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Host Defenses to *Rickettsia rickettsii* Infection Contribute to Increased Microvascular Permeability in Human Cerebral Endothelial Cells

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Abstract

Rickettsiae are arthropod-borne intracellular bacterial pathogens that primarily infect the microvascular endothelium leading to systemic spread of the organisms and the major pathophysiological effect, increased microvascular permeability, and edema in vital organs such as the lung and brain. Much work has been done on mechanisms of immunity to rickettsiae, as well as the responses of endothelial cells to rickettsial invasion. However, to date, no one has described the mechanisms of increased microvascular permeability during acute rickettsiosis. We sought to establish an in vitro model of human endothelial-target rickettsial infection using the etiological agent of Rocky Mountain spotted fever, *Rickettsia rickettsii*, and human cerebral microvascular endothelial cells. Endothelial cells infected with *R. rickettsii* exhibited a dose-dependent decrease in trans-endothelial electrical resistance, which translates into increased monolayer permeability. Additionally, we showed that the addition of pro-inflammatory stimuli essential to rickettsial immunity dramatically enhanced this effect. This increase in permeability correlates with dissociation of adherens junctions between endothelial cells and is not dependent on the presence of nitric oxide. Taken together, these results demonstrate for the first time that increased microvascular permeability associated with rickettsial infection is partly attributable to intracellular rickettsiae and partly attributable to the immune defenses that have evolved to protect the host from rickettsial spread.

Keywords

Bacterial infection; cytokines; nitric oxide; microvascular permeability; rickettsiae

Introduction

Rickettsioses are arthropod-borne diseases caused by Gram-negative obligately intracellular bacteria that primarily reside in the cytoplasm of an infected host cell [1, 2]. These diseases are prevalent in many parts of the world and include Rocky Mountain spotted fever (RMSF), boutonneuse fever, epidemic and endemic typhus caused by *Rickettsia rickettsii*, *R. conorii*, *R. prowazekii*, and *R. typhi*, respectively. RMSF usually results in an untreated case fatality rate of ~25%, making it the most lethal tick-transmitted bacterial infection in the USA [3].

The main target is the microvascular endothelium, leading to a disseminated infection and severe complications including vasogenic cerebral edema and non-cardiogenic pulmonary edema [4-6]. These two complications are responsible for most of the morbidity and mortality of RMSF. Immunohistochemical studies performed on human tissues and animal models of rickettsioses reveal the presence of rickettsiae in the microvascular endothelium of all major organs [7-9]. The lungs show interstitial pneumonitis and presence of proteinaceous fluid in the alveolar spaces. In severe cases, diffuse alveolar damage occurs [10]. Studies from brain tissues show rickettsial organisms in the microvascular endothelium, perivascular inflammation composed of mononuclear inflammatory cells and parenchymal edema. Presently, the mechanisms responsible for increased microvascular permeability in rickettsioses are completely unknown.

The pro-inflammatory cytokines, TNF- α , IL-1 β , and IFN- γ , are essential mediators of anti-rickettsial immunity partly through the induction of nitric oxide (NO) by rickettsiae-infected endothelial cells [11-14]. In mice, depletion of TNF- α and IFN- γ results in a fatal, overwhelming infection because of decreased NO production and uncontrolled rickettsial proliferation [15]. The importance of CD8+ T-lymphocytes in vivo in clearing rickettsial infections has also been well documented [16]. C3H/HeN mice depleted of CD8 +T-lymphocytes experience a fatal, overwhelming infection when infected with normally sublethal doses of *R. conorii*. Taken together, these results demonstrate the importance of a host inflammatory response in clearing infection. However, the relative influence of the host immune response on the endothelial dysfunction experienced during acute rickettsioses has not been studied.

Endothelial barrier function is regulated by a series of protein complexes that exist at the intercellular borders between cells. In the brain, tight and adherens junctions form the molecular barrier that regulates the flow of solutes from the blood into the parenchyma. Two proteins of particular importance are p120-catenin (p120) and β -catenin. These two proteins serve to regulate the functional interaction of VE-cadherin with the actin cytoskeleton, thereby, establishing a molecular fence at cell junctions [17, 18]. A previous study of *R. conorii* infection of human umbilical vein endothelial cells (HUVEC) demonstrated disassembly of adherens junction after a prolonged period of infection [19]. To our knowledge, no one has ever described the effect of *R. rickettsii* infection on adherens junction stability.

The present study was conducted with the purpose of creating an in vitro model to study mechanisms of permeability across rickettsiae-infected microvascular endothelial cells derived from the human brain. In this paper, we report the effects of *R. rickettsii* and three important cytokines, TNF- α , IFN- γ , and IL-1 β , on the permeability of human brain microvascular endothelial cell monolayers. We were able to demonstrate that *R. rickettsii* directly causes an increase in microvascular permeability very early after invasion of the endothelium. We also demonstrated that the addition of TNF- α , but not IL-1 β or IFN- γ , alone is sufficient to enhance rickettsiae-induced microvascular permeability in a dose-dependent manner. However, a low dose of all three cytokines in combination was also

capable of producing greater permeability than rickettsiae alone. Rickettsial infection and cytokine stimulation were both capable of increasing NO production by a human endothelial cell line, and this was associated with decreased rickettsial titers, whereas NO produced by our cell cultures did not overtly contribute to increased microvascular permeability. Finally, we have demonstrated that both *R. rickettsii* and pro-inflammatory cytokines induce a loss of adherens junction assembly at intercellular borders, and this correlates with an increase in microvascular permeability.

Methods

Reagents

1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, ODQ, was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Recombinant human TNF- α , IL-1 β , and IFN- γ were purchased from R&D Systems (Minneapolis, MN, USA). Unless otherwise indicated, all reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) and cell culture medium and supplements from Invitrogen (Carlsbad, CA, USA).

Rickettsial Stocks and Cell Lines

R. rickettsii (Sheila Smith strain) was originally obtained from the laboratory of Dr. David Walker and was passaged in our laboratory one time in embryonated chicken eggs. Ten percent yolk-sac stocks of *R. rickettsii* suspension were propagated through two passages in Vero cell monolayers, grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% bovine calf serum (Hyclone, Logan, UT, USA) at 34°C and purified by Renografin density gradient centrifugation as described previously [20]. Purified light-band rickettsiae were frozen in SPG (0.128 M sucrose, 0.0038 M KH₂PO₄, 0.0072 M K₂HPO₄, 0.0049 M monosodium L-glutamic acid) buffer at -80°C. Rickettsial titers of the frozen stock were determined by plaque assay on Vero cells (ATCC CCL-81, Manassas, VA, USA).

Immortalized human cerebral endothelial cells (SV-HCEC) were a kind gift from Dr. Som Dasgupta and Dr. Robert Yu of The Medical College of Georgia [21]. This cell line was maintained in M199 medium supplemented with 10% heat-inactivated fetal calf serum, insulin-transferrin-selenium (ITS) supplement and 100 μ g/ml heparin. Endothelial cells were grown on rat-tail collagen I-coated plates (BD Biosciences, San Jose, CA, USA) and used between passages 15–25.

Measurements of Transendothelial Electrical Resistance by Electric Cell-Substrate Impedance Sensing

Electric cell-substrate impedance sensing (ECIS Model 1600R, Applied Biophysics, Troy, NY, USA) was utilized with 8W10E gold-coated electrodes to monitor (TER) in real time. A decrease in TER represents an increase in paracellular permeability of endothelial monolayers and will, therefore, be referred to as an increase in vascular permeability. Electrode arrays were coated with rat-tail collagen and seeded with approximately 100,000 cells per well. The electrode arrays were then connected to the detection system, and resistance was monitored until stabilization of the resistance values was observed for at least 24 h. Typical ohms values observed for stabilized monolayers were between 1,200 and 1,400 Ω . The endothelial cell monolayers were then used for the different experiments described below. Resistance measurements were taken every 2 min, and each measurement represents the average resistance of ten electrodes per well, each measuring 250 μ M in diameter. Each experiment was performed in triplicate, and the values expressed as the average normalized resistance and standard deviations were calculated every 6 h.

Nitrite Measurement

Nitrite was measured with the Greiss assay as described previously [14]. Briefly, 100 μ l of filtered cell-culture supernatant was mixed with an equal volume of 1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine in 2.5% phosphoric acid. A standard curve was generated using a nitrite standard from 100–0.78 μ M, and absorbance was read at 540 nm.

Semi-Quantitative Polymerase Chain Reaction

DNA was isolated from infected cells using the Qiagen DNeasy tissue kit, and all samples were prepared in parallel. For all polymerase chain reactions (PCR), the following primers were used to amplify a 147-bp product of the rickettsial *gltA* gene: forward primer CS-5 (GAGA GAAATTATATCCAAATG-TTGAT) and reverse primer CS-6 (AGGGTCTTCGTGCATTTCTT) [22]. Rickettsial genome copies were normalized to the human β -actin gene using commercially available primers (SuperArray Biosciences). Real-time PCR was performed on an Eppendorf *realplex*² instrument with SYBR Green and the following protocol: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Relative copy numbers of rickettsial genomes were calculated between samples using the $\Delta\Delta$ Ct method.

Fluorescent Confocal Microscopy

SV-HCEC monolayers were grown on plastic chamber slides until 100% confluent. The cells were then infected with *R. rickettsii* at a multiplicity of infection (MOI) of 10 and/or treated with the combination of TNF- α , IL-1 β , and IFN- γ , each at a dose of 10 ng/ml. After 24 h of infection and stimulation, the cells were fixed in fresh 4% paraformaldehyde (Polysciences, Inc., Warrington, PA, USA) for 20 min at 4°C. After fixation, the cells were washed three times in phosphate-buffered saline (PBS) before being permeabilized with 0.05% Tween-20. The cells were blocked in 10% normal goat serum before overnight incubation with the following primary antibodies: mouse-anti-p120 (Invitrogen) and rabbit anti- β -catenin (Santa Cruz, Santa Cruz, CA, USA) both at a dilution of 1:100 in PBS. The cells were washed three times with PBS and incubated with goat-anti-mouse Alexa Fluor-568 and goat-anti-rabbit Alexa Fluor-488 (Invitrogen) both at a dilution of 1:1,000. The cells were mounted in VectaShield Hardset (Vector Laboratories, Burlingame, CA, USA), and images were acquired using an Olympus FV-1000 laser scanning confocal microscope.

Statistical Analysis

Sigma Stat v.2.03 was used for statistical analysis of the results. Differences were analyzed by performing *t* tests or Mann–Whitney rank sum test if indicated.

Results

R. rickettsii Causes Increased Microvascular Permeability in SV-HCEC

The effect of *R. rickettsii* infection on endothelial cell monolayer permeability has never been described. To this end, we sought to investigate the impact of *R. rickettsii* on human brain endothelial cells. Confluent SV-HCEC infected with *R. rickettsii* exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in transendothelial electrical resistance. Figure 1a is a representative electric cell-substrate impedance sensing (ECIS) graph demonstrating the real-time loss of electrical resistance across an SV-HCEC monolayer. Permeability increased steadily after rickettsiae were internalized and continued to decline up to 72 h after infection. At 24 h, increases in permeability ranged from 12.3 $\pm$ 2.7% at 1 MOI to 36.9 $\pm$ 2.4% at 20 MOI relative to controls (Fig. 1b). At 48 h post-infection, monolayers infected with one MOI showed a 23.1 $\pm$ 1.5% increase in permeability

compared to controls. During the first 24 to 48 h, increases in permeability were more pronounced in monolayers infected with the highest doses, clearly demonstrating the dose dependence of the effect. Based on these experiments, further work described in this paper was based on an MOI of 10 plaque forming units (PFU). We found that this dose of rickettsiae consistently resulted in a 100% infection rate of endothelial cells early after infection with a true MOI of ~10 as determined by immunofluorescent staining within 6 h of infection.

Pro-inflammatory Cytokines Regulate Microvascular Permeability in SV-HCEC

Because TNF- α , IL-1 β , and IFN- γ are well-known pro-inflammatory cytokines and have already been demonstrated to be critical to effective rickettsial immunity, we decided to determine what effect these cytokines have on uninfected endothelium. At all concentrations, IL-1 β induced a rapid decline in TER by 10 h after stimulation, resulting in ~20% increase in permeability compared to untreated controls (Fig. 2a). By 24 h after stimulation, this effect ranged from a $15 \pm 5.4\%$ increase at 0.1 ng/ml to $31.8 \pm 13\%$ at 1,000 ng/ml compared to controls (Fig. 2b). The effect of the lowest dose of 0.1 ng/ml was only transient, and those cells recovered to near normal levels of permeability by 72 h after stimulation with an average increase in permeability not statistically different than control cells. However, monolayers treated with higher doses showed a steady increase in permeability, which reached values between 30–45% at concentrations of 1 ng/ml ($29.12 \pm 0.12\%$), 10 ng/ml ($25.3 \pm 2.3\%$), 100 ng/ml ($31.1 \pm 3.1\%$), and 1,000 ng/ml ($44 \pm 7.5\%$), respectively, by 72 h. Overall, increasing doses of IL-1 β tended to induce a progressively greater increase in permeability over control cells.

The effects of TNF- α on uninfected SV-HCEC monolayers were more dramatic and behaved in a dose-dependent manner as demonstrated in a representative ECIS graph (Fig. 2c). Low doses of TNF- α at 0.1 ng/ml showed no significant increase in permeability compared to controls at any time point. A dose of 1 ng/ml showed a slightly higher level of permeability at 48 h ($12.3 \pm 7.1\%$) after stimulation, but this effect was diminished by 72 h; after which, the increase in permeability was not statistically higher than control cells (Fig. 2d). Higher doses caused a steady increase in permeability over the 72-h time course ranging from $33.42 \pm 7.6\%$ at 10 ng/ml, $49.79 \pm 7.0\%$ at 100 ng/ml, and $64.2 \pm 3.2\%$ at 1,000 ng/ml. The highest dose, 1,000 ng/ml, was rapidly cytotoxic and was excluded from further experiments with rickettsiae.

Stimulation of SV-HCEC with IFN- γ alone did not produce a significant increase in permeability of these cells at any time point tested (Fig. 2e).

TNF- α and IL-1 β Augment Rickettsiae-Induced Microvascular Permeability in SV-HCEC

We next decided to investigate the effects of these cytokines on monolayers also infected with *R. rickettsii*. The activity of IL-1 β on rickettsiae-infected endothelial cells produced an increase in permeability relative to cells only infected with rickettsiae (Fig. 3a). There was a dose-dependent response of rickettsiae-infected SV-HCECs to IL-1 β , and by 48 h, there was a significant increase in permeability in cells treated with 10 ng/ml ($13.1 \pm 1.5\%$) and 100 ng/ml ($10.3 \pm 1.3\%$) of IL-1 β compared to those only infected with rickettsiae. No additional effect of IL-1 β was observed at any time point for doses ranging from 0.1–1 ng/ml.

The addition of TNF- α to rickettsiae-infected cells elicited an additional increase in permeability at 10 ng/ml ($15.9 \pm 1.1\%$) or 100 ng/ml ($18.6 \pm 0.4\%$) as early as 24 h after stimulation (Fig. 3b). This trend continued for the course of the experiment with a sustained increase in permeability for those two doses above that of cells only infected with

rickettsiae. Lower doses of 1 or 0.1 ng/ml had no additional impact on permeability over unstimulated infected cells.

Based on previous experiments, we decided to investigate whether low doses of cytokines acted in concert to synergistically activate endothelial cells and cause an increase in permeability. The addition of a combination of TNF- α , IL-1 β , and IFN- γ to rickettsiae-infected cells at doses of 0.1 ng/ml produced a significantly greater increase in permeability compared to cells only infected with rickettsiae (Fig. 3c). This additional increase in permeability was maintained up to 72 h after the start of the experiment. Additional permutations of cytokine combinations had no additional impact on endothelial permeability (data not shown), indicating that the cumulative impact of all three cytokines is needed to produce this effect. Likewise, uninfected monolayers also demonstrated a sustained increase in permeability of approximately 20% greater than controls, although the increase in permeability was lower than either infected cells or infected cells treated with the same dose of cytokines.

Stimulation with Combinations of Pro-inflammatory Cytokines Induces an Anti-Rickettsial Effect in SV-HCEC Associated with NO Production

Because NO has been demonstrated to be an important mechanism of immunity to rickettsiae, we next sought to determine the ability of SV-HCEC to produce NO in response to cytokines and whether this has any effect on the number of viable rickettsial organisms. SV-HCEC infected with *R. rickettsii* and/or stimulated with 10 ng/ml of TNF- α , IL-1 β , and IFN- γ produced high levels of NO as measured by nitrite in the supernatant and was first observed at 24 h after infection and stimulation (Fig. 4a). Surprisingly, *R. rickettsii* was alone capable of inducing NO production by SV-HCEC and was consistently higher than those cells only stimulated with cytokines. The combination of rickettsiae and cytokines produced the greatest NO production throughout the course of the experiment. We also sought to demonstrate that cytokine-stimulated endothelial cells had fewer numbers of intracellular rickettsiae compared to unstimulated controls. As expected, the addition of cytokines to rickettsiae-infected SV-HCEC resulted in a decrease in the number of viable intracellular rickettsiae (Fig. 4b). Cells treated with 0.1 ng/ml of cytokines had a decrease in the number of intracellular bacteria by approximately 23.67 $\pm$ 1.6%. Cells treated with a higher dose of 10 ng/ml had a greater reduction in the number of intracellular rickettsiae to approximately 33.5 $\pm$ 11.2% that of unstimulated controls at 24 h after infection. In both groups, this was a significant decrease in the number of intracellular rickettsiae compared to non-treated cells ($p<0.05$). However, there was not a significant difference between the two doses ($p=0.11$). We also tested whether inhibition of endogenous NO production influenced the number of intracellular rickettsiae. Cells treated with the nitric oxide synthase inhibitor L-NG-nitroarginine methyl ester (L-NAME) showed an overall increase in the number of rickettsiae to 22.5 $\pm$ 10.8%, greater than untreated, infected cells and was significantly greater than either of the cytokine-treated groups ($p<0.05$).

Rickettsiae- and Cytokine-Induced Increases in Microvascular Permeability are not Directly Attributable to NO Production

Because NO has been implicated in microvascular and endothelial barrier regulation, we sought to determine the impact of NO production by SV-HCEC, and its downstream signaling effects on microvascular permeability. The addition of the NO synthase inhibitor L-NAME resulted in decreased production of NO in response to cytokine stimulation. Likewise, the addition of L-NAME decreased the amount of NO being produced in response to rickettsial infection alone (Fig. 5a). The soluble guanylyl cyclase inhibitor ODQ had no impact on the level of NO production, as expected (Fig. 5c). Next, we determined the effect of nitric oxide on the early stages of permeability experienced during cytokine stimulation of

rickettsiae-infected endothelial cells. Inhibition of NO production by rickettsiae-infected SV-HCEC had no impact on the ability of *R. rickettsii* to induce a loss of electrical resistance (Fig. 5b). Additionally, inhibition of NO production after cytokine stimulation had no impact on cytokine-induced microvascular permeability. Finally, inhibition of the downstream signaling activity of NO with ODQ failed to induce any abrogation of the effects of either *R. rickettsii* or cytokine combinations, suggesting alternative mechanisms of rickettsiae- and cytokine-induced permeability (Fig. 5d).

Cytokines and *R. rickettsii* Induce the Loss of Adherens Junction Staining at Intercellular Borders

Uninfected SV-HCEC demonstrated staining of the adherens-junction proteins, β -catenin and p120, at intercellular borders consistent with a confluent monolayer (Fig. 6a,b). The staining of these two proteins overlapped, indicating their association with adherens junctions (Fig. 6c). The addition of cytokines appeared to have the greatest impact on p120 localization resulting in punctuate staining pattern not associated with cell-cell junctions (Fig. 6d-f). Additionally, β -catenin showed diminished association with intercellular junctions. Infection of SV-HCEC with *R. rickettsii* resulted in decreased staining of both proteins at cell-cell junctions and a clear change in cell morphology. Gaps between cells were evident, although the monolayer was still mostly intact (Fig. 6g-i). The p120 staining pattern was not nearly as punctuate as those cells stimulated with cytokines, suggesting an alternative pathway of modification. Also, the overall intensity of p120 staining decreased with very few intercellular junctions being observed. While β -catenin was still associated with intercellular junctions, there was decreased colocalization with p120, and the staining pattern was more diffuse than uninfected cells. The cells developed an elongated morphology as compared to either uninfected cells or those only treated with cytokines, which displayed a traditional cobblestone morphology characteristic of endothelium. As expected, the combination of rickettsial infection and cytokine stimulation had the greatest impact on stability of the adherens junctions, resulting in diminished staining of β -catenin and p120 at cell-cell junctions and an increased cytoplasmic localization of both proteins (Fig. 6j-l). Gaps between adjacent endothelial cells were clearly evident, and there was no clear definition of where adherens junctions were localized.

Discussion

The mechanisms of increased microvascular permeability during acute rickettsioses have not received sufficient attention to date. It is generally assumed that rickettsial pathogenesis is solely dependent on rickettsial proliferation and subsequent cell lysis. What has not been considered is the relative influence of the host's immune response on the integrity of the microvascular barrier. We have demonstrated, in an immortalized human microvascular endothelial cell line, the ability of pro-inflammatory cytokines IL-1 β , TNF- α , and IFN- γ to induce an anti-rickettsial response in the form of NO. Subsequently, we demonstrated that the cytokine stimulus but not NO is more than sufficient to induce a rapid decrease in electrical resistance across endothelial monolayers, reflecting an increase in microvascular permeability.

The role of the host immune response in the pathogenesis of acute rickettsioses is a phenomenon that has not been extensively studied. Clearly, a cellular immune response is critical to the effective clearance of rickettsial organisms from the vasculature, as has been demonstrated in the past [16]. Cytotoxic T-lymphocytes (CTL) are recruited to rickettsiae-infected endothelial cells, in part caused by the upregulation of the intercellular adhesion molecule 1 (ICAM-1) but independent of the CXCL9/10-CXCR3 chemokine system [23]. Presumably, an inflammatory state is initiated in these endothelial cells as a result of cytokine secretion by infiltrating macrophages and CD4+ and CD8+ T-lymphocytes [24,

25]. This inflammatory state is characterized by the transcriptional activation of iNOS, increased NO production, and the inhibition of rickettsial proliferation. Feng and Walker demonstrated in human endothelial cells and hepatocytes that the necessary stimuli included IFN- γ , TNF- α , IL-1 β , and regulated upon activation, normal T cell expressed and secreted (RANTES), but the degree of NO production was variable between pools of HUVEC [26]. Likewise, depletion of TNF- α and IFN- γ in mice infected with *R. conorii* resulted in an increased proliferation of rickettsiae, and the animals are no longer able to control a sublethal infection [15].

The pleiotropic cytokine TNF- α is a vital component of a protective immune response to a number of different pathogens including rickettsiae. Its many different effects include inducing apoptotic cell death or, in other instances inducing cellular proliferation, demonstrating the wide range of often divergent effects associated with this protein. Widely produced throughout the body, TNF- α is perhaps most often associated with localized inflammatory processes, although it has been suggested that high levels of circulating cytokine are responsible for the pathological complications of Gram-negative sepsis and hantavirus pulmonary syndrome, diseases characterized by a rapid and dramatic increase in vascular permeability resulting in generalized vascular leakage and severe pulmonary edema, respectively [27, 28]. RMSF, on the other hand, to the best of our knowledge causes a much slower accumulation of interstitial fluid, suggesting that high numbers of localized inflammatory infiltrates associated with foci of rickettsial invasion might mediate the primary mechanism of fluid accumulation. TNF- α is primarily produced by activated macrophages and T-cells, which are cells known to infiltrate sites of rickettsial invasion. In this instance, the primary function of TNF- α is presumably to activate rickettsiae-infected endothelial cells to induce iNOS transcription and increase NO production to control intracellular rickettsial proliferation. However, TNF- α has also been shown to induce p42 oxidation via a NO-dependent mechanism, activate protein kinase C- α , and induce the production of prostaglandins and leukotrienes, resulting in endothelial barrier dysfunction [29-32]. Previous work in our lab demonstrated that the addition of a NO-donor to *R. conorii*-infected endothelial cells resulted in a stabilization of the barrier early after stimulation, suggesting that NO actually enhanced the barrier properties of endothelial cells [33]. On the other hand, work presented in this paper provides no clear indication about the role of NO in cytokine-mediated endothelial permeability, suggesting that NO-independent effects of TNF- α and/or IL-1 β may be more important in regulation of endothelial barrier function. One possible explanation might be that TNF- α mediated p38 MAPK activation, resulting in redistribution of VE-cadherin as was demonstrated previously [34]. While this response is ultimately protective and survival is dependent on TNF- α activity, it is not unreasonable to conclude that the permeability-enhancing properties of TNF- α do, in fact, contribute to the pathogenesis of acute rickettsiosis, as demonstrated in this work.

IL-1 β is a cytokine associated with an inflammatory state and is often regulated in conjunction with TNF- α . IL-1 β demonstrates a remarkable pyrogenic action and was one of the first molecules identified to be directly responsible for causing fever, a component of the clinical triad associated with RMSF besides rash and headache. Interestingly, IL-1 β generally circulates at very high levels during *R. conorii* infection of mice (unpublished data), suggesting that its mechanism of action in inducing vascular permeability is enhanced at sites of TNF- α production. Another intriguing response to IL-1 β production is the increased transcription and secretion of IL-6 in a dose- and time-dependent manner by cultured intestinal epithelial cells [35]. IFN- γ , on the other hand, has no effect on the level of IL-6 being produced by these cells. Taken together with the observations that IL-6 is produced by rickettsiae-infected endothelial cells in the absence of additional cytokine stimulation, this may suggest a role for IL-6 in rickettsial pathogenesis [36]. In fact, lack of IL-6 was associated with decreased microvascular permeability in an experimental model of

pneumococcal meningitis [37]. Likewise, IL-6 has been shown to induce a loss of TER across human umbilical vein endothelial cells in a protein kinase C (PKC)-dependent manner [38]. Therefore, it is not surprising to find that PKC is also up regulated in *R. rickettsii*-infected endothelial cells [39]. The exact role of IL-6 and/or PKC activation in rickettsiae-induced permeability is an area of current investigation.

These observations present a conundrum when trying to determine the mechanism of disease in acute rickettsiosis. Certainly, direct rickettsial cytotoxicity is a well-known component of rickettsial virulence as indicated by the presence of plaques in cell monolayers infected with *R. rickettsii* [40, 41]. Increased production of reactive oxygen species (ROS) is a recognized pathological consequence of *R. rickettsii* infection in human endothelial cells [42-44]. Interestingly, in one study, NO was shown to enhance hydrogen peroxide-mediated endothelial permeability associated with a significant depletion of intracellular glutathione [45]. This is similar to the effect of *R. rickettsii* infection on human endothelial cells characterized by decreased levels of intracellular reduced glutathione and increased levels of peroxide [46]. However, a direct connection between ROS production and increased microvascular permeability during rickettsioses has yet to be established. Our observation that *R. rickettsii*-infected SV-HCEC produce NO is novel and exciting. Few endothelial pathogens have been shown to induce the levels of NO seen in this study in a human-derived microvascular endothelial cell. For example, virulent Junin virus, a viral hemorrhagic fever, demonstrated the ability to induce NO production by human endothelial cells, and serum from patients acutely ill with Junin virus infection have markedly increased levels of nitrite [47]. Other factors released by endothelial cells in response to infection may also have the ability to induce NO production such as vascular endothelial growth factor, VEGF. High serum levels of VEGF has been associated with dengue virus infection, a disease characterized by general vascular dysfunction, and human endothelial cells infected with dengue virus produce VEGF [48-50]. In this work, we showed that blockade of endogenous NO allowed the rickettsiae to proliferate to higher levels. We also showed that blockade of endogenous NO had no effect on the integrity of the endothelial monolayer despite a greater number of intracellular rickettsiae. Most likely this means that the permeability-inducing effects of rickettsiae are more dependent on the initial response to invading rickettsiae.

Several important signaling pathways activated in response to *R. rickettsii* infection have been described, which may play an important role in microvascular barrier integrity. The p38 MAP kinase was shown to be activated via phosphorylation in response to invasion by live *R. rickettsii*, resulting in increased expression of IL-8 and MCP-1, two important mediators of vascular inflammation [51, 52]. p38 MAP kinase has been implicated in viral hemorrhagic fever-induced endothelial permeability and most likely contributes to rickettsiae-induced endothelial dysfunction in a similar manner [53]. Interestingly, MCP-1 is a demonstrated inducer of blood-brain barrier dysfunction both in vitro and in vivo [54, 55]. Taken together with the given observations of IL-6 and PKC activity during rickettsial infection, we can begin to see that the cellular host response to rickettsial invasion may play a much greater role in microvascular dysfunction than originally thought.

The integrity of the endothelial barrier is maintained by a complex and interwoven network of protein complexes such as the ubiquitously expressed junctions known as adherens junctions. They serve to anchor the endothelial cell to the extracellular matrix as well as neighboring cells. VE-cadherin is linked to the actin cytoskeleton by the catenin class of proteins, namely α - and β -catenin, and p120-catenin. Via confocal microscopy, we have demonstrated that β -catenin and p120 dissociate from the interendothelial cell junctions in response to inflammatory cytokine stimuli and/or infection with *R. rickettsii*. In contrast to the studies performed by Valbuena and Walker in which *R. conorii* induced adherens

junction instability only after a prolonged period of infection, the effect of *R. rickettsii* is fast, occurring within the first 24 h of infection [19].

The data presented in this manuscript demonstrate the ability of *R. rickettsii* alone to cause an increase in microvascular permeability, and this response is further augmented by the presence of the pro-inflammatory cytokines TNF- α , IL-1 β , and IFN- γ . These cytokines are important because of the previously demonstrated protective effect in eliminating intracellular rickettsiae. The absence of this response in vivo results in unchecked rickettsial proliferation and severe disease supporting the role for these cytokines in adaptive immunity. Taken together, it is clearly to the benefit of the host to possess this defense mechanism; however, the effects of endothelial activation more than likely contribute to the pathogenesis of RMSF. Future work may reveal novel pathways that can be modified to retain the protective effects of endothelial cell activation while reducing or ablating the potentially detrimental effects of cytokine stimulation.

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Fig. 1.

R. rickettsii caused increased microvascular permeability in human brain endothelial cells. SV-HCEC were grown to confluence on 8W10E gold-coated electrodes, and resistance was monitored by ECIS after infection with *R. rickettsii* at doses ranging from 1–20 MOI. A representative ECIS graph (a) and bar graphs indicating the mean $\pm$ standard deviation of three independent experiments (b).

Fig. 2.

The pro-inflammatory cytokines IL-1 β , TNF- α , and IFN- γ had differential effects on SV-HCEC monolayers. SV-HCEC monolayers were cultured on 8W10E ECIS arrays until confluent at which time they were stimulated with doses of cytokines ranging from 0.1–1,000 ng/ml of IL-1 β (a, b), TNF- α (c, d) and IFN- γ (e). Representative ECIS graphs (a, c, e) demonstrate the time-course of responses to cytokines, whereas *bar graphs* (b, d) indicate the mean $\pm$ standard deviation of normalized resistance values at designated time points.

* $p < 0.05$ compared to uninfected controls.

Fig. 3.

Addition of pro-inflammatory cytokines to rickettsiae-infected SV-HCEC enhanced rickettsiae-induced permeability. Cells were infected with 10 MOI of *R. rickettsii* and stimulated with varying concentration of IL-1 β (a) or TNF- α (b) or 0.1 ng/ml of TNF- α , IL-1 β , and IFN- γ in combination with and without rickettsiae (c). Data is presented as the mean $\pm$ standard deviation of three independent experiments. * $p < 0.05$ compared to *R. rickettsii*-infected cells.

Fig. 4.

Combinations of pro-inflammatory cytokines induced an increase in microvascular permeability associated with an anti-rickettsial state in endothelial cells. SV-HCEC were infected with 10 MOI of *R. rickettsii* and/or stimulated with 10 ng/ml of TNF- α , IL-1 β , and IFN- γ , and supernatants were collected at the given time points. The concentration of nitrite was determined using the Greiss assay (**a**). * $p < 0.05$ compared to Mock-treated cells. SV-HCEC were seeded in 24-well plates and grown to confluence at which time they were infected with 10 MOI of *R. rickettsii* and then stimulated with 0.1 or 10 ng/ml of all three cytokines or treated with 100 μM L-NAME. The percent change in rickettsial genome copies was determined by real-time PCR using the $\Delta\Delta\text{Ct}$ method with primers to rickettsial *gltA* and human β -actin (**b**). * $p < 0.05$ compared to cells only infected with rickettsiae.

Fig. 5. Rickettsiae- and cytokine-induced increases in microvascular permeability are not directly attributable to increased nitric oxide production in SV-HCEC. Nitrite concentrations were measured in cell culture supernatants of infected and uninfected cells treated with or without 10 ng/ml of cytokines or untreated plus 100 μ M L-NAME (a) or 100 μ M ODQ (c). Similarly treated cells were monitored by ECIS for changes in permeability in response to L-NAME (b) and ODQ (d).

Fig. 6. *R. rickettsii* and cytokines induced a loss of β -catenin (**a, d, g, j**) and p120 (**b, e, h, k**) staining at intercellular junctions. Cells were infected with 10 MOI of *R. rickettsii* and/or stimulated with 10 ng/ml of TNF- α , IL-1 β , and IFN- γ and incubated at 37°C for 24 h. Overlaid images indicate the colocalization of β -catenin and p120 (**c, f, i, l**). Arrowheads indicate proper localization of adherens junctions at cell–cell contacts.