Lymphatic endothelial cells – key players in regulation of tolerance and immunity

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The lymphatic vasculature provides routes for dendritic cell and lymphocyte migration into and out of lymph nodes. Lymphatic endothelial cells (LEC) control these processes by expression of CCL21, sphingosine-1-phosphate, and adhesion molecules. LEC express MHC-I and MHC-II, but not costimulatory molecules, and present antigen on MHC-II via both direct and cross-presentation. Whether LEC present to CD4+ T cells on MHC-II is unknown. Interestingly, LEC express antigens otherwise restricted to a small number of peripheral tissues in an autoimmune regulatory element-independent manner. Direct presentation of peripheral tissue antigens (PTA) to CD8+ T cells results in abortive proliferation and deletion, due to both a lack of costimulation and active PD-1 engagement. Autoimmunity develops when deletion is subverted, suggesting that LEC presentation of PTA could lead to human disease if PD-1 signaling were impaired by genetic polymorphisms, or aberrant costimulation occurred during inflammation. The expression of additional inhibitory molecules, which are not involved in LEC-mediated deletion, suggests that LEC may have additional immunoregulatory roles. LEC express receptors for several immunomodulatory molecules whose engagement alters their phenotype and function. In this review we describe the role of LEC in distinct anatomical locations in controlling immune cell trafficking, as well as their emerging role in the regulation of T cell tolerance and immunity.

Keywords: lymphatic endothelial cells, tolerance, trafficking, inflammation, antigen presentation

**LYMPHATIC ENDOTHELIAL CELLS REGULATE THE TRAFFICKING OF DENDRITIC CELLS AND LYMPHOCYTES BETWEEN TISSUES AND SECONDARY LYMPHOID ORGANS**

Lymphatic endothelial cells (LEC) compose the lymphatic vasculature, which maintains tissue fluid balance and transports antigen and dendritic cells (DC) to the lymph node (LN). Lymphatic vasculature in tissues is composed of blind-ended capillary-like structures, termed initial lymphatics (Leak, 1976), which join to form larger collecting lymphatic vessels (Schmid-Schönbein, 1990) and ultimately feed into the LN subcapsular sinus. Within the LN, LEC are localized to the subcapsular, cortical, and medullary sinuses, where they interact with incoming and exiting leukocytes (Grigorova et al., 2010).Whereas the blood vasculature in peripheral tissues attracts leukocytes to inflamed sites to exert effector functions, the lymphatic vasculature facilitates the induction of immunity and tolerance. DC enter the initial lymphatics through portals in the basement membrane (Lämmermann et al., 2008), in tight clusters on the abluminal surface (Vasileva et al., 1999, Tal et al., 2011). CCL21-Leu is the primary determinant for DC entry through engagement of CCR7, but it is not expressed by LEC in LN and does not mediate migration to the node itself (Vasileva et al., 1999, Luther et al., 2008, Nakano and Gunn, 2001). Humans express a single CCL21 isoform, which encompasses the functions of both murine isoforms. LEC that form dermal lymphatics also express CXCL12, which mediates DC entry via CXCR4 (Kobusch et al., 2007).

Extravasation of lymphocytes from blood vasculature is highly integrin-dependent; however, the requirement for integrin-mediated entry into the initial lymphatics is controversial. Although LEC in the initial lymphatics express ICAM-1, and engagement of immobilized CCL21 promotes DC integrin activation and adhesion to ICAM-1 in vitro (Schumann et al., 2010), steady-state migration of DC into LN in vivo does not require integrin engagement (Lämmermann et al., 2008). This suggested other adhesion molecules may be involved. Recently, it was discovered that DC migration into lymphatic vessels and into the LN requires CLEC-2 binding to podoplanin, a glycoprotein expressed by lymphatic vessel and LN-LEC as well as fibroblastic reticular cells (FRC; Acton et al., 2012). Other potential candidates include the scavenger receptor CLEVER-1, which has been implicated in the transmigration of T cells into the lumen of initial lymphatic vessels (Salini et al., 2004). Thus, LEC-mediated entry into the afferent lymphatics is distinct from blood vascular endothelium-mediated entry of leukocytes into tissues.

Lymphatic endothelial cells also mediate the migration of DC into the LN. Once inside the collecting vessels, DC, and presumably T cells, detach from LEC and rhythmic vessel contractions propel DC toward the LN (Randolph et al., 2005). LN-LEC as well as FRC make CCL19 and CCL21-Ser, which mediate direct entry...
into the LN (Vassilieva et al., 1999; Luther et al., 2000; Nakano and Gunn, 2001). It has been hypothesized that LEC in the collecting lymphatics also make these chemokines (Randolph et al., 2005). Additionally, LEC in the subcapsular sinus express CCL1, which can facilitate cell entry into LN (Qu et al., 2004; Kabashima et al., 2007). Once in the subcapsular sinus, DC enter the LN cortex immediately, while T cells enter the LN paracortex via medullary lymphatic sinuses (Beaun et al., 2011). It is unclear how these different routes of entry are regulated. We have found that medullary and subcapsular LEC differentially express Mac-1 integrins (unpublished). These results suggest that cellular trafficking into and through the lymphatics is based on anatomically and molecularly distinct subpopulations of LEC that have different functional properties.

Lymphatic endothelial cells also control egress of lymphocytes from the LN. Upon LN entry, lymphocytes downregulate CCR7 and exit the LN through cortical and/or medullary lymphatic sinuses (Braun et al., 2011). It is unclear how these different routes of entry are regulated. We have found that medullary and subcapsular LEC differentially express Mac-1 integrins (unpublished). These results suggest that cellular trafficking into and through the lymphatics is based on anatomically and molecularly distinct subpopulations of LEC that have different functional properties.

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analogueous to that of mTEC in the thymus in promoting systemic tolerance. The molecular mechanisms controlling PTA expression by LEC have not yet been established. PTA expression by LEC is not dependent on the Aire, which controls PTA in mTEC and eTAC (Anderson et al., 2002; Gardner et al., 2008; Cohen et al., 2010). One LEC-expressed PTA, Ppy, is regulated by Deaf-1, a member of the SAND transcription factor family that includes Sp100, Aire, and NucP4175 (Yip et al., 2009). However, Deaf-1 has not been shown to regulate other LEC-expressed PTA. Deaf-1 and other SAND family members are expressed at comparable levels in all LNSC subsets (Fletcher et al., 2010; unpublished), so it is unclear how Deaf-1 would regulate the expression of non-overlapping PTA in different LNSC populations. However, it is also unknown how Aire controls distinct PTA repertoires in mTEC and eTAC. It is possible that the control of non-overlapping PTA repertoires in different cells by the same transcriptional regulator is due to differences in chromosomal positioning and/or epigenetic modifications. Another possibility is that multiple transcription factors play a role in LNSC PTA expression.

**CONSEQUENCES OF CDS ANTIGEN PRESENTATION BY LEC**

As mentioned above, despite sharing some characteristics with professional APC, antigen presentation by LEC leads to tolerance. Direct presentation of tyrosinase by LN-LEC induces abortive proliferation and deletion of tyrosinase-specific T cells in vivo (Nichols et al., 2007; Cohen et al., 2010; Figure 1). Utilizing β-gal driven under control of the LEC specific Lyve-1 promoter, LEC also induce abortive proliferation and deletion of β-gal specific CD8 T cells (unpublished). Presentation of exogenous antigen by LEC was also shown to induce CD8 apoptosis in vitro (Land et al., 2012). In other models, antigen level determines whether CD8 T cells undergo anergy or deletion (Redmond et al., 2005). It remains to be clarified whether LEC can induce outcomes other than deletion.

We have recently elucidated the mechanism by which LEC induce abortive proliferation and deletion of PTA-specific CD8 T cells (Tewalt et al., 2012). LEC-mediated deletion requires both a lack of costimulation and signaling through the PD-L1-PD-1 pathway (Figure 1). Lack of costimulation leads to rapid and elevated expression of PD-1 on T cells. Signaling through PD-1 blocks upregulation of IL-2R, which is at least in part responsible for apoptotic death (Tewalt et al., 2012). PD-1 signaling had previously only been associated with downregulation of IL-2 itself (Carter et al., 2002; Chikuma et al., 2009). These results integrate previous demonstrations that tolerance is due either to a lack of costimulation (Harding et al., 1992; Hawiger et al., 2001; Hernandez et al., 2002) or to engagement of inhibitory molecules (Martin-Orozco et al., 2006; Nurieva et al., 2006; Goldberg et al., 2007; Tsuchima et al., 2007; Liu et al., 2009; Reynoso et al., 2009), and shows that they are actually interdependent pathways. Importantly, antigen presentation by LEC leads to the development of autoimmune disease when PD-L1 is blocked or exogenous costimulation is provided (Tewalt et al., 2012). Based on previous findings that LEC express multiple PTA (Cohen et al., 2010; Fletcher et al., 2010), this opens the possibility that dysregulation of their tolerance inducing capability might influence the development of some human autoimmune diseases. Finally, LEC express ligands for additional inhibitory pathways, including HVEM:BTLA/CD160, HLA-IL2:LAG-3, and CD48:2B4. These pathways are known to induce additional forms of tolerance, including anergy and Treg formation (Huang et al., 2004; Grosio et al., 2007; Liu et al., 2009). They are not involved in LEC-mediated abortive proliferation and deletion, but their expression suggests that LEC may have additional immunoregulatory roles under steady-state conditions.

We have also investigated the anatomical basis of CDS abortive proliferation and deletion. LN-LEC express higher levels of PD-L1 than other LNSC populations or tissue lymphatic LEC (Tewalt et al., 2012; unpublished). The low level of PD-L1 and PTA expression by tissue LEC suggests that they are unlikely to induce tolerance. In addition, medullary and subcapsular sinus LEC express higher levels of PD-L1 than those in the cortical sinus.

**Figure 1** Peripheral tolerance induction by anatomically distinct subsets of lymphatic endothelial cells during T cell trafficking through lymph nodes. Naive T cells enter the LN through high endothelial venules, and exit via cortical and/or medullary sinuses. Tyrosinase presentation occurs on medullary but not cortical sinus LEC, leading to proliferation and PD-L1 mediated deletion of tyrosinase-specific T cells. Deletion may occur based on engagement of PD-L1+ medullary sinus LEC in the same LN as activation occurs, and/or on PD-L1+ subcapsular sinus LEC in downstream LN.
Importantly, tyrosinase epitope presentation to CD8 T cells is confined to medullary sinus LEC, consistent with their higher expression of tyrosinase message (unpublished; Figure 1). This suggests that abortive proliferation and deletion occur as T cells attempt to exit the LN. Whether the lower level of PD-L1 expressed by cortical sinus LEC is also capable of inducing tolerance to antigens expressed at a higher level than that of tyrosinase remains to be examined. However, the high-level expression of PD-L1 by medullary sinus LEC suggests they may also induce the deletion of egressing T cells activated by other LN-resident tolerogenic APC that express low levels of PD-L1, such as FRC.

**OTHER FACTETS OF IMMUNE CROSS-TALK BETWEEN LEC AND LEUKOCYTES**

Lymphatic endothelial cells express multiple TLR, as well as receptors for inflammatory cytokines (Link et al., 2007; Pego et al., 2008; Kataru et al., 2011), which enable them to respond to changes in tissue and LN microenvironments. Stimulation of cultured tissue or LN-LEC with TLR agonists, TNFα, IL-1, or infection with cytomegalovirus induces the expression of numerous chemokines (Pego et al., 2008; Sawa et al., 2008a,b; Fiorentini et al., 2011; Garrafa et al., 2011), but the role of this enhanced expression in vivo has not been established. In contrast to the steady-state, DC entry into LN under inflammatory conditions is dependent upon ICAM-1 and VCAM-1, which are also upregulated on LEC by proinflammatory agents (Johnson et al., 2006; Pego et al., 2008; Sawa et al., 2008a,b; Fiorentini et al., 2011; Garrafa et al., 2011). Inflammation also leads to proliferation and sprouting of LEC, a process known as lymphangiogenesis, by inducing the production of ligands for VEGfR2, VEGfR3, and the lymphotactin β receptor (LiP; Angeli et al., 2006; Furtado et al., 2007; Kim et al., 2009; Fuster et al., 2010; Mounzer et al., 2010). Lymphangiogenesis may also provide skin inflammation aids in the resolution of inflammation by increasing lymph flow and cell migration to the draining LN, but lymphangiogenesis following peritoneal inflammation reduces lymphatic drainage (Kataru et al., 2009; Kim et al., 2009). LN lymphangiogenesis has been shown to promote lymphocyte egress during prolonged inflammation (Tan et al., 2012). This suggests that one of the primary functions of LEC exposed to inflammatory agents is to attract a range of innate and adaptive immune cells into lymphatics to broaden and sustain ongoing immune responses.

In addition to enhancing leukocyte migration during inflammation, LEC attenuate T cell responses. TNFα activated LEC downregulate CD86 on DC, impairing their ability to induce T cell proliferation (Podgrabińska et al., 2009). LEC also limit T cell proliferation (Khan et al., 2011; Lukacs-Kornek et al., 2011) through release of NO in response to IFNγ and TNFα (Lukacs-Kornek et al., 2011). However, T cells undergoing LEC-mediated abortive proliferation and deletion produce little to no IFNγ and TNFα (unpublished). Thus, NO is unlikely to participate in LEC-mediated peripheral tolerance, but may limit the size of an immune response. Cortical sinus LEC, which express an intermediate level of PD-L1, upregulate PD-L1 in response to TLR3 ligation and IFNγ to match the high levels seen on medullary and subcapsular sinus LEC (unpublished). This could broaden the anatomical locations in the LN in which T cell tolerance occurs, or provide a means to protect cortical sinus LEC from being destroyed by emigrating effector T cells.

**CONCLUDING REMARKS**

Recent work has conclusively demonstrated that LEC play a variety of active roles in shaping immune responses and tolerance. LEC guide lymphocyte and DC trafficking into and out of the LN, and inflammation increases their ability to attract cells. LEC also actively enforce CD8 T cell tolerance to PE through their high-level expression of PD-L1 and lack of costimulatory molecules. It will be immensely interesting to determine the ways in which other inhibitory molecules expressed by LEC control T cell fate. In addition, the general immunoregulatory role of LEC will be more definitively established by understanding their ability to directly induce CD4 tolerance or to serve as a reservoir of PTA for presentation by DC. Furthermore, the identification of a second transcriptional control mechanism, in addition to Aire, will provide the possibility to understand the basis for additional human autoimmune diseases. Finally, LEC represent attractive therapeutic targets to control autoimmunity and prevent transplant rejection or to enhance tumor immunotherapy.

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