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Mechanisms of Increased Microvascular Permeability during Acute Rickettsiosis

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Mechanisms of Increased Microvascular Permeability during Acute Rickettsioses

by

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Dedication

To Sara and Jacob

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Mechanisms of Increased Microvascular Permeability during Acute Rickettsiosis

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Rickettsial diseases represent some of the most severe bacterial infections in man including Rocky Mountain spotted fever and epidemic typhus. Rickettsiae primarily target the microvascular endothelium leading to increased microvascular permeability, the mechanisms of which are completely unknown. We sought to determine the impact of host responses to infection on increasing microvascular permeability both *in vitro* and *in vivo*. Our work has revealed a role for TNF-α, IL-1β, and IFN-γ as mediators of anti-rickettsial immunity that contribute to increased microvascular permeability in a dose-dependent manner by modulating the function of interendothelial adherens junctions. The permeability-inducing effects of these cytokines appear to occur independently of nitric oxide production since inhibition of iNOS does not prevent cytokine-mediated increases in permeability. Additionally we have shown that iNOS expression *in vivo* is associated with sites of rickettsial invasion, which also correlates with the leakage of endogenous serum protein. The lack of significantly higher levels of serum cytokines

suggests this is primarily a localized response confined to areas of leukocyte infiltration. Likewise we have demonstrated a role for innate endothelial cell responses in modulating adherens junctions following rickettsial invasion. Human endothelial cells infected with rickettsiae produced significantly higher levels of VEGF and IL-6, two cytokines which can have a profound impact on adherens junction stability. This was associated with increased kinase activity in the form of protein kinase C, Src, and focal adhesion kinase. Inhibition of Src during *R. rickettsii* infection led to a decreased rate of endothelial permeability however this did not prevent rickettsiae-mediated cell death. Finally, we have identified several novel pathways modulated after rickettsial infection that were not previously thought to be important to rickettsial pathogenesis. Future work will be aimed at determining the relative contribution of these pathways to the endothelial dysfunction accrued during rickettsial infection. The work generated here provides a solid foundation for future endeavors aimed at alleviating the vascular dysfunction experienced during severe rickettsial infection.

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Chapter 1: Introduction

RICKETTSIAE AND RICKETTSIAL DISEASES

The Genus *Rickettsia*

Rickettsia spp. are arthropod-borne, obligate intracellular, Gram-negative bacteria that primarily infect endothelial cells. These organisms represent classic examples of emerging infectious diseases due to their now recognized presence in all continents except Antarctica. Historically speaking, members of this genus are the etiologic agents of some of the most significant infections known to man, including the first description of epidemic typhus in 16th century Europe, and more recently during World War I, where an estimated 3 million people died in Russia from louse-borne typhus. An estimated 200,000 Serbian soldiers died from typhus in November, 1914 alone (Raoult and Roux, 1997;Karatepe, 2002). Today these organisms still cause some of the most severe human bacterial infections known including Rocky Mountain spotted fever, Boutonneuse fever, and epidemic typhus whose etiologic agents are *R. rickettsii, R. conorii,* and *R. prowazekii*, respectively. The advent of antibiotics has decreased the mortality associated with rickettsial infections although Rocky Mountain spotted fever is still responsible for more deaths annually in the United States than any other tick-borne bacterial infection (Paddock *et al.*, 2002). Although genetically very similar, these organisms differ in their association with vectors of disease, as well as their clinical presentations.

Genome Structure and Phylogeny

Rickettsiae invade host cells and reside primarily in the cytoplasm but can also invade the nucleus (Burgdorfer *et al.*, 1968). Due to this specific intracellular niche rickettsiae have undergone a series of significant changes in the composition of their genomes. Rickettsiae are classic examples of reductive evolution, where specific genes

no longer in use are slowly eliminated from the genome (Blanc *et al.*, 2007). Remnants of genes involved in biosynthesis of cofactors are found in various stages of degradation throughout the genome of different rickettsial species. Rickettsiae parasitize the host cell utilizing precious host cell nutrients for their own growth and replication eventually leading to death of the cell.

Rickettsiae are believed to be the closest living relative to mitochondria, and a compelling case can be made for this based on their intracellular niche and genetic composition (Andersson *et al.*, 1998;Emelyanov, 2001). *R. prowazekii* has demonstrated the ability to import mitochondrial porin molecules, and possesses the ability to cleave signal sequences of proteins targeting mitochondria (Emelyanov and Vyssokikh, 2006;Kitada *et al.*, 2007). The presence of these similarities supports the hypothesis that certain eukaryotic organelles evolved from prokaryotic organisms, an intriguing evolutionary question.

Rickettsial genomes typically range in size from 1-1.5x10^6 base pairs and encode anywhere from 800 to >1300 open reading frames. Typical G + C content ranges around 30% with approximately 80% coding content in all species of *Rickettsia*. *R. conorii* possesses 137 unique genes not found in *R. prowazekii,* and the significance of these unique genes is an area of current interest (Ogata *et al.*, 2001). The first sequences of rickettsial genomes have only become available within the past 5-10 years, and it will take much more time, effort and attention to fully unravel the secrets that may lie within them.

Rickettsia spp. are subdivided into two groups; spotted fever group (SFG) and typhus group (TG). Genetically, these two groups are distinct since spotted fever group rickettsiae possess the *ompA* gene and typhus group rickettsiae do not. To date there are only two members of the TG, *R. prowazekii* and *R. typhi*. SFG rickettsiae use actin polymerization as a means of cell-cell spread, but TG rickettsiae do not. *R. australis*, the etiologic agent of Queensland tick typhus, is the most divergent of all spotted fever group

rickettsiae as demonstrated by genetic analysis of the *rompA* and *rompB* genes (Stenos and Walker, 2000). However, it is still more closely related to SFG rickettsiae than TG rickettsiae. Rickettsiae are distributed worldwide and can be found in almost all ecological habitats, demonstrating the relative importance of their intracellular niche in determining their genetic makeup.

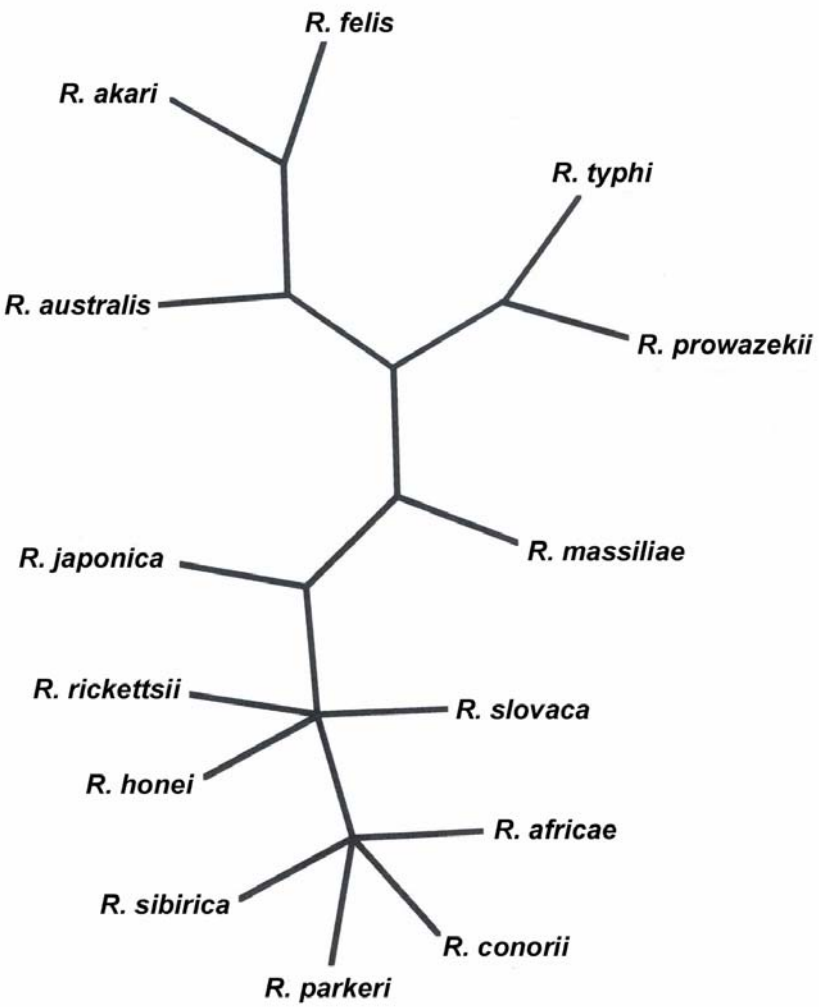


Illustration 1.1: Un-rooted phylogenetic tree of human pathogens from the genus *Rickettsia.*

The Lifestyle of an Obligate Intracellular Pathogen

As mentioned briefly before, rickettsiae have and are adapting to their unique intracellular niche. Not only can rickettsiae utilize host cell nutrients for their own purposes, but it leaves them relatively protected from many of the host's innate defense mechanisms. Rickettsiae adhere to host cells via the interaction of the rickettsial OmpB protein with its cellular receptor, the Ku70 subunit of DNA-dependent protein kinase (Martinez *et al.*, 2005). Ku70 is recruited to cholesterol-rich microdomains at sites of rickettsial invasion and is subsequently ubiquitinated following rickettsial infection. Once engulfed in a phagosome, the rickettsiae quickly escape prior to phagolysosomal fusion, therefore avoiding lethal activated lysosomal enzymes. In a set of simple, yet elegant, experiments Whitworth et al. demonstrated that two rickettsial proteins were responsible for phagosomal escape. Within 30-50 minutes after invasion, *pld* and *tlyC* (phospholipase D, hemolysin C) are transcribed at the peak of rickettsial escape from the phagosome. Transfer of either gene to *Salmonella*, normally an endosomal inhabitant, conferred the ability of these bacteria to escape the phagosome into the host cell cytoplasm (Whitworth *et al.*, 2005). Rickettsiae also upregulate the expression of the invasion gene, *invA*, to buffer the effects of stress-induced toxic nucleotide production. Dinucleoside oligophosphates are converted into a useful molecule, ATP, which promotes the survival of the organism and provides it with a constant source of co-factors (Gaywee *et al.*, 2002a;Gaywee *et al.*, 2002b;Gaywee *et al.*, 2003).

Once inside the cytoplasm SFG rickettsiae use host cell actin as a means of motility and cell-to-cell spread. Elegant studies performed by Heinzen using live cells (Heinzen *et al.*, 1999;Heinzen, 2003) demonstrated that *R. rickettsii* moves at an average rate of 4.8 ± 0.6 μm/min inside the cell. Unlike *Shigella* or *Listeria*, rickettsiae do not utilize the host cell proteins N-WASP or the Arp2/3 complex to polymerize actin (Harlander *et al.*, 2003), although the Arp2/3 complex is needed for binding and entry of rickettsiae into the host cell (Martinez and Cossart, 2004). Instead it appears that SFG

rickettsiae use the protein RickA which is expressed on the cell surface to polymerize actin (Gouin *et al.*, 2004). Rickettsial actin tails do associate with the cytoskeletal proteins profilin, vinculin, and filamin; however, the importance of this interaction is not well understood (Van Kirk *et al.*, 2000). This characteristic of rickettsiae demonstrates one of the challenges faced in treating rickettsial infection; specifically that organisms can spread from cell to cell without ever venturing into the blood, meaning they are hidden from many antibiotics that can't cross the lipid bilayer of mammalian cells. Actin based motility is generally only associated with SFG rickettsiae. Because of the inability of *R. prowazekii* to polymerize actin and the poorly developed actin tails induced by *R. typhi* leading to inefficient cytoplasmic locomotion, TG rickettsiae divide in the cytoplasmic compartment until the host cell bursts, releasing numerous rickettsiae into blood stream which in turn invade endothelial cells downstream.

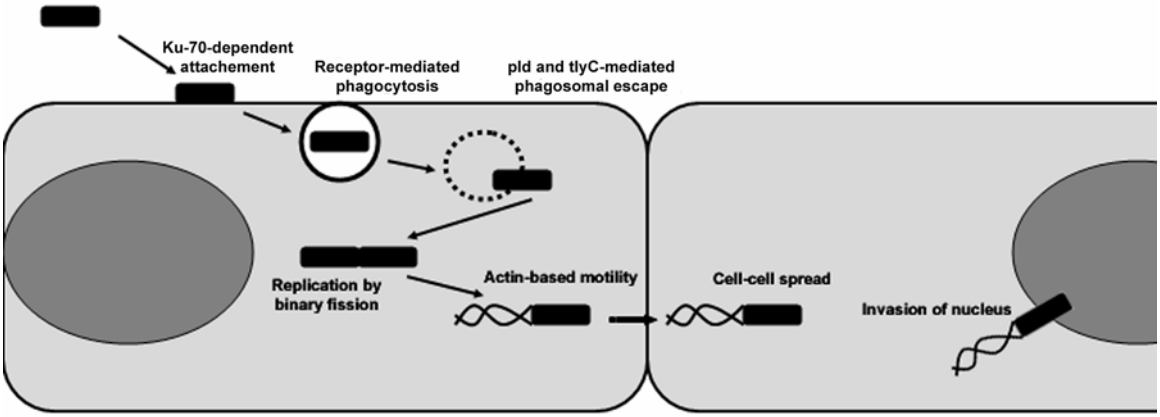


Illustration 1.2: The intracellular life-cycle of spotted fever group rickettsiae.

Rocky Mountain spotted fever: An American Disease

History in North America: "A Blight on the Bitterroot"[3]

First recognized in 1896 in the Snake River Valley of Idaho, Rocky Mountain spotted fever (RMSF) was first referred to as "black measles", undoubtedly due to the non-specific symptoms and characteristic rash. It wasn't until 1904 that Wilson and Chowning first described "spotted fever" in their seminal work from the Bitterroot Valley of Montana. They were the first to describe the clinical features, gross pathology, geographic localization to the western side of the Bitterroot River, and the lack of any culturable bacteria (Wilson and Chowning, 1904). They went on to describe the "increased exposure to infection through their occupation or pleasure taking them outdoors in the foothills and mountains in the spring of the year" as an explanation for the seasonality of the disease and the high degree of association with males between the ages of 20 and 40 years.

Perhaps the greatest contribution to the early study of these organisms came from a young pathologist by the name of Howard Taylor Ricketts. He arrived in Missoula, Montana in the spring of 1906 where he established a camp and laboratory composed mostly of tents (Walker, 2004). Ricketts' biggest contributions came from his description of the wood-tick, *Dermacentor occidentalis*, as the vector of disease (Ricketts, 1906b). He also demonstrated experimental transmission of the disease from the blood of patients to guinea pigs and monkeys, maintenance of the agent via serial passages from monkey to guinea pig and back, and the ability of female ticks to acquire the organism by feeding on an infected animal and to transmit the disease to a naive animal (Ricketts, 1906a;Ricketts, 1907). Based on observations of Wilson and Chowning, Ricketts was the first to identify a new class of pathogens: obligate intracellular bacteria. It was clear that the "protozoon" associated with erythrocytes could be found intracellularly. The agent

[3] From Harden VA., *Rocky Mountain Spotted Fever. History of a Twentieth-Century Disease*. Baltimore, MD: Hopkins University Press; 1990:1–375.

was filterable unlike most viruses, and was not culturable given the state of the art at the time (Ricketts, 1909). In retrospect, Ricketts was the first to connect obligate intracellular bacterial organisms with disease, in this case Rocky Mountain spotted fever.

Ricketts' career in what would become rickettsiology was short lived. He died from laboratory-acquired typhus in Mexico City in May 1910. This was not an unusual outcome of early rickettsial research as some of the greatest contributors to fundamental rickettsiology became victims of their own work. Laboratory acquired infections were common and in the age before antibiotics, the probability of dying from infection with *R. rickettsii* or *R. prowazekii* was ~30%. Today these agents are handled in Biological Safety Level-3 laboratories where the organism is confined to biological safety cabinets with laminar flow and researchers wear N-95 respirators, a far cry from the open air, bench-top research performed in the early 20th Century.

Epidemiology and Ecology of Rocky Mountain spotted fever

RMSF truly is a disease where the classic epidemiological triad (host, infectious agent and environment) interact very closely. *R. rickettsii* is maintained in nature by its tick vector via transstadial and transovarial transmission with small mammals serving as amplifying hosts (Burgdorfer and Varma, 1967). The primary vector of disease in the western United States is the Rocky Mountain wood tick, *Dermacentor andersoni,* whereas the primary vector in the eastern and Midwestern states is the American dog tick, *D. variabilis*. Despite the wide distribution of vectors, less than 1% of wood ticks are infected, possibly due to rickettsiae-mediated pathology in the tick (Niebylski *et al.*, 1999). The question of why virulent *R. rickettsii* has been confined geographically to only the western slopes of the Bitterroot Valley for over a century is fascinating, and may be related to exclusion mediated by the symbiotic rickettsiae *R. peacockii*.

Despite its name, Rocky Mountain spotted fever is found throughout North and South America including Colombia (Hidalgo *et al.*, 2007) and Brazil where it is referred to as Brazilian or Sao Paulo spotted fever (Davis and Parker, 1933;Labruna *et al.*, 2004). In the United States most cases are reported in North Carolina, Oklahoma, Missouri, Arkansas, and Tennessee. The vast majority of cases occur between the months of April and September when the potential for human interaction with ticks is at its highest. Two-thirds of all RMSF cases occur in children under the age of 15, with the peak age between 5 and 9 years. Males and Caucasians are also infected at a higher incidence than any other group. The annual number of reported cases is typically around 1,000 individuals with an overall case fatality rate of approximately 5%. Compare this to the untreated case fatality rate of ~30% and it should be evident that early diagnosis is critical to the successful treatment of RMSF.

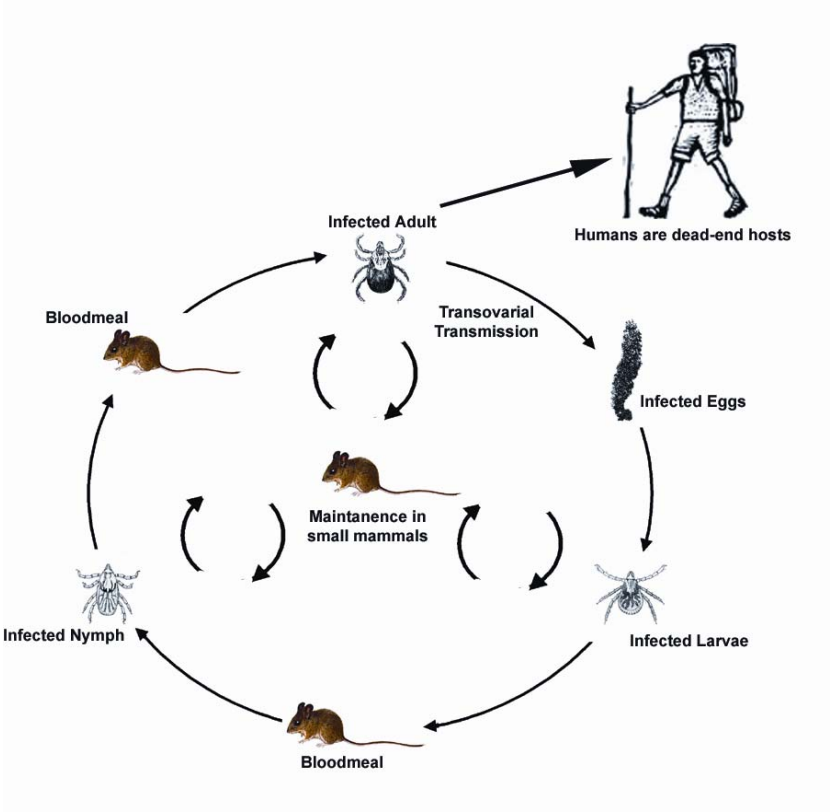


Illustration 1.3: Natural transmission cycle of RMSF

Other Rickettsial Diseases

Rickettsiae are a diverse group of organisms not all of which are pathogenic to humans. Many rickettsiae are inhabitants of arthropods and are not known to cause disease (Niebylski *et al.*, 1997). Still, there is a significant number of rickettsiae that do cause serious and potentially life threatening disease (Table 1.1). In fact, many rickettsiae that were once thought not to cause disease have only recently been associated with human infection. An example of this is *R. parkeri*, a SFG rickettsiae first identified in Gulf Coast ticks outside of Houston, Texas in 1939 (Parker, 1939). The first confirmed case of *R. parkeri* infection in humans was described very recently, leading scientists and health professionals to reassess the public health importance of rickettsiae not previously recognized as causes of human spotted fever (Paddock *et al.*, 2004).

From a practical standpoint, the two most virulent human rickettsial infections are Rocky Mountain spotted fever and epidemic typhus caused by *R. rickettsii* and *R. prowazekii*, respectively. *R. prowazekii,* as opposed to all other rickettsiae, is transmitted by the human body louse *Pediculus humanus humanus*. The other member of the TG is *R. typhi*, which is transmitted by the cat flea, *Ctenocephalides felis*, or the oriental rat flea, *Xenopsylla cheopis*. Transmission usually does not occur by the bite of an infected louse, but rather by self-inoculation of rickettsiae at the time humans scratch the bite site. In situations of especially poor hygiene such as famine or war, *R. prowazekii* is transmitted person-to-person by the transfer of infected lice. As discussed previously, *R. prowazekii* has been responsible for large scale epidemics associated with high mortality rates throughout history, and as recently as the 20th Century. *R. prowazekii* has been designated as a Category B Priority Pathogen and a CDC Select Agent. *R. prowazekii* was weaponized by the Soviet Union during the Cold War and makes an ideal biological weapon due to its low infectious dose, high potential for aerosol infection and high level of pathogenicity.

Table 1.1: Important Human Rickettsioses

Rickettsiae	Disease	Vector	Geographic Distribution	Case Fatality Rate*
Spotted-fever Group				
R. rickettsii	Rocky Mountain spotted fever	Tick	North and South America	25-30%
R. conorii	Boutonneuse fever	Tick	Mediterranean, Africa, India	5%
R. australis	Queensland tick typhus	Tick	Australia	<1%
R. akari	Rickettsialpox	Mite	North America, Europe and Korea	None reported
R. sibirica	North Asian tick typhus	Tick	Russia, China, Mongolia, Pakistan	None reported
Typhus Group				
R. prowazekii	Epidemic typhus	Louse	Worldwide	25-30%
R. typhi	Murine typhus	Flea	Worldwide	<5%

* Untreated case fatality rate. With early and appropriate therapy most rickettsial infections are curable.

Pathogenesis of Rocky Mountain spotted fever

Transmission

As discussed previously, *R. rickettsii* is transmitted by the bite of an infected tick, and in North America the primary vectors are the *Dermacentor* ticks. However, outbreaks of RMSF have now been associated with *Rhipicephalus sanguineus* ticks in the southwestern United States (Demma *et al.*, 2006;Wikswo *et al.*, 2007). In South America, *R. rickettsii* is transmitted primarily by *Amblyomma cajennense* and *A. aurolatum* (Labruna *et al.*, 2004). Unfortunately, the lack of a practical tick-transmitted model of RMSF has left many questions unanswered. Specifically, what is the effect of tick salivary proteins on disease transmission and pathogenesis?

Another potential mode of transmission is inhalation of organisms following aerosol exposure. This is especially evident given the high number of laboratory

acquired infections with RMSF. Current estimates place the number of viable organisms needed to produce disease in humans at fewer than 10 organisms delivered via aerosol (Saslaw and Carlisle, 1966;Saslaw *et al.*, 1966;Bouyer and Walker, 2007).

Entry

RMSF infection is the result of bacterial invasion of the body following a tick bite Aerosol exposure results in entry of the organisms via the lungs as would be experienced in a laboratory accident or a bioterrorism event. Other possible routes of entry include mucous membranes exposed to the environment, especially the conjunctiva.

Spread

The very early events in rickettsial spread are not well understood. However, very recent work by Jordan *et al*. has addressed the role of dendritic cells (DCs) as the immediate target of infection following intradermal inoculation (Jordan *et al.*, 2007). This work demonstrated that DCs are productively infected by *R. conorii* and undergo maturation and migration from the skin to the draining lymph nodes. This response is very important to the development of a protective immune response since mice vaccinated with *R. conorii*-infected DCs exhibit complete protection from lethal rickettsial challenge. The fact that these mice are protected demonstrates the need for a tick-transmitted model of infection, as it is likely that tick saliva-derived factors are very important to host immune suppression and the establishment of a productive infection. Nonetheless, once in the lymph node, rickettsiae have access to the host's vascular system leading to systemic dissemination.

Target Cells and Organs

As mentioned, the primary target cell for most rickettsiae is the endothelial lining of the microcirculation (arterioles, capillaries and venules). Secondary target cells include macrophages and to a lesser extent hepatocytes. In animal models and humans with RMSF spotted fever group rickettsiae have been observed in vascular smooth muscle cells in the media of arterioles. This is most likely due to actin-based motility pushing the rickettsiae out of the endothelium and into an adjacent cell (Feng *et al.*, 1993).

Evasion of Host Defenses

The primary concern following engulfment by a host cell is to escape the phagocytic vacuole. Whereas organisms like *Mycobacterium tuberculosis* or *Ehrlichia chaffeensis* thrive in the endosomal compartment, rickettsiae do not possess a mechanism to counteract the acidification pathways used to destroy many pathogens. Instead, rickettsiae avoid these pathways altogether by degrading the membrane of the phagocytic vacuole through the activity of phopsholipase D and hemolysin C, the products of *pld* and *tlyC* (Whitworth *et al.*, 2005). Once in the cytoplasm, rickettsiae avoid the harsh environment created by activated lysosomal enzymes. Instead, they face different intracellular defense mechanisms such as nitric oxide (NO) production and others. Cytokine stimulation of the endothelium has been shown to be essential to the elimination of rickettsiae. However, persistent cultures of *R. prowazekii* in L929 cells treated with IFN-γ eventually select for rickettsiae that are resistant to the effects of IFN-γ (Turco and Winkler, 1989;Turco and Winkler, 1990). The available *in vivo* data demonstrate that IFN-γ is a critical host defense factor and contributes to the elimination of rickettsiae partly through the induction of nitric oxide (Walker *et al.*, 1997b).

Animal Models of Rickettsioses

The availability of accurate and dependable small animal models of disease is critical to the proper understanding of immunity and pathogenesis in any condition. Fortunately, there are several mouse models of human rickettsioses that have been established to accurately represent human infection (Table 1.2). C3H/HeN mice are susceptible to infection with either *R. conorii* or *R. typhi* and are good models for human infection with *R. rickettsii* and *R. prowazekii*, respectively (Walker *et al.*, 1994b;Walker *et al.*, 2000). These two models have been used extensively to demonstrate the role of the cellular immune response in clearance of rickettsial organisms from the vasculature. The lack of knockout animals in the C3H/HeN background makes mechanistic studies of specific pathways difficult and inconclusive, though. Therefore, *R. australis* infection of C57BL/6 mice provides us with a model capable of utilizing gene knockout mice to investigate the mechanisms of lymphocyte recruitment to sites of infection, mediators of CTL activity, and the role of specific cytokines in mediating immunity to rickettsiae (Walker *et al.*, 2001b;Valbuena and Walker, 2004).

Table 1.2: Mouse models of human rickettsioses

	R. conorii	***R. australis***	***R. typhi***
Mouse strain	C3H/HeN	C57BL/6 or Balb/c	C3H/HeN
Route of inoculation	i.v.	i.v.	i.v.
Outcome	Dose-dependent mortality	Dose-dependent mortality	Dose-dependent mortality
Primary target cell	Endothelium	Endothelium	Endothelium

MECHANISMS OF IMMUNITY TO RICKETTSIAE

Cellular Immune Responses

As an obligate intracellular pathogen, it should be evident that an effective cell-mediated, Th-1 response is critical to the effective clearance of rickettsial organisms from the body. This is in fact the case, and much work has gone into understanding the events

leading up to a $CD8^+$ lymphocyte response during acute rickettsiosis. In fact, perivascular inflammation resulting in meningoencephalitis and interstitial pneumonitis are classic hallmarks of rickettsial infection. Rickettsial vasculitis and perivasculitis are the morphologic manifestation of the immune response to the infection and are therefore necessary for rickettsial clearance. However, this inflammatory response is thought to be responsible for the microcirculatory dysfunction seen during acute rickettsioses.

Infection of C3H/HeN mice with *R. conorii* results in a 5-6 day course of infection when given a lethal dose. Rickettsiae are found throughout the body with particularly high titers in the lungs and testes. Early signs of infection include granulomas in the liver as well as margination and emigration of mononuclear cells in the brain, lung, heart and testis to the vascular wall and perivascular compartments. By day 6 of infection most rickettsial foci are associated with lymphohistiocytic infiltration, and in sub-lethal infection the inflammation persists for up to 15 days after infection (Walker *et al.*, 1994a). Later work using this model demonstrated the importance of $CD8^+$ lymphocytes in clearing infection. Mice depleted of CD8 lymphocytes and infected with a sub-lethal dose either died, or remained persistently infected through day 15. Conversely, mice depleted of $CD4^+$ lymphocytes still cleared a sublethal infection and recovered. Immune $CD4^+$ and $CD8^+$ lymphocytes were both capable of protecting mice from a lethal dose of rickettsiae (Feng *et al.*, 1997). Further work supports this idea by demonstrating the critical importance of major histocompatibility complex (MHC) class I-matched cytotoxic T-lymphocyte (CTL) activity in rickettsial infection (Walker *et al.*, 2001a). CTLs from *R. conorii*-immune mice demonstrate MHC-I restricted activity against infected endothelial cells with a peak activity after 10 days of infection. Likewise, spleen cells from *R. australis*-immune mice show CTL activity against *R. australis*-infected macrophage-like cells. MHC-I knockout mice were more than 50,000-fold more susceptible to a lethal infection than normal mice.

Specific effector functions were also studied demonstrating the critical importance of IFN-γ and perforin in CTL-mediated clearance of rickettsiae. IFN-γ knockout mice were only 100-fold more susceptible to a lethal outcome as were perforin knockout mice, demonstrating the importance of these two pathways. Adoptive transfer of IFN-γ secreting $CD8^+$ cells into IFN-γ knockout mice reduced the organ titers of rickettsial organisms. Additionally, adoptive transfer of $CD8^+$ cells from IFN-γ knockout mice also reduced the rickettsial load, indicating the importance of CTL activity in clearing infection. Clearly, both mechanisms are important in the effective clearance of rickettsial infection.

The next most logical question was to understand the signals needed to recruit CD8 lymphocytes to sites of rickettsial infection, and this remains an area of active investigation. The CXCR3 ligands CXCL9 and CXCL10 are significantly upregulated in the lungs of *R. conorii*-infected mice and in human autopsy material (Valbuena *et al.*, 2003). However, the neutralization of these chemokines with antibody has no effect on the outcome of infection (Valbuena and Walker, 2004). Perhaps in the future we will better understand the chemotractant gradients that attract lymphocytes to sites of rickettsial infection.

Very recent work has been aimed at understanding the importance of dendritic cells in the very early stages of rickettsial infection. Dendritic cells are often termed the orchestrators of the immune response by helping to direct and polarize the immune system towards one particular pathway of response. As a likely target for rickettsiae following the bite of an infected arthropod, it should come as no surprise that rickettsiae can productively infect dendritic cells resulting in the upregulation of specific activation markers on the cell surface (Jordan *et al.*, 2007). This response is also associated with the increased production of certain cytokines including IL-1, IL-12(p40), IL-23 and IL-6. Dendritic cells infected with *R. conorii* and then transferred into naïve mice one day later confer protection from lethal infection, demonstrating the importance of a properly

coordinated immune response very early after infection. However, it is still challenging to understand the true role of dendritic cells in natural rickettsial infection in a model not based on tick transmission. .

Another cell type activated early in infection is natural killer (NK) cells. Depletion of NK cells with monoclonal antibodies enhanced the susceptibility of mice to rickettsial infection and was associated with decreased levels of serum IFN-γ (Billings *et al.*, 2001). The importance of IFN-γ in rickettsial immunity has been well defined, and NK cells are likely an early source of this critical cytokine.

The role of neutrophils in rickettsial infection has not been defined, nor is it clear that these professional phagocytes have any role in rickettsial immunity at all. However, one report has demonstrated that productive infection of endothelial cells with *R. rickettsii* results in the E-selectin-dependent adhesion of neutrophils to the cell surface (Sporn *et al.*, 1993). This effect peaks at 6-8 hours after infection and is diminished by 24 hours.

Perhaps one of the most important discoveries in rickettsiology of the past 20 years is that sublethal infection in mice results in cross-protective immunity to all other rickettsiae. Surprisingly, heterologous immune serum does not confer protection, questioning the role of antibody in protection from future infections (Feng and Walker, 2003). It has now been shown that protection is mediated by T cells (Valbuena *et al.*, 2004). Mice previously infected with *R. conorii* are completely immune to *R. typhi* infection, and vice-versa. Although heterologous infection causes clinical manifestations in mice, they are 100% protected from lethal infection. Adoptive transfer of T-lymphocytes from immune mice confers protection from homologous and heterologous infection and is associated with IFN-γ production by stimulated T-lymphocytes. Likewise, human volunteers previously immunized to *R. conorii* possess both $CD4^+$ and $CD8^+$ cells that produce similar levels of IFN-γ when challenged with *R. conorii* or *R. typhi* antigen. In conclusion, not only is a cellular immune response critical to protection

from primary infection, but $CD4^+$ and $CD8^+$ cells are also important in conferring lifelong protection.

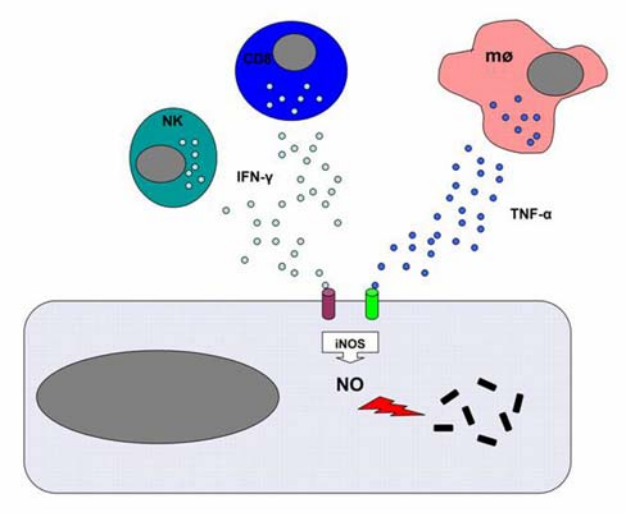
Still, the effective clearance of rickettsial infection results in life-long immunity and is associated with relatively high IgG titers specific for rickettsiae. In fact, people infected with *R. rickettsii* during childhood occasionally exhibit high antibody titers even after more than 20 years. It may seem counterintuitive that antibody would play a direct role in inhibiting obligately intracellular bacterial pathogens. However, it is now known that polyclonal antibody or monoclonal antibodies to OmpA and OmpB inhibit the ability of rickettsiae to invade host cells by blocking phagosomal escape (Feng *et al.*, 2004b), and confer protection to *R. conorii*-infection in severely immunocompromised SCID mice (Feng *et al.*, 2004a). Although it is still not clear whether antibody plays any role in primary infection, it would be prudent to include B-cell epitopes in any future subunit vaccine.

Innate Cellular Responses

As the primary target cell of rickettsiae, endothelial cells respond to infection by increasing the secretion of specific cytokines, and by responding to inflammatory stimuli directed at the infection. Perhaps the best studied response of endothelial cells involving immunity to rickettsiae is the production of NO. NO is a widely studied molecule with broad effects on vascular tone, coagulation and hemostasis. Activation of nitric oxide synthase (NOS) activity results in the production of nitric oxide via an enzymatic reaction involving the conversion of $_L$-arginine into $_L$-citrulline, resulting in the liberation of one molecule of nitric oxide and a molecule of water (Knowles *et al.*, 1989; Palacios *et al.*, 1989). We now know that NO production is an important effector function used by many cell types to control the number of intracellular pathogens including viruses,

parasites and rickettsiae (Karupiah *et al.*, 1993; MacMicking *et al.*, 1997;Nathan and Shiloh, 2000;Ismail *et al.*, 2002).

For many years it was well known that TNF-α and IFN-γ were crucial host defenses to rickettsial infection and were associated with inhibition of *R. conorii* growth both *in vitro* and *in vivo* (Li *et al.*, 1987; Manor and Sarov, 1990). However, it wasn't until later that scientists fully understood the mechanism of inhibition of rickettsial growth by these cytokines when it was demonstrated that they exert their anti-rickettsial effect via production of NO (Feng and Walker, 1993). Murine endothelial cells stimulated with the pro-inflammatory cytokines TNF-α and IFN-γ up-regulate the expression iNOS in a dose- and time-dependent manner and this response in associated with decreased numbers of intracellular rickettsiae (Walker *et al.*, 1997). Further, depletion of IFN-γ and TNF-α in mice infected with *R. conorii* results in a fatal, overwhelming infection associated with increased numbers of rickettsiae in spleen, liver, and lungs. Additionally, treatment of mice with an L-arginine analog reduced the amount of NO by-products in the urine and impaired rickettsial killing in mice (Feng *et al.*, 1994). Concurrent work by others demonstrated similar effects with *R. prowazekii* infection of fibroblasts and macrophage-like cells (Turco and Winkler, 1990;Turco and Winkler, 1994;Turco *et al.*, 1998). This rickettsicidal mechanism was eventually demonstrated in human cells, including endothelial cells, hepatoctyes and macrophages (Feng and Walker, 2000). What was interesting about this work was that it also attributed some anti-rickettsial activity to reactive oxygen species (ROS), specifically hydrogen peroxide. The importance of hydrogen peroxide in rickettsial pathogenesis will be discussed in much detail later. This work also showed that tryptophan depletion by indoleamine-2,3-dioxygenase (IDO) expression prevented the growth of rickettsiae. IDO is a well described host defense pathway to a number of bacteria and parasites which acts to starve infectious agents of important building blocks (Daubener and MacKenzie, 1999;MacKenzie *et al.*, 1999;MacKenzie *et al.*, 2007). Very recent evidence



demonstrates a positive correlation between the intensity of TNF-α and IL-1β production and disease severity in human Mediterranean spotted fever patients (Sousa *et al.*, 2007). This report also confirms a role for IDO activity in human rickettsial infection.

Illustration 1.4: The anti-rickettsial effects of TNF-α and IFN-γ are partly dependent on iNOS-dependent NO production.

INTERACTION OF RICKETTSIAE AND THE MICROVASCULAR ENDOTHELIUM

Rickettsiae-Mediated Host Cell Injury

Rickettsiae-mediated cytotoxicity has been documented for well over twenty-five years, and is the result of necrotic cell death 4-6 days after infection *in vitro* (Walker and Cain, 1980;Walker *et al.*, 1982). In fact, plaque assays are a standard laboratory technique for the quantification of infectious rickettsiae in working stocks. *In vitro*, host cells infected with *R. rickettsii* undergo a number of changes associated with cell damage that have been observed ultrastructurally. Mitochondria are often swollen, potentially causing significant alterations of host cell metabolism. The outer nuclear envelope becomes distended and the rough endoplasmic reticulum (ER) is dilated. Very late in infection, cells demonstrate large numbers of intracisternal cytoplasmic islets, and the remaining rickettsiae are often enclosed by host cell membrane (Silverman and Wisseman, Jr., 1979). *R. prowazekii*, on the other hand, causes less morphological changes in infected cells, instead replicating to very high levels prior to inducing cell

lysis (Silverman *et al.*, 1980). Unfortunately, the importance of endothelial cell death *in vivo* during acute rickettsioses is still not well understood and has not received sufficient attention despite the availability of several small-animal models of infection.

The primary mechanism of rickettsiae-mediated cell injury, to the best of our knowledge, is the formation of reactive oxygen species (ROS) resulting in lipid peroxidation (Silverman and Santucci, 1988). Furthermore, it has been demonstrated that the primary ROS produced by *R. rickettsii*-infected endothelial cells are the superoxide anion and its downstream decomposition product hydrogen peroxide which can have profound effects on cellular adhesion. Superoxide dismutase (SOD), the enzyme responsible for superoxide decomposition, is upregulated very early after internalization of *R. rickettsii* (Hong *et al.*, 1998), explaining the presence of hydrogen peroxide both intracellularly and in cell culture supernatants. Infection is also associated with decreased levels of intracellular reduced glutathione (GSH) and glutathione peroxidase (GPX) activity (Eremeeva and Silverman, 1998a;Eremeeva and Silverman, 1998b). The anti-oxidant, α-lipoic acid, was shown to increase cell viability and glutathione peroxidase activity and decrease peroxide levels. This was associated with reduced ultrastructural changes compared to non-treated, infected cells.

It has now been shown in a mouse model of infection that these key antioxidant enzymes are modulated in host tissues during *R. conorii* infection (Rydkina *et al.*, 2004). Specifically, in the lungs the activity of GPX, SOD and GSH increases during the first 48 hours of infection. Surprisingly, the activities of all three enzymes decrease relative to controls in high-dose infected animals at 96 hours. The administration of α-lipoic acid to these animals ablates this drop in activity at 96 hours and also results in dramatically higher numbers of rickettsiae, indicative of the anti-rickettsial activity of ROS described by Walker.

Rickettsiae-induced Inhibition of Apoptosis

As an obligate, intracellular pathogen it is to the benefit of rickettsiae to delay cell death for an amount of time sufficient to allow intracellular replication and cell to cell spread. To this end, *R. rickettsii* inhibits host cell apoptosis via an NF-κB-dependent pathway (Clifton *et al.*, 1998). Pharmacologic inhibition of NF-κB results in increased levels of apoptotic cells very early after infection because of a lack of NF-κB translocation to the nucleus. Additional work demonstrated that the mechanism of action of NF-κB-mediated inhibition of apoptosis is through blockade of apical and effector caspases and the maintenance of mitochondrial integrity (Joshi *et al.*, 2003). Inhibition of NF-κB activation during *R. rickettsii* infection results in decreased Bcl-2 levels, an inhibitor of apoptosis. Likewise, mitochondrial proteins are released into the cytoplasm in the presence of the NF-κB inhibitor confirming the importance in maintaining mitochondrial integrity during invasion by rickettsiae (Joshi *et al.*, 2004).

At this time we have not identified any bacterial virulence factors that specifically target the NF-κB pathway, or any other host cell pathway, that may explain the mechanism of rickettsiae-mediated host cell gene transcription.

Endothelial Cell Response to Infection

Upon invasion by rickettsiae endothelial cells undergo a series of changes involving the activation of host signaling pathways resulting in the expression and secretion of cytokines, chemokines and regulators of hemostasis. Early work demonstrated the release of von Willebrand factor (vWF) from Weibel-Palade bodies in response to *R. rickettsii* infection, and this was dependent on the intracellular presence of rickettsiae (Sporn *et al.*, 1991). Later it was demonstrated that human endothelial cells also produce tissue factor (TF) very quickly after invasion by *R. rickettsii*, possibly leading to the activation of coagulation in infected vessels (Sporn *et al.*, 1994). This

response was demonstrated to be dependent on autocrine cell stimulation via the production of cell-associated IL-1α (Sporn and Marder, 1996). Additionally it was shown that protein kinase C (PKC) activation during infection is a downstream activator of TF expression, presumably in response to IL-1α stimulation (Sahni *et al.*, 1999). While TF and vWF production by endothelial cells likely does not contribute directly to increased microvascular permeability, the observed PKC activity in rickettsiae-infected endothelial cells is exciting given the wealth of evidence linking PKC to the regulation of endothelial permeability. On the other hand, the significance of the changes observed in TF levels and vWF production *in vitro* is not known in humans or mice since thrombotic complications in rickettsiosis are infrequent (Elghetany and Walker, 1999).

Others went on to describe the cytokines secreted by endothelial cells in response to rickettsial infection. *R. conorii* infection of human endothelial cells leads to the production of IL-6 and IL-8, again via a cell-associated IL-1α-dependent pathway much like TF expression (Kaplanski *et al.*, 1995). IL-6 and IL-8 are two important regulators of inflammation that help recruit immune cells to sites of infection and this provides a possible explanation for the vasculitic phenomena observed during the acute phase of the disease. IL-8 was also shown to be produced by *R. rickettsii* infected human endothelial cells and was shown to be regulated by NF-κB activation of the p38 MAP kinase cascade (Clifton *et al.*, 2005;Rydkina *et al.*, 2005). However, this work also detected the expression of monocyte chemoattractant protein-1 (MCP-1) in addition to IL-8. MCP-1 serves to activate and recruit monocytes to sites of infection, but it also has been shown to be capable of inducing blood-brain barrier breakdown resulting in increased microvascular permeability and vasogenic cerebral edema (Stamatovic *et al.*, 2003;Stamatovic *et al.*, 2005). This is particularly intriguing given the similar evidence with IL-6 that demonstrates this cytokine is able to induce endothelial permeability *in vitro* and is associated increased vascular permeability in a mouse model of bacterial meningitis (Maruo *et al.*, 1992;Paul *et al.*, 2003). Taken together we can begin to see that

host cell response to infection may play an important role in the increased vascular permeability experienced during acute rickettsioses.

Clinical Complications Associated with Vascular Dysfunction during Acute Rickettsioses

The two most important clinical features associated with acute rickettsial infections like RMSF are acute interstitial pneumonitis and meningoencephalitis. While inflammation is generally regarded as a protective response aimed at clearing the infection, we still do not fully understand the consequences of widespread perivascular inflammation with respect to disease severity or potential immunopathogenesis.

Analysis of human autopsy cases of fatal RMSF demonstrates a correlation between the distribution of rickettsiae and the presence of vasculitis. In the lungs, infection of the microvasculature is associated with alveolar septal congestion and interstitial edema, alveolar edema, fibrin deposition, the presence of macrophages and in fatal cases, hemorrhage (Walker *et al.*, 1980). Clinically we see the consequences of pulmonary involvement through the high number of critically ill patients that require mechanical ventilation (Buckingham *et al.*, 2007).

Meningoencephalitis and encephalitis are two important manifestations of severe RMSF (Horney and Walker, 1988). Infection of the brain microvasculature results in perivascular inflammation associated with foci of infection leading to dramatic clinical features indicating neurological involvement. Coma can occur in up to 10% of patients and neurological deficits after discharge occur in up to 15% of patients. These can include speech and/or swallowing problems, global encephalopathy, gait pattern disturbances, and rare incidence of cortical blindness (Buckingham *et al.*, 2007).

There is clear evidence that endothelial cells are damaged during rickettsial infection *in vivo*. In guinea pigs infected with *R. rickettsii*, we see cytopathic changes associated with endothelial cell damage including detachment of endothelial cells from

the basement membrane (Walker *et al.*, 1977). We also know that human patients with Mediterranean spotted fever possess a higher number of circulating endothelial cells and cell fragments and this is associated with increased plasma levels of thrombomodulin and von Willebrand Factor (George *et al.*, 1993). Still, a mechanism for endothelial cell release from infected vessels has yet to be described.

There has been very little work directly addressing the presence and severity of vascular permeability during rickettsial infections. Dogs experimentally infected with *R. rickettsii* demonstrate areas of retinal vasculitis and vascular leak within 1-2 days after the onset of fever (Davidson *et al.*, 1990). This change has also been observed in *R. conorii* infected mice (Valbuena *et al.*, 2002). Likewise, Rhesus monkeys infected with *R. rickettsii* demonstrate alterations in body fluid distribution including increased water content of the liver (Liu *et al.*, 1978). Clearly, the amount of information addressing increased microvascular permeability during acute rickettsial infection is very scant.

OUTSTANDING QUESTIONS RELATED TO SPOTTED FEVER PATHOGENESIS

Despite one-hundred years of research on Rocky Mountain spotted fever, there is still a lot we do not know about this disease. The lack of an adequate animal model of tick-transmitted rickettsiosis has limited our understanding of the disease to models of direct i.v. injection. Similarly, we do not know what the effects of tick salivary components are on disease transmission and pathogenesis. Perhaps the biggest question that has gone unanswered is what the mechanisms of increased microvascular permeability are during rickettsial infection. The available evidence points to rickettsiae-mediated cell death as the decisive outcome of infection and to a certain extent this is true. However, the role of the host immune response has been grossly underappreciated as a potential cause for the vascular damage experienced in this disease. With respect to

potential mechanisms of increased microvascular permeability we feel that the following need to be addressed:

- The role of innate host cell responses to infection that may contribute to endothelial dysfunction and increased microvascular permeability
- The role of lymphocyte-mediated denudation of the endothelium in vascular leakage
- The role of circulating cytokines in the induction of vascular leakage

The work presented herein represents the most in-depth investigation to date that directly addresses mechanisms of increased microvascular permeability during acute rickettsial infection, specifically of models of spotted fever rickettsioses. We have addressed the role of the host immune response in modulating microvascular permeability both *in vitro* and *in vivo* and have demonstrated that immunopathogenic responses to rickettsial infection probably play a role in the disease. Additionally we have identified innate pathways activated in rickettsiae-infected endothelial cells that contribute to the loss of interendothelial junction integrity leading to increased microvascular permeability, thus contributing to the disease manifestations. We feel that the knowledge gained by this work represents the first step towards a better understanding of rickettsial pathogenesis during severe disease and will one day lead to novel therapeutics for the treatment of severe, life-threatening forms of human spotted fever.

Chapter 2: Materials and Methods

RICKETTSIAE

All rickettsial stocks were transferred to Dr. Olano's laboratory from Dr. David H. Walker's laboratory (University of Texas Medical Branch) in the form of 10% yolk sac suspensions in sucrose-phosphate-glutamate (SPG) buffer.

Renografin density gradient purification of *Rickettsia*

For all *in vitro* experiments described in chapters three and four, we utilized renografin-purified rickettsiae that had been propagated in Vero cells according to the protocol of Hanson *et al* (Hanson *et al.*, 1981). Briefly, heavily infected Vero cells were collected by high speed centrifugation and sonicated to release intracellular rickettsiae. The rickettsiae contained in the supernatant were collected by low-speed (2000 rpm) centrifugation to remove cell debris followed by high-speed (13,000 rpm) centrifugation to pellet the rickettsiae. This crude rickettsial preparation was overlaid on a 30%/36%/42% discontinuous gradient of renografin-76. This was centrifuged at 22,000 rpm for 75 minutes after which time the viable rickettsiae at the 36%/42% interface were collected. The rickettsiae were washed one more time in SPG buffer and frozen at -80°C. Viable rickettsial content of the frozen stock was determined by plaque assay on Vero cells and was determined to be ~1 $x10^9$ plaque forming units (pfu) per ml. A summary of the different rickettsial species and strains used for these experiments is listed in Table 2.1.

Table 2.1: Summary of rickettsial species and strains utilized for in vitro experiments

Rickettsiae	Strain	Original Stock	Passage History	Purification	Working Stock Titer (PFU/ml)
R. rickettsii	**Sheila Smith**	**Infected SVEC**	**2-4x Vero 34°C**	**Renografin**	**1.5×10^8**
R. rickettsii	**Iowa**	**10% Yolk Sac**	**2x Vero 34°**	**Renografin**	**1.1×10^8**
R. conorii	**Malish 7**	**10% Yolk Sac**	**2x Vero 32°C**	**Renografin**	**2×10^9**
R. australis	**Cutlack**	**10% Yolk Sac**	**2x Vero 34°**	**Renografin**	**1×10^8**

Preparation of 10% yolk-sac suspensions for animal inoculations

For all experiments involving animal inoculations, we utilized 10% yolk-sac suspensions of *R. conorii* (Malish 7 strain) and *R. australis* (Cutlack strain). Briefly, rickettsiae were grown through two passages in embryonated chicken eggs. Yolk sacs from infected eggs with dead embryos were homogenized using a Waring blender in SPG buffer to create a 10% suspension. Following low-speed removal of cell debris, the rickettsial suspension was frozen at -70°C. For all animal experiments the LD_{50} of these stocks was determined in C3H/HeN mice for *R. conorii* and C57BL/6 mice for *R. australis* with a 300 µl volume dose.

Plaque Assay

Viable rickettsial content of our rickettsial stocks was determined by plaque assay. Briefly, we seeded 2×10^5 Vero cells per well on 24 well plates. At this seeding density the cells formed a confluent monolayer within 16-24 hours at 37°C + 5% CO_2. Frozen rickettsial stock was thawed quickly at 37°C, and 10-fold dilutions were made in Dulbecco's Modified Eagles Medium (DMEM) plus 5% bovine calf serum (BCS) (v/v)

and 1% HEPES (v/v). Exactly 200 µl of each dilution was overlaid on the Vero cells, and the plate was centrifuged at 400 x g for 5 minutes. The cells were then incubated at 37°C for 2 hours. After the rickettsiae were allowed to invade, the cells were then overlaid with 1 ml of Modified Eagle Medium (MEM) plus 0.5% agarose, 1 µg/ml cycloheximide, 1% HEPES, and 1% BCS. The cells were incubated at 37°C for 72 hours after which time plaques became visible and were counted to determine the content of the frozen stock.

CELL LINES

Vero Cells

Vero cells were used for propagation of all rickettsiae species. Vero cells were maintained in DMEM supplemented with 5% BCS and 1% HEPES buffer.

HMEC-1

The Human Microvascular Endothelial Cell Line (HMEC-1) was a kind gift from Francisco Candal (CDC, Atlanta, GA) (Ades *et al.*, 1992). This immortalized endothelial cell line was originally derived from dermal microvascular endothelial cells obtained from human foreskin. HMEC-1 were grown on collagen I-coated plates in MCDB 131 medium supplemented with 10% fetal calf serum (FCS), 100ug/ml heparin and 10ng/ml of epidermal growth factor (EGF).

SV-HCEC

SV-40-transformed human cerebral endothelial cells (SV-HCEC) were a kind gift from Drs. Som Dasgupta and Robert Yu of The Medical College of Georgia (Duvar *et al.*, 2000). This cell line was maintained in M199 medium supplemented with 10% heat-

inactivated FCS, Insulin-Transferrin-Selenium (ITS) supplement and 100 μg/ml heparin. Endothelial cells were grown on rat-tail collagen I-coated plates (BD Biosciences, San Jose, CA) and used between passages 15-25.

IN VITRO CELL CULTURE ASSAYS

Measurement of Trans-endothelial Electrical Resistance (TER)

Measurements of microvascular permeability were accomplished through the use of Electric Cell-Substrate Impedance Sensing, otherwise known as ECIS. All experiments were performed using the ECIS™ Model 1600R providing the capability to monitor 16 individual samples simultaneously on two individual 8-well arrays. We utilized 8W10E gold-coated electrodes composed of 8 chambered wells each containing ten 250 μm electrodes. For most experiments measurements of resistance were acquired every two minutes, and each value represents the average resistance reading for all ten electrodes. We found that artificial fluctuations in resistance were commonplace immediately after attaching the arrays to the array holder. To the best of our knowledge this is a result of temperature fluctuations of the gold-coated electrodes after being placed in the incubator. We found that a short incubation period of approximately five minutes prior to the initiation of measurements was efficient to minimize these artifacts.

Most experiments were performed in triplicate, and the absolute resistance values were normalized to time zero, or the very first measurement taken. The average normalized resistance was determined ± standard deviation. Any drop below 1.0 represents a decrease in transendothelial electrical resistance (TER) indicating an increase in permeability across the endothelial monolayer.

Endothelial cells were grown on 8W10E electrodes that had previously been coated with rat tail collagen for two hours. TER was monitored from the time of seeding,

and experimental conditions were not initiated until a steady state of resistance had been achieved and maintained for at least 24 hours.

Bio-Plex® Analysis of Cytokine Concentrations

We utilized multiplexed bead-based arrays for the determination of cytokine concentrations in serum and cell culture supernatants. The human group one 27-plex cytokine array (Bio-Rad, Hercules, CA) was used for the analysis of SV-HCEC supernatants in chapter 4 and human serum samples in chapter 5. Cell culture supernatants were first filtered through 0.22 μm syringe filters to remove rickettsiae and cell debris and assayed directly without any dilution according to the manufacturer's instructions. Human serum was diluted four-fold in human serum diluent before incubation with the beads. For the analysis of mouse serum, we utilized the mouse 23-plex and group two 9-plex cytokine arrays. Mouse serum was diluted four-fold in mouse serum diluent. We collected 100 beads per analyte, and data were extrapolated off of an 8-point standard curve.

Greiss Assay

Nitrite was measured with the Greiss assay as described previously (Walker *et al.*, 1997). Briefly, 100 μl of filtered cell-culture supernatant was mixed with an equal volume of 1% sulfanilamide, 0.1% N-(1-napthyl)ethylenediamine in 2.5% phosphoric acid. A standard curve was generated using a nitrite standard from 100 μM-0.78 μM, and absorbance was read at 540 ηm.

Quantification of Rickettsiae

DNA was isolated from infected cells using the DNeasy Tissue Kit (Qiagen). For all PCR reactions the following primers were used to amplify a 147 bp product of the

rickettsial *gltA* gene: forward primer CS-5(GAGAGAAATTATATCCAAATGTTGAT) and reverse primer CS-6(AGGGTCTTCGTGCATTTCTT) (Labruna *et al.*, 2004). For normalization to a human housekeeping gene, we amplified a fragment of the human β-actin gene using commercially available primers (SuperArray Bioscience). Real-time PCR was performed on an Eppendorf *realplex*^{2} instrument using SYBR Green Supermix (Bio-Rad) and primers at a concentration of 20 uM with the following protocol: 95ºC for 5 min, followed by 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec. Melting curves were run to ensure single product amplification and the absence of primer dimers. Where indicated standard curves were generated by cloning the PCR products into the TOPO TA-2.1 vector followed by ampicillin selection. Plasmid DNA was then purified using a Mini Prep kit (Qiagen) and individual clones were assayed for efficiency using the designated primers.

Determination of Protein Kinase C Activity

SV-HCECs were cultured in 60 mm tissue culture dishes until 100% confluent. The monolayers were then infected with approximately 1x10^7 pfu of *R. conorii* (Malish 7) or *R. rickettsii* (Sheila Smith) and incubated for 24 hours at 37°C. The supernatants were aspirated and the cells were lysed for 10 minutes on ice using 500 ul of NP-40 lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride and 10 μg/ml leupeptin and aprotinin). The cells were then collected by scraping of the flask with a rubber cell scraper. The lysates were then centrifuged for 15 minutes at 13,000 rpm and the supernatant was removed to a fresh pre-chilled microcentrifuge tube. The protein content of the lysates was measured using Bradford's reagent. Protein kinase C (PKC) activity was measured using a PKC kinase activity assay kit (Stressgen, Ann Arbor, MI). This kit

measures the ability of crude lysates to phosphorylate plate-bound substrate in the presence of ATP. Then a primary antibody specific for the phosphorylated peptide is bound followed by an HRP-conjugated secondary antibody. The plate is reacted with 3,3',5,5'-tetramethylbenzidine (TMB), and the reaction product was read at 450 ηm. Data were expressed as the fold-change in kinase activity relative to mock-infected cells.

Western Blotting

We utilized western blotting to investigate the expression levels and post-translational modifications of endothelial cell proteins that occured following infection with various rickettsiae. Cells were grown on rat tail collagen-coated tissue culture plates to 100% confluence and allowed to rest as a confluent monolayer for at least 24 hours. Following infection the cells were harvested by lysis with NP-40 lysis buffer for 10 minutes in the plate. The cell monolayer was scraped off the plate and placed into a pre-chilled microcentrifuge tube. The samples were vortexed briefly then centrifuged at ~13,000 x g for 15 minutes. The supernatant was removed and stored at -70°C. Protein concentrations were measured using Bradford reagent and diluted to equal concentrations of protein with lysis buffer. Equal amounts of proteins were then heated for 10 minutes at 70°C in LDS sample buffer (Invitrogen) and reducing agent. The samples were loaded onto a 10% Bis-Tris NU-PAGE gel in MOPS SDS buffer and electrophoresis was performed at 200 volts for 50 minutes. Following electrophoresis the proteins were transferred to a nitrocellulose membrane for 30 minutes at 15 volts. The membranes were blocked for 20 minutes in Tris-buffered saline (TBS) containing 2% non-fat dry milk (NFDM) and 0.2% Tween-20. Following the blocking step the membranes were incubated in primary antibody (Table 2.1) in TBS plus 1% NFDM and 0.1% Tween-20 at 4°C overnight with gentle agitation. The following day the membranes were washed three times and then incubated in horseradish peroxidase (HRP)-conjugated secondary

antibody at room temperature for 1 hour. Following three final washes in TBS, the membranes were incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 minutes for peroxidase detection. Chemiluminescent detection was performed by exposing the blots to ECL Hyperfilm and developed in an automated x-ray film developer.

Fluorescent Confocal Microscopy

Laser scanning confocal microscopy with an Olympus FV-1000 inverted microscope was used for all fluorescent image acquisition described in this work. Cells were grown on plastic chamber slides coated with rat tail collagen as described previously. Samples were fixed in methanol or 4% paraformaldehyde depending on the antibody for 20 minutes at 4°C. Following three washes in PBS the cells were permeabilized with 0.05% Tween-20 and blocked in 5% normal goat serum (NGS) for 20 minutes at room temperature. Most primary antibodies were incubated overnight at 4°C with gentle agitation in PBS containing 1% NGS. Following three washes in PBS the cells were incubated for 1 hour at room temperature with the appropriate secondary antibody. Cells were then mounted using Vectashield HardSet with DAPI (Vector Laboratories) and allowed to sit overnight at 4°C prior to image acquisition. Designated primary and secondary antibodies along with the working dilutions are listed in Table 2.1.

Target	Species	Specificity	Clone #	Stock Concentration	Vendor	Working Dilution	Use	Secondary Reagent
eNOS	Mouse	m,h	3	50 ug	BD Biosciences	1:100	IF	anti-mouse Alexa Fluor-568
F4/80 - macrophage	Rat IgG2a,κ	m	BM8	500 ug/ml	eBioscience	1:50	IHC	anti-rat biotin - ABC-AP w/ Vector Red
IL-6	Mouse	h	1936	500 ug/ml	R&D Systems	1:500	Neut.	-
iNOS	Mouse	m,h	54	50 ug	BD Biosciences	1:1000	WB	anti-mouse HRP
						1:100	IHC	anti-mouse HRP - TSA Alexa Fluor-488
MCP-1	Mouse	m,h	5D3-F7	1 mg/ml	BD Biosciences	1:50	IHC	anti-mouse HRP - TSA Alexa Fluor-488
p120	Rabbit	m,h	H-90	200 ug/ml	Santa Cruz Biotech	1:1000	WB	anti-rabbit HRP
p120 (NT)	Mouse	m,h	6H11	n.a.	Zymed	1:100	IF	anti-mouse Alexa Fluor-568
p-eNOS (Ser1177)	Rabbit	h		200 ul serum	Upstate	1:1000	WB	anti-rabbit HRP
p-β-catenin (Tyr654)	Mouse	h	1B11	100 ug/ml	Santa Cruz Biotech	1:1000	WB	anti-mouse HRP
						1:1000	WB	anti-mouse HRP
p-β-catenin (Tyr86)	Mouse	h	24E1	100 ug/ml	Santa Cruz Biotech	1:1000	WB	anti-mouse HRP
						1:1000	WB	anti-mouse HRP
R. conorii	Rabbit	SFG rickettsiae				1:2000	IHC	anti-rabbit biotin - ABC-AP w/ Vector Red
TNF-α	Goat	m	Lot #NQ11	200 ug/ml	R&D Systems	1:50	IHC	anti-goat HRP - TSA Alexa Fluor-488
VEGF	Rat	m,h	Lot# DPA02	100 ug/ml	R&D Systems	1:20	IHC	anti-rat HRP - TSA Alexa Fluor-488
						1:100	Neut.	
α-Tubulin	Mouse	m,h	B-5-1-2	500 ug/ml	Zymed	1:1000	WB	anti-mouse HRP
β-catenin	Rabbit	m,h	H-102	200 ug/ml	Santa Cruz Biotech	1:100	IF	anti-rabbit Alexa Fluor-488
						1:1000	WB	anti-rabbit HRP
Mouse IgG - AP	Rabbit	m			Sigma	1:100	IHC	None – Vector Red

Table 2.2: List of primary antibodies and secondary detection reagents utilized in this work.

RNA MICROARRAYS AND REAL TIME RT-PCR

RNA Isolation

For the isolation of RNA for gene expression analysis we utilized the high efficiency RNAqueous 4-PCR kit (Ambion) for the DNA-free purification. Briefly, the cell culture medium was aspirated from culture plates, and the cells were lysed in 500 µl of lysis buffer for 5 minutes on ice. The lysates were then centrifuged at ~13,000 x g for 2-3 minutes to help clarify the sample. Total RNA was then isolated according to the manufacturer's protocol. Genomic DNA was removed via incubation with DNAse I for 30 minutes at 37°C. Finally the DNA-free RNA was concentrated and desalted by alcohol precipitation. RNA concentration and purity were determined by spectrophotometry. For efficient downstream applications it was essential to achieve a 260/280 ratio of at least 2.0 and a 260/230 ratio of at least 1.7. If not used immediately the RNA was stored at -80°C.

Generation of cRNA for Microarray Analysis

For microarray hybridization it was necessary to create a biotin-UTP-labeled copy of total cellular RNA. To this end we utilized the TrueLabeling-AMP 2.0 kit (Superarray Biosciences Corp.) to first generate a cDNA copy of the transcriptome using 1.0 µg of purified RNA. This was followed by overnight labeling and amplification of the cDNA with biotin-11-UTP, which generally yielded 5-10 µg of labeled RNA. cRNA was further purified using the ArrayGrade™ cRNA cleanup kit, and the concentration was determined by spectrophotometry.

Microarray Hybridization and Analysis

For the analysis of endothelial cell transcriptional responses to rickettsial infection, we utilized the Endothelial Cell Biology Oligo GEArray (SuperArray

Biosciences), which measures the relative transcriptional activity of 113 genes associated with endothelial cell biology. All array hybridizations were performed in parallel to minimize intra-assay variability. Briefly, 4 μg of labeled cRNA was diluted in hybridization buffer and incubated in rolling hybridization tubes overnight at 60°C. Each array was then washed once in a low-stringency buffer for 10 minutes followed by 10 minutes in a high-stringency buffer with gentle agitation. The arrays were then blocked for 40 minutes at room temperature followed by incubation with alkaline phosphatase-labeled streptavidin for 10 minutes. The arrays were then developed for 5 minutes in CDP-Star before exposing to ECL Hyperfilm for 2 minutes. The film was converted to digital format and uploaded to the GEArray Expression Analysis Suite at 600 dpi. Individual arrays were then digitally aligned, and gene expression was normalized to a series of housekeeping genes and internal controls assigned by the manufacturer.

Generation of cDNA for Real Time RT-PCR

cDNA was created from the same RNA samples utilized above for microarray analysis of gene expression. One microgram of unlabeled RNA was reverse transcribed using the RETROscript® First Strand Synthesis Kit for RT-PCR (Ambion) for 1 hour at 44°C followed by 10 minutes at 95°C to deactivate all enzymatic activity in the reaction. cDNA products were stored on ice or at -20°C until analysis by real time PCR.

Real Time RT-PCR and Analysis

Real-time RT-PCR was performed using pre-designed commercially available primers (Superarray Bioscience). SYBR Green Supermix (Bio-Rad) was used to determine the relative expression levels of the designated genes by normalization to the human housekeeping gene β-actin. Real-time RT-PCR was performed on cDNA products with the following protocol: 95°C for 10 minutes, followed by 40 cycles of

95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A melt-curve was performed after each reaction to verify amplification of a single product, and the absence of primer dimer formation. Relative expression levels were determined using the ΔΔCt method.

ANIMALS

Mouse Strains and Infections

All animal experiments were performed in accordance with the rules and regulations of the University of Texas Medical Branch Institutional Animal Care and Use Committee (IACUC) in an Animal Biosafety Level-3 Laboratory.

To better document the microvascular changes occurring during spotted fever rickettsioses, we utilized two previously described murine models of spotted fever rickettsioses using *R. conorii* (Malish 7 strain) in male C3H/HeN mice and *R. australis* (Cutlack strain) in C57BL/6 male mice (Feng *et al.*, 1993;Walker *et al.*, 1994). All mice were between the ages of 6 and 8 weeks at the time of infection. Rickettsiae were administered via i.v. injection into the tail vein of restrained mice. No anesthesia was used during the inoculations. The LD_{50} of *R. conorii* was determined to be approximately $1x10^4$ PFU of 10% yolk sac suspension in 300 µl of PBS. Likewise, the LD_{50} of *R. australis* was determined to be approximately $7.5x10^5$ PFU of 10% yolk sac suspension in 300 µl of PBS. Animals were injected with varying doses of rickettsiae, and some mice were serially sacrificed at the indicated time point whereas other mice were monitored daily for signs of illness and sacrificed once it was determined that they were moribund.

Histopathology and Immunohistochemistry

Tissue samples obtained from mice at the time of sacrifice were fixed by immersion in Zinc fixative (BD Biosciences) at room temperature for 3-5 days. Tissue specimens were then processed by the UTMB Research Histopathology Core Laboratory and sections from each paraffin-embedded block were stained with hematoxylin and eosin.

We used immunohistochemical staining of paraffin-embedded tissues to localize the presence of various targets in the tissues. First, the sections were de-paraffinized by heating at 60°C for 1 hour followed by three, five minute washes in xylene. The slides were then hydrated with two 5 minute incubations in 100% ethanol, 5 minutes in 95% ethanol, 5 minutes in 70% ethanol followed by two washes in deionized water. Samples were then equilibrated in PBS for 5 minutes. In the case of peroxidase-labeled antibodies the samples were at this point incubated in 3% hydrogen peroxide diluted in deionized water for 10 minutes to deactivate any endogenous peroxidase activity.

A list of primary antibodies used in these experiments is included in Appendix I. For dual-label staining the targets were labeled individually, often on succeeding days. Spotted fever group rickettsiae, F4/80 macrophage marker and endogenous IgG were labeled using the ABC Alkaline Phosphatase (AP) system (Vector Laboratories) according to the manufacturer's recommendations. Briefly, tissue sections were blocked in normal blocking serum for 20 minutes at room temperature followed by incubation with primary antibody in PBS for 1 hour at room temperature or overnight at 4°C. The sections were washed three times in PBS and then incubated with biotinylated secondary antibody in PBS for 30-60 minutes at room temperature. Following another wash step the sections were incubated with ABC-AP reagent for 30 minutes at room temperature before reaction with Vector Red substrate for 20 minutes. Dual labeling of secondary targets was accomplished using horseradish peroxidase (HRP)-based tyramide signal amplification (AlexaFluor 488-tyramide, Molecular Probes). Mouse monoclonal

antibodies were hybridized using the Mouse-on-Mouse Kit (Vector) to prevent cross-reaction with endogenous mouse IgG.

Measurements of Tissue Water Content

To assess the level of interstitial edema that occurs during acute rickettsioses, we decided to measure the amount of water that is present in the vital organs at different stages of infection. We expected that this would give us a good idea of the intensity and temporal characteristics of increased microvascular permeability. Mice were infected i.v. with *R. conorii* at 1, 3, and 10 LD_{50} or *R. australis* at ~1 LD_{50} and sacrificed between days 5 and 7 after infection. Whole intact brain and lungs were removed and stored in enclosed plastic tissue culture dishes. Shortly after collection the organs were weighed to determine their wet weight. The tissues were then dried by placing them in a 60°C oven for 48 hours. Following the drying procedure the tissues were then re-weighed to determine the dry weight. For this method it was essential to use an analytical quality scale to detect relatively minute changes in tissue weight. Percent water content was calculated using the following formula:

$$\% \text{ Water Content} = ((\text{Wet Weight} - \text{Dry Weight})/\text{Wet Weight}) \times 100$$

Quantification of Rickettsiae in Tissues

Real time quantitative PCR was utilized to determine the content of rickettsiae in organs from infected mice. At the time of sacrifice mice were asphyxiated with carbon dioxide and euthanized by cervical dislocation. Tissue pieces of <25 mg in size were removed from the brain, lungs, liver and spleen and stored in tissue lysis buffer until processing. We first added 20 µl of Proteinase K solution to each sample and incubated at 56°C overnight to homogenize the tissue. DNA was then purified according to the

manufacturer's directions using the DNeasy Tissue Kit (Qiagen). DNA was eluted in 200 µl of elution buffer and stored at -20°C until analysis.

For the quantification of rickettsial genomes, we generated two plasmids containing the PCR products of rickettsial *gltA* and mouse *β-actin*. Briefly, the PCR products were cloned into the TOPO TA-2.1 cloning vector and chemically transformed into Top10 *E. coli*. *E. coli* were then cultured overnight via ampicillin selection and positive colonies were further propogated in ampicillin-containing LB broth. Plasmid DNA was purified using the DNA mini kit (Qiagen), and the concentration was determined by spectrophotometry.

Measurement of Serum Cytokine Content

Serum cytokine concentrations were determined using Bio-Plex bead-based multiplexed cytokine arrays (Bio-Rad). Mouse serum samples were acquired by cardiac puncture of CO_2-asphyxiated mice at the indicated time points after infection. Whole blood was allowed to rest at room temperature and clot. The clotted whole blood was then centrifuged at ~13,000 x g for 15 minutes, and the supernatant containing serum was carefully aspirated and stored at -20°C until use. Serum samples were diluted 1:4 in serum diluent and incubated with cytokine-specific beads according to the manufacturer's recommendations. Serum samples were analyzed with the mouse 23-plex Group I cytokine array (Bio-Rad).

Chapter 3: Host Immune Responses to Rickettsial Infection Contribute to Increased Microvascular Permeability

INTRODUCTION

Cytokine Modulation of Microvascular Permeability

The endothelial barrier is a highly complex and regulated system evolved to control the flux of solutes and fluid from the circulation into specific tissues. In the brain, the endothelium is highly specialized earning itself the moniker blood-brain barrier (BBB). Designed to protect the brain from typical solutes present in circulating blood, the BBB possesses complex intracellular junctions called tight- and adherens-junctions which regulate what can and cannot proceed across the endothelial barrier (Copin and Gasche, 2003).

Under pathologic conditions such as infection or trauma, increased levels of circulating cytokines can influence the integrity of the microvascular barrier resulting in a number of effects including alterations in the endothelium's ability to function as a protective barrier. Certain cytokines like tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) can have profound influence on the integrity of the endothelial barrier, leading to biochemical and conformational changes in junctional proteins that negatively affect the function of the endothelial barrier (de Boer and Breimer, 1998).

TNF-α is a pluripotent cytokine shown to be an important mediator of many biological processes. Among its most well known affects is the ability of this cytokine to induce endothelial barrier dysfunction by acting directly on endothelial cells. TNF-α has been shown to induce endothelial barrier breakdown through the tyrosine phosphorylation of endothelial cell junction proteins including VE-cadherin, β-catenin and p120 catenin (Angelini *et al.*, 2006). Additionally, TNF-α barrier dysfunction has been linked to NADPH oxidase activity, F-actin depolymerization and the presence of peroxynitrite, a highly reactive molecule produced by the combination of the superoxide anion and nitric oxide (Goldblum *et al.*, 1993; Gertzberg *et al.*, 2004; Neumann *et al.*,

2006). TNF-α is associated with a number of pathologic conditions including acute respiratory distress syndrome (ARDS), sepsis, and has been implicated in the pathogenesis of hantavirus pulmonary syndrome (Tracey *et al.*, 1987; Roten *et al.*, 1991; Khaiboullina and St Jeor, 2002). Perhaps not surprisingly these conditions are all characterized by the presence of dramatic increases in vascular permeability.

IL-1β is another cytokine with a broad spectrum of effects on the host; perhaps the most notable being the effect of IL-1β on the thermal response during infection and sepsis. However, IL-1β has also been demonstrated to produce changes in endothelial cells that can result in barrier dysfunction and increased vascular permeability. *In vitro* IL-1β increases permeability across endothelial and epithelial monolayers in a dose- and time-dependent manner (Abe *et al.*, 2003;Wong *et al.*, 2004). IL-1β has also been shown to induce expression of other pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1 (Holtkamp *et al.*, 1998;Holtkamp *et al.*, 1999). This effect might explain the permeability-inducing properties of IL-1β or may serve to amplify the level and intensity of inflammation produced in response to IL-1β.

Nitric Oxide as a Mediator of Microvascular Permeability

The evidence surrounding nitric oxide as a pathologic mediator of increased microvascular permeability is controversial, to say the least. Several *in vivo* studies have implicated NO production in response to cytokines like IFN-γ and TNF-α as the central mediator of increased microvascular permeability (Mayhan, 2002). The authors demonstrated that they could reduce the leakage of FITC-dextrans out of rat meningeal vessels through the application of the inhibitor of soluble guanylate cyclase, ODQ. Soluble guanylate cyclase (sGC) is upregulated in response to NO production resulting in an increase in cGMP production and protein kinase G activity. However, others have shown that NO may actually enhance the barrier properties of endothelial cells, and the

inhibition of sGC results in increased permeability of endothelial cells in culture (Wong *et al.*, 2004). What is totally not known at this time is how NO modulates endothelial cells in the way that it does. Protein s-nitrosylation is often used as a marker of NO availability and protein modification; however, it is not known whether s-nitrosylation of proteins has a detrimental impact on their form or function. Consequently, the impact of nitric oxide on endothelial cell permeability is still not completely defined.

Mechanisms of Immunopathogenesis during Infection

Many infectious diseases are characterized by the presence of pathological consequences of the immune response to the pathogen. Potential mechanisms of immunopathogenesis include the overproduction of inflammatory cytokines, deposition of immune complexes in host tissues, or leukocyte mediated tissue damage. In some cases this may be a benefit to the pathogen, whereas in other cases it is merely an unfortunate consequence of the immune response to infection.

Perhaps the most well described immunopathologic condition is gram-negative bacterial sepsis. High levels of circulating bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa* lead to an overproduction of pro-inflammatory cytokines like TNF-α and IL-1β by LPS-stimulated monocytes, macrophages and specific lymphocyte subsets, eventually leading to systemic vascular dysfunction accompanied by dramatic increases in microvascular permeability with a sudden onset (Gutierrez-Ramos and Bluethmann, 1997). TNF-α has been shown to be responsible for BBB permeability during sepsis in animal models (Tsao *et al.*, 2001). Likewise, TNF-α and IL-1β from the serum of LPS-treated human volunteers are responsible for increased microvascular permeability across human endothelial cell monolayers (Nooteboom *et al.*, 2002). Neutralization of these two cytokines alleviates the detrimental effects on vascular integrity without affecting the outcome of the infection.

TNF-α has also been hypothesized to be responsible for the non-cardiogenic pulmonary edema observed during hantavirus pulmonary syndrome in humans. Fatal human cases demonstrate TNF-α-containing cells in the lungs, and a significant increase of serum TNF-α levels occurs during the acute stage of infection (Krakauer *et al.*, 1995;Mori *et al.*, 1999). In this case, much like bacterial sepsis, it appears that the levels of circulating cytokines are high enough to induce systemic changes in vascular permeability, contributing to the severe nature of this disease.

Cerebral malaria (CM) is a severe manifestation of *Plasmodium falciparum* infection which primarily affects children under the age of 6 years. CM is characterized by a loss of blood-brain barrier integrity and is associated with adherence of parasitized red blood cells and leukocytes (Berendt *et al.*, 1994;Neill and Hunt, 1992). It has been hypothesized that cytokine stimulation of the endothelium leads to an increase in BBB permeability via an indoleamine oxidase (IDO)-dependent mechanism (Hunt *et al.*, 2006). IDO is responsible for the enzymatic reaction that converts tryptophan to kynurenine, which is further metabolized by glial cells into quinolinic acid and kynurenic acid. An imbalance in the ratio of these two compounds leads to astrocyte-mediated damage of the brain endothelium, an increase in microvascular permeability, and cerebral edema.

Human monocytotropic ehrlichiosis (HME) is an example of an infection characterized by an overactive immune response failing to clear the infection leading to severe disease manifestations, which is supported by data from an animal model of HME using *Ixodes ovatus Ehrlichia* (IOE) in C57BL/6 mice. Decreased IFN-γ production by CD4 T-cells is associated with a failure to clear the infection whereas increased TNF-α production by CD8 T-cells leads to a systemic toxic shock-like syndrome (Ismail *et al.*, 2004). Bacteria are found throughout the body but primarily cause an interstitial pneumonitis in the lungs and hepatic granulomas accompanied by necrosis and apoptosis

of hepatocytes (Sotomayor *et al.*, 2001). Severe cases of HME are often comparable in severity to RMSF although the case-fatality ratio is much lower in HME.

Clearly, pro-inflammatory cytokines have been linked to several conditions characterized by a general increase in microvascular permeability and/or endothelial denudation. However, the available data do not suggest the presence of significantly higher levels of TNF-α or IFN-γ in the serum of lethally infected mice. This supports a role for these cytokines as localized activators of endothelial cells, rather than systemic mediators.

Potential Mechanisms of Immunopathogenesis in Rickettsial Infection

It is well understood that rickettsiae cause necrotic cell death in infected endothelial cells *in vitro*. However, to prevent widespread endothelial destruction the host's immune response activates endothelial cells to kill intracellular rickettsiae via TNF-α, IFN-γ and IL-1β stimulation of infected endothelial cells and subsequent iNOS and IDO upregulation. Endothelial-derived nitric oxide has been hypothesized to contribute to vascular leakage *in vivo,* perhaps through the formation of peroxynitrite, during inflammatory conditions including bacterial infection and acute inflammation (Bucci *et al.*, 2005;Winkler *et al.*, 2001). What is not known is the relative contribution of this response to the vascular dysfunction experienced during acute rickettsioses. A better understanding of the relationship between endothelial cell activation and increased microvascular permeability during rickettsial infection may reveal novel targets of therapy for severe cases of human rickettsioses.

RESULTS

The effects of exogenous nitric oxide (NO) on rickettsiae-infected endothelium

Exogenous NO limits intracellular rickettsial viability

To study the effects of exogenous NO on microvascular permeability, we decided to first determine whether NO-donors would be a suitable model for this purpose. To this end, we first determined the impact of Diethylenetriamine NONOate (DETA NONOate), a NO-donor, on intracellular rickettsial viability. The addition of DETA NONOate at either 100 μM or 500 μM resulted in a significant decrease in the number of intracellular rickettsiae in HMEC-1 endothelial cells (Figure 3.1). The effect was time-dependent with the greatest differences being measurable at 72 hours after infection and stimulation.

To confirm the presence of nitric oxide in this system, we performed a Greiss assay to measure the concentration of nitrite in the cell culture supernatants. Mock infected cells produced undetectable amounts of nitrite, and *R. conorii*-infected cells produced very minute quantities, as well. However, the presence of DETA NONOate correlated with a significant increase in nitrite concentration to over 30 μM by 24 hours after infection and stimulation (Figure 3.2). The concentration was unchanged between 24 and 48 hours, suggesting the donor activity was depleted after 24 hours.

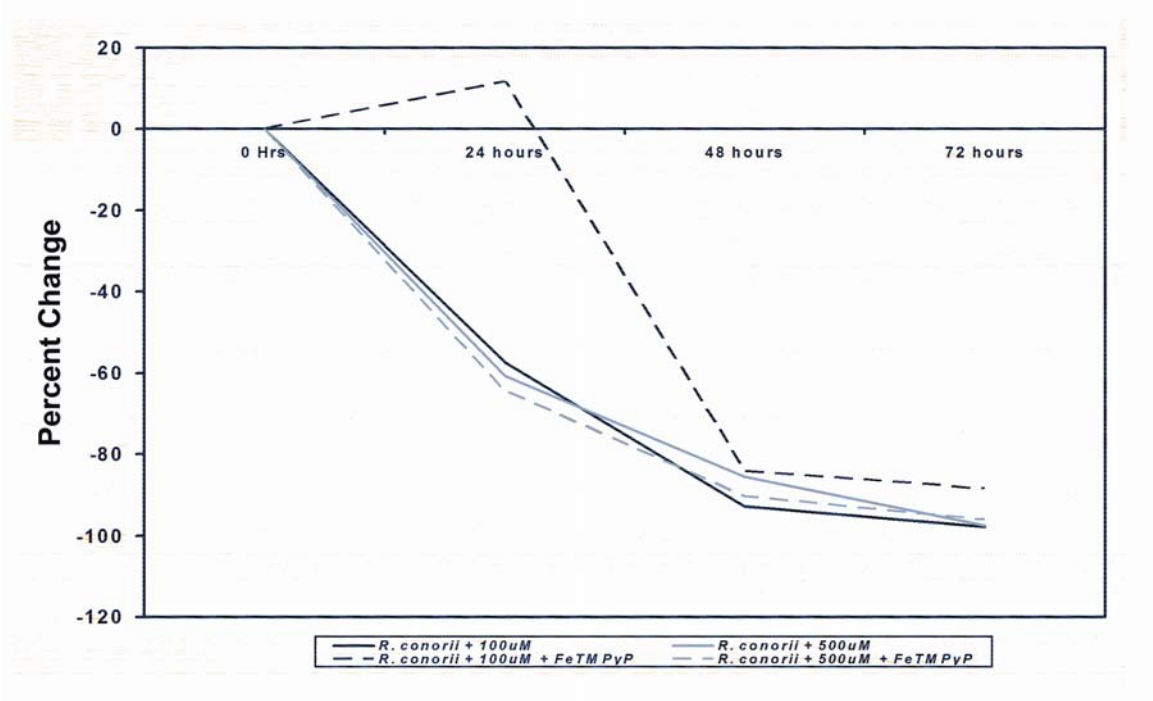


Figure 3.1: Exogenous nitric oxide decreases the number of intracellular rickettsiae in human microvascular endothelial cells. HMEC-1 were infected with 10 moi of *R. conorii* and treated with DETA NONoate at 100 μM or 500 μM. Additionally some cell monolayers were treated with the peroxynitrite decomposition catalyst FeTMPyP (100 μM). DNA was purified from infected cells at 24, 48 and 72 hours and relative copies of rickettsial genomes were determined using real-time PCR and the ΔΔCt method. Data are presented at the percent decrease in relative rickettsial genomes compared to infected, untreated cells.

Exogenous nitric oxide affects microvascular permeability in rickettsiae-infected endothelial cells through the modulation of adherens junctions

We next sought to determine what the effect was of exogenous NO on human endothelial cell monolayer permeability as measured by ECIS. Cells were infected with approximately 10 moi of *R. conorii* and stimulated with two different concentrations of DETA NONOate. Mock infected cells maintained a relatively stable level of permeability over the course of the 5-day experiment (Fig 3.3). Cells infected with *R. conorii* maintained a steady level of permeability for the first 2-3 days of infection, after which time they began to experience an increase in permeability as measured by a drop in

resistance. This slow increase in permeability became progressively worse over the final two days of the experiment. Surprisingly, cells infected with *R. conorii* but also stimulated with the NO-donor at 100 μM maintained a level of permeability not significantly different than mock-infected cells for the full five days of the experiment. On the other hand, a dose of 500 μM had a profound impact on microvascular permeability. By 24 hours these cells were experiencing a stabilization of the monolayer resulting in decreased permeability. This effect was only transient, after which time the infected monolayers experienced a slow and steady increase in permeability over the next four days, eventually reaching levels of permeability significantly greater than either those that are infected, or infected and stimulated, by the 60 hour time point.

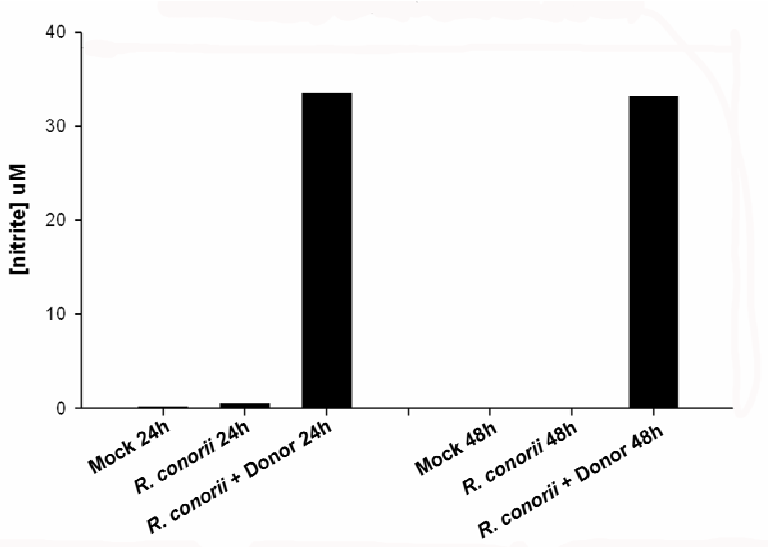


Figure 3.2: DETA NONOate released high levels of NO as measured by nitrite in cell culture supernatants. HMEC-1 were infected with *R. conorii* and treated with 500 μM of DETA NONoate. Cell culture supernatants were collected at 24 and 48 hours and analyzed for the presence of nitrite by the Greiss assay.

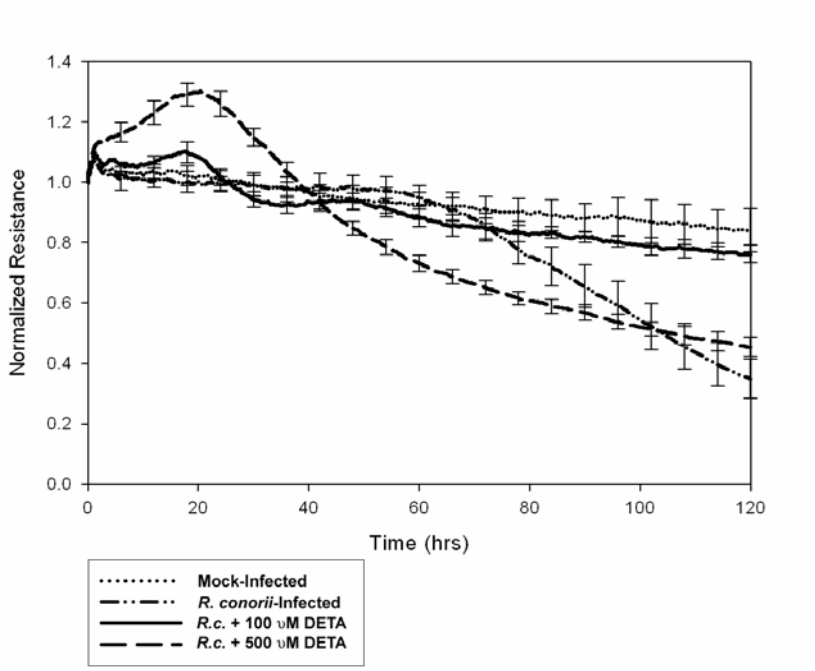


Figure 3.3: Exogenous NO exerts a dose-dependent effect on microvascular permeability of *R. conorii*-infected endothelial cells. HMEC-1 cells were infected with 10 moi *R. conorii* and treated with 100 μM or 500 μM of DETA-NONOate. The cells were monitored by ECIS for changes in endothelial permeability. Data is expressed as the average normalized resistance and the standard deviation was determined every 6 hours.

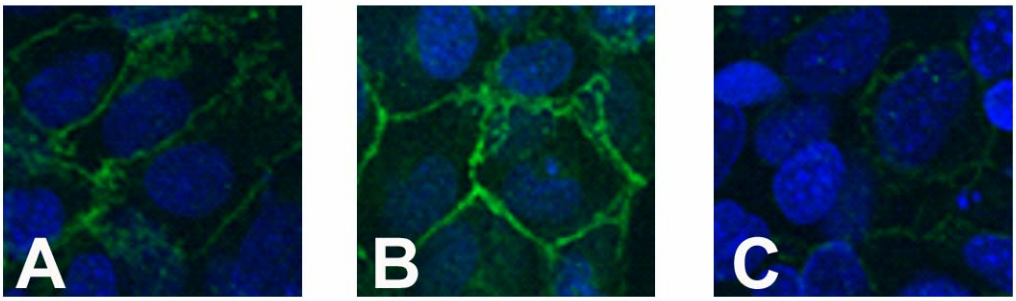


Figure 3.4: High levels of exogenous NO ultimately result in adherens junction disassembly in HMEC-1. HMEC-1 were infected with 10 moi of *R. conorii* and treated with the NO-donor DETA NONOate (500 μM). After 48 hours of infection and stimulation, the cells were fixed in 4% paraformaldehyde and stained with a polyclonal antibody to β-catenin (green). Nuclei were stained with DAPI (blue). Uninfected (A) and *R. conorii*-infected (B) cells retained staining of β-catenin at intercellular borders whereas infected cells treated with the NO-donor (C) exhibited decreased staining indicating loss of adherens junctions function.

The Effects of Exogenous NO are Dependent on Peroxynitrite

In order to elucidate the source of action of exogenous nitric oxide, we decided to investigate the role of peroxynitrite in the regulation of microvascular permeability. HMEC-1 were infected with 10 moi of *R. conorii* and stimulated with 500 μM DETA NONOate and 100 μM of the peroxynitrite decomposition catalyst iron tetrakis (N-methyl-4'-pyridyl) porphyrinato (FeTMPyP) (Figure 3.5). The increased rate of peroxynitrite decomposition resulted in an abolished response to the high dose of the NO-donor. Specifically we no longer saw a stabilization of the endothelial monolayer within the first 24 hours. While there was a significant increase in permeability compared to infected cells, the monolayers treated with the NO-donor and the scavenger behaved more similarly to the infected monolayers by experiencing a steady increase in permeability beginning at ~65 hours. At this point the level of permeability was not significantly greater than normally infected cells.

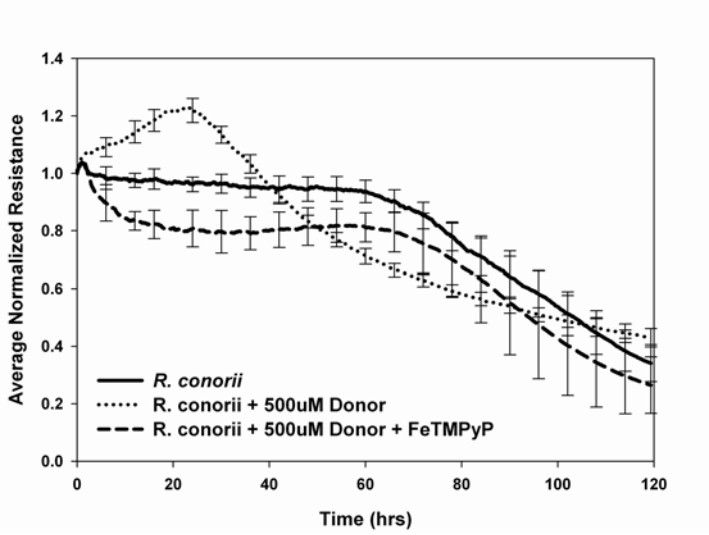


Figure 3.5: Exogenous nitric oxide exerts its permeability modulating effects through the action of peroxynitrite. HMEC-1 were infected with *R. conorii*, stimulated with 500 μM of DETA NONoate, and treated with 100 μM of FeTMPyP, a peroxynitrite decomposition catalyst. Electrical resistance was monitored by ECIS to indicate changes in the permeability of the treated monolayers. Whereas infected cells only treated with the NO-donor showed the characteristic increase in resistance followed by a slow and steady loss of integrity, the cells also treated with FeTMPyP behaved more similar to cells only infected with *R. conorii*.

The Effects of Cytokine Stimulation on Rickettsiae-infected Human Cerebral Endothelial Cells

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-HCECs infected with *R. rickettsii* exhibited a dose-dependent increase in endothelial permeability reflected as a decrease in transendothelial electrical resistance. Figure 3.6A is a representative 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 hours after infection. At 24 hours, increases in permeability ranged from 12.3±2.7% at 1 MOI to 36.9±2.4% at 20 MOI relative to controls (Figure 3.6B). At 48 hours post-infection, monolayers infected with 1 MOI showed a 23.1±1.5% increase in permeability compared to controls. During the first 24 to 48 hours increases in permeability were more pronounced in monolayers infected with the highest doses, clearly demonstrating the dose dependence of the effect.

Pro-inflammatory cytokines regulate microvascular permeability in SV-HCEC

Since TNF-α, IL-1β 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-1β induced a rapid decline in TER by 10 hours after stimulation resulting in ~20% increase in permeability compared to untreated controls (Figure 3.7A). By 24 hours after stimulation this effect ranged from a 15±5.4% increase at 0.1 ηg/ml to 31.8±13% at 1000 ηg/ml compared to controls (Figure 3.7B). The effect of the lowest dose of 0.1 ηg/ml was only transient, and those cells recovered to near normal levels of permeability by 72 hours after stimulation with an average increase in permeability not

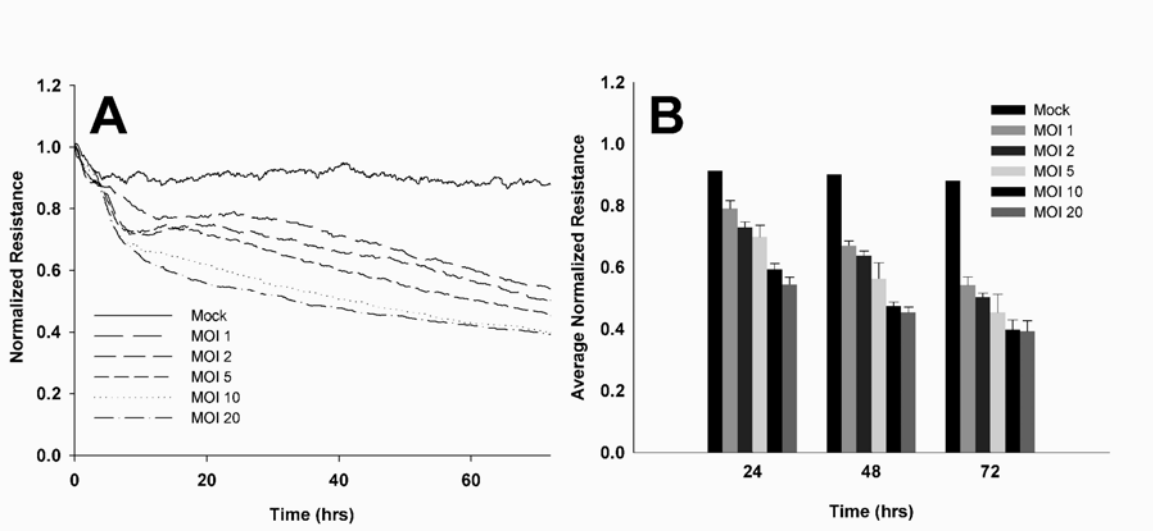


Figure 3.6: *Rickettsia rickettsii* caused increased microvascular permeability in human brain endothelial cells. SV-HCEC were grown to confluence on 8W10E gold-coated electrodes, and resistance was monitored by ECIS following infection with *R. rickettsii* at doses ranging from 1-20 MOI. A representative ECIS graph (A), and bar graphs indicating the mean ± standard deviation of three independent experiments (B).

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 ηg/ml (29.12±0.12%), 10 ηg/ml (25.3±2.3%), 100 ηg/ml (31.1±3.1%)and 1,000 ηg/ml (44±7.5%), respectively, by 72 hours. Overall, increasing doses of IL-1β 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 (Figure 3.7C). Low doses of TNF-α at 0.1 ηg/ml showed no significant increase in permeability compared to controls at any time point. A dose of 1 ηg/ml showed a slightly higher level of permeability at 48 hours (12.3±7.1%) after stimulation, but this effect was diminished by 72 hours after which the increase in permeability was not statistically higher than control cells (Figure 3.7D). Higher doses caused a steady increase in

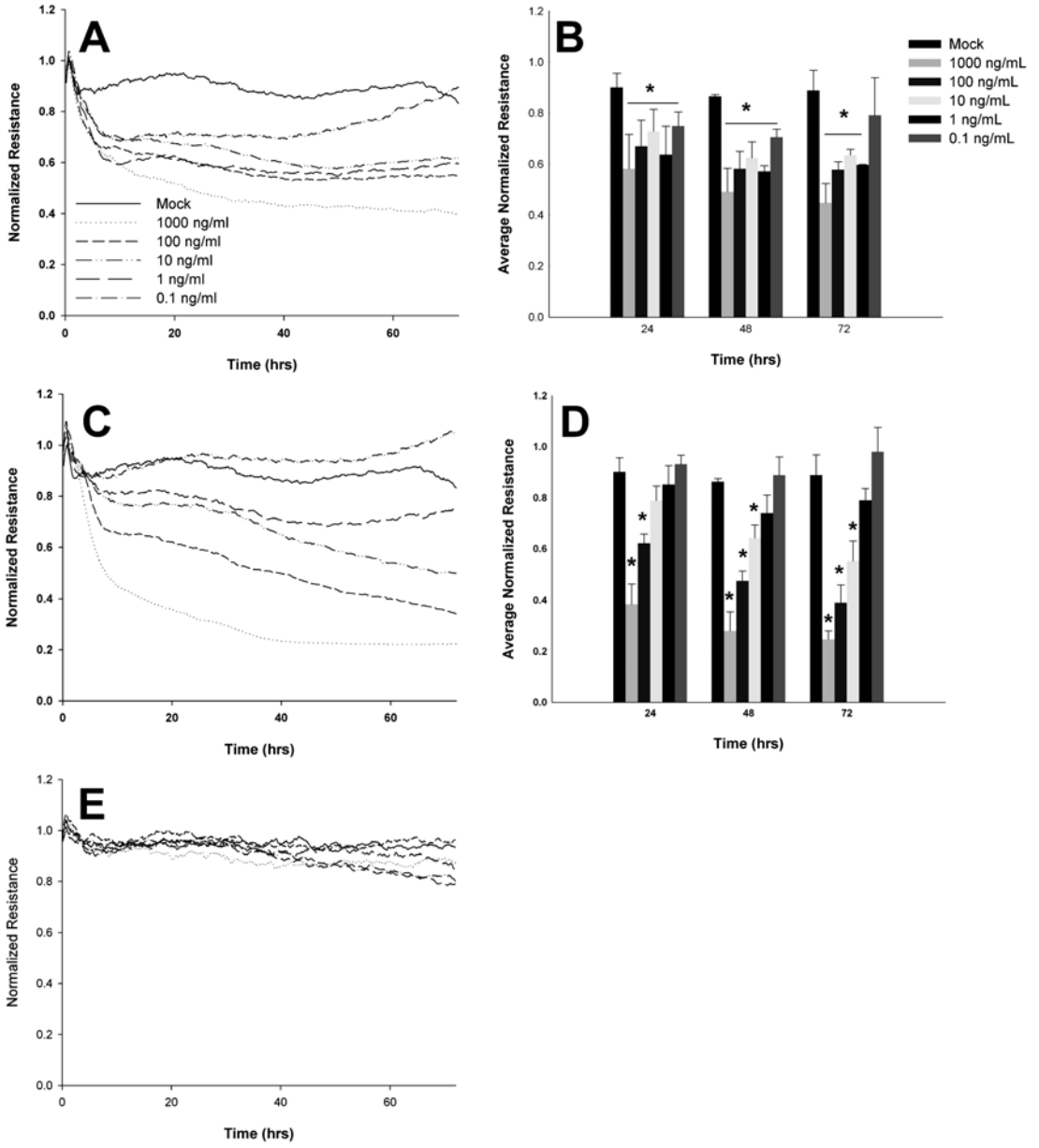


Figure 3.7: The pro-inflammatory cytokines IL-1β, TNF-α and IFN-γ had differential effects on SV-HCEC monolayers. SV-HCEC monolayers were cultured on 8W10E ECIS arrays until confluent at which time they were stimulated with doses of cytokines ranging from 0.1-1,000 ηg/ml of IL-1β (A,B), TNF-α (C,D) and IFN-γ (E). Representative ECIS graphs (A,C,E) demonstrate the time-course of responses to cytokines whereas bar graphs (B,E) indicate the mean ± standard deviation of normalized resistance values at designated time-points. *P<0.05 compared to uninfected controls.

permeability over the 72 hour time course ranging from 33.42±7.6% at 10 ηg/ml, 49.79±7.0% at 100 ηg/ml, and 64.2±3.2% at 1000 ηg/ml. The highest dose, 1000 ηg/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 (Figure 3.7E).

TNF-α and IL-1β 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-1β on rickettsiae-infected endothelial cells produced a greater increase in permeability than cells only infected with rickettsiae (Figure 3.8A). There was a dose-dependent response of rickettsiae-infected SV-HCECs to IL-1β and by 48 hours there was a significant increase in permeability in infected cells treated with 10 ηg/ml (13.1±1.5%) and 100 ηg/ml (10.3±1.3%) of IL-1β compared to those only infected with rickettsiae. No additional effect of IL-1β was observed at any time point for doses ranging from 0.1-1 ηg/ml.

The addition of TNF-α to rickettsiae-infected cells elicited an additional increase in permeability at 10 ηg/ml (15.9±1.1%) or 100 ng/ml (18.6±0.4%) as early as 24 hours after stimulation (Figure 3.8B). 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 ηg/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 modify endothelial cells and cause an increase in permeability. The addition of a combination of TNF-α, IL-1β and IFN-γ to rickettsiae-infected cells at doses of 0.1 ηg/ml produced a significantly greater increase in permeability compared to cells only infected with rickettsiae (Figure 3.8C). This

additional increase in permeability was maintained up to 72 hours 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.

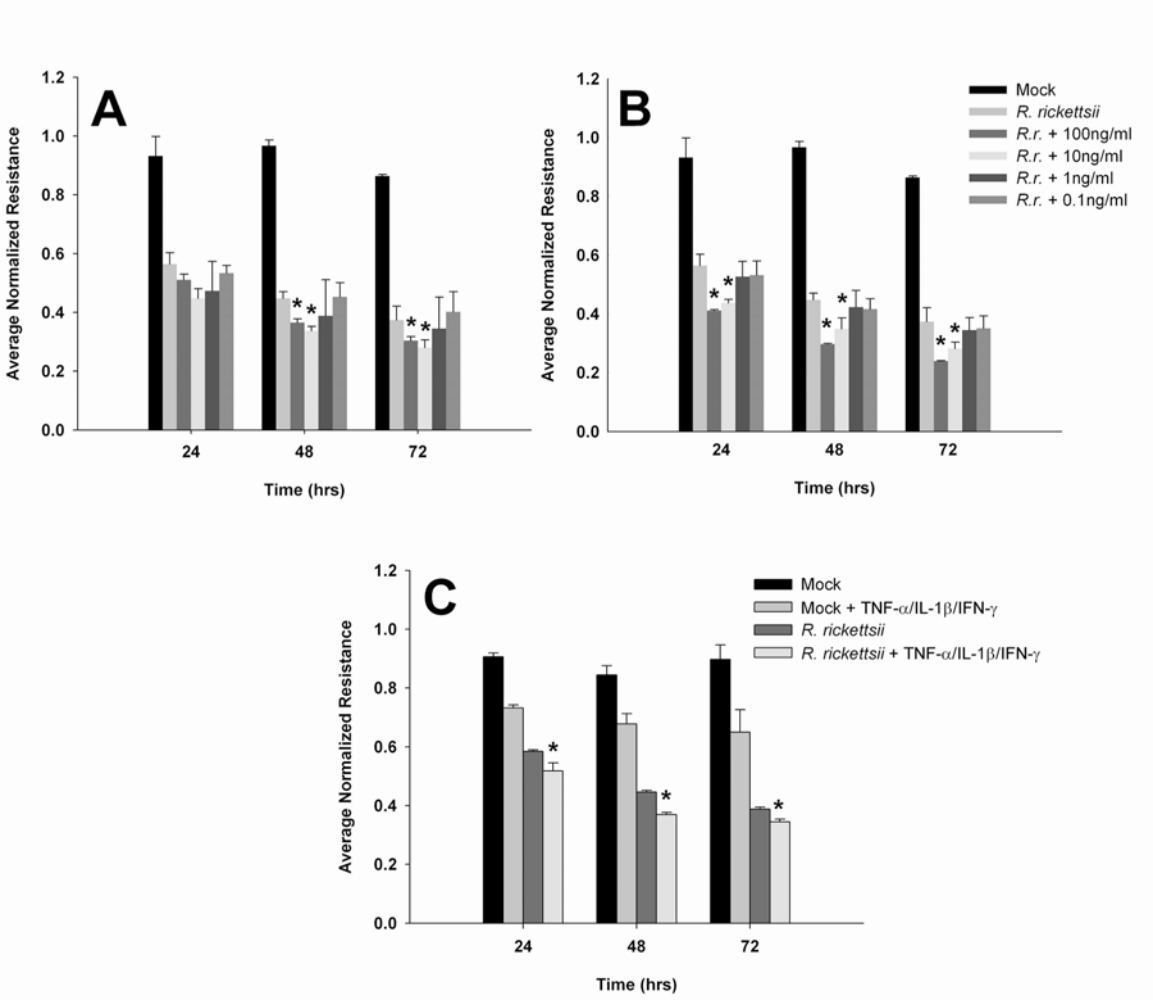


Figure 3.8: Addition of pro-inflammatory cytokines to rickettsiae-infected SV-HCEC enhanced rickettsiae-induced permeability. Cells were infected with 10 moi of *R. rickettsii* and stimulated with varying concentration of IL-1β (A) or TNF-α (B) or 0.1ηg/ml of TNF-α, IL-1β and IFN-γ in combination with and without rickettsiae (C). Data is presented as the mean ± standard deviation of three independent experiments. *P<0.05 compared to *R. rickettsii*-infected cells.

Stimulation with combinations of pro-inflammatory cytokines induces an anti-rickettsial state in SV-HCEC associated with nitric oxide production

Because NO has been demonstrated to be an important mechanism of immunity to rickettsiae, we next sought to determine the ability of SV-HCECs 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 ηg/ml of TNF-α, IL-1β, and IFN-γ produced high levels of NO as measured by nitrite in the supernatant and was first observed at 24 hours after infection and stimulation (Figure 3.9A). 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 (Figure 3.9B). Cells treated with 0.1 ηg/ml of cytokines had a decrease in the number of intracellular bacteria by approximately 23.67±1.6%. Cells treated with a higher dose of 10 ηg/ml had a greater reduction in the number of intracellular rickettsiae to approximately 33.5±11.2% that of unstimulated controls at 24 hours 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-NAME showed an overall increase in the number of rickettsiae to 22.5±10.8% greater than untreated, infected cells, and was significantly greater than either of the cytokine-treated groups ($p<0.05$).

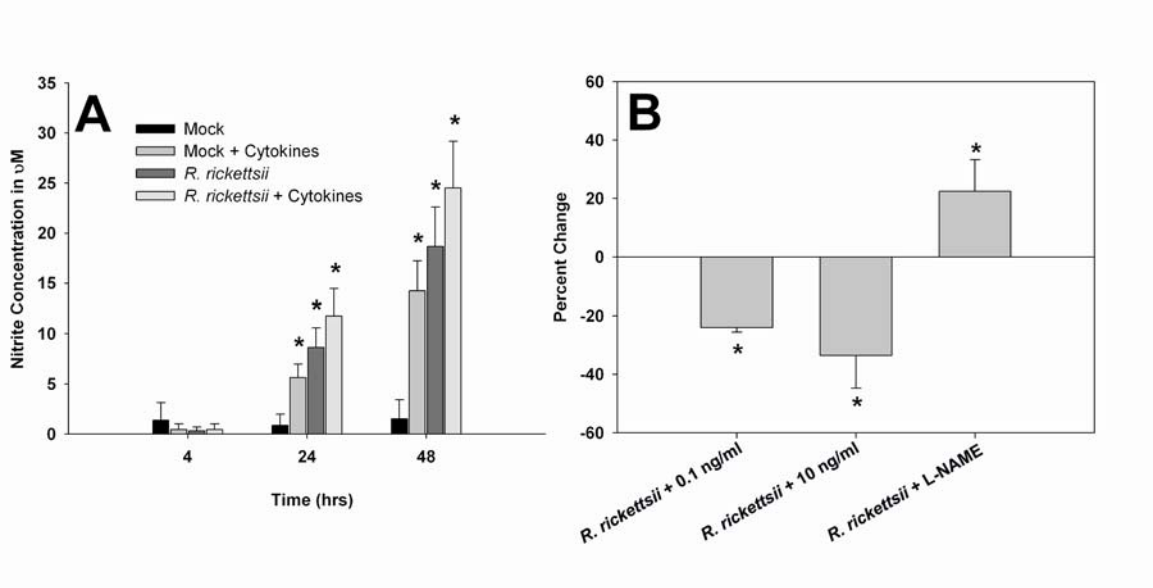


Figure 3.9: Combinations of pro-inflammatory cytokines induced an increase in microvascular permeability associated with an anti-rickettsial state in endothelial cells. SV-HCEC were infected with 10 MOI of *R. rickettsii* and/or stimulated with 10 ηg/ml of TNF-α, IL-1β and IFN-γ and supernatants were 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 ηg/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 ΔΔCt method with primers to rickettsial *gltA* and human *β-actin* (B). *P<0.05 compared to cells only infected with rickettsiae.

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-N^G-nitroarginine methyl ester (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 (Figure 3.10A). The soluble guanylate cyclase inhibitor ODQ had no impact of the

level of NO production, as expected (Figure 3.10C). 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 (Figure 3.10B). Additionally, inhibition of NO production following cytokine stimulation had no impact on cytokine-induced microvacsular permeability. Finally, inhibition of the downstream signalling 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 (Figure 3.10D).

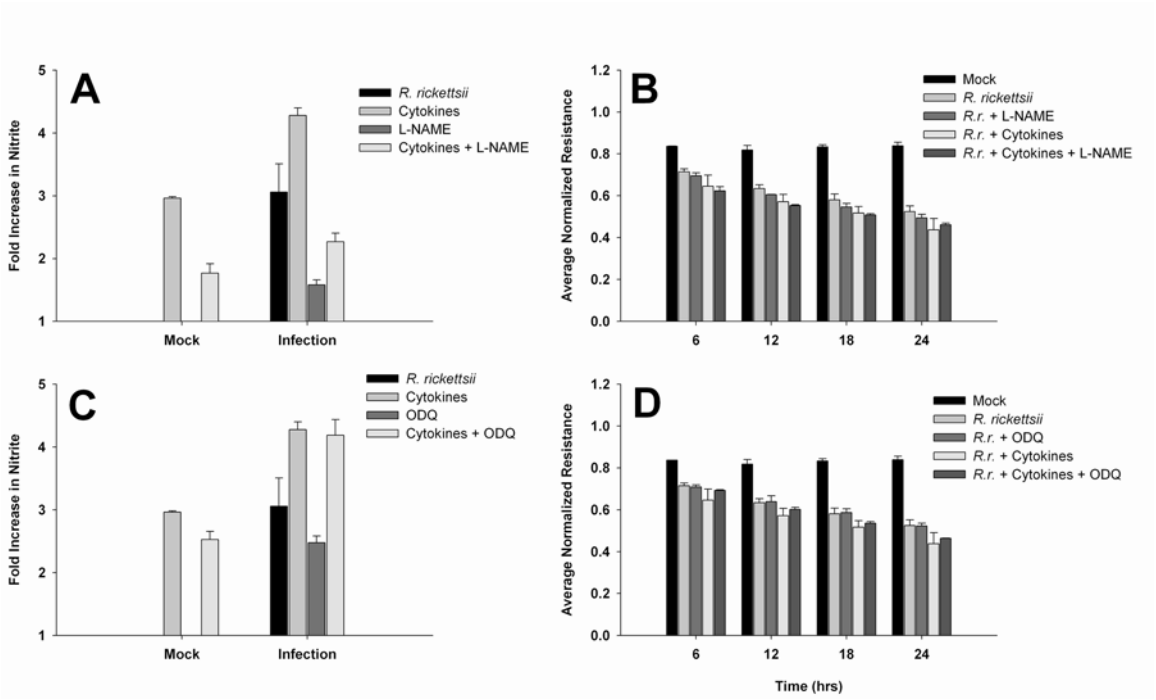


Figure 3.10: Rickettsiae- and cytokine-induced increases in microvascular permeability are not directly attributable to increased nitric oxide production in SV-HCEC. Nitrite concentrations were measured in cell culture supernatants of infected and uninfected cells treated with or without 10 ηg/ml of cytokines or untreated plus 100 μM L-NAME (A) or 100 μM ODQ (C). Similarly treated cells were monitored by ECIS for changes in permeability in response to L-NAME (B) and ODQ (D).

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 (Figure 3.11A,B). The staining of these two proteins overlapped indicating their association with adherens-junctions (Figure 3.11C). The addition of cytokines appeared to have the greatest impact on p120 localization resulting in punctuate staining pattern not associated with cell-cell junctions (Figure 3.11D-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 (Figure 3.11G-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 (Figure 3.11J-L). Gaps between adjacent endothelial cells were clearly evident, and there was no clear definition of where adherens-junctions were localized.

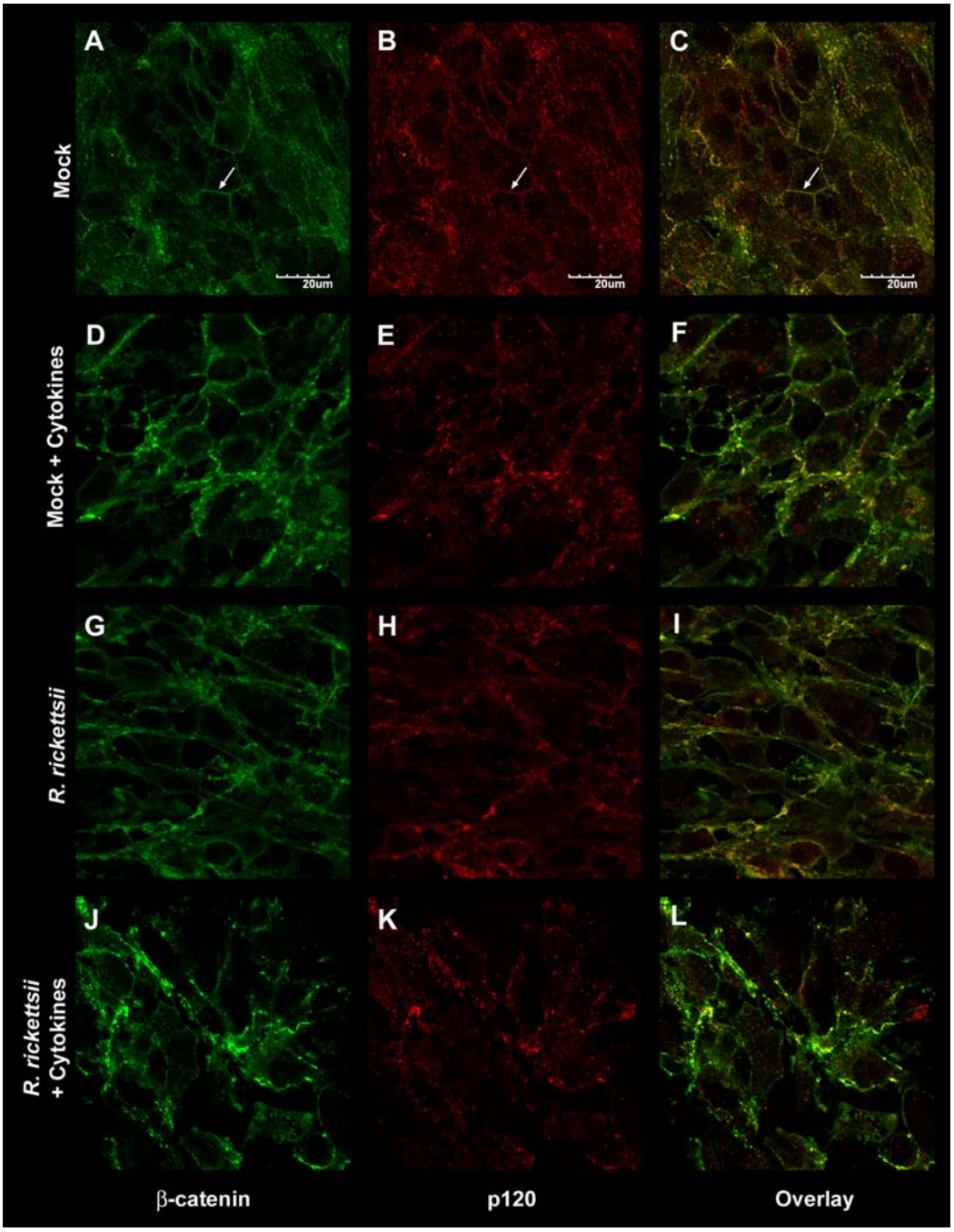


Figure 3.11: *R. rickettsii* and cytokines induced a loss of β-catenin and p120 staining at intercellular junctions. Cells were infected with 10 MOI of *R. rickettsii* and/or stimulated with 10 ηg/ml of TNF-α, IL-1β and IFN-γ and incubated at 37°C for 24 hours. Overlaid images indicate the colocalization of β-catenin and p120 (C,F,I,L). Arrowheads indicate proper localization of adherens junctions at cell-cell contacts.

DISCUSSION

The role of the host's immune response in contributing to disease processes is an often overlooked component of the pathogenesis of infectious diseases. Here, we report credible evidence that responses designed to limit the spread of rickettsial infection may actually contribute to the microvascular dysfunction experienced during Rocky Mountain spotted fever. Specifically, cytokine stimulation of the endothelium results in an increase in NO production that leads to a decrease in the number of viable, intracellular rickettsiae. However, a potential side effect of this response is an increase in microvascular permeability due to high levels of TNF-α and IL-1β. Investigation into the role of NO in this response has proven inconclusive as to whether NO mediates any of the permeability-enhancing effects of these cytokines.

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 (Walker *et al.*, 2001). Cytotoxic T-lymphocytes (CTLs) are recruited to rickettsiae-infected endothelial cells in part due to upregulation of intercellular adhesion molecule 1 (ICAM-1) but independent of the CXCL9/10-CXCR3 chemokine system (Valbuena and Walker, 2004). 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 (Blann *et al.*, 2005;Constans and Conri, 2006). 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-1β and RANTES, but the degree of NO production was variable between pools of HUVECs (Feng and Walker, 2000). 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 sub-lethal infection (Feng *et al.*, 1994).

The pluripotent cytokine TNF-α is a vital component of a protective immune response to a number of different pathogens including rickettsiae. Its many different activities 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 non-cardiogenic pulmonary edema, respectively (Peters and Khan, 2002; Lorente and Marshall, 2005). 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 (Spellerberg and Tuomanen, 1994; Rothwell and Hopkins, 1995; Ferro *et al.*, 1997; Ferro *et al.*, 2000). However, work presented here demonstrates that the addition of a NO-donor to *R. conorii*-infected endothelial cells results in a stabilization of the barrier early after exposure, suggesting that NO actually enhanced the barrier properties of endothelial cells (Woods *et al.*, 2005). On the other hand, our studies utilizing a

physiologically relevant model of NO production indicate that cytokine-mediated endothelial permeability occurs independently of NO production and the alternative effects of TNF-α may be more important in regulation of endothelial barrier function. While this response is ultimately protective and survival is dependent on cytokine 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 here.

IL-1β is cytokine associated with an inflammatory state and is often regulated in conjunction with TNF-α. IL-1β 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 including rash, headache and fever. Interestingly, IL-1β generally circulates at very high levels during *R. conorii* infection of mice (unpublished data), suggesting that its mechanism of action in inducing vascular permeability could be enhanced at sites of TNF-α production. Another intriguing response to IL-1β production is the increased transcription and secretion of IL-6 in a dose- and time-dependent manner by cultured intestinal epithelial cells (Parikh *et al.*, 1997). 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, a role may be suggested for IL-6 in rickettsial pathogenesis (Kaplanski *et al.*, 1995). In fact, lack of IL-6 was associated with decreased microvascular permeability in an experimental model of pneumococcal meningitis (Paul *et al.*, 2003). Likewise, IL-6 has been shown to induce a loss of TER across human umbilical vein endothelial cells in a PKC-dependent manner. Therefore it is not surprising to find that PKC is also up-regulated in *R. rickettsii*-infected endothelial cells (Sahni *et al.*, 1999). The exact role of IL-6 and/or PKC activation in rickettsiae-induced permeability is an area of current investigation.

TNF-α and IL-1β along with IFN-γ come together to induce NO production by endothelial cells, resulting in a decrease in the number of intracellular rickettsiae. NO itself has been implicated in the regulation of vascular permeability in several different models. One example of this is bacterial meningitis in which the inducible nitric oxide synthase (iNOS) enzyme is associated with blood-brain barrier (BBB) breakdown. iNOS-deficient mice experience reduced BBB disruption, lack of nitrotyrosine immunoreactivity in the brain and decreased levels of certain pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α (Winkler *et al.*, 2001). Therefore, it seemed reasonable to hypothesize that iNOS and NO may play a similar role in rickettsial pathogenesis. The production of NO in the presence of superoxide generation results in the formation of the highly reactive molecule peroxynitrite. Peroxynitrite appears to be the primary effector of NO-mediated cellular changes, most likely through nitration of amino acids in proteins. Previous work by others has demonstrated that TNF-α-induced endothelial barrier dysfunction occurs partly through the combined effects of endothelial nitric oxide synthase (eNOS) and NAD(P)H-oxidase activity leading to peroxynitrite accumulation (Ferro *et al.*, 1997; Bove *et al.*, 2001;Gertzberg *et al.*, 2004). This results in nitration of β-actin which is prevented by the addition of urate, an anti-peroxynitrite compound (Neumann *et al.*, 2006). These results demonstrate that TNF-α-induced barrier dysfunction can be highly dependent on NO production.

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* (Walker and Cain, 1980; Walker *et al.*, 1982). Increased production of reactive oxygen species (ROS) is a recognized pathological consequence of *R. rickettsii* infection in human endothelial cells (Silverman and Santucci, 1988; Devamanoharan *et al.*, 1994; Eremeeva and Silverman, 1998a). Interestingly, in one study NO was shown to enhance hydrogen peroxide-mediated endothelial

permeability associated with a significant depletion of intracellular glutathione (Okayama *et al.*, 1997). 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 (Eremeeva and Silverman, 1998b). 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-HCECs produce NO is novel and exciting. Few endothelial pathogens have been shown to induce the levels of NO seen here. For example, virulent Junin virus, a viral hemorrhagic fever agent, has 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 (Gomez *et al.*, 2003). 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 have been associated with dengue virus infection, a disease characterized by general vascular dysfunction, and human endothelial cells infected with dengue virus produce VEGF (Tseng *et al.*, 2005; Azizan *et al.*, 2006;Srikiatkhachorn *et al.*, 2007). In this work we showed that blockade of endogenous NO production had little effect on the permeability of rickettsiae-infected endothelial cells, despite the ability of endogenous NO to reduce the number of intracellular rickettsiae. Given the data demonstrating a dose-dependent effect of *R. rickettsii* on SV-HCEC, one might think that inhibition of this endogenous NO might actually enhance permeability. Our data do not support this conclusion, suggesting that the permeability-enhancing effects of *R. rickettsii* may actually be dependent on the number of invading rickettsiae. Likewise, several important signaling pathways activated in response to *R. rickettsii* infection have been described and 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 (Clifton *et al.*, 2005;Rydkina *et al.*, 2005). 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 (Chiang *et al.*, 2006). Interestingly, MCP-1 is a demonstrated inducer of blood-brain barrier dysfunction both *in vitro* and *in vivo* (Song and Pachter, 2004;Stamatovic *et al.*, 2005). Taken together with the 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 hours of infection. It should also be noted that these previous studies were performed in HUVECs which have been used extensively as an *in vitro* model to study endothelial permeability. However, the origin of these cells means they are of little physiological importance during rickettsial infection, and numerous studies have now demonstrated that endothelial cells are actually quite heterogeneous in their response to different stimuli. Nevertheless, our observations suggest an increased level of virulence for *R. rickettsii* in endothelial cells, which might explain the increased virulence seen in human disease during *R. rickettsii* and *R. conorii* infections. Adherens junctions are regulated mostly via phosphorylation of adherens junction proteins. For example, p120 was first identified as

a phosphorylation substrate for Src kinase, which has been implicated in a number of pathologic inflammatory conditions. Additionally these proteins seem to be regulated by a number of different PKC-dependent pathways as has been addressed earlier. Importantly it should be noted that treatment of endothelial cells with exogenous peroxynitrite leads to the nitration of β-catenin leading to increased microvascular permeability (Knepler, Jr. *et al.*, 2001). However, we demonstrated that blockade of NO production did not prevent increased microvascular permeability in rickettsiae-infected endothelial cells. This indicates an additional impact by rickettsiae that overrides the effects of NO and peroxynitrite produced in response to TNF-α. In fact, some of our evidence supports this since *R. conorii*-infected endothelial cells still experienced an increase in permeability in the presence of the peroxynitrite scavenger after three days of infection.

The complexity of this system should be evident. Clearly rickettsiae have a tremendous impact on endothelial cell function through the activation of innate host cellular pathways and eventually cause cell death. In order to limit the destructive spread of intracellular rickettsiae, the host's immune response acts to kill these intracellular rickettsiae, partly through the activation of iNOS in endothelial cells. However this response does not come without a cost. Since rickettsiae are not confined to one particular organ or compartment and are distributed systemically, the cumulative impact of a potentially very large number of focal inflammatory foci could lead to a widespread increase in microvascular permeability, eventually causing the accumulation of extracellular fluid and protein. In an organ such as the lung or brain, this could be severely detrimental to the health of the infected individual. The work presented in this chapter demonstrates for the first time that this is a real possibility during rickettsial infection. However, more work using animal models of infection is needed in order to demonstrate the true impact of these responses. Additionally we need a better understanding of the innate host cell responses that are activated in response to rickettsial

infection independent of any outside stimuli. Hopefully a better understanding of these factors will allow us to design novel therapeutics to counteract the potentially detrimental effects of these responses while retaining the beneficial effects.

Chapter 4: Rickettsiae-Mediated Host Cell Signaling Related to Regulation of Microvascular Permeability

INTRODUCTION

Adherens Junctions as Regulators of Microvascular Permeability

Endothelial cells are held together via a complex system of protein complexes including tight junctions, adherens junctions and gap junctions. These serve to regulate the flow of solutes through the paracellular pathway between endothelial cells, effectively blocking components in the blood from interacting unnecessarily with cells of the parenchyma. These junctions serve important functions in development, regulation of angiogenesis and inflammation by helping to orchestrate cellular adherence to neighboring cells and the extracellular matrix.

Adherens junctions are ubiquitously expressed throughout the body by endothelial cells and are formed by transmembrane adhesive proteins, the most important being VE-cadherin. VE-cadherins bind each other on neighboring cells through homophilic-type binding of the extracellular region of the protein. The short cytoplasmic domain binds cytoplasmic proteins called catenins, in particular β-catenin and p120, two members of the Armadillo family of proteins (Peifer, 1995). β-catenin, but not p120, in turn associates with α-catenin which binds to the actin cytoskeleton promoting junction stabilization (Aberle *et al.*, 1996). β-catenin can also act as a signaling molecule via its cytoplasmic form which directly participates in the Wnt growth factor signaling cascade. Glycogen synthase kinase-3 (GSK-3) phosphorylates β-catenin leading to rapid inactivation via proteosomal degradation. Wnt is responsible for the inactivation of GSK-3 through receptor binding indicating the importance of Wnt in stabilization of cytoplasmic β-catenin (Nusse, 1997). Cytoplasmic free β-catenin binds other cellular proteins including the actin fasciculation protein fascin, suggesting that free β-catenin may affect the cytoskeletal organization in a cadherin-independent fashion (Tao *et al.*, 1996).

p120 is another important molecule whose role in adherens junction biology is much less well defined. First identified as a substrate for Src, p120 binds VE-cadherin but does not directly bind actin. Instead, p120 has a diverse range of functions that are just now beginning to be understood. Among those include the ability of p120 to regulate the endocytosis and internalization of VE-cadherin (Xiao *et al.*, 2007). Additionally, it is now well understood that p120 serves as the switchboard for a number of signaling pathways including tyrosine phosphorylation-based pathways and signaling mediated by the Rho family of small GTPases (Alema and Salvatore, 2007;Anastasiadis, 2007). Taken together we begin to appreciate that p120 may regulate endothelial barrier function in a manner not usually associated with other adherens junction proteins.

Signaling Pathways that Control Adherens Junction Function

Protein kinase C

Protein kinase C is a family of structurally similar serine/threonine kinases that are dependent on Ca^{+}, phospholipids and diacylglycerol for activation (Hug and Sarre, 1993). The distribution of specific isoenzymes varies widely by tissue and cell type, and as a result the phosphorylation targets of PKC are diverse. PKC is an important mediator of microvascular permeability and has a direct effect on the organization and function of adherens junction proteins. N-terminal serine phosphorylation of β-catenin by PKC-α targets β-catenin for proteosomal degradation and subsequent inhibition of the Wnt/β-catenin pathway (Gwak *et al.*, 2006). A similar response occurs in response to a related kinase, glycogen synthase kinase 3β (GSK-3β) in which β-catenin is degraded following GSK-3β-mediated phosphorylation in response to virulent *Salmonella typhimurium* infection both *in vitro* and *in vivo* (Duan *et al.*, 2007). Degradation of β-catenin in this system is associated with a decreased physical association of β-catenin and NF-κB, decreased expression of c-myc, and increased levels of IL-6 and TNF-α. Constitutive

over-expression of β-catenin led to a stabilization of IκBα and NF-κB inhibition associated with decreased IL-8 secretion. These results demonstrate an important role for β-catenin as a negative regulator of inflammation, likely in response to PKC and/or GSK-3β activation.

PKC has also been shown to modify the adherens junction proteins VE-cadherin and p120 in response to thrombin, a potent permeability-inducing factor (Konstantoulaki *et al.*, 2003). Thrombin-induced dephosphorylation of VE-cadherin was associated with the formation of intercellular gaps. This was accompanied by dephosphorylation of β-catenin, phosphorylation of p120, and dissociation of both proteins from the cytoplasmic tail of VE-cadherin. The authors went on to demonstrate that blockade of PKC activity restored barrier function and abrogated post-translational modification of the cadherin/catenin complex.

Sahni et al. demonstrated a role for non-classical PKC isoforms in *R. rickettsii*-induced NF-κB activation and subsequent tissue factor expression (Sahni *et al.*, 1999). However, no connection has been established between rickettsiae-induced PKC activation and vascular dysfunction during rickettsial infection.

Src kinase

The Src family of protein tyrosine kinases is an important mediator of vascular permeability. Src is activated via phosphorylation of Tyr 416 and targets a number of proteins including p120 and β-catenin (Alema and Salvatore, 2007). Recent work has shown Src-dependent endothelial permeability changes in response to VEGF, TNF-α, and ROS through phosphorylation of adherens junctions (Eliceiri *et al.*, 1999;Kevil *et al.*, 2001;Nwariaku *et al.*, 2002). Src has been shown to directly associate with and phosphorylate β-catenin during neutrophil-mediated venular hyperpermeability responses, which are abolished with blockade of Src (Tinsley *et al.*, 2002). Work in

macrophages has shown that Hck, a Src family kinase, associates with the β-chain of the IL-6 receptor although the downstream effects of this interaction are not well understood (Schaeffer *et al.*, 2001). Likewise it is not clear whether Hck mediates IL-6 induced changes in endothelial cells. Finally Src has been shown to directly phosphorylate focal adhesion kinase (FAK) and paxillin, which bind $\alpha v\beta_5$-integrins and regulate the formation of focal adhesions which bind the extracellular matrix (Eliceiri *et al.*, 2002). *In vivo* Src has been shown to mediate pathologic changes during acute inflammatory response including LPS-induced acute lung injury (ALI), ischemic brain injury and myocardial infarction (Okutani *et al.*, 2006). Clearly, Src is a very important component of vascular integrity; however, the relative contribution to vascular hyperpermeability during viral or bacterial infection is completely unknown.

Interleukin-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine associated with a number of infections and has been associated with adverse outcomes in many. For example, the levels of IL-6 are statistically higher in human serum samples from fatal cases of yellow fever, compared to nonfatal cases (ter Meulen *et al.*, 2004). Experimentally, IL-6 knockout mice experience decreased vascular permeability during pneumococcal meningitis (Paul *et al.*, 2003). Therefore, it is not surprising that IL-6 is capable of increasing endothelial permeability *in vitro* (Maruo *et al.*, 1992). More importantly it has also been demonstrated that this occurs via a PKC-dependent pathway (Desai *et al.*, 2002). IL-6 induced permeability is associated with redistribution of the tight junction protein ZO-1 as well as actin and increased cell contraction. PKC-inhibition reverses IL-6-mediated permeability changes, demonstrating the importance of this kinase system in mediating changes in vascular permeability.

The importance of IL-6 in rickettsial infection is relatively unknown. However, it has been shown that human endothelial cells infected with *R. conorii* secrete IL-6 and IL-8 via a cell-associated IL-1α-dependent pathway (Kaplanski *et al.*, 1995). Beyond this it is not clear what role IL-6 plays in rickettsial pathogenesis.

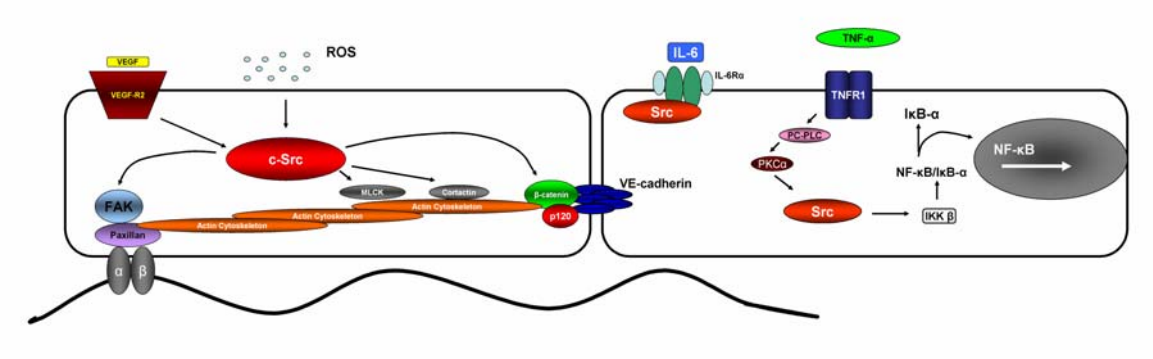


Illustration 4.1: Src-mediated signaling pathways present in endothelial cells that may be important to rickettsial pathogenesis.

Vascular endothelial growth factor

Vascular endothelial growth factor, VEGF, was the first permeability-inducing factor to be identified (Senger *et al.*, 1990). We now know VEGF as a class of alternative-splicing variants of a single mRNA consisting of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PIGF) (Kosmidou *et al.*, 2007). By far the most well studied variant is VEGF-A, a 38kDa homodimeric peptide capable of inducing increased permeability of endothelial monolayers. This appears to occur through binding to the cellular receptor VEGF-R2 resulting in stimulation of phospholipase C, DAG production and subsequent calcium influx through store-independent calcium channels.

One of the most important downstream pathways activated in response to VEGF stimulation is the endothelial nitric oxide synthase (eNOS) pathway. Hood et al.

demonstrated an increase in eNOS mRNA, protein and NO production in human endothelial cells stimulated with VEGF (Hood *et al.*, 1998). They also showed the calcium-dependence of this effect by stimulating VEGF treated cells with the calcium ionophore A-23187, which resulted in a doubling in the amount of NO produced. The available data also shows that eNOS is actually a very important mediator of microvascular hyperpermeability and acute inflammation *in vivo*. eNOS-deficient mice exhibit a markedly reduced permeability response to platelet-activating factor (PAF) when compared to wild-type or iNOS-deficient animals (Hatakeyama *et al.*, 2006). Likewise, eNOS-deficient mice experience less edema in response to subplantar carrageenan injection (Bucci *et al.*, 2005).

The relationship between VEGF-induced NO production was later determined to have a direct impact on endothelial permeability through a cGMP-dependent pathway (Mayhan, 1999). Perfusion of VEGF increases the rate of FITC-dextran clearance from meningeal vessels of treated rats indicating a loss of blood brain-barrier integrity in response to VEGF. The pharmacological inhibition of NO-production or soluble guanylate cyclase with L-NMMA and ODQ, respectively, abolished this increase in permeability. It was later shown that this is most likely a result of reduced expression of occludin, an important component of endothelial tight junctions (Wang *et al.*, 2001).

VEGF is also known to negatively affect endothelial permeability via a pathway involving ERK-1/2 phosphorylation by PKC (Breslin *et al.*, 2003). Additionally VEGF has been shown to induce PKC-dependent tyrosine phosphorylation of β-catenin resulting in increased endothelial permeability in response to binding to VEGF-R2 (Cohen *et al.*, 1999). This collection of data demonstrates the multifaceted impact of VEGF on endothelial permeability and supports a role for NO and PKC activity in mediating these effects.

A number of vascular disorders are associated with increased VEGF production. For example, increased BBB permeability during acute lead exposure has been shown to

be caused by a two-fold increase in the amount of cerebellar VEGF associated with vasogenic cerebellar edema (Hossain *et al.*, 2004). VEGF has also been shown to be important in pulmonary edema although the available data points more towards a dual role for VEGF in mediating endothelial barrier function. Karmpaliotis et al. have demonstrated increased levels of VEGF in mice receiving an inhalational dose of LPS (Karmpaliotis *et al.*, 2002). It has also been demonstrated that patients with acute respiratory distress syndrome have significantly higher levels of plasma VEGF than control patients (Thickett *et al.*, 2001). Conversely Maitre et al. demonstrated decreased levels of VEGF in a rat model and in humans experiencing acute lung injury (Maitre *et al.*, 2001). One possible explanation for this disconnection is that VEGF may act very early after an insult or injury producing an increase in vascular permeability, but then over the long term healing process VEGF contributes to angiogenic vascular remodeling. This might be explained by different receptor expression and interaction resulting in different and contrasting effects of VEGF.

There are also data to suggest that VEGF is an important biological mediator during dengue virus infection. Dengue is an emerging infectious disease endemic to many tropical regions of the world that usually causes a mild, self-limiting disease. Recently, however, we have observed a startling increase in the severe form of dengue referred to as dengue hemorrhagic fever (DHF). DHF is characterized by a generalized increase in vascular permeability that can eventually lead to hemorrhage and death in about 5% of patients. Recently it was reported that DHF patients have significantly higher levels of circulating VEGF compared to non-hemorrhagic dengue fever (DF) patients or case controls (Tseng *et al.*, 2005). *In vitro* data demonstrate that human endothelial cells infected with dengue virus produce VEGF in addition to cytokines like IL-6, IL-8, TNF-α, and IFN-γ (Azizan *et al.*, 2006). Additionally, data from human DHF patients show that soluble VEGF-R2 decreases during DHF and this is associated with leakage from the vasculature (Srikiatkhachorn *et al.*, 2007). Presumably this results in

engagement of the cell-bound form of the receptor producing changes in the endothelium resulting in disassembly of the adherens junctions and increased microvascular permeability. This latter observation should underscore the importance of receptor expression in regulation of VEGF signaling and should not be ignored.

Clearly, VEGF-mediated NO signaling appears to be an important mechanism of increased microvascular permeability during acute inflammatory conditions. However, the importance of this pathway in rickettsial pathogenesis is completely unknown.

Innate Host Cell Responses to Rickettsial Infection as Mediators of Increased Microvascular Permeability

Rickettsial infection of endothelial cells results in a very dynamic environment despite the relative sequestration of rickettsiae in the host cell cytoplasm. Previous work has demonstrated a number of pathways activated in response to rickettsial invasion ranging from cellular stress responses to the secretion of chemotactic cytokines. Unfortunately, no one has been able to directly link any of these responses to pathologic changes in the endothelium that might contribute to the overall pathogenesis of rickettsial infection. We have addressed this major gap in knowledge with a particular emphasis on autocrine cell signaling by cytokines secreted in response to rickettsial infection. We hypothesized that rickettsiae-mediated increase in microvascular permeability is partly a result of increased secretion of IL-6 and/or VEGF by rickettsiae-infected endothelial cells leading to the post-translational modification of the adherens junction proteins β-catenin and/or p120. This will help address a major gap in knowledge concerning rickettsial pathogenesis and may perhaps present us with a novel mode of therapy for the treatment of severe cases of acute rickettsial infection.

RESULTS

Rickettsia rickettsii Infection Induces NO Production Independent of iNOS Activation in Human Cerebral Endothelial Cells

Nitric oxide production is an important component of VEGF signaling in the endothelium. Therefore we decided to test whether rickettsial infection leads to the increased production of NO by human cerebral endothelial cells. SV-HCEC infected with *R. rickettsii*, but not *R. conorii*, led to the accumulation of significant levels of nitrite in the supernatant, indicating that NO was being produced by these cells (Figure 4.1A). It was also determined that this was not a result of increased iNOS activity, since there was no iNOS detectable in lysates from infected cells (Figure 4.1B). This indicates that rickettsiae-mediated NO production is occurring as a result of an alternative NOS enzyme.

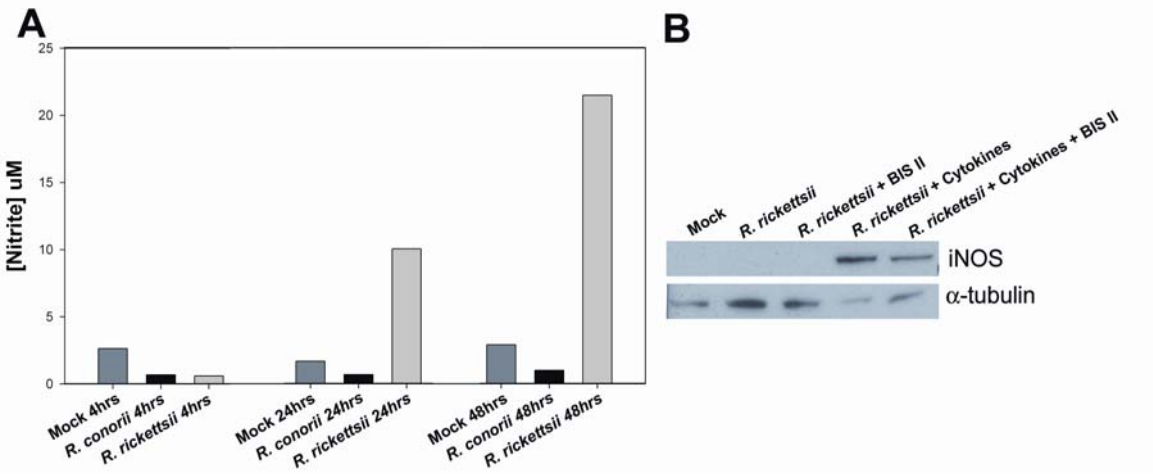


Figure 4.1: iNOS-independent NO production by *R. rickettsii*-infected SV-HCEC. SV-HCEC infected with *R. rickettsii* but not *R. conorii* produce high levels of nitrite by 24 hours after infection (A). This is not due to transcriptional activation of iNOS since no iNOS protein was detectable in cell lysates (B). Likewise, PKC inhibition with BIS II did not appear to have an effect on the level of iNOS activation.

Rickettsial Infection Induces IL-6 and VEGF Production Both *In Vitro* and *In Vivo*

Since VEGF and IL-6 are such important mediators of vascular permeability in a number of conditions, we decided to determine whether these two cytokines are produced at higher amounts by rickettsiae-infected human cerebral endothelial cells and whether or not they act in an autocrine fashion altering microvascular permeability. Additionally we compared the responses to two closely related rickettsial species, *R. conorii* and *R. rickettsii*, to determine whether host cell responses may potentially translate into the increased virulence seen during human *R. rickettsii* infection. SV-HCEC infected with ~1 moi of rickettsiae began secreting VEGF and IL-6 at levels above that of mock-infected cells as early as 6 hours after infection (Figure 4.2). VEGF was produced at higher amounts by *R. rickettsii*-infected cells resulting in a supernatant concentration of approximately 110 pg/ml or about 4.6-times that of uninfected cells. Conversely, cells infected with *R. conorii* only doubled their production of VEGF with a supernatant concentration of approximately 41 pg/ml. IL-6 was also produced at significantly higher levels by those cells infected with *R. rickettsii*, with a concentration of 51.62 pg/ml compared to *R. conorii*-infected cells at 5.76 pg/ml or mock-infected cells at 3.11 pg/ml. We also showed that IP-10 was significantly upregulated by both rickettsial species, with endothelial cells producing comparable levels after 6 hours of infection. Additionally we showed that the two important mediators of microvascular permeability addressed in Chapter 3, namely TNF-α and IL-1β, were not produced at higher levels in SV-HCEC infected with rickettsiae.

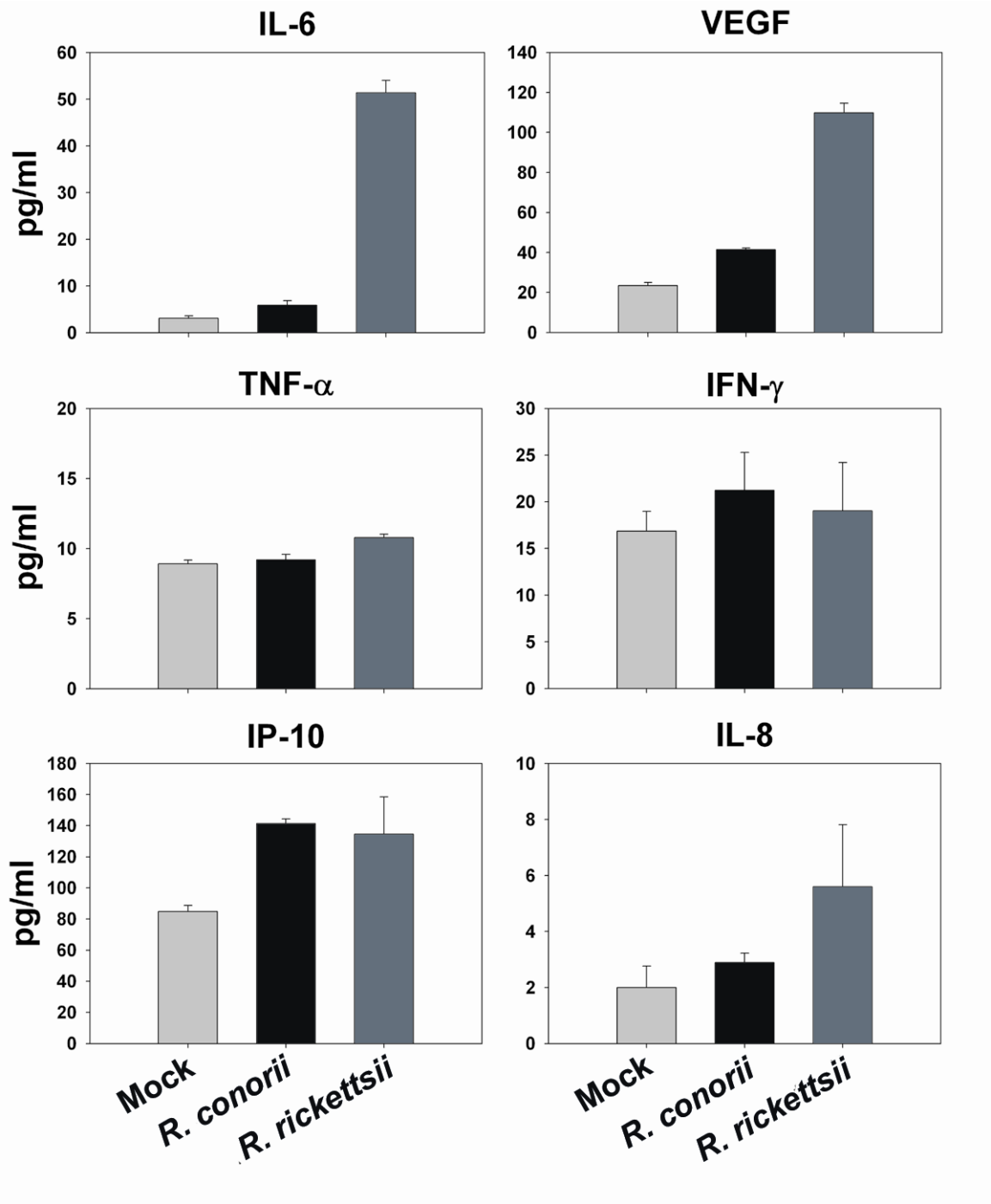


Figure 4.2: Cytokine production by rickettsiae-infected SV-HCEC 6 hours after infection. Cells were infected with 1 moi of *R. conorii* or *R. rickettsii* for six hours at which time the supernatants were filtered and assayed via Bio-Plex for supernatant cytokine concentrations. Data are expressed as the mean cytokine concentration in pg/ml ± standard deviation.

Supernatant from *R. rickettsii*-infected Endothelial Cells Causes Increased Microvascular Permeability

Next we measured the ability of these secreted cytokines to act on uninfected endothelial monolayers and induce an increase in microvascular permeability. SV-HCECs were infected with *R. conorii* or *R. rickettsii* for 24 hours, and the cell culture supernatant was filtered through a 0.22 μm membrane to remove viable rickettsiae. Cell culture supernatants from *R. rickettsii*-infected endothelial monolayers induced a decrease in TER across uninfected SV-HCEC monolayers indicating an increase in microvascular permeability (Figure 4.3). However, supernatants from *R. conorii*-infected endothelial cells did not have the same effect. Endothelial monolayers maintained a level of resistance equal to that of cells treated with supernatants from uninfected cells. Given that IL-6 and VEGF were secreted by SV-HCEC infected with rickettsiae, we attempted to neutralize these two cytokines with the expectation that this would mitigate some of the effects of the supernatants on uninfected cells. IL-6 and/or VEGF neutralization did not block any of these permeability-inducing effects. This could be due to inefficient neutralizing activity despite the manufacturer's claims, or it could suggest the presence of other, unknown factors acting on the endothelium.

VEGF is expressed by Rickettsiae-infected Cells *In Vivo*

Since VEGF was produced by rickettsiae-infected endothelial cells *in vitro*, we wished to determine whether this also occurs *in vivo* and whether this correlates with pathologic changes associated with infection. Immunohistochemical staining of tissues from *R. australis*-infected mice revealed VEGF production by rickettsiae-infected cells in the spleen (Figure 4.4A-C). A great majority of infected cells stained positively for VEGF, although we also observed infected cells that did not stain for VEGF. VEGF-producing cells that were uninfected were not detected. In the brain rickettsial antigen localized to sites of vascular inflammation; however, there was no detectable VEGF

expression (Figure 4.4D). In the lung, rickettsial antigen was detected in the alveolar septa (Figure 4.4E) although due to the high degree of interstitial inflammation we were unable to determine whether these were in fact endothelial cells. In the liver, rickettsial antigen associated with infiltrates of mononuclear cells and few neutrophils (Figure 4.4F).

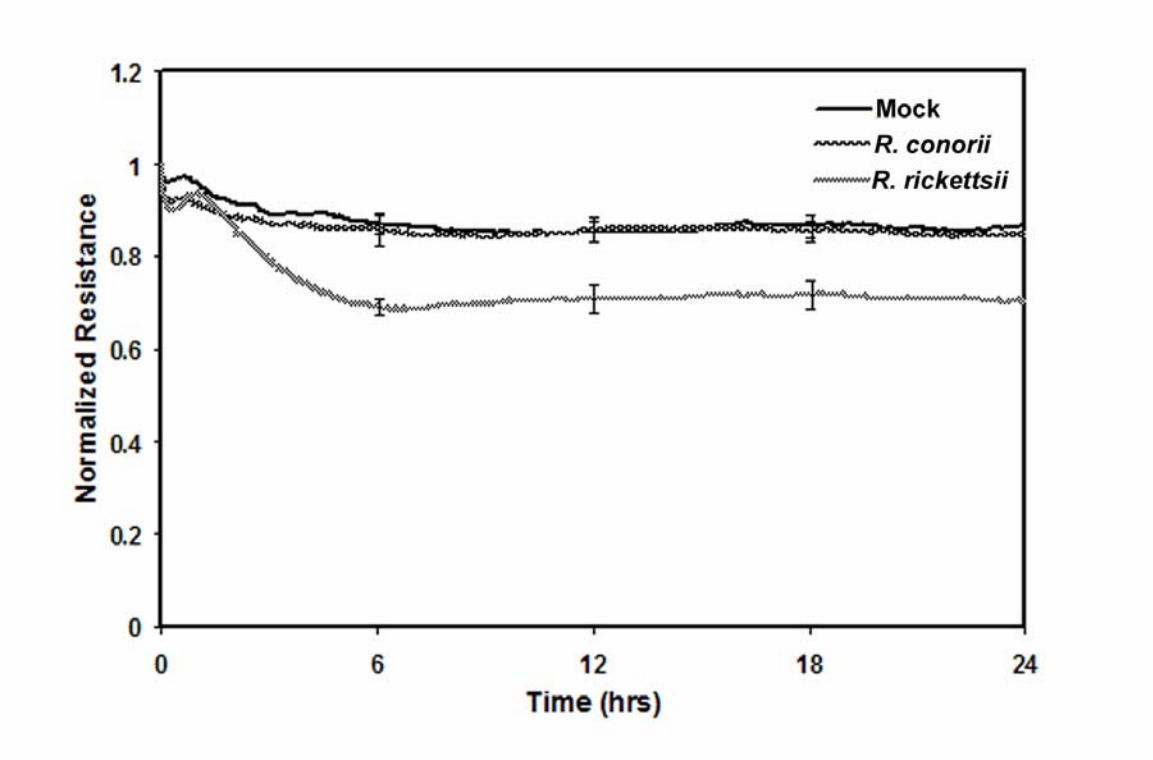


Figure 4.3: Supernatant from *R. rickettsii*-infected SV-HCECs causes an increase in microvascular permeability across uninfected endothelial monolayers. SV-HCECs were infected with *R. conorii* or *R. rickettsii* (moi 10). After 24 hours the supernatants were removed and sterile-filtered through 0.22 μm syringe filter. These supernatants were then added to a fresh monolayer of SV-HCEC and monitored for changes in electrical resistance for 24 hours.

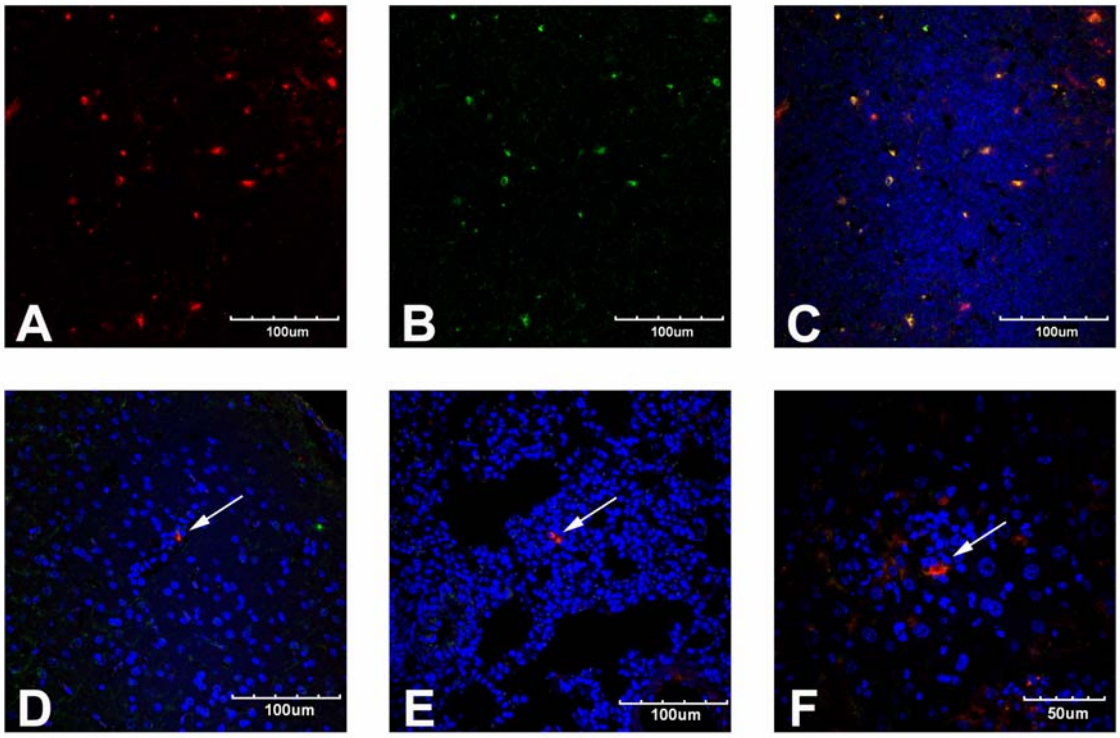


Figure 4.4: VEGF is produced by rickettsiae-infected cells *in vivo*. Tissues from *R. australis*-infected C57BL/6 mice were stained for SFG rickettsiae (red) and VEGF (green). In the spleen, rickettsial antigen (A) and VEGF (B) co-localized (C, yellow) to the same cells at day 6 after infection. There was no detectable VEGF staining in rickettsiae-infected cells in the brain (D), lung (E) or liver (F) of these mice (arrows).

Rickettsial Infection Activates Kinase Signaling in Human Endothelial Cells

Protein kinase C activation is a recognized consequence of both VEGF and IL-6 signaling. Therefore we wished to determine the intensity of PKC activation during rickettsial infection and whether or not this plays any role in rickettsiae-mediated permeability. Crude lysates of SV-HCEC infected with *R. conorii* or *R. rickettsii* possessed greater PKC activity compared to uninfected controls (Figure 4.5A). Additionally we showed that *R. rickettsii* induced a greater increase in relative activity than *R. conorii* at 24 hours after infection. To determine the impact of PKC inhibition on

rickettsiae-induced permeability, we treated endothelial cells with bisindolylmaleimide II, a cell permeant, selective inhibitor of PKC. Once again we observed that PKC inhibition had no effect on permeability (Figure 4.5B). However, inhibition of Src-kinase with SU6656 did have an impact on the level of permeability experienced in *R. rickettsii*-infected cells. While we were able to demonstrate a significantly higher level of TER (decreased permeability) in these treated cells, inhibition of Src did not prevent rickettsiae-mediated cell death after 24 hours of infection.

We also investigated the regulation of another important signaling molecule, focal adhesion kinase (FAK). Phosphorylation of FAK at specific tyrosine and serine residues has been shown to be important in regulating FAK function. Using a phospho-specific antibody to Ser722, we have been able to demonstrate that FAK is dephosphorylated in response to rickettsial infection while the overall levels of FAK do not change (Figure 4.6). Although this occurred in cells infected with both *R. conorii* and *R. rickettsii*, the effect of *R. rickettsii* seemed to be the greater with virtually no pFAK(Ser722) being detectable after 24 hours of infection.

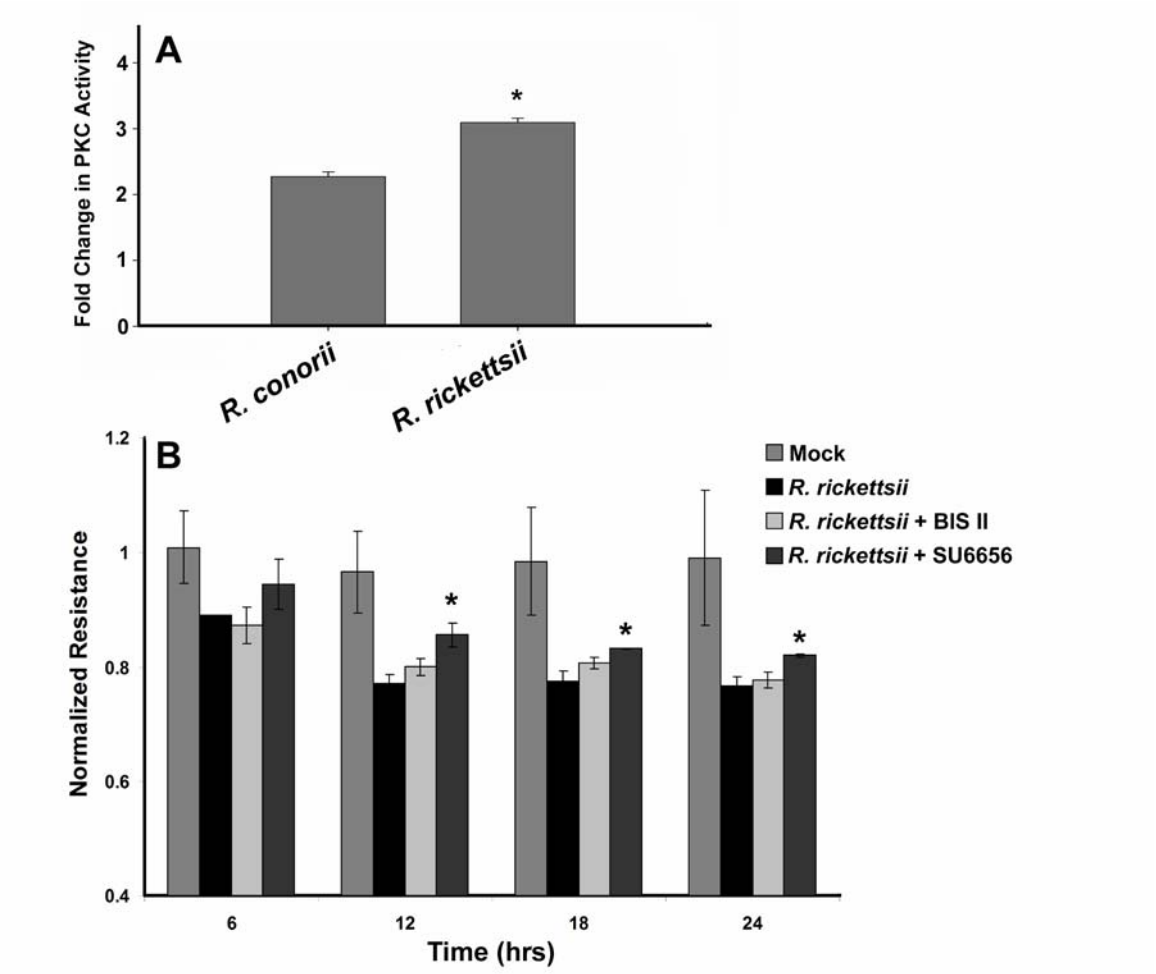


Figure 4.5: Rickettsial infection activates PKC, but this is not related to rickettsiae-induced microvascular permeability. SV-HCECs were infected with *R. conorii* and *R. rickettsii* (moi 1). After 24 hours crude lysates were measured for PKC activity by the ability of the lysates to phosphorylate plate-bound PKC substrate (A). *R. rickettsii*-infected endothelial cells had a significantly higher level of PKC activity than *R. conorii*-infected cells. Additionally cells were treated with inhibitors of PKC and Src at the time of infection and monitored by ECIS for changes in microvascular permeability. Src-inhibition with SU6656 (1μM) resulted in a significantly smaller increase in permeability compared to infected, untreated cells. Inhibition of PKC with bisindolylmaleimide II (BIS II, 1 μM) did not have any effect on the level of permeability of infected cells. *p=<0.05.

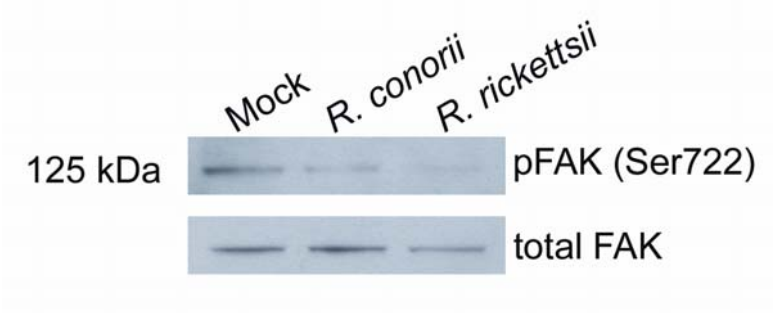


Figure 4.6: Rickettsial infection results in dephosphorylation of focal adhesion kinase at Serine 722. SV-HCECs were infected with *R. conorii* or *R. rickettsii* (moi 1). After 24 hours of infection the cells were washed once in ice-cold PBS before being homogenized in ice-cold lysis buffer. Equal amounts of protein were loaded onto a 10% Bis-Tris gel and separated by SDS-PAGE electrophoresis. The proteins were transferred to a nitrocellulose membrane and probed with monoclonal antibodies to FAK and pFAK(Ser722). The blots showed a significant decrease in pFAK levels in rickettsiae-infected cells compared to mock-infected controls.

Rickettsial Infection Produces Post-translational Modification of the Adherens Junction Protein β-catenin in Human Cerebral Endothelial Cells

Next we investigated the functional implications of rickettsial infection with regards to modification of the adherens junction proteins β-catenin and p120. Western blotting after 24 hours of *R. rickettsii* infection revealed a change in the migration pattern of β-catenin associated with the appearance of several distinct bands with an apparent molecular weight less than that of intact β-catenin (Figure 4.7). These bands appeared as early as 12 hours after infection and increased in both number and intensity by 24 hours after infection. These changes appeared to reflect cleavage of the β-catenin protein and did not occur with p120. Also noteworthy, the expression levels of β-catenin and p120 did not change significantly, supporting the contention that post-translational modifications, potentially via phosphorylation are mediating disassembly of adherens junctions in *R. rickettsii*-infected cells.

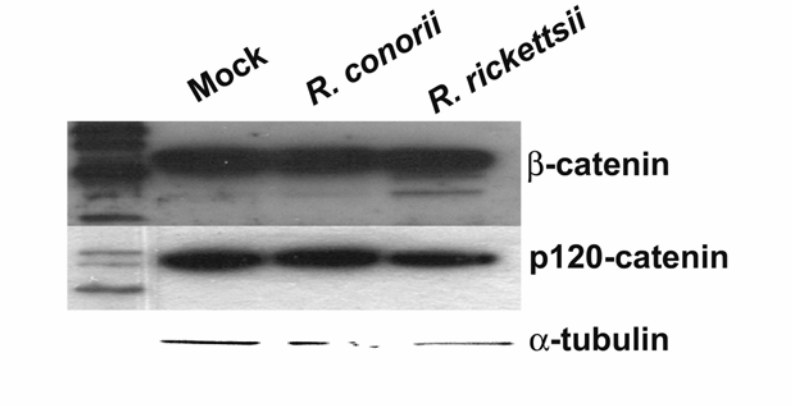


Figure 4.7: Apparent cleavage of β-catenin after 24 hours of *R. rickettsii* infection. SV-HCEC infected for 24 hours with *R. conorii* or *R. rickettsii* (moi 10) were blotted and probed for β-catenin, p120, and α-tubulin. β-catenin staining revealed the presence of smaller molecular weight bands indicating cleavage of the protein. Overall expression levels were not altered.

RNA Microarray Analysis of Human Endothelial Cell Responses to Rickettsial Infection

Finally, we decided to analyze the transcriptional profile of host cell genes related to endothelial cell biology, with the expectation that this would reveal novel pathways of endothelial dysfunction during rickettsial infection that have not been previously identified. Secondly, we saw this as an opportunity to compare the host cell response to different rickettsial species with varying levels of virulence in humans. With this intention we assessed the mRNA profile of 113 genes in response to infection with *R. conorii* (Malish 7 strain), *R. australis* (Cutlack strain), *R. rickettsii* (Sheila Smith strain), and *R. rickettsii* (Iowa strain). Infection of SV-HCEC cells with each strain revealed the modulation of several genes including those important in regulation of apoptosis, cell growth, and the ability to respond to stimuli such as pro-inflammatory cytokines (Figure 4.8). There was a strong modulation of genes involved in control of apoptosis including BCL2-associated X protein and BCL2-like 1, which were initially upregulated

within 4 hours after infection, but were downregulated by all rickettsiae after 24 hours of infection (Table 4.1). Colony stimulating factor 3 (CSF-3), interferon beta 1 (IFN-β1), RhoB and thrombospondin-1 (THBS-1) were also upregulated by some rickettsiae (*R. australis* and *R. rickettsii* (Iowa)) four hours after infection, but overall underwent a decreased level of transcription after 24 hours of infection. The only gene shown to be upregulated both at 4 and 24 hours was angiotensin I converting enzyme (ACE), although *R. rickettsii* (Sheila Smith) did not seem to stimulate upregulation of ACE at 24 hours after infection, but instead ACE was suppressed. Matrix metallopeptidase 2 (MMP2) was suppressed by all rickettsiae except for *R. rickettsii* (Sheila Smith). Finally, the TRAIL decoy receptor, TNFRSF10c, showed a number of differences in response to rickettsial infection. Whereas *R. australis* showed an early suppression of expression followed by a return to normal levels, *R. rickettsii* (Iowa) initially upregulated expression of TNFRSF10c at 4 hours followed by a suppression at 24 hours. Similarly, *R. conorii* and *R. rickettsii* (Sheila Smith) caused an overall suppression of this gene after 24 hours of infection. Finally, we observed upregulation of the gene for tissue factor pathway inhibitor (TFPI) after 4 hours of infection with *R. australis* or *R. rickettsii* (Iowa). By 24 hours, however, this response had diminished, and expression levels were normal for those two strains of rickettsiae. This was also true for *R. conorii*; however, *R. rickettsii* (Sheila Smith) appeared to cause a down-regulation of TFPI after 24 hours of infection.

We validated the results obtained from this microarray analysis using real time RT-PCR to measure the relative expression levels of selected genes at 4 and 24 hours of infection. Analysis of RhoB expression revealed an initial decrease in response to *R. conorii* or *R. rickettsii* (Iowa) infection, whereas *R. australis* appeared to cause an initial increase in expression after 4 hours (Figure 4.9). However, after 24 hours of infection all three rickettsiae caused a decrease in relative RhoB expression of between 70-95%.

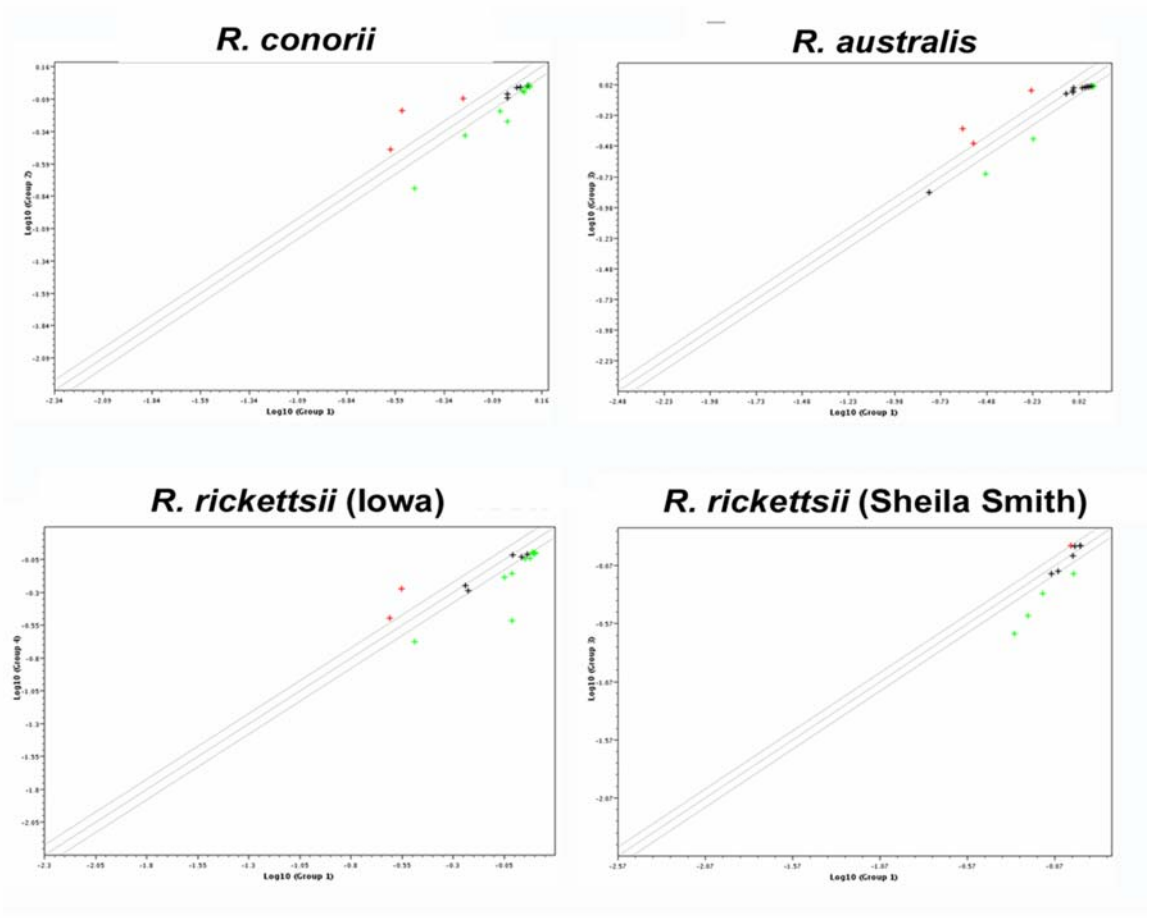


Figure 4.8: Rickettsial infection leads to transcriptional modulation of several important host genes. Representative dot-plots of host cell gene expression in rickettsiae-infected SV-HCEC compared to mock-infected cells at 24 hours of infection. Green stars indicate genes that are down-regulated in relation to controls whereas red stars indicate genes that are up-regulated in relation to controls.

Table 4.1: Relative gene expression of select genes in rickettsiae-infected SV-HCEC

Gene	*R. australis* 4hrs	*R. rickettsii* (Iowa) 4hrs	*R. conorii* 24hrs	*R. australis* 24hrs	*R. rickettsii* (Iowa) 24hrs	*R. rickettsii* (SS) 24hrs
Angiotensin I Converting Enzyme	3.12	4.51	1.36	1.88	1.3	0.03
BCL2-associated X Protein	1.16	1.13	0.9	0.88	0.8	0.89
BCL2-like 1	2.25	2.16	0.92	0.91	0.86	0.66
Burkitt Lymphoma Receptor	0.96	1.15	1.44	1.65	0.98	0.86
Colony Stimulating Factor 3	5.27	7.43	0.51	0.6	0.65	0.26
Interferon, beta 1, fibroblast	11.51	7	0.73	0.64	0.87	0.15
Interleukin 1, beta	0.93	0.91	0.84	0.82	0.81	1.07
Matrix Metallopeptidase 2	0.92	0.91	0.83	0.81	0.78	1.07
Ras Homolog, gene family, member B	1.38	1.33	0.82	0.88	0.85	0.06
Thrombospondin-1	1.48	1.31	0.57	0.94	0.32	0.11
Tissue Factor Pathway Inhibitor	1.52	1.22	0.92	1.01	0.99	0.53
TNFRSF10c	0.86	1.18	0.74	1	0.74	0.45

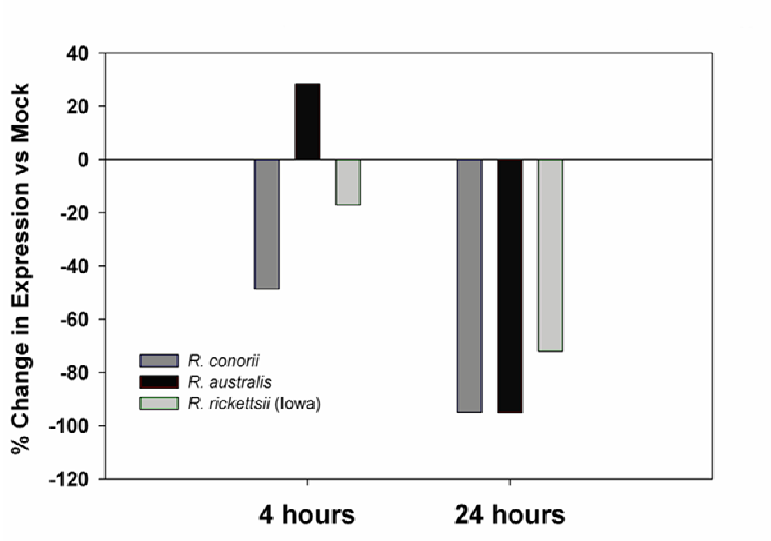


Figure 4.9: Relative *RhoB* expression in SV-HCEC infected with three different strains of rickettsiae. RNA from rickettsiae-infected SV-HCEC was reverse transcribed and used for real-time PCR using commercially available primers for *hRhoB*. Data were normalized to *hβ-actin,* and relative expression versus uninfected controls was determined using the ΔΔCt method.

DISCUSSION

Modulation of host cell gene expression in response to infection is an important area of interest, and unfortunately has been, until recently, underappreciated by many in the field of rickettsiology as a contributor to pathogenesis. Endothelial cells must respond to invading rickettsiae in order to activate an inflammatory response and recruit leukocytes into regions of invasion to help clear the infection. It has been well demonstrated that this is essential to the development of an effective anti-rickettsial response in the form of iNOS and IDO activation, as well as potential clearance of infected cells through CTL activity. Past work has demonstrated the release of chemokines such as MCP-1, IL-8, CXCL9/10 and fractalkine (Valbuena *et al.*, 2003;Clifton *et al.*, 2005;Valbuena and Walker, 2005), although it is unclear at this point what affect these proteins have on the endothelial cells themselves. To this end, we sought to identify novel pathways activated in rickettsiae-infected human endothelial cells that may negatively affect the structure and function and interendothelial adherens junctions, thus leading to increased microvascular permeability. We have identified VEGF as a mediator released by rickettsiae-infected endothelial cells that may potentially act through PKC- and/or Src-mediated phosphorylation of the adherens junction proteins β-catenin and p120. Additionally, through the use of microarray analysis of rickettsiae-infected human endothelial cell gene expression, we have identified novel pathways that may serve an important role in the pathogenesis of human spotted fever.

VEGF production by rickettsiae-infected endothelial cells is a novel observation and could have potentially dramatic implications in the vascular damage and hyperpermeability experienced during acute rickettsioses. The fact that VEGF was produced by both *R. rickettsii*- and *R. conorii*-infected cells suggests that this is a conserved response initiated in response to rickettsial infection with different species, even though the intensity of the response was much greater in *R. rickettsii*-infected cells. This may also explain why we observed nitrite accumulation in *R. rickettsii*-infected cell

culture supernatants. VEGF potently activates eNOS via phosphorylation at Serine 1177 leading to NO production, activation of soluble guanylate cyclase (sGC) and subsequent production of cGMP. This process is blocked through the action of thrombospondin-1, which we demonstrated is downregulated in response to rickettsial infection (Isenberg *et al.*, 2005). However, as demonstrated by other work in our laboratory, inhibition of this NO production does not necessarily alleviate the permeability-inducing properties of rickettsiae. Most likely this is a result of the active infection, since intracellular rickettsiae utilize host cell nutrients eventually leading to starvation of the endothelium.

However, we must also consider alternative pathways of VEGF signaling, particularly through PKC and/or Src kinase. In support of this, we have demonstrated that PKC kinase activity is increased in rickettsiae-infected endothelial cells, as has been described previously (Sahni *et al.*, 1999). However, inhibition of PKC with an inhibitor of classical PKC isoforms did not reverse the effects *R. rickettsii* had on permeability of human cerebral endothelial cell monolayers. We were able to show that inhibition of Src kinase with SU-6656 resulted in a moderated increase in permeability, less than that of untreated cells. However, this did not prevent endothelial cell death in response to infection since rickettsiae were still allowed to perpetuate. Based on these statements it could be construed that kinase activity plays no role in rickettsial pathogenesis since cells would be overwhelmed by rickettsial division anyway. On the contrary, not every endothelial cell is infected *in vivo*, and given that those that are infected are producing cytokines capable of inducing microvascular permeability in neighboring cells, it is quite possible that cytokines such as VEGF and/or IL-6 could be acting in a paracrine fashion. Therefore, Src-inhibition therapy might be appropriate during severe, late-stage rickettsiosis when administered concurrently with appropriate antibiotic therapy. In fact, SU-6656 has been tested for therapeutic potential in murine models of sepsis. Src inhibition was shown to decrease the level of mortality induced by LPS and also shown to protect against LPS-induced ALI (Severgnini *et al.*, 2005). The availability of several

mouse models of rickettsial infection presents the opportunity to explore the role of Src and/or PKC activity as pathologic mediators of rickettsial pathogenesis.

We also have explored the effects of rickettsial infection on directly modulating adherens junction structure. Analysis of β-catenin and p120 expression did not reveal any significant alterations in the level of expression of these two cytokines. This supports a role for post-translational modification in the form of phosphorylation or dephosphorylation of adherens junction components, probably in response to PKC or Src activity. The appearance of smaller molecular weight bands of β-catenin after *R. rickettsii* infection suggests proteolytic cleavage of the protein in a subset of endothelial cells. This could represent changes to the cytoplasmic form of β-catenin that is not necessarily related to the interaction of β-catenin with the cytoskeleton-bound form found at adherens junctions. Efforts in our laboratory to identify specific sites of tyrosine and/or serine/threonine phosphorylation on β-catenin have been met with many challenges and are an area of future focus.

Another major component of this work was to identify novel pathways of endothelial cell activation that might contribute to rickettsial pathogenesis. We also saw this as an opportunity to explore differences in rickettsial virulence between different rickettsial species that demonstrate varying levels of virulence both in humans and in animal models of infection. *R. rickettsii* is generally regarded as the most virulent spotted fever group rickettsia in man, although it does not efficiently infect mice. The Sheila Smith strain is the prototype strain for this species of rickettsiae and has been associated with a greater degree of endothelial cell damage than other strains of *R. rickettsii* (Eremeeva *et al.*, 2001). However, the Iowa strain of *R. rickettsii* has been suggested to be an attenuated variant of Sheila Smith due to a deletion in the *ompB* gene (Hackstadt *et al.*, 1992). *R. conorii* (Malish 7 strain) is generally less virulent than *R. rickettsii*, but is still capable of causing fatal disease. Finally, *R. australis* rarely causes lethal disease in humans.

Transcriptional analysis of SV-HCECs infected with comparable doses of all four rickettsial strains revealed a consistent modulation of several genes important to endothelial cell homeostasis. Most of the genes we saw modulated are related to control of apoptosis, consistent with rickettsiae-mediated inhibition of apoptosis. However, we noticed down-regulation of several genes with important roles in inflammatory responses. Specifically we saw consistent down-regulation of RhoB, THBS-1 and the TRAIL decoy receptor, TNFSFR10c, after 24 hours of infection. Earlier time points revealed that there was some degree of increased expression of these genes after 4 hours of infection with *R. australis* or *R. rickettsii* (Iowa); however the long-term effect was to down-regulate their expression.

Ras homolog gene family, member B (RhoB), plays an important role in responses to pro-inflammatory stimuli, especially TNF-α, IFN-γ and IL-1β. Specifically, RhoB has been linked to the ability of cells to respond to cytokine stimulation and induce transcription of iNOS (Delarue *et al.*, 2001). Overexpression of RhoB was shown to increase iNOS promoter activity in response to cytokine stimulation, whereas RhoA suppressed this effect. This has tremendous implications for rickettsial pathogenesis and suggests that rickettsiae-infected endothelial cells may actually have a decreased ability to respond to cytokine stimulation relative to their uninfected counterparts.

THBS-1 was first identified to be an anti-angiogenic factor important in regulating the interaction of cells with the cellular matrix. However, work by Isenberg *et al.* demonstrated a role for THBS-1 in VEGF-mediated NO production and the subsequent downstream signaling events (Isenberg *et al.*, 2005). It appears that THBS-1 can block the activation of sGC preventing the production of cGMP and the responses associated with it. Evidence also suggests that THBS-1 can be regulated by the levels of NO present in a cell through a negative feedback mechanism. The importance of THBS-1 as a regulator of cell-cell adherence is well documented and should be the focus of future work on rickettsial pathogenesis.

TNF Superfamily Receptor 10c, TNFSFR10c, is a cell-bound receptor for TRAIL; however, it lacks an intracellular death domain. Therefore, TNFSFR10c acts as a decoy receptor for TRAIL preventing TRAIL-mediated apoptosis. A lack of decoy receptor expression by rickettsiae-infected endothelial cells may make them more prone to TRAIL-mediated apoptosis. Very little evidence exists which links TRAIL to infectious disease pathogenesis, but TRAIL has been mostly described as a method for clearance of cancer cells. Our observations may be a side-effect of working with immortalized cell lines and should be taken into consideration for any future work on the matter.

Our analysis also revealed the modulation of angiotensin-I converting enzyme (ACE) after rickettsial infection. Unlike other genes, ACE was primarily upregulated in response to infection. ACE is responsible for the conversion of angiotensin I into angiotensin II, a key component of the renin-angiotensin system. Binding of angiotensin II to the type 1 receptor has been associated with increased microvascular permeability in rat mesenteric venules (Newton *et al.*, 2005). Additional work has demonstrated that this is mediated via cGMP activity (Chong and Victorino, 2006). Based on these data we feel that angiotensin II should be explored as a possible contributor to vascular dysfunction during rickettsial infection.

Finally, the observation of increased TFPI expression within hours of infection with *R. australis* or *R. rickettsii* was interesting given the fact that rickettsial infection leads to tissue factor (TF) expression by human endothelial cells. This transient increase in TFPI expression ultimately may not affect the production of TF, especially given the return to normal or below normal levels of TFPI.

Based on the work presented here, we feel it is evident that endothelial cell responses to infection can have a dramatic impact on microvascular function. Chemokine and cytokine secretion by infected endothelial cells may potentially act in a paracrine manner on neighboring endothelial cells leading to disassembly of adherens

junctions and increased microvascular permeability. It would be wise for these interactions to be explored in a model subject to shear-stress and fluid movement which would more accurately represent the intravascular environment. Additionally we must consider changes in host cell gene expression that alter the ability of endothelial cells to respond to external stimuli. Down-regulation of genes such as RhoB may ultimately benefit the rickettsiae by dampening the iNOS response and allowing the rickettsiae to grow better, even in activated endothelial cells. Clearly, the intracellular environment is a complex interwoven network of responses and will need significantly more attention and work to fully understand the implication of rickettsial invasion, and how this relates to the overall health of the host.

Chapter 5: Characterization of Microvascular Dysfunction during Acute Rickettsioses *In Vivo*

INTRODUCTION

Endothelial Target Infections

Very few pathogens target the microvascular endothelium as the primary site of infection. Among those that do are bacterial pathogens from the genera *Bartonella, Orientia,* and *Rickettsia.* Several viral agents target the microvasculature including Junin virus and other hemorrhagic fever viruses, human herpesvirus 8, hantaviruses and Nipah virus, an emerging pathogen that causes encephalitis. A common theme of many of these infections is altered endothelial cell function resulting in leakage of fluid and proteins from the vasculature, and edema. Unfortunately, an insufficient amount of information is available addressing the mechanisms of increased microvascular permeability observed in these infections, which has limited our understanding and ability to treat them. This is mostly due to a lack of animal models that closely mimic the human conditions and lack of interaction between leading scientists in the infectious disease and endothelial pathobiology arenas.

Three potential mechanisms of increased microvascular permeability during infections of the endothelium are 1) direct cytotoxicity to the endothelium by an invading pathogen, 2) leukocyte-mediated denudation of the endothelium or 3) elevated serum levels of pro-inflammatory cytokines such as TNF-α and IL-1β. However, it is possible for a disease to be characterized by the presence of all three mechanisms, especially when an organism is highly cytotoxic and does not possess mechanisms to block host immune responses.

Host Inflammatory Responses and Vascular Dysfunction

Inflammation is a complex, highly evolved response to tissue damage within the host. Damage can be caused by many effecters including infection, trauma or other factors leading to a predictable pattern of events aimed at minimizing additional damage. The key events experienced during the inflammatory process are endothelial cell activation leading to the increased recruitment of leukocytes to sites of damage accompanied by activation of the coagulation system in an attempt to block off the damaged region from the rest of the body and contain any potentially harmful effects of tissue damage. Infiltrating leukocytes then engage in a complex system of cross-talk with endothelial cells in which both cell types can release cytokines that regulate the intensity and duration of the inflammatory process (Levi *et al.*, 2002).

In order for leukocytes to access specific regions of the body, they must first traverse the endothelial layer. Through a series of events involving adhesion to and rolling along the endothelium, leukocytes must pass between tightly opposed endothelial cells potentially exposing the underlying basement membrane to coagulation factors present in the blood circulation. Typically this process is highly regulated, and no significant detrimental effect is observed in response to diapedesis of the infiltrating leukocytes (Muller, 2003). However, in cases of extreme tissue damage or severe infection, the amount of infiltration occurring can have a significant impact on the health and well being of the host.

$CD8^+$ T-Lymphocytes as mediators of microvascular permeability

$CD8^+$ T-lymphocytes are a critical component to the effective clearance of intracellular pathogens such as rickettsiae. In fact, CTL action is essential for survival during rickettsial infection and is partly dependent on perforin-mediated clearance of infected endothelial cells (Walker *et al.*, 2001). However, current evidence demonstrates

that $CD8^+$ T-lymphocytes can negatively impact the microvasculature leading to increased vascular permeability (Suidan *et al.*, 2006). Rapid expansion of $CD8^+$ T-lymphocytes following dengue virus infection in children has been associated with high levels of soluble IL-2 receptor, soluble CD4 and CD8, IL-2 and IFN-γ and correlates with the appearance of widespread tissue damage and hemorrhage (Kurane *et al.*, 1991;Kurane *et al.*, 1995). Additionally, negative clinical outcome during DHF is associated with expression of a specific class I HLA type, HLA-A*0207 in ethnic Thais (Stephens *et al.*, 2002;Zivna *et al.*, 2002). There is also a correlation between HLA type and susceptibility to hantavirus pulmonary syndrome, a disease characterized by a rapid and severe increase in vascular permeability. Clearly there is strong evidence linking $CD8^+$ T-lymphocytes to conditions involving increased vascular permeability.

A pathogenic role for $CD8^+$ T-lymphocytes has been demonstrated experimentally in several model systems. Circulatory shock during *Plasmodium berghei* infection is reversed with the depletion of $CD8^+$ T-lymphocytes. Likewise, a Theiler's murine encephalomyelitis virus (TMEV) model of MS has demonstrated peptide-specific responses in the CNS resulting in increased vascular permeability associated with a fatal outcome (Johnson *et al.*, 2005). Given this information it seems quite feasible that $CD8^+$ T-lymphocytes may play a role in immune-mediated pathology during acute rickettsial infection, despite the fact that these cells are critical to anti-rickettsial immunity.

Polymorphonuclear leukocytes (PMNs) as mediators of microvascular permeability

PMNs are another important component of the inflammatory response and are often the first leukocytes to respond to injury or infection. Their role in rickettsial pathogenesis has not been studied extensively; however, we do know that *R. rickettsii* infection of endothelial cells results increased E-selectin-dependent adhesion of neutrophils (Sporn *et al.*, 1993). In one study the binding of neutrophils to endothelial

cells resulted in a $[Ca^{2+}]_i$ –dependent reorganization of intracellular junctions which aided in the transmigration of these cells across the vascular barrier (Gautam *et al.*, 1998;Huang *et al.*, 1993). This process is a normal physiological response that does not normally result in any significant increase in vascular permeability due to the close opposition (<150 Å) of cell membranes. However, under conditions of acute inflammation this process was shown to result in the loss of close associations between cells, diminished transendothelial electrical resistance, and increased permeability to albumin (Huang *et al.*, 1988). These observations provide a hypothetical mechanism for the changes that occur during acute inflammation that lead to tissue edema. Similar responses have been observed for natural killer (NK) cells added to IL-1 or TNF-α activated HUVECs. This interaction occurs via β_2-integrin-ICAM-1 interaction (Pfau *et al.*, 1995).

Soluble mediators of vascular permeability during inflammation

During vascular inflammatory processes endothelial cells and leukocytes communicate by secreting and responding to cytokines and chemokines. This often results in increased expression of adhesion molecules by endothelial cells followed by increased production, activation and recruitment of leukocytes to sites of injury (Levi *et al.*, 2002). Coincidentally, during severe infections or trauma the intensity of the cytokine response is so great that this ultimately results in systemic endothelial dysfunction associated with increased microvascular permeability leading to edema.

Monocyte chemoattractant protein-1 (MCP-1 or CCL-2) is a member of the C-C chemokine family and plays a vital role in recruiting mononuclear cells into sites of infection and inflammation. Recent work demonstrates that MCP-1 is also quite capable at altering the expression of tight junction-associated proteins in brain microvascular endothelial cells *in vitro* leading to increased permeability of the blood brain-barrier (BBB) in mice receiving an intravenous injection of MCP-1 (Song and Pachter,

2004;Stamatovic *et al.*, 2005). This change in tight junction structure appears to be related to Rho and Rho kinase activity (Stamatovic *et al.*, 2003). Recently MCP-1 was shown to be upregulated in cases of DHF and human monocytes infected with dengue virus release MCP-1 at levels high enough to alter the permeability of human endothelial cell monolayers (Lee *et al.*, 2006). This is interesting given the available data that demonstrates increased expression of MCP-1 by *R. rickettsii*-infected endothelial cells, possibly implicating MCP-1 as a mediator of increased microvascular permeability during acute rickettsioses (Clifton *et al.*, 2005).

Other proinflammatory cytokines also have the ability to cause increased microvascular permeability. As demonstrated in Chapter 1, TNF-α and IL-1β have a potent effect on endothelial cells leading to reorganization of interendothelial adherens junctions and increased endothelial permeability. The primary beneficial effect of these two cytokines to the best of our knowledge is the increased expression of iNOS leading to NO production and killing of intracellular rickettsiae. While it is not clear whether NO is the primary mediator of TNF-α/IL-1β-induced permeability as has been suggested in other model systems, we can conclude that this response does in fact affect the ability of the endothelium to function as an efficient barrier. However what is not clear is whether these two cytokines act locally or systemically to stop the spread of rickettsiae, and how this relates to the changes in microvascular permeability experienced during rickettsial infection. Knowing the source of these cytokines would give us a far better understanding of the mechanisms in action. One potential source is cells of the monocyte/macrophage lineage. In fact, LPS-stimulated monocytes have been shown to release high amounts of TNF-α, IL-6 and IL-10, which when added to cultures of human brain endothelial cells result in the formation of intercellular gaps and enhanced migration of monocytes (Persidsky *et al.*, 1997). Additionally it has been demonstrated in other models that IL-1β and TNF-α are responsible for neutrophil-mediated microvascular permeability *in vivo* (Yi and Ulich, 1992).

Given this information it seems reasonable for us to conclude that leukocyte transmigration across microvascular barriers coupled with cytokine release by the invading cells can have a tremendous impact on the barrier properties of the microvasculature. In fact, recent work by Souza *et al.* has shown a direct correlation between disease severity and the level of TNF-α and IL-1β mRNA present in skin biopsies of patients with Mediterranean spotted fever (Sousa *et al.,* 2007). Characterization of the local environment around sites of rickettsial invasion should give us a better understanding of the mechanisms of increased microvascular permeability during rickettsioses.

Microvascular Permeability in Rickettsioses

To date there are very few data concerning the events leading up to vascular damage and increased microvascular permeability during severe acute rickettsial infection. There are even fewer reports actually documenting vascular dysfunction at the microvascular level either in human infection or in animal models of disease. Valbuena *et al.* demonstrated increased vessel permeability to Evans' blue dye in the retina of *R. conorii*-infected mice (Valbuena *et al.*, 2002). However, it is not clear whether the sites of leakage are also sites of infection and/or inflammation. Retinal vessel permeability was also demonstrated during *R. rickettsii*-infection of dogs (Davidson *et al.*, 1990), although these studies were also limited by their inability to demonstrate increased permeability associated with sites of infection. The association between increased microvascular permeability and sites of infection was addressed by the observation of colloidal gold leakage in guinea pig cremaster muscles although there were no rickettsiae found in the endothelial cells of inflamed vessels (Moe *et al.*, 1976). Unfortunately, these observations do not address changes associated with severe clinical disease, specifically increased microvascular permeability in the lungs and brain.

Earlier work by Liu et al. did address this problem in *R. rickettsii*-infected rhesus monkeys (Liu *et al.*, 1978). It was determined that the tissue water content increased in the medulla oblongata and the lungs of infected animals, although the authors concluded that increased capillary permeability was unlikely the cause of this shift in fluid distribution. Instead the authors suggested that intracellular overhydration of the medulla oblongata led to cardiovascular and respiratory depression eventually culminating in shock. However, analysis of human autopsy material demonstrates consistent interstitial pneumonitis characterized by mononuclear inflammatory cells in the pulmonary interstitium, alveolar edema (in some cases admixed with alveolar hemorrhage), septal congestion, and in severe cases, diffuse alveolar damage (Walker *et al.*, 1980). Taken together it is evident that vascular dysfunction is most likely a consequence of severe RMSF; however, the mechanisms leading up to it are still not clear.

The present study was undertaken to characterize the inflammatory process during rickettsial infection in mice as it relates to changes in vascular permeability. Specifically, we have addressed the localized response to sites of rickettsial infection by characterizing the degree and timing of inflammatory cell infiltration into infected regions. Additionally, we have attempted to demonstrate localized cytokines acting to upregulate iNOS expression at sites of infection and/or permeability. We have also addressed the role of specific vasoactive cytokines and chemokines during severe infection. It is anticipated that the work presented herein will give us a better understanding of the histopathological findings in rickettsial infection. Additionally, we hope that the information gained here may identify potential therapeutic targets for the treatment of severe, late-stage rickettsiosis.

RESULTS

Rickettsia conorii Infection of C3H/HeN Mice Leads to a Vasculitis of the Lungs and Brain Associated with Increased Microvascular Permeability

Our initial goal was to characterize the inflammatory response to endothelial-target rickettsial infection with respect to time and intensity. Intravenous administration of *R. conorii* to 6-8 week old male C3H/HeN mice led to a widespread dissemination of rickettsiae throughout the brains and lungs. Rickettsial organisms were detectable after one day of infection, although at very low levels. The amount of organisms increased after three days of infection, but there was a marked increase in the number of infected vessels by the fifth day of infection. Rickettsiae were present in a large number of vessels throughout the brain (Figure 5.1A) and were contained within the endothelial lining of the vasculature (Figure 5.1B). Note that at day five of infection, sites of invasion were not associated with any appreciable amount of inflammatory cells despite the high number of invading organisms. At this time point mice were demonstrating classic signs of illness including ruffled fur, lethargy, hunched posture and an apparent sensitivity to light.

Mice receiving a dose of 3 LD_{50} of *R. conorii* exhibited a course of illness of approximately six days. Signs of illness arose on day five and progressed throughout the night until the mice were moribund beginning on day six. Severely ill animals were sacrificed at day six, and the tissues were processed and stained for histological analysis. A large number of vessels in the brain showed perivascular lymphohistiocytic infiltration associated with sites of rickettsial endothelial infection (Figure 5.2A-D). Occasionally, we found typhus nodules composed primarily of lymphocytes, but it was not clear if these were associated with a blood vessel. The lungs showed interstitial pneumonitis that consisted of lymphohistiocytic infiltration of the pulmonary interstitium (Figure 5.2 E-F). Rarely did we find alveolar edema or diffuse alveolar damage despite the presence of large numbers of rickettsiae in the pulmonary parenchyma.

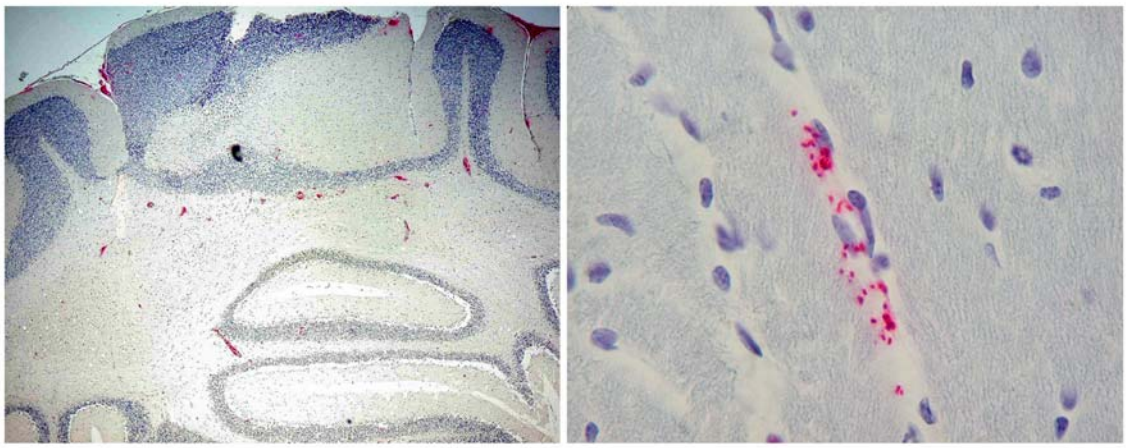


Figure 5.1: *R. conorii* infects the microvascular endothelium of the brain in C3H/HeN mice. Mice were injected i.v. with $1x10^5$ PFU of *R. conorii,* and tissues were collected on days 1, 3, and 5 after infection. The tissues were processed and stained using rabbit polyclonal antiserum to spotted fever-group rickettsiae and counterstained with hematoxylin. A) Red product demonstrates widespread invasion throughout the vasculature of the brain, 400X magnification. B) Individual rickettsiae are visible in the endothelial lining of a capillary vessel in the brain, 1000X.

We next wished to demonstrate that sites of inflammation were associated with loss of the endothelial barrier resulting in increased microvascular permeability. Immunohistochemical staining for endogenous IgG revealed the presence of increased staining in the brain parenchyma around regions with presence of inflammatory cells in perfused mice (Figure 5.3A). Conversely, vessels of uninfected mice displayed a consistent intravascular staining pattern indicating an intact vascular barrier (Figure 5.3B). The absence of demonstrable leakage in the lungs probably reflects compensatory mechanisms in the lungs such as markedly increased lymphatic drainage that would prevent accumulation of fluid in the alveolar spaces. (Data not shown).

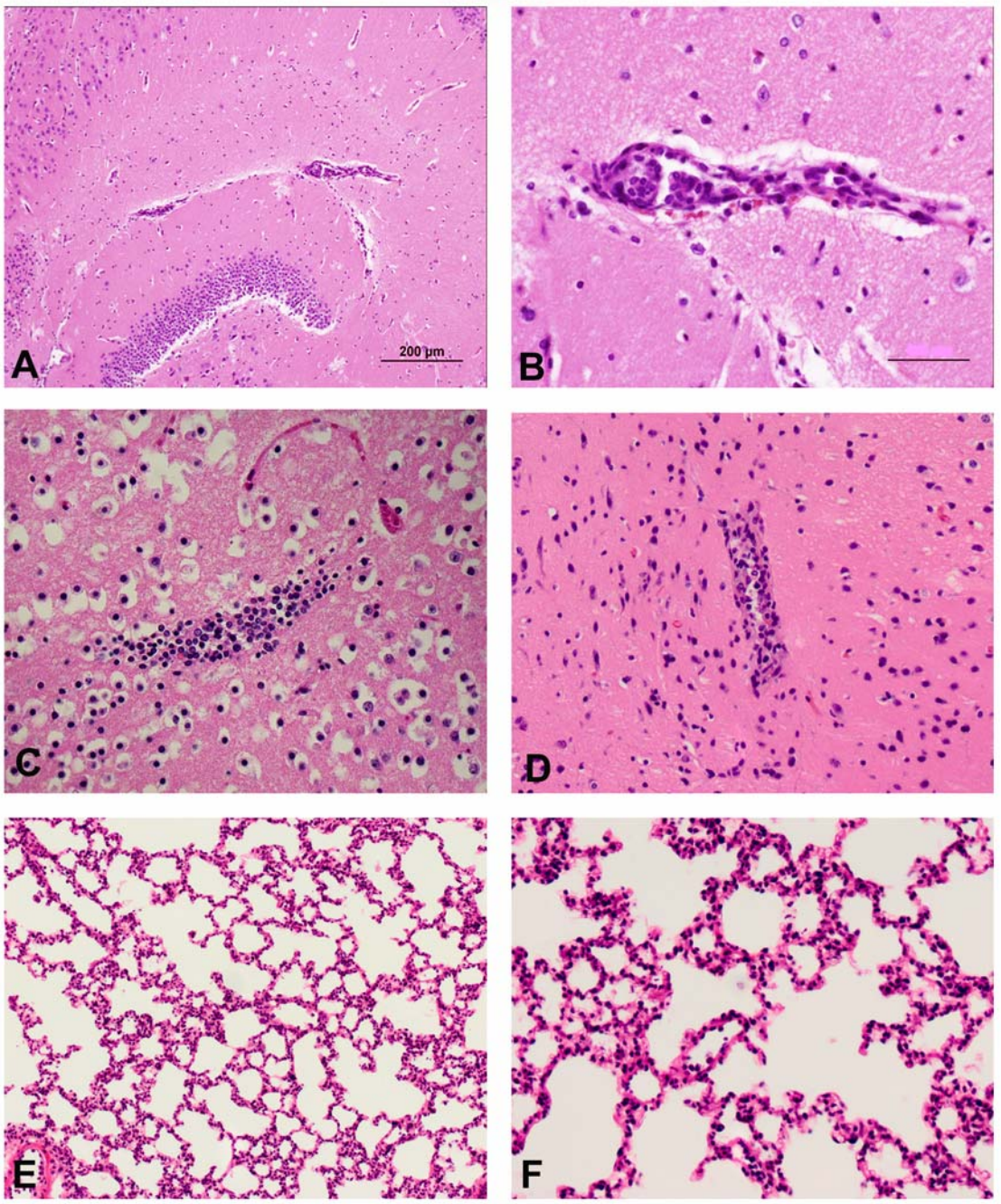


Figure 5.2: Vascular inflammation associated with immune cell infiltration in the brain and lungs of *R. conorii*-infected mice. Mice receiving a dose of 3 LD_{50} were sacrificed after six days of infection, and the tissues were processed for histology and staining with hematoxylin and eosin. Brain tissue (A-D) contained sites of vascular inflammation and lungs (E,F) demonstrated interstitial pneumonitis. (A, E 10x; B,C 40x; D,F 20x)

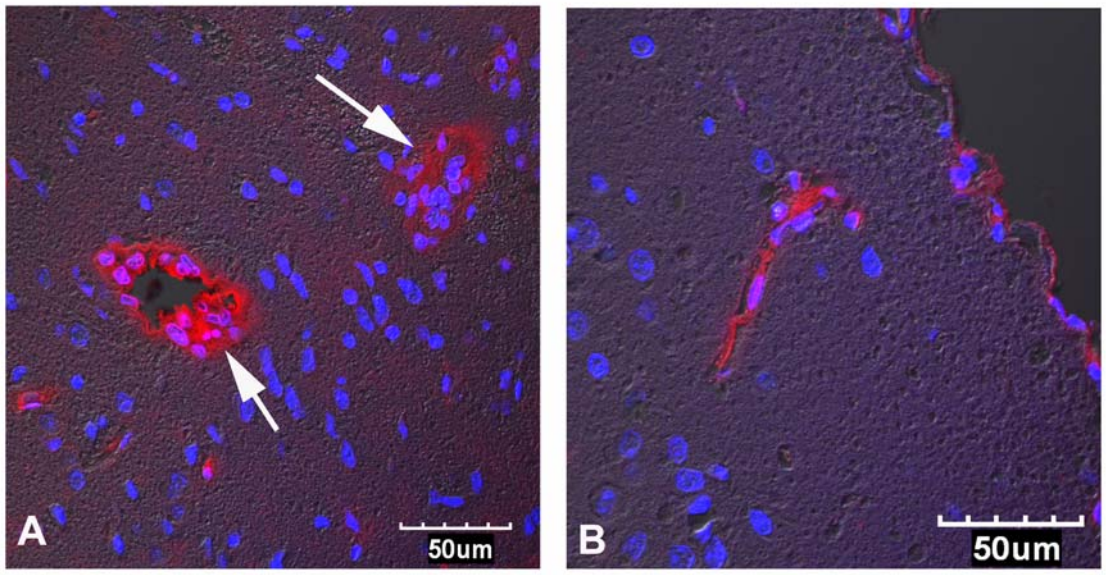


Figure 5.3: Increased extravascular IgG staining at sites of vascular inflammation. Prior to sacrifice mice were perfused with lactated Ringer's solution to remove the intravascular components, and then the brain was removed and fixed in zinc fixative. Paraffin-embedded tissues were stained for mouse IgG. Leakage of IgG (red) was associated with sites of inflammation (A, arrows) while uninfected vessels retained IgG intravascularly (B). Nuclei were stained with DAPI (blue).

Rickettsial Infection is Associated with Interstitial Edema of the Lungs of Infected Mice

Our next goal was to determine whether these changes occurring in these mice resulted in a significant amount of interstitial fluid accumulation in vital and target organs, namely the brain and lungs. To accomplish this goal, we calculated the water content in the brain and lungs from infected and uninfected mice. C3H/HeN mice infected with varying doses of *R. conorii* (Figure 5.4A-C) demonstrated increased percent water content in the lungs, but not the brain, at the peak of the disease. Likewise, C57BL/6 mice infected with 1 LD_{50} of *R. australis* showed a significant increase in tissue water content at day seven of infection. This equated to approximately a 3.3% increase

in lung water content at this time-point. Additionally we calculated that in this set of mice this equals a total additional fluid load of 40.02 µliters. These observations correlate well with the signs of illness observed in the mice at this time-point, including ruffled fur, lethargy, and heavy breathing patterns. Due to the absence of any histological observation of significant alveolar edema, we believe that this represents interstitial fluid accumulation.

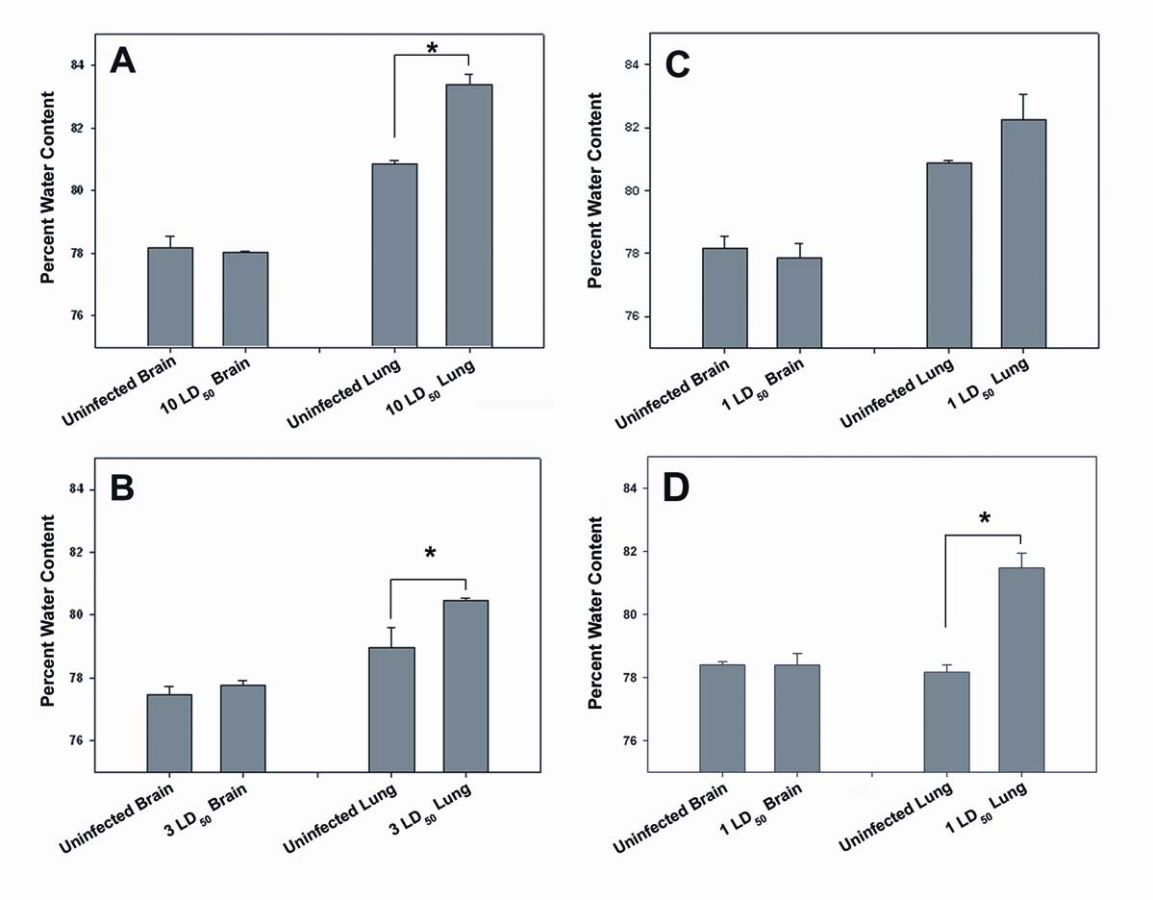


Figure 5.4: Rickettsiae-infected mice experience significantly higher pulmonary fluid accumulation. C3H/HeN mice infected with 10 LD_{50} (A), 3 LD_{50} (B) and 1 LD_{50} (C) of *R. conorii* experienced significant additional tissue water content in the lungs at days 5, 6, and 7, respectively. C57BL/6 mice infected with 1 LD_{50} of *R. australis* (D) experienced a similar effect on day 7. The tissue water content of the brain did not change for any group (*=$P<0.05$). (n=3).

Inducible Nitric Oxide Synthase Associates with Regions of Rickettsial Vasculitis and is Essential for Survival

We next wished to determine whether the NO response to rickettsial infection is a localized inflammatory response, or whether it is dependent on a systemic activation of iNOS by cytokine stimulation. Fluorescent staining of brain tissue from *R. conorii*-infected mice revealed the presence of significant iNOS staining at sites of vascular inflammation associated with the presence of rickettsial organisms (Figure 5.5). iNOS reactivity was present throughout the areas with inflammatory infiltrates but did not appear to localize to anyone particular cell type as staining was associated with multiple cells in the vicinity. Occasionally, we found foci of rickettsiae not associated with vascular inflammation. In this case the rickettsial staining did not colocalize with iNOS staining, suggesting an important role for the inflammatory cells in inducing iNOS activity (Figure 5.5B, arrowhead). Normal brain tissue from uninfected mice revealed occasional iNOS staining of endothelial cells albeit at a much lower level (Figure 5.5A, asterisk).

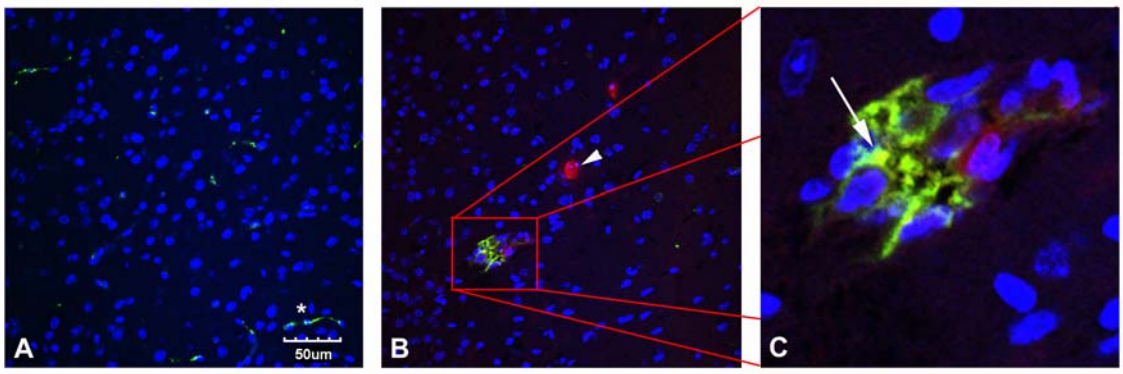


Figure 5.5: Increased iNOS activity at sites of rickettsiae-induced vasculitis. Brains from *R. conorii*-infected C3H/HeN mice exhibit regions of increased cellularity indicative of a cellular inflammatory response to sites of rickettsial infection (red) (B). These regions (C) demonstrate a higher expression of iNOS (green) compared to normal vessels (*) in uninfected mice (A) or infected foci not associated with inflammatory infiltrates. (arrowhead, B). Nuclei are stained with DAPI (blue).

Given the available data indicating that NO can negatively affect the function of the microvascular barrier, we tested whether the lack of iNOS activity would result in a decreased disease severity. iNOS$^{-/-}$ mice infected with *R. australis* demonstrated a dramatic increase in the susceptibility to infection compared to wild type mice (Figure 5.6). The genetically deficient mice died from rickettsial infection between days six and seven whereas there were no fatalities in wild type mice. Both groups demonstrated similar courses of disease with signs of infection first occurring on day four post-infection. Wild type mice recovered by days seven and eight post-infection.

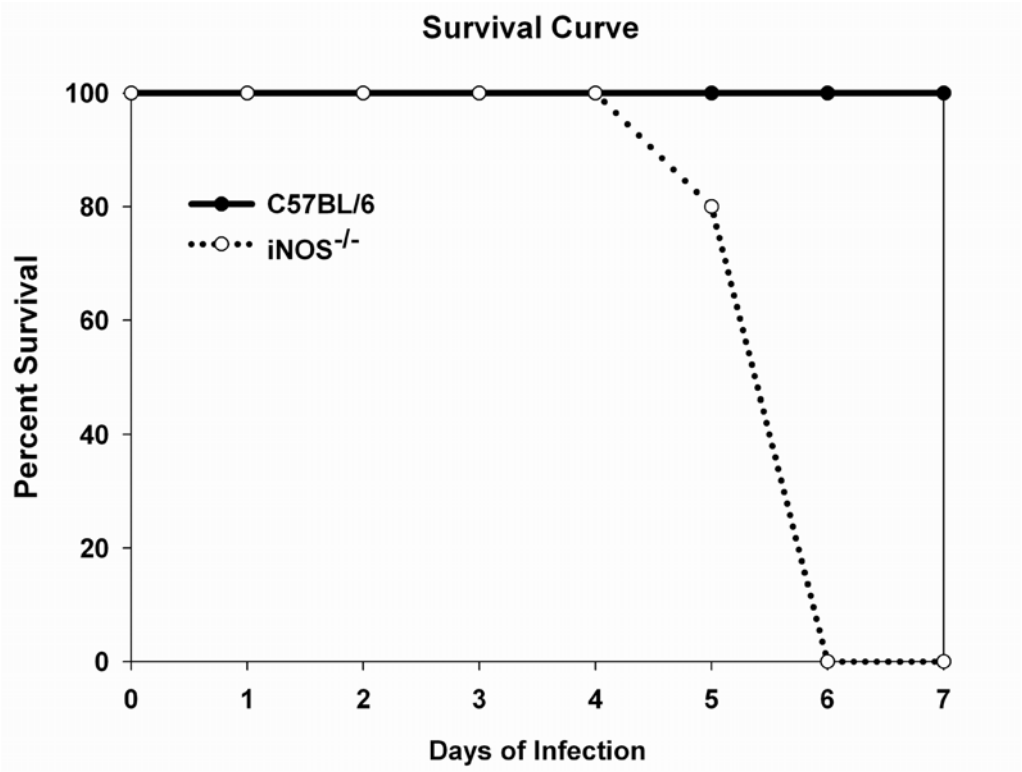
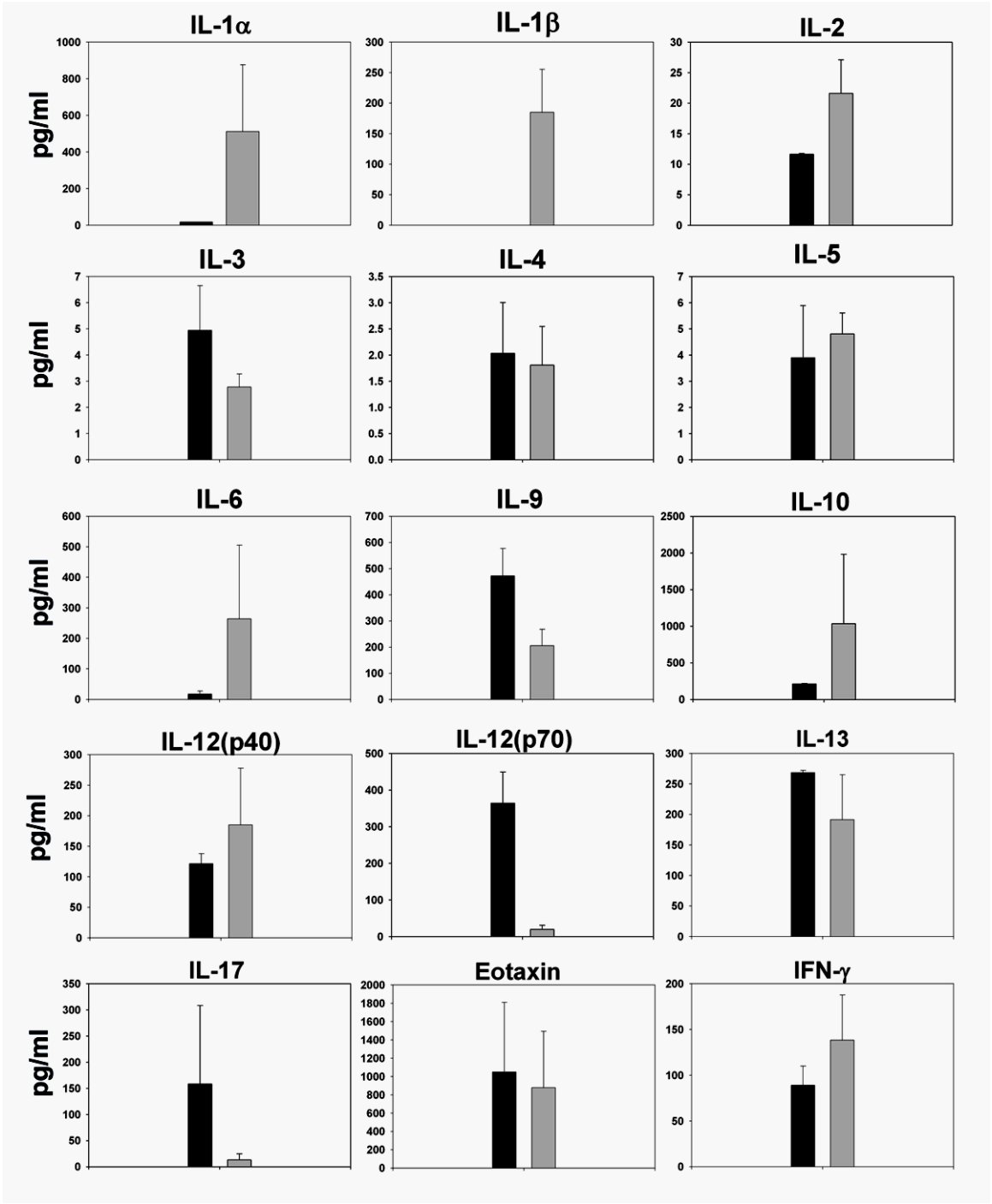


Figure 5.6: iNOS-deficient mice are more susceptible to fatal rickettsial infection. Wild type and iNOS$^{-/-}$ C57BL/6 mice were infected i.v. with 3.3x10^5 PFU of *R. australis*. All iNOS-deficient mice died between days 5 and 6 whereas all wild type mice survived the infection. (n=5)

Acutely Ill Mice and Humans Have High Levels of Circulating Cytokines Related to Vascular Permeability

Due to the wealth of evidence linking soluble cytokines to the regulation of microvascular permeability, we next wished to characterize the cytokine response to rickettsial infection in a mouse model. C3H/HeN mice infected with 3 LD_{50} of *R. conorii* possessed significantly higher levels of certain pro-inflammatory cytokines at day six after infection. The cytokines with overall increased serum levels in infected mice compared with uninfected mice include IL-1α (17.24 pg/ml vs 512.36 pg/ml), IL-1β (n.d. vs 184.99 pg/ml), IL-2 (11.65 pg/ml vs 21.6 pg/ml), IL-6 (17.4 pg/ml vs 263.7 pg/ml), IL-10 (213.62 pg/ml vs 1034.63 pg/ml), KC (IL-8) (42.82 pg/ml vs 219.65 pg/ml), and MCP-1 (191.92 pg/ml vs 1141.92 pg/ml) (Figure 5.6). We also documented decreased serum levels of the cytokines IL-9 (472.45 pg/ml vs 205.14 pg/ml), IL-12(p70) (364.115 pg/ml vs 19.79 pg/ml), and IL-17 (158.49 pg/ml vs 13.47 pg/ml). Cytokines which we could not detect changes in serum included IL-3, IL-4, IL-5, IL-12(p40), IL-13, eotaxin, IFN-γ, RANTES, and TNF-α.

We wished to compare mouse cytokine levels to those in human patients infected with spotted fever group rickettsiae. Therefore, we acquired human serum samples from a series of South American patients infected with *R. rickettsii*, of whom two were fatal cases. These were compared against acute and convalescent sera from European patients infected with *R. conorii* (Figure 5.7). Both case fatalities from South America had overall higher levels of the cytokines IL-6, IL-10, G-CSF, IFN-γ, and MCP-1 compared to any other group. At least one of these patients also had much higher levels of IL-1rα compared to the other groups. RANTES was elevated in the non-fatal *R. rickettsii* cases and the convalescent *R. conorii* infections. TNF-α was elevated in both fatal cases; however, one of the non-fatal cases also had very high levels of this cytokine. Finally, VEGF was expressed at much higher levels during *R. conorii* infection.


IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-9
IL-10
IL-12(p40)
IL-12(p70)
IL-13
IL-17
Eotaxin
IFN-γ
pg/ml

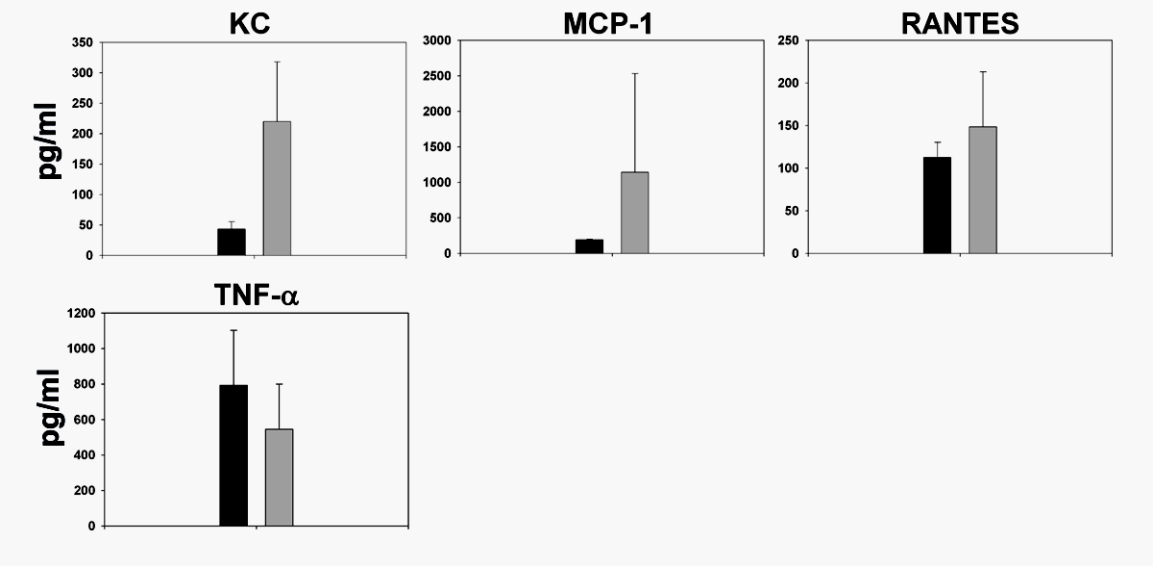


Figure 5.7: *R. conorii*-infected mice exhibit altered serum levels of pro-inflammatory cytokines 6 days after infection. Whole blood was collected via cardiac puncture from uninfected mice (dark bars) or mice receiving a dose of 3 LD_{50} of *R. conorii* (light bars) six days after infection. The serum was harvested and analyzed using multiplexed bead arrays. Data are expressed as mean ± standard deviation.

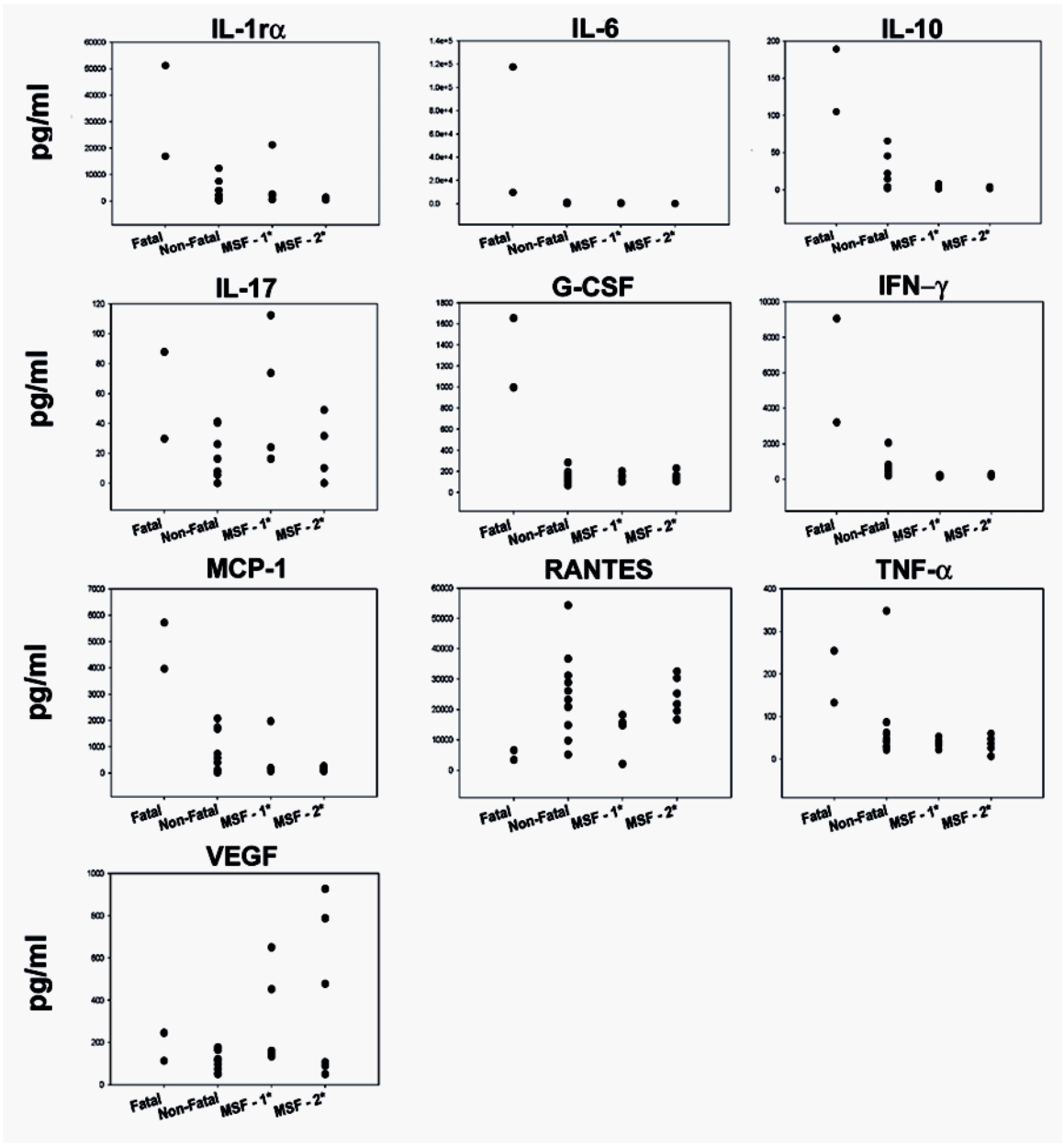


Figure 5.8: Bio-plex® analysis of human serum samples from fatal and non-fatal *R. rickettsii* infection and acute and convalescent *R. conorii* infection. Human serum samples were analyzed using Bio-Plex® bead-based arrays to determine the levels of specific pro-inflammatory cytokines. Fatal (*R. rickettsii*, n=2), Non-Fatal (*R. rickettsii*, n=5), MSF-1* (*R. conorii*, acute, n=4), MSF-2* (*R. conorii*, convalescent, n=6). Samples were run in duplicate and data is expressed as the mean value in pg/ml.

Rickettsial Infection is Associated with Increased MCP-1 Expression Resulting in Monocyte Recruitment to Sites of Infection

Given the available data presented both here and elsewhere regarding the relationship between rickettsial infection and MCP-1 production, we determined whether MCP-1 localized to sites of rickettsial infection as well as correlated this inflammation with the cellular inflammatory response. C3H/HeN mice receiving a dose of 1 LD_{50} of *R. conorii* showed a correlation between the intensity of cellular infiltration to sites of infection and MCP-1 expression. After seven days of infection these mice showed very intense staining for MCP-1 around regions of inflammation associated with rickettsial antigen in brain microvessels. While the staining primarily localized to the inflammatory foci, we could also see staining in the vasculature within the vicinity of the inflamed vessel. The staining appeared to associate with cell membranes as well as co-localizing to regions of rickettsial antigen indicating an infected vessel (Figure 5.8B). Uninfected vessels, on the other hand, showed very low levels of MCP-1 staining (Figure 5.8A).

In the lungs, MCP-1 did not associate with sites of rickettsial antigen but instead was distributed throughout the lung (Figure 5.8D). The staining pattern was more focal indicating expression by specific cell types although we could not determine specifically which cells these were. Uninfected lung tissue contained very little MCP-1 staining (Figure 5.8C).

Next we decided to determine the role of MCP-1 in rickettsiae-induced vascular inflammation using mice genetically deficient for MCP-1. Histologically, the most striking differences between wild type and deficient mice were visible in the liver. Wild type mice developed lymphohistiocytic infiltrates in the liver parenchyma beginning two days after infection (Figure 5.10A). These lesions progressed in both size and number by six days after infection (Figure 5.10C) and were characterized by the presence of mononuclear inflammatory cells and scattered PMN's (Figure 5.10E). MCP-1-knockout mice, on the other hand, did not exhibit these lesions after two days of infection (Figure

5.10B). Inflammatory foci began to appear by day six in MCP-1 kncokout mice (Figure 5.10D); however, they were much less well defined, were smaller, and were much less prevalent throughout the liver (Figure 5.10F). Morphologically, the lesions were composed primarily of PMNs although we occasionally observed macrophage-like cells.

Immunofluorescent staining of the lungs revealed F4/80-positive cells in the lungs of *R. australis*-infected wild-type mice as well as distinct MCP-1 expression that did not colocalize with the F4/80-positive cells (Figure 5.11A). MCP-1-knockout mice had far fewer F4/80-positive cells as well as no MCP-1 staining, as was to be expected (Figure 5.11B). Because the levels of MCP-1 expression were not high in other organs, we examined the spleen as a possible source of circulating cytokine. Macrophages were present in the red pulp of the spleen, and no differences were noted in MCP-$1^{-/-}$ mice (Figure 5.11C,D). However, we did observe significant MCP-1 expression in and throughout regions of high macrophage density in wild-type mice.

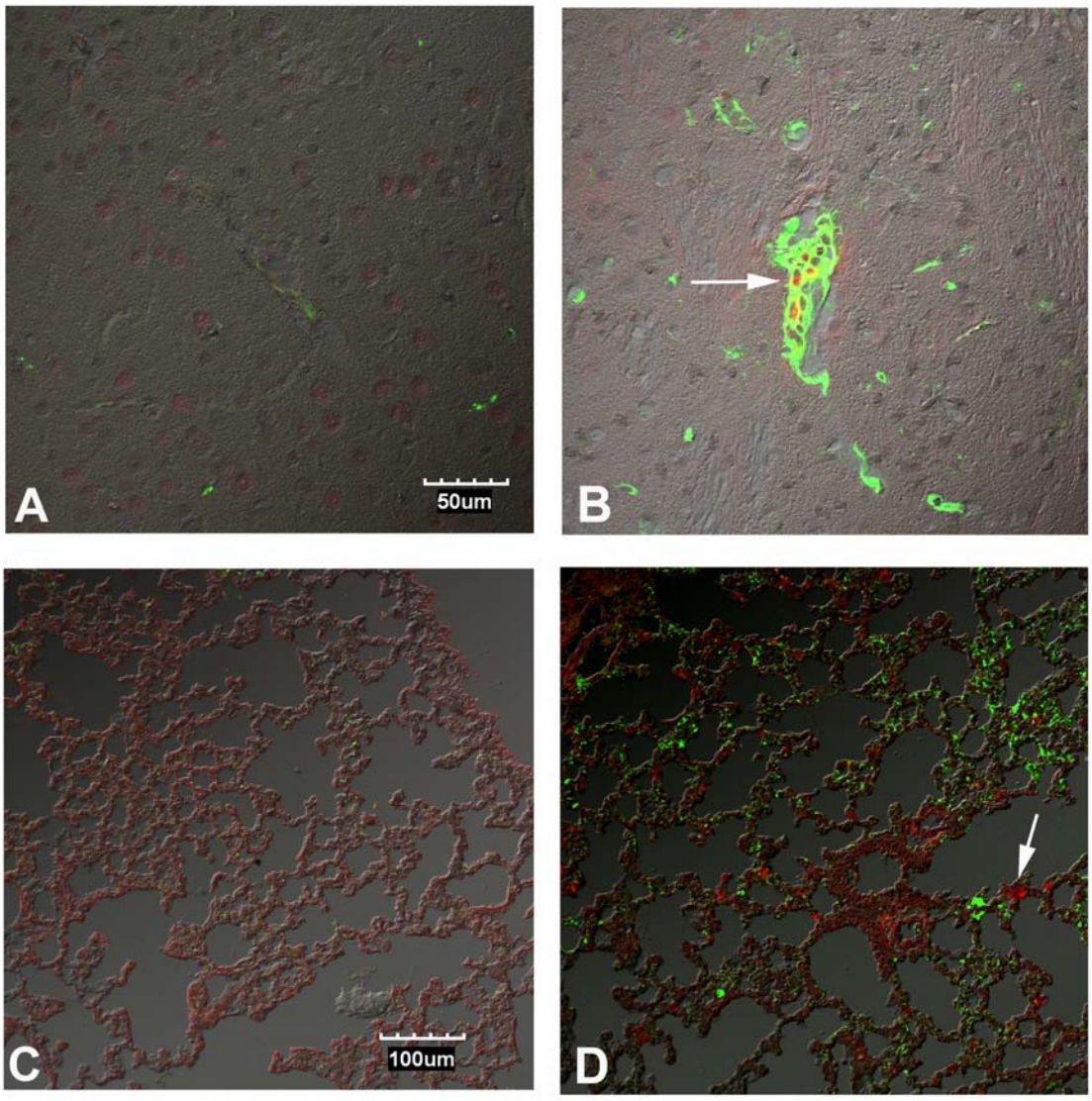


Figure 5.9: Rickettsial vasculitis is associated with increased MCP-1 expression in *R. conorii*-infected mice. Brain (A,B) and lung (C,D) from *R. conorii*-infected C3H/HeN mice were harvested after 7 days of infection, fixed and embedded in paraffin. 4 μm sections were stained with a monoclonal antibody to MCP-1 and polyclonal antiserum to SFG rickettsiae. MCP-1 (green) localized in the brain to sites of rickettsial vasculitis associated with rickettsial antigen (red). In the lung MCP-1 was expressed focally by specific cells not consistently associated with rickettsial antigen.

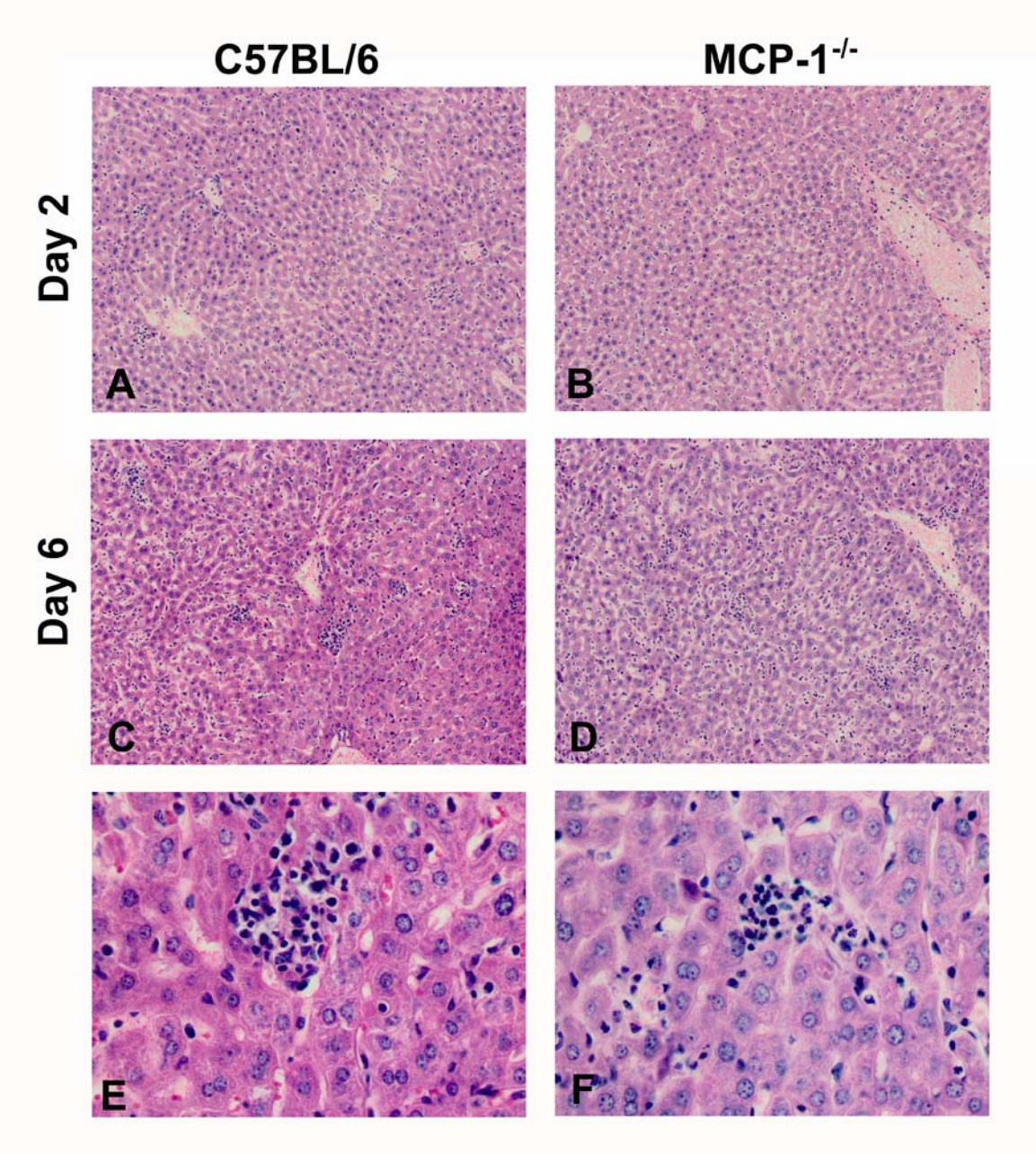


Figure 5.10: Liver pathology in wild type and MCP-1-knockout mice. Wild-type C57BL/6 mice infected with *R. australis* developed lymphohistiocytic infiltrates throughout the liver beginning on day 2 and progressing to day 6 (A,C,E). MCP-1-knockout mice did not develop these lesions at two days after infection. However, they did appear by day six, although morphologically they were different when compared to lesions seen in wild type mice (B,D,F). (A-D 10X; E,F 40x).

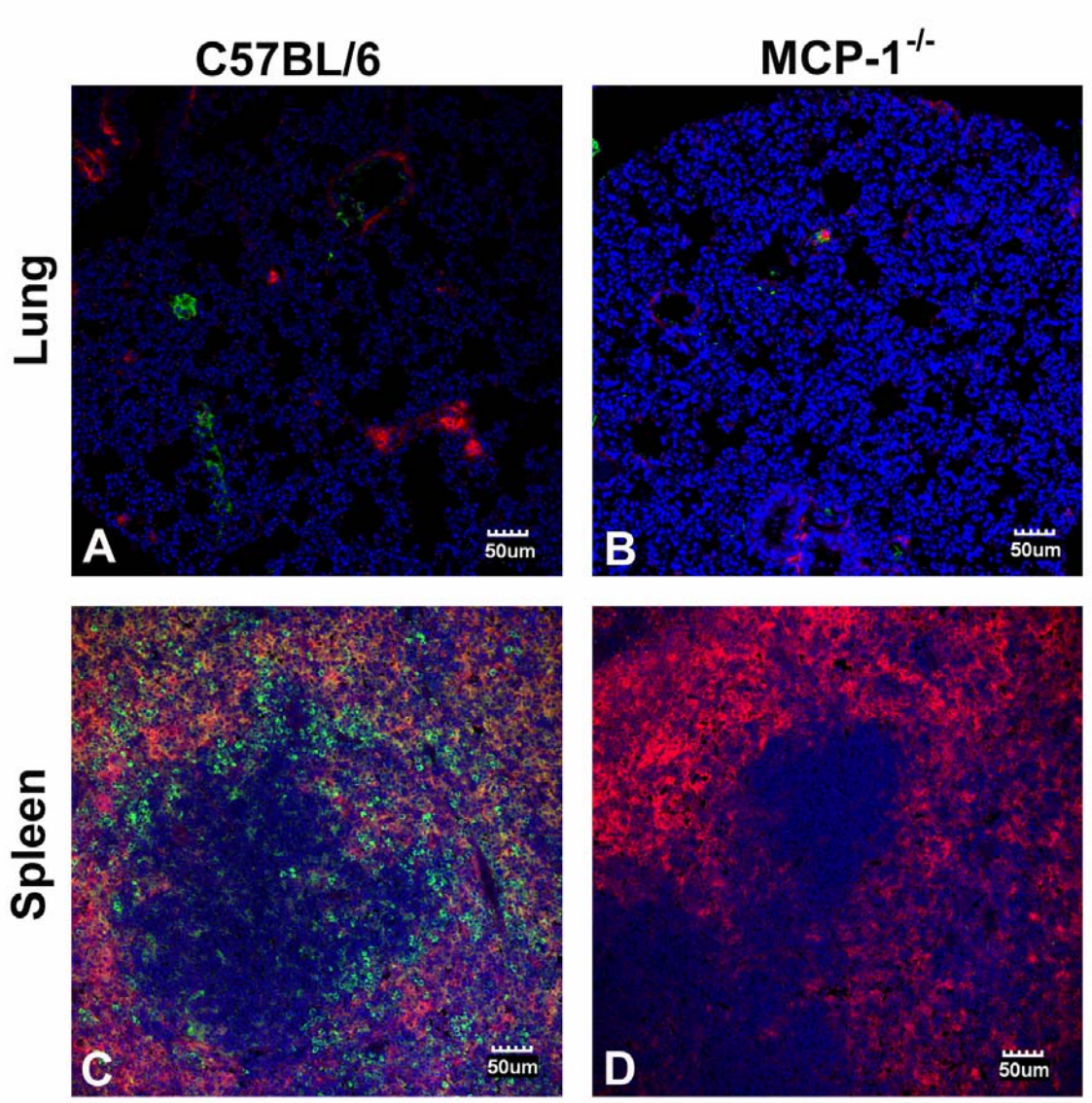


Figure 5.11: MCP-1-knockout mice have fewer infiltrating macrophages in the lungs whereas the spleen appears to be a major source of MCP-1. Wild-type and MCP-1-knockout mice were infected i.v. with *R. australis*. Six days after infection the organs were collected and stained for F4/80 (pan-macrophage, red) and MCP-1 (green). Nuclei were stained with DAPI (blue).

DISCUSSION

The goal of this work was to study the characteristics of rickettsial pathogenesis that relate to endothelial dysfunction and increased microvascular permeability in mouse models of infection. These models have been used extensively in the past to study mechanisms of immunity to *R. conorii* and *R. australis*; however, little attention has been paid to the pathophysiological consequences of endothelial target rickettsial infection. Specifically we wished to determine the role of the host inflammatory response in regulating microvascular permeability in the vital organs, namely the brain and lungs. The presence of inflammatory infiltrates in sites of infection is critical to the effective clearance of rickettsiae from the vasculature. The role of $CD8^+$ T-lymphocytes in this process has been well characterized by others and based on the available data appears to occur through perforin-mediated CTL activity (Walker *et al.*, 2001). It was also demonstrated by Valbuena that T-lymphocytes mediate protective immunity, even against non-homologous rickettsial species (Valbuena *et al.*, 2004). Likewise, it is well understood that the cytokines TNF-α, IFN-γ, and to a certain extent IL-1β exert an antirickettsial effect through the induction of NO by endothelial cells; however, the source of these cytokines during infection is not well understood (Feng *et al.*, 1994;Feng and Walker, 1993;Feng and Walker, 2000;Walker *et al.*, 1997). Therefore, we hypothesized that the cellular immune response is responsible for endothelial cell activation during infection which leads to increased iNOS activity. However, we also believe that this response comes with a significant "side effect" to the host in the form of increased microvascular permeability around sites of inflammation.

Initially we demonstrated that i.v. inoculation of rickettsiae results in disseminated multifocal infection of the vasculature. While the relative abundance of rickettsial antigen is quite low through the first few days of infection, we noticed a sharp rise in the amount of rickettsial organisms detected around day five of infection. This correlated well with the appearance of vascular inflammation in the brain and lungs in the

form of mononuclear inflammatory cells. We also showed that this inflammatory response was associated with increased iNOS activity, although it did not localize strictly to endothelial cells. The increased staining of iNOS at this time of infection argues that the necessary stimuli for transcriptional iNOS activation are also present, although we can not rule out the presence of alternative means of activation. It should also be noted that high levels of NO have been implicated as a cause for increased vascular permeability in a number of inflammatory disorders. iNOS is shown to be essential for BBB breakdown during bacterial meningitis in which iNOS-deficient mice experience lower increases in vascular permeability to Evans blue dye compared to wild-type mice (Winkler *et al.*, 2001). However, this response is also associated with decreased levels of IL-1β, IL-6 and TNF-α in the brain of these mice. In our model of infection, iNOS is essential to surviving an infection as we have demonstrated using iNOS-knockout mice infected with *R. australis*. Given the cytopathic nature of rickettsiae these mice most likely succumbed from endothelial damage directly related to rickettsiae. Nevertheless, we cannot rule out a role for NO in mediating increased vascular permeability during rickettsial infection. In fact, Bucci *et al.* demonstrated that the constitutive enzyme endothelial nitric oxide synthase (eNOS) was responsible for increased microvascular permeability during an acute inflammatory response *in vivo*. Mice receiving an intraplantar injection of carrageenan experience a biphasic inflammatory response characterized by edema formation and increased leukocyte extravasation. However, eNOS-knockout mice experience much less edema formation without experiencing any change in the level of cellular inflammation. Inhibition of the upstream eNOS signaling components phosphatidylinositol 3-kinase or hsp90 also reduced edema formation indicating a vital role for this pathway in regulation of vascular permeability (Bucci *et al.*, 2005). This was also demonstrated for TNF-α-mediated BBB-breakdown in rat meningeal vessels. Inhibition of the downstream signaling component of the eNOS pathway, soluble guanylate cyclase, with ODQ decreased the level of fluorescent dextran

leakage from the vasculature in TNF-α-treated rats (Mayhan, 2002). Given these data, it is still possible that NO may play an important role in mediating vascular permeability during acute rickettsial infections, perhaps through the activity of eNOS rather than iNOS.

Serum cytokine analysis revealed that the levels of TNF-α and IFN-γ were not significantly different than normal, healthy mice. This, we feel, supports the role of inflammatory cells as the source of these two cytokines in endothelial cell activation and NO production. Localized secretion of these two cytokines in vascular and perivascular inflammatory foci around infected endothelial cells potentially limits the effects of these two cytokines on a systemic level. However, we cannot ignore the fact that IL-1β was present systemically at very high levels in these mice. Given our data regarding the synergistic action of these cytokines on the regulation of microvascular permeability, it is plausible that increased IL-1β is the molecular "switch" needed to activate iNOS and other host defense mechanisms in conjunction with TNF-α and IFN-γ.

Still, we must also acknowledge the presence of additional cytokines in the serum of infected mice that may be affecting microvascular integrity on a systemic level. IL-1α, IL-6, and MCP-1 have all been shown to be capable of inducing vascular leakage both *in vitro* and *in vivo*. One particularly intriguing report was the work of Dube *et al*. that demonstrated a role for IL-1α in inducing a pathologic inflammatory state during *Yersinia enterocolitica* infection (Dube *et al.*, 2001). The authors demonstrated that the presence of IL-1α is essential for severe intestinal inflammation during this infection. Animals with infection that did not induce IL-1α production failed to develop severe inflammation despite the presence of TNF-α, IL-1β, and IFN-γ at normal levels. This report demonstrates the importance of distinguishing between IL-1α and IL-1β during inflammatory processes and supports a similar role for IL-1α-mediated disease during rickettsial infection.

Similarly, IL-6 has been shown to cause increased microvascular permeability (Maruo *et al.*, 1992). This has been demonstrated *in vitro* to occur via a PKC-dependent pathway associated with reorganization of cellular junctions (Desai *et al.*, 2002). We and others have shown that IL-6 is secreted by rickettsiae-infected endothelial cells. However, it was not clear what the effect might be *in vivo*. Here we show that IL-6 is present at very high levels both in mice and humans during lethal rickettsial infection. The obvious question is whether IL-6 is present at higher levels because of the severity of the infection, or whether the disease is severe because of high IL-6 levels. More work is needed to understand the role of IL-6 in vascular dysfunction during acute rickettsiosis.

Finally, we have addressed the role of MCP-1 in rickettsial pathogenesis. MCP-1 is produced by rickettsiae-infected endothelial cells via a p38 MAPK-dependent pathway and has been hypothesized to be an important mediator of rickettsial vasculitis. We have demonstrated that MCP-1 is produced at very high levels both locally and systemically during rickettsial infection. We have also shown that MCP-1 is present at very high levels in the serum of individuals with a lethal *R. rickettsii* infection. In the brain of infected mice, MCP-1 is present in regions of vascular inflammation and infection whereas the distribution is much less localized in the lungs. This is interesting given that MCP-1 has only been shown to cause increased microvascular permeability in brain-derived endothelial cells. Song and Pachter demonstrated that MCP-1 alters the expression of the tight junction-associated protein ZO-1 in brain microvascular endothelial cells as well as intact microvessels (Song and Pachter, 2004). Later it was shown that MCP-1 injected intracerebrally in mice causes a loss of BBB integrity and increased permeability to FITC-albumin. This effect was ablated in CCR2 receptor knockout mice. Our data demonstrate that MCP-1 plays an important role in recruitment of monocytes to sites of rickettsial infection, especially in the liver as MCP-$1^{-/-}$ mice had fewer and smaller granulomatous-like lesions. An end-point analysis of susceptibility to

infection accompanied by histological analysis of capillary leakage we hope will delineate the importance of MCP-1 in pathologic inflammation during acute rickettsiosis.

It was surprising to us that we did not detect significant changes in the tissue water content of the brain during lethal rickettsial infection. This, we feel, underscores one of the major shortcomings of the available small animal models of infection. Intravenous administration of rickettsiae is essential to establish a systemic infection comparable to human infection. However this results in a compressed disease course with the outcome being determined prior to the development of a significant adaptive immune response. Additionally, a relatively high percentage of rickettsiae is deposited in the lungs of these mice as it is the first capillary bed encountered by rickettsiae administered via the tail vein. This might explain our observations that these mice experienced significant pulmonary edema and inflammation while the inflammation in the brain is often mild compared to fatal human autopsy material. These models also lack the influence of tick-derived immune modulators, which have been shown to have a tremendous impact on disease transmission and pathogenesis for conditions such as Lyme disease (Wikel, 1999). A clear understanding of rickettsial pathogenesis *in vivo* will not be achievable until these factors are resolved.

The work presented here demonstrates for the first time that vascular dysfunction during acute rickettsial infection is associated with vascular inflammation including cellular infiltration and cytokine secretion. These observations coincide with the appearance of clinical signs of disease, supporting a role for the host immune response in mediating the disease manifestations although we acknowledge that this could also be due to increased numbers of rickettsiae present in the vasculature. Herein lies the challenge of studying immunopathogenesis during acute rickettsioses. While we do not claim that the lack of an inflammatory response would mitigate disease, we must also consider the impact of this response in a clinical setting. Often infected individuals do not receive appropriate antibiotic therapy until the late stages of disease, which usually

results in death. Given the histological characteristics of human autopsy material, it is clear that at this point these individuals are experiencing an intense and widespread vasculitis. This response has clearly been shown to be able to clear rickettsiae from the vasculature however rickettsial titers in these tissues are very high. Still, it is possible that the intensity of this response and high number of inflammatory foci is a major contributor to disease, likely due to one of the responses demonstrated in this work. Future work should address potential anti-inflammatory therapies that retain the beneficial effects of CTL activity and/or nitric oxide production but limit the detrimental effects of circulating cytokines and/or endothelial derived factors on microvascular function.

Chapter 6: Discussion and Conclusions

DISCUSSION

More than a century after the description of "black measles" (renamed soon thereafter Rocky Mountain spotted fever) in the Bitterroot Valley in Montana and the description of *Rickettsia rickettsii* as its etiologic agent, there are great gaps in our knowledge on the pathogenetic mechanisms responsible for the potentially lethal clinical manifestations of this important rickettsiosis. Foremost among these is the lack of a clear understanding of the mechanisms of increased microvascular permeability during severe RMSF. This has not only limited our understanding of rickettsial pathogenesis, but has also hampered our ability to treat severe cases of the disease. Despite the availability of effective antibiotic therapy it is not uncommon for people to succumb to infection at a rate of approximately 5%, mostly due to delayed diagnosis. What is not known is whether individuals receiving treatment late in the disease process die as a result of microcirculatory alterations due to extensive rickettsiae-mediated endothelial cell death, or rather the development of a "leaky" microcirculation due to the vasculitic and/or perivasculitic lesions as part of the immune response to the infection. This work attempts to address this question by examining the role of host immune factors in regulating the ability of the microvascular endothelium to function as an effective barrier to fluid and protein flux between the intravascular and extravascular compartments.

Host Inflammatory Responses and Endothelial Permeability

It is important to understand that increased microvascular permeability is part of the inflammatory response to chemical, physical or infectious agents. Furthermore, it is also present during angiogenesis as part of the healing response to tissue injury and during embryonic development. Interendothelial junctions are very dynamic cellular structures that are highly regulated and can undergo transient destabilization to allow the

transmigration of infiltrating leukocytes, or to allow the differentiation and growth of new blood vessels. However, infections are not physiological processes, and very frequently the evolutionarily conserved responses to a pathogen ultimately result in damage to the host. Leukocyte infiltration into sites of rickettsial invasion is critical to the clearance of this obligate intracellular bacterium from the endothelial lining of the microvasculature, and the lack of $CD8^+$ CTL action dramatically increases the susceptibility to fatal infection. Previous work demonstrated that rickettsial clearance is partly due to the action of perforin which presumably results in destruction of infected endothelial cells (Walker *et al.*, 2001). However, there is also strong evidence that TNF-α, IL-1β, and IFN-γ are essential mediators of immunity through the induction of nitric oxide by cytokine-stimulated endothelial cells (Feng and Walker, 1993), something which we have demonstrated convincingly happens in human cerebral endothelial cells. Previous work has demonstrated that this most likely occurs through autophagy. However, what had not been addressed was the impact of cytokine stimulation on the ability of an endothelial barrier to function effectively. We have shown that in fact these cytokines do have an impact on microvascular permeability partly through the modulation of adherens junctions. More *in vivo* experiments will be needed to ascertain the source of these cytokines.

NK cells are known to produce high quantities of IFN-γ very early after rickettsial infection *in vivo* resulting in anti-rickettsial activity (Billings *et al.*, 2001). At this time, though, it is not clear whether these cells are the primary sources for IFN-γ that induces NO production. Likewise, the precise source of TNF-α during rickettsial infection is not known, although it has been suggested that macrophages and certain lymphocyte subsets play an important role in this response. Given that infiltration of leukocytes around infected vessels is necessary to clear a systemic infection, we wished to determine whether this cellular response is associated with changes in microvascular permeability. We demonstrated that serum protein leakage occurs at sites of rickettsial

vasculitis/perivasculitis and is associated with increased activity of the iNOS enzyme, which we have previously shown to be activated by TNF-α, IL-1β, and IFN-γ.

Chemokine production is a common response of many cell types during infection. Proteins such as IL-8 and MCP-1 recruit neutrophils and monocytes, respectively, to sites of infection in an effort to phagocytose invading pathogens. MCP-1 in particular has been shown to have a potent effect on interendothelial junctions *in vitro* and has also been shown to cause a loss of blood-brain barrier integrity after prolonged exposure (Stamatovic *et al.*, 2005). Presumably this response has evolved to mediate the emigration of monocytes from the intravascular into the extravascular compartment. While we did see high levels of MCP-1 produced locally at sites of rickettsial infection and vasculitis, it was also present at very high levels in the blood of fatally-infected mice and humans. Overexpression of MCP-1 in transgenic mice has actually been shown to increase the susceptibility to the intracellular pathogens *Listeria monocytogenes* and *Mycobacterium tuberculosis* due to deficiencies in monocyte homing, most likely due to monocyte nonresponsiveness to locally produced gradients (Rutledge *et al.*, 1995). These two observations taken together suggest that not only could high serum levels of MCP-1 mediate vasogenic brain edema, but it may also inhibit the ability of monocytes to migrate to specific sites of infection.

Rickettsiae-mediated Host Cell Responses to Infection

We must also consider the role of rickettsiae-induced host cell signaling which may result in changes in the endothelium. There is a significant amount of data describing endothelial cell responses to infection that may or may not be important in regulation of microvascular permeability. Indeed, direct rickettsial cytotoxicity to the endothelium has a tremendous impact on the integrity of the microvascular barrier. Reactive oxygen species (ROS) have been hypothesized to be the major effecter of

cellular damage, but a connection has yet to be established linking ROS generation with increases in microvascular permeability. Our work intended to directly address mechanisms of increased microvascular permeability during rickettsial infection of microvascular endothelial cells and has revealed a potential role for VEGF as a mediator. However we were challenged to directly link rickettsiae-induced VEGF production to changes in permeability. Additionally serum VEGF levels of human RMSF patients were actually lower in fatal infections. This suggests a role for VEGF as a localized mediator. Also, it would be prudent to investigate the levels of specific VEGF receptors to help clarify the importance of VEGF as a mediator of vascular permeability during acute rickettsiosis. We must also consider the role of other cytokines such as IL-6, IL-8, or other unidentified factors that may also influence microvascular permeability.

We did however demonstrate a role for Src-mediated changes in endothelial permeability, which may explain the loss of p120 and/or β-catenin staining at endothelial adherens junctions.

Adherens Junctions as Therapeutic Targets

Our work addressing both host immune factors as well as direct endothelial responses to rickettsial infection demonstrated an important role for adherens junction disassembly as the primary functional deficit resulting in increased microvascular permeability. Loss of β-catenin and p120 association with adherens junctions results in decreased cellular adhesion, although this is not necessarily a result of decreased expression of these two proteins. Post-translational modifications in the form of phosphorylation play an important role in modulating the activity of β-catenin and p120 and are regulated by a variety of stimuli including VEGF and TNF-α. Considering the impact of both exogenous and endogenous signals on adherens junction stability during

rickettsial infection, these important regulators of permeability may serve as a potential therapeutic target in rickettsiosis as well as other inflammatory disorders.

Sphinogsine 1-phosphate and adherens junction stability

Sphingosine 1-phosphate (S1P) is a biologically active sphingolipid produced by the phosphorylation of sphingosine, which is derived from the degradation of sphingomyelin, a component of the plasma membrane (Hla, 2003). Extensive studies by Garcia *et al.* have demonstrated that S1P possesses very potent barrier-enhancing properties through the ligation of the endothelial differentiation gene (Edg) family of receptors (Garcia *et al.*, 2001). *In vitro* this results in a dose-dependent increase in TER across human lung microvascular endothelium that is maintained for an extended period of time. Further work has demonstrated that S1P activates Rac GTPase, which induces the formation and stabilization of adherens junctions by promoting the linkage of VE-cadherin with β-catenin (Lee *et al.*, 1999).

Thrombocytopenia causes an increase in microvascular permeability which is reversed through the infusion of platelets or platelet-derived products via the stabilization of endothelial monolayers (Gimbrone, Jr. *et al.*, 1969;McDonagh, 1986;Lo *et al.*, 1988). Platelets are a significant source of S1P due to their relatively high expression of sphingosine kinase so it is not surprising that the vascular protective effects of platelets have been linked to S1P release (Pyne and Pyne, 2000;Schaphorst *et al.*, 2003). S1P has also been tested for its therapeutic potential during LPS-induced vascular permeability in mice. Infusion of LPS-treated mice with S1P results in a significantly decreased level of permeability and lung weight gain as well as less overall inflammation (Peng *et al.*, 2004). The immunosuppressive agent FTY720 is currently in Phase III trials for the treatment of multiple sclerosis. FTY720 undergoes phosphorylation by sphingosine kinase to generate an analog of S1P which selectively binds S1P receptors (Brinkmann *et*

al., 2002). One mechanism of action of FTY720 is to sequester lymphocytes in lymph nodes resulting in decreased inflammation. This compound also attenuates LPS-induced vascular permeability and protects endothelial barriers *in vitro* (Peng *et al.*, 2004).

During human RMSF infection, approximately 30-50% of patients experience thrombocytopenia (Elghetany and Walker, 1999). As described above this could have a tremendous impact on the systemic integrity of the microvasculature during rickettsial infection, and we cannot rule out the possibility that administration of S1P could serve a protective role through the stabilization of adherens junctions. We must also consider that S1P acts to sequester lymphocytes away from the sites of infection, which may not necessarily be detrimental to the individual if they also receive appropriate antibiotic therapy. Given the data presented in this work, we feel that an anti-inflammatory therapy could serve a role in treating not only severe rickettsial infections but also other infectious diseases leading to increased microvascular permeability, by limiting the deleterious impact of the host's immune response on the endothelium.

Other novel therapies

Given the tremendous power of recent advances in genomics and proteomics, we will soon have the ability to design highly specific therapies which target individual pathways. However we must first understand the pathways that are important to a particular disease and where in the signaling cascade is an appropriate intervention point. Information learned from this and other studies has demonstrated the importance of adherens junctions, and the interrelated signaling pathways that regulate their function, in the pathogenesis of inflammatory disorders. For example, a therapy which could directly target a specific kinase such as c-Src, or PKC-α, could be used to directly intervene in the phosphorylation of adherens junction components like p120 or β-catenin preventing their disassociation from the junctions. Small peptide inhibitors which bind with a very high

affinity to individual kinase domains on these enzymes could be delivered systemically and at a relatively low cost to patients experiencing severe alterations in microvascular permeability during RMSF, viral hemorrhagic fevers, or sepsis. In fact, thioaptamers to the AP-1 family of transcription factors have already been used to reduce mortality in guinea pigs infected with Pichinde virus, an arenavirus very similar to the human pathogen Lassa virus (Fennewald *et al.*, 2007). However, these therapies will not come to fruition without a thorough understanding of the pathways and proteins active during these infections.

FUTURE DIRECTIONS

More Accurate Models of Microvascular Permeability

Fluid shear stress

Mechanistic basic research in biomedical sciences, and any other branch of science, relies on a reductionistic approach to solve scientific questions. Endothelial pathobiology is not the exception. Most *in vitro* models of permeability suffer from an oversimplification of important mechanisms to define the importance of a specific pathway. However, we must acknowledge that the endothelium is a very complex and protean component of homeostasis. Several "systems" converge on the microvascular endothelium such as the circulatory system in the form of fluid shear stress, the immune system, the coagulation and fibrinolytic systems, and so on. Needless to say, the microvascular endothelium is a highly dynamic tissue. Fluid shear stress, or the force of fluid flow across an endothelial barrier, has a constant impact on the microvascular barrier and is responsible for establishing chemotactic gradients. The endothelium is also constantly being replenished with fresh nutrients and growth factors. Through the use of ECIS, others have demonstrated an important role for fluid shear stress in modulating the level of permeability of endothelial monolayers (Depaola *et al.*, 2001). More recent work

has demonstrated that endothelial cells exposed to low versus physiological shear stress have a decreased level of occludin mRNA and protein expression which could lead to tight junction destabilization and increased permeability (Conklin *et al.*, 2007). Shear stress has also been shown to induce peripheral focal adhesion assembly through the site-specific phosphorylation of FAK at tyrosine 576, and activation of the small GTPase Rac (Shikata *et al.*, 2005). Based on these data it would be prudent to develop a model of rickettsial infection which is exposed to fluid shear stress.

Pathways dependent on exogenous stimuli

Further demonstrating the importance of accurate *in vitro* models is our observation that ACE, or angiotensin I converting enzyme, is modulated in endothelial cells infected with rickettsiae. ACE is usually expressed in high levels in the endothelial lining of blood vessels and catalyzes the conversion of the biologically inert angiotensin I (Ang I) into the highly active angiotensin II (Ang II). Ang I is normally produced by the conversion of angiotensinogen into Ang I through the action of renin and is supplied to the endothelium via the circulation. Ang I is then subject to ACE-mediated conversion into Ang II, primarily in the pulmonary microcirculation. This pathway is a fundamental regulator of blood pressure. However, it is now clear that Ang II can play a role in inducing vascular inflammation during such conditions as atherosclerosis, diabetes, and sepsis (Cheng *et al.*, 2005). In fact, Ang II has been shown to cause an increase in microvascular permeability via a PKC-β-dependent pathway *in vitro* (Idris *et al.*, 2004). *In vivo,* perfusion of rat mesenteric venules with Ang II significantly increases permeability (Victorino *et al.*, 2002). Work by Sano *et al.* has shown that blockade of the Ang II type 1 receptor (AT-1) in mice ameliorates VEGF-induced vascular leakage (Sano *et al.*, 2006). Likewise, MCP-1 has been implicated as the central inflammatory mediator during Ang II-induced vascular inflammation (Ni *et al.*, 2004). Ang II also increases IL-

6 production in vascular smooth muscle cells, macrophages and mesangial cells (Han *et al.*, 1999;Schieffer *et al.*, 2000). Additionally, IL-6 has also been shown to induce angiotensinogen in the vascular wall which perpetuates vascular inflammation through increased local Ang II production (Brasier *et al.*, 2002). Given that MCP-1 and IL-6 are present at very high levels in the serum of patients with severe RMSF, and that ACE is upregulated early after rickettsial infection in human endothelial cells, there may be a role for Ang II-mediated pathology during human rickettsial infection, and this hypothesis should be pursued more closely. As suggested earlier, this would only be possible in a model system containing the necessary stimuli, in this case Ang I. This is only one example of the drawbacks of *in vitro* models in which the endothelium is functioning in an environment reduced purely to endothelial invasion by rickettsiae.

CONCLUSIONS

This work represents the first in-depth investigation into mechanisms of increased microvascular permeability during acute rickettsial infection. The information gained here demonstrates a role for the host's response to infection in mediating changes at the microvascular level, while confirming the importance of rickettsiae-mediated cell death as a contributor to pathogenesis. Further studies are needed to determine the precise role of proteins such as VEGF, MCP-1, and IL-6 in rickettsial pathogenesis *in vivo*. However, the work presented here provides a solid foundation and springboard for those studies. The questions generated as a result of these studies will lead to an even deeper understanding of rickettsial pathogenesis and may one day lead to the development of novel therapeutics not only for RMSF but also other rickettsioses, viral and bacterial diseases whose "final pathogenetic common pathway" is profound alterations in the microvasculature leading to generalized microvascular hyperpermeability and eventually multi-organ failure.

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