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Pathogenesis of Rickettsial Diseases: Pathogenic and Immune Mechanisms of an Endotheliotropic Infection

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Abstract

Obligately intracytosolic rickettsiae that cycle between arthropod and vertebrate hosts cause human diseases with a spectrum of severity, primarily by targeting microvascular endothelial cells, resulting in endothelial dysfunction. Endothelial cells and mononuclear phagocytes have important roles in the intracellular killing of rickettsiae upon activation by the effector molecules of innate and adaptive immunity. In overwhelming infection, immunosuppressive effects contribute to the severity of illness. *Rickettsia*–host cell interactions involve host cell receptors for rickettsial ligands that mediate cell adhesion and, in some instances, trigger induced phagocytosis. Rickettsiae interact with host cell actin to effect both cellular entry and intracellular actin-based mobility. The interaction of rickettsiae with the host cell also involves rickettsial evasion of host defense mechanisms and exploitation of the intracellular environment. Signal transduction events exemplify these effects. An intriguing frontier is the array of rickettsial noncoding RNA molecules and their potential effects on the pathogenesis and transmission of rickettsial diseases.

INTRODUCTION

What Are Rickettsiae?

Rickettsiae are commonly defined as genetically related, obligately intracellular bacteria that reside in an arthropod host during a part of their zoonotic cycle. By this definition, rickettsiae are members of at least seven genera: *Rickettsia*, *Orientia*, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, *Neoehrlichia*, and *Wolbachia*. This review addresses the diseases caused by *Rickettsia*. Pathogenic *Rickettsia* are associated with hematophagous arthropods: ticks, mites, fleas, and lice. However, organisms belonging to the genus are found in a vast array of hosts, including herbivorous arthropods, leeches, and amoebae (1). *Rickettsia* that are established pathogens have evolved into the obligately intracellular lifestyle with an evolutionarily reduced genome. Residing free in the cytosol of the host cell, they acquire many necessary components via transport mechanisms instead of maintaining genes for synthesizing sugars, lipids, nucleotides, and amino acids. Indeed, *Rickettsia* can parasitize host ATP via the exchange of ADP through translocation systems, yet when the host cell ATP is depleted, rickettsiae can also synthesize ATP.

The contemporary phylogeny of *Rickettsia* recognizes three pathogenic clades: the classic spotted fever and typhus groups (TG), and a transitional group (TRG), in addition to numerous other clades of organisms of undetermined pathogenicity that are ancestral (2) to the pathogens. The spotted fever group (SFG) contains many closely related organisms, such as *R. rickettsii* and *R. conorii*, that reside in a limited number of tick species. The TG contains *R. prowazekii* and *R. typhi*, the etiologic agents of, respectively, louse-borne epidemic typhus and murine typhus. The TRG contains *R. akari*, *R. australis*, and *R. felis*, a ubiquitous organism found throughout the world in cat fleas, other fleas, ill persons, and in some reports, also in healthy persons and mosquitoes (3–6).

The Spectrum of Rickettsial Diseases

The organisms and diseases of importance in North America are *R. rickettsii* (Rocky Mountain spotted fever) (7), *R. parkeri* (maculatum disease) (8), *R. akari* (rickettsialpox), *Candidatus* R. phillipi (an eschar-associated illness in California) (9), *R. typhi* (10), *R. prowazekii* (in the United States, a zoonosis spread to humans from infected flying squirrels by their ectoparasites) (11), and *R. africae* (a disease frequently associated with travel to sub-Saharan Africa) (12). *Candidatus* refers to the taxonomic status of a proposed species designation that has yet to be officially adjudicated.

In general, rickettsioses comprise diseases with a continuous spectrum of severity of illness and overlapping clinical manifestations. *R. rickettsii*, *R. prowazekii*, *R. conorii*, and *R. typhi* can cause life-threatening diseases. The case fatality rate for Rocky Mountain spotted fever in the preantibiotic era was 20–25% and remains 3–4% (13, 14). In Latin America, the current case fatality rate is an amazing 30–40%, apparently owing to more virulent strains of *R. rickettsii*. The case fatality rate of *R. prowazekii* infection is 15%, with much higher rates in the settings of extreme poverty and lack of adequate supportive care, a common condition under circumstances of natural disasters, war, and famine. There have been no fatalities in flying squirrel–associated *R. prowazekii* infections in the United States, and whether this can be attributed to reduced virulence or the better general health of infected persons remains undetermined. Human rickettsioses progress from chills, fever, headaches, myalgia, nausea, and vomiting in the first few days to the appearance of a rash after 3 to 5 days and, in the most severe cases, progression to respiratory failure, hypotensive shock, oliguric acute kidney injury, jaundice, hemorrhagic lesions, coma, and seizures. Patients with non-life-threatening rickettsioses due to *R. parkeri* or *R. africae* infection usually develop a focus of epidermal and dermal necrosis (eschar) at the site of tick feeding and inoculation of rickettsiae

prior to the onset of fever, headache, myalgias, and draining lymphadenopathy (8, 12). Rash occurs in a smaller percentage of patients, tends to be sparser, and is sometimes vesicular or pustular. Tick-borne lymphadenopathy, a disease in Europe caused by *R. slovaca*, is characterized by an eschar usually occurring at the site of a tick bite on the scalp that is followed in 7 to 9 days by painful draining cervical lymphadenopathy, but it rarely manifests as fever or rash (15, 16). Strong circumstantial evidence suggests that *R. amblyommatis*—which is carried by approximately 50% of *Amblyomma americanum* (lone star ticks), the most prevalent human biting ticks in the southeastern and south–central United States—is spreading steadily northward and causes subclinical infection in the majority of persons who asymptomatically develop anti-SFG antibodies (17).

Vector Biology of Rickettsial Diseases

The vector biology of rickettsial diseases is only partially known. The human body louse vector of *R. prowazekii* is the least successful rickettsial host; 100% of infected lice are killed by the rickettsiae. Similarly, the most pathogenic organism, *R. rickettsii*, is found in fewer than 0.1% of its vector ticks (*Dermacentor variabilis* in the United States) compared with high carriage rates for the much less pathogenic *R. africae* and *R. amblyommatis* in their tick vectors. Adult and nymphal ticks feed for several days, often more than a week, while usually remaining unnoticed. Studies of the salivary gland secretions of *Ixodes scapularis*—which transmits Lyme borreliosis, anaplasmosis, babesiosis, Powassan virus encephalitis, and *Ehrlichia muris eauclairensis* infections—have identified a veritable salivary pharmacy of anticoagulants that maintain blood flow in the tick bite lesion, immunomodulators that suppress inflammation and rejection, and pain suppressors that prevent detection (18). Studies of the salivary secretions of other evolutionarily divergent tick vectors of rickettsioses are not as advanced, but the existence of similar phenomena, likely mediated by different molecules, seems likely. Vector biology of *Dermacentor*, *Amblyomma*, and *Rhipicephalus* ticks represents a gap in knowledge that is relevant to the transmission of *Rickettsia* species, including phenomena such as the reactivation of rickettsial virulence from organisms in unfed ticks that do not cause disease but are reactivated to virulence during tick feeding (19).

Pathology and Pathophysiology of Rickettsial Diseases

The pathogenic sequence of events that occurs in rickettsial infection begins with the entry of organisms inoculated by the feeding tick or mite or scratched into the skin from infected louse or flea feces deposited on the skin. The initial target cells of infection are $CD68^+$ cells (macrophages or dendritic cells, or both) (20). The rickettsiae then spread via lymphatic vessels to the regional lymph nodes, as has been observed vividly in the lymphangitis associated with *R. sibirica mongolitimonae* infection (21). Rickettsiae then spread hematogenously throughout the body and infect mainly endothelial cells but also, to a lesser extent, macrophages, in the skin, lungs, brain, liver, gastrointestinal tract, kidneys, heart, and other organs.

The fundamental lesion of rickettsial diseases is vasculitis (22–24), which comprises the events that follow rickettsial infection and the activation of endothelium and the subsequent spread to involve focal continuous networks of endothelial cells, mainly of the microcirculation. Rickettsial infection of endothelium stimulates cell signaling cascades, leading to the secretion of cytokines and chemokines and other events, as described in the section titled Innate Immune Signaling. What pathologists visualize in the foci of vascular infection by rickettsiae is, in fact, the cellular host defense comprising CD8 and CD4 T lymphocytes and macrophages that infiltrate both the vascular wall and perivascular space (25, 26). A small minority of vascular lesions contain nonocclusive hemostatic plugs, and the pathophysiologically important interstitial edema that

occurs owing to increased vascular permeability is usually obscured by the artifact of formalin-induced tissue shrinkage (27–29).

These lesions form the basis for the rash, which progresses from macules to maculopapules with the accumulation of interstitial edema and subsequently to petechial lesions with the extravasation of blood around the most intensely infected networks of microcirculation in the center of the maculopapules (**Table 1**). In the lungs, this sequence of events leads in the most severe cases to interstitial pneumonia, noncardiogenic pulmonary edema, and diffuse alveolar damage (30). In the central nervous system, rickettsial meningoencephalitis consists of perivascular infiltrates of CD8 and CD4 T cells and macrophages, classically termed glial nodules, in the neuropil and also in the subarachnoid space (25, 31). Microinfarcts and perivascular hemorrhages occur in some severe cases, particularly in white matter. The liver shows vasculitis in portal triads and multiple randomly distributed small foci of hepatocellular death and lymphocytic inflammation that are associated with mildly elevated hepatic enzymes and, in some cases, hyperbilirubinemia, but massive hepatic necrosis and hepatic failure do not occur (32, 33). Focal lesions in the wall of the gastrointestinal tract, gallbladder, and pancreas, although not extensive, are observed not only in fatal cases at autopsy but also in resected tissues from patients in whom abdominal pain and tenderness lead to exploratory laparotomy with appendectomy or cholecystectomy (34–36). Acute kidney injury is usually a result of prerenal azotemia associated with hypovolemia and, rarely, acute tubular necrosis. The multifocal vasculitis observed typically in the renal cortical medullary junction is unlikely to contribute significantly to the renal pathophysiology (37). The heart manifests interstitial lymphohistiocytic infiltrates without necrosis of cardiac myocytes, which is consistent with an appropriate cellular host defense against the rickettsiae infecting the cardiac microcirculation (27). Echocardiography usually demonstrates adequate cardiac function. In most fatal cases, death is associated with respiratory failure, encephalitis, and hypotensive shock.

The pathophysiologic events can be attributed principally to rickettsia infection–associated increased vascular permeability leading to edema, hypovolemia, hypotension, reduced perfusion of organs, acute respiratory distress syndrome, and central nervous system abnormalities. The increased permeability of endothelial cell monolayers depends on the quantity and virulence of the rickettsiae and the duration of intracellular rickettsial growth, and it is enhanced by the presence of proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (38–40). The infection of endothelial monolayers in the presence of proinflammatory cytokines is associated with the disruption of intercellular adherens junctions and the redistribution of P120 and β-catenin proteins, which attach endothelial cells to the extracellular matrix and regulate the interaction of vascular endothelial (VE)-cadherin with the actin cytoskeleton. Rickettsial activation of endothelial cells causes phosphorylation of VE-cadherin, resulting in decreased VE-cadherin homotypic interactions at adherens junctions. A further mechanism related to increased vascular permeability is mediated by the ribonuclease activity of cytoplasmic angiogenin, which yields 5′ transfer RNA fragments associated with tyrosine phosphorylation and internalization of VE-cadherin and decreased function of the endothelial cell barrier (41). The functional contributions remain unknown of vasoactive prostaglandins (PGs), vascular endothelial growth factor (VEGF), thrombin, and other potential mediators.

Host factors often play an important part in the severity of rickettsial infections. A greater risk of a fatal outcome is associated with older patients; males; and patients with diabetes, alcoholism, or glucose-6-phosphate dehydrogenase deficiency, which predisposes patients to fulminant Rocky Mountain spotted fever and results in death within 5 days of the onset of illness (42–45).

The most valuable animal models that represent the target cells, organ involvement, and pathologic lesions of human rickettsioses with dose-dependent lethality are C3H/HeN mice infected intravenously with *R. conorii* for SFG rickettsioses or *R. typhi* for TG rickettsioses

Table 1 Clinicopathologic correlations in spotted fever rickettsioses

Organ	Signs, symptoms, and clinical manifestations	Pathologic lesions	Key references
Skin	Macules, maculopapules, petechial maculopapules, ecchymoses, involvement of palms and soles (late), edema, gangrene Eschar (frequent, except in Rocky Mountain spotted fever)	Rash: dermal capillaritis and venulitis, lymphohistiocytic and leukocytoclastic vasculitis with polymorphonuclear leukocytes, hemorrhages, edema, focal fibrin thrombi Eschar: dermal and epidermal coagulative necrosis, lymphohistiocytic vasculitis, edema, focal hemorrhage, thrombosis (rare)	22, 25, 146, 147
Lung	Cough, usually nonproductive (33% of cases); dyspnea (25%); chest pain (12%); rales; impaired gas exchange; hemoptysis (rare)	Interstitial pneumonia, alveolar septal congestion, interstitial edema, alveolar edema, diffuse alveolar damage, hemorrhage	22, 24, 30, 147, 148
Central nervous system	Headache (80% of cases), lethargy (frequent), confusion (28%), stupor or delirium (21%), coma (10%), seizures (8%), ataxia (5%), focal neurologic signs (2%), CSF pleocytosis (38% of those tested), increased CSF protein (35% of those tested), permanent neurologic sequelae, particularly in the preantibiotic era	Perivascular lymphocytes and macrophages, edema, punctate perivascular hemorrhages, arteriolar thrombosis, and small white matter infarcts	22, 31, 147
Liver, pancreas, and gastrointestinal tract	Anorexia, nausea, or vomiting (56% of cases); diarrhea (20%); abdominal pain (34%); abdominal tenderness; gastrointestinal hemorrhage; hyperbilirubinemia; elevated hepatic transaminases	Rocky Mountain spotted fever: hepatomegaly, large mononuclear cell- and polymorphonuclear leukocyte-rich portal triaditis, portal vasculitis (*Rickettsia rickettsii* in endothelium of portal vessels), sinusoidal erythrophagocytosis, sinusoidal leukocytosis (children) Mediterranean spotted fever: foci of hepatocellular cell death and mononuclear cell reaction (*R. conorii* in sinusoidal lining) Stomach, small and large intestines, gallbladder, and pancreas: focal capillaritis, venulitis, and arteriolitis in stomach, small and large intestines, gallbladder, and pancreas	24, 22, 32–36, 147, 149, 150
Kidney	Oliguria, prerenal azotemia, anuria	Multifocal perivascular interstitial nephritis (lymphohistiocytic vasculitis of intertubular capillaries and venules), predominantly in the outer medulla and at the corticomedullary junction, acute tubular necrosis	22, 24, 37, 147, 151
Cardiovascular system	Edema, hypotension, shock, arrhythmias	Increased systemic and pulmonary vascular permeability and edema; patchy myocardial interstitial infiltrate of lymphocytes, macrophages, and plasma cells; interstitial myocardial edema	22, 24, 26, 147, 152
Coagulation	Bleeding (petechiae, ecchymoses), gastrointestinal hemorrhage, hemoptysis (rare), consumption coagulopathy	Few, seldom occlusive, hemostatic plugs located in the foci of severe rickettsial infection and damage	29

Abbreviation: CSF, cerebrospinal fluid.

(46, 47). Intravenous infection of C57BL/6 mice with *R. australis* has allowed for studies of specific immune mechanisms in gene knockout mice (48, 49). Guinea pigs and nonhuman primates are susceptible to *R. rickettsii* and *R. prowazekii*, providing models for the preclinical testing of vaccines and novel therapeutics.

THE PATHOGENESIS OF SEVERE RICKETTSIOSES

The pathogenesis of rickettsioses is incompletely understood, although the immune system is known to play an important part. Both human and animal studies have demonstrated that numerous elements of the immune system—including dendritic cells, innate immune signaling, macrophages, natural killer (NK) cells, CD8 T cells, CD4 T cells, endothelial cells, antibodies, and inflammatory cytokines and chemokines—have strong impacts on controlling rickettsial infection, predominantly via mechanisms that facilitate the intracellular killing of rickettsiae (**Figure 1**). Apparently, if these components of protective immunity ineffectively engage in the host response or rickettsiae modify or manipulate the response of these immune elements, the disease will progress with increased severity, leading to a fatal outcome. Moreover, other immune components, such as inducible T regulatory cells, contribute to the progression and pathogenesis of rickettsial diseases. Here, we address how rickettsiae subvert or evade host immune mechanisms to promote the disease.

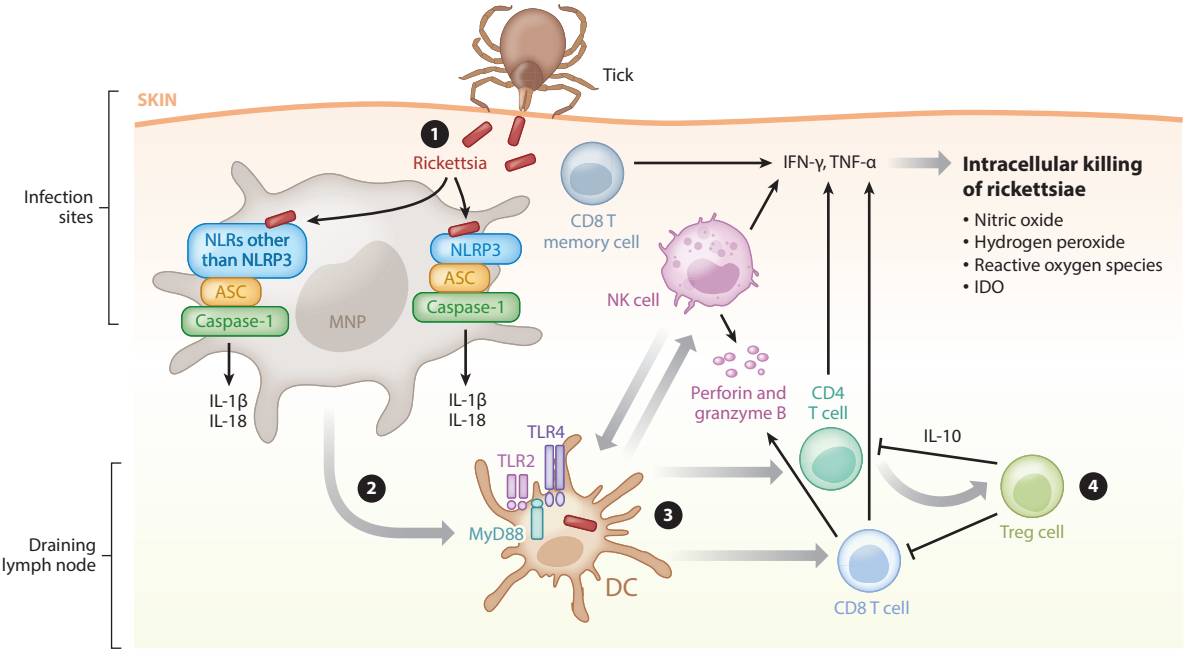

Figure 1

Schematic of interactions of spotted fever group rickettsiae with different components of the host immune system. After a tick bite, the inoculated rickettsiae initially target MNPs (❶) and then activate DCs (❷). Rickettsial antigen is presented by DCs and subsequently activates NK cells, CD4 T cells, and CD8 T cells (❸). Simultaneously, IL-10-producing inducible $CD4^+CD25^+$ Treg cells are generated, leading to an immunosuppressive T cell response (❹). Abbreviations: DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; MNP, mononuclear phagocyte; NK, natural killer; NLR, Nod-like receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor; Treg, T regulatory.

Mechanisms Involved in Intracellular Killing of Rickettsiae

Upon activation by immune stimuli, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and RANTES, or through an Fc-dependent antibody effect, human endothelial cells and macrophages elicit intracellular killing of rickettsiae (50, 51). Mouse endothelial cells in a model of spotted fever rickettsiosis also exhibit antirickettsial activity under cytokine stimulation. The mechanisms of rickettsial killing in these cells involve one or a combination of three possibilities: inducible nitric oxide (NO) synthesis, hydrogen peroxide and reactive oxygen species production, and limiting the availability of tryptophan via its degradation by indoleamine 2,3-dioxygenase (IDO) (52). Although in vivo rickettsicidal activity is not completely understood in humans, increased mRNA levels of IDO and inducible NO synthase (iNOS) have been detected in the infection sites of patients with mild-to-moderate Boutonneuse fever due to *R. conorii* infection (53). However, further experimental studies using iNOS knockout mice suggest that NO is partially involved in CpG type B-induced death of mice infected with *R. australis* (54). Interestingly, IDO and NO may not be involved in protection conferred by pretreatment with CpG type B against a lethal-dose infection with *R. australis*, as demonstrated in a study using IDO knockout and iNOS knockout mice. Thus, IDO is not the exclusive mechanism of protection conferred by treatment with CpGs before infection (54).

Another mechanism related to the degradation of cytosolic rickettsiae is autophagy. As shown by electron microscopy, in mouse brain endothelial cells activated by IFN-γ and TNF-α, *R. conorii* are destroyed in structures resembling autophagolysosomes (55). However, the question of whether autophagy really plays a part in clearing rickettsiae in immune cells other than endothelial cells—particularly before being activated by immune effector cytokines produced by the major players in adaptive immunity—requires further studies using both in vitro and in vivo approaches.

Our studies have demonstrated that endothelial cell responses to rickettsiae, including a proinflammatory cytokine profile and endothelial permeability, have a role in the pathogenesis of rickettsial diseases. The secretion of monocyte chemoattactant protein-1 (MCP-1) by human endothelial cells upon infection with rickettsiae, which is accompanied by a minimum level of cell death, prognosticates a mild rickettsial disease, as demonstrated by *R. massiliae* infection (40). In contrast, human endothelial cells infected with the Israeli spotted fever strain of *R. conorii* manifest a proinflammatory profile characterized by IL-8 and IL-6 and a significant level of cell death that predicts a severe infection outcome. The endothelial dysfunction caused by infection with this strain of *R. conorii* leads to increased permeability that is at least partly mediated by caspase-1.

Although endothelial cells are the primary target cells for rickettsial infection, pathogenic rickettsiae also infect monocytes, macrophages, and hepatocytes, as has been illustrated in both humans and established animal models. Upon infection, the perivascular infiltration of lymphocytes and macrophages comprises what is called rickettsial vasculitis. In response to IFN-γ and TNF-α, macrophages are activated and serve as crucial effector cells, mediating the clearance of pathogens within these macrophages. As discussed earlier, macrophages are the initial targets of rickettsiae in the inoculation site of infection in the skin. Mononuclear cells are one of the major inflammatory cells in the cutaneous inoculation sites in patients infected with *R. parkeri* (56). $CD68^+$ macrophages are the predominant infected cells in the skin lesions of patients with rickettsialpox (20). Furthermore, the severity of rickettsial diseases has been reported to clearly correlate with whether these rickettsiae can survive and proliferate in human macrophage-like cells (57). Thus, further studies are required to determine how macrophages contribute to the pathogenesis of these diseases.

Innate Immune Effectors in Host Defense

Animal models of rickettsial infection provide useful tools to investigate host immune effectors against and the pathogenesis of severe rickettsioses. Currently, mouse models of *R. australis*, *R. conorii*, and *R. typhi* infections have been well described and exploited (46, 47, 49). The mouse models mimic human pathology; *R. conorii*-infected C3H/HeN mice provide one of the best models because endothelial cells are the major targets for rickettsiae in vivo. It is worth noting, however, that C57BL/6 mice, the background of most knockout mice, are susceptible to infection only with *R. australis* and are highly resistant to *R. conorii* and other rickettsiae. Although *R. australis* is not the most virulent rickettsial species in humans, *R. australis*–infected B6 mice allow us to mechanistically study the critical components of the host immune system by employing a variety of knockout mice on the B6 background.

Understanding the mechanisms of host resistance against rickettsial infection advances our knowledge of the innate effectors of host defense. During the early stage of infection, $Rag^{-/-}$ mice, lacking acquired immunity, develop significantly greater rickettsial loads in tissues compared with wild-type controls, which may be attributed to an expanded population of IFN-γ-producing memory CD8 T cells (58). Although a deficiency of CD8 T cells does not alter the innate resistance to *R. conorii* infection in B6 mice, the contribution of CD8 T cells to innate resistance has been demonstrated with a variety of other pathogens, such as murine cytomegalovirus and *Listeria monocytogenes* (59–61). The depletion of NK cells enhances the susceptibility of mice to *R. conorii* infection in a background in which acquired immunity is intact (62). Further investigations using $Rag^{-/-}$ mice revealed that NK cells, as the major effector cells of innate immunity, mediate host resistance against rickettsial infection independently of B and T cells via modulation of IFN-γ (58). Furthermore, NK cells contribute to the prevention of vascular damage by secreting perforin.

Dendritic Cells

As the most potent professional antigen-presenting cells, dendritic cells have a crucial role in bridging innate and adaptive immune responses against rickettsial diseases. In contrast with other intracellular pathogens, such as *Leishmania*, rickettsiae efficiently infect bone marrow–derived dendritic cells (BMDCs) (63). After undergoing phagocytosis, rickettsiae are localized both in phagosomes and in the cytosol of BMDCs compared with residing freely in the cytosol of endothelial cells. The distinct intracellular location of rickettsiae both in cytosol and vacuoles in BMDCs may be the basis for processing and presenting rickettsial antigen to both CD4 and CD8 T cells through, respectively, the major histocompatibility complex (MHC) class II and MHC class I pathways. Indeed, *R. conorii* drives the maturation of BMDCs, which prime both naive CD4 and CD8 T cells though T cell receptor stimulation (signal 1), costimulatory molecules (signal 2), and cytokines (signal 3). Rickettsiae-infected dendritic cells activate naive CD8 T cells in vitro. Rickettsiae activate dendritic cells through mechanisms involving Toll-like receptor 4 (TLR4) and MyD88 (64, 65), both of which have critical roles in mediating host protection in vivo. The adoptive transfer of rickettsiae-stimulated dendritic cells provides host protection against an ordinarily lethal challenge through increased activity of $CD4^+$, $CD8^+$, and NK cells (64, 66, 67).

Innate Immune Signaling

The first line by which host immune cells recognize the invasion of pathogens is pattern recognition receptors (PRRs), which perceive molecular signatures characteristic of a whole class of microbes, termed pathogen-associated (or microbe-associated) molecular patterns or PAMPs (68). PRRs

are germline-encoded receptors, including transmembrane TLRs, cytosolic nucleotide-binding oligomerization-domain-(Nod)-like receptor (NLR) family proteins, and retinoic acid–inducible gene (RIG)-I (69). TLR4 signaling is one of the molecular mechanisms by which rickettsiae activate the host immune system. Among the important components of PRRs, TLRs trigger signaling through adaptor molecules, including MyD88 and Toll-receptor-associated activator of interferon (known as TRIF), mediating the production of inflammatory cytokines as well as host cellular immunity (70, 71). Lipopolysaccharide is one of the TLR4 ligands. Indeed, TLR4 activation has been reported to mediate host protection against other Gram-negative bacteria, such as *Salmonella typhimurium* (72). Mice naturally defective in TLR4 signaling (C3H/HeJ) succumb to a dose of *R. conorii* that is sublethal for TLR4-competent mice (63). The immune response attributed to TLR4 signaling during rickettsial infection in vivo is associated with greater concentrations of inflammatory cytokines (IFN-γ, IL-6, IL-12, and TNF-α) and increased expansion of activated NK cells, and CD4 and CD8 T lymphocytes. Further studies investigating dendritic cell maturation upon rickettsial infection in mice deficient in TLRs will reveal the molecular mechanisms by which dendritic cells polarize and activate NK cells and T lymphocytes (66, 67). In addition, *R. akari* triggers cell activation via TLR2 or TLR4 (73). Live and heat-killed *R. akari* cause phosphorylation of interleukin-1 receptor-associated kinase-1 (IRAK1) and p38 mitogen-activated protein kinase (MAPK) in 293/TLR4/MD-2 or 293/TLR2 stable cell lines, whereas only live bacteria elicit responses in TLR2- and TLR4-negative HEK293T cells. These results suggest that live *R. akari* are recognized by additional TLRs or NLRs, or both, in addition to TLR2 and TLR4.

Upon activation by microbes, TLRs transduce signals mostly via an adaptor molecule, MyD88. MyD88 controls downstream signaling of the IL-1R family and most TLRs, except for TLR3 and only partly for TLR4 (74). Our studies demonstrate that MyD88 is essential for host resistance to both *R. australis* and *R. conorii* (65). During rickettsial infection, MyD88 is required for inducing a protective inflammatory cytokine response consisting of IFN-γ, IL-6, and IL-1β, accompanied by inflammatory infiltrates of macrophages and neutrophils in vivo. In dendritic cells infected with *R. australis*, MyD88 is responsible for the increased expression of MHC class II^{high} and production of IL-12p40, which are the two major signals mediating the priming and educating CD4 T cells to differentiate into type 1 effector T cells.

As cytosolic bacteria, rickettsiae escape from the endocytic pathway and replicate in the host cytosol. It is hypothesized that rickettsiae not only evolve a variety of strategies to escape the host cytosolic surveillance systems but also activate innate immune responses through receptors in the cytosol. PRRs, including the membrane-bound receptors, such as TLRs, and the cytosolic receptors, such as NLRs, are the major sensors recognizing the invading pathogens. Inflammasomes are multiprotein signaling platforms that control the inflammatory response and coordinate host antimicrobial immunity. The inflammasome detects danger signals and infectious pathogens in the cytosol through NLRs and subsequently activates caspase-1 (75). In turn, activated caspase-1 cleaves pro-IL-1β/IL-18 into biologically functional IL-1β/IL-18, which is released extracellularly. Our research has demonstrated that the inflammasome is activated by *R. australis* in both mouse and human macrophages (76). Although NLRP3 significantly contributes to cytosolic sensing of *R. australis* in mouse macrophages, ASC is essential for inducing inflammasome activation by these bacteria. Interestingly, further in vivo studies suggest a minimal contribution of the NLRP3 inflammasome to host clearance of *R. australis* in tissues of infected animals. In BMDCs, *R. australis* induces MyD88-dependent IL-1β secretion, suggesting that MyD88-dependent TLR signaling mediates inflammasome activation. In line with these in vitro results, the levels of IL-1β in the serum of *R. australis*–infected mice are also MyD88 dependent. Our studies have further demonstrated that MyD88 signaling mediates the secretion of IL-1β and type 1 immune cytokines, which may account for the protective inflammatory response during rickettsial infection.

Furthermore, compared with other intracellular pathogens, *R. australis* activates the inflammasome via a distinct kinetic mechanism. In mouse macrophages, the inflammasome is activated by *Listeria* at 5 h post-infection (p.i.) (77), by *Shigella* at 6 h p.i. (78), by *Burkholderia* at 4 h p.i. (79), and by *Francisella* at 5 h p.i. (80), as detected by secretion of IL-1β and IL-18. In mouse macrophages, *R. australis* activates the inflammasome as late as 8 h after a high dose of infection and at 12 h after a low dose of infection, resulting in the secretion of IL-1β and IL-18. Regardless of the dose, the levels of inflammasome activation by *R. australis* increase progressively as the infection progresses and reach a peak at 24 h p.i. Apparently, the activation of the inflammasome by rickettsiae in macrophages is significantly delayed compared with the several facultative cytosolic bacteria mentioned above. These results suggest that *R. australis* employs a strategy to evade the early activation of the inflammasome. Although it is not yet known how rickettsiae escape the early activation of the inflammasome, it is likely that rickettsiae might benefit from delaying the cytosolic recognition by the inflammasome to establish their infection niche in the cytosol and further cause disease. The inflammasomes responsible for recognizing these intracellular bacteria at a late stage of infection might be ideal candidates for future vaccine development targeting inflammasome activation.

Acquired Immunity Against Rickettsiae and CD8 T Cells

MHC class I, IFN-γ, and CD8 T cells are well described as the major effectors of effective adaptive immune response to rickettsial infection (48). Mice deficient in MHC class I are 50,000-fold more susceptible to a lethal infection with *R. australis* than wild-type B6 mice. Rollwagen et al. (81) showed that MHC class I–matched, but not MHC class II–matched, *R. typhi*-infected fibroblasts are targets of immune T lymphocyte–mediated cytotoxicity. In line with these findings, splenocytes from mice immune to *R. conorii* elicit cytotoxicity on specific MHC class I–matched *R. conorii*–infected SVEC-1 endothelial cells, while splenocytes from mice immune to *R. australis* exert cytotoxic activity against an MHC class I–matched *R. australis*–infected macrophage-like cell line (48). These results suggest that the MHC class I–matched cytotoxicity of CD8 T lymphocytes has an important role in host immunity against rickettsiae. The critical role for CD8 T lymphocytes in clearing rickettsial infections has also been demonstrated by CD8 T cell depletion, immune CD8 T cell adoptive transfer, and experiments in mice with knockout of selected immune response genes (48, 82). Furthermore, following infection with *R. australis*, IFN-γ knockout mice are more than 100-fold more susceptible than wild-type mice, while perforin gene knockout mice are 1,000-fold more susceptible than wild-type mice (48). Thus, IFN-γ does not account for all of the antirickettsial activity mediated by CD8 T cells. Indeed, the adoptive transfer of immune CD8 T lymphocytes from IFN-γ knockout mice into *R. australis*–infected IFN-γ knockout mice dramatically reduced the infectious rickettsial content in the organs, indicating that cytotoxic activity is an effective mechanism of rickettsial elimination. Thus, in addition to perforin-mediated cytotoxicity, other mechanisms such as the release of granulysin or Fas/Fas ligand interactions could also be actively involved in CD8 T cell–mediated cytotoxicity in clearing rickettsiae. The clearance of *R. australis* by CD8 T cells is mediated at least partly by cytotoxic apoptotic elimination of *R. australis*–infected endothelial cells (48).

Immunosuppression and CD4 T Cells

CD8 cell depletion enhances the susceptibility of the murine host to *R. conorii* infection, while depletion of CD4 cells has no effect on the outcome of infection. Upon infection with 0.07 of the ordinarily median lethal dose of rickettsiae, both CD4 T cell–depleted and sham depleted mice become ill, but they clear the infection by day 10 (82). Our studies have demonstrated that

IFN-γ produced by CD4 T cells is important in host protection, as evidenced by the observation that a greater percentage of IFN-γ-producing CD4 T cells correlates with protective immunity in mice with a sublethal infection compared with mice infected with a lethal dose of *R. conorii* (83). Furthermore, we employed susceptible and resistant mouse models of rickettsial infection to investigate the immune mechanisms involved in the pathogenesis of severe rickettsioses. In dendritic cells from both resistant B6 mice and susceptible C3H mice, *R. conorii* infection in vitro upregulates their expression levels of maturation markers, including MHC class II, MHC class I, CD40, CD86, and CD80 (63). However, dendritic cells from susceptible mice express a significantly lower level of MHC class II and a significantly higher level of CD40 compared with resistant mice. The distinct expression pattern of dendritic cell maturation markers is likely associated with host resistance versus susceptibility to severe rickettsial disease. *R. conorii*–infected susceptible dendritic cells prime CD4 T cells with delayed activation kinetics as well as significantly impairing IFN-γ production. Strikingly, these in vitro findings are further confirmed in vivo by the experimental observation that an immunosuppressed CD4 T cell response is a component of the immune pathogenesis of severe rickettsioses (83). The impaired host immune response in a lethal model of rickettsial infection was characterized by suppressed CD4 T cell proliferation, significantly reduced levels of systemic and splenic CD4 T cell–specific IFN-γ, as well as high levels of IL-10. CD4 T cells from mice infected with a sublethal dose of *R. conorii* undergo proliferation in response to in vitro polyclonal and *Rickettsia*-specific stimulation. In contrast, lethal rickettsial infection results in suppressed CD4 T cell proliferation or even a lack of responsiveness to T cell receptor and rickettsial stimuli compared with the proliferation of CD4 T cells from uninfected and sublethally infected mice. The suppressed $CD4^+$ T cell proliferation in lethal infection is linked to the failure to produce IL-2. Our studies suggest that CD4 T cell anergy and suppressed CD4 T cell response play critical parts in severe or even fatal rickettsial diseases. Interestingly, human rickettsial infection has been characterized as having a dysregulated immune response, including a reduction in peripheral CD4 T lymphocytes (84). Our studies have further demonstrated that $CD4^+CD25^+$Foxp3-inducible T regulatory cells are most likely responsible for inducing immunosuppression during fatal rickettsioses.

RICKETTSIA–HOST CELL INTERACTIONS

Pathogenic rickettsiae replicate in many different cell types, including monocytes, macrophages, and hepatocytes, but preferentially target the vascular endothelial cell lining of small- and medium-sized vessels as places to multiply and establish infection in their mammalian hosts. Consequently, interactions of pathogenic rickettsiae with vascular endothelium constitute the major determinants of the mechanisms underlying rickettsial pathogenesis, host defense and immunity, and the manifestations as well as complications of disease. As such, the pathogenesis of rickettsial infections primarily involves a series of events leading to vascular damage and dysfunction, characterized by significant changes in the expression of hemostatic proteins (29), altered vascular permeability that causes pulmonary and cerebral edema (85), and in severe cases, abnormalities of the coagulation system (86).

Adhesion to and Entry into Host Cells

As obligately intracellular bacteria, rickettsiae need to adhere to and invade target cells to gain access to a nutrient-rich cytoplasmic niche, which facilitates their proliferation, replication, and dissemination, thus leading to the successful establishment of infection in the host. Analyses of rickettsial genomes utilizing bioinformatics-based approaches have identified multiple surface cell

antigens (Sca) belonging to the family of rickettsial autotransporters and involved in adhesion to host cell receptors. Although 17 Sca proteins have been identified, only 5—namely, Sca0 (outer membrane protein A, or OmpA), Sca1, Sca2, Sca4, and Sca5 (OmpB)—are active and functional in the SFG. The Sca proteins are localized within the surface layer of the rickettsial membrane and aid in adhesion to the host cell. Among these, OmpA and OmpB are conserved throughout the SFG and are thought to be fundamental to pathogenesis, whereas OmpB, which is conserved in all *Rickettsia* species except *R. canadensis*, plays a major part in the TG species (87–89).

OmpA of *R. rickettsii* is expressed as a 247-kDa protein, while OmpA of *R. conorii* is approximately 224 kDa (90) and is further processed to a 190-kDa protein. The OmpB protein is composed of three different domains, namely signal peptide, passenger domain, and a β-barrel transmembrane domain. OmpB, expressed as a 168-kDa protein, is also proteolytically processed into a 135-kDa passenger domain and a 32-kDa β-barrel domain (91). The signal peptide translocates the protein across the inner membrane and deposits it into the periplasmic space, and the passenger domain remains attached to the outer membrane. Following this event, the β-barrel transmembrane domain implants into the bacterial outer membrane to form a pore, the mechanism for which is not yet fully elucidated. The passenger domain is then fed through the β-barrel pore and subsequently cleaved (90). The first identified host cell–specific receptor, Ku70 (a subunit of a nuclear DNA-dependent protein kinase with subcellular localization in the cytoplasm and plasma membrane), interacts with rickettsial OmpB (92). Ubiquitination of the cholesterol-rich microdomains containing Ku70, which is mediated by the recruitment of the ubiquitin ligase c-Cbl, is required for internalization of rickettsiae into host cells (92). Ku70–OmpB interactions and clathrin and caveolin-2 contribute to endocytosis and rickettsial internalization (92). OmpA was initially shown to be critical for *R. rickettsii* adhesion to host cells (93), and later it was suggested that rickettsial OmpA interacts with $\alpha 2\beta 1$ integrin to promote invasion of the bacteria into the host cells (94). Recent work has further demonstrated that OmpA is not critical for virulence in the guinea pig model, but it may play a part in the survival of *R. rickettsii* or transmission from the tick vector (95).

Autotransporter Sca1 is present in all rickettsial species; full length Sca1 is expressed in *R. conorii* and *R. typhi*, while it is split in *R. prowazekii* and *R. canadensis* and presents as a pseudogene (88, 89, 96). Sca1 is localized on the bacterial membrane and contains a β-barrel transmembrane domain similar to OmpA and OmpB (87). Sca1 has also been implicated in rickettsial attachment to mammalian host cells, with no demonstrated role in invasion (96).

Another autotransporter, Sca2, which is highly conserved in SFG *Rickettsia* (97), is fragmented in *R. prowazekii* (87–89). Initially, *R. typhi* was reported to have a fragmented *sca2*, but later data showed that a complete *sca2* is expressed (88, 98). Sca2 in *R. conorii* is expressed as a 150-kDa protein consisting of a short N-terminal signal sequence, a passenger domain, and an autotransporter domain. In *R. parkeri*, *R. typhi*, *R. prowazekii*, and *R. bellii*, Sca2 contains five Wasp (Wiskott–Aldrich syndrome protein) homology-2 (WH2) domains, and their location is different than in most SFG rickettsiae. Sca2 mediates adherence to and invasion of target host cells (97). In mammals, the receptors that interact with Sca1 and Sca2 are unknown.

Sca4, another surface antigen, is present in all rickettsial groups; initial sequencing data demonstrated that *Sca4* was split into two open reading frames, but later, independent sequencing of this gene in *R. prowazekii* Madrid E revealed a complete sequence (UniProt number Q9ZD49.2) (87). Sca4 is complete in the virulent strain of *R. prowazekii* Rp22 (88). Sca4 is expressed *R. bellii*, but appears to be absent in *R. canadensis* (87). Sca4 is active in L929 cells infected with *R. typhi* in vitro and in cat fleas in vivo (88). Sca4 lacks an autotransporter domain but is still transported to the rickettsial surface by an unknown mechanism (87, 99). Surface antigen Sca4 colocalizes with and activates host vinculin at focal adhesion sites, suggesting a potential role in host cell invasion (100).

Another host protein, exchange protein directly activated by cyclic AMP (Epac), is also involved in rickettsial entry into host endothelial cells, and inhibiting Epac using a small molecule inhibitor significantly diminishes morbidity and mortality in a murine model of infection with *R. australis*, the causative agent of Queensland tick typhus (101). More recently, yet another host cell receptor, fibroblast growth factor receptor-1 (FGFR1), has been implicated in the internalization of SFG rickettsiae and shown to interact with rickettsial OmpA in a caveolin-1-dependent manner (102). Mass spectrometry and biochemical techniques reveal that a 32-kDa fragment of OmpA interacts with FGFR1, mediating the internalization of SFG rickettsiae into host endothelium (102). Previously, OmpB was shown to be proteolytically processed at the C-terminal end of the protein, cleaving the 168-kDa precursor into 135-kDa and 32-kDa fragments, implying that the 32-kDa fragment functions as the membrane anchor domain (91). Similarly, a recent study has provided evidence for posttranslational processing of OmpA, revealing a 32-kDa fragment at the C-terminal end of the protein during surface proteome analysis (103). Once rickettsiae are intracellular, they escape from phagosomes prior to their fusion with lysosomes to free themselves into the cytosol. This is accomplished through a number of membranolytic activities, including the release of phospholipase A_2, phospholipase D, and hemolysin C (86). Due to their absolute dependence on the host's intracellular machinery for replication and spread, it is not surprising that *Rickettsia* species exploit redundant mechanisms for host cell adhesion and invasion (**Figure 2**).

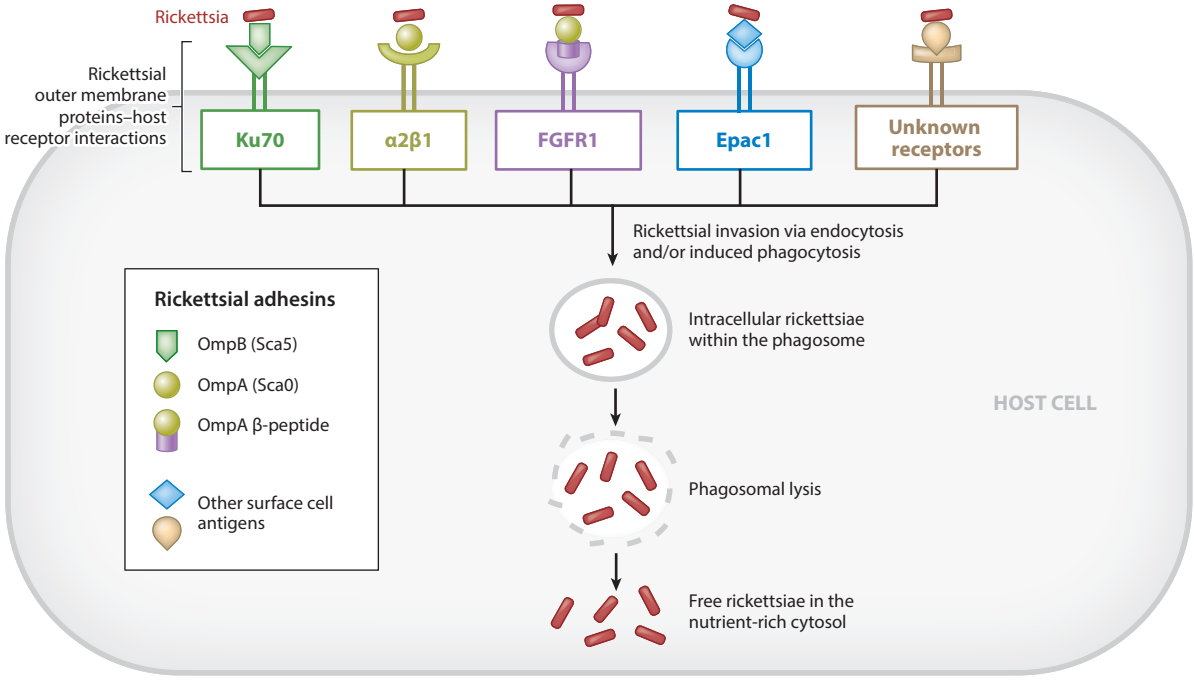

Figure 2

A model of rickettsial invasion into host cells. The mammalian receptors interact with rickettsial outer membrane proteins, resulting in the internalization of rickettsiae into the cells via endocytosis or induced phagocytosis, or both. Internalized rickettsiae quickly escape from the phagosomes using membranolytic activities to establish free—that is, nonvacuole-bound—parasites in the cytosol.
Abbreviations: Epac1, exchange protein directly activated by cyclic AMP-1; FGFR1, fibroblast growth factor receptor-1; Omp, outer membrane protein; Sca, surface cell antigen.

Actin-Based Motility

After invasion, rapid microbial dissemination between host cells is a critical step for spreading intracellular bacterial pathogens and involves a phenomenon called actin-based motility. Bacterial motility between the cells requires the polymerization of actin filaments derived from the host's cytoskeleton to form the typical F-actin comet tail. Host-mediated motility allows *Rickettsia* to move from cell to cell while avoiding the immune system of the host. Using dual fluorescence staining, polar actin tail formation was first observed in SFG rickettsiae within 30 min p.i. (104). Double fluorescence labeling of F-actin revealed comet-like structures extending from a polar end of *R. conorii*. In contrast, the formation of actin tails was observed infrequently in *R. typhi*. Also, it was shown that *R. conorii* were randomly dispersed throughout the cell, whereas *R. typhi* remained confined to the same location (105). *R. prowazekii* does not produce actin tails in comparison with the short tails produced by *R. typhi*. Actin-based motility has been documented in all SFG rickettsiae except *R. peacockii*.

Host cell actin polymerization also has an important role in rickettsial internalization, as indicated by the potential contributions of upstream signaling mechanisms involving Cdc42 (a GTPase), phosphoinositide 3-kinase, c-Src, and possibly other protein tyrosine kinases that result in the activation of the Arp2/Arp3 complex (106). RickA, a WASP, is sufficient to direct actin-based motility via activation of actin nucleation and recruitment of the Arp2/Arp3 complex (107, 108). RickA (RC0909) is found in *R. conorii*, but it is not present in *R. prowazekii* (107). *R. bellii*, an ancestral group (AG) member, encodes an ortholog of RickA and forms actin tails for movement into the host cell nucleus. Upon entering the nucleus, the bacterium becomes trapped due to the lack of the Arp2/Arp3 complex required for actin tail formation. *R. peacockii*, an SFG species, lacks actin-based motility due to the disruption of the *rickA* gene (109). TRG members *R. australis* (104) and *R. felis* (110) both form actin tails, and RickA expression in *R. raoultii* has been demonstrated by Western blotting (108). These findings demonstrate the presence of actin-based motility in *Rickettsia* belonging to different groups (SFG, TRG, and AG), with the exception of *R. prowazekii*, and illustrate that actin-based motility represents an important lifestyle feature for the genus *Rickettsia*, which may have been lost during the course of evolution in *R. prowazekii* while being retained in the other rickettsiae.

Interestingly, further research on this topic has revealed biphasic rickettsial motility with distinct movement parameters (111). Motility is slow and meandering right after invasion (within 15–30 min) due partly to the short, curved actin tails, which consist of the Arp2/Arp3 complex and cofilin, and require the activities of RickA and the Arp2/Arp3 complex (111). In contrast, motility is faster and directionally persistent later in the infection (within 24–48 h), resulting in longer, straight actin tails. In the late phase, motility is independent of the Arp2/Arp3 complex and RickA, and it requires rickettsial Sca2 protein. The N-terminal domain of Sca2 is predicted to share secondary structural similarity with the formin homology-2 (FH2) domains of formins (112), and a central region in Sca2 has amino acid similarity to formin homology-1 (FH1) domains (113). Sca2 also has three WH2 domains, which may bind to actin monomers (112, 113). Sca2 nucleates the assembly of linear actin filaments and promotes filament elongation by using profilin and inhibiting the activity of capping proteins (114). A number of SFG and TRG species, including *R. africae*, *R. akari*, *R. australis*, *R. conorii*, and *R. rickettsii*, encode full-length Sca2 that contains an N-terminal Sec-dependent secretion signal, four putative WH2 domains, proline-rich regions (similar to formins), and an autotransporter domain in the C terminus. In contrast, Sca2 from *R. prowazekii* is truncated, lacking the first three WH2 domains, the proline-rich segments, and the secretory signal sequence. Sca2 from *R. typhi* is shortened, with overlapping autotransporter and WH2 domains, but a divergent FH1 domain and secretion signal (113). The *R. prowazekii*

effector RalF also contains a proline-rich region that can serve as a formin modulator to regulate actin polymerization (115). These findings suggest that SFG rickettsiae utilize the eukaryotic formin-like properties of Sca2 as a primary mechanism for moving intracellularly and spreading from cell to cell.

Signal Transduction Events

An in vitro model of infection of cultured endothelial cells with *R. rickettsii* demonstrated early cell-to-cell spread without noticeable host cell injury that was accompanied by membrane damage and led to cell death. *R. rickettsii* and *R. conorii* infection of endothelial cells results in increased expression of tissue factor, IL-1α, intercellular and vascular cell adhesion molecules, thrombomodulin, and E-selectin; increased synthesis of plasminogen activator inhibitor-1; von Willebrand factor release from Weibel–Palade bodies; and altered adhesiveness of platelets to the endothelial cell surface (86). Infection of cultured cells with *R. prowazekii* leads to increased secretion of the prostaglandins PGE2 and PGI2 (116), enhanced synthesis of platelet activating factor (117), and the transmigration of leukocytes (118). *R. rickettsii* and *R. conorii* infection in vitro also activates the fibrinolytic system, leading to elevated levels of plasma fibrin(ogen) degradation products (119).

The mechanisms underlying early host cell responses to rickettsial infection are mediated by the activation of nuclear transcription factor κB (NF-κB), MAPK pathways, and phosphorylation–dephosphorylation mechanisms, leading to the activation of protein kinase pathways. Infection of vascular endothelial cells with *R. rickettsii* or *R. conorii* results in the activation of NF-κB and p38 MAPK and activating transcription factor-2, as well as the expression and secretion of several cytokines and chemokines (120, 121). Infection of endothelial cells with *R. rickettsii* or *R. conorii* stimulates the secretion of high levels of IL-6 and IL-8, increases the expression of adhesion molecules on the cell surface, and increases the adhesion of leukocytes in an IL-1α pathway–dependent manner (122). *R. conorii* infection of endothelium induces the expression of chemokines, namely CCL2, CCL3, CCL4, CCL5, CCL12, CCL19, CCL21, CX3CL1, CXCL1, CXCL9, and CXCL10 (123). The genes expressed in response to early rickettsial infection include those encoding for the proteins IL-8 and monocyte chemoattractant protein, both of which contain NF-κB binding sites in their promoter regions, indicating that infection-induced alterations in gene expression may be regulated partly by activation of NF-κB (124). Apparently, *R. rickettsii* is also capable of directly interacting with NF-κB in the cytoplasm by an unidentified bacterial protease activity (125). Rickettsiae also manipulate host cell–death mechanisms, as illustrated by the apoptosis of infected cells consequent to the inhibition of infection-induced activation of NF-κB (124). Follow-up studies to identify additional upstream signaling mechanisms during *R. rickettsii* infection of cultured human endothelial cells revealed an essential role for NF-κB activation in maintaining host cell mitochondrial integrity, maintaining the balance between pro- and antiapoptotic proteins, and preventing activation of the caspase cascade (126, 127).

The activation of various STAT proteins is required for the antiviral activities of interferons. Infection of endothelial cells by *R. rickettsii* and *R. conorii* triggers differential activation of STAT proteins (128, 129). Notably, STAT1 activation is mainly attributed to the secretion of IFN-β by infected endothelium, and IFN-β-mediated inhibition of rickettsial replication in host cells depends on STAT 1 (129). Because the transcription enhancer of IFN-β promoter binds to NF-κB, ATF-2, and the interferon regulatory factors IRF3 and IRF7 (128), *Rickettsia*-induced IFN-β could be the outcome of one specific mechanism or a combination of mechanisms. In this regard, although there is no evidence for serine 396 phosphorylation as an indicator of IRF3 activation (129), there is increased expression of IRF7 and IRF9, suggesting a possible role for either one or potentially both of these interferon regulatory factors during *R. conorii* infection.

An analysis of downstream targets regulated by the JAK–STAT pathway further revealed that *R. conorii* infection induces the expression of interferon-regulated genes, such as interferon stimulated gene 15 (*ISG15*), oligoadenylate synthetase 1 (*OAS1*), and guanylate binding protein 1 (*GBP1*) (130). Among these, ISG15 is mainly induced by type 1 interferons, whereas GBP1 and OAS1 are stimulated by both type 1 and type 2 interferons. Intriguingly, there is unequivocal evidence for the robust expression of *ISG15* mRNA and protein in *R. conorii*–infected endothelial cells, providing solid and novel evidence for its participation in posttranslational modification of other cellular proteins via ISGylation (130, 131). Since ISG15 has been implicated in the regulation of antiviral immune responses, it will be highly informative to determine its role (or roles) in bacterial infections of vascular endothelial cells.

R. rickettsii infection of endothelial cells causes extensive membrane damage that leads to changes in the cytoskeleton and suggests the involvement of oxidative stress in the pathogenesis of rickettsioses. Infected endothelial cells undergo oxidative stress, with the accumulation of intracellular superoxide anion (O_2^-) and extracellular hydrogen peroxide and with marked reductions in both the levels of the protective intracellular thiols and activities of antioxidant enzymes (132). Further, in patients, genetic deficiency of the enzyme glucose-6-phosphate dehydrogenase results in more severe rickettsioses, suggesting the involvement of redox systems in combating the disease (43, 133). In this regard, higher expression of heme oxygenase (HO)-1, a rate-limiting enzyme in heme catabolism that has established antioxidant functions, is an important cellular response to oxidative stress caused by the infection (134, 135). Bilirubin and carbon monoxide, generated as end products of HO-1 activity, decrease oxidative stress, reduce vascular constriction, attenuate inflammation, and inhibit apoptosis. Another important function of the heme–HO system is to influence the synthesis and secretion of prostaglandins by regulating vascular cyclooxygenase (COX) activity. Marked induction of COX-2 and increased secretion of PGE2 and PGI2 occur in rickettsial infection of endothelial cells, and the biphasic increase in COX-2 expression is similar to the activation of NF-κB (116, 136). The induction of COX-2 and release of prostaglandin in the vasculature may also contribute to increased vascular permeability during *Rickettsia* infection.

Noncoding RNAs

The term noncoding RNAs refers to those that do not encode a protein, and it has been assumed that most genetic information is transacted by proteins. However, recent evidence suggests that a significant portion of the genomes of prokaryotes and eukaryotes is transcribed into noncoding RNAs, many of which are alternatively spliced or processed into smaller products. Noncoding RNAs include microRNAs (miRNAs), small RNAs (sRNAs), long noncoding RNAs, small interfering RNAs, and small nucleolar RNAs. Noncoding RNAs are generally involved in regulating gene expression at the transcriptional and posttranscriptional levels. Small RNAs (sRNAs) include true antisense RNAs synthesized from the strand complementary to the mRNA that they regulate, sRNAs with limited complementarity with their targets, and sRNAs that bind to proteins and regulate protein activity. The sRNAs with limited complementarity are similar to eukaryotic miRNAs in their ability to modulate the activity and stability of multiple mRNAs. Recently, differentially expressed miRNAs have been identified in *Rickettsia*-infected endothelial cells (137).

MicroRNAs

miRNAs are single-stranded, typically 21 to 23 nucleotides long, noncoding RNAs that are expressed by eukaryotic cells and that function by degrading or suppressing protein translation based on sequence complementarity between the miRNA and the targeted mRNA. As novel

posttranscriptional regulators of gene expression, miRNAs have also been implicated as important determinants of responses during normal and pathogenic immunological responses (138). The therapeutic applications of miRNAs include a novel anti-miRNA treatment, miravirsen, currently in Phase IIb clinical trials, and viral vaccines in the preclinical testing phase in which the virus is attenuated through the incorporation of miRNA target sequences; miRNAs also hold substantial promise for use as biomarkers of infection.

It is now well established that one of the strategies adopted by bacterial pathogens to survive inside host cells is to modulate the miRNAs associated with biological processes. Pathogens alter the expression pattern of cellular miRNAs during infection, and it has been experimentally validated that intracellular bacterial pathogens—such as *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis*, and others—have the ability to alter the host's cellular miRNAs for their own survival (139). A recently published study has documented the differential expression of several miRNAs in endothelial cells infected with *R. rickettsii* (137). Specifically, miRNA 200a-3p is highly upregulated both in infected endothelial cells in vitro and in vivo in the lungs of *R. conorii*–infected mice. These findings further suggest that there is inhibitory cross talk between miR-200a-3p and NOTCH1 expression, which indicates a potentially important role for this interrelationship in rickettsial biology because NOTCH1 signaling, along with other signaling mechanisms, regulates endothelial proliferation and angiogenesis. Ongoing studies of mechanisms of altered endothelial miRNA expression are expected to provide new insights into the regulatory elements governing host responses and pathogenesis during human rickettsial infections.

Small RNAs

Bacterial sRNAs are typically 50- to 500-bp-long noncoding RNAs and comprise three different types, namely riboswitches, which are located upstream of mRNAs; *cis*-acting sRNAs, synthesized from the complementary strand of an open reading frame; and *trans*-acting sRNAs, transcribed from the intergenic regions, with only partial complementarity to their target genes. Bacterial sRNAs can activate or inhibit translation either by stabilizing the mRNA and opening the ribosome binding site or by degrading the target mRNA, respectively (140). *R. prowazekii* carries a large portion (approximately 24%) of noncoding DNA and an adenine and thymine–rich genome with a guanine and cytosine content of 29.1% (141). A number of sRNAs were predicted in rickettsial genomes using complementary computational approaches (142). An analysis utilizing next-generation sequencing identified 35 novel *trans*-acting and 23 *cis*-acting sRNAs, and the expression of four novel sRNAs was confirmed by Northern blotting (143). The transcription start sites of five novel rickettsial sRNAs were also determined. These were further characterized using computational approaches to determine the location of −10 and −35 promoter motifs, secondary structure, and potential targets (143). These noncoding RNAs are hypothesized to be potentially critical posttranscriptional regulators involved in bacterial virulence, survival, stress response, metabolism, and other pathways. A recent report has further demonstrated the sRNA expression profile of *R. prowazekii* during infection of arthropod host cells in vitro (144). An analysis of the *R. prowazekii* transcriptome by strand-specific RNA sequencing has identified 67 *cis*-acting (antisense) and 26 *trans*-acting (intergenic) sRNAs expressed during the infection of *Amblyomma americanum* tick (AAE2) cells. Comparative expression during *R. prowazekii* infection of endothelial cells and AAE2 cells demonstrated significantly higher expression of four selected sRNAs in the AAE2 cells. Examination of the coding transcriptome revealed differential upregulation of more than 150 rickettsial genes in both the endothelial cells and AAE2 cells and yielded evidence for host cell–dependent utilization of alternative transcription start sites by 18 rickettsial genes

(144). These results suggest that there are differences in the expression of sRNAs, as well as in the coding transcriptome, during the infection of human endothelial cells and arthropod cells, and these results offer new insights into rickettsial virulence and transmission mechanisms. Investigations are under way to further elucidate the importance of these novel sRNAs in regulating rickettsial pathogenesis.

Strand-specific RNA sequencing of infected endothelial cells has also identified 4 riboswitches and 13 *trans*-acting and 22 *cis*-acting sRNAs in *R. conorii* (145). The expression of four selected novel *trans*-acting sRNAs (*Rc*_sR31, *Rc*_sR33, *Rc*_sR35, and *Rc*_sR42) was further confirmed by Northern hybridization. Comparative analysis during the infection of endothelial cells and AAE2 cells revealed significantly higher expression of *Rc*_sR35 and *Rc*_sR42 in endothelial cells, whereas *Rc*_sR31 and *Rc*_sR33 were expressed at similar levels in both cell types. Further, bioinformatics analysis predicted that several genes are involved in important biological processes as potential targets of *Rc*_sRs, and the interaction of *Rc*_sR42 with cytochrome d ubiquinol oxidase subunit I (*cydA*) was validated (145). *Rc*_sR42 could potentially be involved either in stabilizing the *cydA* transcript as a result of the cleavage of the polycistronic *cydAB* transcript or in the degradation of the *cydAB* transcript by forming double-stranded RNA. Rickettsiae are capable of synthesizing ATP during later stages of infection when the ATP supply in the host cytosol is exhausted. Significantly higher expression of *Rc*_sR42 at 24 h p.i. likely suggests that *cydA* mRNA is regulated at later stages of infection, which may facilitate the survival of the bacteria in the intracellular niche. Differentially expressed sRNAs during vertebrate host–pathogen and vector–pathogen interactions suggest their role in survival and transovarial and transstadial transmission in arthropod vectors and in regulating virulence in the human host. Comprehensive molecular studies employing appropriate heterologous model systems to generate knockout mutants may reveal the functional implications of these regulatory sRNAs during rickettsial pathogenesis. The identification of novel immune evasion strategies employed by bacterial sRNAs will significantly contribute to understanding the pathogenesis of infection and designing new and effective therapeutic approaches to combat diseases caused by intracellular pathogens.

CONCLUSIONS

Despite the small number of scientists pursuing knowledge of these extremely recalcitrant bacteria, the findings continue to surprise researchers and emphasize the rich opportunities for scientific discovery that the field offers.

SUMMARY POINTS

1. Arthropod-borne pathogenic *Rickettsia* species capable of causing significant morbidity and mortality in humans remain a major health issue across the globe.
2. The major rickettsial surface proteins outer membrane protein A (OmpA), OmpB, and the β peptide of OmpA interact with the host receptors $\alpha 2\beta 1$, Ku70, and fibroblast growth factor receptor-1 (FGFR1), respectively, for internalization, indicating the strategic importance of redundant entry mechanisms for achieving an intracytosolic niche in the host cells.
3. Spotted fever group rickettsiae form polar actin tails and exploit biphasic actin-based motility for intracellular movements and intercellular spread. The representative typhus group rickettsiae either display erratic motility (*R. typhi*) or lack this activity (*R. prowazekii*).

4. Endothelial cells are the preferred target cells in humans and in vivo animal models of *Rickettsia* infection, and these cells display proadhesive, procoagulant, and proinflammatory properties. The antiapoptotic functions of infection-induced nuclear transcription factor κB either prevent or delay host cell death to allow rickettsiae to maintain their intracellular niche. *R. rickettsii* and *R. conorii* infection of endothelial cells trigger antiviral-like host responses including interferon (IFN)-β-mediated activation of JAK–STAT signaling and activation of downstream IFN-stimulated genes.
5. The infection-induced regulatory effects of certain host microRNAs (miRNAs) and the expression of bacterial small RNAs (sRNAs) have recently been demonstrated in *Rickettsia*-infected endothelial cells. Further in-depth investigations of the mechanisms of miRNA and sRNA expression in the host and invading pathogens, and their downstream regulatory effects, are expected to reveal insights into host–pathogen interactions and determinants of host responses during human rickettsioses. In concert with established fundamentals of rickettsial pathogenesis, this new knowledge will facilitate the development of new and improved diagnostics, additional therapeutic remedies, and preventive vaccines.
6. As cytosolic sensors, ASC- and caspase-1-dependent inflammasomes contribute to detecting intracellular rickettsiae and overall host protection in vivo. Compared with other cytosolic bacteria, rickettsiae activate the inflammasome with delayed kinetics. Identifying Nod-like receptors (NLRs) other than NLRP3 that are responsible for recognizing rickettsiae and gaining understanding of the delayed kinetic mechanisms modified by rickettsiae could contribute to the design of an inflammasome-targeted vaccine or therapeutic approach.
7. Toll-like receptor (TLR) 2, TLR4, and MyD88 signaling play a critical part in host protection against rickettsiae by mediating efficient antigen presentation by dendritic cells to immune effector cells of both the innate and adaptive immune systems.
8. In an overwhelming rickettsial infection, rickettsiae activate and differentiate CD4 T cells into both protective IFN-γ-producing T helper-1 cells and $CD4^+CD25^+$IL-10-producing T regulatory cells. These inducible T regulatory cells suppress the protective response and, at least in part, contribute to the pathogenesis of fatal rickettsioses.

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