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Host-cell interactions with pathogenic *Rickettsia* species

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Abstract

Pathogenic *Rickettsia* species are Gram-negative, obligate intracellular bacteria responsible for the spotted fever and typhus groups of diseases around the world. It is now well established that a majority of sequelae associated with human rickettsioses are the outcome of the pathogen's affinity for endothelium lining the blood vessels, the consequences of which are vascular inflammation, insult to vascular integrity and compromised vascular permeability, collectively termed 'Rickettsial vasculitis'. Signaling mechanisms leading to transcriptional activation of target cells in response to Rickettsial adhesion and/or invasion, differential activation of host-cell signaling due to infection with spotted fever versus typhus subgroups of *Rickettsiae*, and their contributions to the host's immune responses and determination of cell fate are the major subtopics of this review. Also included is a succinct analysis of established *in vivo* models and their use for understanding Rickettsial interactions with host cells and pathogenesis of vasculotropic rickettsioses. Continued progress in these important but relatively under-explored areas of bacterial pathogenesis research should further highlight unique aspects of Rickettsial interactions with host cells, elucidate the biological basis of endothelial tropism and reveal novel chemotherapeutic and vaccination strategies for debilitating Rickettsial diseases.

Keywords

apoptosis; chemokine; cytokine; endothelium; NF- κ B; p38 MAPK; *Rickettsia*; spotted fever; rickettsioses; typhus; rickettsioses; vasculitis

The Gram-negative α -proteobacteria belonging to *Rickettsia* species include two major antigenically defined groups, namely the spotted fever group (SFG) and the typhus group (TG), although tick-associated *Rickettsia bellii* and *Rickettsia canadensis* have been categorized as an 'ancestral' group based on neighbor-joining phylogenetic analysis [1,2] and *Rickettsia akari*, *Rickettsia australis* and *Rickettsia felis* have been grouped together as another distinct 'transitional group' that shares immediate ancestry with the members of SFG *Rickettsiae* [3, 4]. Caused by *Rickettsia rickettsii*, Rocky Mountain spotted fever (RMSF) is one of the most

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severe spotted fever group rickettsiosis in the western hemisphere owing to the greater than 20% mortality without adequate antibiotic treatment [5]. In general, Mediterranean spotted fever (MSF) due to *Rickettsia conorii* is considered to be a milder rickettsiosis in humans with a lower mortality rate than RMSF [6], but accumulating recent evidence for much wider geographic distribution and increased severity raises important new questions and challenges posed by human infections with *R. conorii* [7]. A prospective study of a population of Portuguese patients with confirmed *R. conorii* infection documents the Rickettsial strain virulence and host alcoholism as aggravating risk factors for disease severity and association with acute renal failure and hyperbilirubinemia as important determinants of mortality [8]. The pathogenic potential of *Rickettsia parkeri* and *Rickettsia amblyommii* to cause human rickettsioses has also been realized fairly recently [9,10]. Transmission of Rickettsial diseases by previously unknown, unexpected arthropod vectors further attests to the pathogen's ability to adapt to new ecological niches and maintain virulence [11].

Available estimates attribute more than 3 million human deaths during the last century to louse-vectored *Rickettsia prowazekii*, the etiological agent of epidemic typhus. Despite the fact that a majority of these deaths occurred during or immediately after World Wars I and II, epidemic typhus outbreaks in disparate global communities have been known to occur fairly recently [12]. With a mortality rate of 10–60%, epidemic typhus is one of the most severe infectious diseases of humans, the differential diagnosis of which can often be difficult due to nonspecific early symptoms such as fever, headache, malaise and myalgia. Additionally, *R. prowazekii* is the only pathogen among various Rickettsial species with a recognized capacity to maintain persistent subclinical infection in convalescent patients, which can later manifest as Brill-Zinsser disease or recrudescent typhus. Another major TG species *R. typhi*, transmitted to humans by fleas, is responsible for endemic or murine typhus, a relatively mild acute febrile illness [13]. The potential for deliberate use of pathogenic *Rickettsia* species is abundantly clear from the development of *Rickettsia prowazekii* as a battlefield weapon [14]. The possibility that antibiotic-resistant strains may have been or can be developed also remains a matter of grave concern [1]. Thus, considering the potential for illegitimate use to inflict severe disease and associated morbidity/mortality, *R. prowazekii* and other *Rickettsia* species have been classified as select agents and priority pathogens for biodefense-related research [1,14,15].

Primary/preferred target cell(s) during Rickettsial infections

Some of the most common phenotypic characteristics of *Rickettsia* include their strict intracellular location and lifecycle association with arthropods. Although pathogenic *Rickettsiae* are able to infect and replicate in a number of different cell types *in vitro*, a unique property during *in vivo* infection is their affinity for vascular endothelial cells (ECs) lining small and medium-sized blood vessels in humans and in established animal models of infection [16–18]. Attributed to their ability to invade, colonize and disseminate through the endothelium culminating in damage to vascular networks, the typical tell-tale symptoms of RMSF, MSF and epidemic as well as endemic typhus manifest as dermal lesions of various types such as maculopapular and petechial rash in a majority of patients. The recently emerging concept of 'EC heterogeneity' dictates the existence of significant morphological and functional differences between large and small vessels and between cells derived from various microvascular endothelial beds [19], necessitating the need for comprehensive analysis of the biological basis of Rickettsial affinity for vascular ECs. However, it is also important to consider that infection of underlying vascular smooth muscle cells following dissemination to distant endothelium and disruption of its uniform monolayer, and infection of perivascular cells such as monocytes and macrophages is also evident [20], which may also contribute to the pathogenesis and complications of Rickettsial diseases. In this context, a notable exception to

the vasculotropic nature of *Rickettsiae* is *R. akari*, the causative agent of Rickettsialpox, for which the determined predominant target cell type is CD68⁺ macrophages [21].

Salient pathologic features of Rickettsial diseases

As the major target of pathogenic *Rickettsiae* is the endothelium lining of vital organs in humans, a majority of sequelae reflect infection-induced damage to the vascular system. As crucial components of the vasculature, ECs exhibit a number of unique properties and serve to maintain vascular homeostasis. Known to both produce and react to a wide variety of mediators including cytokines, growth factors, adhesion molecules, vasoactive substances and chemokines, with effects on many different cell types, ECs have recently emerged as key immunoreactive cells involved in host defense and inflammation [17,22]. Accordingly, a majority of salient pathologic features associated with RMSF and MSF are attributed to widespread infection of endothelium resulting in damage to blood vessels, altered vascular permeability and vascular inflammation/dysfunction collectively termed 'Rickettsial vasculitis' [23–25]. The clinical manifestations in severe, untreated cases often manifest as encephalitis leading to neurological symptoms such as delirium, coma and seizures; pulmonary edema, interstitial pneumonia and acute respiratory distress syndrome; hypovolemic hypotension leading to acute renal failure, occasionally as multiple organ failure, and rarely as disseminated intravascular coagulation [5,6,16,18,25]. Similarly, vascular dysfunction and damage are the major contributing factors to the complications of human TG rickettsioses as well. From a physician's perspective, considerations of epidemiologic aspects of Rickettsial diseases and travel history of patients in conjunction with thorough clinical examination for the presence of specific rashes (although rash may develop very late during the disease or may not be identified in some cases) are critically important, since patients with rickettsioses usually present with nonspecific 'flu-like' symptoms such as malaise, myalgia, headache, anorexia and chills.

Rickettsial interactions with host cells *in vitro*: adhesion & invasion

For pathogenic *Rickettsiae* dependent upon a nutrient-rich intracytoplasmic niche and fastidious growth requirements within the host cell, target-cell invasion is an essential prerequisite for subsequent intracellular replication and intercellular spread. Manipulations of the host-cell cytoskeleton or loss of Rickettsial viability adversely affect entry into human ECs via induced phagocytosis, implicating that adherence of a viable bacterium to the cell surface triggers intracellular uptake by a metabolically active host cell [26]. Among well-characterized major surface-exposed outer membrane proteins, rOmpA and rOmpB are present in SFG *Rickettsiae* whereas TG organisms only possess rOmpB. Although initial inhibition studies identified rOmpA as a protein critical for *R. rickettsii* adhesion to host cells [27], recent proteomics-based analysis has revealed two additional putative Rickettsial adhesins, one of which is the C-terminal β -peptide of rOmpB and the other is encoded by the gene *RC1281* in *R. conorii* and the gene *RP828* in *R. prowazekii* [28]. Interestingly, rOmpB interacts with Ku70, a predominantly nuclear DNA-dependent protein kinase, which is also present in cytoplasm and plasma membrane, and this interaction has been implicated in *R. conorii* internalization into host Vero and HeLa cells. Rickettsial invasion requires the presence of cholesterol-rich microdomains containing Ku70 and recruitment of a ubiquitin ligase, c-cbl, to the entry foci for ubiquitination of Ku70 [29]. Additional evidence for potential coordinated involvement of upstream signaling mechanisms via Cdc42 (a GTPase), phosphoinositide 3-kinase, c-Src and other protein tyrosine kinases in the activation of the Arp2/3 complex or other as yet unknown pathways in the activation of p38 MAPK suggests an important role for host-cell actin polymerization in Rickettsial internalization [29–31]. In this regard, recent evidence further indicates that Ku70–rOmpB interactions are sufficient to mediate Rickettsial invasion of nonphagocytic host cells and the process of internalization also involves contributions of

clathrin and caveolin-2-dependent endocytosis [32]. Detailed investigations by electron microscopy indicate that Rickettsial entry into mammalian cells occurs within minutes after bacterium–host-cell contact and, thus, is almost instantaneous and once internalized, *Rickettsiae* are capable of quickly escaping into the cytoplasm, presumably prior to phagolysosomal fusion [33] and likely via a phospholipase activity [34].

Rickettsial interactions with host cells *in vitro*: activation of intracellular signaling mechanisms

It is now widely accepted that interactions between host ECs and invading *Rickettsiae* constitute one of the most fundamental and important aspects underlying the onset and progression of infection, replication within the intracytoplasmic niche, dissemination through the host and pathogenesis of resultant diseases. A major breakthrough in this area was the first description of an *in vitro* model of infection of cultured human ECs with *R. rickettsii*, documenting early cell-to-cell spread without detectable host-cell injury, culminating in extensive membrane damage and eventual cell death [35]. This was soon followed by a series of findings that ECs are not simply injured by infection, but also launch distinct cellular responses including functional changes indicative of an activated phenotype – a phenomenon referred to as ‘endothelial activation’. Specifically, *in vitro* endothelial responses to *R. rickettsii* and *R. conorii* include changes in the surface adhesiveness for platelets [36]; increased expression of tissue factor [37,38], IL-1 α [39,40], intercellular and vascular cell-adhesion molecules [41], and E-selectin [42]; increased synthesis of plasminogen activator inhibitor-1 [43,44]; and release of von Willebrand factor from Weibel–Palade bodies [37,45]. In addition, infection with the TG species *R. prowazekii* results in enhanced secretion of the arachidonate-derived autocooids PGE₂ and PGI₂ [46]. Thus, unlike the normal, quiescent state of endothelium yielding an anticoagulant and antithrombotic barrier between the blood and tissue, ECs infected with *Rickettsiae* exhibit procoagulant and proinflammatory properties, likely determinants of the manifestations and severity of vasculotropic rickettsioses.

An impressive variety of pathophysiological situations affecting ECs of the vasculature leads to the expression of genes dependent on nuclear factor (NF)- κ B family of transcription factors [47]. Listed in Table 1, a majority of early response genes transcriptionally up-regulated in response to *R. rickettsii* and *R. conorii* infection *in vitro* contain NF- κ B binding sites in their promoter regions, indicating that infection-induced alterations in the pattern of gene expression may be governed, at least in part, by activation of NF- κ B. NF- κ B is a dimeric transcription factor composed of homo- and heterodimers of the Rel family of proteins, of which there are five members in mammalian cells; Rel A or p65, c-Rel, Rel B, NF- κ B1 or p50, and NF- κ B2 or p52. These dimers are maintained in the cytoplasm in an inactive form associated with regulatory proteins termed inhibitors of NF- κ B (I κ Bs). Amongst the seven known I κ Bs, I κ B α , I κ B β and I κ B ϵ preferentially associate with Rel protein dimers. In ECs, I κ B ϵ is associated with p65 and to a lesser extent with c-Rel, whereas I κ B α and I κ B β associate with p65, but not c-Rel [48]. An important step in NF- κ B activation, phosphorylation of I κ B is mediated by a high-molecular weight I κ B kinase (IKK) complex composed of two kinase subunits, IKK- α and IKK- β , and a regulatory subunit IKK- γ . As well as phosphorylating I κ B β and I κ B ϵ , IKK activation results in phosphorylation of I κ B α at specific amino-terminal serine residues, a prerequisite for degradation by 26S proteasome [49].

Human ECs infected with *R. rickettsii* display nuclear translocation of NF- κ B (a marker of its activation as a consequence of degradation of masking I κ B and exposure of nuclear location sequences) as early as 1.5 h postinfection and characterized by an early, transient peak at 3 h, followed by a later, sustained phase of more robust activation at 18–24 h with activated NF- κ B species primarily composed of RelA (p65)–p50 heterodimers and p50 homodimers [50]. Such a unique, biphasic pattern of activation in response to an intracellular pathogen involves

activation of both IKK- α and IKK- β , and can be effectively inhibited by specific inhibitors of activities of IKK as well as the proteasome [51]. Intriguingly, however, *R. rickettsii* is also capable of directly interacting with NF- κ B present in its inactive form in EC cytoplasm [52]. Activation of NF- κ B in this 'cell-free' system lacking signaling interactions originating at the cell membrane occurs in a proteasome-independent fashion and appears to be dependent on an as yet unidentified protease activity of *Rickettsiae* [52,53].

The general characteristics of SFG and TG of *Rickettsiae* reveal so many common features that it is easy to forget that there may be significant differences in the pathogenesis of the resulting diseases. As discussed previously, the common features include the fact that both spotted fever and typhus *Rickettsiae* are typical Gram-negative bacteria exhibiting extreme fastidiousness in their growth requirements and that both possess outer membrane proteins that are required for bacterial attachment to, and subsequent infection of, vascular endothelium. There are, however, several interesting contrasts in their patterns of intracellular growth and motility. While SFG *Rickettsiae* (*R. rickettsii* and *R. conorii*) use actin-based motility for intracellular movements and intercellular dissemination, TG organisms display either no (*R. prowazekii*) or very short (*R. typhi*) actin tails [54,55]. Likewise, TG *Rickettsiae* tend to accumulate within the cytoplasm until cell lysis while SFG organisms spread rapidly from cell to cell and usually accumulate in significantly lower numbers (~5–8 times) than TG *Rickettsiae* [23]. Comparative genomics of *R. prowazekii* and *R. conorii*, the representative species of TG and SFG subgroups, respectively, the entire genomes of which were the first to be sequenced, annotated and published [56,57], reveals the presence of about 500 genes unique to *R. conorii*, but very few in *R. prowazekii*. *R. conorii* also has as many as 400 orphan genes with no homologs outside of the *Rickettsiae* [58]. To date, only a small number of Rickettsial genes, which can be annotated confidently by their extensive similarity to proteins of known function in other organisms, have been characterized in some detail. Thus, considering several distinct differences in the intra- and intercellular motility and intracellular growth patterns of spotted fever and typhus *Rickettsiae*, it would be reasonable to hypothesize that the host cell is able to sustain the growth and multiplication of infective organisms and accumulation of progeny during infection with TG owing to significant differences in signaling responses in comparison to those triggered by SFG organisms. In this context, although NF- κ B activation response to infection with *R. rickettsii* and *R. conorii* is almost identical, as expected, comparative analysis further discerns noticeable differences in the intensity and/or kinetics of NF- κ Bs activation pattern in *R. conorii*- versus *R. typhi*-infected host ECs [59,60].

Several studies have suggested a role for protein kinase C (PKC), a family of structurally related, phospholipid-dependent, serine–threonine kinases, in the activation of NF- κ B by infectious agents and other stimuli [61]. Activated PKC itself is also sufficient to induce NF- κ B activation *in vitro* [62]. Downregulation of phorbol ester-sensitive, calcium-dependent classical PKCs (α , β and γ) by either prolonged treatment with high concentrations of phorbol 12-myristate 13-acetate (PMA) or specific inhibition by bisindolylmaleimide I hydrochloride has no effect on NF- κ B activation, but indicates the possibility of potential involvement of a non-PMA responsive, calcium-independent atypical PKC isoform (ζ or δ) in post-transcriptional control of *R. rickettsii*-induced tissue factor expression [63].

MAPKs also play a critical role in signal transduction events. Three major MAPK cascades, namely extracellular signal-regulated kinases, c-Jun-N-terminal kinases, and p38 MAPK, have been identified and characterized. These MAPK subfamilies can be activated simultaneously or independently to constitute a critical component of a central regulatory mechanism that coordinates signals originating from a variety of extracellular and intracellular mediators. Specific phosphorylation and activation of enzymes in a MAPK module transmits the signal down the cascade resulting in phosphorylation of many proteins with substantial regulatory functions throughout the cell. Specifically, MAPKs have been implicated in many

physiological functions including regulation of immune responses, cytoskeletal organization and transcriptional activity of NF- κ B [64,65]. In the vasculature, MAPKs regulate diverse physiological activities including proliferation, migration, apoptosis and barrier function of ECs. In addition, proinflammatory cytokines, shear stress and reactive oxygen species (ROS) also induce signaling mechanisms resulting in the activation of selective MAPKs in a cell-type dependent manner [66]. As evidenced by its increased phosphorylation and enzymatic activity, *R. rickettsii* infection selectively induces activation of p38 MAPK in ECs. Activation of p38 kinase depends on active cellular invasion by viable *Rickettsiae*, appears to involve generation of ROS, and subsequently facilitates host-cell invasion by *R. rickettsii* [67]. *In vitro* experimental evidence further suggests the lack of cross-talk between NF- κ B and p38 signaling mechanisms, because inhibition of p38 does not affect the NF- κ B response of *R. rickettsii*-infected cells [67]. Similar to their effect on NF- κ B signaling, comparative studies during *R. conorii* and *R. typhi* infection also yield evidence for unique differences in signaling through p38 MAPK, which include delayed activation of comparatively less intensity in *R. typhi*-infected host ECs [59,60].

Rickettsial interactions with host cells: oxidative stress

Virtually all types of vascular cells generate superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), the ROS implicated in a variety of vascular diseases, including atherosclerosis. Although ROS are known to contribute to distinct cellular stresses such as heat shock, UV irradiation and so on, it has now become apparent that ROS in the vasculature may even serve second messenger functions. The injury to EC following infection with *R. rickettsii* culminates in widespread dilatation of intracellular membranes, most conspicuously of rough endoplasmic reticulum, loss of osmoregulatory control and cell lysis at 5 or 6 days postinfection [35]. The observation that cells infected with this organism exhibit striking changes in the structural organization of the endoplasmic reticulum–nuclear envelope complex and cytoskeleton led to the hypothesis of induction of oxidative stress during intracellular infection. Indeed, subsequent biochemical studies confirmed the accumulation of intracellular peroxides and superoxide radicals, detection of higher amounts of extracellular H_2O_2 , reduction in the levels of cellular thiols and alterations in the activities of important antioxidant enzymes [68,69]. However, interestingly, cells infected with TG *Rickettsia* show no detectable morphological cytopathic changes prior to cell death [70]. Accruing evidence further indicates an important role for adaptive alterations in oxidant-scavenging mechanisms and beneficial effects of antioxidant compound α -lipoic acid in *R. rickettsii*-infected endothelium *in vitro* [68,71,72]. In an *in vivo* mouse model of RMSF, the activities of important antioxidant enzymes, namely glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and superoxide dismutase, display differential and selective modulation in apparent correlation with Rickettsial titers in target host tissues and yield indirect evidence for protection against infection-induced oxidative injury by α -lipoic acid treatment [73]. Physiologically relevant responses of host cells to infection-induced oxidative stress include increased expression of an isoform of heme oxygenase (HO-1) and ferritin, a multimeric protein capable of storing free intracellular iron and performing potent antioxidant and anti-apoptotic functions in the vasculature [59,69]. HO, a rate-limiting enzyme, oxidatively cleaves heme resulting in the release of ferrous iron and generation of carbon monoxide (CO) and biliverdin, the latter is subsequently converted to bilirubin. Recent discoveries of diverse biological activities of HO metabolites have led to the paradigm shift from HO's role as a 'molecular wrecking ball' to a 'mesmerizing trigger' of cellular events [74]. Vascular ECs contain two distinct HO isozymes HO-1 and HO-2, which are encoded by different genes. Several lines of evidence suggest that the products of HO activity play an important role in the vascular endothelium by the reduction of oxidative stress, diminution of vascular constriction, attenuation of inflammation, decrease in smooth muscle cell proliferation and inhibition of apoptosis. The heme–HO system also functions as a cellular regulator of vascular cyclooxygenase (COX) and in doing so influences

the generation of prostaglandins. Functional interplay between HO-1 and COX signaling mechanisms in the vasculature likely results in increased expression of the inducible COX isozyme COX-2 and significantly higher levels of prostaglandin secretion [75]. Of these, PGE₂ and PGI₂ are known to cause increased vascular permeability and edema, considered to be cardinal features of acute inflammation during Rickettsial infections. Consequently, a promising rationale for new and improved interventions are the agents that can either quench harmful ROS or potentiate cellular protective mechanisms against oxygen radicals. In this context, we hypothesize that use of naturally occurring biologic antioxidants to preserve homeostasis at sites of vascular injury and pharmacologic or genetic approaches targeting HO-1 or COX-2 in the vessel wall may represent novel therapeutic approaches in treating *Rickettsia* infections and vascular diseases in general.

Rickettsial interactions with host cells: expression and/or secretion of mediators of inflammation

As essential components of the immune system, interferons (IFNs) are key immunoregulatory proteins known to play a major role in the host innate and adaptive immune response. Type I IFNs in humans and mice include a number of IFN- α subtypes and a single species of IFN- β . IFN- γ , a type II IFN produced by activated natural killer and Th1 cells, not only induces antiviral function, but also activates macrophages and dendritic cells in the presence of IL-12 and IL-18 to strengthen innate immune responses to microorganisms. Initial studies on *R. prowazekii* interactions with fibroblast-like L929 cells *in vitro* demonstrated the production of IFNs α and β , but not IFN- γ . Consistently, secreted IFN- α as well as IFN- β were found to have inhibitory activity on the growth of *R. prowazekii* [76]. However, IFN- γ also suppresses the growth of *R. prowazekii* in mouse L929 cells, macrophage-like RAW264.7 cells and human fibroblasts [77–79]. These observations subsequently led to the isolation and characterization of IFN-sensitive and IFN-resistant strains of *R. prowazekii* [79,80]. In addition, IFN- γ also potentiates the inhibitory effect of recombinant human TNF- α on *R. conorii* growth in HEp-2 cells [81]. The ability of primary target cell -type, for instance ECs, and other potential targets of infection such as macrophages and hepatocytes to kill intracellular *Rickettsiae* is dependent on the differential stimulatory effects of IFN- γ , IL-1 β , RANTES and TNF- α . Activation of host cells by these cytokines or RANTES has been shown to be responsible for three predominant rickettsicidal mechanisms, which include either alone or a combination of synthesis of nitric oxide, production of H₂O₂ and degradation of tryptophan via indoleamine-2,3-dioxygenase [82]. *In vivo* studies in animal models have also indicated that IFN- γ has an important protective role in host defense during infection with *R. conorii*, *R. australis* and *R. typhi* [83–85]. Available evidence further suggests that the early immune response against *R. conorii* is associated with natural killer cells and is most likely governed by IFN- γ [86].

Infection of host ECs with *R. rickettsii* or *R. conorii* results in increased expression and secretion of interleukins IL-1 α (but not IL-1 β) and IL-6, chemokines IL-8 and monocyte chemoattractant protein-1 [39,40,60,67,87], and fractalkine [88]. Moreover, infection of P388D1 macrophages with *R. akari* and *R. typhi* results in differential synthesis and expression of IL-1 β and IL-6, indicating a potential correlation with the existence of biological differences between these two *Rickettsia* species [89]. Induced expression of a number of genes encoding these proinflammatory molecules occurs through signaling mechanisms that require activation of NF- κ B, a ubiquitous nuclear transcription factor involved in the control of apoptosis, inflammation and stress response, and p38 kinase, the downstream effector component of a stress-activated MAPK cascade [67,87]. Expression analysis of Mig and IFN- γ inducible protein-10 in various tissues of *R. conorii*-infected mice and brainstem specimens of RMSF patients further indicates a critical role for these T-cell targeting chemokines in an *in vivo* model of infection and in the human disease process [90]. *R. prowazekii*-infected cells display

increased platelet activating factor synthesis and prostaglandin secretion [46,91,92]. A recent study reports that *R. prowazekii* infection of both murine and human ECs *in vitro* results in increased transmigration of leukocytes, most likely through an exacerbated inflammatory response [93]. However, further detailed studies to discern the mechanisms responsible for the expression and secretion of cytokines, chemokines and other immune mediators during *Rickettsia* infection of ECs under constant fluid flow conditions mimicking physiologically relevant perfusion rates and shear stress environments are warranted. This should yield important new information regarding the recruitment of inflammatory cells to the site of infection, a critical determinant of the pathogenesis and outcome of disease.

Rickettsial interactions with host cells *in vitro*: regulation of programmed cell death

Host-cell death is one of the most critical determinants of the progression and outcome of disease during microbial infections. The pathophysiological implications of EC involvement in Rickettsial pathogenesis warrant investigation into the mechanisms of cell death. There is increasing recognition that apoptosis, a tightly regulated process of altruistic suicide, plays a central role in the complex interactions between an invading pathogen and host-cell defense. Regulation of apoptosis becomes even more critical in determining the outcome of infection in intracellular pathogens, which depend on their hosts to survive and propagate. While apoptosis of infected cells is an important host defense mechanism for limiting the spread of infection, viruses and bacteria employ a variety of strategies to inhibit host cell's apoptotic machinery to ensure and enhance the survival of the host as a cell that dies upon infection is a cell that does not aid and abet the intracellular pathogen's multiplication. Thus, depending on the nature of infection, apoptosis can be either advantageous or detrimental to the host. ECs undergoing apoptosis *in vivo* detach from the vessel basement membrane and enter the circulation, where they are rapidly cleared by phagocytes. Such an occurrence at early stages of infection would, indeed, be detrimental for the growth and proliferation of intracellular *Rickettsiae*. Hence, it is possible that, as in other intracellular pathogens such as *Chlamydia* species, successful establishment and progress of infection is contingent on the Rickettsial ability to suppress apoptosis in host cells. The first evidence in support of this hypothesis was the demonstration that the anti-apoptotic functions of NF- κ B are essential for the survival of ECs during *R. rickettsii* infection (Figure 1) [94].

A plethora of apoptotic responses are known to involve the activation of apical caspase-8 or -9: the former by the TNF-receptor family and the latter by release of cytochrome *c* as a consequence of mitochondrial damage. Activation of either of these two initiator caspases can lead to the activation of downstream effector caspases -3, -6, or -7 [95]. Infection of cultured human ECs with *R. rickettsii* with simultaneous inhibition of NF- κ B induces the activation of apical caspases-8 and -9, and also the executioner enzyme, caspase-3. Inhibition of either caspase-8 or -9 using specific cell-permeating peptide inhibitors causes a significant decline in the extent of apoptosis. The peak activity of caspase-3 coincides with the cleavage of poly-(ADP-ribose)-polymerase, followed by DNA fragmentation and apoptosis. As evidenced by the loss of transmembrane potential and release of cytochrome *c* into the cytosol, caspase-9 activation is mediated through the mitochondrial pathway of apoptosis. Thus, activation of NF- κ B is required for the maintenance of mitochondrial integrity of host cells and protects against infection-induced apoptotic death by preventing the activation of caspase-8 and -9 mediated pathways [96].

The B-cell lymphoma-2 (Bcl-2) family of proteins, which includes both pro- and anti-apoptotic factors, play a critical role in the regulation of apoptotic cell death by controlling mitochondrial permeability [97]. The anti-apoptotic proteins Bcl-2 and Bcl-xL reside in the outer membrane of mitochondria and inhibit the release of cytochrome *c*. On the other hand, Bad, Bid and Bax

are cytosolic pro-apoptotic proteins and their translocation to mitochondria promotes cytochrome c release. The phosphorylation of Bad is necessary for its sequestration in the cytosol. The effects of Bid are dependent on the generation of tBid, the active truncated fragment capable of translocating into mitochondria [98]. Determination of the effects of NF- κ B inhibition on proteins of the Bcl-2 family and x-linked inhibitor of apoptosis protein during *in vitro* EC infection by *R. rickettsii* further reveals significant changes in the expression of various pro- and anti-apoptotic proteins (summarized in Table 2), the ultimate outcome of which is the 'equilibrium-shift' towards inhibition of apoptosis [99]. Subsequent findings from the infection of T24-bladder carcinoma cells expressing a super-repressor mutant of I κ B α to inhibit NF- κ B activity and recent evidence documenting resistance of *R. rickettsii*-infected human microvascular ECs to staurosporine-induced apoptosis lend further support to the emerging concept of regulation of mechanisms of programmed host-cell death by intracellular *Rickettsiae* [99,100].

Rickettsial interactions with host cells *in vitro*: alterations in vascular permeability

A widely accepted concept with regards to the pathogenesis of Rickettsial diseases is that increased vascular permeability resulting in fluid imbalance and edema of vital organ systems is a major feature of acute inflammation. The mechanisms underlying compromised permeability of the vasculature are now beginning to be elucidated. *R. conorii* infection of human ECs induces changes in the localization and staining patterns of adherens junction proteins and discontinuous adherens junctions lead to the formation of interendothelial gaps. Interestingly, such changes occur late during *in vitro* infection even if cells are heavily infected at earlier times and are apparently unrelated to the manipulation of host-cell actin cytoskeleton by *Rickettsiae* [101]. As suggested by a noticeable dose-dependent decrease in *trans*-endothelial electrical resistance, *R. rickettsii* infection of human cerebral ECs also results in increased vascular permeability associated with the disruption of adherens junctions. Interestingly, the presence of proinflammatory cytokines IL-1 β , IFN- γ and TNF- α , but not nitric oxide, during infection exacerbates the effects of *Rickettsia* on endothelial permeability, further suggesting that the changes in the barrier properties of vascular endothelium are most likely due to a combinatorial effect of the presence of intracellular *Rickettsiae* and immune responses of the host cell [102]. Another possibility in this regard is the potential contributions of prostaglandins, which are secreted as a result of increased expression of COX-2 and are also known to cause increased vascular permeability and edema [75,103].

Pathogenesis in established *in vivo* models of infection

Vascular dysfunction and damage are the major pathologic sequelae responsible for complications in human disease. Therefore, studying signaling interactions between the vascular endothelium of vital organ systems and different *Rickettsia* species or strains of varying virulence using disseminated *in vivo* vasculature infection represents a major milestone in advancing our understanding of host-pathogen interactions during Rickettsial diseases. Although experimental infection of birds, rodents, lagomorphs and guinea pigs has been attempted and susceptibility of a number of mouse strains to *R. conorii* delivered via intraperitoneal and subcutaneous routes has been established [104], infection of C3H/HeN mice by intravenous administration is reportedly one of the best available animal model of endothelial injury and closely parallels many aspects of human SFG rickettsiosis [105]. With an infectious dose higher than the LD₅₀ (2.25×10^5 pfu), infection of endothelium is established at 24 h with little evidence of cellular injury. Phenotypic perturbances of endothelium, emigration of mononuclear cells to various organ systems and hemostatic/fibrinolytic changes are also apparent during the course of the disease. Disseminated vascular injury during infection affects nearly all organ systems as evidenced by interstitial pneumonia and vasculitic

inflammation of lungs, granulomatous inflammation in the liver, meningoencephalitis in the cerebrum, moderate reticuloendothelial hyperplasia in the spleen and perivascularitis in the kidneys and testes. Expression of the chemokines Mig (CXCL9) and IP-10 (CXCL10), known to target activated T cells through CXCR3, is significantly increased in the lungs, brain and liver of mice infected with sublethal and lethal doses of *R. conorii* [90]. Furthermore, increased expression of fractalkine (CXCL1) appears to coincide with the infiltration of macrophages into *R. conorii*-infected tissues [88]. Selective modulation of superoxide dismutase and glutathione redox pathways in different organs of the infected host is also evident during *in vivo* infection, the extent of which varies depending on the titer of viable *Rickettsiae* in different organs of the host [73]. Similarly, intravenous administration of *R. australis*, the causative agent of Queensland tick typhus, results in disseminated endothelial infection of BALB/c and C57BL/6 mice with involvement of nearly all vital organ systems including the lungs, liver, testes, kidneys and spleen [106]. Surprisingly, neutralization of CXCL9 and CXCL10 through function blocking antibodies or infection of CXCR3 knockout mice suggests no survival advantage or beneficial effects on tissue bacterial titers during *R. conorii* or *R. australis* infection [107]. Capable of recapitulating alterations in the expression of antioxidant enzymes, infection of the pine vole (*Microtus pinetorum*) has also been recognized as a valuable *in vivo* experimental model for studying the pathogenesis of RMSF [108], but a direct and more convenient murine model of *R. rickettsii* infection facilitating the use of well-characterized knockout strains deficient in a particular gene locus has yet to be established.

Experimental *R. prowazekii* infection in small laboratory animals such as cotton rats, gerbils and guinea pigs has also been attempted [109], but the only well-established model for an endothelial target model of typhus rickettsiosis exploits the infection of C3H/HeN mice with *R. typhi*, the etiologic agent of endemic or murine typhus [85]. However, prior to this development, disease characteristics and mechanisms of immunity were investigated by *R. typhi* infection of either guinea pigs or BALB/c mice [110]. Capable of producing the signs and pathologic features similar to human disease, a direct model of epidemic typhus describes the infection of cynomolgus monkeys with the virulent Breinl strain of *R. prowazekii* [111]. A noteworthy recent development is the description of a murine model of *R. prowazekii* infection amenable to laboratory investigations of pathogenetic mechanisms of epidemic typhus [112, 113]. In this model, *R. prowazekii* can be detected in the blood, brain, liver and lungs of infected mice at 24 h and persists in these tissues for up to 9 days. The disease is characterized by interstitial pneumonia, hemorrhages in the lungs and brain, multifocal granulomatous inflammation of the liver and elevated expression of IFN- γ , TNF- α and RANTES in infection-induced tissue lesions. However, whether or not this *in vivo* model of epidemic typhus emulates the characteristic features of human disease, for instance disseminated endothelial-targeted infection, endothelial activation and vascular inflammation, remains a critically important open question.

Correlations or lack thereof with the findings from the clinic

Attributed mainly to the vasculotropic nature of *Rickettsiae* and vascular dysfunction and/or damage, Rickettsial diseases are generally associated with significant changes in the levels of hemostatic/fibrinolytic proteins and mediators of inflammation. Spotted fever rickettsioses due to *R. conorii* and *R. rickettsii* often lead to coagulation abnormalities as demonstrated by pronounced alterations in plasma concentrations of coagulation factors, natural anticoagulants and components of fibrinolytic system [114,115]. An elevation in the levels of plasma fibrin (ogen)-degradation products during *R. rickettsii* and *R. conorii* infection of humans indicates activation of the fibrinolytic system. At the same time, increased levels of circulating fibrinogen likely reflect enhanced synthesis of this acute phase protein. The presence of circulating C1 inhibitor-kallikrein complex and lower concentrations of prekallikrein indicate activation of the kallikrein-kinin system during spotted fever syndromes, for which the evidence for *in*

vivo vascular injury/inflammation and onset of disseminated intravascular coagulation has also been described [116]. Enhanced urinary secretion of 11-dehydro-thromboxane B₂, a major enzymatic metabolite of thromboxane A₂, may largely be due to increased platelet thromboxane synthesis and activation during MSF [117]. Furthermore, in patients with acute MSF (also known as boutonneuse fever), serum levels of IFN- γ , TNF- α , IL-6 and IL-10 exhibit significant increases, whereas IL-1 α and IL-8 are not detected in the blood during any phase of infection [118,119]. The absence of circulating IL-1 α may be explained by *in vitro* findings suggesting that a major portion of newly synthesized IL-1 α remains cell-associated during *in vitro* activation of ECs with *R. conorii* and *R. rickettsii* [39,40]. Although IL-8 has been shown to be present in the Weibel–Palade bodies of endothelium from where it can be released rapidly and *in vitro* interactions between ECs and *R. conorii* or *R. rickettsii* induce the release of von Willebrand factor from Weibel–Palade bodies [37,45,87], it is intriguing that there are no changes in the IL-8 level in blood samples of human subjects with a confirmed diagnosis of *R. conorii* infection. In contrast, a patient with fatal fulminant RMSF was reported to have increased levels of serum IL-8 despite therapy with antibiotics and vaso-pressors [120]. Clinically, plasma levels of IFN- γ in serologically confirmed cases of MSF are significantly higher during acute phase in comparison to the convalescent phase of the disease [119]. Although a similar pattern of increased serum IFN- γ is also seen during the acute phase of fulminant Japanese spotted fever, caused by *Rickettsia japonica* [121,122], its level did not exhibit any changes during the course of African tick-bite fever caused by *Rickettsia africae*, a newly identified SFG species [123]. The inflammatory response to tick-bite fever due to *R. africae* also involves systemic increases in the levels of TNF- α , IL-6, IL-8 and IL-10. Another recent study evaluating serum chemokine (IL-8 and monocyte chemoattractant protein-1) and adhesion molecules (E-selectin and vascular cell adhesion molecule-1) levels in patients with African tick-bite fever and MSF suggests inflammation of higher severity during MSF, indicating the superior inflammatory potential of *R. conorii* compared with *R. africae* [124]. Therefore, it appears that alterations in the balance between inflammatory and anti-inflammatory networks vary in response to infection with different species of SFG *Rickettsiae* and likely correspond to the level of *in vivo* endothelial activation and the virulence potential of the infectious agent. Also, recent examination of mediators of inflammation or the immune response at the site of infection shows elevated intralesional expression of IFN- γ mRNA, particularly in patients with mild/moderate MSF. In addition, there is a positive correlation between high levels of IFN- γ mRNA, the absence of *Rickettsiae* in the blood, and the production of enzymes such as inducible nitric oxide synthase and indoleamine-2,3-dioxygenase, known to be involved in limiting the growth of *Rickettsiae* [125]. Also, severe cases of human Rickettsial infection are associated with acute renal failure and respiratory distress syndrome and the prognosis of spotted fever and typhus patients with genetic glucose-6-phosphate dehydrogenase deficiency is poor due to enhanced severity, indicating the potential importance of redox systems in combating the disease [126]. Importantly, transcriptional responses of the pathogen itself at the point of entry are also beginning to be elucidated and reveal that *R. conorii* in inoculation eschars modulates its gene expression profile to overcome or escape host-defense mechanisms and employs other survival strategies such as adaptive alterations to osmotic stress, modification in cell-surface antigens and regulation of virulence factor expression [127]. As this study shows that *R. conorii* in skin biopsies of MSF patients is mainly associated with inflammatory cells in necrotic areas of the dermis, a potential complicating factor in such analyses is the loss of viable host-cell niche and consequential adverse effects on Rickettsial viability, metabolism and gene expression.

Conclusion & future perspective

Rickettsiae are Gram-negative bacilli characterized by their strict intracellular location, fastidious growth requirements, association with arthropods and tropism for vascular endothelium in mammalian hosts. Rickettsial diseases have been a scourge of humankind

throughout history and infection with pathogenic *Rickettsiae* remains a major health issue, causing significant morbidity and mortality all over the world, including regions where rickettsioses are either re-emerging or remained previously unrecognized likely due to the lack of in-depth epidemiologic surveys and molecular tools for accurate detection. Typhus epidemics due to louse-borne *R. prowazekii* have been estimated to cause more deaths than all wars combined and re-emergence of epidemic typhus in different geographic locations of the world is being described. No vaccines are currently available to prevent the detrimental effects of major human rickettsioses and present day threats posed by *Rickettsia* species include the potential for illegitimate use as bioweapons. This has warranted the designation of *R. prowazekii* as category B and other *Rickettsia* species as category C select agents and priority pathogens for biodefense research. Since *Rickettsiae* exhibit a unique tendency of preferentially targeting vascular endothelium in humans and in animal models of infection, interactions between invading/intracellular *Rickettsiae* and ECs and elucidation of the mechanisms underlying host defense mechanisms represent important avenues for expanding our knowledge of both Rickettsial pathogenesis as well as vascular cell biology. Pathogen-induced cellular signaling represents an important host response that may be involved in changes in gene expression patterns and determination of cell fate. Therefore, continued efforts to decipher the potential roles of upstream signaling via membrane receptors and intracytoplasmic proteins and the downstream effects of NF- κ B and MAPK pathways in determining host cells' inflammatory and apoptotic responses to infection with pathogenic *Rickettsiae* should yield critical insights into signaling mechanisms for *Rickettsia*-induced endothelial activation. It is anticipated that new knowledge from such an approach will unravel novel postulates for immunopathologic mechanisms and intervention strategies for infection-induced vascular inflammation. A promising rationale for new and improved supplemental interventions are agents that can either quench harmful ROS or potentiate cellular protective mechanisms against oxygen radicals. In this context, use of naturally occurring biologic antioxidants to preserve homeostasis at sites of vascular injury and pharmacologic or genetic approaches targeting HO-1 or COX-2 in the vessel wall represent novel therapeutic approaches in treating *Rickettsia* infections and vascular diseases in general. Yet another, hitherto not addressed, aspect of rickettsioses is the definition of Rickettsial interactions with host cells to determine the extent to which SFG and TG species induce anti-apoptotic mechanisms and to identify the apoptosis modulating factors involved. The dissection of the influences that *Rickettsiae* may have upon host gene expression and defense mechanisms favoring either the host or the pathogen has tremendous applications for clinical therapeutic modalities aimed at early clearance of infection.

Despite considerable recent progress in genomics, proteomics and other important aspects of Rickettsial biology, the relative paucity of knowledge of basic cellular and molecular concepts of epidemic typhus pathogenesis is rather astonishing. Specifically, our current understanding of the interactions of typhus *Rickettsiae* with vascular endothelium still remains in its infancy and the first description of a convenient, small animal model of *in vivo* *R. prowazekii* infection has only become available fairly recently. Since it is well established that vascular dysfunction/damage is the major cause of complications of human rickettsioses, obtaining a definition of *in vitro* and *in vivo* signaling interactions between ECs and *R. prowazekii* strains of varying virulence represent important major steps in advancing our understanding of epidemic typhus pathogenesis. These studies will ultimately lead to the development of novel therapeutic/immunologic strategies to combat this debilitating disease.

Executive summary

Rickettsiae & Rickettsial diseases

- *Rickettsia* species are Gram-negative, obligate intracellular bacteria known to derive their nutrition primarily from the cytoplasm of the host cell.

- Two major subgroups of pathogenic *Rickettsiae* are the spotted fever and typhus groups.
- The prototypical spotted fever species, *Rickettsia rickettsii* and *Rickettsia conorii*, are etiological agents of Rocky Mountain spotted fever and Mediterranean spotted fever, respectively.
- The prototypical typhus group species *Rickettsia prowazekii* and *Rickettsia typhi*, are the causative agents of epidemic typhus and endemic or murine typhus, respectively.
- *R. prowazekii* was one of the major killers during World War I and II and is the only Rickettsial species capable of latent infection and manifestation as recrudescent typhus years later.

Primary/preferred target cell(s) during Rickettsial infections

- *Rickettsiae* target vascular endothelial cells lining the small and medium-sized blood vessels during human infections, but can invade underlying tissue such as smooth muscle cells, perivascular macrophages and monocytes.
- CD68⁺ macrophages are the primary targets cells of *Rickettsia akari*, the causative agent of Rickettsialpox.

Clinical & pathological features of rickettsial diseases

- Somewhat nonspecific 'flu-like' initial symptoms include frontal headache, myalgia, fever, fatigue and restlessness/insomnia.
- Maculopapular and petechial rash with centripetal spread (extremities to trunk) for Rocky Mountain spotted fever and progression from the trunk to extremities for louse-borne epidemic typhus.
- Manifestations include gastrointestinal symptoms, respiratory/renal failure, encephalitis, and disseminated intravascular coagulation in rare, severe cases of Rickettsial disease.

***In vitro* interactions with host cells**

- Contact of viable *Rickettsiae* with a metabolically active host cell initiates their uptake via induced phagocytosis.
- Ku70, a DNA-dependent protein kinase, interacts with Rickettsial outer membrane protein B and serves as a receptor in the process of Rickettsial internalization into host Vero or HeLa cells.
- Infection of host endothelial cells with *R. rickettsii* and *R. conorii* and *R. typhi* induces the activation of nuclear factor- κ B (NF- κ B) and p38 MAPK.
- *Rickettsiae* likely interact with inactive NF- κ B in endothelial cell cytoplasm and proteolytically cleave I κ B to expose the DNA-binding domains.
- Endothelial cells infected with *R. rickettsii* experience oxidative stress and induce the expression of antioxidant enzyme heme oxygenase-1.
- Increased secretion of vasoactive prostaglandins by endothelial cells infected with *R. rickettsii* and *R. conorii* may be attributed to increased expression of cyclooxygenase-2.
- Endothelial cells, infected *in vitro*, express and secrete a variety of inflammatory and chemotactic cytokines, the upstream signaling mechanisms for which involve activation of NF- κ B and p38 MAPK.

- Infection-induced NF- κ B activation prevents the endothelial cells from undergoing apoptotic death early during the infection, thereby providing the intracellular environment essential for Rickettsial replication and spread.
- Proinflammatory cytokines exacerbate the effects of *R. conorii* infection on the permeability of endothelial cell monolayers.

Models to study Rickettsial pathogenesis *in vivo*

- Experimental models to study the pathogenesis of Rocky Mountain spotted fever and epidemic typhus exploit the infection of susceptible strains of mice with *R. conorii* and *R. typhi*.
- Disseminated endothelial infection as seen during human disease and presence of *Rickettsiae* in nearly all vital organ systems including the lungs and the brain is evident in *in vivo* models of infection.
- Infection of BALB/c mice with *R. prowazekii* has recently been shown to produce a disease that mimicks epidemic typhus in humans.

Clinical correlates of *in vitro* & *in vivo* findings

- Inflammatory responses of humans (serum levels of endothelial activation markers and cytokines) appear to coincide with the disease severity and inflammatory potential of pathogenic *Rickettsiae*.

In summary, the past few years have witnessed several important breakthroughs in the understanding of the molecular basis of host-cell interactions with pathogenic *Rickettsiae*. The presence of RickA, a protein with similarity to the Wiskott–Aldrich syndrome protein family of Arp2/3-complex activators, has been established as an important molecular switch for actin-based motility exploited by the SFG *Rickettsiae*, *R. rickettsii* and *R. conorii* for cell–cell spread [128,129]. Comparison of the genome sequences of *R. prowazekii*, *R. conorii* and *R. typhi* has revealed the presence of a set of virulence-related Type IV secretion system proteins and application of an approach based on a bacterial two-hybrid system in combination with the whole genome shotgun sequencing of *Rickettsia sibirica* has further identified a number of unique protein–protein interactions involving subunits of the type IV secretion system [130, 131]. Similar to RalF protein, a substrate translocated into host cells by the dot/icm-encoded secretion system of *Legionella pneumophila*, the Sec7 homology domain-containing orthologs are present in *R. prowazekii* and *R. typhi* [131,132]. Another important molecular breakthrough is the demonstration of the presence of pRF and pRM plasmids in *R. felis* [3,133], *R. monacensis* [134], and other *Rickettsia* species [135]. Indeed, the potential development of Rickettsial plasmids as transformation vectors for genetic manipulation of *Rickettsiae* in conjunction with a ‘compare and contrast’ approach to benefit from the knowledge of pathogenesis and immune evasion mechanisms employed by other Gram-negative bacterial pathogens should facilitate the description of as yet unknown facets of host cell interactions with pathogenic *Rickettsiae*.

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Figure 1. NF- κ B inhibition via attenuation of proteasome activity by MG132 treatment during *Rickettsia rickettsii* infection of human umbilical vein endothelial cells induces apoptotic cellular events

Endothelial cells (ECs) were either incubated for 6 h with culture medium alone and medium containing MG132 or were infected in the absence and presence of MG132. Treatment with staurosporine or etoposide was used as a positive control for induction of apoptosis. **(A & B)** show fragmentation of cellular DNA and changes in nuclear morphology as observed under a fluorescent microscope after TUNEL and Hoechst 33258 staining, respectively. Yellow arrows point towards cells with normal nuclear structure while white arrows indicate TUNEL-positive cells in **(A)** and condensed nuclei correlating with induction of apoptosis in **(B)**.

Original magnification $\times 20$. Bar = 10 μ m.

C: Culture medium alone; ETP: Treatment with etoposide; MG: Medium containing MG132; Rr: Infected in the absence of MG; STS: Treated with staurosporine. Reproduced with permission from [99].

Effects of *Rickettsia rickettsii* infection on the expression, synthesis and release of endothelial cell products

Protein/enzyme	mRNA expression	Protein synthesis	Time (postinfection)	Regulation mechanism(s)	Ref.
von Willebrand factor	ND	Increase	24 h	Post-translational; release from Weibel-Palade bodies	[45]
E-selectin	ND	Increase	6–8 h	ND	[42]
IL-1 α *	Increase	Increase	4–8 h	ND	[40]
IL-8 †	Increase	Increase	3–21 h	Transcriptional; dependent on NF- κ B and p38 kinase [67,87]	[67,87]
MCP-1 †	Increase	Increase	3–21 h	Transcriptional; dependent on NF- κ B and p38 kinase [67,87]	[67,87]
Tissue factor	Increase	Increase	4–8 h	Transcriptional	[38]
Heme oxygenase-1	Increase	Increase	3–7 h	Transcriptional	[69]
PAI-1	Increase	Increase	24 h	Post-transcriptional (increased mRNA stability)	[44]
Cyclooxygenase-2	Increase	Increase	3–21 h	Transcriptional; dependent on p38 MAPK	[75]

* Indicates that a majority of IL-1 α synthesized remains associated with the cells with only a fraction secreted into the medium.

† Indicates time-dependent secretion and accumulation in the culture medium.

MCP: Monocyte chemoattractant protein; ND: Not determined.

Table 2
Effects of NF- κ B inhibition on the regulation of important pro- and anti-apoptotic proteins of the Bcl-2 family and x-linked inhibitor of apoptosis protein during *Rickettsia rickettsii* infection of endothelial cells

Protein	Cellular fraction used for analysis	Technique(s) employed	Level of expression	Potential mechanism(s)
Bid	Total protein lysates	Immunoblotting	Decrease	Cleavage to tBid by caspase-8
Bad (dephosphorylated)	Total protein lysates, fixed cells	Immunoblotting, immunocytochemistry	Increase	Enhanced dephosphorylation and translocation to mitochondria
Bax	Cytosolic preparations	Immunoblotting	Decrease	Translocation to mitochondria
Bcl-2	Total protein lysates	Immunoblotting	Decrease	Dependence on NF- κ B response
xIAP	Total protein lysates	Immunoblotting	Decrease	Dependence on NF- κ B response
Cytochrome c	Cytosolic preparations, fixed cells	Immunoblotting, immunocytochemistry	Increase	Loss of mitochondrial integrity and release into cytosol
SMAC (Diablo)	Cytosolic preparations, fixed cells	Western blotting, immunocytochemistry	Decrease	Loss of mitochondrial integrity and release into cytosol

Bcl: B-cell lymphoma; NF- κ B: Nuclear factor- κ B; SMAC: Second mitochondria-derived activator of caspases; xIAP: X-linked inhibitor of apoptosis protein.