Human Lymphatic Endothelial Cells Express Multiple Functional TLRs

Amarendra Pegu, Shulin Qin, Beth A. Fallert Junecko, Riccardo E. Nisato, Michael S. Pepper and Todd A. Reinhart

*J Immunol* 2008; 180:3399-3405; doi: 10.4049/jimmunol.180.5.3399

http://www.jimmunol.org/content/180/5/3399

---

**References**  This article cites 44 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/180/5/3399.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human Lymphatic Endothelial Cells Express Multiple Functional TLRs

Amarendra Pegu,* Shulin Qin,* Beth A. Fallert Junecko,* Riccardo E. Nisato,† Michael S. Pepper,‡ and Todd A. Reinhart2*

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 Ag-loaded DCs to lymphatic vessels and therefore are likely to regulate the migration of DCs to lymph nodes. TLRs are immune receptors that recognize pathogen associated molecular patterns 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 (HD) and lung LECs as well as in immortal HDLECs, which display a longer life-span than HDLECs. 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 Ag and environmental cues. The Journal of Immunology, 2008, 180: 3399–3405.

One mechanism by which DCs receive information about the peripheral environment and acquired Ags is via 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 (6, 7). To date, 13 mammalian TLRs have been identified, of which the expression of only TLR1–10 has been observed in humans (8). 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 costimulator 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 Ags of pathogens or cancer cells to the immune system to induce appropriate Ag-specific immune responses, and adjuvants have been used to increase the immunogenicity of Ags and the efficacy of vaccines (9, 10). 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 (11, 12). The contribution of TLR responsiveness in vaccine approaches has focused primarily on DCs, which expresses multiple TLRs (13, 14), 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 outcomes from infection or vaccination 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 (15). 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.

*Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261; and Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland
†Address correspondence and reprint requests to Dr. Todd A. Reinhart, Department of Infectious Diseases and Microbiology, Graduate School of Public Health, 606 Parran Hall, University of Pittsburgh, 130 DeSoto Street, Pittsburgh, PA 15261. E-mail address: reinhar@pitt.edu
‡Abbreviations used in this paper: LE, lymphatic endothelium; PAMP, pathogen-associated molecular patterns; HD, human dermal; OSM, Oncostatin M; ODN, oligodeoxynucleotides; HMVEC, human lung microvascular endothelial cell; iDC, immature DC.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

Received for publication June 25, 2007. Accepted for publication December 27, 2007.
(16). Understanding TLR expression and responsiveness profiles in LECs could potentially 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 insight into how these cells that line the conduit draining peripheral tissues might participate in host responses to infection and vaccination.

Materials and Methods

LEC cultures

Primary human dermal (HD) (human lung microvascular endothelial cell (HMVEC)-dLy) and lung (HMVEC-LLy) LECs (Cambrex) were cultured in EGM-2MV medium (Cambrex) according to the supplier’s suggestions. hertt-HDLECs were also cultured in EGM-2MV medium as previously described (17).

Stimulation of LECs with TLR agonists and cytokines

Agonists for TLR1–9 (TLR agonist kit, InvivoGen) and cytokines (PeproTech) were used to treat confluent monolayers of HMVEC-dLy, HMVEC-LLy, and hertt-HDLECs. The TLR agonists were: TLR1/2, Pam3CSK4 (1 μg/ml); TLR2, heat killed Listeria monocytogenes (HKLM, 10^7 cells/ml); TLR3, poly(I:C) (25 μg/ml); TLR4, Escherichia coli K12 LPS (100 ng/ml); TLR5, Salmonella typhimurium flagellin (1 mg/ml); TLR6/2, FSL1 (1 μg/ml); TLR7, imiquimod (2.5 μg/ml); TLR8, ssRNA40 (2.5 μg/ml); and TLR9, oligodeoxynucleotide 2216 (ODN2216), ODN2006, or ODNM362 (10 μg/ml). The cytokines used were IL-1β (10 ng/ml), TNF-α (1 ng/ml), and Oncostatin M (OSM, 100 ng/ml). After 24 h of treatment, culture supernatants were cryopreserved and the cells were lysed with Trizol (Invitrogen Life Technologies) to isolate total RNA.

Measurement of TLR and LEC marker 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, podoplanin, LYVE-1, Prox-1, and VEGFR3 (Applied Biosystems) on an ABI Prism 7000 sequence detection system (Applied Biosystems) as described (18). 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 detached from culture flasks by trypsinization (trypsin/EDTA; Invitrogen Life Technologies) for 3–4 min, immediately washed with RPMI 1640 medium with 10% FBS, and then washed with PBS. Detached cells were stained with either PE-conjugated Abs or a panel of PE-conjugated mAbs (BD Pharmingen) against cell surface CD31 (PE-conjugated clone WM59; BD Pharmingen) and pericellular CD301 (PE-conjugated clone NZ-1; AngioBio). Stained cells were analyzed using an XL flow cytometer (Beckman Coulter). Flow cytometry was used next to measure TLR protein levels on cell surfaces and in intracellular compartments of all three cell types (Fig. 1D), 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 hertt-HDLECs, and that the surface expression levels for TLR3 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 with 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. At sites of inflammation, the engagement of TLRs by their ligands leads to signal transduction events which activate transcription of inflammatory genes (8), including chemokines.

Chemotaxis

The chemotactic responses of L1.2 murine pre-B cells engineered to express CXCR3 were measured as described (19). Chemotaxis toward 31 μl of culture supernatants from LEC populations treated with TLR ligands or cytokines was measured by counting the numbers of migrating cells on a hemacytometer after 3 h of migration.

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, hertt-HDLECs, which are dermal in origin and display a longer life-span due to the ectopic expression of human telomerase reverse transcriptase (17). All three cell populations expressed mRNAs for the lymphatic markers podoplanin, LYVE-1, Prox1, and VEGFR3 as measured by real-time RT-PCR (Fig. 1A). In addition, they expressed the lymphatic marker podoplanin along with the pan-endothelial marker CD31 on their surfaces as detected by flow cytometry (Fig. 1B). These findings, therefore, provided 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. 1C). Considering all three cell populations together, TLR1–3 and TLR6 mRNAs were expressed to similar levels, whereas TLR5 and TLR9 mRNAs were ~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 hertt-HDLECs relative to the primary dermal LECs and TLR6 mRNA ~10-fold lower in the primary dermal LECs. Overall, however, TLR mRNA expression profiles were highly similar among 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. 1D), 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 hertt-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 with their surface expression on all three LEC types.

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. At sites of inflammation, the engagement of TLRs by their ligands leads to signal transduction events which activate transcription of inflammatory genes (8), including chemokines.
The findings from this comprehensive set of studies are shown collectively in Fig. 2. We found that 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. 2A). The primary dermal LECs responded to the largest number of TLR ligands and also exhibited a higher induction of inflammatory chemokine mRNA expression. Among 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 most potent inducer followed by the TLR8 ligand ssRNA40 and then the TLR4 ligand LPS (Fig. 2A). 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 (20), 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 (4). Therefore, we examined whether TLR ligands affect the expression of DC-recruiting CCR6 and CCR7 ligands by LECs (Fig. 2A). 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 (21), 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. 2A).

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, and
FIGURE 2. Induction of mRNAs encoding chemokines, cytokines, and adhesion molecules in LECs by TLR ligands and cytokines. Confluent monolayers of HMVEC-dLy (De), HMVEC-LLy (Lu), and hert-HDLECs (hT) cells were treated with TLR ligands (A and B) or cytokines (C) for 24 h and total RNA from the cells was analyzed for the induction of mRNAs for chemokines (A and C), cytokines (B), and adhesion molecules (B and C) by real-time RT-PCR. The relative fold-change for each gene is indicated by the shade of gray according to the map in the lower right. The asterisk indicates that the mRNA for CCL21 was not detected by real-time RT-PCR in total RNA from hert-HDLECs (threshold cycle values > 45). These data were compiled from separate experiments performed with duplicate cultures and with duplicate real-time RT-PCR performed on each total RNA preparation.

All three LEC populations secreted nearly undetectable levels of CCL20 and CXCL10 secreted by the LEC populations after treatment with TLR ligands (Fig. 3A, and B). We found that the levels of CCL20 generally paralleled the induction of its mRNA as observed by real-time RT-PCR (Fig. 3A). 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 hert-HDLECs. Similar to the induction of CXCL20 mRNA observed in mainly the primary dermal LECs by the TLR2/6 ligand FSL1, we found high levels of CXCL20 secreted only by the primary dermal LECs and the induction in its mRNA levels in all three cell types which was consistent with the levels of induction of its mRNA. Similarly, only supernatants from poly(I:C)-treated LECs led to strong chemotactic responses by cells engineered to express CXCR3, the receptor for CXCL10, which was consistent with the levels of induction of its mRNA.

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

TLR stimulation can also induce expression of inflammatory cytokines (6). 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. 2B). As with chemokines, the levels of induction of these cytokines were highest in the primary dermal LECs compared with 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. 3C). 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 (22, 23). These molecules could regulate the trafficking of immune cells into and through the lymphatics during inflammation. In all three 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. 2B). Although the primary lung LECs were generally less prone to express inflammatory molecules upon TLR stimulation, they universally up-regulated 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. 4). 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. 2B). 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 (22, 23). In addition, injection of TNF-α into skin can lead to increased expression of CCL21 by lymphatic vessels (16), although the mechanism of this induction is a matter of debate since in a later report, a pleiotropic cytokine, OSM, and not TNF-α, directly enhanced expression of CCL21 by endothelial cells (24). 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, 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. 2C and 3). 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 (22, 23).

**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 (25) and suggest that there are defects in ligand uptake or postbinding 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 (26, 27); gram-negative bacteria through TLR4 (28, 29); mycobacteria through TLR2/TLR6 heterodimers (30); and viral replication intermediates through TLR3 (31). The lack of responsiveness of dermal and lung LECs to TLR5 ligand might be related to the expression of flagellin by a primordial motile, enteric bacteria (32), 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, because 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 nonfunctional TLR9 protein, similar to TLR9 expression in some instances by keratinocytes, myeloid DCs (33), and HMVECs (25). 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.

Afferent lymphatics are a conduit for the migration of Ag-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, because mature DCs express high levels of CCR7 (4). Treatment of skin with inflammatory cytokines can lead to increased production of CCL21, which in turn enhances migration of mature DCs to draining LNs (16), although the exact mechanism of this induction is not clear (24). 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 (24). 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, as has been suggested previously (34). Additionally, in LNs, CCL21 is expressed mainly by cells other than LECs (21).

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 Ag, mature through perhaps the same signals that were received by the LECs or alternatively produced by the LECs, and carry Ag 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 Ag-loaded DCs across the endothelial lining (35). The CCL20/CCLR6 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 (21). Previous findings suggest that CCL20 is involved in recruitment of CCR6+ DCs into LNs (36). Apart from iDCs, CCR6 is also present on effector and memory T cells (37), a major proportion of regulatory T cells (38, 39), and naive and memory B cells (40). 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 (41, 42).

TLR ligands induced expression by LECs of additional inflammatory chemokines including CXCL9–11, 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 (43) 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 (44). 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 among 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 up-regulating 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.

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 Ags 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.

Acknowledgments

We thank Dr. Tianyi Wang for helpful discussions and critical reading of the manuscript. Dr. David Finogeal for helpful discussions, Dr. James Pease for advice and assistance with the generation of the L1.2 cell line, and Dr. Joanne Flynn for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References