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Infection of the endothelium by members of the order Rickettsiales

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Summary

The vascular endothelium is the main target of a limited number of infectious agents; *Rickettsia*, *Ehrlichia ruminantium*, and *Orientia tsutsugamushi* are among them. These arthropod-transmitted obligately-intracellular bacteria cause serious systemic diseases that are not infrequently lethal. In this review, we discuss the bacterial biology, vector biology, and clinical aspects of these conditions with particular emphasis on the interactions of these bacteria with the vascular endothelium and how it responds to intracellular infection. The study of these bacteria in relevant *in vivo* models is likely to offer new insights into the physiology of the endothelium that have not been revealed by other models.

Keywords

Rickettsia; *Orientia tsutsugamushi*; *Ehrlichia ruminantium*; endothelium; blood circulation

General biological and ecological considerations

Rickettsia, *Ehrlichia*, and *Orientia* are closely related bacteria of the family Rickettsiaceae (genera *Rickettsia* and *Orientia*) and Anaplasmataceae (includes several genera but only one species of the genus *Ehrlichia* infects endothelial cells) that can only survive inside eukaryotic cells as a result of a reductive process of evolution that led to the loss of many genes encoding proteins that participate in several biosynthetic pathways (1–3); thus, these microorganisms are strictly intracellular parasites that reside free in the cytoplasm (*Rickettsia* and *Orientia*) or in cytoplasmic vacuoles (*Ehrlichia*) of their eukaryotic hosts (4). Their intimate relationship with endothelial cells in the infected host begins early for *Ehrlichia* and some *Rickettsia* since they are inoculated by haematophagous arthropod vectors and circulate in the bloodstream (5).

Infections caused by *Orientia tsutsugamushi*, most *Rickettsia*, and some *Ehrlichia* are interesting because of the target cells (endothelium) and the nature of the pathogen (obligately intracellular bacterium). Indeed, of the infectious agents that have been demonstrated to infect the endothelium (6), only hantaviruses (7,8), human herpes virus-8 (9,10), *Orientia tsutsugamushi* (11), *Ehrlichia ruminantium* (12), and rickettsiae (13–17) use endothelial cells as their main targets.

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Orientia tsutsugamushi, the agent of scrub typhus, is transmitted by the larvae of mites belonging to the family Trombiculidae (commonly known as chiggers or red bugs), particularly members of the genus *Leptotrombidium* (18). Larvae are the only parasitic stage of these mites; they feed preferentially on small animals and infest humans only accidentally. They pierce the epidermis with their chelicerae in thin areas of the skin (e.g., around hair follicles) where they insert their capitulum (including the mouth parts) to form a feeding tube (stylostome) through which they feed on skin cells, tissue fluid, and lymph. If an infection is established, an eschar usually forms around the feeding area as a consequence of local infection and replication. This is subsequently followed by systemic spread. *O. tsutsugamushi* binds fibronectin through the *Orientia* membrane protein TSA56, and this enhances invasion of host cells (19). The mechanism of entry is a clathrin-dependent endocytosis pathway, which is followed by escape of the bacteria from late endosomes (as inferred from co-localisation with Lysosomal-associated membrane protein 2 [LAMP2]); also, acidification appears to be essential in this process since increasing the vacuolar pH inhibits *Orientia* replication (20).

A modern classification of *Rickettsia*, based on whole-genome analysis data (3,21–23), divides them in four groups: ancestral group (*R. bellii* and *R. canadensis*; not known to be pathogenic), typhus group (*R. typhi* and *R. prowazekii*), spotted fever group (*R. rickettsii*, *R. parkeri*, *R. conorii*, and several others), and transitional group (*R. akari*, *R. australis*, and *R. felis*). The spotted fever group rickettsiae differ from the typhus group rickettsiae in their capacity to stimulate host cell actin polymerisation for directional cell-to-cell movement (24–26), a lipopolysaccharide (LPS) that contains antigens specific for each group (27), and the presence of the rickettsial outer membrane protein OmpA (28). Until recently, the serological response was the main criterion used to classify rickettsiae in only two groups, spotted fever and typhus.

Typhus group rickettsiae are transmitted mainly by insect vectors. The agent of epidemic typhus, *R. prowazekii*, is transmitted in the excrement of the human body louse (*Pediculus humanus corporis*). It was thought to be confined to this vector and the human host until its presence was documented in flying squirrels (*Glaucomys volans*) and their ectoparasites (lice and fleas) in North America (29–32). Furthermore, there is evidence implicating ticks as a natural niche (33). Lice acquire *R. prowazekii* upon feeding on persons suffering from epidemic typhus. Only epithelial cells lining the louse gut become infected and not the salivary glands; for this reason, *R. prowazekii* is not transmitted through direct inoculation into the infected person's bloodstream, but by inoculation of infected louse feces into small open wounds created by scratching (34).

The agent of murine typhus, *R. typhi*, exists in nature in an enzootic cycle involving rodents and their ectoparasites (fleas and lice). It is transmitted to humans mainly by the excrement of *Xenopsylla cheopis*, the oriental rat flea, although other species of fleas, as well as lice and mites, have occasionally been implicated (35,36). Murine typhus is transmitted to humans by inoculation of infected flea feces into skin abraded by scratching; interestingly, *X. cheopis* can also transmit via its bite under experimental conditions (36–38). Fleas, like lice, acquire *Rickettsia* upon feeding on an infected host (rats, opossums, and other small mammals). *R. typhi* proliferates almost exclusively in the epithelial cells of the flea's gut and then is excreted in the feces. While *R. prowazekii* is lethal to lice, *R. typhi* does not have pathological effects on fleas.

Spotted fever group rickettsiae are transmitted by the bite of any developmental stage (larva, nymph, and adult) of hard ticks (family Ixodidae), particularly the genera *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. Rickettsiae infect multiple tissues of the tick, including salivary glands and ovaries. For this reason, *Rickettsia* can be maintained by transovarial

(adult female to egg) and transstadial (egg to larva to nymph to adult) transmission (39). Ticks puncture the skin with their chelicerae and anchor themselves using the hypostome; such anchoring is reinforced through salivary secretion of cement. The chelicerae rupture superficial small vessels in order to create a small pool of blood, a cavity from which female ticks feed for several days; unlike females, male ticks feed repeatedly in small amounts. The fluidity of the blood at the feeding site is maintained through periodic injection of saliva containing anticoagulant substances into the bite site (40).

Of the transitional group of *Rickettsia*, *R. felis* and *R. australis* infect endothelial cells, while *R. akari*, the agent of rickettsial pox, also infects macrophages. One of the cellular receptors for *Rickettsia* (identified with *R. conorii*) is the multifunctional Ku70 protein, which, although ubiquitous, is very abundant on the surface of endothelial cells. It was determined through a biochemical approach with affinity chromatography that the rickettsial ligand of Ku70 is the outer membrane protein OmpB, also known as Sca5 (41). Moreover, heterologous expression of OmpB in *E. coli* demonstrated that the interaction of OmpB with Ku70 triggers not only adhesion but also invasion through a zipper mechanism dependent on regulation of microtubules and the actin cytoskeleton (through multiple pathways that activate the Arp2/3 complex) (42) with the participation of c-Cbl, clathrin, and caveolin 2 (43). Spotted fever group *Rickettsia* also use OmpA to adhere to host cells (44); however, it is not known whether this rickettsial protein also participates in the entry mechanism. *Rickettsia* may also enter phagocytic cells such as macrophages (which are a secondary target of most *Rickettsia*) by antibody-mediated opsonisation (45).

After internalisation, unopsonised rickettsiae escape from the phagosome into the cytosol. This activity is most likely mediated by the rickettsial genes *pld*, which encodes an enzyme with phospholipase D activity (46), and *tlvc*, which encodes a haemolysin (47). When rickettsial *tlvc* or *pld* (which is more potent) is expressed in *Salmonella enterica*, this vacuolar intracellular parasite becomes capable of escaping into the host cytosol (48).

Ehrlichia (previously known as *Cowdria*) *ruminantium* is the only *Ehrlichia* that primarily infects endothelial cells; it is transmitted by several species of *Amblyomma* ticks (39). The disease, heartwater (named after the prominent pericardial oedema that is frequently present), occurs in large ungulates (including livestock) of sub-Saharan Africa and three Caribbean islands. Although there is a mouse model of heartwater in which endothelial cells are infected (49), the number of publications addressing the endothelium-*Ehrlichia* relationship is limited. Thus, much of the information discussed in this review pertains to *Rickettsia* and, secondarily, to *Orientia*.

Clinical considerations

The bacteria described in this review cause severe febrile illnesses with systemic multi-organ involvement; cases are not infrequently fatal even in young immunocompetent individuals if appropriate antibiotics are not administered early (50–53). Damage to endothelial cells, the main target cells, is reflected in the clinical features of all rickettsial diseases (54) and the circulation of detached infected endothelial cells in patients infected with spotted fever group *Rickettsia* (55,56). Injury to endothelial cells in the lungs and brain results in the most severe manifestations of these diseases including non-cardiogenic pulmonary oedema, interstitial pneumonia, adult respiratory distress syndrome, meningoencephalitis, seizures, and coma (54,57–59). Other severe manifestations can develop including acute renal failure, haemorrhagic phenomena, focal neurological deficits, peripheral oedema and hypovolemic hypotension due to leakage of intravascular fluid into the extravascular space. The initial symptoms are, however, those of a flu-like syndrome. Such non-specific initial clinical presentation and the lack of commercially available

diagnostic methods that are useful during the acute stage (60,61) partially explain the underreporting of these conditions.

Epidemic typhus is among the most severe infectious diseases affecting mankind. It has, in fact, changed the course of history by decimating armies and civilian populations during times of war (62,63). Clinically, it is similar to murine typhus, although more severe, including serious neurological manifestations (e.g., deafness, delirium, confusion and even coma). Furthermore, murine typhus has a mortality rate of less than 2%, while epidemic typhus' mortality ranges from 10 to 60%. The geographic distribution in underdeveloped regions and unavailability of effective diagnostic tests for murine typhus partly explains its severe underreporting despite its widespread occurrence (35). Interestingly, human epidemic typhus associated with flying squirrels is clinically indistinguishable from murine typhus (59). Also, epidemic typhus is the only known rickettsiosis that can present in a recrudescence form years after the primary infection (Brill-Zinsser disease). This form of epidemic typhus is a source of new epidemics if it occurs at a place and time with deteriorated living and hygiene conditions that would favor the presence of the human body louse. The current low level of immunity to *R. prowazekii* in the general population, the history of its development as a bioweapon, transmissibility by aerosol, prolonged infectious stability in louse feces (its vector), high infectivity (fewer than 10 organisms), and potential for human-to-human transmission in louse-infested populations (64,65) justify its designation, together with *R. rickettsii* (the agent of Rocky Mountain spotted fever), as category B and C (respectively) select agents with potential use for bioterrorism.

Although all the bacteria discussed here produce similar symptoms early during the course of the disease, some lead to more severe and potentially fatal diseases (e.g. Rocky Mountain spotted fever and epidemic typhus). In several infectious diseases, bacterial load correlates with disease severity, and this has been directly demonstrated for scrub typhus (66). However, some of the clinical variability observed among the rickettsioses in terms of severity could also be explained by several mechanisms that are not necessarily mutually exclusive:

- Cross-protecting immunity owing to infections with other rickettsiae that cause cross-reactive humoral or cell-mediated immune responses (67).
- Infections with strains of *Rickettsia* (of the same species) of variable virulence (68,69).
- Host factors conferring variable resistance (the ability to limit pathogen burden) to virulent *Rickettsia* among heterogeneous human populations.
- Variable resilience (the ability to limit the health impact of a given pathogen burden) (70) to the pathogenic effects of *Rickettsia* among heterogeneous human populations; this is in agreement with the evidence that resistance against intracellular bacteria is genetically controlled and highly polygenic (71).
- Environmental factors (which may be different among different populations) affecting the anti-rickettsial immune response (e.g., nutritional status, concurrent infections, use of drugs of abuse such as alcohol and medications such as sulfonamides, and exposure to vectors with immunomodulatory activities in their saliva).
- Different types of relationship with endothelial cells and different responses of those cells may underlie the observed clinical spectrum. For instance, the dynamics of activation of NF- κ B and p38 MAPK (as well as the production of CXCL8 and CCL2) as a consequence of rickettsial infection differs between *R. typhi* and *R. conorii* in experiments performed with cultured human umbilical vein endothelial

cells (HUVECs) (72). Similarly, spotted fever group rickettsiae associated with clinical entities of contrasting severity induce equally contrasting inflammatory responses in cultured endothelial cells and the sera of patients with spotted fever group rickettsioses (73). One must also remember that the serological responses against rickettsiae are highly cross-reactive within each group. Thus, the high seroprevalence reported in some endemic areas (74) might be explained by the circulation of other less virulent, yet pathogenic, rickettsiae such as *R. parkeri* in the Americas (75), or even *Rickettsia* of undetermined pathogenicity such as *R. amblyommii*.

- Lastly, the initial path of infection may be a contributing factor in determining the severity of the disease, particularly for spotted fever group rickettsioses. Some rickettsiae proliferate at the site of inoculation eventually leading to local necrosis (an eschar) (76). This period might buy time for the adaptive immune response against *Rickettsia* to develop, particularly if the path of initial spread involves lymphatic vessels with immune events in the draining lymph nodes preceding haematogenous spread. This hypothesis would be in agreement with the observation that those rickettsioses associated with an eschar and regional lymphadenitis are not the ones producing the highest mortality. On the other hand, rickettsioses that are rarely associated with the formation of an eschar (such as Rocky Mountain spotted fever) are the ones that produce the highest mortality. Perhaps, in those more severe rickettsioses there is earlier vascular dissemination. Of course, the ultimate determinant of clinical outcome might actually be the species and developmental stage of the vector inoculating the *Rickettsia* or *Orientia* because of the effects of immunomodulatory substances in the arthropod saliva. This aspect is just now beginning to be explored for rickettsioses.

More detailed information about the clinical aspects of the diseases discussed here can be found in excellent recent reviews (77–82).

Response of the infected endothelium

The target cells of *Rickettsia*, *O. tsutsugamushi*, and *E. ruminantium*, the endothelial cells, form a layer of flat cells that line the vasculature. Such a critical location allows the endothelium to serve regulatory functions in angiogenesis, haemostasis, permeability and solute exchange, vascular tone, immunity, and inflammation (83–87). Infection of endothelial cells with these bacteria affects all of these functions.

Mechanisms that may participate in the increased vascular permeability observed in humans infected with *Rickettsia* or *Orientia* include endothelial detachment and denudation of vessels, the production of vasoactive prostaglandins as a consequence of increased expression of COX-2 (88), endothelial production of nitric oxide (89), effects of inflammatory cells and their mediators (90), and possible changes in the inter-endothelial junctions. *In vitro*, rickettsial infection induces a change into a spindle shape and the formation of actin stress fibers and discontinuities in the inter-endothelial adherens junctions, although this occurs only at later time points when endothelial cells are completely filled with rickettsiae (91). However, in a more recent study using electric cell-substrate impedance sensing, a very sensitive method, it was demonstrated that *R. rickettsii* increases the permeability of a human brain endothelial cell line (i.e. decreased transendothelial electrical resistance) starting within a few hours after infection (90). This change was amplified by addition of tumour necrosis factor (TNF)- α and interleukin (IL)-1 β . Whether the actin polymerisation activity of most *Rickettsia* (but not *R. prowazekii*, which does not polymerise actin) directly affects endothelial functions, particularly the regulation of vascular permeability, remains to be determined experimentally.

Vascular denudation of the endothelium may result from direct damage to host cells. One of the possible mechanisms of damage is a rickettsial phospholipase A2 activity that was identified through the use of the inhibitor p-bromophenacyl bromide and antibody to king cobra venom phospholipase A2 (92); although a gene with this predicted function has not been identified in any of the sequenced rickettsial genomes, a large proportion of those sequenced genomes consists of genes of unknown function. On the other hand, most rickettsiae have a phospholipase D that may contribute to direct host damage. Spotted fever group *Rickettsia* may also damage cells at the time of exiting when directional actin polymerisation (the rickettsial gene *rickettsial actin* [93] regulates actin polymerisation) is followed by membrane rupture (94). On the other hand, typhus group *Rickettsia*, which lack directional actin polymerisation (*R. typhi* polymerises actin, but not directionally) (95), grow inside infected cells until the cells burst; two genes encoding proteins with possible membranolytic activities, *pat1* and *tlyA*, could play a role here, and this needs to be investigated. Oxidative stress also likely contributes to endothelial damage through lipid peroxidation (96–100). This mechanism is further supported by *in vitro* data indicating that antioxidants reduce the *Rickettsia*-induced damage (101). Moreover, endothelial cells respond to rickettsial infection with the upregulation of protective mechanisms including haeme oxygenase 1 (102), an enzyme that regulates many vascular processes including antioxidant mechanisms.

Rickettsia-infected endothelial cells also acquire a procoagulant phenotype. Experiments with cultured human endothelial cells infected with various rickettsiae show that rickettsial infection induces the expression of tissue factor (103), production of Platelet Activating Factor (104), expression of thrombomodulin (105,106), secretion of plasminogen activator inhibitor (107), and release of von Willebrand factor (103,108). Disseminated intravascular coagulation occurs only rarely in lethal cases and is not a common feature of rickettsioses (109). The regulation of haemostasis during endothelial cell infections was recently reviewed (110).

Relatively little is known about the endothelial inflammatory phenotype in response to rickettsial infection. Cultured human endothelial cells become activated upon rickettsial infection resulting in the secretion of cytokines and chemokines such as IL-1 β , IL-6, and CXCL8 (111,112), the expression of adhesion molecules such as E-selectin (CD62E), VCAM-1 (CD106), and ICAM-1 (CD54) (113,114), and the secretion of prostanoids (115) as a consequence of the induction of COX-2 via signaling through p38 MAPK (88). *Ehrlichia ruminantium* also triggers the production of IL-1 β , IL-6, and CXCL8 from infected cultured endothelial cells (116). In a mouse model of *R. conorii* infection of the endothelium (117), there is prominent expression of the chemokines CCL2, CCL3, CCL4, CCL12, CX3CL1, CXCL9, and CXCL10 (118,119). Most of these responses may be initiated by direct *Rickettsia*-triggered activation of the transcription factor NF- κ B (120–122), the activation of p38 MAP-kinase (123), and/ or the activation of TLR4 (106). Interestingly, activation of NF- κ B is also very important in maintaining the rickettsial cellular niche through inhibition of apoptosis (124). *O. tsutsugamushi*-susceptible mice also produce cytokines (TNF- α , IFN- γ , IL-10, and IL-12) and chemokines (CCL2, CCL3, and CXCL1) in response to infection; however, these data are derived from an intraperitoneal model of infection that does not replicate the clinical and pathological characteristics of human scrub typhus (125).

Although much is known about the mechanisms of transendothelial migration of leukocytes (126), the interactions between leukocytes and endothelial cells during rickettsial infections have not been extensively studied. Only recently, it was demonstrated that *R. prowazekii* infection of cultured endothelial cell lines grown in transwell inserts favors transmigration of peripheral blood mononuclear cells (127). Such a transmigration of leukocytes was greater when endothelial cells were infected with virulent *R. prowazekii* as compared to the

attenuated Madrid E strain. It was also shown that the inflammatory phenotype of the transmigrated cells depends on soluble factors produced by *Rickettsia*-infected endothelial cells.

The demonstrated role of CD8+ T cells in anti-rickettsial immunity in animal models (128) and the perivascular lymphocytic infiltrates containing CD8+ T cells in *Rickettsia*-infected humans (14,118,129) suggest that endothelial cells play an important role in antigen presentation and activation of CD8+ T cells. Although not yet proven for rickettsial infections, this should not be surprising in light of recent evidence. Endothelial cells can cross-present exogenous antigens on class I MHC (130). What is more, MHC class I and class II molecules (only class I for mice) are constitutively expressed by endothelial cells *in vivo* (131,132). The functional significance of this expression is highlighted by evidence that endothelial antigenic presentation triggers memory T cell activation and proliferation (133,134) as well as antigen-specific translocation of T cells into the perivascular space (135–137). On the other hand, the capacity of endothelial cells to activate secondary (memory) T cell responses, which has been demonstrated in other models (131,132,138), has not been verified in infectious diseases. Given the fact that endothelial cells can express an array of T cell co-stimulatory molecules (e.g., CD58, CD80, CD86, CD2, CD40, CD154, CD134L, and ICOS-L) (139–149), it is possible that they could play an important role in regulating the primary cellular immune response against endothelium-target pathogens. This aspect merits attention given the evidence suggesting that endothelial cells can activate naïve alloreactive CD8+ T cells *in vivo* (150,151) as well as regulatory CD4+ T cells (152). Such studies will have to consider the evidence, although provided only for *E. ruminantium*, that infection of primary endothelial cells inhibits MHC class I and II expression (153). For *Rickettsia*, we only know that the mouse endothelial cell line SVEC4–10 does not downregulate MHC class I expression after *R. conorii* infection (unpublished observation).

As a consequence of endothelial activation by cytokines such as IFN- γ and TNF- α , presumably produced by NK cells and lymphocytes after cognate interactions with infected endothelial cells, endothelial cells acquire the ability to kill rickettsiae through nitric oxide produced by iNOS (particularly in mouse endothelial cells) (154) and hydrogen peroxide (tested in human endothelial cells) (155). Nitric oxide is also bactericidal for *Ehrlichia ruminantium* growing in endothelial cells (156). In an experiment with an immortalised human brain endothelial cell line, *R. rickettsii* alone induced the production of nitric oxide. Such effect was further enhanced by TNF- α , IL-1 β , and IFN- γ (90). It is interesting to note that doxycycline, the recommended first-line treatment for rickettsioses and scrub typhus (157), decreases the expression of iNOS (158). On the other hand, doxycycline counteracts the procoagulant phenotype acquired by *Rickettsia*-infected endothelial cells (106). The combined effect of these activities on the final outcome of clinical rickettsial infections warrants further investigation.

The role of vectors

Ticks are not passive vehicles of transmission of infectious agents in their saliva (159–161). Tick saliva induces changes in the host environment that are critical for both successful blood feeding as well as pathogen transmission and establishment in the host (162,163). Ticks do this by modulating or down-regulating host haemostasis, innate and specific acquired immune defenses, complement activation (164), angiogenesis, and extracellular matrix regulation (163,165,166). Many of these aspects were recently reviewed (5,163).

Very little research has directly explored the influence of tick salivary proteins on endothelial biology. We know that expression of endothelial cell adhesion molecules is significantly down-regulated by tick salivary gland proteins (167). In this study, salivary

gland extracts from *Dermacentor andersoni* (the wood tick) reduced the upregulation of ICAM-1 (but not other adhesion molecules) induced by TNF- α on a mouse endothelial cell line. On the other hand, salivary gland extracts from *Ixodes scapularis* ticks reduce the expected upregulation of VCAM-1 and P-selectin, but not ICAM-1, induced by TNF- α upon treatment of the same mouse endothelial cell line. These changes have important implications for reduced leukocyte migration into tick bite sites and for host responses to rickettsiae in endothelial cells. Furthermore, the regulatory mechanisms appear to be different for different species of ticks and may determine the composition of the leukocytic infiltrate at bite sites and affect the ability of antigen-specific T cells to engage with endothelial cells as antigen presenting cells.

Much research remains to be done in order to understand the interactions between vectors of rickettsial diseases and the host endothelium. Indeed, we could not find publications addressing this important aspect. For now, some clues may be inferred from alternative models. As explained above, female ticks feed for several days by maintaining a blood-filled feeding cavity. One mechanism by which this may be accomplished is through inhibition of endothelial proliferation and the angiogenic reparative response. For example, salivary gland extracts of *I. scapularis* (but not from mosquitoes) inhibit the proliferation of human dermal microvascular endothelial cells *in vitro* and decrease endothelial cell attachment through proteolysis of the integrins $\alpha 5\beta 1$ and $\alpha 1\beta 1$ (168). Furthermore, those tick salivary gland extracts inhibit the sprouting of vessels in an *in vitro* angiogenesis assay with aortic rings in Matrigel. A troponin I-like molecule has been suggested as the mediator of this inhibitory activity (169).

The future

Much of the molecularly detailed data obtained thus far in regard to infection of the endothelium with *Rickettsia*, *Orientia*, and *E. ruminantium* has been derived from *bona fide* endothelial cell lines, endothelial cell lines produced by fusion with epithelial cells (such as EA.hy926), cell lines misidentified as endothelial (such as ECV304) (170), and primary endothelial cells derived from large vessels (such as HUVECs) cultured under static conditions. Moreover, identification and purification of primary endothelial cells has sometimes been performed with non-specific markers, such as lectins, which also mark monocytes and macrophages. This confusion is particularly troublesome in light of the evidence indicating that myelocytic lineage cells can transdifferentiate into endothelial cells under certain conditions (171–173). Of course, this possibility should not be a cause of surprise given the intimate developmental connections between endothelium and haematopoietic cells (174,175).

Endothelial cells cultivated *in vitro* are very different from endothelial cells *in vivo* due to dynamic tissue microenvironment-mediated regulation (including the constantly changing environment of inflammation) (176–180), blood flow-mediated regulation, particularly its anti-inflammatory effect (181–183), and differences that result from the remarkable heterogeneity of the endothelium throughout the different anatomic portions of the vasculature (184–187). This evidence highlights the importance of studying endothelium-target infections using *in vivo* models whenever possible. Alternatively, if the use of *in vitro* models becomes necessary, the endothelium should be studied under laminar fluid flow since this force is a major regulator of the endothelial phenotype. For instance, steady laminar shear stress applied to cultured endothelial cells induces the upregulation of COX-2 (7-fold over controls under static conditions), which, in turn, induces haeme oxygenase 1 (HO-1) whose activity stops TNF- α generation in endothelial cells (188). In light of the involvement of all these pathways in rickettsial diseases, it will be very important to explore their role in a truly physiological context. Such exploration should focus on endothelial cells

of the microvasculature, which constitutes the bulk of the cells infected during clinical rickettsial infections.

Many relevant aspects of endothelial pathophysiology during endothelium-target bacterial infections remain to be explored; among them, we would like to highlight some of the most intriguing ones: 1) exosomes and microparticles produced by activated endothelial cells; 2) gap junction communication between endothelial cells; 3) alterations of the endothelial glycocalyx; 4) the role of nitric oxide as an immunological effector in human endothelial cells; 5) the regulatory role of other vessel wall cells such as smooth muscle cells, mast cells, and pericytes; 6) the consequences of a disrupted polarised endothelial response; 7) the role of microRNA-mediated regulation; 7) the role of endothelial progenitor cells (bone marrow-derived circulating endothelial precursors) in repairing *Rickettsia*-mediated vascular damage; 8) the role of caveolae in permeability changes; and 9) the role of infected endothelial cells in orchestrating and regulating the anti-microbial immune response. Even some aspects that at first glance may seem unconnected, such as the regulation of expression of ion channels in endothelial cells, may play an important role in explaining some elements of the pathophysiology of rickettsioses during severe haemorrhagic presentations (189). In conclusion, much remains to be investigated in order to understand the role of endothelial cells in the pathogenesis of endothelium-target bacterial infections. A better understanding of this aspect through the study of these pathogens as alternative experimental models will offer new insights into the physiology of the endothelium that have not been revealed by other models, particularly those of autoimmunity, atherosclerosis, and allograft rejection. Finally, the results of the proposed new lines of investigation will be applicable to other endothelial-target infectious agents (including hantaviruses, cytomegalovirus, Nipah virus, and Kaposi's sarcoma-associated herpesvirus) as well as non-infectious conditions in which endothelial cells play an important immunological role such as atherosclerosis, cancer, autoimmune vasculitides, and solid organ transplantation.

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