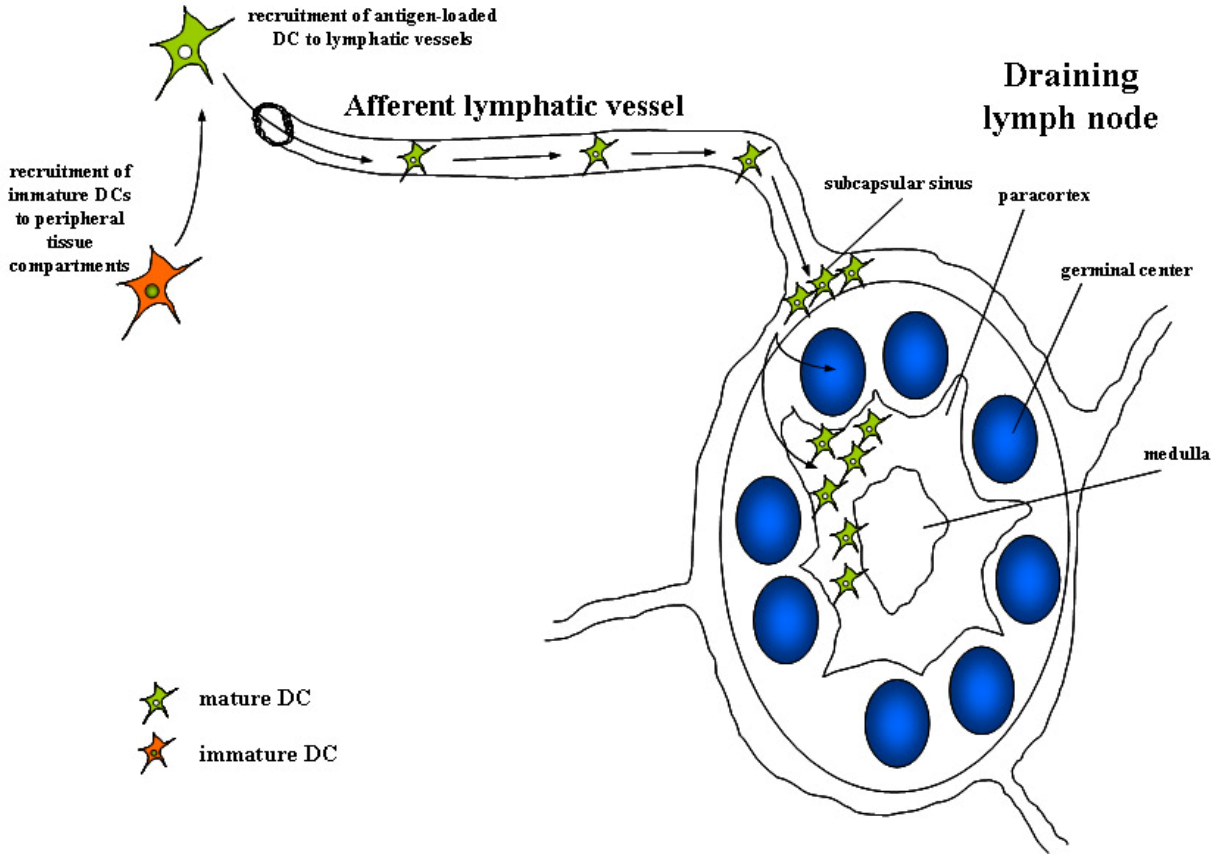

Figure 1. Role of lymphatic vessels in the migration of mature DCs from the peripheral tissues to the draining LNs. Immature DCs are recruited to peripheral tissues, where they take up antigen and undergo maturation. These antigen-loaded mature DCs express CCR7 and are recruited by peripheral lymphatic vessels that secrete the CCR7 ligand CCL21. These DCs then transit through the lymphatic vessels to reach the subcapsular sinus of draining LNs, where they further migrate to the paracortical regions to stimulate naïve lymphocytes.

of DCs can be further increased under inflammatory conditions (57,60-63). Therefore lymphatic vessels serve as a conduit for the migration of DCs from peripheral tissues to the draining LNs.

In addition to DCs, other cell types have also been described that traffic through the lymphatic vessels from the periphery to the draining LNs. A subset of effector T cells that express CCR7 exit peripheral tissues and enter lymphatic vessels to migrate to the draining LNs (38,64). These studies have shown that in two different tissue compartments - skin and lung - the exit of T cells from these tissues occur via the afferent lymphatic vessels and is dependent on

the expression of CCR7 by the T cells. It has also been shown that $CD4^+$ memory T cells traffic through lymphatic vessels (65).

A large influx of neutrophils has been observed in lymphatic vessels during inflammatory conditions (48,66,67). In addition neutrophils have been shown to rapidly migrate via lymphatic vessels after inoculation with *M. bovis* BCG (68). These neutrophils were also shown to shuttle live bacilli to the draining LNs from the site of inoculation via the lymphatic vessels. Similarly other pathogens have been shown to use the lymphatic vessels to reach the draining LNs from peripheral sites of infection and disseminate into other parts of the body (69-72).

The role of lymphatics in tumor metastasis has begun to gain prominence. It is now widely accepted that cancerous cells can transit through lymphatic vessels and spread to other tissue compartments like the LNs (73). Tumor metastasis to regional LNs represents the first step in the dissemination of cancers, particularly skin cancers (2). Several studies have shown that increased levels of the lymphatic growth factors VEGF-C and VEGF-D in tumors promote growth of lymphatic vessels and lymphatic tumor spread to the regional LNs (74-76). The inhibition of tumor metastasis by targeting VEGFR-3, the receptor for VEGF-C/D and/or blocking its signaling pathway provides further evidence in support of the essential role for these lymphatic growth factors in the spread of tumors (77-81). It has also been noted that lymphatic vessels can actively recruit tumor cells and participate in metastasis formation by their secretion of CCL21 since some tumor cells are known to express CCR7 (82).

Lymphatic vessels serve as a conduit for multiple cell types, although the exact mechanisms by which they recruit these cell types and the functional significance for the subsequent migration of these cell types to the draining LNs have not been identified. In addition these lymphatic vessels can also be subverted by pathogens and cancerous cells for the

dissemination of infection or metastasis of tumors. Therefore, there is a need for better understanding of the factors involved in the regulation of cellular traffic through lymphatic vessels under both homeostatic and inflammatory conditions.

1.3 THE LYMPHATIC ENDOTHELIUM IN INFLAMMATION

The response of the blood endothelium to inflammation has been widely studied and well characterized. In contrast, the effects of inflammation on LECs have not been very well studied. For example, there have been conflicting reports on expression of adhesion molecules by LECs under inflammatory conditions (83-88). Although in recent studies that used well characterized primary LECs, increased expression of adhesion molecules ICAM-1 and VCAM-1, key molecules that control leukocyte trafficking, by LECs after stimulation with inflammatory cytokines was observed (89,90). Therefore, these data taken together with the earlier observations of increased cellular traffic through lymphatic vessels under inflammatory conditions suggest that LECs respond to inflammatory conditions by increasing expression of adhesion molecules that enable a larger number of cells to adhere to LECs and migrate via the lymphatic vessels. The lymphatic vessels might also be stimulated by other inflammatory mediators to secrete chemokines and cytokines, which might have effects on the cellular traffic in these vessels by the recruitment of specific cell types and affecting the phenotype of trafficking cells. Recent studies have shown that inflammatory cytokines can lead to production of chemokines and regulators of leukocyte migration by LECs both *in vitro* and *in vivo* which might play important roles in regulating the availability of the lymphatic vessels for cellular traffic (57,89). The effects of other inflammatory molecules such as pathogens and their

components on LECs remains largely unknown and merits further exploration to understand the effects of pathogenic infections on LEC function.

1.3.1 Toll-like receptors

Toll-like receptors (TLRs) belong to a family of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and play important roles in the innate and adaptive immune responses to pathogens. To date, 13 mammalian TLRs have been identified, out of which the expression of TLRs 1-10 has been observed in humans (91). TLRs are type I transmembrane proteins with a cytoplasmic Toll/IL-1R domain involved in signal transduction and activation of transcription factors leading to induction of proinflammatory cytokines, chemokines and costimulatory molecules. They can be broadly classified into two groups based on their subcellular localization with TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are localized to intracellular vesicles (92). TLRs recognize conserved signature molecules from a wide range of pathogens including bacteria, viruses, fungi and parasites. TLR2 forms heterodimers with either TLR1 or TLR6 and is involved in detection of lipoproteins from gram-positive bacteria and mycobacteria (93-95). TLR2 alone can recognize lipoteichoic acid (LTA) (96), whereas TLR2 and TLR1 together detect triacylated lipoproteins (94) and TLR2 and TLR6 associate to recognize diacylated lipoproteins and peptidoglycan (93,97). TLR4 recognizes lipopolysaccaride (LPS), a characteristic component of the cell wall of gram negative bacteria (98,99), and TLR5 is involved in the detection of bacterial flagellin (100). TLR3, TLR7, TLR8 and TLR9 are involved in the recognition of viral and bacterial nucleic acids (101). TLR3 recognize viral double stranded RNA, whereas TLR7 and TLR8 detect viral single stranded

RNA. TLR9 is involved in the detection of DNA containing unmethylated CpG motifs (CpG), which are abundant in bacterial DNA. The ligand for TLR10 has not been identified yet (102,103). The expression of TLRs by LECs and their responsiveness to PAMPs have not been well studied, with only limited analysis of the expression of TLR2 and TLR4 in human intestinal lymphatic vessels, although the functional significance of this expression was not examined (104). The expression of TLRs by LECs could play an important role in regulation of the cellular traffic through the lymphatic vessels by the production of chemokines and cytokines upon engagement of these TLRs.

Among other inflammatory mediators, arachidonic acid and its metabolites have been shown to directly act on lymphatic vessels and are important modulators of lymphatic function (105,106). The use of cyclooxygenase (COX) inhibitors and inhibitors of other pathways of arachidonate metabolism led to impairment of lymphatic vessel contractility indicating the involvement of arachidonate and its metabolites in lymphatic vessels function (106). Other studies have also demonstrated the regulatory effects of prostaglandins and related metabolites on lymphatic function (107,108). These studies indicate that LECs are regulated by multiple immuno-modulators that are generated during inflammation and therefore understanding the effects of inflammation on these vessels should include consideration of the net effects of these molecules.

1.3.2 Lymphangiogenesis and inflammation

Lymphangiogenesis or the growth of new lymphatic vessels has been associated with inflammatory processes that can occur during both acute/chronic inflammation and cancer. Lymphatic vessels are known to proliferate under inflammatory conditions (109) and pro-

inflammatory cytokines induce VEGF-C, a lymphatic growth factor, supporting the involvement of inflammation in lymphatic vessel growth (110). Another vascular growth factor VEGF-A has been shown to be involved in inflammation-driven lymphangiogenesis both in a model of corneal neovascularization (111) and a model of delayed-type hypersensitivity reaction (112). Lymphangiogenesis also occurs in LNs in response to inflammation, which was associated with an increase in DC migration to the inflamed LNs (113). In this study B cells were shown to be essential for the induction of lymphangiogenesis by their secretion of VEGF-A, thus providing further evidence for the role of VEGF-A as a lymphangiogenic factor and the involvement of immune cells in inducing lymphangiogenesis during inflammation. Other lymphangiogenic factors that have been identified include neurophilin-2 (114), angiopoietin-1/2 (115-117), hepatocyte growth factor (HGF) (118,119), fibroblast growth factor-2 (FGF-2) (120-122), platelet-derived growth factor-BB (123,124) and the insulin-like growth factors 1 and 2 (125). Therefore, it appears that several lymphangiogenic factors either alone or in complex with each other can lead to the growth and proliferation of lymphatic vessels in health and disease (2).

The role of lymphangiogenesis in the development and maintenance of chronic inflammatory diseases has now begun to be more widely studied. Lymphangiogenesis has been observed in psoriasis (112,126,127), human renal transplants undergoing rejection (128,129) and in a mouse model of chronic respiratory tract infection with *Mycoplasma pulmonis* (130). It has been proposed that lymphangiogenesis contributes to and maintains the inflammation rather than resolves it (83) and that inflammation-driven lymphangiogenesis leads to the development of dysfunctional lymphatic vessels (131,132). These dysfunctional lymphatic vessels might also negatively impact DC migration to LNs and inhibit the regulation of the immune response. Impaired DC migration has been associated with chronic inflammatory diseases since it has been

reported that in Crohn's disease there was an increased retention of mature DCs in the gut (133). Similarly, in psoriasis the ability of DCs to migrate was shown to be impaired (134). In murine models of atherosclerosis, a chronic inflammatory disease of the aorta and arteries, impaired DC migration has been demonstrated accompanied by dysfunctional lymphatic vessels (113,135). The retention of DCs in inflammatory sites in tissues can lead to increased levels of chemokines and cytokines secreted by these DCs that can recruit more leukocytes, thereby contributing to the maintenance of the local inflammation and lead to further amplification of lymphangiogenesis (83). The influx of cells and expansion of the lymphatic network at these local sites might ultimately lead to the establishment of tertiary lymphoid organs or lymphoid neogenesis, which has been reported in several chronic inflammatory diseases (136,137).

Inflammation can have a multitude of effects on the lymphatic system and depending on a variety of factors can either promote the expansion of the lymphatic network or lead to disruption in lymphatic function. The lymphatic vessels not only play an essential role in the clearance of excess interstitial fluid from local inflammatory sites, but are also likely to be intimately involved in the regulation of the inflammatory process and maintenance of immune responses. They also express chemokine scavenger receptors that can contribute to the clearance of inflammatory chemokines from inflammatory sites and help in the resolution of the inflammation (41,42,138). Therefore further study of the lymphatic vessels during inflammation and pathogenic infections need to be pursued to obtain a better understanding of the involvement of lymphatic vessels in disease processes.

1.4 LYMPHATIC DISEASES

A chronic disfiguring and disabling swelling of one or several limbs due to insufficient lymphatic drainage characterizes lymphedema. Lymphedema can interfere with wound healing and enhances susceptibility to infection. Primary or hereditary lymphedema is a condition with no identifiable cause and is generally due to genetic mutations in genes involved in the development and regulation of the lymphatic system like missense mutations in the VEGFR-3 gene, which is the receptor for the lymphatic growth factor VEGF-C (12,139). In contrast, secondary or acquired lymphedema develops when the lymphatic vessels are damaged by infection, radiation therapy or when lymph nodes are surgically removed. The accumulation of protein-rich interstitial fluid in tissue is associated with increased recruitment of leukocytes including neutrophils, macrophages and DCs (140,141). It has been observed that patients with lymphedema are prone to developing chronic infections and various tumors in the lymphedematous limb, suggesting that regional immune surveillance is impaired (142), which supports the role of lymphatics as an important contributor to immune responses. The disruption of afferent lymphatic vessels in LNs resulted in the loss of high endothelial venules (HEVs) from the LNs and reduced lymphcotye trafficking (143) further indicating the importance of lymphatic vessels in multiple aspects of the regulation of cellular traffic to LNs.

However, the most common form of lymphoedema results from lymphatic filariasis, which is caused by the parasitic filarial worms *Wuchereria bancrofti* and *Brugia malayi* (144,145). Lymphatic filariasis is a mosquito-transmitted infection that is endemic to the tropical areas of the world. The parasitic worms establish themselves in lymphatic vessels during infection, causing disruption to the lymphatic system often leading to development of lymphedema and other related pathology. It has been proposed that filarial lymphedema

develops due to the interactions between filarial infection and host genetic risk factors in which heightened inflammatory responses and lymphatic drainage following filarial infection combined with mutations in genes involved in lymphangiogenesis like VEGFR-3 might lead to the development of filarial lymphedema (146).

Kaposi's sarcoma (KS) is a vascular tumor that consists of sheets of proliferating spindle cells and infection with KS-associated herpesvirus/human herpesvirus-8 (KSHV/HHV-8) is essential for KS tumor formation (2,147). The origin of KS tumor cells is thought to be LECs since these spindle cells express multiple lymphatic markers and KSHV infection can reprogram differentiated BECs into a LEC phenotype with expression of approximately 70% of the major lymphatic lineage specific genes and downregulation of blood vascular genes (148-151). Therefore, LECs are not only associated with the metastasis of cancerous cells, but they themselves can get infected and lead to the development of vascular tumors like KS.

1.5 TUBERCULOSIS AND THE LYMPHATIC ENDOTHELIUM

Tuberculosis (TB) is a chronic infectious disease of the respiratory tract, which kills approximately 2 million people each year (152). Overall one-third of the world's population is currently infected with the TB bacillus, *M. tuberculosis*, with someone becoming newly infected every second. Only about 5-10% of those infected with the mycobacterium develop disease, although due to the breakdown in health services, the spread of HIV/AIDS and the emergence of multidrug-resistant TB, there has been resurgence in the number of TB cases resulting in a growing global epidemic. Therefore, renewed efforts to completely understand the various steps involved in the disease process are required.

1.5.1 Natural history of infection

Tuberculosis is spread through the air and a person needs to inhale only a small number of *M. tuberculosis* bacilli, expelled by an infectious individual with pulmonary tuberculosis, to get infected. Alveolar macrophages lining the respiratory tract are one of the first cell types to encounter and engulf the bacilli by phagocytosis. Many of these cells are able to kill the phagocytosed microorganism, but there remain resting, non-activated macrophages that are not able to eradicate the bacilli (153). These infected macrophages permit the intracellular replication of mycobacteria, which then lyse the cells and infect more macrophages. This cycle of infection continues leading to the activation of macrophages, which then secrete inflammatory cytokines and chemokines. The inflammatory chemokines like CCL2, CCL3, CCL4, CCL5, CCL7, CXCL8, CXCL9, CXCL10 and CXCL11 lead to the recruitment of inflammatory cells like monocytes, neutrophils and activated lymphocytes to the site of infection (154). The accumulation of cells at the site of infection culminates in the formation of structures called granulomas. The granulomatous structures are focal collections of the recruited cells, composed of infected and uninfected macrophages, including epitheloid macrophages and giant cells, DCs, T cells, B cells, and fibroblasts, and they function as physical barriers to prevent the spread of infection. The cells present within these structures secrete inflammatory mediators, form a focus of chronic inflammation and help maintain the granuloma. For example, TNF-α, a pro-inflammatory cytokine, affects the expression of chemokines by macrophages, which probably influences the local chemokine gradients but certainly affects granuloma formation and maintenance (155). Also, the localized expression of IFN-γ and IFN-γ inducible chemokines in granulomatous lung tissues obtained from *M. tuberculosis* infected cynomolgus macaques, suggest a role for these molecules in granuloma formation and maintenance (156).

1.5.2 Dendritic cells

There is an increased accumulation of DCs within the granuloma in tuberculosis (157,158), although their exact role in these structures has yet to be conclusively defined. DCs are important for a proper immune response as they can activate naïve T cells in draining LNs for which they need to migrate to the lymph nodes from the periphery. Therefore, there might be a need for an increased concentration of lymphatic vessels around tuberculous granulomas to facilitate the migration of DCs that are known to accumulate within these structures. The increased concentration of lymphatic vessels around tuberculous granulomas might be induced by activated macrophages present in these structures, since they have been shown to secrete VEGF-C, which signals through VEGFR-3 on lymphatic cells and promotes lymphangiogenesis, on stimulation with inflammatory mediators like TNF-α and LPS (159).

In inflammatory bowel disease (IBD) it has been observed that there is a proliferation of lymphatic vessels under the chronic inflammatory conditions in the gut suggesting the involvement of lymphatic vessels in development of IBD (160-162). It was also shown that in Crohn's disease (CD) there was an association of lymphatic vessels with the granulomas that developed during disease (163). Therefore, in tuberculosis, another chronic inflammatory disease characterized by granuloma formation, there might also be an association of lymphatic vessels with granulomas. The inflammatory environment of these granulomas might lead to an increased concentration of lymphatic vessels around it, which will facilitate the migration of local DCs from the granuloma. Additionally, in a mathematical model of tuberculosis, it was found that delays in DC migration to the draining lymph node could alter the outcome of *M. tuberculosis* infection, defining progression to primary disease or latent infection and reactivated tuberculosis (164). It was seen that when there was no trafficking of DCs, progression to

primary disease or reactivation of latent tuberculosis occurred. Therefore, either the increased presence or relative absence of lymphatic vessels around granulomas might alter disease outcome since these lymphatic vessels are involved in the trafficking of DCs to the draining lymph nodes.

1.5.3 Co-infection with HIV

It has also been shown that human immunodeficiency virus type 1 (HIV-1) infection is one of the most important risk factors for susceptibility to tuberculosis and reactivation tuberculosis (165). Therefore, co-infections of HIV-1 and *M. tuberculosis* can accelerate or reactivate tuberculosis disease, which could be a result of the active immune suppression caused by HIV-1. In addition HIV might cause an impairment of the lymphatic endothelium and its function, which could contribute to the acceleration of tuberculosis disease progression in a co-infection model, since HIV-1 components can induce apoptosis in endothelial cell cultures (166,166,167,167-169). It has been also seen that during simian immunodeficiency virus (SIV) infection in monkeys, a nonhuman primate model for HIV-1 infection and disease, there were changes in DC migration and activation which suggested a role in initial viral spread and eventual immunosuppression (40,170). Therefore in the case of tuberculosis, a HIV-1 co-infection could aid in the spread of the mycobacteria by DCs early in the infection, which could accelerate disease progression. By examining the effects of viral co-infection on the LE and its function in DC migration during the development of tuberculosis, we would have a better understanding about cellular trafficking through the lymphatic network in HIV-1/*M. tuberculosis* co-infections. Thus by obtaining a better understanding of the lymphatic network and its role in trafficking of cells in tuberculosis disease, we will perhaps be better positioned to elucidate the process of disease development.

1.6 SUMMARY

The LE is important for maintaining fluid balance and regulating cellular traffic from the periphery to the LNs. The study of the role of the LE during pathogenic infections will be important in understanding the dynamics of cellular trafficking, lymphatic function and inflammatory reactions in development and elimination of infections. Tuberculosis is a global health problem and defining the role of the LE in the development of disease may aid in the discovery of novel therapeutic targets for ameliorating infection and the pathology associated with it. Also a better understanding of the role of the LE in regulating cellular traffic to LNs will aid in the design of better vaccine strategies for cancer and infectious diseases.

2.0 SPECIFIC AIMS

The lymphatic endothelial network plays an important role during immune responses to pathogens and during vaccinations. The afferent lymphatic vessels act as a conduit for dendritic cells (DCs) to migrate from a site of infection to the draining lymph node to activate immune cells, whereas the efferent lymphatic vessels are used by activated immune cells to reach the systemic circulation from the lymph node. Therefore, in a *Mycobacterium tuberculosis* infection, the lymphatic endothelial network might play an important role in determining disease progression by regulating the trafficking of immune cells to and from the site of infection. An increased concentration of lymphatic vessels at the site of *M. tuberculosis* infection, characterized by granulomatous structures, might help in the trafficking of cells like DCs from within these structures to the lymph nodes leading to activation of *M. tuberculosis* specific immune cells. This increased concentration of lymphatic vessels might be induced by macrophages and DCs present within granulomas, since these cell types have been shown to secrete inflammatory mediators that can stimulate lymphangiogenesis or the growth of new lymphatic vessels. Also, the LECs might contribute to the immune response by increasing secretion of inflammatory molecules in response to the inflammatory environment as well as mycobacterial components present at sites of infection. **My hypothesis for this project was that during a *M. tuberculosis* infection, there is an increased concentration of lymphatic vessels around granulomas, which is induced by lymphangiogenic factors secreted by macrophages and DCs present within these structures, and that the inflammatory**

environment including mycobacterial components present in granulomas and other sites of infection elicit an inflammatory response from these lymphatic vessels which could ultimately contribute to the overall immune response to *M. tuberculosis* infection.

This hypothesis was addressed by the specific aims listed below.

1. **To evaluate the response of lymphatic endothelial cells to mycobacterial components, cytokines and other inflammatory mediators.** The effects of model *M. tuberculosis* components, cytokines and other inflammatory mediators on cultured model LECs were studied by flow cytometry, real-time RT-PCR, and ELISA.

2. **To determine the effects of *M. tuberculosis*, SIV or *M. tuberculosis*/SIV co-infection on the expression of multiple endothelial markers and chemokines by the lymphatic endothelial network present in lymph nodes.** Lymph node tissues obtained from cynomolgus macaques (*Macaca fascicularis*), infected with *M. tuberculosis*, SIV or co-infected with *M. tuberculosis* and SIV, were used to assess the distribution of lymphatic vessels in LNs by *in situ* hybridization and immunohistochemical approaches.

3. **To study the distribution of the lymphatic endothelial network in pulmonary granulomas formed during *M. tuberculosis* infection or *M. tuberculosis*/SIV co-infection.** Granulomatous lung tissues obtained from cynomolgus macaques (*Macaca fascicularis*), infected with *M. tuberculosis* or co-infected with *M. tuberculosis* and SIV,

were used to assess the distribution of lymphatic vessels and lymphangiogenic factors in granulomas by *in situ* hybridization and immunofluorescence approaches.

These studies provide insight into the basic aspects of the LE and their response to inflammation and infection. I have organized the experimental data into three main chapters, which is followed by a final discussion on the important findings of this dissertation. At first I have presented a basic characterization of primary human LECs in terms of their expression of TLR molecules and their responsiveness to TLR ligands and inflammatory cytokines. The next chapter contains an evaluation of the expression of lymphatic markers and chemokines by the lymphatic endothelial network present in LNs, an important secondary lymphoid organ, both in health and infectious disease. Finally I have presented a study of the lymphatic endothelial and DC networks present in pulmonary granulomas formed in cynomolgus macaques infected with *M. tuberculosis*.

3.0 HUMAN LYMPHATIC ENDOTHELIAL CELLS EXPRESS MULTIPLE FUNCTIONAL TOLL-LIKE RECEPTORS

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This chapter includes a manuscript that has been submitted to a peer-reviewed journal for publication and is currently under review.

All the experiments in this manuscript were performed by Amarendra Pegu.

(Submitted manuscript)

3.1 PREFACE

In these studies, primary human LECs were evaluated for their expression of innate immune receptors and responsiveness to inflammatory mediators using *in vitro* model systems. In this chapter I have described experiments and data related to the expression of TLRs by human LECs and the responsiveness of human LECs to a panel of ligands for TLR 1-9. These studies have shown that human LECs express all TLRs except for TLR7, TLR8 and TLR10 at both mRNA and protein levels. These data have also revealed that LECs are responsive to the ligands for most of the TLRs, although there were some key exceptions. Most notably the model ligands for TLR9 did not induce any response from the human LECs despite the expression of both mRNA and protein for TLR9 in these cells. Amongst the TLR ligands that induced responses from the LECs, the TLR3 ligand poly(I:C) led to the highest induction in multiple chemokine and cytokine genes in the LECs. I have shown that LECs respond to TLR ligands by increasing expression of multiple inflammatory molecules including chemokines, cytokines and adhesion molecules. These data reveal that LECs can be actively involved in the local inflammatory reactions by production of inflammatory molecules and they likely can regulate the trafficking of different cell types through lymphatic vessels by production of chemokines.

3.2 ABSTRACT

The lymphatic endothelium is the preferred route for the drainage of interstitial fluid from tissues and also serves as a conduit for peripheral dendritic cells (DCs) to reach draining lymph nodes. Lymphatic endothelial cells (LECs) are known to produce chemokines that recruit antigen-loaded DCs to lymphatic vessels and therefore are likely to regulate the migration of DCs to lymph nodes. Toll-like receptors (TLRs) are immune receptors that recognize pathogen associated molecular patterns (PAMPs) and then signal and stimulate production of inflammatory chemokines and cytokines that contribute to innate and adaptive immune responses. TLRs are known to be expressed by a wide variety of cell types including leukocytes, epithelial cells and endothelial cells. Because the TLR expression profile of LECs remains largely unexamined, we have undertaken a comprehensive study of the expression of TLR1-10 mRNAs and protein in primary human dermal and lung LECs as well as in hTERT-HDLECs, which display a longer life-span than human dermal LECs. We found that all three cell types expressed TLR1-6 and TLR9. The responsiveness of these LECs to a panel of ligands for TLR1-9 was measured by real-time RT-PCR, ELISA and flow cytometry, and revealed that the LECs responded to most but not all TLR ligands by increasing expression of inflammatory chemokines, cytokines and adhesion molecules. These findings provide insight into the ability of cells of the lymphatic vasculature to respond to pathogens and potential vaccine adjuvants and shape peripheral environments in which DCs will acquire antigen and environmental cues.

3.3 INTRODUCTION

The lymphatic endothelium (LE) lines the lymphatic vascular system, which drains interstitial fluid from tissues and provides the conduit through which immune cells traffic to draining lymph nodes (LNs) constitutively and during infection or vaccination. Lymphatic endothelial cells (LECs) are known to produce chemokines, including CCL21, that can draw cells such as antigen loaded dendritic cells (DCs) into the draining lymphatic vessels which they traverse to reach secondary lymphoid organs where they stimulate and regulate immune responses (3). Given the critical role DCs play in antigen sampling and regulation of immune function, lymphatic vessels clearly have supporting functions in the movement of DCs from the periphery to LNs, but they might also have more direct roles in modulating the environments in which DCs capture antigen and are shaped to provide environmental instruction (171) to T-lymphocytes in draining LNs.

One mechanism by which DCs receive information about the peripheral environment and acquired antigens is via Toll-like receptor (TLR) signaling. TLRs belong to a family of pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and contribute to the innate and adaptive immune responses to pathogens (92,172). To date, 13 mammalian TLRs have been identified, of which the expression of only TLR1-10 has been observed in humans (91). TLRs are type I transmembrane proteins with a cytoplasmic Toll/IL-1R domain involved in signal transduction and activation of transcription factors leading to induction of proinflammatory cytokines, chemokines and costimulatory molecules. TLRs recognize a wide range of PAMPs and many different cell types express subsets of TLRs, which may reflect the pathogens that they are likely to encounter.

Vaccines deliver target antigens of pathogens or cancer cells to the immune system to induce appropriate antigen-specific immune responses, and adjuvants have been used to increase the immunogenicity of antigens and the efficacy of vaccines (173,174). The ligands for TLRs present attractive candidates for use as vaccine adjuvants given their generally proinflammatory properties, and a number have already been used for this purpose in clinical trials (175,176).

The contribution of TLR responsiveness in vaccine approaches has focused primarily on DCs, which expresses multiple TLRs (177,178), whereas TLR expression and responsiveness has not been comprehensively examined in LECs. The LECs in the draining lymphatic vasculature might have more active roles in vaccination outcomes than heretofore anticipated. To date, analysis of the expression and function of TLRs in LECs has been limited, and has focused on TLR2 and TLR4 in human intestinal lymphatic vessels (104). The expression and engagement of TLRs by LECs could affect the trafficking and modulation of DCs by producing chemokines and cytokines. Increased LEC production of chemokines that can recruit DCs could lead to increased DC migration and subsequently stronger immune responses (57). Understanding TLR expression and responsiveness profiles in LECs will improve vaccine and adjuvant designs that target TLRs. Toward this end, we have analyzed the expression of TLR1-10 in primary human LECs from skin and lung and have examined their responsiveness to a full panel of TLR ligands by measuring the induction of inflammatory chemokines, cytokines and adhesion molecules. These studies identified subsets of TLRs expressed by LECs in these different anatomic compartments and revealed an unanticipated lack of induction of CCL21 expression. The patterns of responsiveness of these LECs to TLR ligands provide a foundation for formulating vaccines that increase the DC attractive and modulating forces of draining lymphatics.

3.4 MATERIALS AND METHODS

LEC cultures

Primary human dermal (HMVEC-dLy) and lung (HMVEC-LLy) LECs (Cambrex Bio Science) were cultured in EGM-2MV medium (Cambrex Bio Science) according to the supplier's suggestions. hTERT-HDLECs were also cultured in EGM-2MV media as previously described (179).

Stimulation of LECs with TLR agonists and cytokines

Agonists for TLR1-9 (TLR Agonist Kit, InvivoGen) and cytokines (Peprotech Inc.) were used to treat confluent monolayers of HMVEC-dLy, HMVEC-LLy and hTERT-HDLECs. The TLR agonists were: TLR1/2, Pam3CSK4 (1 ug/ml); TLR2, heat killed *Listeria monocytogenes* (HKLM, 10^8 cells/ml); TLR3, poly(I:C) (25 ug/ml); TLR4, *Escherichia coli* K12 lipopolysaccharide (LPS, 100 ng/ml); TLR5, *S. typhimurium* flagellin (1 ug/ml); TLR6/2, FSL1 (1 ug/ml); TLR7, imiquimod (2.5 ug/ml); TLR8, ssRNA40 (2.5 ug/ml); and TLR9, ODN2216, ODN2006, or ODNM362 (10 ug/ml each). The cytokines used were IL-1β (10 ng/ml), TNF-α (1 ng/ml) and Oncostatin M (OSM, 100 ng/ml). After 24 hr of treatment, culture supernatants were cryopreserved and the cells were lysed with Trizol (Invitrogen) to isolate total RNA.

Measurement of TLR expression by real-time RT-PCR and flow cytometry

Real-time RT-PCR was performed on total RNA samples using commercially available TaqMan assays for TLR1-10 (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System

(Applied Biosystems) as described (180). The level of expression for each TLR was measured relative to the expression of the endogenous control gene β-glucuronidase. For flow cytometric analysis, cells were stained with either phycoerythrin (PE)-conjugated monoclonal antibodies (eBioscience) against TLR1 (GD2.F4, 2.5 μg/ml), TLR2 (TL2.1, 5 μg/ml), TLR3 (TLR3.7, 4 μg/ml), TLR4 (HTA125, 2.5 μg/ml) and TLR9 (eB72-1665, 4 μg/ml), or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (Imgenex) against TLR5 (85B152.5, 10 μg/ml) and TLR6 (86B1153.2, 10 μg/ml) for surface expression, or for surface and intracellular expression after fixation and permeabilization with Cytofix-Cytoperm (BD Biosciences). Stained cells were analyzed using an XL flow cytometer (Beckman Coulter). Staining with isotype control antibodies was performed in parallel in all studies. Flow cytometry data were analyzed using the Expo32 software package (Beckman Coulter).

Measurement of TLR responsiveness of LECs

Real-time RT-PCR (comparative Ct method) was performed as described (180) using commercially available TaqMan assays for chemokines, cytokines and adhesion molecules (Applied Biosystems). Culture supernatant levels of CCL20 and CXCL10 (R & D Systems), and IL-6 (BD Biosciences), were measured by ELISA. Flow cytometric analysis was performed to detect the expression of VCAM-1 and ICAM-1 on the surface of LECs using FITC- or PE-conjugated monoclonal antibodies (BD Biosciences) against VCAM-1 (51-10C9, 0.125 μg/ml) and ICAM-1 (HA58, 5 μg/ml) respectively, after treatment of the LECs with TLR ligands for 24 hr.

3.5 RESULTS

TLR expression profiles of primary LECs

To define the expression of TLRs 1-10 in primary LECs, we first used real-time RT-PCR to measure TLR mRNA levels in primary dermal (HMVEC-dLy) and lung (HMVEC-LLy) LECs. These were examined in parallel with model LECs, hTERT-HDLECs, which are dermal in origin and display a longer life-span due to the ectopic expression of human telomerase reverse transcriptase (179). All three cell populations expressed the lymphatic markers podoplanin and VEGFR-3 along with the pan-endothelial marker CD31 on their surface as detected by flow cytometry (>95% antigen positive) and they expressed mRNAs for the lymphatic markers LYVE-1 and Prox1 as measured by real-time RT-PCR (data not shown) providing confidence in the lymphatic lineage of these cells.

All three LEC populations expressed mRNAs for TLR1-6 and TLR9, whereas the mRNAs for TLR7, TLR8 and TLR10 were not detected (threshold cycle values >45). The mRNA encoding TLR4 had the highest level of expression relative to the endogenous control gene in all three cell populations (Fig. 2A). Considering all three cell populations together, TLR1-3 and TLR6 mRNAs were expressed to similar levels, whereas TLR5 and TLR9 mRNAs were approximately 10-fold lower. TLR2 and TLR6 mRNAs were expressed the most disparately among the three cell populations with TLR2 mRNA 10-fold less abundant in the primary lung LECs and hTERT-HDLECs relative to the primary dermal LECs, and TLR6 mRNA approximately 10-fold lower in the primary dermal LECs. Overall, however, TLR mRNA expression profiles were highly similar amongst the three LEC populations.

Flow cytometry was used next to measure TLR protein levels on cell surfaces and in intracellular compartments of all three cell types (Fig. 2B), focusing on the TLRs that had

detectable mRNA expression. We found that TLR4-6 were expressed on the surface of all three cell types although minimally so on the hTERT-HDLECs, and that the surface expression levels for TLR5 and TLR6 were similar and higher than those of TLR4. Interestingly, both TLR3 and TLR9 were expressed on the surfaces of all three LEC populations in addition to being expressed intracellularly and the levels for TLR9 were higher than TLR3. We found a surprising lack of expression of TLR1 and TLR2 on the surfaces of all three LEC populations, whereas these TLRs were expressed intracellularly. Except for TLR5, we observed higher levels of intracellular expression for all TLRs compared to their surface expression on all three LEC types.

Induction of inflammatory chemokines in primary LECs by TLR ligands

To measure the responsiveness of LECs to TLR stimulation, we treated each cell population with a panel of ligands for each of TLR1-9 and measured changes in expression of inflammatory molecules. The findings from this comprehensive set of studies are shown collectively in Fig. 16. At sites of inflammation, the engagement of TLRs by their ligands leads to signal transduction events which activate transcription of inflammatory genes (91), including chemokines.

We found that out of a total of nine TLR ligands, the LECs responded strongly to five of them, which were Pam3CSK4, poly(I:C), LPS, FSL1 and ssRNA40, by increasing expression of mRNA (>2 fold change in mRNA) for at least one chemokine (Fig. 3A). The primary dermal LECs responded to the largest number of TLR ligands and also exhibited a higher induction of inflammatory chemokine mRNA expression. Amongst the inflammatory chemokines, the mRNAs for the CXCR3 ligands, CXCL9, CXCL10 and CXCL11, were the most highly induced by TLR ligands in all three LEC populations, with the model TLR3 ligand, poly(I:C), being the

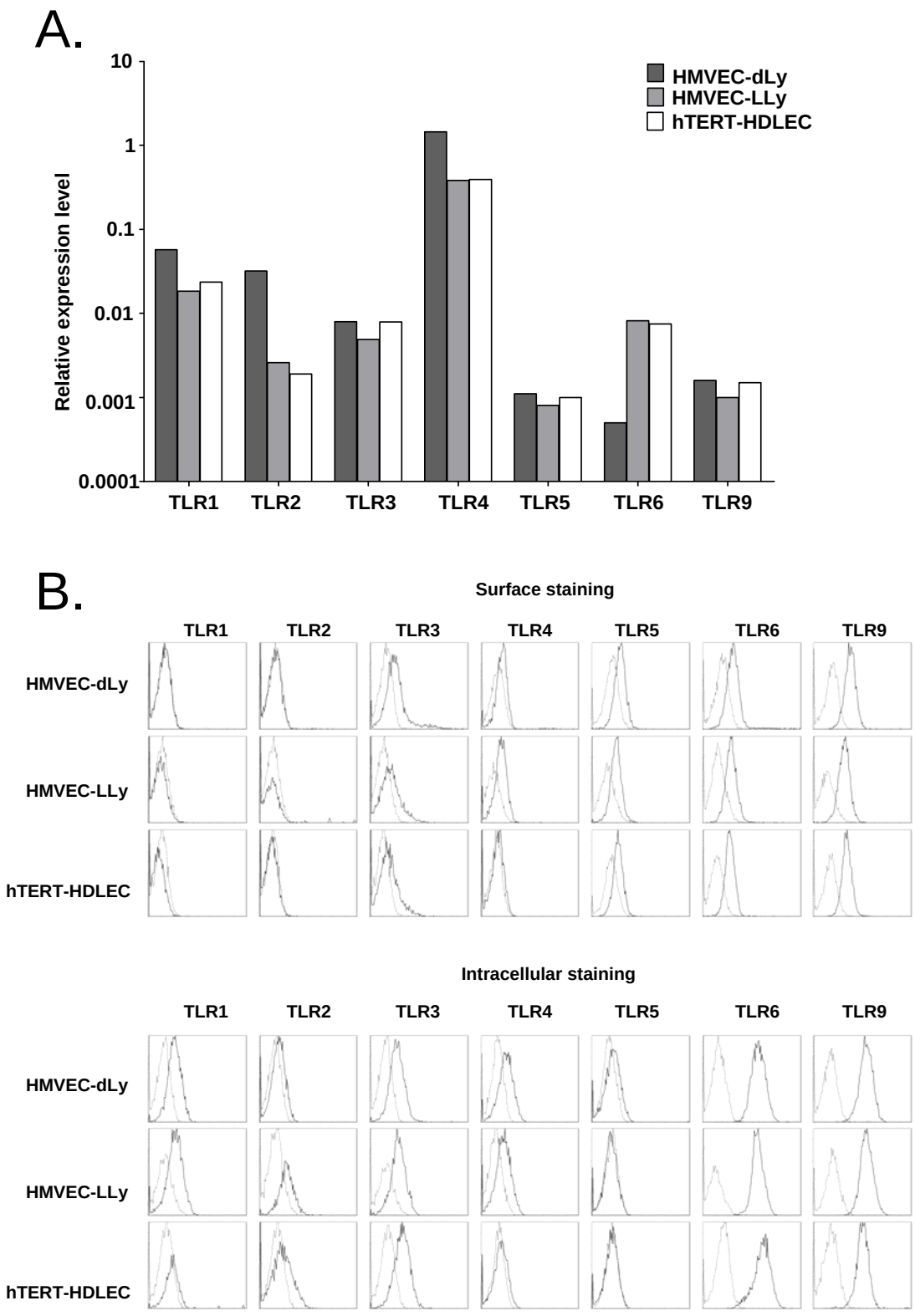

Figure 2. TLR expression by LECs. (A) Total RNAs from confluent monolayers of HMVEC-dLy, HMVEC-LLy and hTERT-HDLEC were analyzed for the expression of mRNAs for TLRs 1-10 by real-time RT-PCR. (B) HMVEC-dLy, HMVEC-LLy and hTERT-HDLECs were also assayed for surface and intracellular expression of TLR1-6 and 9 by flow cytometry. Parallel staining with isotype control antibody is represented by the grey histograms, whereas staining with TLR-specific antibodies is represented by the black histograms. The results in (A) and (B) are both from one experiment performed twice with similar results.

most potent inducer followed by the TLR8 ligand, ssRNA40, and then the TLR4 ligand, LPS (Fig. 3A). In the primary dermal LECs, the TLR2/6 ligand, FSL1, which is a synthetic lipoprotein that represents the N-terminal part of the 44-kDa lipoprotein LP44 of *Mycoplasma salivarium* (181), also increased expression of the mRNAs for CXCR3 ligands, whereas in the primary lung LECs and hTERT-HDLECs, FSL1 had only minimal effects on the expression of CXCR3 ligand mRNAs.

The chemokine receptors CCR6 and CCR7 play important roles in the migration of DCs via the lymphatics and are expressed on immature and mature DCs, respectively (3). Therefore, we examined whether TLR ligands affect the expression of DC-recruiting CCR6 and CCR7 ligands by LECs (Fig. 3A). The mRNA encoding the CCR6 ligand CCL20 was highly induced in all three LEC types by most TLR ligands, whereas the mRNA encoding the CCR7 ligand CCL21 remained at basal levels or was induced up to 3-fold in the primary LEC populations after TLR ligand treatment. CCL21 was not detected by real-time or standard RT-PCR in hTERT-HDLECs, which is consistent with our earlier observations with LECs within LNs (182), whereas the other CCR7 ligand, CCL19 was not detected by real-time RT-PCR in any of the three cell types (data not shown). Similar to our findings for the CXCR3 ligands, the TLR ligands poly(I:C), LPS and Pam3CSK4 induced high levels of expression of CCL20 mRNA, whereas the rest of the TLR ligands had minimal to moderate effects on CCL20 mRNA levels. The TLR2/6 ligand, FSL1, also induced expression of CCL20 mRNA in mainly the primary dermal LECs. In contrast to its effects on CXCR3 ligands, the TLR8 ligand ssRNA40 had no appreciable effects on CCL20 expression (Fig. 3A).

We also observed increased expression of mRNAs for the inflammatory chemokines CCL5 and CXCL8 generally in all three LEC types treated with the TLR ligands poly(I:C), LPS

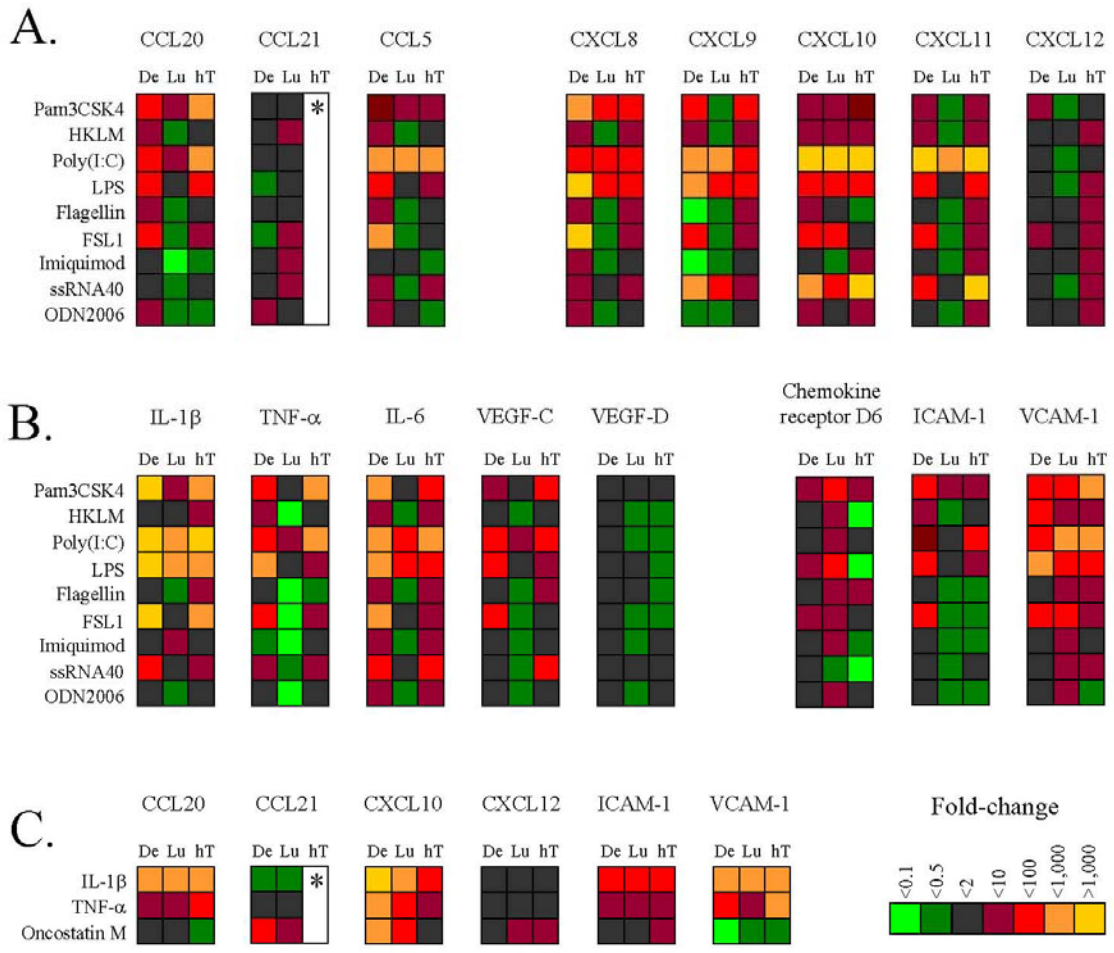

Figure 3. Induction of mRNAs for chemokines, cytokines and adhesion molecules in LECs by TLR ligands and cytokines. Confluent monolayers of HMVEC-dLy (De), HMVEC-LLy (Lu) and hTERT-HDLECs (hT) cells were treated with TLR ligands (A-B) or cytokines (C) for 24 hr and total RNA from the cells was analyzed for the induction of mRNAs for chemokines (A, C), cytokines (B) and adhesion molecules (B, C) by real-time RT-PCR. The relative fold-changes for each gene are indicated by their color according to the heat map legend. The "*" indicates that the mRNA for CCL21 was not detected by real-time RT-PCR in total RNA from hTERT-HDLECs (threshold cycle values >45). These data represent data compiled from separate experiments performed with duplicate cultures and with duplicate real-time RT-PCR reactions performed on each total RNA preparation.

and Pam3CSK4, with FSL1 also inducing similar changes mainly in the primary dermal LECs (Fig. 3A). The expression of CXCL12 mRNA changed only minimally in the three LEC types on treatment with the individual TLR ligands.

A surprising observation from our studies was the unresponsiveness of all three cell types to the TLR9 ligand, ODN2006. In all three cell types, treatment with ODN2006 led to only

minimal changes in the expression of mRNAs encoding any of the chemokines examined (Fig. 3A). Since ODN2006 is representative of one of three different types of CpG oligodeoxynucleotides (ODNs) that can interact with TLR9, we subsequently examined the effects of representative CpG ODNs of the other two types (ODN2216 and ODNM362). Similar to ODN2006, there were minimal changes in expression of the mRNAs for chemokines in all three cell types after treatment with either ODN2216 or ODNM362 (data not shown). These results indicate that the TLR9 expressed in primary human LECs is not responsive to model TLR9 ligands under the conditions we used.

To extend the mRNA measurements, we also measured the levels of CCL20 and CXCL10 secreted by the LEC populations after treatment with TLR ligands (Fig. 4A and B). We found that the levels of CCL20 generally paralleled the induction of its mRNA as observed by real-time RT-PCR (Fig. 4A). The TLR ligands poly(I:C), LPS and Pam3CSK4 led to the highest levels of CCL20 secreted by all three cell types with the primary dermal LECs having higher values than both the primary lung LECs and hTERT-HDLECs. Similar to the induction of CCL20 mRNA observed in mainly the primary dermal LECs by the TLR2/6 ligand, FSL1, we found high levels of CCL20 secreted only by the primary dermal LECs and not the other two cell types when treated with FSL1. The rest of the TLR ligands did not lead to increased secretion of CCL20 by any of the three cell types when compared to their respective mock control cultures.

All three LEC populations secreted nearly undetectable levels of CXCL10 at baseline with minimal induction in response to most of the TLR ligands (Fig. 4B). Only the TLR3 ligand poly(I:C) stimulated high levels of CXCL10 secretion by all three cell types, which was consistent with the levels of induction of its mRNA. In addition, TLR ligands Pam3CSK4, LPS and FSL1 led to increased secretion of CXCL10 only by the primary dermal LECs, whereas the

TLR8 ligand, ssRNA40 led to increased CXCL10 secretion only by the hTERT-HDLECs, despite the induction observed in CXCL10 mRNA in both the primary dermal LECs and hTERT-HDLECs treated with ssRNA40. Overall, these data demonstrate that there is a high degree of concordance in the induction of both mRNA and protein for chemokines produced by LECs, but that there are also important differences in the levels of mRNA and secreted protein.

Induction of cytokines and adhesion molecules in primary LECs by TLR ligands

TLR stimulation can also induce expression of inflammatory cytokines (92). Therefore, we examined the expression levels of the inflammatory cytokines IL-1β, TNF-α and IL-6, in all three LEC populations following treatment with TLR ligands. Similar to the induction of chemokines, we detected increased expression of the mRNAs for all three inflammatory cytokines in response to the TLR ligands poly(I:C), LPS and Pam3CSK4. FSL1 led to induction again mainly in the primary dermal LECs (Fig. 3B). As with chemokines, the levels of induction of these cytokines were highest in the primary dermal LECs compared to the other two LEC populations. We also measured the amount of IL-6 secreted by the LECs after treatment with the TLR ligands and found that there was concordance in the amount of protein secreted by the LECs and the induction in its mRNA levels in all three cell types (Fig. 4C). We also found that in all three cell types, the TLR ligands led to moderate changes in the expression of mRNA encoding the lymphatic growth factor VEGF-C and no changes in the expression of mRNA encoding VEGF-D.

Lymphatic endothelium can express leukocyte adhesion molecules such as ICAM-1 and VCAM-1 after treatment with inflammatory cytokines (89,90). These molecules could regulate the trafficking of immune cells into and through the lymphatics during inflammation. In all three

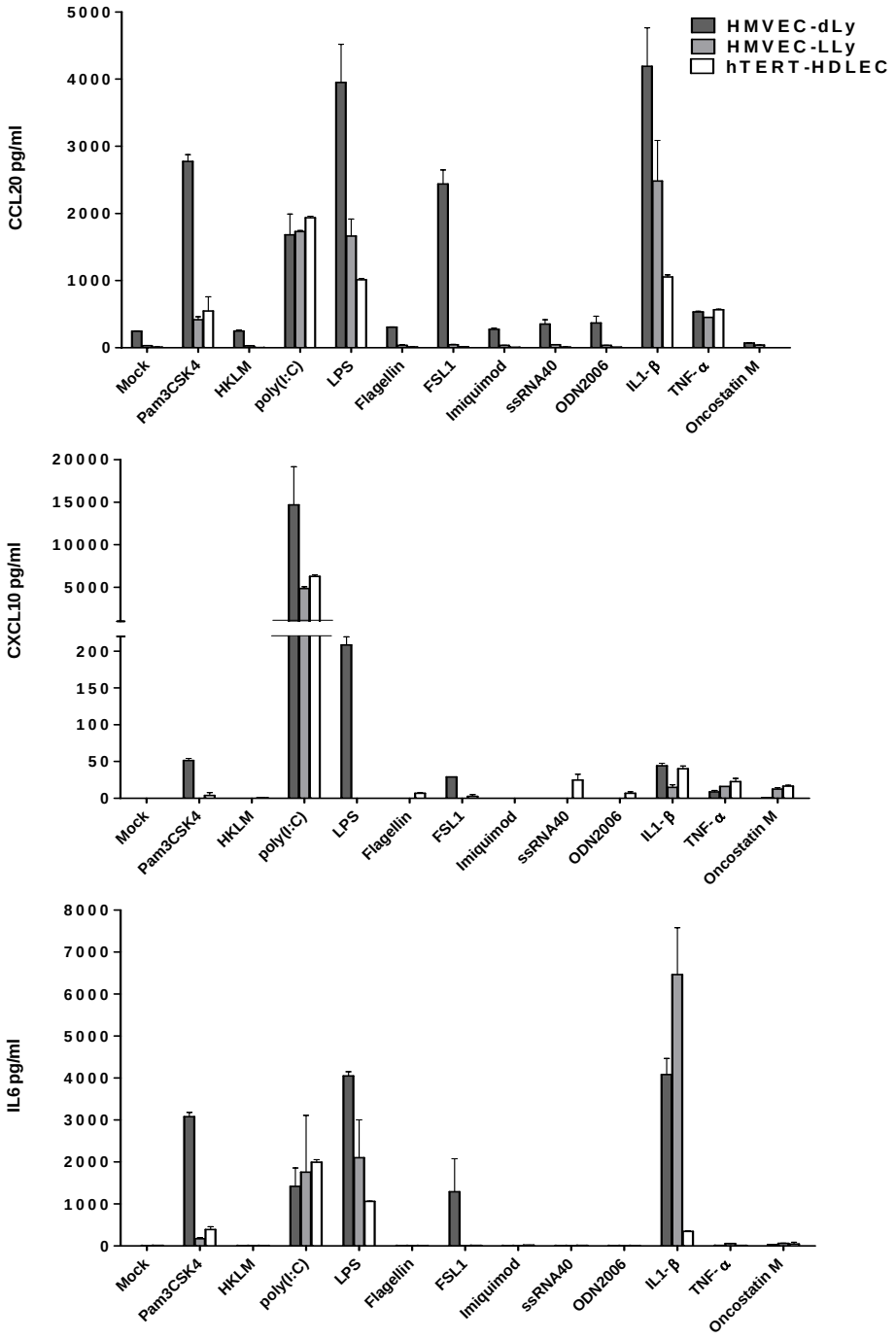

Figure 4. Production of CCL20, CXCL10 and IL6 by LECs after treatment with TLR ligands. Confluent monolayers of HMVEC-dLy, HMVEC-LLy and hTERT-HDLECs, were treated with TLR ligands for 24 hr and the amount of CCL20 (A), CXCL10 (B) and IL-6 (C) released into the culture supernatants by each cell type were measured by ELISA. These results represent the average (+/- SEM) of duplicate cultures with each supernatant measured in duplicate by ELISA.

LEC populations, ICAM-1 and VCAM-1 mRNA levels were increased in response to the TLR ligands poly(I:C), LPS and Pam3CSK4, with FSL1 again inducing expression mainly in primary dermal LECs (Fig. 3B). Although the primary lung LECs were generally less prone to express inflammatory molecules upon TLR stimulation, they universally upregulated VCAM-1 expression after treatment with any of the TLR ligands. In concordance with the induction in their mRNA levels, there was an increase in the expression of ICAM-1 and VCAM-1 on the surfaces of all three cell types in response to poly(I:C) and LPS, whereas ODN2006 did not lead to changes in their surface expression (Fig. 5). Finally, in all three cell types, TLR ligands led to minimal changes in the expression of the mRNA for LE-specific scavenger receptor, chemokine receptor D6, except for moderate induction in the primary lung LECs (Fig. 3B). These data indicate that LECs respond to TLR ligands by increasing production of inflammatory cytokines and expression of adhesion molecules on their surface, whereas the expression of lymphatic growth factors and scavenger receptors by LECs are minimally affected.

Induction of inflammatory molecules in primary LECs by cytokines

Cytokines produced as a result of inflammation can have effects on the phenotype and function of local lymphatic vessels (89,90). In addition, injection of TNF-α into skin can lead to increased expression of CCL21 by lymphatic vessels (57), although the mechanism of this induction is a matter of debate since in a later report, a pleiotropic cytokine, oncostatin M (OSM), and not TNF-α, directly enhanced expression of CCL21 by endothelial cells (183). Therefore, to understand better the effects of cytokines on LECs, we treated the three LEC populations with the cytokines IL-1β, OSM and TNF-α and measured changes in the expression of a subset of chemokines, cytokines and adhesion molecules. With all three cell populations,

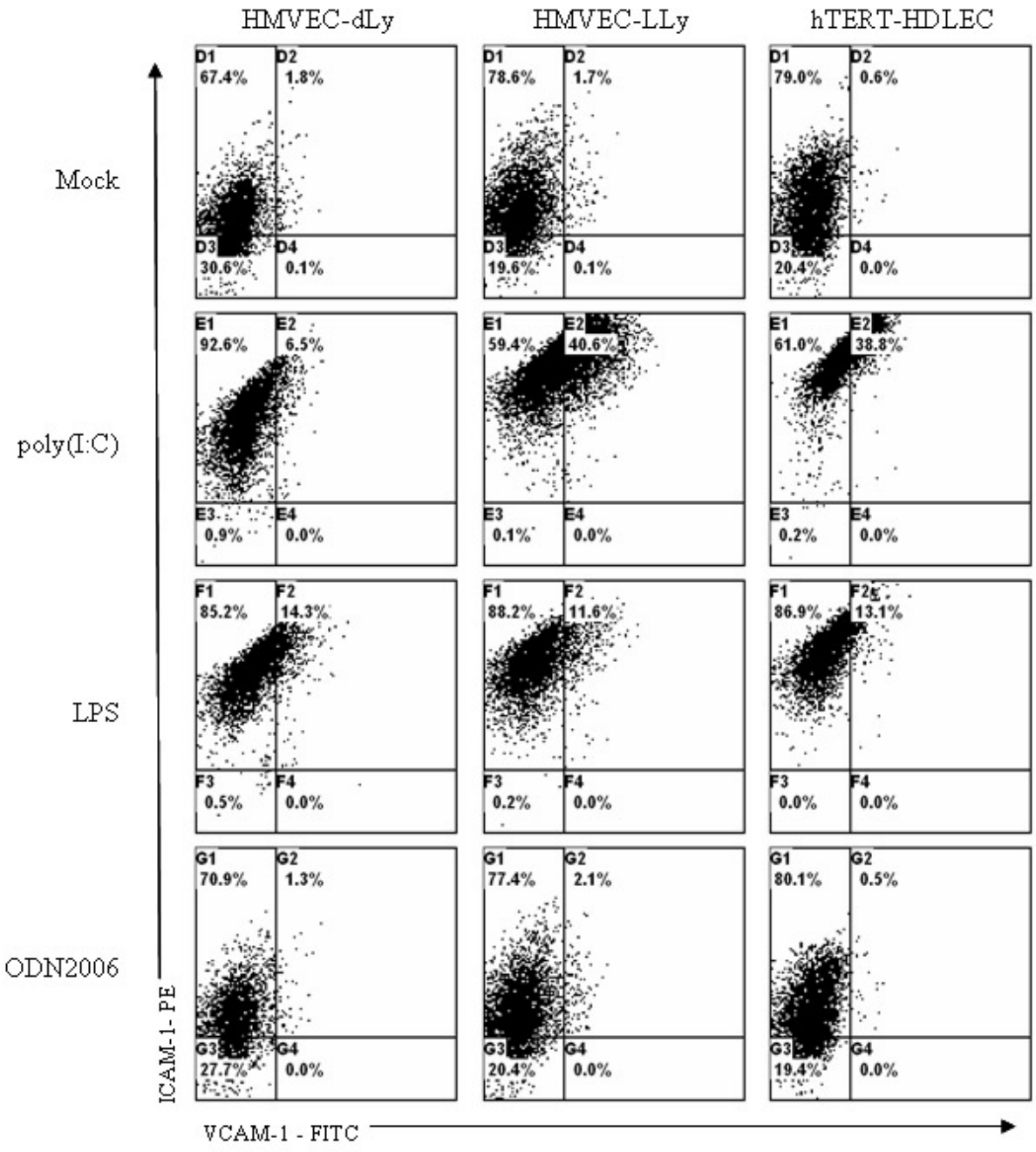

Figure 5. Surface expression of adhesion molecules by LECs after treatment with TLR ligands. Confluent monolayers of HMVEC-dLy, HMVEC-LLy and hTERT-HDLECs were treated with the indicated TLR ligands for 24 hr and the level of surface expression of ICAM-1 and VCAM-1 in these cells was then assayed by flow cytometry. The quad gates were set based on analysis of parallel staining with isotype control antibodies.

IL-1β led to the highest level of induction of CCL20 and CXCL10 mRNAs, whereas TNF-α and OSM had more moderate effects on the levels of these immunomodulators (Fig. 3C and Fig. 4). Interestingly, only OSM induced the expression of CCL21 and only in the primary LEC populations. We also found that there were minimal changes in the expression of CXCL12 by all three cell types in response to IL-1β and TNF-α, whereas OSM led to a slight increase in the mRNA levels for CXCL12. The adhesion molecules ICAM-1 and VCAM-1 were both highly induced in all three cell types in response to IL-1β and TNF-α but not OSM, which is similar to earlier reports of increased expression of these adhesion molecules in TNF-α treated LECs (89,90).

3.6 DISCUSSION

Here we present a comprehensive analysis of the expression of TLRs by primary LECs, as well as the cell line hTERT-HDLEC, and the effects of TLR stimulation on the induction of key molecules involved in regulating cellular traffic through lymphatics. Our findings provide insight into PAMP-specific induction of immunomodulatory molecules by LECs and their ability to contribute to shaping the immune response by helping to move DCs to draining LNs and by exposing DCs to cytokines, chemokines, and adhesion molecules during transit through the lymphatics.

Our studies revealed that LECs expressed a large repertoire of TLR molecules comprised of TLR1-6 and TLR9, and identified a number of interesting aspects of TLR biology in these cells. The expression of a large set of TLRs suggests that LECs have the ability to respond to many microbes that could be encountered in peripheral tissues. Interestingly, despite expression of low levels of TLR5 and high levels of TLR9 by all three LEC populations examined, treatment with their respective ligands did not induce proinflammatory mediators. These findings are consistent with studies performed with human vascular endothelial cells from the same supplier as our LECs (184) and suggest that there are defects in ligand uptake or post-binding signaling in these cells that might otherwise be intact in the context of whole tissues.

The responsiveness of the three LEC populations to specific PAMPs suggests that these cells will respond to the corresponding pathogens including: gram-positive bacteria through TLR1/2 (94,95); gram-negative bacteria through TLR4 (98,99); mycobacteria through TLR2/TLR6 heterodimers (97); and viral replication intermediates through TLR3 (101). The

lack of responsiveness of dermal and lung LECs to TLR5 ligand might be related to the expression of flagellin by primarily motile, enteric bacteria (100), which will not be encountered in the skin and lung at the same frequency as in the gut. More generally, the higher overall responses of the primary dermal LECs to PAMPs and cytokines might be related to the anatomic origin of the cells, since excessive inflammatory responses in the lungs would likely be more detrimental than similar responses in the skin.

The responsiveness of the LECs to TLR ligands did not completely correlate with the levels of expression of the corresponding TLRs, as determined by flow cytometry. For example, total cellular TLR4 levels were not abundant, yet LPS potently induced chemokines, cytokines, and adhesion molecules. In contrast, TLR9 was abundantly expressed on cell surfaces and intracellularly, yet multiple CpG ODNs only minimally induced a limited number of targets. LECs, therefore, appear to express a non-functional TLR9 protein, similar to TLR9 expression in some instance by keratinocytes, myeloid DCs (185), and HMVECs (184). Despite our inability to detect TLR8 mRNA, the model TLR8 ligand, ssRNA40, nevertheless induced CXCR3 ligand expression, suggesting that ssRNA40 might be recognized by another PRR such as RIG-I (172,186).

Afferent lymphatics are a conduit for the migration of antigen-loaded DCs from the periphery to draining LNs where they stimulate and shape immune responses. The expression of CCL21 by LECs is involved in this DC migration, since mature DCs (mDCs) express high levels of CCR7 (3). Treatment of skin with inflammatory cytokines can lead to increased production of CCL21, which in turn enhances migration of mDCs to draining LNs (57), although the exact mechanism of this induction is not clear (183). We did not observe increased expression of CCL21 mRNA above baseline levels in primary LECs in response to any TLR ligand, and

CCL21 was not expressed to detectable levels by the hTERT-HDLECs. In addition, the only cytokine in our studies that led to induction of CCL21 by LECs was OSM, which is consistent with previous findings in mixed endothelial cell populations (183). The lack of CCL21 expression by hTERT-HDLECs and the lack of its induction in the primary dermal and lung LECs suggest there might possibly be environmental cues from neighboring cells in these tissues that provide signals for basal and increased CCL21 expression. Additionally, in LNs CCL21 is expressed mainly by cells other than LECs (182).

In contrast, the CCR6 ligand CCL20 was induced upon stimulation of most TLRs, particularly by the dermal LECs. CCL20 could recruit $CCR6^+$ immature DCs (iDCs) to tissues harboring stimulated afferent lymphatic vessels. The recruited iDCs could acquire antigen, mature through perhaps the same signals that were received by the LECs or alternatively produced by the LECs, and carry antigen to draining LNs. TLR-mediated induction of ICAM-1 and VCAM-1, which bind to the integrins LFA-1/Mac-1 and VLA-4, respectively, will also contribute to the movement of antigen-loaded DCs across the endothelial lining (39). The CCL20/CCR6 axis might also have a role in this movement of DCs into lymphatic vessels during inflammation, as well as movement to the interface between the afferent lymphatics and LN parenchyma (182). Previous findings suggest that CCL20 is involved in recruitment of $CCR6^+$ DCs into LNs (187). Apart from iDCs, CCR6 is also present on effector and memory T cells (188), a major proportion of regulatory T cells (189,190), and naïve and memory B cells (191). Thus CCL20 expressed by afferent LECs could contribute to the trafficking of multiple cell types and thereby help determine the net effects on cell influx and egress in peripheral tissues and associated draining LNs. Finally, increased production of CCL20 and other chemokines will

also comprise an innate immune response at the site of TLR stimulation due to the direct antimicrobial properties of these proteins (192,193).

TLR ligands induced expression by LECs of additional inflammatory chemokines including CXC9-11, CXCL8, and CCL5. Cells expressing receptors for these chemokines could be recruited into environments containing stimulated lymphatic vessels, and this may represent a pathway for the recirculation and clearance of immune effector cells during inflammation. Basal production of inflammatory chemokines by LECs has been reported (194) and our findings here demonstrate that PAMPs induce high levels of these inflammatory mediators. Such pathogen-driven inflammatory responses could contribute to dissemination of organisms, such as by CXCL8-mediated recruitment of neutrophils harboring live bacteria (68). TLR stimulation of LECs also induced expression of the inflammatory cytokines IL-1β, TNF-α and IL-6, with IL-1β induced to the highest levels amongst these cytokines. Therefore, LECs are likely to be an important source of inflammatory cytokines during pathogen-driven inflammation. LECs in turn respond to inflammatory cytokines by upregulating chemokines, adhesion molecules, and other cytokines, indicating that LECs are also affected by the local inflammatory milieu present at sites of infection or vaccination. LECs could contribute to amplification of inflammatory processes at sites of infection or vaccination, both by producing and responding to inflammatory modulators. Overall these data indicate that LECs recognize PAMPs, actively contribute to the inflammatory process, and are not simply a passive conduit for cellular and fluid traffic.

Our findings suggest that LECs might have active roles in chronic inflammatory diseases. For example, in inflammatory bowel disease (IBD) there is increased proliferation of intestinal lymphatic vessels, which was suggested to be the result of chronic inflammation (161,162). Increased numbers of intestinal lymphatic vessels could represent a potent source of

inflammatory mediators that contribute to chronic inflammation in the gut. Similarly, in a transgenic model of psoriasis it has been suggested that lymphatic hyperplasia contributes to inflammation (112).

Defined TLR ligands are attractive vaccine adjuvants. Our findings indicate that LECs will actively respond to such adjuvants and contribute to shaping the environment in which local DCs acquire antigens and begin to undergo a maturation program. We have shown here that not all TLR ligands are equivalent in modulating expression of molecules that impact on the recruitment and trafficking of DCs and other immune cells by LECs, indicating that further study of the impact of individual or combined TLR ligands on vaccine potency should prove valuable. Finally, our findings underscore the need to recognize the lymphatic endothelium as an important active participant in host responses to infection and vaccination.

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4.0 AFFERENT AND EFFERENT INTERFACES OF LYMPH NODES ARE DISTINGUISHED BY EXPRESSION OF LYMPHATIC ENDOTHELIAL MARKERS AND CHEMOKINES

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All the experiments in this manuscript were performed by Amarendra Pegu, while all the animal care needs and medical services were performed by the staff in Dr. JoAnne Flynn's laboratory and by the Division of Laboratory Animal Resources (DLAR).

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4.1 PREFACE

After the phenotypic characterization of LECs, I next analyzed the distribution of the lymphatic endothelial network in immune inductive sites during health and infection. In this section I have described published data related to expression of LEC markers and chemokines by the lymphatic endothelial network of lymph nodes. This chapter contains data from ISHs for LEC marker and chemokine mRNAs that were detected in LNs from a cohort of healthy animals and animals infected with either *M. tuberculosis*, SIV or both. At the start of this project I obtained partial cDNA clones for endothelial specific markers to generate riboprobes for use in the ISH studies (Appendix A). These studies have revealed that there is heterogeneity in the expression of lymphatic markers and chemokines by the lymphatic endothelial network present in LNs. In LNs from healthy animals, I observed the expression of the LEC marker LYVE-1 mRNA almost exclusively by the LECs present at the efferent interfaces of LNs, whereas mRNAs for the other LEC markers podoplanin, prox1 and VEGFR3 were expressed by LECs present at both the afferent and efferent interfaces of LNs. I also found that CCL20 mRNA was expressed only by LECs at the afferent interfaces of LNs and at very low levels in LNs from healthy animals. In LNs from infected animals, there was an increased expression of CCL20 mRNA by the LECs at the afferent interfaces of LNs indicating that either the infection or the inflammatory environment due to the infection lead to the increased expression of CCL20 by these LECs. This was further supported by the *in vitro* data in which I found that both poly(I:C) and γ-irradiated *M. tuberculosis* lead to increased production of CCL20 by model LECs. Overall these studies provide a comprehensive definition of the lymphatic endothelial network of LNs.

4.2 ABSTRACT

Background: Lymph nodes (LNs) are important sites of connection between the sampled peripheral tissues, the many cells of the immune system, and the blood. The organization of the interface between the afferent and efferent lymphatic vasculature and LN parenchyma is incompletely understood, and obtaining a better understanding of these tissue microenvironments will contribute to an improved understanding of overall lymphatic function. *Methods and Results:* We used histologic approaches to define the distributions of cells expressing lymphatic endothelial cell (LEC) markers in LNs from healthy, simian immunodeficiency virus (SIV) infected, or *Mycobacterium tuberculosis* infected cynomolgus macaques. Cells at the afferent and efferent interfaces of LNs from all animals showed differential expression of LEC markers, with podoplanin, Prox-1 and VEGFR3 expressed in both microenvironments, but with LYVE-1 expressed only at the efferent interface. The chemokine CCL20 was uniquely expressed at the afferent interface by cells co-expressing podoplanin, and this expression was increased during SIV or *M. tuberculosis* infection. In contrast, only a small proportion of cells expressing the CCR7 ligand CCL21 co-expressed podoplanin. Treatment of model LECs with the TLR3 ligand poly(I:C) or γ-irradiated *M. tuberculosis* increased production of CCL20 without altering CCL21 or LEC marker expression. *Conclusions:* This study provides a comprehensive mapping of the organization of the lymphatic endothelial network entering and exiting LNs in health and in chronic infectious diseases in a nonhuman primate model. The differences we have defined between the afferent and efferent interfaces of LNs could inform the future design of vaccines and immunotherapies.

4.3 CONDENSED ABSTRACT

Lymph nodes (LNs) are important sites of connection between peripheral tissues, immune cells, and blood. Histologic approaches were used to define the distributions of cells expressing lymphatic endothelial markers in LNs from healthy, simian immunodeficiency virus (SIV) infected, or *Mycobacterium tuberculosis* infected cynomolgus macaques. Cells at the afferent and efferent interfaces of LNs showed differential expression of a subset of lymphatic markers and the chemokine CCL20. CCL20 and LYVE-1 expression at the afferent interface was increased during infection. This study provides a comprehensive mapping of the afferent and efferent interfaces in LNs in health and in models of chronic infectious diseases.

4.4 INTRODUCTION

The lymph node (LN) is an important secondary lymphoid organ involved in the initiation and maintenance of humoral and cellular immune responses (195-197). LNs are components of the lymphatic system that drain interstitial fluid and immune cells from peripheral tissues and back into the blood. The lymph enters a LN via the afferent lymphatic vessels which either terminate at the subcapsular sinus or penetrate into the cortex, and leaves by the efferent lymphatic vessels that collect in the medullary sinus (3,197). These lymphatic vessels have been collectively referred to as the lymphatic endothelium although there likely are phenotypic differences between afferent and efferent lymphatic vessels due to their different roles in moving things to or from LNs.

Lymphatic vessels contribute to the migration of cells out of peripheral tissues and into LNs due in part to the production of chemokines. Lymphatic endothelial cells (LECs) can produce and secrete the homeostatic chemokine CCL21, a CCR7 ligand most recognized for its control of the recruitment of mature DCs and naïve T cells into the paracortices of LNs (198,199). LECs can also secrete inflammatory chemokines including CXCL9, CCL6 and XCL1,(194) and their treatment with inflammatory cytokines leads to increased expression of chemokines CCL21, CCL20 and CCL2 (6,57,200).

Lymphatic vessels have been identified using a number of recently discovered markers, which include the vascular endothelial growth factor receptor 3 (VEGFR3) (9,201,202), the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (24,26), podoplanin (34,203), and Prox-1 (19,20,204). Although these markers have been used to identify lymphatic vessels in

studies focused on the role of lymphatic vessels in LN tumor metastasis, there has not been a comprehensive analysis of their relative expression patterns at the afferent and efferent interfaces of LNs, nor of the effects of infectious agents on these patterns of expression in vivo. In the current study we have addressed these issues and have determined that both LEC marker and chemokine expression distinguish the afferent and efferent interfaces of LNs and that these patterns are modulated by the persistent pathogens simian immunodeficiency virus (SIV) and *Mycobacterium tuberculosis*.

4.5 MATERIALS AND METHODS

Animals and tissue processing

All animal studies were performed under the guidance and approval of the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Adult cynomolgus macaques (*Macaca fascicularis*) were inoculated with virulent *M. tuberculosis* (Erdman strain) via bronchoscope into the lower right lobe or intravenously (I.V.) with SIV/DeltaB670 primary isolate as described (40,156,205-207). At necropsy, the LN tissue specimens were fixed by immersion in fresh 4% paraformaldehyde (Sigma Co., St. Louis, MO)/phosphate buffered saline (Biowhittaker, Walkersville, MD) (PF/PBS) and processed as described (207,208).

Cloning of endothelial cell markers

Partial cDNA fragments were generated from rhesus macaque total cellular RNA by RT-PCR as described (209). Total cellular RNA was obtained from snap-frozen tissues using Trizol (Life Technologies, Rockville, MA) and reverse transcription was performed using oligo-dT primers (Reverse Transcription System, Promega Corp., Madison, WI). The cDNAs generated were amplified by PCR using gene-specific primers designed from human sequences available in the GenBank database. The amplified products were ligated into pGEMT vector (Promega Corp.) and DNA sequenced. The Vector NTI Advance software package (Invitrogen) was used for analysis of the resulting DNA sequences. The probes used here ranged in size from 530nt to 629nt (45-62% GC content) for LEC markers, and 291nt to 396nt (45-58% GC content) for chemokines.

In situ *hybridization and immunohistochemical staining*

In situ hybridization (ISH) with [^{35}S]-UTP-labeled riboprobes, immunohistochemical staining (IHC), and combined ISH/IHC were performed as described (40,207,208). Autoradiographic exposure times were 21 days for the Prox-1, LYVE-1, VEGFR3 ISHs; 7 days for CCL20 and CCL21 ISHs; and 14 days for *M. tuberculosis* 16s rRNA ISHs. An anti-human podoplanin antibody was used for IHC detection of podoplanin (AngioBio, Del Mar, CA).

Cells and culture

hTERT-HDLECs (kindly provided by Dr. M.S. Pepper of The University of Geneva) were cultured in EGM-2MV media (Cambrex Bio Science Inc, Walkersville, MD, USA) as previously described (179). Poly(I:C) (InvivoGen; 25 μg/ml) and γ-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO; 500 μg/ml) were used to treat confluent monolayers of hTERT-HDLECs. At specified time intervals, the cells were lysed with Trizol (Invitrogen) to isolate total RNA and the culture supernatants were cryopreserved. Total RNAs were then treated with DNase (Ambion) and further purified with RNeasy columns (Qiagen) as described (180). Real-time RT-PCR (comparative Ct method) was performed as previously described using predeveloped assays for CCL20 and CCL21 (Applied Biosystems) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) (180). CCL20 protein levels in the culture supernatants were measured by ELISA (R & D systems).

4.6 RESULTS

Differential distribution of lymphatic endothelial markers in LNs

To determine the relative distributions of multiple LEC markers in LNs in health and during persistent infections, we examined LNs obtained from cynomolgus macaques that were otherwise healthy or infected with either SIV, *M. tuberculosis* or both (Table 1). We determined the patterns and levels of LEC marker expression directly in sections of LNs that drained two different mucosal sites, including hilar LN (HLN), which drains the lungs, and mesenteric LN (MLN), which drains the gastrointestinal tract.

Table 1. STUDY ANIMALS

Animal	*Pathogen*	*Duration of infection (wk)*	*Clinical Status*[a]
M6802	None	-	Healthy
M6202	None	-	Healthy
M5602	None	-	Healthy
M7102	None	-	Healthy
M7100	*M. tuberculosis*	9	Moderate disease
M7200	*M. tuberculosis*	41	Advanced disease
M11301	*M. tuberculosis*	33	Advanced disease
M15100	*M. tuberculosis*	17	Moderate disease
M15300	*M. tuberculosis*	11	Advanced disease
M3001	SIV and *M. tuberculosis*	9	Advanced disease
M3002	SIV and *M. tuberculosis*	8	Advanced disease
M3101	SIV and *M. tuberculosis*	11	Advanced disease
M3202	SIV and *M. tuberculosis*	7	Advanced disease
M2601	SIV and *M. tuberculosis*	8	Advanced disease
M6002	SIV	2	Acute infection
M7402	SIV	2	Acute infection
M7802	SIV	2	Acute infection
M5702	SIV	2	Acute infection

[a]Cynomolgus macaques infected with M. tuberculosis for the indicated time period were considered to have moderate or advanced disease based on antemortem chest radiographs and postmortem gross and microscopic pathological evaluation. Cynomolgus macaques infected with pathogenic SIV were classified as in the midst of acute infection, as virus replication typically undergoes and initial systemic burst between days 7-21 PI.

Cells expressing LEC markers were identified by ISH using radioactively-labeled, gene-specific riboprobes for mRNAs encoding LYVE-1, Prox-1 or VEGFR3. In both the HLN and MLN tissue compartments of uninfected animals, we observed expression of mRNA for all three markers, although their distributions and levels of expression differed from each other (Fig. 6). LYVE-1 mRNA was expressed to the highest level on a per cell basis among the three markers, whereas Prox-1 mRNA expression was more moderate, and VEGFR3 mRNA expression was lowest in these LN cells. Cells expressing Prox-1 and VEGFR3 mRNAs were found in the subcapsular, cortical and medullary regions of LN (Fig. 6A, B, E, and F), whereas those expressing LYVE-1 mRNAs were localized nearly exclusively in medullary and discrete cortical regions (Fig. 6C and D). These data indicate that in LNs from healthy animals, Prox-1 and VEGFR3 were expressed by cells at both the afferent and efferent interfaces in LNs, whereas LYVE-1 was selectively expressed by cells at the efferent interface.

Cells expressing Prox-1 and VEGFR3 mRNAs were similarly distributed within HLN and MLN tissue compartments even after infection with either SIV, *M. tuberculosis*, or both pathogens. With the exception of a subset of cells expressing LYVE-1 mRNA in subcapsular regions in LNs from infected animals, nearly all of the cells expressing LYVE-1 mRNA were localized to medullary regions, as was observed in uninfected animals.

Podoplanin is an additional marker used to identify LECs (34,37,210,211). To provide further confidence of the lymphatic lineage of cells expressing Prox-1, VEGFR3, or LYVE-1 mRNAs, we performed simultaneous ISH and IHC using riboprobes specific for these mRNAs and a podoplanin-specific antibody on the same LN tissue sections. Subsets of cells in subcapsular, cortical and medullary regions of LNs expressed podoplanin (Fig. 7). Among the podoplanin^{+} cells, nearly all of them expressed Prox-1 mRNA, whereas only a subset expressed

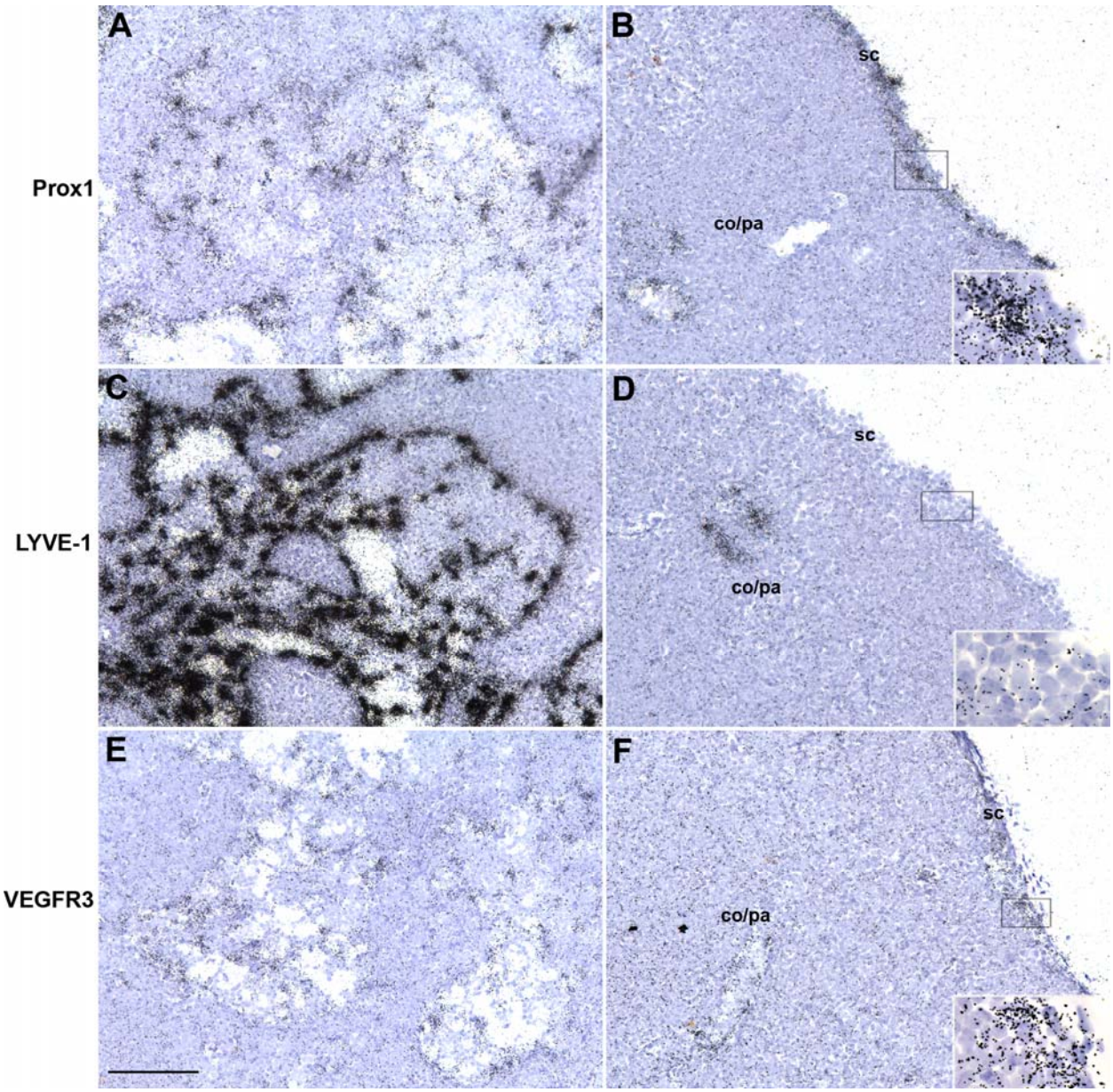

Figure 6. In situ hybridization detection of LEC marker mRNAs in normal macaque LNs. LN tissue sections from an uninfected cynomolgus macaque were hybridized in situ with [^{35}S]-labeled riboprobes specific for Prox-1, LYVE-1 or VEGFR3 mRNAs, as indicated. (A-B), (C-D) and (E-F) are different regions on the same tissue section, with (A), (C) and (D) representing medullary regions of LN, whereas (B), (D) and (F) show cortical and subcapsular regions. The boxes in (B), (D) and (F) indicate the regions of magnification (X400) represented in the corresponding insets. co/pa, cortex/paracortex; sc, subcapsular region. Size bar = 100 μm (A-F).

LYVE-1 mRNA. The distributions of podoplanin^{+} cells that expressed Prox-1 mRNA were similar between uninfected and infected animals (Fig. 7C and E). In both HLN and MLN tissue

compartments of healthy animals, LYVE-1 mRNA was expressed by podoplanin$^+$ cells localized to medullary regions or in rare discrete cortical regions, whereas podoplanin$^+$ cells localized to the subcapsular regions were devoid of LYVE-1 mRNA expression (Fig. 7B and D), although these subcapsular cells expressed Prox-1 mRNA (Fig. 7A and C). However, in HLNs and MLNs from animals infected with either SIV, *M. tuberculosis* or both, subsets of podoplanin$^+$ cells present in subcapsular regions expressed LYVE-1 mRNA (Fig. 7F). These data demonstrate that there is heterogeneity in the expression of LYVE-1 by cells at the afferent and efferent interfaces of LNs and that the expression of this LEC marker is affected by local infections.

We also found granulomatous structures in some LNs from animals infected with *M. tuberculosis*. In these LNs, the granulomas disrupted the normal LN tissue architecture and often occupied the majority of the tissue space. We performed ISH on these LNs for LEC markers, the CCR7 ligand CCL21, and *M. tuberculosis* 16s rRNA. CCL21 and podoplanin mRNAs were the most abundantly expressed mRNAs in these LNs. CCL21 mRNA was expressed by cells predominantly in non-granulomatous regions of LN likely to be compressed LN cortex, whereas podoplanin mRNA was expressed by cells that formed a thick cellular band around the granulomas (Fig. 8A and E). We also found that mRNAs for podoplanin, VEGFR3, Prox-1 and LYVE-1 were expressed by subsets of cells at the outer borders of the granulomas (Fig. 8B, C, D and E). *M. tuberculosis* 16s rRNA was used as a surrogate marker for the presence of *M. tuberculosis* organisms/antigens and was localized primarily within the centers of the granulomas, although some 16s rRNA, and presumably antigen, was located within the cellular layer containing abundant podoplanin expression. These data indicate that *M. tuberculosis* induced granulomas in LNs harbor cells expressing lymphatic markers and disrupt LN architecture and lymphatic marker distribution.

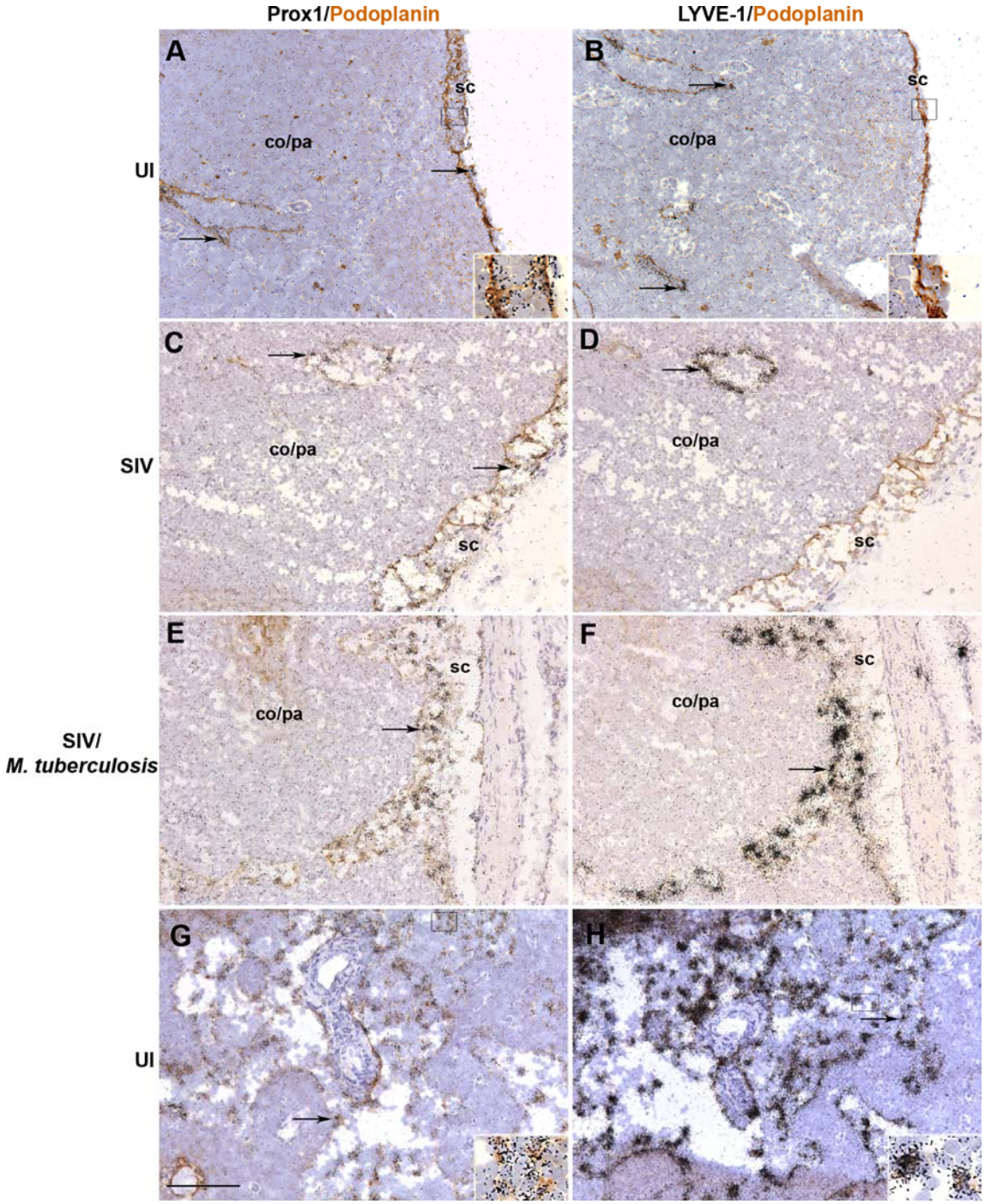

Figure 7. Simultaneous in situ hybridization detection of LEC marker mRNAs combined with immunohistochemical staining for podoplanin in normal and infected macaque LNs. LN tissue sections from uninfected, SIV infected and SIV/*M. tuberculosis* co-infected cynomolgus macaque were hybridized in situ with [^{35}S]-labeled riboprobes specific for Prox-1 and LYVE-1 mRNAs, and immunohistochemically stained for podoplanin as indicated. (A) and (G), and (B) and (H), are different regions on the same tissue section from an uninfected animal, with (G) and (H) representing medullary regions. The boxes in the panels indicate the regions of magnification (X400) represented in the corresponding insets. Arrows highlight select double-positive cells in each section. co/pa, cortex/paracortex; sc, subcapsular region. Size bar = 100 μm (A-H).

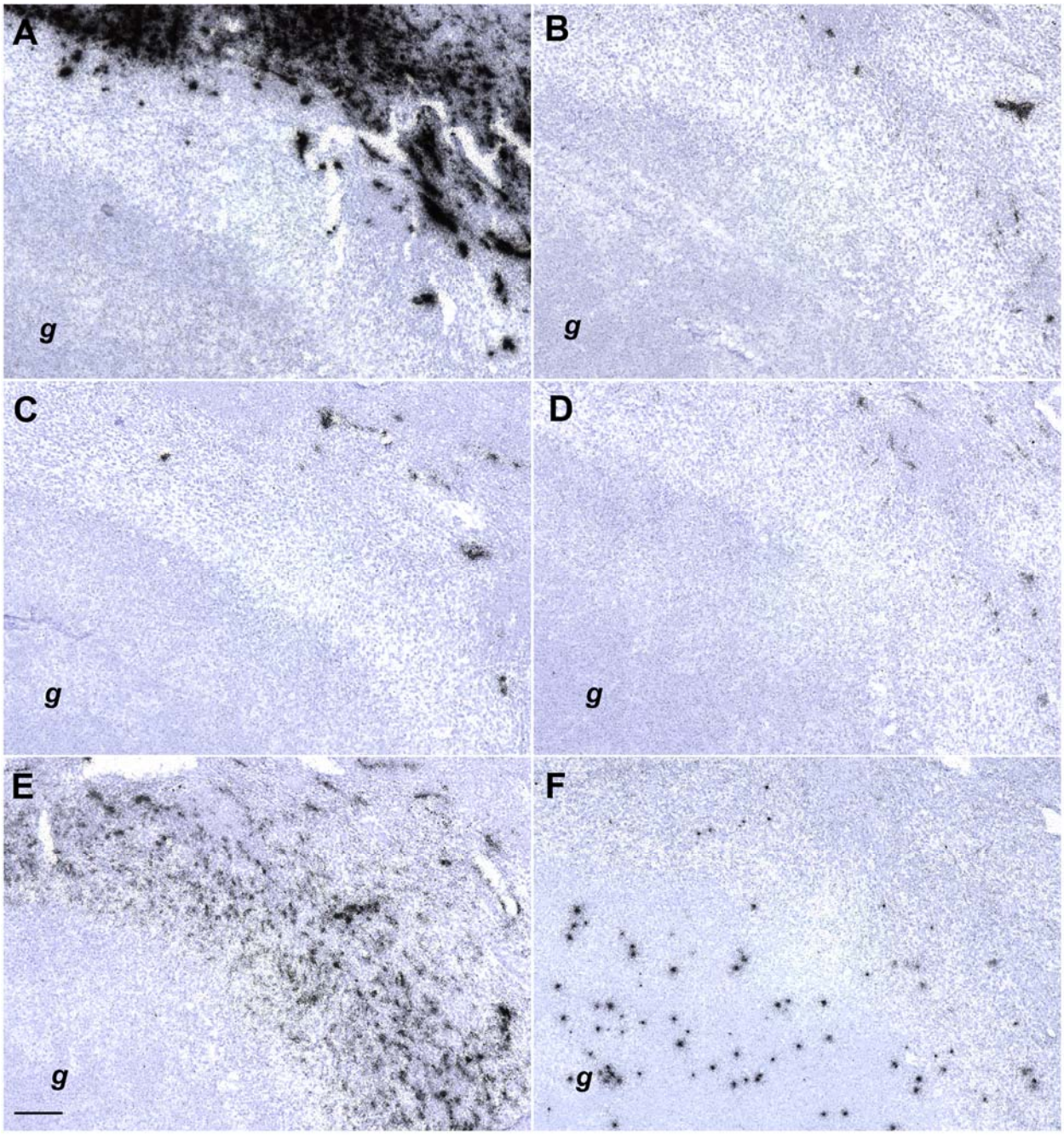

Figure 8. In situ hybridization detection of LEC and *M. tuberculosis* marker RNAs in granulomas circumscribed within LNs from *M. tuberculosis* infected macaques. LN tissue sections from a *M. tuberculosis* infected cynomolgus macaque were hybridized in situ with [^{35}S]-labeled riboprobes specific for CCL21 (A), VEGFR3 (B), Prox-1 (C), LYVE-1 (D), podoplanin (E), and *M. tuberculosis* 16s rRNA (F) RNA targets. *g*, granuloma center. Size bar = 100 μm (A-F).

Induction of CCL20 expression in lymphatic endothelial cells under inflammatory conditions in vivo *and* in vitro

LECs can express CCL21 (57,59,199) and can produce CCL20 upon stimulation with inflammatory cytokines *in vitro* (6). Both of these chemokines are expressed in LNs (40) and expression in this tissue compartment might be contributed by LECs. To address this issue we performed simultaneous ISH and IHC with CCL20- or CCL21-specific riboprobes and anti-podoplanin antibody, respectively. Cells expressing CCL20 mRNA were localized almost exclusively to subcapsular regions of HLNs and MLNs, and all of these cells were podoplanin$^+$ (Fig. 9A). There was a large increase in CCL20 mRNA expression by these cells in LNs of animals infected with either SIV, *M. tuberculosis* or both (Fig. 9C and E). In contrast, in LNs from both healthy and infected animals, only a subset of the podoplanin$^+$ cells present in cortical and some subcapsular regions of LNs expressed mRNA for CCL21 (Fig. 9B). The majority of the CCL21 mRNA expressing cells in LNs did not stain for podoplanin and were localized to the paracortex where few podoplanin$^+$ cells were present (Fig. 9B and D). These findings demonstrate that CCL20 is uniquely expressed by LECs and at the afferent interface of LNs, whereas CCL21 is expressed predominantly in the paracortex and by cells other than LECs.

To determine whether increased expression of CCL20 mRNA by LECs in infected LNs could be a consequence of their interaction with components of *M. tuberculosis* or SIV, we treated hTERT-HDLECs, an *in vitro* LEC model (179) with γ-irradiated *M. tuberculosis* H37Rv to mimic exposure to mycobacteria or with TLR3 ligand poly(I:C) to mimic exposure to viral RNA. Treatment of hTERT-HDLECs with γ-irradiated *M. tuberculosis* H37Rv led to a dramatic increase in CCL20 mRNA, which was accompanied by a concomitant increase in CCL20 protein levels in culture supernatants (Fig. 10A and B). An even greater increase in expression of

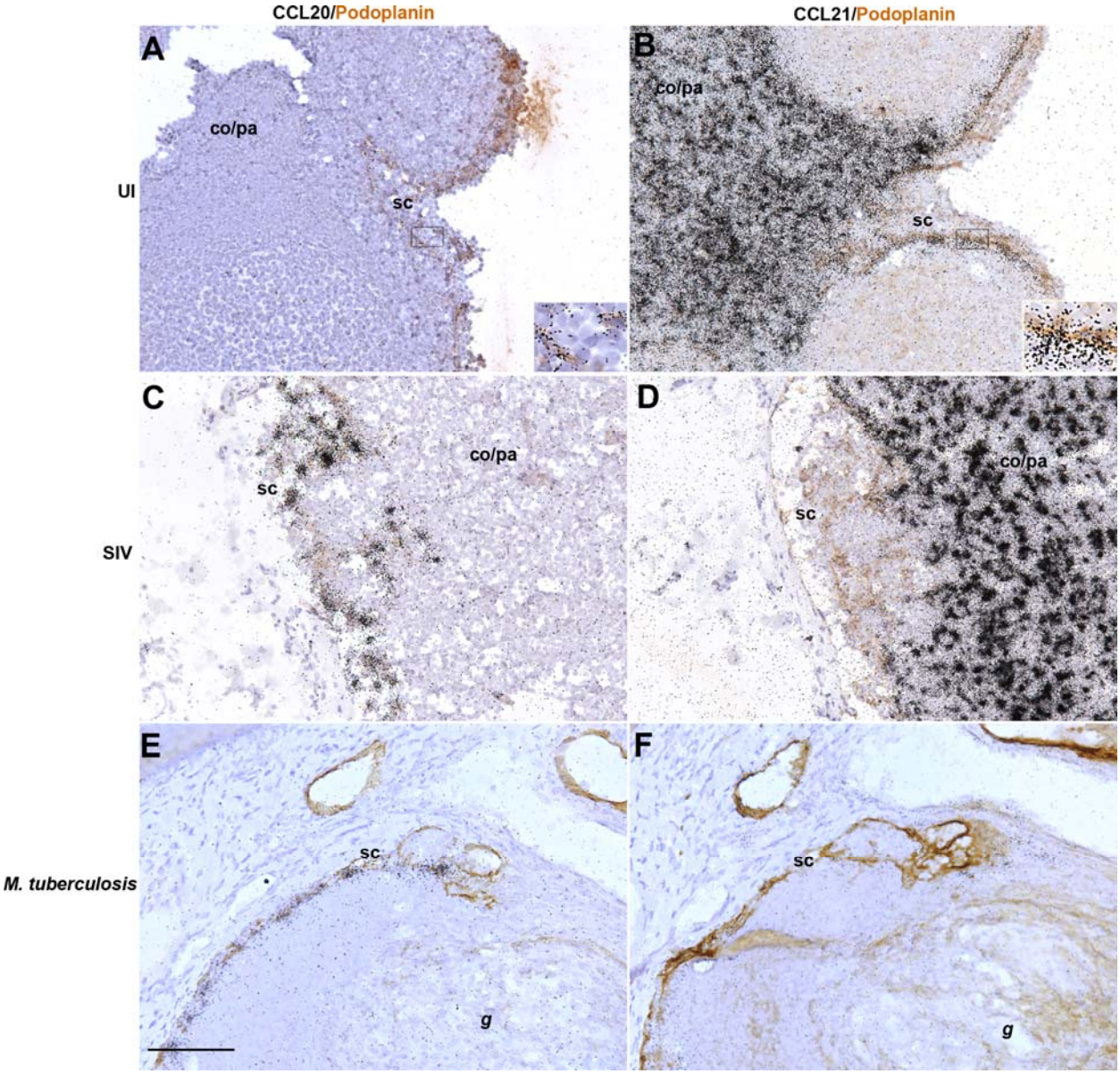

Figure 9. Simultaneous in situ hybridization detection of chemokine mRNAs combined with immunohistochemical staining for podoplanin in macaque LNs. LN tissue sections from uninfected, SIV infected and *M. tuberculosis* infected cynomolgus macaques were hybridized in situ with [^{35}S]-labeled riboprobes specific for the chemokines, CCL20 and CCL21, and immunohistochemically stained for podoplanin as indicated. (A-B), (C-D) and (E-F) are serial tissue sections. The boxes in panels (A) and (B) indicate the regions of magnification (X400) represented in the corresponding insets. co/pa, cortex/paracortex; sc, subcapsular region; *g*, granuloma center. Size bar = 100 μm (A-F).

CCL20 mRNA and protein was observed when hTERT-HDLECs were treated with poly(I:C) (Fig. 10C and D). Interestingly, these model LECs did not express CCL21 mRNA endogenously nor after treatment with either γ-irradiated *M. tuberculosis* or poly(I:C) (data not shown). These

in vitro data are consistent with our findings that CCL20 was endogenously and inducibly expressed in LNs uniquely by LECs, and that although CCL21 is expressed by LECs in peripheral tissues (57), LECs are not the major producers of CCL21 in LNs.

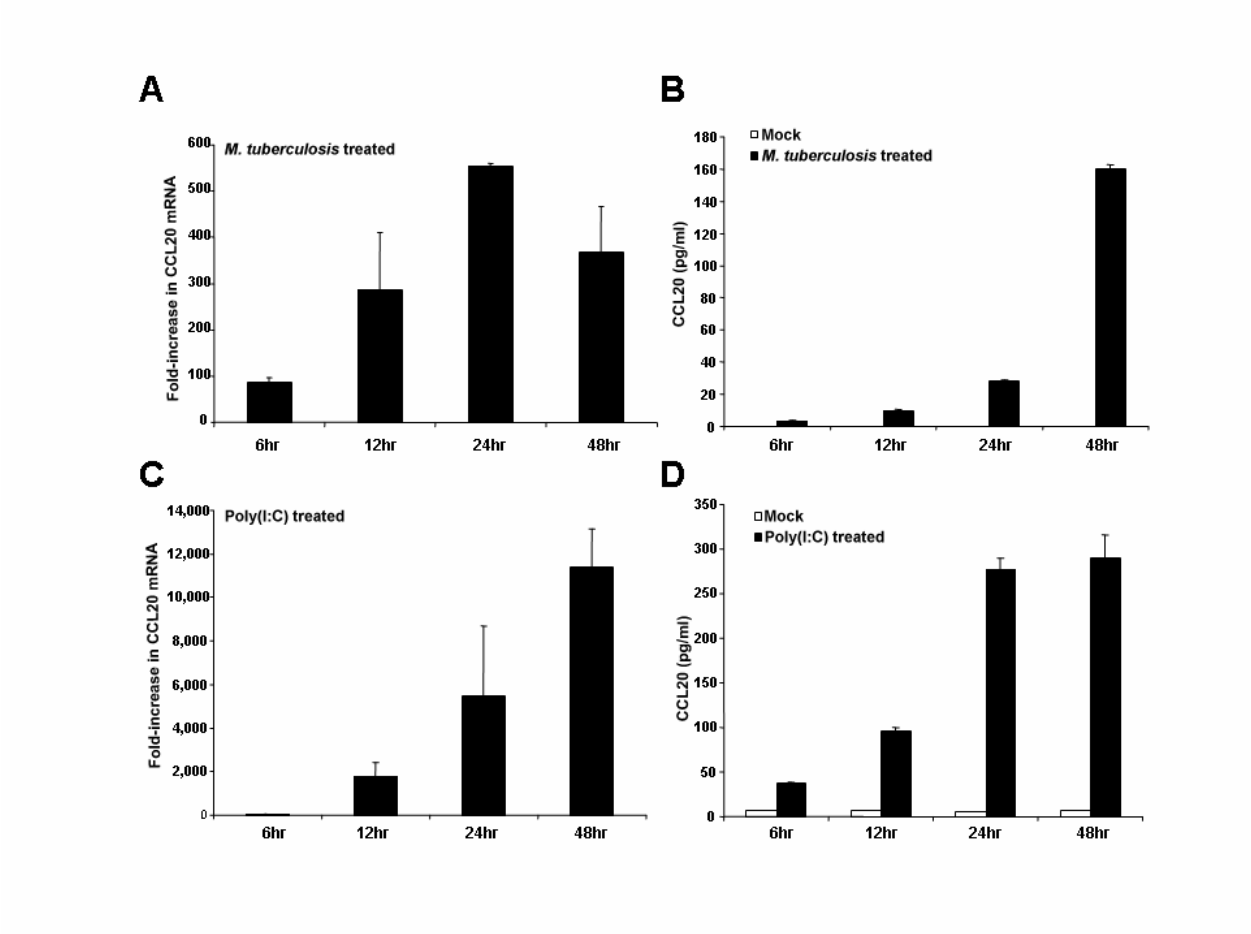

Figure 10. Increased expression of CCL20 mRNA and protein by hTERT-HDLECs treated with model pathogen components. Confluent monolayers of hTERT-HDLECs were treated with γ-irradiated *M. tuberculosis* H37Rv (A-B) or poly(I:C) (C-D) and total RNAs from the cells were analyzed for the relative induction of CCL20 mRNA over time by real-time RT-PCR (A, C). The amount of CCL20 released into the culture supernatants by the same cells was measured by ELISA (B, D).

4.7 CONCLUSIONS

We have presented a comprehensive analysis of the distributions and levels of expression for four LEC markers and two chemokines in nonhuman primate LNs that drain two mucosal sites, and have examined the effects of SIV and *M. tuberculosis* infection on their expression *in vivo*. These findings (summarized in Table 2) provide insight into the structures and phenotypes of the conduits leading into and out of LNs, the organization of the interfaces between the conducting lymphatics and LN parenchyma, and the effects of persistent pathogens of tremendous public health importance on this organization.

This study represents one of the first and most comprehensive examinations of the expression of multiple LEC markers in LNs and the effects of pathogenic infections on these patterns. One key finding from these studies is that LECs at the afferent and efferent interfaces of the same LN are heterogeneous in lymphatic marker and chemokine expression. During health the lymphatic marker LYVE-1 was expressed almost exclusively by LECs localized to LN medullary sinuses, similar to human LNs (212,213). These findings underscore the heterogeneity of populations of LECs, not only throughout the body (7,214,215), but also within LNs.

Interstitial fluid and cells transiting from the peripheral tissues reach the LN via the afferent lymphatic vessels (3,197). In contrast, effector cells and fluids leave the LN via the efferent lymphatic vessels present in the medullary sinuses on their way to the blood. Our demonstration that LYVE-1 is expressed almost exclusively in LN medullary sinuses, combined with previous reports (25,59,212) suggests that LYVE-1 might have a role in cellular egress from

LNs. These interactions are especially important in the trafficking of effector T cells from this immune inductive site into the blood and to the necessary immune effector site.

Table 2. SUMMARY OF LEC MARKER AND CHEMOKINE LOCALIZATON IN MACAQUE LNS

		LN microenvironment		
Marker	*Infection status*	*Subcapsular*	*Cortex/ Paracortex*	*Medulla*
Podoplanin, VEGFR3, Prox-1	None	++[a]	++	+
	SIV	++	++	+
	M. tuberculosis	++	++	+
	SIV and *M. tuberculosis*	++	++	+
LYVE-1	None	-	+++	+++
	SIV	+/-	+++	+++
	M. tuberculosis	+/-	+++	+++
	SIV and *M. tuberculosis*	+/-	+++	+++
CCL20	None	+/-	-	-
	SIV	+++	-	-
	M. tuberculosis	+++	-	-
	SIV and *M. tuberculosis*	+++	-	-
CCL21	None	+	+++	+
	SIV	+	+++	+
	M. tuberculosis	+	+++	+
	SIV and *M. tuberculosis*	+	+++	+

Summarized here are the ISH findings in different LN microenvironments, with the six LEC marker- or chemokine-specific probes in LNs from the animals infected with the indicated persistent pathogens. [a]The scoring here represents a summary of the levels of expression of the indicated mRNA in the different LN microenvironments based on the numbers of cells showing signal and the signals per cell.

One challenge in understanding the implications of CCL20 and CCL21 expression in LNs comes from their differential expression in peripheral and lymphoid tissues. For example, CCL21 is expressed by peripheral tissue afferent LECs and is important in the trafficking of $CCR7^+$ DC to LNs (57), yet we have demonstrated that in LNs it is not expressed at the afferent interface, but rather primarily by cells in the paracortex that do not express podoplanin. Similarly, CCL20 is poorly expressed in skin tissue except during inflammation (216), but we

have shown that it is uniquely expressed at the afferent surface of LNs by podoplanin$^+$ cells. It is conceivable that the environmental context in which the LECs reside contributes to their chemokine expression patterns. Based on these data, we propose that the current model of cell trafficking from peripheral tissues to LNs should potentially include a role for CCL20 and CCR6. Cells trafficking from peripheral tissues, such as DCs, will use CCR7 to respond to LEC-expressed CCL21 in those tissues, move into and through the lymphatic vasculature and encounter CCL20 at the afferent surface of the LN, perhaps responding to this signal due to sustained expression of CCR6. Interestingly, CCR6 is involved in the trafficking of intestinal DCs to draining LNs (187). The trafficking of cells then to the paracortical regions of LNs will be fine-tuned in large part by CCL21 expressed by non-LECs. CCL20 expressed at the afferent face of the LN might also contribute to the localization of subsets of CCR6+ cells coming through high endothelial venules (HEVs) to the afferent/parenchyma interface.

CCL20 is one of two known ligands for CCR6 and is involved in the recruitment of CCR6$^+$ cells such as immature DCs (iDCs) into inflammatory sites (53,217-222). The other known CCR6 ligand is the human anti-microbial peptide β-defensin 2, which is also chemotactic for CCR6$^+$ cells (223). Given that CCR6 can contribute to the migration of DCs to draining LNs (187), increased expression of CCL20 at the interface between the afferent lymphatics and LN parenchyma during a pathogenic infection could lead to enhanced recruitment of immature or transitional DCs into LNs. Apart from iDCs, CCR6 is also present on effector and memory T cells (188), regulatory T cells (189,190), and naïve and memory B cells (191), and CCL20 expressed by LECs in LNs likely recruits these cell types into LNs, especially during inflammatory conditions. Recently it has been shown that CCR6$^+$ cells accumulate in spleen during HIV-1 infection (224). Therefore, CCL20 might contribute to recruitment of effector and

regulatory lymphocyte populations into lymphoid tissues that are characterized by an inflammatory environment due to infection, thereby ultimately affecting the regulation and maintenance of immune responses. Interestingly the structural and functional properties that CCL20 shares with β-defensins includes potent antimicrobial properties (192,225,226). Since pathogens can arrive at LNs from peripheral sites via the afferent lymphatics (69-72), the increased expression of CCL20 at this site during an infection might be a mechanism employed by the host for immediately attacking pathogens that arrive at the LN surface in afferent lymphatic fluid.

We used an *in vitro* model of LECs and observed induction of CCL20 expression by LECs after treatment with either poly(I:C) or γ-irradiated *M. tuberculosis* H37Rv. Poly(I:C) was used to model the double stranded RNA present in tissue during a local viral infection that could signal through TLR3 (227). The dramatic induction of CCL20 after treatment with poly(I:C) indicates that LECs can bind and respond to viral components. Similarly, treatment of LECs with γ-irradiated *M. tuberculosis* also led to dramatic increases in CCL20 expression, indicating that LECs can also bind and respond to mycobacterial components. Therefore, during SIV or *M. tuberculosis* infection, both viral and bacterial components can induce CCL20 expression by LECs at the afferent interface in LNs. Interestingly, even within the same LN, though, CCL20 was not induced in cells at the efferent interface.

We observed classical granulomas in some LNs from animals infected with *M. tuberculosis*. These granulomas severely disrupted the LN architecture, which might in itself contribute to deficits in the generation and maintenance of immune responses. Interestingly, the LN granulomas also contained lymphatic vessels within them. These lymphatic vessels might not be contiguous with the proper lymphatic network since the granuloma itself is within the LN.

This organization might have adverse effects on the trafficking of cells and fluids to, from, and within LNs, since lymphocytes that normally exit the LN via the efferent lymphatics could be misguided by the lymphatic vessels present within these granulomas.

In summary, these findings illustrate the heterogeneity of the LEC populations present at the afferent and efferent interfaces in LNs in health, and the alterations of the phenotypic characteristics of LEC populations at the afferent interface of LNs due to persistent infections. Since the LN has a central role in the generation and maintenance of immune responses, these findings can provide direction in the development of therapeutic or immunization strategies for combating infectious diseases or cancer metastasis in which cellular movement to or from LNs would need to be enhanced or inhibited, respectively.

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We thank Dr. Philana Ling Lin for assistance with project coordination and animal care, Dr. Michael S. Pepper for providing the hTERT-HDLECs, Dr. Craig L. Fuller for helpful discussions, Dr. David N. Finegold for helpful discussions and critical reading of the manuscript, and Beth Fallert for expert technical training and helpful discussions.

5.0 NETWORKS OF LYMPHATIC ENDOTHELIAL CELLS AND DENDRITIC CELLS WITHIN PULMONARY GRANULOMAS IN CYNOMOLGUS MACAQUES INFECTED WITH *MYCOBACTERIUM TUBERCULOSIS*

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This chapter is comprised of data that are being prepared as a manuscript for submission to a peer reviewed journal.

The majority of this work was performed by Amarendra Pegu. The ISHs and IHCs performed for DC-associated markers that included DC-LAMP, DC-SIGN, DC-STAMP, B7-DC, CD11c, CD123, fascin, CCR6, CCR7, CCL19, CCL20 and CCL21 were performed by Craig Fuller, with some initial work performed by Kelly Whelton. This chapter contains work that has been expanded from the initial findings of Craig Fuller's research and as such, includes two figures (Figs. 12 and 13) essentially unmodified from his dissertation.

(Manuscript in preparation)

5.1 PREFACE

Following the examination of the LE network in immune inductive sites, I then focused on analysis of the distribution of the LE network in immune effector sites. In this section I have described experiments and data related to the expression of LEC and DC associated markers in pulmonary granulomas from *M. tuberculosis* infected cynomolgus macaques. This chapter contains data from tissue-based analyses of chemokine, LEC and DC marker expression in pulmonary granulomatous tissues from *M. tuberculosis*-infected animals and normal lung tissues from healthy animals. I have also performed *in vitro* studies using primary LEC and DC cultures to further study their interactions with *M. tuberculosis*. These studies have revealed that a network of lymphatic vessels and DCs is present within granulomas that form during *M. tuberculosis* infection. The *in vitro* experiments have revealed that LECs and DCs respond to mycobacterial components in a similar fashion and interestingly share a number of phenotypic characteristics. I also performed a single chemokine microarray experiment using RNAs from primary lung LECs that were either treated with mycobacterial components or left alone (Appendix B). The results from that array correlated with the changes in gene expression of chemokines as observed by real time RT-PCR in the same RNA samples. Overall these studies provide evidence for the presence of lymphatic vessels within granulomas that form during *M. tuberculosis* infections and the potential interactions between the LE and *M. tuberculosis* during disease development.

5.2 ABSTRACT

Dendritic cells (DCs) are professional antigen presenting cells that are responsible for surveillance of mucosal surfaces. Antigen-loaded DCs migrate through lymphatic vessels from peripheral tissues to the draining lymph nodes (LNs), where they stimulate and regulate immune responses. The lymphatic endothelial cells (LECs) that line the lymphatic vessels secrete the CC chemokine CCL21, which plays an essential role in the recruitment and migration of $CCR7^+$ DCs from the peripheral tissues to the draining LNs. Thus, interactions between DCs and LECs are inevitable and play a critical role in the initiation and regulation of immune responses. DCs have been previously observed in granulomas, including tuberculous granulomas, whereas LECs have been minimally studied in these granulomas. In this study, we used a repertoire of LEC- and DC-associated markers to define the composition of LEC and DC populations within tuberculous granulomas. In an experimental nonhuman primate model of tuberculosis, we found abundant local expression of multiple DC-associated genes and focal expression of LEC markers that localize to likely lymphatic vessels in these granulomas. Additionally, we found that monocyte-derived DCs and LECs responded to γ-irradiated *M. tuberculosis* in a similar fashion and shared multiple phenotypic characteristics. Taken altogether, these findings reveal a network of DCs and lymphatic vessels within pulmonary granulomas during active *M. tuberculosis* infection and suggest that LECs could potentially contribute to the inflammatory processes during infection.

5.3 INTRODUCTION

Tuberculosis causes approximately 2 million deaths each year and since 1993 has been classified as a global health emergency by the World Health Organization (WHO). Tuberculosis is a preventable disease and often curable, although multiple demographic and socioeconomic factors render prevention and treatment difficult. One-third of the world's population is infected, with 10% of the infected individuals developing active disease, usually within the first two years (228). The remaining 90% of the infected population contain the infection and this clinical latency may persist throughout the life of the individual. Acquired immunity to *M. tuberculosis* involves multiple T cell subsets, including $CD4^+$ and $CD8^+$ T cells directed against mycobacterial antigens (229,230). These T cells are stimulated against mycobacterial antigens by antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs).

DCs are a heterogeneous population of APCs that are central to the integration of innate and adaptive immunity. Immature DCs, which survey the environment, have high phagocytic activity, but are inefficient at stimulating T cells due to their expression of MHC and costimulatory molecules at low levels (231). In contrast, mature DCs have increased expression of MHC and costimulatory molecules and are able to migrate via lymphatic vessels from peripheral tissues to the regional lymph nodes (LNs), where they efficiently present the captured antigen to naïve T and B cells. Immature DCs express multiple chemokine receptors, but are recruited primarily through CCR6 by CCL20, but also respond to CCL5 and CCL3 (232). Upon maturation of DCs, CCR6 expression is downregulated and the increased expression of CCR7 on

mature DCs sensitizes these cells to migrate to the regional LNs via CCL21 secreted by the lymphatic vessels (54).

DCs have been broadly classified into two subsets, plasmacytoid and myeloid DCs based on their surface expression of either CD123 (233) or CD11c, respectively. The surface marker DC-SIGN (DC-specific ICAM-3 grabbing nonintegrin/CD209) has been used to classify DCs as immature, and functions as an adhesion molecule. A characteristic of mature DCs is the expression of DC-LAMP/CD208, a lysosome-associated membrane glycoprotein homologous to CD68 (234). Another group of surface markers associated with DC maturation/activation is the B7 family, out of which the expression of only B7-DC has been reported to be restricted to DCs, whereas the other B7 family members are broadly distributed on multiple hematopoietic and nonhematopoietic tissues (235).

Interestingly, $CD11c^+$ DCs have been observed within the granulomatous structures in patients with tuberculosis with a simultaneous reduction in $CD11c^+$ DC in the blood (157). In another study, DCs pretreated with bacillus Calmette-Guerin (BCG) augmented the polarization of Th1 cells (236), suggesting that trafficking of DCs to the site of the granuloma further skewed the immune response towards a type 1 response.

The lymphatic endothelium (LE) is part of the lymphatic system that is involved in the trafficking of cells like DCs to secondary lymphoid organs and transport of interstitial fluid or lymph in the body. Lymphatic endothelial cells (LECs) that make up the LE are thought to constitutively secrete the CCR7 ligand CCL21, which recruits $CCR7^+$ cells, primarily mature DCs, to lymphatic vessels in the peripheral tissues (3). These mature DCs then subsequently traffic through the lymphatic network to reach the draining LNs, where they can stimulate and regulate antigen-specific immune responses. LECs have also been shown to respond to

inflammatory molecules by increasing expression of molecules involved in cellular trafficking like chemokines and adhesion molecules (57,89,90,183), which suggests that local factors can influence the accessibility of the lymphatic vessels for cellular traffic. An increased presence of lymphatic vessels was observed in the intestine during inflammatory bowel disease suggesting involvement of LECs in chronic inflammatory processes (161,162). Therefore, in pulmonary tuberculosis, another chronic inflammatory disease, lymphatic vessels might be involved in the local inflammatory processes that take place in the lung compartment.

In this study, we sought to characterize the LEC and DC populations within the granulomatous lesion using LEC and DC-associated markers not previously examined in tissues of an experimental nonhuman primate model of tuberculosis. We examined the expression of inflammatory chemokines by LECs and DCs present within these granulomas and also studied the response of the LECs and DCs to *M. tuberculosis in vitro*. These studies have revealed the presence of CCL21 expressing lymphatic vessels within pulmonary granulomas and an abundance of DC-associated molecules at these sites that include fascin (p55), an actin bundling protein known to be expressed by DCs, CD11c, DC-LAMP and IL-4-INDucible protein (FIND). Additionally, we found that both LECs and DCs responded to *M. tuberculosis* by increasing expression of inflammatory molecules and that LECs and DCs share phenotypic similarities, which suggest an interesting relationship between DCs and their conduit, the lymphatic vessels. Overall this study demonstrates the presence of a network of DCs and lymphatic vessels within pulmonary granulomas formed during *M. tuberculosis* infection and that LECs may be more actively involved during infection than previously envisaged.

5.4 MATERIALS AND METHODS

Animals and tissue processing

All animal studies were performed under the guidance and approval of the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Adult cynomolgus macaques were inoculated with a low dose (approximately 25 colony forming units) of virulent *M. tuberculosis* (Erdman strain) via bronchoscope into the lower right lobe, as described elsewhere (205). Infection was allowed to proceed until macaques reached disease states that spanned a spectrum from no apparent disease to advanced disease. At necropsy, tissues were collected and fixed in 4% paraformaldehyde/1X phosphate buffered saline (PF/PBS) for 5 hr at 40^{o}C, as previously described (208). After fixation, the tissues were cryoprotected and snap-frozen in isopentane cooled on dry ice to -65^{o}C.

Immunohistochemistry (IHC)

Immunohistochemical staining of 14μm tissue sections was performed using cell-type specific monoclonal antibodies for fascin (clone 55K-2, Dako, Carpinteria, CA), CD68 (clone KP1, Dako) and Podoplanin (Angiobio, Del Mar, CA). Tissue sections were pretreated in 0.01M sodium citrate (pH 6.0) by microwaving followed by application of the primary antibody (diluted in 1X PBS) to the tissues for 1 hr in a humid chamber at room temperature. Primary antibodies were detected with the SuperPicTure^{TM} detection system (Invitrogen), using 3,3'-diaminobenzidine (DAB) as the final substrate.

In situ *hybridization (ISH)*

Riboprobe syntheses and ISHs were performed on 14μm tissue sections as described (40,207,208). Cytokine, chemokine, LEC and DC-associated mRNAs were detected by ISH using gene-specific riboprobes. Autoradiographic exposure times were 7 days for CCL20, CCL21 targets, 8 days for DC-LAMP, DC-SIGN, B7-DC, DC-STAMP targets, 21 days for CCR6, CCR7, CD123, CD11c, Podoplanin, VEGFR3, Prox1, LYVE-1 and VEGF-C targets.

Combined In situ *hybridization and immunohistochemistry*

Simultaneous ISH and IHC were performed as described (207,208).

Immunofluorescent staining

Immunofluorescent staining of 14μm tissue sections was performed using cell-type specific monoclonal antibodies for fascin (clone 55K-2, Dako, Carpinteria, CA), Ki67 (clone MM1, Novacastro) and Podoplanin (Angiobio, Del Mar, CA). Tissue sections were pretreated in 0.01M sodium citrate (pH 6.0) by microwaving followed by application of the primary antibody (diluted in 1X PBS) to the tissues for 1 hr in a humid chamber at room temperature. Primary antibodies were detected with isotype-specific antibodies conjugated to fluorescein or biotin. Subsequently fluorescein and biotin conjugates were detected with an Alexa Fluor 488 signal-amplification kit for fluorescein-conjugated probes (Invitrogen), and an Alexa Fluor 746 conjugate of streptavidin (Invitrogen), respectively. The slides were mounted with prolong antifade reagent (Invitrogen) and visualized using the Olympus Fluoview 500 confocal microscope (Center for Biologic Imaging, University of Pittsburgh).

Isolation of monocyte-derived DC

Peripheral blood mononuclear cells were isolated from human buffy coats (Central Blood Bank) by Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich, ST. Louis, MO). $CD14^+$ monocytes were isolated by positive selection using anti-CD14 microbeads, per the manufacturer's recommendations (Miltenyi Biotech, Auburn, CA). The purity of $CD14^+$ cells after enrichment was >90% as determined by flow cytometry. $CD14^+$ cells were cultured in RPMI 1640 media supplemented with L-glutamine (2 mM, Invitrogen), nonessential amino acids (100 μM, Invitrogen), 1M HEPES (Invitrogen), 10% heat-inactivated fetal calf serum (Hyclone), penicillin/streptomycin (100 U/100 μg, Invitrogen), IL-4 (25 ng/ml, Peprotech Inc) and GM-CSF (50 ng/ml, Bayer); the medium was changed on days 2 and 4.

Culture of LECs

Primary human dermal (HMVEC-dLy) and lung (HMVEC-LLy) LECs (Cambrex Bio Science) were cultured in EGM-2MV medium (Cambrex Bio Science) according to the supplier's suggestions. hTERT-HDLECs, kindly provided by Dr. M. S. Pepper (University of Geneva), were also cultured in EGM-2MV media as previously described (179).

Treatment of LECs and DCs with γ-irradiated M. tuberculosis

Confluent monolayers of LECs and immature human DCs (day 6 culture of monocyte-derived DC) were treated with γ-irradiated *M. tuberculosis* H37Rv (Colorado State University, Fort Collins, CO; 500 μg/ml). This concentration has been used previously for the treatment of DCs (237). The cultures were incubated in 5% CO_2 at 37°C. At the indicated time points the cells were harvested and the supernatants were saved. The cells were then used for phenotypic

analysis that included real-time RT-PCR for multiple immune-related genes, flow cytometry for multiple phenotypic markers, or antigen processing assays.

Phenotypic analysis of LECs and DCs

Real-time RT-PCR (comparative Ct method) was performed as described (180) using commercially available TaqMan assays for multiple chemokines, cytokines and adhesion molecules (Applied Biosystems). Culture supernatant levels of CCL20 and CXCL10 (R & D Systems), and IL-6 (BD Biosciences), were measured by ELISA. Flow cytometry analysis was performed to detect the expression of CD31 (clone WM59, BD Biosciences), CD34 (clone 563, BD Biosciences), HLA-DR/DP/DQ (clone TU39, BD Biosciences), HLA-A,B,C (clone G46-2.6, BD Biosciences), CD80 (clone L307.4, BD Biosciences), CD83 (clone HB15e, BD Biosciences), CD86 (clone FUN1, BD Biosciences), CD40 (clone MAB89, Beckman Coulter), CD123 (clone 7G3, BD Biosciences), DC-LAMP (CD208, clone 104.G4, Beckman Coulter), VEGFR3 (clone 54733, R&D Systems), VCAM-1 (CD106, clone 51.10C9, BD Biosciences) and ICAM-1 (CD54, clone HA58, BD Biosciences). DQ-Ovalbumin (DQ-Ova, Invitrogen) was used for assaying the antigen uptake and processing ability of the cells. In brief, cells (10^5 cells) were pulsed with DQ-Ova (5 μg/ml in phosphate-buffered saline) in 96-well cell culture plates (total volume of 100 μl) either at 37°C or 4°C for 1 hr and then the breakdown of DQ-Ova was assessed by flow cytometry.

5.5 RESULTS

To characterize the types and locations of LEC and DC populations within tuberculous granulomas, we examined pulmonary granulomas from cynomolgus macaques that were inoculated intrabronchially with a low dose of virulent *M. tuberculosis* (Erdman strain). All animals examined in this study were successfully infected and detailed clinicopathological and bacteriological findings have been presented elsewhere (205). The pulmonary granulomas had abundant expression of IFN-γ-inducible chemokines and proinflammatory cytokines (156) and in this study we sought to define the LEC and DC populations present in granulomas in a relevant nonhuman primate model of tuberculosis.

Expression of CCR6, CCR7 and their ligands in pulmonary granulomas

Surveillance of the pulmonary mucosal interface with the external environment is performed by DCs and is characterized by their rapid turnover (238). One of the mechanisms influencing the movement of DCs is the set of interactions between chemokines and chemokine receptors. Immature DCs express abundant CCR6, which binds CCL20, whereas mature DCs express CCR7, which binds CCL19 and CCL21 (239). Therefore, we sought to determine the mRNA expression patterns of these two chemokine receptors within pulmonary granulomatous tissues using ISH. We detected CCR7 mRNA and its expression was abundant in the lung tissues, specifically within the granulomas (Fig. 11A). In contrast, CCR6 mRNA expression was dispersed diffusely around the granuloma (Fig. 11B).

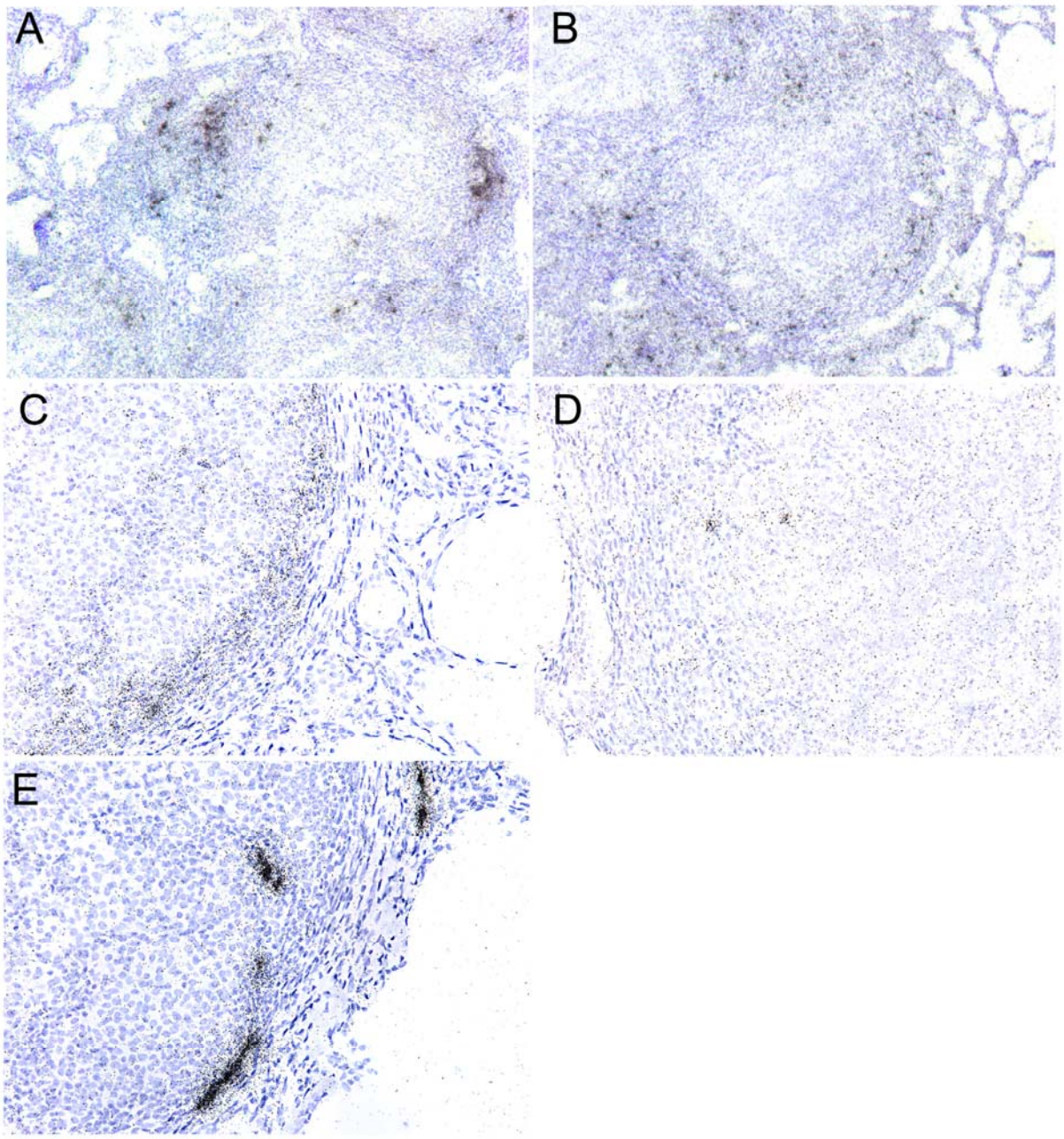

Figure 11. In situ hybridization for CCR6, CCR7 and their ligand mRNAs in pulmonary granulomatous tissues from cynomolgus macaques infected with virulent *M. tuberculosis*. CCR6 mRNA (B) was spread out over the granuloma, whereas CCR7 mRNA (A) was more focalized to distinct regions of the granuloma. CCL19 mRNA (C) was abundant and expressed as a ring on the internal portion of a cellular band ringing the granuloma, whereas CCL21 mRNA (E) was present as intense focal, vascular collections in the granuloma. CCL20 mRNA (D) was relatively rare and localized within the cellular region of the granuloma. Original magnifications x100 (A, B), x200 (C-E).

We also sought to determine if chemokines that recruit immature or mature DCs were present in granulomas. Thus, we performed ISH for CCL20, CCL19 and CCL21 mRNA in the granulomatous tissues of cynomolgus macaques infected with *M. tuberculosis*. Interestingly, we found very few $CCL20^+$ cells expressed within granulomas (Fig.11D), whereas the CCR7 ligands (CCL19 and CCL21) were more abundant (Fig. 11C and E). CCL19 mRNA was expressed as a dispersed signal in a cellular band ringing the granulomas and was concentrated just outside the acellular necrotic region (Fig. 11C). In contrast, ISH signal for CCL21 mRNA was intense and localized to specific sites along the outer portion of the granuloma, which appeared to be thin-walled vessels (Fig.11E), which were likely lymphatic vessels. The distribution of CCL21 mRNA is consistent with CCR7 mRNA expression as described above, suggesting that $CCR7^+$ mature DCs are present in granulomas and are localized by $CCL21^+$ lymphatic vessels in these granulomas.

Expression of DC-associated markers in pulmonary granulomas

To further define the DC populations present in the pulmonary granulomas, we performed IHC and ISH for multiple DC targets. We performed ISH for CD11c and CD123, to identify myeloid and plasmacytoid DCs respectively. Although CD11c could be expressed by alveolar macrophages, we found that its mRNA (Fig. 12A and B) was abundant and dispersed throughout the granuloma indicating that a proportion of these cells might be myeloid DCs. CD123 mRNA (Fig. 12C and D) was also expressed in granulomas and the expression was dispersed throughout the granulomas, but at a lower intensity than CD11c (Fig. 12).

Fascin (p55) is a 55kDa protein involved in the formation of microfilament bundles and is expressed by interdigitating and follicular DCs within LNs (240), and by DCs in thymus,

spleen and peripheral blood (241,242). Fascin can also be expressed by fibroblasts (243) and neuronal cells (244). Fascin protein (Fig. 12G and H) and mRNA (Fig. 12E and F) had relatively the same distribution patterns in these granulomas, with staining observed in cells throughout the entire lung tissue. The fascin^{+} cells were distinct from pulmonary macrophages as shown by simultaneous IHC for fascin and CD68 (Fig. 12I and J). Taken together, the expression pattern of fascin antigen and mRNA suggests that cells expressing myeloid and plasmacytoid DC markers are present in granulomas.

In addition to identifying DCs expressing lineage-specific (CD11c, CD123) and pan-DC (fascin) markers, we sought to obtain data regarding the maturation/activation states of the DC populations in granulomas. We performed ISH for DC-SIGN, DC-LAMP and B7-DC mRNAs on these granulomatous tissues. Although previous studies in rodents have shown that pulmonary DC populations have functional characteristics of an immature DC (231), we did not detect any DC-SIGN mRNA within these granulomatous tissues. This is further supported by our earlier observation where we found very little CCL20 mRNA within granulomas, suggesting that immature DCs were not recruited to granulomas. However, we were able to detect DC-SIGN mRNA in some submucosal areas of the lung tissues (Fig. 13A and B). In situ hybridizations for mRNA encoding the lysosomal marker DC-LAMP revealed that DC-LAMP^{+} cells encircled the granulomas (Fig. 13C) and the ISH signal patterns in the normal lung architecture were consistent with our previous findings in rhesus macaques (245). The cells expressing DC-LAMP mRNA were abundant on the outside surfaces of the granulomas with a small number of DC-LAMP mRNA^{+} cells also observed within the cellular portion of the granuloma. These findings suggested that DCs were present in and around the granulomatous structure. However, two reports have shown that DC-LAMP protein co-localizes with markers

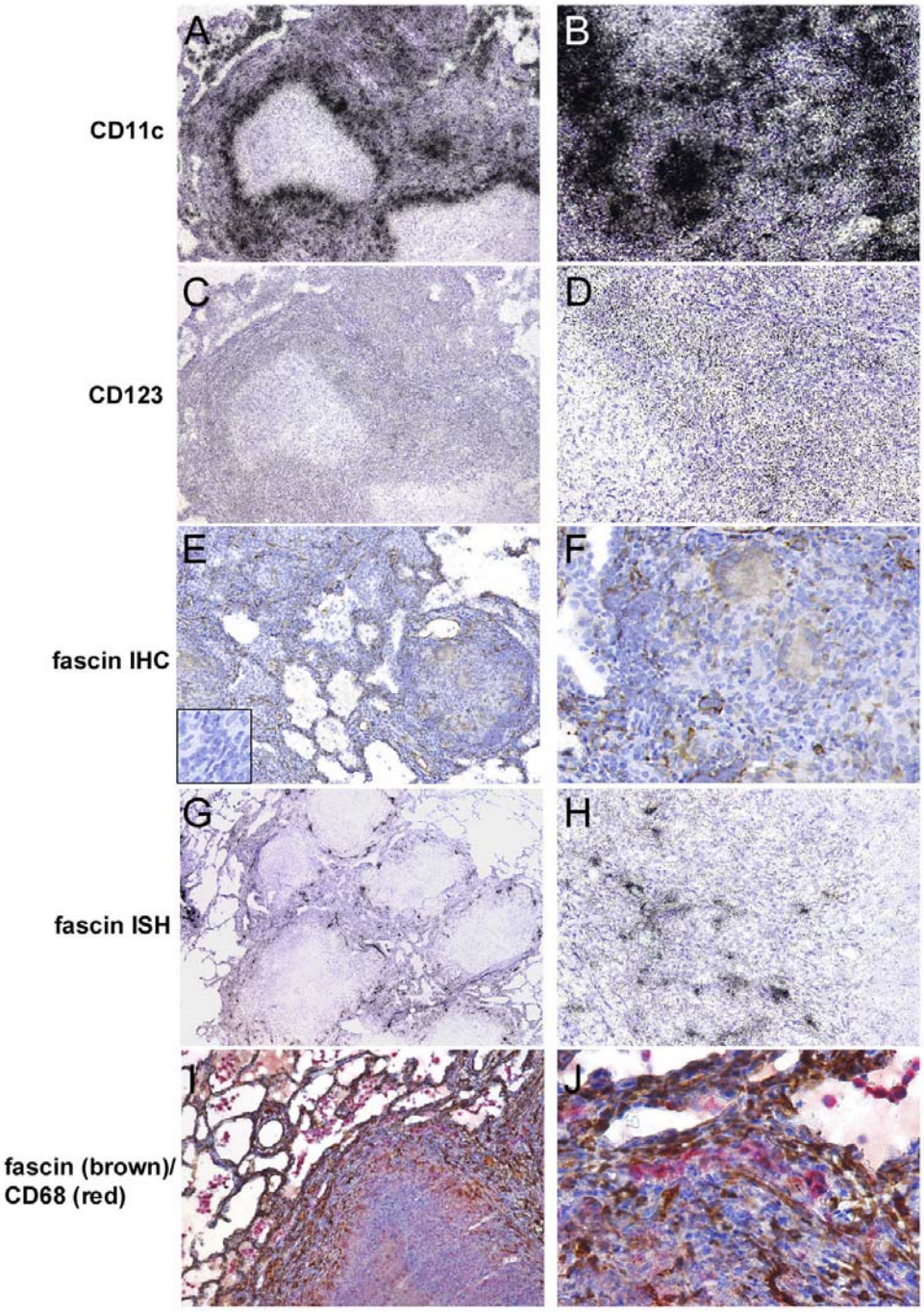

Figure 12. In situ hybridization for DC-associated mRNAs in pulmonary granulomatous tissues from cynomolgus macaques infected with virulent *M. tuberculosis*. The expression of CD11c (A, B) and CD123 (C, D) mRNAs were examined to determine the presence of myeloid and plasmacytoid DC. Fascin-positive cells were also identified in the granulomatous tissues by IHC (E, F) and ISH (G, H), with the isotype control shown (inset, C). The expression of fascin was restricted to DC as shown by double IHC (I, J), with fascin-positive cells (DC, brown) and CD68-positive cells (macrophages, red). Original magnifications, x40 (D, G), x100 (A, C, I), x400 (B, D, F, H), x600 (J).

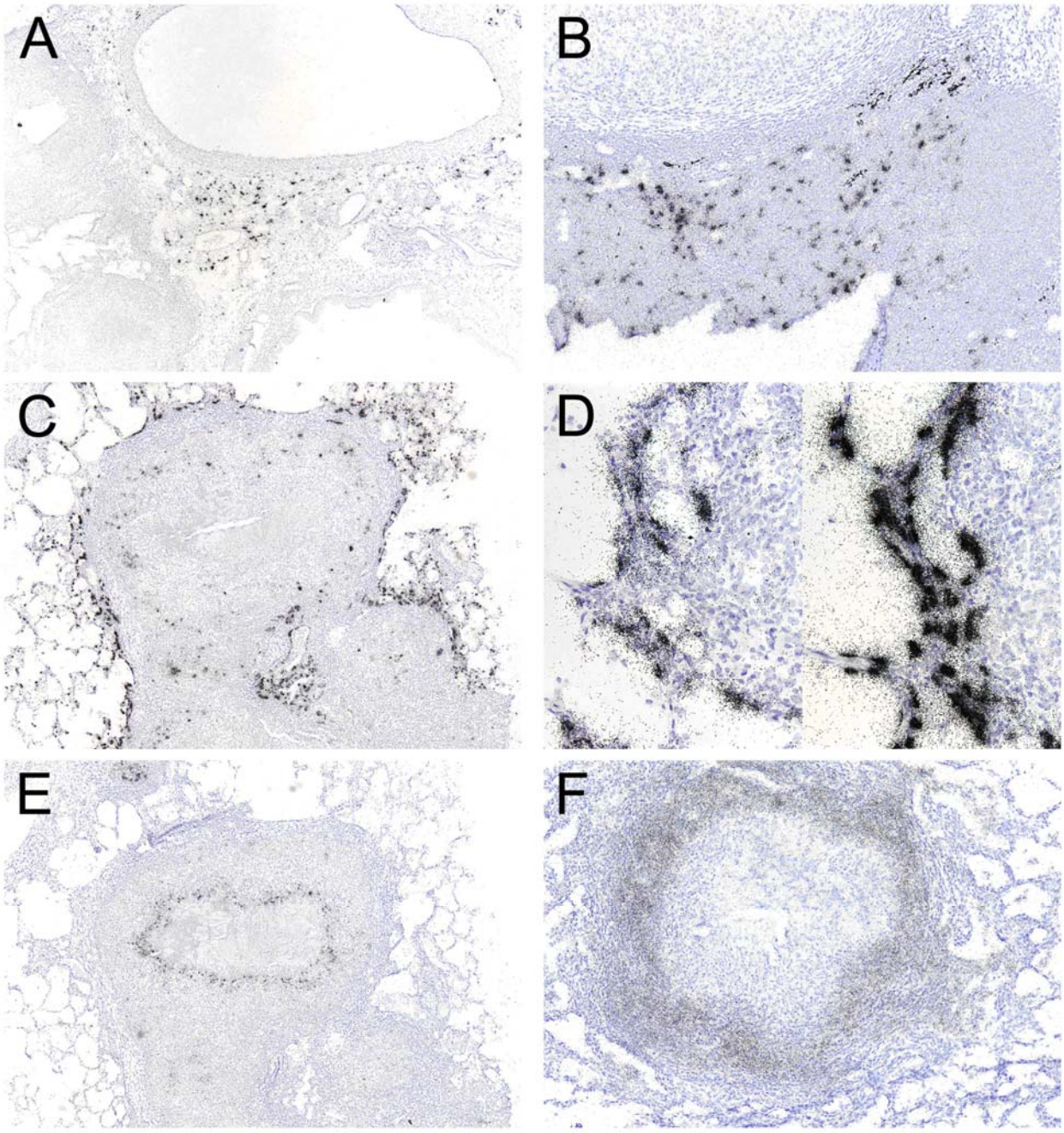

Figure 13. In situ hybridization for DC-associated mRNAs in pulmonary granulomatous tissues from cynomolgus macaques infected with virulent *M. tuberculosis*. The expression of DC-SIGN was rare in lung tissues (A) and was more abundant in subregions of hilar lymph nodes (B). DC-LAMP mRNA^{+} cells were abundant surrounding the granuloma and dispersed within the cellular region of the granuloma (C). Further characterization of these cells showed that the DC-LAMP mRNA^{+} cells (D, left) were colocalized with type II pneumocytes expressing surfactant B mRNA (D, right). DC-STAMP mRNA expression (E) was confined to the interface between the caseation and cellular region of the granuloma, whereas B7-DC mRNA (F) was dispersed throughout the entire granuloma structure, but with increased intensity near the same interface. Original magnifications x40 (A, C, E and F), x100 (B), x400 (D).

for human type II pneumocytes (246,247). Thus, we examined whether the DC-LAMP ISH signal within the lung was consistent with these previous reports by performing ISH for surfactant B mRNA, which is expressed by type II pneumocytes (248). ISH on subjacent sections for DC-LAMP (Fig. 13D, left) and surfactant B (Fig. 13D, right) mRNAs exhibited the same distribution patterns, confirming the previous findings (246,247). These data indicate that the DC-LAMP mRNA expression observed in these tissues is predominantly contributed by type II pneumocytes that line the alveolar spaces or that are trapped within granulomas that form around them, and suggest that there are few DC-LAMP$^+$ mature DCs in the pulmonary granulomas. Another marker that is expressed by activated DCs is the B7 family costimulatory molecule, B7-DC, which is considered to be DC-restricted. Unlike DC-LAMP, B7-DC ISH signal (Fig. 13F) is highly dispersed throughout and did not label distinctive cells, consistent with the cells potentially having numerous intertwining DC processes.

DC-STAMP, a DC-specific transmembrane protein, is a multi-membrane spanning molecule that was initially described as being expressed preferentially by DC (249). Our analysis of related sequences in the GenBank database revealed that FIND was identical in nucleotide sequence to DC-STAMP. FIND (IL-Four INDuced) was initially described as a putative transmembrane molecule expressed by mononuclear phagocytes deactivated by IL-4 and not IL-10 treatment (250). Next, we examined the expression of DC-STAMP/FIND mRNA in granulomatous tissues and found that cells expressing this IL-4-inducible mRNA were exquisitely localized to the cellular and acellular interface of necrotic granulomas (Fig. 4E), whereas DC-STAMP mRNA was not observed in solid granulomas or in normal lung tissue. Given that DC-STAMP and FIND are the same molecule, and that FIND was known to be induced by IL-4 (250), we suggest that DC-STAMP could potentially serve as a surrogate

marker for local IL-4 production in granulomas from cynomolgus macaques since we were not able to detect IL-4 mRNA in these granulomas although previously IL-4 mRNA has been detected in subsets of human granulomas (251),

Expression of LEC markers in pulmonary granulomas

We found expression of CCL21 mRNA in focal collections within pulmonary granulomas from *M. tuberculosis*-infected cynomolgus macaques (Fig. 11E). Since lymphatic vessels are known to produce CCL21 in peripheral tissues, which is instrumental in the migration of DCs from peripheral tissues to draining LNs (3,59), we examined the expression of the lymphatic marker podoplanin by the CCL21 and CCL20 mRNA expressing cells. We performed simultaneous ISH for CCL20 or CCL21 mRNA and IHC for podoplanin protein in both normal lung and granulomatous lung tissue sections from uninfected, healthy animals and *M. tuberculosis*-infected animals. We found that all the $CCL21^+$ cells stained for podoplanin although not all $podoplanin^+$ cells expressed CCL21 mRNA (Fig. 14C and D). Podoplanin protein was expressed at high levels by the cells that expressed CCL21 mRNA and that had the morphology of loosely structured vessels that were indicative of lymphatic vessels in both normal and granulomatous lung tissues, whereas it was expressed at low levels by cells that did not express CCL21 mRNA but were abundantly present in granulomas. We also found that there was minimal expression of CCL20 mRNA by $podoplanin^+$ cells in both normal and granulomatous lung tissues (Fig. 14A and B). To further confirm the identity of lymphatic vessels, we examined the expression of multiple LEC marker mRNAs by the $podoplanin^+$ cells in these lung tissues. We performed simultaneous ISH/IHC for detecting the mRNA for the LEC markers podoplanin, Prox1 and LYVE-1 along with podoplanin protein in these tissue sections. These markers have been

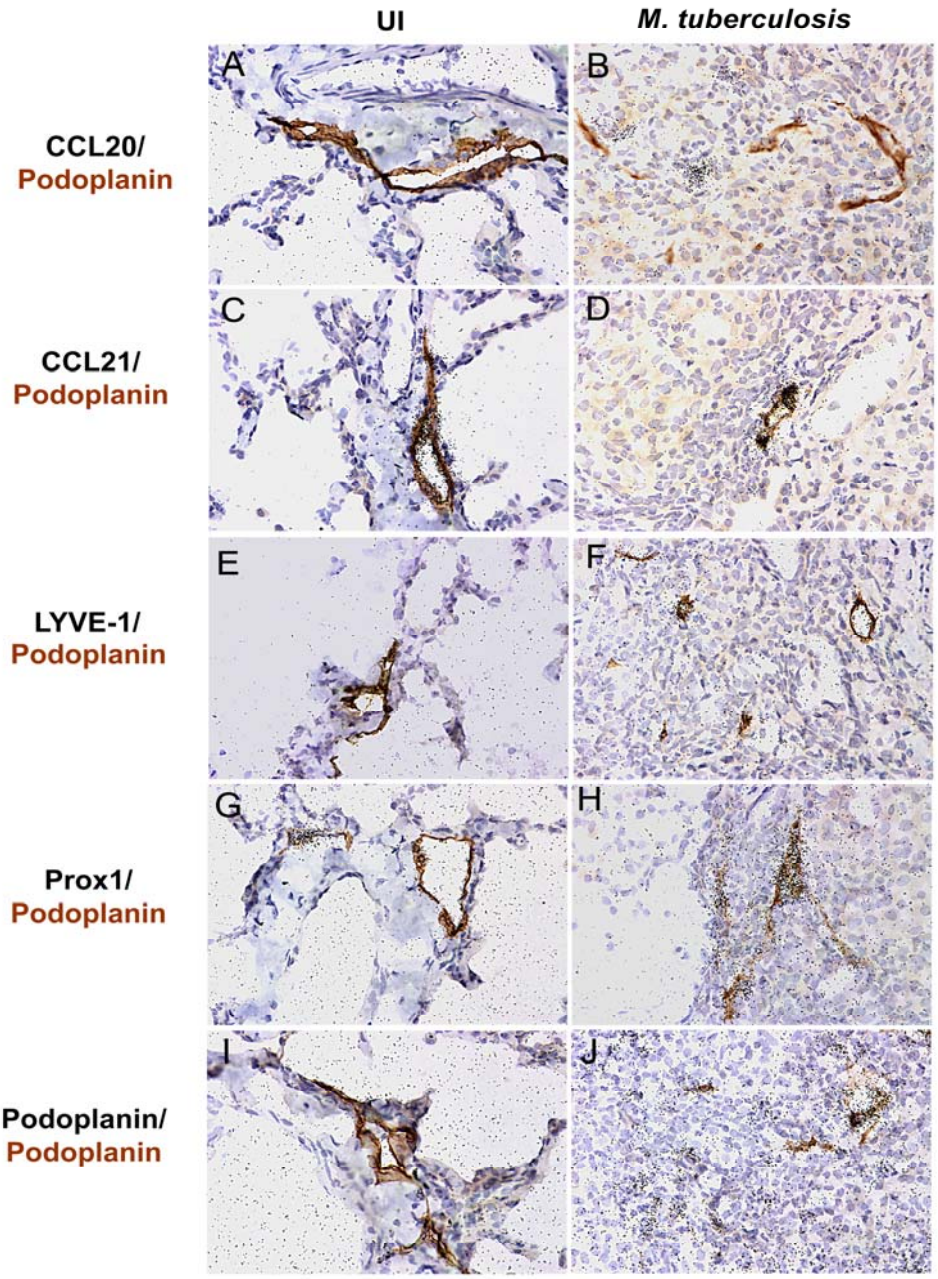

Figure 14. Simultaneous in situ hybridization detection of chemokine and LEC marker mRNAs combined with immunohistochemical staining for podoplanin in normal lung and pulmonary granulomatous tissues from cynomolgus macaques that were either uninfected (UI) or infected with virulent *M. tuberculosis*. CCL20 mRNA was undetectable in uninfected lung sections (A), whereas in granulomatous lung it was not expressed by podoplanin$^+$ vessels (B). CCL21 mRNA was expressed by podoplanin$^+$ vessels in both uninfected and granulomatous lungsC, D). LYVE-1 and Prox1 mRNAs were minimally expressed by podoplanin$^+$ vessels in uninfected lung (E, G), whereas they were highly expressed by podoplanin$^+$ vessels in granulomatous lung (F, H). Podoplanin mRNA was minimally expressed by podoplanin$^+$ vessels in uninfected lung (I), whereas it was highly expressed by podoplanin$^+$ vessels and other cells present within granulomas in granulomatous lung (J). Original magnification x400 (A-J).

widely used to identify lymphatic vessels in tissues and are known to be expressed by LECs (2). We found that podoplanin, Prox1 and LYVE-1 mRNAs localized to the same regions in these granulomas as the podoplanin$^+$ cells that expressed CCL21 mRNA indicating that lymphatic vessels were present in these granulomas and that there was an increased expression of these marker mRNAs by the lymphatic vessels in granulomatous lung tissues compared to those in normal lung tissues (Fig. 14E-J). In addition we found that both podoplanin mRNA and protein were also abundantly expressed by cells other than LECs in the cellular region of these granulomas (Fig. 14J). These data indicate that lymphatic vessels are present within pulmonary granulomas of *M. tuberculosis*-infected cynomolgus macaques and that granulomas had high levels of lymphatic marker expression compared to lymphatic vessels in normal lung tissues. Interestingly, we also found that podoplanin$^+$ lymphatic vessels often localized adjacent to blood vessels in normal lung tissues (Fig. 14A, C, E, G and I).

Inflammation leads to production of the lymphangiogenic factors VEGF-C and VEGF-D that promote the growth and proliferation of lymphatic vessels (130). Since granulomas formed during *M. tuberculosis* infection are an inflammatory environment, we examined the expression of VEGF-C mRNA in these granulomas. The expression of VEGF-C mRNA was limited in these granulomas to a handful of cells in the cellular regions of granulomas. The VEGF-C mRNA was predominantly expressed by fascin$^+$ cells and rarely by CD68$^+$ macrophages or podoplanin$^+$ lymphatic vessels in these granulomas (Fig. 15A). This indicates that there is limited expression of the lymphangiogenic factor VEGF-C in these granulomas and that fascin$^+$ cells are the primary source of VEGF-C. We also examined the proliferation states of the lymphatic vessels in these granulomas and found that most of the LECs did not stain for Ki67, which is a known proliferation marker (Fig. 15B). These data suggest that there is limited

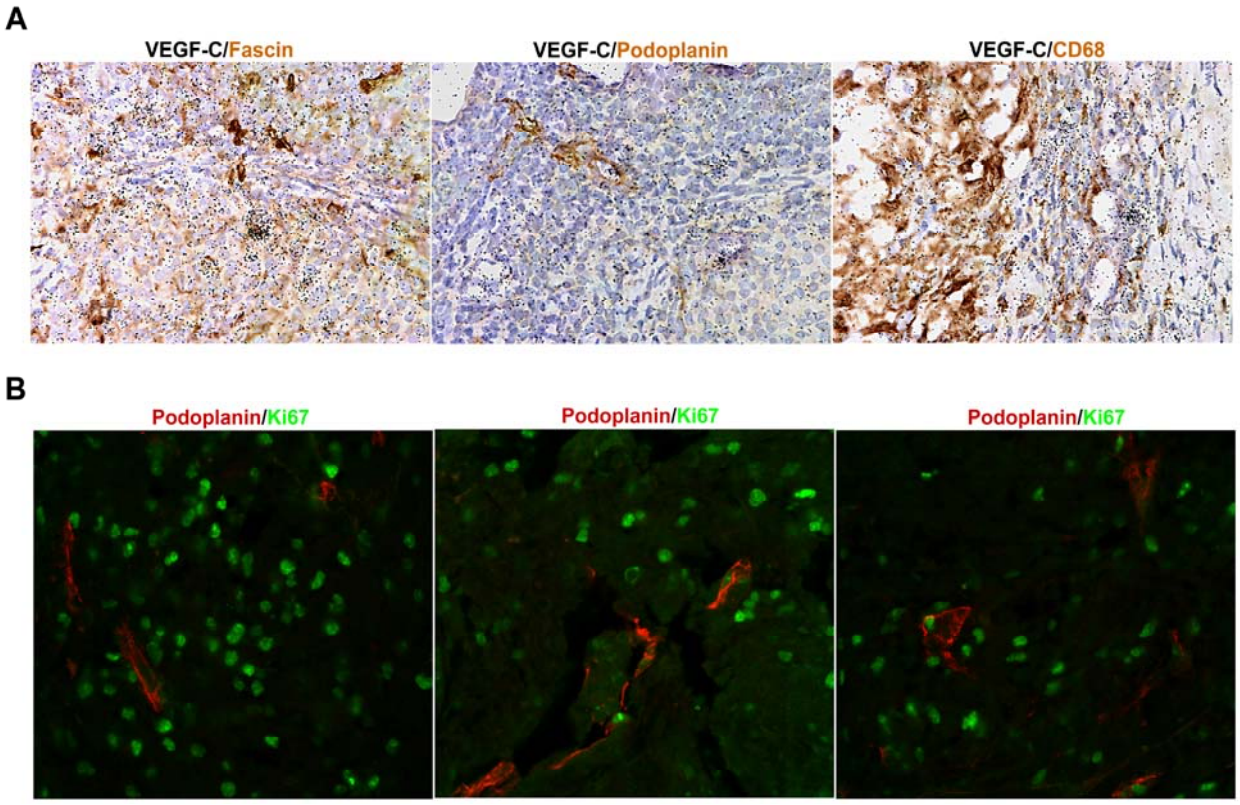

Figure 15. Simultaneous in situ hybridization detection of VEGF-C mRNAs combined with immunohistochemical staining for fascin/podoplanin/CD68 and double immunofluorescent staining for Ki67 and podoplanin in pulmonary granulomatous tissues from cynomolgus macaques infected with virulent *M. tuberculosis*. VEGF-C mRNA was expressed by fascin$^+$ cells and not by both podoplanin$^+$ vessels and CD68$^+$ macrophages in the granuloma(A). Confocal images showing Ki67$^+$ cells are present in granulomas but rarely colocalize with podoplanin$^+$ vessels (B). Original magnification x400 (A), x600 (B)

expression of a lymphangiogenic factor in these granulomas, which is corroborated by the lack of proliferating lymphatic vessels in these granulomas.

Phenotypic similarities between LECs and DCs

Lymphatic vessels recruit CCR7$^+$ mature DCs by the production of the CCR7 ligand CCL21 and act as a conduit for these DCs to reach the draining LNs. Since we observed lymphatic vessels that expressed CCL21 in granulomas, we examined the distribution of DCs relative to these lymphatic vessels in granulomas. We performed simultaneous ISH/IHC for CCL21 mRNA and

fascin protein to detect lymphatic vessels and purportedly DCs, respectively, in the same granulomatous sections. Unexpectedly, we found that fascin was itself expressed by the LECs expressing CCL21 mRNA in granulomas suggesting co-expression of fascin by both DCs and LECs (Fig. 16A). To further evaluate the expression of fascin by LECs, we performed double immunofluorescent staining of granulomatous sections using fascin and podoplanin specific antibodies. Similar to the earlier observations, we found that the podoplanin^{+} lymphatic vessels stained for fascin providing evidence for the expression of fascin by LECs (Fig. 16B). These results indicate that DCs and LECs express a common marker and suggest that they might have phenotypic similarities.

Since lymphatic vessels express TLR molecules (104), they can directly bind and respond to pathogens. Therefore, we evaluated the response of LECs to γ-irradiated *M. tuberculosis* as a surrogate for the live organism and its components that LECs might encounter in the granulomas formed during *M. tuberculosis* infections. For *in vitro* studies, we used primary dermal (HMVEC-dLy) and lung (HMVEC-LLy) LECs along with model LECs, hTERT-HDLECs, which are dermal in origin and display a longer life-span due to the ectopic expression of human telomerase reverse transcriptase (179). We observed increased expression of a panel of inflammatory chemokine and cytokine mRNAs in the treated LECs compared to the untreated control cell populations (Fig. 17A). This response of LECs paralleled DC responses to γ-irradiated *M. tuberculosis* in terms of induction of the same inflammatory molecules (Fig. 17B). We also found an induction in the expression of podoplanin mRNA by the DCs suggesting that the expression of podoplanin by cell types other than LECs in granulomas could have been induced due to direct interaction with the pathogen and/or the inflammatory conditions present in granulomas. These data provide further evidence for the phenotypic similarities between LECs

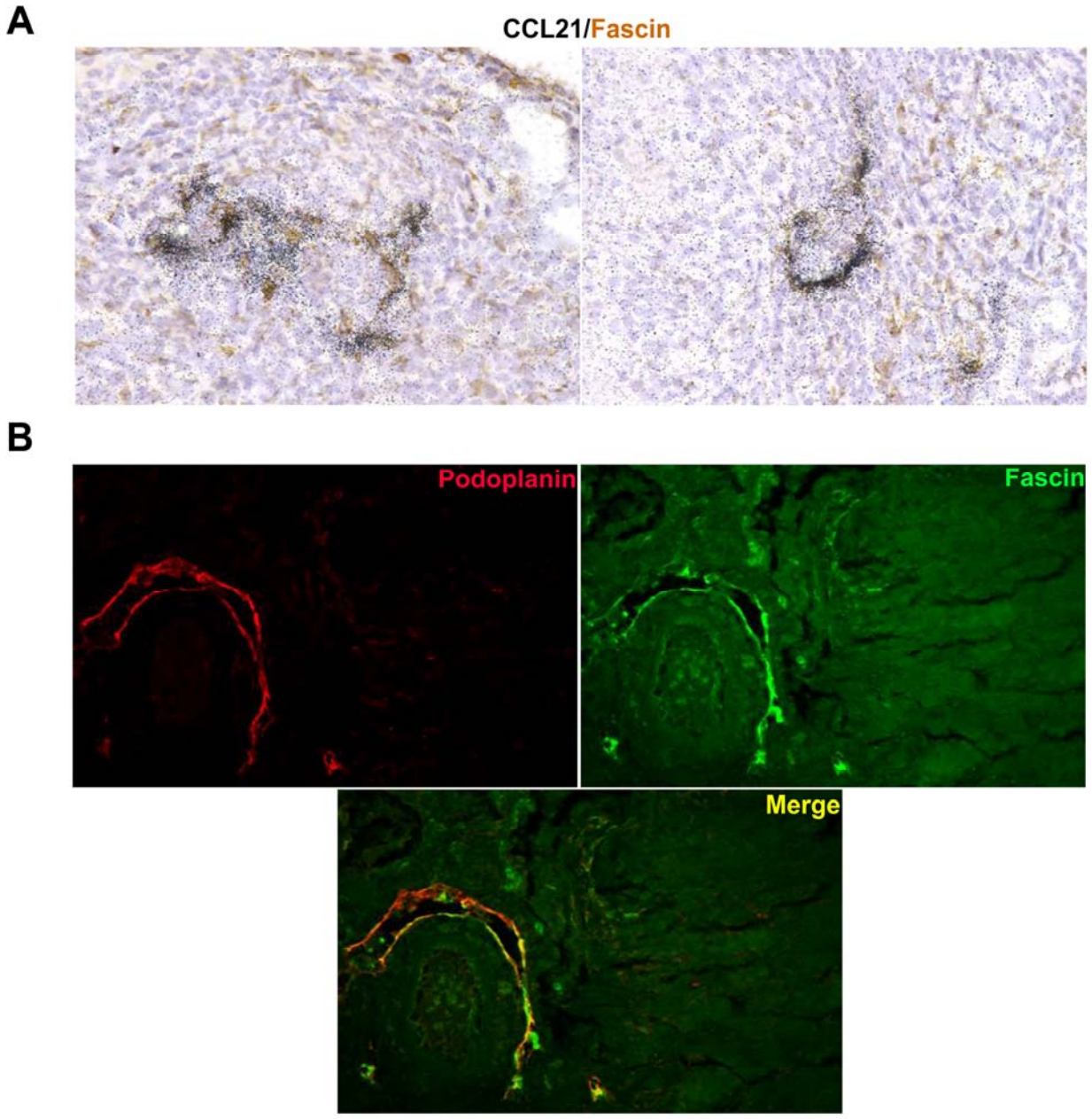

Figure 16. Simultaneous in situ hybridization detection of CCL21 mRNAs combined with immunohistochemical staining for fascin and double immunofluorescent staining for fascin and podoplanin in pulmonary granulomatous tissues from cynomolgus macaques infected with virulent *M. tuberculosis*. CCL21 mRNA expressing cells stained for fascin (A) and podoplanin^{+} vessels also stained for fascin (B). Original magnification x600 (A), x400 (B).

and DCs. We also found that these DCs did not express detectable

levels of VEGF-C mRNA even after treatment with γ-irradiated *M. tuberculosis* (data not shown) and that there was a minimal induction in their expression of VEGF-D mRNA after treatment suggesting that these DCs do not produce lymphangiogenic factors in response to direct treatment with *M. tuberculosis.*

Next we examined the expression of a subset of DC-associated molecules that included MHC class I/II, costimulatory molecules CD40/80/83/86, DC-LAMP and CD123 by LECs and found that LECs did express both MHC class I and II, CD40, DC-LAMP and CD123 (Fig. 17C) although the levels of MHC class II were minimal these cells also expressed negligible amounts of the costimulatory molecules CD80, CD83 and CD86 (data not shown). After treatment with γ-irradiated *M. tuberculosis*, there was a slight increase in the expression level of MHC class I and a greater increase in CD123 by the LECs, which indicates that there are minimal changes in the expression levels of DC-associated molecules by LECs (Fig. 17C). Therefore, these data identify similarities between LECs and DCs in both the expression of phenotypic markers and in some of their responses to interaction with pathogens.

The adhesion molecules, ICAM-1 and VCAM-1 are involved in the adherence of immune cells to lymphatic vessels and are important for the migration of these cells through the lymphatic vessels (89). We found that the LECs increased surface expression of both ICAM-1 and VCAM-1 in response to the γ-irradiated *M. tuberculosis* (Fig. 17D). These data further support the notion that lymphatic vessels have an important role in regulation of cellular traffic from the periphery to LNs.

Endothelial cells can exhibit functions that are exhibited more typically by APCs, which include antigen uptake, processing and presentation to T cells (252-256). Therefore, we compared the LECs and DCs in their ability to take-up and process antigen using a model

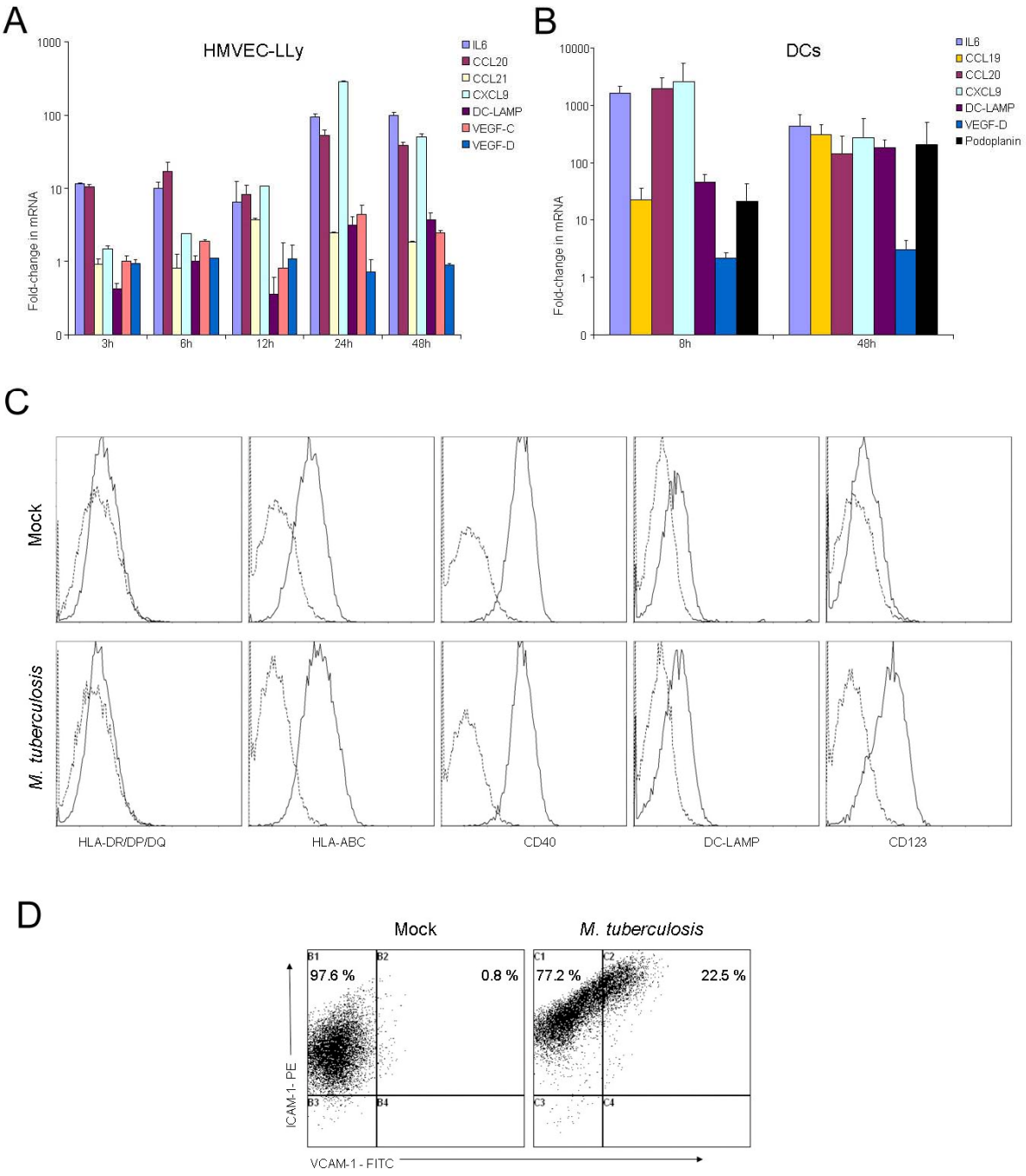

Figure 17. Phenotypic similarities between LECs and DCs. Confluent cultures of HMVEC-LLy (A) and monocyte-derived DCs (day 6, B) were either treated with γ-irradiated *M. tuberculosis* H37Rv (500 μg/ml) or medium alone (Mock). At the indicated time points total RNA was analyzed for the induction in mRNA of a panel of chemokine, cytokine and LEC marker genes (A and B). The data are presented as fold-change in mRNA levels between the treated and mock groups for each time point and were normalized to an endogenous control mRNA. Also after 48 hours, the expression of the indicated immune related markers (C) and ICAM-1/VCAM-1 (D) by HMVEC-LLys was analyzed by flow cytometry. Isotype controls are indicated by a dashed line (C) and the quadrants were set on the isotype control (D). Similar results were obtained with HMVEC-dLys and hTERT-HDLECs.

antigen DQ-Ovalbumin (DQ-Ova). We observed that both immature DCs and LECs were able to process antigen and that LECs were at least as efficient at processing this model antigen compared to immature DCs (Fig. 18). We also found that after treatment with γ-irradiated *M. tuberculosis*, the DCs greatly lost their ability to process antigen, whereas there was only a slight reduction in the LECs' abilities to process antigen. In contrast, HEK293 cells did not take-up and process this model antigen either before or after treatment with γ-irradiated *M. tuberculosis* suggesting that this phenomenon might be restricted to endothelial cells and APCs.

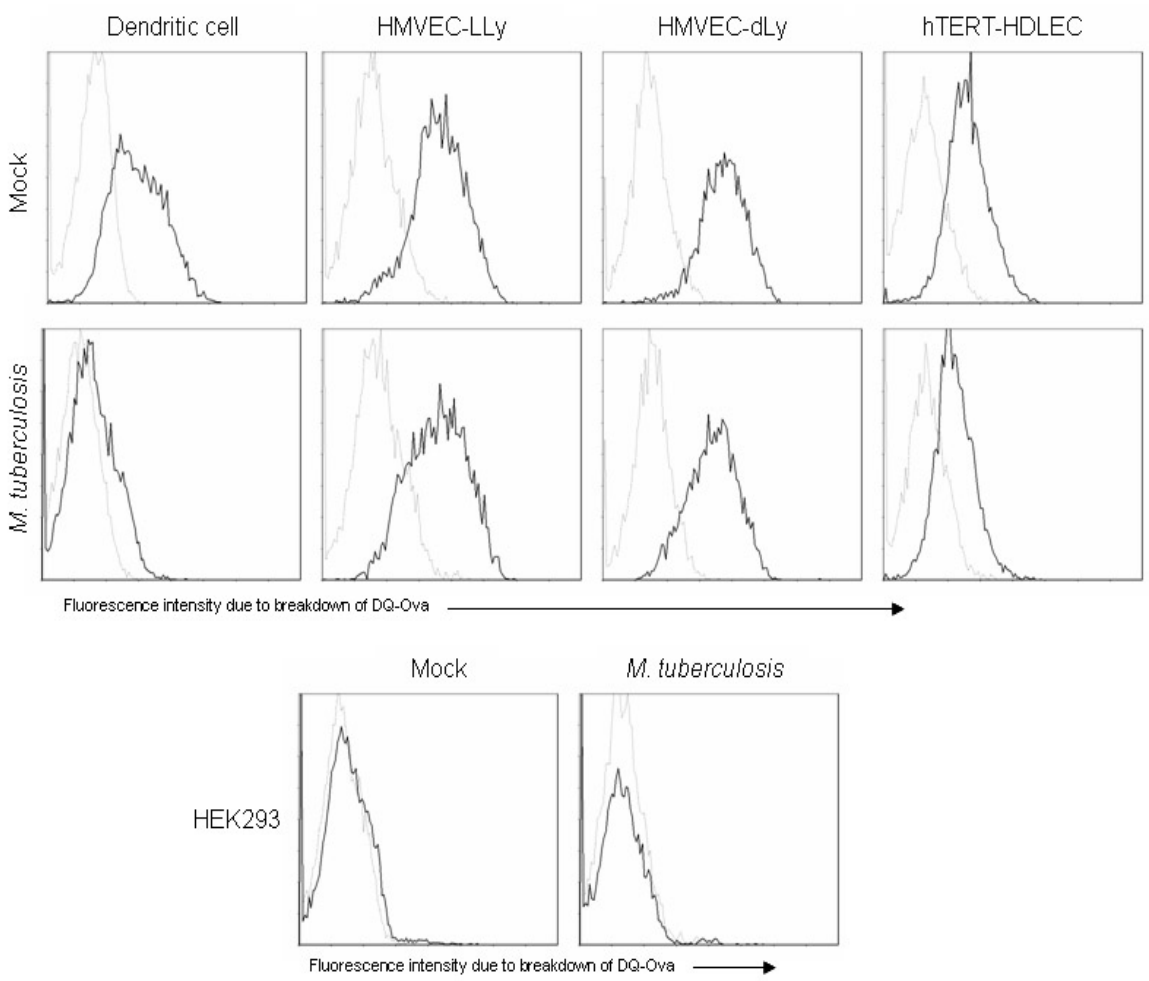

Figure 18. Antigen uptake and processing assay using DQ-Ova. Confluent cultures of HMVEC-LLy, HMVEC-dLy, hTERT-HDLECs and HEK293 alongwith monocyte-derived dendritic cells (day 6) were either treated with γ-irradiated *M. tuberculosis* H37Rv (500 μg/ml) or left alone. After 48 hours, the cells were incubated with DQ-Ova either at room temperature (black lines) or 4°C (grey lines) for 1 hr and fluorescence intensity in the FITC channel was measured by a flow cytometer. Representative histograms are shown for one experiment out of a total of two independent experiments.

The data presented here indicate that lymphatic vessels directly respond to *M. tuberculosis* by increasing production of inflammatory chemokines and cytokines that can recruit and affect immune cells, and that they also increase expression of adhesion molecules that are involved in the migration of these cells through these vessels. Therefore, lymphatic vessels have more than a structural role to play during infection and can impact the overall outcome of the immune response by regulating the cellular traffic from peripheral sites of infection to LNs.

5.6 DISCUSSION

In this study we have examined the local distributions of LECs and DCs in pulmonary granulomas resulting from intrabronchial infection of cynomolgus macaques with a low dose of virulent *M. tuberculosis*. We have also characterized the *in vitro* response of LECs to *M. tuberculosis* and found that LECs share phenotypic similarities with DCs in both the expression of common markers and in their response to *M. tuberculosis*.

Fascin$^+$ cells were abundant in the granulomatous lesions and most of these cells are likely DCs. Previous studies have shown that fascin is present in the dendritic portions and cell bodies of cultured Langerhans cells and the expression of fascin increased in concordance with the maturation of the DC (244,257). Fascin can also be expressed by fibroblasts (243) and neuronal cells (244). Although we cannot definitively state that these fascin$^+$ cells are DCs, the morphological structure of the majority of these cells is consistent with their being DCs and less so with the fibrous-like structure of fibroblasts encircling the granuloma. We also found that a minor population of fascin$^+$ cells localized to lymphatic vessels as revealed by their co-expression of fascin and lymphatic markers.

Previously, we have shown that DC-LAMP mRNA expression is present in cells lining the alveolar walls, consisting of approximately 10-15% of the total cells (245). We have also shown previously that DC-LAMP mRNA was abundant in lymphoid tissues and distributed distinctly from DC-SIGN mRNA signals (40). Other groups have recently shown that type II pneumocytes also express DC-LAMP in association with MHC class II molecules, suggesting a similar function in both cell types (246,247). In the macaque tissues examined here we found

that DC-LAMP co-localizes in cells also expressing surfactant B, which is a type II pneumocyte marker in the lungs. This suggests that the DC-LAMP mRNA we observed in macaque lung tissues and surrounding the granulomatous lesions are likely to be type II pneumocytes and not DCs. On the other hand, the expression of the mRNA for the costimulatory molecule B7-DC in granulomas suggests that mature DCs are present in these structures since B7-DC expression is thought to be restricted to activated or mature DCs (235). With the lack of DC-SIGN mRNA, a marker for immature DCs and relative abundance of B7DC mRNA in granulomas, we suggest that mature DCs are more abundantly present in granulomas than immature DCs.

Mathematical modeling of *M. tuberculosis* infection suggests that DCs are necessary for the development of protective immunity against *M. tuberculosis* (164) and are likely involved in granuloma formation. DC-recruiting chemokines have been observed in granulomas of giant cell arteritis and potentially are responsible for the sustained presence of DCs at the site of inflammation (258). We have observed that tuberculous granulomas have cells expressing CCL19 and CCL21 mRNAs and they also have numerous cells expressing mature DC markers like B7-DC and CCR7, the receptor for CCL19/21. These data suggest that CCR7 ligands are abundantly present in granulomas and are responsible for an increased presence of $CCR7^{+}$ mature DCs in these granulomas.

Lymphatic vessels produce CCL21, in part to recruit CCR7 expressing cells like mature DCs, which has been shown to be important for regulation of immune responses (3). We identified the cells expressing CCL21 mRNA in granulomas as lymphatic vessels since these cells also expressed the LEC markers podoplanin, VEGFR-3, LYVE-1, and Prox1. We also found that there was increased localization of lymphatic vessels that expressed CCL21 mRNA in these granulomas compared to normal lung tissue. These data suggest that there is a network of

lymphatic vessels present within granulomatous regions, which might be an outcome of the inflammatory conditions within these regions.

Increased proliferation of lymphatic vessels associated with granulomas has been observed in other chronic inflammatory diseases (161-163) suggesting that it is a phenomenon associated with chronic inflammation. The increased density of lymphatic vessels might aid in the trafficking of cells like DCs from granulomas to the draining LNs, which might be important for regulation of immune responses and antigenic surveillance of granulomas. It has also been suggested that inflammation-driven lymphatic growth might lead to development of dysfunctional lymphatic vessels, which could further contribute to and maintain the inflammation rather than help in its resolution (83). Therefore, lymphatic vessels in granulomas might be impaired in their ability to drain interstitial fluid and may not allow for migration of recruited DCs to draining LNs. This might explain the accumulation of fluid and retention of mature DCs that is observed within these granulomas. Further evaluation of the involvement of the lymphatic vessels in granulomas in trafficking of cells and drainage of interstitial fluid from granulomas will help in resolving this issue.

The presence of lymphatic vessels in granulomas coupled with the expression of VEGF-C mRNA in these granulomas suggest that the inflammatory conditions within granulomas leads to the development of a lymphangiogenic environment supporting the growth and proliferation of lymphatic vessels. Although we found that most of the lymphatic vessels in these granulomas did not express the proliferation marker Ki67, it could be possible that during the initial stages of granuloma formation there was a generation of newly formed lymphatic vessels within it and afterwards these vessels then lost their proliferative capacity.

The co-expression of fascin by LECs and DCs in granulomas indicated phenotypic similarities between these two cell types. Therefore, we further investigated the similarities between DCs and LECs using *in vitro* models for both cell types. The ability of both LECs and DCs to take-up and process antigen, and the increased expression of inflammatory molecules by both cell types in response to *M. tuberculosis* provided further evidence in support of their phenotypic similarities. DCs have been shown to express lymphatic markers like VEGFR3 (16,17) and differentiate into endothelial-like cells (259). The data presented here shows that LECs in turn could express DC-associated markers which suggest functional plasticity between these two cell lineages and therefore, a more detailed analysis of their functional properties and interactions with each other is needed.

Since endothelial cells have been previously shown to process and present antigen (252-256), the data presented here suggest that similarly LECs can process antigen and as they also express other immune related molecules on their surface, they may also be able to present antigen to relevant lymphocyte populations. Preliminary experiments indicate that the LECs are not as potent in activating T cells in mixed- leukocyte reactions (MLRs) as mature DCs (data not shown). Therefore, assessing the capability of LECs to present antigens in multiple model systems needs to be explored further.

Overall the data presented here argue for a more active role for the lymphatic endothelium in the inflammatory processes that occur during *M. tuberculosis* infections. The association of lymphatic vessels with granulomas suggests the involvement of these vessels in regulating cellular traffic from the granuloma and may be important for the regulation of the immune response to *M. tuberculosis*. These data also shed light on the interesting relationship between DCs and LECs by their expression of common phenotypic markers and may present

new avenues for the development of therapeutics that target DCs and require their migration through lymphatic vessels such as vaccines for cancer and other infectious diseases.

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6.0 FINAL DISCUSSION

The LE is an essential component of the mammalian vascular system. Its vital functions include drainage of interstitial fluid and trafficking of immune cells to secondary lymphoid organs. Due to the paucity of reliable lymphatic markers until recently, the study of the LE has been lagging behind that of the blood endothelium. In the recent past, the identification of a number of specific molecular markers for the LE has led to better characterization of the LE in tissues and cultures of relatively pure LEC cultures (2). From the perspective of the immune system, the LE has been studied in the development of multiple cancer types and some inflammatory diseases. In infectious diseases, the LE has generally been given a passing mention with its role as a conduit for DCs to migrate to LNs from sites of infection recognized but poorly studied. *Mycobacterium tuberculosis* and HIV-1 infections cause two major infectious diseases that have been declared as global pandemics and collectively cause more than 4 million deaths annually (152). There has been a growing knowledge about the pathogenic mechanisms of these inflammatory infections, although a complete understanding of their pathogenesis is still lacking. Therefore, the objective of this study was to determine the response of the LE to inflammation and define its role during pathogenic infections using nonhuman primate models of *M. tuberculosis* and SIV infections. We have used in vitro models of LECs to study their response to inflammatory mediators that included multiple TLR ligands and *M. tuberculosis* components. We have also used single-cell approaches to identify and characterize the LE networks in the lung and LN tissue compartments of normal and infected animals. In addition, we examined the

relationship between LECs and DCs by analyzing their expression of overlapping sets of phenotypic markers.

6.1 TOLL-LIKE RECEPTORS

We found that primary LECs express most known TLR molecules except for TLR7, TLR8 and TLR10. We also found that LECs are responsive to the ligands for most of these TLRs and respond by increasing expression of inflammatory molecules. The expression of TLRs by LECs has not been studied extensively and only a limited study on the expression of TLR2 and TLR4 by intestinal lymphatic vessels has been reported (104). We examined primary dermal and lung LECs for their TLR expression and found that overall they had similar expression profiles for the TLR molecules. The relatively high expression of TLR4 mRNA compared to the expression of TLR4 protein on the surface was a surprising result and may indicate greater stability for the mRNA compared to the protein for TLR4. In contrast TLR9 protein was expressed at the highest levels amongst all TLRs although its mRNA levels were lower than that of most TLRs. These data show that mRNA levels for TLRs do not necessarily correlate with the expressed protein levels suggesting different stabilities for mRNA and protein and may indicate different mechanisms for regulation of the expression of the TLR molecules.

We observed that the primary LECs were responsive to five out of a panel of nine known ligands for TLR1-9. The TLR3 ligand poly(I:C) induced increased expression of the maximum number of inflammatory genes amongst all the TLR ligands followed by the TLR4 and TLR2 ligands LPS and Pam3CSK4 respectively. These results indicate that LECs can recognize and respond to viral and bacterial components. We also found that the primary dermal LECs were

more responsive than the primary lung LECs which might be reflective of their anatomic origin since inflammatory responses will be more tolerated in skin than in lung. A surprising result we obtained was the non-responsiveness of the primary LECs to model TLR9 ligands despite their expression of TLR9 protein. This suggests that these LECs express a non-functional form of TLR9 similar to TLR9 expression by keratinocytes and myeloid DCs (185), and HMVECs (184). Amongst the other TLR ligands, the TLR6 ligand FSL1 induced responses in only the primary dermal LECs suggesting that TLR6 expressed by the primary lung LECs might be non-functional, whereas the TLR8 ligand ssRNA40 induced increased gene expression for only the CXCR3 ligands in primary LECs despite their lack of TLR8 expression indicating that maybe another PRR for single-stranded RNA like Retinoic acid inducible gene-I (RIG1) (172,186) may be involved in recognizing ssRNA40.

Since lymphatic vessels are conduits for DCs to migrate from the periphery to draining LNs, we also evaluated the effects of TLR ligands on the expression of DC recruiting chemokines CCL19, CCL20 and CCL21 by LECs. To our surprise we found that none of the TLR ligands induced increased expression of the CCR7 ligand CCL21 by the primary LECs and only the cytokine OSM led to increased expression of CCL21 by the LECs as previously shown with mixed endothelial cell populations (183). In contrast, we found that the CCR6 ligand CCL20 was induced upon stimulation with most TLR ligands suggesting that LECs could play an important role in the recruitment of $CCR6^+$ immature DCs to inflamed sites. These data are further supported by the increased expression of CCL20 by afferent LECs we earlier observed in inflamed LNs (Fig. 9C and E). Also the close juxtaposition of blood vessels with lymphatic vessels as observed in normal lung tissue (Fig. 14A) suggests that CCL20 secreted by LECs might be able to recruit $CCR6^+$ immature DCs from the systemic circulation, although in

granulomatous lung tissues we found that lymphatic vessels were not the major cell types that expressed CCL20 mRNA.

Apart from immature DCs, CCR6 is expressed by effector and memory T cells (188), a major proportion of regulatory T cells (189,190), and naïve and memory B cells (191). Thus CCL20 expressed by LECs could contribute to the trafficking of multiple cell types through the lymphatic endothelial network. In addition, the increased expression of other inflammatory chemokines including the CXCR3 ligands, CXCL8 and CCL5 by LECs after TLR engagement provides further evidence for the involvement of the lymphatic vessels in the regulation of trafficking of multiple cell types and for the recirculation of immune effector cells during inflammation. Inflammatory chemokines have been earlier shown to be produced at basal levels by LECs (194) and our data further expand on these findings by demonstrating the increased production of these chemokines by LECs on TLR engagement.

6.2 THE LYMPHATIC ENDOTHELIUM IN GRANULOMAS

We detected lymphatic vessels in pulmonary granulomas that formed during experimental infection with *M. tuberculosis*. These lymphatic vessels were initially identified by their expression of CCL21 mRNA and further confirmed by their expression of multiple lymphatic markers. These data suggest that there is an association of lymphatic vessels with granulomatous structures during tuberculosis. Furthermore, we observed that lymphatic vessels within pulmonary granulomas had higher expression levels for lymphatic marker mRNAs, particularly that of Prox1, than lymphatic vessels from normal lung tissues suggesting that these lymphatic vessels are newly formed within granulomas. The increased expression of lymphatic markers

has been correlated with the proliferation of new lymphatic vessels and is used as a marker for lymphangiogenesis. Therefore, the inflammatory conditions of granulomas might create a lymphangiogenic environment that leads to the growth and proliferation of new lymphatic vessels in these structures. It is also possible that pre-existing lymphatic vessels were entrapped in a developing granuloma and the inflammatory environment present in the granuloma led to increased expression of these lymphatic markers.

We found that there was minimal expression of the lymphangiogenic factor VEGF-C mRNA in pulmonary granulomas from *M. tuberculosis* infected animals and that its expression was mostly limited to fascin$^+$ cells rather than CD68$^+$ macrophages. We also observed that most of the lymphatic vessels within granulomas did not express the proliferation marker Ki67. Therefore it appears that ongoing lymphangiogenesis is not a prominent feature of the granulomas that we examined. Since the animals in these study all had active tuberculosis and some were in advanced stages of disease progression, lymphangiogenesis might have taken place during the initial stages of *M. tuberculosis* infection and development of disease. Also other lymphangiogenic factors such as VEGF-A/D, neurophilin-2 or angiopoietins might be present more abundantly than VEGF-C, which could contribute to the growth and proliferation of lymphatic vessels in granulomas. An intriguing possibility could be that due to the lack of lymphangiogenesis and increased lymphatic network in these granulomas, there was a deficiency in the drainage of interstitial fluid and migration of cells from granulomas which led to the accumulation of both fluid and cells in these structures. This might contribute to the pathogenesis of the infection and progression to active disease in these animals. In a model of airway inflammation, blocking lymphangiogenesis led to bronchial lymphedema and exaggerated airflow obstruction (130) which supports the importance of lymphangiogenesis for

the resolution of inflammatory responses in pulmonary compartments. Therefore, the analysis of the expression of lymphangiogenic factors and evaluation of lymphatic vessels for expression of proliferation markers in pulmonary granulomas at different stages particularly early on during *M. tuberculosis* infections will be essential for a better understanding of the role of lymphangiogenesis in development and maintenance of granulomas.

In other chronic inflammatory diseases, proliferating lymphatic vessels have been associated with granulomatous structures. Most notably in IBD, chronic inflammation and granulomatous structures were associated with increased density of lymphatic vessels (160-162). Therefore, our data provide further evidence for the association of lymphatic vessels with granulomas during chronic inflammatory diseases and suggest that these vessels might have important roles to play during the inflammatory process. Inflammation-driven lymphangiogenesis might lead to development of dysfunctional lymphatic vessels, which could further contribute to and maintain the inflammation rather than help in its resolution (83). Therefore, examining the functional characteristics of lymphatic vessels found in granulomas will be crucial to determining their role in the development and maintenance of the inflammation during infection.

These studies also indicate that LECs respond to multiple inflammatory mediators that include TLR ligands, cytokines and mycobacterial components by increasing expression of inflammatory chemokines and cytokines. These data indicate that the LE can directly interact with and respond to these inflammatory molecules and pathogenic components. Since granulomas form at sites of *M. tuberculosis* infection, they contain mycobacterial components that can directly interact with lymphatic vessels found in these granulomas. Therefore, these lymphatic vessels can respond to the mycobacterial components by increasing expression of

inflammatory cytokines and chemokines, thus contributing to the inflammatory environment of these structures. Increased production of inflammatory chemokines and cytokines will aid in the recruitment of immune cells to the site of infection and increased activation of cells present at these sites. Further analysis of the expression of inflammatory cytokines and chemokines by the lymphatic vessels in granulomatous tissues will help elucidate the contribution of the LE to the inflammatory process during *M. tuberculosis* infection.

Interestingly we found that a widely used lymphatic marker podoplanin was expressed by cell types other than LECs within these granulomas. In addition, we also observed an induction in the expression of podoplanin mRNA by monocyte-derived DCs *in vitro* on treatment with γ-irradiated *M. tuberculosis*. These data indicate that podoplanin can be expressed by non-LEC type cells that are most likely DCs or other monocytic cells in granulomas. DCs and other monocyte lineage cells have been shown to express lymphatic markers like VEGFR-3 and LYVE-1 under inflammatory conditions (16,17,260) which is consistent with our observations. Therefore, the expression of lymphatic markers by cells of the monocytic lineage suggests that these cells may act as a reservoir of LEC progenitor cells, which seeds the development of new lymphatic vessels in presence of lymphangiogenic factors at sites of infection like granulomas in the case of *M. tuberculosis* infection.

Overall we found that lymphatic vessels were found in granulomas that contained abundant mature DCs with limited numbers of immature DCs and I present a model for the organization of a granuloma based on our *in situ* findings (Fig. 19). These granulomas contained *M. tuberculosis* and *M. tuberculosis*-infected macrophages at their center with a surrounding ring of lymphocytes and macrophages. These immune cells secrete numerous inflammatory cytokines and chemokines (156), which leads to the establishment of an inflammatory

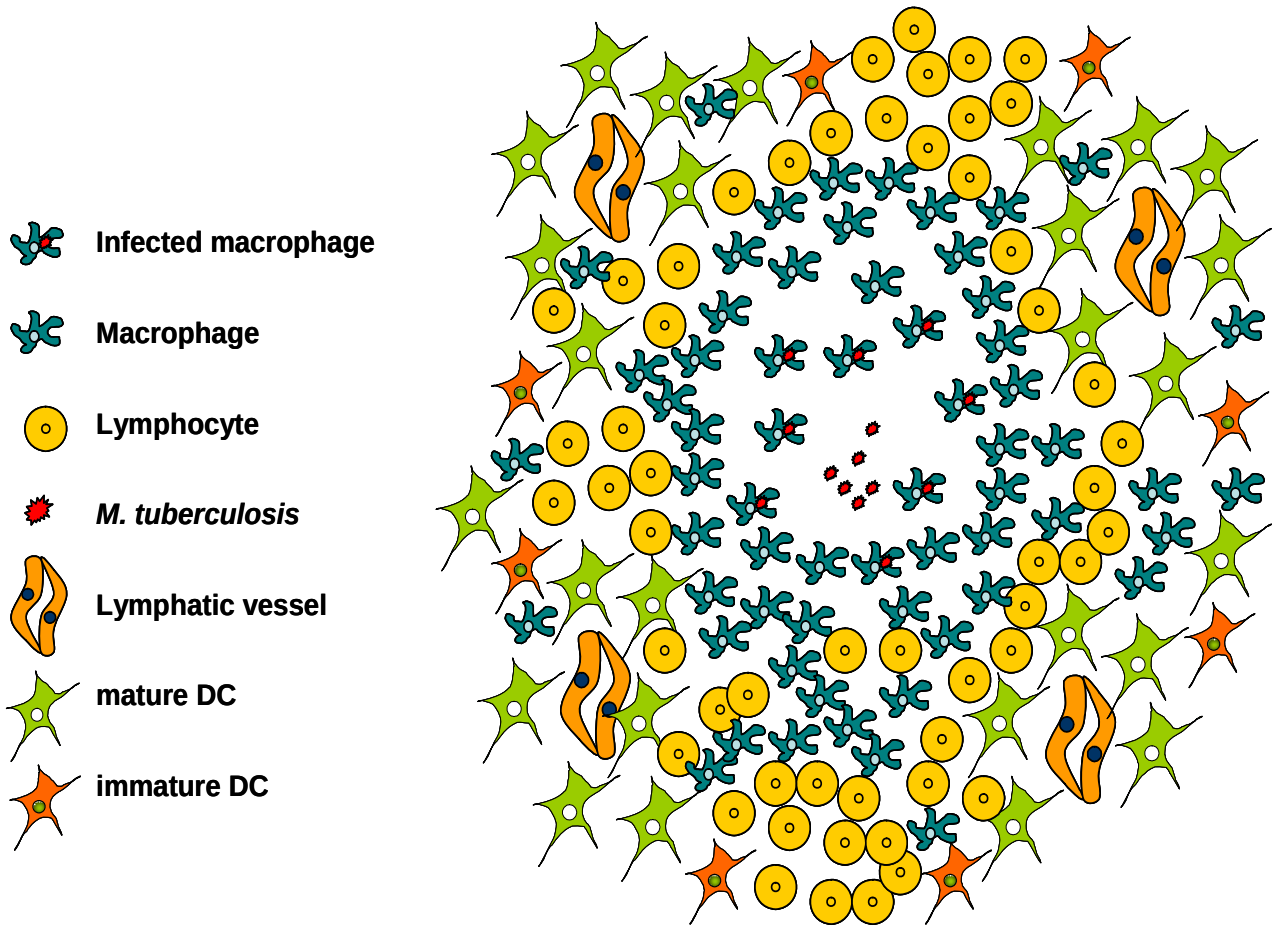

Figure 19. A schematic representation of a granuloma. *M. tuberculosis* and *M. tuberculosis*-infected macrophages are present in the center of granulomas, with a ring of lymphocytes and macrophages surrounding them. Lymphatic vessels are present on the outer ring of the granuloma and mature DCs are concentrated around these lymphatic vessels, whereas very few immature DCs are present in these granulomas.

environment within these granulomas. The inflammatory environment also results in production of lymphangiogenic factors like VEGF-C primarily by DCs that causes limited growth and proliferation of the lymphatic vessels in these granulomas. Since lymphatic vessels constitutively secrete the chemokine CCL21, an increased number of $CCR7^{+}$ mature DCs are recruited to these granulomas due to the presence of these lymphatic vessels in granulomas.

6.3 HETEROGENEITY OF LYMPHATIC ENDOTHELIAL CELLS

We have studied the lymphatic endothelial network of LNs and characterized their expression of multiple lymphatic markers and chemokines (summarized in Fig. 20). We found that the LECs in a LN are heterogeneous in their expression of the lymphatic marker LYVE-1 and the chemokines CCL20 and CCL21. Since the afferent and efferent lymphatics of a LN perform different functions in their localization and in the regulation of cellular traffic going into and out of LNs, our results further illustrate the differences amongst these two interfaces of the LNs. The expression of LYVE-1 mRNA almost exclusively by the LECs present at the efferent interfaces of LNs suggests that LYVE-1 may have a role to play in the egress of cells from LNs. Although studies in LYVE-1 deficient mice have shown no obvious abnormalities in terms of normal lymphatic function and only minimal functional alterations in lymphatic vessels were observed (29,30), there might be other compensatory molecules that can carry out its functions. Therefore, further characterization of the function of LYVE-1 in lymphatic vessels is needed.

The expression of the chemokine CCL20 exclusively by the LECs present at the afferent interface of LNs suggests that it is involved in the recruitment of $CCR6^+$ cells from the periphery. Furthermore we found increased expression of CCL20 in inflamed LNs by the same LECs at the afferent interface of LNs, which further allude to the responsiveness of LECs to inflammatory signals and their production of chemokines involved in the recruitment of inflammatory cells. These data demonstrate that the lymphatic network can play a more active role than earlier envisaged in the recruitment of immune cells and regulation of cellular traffic going into and out of LNs.

The lack of expression of CCL21 mRNA by LECs in LNs compared to its high

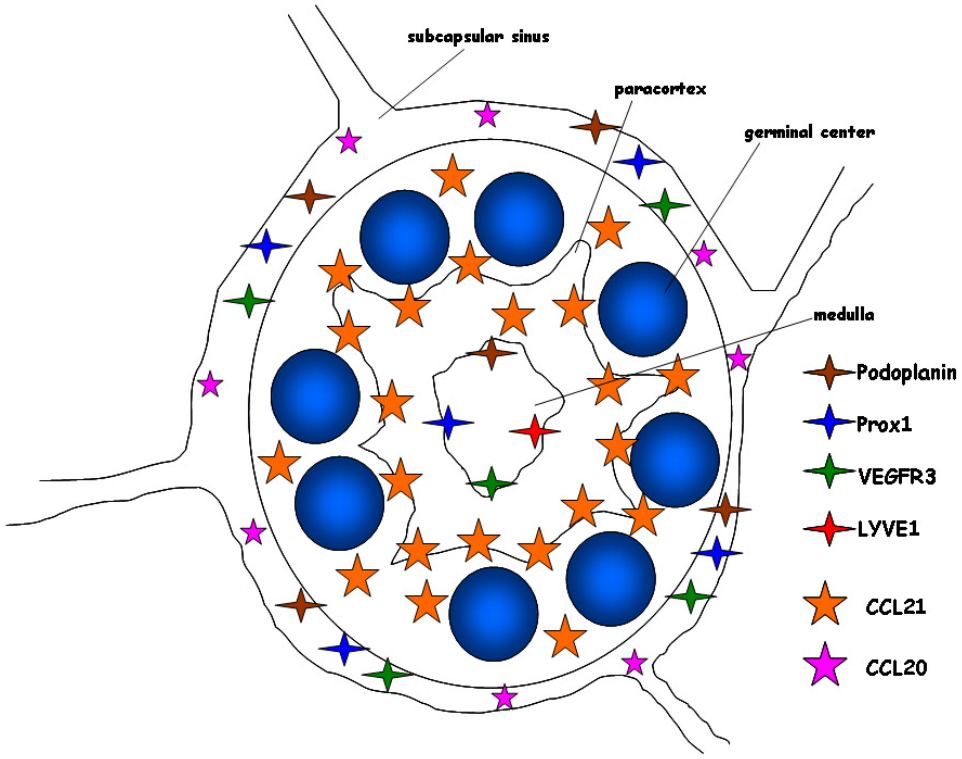

Figure 20. Expression of chemokines and lymphatic markers in a representative lymph node.

expression seen in LECs present in the lung reflects the heterogeneity in the LEC populations that make up the lymphatic vascular network. Combined with the selective expression of CCL20 by the afferent lymphatics and LYVE-1 by the efferent lymphatics in LNs, the heterogeneous characteristics of LECs are further highlighted. These data are in concordance with earlier reports that have also observed heterogeneity in the expression of lymphatic markers by LECs (7,214,215).

The heterogeneity observed in LEC populations may be a reflection of their different functional and anatomical origins. For example in LNs, the lymphatic endothelial network is present at both the entry and exit routes for cellular and fluid traffic. Therefore, LECs at the afferent interface will be involved in the recruitment of cells and drainage of fluid from the

peripheral tissues via the collecting vessels of the lymphatic network and as we have observed inflammation can affect the expression of chemokines like CCL20 by these LECs thereby ultimately affecting the recruitment of the cells from the periphery into the LNs. In contrast LECs at the efferent interfaces of the LNs are involved in the egress of cells from LNs. These efferent LECs did not express either CCL20 or CCL21 under normal and inflammatory circumstances suggesting that these LECs may have a more passive role in the regulation of cellular egress from LNs. Even in the lung tissue compartments we found that lymphatic vessels had variable expression of mRNAs for multiple lymphatic markers depending on the immune status of the lung. Lungs from *M. tuberculosis*-infected animals had lymphatic vessels concentrated in granulomas and these vessels had increased expression of mRNAs for multiple lymphatic markers suggesting that the inflammatory environment of granulomas has specific effects on the expression of these markers. We also found that lymphatic vessels in the lung did not express CCL20 under either normal or inflamed conditions in contrast to the increased expression of CCL20 observed in the afferent lymphatics of inflamed LNs. We did observe increased expression of CCL20 in pulmonary granulomas in comparison to normal lung tissues but it was expressed by cell types other than LECs. Overall these observations strengthen the argument for the prevalence of multiple LEC subtypes with different phenotypic properties depending on their anatomic localization and their responsiveness to environmental cues.

6.4 PHENOTYPIC SIMILARITIES WITH DENDRITIC CELLS

Another interesting outcome of this study has been the observation of phenotypic similarities between LECs and DCs. We found that LECs express DC-associated molecules and show some

similar functional characteristics as DCs (Fig. 16-18). In addition, both LECs and DCs respond to mycobacterial components by increasing expression of the same inflammatory molecules. These results highlight the intriguing possibility of functional plasticity between LECs and DCs. A growing body of literature has provided evidence of the expression of LEC markers by DCs and cells of the monocytic lineage under healthy and inflammatory conditions (16,17,260). In addition, monocytes have been shown to transdifferentiate into endothelial-like cells that have properties of LECs (129,259). Therefore, it appears that monocytes or DCs can serve as endothelial progenitor cells that can seed the formation of new lymphatic vessels at sites of inflammation and help in increased drainage of accumulated interstitial fluid and trafficking of recruited cells at these sites.

The expression of DC-associated molecules involved in immune recognition and activation by LECs (Fig. 17) suggests that the interactions between LECs and immune cells are not limited to those involved in migration but also could be involved in providing signals that could lead to either further activation or regulation of effector immune responses. We have shown that LECs are able to take up and process a model antigen much like a DC (Fig. 18) which presents the interesting possibility of LECs presenting antigens to immune cells. Previously endothelial cells have been shown to process and present antigens to immune cells (252-256) and our findings provide evidence that LECs might also be able to perform similar functions. Therefore, evaluation of the antigen processing and presentation capabilities of LECs using multiple antigenic models will help in understanding the interactions between LECs and immune cells.

The increased expression of inflammatory molecules like CCL20 and IL-6 by LECs and DCs in response to mycobacterial components illustrates the phenotypic similarities between the

two cell populations. The increased expression of the inflammatory chemokine and CCR6 ligand CCL20 by the treated DCs suggests that immature DCs that encounter mycobacterial components produce chemokines that can further recruit more $CCR6^+$ immature DCs to the site of infection. Similarly LECs also can produce CCL20 which will aid in the recruitment of immature DCs and other $CCR6^+$ immune cells to the site of infection. Besides CCL20, both LECs and DCs increased expression of the CXCR3 ligands CXCL9 and CXCL10 in response to mycobacterial components. Since these chemokines are known to recruit effector T cells, both LECs and DCs can contribute to the recruitment of effector cells to sites of infection. Overall both LECs and DCs produce chemokines that can recruit immune cells to sites of infection, which provides evidence for not only phenotypic similarities between LECs and DCs but also their contribution to the trafficking of immune cells during infection or inflammation.

6.5 ROLE IN INFECTION AND INFLAMMATION

The findings presented in this dissertation provide evidence in support of a role of the LE in infection and inflammation. In addition, phenotypic similarities between LECs and DCs were also observed. Therefore, we propose a model in which the role of LECs parallels that of DCs during infection or inflammation (Fig. 21). DCs have been widely studied and their role during infection and inflammation has been well established (52,261). In contrast, LECs have been minimally studied in infection and inflammation. Our findings suggest that during pathogenic infections, pathogens or their components can directly interact with LECs most probably through PRRs like TLR molecules. In turn, LECs respond by increasing expression of inflammatory cytokines and chemokines that can contribute to the overall inflammatory response. LECs might

also contribute to the control of infection by their production of the chemokine CCL20 and other such molecules, which have β-defensin–like antimicrobial properties (192,223). In addition, LECs can recruit $CCR6^+$ cells like immature DCs by their production of the CCR6 ligand CCL20 and lead to maturation of these immature DCs by their production of inflammatory cytokines. The DCs can also affect LECs and their function by secreting lymphangiogenic factors that will promote lymphatic vessel growth. Also, our findings indicate that DCs and other monocytic cells can express LEC markers during inflammatory conditions and that these cell types may infact act as progenitor cells for the seeding of new lymphatic vessels at sites of infection or inflammation. Overall our findings suggest a more active role for the LE during infection and inflammation than previously envisaged.

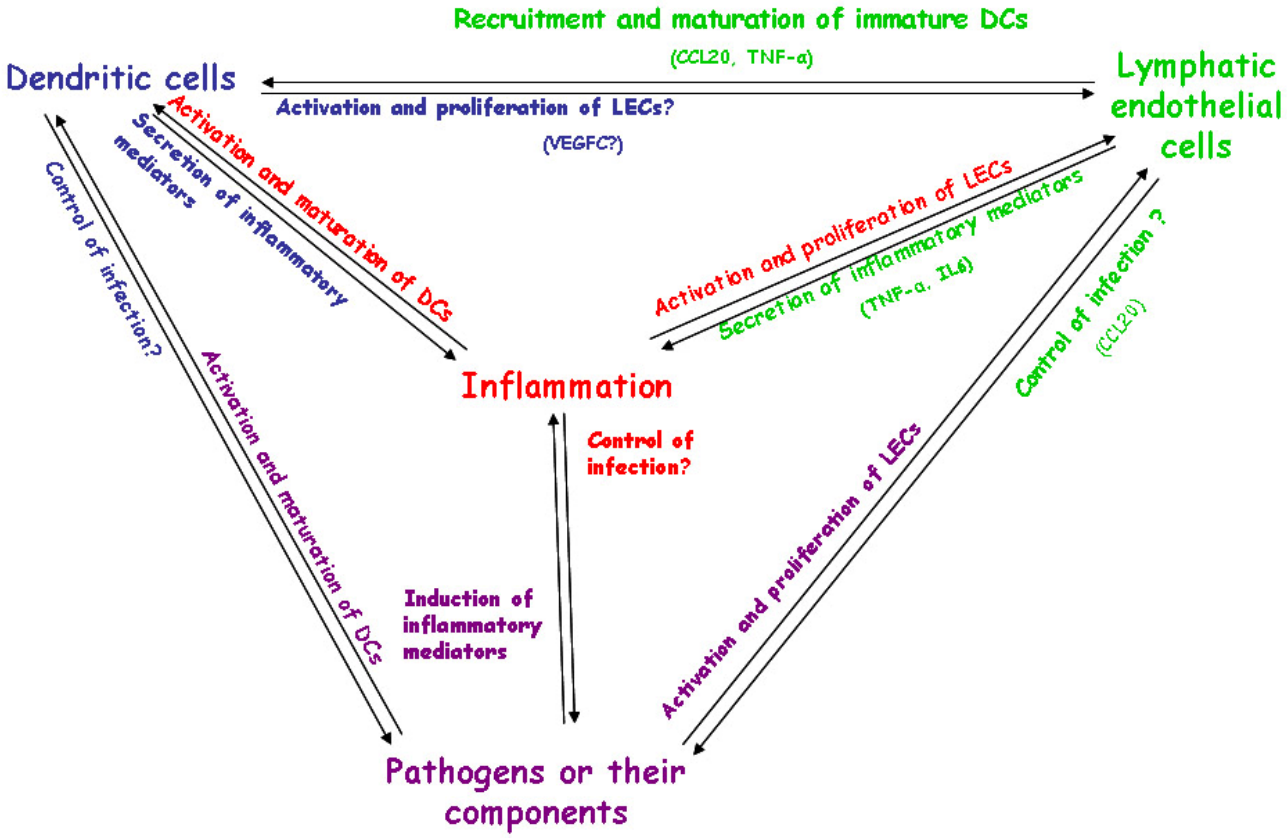

Figure 21. A schematic representation of the key interactions between LECs, DCs and infectious agents.

6.6 PUBLIC HEALTH SIGNIFICANCE

The LE is an essential component of the mammalian vascular system and is at the crucial interface between peripheral organs and secondary lymphoid organs. The lymphatic vascular network can affect the drainage of interstitial fluid from peripheral sites as well as regulate the cellular traffic from peripheral sites to draining LNs. Therefore, it has an important role to play during inflammation and vaccination by enabling the drainage of accumulated interstitial fluid at inflammatory sites and also in the delivery of antigenic material from sites of infection or vaccination. A thorough understanding of the regulation of these processes by the LE will aid in the development of better vaccine and immunotherapeutic strategies for cancer and infectious diseases.

Tuberculosis is a chronic inflammatory disease that affects more than one-thirds of the world population. Basic aspects of the development and pathogenesis of *M. tuberculosis* infection are known, although a comprehensive knowledge about the various players involved in the response to infection is still lacking. The association of lymphatic vessels with granulomas that form during *M. tuberculosis* infection presents a new avenue to study in terms of disease development and pathogenesis. Since lymphatic vessels are crucial for drainage of interstitial fluid and trafficking of cells, they could have considerable impact on the development, maintenance and resolution of granulomas. Our results provide insight into the potential role of LECs during *M. tuberculosis* infection and will aid in the further exploration of the importance of the lymphatic vascular system in tuberculosis. These could ultimately lead to development of novel therapeutics that targets the lymphatic endothelial network and its multifaceted functions.

6.7 SUMMARY

In summary, these studies have shown that there is an association of lymphatic vessels with granulomatous structures that form during *M. tuberculosis* infection and that LECs that make up these vessels might have a more active role in the development, maintenance and resolution of inflammatory processes during infection. In addition we have presented a study of heterogeneous expression of chemokines and lymphatic markers by the lymphatic endothelial network of LNs in health and infection. We have also presented for the first time a comprehensive study of the expression of all known TLRs by primary human LECs and assessed the responsiveness of LECs to a panel of TLR ligands. These studies taken together provide evidence for a more active role than previously appreciated for the lymphatic endothelium during inflammation and infection (Fig. 20). In conclusion, this body of work provides insight into the functioning of the lymphatic endothelium during health and disease, and presents exciting new opportunities to further explore the lymphatic vascular system.

7.0 FUTURE DIRECTIONS

This dissertation has expanded on the understanding of the lymphatic endothelium and its response to inflammation and infection in *in vivo* and *in vitro* systems. Through the course of these studies many pertinent questions have arisen that could not be addressed due to constraints of time. The following section lists some of the key areas that could be studied further, which would address some of the questions that have remained unanswered.

7.1 QUANTITATIVE AND QUALITATIVE ANALYSIS OF LYMPHATIC VESSELS PRESENT IN PULMONARY GRANULOMAS

I have investigated the presence of lymphatic vessels in granulomas arising during *M. tuberculosis* infection in a cohort of animals that were in the active stages of disease progression. Therefore, further study needs to be performed to evaluate the lymphatic vessels in newly formed or early granulomatous structures in animals in the early and latent stages of infection. Also a quantitative estimation of lymphatic vessel density in granulomas as has been used in many cancer models will aid in the defining the requirement of increased lymphatic endothelial network for resolution or progression of infection. Since it has been observed that chronic inflammation could lead to development of dysfunctional lymphatic vessels, assessing the normal functioning of the lymphatic vessels found in granulomas will be important.

7.2 EXPRESSION OF CHEMOKINES, CYTOKINES AND OTHER INFLAMMATORY MOLECULES BY LECS *IN VIVO*

The studies presented here have shown that LECs can express inflammatory mediators on stimulation with *M. tuberculosis* components and cytokines. All these studies have primarily been done using *in vitro* models with limited analysis of expression of inflammatory chemokines by LECs in tissues. Therefore, the examination of the expression of inflammatory modulators by lymphatic vessels present in tissues obtained from animals or individuals having infection or other forms of inflammatory diseases would be valuable in validating the results obtained using *in vitro* models for LECs.

7.3 EXPRESSION OF INNATE IMMUNE MOLECULES BY LECS *IN VIVO* AND *IN VITRO*

I have evaluated the expression of TLR molecules by LECs *in vitro*. Since TLRs represent one subset of a growing number of PRRs that have been identified and shown to be important mediators of immune responses, further study of the expression of other functional PRRs by LECs would help in understanding the importance of the LECs in sensing pathogens and modulating immune responses against them. The expression of TLRs by lymphatic vessels and assessment of their responsiveness to TLR ligands in an *in vivo* setting will help further expand on the findings in my studies. These would also aid in the development of better vaccine adjuvants that target TLRs and other PRRs.

7.4 EVALUATION OF INTERACTIONS BETWEEN LECS AND IMMUNE CELLS

I have shown here that LECs can recruit multiple cells types and also exhibit phenotypic characteristics of DCs. Further exploration of the phenotypic similarities between LECs and DCs in terms of antigen processing and presentation will help expand the understanding of this interesting phenomenon. LECs have been shown to form tubules in 3-D matrices of collagen. Therefore, study of LEC tubules and immune cells in these matrices will help recreate the potential interactions that take place between them *in vivo* and may provide insight into functioning of the lymphatic endothelium in regulating cellular traffic.

Overall this dissertation has led to some interesting questions and provides additional opportunities for the further study of the lymphatic endothelium in health and disease. These studies will help in expanding our knowledge about the lymphatic endothelium and its underappreciated role in the host response to pathogens and their components.

APPENDIX A

Table 3. ENDOTHELIUM SPECIFIC PARTIAL cDNAS.

Gene Name	Partial cDNA size (bp)	% homology to human sequence	Tissue from which it was cloned	Note
Pan endothelial marker				
CD31	606	95	Lung	PECAM-1, Adhesion molecule
Blood endothelial marker				
CD34	443	94	Lung	Ligand for CD62L (L-selectin)
vWF	550	97	Lung	Mediates platelet adhesion to injured endothelium
Lymphatic endothelial markers				
LYVE-1	530	94	Lung	Hyaluronan receptor
VEGFR-3	550	97	Lung	Receptor for VEGF-C/D
Podoplanin	560	90	Lymph node	Glomerular podocyte membrane protein
Prox-1	550	97	Lung	Homeobox gene product
Miscellaneous markers				
VEGF-C	636	98	Jejunum	Lymphatic growth factor
MR	638	97	Lymph node	Mannose receptor
Chemokine R D6	555	98	Lymph node	Chemokine scavenging receptor
Desmoplakin	629	95	Lymph node	Intercellular junction protein
Reelin	669	98	Brain	Involved in neuronal development
Plakoglobin	599	98	Lymph node	Involved in cell-cell adhesion

These cDNAs were cloned using gene-specific primers and total RNA from the indicated tissues obtained from healthy cynomolgus macaques. Gene-specific primers were designed using conserved regions amongst human and other known mammalian sequences for each gene. All sequence analysis were done using the Vector NTI software package (Invitrogen)

APPENDIX B

CHEMOKINE ARRAY EXPERIMENT

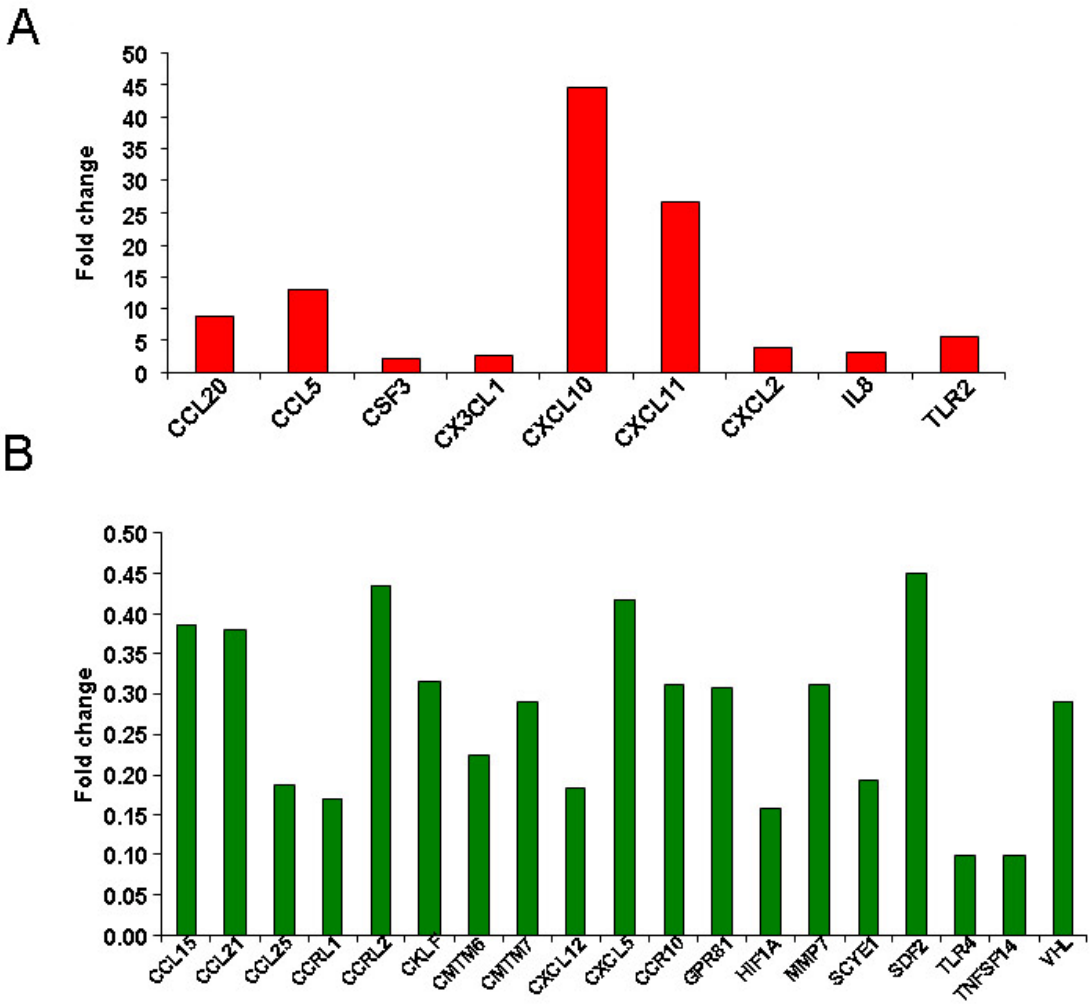

Figure 22. Changes in the expression of a panel of chemokine and their related mRNAs by *M. tuberculosis* treated HMVEC-LLys. (A) Upregulated genes (fold-change>2) and (B) down regulated genes (fold-change <0.5). HMVEC-LLys were either left in culture (mock) or treated with γ-irradiated *M. tuberculosis* for 24 hrs. Total RNAs were then extracted and hybridized to chemokine oligo GEarrays (SuperArray) using manufacturer's protocol. The oligo GEarrays were subsequently analyzed using the GEArray Expression Analysis Suite (SuperArray) and fold-changes were calculated using the mock as the control group.

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