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Pathogen interactions with endothelial cells and the induction of innate and adaptive immunity.

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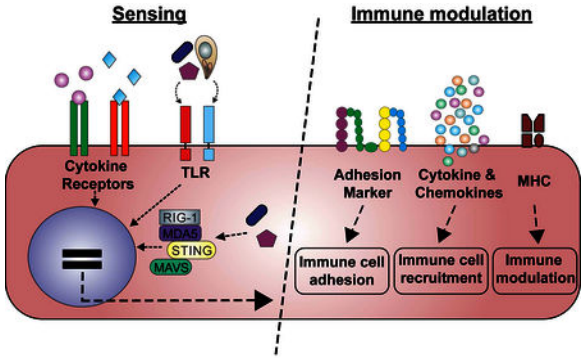
Abstract

There are over 10 trillion endothelial cells (EC) that line the vasculature of the human body. These cells not only have specialized functions in the maintenance of homeostasis within the circulation and various tissues but they also have a major role in immune function. EC also represent an important replicative niche for a subset of viral, bacterial and parasitic organisms that are present in the blood or lymph; however, there are major gaps in our knowledge regarding how pathogens interact with EC and how this influences disease outcome. In this article, we review the literature on EC-pathogen interactions and their role in innate and adaptive mechanisms of resistance to infection and highlight opportunities to address prominent knowledge gaps.

Graphical Abstract



Through their ability to express cytokine and toll like receptors Endothelial cells can sense microbial threats and respond to inflammatory stimuli. This can lead to the release of chemokines, expression of adhesion molecules and upregulation of MHC molecules, which can have a crucial impact on the immune response to infection.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

A. Introduction

The circulatory system in vertebrates consists of a network of vessels that can be broadly divided into vascular and lymphatic arms. The vascular system has an important role in thermoregulation, fluid balance and provides a conduit for the delivery of oxygen and nutrition to, and waste products and carbon dioxide away from, organs [1]. The lymphatic system functions to remove interstitial fluid from tissues and provides a conduit for antigen transport and immune-cell trafficking from peripheral tissues to secondary lymphoid organs. It is typically assumed that the lymphatics represent the primary route for pathogen dissemination after initial breach of mucosal or surface barriers, but there are few studies that directly address this issue. For vector-borne pathogens, the trauma and anti-coagulants associated with insect feeding will likely provide infectious stages with direct access to the blood. For example, the motile sporozoites of malaria that are deposited in the skin by mosquitoes can directly access the blood [2]. In contrast, for pathogens that enter their hosts through mucosal surfaces in the absence of obvious trauma, the involvement of the lymphatics for dissemination would seem likely; however, following oral challenge with *Yersinia enterocolitica*, the lymphatics were found not to be a major route for bacterial spread, rather small scale breaches of the intestine allowed systemic dissemination [3]. Another study has highlighted the importance of a gut-vascular barrier that limits entry of commensals into the blood, but *Salmonella typhimurium*, for example, utilizes its type III secretion system to cross this barrier [4]. These examples illustrate bacterial pathogens that have evolved strategies to directly access the vascular compartment and bypass the lymphatics, which presumably delays their recognition by the immune system. While, mainly due to technical challenges, the interactions of EC with pathogens is certainly understudied, these reports highlight that vascular EC are involved in many of the earliest events associated with infections that access the blood.

There are approximately 10 – 60 trillion endothelial cells (EC) in the human body that cover approximately 4000 m^2 [1]; they line the blood and lymphatic vessels with the exception of the placenta where EC are replaced by trophoblasts. EC provide a physical barrier between the circulation and tissues, and have functional specializations related to tissue location and activation status that affect function [1],[5]. The endothelium can be categorized into the macrovasculature composed of arteries and veins, and the microvasculature that includes arterioles, capillaries and venules. The macrovascular endothelium is non-fenestrated (lack pores) and continuous with limited vascular permeability whereas the microvasculature can be either continuous, fenestrated or discontinuous depending on the type of capillary bed. Fenestrated endothelium allows the rapid exchange, uptake and secretion of fluids, solutes and molecules and is present in tissues involved in filtration and secretion such as exocrine and endocrine glands, kidney glomeruli and the intestinal mucosa. Discontinuous endothelium is found in sinusoidal vascular beds such as those in the liver and bone marrow where the ability of cells to readily enter and exit the circulation is relevant [6].

EC in the blood-brain barrier (BBB) exhibit unique features such as intercellular tight junctions, the absence of fenestrae and a reduced level of pinocytic activity, asymmetrically-localized enzymes and carrier-mediated transport systems that distinguish them from peripheral EC [1],[7]. These specialized features protect the CNS from pathogens, toxic

molecules and limits access of antibodies and immune cells (discussed in more detail in Section CIV, EC of the BBB and infection). Thus, EC are a heterogeneous group of cells that are responsive to signals from the microenvironment, which include metabolic and mechanical stimuli, and interactions with other cells. The basis for this heterogeneity is the subject of other reviews [6],[8] and will not be discussed further here.

Given the inherent heterogeneity of EC populations in different tissues, it would be expected that EC in distinct compartments had unique responses and functions associated with diverse infections. For example, EC in the bone marrow that are involved in platelet formation would have a role in vivo distinct from those that form the BBB, but there is a lack of studies that systematically compare how EC in different tissues respond to infection. This represents a major knowledge gap and is, in part, a reflection of several technical issues. Cultures of EC (most notably Human Umbilical Vein Endothelial cells, HUVECs) have been used extensively to study how these cells respond to different microbes, but EC tend to lose their specialized properties in culture and obtaining differentiated EC from human adults is a challenge. The ability to use induced pluripotent stem cells to generate EC that resemble mature primary vascular endothelium and which can adopt an activated phenotype that supports cytokine production, leukocyte adhesion and transmigration will help address these issues [9]. This approach should also be useful to assess the impact of host genetics on EC responses to infection and provide the opportunity to study the influence of different EC subtypes on the immune response. There are also relevant concerns that while it is straightforward to expose EC to pathogens in vitro, often under non-physiological conditions, the in vivo relevance can be uncertain. The application of intravital imaging approaches that allow the visualization of EC-pathogen interactions in real time has been important but extended live imaging of deep tissues in the context of infection is not trivial. Despite these challenges, there continues to be remarkable progress in addressing various aspects of EC-pathogen interactions as discussed in this review, which concentrates on the response of the endothelium system as a whole to infection, calling out sub-location differences where known and focusing on micro-organisms where the pathophysiology of the infection is closely linked to EC biology.

B. Homeostatic and Inflammatory Functions of EC

While EC are a key structural component of the vascular system they also have functions that revolve broadly around the maintenance of immunological homeostasis versus the initiation and amplification of the inflammatory response to vascular insults such as infection or trauma. EC also have mechanisms that act to prevent aberrant inflammation and their ability to produce prostaglandin I_2 and constitutive expression of endothelial nitric oxide synthase (eNOS) both antagonize cytokine mediated upregulation of adhesion molecules [10]–[12]. Similarly, basal expression of tissue factor pathway inhibitors (TFPIs) block the initiation of the coagulation cascade and inhibit platelet adhesion and aggregation (Figure 1A) [13]. In addition, during infection with a human pathogenic strain of influenza virus, EC expression of the S1P receptor limits the early innate immune response and results in reduced mortality [14]. This finding indicates that the ability of EC to respond to bioactive sphingolipid sphingosine 1-phosphate is important to limit systemic pathological responses [14]. EC also synthesize Weibel–Palade bodies (WPB), which are specialized

storage vesicles containing von Willebrand factor, P-selectin, Angiopoetin-2 and chemokines which can mediate an immediate response to inflammatory signals. However, the number of WPBs and their precise content vary between endothelial tissues [15]. Thus, at sites of discrete vascular injury, the ability of EC to release von Willebrand factor supports the local recruitment of platelets that plug damaged vessels and thereby avoids the induction of systemic pro-coagulant events (Figure 1B) [16].

In steady state, EC express basal levels of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and, additionally, E-selectin in the skin, and vascular cell-adhesion molecule 1 (VCAM1) in the brain and bone marrow; however, during inflammation activated EC universally upregulate the expression of adhesion molecules (Figure 1C). EC can also bind chemokines on their luminal surface via cell surface heparan sulphate proteoglycans [17]. The activation of EC in response to damage, infection or immune stimuli (Figure 1B-D) also has profound physiological consequences that include increased blood flow and the formation of gaps between EC which allows leakage of plasma proteins [5]. The complement system represents an important mechanism for pathogen control [18], and the binding of C1q to the C1qRs promotes EC expression of adhesion molecules [19], and the release of cytokines and chemokines such as interleukin (IL)-6, IL-8 and monocyte chemoattractant peptide-1 (MCP-1) [20]. The binding of C5a to C5aR also stimulates EC to express P-selectin, release von Willebrand factor [21],[22], and to upregulate expression of adhesion molecules, vascular endothelial growth factor (VEGF)-C, IL-1β, IL-8 and RANTES [22], which promote immune cell access to damaged tissues. Furthermore, EC express receptors for the cytokines interferon (IFN)-γ, tumor necrosis factor (TNF), IL-1 and IL-6 [5],[23], which allows them to sense inflammatory signals derived from cells in the vascular compartment or inflamed tissues. EC responses to these signals are characterized by the up-regulation of ICAM1, VCAM1, E- and P-selectin and the secretion of pro-inflammatory cytokines and chemokines that promote leukocyte rolling and adherence to the vessel wall. These events are typically associated with extravasation into inflamed tissues to control infection [24], but it is unclear if these processes are directly relevant for the clearance of infected EC.

C. The vascular system and pathogens

For many pathogens, after initial invasion, the ability to access the vascular compartment is essential not only for dissemination in the new host but also for transmission to insect vectors or blood borne transmission. To limit systemic spread of infection, there are multiple anti-microbial immune effectors in the blood that include macrophages, lipoproteins as well as complement- and coagulation-mediated pathways [18],[25],[26]. The importance of complement in resistance to infection is illustrated by the increased incidence of *Neisseria meningitidis* in patients with primary genetic deficiencies in the complement pathway [27]. Further, it is well accepted that the presence of pathogen-specific antibodies that activate complement or promote phagocytosis can curtail infections in the blood. The significance of these immune-mediated mechanisms of resistance is apparent by the evolution of vascular pathogens that express surface coats that mitigate the effects of complement activation or which have proteins that deactivate complement [18],[28]. For a sub-set of micro-organisms, the ability to invade EC not only allows them to evade humoral immunity but also provides

ready access to the vasculature. In some cases, EC represent a site of microbial persistence and a recent study has highlighted lymphatic EC as a niche for *Mycobacterium tuberculosis* [29]. Similarly, the ability of *Chlamydia pneumoniae* to persist in EC may be involved in the induction and acceleration of atherosclerosis [30], a process with an immune component characterized by the formation of plaques in arteries. Nevertheless, it is relevant to recognize that EC are not simply a replicative niche for diverse organisms but there is a growing appreciation that EC influence the outcome of infections that affect the circulatory system. The examples provided below illustrate some of the most important pathogens, which infect or interact with EC and highlights the potential role of EC in microbial detection, as well as illustrating how EC-pathogen interactions affect disease manifestation (Figure 2).

CI. Interactions of Endothelial Cells with Pathogens.

Pathogen Detection—Given that the appearance of micro-organisms within the vascular system can have life threatening consequences, there is a need to mount vascular-specific anti-pathogen responses. The location of EC at the interface between the blood flow and tissues, and EC expression of diverse pattern recognition receptors (PRRs) that recognize pathogen-associated molecular pattern molecules (PAMPs) and damage-associated molecular pattern molecules (DAMPs) makes them ideal sentinel cells [31]–[33] (Figure 1). This concept becomes more relevant with the recognition that some pathogens such as *S. typhimurium* and *Y. enterocolitica* appear capable of directly accessing the vasculature [3], [4]. However, EC are highly polarized cells with significant differences in membrane composition on the apical and abluminal sides and it is not clear whether the distribution of PRRs allow EC to distinguish microbial threats present in tissues or the vascular lumen. A more likely scenario is that for those organisms in the blood the direct interaction with EC engages conserved pro-inflammatory pathways, dominated by the activation of NF-κB signaling (summarized in Table 1), and it is assumed that this process contributes to pathogen control. Indeed, there are multiple illustrations that support this concept. For example, Listeriolysin O, a pore forming toxin from *Listeria monocytogenes,* induces increased EC expression of surface E-selectin and ICAM1 [34]. VirB is a *Bartonella* sp virulence factor that not only inhibits apoptosis of infected EC, but also activates NFκB that leads to increased EC adhesion molecule expression and chemokine secretion [35],[36]. *Chlamydia pneumoniae* can infect microsvascular EC and the TLR4-MD2 complex provides a PRR that recognizes Chlamydial heat shock protein 60 (cHSP60) and activates NF-κB, which promotes inflammatory responses similar to those that contribute to atherogenesis [37]. Similarly, EC recognition of *Candida albicans* through TLR3 results in the activation of NF-κB and the p38 MAPK pathway which engages a pro-inflammatory transcriptome associated with resistance to this organism [38].

EC also express cytosolic PRRs which allows them to respond to viral invasion and the DNA sensor Stimulator of interferon genes (STING) is required in EC for optimal HCMV-induced Type I IFN production and inhibition of viral replication [39]. Other relevant cytosolic receptors include Retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) that can detect viral products such as genomic RNA [40],[41]. In EC, double-stranded RNA (dsRNA) induces the upregulation of Type I IFN, MHC-I genes and the antiviral genes protein kinase R (PKR) and 2’,5’-oligoadenylate

synthetase (2',5'-OAS), as well as cytokines and adhesion molecules [42], which together restrain viral replication. Dengue Virus (DV) infection of human EC in vitro causes apoptosis but also the expression of a range of cytokines including IFN-β [43]-[45]. In human brain micro-vascular endothelial cells (HBMECs), RIG-1 is required for DV-induced production of type I IFN and proinflammatory cytokines [46]. The importance of these mechanisms is illustrated by the observation that the blockade of IFN-β during infection in vitro leads to increased DV replication in EC [47]. The relevance of these sensors is not restricted to viral products, and EC stimulated with LPS can utilize RIG-1, and MAVS to induce NF-κB–mediated production of adhesion molecules and cytokines [48], Overall, these examples highlight the existence of conserved responses of EC to infection that form the basis for the idea that EC may not just be a substrate to recruit inflammatory cells to sites of infection, but rather have a crucial role in sensing as well as in combating infection.

EC control of pathogen replication—In vitro studies have established that EC can support the replication of a wide array of pathogens, but EC also have a number of mechanisms that can restrict the growth of micro-organisms. Thus, the ability of EC to detect the presence of pathogens (described above) is usually accompanied by enhanced anti-microbial activities as a consequence of EC-intrinsic pathways or by secondary signals derived from immune cells. Multiple *in vitro* studies demonstrate that EC can be activated by IFN-γ or the type I IFNs to limit replication of pathogens such as *T. gondii*, *S. aureus* and *HCMV* [39],[49],[50] (Figure 1D). Indeed, the combination of IFN-γ alone or in combination with TNF can activate EC to upregulate indoleamine 2,3-dioxygenase (IDO) which in turn leads to tryptophan degradation and starvation of *T. gondii* [49], *S. aureus* [50] and Rickettsia [51]. For Rickettsia and *M. tuberculosis,* EC production of nitric oxide (NO) also inhibits microbial growth [29],[51] and, in IFN-γ–activated EC, *M. tuberculosis* and eNOS co-localize [29]. More recent studies with EC have also highlighted that autophagy is a component of the cell intrinsic pathways that EC use to control *T. gondii* [52]. Thus, EC engage multiple mechanisms to limit pathogen replication, but the extent to which these vary with EC subtype and across host species is unclear. Indeed, because of the lack of tractable model systems to target EC-specific pathways in vivo, there remains a significant knowledge gap in defining the core anti-microbial pathways utilized by different EC cell types and their relevance to different micro-organisms. The field would benefit from the application of standardized approaches for in vitro studies, combined with a way to reliably and specifically target EC in vivo, in order to identify core EC responses that mediate resistance to different classes of pathogens.

Microbial influence on EC homeostasis—Interestingly, EC not only respond to microbial threats but there are examples of how the microbiome can influence EC activity, reinforcing the role of EC as sentinels that monitor their environment. In germ free mice, the BBB has reduced expression of tight junction proteins and increased permeability which can be reversed by the colonization of these mice with a pathogen-free microbiota [53]. Interestingly, cerebral cavernous malformations (CCM) are vascular abnormalities that have a genetic basis, likely are formed during development and are prone to leakage. When this occurs in the CNS, it predisposes to haemorrhagic stroke and seizures. The observation that the bacterial microbiome is the primary source of TLR4 ligand that stimulates CCM

formation in mice [54] highlights the complex interplay between EC, PRR and microbes that can result either in disease or promote normal developmental processes.

CII. EC interactions with infections that underlie pathogenesis

Most of the infections discussed in this review are associated with the ability to injure EC as a result of direct invasion and, given that EC are prominently infected in vivo with Human cytomegalovirus (HCMV), Dengue virus (DV), Nipah, Ebola or West Nile Virus, it suggests that there may be a viral tropism for these host cells. However, there can also be bystander effects that lead to disease and here, we will focus on some examples where the involvement of EC appear to directly contribute to distinct pathologies. HCMV is a ubiquitous herpesvirus with a broad host cell range and the HCMV genes UL128 to UL150 facilitate adsorption and entry into EC via endocytosis followed by fusion with endosomal membranes [55]. In vitro, HCMV replication in EC promotes the secretion of von Willebrand factor accompanied by platelet adherence and aggregation. These processes may explain the links of HCMV to the development of transplant vascular sclerosis, restenosis following angioplasty, and atherosclerosis [56]. Similarly, the Rickettsia genus is composed of Gram-negative, obligate, intracellular bacteria that are associated with generalized vascular inflammation [57]. In vitro, Rickettsia replicates in human and mouse EC and the increased permeability of endothelial monolayers infected with Rickettsia is associated with disruption of intercellular adherens junctions [57]-[59]. EC stimulation with Rickettsia induces a rapid up-regulation of the inducible isoenzyme cyclooxygenase (CoX) which catalyses the production of prostaglandins and leukotrienes which in turn influence inflammatory responses and vascular permeability [58]. These observations may provide a mechanistic explanation into how rickettsial infection of the endothelium leads to local dermal and epidermal necrosis, vasodilation and fluid leakage into the interstitial spaces [59].

The ability of Gram-negative *Bartonella* sp. to infect EC is associated with multiple effects which includes the activation of the host GTPases Rho, Rac and Cdc42 which mediate rearrangement of the actin cytoskeleton leading to the uptake of the bacteria [60],[61]. *Bartonella* sp. also encode two type IV secretion systems (T4SS) and in EC one of these inhibits apoptosis of infected cells [62], and induces activation of NFκB which leads to adhesion molecule expression and chemokine secretion [35],[36]. In humans, infection of the endothelium with *Bartonella sp.* can lead to vaso-proliferation, and the formation of vascular tumors is associated with the influx of monocytes/lymphocytes that secrete pro-angiogenic factors that support vasoproliferative growth [60]. Since Kaposi sarcoma–associated herpesvirus is also linked with the formation of vascular tumors [63], it suggests that aberrant inflammation may provide a common mechanism that underlies tumor formation.

There are also several eukaryotic pathogens that can cause significant disease in the vasculature and there are a select group of fungi whose interactions with EC are well studied. For example, during the hematogenous spread of *Candida albicans* the ability to adhere to EC is important for transmigration into tissues where *C. albicans* causes disease. Consequently, the molecular basis for *C. albicans* adherence to, and invasion of, EC through

candidal adhesins is well characterized [64]. It is also known that the uptake of *C. albicans* can lead to EC death through a process dependent on EC iron metabolism [65]. Similar to the studies described for *S. typhimurium* and *Yersinia* [3],[4] the hyphae of *Aspergillus fumigatus* within the lung parenchyma can enter EC from the abluminal side and then transverse into the vascular compartment for hematogenous dissemination. Interestingly, abluminal invasion causes less EC damage than luminal invasion but also greater induction of EC genes encoding cytokines and adhesion molecules [66]. For *Plasmodium falciparum,* a causative agent of malaria, as the parasite matures within erythrocytes these cells can adhere to EC. These events allow the mature parasite to avoid passage through the spleen, where macrophages remove infected RBC [67]. At the molecular level, it is the ability of infected erythrocytes to form 'knobs' at their surface, where parasite-derived surface receptors such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) are concentrated [68],[69], that facilitates the adhesion of infected RBCs to EC via EC surface binding molecules including ICAM-1, CD31, CD36, CSA, gC1qR and the Protein C receptor [68], [70]. One consequence of this evasion strategy is that these events provoke localized inflammation within the capillaries of many tissues and, when this occurs in the CNS, it leads to cerebral malaria. Cytoadherence of infected cells to EC is also seen with other species of malaria and related parasites, which indicates that this is an evolutionary conserved strategy to avoid immune-mediated clearance mechanisms in the spleen.

CIII. Infections and coagulation

The coagulation process (discussed earlier in the section "Homeostatic and inflammatory functions of EC") has an important role in the repair of local damage in the vascular system and resistance to infection. This is illustrated by the essential role for fibrin in limiting infection-induced blood loss in mice infected with *T. gondii* [71] and also by examples where fibrin deposition has anti-microbial activities e.g. in listeriosis [72]. However, extensive EC damage can result in systemic induction of the coagulation cascade which, in the context of sepsis, leads to disseminated intravascular coagulation (DIC), widespread micro-vascular thrombosis and hemorrhage [25]. Similarly, a mutation in the protein C gene, which encodes a protein that regulates coagulation by degradation of factor Va and factor VIIIa, results in increased bleeding or thrombosis during acquired disturbances such as infectious disease or cancer [73],[74].

For *N. meningitidis*, a crucial step in the pathogenesis of this infection is the adhesion of meningococci to EC located in regions of low blood flow. The meningococci interact with the human receptor CD147 which is expressed by EC in capillaries [75] and, after initial attachment, *Neisseria* forms micro-colonies. This attachment is accompanied by EC cytoskeletal modifications and the formation of microvilli-like protrusions that stabilize bacterial adhesion, and altered localization of junctional proteins allows these bacteria to penetrate into tissues [76]. This intimate interaction of meningococci with EC engages local inflammatory and coagulation processes which lead to skin associated purpura fulminans, a disorder characterized by coagulation in small blood vessels that can result in necrosis and DIC.

Although DV can infect EC in vitro, EC are not considered a major cellular target in humans. Nonetheless this infection is responsible for dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), the cardinal signs of which include hemorrhage, vascular leakage, and shock, accompanied by severe thrombocytopenia and systemic complement activation. In mouse models, DV infection of EC is linked to release of oxygen free radicals that mediate vascular leakage [47],[77]. Ebola virus (EV) also infects EC and causes severe disease characterized by hypotension, lymphopenia, coagulative disorders, and hemorrhage with high mortality rates in humans and nonhuman primates [78],[79]. Several EV-derived proteins can induce a decrease in EC barrier function or are cytotoxic to EC and thus may contribute to endothelial damage [79]. However, there is evidence that EV-induced coagulopathy results primarily from vascular disruption induced by factors secreted from infected monocytes/macrophages and dendritic cells, and that virus-induced EC damage has a secondary role in the increased susceptibility to hemorrhage [80]. Given that changes in coagulation and thrombosis formation are a common feature of the examples discussed above understanding the events that underlie the development of the clinical diseases associated with these hemorrhagic viruses (and other pathogens) may provide the opportunity to restore haemostasis and better manage these conditions.

CIV. EC of the Blood Brain Barrier and infection

Systemic inflammation-induced changes in EC may allow microbial access to tissues and, although there is considerable EC heterogeneity, there are no systematic comparisons of how different EC sub-types respond to various infections. The major exception may be the EC of the brain, which are highly specialized and provide a barrier function that limits microbial access to the CNS. Certain micro-organisms appear to have a *bona fide* tropism for neural tissues [81] and there has been a significant focus on pathogen interactions with respect to EC as a component of the BBB. *Cryptococcus neoformans* is an encapsulated budding yeast that can invade EC and causes meningoencephalitis in humans and animals. *S*everal microbial factors, including urease and phospholipase B1 [82], as well as host plasmin [83], have been reported to be involved in adherence of *C. neoformans* to brain EC. The fungal inositol transporters Itr1a and Itr3c mediate increases in inositol which promotes Cryptococcus production of hyaluronic acid [84], which is a ligand for host CD44. The interactions of *C. neoformans* with microvascular EC membrane lipid rafts promotes invasion in a CD44-dependent manner [85]-[87]. Adherence of *C. neoformans* to EC leads to EC structural changes that ultimately result in EC necrosis and which may facilitate *C. neoformans* CNS invasion [88]. The protozoan *T. gondii* is clinically relevant in humans where it can cause toxoplasmic encephalitis. Mice are natural hosts for this parasite and acute infection results in a transient parasitemia that precedes access to the CNS [89],[90]. There are reports that cells infected with *T. gondii* can cross the BBB [89],[91] or that infected cells release parasites when they make contact with EC [92]. In vitro experiments have shown that extracellular parasites can readily infect EC under flow conditions and can perform paracellular migration across EC monolayers [93]. In vivo, EC are readily infected and replication in, and lysis of, EC is required for the ability of *T. gondii* to access the CNS [90]. This mechanism is likely relevant for other CNS-tropic pathogens such as *L. monocytogenes* that can accesses EC either by direct invasion or by cell-to-cell spread from infected mononuclear phagocytes or other EC [94]-[97].

There are also examples where the host receptors that allow pathogen attachment and entry to EC of the BBB have been identified and these may provide targets for therapies. Thus, Nipah virus (NiV) initially infects epithelial cells in the lungs but its ability to disseminate and infect EC in the small blood vessels supplying the CNS is the basis for the development of fatal encephalitis in humans [98],[99]. Consistent with its cellular tropism, the attachment glycoprotein of NiV binds to ephrinB2, the membrane bound ligand for the EphB class of receptor tyrosine kinases (RTKs), which is expressed by EC. In EC, the fusion and attachment proteins of NiV mediate syncytia formation, and multinucleated giant EC are a characteristic feature of affected tissues [100]. For *Escherichia coli* K1 (the most common Gram-negative cause of neonatal meningitis), the ability to adhere to and invade EC is required for this bacterium to cross the BBB [101],[102]. *E. coli* adherence to EC is initiated by the Type 1 fimbrial adhesive protein FimH, which can interact with CD48 on EC, and by the outer membrane protein OmpA, which interacts with the glycoprotein gp96 present on the surface of human brain micro-vascular EC and which may influence neuro-tropism [103],[104]. Regarding *Streptococcus pneumoniae,* the analysis of brain biopsies from patients who died of pneumococcal meningitis revealed that pneumococci co-localize with the polymeric immunoglobulin receptor (pIgR) and with platelet endothelial cell adhesion molecule (PECAM-1) [105]. Moreover, through the use of a murine model, it was shown that antibodies against these molecules synergize with anti-bacterial drugs to reduce bacterial invasion of the brain [105]. This is an important proof of concept for a treatment strategy that is relevant to other pathogens with well-defined host receptors that mediate entry to the brain.

West Nile virus (WNV) is an important human pathogen that targets neurons and can cause encephalitis characterized by disruption of the BBB, enhanced infiltration of immune cells into the CNS, microglial activation and eventual loss of neurons. WNV can infect EC in vitro, which in turn up-regulate claudin-1, ICAM1, VCAM1 and E-selectin expression and multiple mechanisms have been proposed for how it crosses the BBB [106]. In vivo during infection, TLR3 engagement leads to the secretion of TNF and this induces a transient change in the permeability of the BBB [107], which may allow virus to cross into the CNS [108],[109]. Similarly, African trypanosomes, which exist as motile extracellular forms present in the blood, can access the brain, most likely through the post-capillary venules or via the Virchow Robbins space (fluid filled perivascular gap) [110],[111]. While a parasite-derived protease is implicated in this process, host IFN-γ and G protein–coupled receptors (GPCR) contribute to the ability of trypanosomes to cross the BBB, presumably through loosening of the EC tight junctions [112],[113]. Thus, infection-induced systemic inflammation can alter BBB permeability and thereby increase microbial access to the CNS

D. EC interaction with Immune Cells During Infection.

Monocytes

Monocytes play an important role in microbial control and help to remove dead cells or damaged tissues. As noted above (Table 1, Figure 1), a common innate response of EC to infection is the production of chemokines and the upregulation of adhesion molecules that promotes adherence of immune cells to EC and extravasation of neutrophils, monocytes and

lymphocytes into inflamed tissue. EC also constitutively express the chemokine fractalkine (CX3CL1) and, while fractalkine levels are upregulated in response to inflammation, constitutive levels enable rolling of monocytes along EC surfaces. Intravital imaging studies have shown that monocytes which express the fractalkine receptor (CX3CR1) adhere to and patrol the luminal side of the entire microvasculature at homeostasis and, in CX3CR1-KO mice, monocyte adherence is reduced six-fold [114]. The authors proposed that this behavior provides a mechanism that allows the monocytes resident in the blood to rapidly access the site of an infection where they mediate anti-microbial activities but it also seems possible that monocytes may interact with infected EC. The same group have suggested a role for a sub-population of these patrolling monocytes as intravascular housekeepers which phagocytose EC that have been damaged by local neutrophil responses [115]. Whether this subset of monocytes is involved in the resolution of infection-induced damage is uncertain but, given that many of the EC chemokines that are upregulated by *C. albicans* are involved in the recruitment of neutrophils, which play a pivotal role in the resolution of fungal infections [116], EC damage by neutrophils may be widespread.

T cells and NK cells

Although it is recognized that there are subsets of tissue-resident T cells that are specialized for many different tissues, it is not yet clear whether there are T cells that are specialized for surveying the vascular compartment. Nonetheless, EC do express MHC class I and II, are able to present class I and II restricted antigens and can interact with cells of the adaptive immune system [5],[24],[117]. EC are also implicated in cross-presentation of antigen [118], [119] but whether these antigen-restricted EC events impact on the trafficking, activation, and differentiation of lymphocytes during infection is understudied but of interest. There is also growing interest in the role of the lymphatic endothelium as an archive of antigen following vaccination or infection that can be accessed by DC and used to maintain memory T-cell responses [120]-[122]. Interestingly, a subset of activated T and NK cells express the chemokine receptor CX3CR1 and there is in vitro evidence that the ability of NK cells to injure EC is CX3CR1-dependent [123]. In response to several infections (Listeria, LCMV, TB) a subset of pathogen-specific effector or memory T cells located within the vascular compartment express CX3CR1 [124],[125]. Current paradigms suggest that this pathway is likely important for T cells to extravasate and access inflamed tissues; however, it is also possible that CX3CR1 expression might be involved in T-cell surveillance of the vasculature lumen for infected ECs or to promote T-cell interactions with the lymphatic endothelium.

While it is not clear if there is specific immune surveillance of the endothelium, it provides a potential mechanism for resistance to infection but such a mechanism can also have adverse consequences. In a model of cerebral malaria, although EC are not infected with Plasmodium, the production of IFN-γ enables brain EC to cross-present parasite antigens and become susceptible to killing by $CD8^+$ T cells [118]. In other reports, malaria specific $CD8^+$ T cell responses lead to changes in tight junction function and breakdown of the BBB that leads to cerebral herniation [126],[127], with pathology analogous to the human disease [128]. Similarly, following intra-cerebral challenge with LCMV, the interaction of $CD8^+$ T cells with EC leads to vascular injury in the meningeal compartment and the recruitment of neutrophils and monocytes that mediate the breakdown of the BBB [129].

An additional mechanism that allows EC to exert a regulatory activity on T-cell activities includes the presentation of viral antigen by liver sinusoidal EC to $CD8^+$ T cells, thereby inducing tolerance [130]. In addition, IFN-γ stimulation of EC leads to expression of the inhibitory receptors PD-L1 and PD-L2 which can induce PD-1^+ $CD8^+$ T cells to limit their production of IFN-γ and cytolytic activity. Indeed, after systemic LCMV infection in the absence of PD-1, $CD8^+$ T cells killed infected vascular EC and compromised the vascular integrity [131]. Thus, EC expression of PD-L1 and PD-L2 may be a mechanism that allows the activation and extravasation of T cells without excessive vessel damage [132].

F. Conclusions

The sections above have highlighted the significant impact of EC on a variety of immune processes, such as innate recognition, anti-microbial activity, antigen presentation and immune regulation, that govern the outcome of EC-pathogen interactions; however, one of the major challenges of studying EC during infection in vivo is the ability to alter gene expression in EC specifically in order to test which cell-intrinsic immune pathways are involved in mediating resistance to infection versus contributing to the development of disease. For example, the use of promoters for Tie2 and Cdh5 (the gene for VE-cadherin) have proven useful to target EC but Tie2 is also expressed in hematopoietic cells [133]. Further, our own experience with the Cdh5 promoter is that it drives Cre expression in approximately 30% of hematopoietic cells, presumably because Cdh5 is expressed early in development. These technical challenges make it difficult to isolate the immunological activities of EC in vivo in systems where canonical immune populations have a role. While bone marrow chimeras can help address this problem, there remains a need to apply inducible Cre systems to target immunologically relevant pathways in EC of adult mice that have minimal off-target effects. Ideally, these genetic approaches could be combined with intravital imaging to detect rare encounters of EC with pathogens or immune cells. Advances in this field include the development of approaches that allow long-term imaging of the vasculature, in combination with the ability to visualize monocytes, T cells or micro-organisms engineered to express fluorescent reporters, lacZ, Cre or calcium signaling reporters. These approaches have already changed how we view the patrolling behavior of immune cells and their ability to monitor EC integrity and have allowed the visualization of pathogen interactions with EC in real time [90],[134]-[138]. The continued application of these technologies should help to distinguish the circumstances in which EC act either as innate sensors of pathogens or regulators of protective and pathological immune responses that dictate the outcome of infection.

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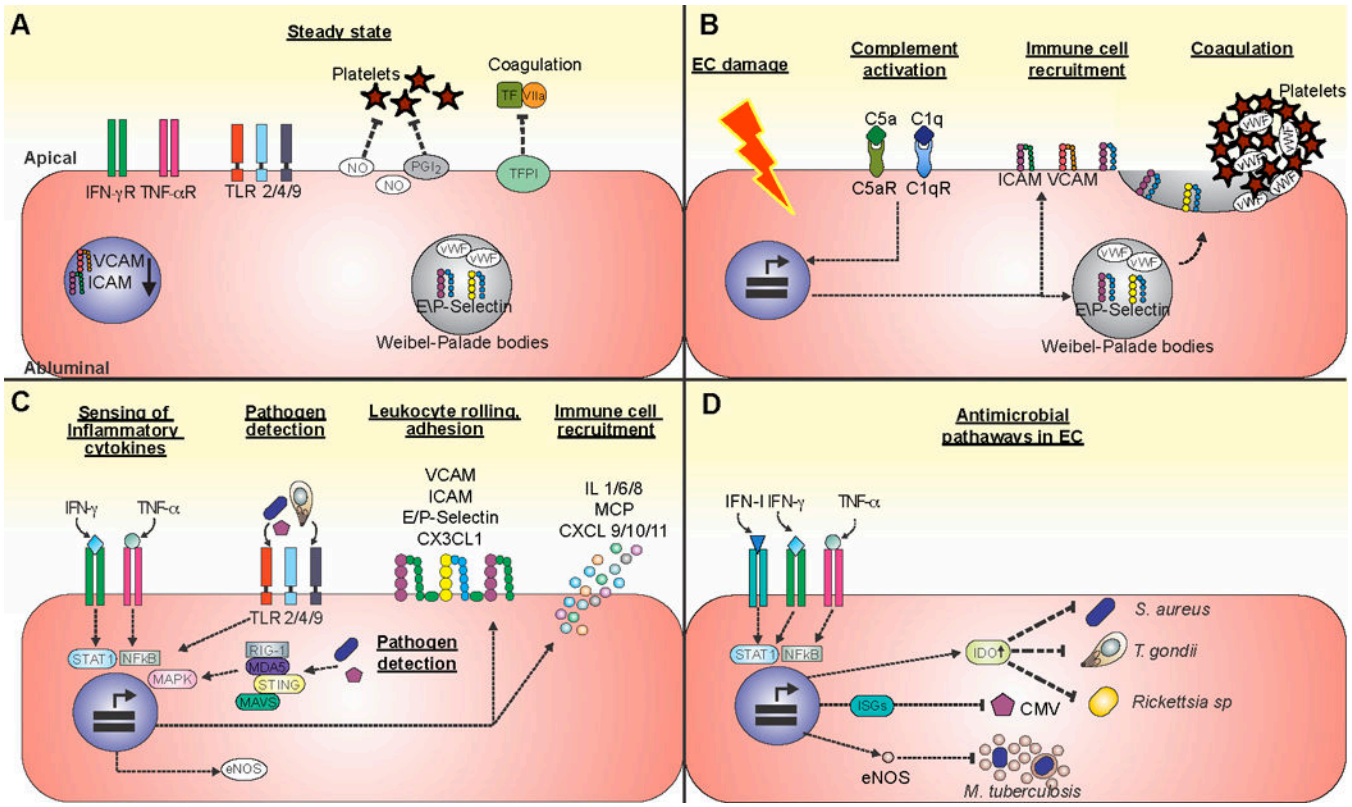

Figure 1: EC functions associated with homeostasis and resistance to infection.

(A) In steady state EC have mechanisms that act to prevent inflammation through their ability to produce prostaglandin I_2, which together with the constitutive expression of endothelial nitric oxide synthase (eNOS), antagonizes cytokine mediated upregulation of adhesion molecules. Basal expression of tissue factor pathway inhibitors (TFPIs) block the initiation of the coagulation cascade and inhibit platelet adhesion and aggregation. **(B)** EC contain Weibel–Palade bodies (WPB), which are cytoplasmic storage vesicles that contain von Willebrand factor (vWF) and P-selectin, in response to vascular injury, complement activation via the C1qRs and C5aR, EC release von Willebrand factor that supports the local recruitment of platelets and coagulation to prevent blood loss. EC damage can also promotes expression of adhesion molecules. **(C)** EC express cytokine receptors (eg IFN-γR and TNFR) and many classes of PRRs (e.g. TLR2, TLR4, TLR9, RIG-1, and STING) relevant to bacterial and viral infection, which allow EC to respond to infection by the activation of NF-κB and MAPK. Further, EC activation by pathogen leads to the up-regulation of adhesion molecules such as ICAM1, VCAM1, Selectins and CX3CL1, and the release of chemokines and pro-inflammatory cytokines such as IL-1/6/8, MCP, and CXCL9/10/11. **(D)** EC activation by IFN-γ, TNF-α or Type I IFNs induce a number of mechanisms that can restrict the growth of micro-organisms. IFN-γ and TNF-α activate EC to upregulate indoleamine 2,3-dioxygenase (IDO) leading to tryptophan degradation and starvation of *T. gondii*, *S. aureus* and Rickettsia. Moreover, EC production of nitric oxide (NO) inhibits microbial growth of Rickettsia and *M. tuberculosis*. Further, Type I IFNs induced expression of interferon-stimulated genes (ISG) inhibit viral replication during HCMV infection.

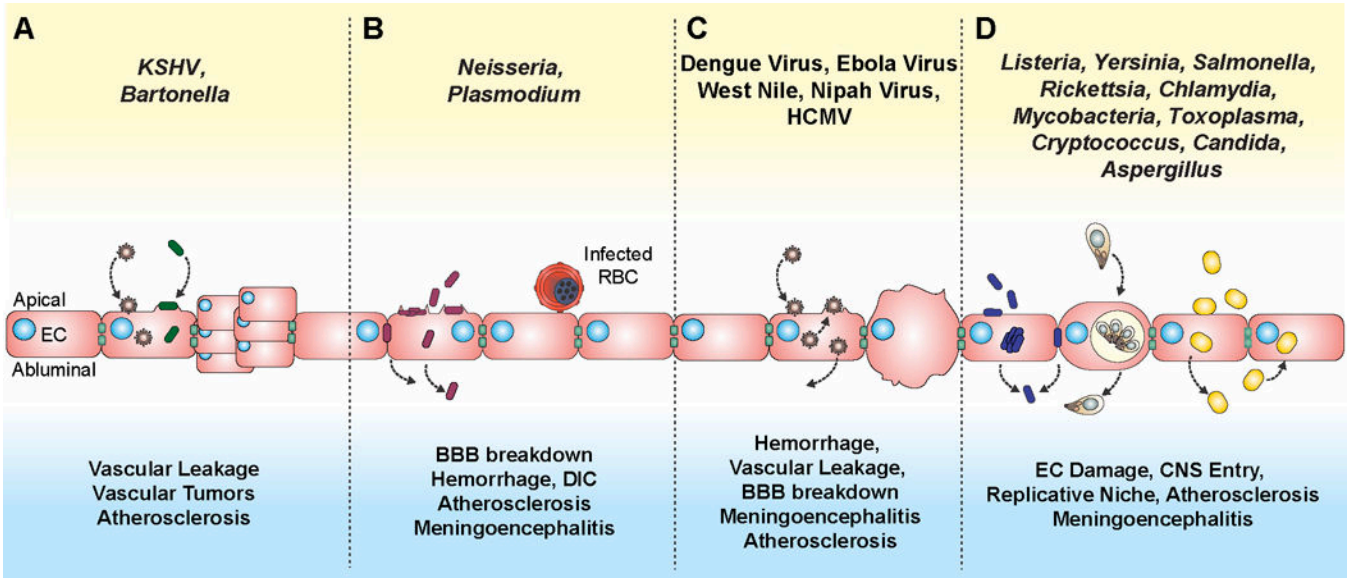

Figure 2: EC-pathogen interactions that lead to disease.

(A) Infection of the endothelium by *Bartonella sp. and* Kaposi sarcoma–associated herpesvirus (KSHV) are associated with the influx of monocytes/lymphocytes that secrete pro-angiogenic factors that can lead to vaso-proliferation and the formation of vascular tumors. **(B)** For *N. meningitidis*, the adhesion of the meningococci to EC is accompanied by host cell cytoskeletal modifications and the formation of microvilli-like protrusions; these changes are associated with increased coagulation in small blood vessels. Plasmodium matures in erythrocytes and within these cells can adhere to EC. These events provoke localized inflammation within the capillaries and can lead to cerebral malaria. **(C)** For Human cytomegalovirus (HCMV), Dengue virus (DV), Nipah, Ebola and West Nile Virus there may be a viral tropism for EC and this viral presence is associated with a number of pathological symptoms; for some of these pathogens, this might allow their access to the CNS. **(D)** EC can serve as an important replicative niche for a subset of bacterial, parasitic and fungal pathogens. The infection of, replication in and lysis of EC can be critical for pathogen access to tissues like the CNS.

Table 1:

This table summarizes some of the literature on diverse pathogens which infect or interact with EC and their response. The recognition of PAMPs or DAMPs by EC leads to their activation and the initiation of conserved pro-inflammatory pathways, dominated by the activation of NF-κB signaling. This leads to the production of chemokines and the upregulation of adhesion molecules that promotes adherence of immune cells to EC and extravasation of neutrophils, monocytes and lymphocytes into inflamed tissue.

Pathogen	Recognition	Signaling	Increased expression	Induction	Suppression	References
Dengue Virus	RIG-1	NFκB	ICAM-1	IFN-β BAFF, CXCL9/10/11, RANTES IL-6/7/8		[43]-[46]
West Nile Virus	TLR3		ICAM1, VCAM1, E-selectin	TNF-α		[107]
Ebola virus					MHC-I, IL-6, ICAM1, PKR, IRF-1	[78]
HCMV	STING			Type I IFNs		[39]
Listeria monocytogenes			ICAM1, VCAM1, E-selectin			[34],[139]
Bartonella sp		NFκB	ICAM1	IL-8, MCP-1		[35],[36]
Staphylococcus aureus			ICAM1, VCAM1	IL-1β, IL-6/8, MCP-1		[140]-[143]
Rickettsia sp		NFκB	E-selectin	IL-1/6/8,		[144]
Chlamydia pneumoniae	TLR4, MD2	NFκB, MAPK	ICAM1, VCAM1, E-selectin	IL-8, MCP-1		[145]-[147]
Toxoplasma gondii			ICAM1, VCAM1, E-selectin, P-selectin, CX3CL1	IL-6, MCP-1		[148],[149]
Trypanosoma cruzi		NFκB	ICAM1, VCAM1, E-selectin	IL-1β, IL-6, CSF-1		[150]-[152]
Candida albicans	TLR3	NFκB, MAPK		IL-8, CXCL8		[38]