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The committee for Jeffrey M. Jordan certifies that this is the approved version of the following dissertation:

THE ROLE OF DENDRITIC CELLS IN RICKETTSIAL INFECTION; INITIATION OF EARLY IMMUNITY TO SPOTTED FEVER GROUP RICKETTSIAE

Committee:

David H. Walker, MD, Supervisor

Lynn Soong, MD, PhD

D. Mark Estes, PhD

B. Mark Evers, MD

Abul Abbas, MD

Dean, Graduate School

THE ROLE OF DENDRITIC CELLS IN RICKETTSIAL INFECTION; INITIATION OF EARLY IMMUNITY TO SPOTTED FEVER GROUP RICKETTSIAE

by
Jeffrey M. Jordan, BS.

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Approved by the Supervisory Committee

David H. Walker, MD
D. Mark Estes, PhD
B. Mark Evers, MD
Lynn Soong, MD, PhD
Abul Abbas, MD

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Galveston, Texas

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To my Family:
For their constant support that made this work possible

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Jeffrey M. Jordan, MD, PhD
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Supervisor: David H. Walker

Identifying the role(s) dendritic cells play in rickettsial infection is important in determining the characteristics which elicit protective immunity. Previous data imply that Th1 responses are essential for immunity to rickettsiae; however, they do not address mechanisms important in initiating early immunity, particularly those involving dendritic cells. Dendritic cells are instrumental in initiation and control of a strong Th1 response towards invading pathogens. I hypothesize that cutaneous dendritic cells comprise an important initial target for rickettsial infection. Additionally, activation and migration of rickettsiae-infected dendritic cells to draining lymph nodes may be critical to activation of NK and CD8 T-cells. Lastly, preliminary data suggest that rickettsiae act as TLR4 agonists on dendritic cells, resulting in their activation. To elucidate the significance of dendritic cells in rickettsioses, I developed a model of dermal infection to demonstrate that cutaneous dendritic cells comprise an important initial target cell. Furthermore, I demonstrated that rickettsiae-infected dendritic cells were capable of inducing protective immunity in naïve mice to an ordinarily lethal challenge. This protection was correlated with significantly elevated levels of IFN-γ producing CD4 and CD8 T-cells as well as NK cells, indicating that rickettsiae-infected dendritic cells are capable of inducing protective Th1 responses and NK cell mobilization. Lastly, we demonstrated that mice defective in TLR4 signaling were more susceptible to lethal rickettsial infection. This susceptibility was correlated with a significant decrease in Th1 immune responses. Additionally, TLR4 was shown to be critical towards the generation of Th17 responses. TLR4 ligation in dendritic cells also appears important in augmenting NK cell activation *in vivo*.

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LIST OF ABBREVIATIONS

2-ME	2-β-mercaptoethanol
Ab	Antibody
APC	Antigen presenting cell
BBB	Blood-brain barrier
BCS	Bovine calf serum
BMDC	Bone-marrow-derived dendritic cell
CFSE	carboxy-fluorescein diacetate succinimidyl ester
CTL	Cytotoxic T-lymphocyte
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
ECGS	Endothelial cell growth supplement
E:T	Effector-to-target ratio
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneal
i.v.	Intravenous
KO	Gene knockout
LPS	Lipopolysacharide
mAb	Monoclonal antibody
MAPK	mitogen-activated protein kinase
MBEC	Mouse brain endothelial cells
MFI	Mean fluorescence intensity

moi	Multiplicity of infection
NGMA	NG-monomethyl-L-arginine
NHS	Normal horse serum
NK	Natural killer cell
NO	Nitric oxide
NOS2	Inducible nitric oxide synthase
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular pattern
PAMPR	Pathogen-associated molecular pattern receptor
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cells
PFU	Plaque forming units
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
plc	Phospholipase c
pld	Phospholipase d
OmpA	Outer membrane protein A
OmpB	Outer membrane protein B
RMSF	Rocky Mountain spotted fever
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SPG	Sucrose-phosphate-glutamic acid buffer
TCR	T-cell receptor
TLCK	N_{α}-toxyl-L-lysine chloromethyl ketone
TLR	Toll-like receptor
$TLR4^{(LPS\text{-}d)}$	Mice defective in TLR4 signaling (C3H/HeJ)
TNF	Tumor necrosis factor
YF17D	Yellow Fever 17D vaccine strain

CHAPTER 1: SPOTTED FEVER RICKETTSIOSES

Rickettsial diseases are widely distributed throughout the world, with human rickettsioses being described on every continent except Antarctica. *Rickettsia* are Gram-negative, obligate-intracellular zoonotic pathogens and are segregated into two antigenically distinct groups: The typhus group consists of *R. prowazekii* and *R. typhi*, the etiologic agents of epidemic typhus and murine typhus, respectively, and the spotted fever group is characterized by *R. rickettsii* and *R. conorii*, the etiologic agents of Rocky Mountain spotted fever and Mediterranean spotted fever (boutonuese fever), respectively. Additionally, numerous other spotted fever group rickettsiae have also been implicated in human disease. The reported annual incidence of Rocky Mountain spotted fever is typically about 2.2 cases per million people in the United States, although this trend has experienced a dramatic increase of over 300% in the last reportable year (2005) (1;2). In the United States in 2005, Rocky Mountain spotted fever accounted for at least 1,936 reported cases (2). These figures are further startling, due to the severe virulence of *R. rickettsii* (nearly 5% of previously healthy children and adults succumb to the disease despite treatment whereas 20-25% of adults succumb without appropriate antibiotic treatment) as well as the fact that rickettsioses are often under-reported.

SPOTTED FEVER RICKETTSIOSES

Rocky Mountain spotted fever (RMSF), the prototypical and most virulent of the spotted fever rickettsioses, was first observed by an Army physician, Marshall H. Wood, in Boise, Idaho in 1896 (3). Afterwards, RMSF was reported in the medical literature by Edward Ernest Maxey, who described human cases that occurred in the Snake River Valley in Idaho in 1899 (4). The initial important findings of RMSF were made by Wilson and Chowning in 1902 and published in 1904 (5). Wilson and Chowning closely observed seven fatal infections in the Bitter Root Valley in Montana while studying over 100 presumptive cases of RMSF. In their study, they witnessed the extreme virulence of infection, noting a 69% fatality rate and additionally hypothesized that the disease was

spread by the wood tick given the history of tick exposure in afflicted patients and seasonality of infection.

After Wilson and Chowning's initial observations, the etiologic agent of RMSF was described by the preeminent rickettsiologist, Howard T. Ricketts, in 1906 (6). In several noteworthy publications, Ricketts systematically elucidated important aspects of RMSF (7-10). Ricketts followed up on the hypothesis of Wilson and Chowning and demonstrated conclusively that the agent of RMSF could be transmitted to guinea pigs and monkeys by the wood tick; moreover, Ricketts demonstrated that rickettsiae could be seen in the freshly laid eggs of infected ticks and that the etiologic agent could be obtained from the blood of infected patients and also be removed by filtration.

Since the Ricketts' initial discovery, many other spotted fever rickettsioses have been described throughout the world (**Table 1.1**); notable are recently emergent pathogens, *R. africae, R. japonica, R. honei,* and *R. slovaca* (11). Nearly all spotted fever rickettsiae share similar vectors of transmission (an ixodid tick), with the notable exception of *R. akari* and *R. felis*, which are transmitted by a mite and flea, respectively, as well as similar manifestations of disease. However, despite extremely similar genomes, spotted fever rickettsiae possess differences in virulence and extent of pathologic lesions in patients. The most virulent spotted fever rickettsiae still remain *R. rickettsii*. The virulence and clinical pictures of other common spotted fever rickettsiae vary by species, suggesting that, notwithstanding significant genetic similarity, differences in unknown virulence factors or other factors such as vector or host susceptibility may influence pathogenesis.

***Rickettsia* Species.**	**Disease**	**Transmission to Humans**	**Geographic distribution**
R. rickettsii	Rocky Mountain spotted fever	Tick bite	North & South America
R. conorii	Mediterranean spotted fever	Tick bite	Africa, Southern Europe, Asia
R. sibirica	North Asia tick typhus	Tick bite	Eurasia, Africa
R. africae	African tick bite fever	Tick bite	Africa, Caribbean Islands
R. japonica	Japanese spotted fever	Tick bite	Japan
R. honei	Flinders Island spotted fever	Tick bite	Flinders Island, Tasmania
R. akari	Rickettsialpox	Mite bite	Worldwide
R. felis	Flea-borne spotted fever	Flea	Worldwide

Table 1.1: Spotted Fever Rickettsiae

Despite the fact that over one-hundred years have passed since the initial description of RMSF, Ricketts' elucidation of the etiologic agent, *R. rickettsii*, and others' subsequent vigorous study of numerous rickettsial pathogens, there still exist large gaps in knowledge of the virulence mechanisms of infection and the requirements necessary for protective immunity.

Epidemiology and Ecology of Spotted Fever Rickettsioses

Rickettsial diseases occur throughout tropical, subtropical and temperate regions of the world. Transmission of rickettsiae to humans is not a requirement for the maintenance of spotted fever group rickettsiae in nature as spotted group rickettsiae are maintained in various Ixodid tick or mite vectors through transovarial transmission as well as horizontal transmission to small animals. As a result, rickettsial diseases are classified as zoonoses, and the global distribution of spotted fever group rickettsiae is dictated by the availability of a small animal host and the blood-sucking arthropod important for transmission and maintenance of rickettsiae in nature. In the United States, *R. rickettsii* is primarily transmitted from the east coast to the central prairie states by the American dog tick, *Dermacentor variabilis* and in the west by the Rocky Mountain wood tick, *D. andersoni* (12). Additionally, *Amblyomma cajennense*, *A. aureolatum*, and *Rhipicephalus sanguineus* have also been implicated in transmission of *R. rickettsii* in Central and South America (13-15).

Rickettsiae infect ticks by feeding on the blood of infected animals or are passed transovarially. It is important to note that, although vertical transmission by maintenance of *R. rickettsii* within infected ovaries in ticks is known to occur, *R. rickettsii* infection eventually exacts a toll on the tick, decreasing both the survival of female engorged ticks and the ability to lay fertilized eggs (16). Therefore, horizontal transmission to small mammals is most-likely important in maintenance of *R. rickettsii* in nature (**Figure 1.1**).

In the arthropod host, rickettsiae reside in the salivary glands and are transmitted into the dermis in salivary secretions during tick feeding. Rickettsiae may be released from the salivary glands as soon as 6-10 hours after tick attachment, although transmission may take 24 hours or longer (12). Spotted fever rickettsial infections in humans occur when rickettsiae are transmitted through the bite of an infected tick, culminating in rickettsial infection of vascular endothelium. As a result of ixodid ticks being the vectors responsible for human transmission, reported cases of spotted fever rickettsiae coincide temporally with times when human activity brings them in close proximity with infected ticks, specifically late spring to early fall. The highest incidence of spotted fever group rickettsiae occurs in two groups: children aged 5-9 years old and older persons aged 40-64 years old (1). Despite its name, most cases of RMSF in the United States occur within the southeastern states, with the greatest number of cases occurring in North and South Carolina as well as Oklahoma, Arkansas, and Missouri (17); additionally, *R. rickettsii* is an important rickettsial disease in South America (18-20). In addition to the arthropod transmission cycle of human rickettsioses, aerosol infections have also been reported in the laboratory (21).

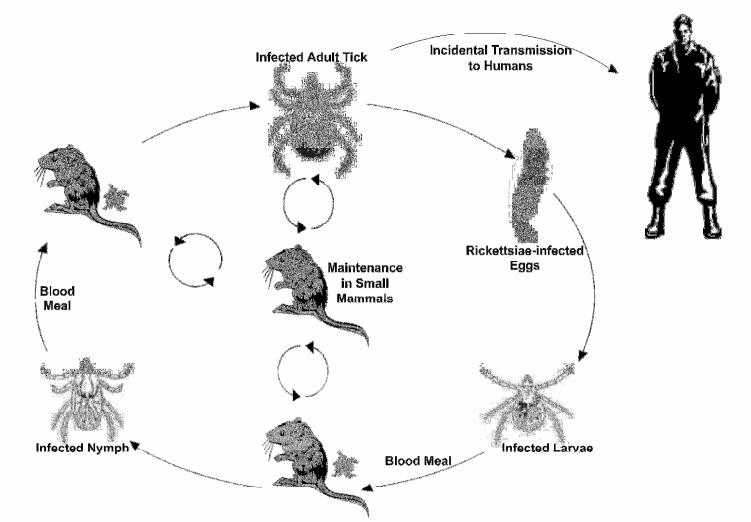


Figure 1.1: Transmission Cycle of Rocky Mountain spotted fever. *R. rickettsii* is maintained in ixodid ticks in nature transovarially as well as horizontal transmission of tick to small mammals to tick. Rickettsial infection of humans occurs incidentally.

Clinical Manifestations, Pathogenesis, and Diagnosis of Spotted Fever Rickettsioses

Clinical Manifestations

The early symptoms of rickettsial infection are similar to many other infections, particularly viral syndromes, making a clinical diagnosis difficult (22). Diagnosis is further complicated by the fact that tick bites in spotted fever rickettsioses are only reported about 60% of the time. Additionally, patients infected through the bite of an infected tick may or may not develop an eschar at the site of tick feeding, depending mainly on the particular rickettsial species. After a 7-10 day incubation period following the bite of an infected tick, the initial symptoms of rickettsioses are exemplified by fever, severe headache, myalgias, malaise, nausea, vomiting, and after several days a macular rash. It should be noted that although the aforementioned symptoms are present in a majority of the patients, numerous other non-specific symptoms may be present in a minority of patients. As the disease progresses in a patient, the macular rash may progress to a macular-papular rash and later to a petichial rash as rickettsial infection of the vascular endothelium progresses leading to extensive vascular damage.

Later in the course of infection more serious organ-specific symptoms occur due to widespread vascular damage. Clinically, patients may present with abdominal pain, presumably due to decreased perfusion and endothelial damage to the vasculature supplying the abdominal organs. The most severe clinical symptoms are generally difficulty breathing due to damage to the endothelium of the lungs as well as confusion, stupor and coma associated with involvement of the microvasculature of the brain. Severe cases of *R. rickettsii* infection are characterized by noncardiogenic pulmonary edema, hemorrhagic rash, renal failure, meningoencephalitis, and hypovolemic hypotension (23).

Pathogenesis

The pathogenic sequence of spotted fever rickettsioses begins with infection of the vascular endothelium or possibly lymphatic endothelial vessels after dermal inoculation. Rickettsiae attach to endothelium through adhesins—outer membrane

protein A (OmpA) has been described as an adhesion protein for *R. rickettsii*, whereas Omp B has been shown to be an adhesin of *R. conorii* (through ligation of Ku70 on mammalian cells) and *R. japonica* (24-26). After attachment to host-cells, rickettsiae induce cytoskeletal rearrangements in the host cell at the site of attachment resulting in their uptake into endothelium possibly in a phosphoinositide 3-kinase (PI3K)-dependent mechanism (24;27). Upon infection of the endothelium, rickettsiae rapidly escape the phagosome through rickettsial phospholipase d (pld) prior to phagolysosomal fusion and proliferate inside the host-cell cytoplasm (28). Rickettsiae then disseminate throughout the vascular system. Of note, spotted fever group rickettsiae are able to stimulate polymerization of host-cell actin (through RickA-dependent polymerization in *R. conorii*), facilitating cell-to-cell spread through focal lysis of host-cell membranes (**Figure 1.2**)(29;30); therefore, spotted fever group rickettsiae are able to disseminate throughout the endothelium without becoming extracellular, evading many important mechanisms of immunity.

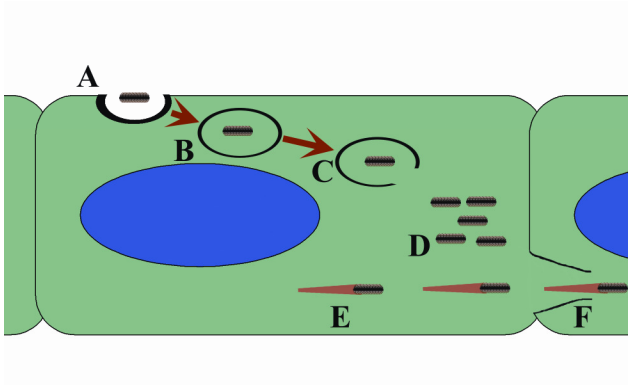


Figure 1.2: Early pathogenic steps of spotted fever rickettsiae. Rickettsial attachment (A) to host-cells through adhesins facilitates uptake into endothelium (B). Rickettsiae escape the phagosome (C) and proliferate intracellularly (D). Polymerization of host-cell actin (E) facilitates cell-to-cell spread through focal lysis of host-cell membranes.

Intracellular rickettsial proliferation is directly cytopathic to cells, although no known virulence factors have been identified to date. Despite rickettsiae lacking any known virulence factors, they do possess the ability to promote host-cell survival, theoretically allowing rickettsiae to proliferate further. *R. rickettsii* has been shown to inhibit host-cell apoptosis by an NF-κB-dependent mechanism (31). However, rickettsiae do induce the production of reactive oxygen species (ROS) in infected cells. *In vitro* culture systems have shown that the production of ROS such as hydrogen peroxide and the concurrent depletion of protective mediators such as glutathione lead to oxidative

damage, characterized by lipid peroxidation of host-cell membranes (32;33). In addition to rickettsiae-specific endothelium damage, immune effector mechanisms may also play a role in vascular damage. Overall, however, the immune response appears beneficial to the host in experimental models, as lymphocyte-depleted animals have greater titers of rickettsiae and similar pathogenic manifestations (34).

Rickettsial infection and subsequent damage of vascular endothelium lead to increased vascular permeability and an accumulation of fluid in the surrounding interstitial space culminating in decreased blood volume and hypovolemic shock. Additionally, extensive lymphohistiocytic infiltrates are frequently visualized around and involving small vessels which are infected with rickettsiae (35). Major vital organs affected are the brain and lungs manifesting as meningoencephalitis and cerebral edema as well as interstitial pneumonitis, intra-alveolar hemmorhage and vascular leakage into alveoli, respectively. Focal myocarditis is also observed. Decreases in kidney perfusion owing to hypovolemia may also lead to acute renal failure while vascular leakage into the alveolar spaces in the lungs impairs gas exchange leading to hypoxemia.

Diagnosis

The initial diagnosis of rickettsiosis is generally based on the clinical picture and epidemiologic observations (reviewed in 36). To confirm the diagnosis, serologic assays are generally used, although antibodies do not appear until later in the disease course, after empiric treatment should have been initiated. As a result of serologic assays being the main diagnostic modality used to confirm Rocky Mountain spotted fever, the case fatality rate may be underestimated due patients dying before seroconversion or inability to obtain convalescent sera from presumptive patients that survive infection (35). Immunohistochemical detection of rickettsiae in rash biopsies is diagnostically useful after skin lesions appear, although rickettsiae are sporadically distributed throughout the skin making definitive diagnosis difficult because of the low sensitivity of the test before the appearance of a rash (35).

Immunity to Rickettsiae

Early reports investigating the host-cell responses to rickettsiae focused on the responses of macrophages to rickettsial infection, investigators initially believed that the primary anti-rickettsial mechanism(s) centered around macrophages (37). However, given the intracellular nature of rickettsiae, it is logical that a vigorous cell-mediated immune response, characterized by natural killer (NK) cells and cytotoxic T-lymphocytes (CTL), is largely responsible for clearance of the infection (38;39). Through use of accurate murine models of human rickettsioses, many of the anti-rickettsial mechanisms which lead to decreased rickettsial load, protective immunity, and/or resolution of disease have been elucidated.

Deciphering Immunity through Mouse Models of Human Rickettsioses

Understanding the role of immunity, both in terms of protection from rickettsial infection, but also of pathogenesis related to immune-mediated damage, is critical to the development of new efficacious vaccines as well as possible immuno-therapeutics. Through the use of mouse models which closely emulate the pathologic sequences seen in humans, investigators have made strides towards understanding the important immune-effector responses leading to protection. Mouse models utilizing *R. typhi* and *R. conorii* infection in C3H/HeN mice have proved to be beneficial towards demonstrating the many important aspects of immunity and pathogenesis of typhus group (40) and spotted fever group rickettsioses (41) (**Table 1.2**). However, these models do not allow investigators to determine the importance of individual constituents through the use of knockout-mice. The *R. australis* model of spotted fever rickettsioses in the

Agent	*R. conorii*	*R. australis*	*R. typhi*
Mouse strain	C3H	C57BL/6	C3H
Route of inoculation	i.v.	i.v.	i.v.
Outcome	Dose-dependent mortality	Dose-dependent mortality	Dose-dependent mortality
Target cell	Endothelium	Endothelium	Endothelium
Histopathology	RMSF-like	RMSF-like	Epidemic typhus-like

Table 1.2: Mouse models of human rickettsioses

C57Bl/6 mouse is another model that can be used. This model allows the use of knockouts in a C57Bl/6 background; however the invasiveness of *R. australis* does not appear as pathologically similar to human rickettsioses as other cell types in addition to vascular endothelium are infected (42).

Innate Immunity

Classically, investigation of innate immunity has focused on non-anamnestic cellular responses and components such as complement and production of cytokines that induce killing activity in macrophages, monocytes and other cell types. However, recent evidence suggests that immunity—both innate and adaptive—is also regulated through cross-talk by many other cell types not considered as part of the immune system. Therefore, this discussion of innate immunity to rickettsiae will also focus on the cellular responses of many cell types experimentally infected with rickettsiae, with particular focus on the endothelial response to rickettsiae.

Many early *in vitro* studies of rickettsiae were undertaken in immortalized cell lines such as RAW264.7 (mouse macrophage-like), Vero (green monkey kidney epithelial), and various fibroblast cell lines. The advantages of these cell lines were that rickettsiae readily infected them and their culture characteristics were well-established; however, the results obtained using these cells should be interpreted with caution as these cell types do not represent the main cellular targets (vascular endothelium) of infection for human rickettsioses. Nevertheless, many of these studies were integral in demonstrating the importance of soluble immune-mediators (in particular, cytokines) in limiting intracellular rickettsial proliferation and inducing rickettsicidal activity.

The first demonstration that rickettsial infection resulted in the production of interferon-like compounds was accomplished by Kohno et al. Using embryonic chick cells, rickettsiae were shown to produce interferon which correlated with intracellular rickettsial proliferation (43). These studies were subsequently followed by demonstrations of the ability of interferon to inhibit rickettsial proliferation in fibroblasts

in vitro; however, investigators were unable to demonstrate that interferon played a role *in vivo* (44).

These studies led to the investigation of the ability of lymphocytes to produce interferon-like compounds in response to rickettsiae—and to determine their effect on rickettsial infection. In similar experiments, investigators independently demonstrated that cultured lymphocytes secrete soluble mediators into supernatants that were capable of inhibiting rickettsial growth *in vitro* (45;46). Turco and Winkler showed that both ConA-stimulated human lymphocytes and rickettsial-stimulated lymphocytes obtained from mice are capable of inhibiting rickettsial proliferation in cultured fibroblasts in a species-specific pattern (45), whereas Wisseman and Waddell demonstrated similar activity in human endothelial cells, macrophages and fibroblasts (46). Moreover, Turco and Winkler demonstrated that this anti-rickettsial effect was due to protein production in cultured fibroblasts, as addition of cyclohexamide abolished the inhibition of rickettsial proliferation. Lastly, both groups hypothesized that the soluble anti-rickettsial effector may be gamma interferon (IFN-γ), as the mediator was labile at pH 2 and stable at 56^{o} C. Subsequently, Turco and Winkler substantiated their hypothesis, demonstrating that cloned murine IFN-γ was responsible for the inhibition of rickettsial proliferation in mouse fibroblasts (47), and Jerrells et al. demonstrated that blockage of IFN-γ with monoclonal antibody (mAb) treatment inhibited anti-rickettsial activity in macrophages (48).

Later, it was shown that intravenous inoculation of rickettsiae in previously immune mice resulted in a significant production of IFN-γ *in vivo*, peaking at four hours post-inoculation. Further evaluation of the kinetics of IFN-γ production *in vivo* demonstrated that production occurred in a bimodal distribution, with peaks early in infection around day 3, and later around day 9. *In vitro*, investigators demonstrated that antigen presentation was necessary for T-cell specific IFN-γ production(49;50). Further studies demonstrated that human T-lymphocyte clones produced IFN-γ in response to rickettsial antigen (51).

The importance of IFN-γ as an important effector *in vivo* was further shown, as mice which were resistant to rickettsial infection were treated with mAb to IFN-γ. Mice treated with mAb to IFN-γ had 100% morbidity (indicated by prostration and ruffled fur) and 47% mortality, whereas mice treated with control mAb did not become ill after infection; moreover, mice treated with anti-IFN-γ mAb had significantly greater titers of rickettsiae in the liver and spleen (52).

Despite numerous data suggesting that IFN-γ could induce rickettsicidal activity in infected cells, all the data compiled at this point had not demonstrated that this activity could be induced in endothelium; moreover, the mechanism behind the IFN-γ-induced anti-rickettsial activity was still unclear. It had been reported that IFN-γ inhibited the growth of *Toxoplasma gondii* in human fibroblasts by induction of tryptophan degradation through induction of indoleamine 2,3-dioxygenase (53;54). Neither this mechanism nor depletion of other amino acids, however, was responsible for the limitation of rickettsial proliferation in murine fibroblasts (55).

Another cytokine, tumor necrosis factor-alpha (TNF-α) was shown to be important in limiting rickettsial activity *in vitro*. After exposure to rickettsiae, the macrophage-like cell line RAW264.7 as well as human monocyte-derived macrophages were shown to produce significant quantities of TNF-α in a dose-dependent manner (56;57). Moreover, Manor demonstrated that addition of recombinant human TNF-α to rickettsiae-infected HEp-2 cells significantly reduced rickettsial numbers *in vitro*, and this effect was not dependent on *de novo* protein synthesis as the addition of cyclohexamide did not abrogate the anti-rickettsial phenomenon (58). Additionally, the effect of TNF-α on limiting rickettsial load *in vitro* was enhanced by the addition of IFN-γ. In contrast to previous findings regarding IFN-γ-inhibition that suggested tryptophan degradation was not responsible for limiting rickettsial proliferation, these investigators demonstrated that addition of tryptophan to cultures partially restored the proliferative capacity of rickettsiae *in vitro*. Despite this observation, it was clear that the main mechanism by which TNF-α and IFN-γ exert their anti-rickettsial actions was still elusive.

Using 2-dimensional gel electrophoresis, investigators had observed that treatment of cell lines with TNF-α and IFN-γ induced several polypeptides that were not produced after treatment with either cytokine alone, however the significance to anti-rickettsial activities was unclear (59;60). Investigators demonstrated that addition of TNF-α and IFN-γ to cultured cells infected with rickettsiae acted synergistically to induce the production of nitric oxide (NO) (61-63). Nitrate production was significantly enhanced following addition of TNF-α and IFN-γ in fibroblasts; moreover, this enhancement correlated with inhibition of rickettsial proliferation *in vitro.* Lastly, inhibition of this anti-rickettsial action was achieved through the addition of nitric oxide synthase inhibitors, NG-monomethyl-L-arginine (NGMA) and aminoguanidine.

Despite these data suggesting that NO-dependent killing of rickettsiae is important in host-cell protection, the significance of these observations in endothelial cells had not been established. TNF-α and IFN-γ treatment was shown to induce the expression of inducible nitric oxide synthase (iNOS, NOS2) in murine and human endothelial cells. Subsequent production of nitric oxide resulted in intracellular rickettsial killing, which could be inhibited by NGMA (64;65). Investigators also determined that treatment of cells with nitroprusside, resulting in the release of intracellular NO, also was capable of killing rickettsiae. In human endothelium, hydrogen peroxide production—induced by cytokine treatment—was also demonstrated to play a role in intracellular killing of rickettsiae. Together, these data were the first to establish that cytokine-activated endothelium was capable of limiting rickettsial proliferation.

Although clear evidence demonstrated the importance of NO production *in vitro*, the significance of this effector response *in vivo* was unclear. The importance of TNF-α and IFN-γ *in vivo* was substantiated by depletion with mAb. Depletion of either TNF-α or IFN-γ or both together led to widespread rickettsial infection and death in mice given an ordinarily sublethal dose and correlated with inhibition of NO production (66). Moreover, treatment of mice with NGMA significantly affected rickettsial proliferation *in vivo* in association with reducing NO production. However, other effector responses,

in addition to NO production, may play a role in the susceptibility to infection observed in TNF-α and IFN-γ-depleted mice *in vivo*. In another murine model of human rickettsiosis, depletion of IFN-γ alone also resulted in overwhelming infection (40).

IFN-γ was demonstrated to play an important effector function both *in vitro* and *in vivo,* and significant levels of IFN-γ are present in sera of mice infected and humans infected with rickettsiae. Previous reports had demonstrated that IFN-γ production *in vivo* followed a bimodal temporal pattern, with production early after infection during the first three days, then followed by another increase in IFN-γ later around day 9 (50). These data suggested that IFN-γ production *in vivo* was both antigen-dependent and independent. Immune T-lymphocytes produced significant levels of IFN-γ in an antigen-dependent fashion; however, the cellular source for early IFN-γ production remained elusive. Antibody-mediated depletion NK cells resulted in significant depletion of serum IFN-γ early in infection in mice, suggesting that NK cells are responsible for the early antigen-independent production of IFN-γ during infection (67). NK cells may also possess other effector roles in immunity to rickettsiae; however their importance is currently unclear.

Lastly, the role of endothelium—in cooperation with the immune response—in initiating anti-rickettsial actions has been largely ignored. With the exception of a few studies described above, investigators have neglected the importance of endothelial responses in rickettsial immunity. Upon rickettsial infection, human umbilical vein endothelial cells (HUVEC) are capable of significant production

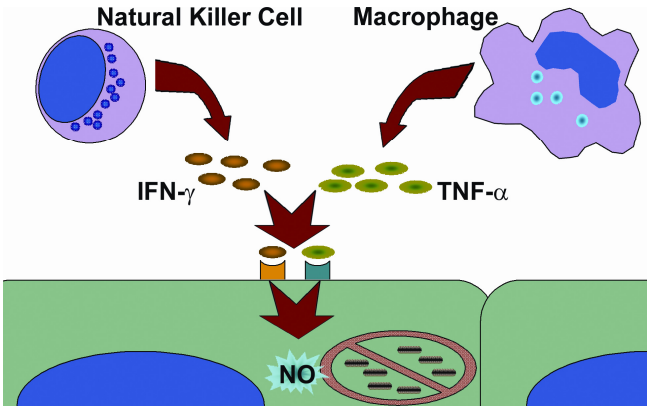


Figure 1.3: Innate immune effector responses to rickettsiae. NK cells and Macrophages produce IFN-γ and TNF-α, respectively, which induce intracellular rickettsial killing through NOS2-dependent NO production.

of interleukin (IL)-6 and IL-8, suggesting that they may participate in pro-inflammatory immunity, although they do not secrete significant quantities of IL-1α, IL-1β, or TNF-α (68). In subsequent experiments, IL-8 and MCP-1 were produced by *R. rickettsii* infected human endothelium via an NF-κB-dependent mechanism, although the importance of these cytokines in immunity to rickettsiae is unknown (69;70).

As a result of the data discussed above, we know that the innate immune response to rickettsiae *in vivo* is characterized by early activation of macrophages and NK cells, leading to the production of TNF-α and IFN-γ, respectively (**Figure 1.3**). These cytokines then induce the expression of NOS2 leading to the production of NO which facilitates killing rickettsiae inside endothelium and macrophages, thereby limiting their dissemination *in vivo*. Currently, the mechanisms by which these responses are mediated are unclear. There exists a need to define the molecular signaling by which these responses are generated and define other cell types important in initiation of innate immunity.

Adaptive Immunity

Humoral Immunity

The importance of humoral immunity in regards to primary rickettsial infection in man is debatable. Once spotted fever group rickettsiae gain access to the vascular endothelium in the dermis of the skin, there is no need for rickettsiae to become extracellular as they can spread throughout the endothelium through actin polymerization and focal lysis of host-cell membranes. In fact, during acute rickettsial infection very few rickettsiae are extracellular in the blood. Nevertheless, antibody may be an important consideration in protection from reinfection as well as protection following successful immunization.

Many early demonstrations were done to show the importance of antibody response to rickettsiae. Kenyon et.al. demonstrated that rickettsiae pre-treated with immune-sera were taken up by peritoneal macrophages and killed, whereas rickettsiae treated with non-immune sera invaded peritoneal macrophages, escaped the phagosome

and proliferated—eventually killing the macrophage (71). These data suggest that antibody may be important in increasing macrophage killing of rickettsiae—most likely through an oposonization mechanism.

The role of antibody in rickettsial infection in non-professional antigen presenting cells (APC) is less clear, however. Hanson demonstrated that treatment of the related rickettsial pathogen, *Orientia tsutsugamushi* (then *R. tsutsugamushi*), with hyperimmune serum prevented attachment and entry of rickettsiae independent of complement presence in suspended embryonic chicken cells (72). On the other hand, in several important studies, pretreatment of rickettsiae with antibody actually increased attachment and entry of rickettsiae into endothelium and macrophages—presumably through Fc-mediated entry; however, rickettsiae were unable to escape the phagosome prior to phagolysosomal fusion leading to intracellular killing (73;74). Although these studies seem to contradict each other, it is likely that the true role of antibody in rickettsioses centers on opsonization of free rickettsiae in the blood as well as inhibition of phagosomal escape, leading to intracellular destruction.

Cellular Immunity

In contrast to humoral immunity, the importance of cellular immunity to rickettsiae is straightforward given the obligate-intracellular lifestyle of rickettsiae. Historically, many of the initial observations of the importance of T-lymphocytes in rickettsial diseases followed similar observations with other pathogens or antigenic stimuli. For instance, Jerrells and Eisemann demonstrated the importance of T-lymphocytes in antibody production during rickettsial infection after the seminal observation on the importance for T-lymphocyte help in the production of IgG to antigens (75).

Early studies of cellular immunity to rickettsiae were undertaken in mice models utilizing intraperitoneal (ip) injection of rickettsiae. Overall, they demonstrated that after infection with *Rickettsia spp.*, mice developed T-lymphocyte responses, and that they T-lymphocytes would proliferate in response to crude rickettsial antigen preparations *in vitro* (76;77). The importance of T-lymphocyte responses in rickettsial infection were

initially shown in mice deficient of T-lymphocytes, B-lymphocytes, or both. Montenegro et.al., demonstrated that mice deficient in T-lymphocytes died after rickettsial infection, whereas mice that were deficient in B-lymphocytes survived infection (78).

The observation of T-lymphocyte proliferation in response to rickettsiae was further corroborated in humans. The presence of cellular immunity (presumably T-lymphocytes) in humans was demonstrated by stimulation of peripheral blood mononuclear cells (PBMC) of rickettsiae-exposed individuals to rickettsial antigens(79). Additionally, they demonstrated that a strong correlation existed between stimulation index of PBMC and ELISA titer of antibody in individuals. At this point, it was becoming clear to researchers that T-lymphocytes played an important role in immunity to rickettsiae in mice and men, although the characterization of these effector cells, and elucidation of the mechanism(s) by which they exerted their anti-rickettsial effects were unclear.

Consecutive studies by Rollwagen helped to clarify the importance of T-lymphocytes. She demonstrated that rickettsiae-infected cells could present rickettsial antigens on MHC-I molecules in fibroblasts, and that cytotoxic T-lymphocytes obtained from immune mice could kill infected cells in an MHC-I-restricted fashion (80;81). In humans, Carl and Dasch similarly demonstrated that human PBMC obtained from rickettsiae-immune individuals could lyse HLA-matched rickettsiae-infected cells; moreover, they demonstrated that the cells responsible for this phenomenon were $CD3^+CD8^+$ T-lymphocytes (82;83). In subsequent experiments, Carl and Dasch demonstrated that this cytotoxic activity could be inhibited by treatment of cells with anti-$CD3^+$ mAb, but not with anti-$CD4^+$ or $CD8^+$ mAb, suggesting the T-cell receptor (TCR) complex is necessary in this process (84;85).

These studies built upon previous work, indicating that $CD8^+$ T-lymphocytes were capable of acting as direct effectors of anti-rickettsial activity *in vitro*. Therefore, at this point, it was clear that T-lymphocytes participated as anti-rickettsial effectors *in vitro* through several mechanisms: production of IFN-γ, leading to the intracellular killing of

rickettsiae, and direct killing of infected cells through TCR-mediated recognition of antigen-expressing cells.

As discussed in the *Innate Immunity* section above, the C3H/HeN mouse model, utilizing intravenous (i.v.) injection, was integral in elucidating important effector responses *in vivo*. As such, this model was also important in elucidating the important effector responses of $CD4^+$ and $CD8^+$ T-lymphocytes. Previous research had demonstrated that T-lymphocytes were important for immunity to rickettsiae in a different mouse model described above, although the importance of T-lymphocyte subsets was not elucidated.

Feng et al. demonstrated through selective depletion and adoptive transfer experiments the importance of different T-lymphocyte subsets. Adoptive transfer of either immune $CD4^+$ or $CD8^+$ T-lymphocytes into naïve mice was sufficient to protect from an ordinarily lethal inoculum of *R. conorii* (86). Interestingly, depletion of $CD4^+$ T-lymphocytes with mAb did not affect the ability of mice to mount an effective immune response, whereas depletion of $CD8^+$ T-lymphocytes led to high fatality in mice given an ordinarily sublethal dose of rickettsiae (40;86). Together, these observations suggest that $CD8^+$ T-lymphocytes are absolutely critical in protection from rickettsioses and they may be activated in a $CD4^+$-independent fashion, possibly through a cross-presentation mechanism. Further demonstration of the critical role of $CD8^+$ T-lymphocytes was shown through perforin-, MHC-I- and IFN-γ-gene knockout mice. All knockouts demonstrated extreme susceptibility to rickettsial infection (66;87).

Summary

In conclusion, based on the intracellular lifestyle of rickettsiae, initiation of cellular immunity is of paramount importance. However, both the innate immune response and humoral immunity also play important roles in protection against rickettsiae. Ultimately, CTL are critical to clearance of rickettsiae, and the cytokines IFN-γ and TNF-α induce intracellular killing in endothelial cells through the activation of iNOS and subsequent rickettsicidal NO production. Natural killer cells also contribute to

immunity against rickettsiae, primarily through the production of IFN-γ. Lastly, the humoral immune response, specifically antibody production against outer membrane proteins, may prevent reinfection by blocking invasion or phagosomal escape.

Knowledge Gaps in Rickettsial Immunity

Despite the understanding of important immune-effector responses to rickettsiae, there still exist many crucial gaps in fully understanding the requirements necessary to induce immunity to rickettsiae and the cell types and signaling necessary to achieve protective immunity. Elucidating these important requirements is necessary to consider when evaluating the requirements of a protective vaccine.

Dendritic cells (DC) in the skin, particularly Langerhans cells and dermal DC, may be important in the initial recognition of rickettsial infection following the bite of an infected tick. These cells may play a role in pathogenesis and immunity in several ways. First, DC in the skin may provide important initial target cells for rickettsial infection. Second, recognition of rickettsiae in the skin by DC may result in migration of rickettsiae-containing DC to draining lymph nodes. This may contribute to systemic rickettsial dissemination by facilitating transport of rickettsiae to lymph nodes; however, infection of DC by rickettsiae may also initiate DC activation leading to protective immunity.

Importantly, DC have been shown in numerous infectious disease models to be important in the initiation and polarization of protective immunity; however, the role of DC in rickettsial infection is unknown. As such, investigating the role of DC in rickettsial infection is a logical next-step towards investigating the requirements necessary to induce protective immunity to rickettsioses. Characterization of the recognition of rickettsiae by DC, including the importance of pathogen-associated molecular pattern receptors (PAMPR) in this process will advance the overall knowledge of inititation of immunity to rickettsiae, and also possibly elucidate important concepts for engineering a vaccine.

CHAPTER 2: MATERIALS AND METHODS

Animals, Bacteria and Cell Lines

Mice

Male C3H/HeN (H-2^k) (Harlan Sprague Dawley, Indianapolis, IN) or C3H/HeJ (H-2^k) (Jackson Laboratories, Bar Harbor, ME) mice between the ages of 6 and 12 weeks were housed under animal biosafety level-3 specific pathogen-free conditions according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (Galveston).

Rickettsia

R. conorii (Malish 7 strain), a human isolate from South Africa, was obtained from the American Type Culture Collection (ATCC VR 613, Manassas, VA) and was cloned by plaque purification in our laboratory.

Rickettsiae for animal experiments

The rickettsial stock for animal infections was prepared as an infected 10% yolk sac suspension and stored at –80°C. Briefly, embryonated eggs (Charles River Laboratories, Wilmington, MA) were sterilized with betadine followed by 70% ethanol. After sterilization, 0.5 ml of rickettsiae in sucrose-phosphate-glutamic acid (SPG) buffer (0.218 M sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM monosodium glutamic acid, pH 7.0) was injected into each egg, and shells were sealed with DUCO cement (Devcon, Danvers, MA). After 72 hrs, eggs were candled, and dead eggs were discarded. Thereafter, eggs were candled daily for focal hemorrhage in the vascular network. When extensive hemorrhage was observed (generally 4-6 days after inoculation), eggs were placed at 4^o C. Vascular bed membranes of the yolk sac were obtained from infected eggs and placed in individual wells of 12-well plates and maintained at 4^o C overnight. Small pieces of membranes were smeared on glass slides or sheep blood agar plates. Slides were fixed in 100% ice-cold methanol, and the extent of rickettsial infection was determined by Diff-Quik stain or rickettsial immunofluorescence with anti-*R. conorii*

rabbit polyclonal antibody (pAb) and FITC-labeled goat anti-rabbit antibody (see appendix). Sheep blood agar plates were incubated overnight at 35° C to ensure a lack of contamination. After determining relative rickettsial quantities and possible contamination, membranes that were contaminated were discarded, and highly infected membranes were combined, diluted to 10% with SPG buffer and homogenized with a Waring blender. Rickettsial stock was then clarified by centrifugation at 130 x g for 3 min. Rickettsial stock was then aliquoted and stored at -80° C. Prior to use, animal stocks were quantitated by plaque assay and qualitatively assessed by determining LD_{50} by intravenous inoculation in male C3H/HeN mice.

Rickettsiae for *in vitro* experiments

Rickettsiae for *in vitro* studies were purified by density gradient centrifugation. Briefly, monolayers of Vero cells were infected with *R. conorii* from the 10% yolk sac stock (10^5 PFU per 150-cm^2 flask). Percent rickettsial infection was assessed by Diff-Quik staining infected Vero cell smears. When extensive infection was observed, generally after 7 to 10 days of incubation at 34°C, cells were harvested and lysed by ultrasonication. Rickettsiae were purified from cell debris by discontinuous density gradient centrifugation in renografin (88). Viable rickettsiae were then collected in SPG buffer and stored at –80°C until used. Prior to use, rickettsiae were quantified by plaque assay.

Heat-killed rickettsiae

Heat-killed rickettsiae were obtained from renografin-purified rickettsial stocks for antigen-presentation assays. Briefly, 50 µl aliquots of renografin-purified rickettsiae in 250 µl Eppendorf tubes were thawed at 37° C and then transferred to a 60° C water bath for 45 min. Death of rickettsiae was corroborated experimentally by plaque assay and evaluation of infectivity in Vero cells *in vitro*.

Cell lines

Vero cells

For progagation of rickettsiae *in vitro*, mycoplasma-free Vero cells (Catalog # CCL-81, ATCC, Manassas, VA) with an unknown passage history were a kind gift from Dr. Gustavo Valbuena. Vero cells were maintained in minimum essential medium (MEM) adjusted to contain 10 mM HEPES and 5% bovine calf serum (BCS) and cultured in a humidified incubator at 37^{o} C at 5% CO_2. When Vero cells were infected with rickettsiae, cells were cultured at 32^{o} C, and medium was replaced every 2 days.

GM-CSF-transfected J558L cells

J558L cells, transfected with the murine *gm-csf* gene (courtesy of Dr. Charles Janeway and Dr. Lynn Soong) under a geneticin (G418, Gibco BRL [Invitrogen], Carlsbad, CA) repressor, were cultivated for the purpose of obtaining GM-CSF-conditioned medium. Cells were maintained in culture in Iscove's modification of DMEM (IMDM) containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 50 μM 2-β-mercaptoethanol (2-ME) and 1 mg/ml geneticin in a humidified incubator at 37^{o} C and 5% CO_2. Cells were passaged and adjusted to 1 x 10^6 cells per ml with fresh geneticin-containing IMDM every two days. To produce GM-CSF-conditioned medium, cells were harvested and washed three times in phosphate-buffered saline (PBS) without magnesium or calcium to ensure absence of geneticin, adjusted to contain 1.5 x 10^6 cells per ml complete IMDM without geneticin, and cultured for 3 days at 37^{o} C in 5% CO_2. After 3 days, culture supernatants were obtained and stored at -80^{o} C until use in bone-marrow-derived dendritic cell (BMDC) cultures.

Yac-1 cells

Yac-1 cells (Catalog # TIB-160, ATCC, Manassas, VA) for NK-cell cytotoxicity assays and NK cell activation assays were obtained from Drs. Edward Curran and D. Mark Estes. Yac-1 cells were maintained in RPMI 1640 with 2 mM L-glutamine, adjusted to contain 10% FCS, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate. Cells were maintained in culture by 1:10 passage every two days in a humidified 37^{o} C incubator at 5% CO_2.

Isolation of Primary Cells

DC culture

BMDC were generated as described with modifications (89;90). Briefly, bone marrow cells were cultured in a 100 mm Petri dish (Fisher Scientific, Houston, TX) at 2 x 10^6 cells per 10 ml of complete IMDM (Iscove's modified DMEM containing 10% FCS, 1 mM sodium pyruvate, 50 µM 2-ME). Recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (eBioscience, San Diego, CA) or 2% culture supernatants of J558L cells that had been transfected with the murine *gm-csf* gene (a gift from Charles Janeway's laboratory, Yale University) were used as the source of GM-CSF. Nonadherent cells were harvested between days 6-8 and analyzed by flow cytometry to ensure phenotypic immaturity. Nonadherent cells were typically >80% $CD11b^+CD11c^+$ DC. Sometimes $CD11c^+$ cells were directly purified from a day 8 culture to >95% purity with microbeads according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA).

Isolation of CD4 and CD8 T-lymphocytes from mice

Naïve and immune T-lymphocytes were obtained from the spleens of C3H/HeN mice. Immune T-lymphocytes were derived from mice previously immunized by intravenous inoculation with a sublethal dose (2 x 10^3 PFU) of *R. conorii* as described previously (41). All mice exhibited signs of illness and were considered immune 1 month post-infection. $CD4^+$ and $CD8^+$ T-lymphocyte subsets were purified using T-cell subset enrichment columns (R & D Systems, Minneapolis, MN), and purity of T-cells was typically > 95% by flow cytometric analysis.

Brain microvascular endothelial cell isolation

Isolation of mouse brain endothelial cells (MBEC) was adapted from previously reported protocols (91;92). Briefly, whole brains from male C3H/HeN or C3H/HeJ mice were removed aseptically and briefly soaked with 70% ethanol to render leptomeningeal vessels non-viable. The fresh brains were stored in ice-cold DMEM/F12 containing 2%

FCS prior to homogenization. Brains were homogenized using a glass Dounce homogenizer and centrifuged. The resulting pellet was resuspended in 15% Dextran (M.W. 70 KDa) and centrifuged at 11,400 x g for 10 minutes at 4º C to remove the myelin-containing cells. The pellet was washed once more in DMEM/F12 with 2% FBS and incubated at 37ºC for 1.5 hours in 1 mg/ml collagenase/dispase containing 10 U/ml DNAse I and 0.147 μg/ml N_{α}-toxyl-L-lysine chloromethyl ketone (TLCK) with constant agitation. Following the digestion step, the crude microvessels were washed in medium and plated on rat-tail collagen-coated plates in growth medium containing DMEM/F12, 10% FCS, 10% normal horse serum (NHS), 100 μg/ml endothelial cell growth supplement (ECGS) (Biomedical Technologies, Stoughton, MA), 100 μg/ml heparin, and 3 μg/ml puromycin. After three days of incubation, the puromycin was removed from the culture medium, and the cultures then consisted of pure brain endothelial cells. The cells were maintained at 37ºC in 5% CO_2.

Cell Culture Assays

DC activation assay

Phenotypically immature BMDC were stimulated with *R. conorii* (multiplicity of infection [moi] = 5) or *E. coli* O111:B4 lipopolysacharide (LPS) (50 ng/ml) (Sigma, St. Louis, MO). To determine cytokine production following rickettsial challenge, DC were cultured in 24-well plates (4 x 10^6 cells/ml). Cell-free supernatants were harvested at 4, 8, 12, and 24 h post-infection, passed through a 0.2 μm filter, and stored at -80^{o} C until assayed. To determine phenotypic status of maturation and activation markers, DC were cultured in 6-well plates (6 x 10^6 cells/well), collected at 24 h post-stimulation, and analyzed by flow cytometry.

Antigen presentation assay

DC-T-cell co-cultures were performed to determine the ability of DC to present rickettsial antigens to naïve and immune $CD4^+$ and $CD8^+$ T-lymphocytes. For comparison, T-lymphocytes were co-cultured with macrophages. 10^6 DC or splenic

macrophages were pulsed with renografin-purified *R. conorii* (moi = 5) and co-cultivated with 5 x 10^6 T-cells in 2 ml in 24-well plates (APC:T cell ratio = 1:5) for assessment of cytokine production. After 48 h culture, supernatant was collected as described above.

NK cell cytotoxicity assay

Cytotoxicity assays were completed using the LIVE/DEAD® Cell-mediated cytotoxicity kit for animal cells (Molecular Probes [Invitrogen], Carlsbad, CA) according to manufacturer's protocol, and percent cytotoxicity was assessed by flow cytometry as described previously (93). Briefly, splenocytes were obtained from mice as effector cells and resuspended in RPMI 1640 with 2 mM L-glutamine, adjusted to contain 10% FCS, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate. Yac-1 target cells were labeled with $DiOC_{18}$ for 15 min at 37° C, washed two times in PBS, resuspended in complete RPMI, and transferred to round-bottom 96 well plates (2 x 10^4 cells per well). Effector splenocytes were obtained by mechanical disruption of spleens and passage through 40 μm filters. After obtaining a single-cell suspension, splenocytes were overlayed onto lympholyte-M (Cedarlane Laboratories, Burlington, NC), a density gradient solution. After centrifugation, lymphocytes were obtained at the interface, enumerated, and aliquoted into 96-well plates with target cells for final effector-to-target (E:T) ratios of 50:1, 25:1, and 12.5:1 in duplicate. Propidium iodide (PI) was added to culture medium to allow for the determination of Yac-1 cell death after 3 hr incubation at 37° C, 5% CO_2. Percent cytotoxicity was assessed by flow cytometric analysis after collecting 3000 $DiOC_{18}^+$ events. Lysed Yac-1 cells were identified by their dual positive characteristic (PI^+, $DiOC_{18}^+$), whereas viable Yac-1 cells were $DiOC_{18}^+$ and PI^-. Total cytotoxicity was determined by: [experimental percent cytotoxicity in E:T wells] – [background % cytotoxicity in control Yac-1 cultures].

NK cell activation assay—determination of IFN-γ production

In order to determine NK-cell derived IFN-γ production, splenocytes, obtained as described above, were co-cultured with Yac-1 cells in 24-well plates (3 x 10^6 splenocytes, 1 x 10^6 Yac-1 cells per well in 2 ml complete RPMI-1640 medium) for 18 hrs at 37° C, then GolgiStop® (BD Pharmingen, San Diego, CA) was added for 6 more

hours in culture. After 24 hours of total culture time, cells were harvested and subjected to intracellular flow cytometric analysis as described below to determine percent IFN-γ- and Granzyme B-positive NK cells.

Animal Experiments

Mouse infections

All animal studies were conducted in a animal biosafety level-3 (ABSL-3) containment facility according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch (Galveston).

Chapter 3

The murine model of human spotted fever rickettsiosis in the C3H/HeN mice has been described previously (41), and was utilized in Chapter 3 in DC transfer experiments. Briefly, mice were infected i.v. with *R. conorii* with a typically uniformally lethal dose (3 LD_{50}, 2.4 x 10^4 PFU *R. conorii*). In some experiments, mice were monitored twice daily for survival, whereas in other experiments, mice were serially sacrificed. In serial sacrifice experiments, sera were obtained by cardiocentesis for determination of cytokine levels. Organs were harvested at necropsy for determination of rickettsial titers by quantitative real-time polymerase chain reaction (RT-PCR) and evaluation of pathologic lesions and extent of rickettsial antigen staining by hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC), respectively. Leukocytes were obtained by mechanical disruption of spleen and peripheral lymph nodes (LN) to attain a single-cell suspension, enumerated, and subjected to culture or flow cytometric analysis.

For intradermal infection of rickettsiae, mice were shaved 24 h prior to inoculation on their ventral thorax and inoculated intradermally in the ventral midline with 1 x 10^6 PFU of renografin-purified *R. conorii* (inoculum volume = 100 µl). Twenty-four h therafter, skin and draining lymph node (axillary lymph nodes) were harvested as for determination of leukocyte numbers by flow cytometry and IHC evaluation.

Chapters 4 and 5

In preliminary experiments determining the role of TLR4, C3H/HeJ and C3H/HeN mice were challenged i.v. with serial dilutions of *R. conorii* to determine relative susceptibilities of the mouse strains. The dose of 8 x 10^3 PFU, approximately 1 LD_{50} in C3H/HeN mice, was determined to cause uniform lethality in C3H/HeJ mice, and was used for subsequent experiments. After infection, mice were monitored twice daily for survival. Serial sacrifice studies were conducted to collect sera by cardiocentesis for determination of cytokine levels, to determine rickettsemia levels and pathologic lesions in target organs, and to determine the relative and absolute numbers of immune effector cells in peripheral lymph nodes and spleen. For experiments determining the recall response after survival from rickettsial infection, mice were inoculated i.v. with 2 x 10^2 PFU *R. conorii*. Both strains of mice survived infection at this dose.

DC vaccination and mouse infection

BMDC were stimulated with *R. conorii* (moi = 5), LPS (50 ng/ml) or mock-infected (SPG buffer diluted similarly in complete IMDM) in a similar manner as described in DC activation assays. DC were harvested at 24 h post-stimulation, washed three times in PBS, and resuspended at a concentration of 5 x 10^6 cells per ml. Mice were injected with 100 µl of DC suspension (5 x 10^5 DC per mouse) into the hind footpads (50 µl per foot), and challenged intravenously 24 h later with 3 LD_{50} (2.4 x 10^4 PFU) of *R. conorii*. Mice were monitored twice daily for survival. Serial sacrifice studies were conducted to collect sera, determine rickettsemia levels and pathologic lesions in target organs, and the relative and absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$, and NK cells in draining lymph node and spleen.

DC migration assay

DC were stimulated and injected as described above with modifications. Prior to injection, DCs were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes), washed three times and resuspended in PBS at a concentration of 10^8 DC per ml. DC (10^7 /mouse) were injected into the hind footpads of mice. To visualize migrated DC, lymph nodes were fixed in 10% paraformaldehyde for

24 h, sectioned, counterstained with DAPI and mounted for confocal microscopy. Photomicrographs were obtained using a FluoView-1000 laser scanning confocal microscope and FluoView software (Olympus, Mellville, NY).

Histopathology and immunohistology

Necropsies were performed on mice sacrificed on days 0, 1, 2, 3, 4, 5, and 6 post-infection. Brain, lung, and spleen were immersed in zinc fixative (BD Biosciences, San Jose, CA) for immunohistology or histopathology, embedded in paraffin, and sectioned at 5 μm thickness. Sections were processed and stained with hematoxylin and eosin for histopathologic evaluation. For immunohistology anti-*R. conorii* polyclonal Ab was used for evaluation of rickettsial antigen (Ag) (41;94) using Vectastain® ABC reagents (Vector Laboratories, Burlingame, CA) and either Vector® Red or DAB (3,3'-diaminobenzidine).

Isolation of skin leukocytes

Isolation of skin leukocytes were obtained using the Medimachine™ (BD Biosciences) mechanical disaggregation system per manufacturer's instructions. Briefly, immediately after intradermal injection, the boundaries of the injection site were marked with a permanent marker. Skin samples were obtained by careful excision along injection site boundaries 24 h after intradermal inoculation. Skin samples were then minced with a scalpel blade and transferred to a 50 μm Medicon™ and disaggregated for 45 s. Cell suspensions were then filtered through a 50 μm Filcon™ and subjected to flow cytometric analysis as described below.

Experimental Techniques

Flow cytometry

Staining of cell surface markers

Phenotypic analysis of cells was accomplished by staining cell suspensions in FACS buffer (PBS containing 1% FCS and 0.09% sodium azide) with fluorochrome-conjugated mAbs. All staining were performed in v-bottom 96 well plates on ice in the

dark and, when possible, approximately 10^6 cells were stained. Prior to staining with fluorochrome-conjugated mAbs, Fc receptors were blocked by incubating cells with anti-CD16/32 (Fc Block, 2.4G2, BD Pharmingen, San Diego, CA) for 20 min. Cell-surface-specific fluorochrome-conjugated mAbs (see appendix) were then added, and cells were incubated for 30 min. Cells were then washed three times in FACS buffer and resuspended in 250 µl. Intensity of fluorescence was measured on a FACScan or FACScalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest (BD Biosciences), FCS express (DeNovo Software, Thornhill, Ontario) or FlowJo (TreeStar, San Carlos, CA) software.

Antibodies used included: PerCP Cy5.5 anti-CD11b (M1/70), FITC or APC anti-CD11c (HL3), PE anti-CD40 (3/23), PE anti-CD80 (16-10A1), PE anti-CD86 (GL1), PE anti-I-A/I-E (M5/114.15.2), PE anti-H-$2d^k$ (15-5-5) (BioLegend, San Diego, CA), FITC anti-CD4 (RM4-5), FITC anti-CD8 (53-6.7), FITC anti-CD49b/Pan-NK cells (DX5), and appropriate isotype controls. Antibodies were obtained from BD Biosciences (San Diego, CA) unless noted.

Staining of intracellular cytokines

For analysis of intracellular cytokines, cell surface molecules were stained essentially as described above. Thereafter, cells were washed twice with FACS buffer and were fixed and permeabilized with 100 µl Cytofix/Cytoperm™ (BD Biosciences) for 20 min on ice. Cells were then washed two times in Perm/Wash™ (BD Biosciences) and resuspended in 50 µl Perm/Wash™. Applicable cytokine-specific fluorochrome-conjugated mAbs were then added, and cells were incubated for 20 min. Subsequently, cells were washed with FACS buffer and analyzed as described above.

Intranuclear staining of FoxP3

For analysis of regulatory T-lymphocytes, intranuclear staining of cells was undertaken to determine the percentage of $FoxP3^+$ $CD25^+$ $CD4^+$ T-lymphocytes according to manufacturer's instructions. Briefly, staining of cell surface molecules (CD4 and CD25) was performed as described above. Thereafter, cells were fixed and

permeabilized by resuspension with fixation/permeabilization buffer (eBioscience, San Diego, CA) for 60 min. Cells were then washed in permeabilization buffer twice and resuspended in 50 µl of permeabilization buffer, and PE-conjugated anti-mouse FoxP3 mAb was added and incubated for 30 min. Cells were then washed twice more in permeabilization buffer and resuspended in FACS buffer for analysis. Cells were analyzed as described above.

Determination of cytokine production by ELISA

The levels of IL-2, IL-4, IL-10, IL-12p40, IL-12p70, IL-23 and IFN-γ in the supernatant of DC activation assays, antigen presentation assays, and sera of animals in animal experiments were quantified using commercial ELISA kits from R&D Systems (Minneapolis, MN) with the exception of IL-23 (eBioscience). Data were collected using a Versa Max microplate reader and SoftMAX pro software (Molecular Devices, Sunnyvale, CA).

Determination of cytokine production by BioPlex assay

Sera cytokine levels in some experiments were quantified using the Bio-Plex system, a bead-based array which measures up to 23 individual cytokines simultaneously, per manufacturer's instructions (Bio-Rad, Hercules, CA). Cytokine concentration was determined in sera in by analyzing serum samples in duplicate wells. Data were collected and evaluated on a Bio-Plex analyzer and associated software (Bio-Rad).

Real time-PCR quantitation of rickettsemia levels

One mm pieces (roughly 2 mg) of brain, lung, and spleen were harvested on days 2, 4, and 6 post-infection from animals that had received DCs and stored at -20°C until processing. Tissue pieces were homogenized, and DNA was purified using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Plasmids containing rickettsial *gltA* and murine *β-actin* PCR products were constructed using the TOPO 2.1 and TOPO 4 cloning kits, respectively. Rickettsial *gltA* was amplified using the forward primer CS-5 (5'-GAGAGAAAATTATATCCAAATGTTGAT) and the reverse primer CS-6 (5'-AGGGTCTTCGTGCATTTCTT). Murine *β-actin* was amplified using the forward

primer (5'-AGAGGGAAATCGTGCGTGAC) and the reverse primer (5'-CAATAGTGATGACCTGGCCGT). Real time PCR was performed using SYBR green Supermix, 1 µl DNA and primers (0.2 µM) on an iCycler real-time PCR apparatus (Bio-Rad, Hercules, CA). Standard curves were generated using plasmids containing cloned PCR products. All PCR reactions were performed using the following protocol: 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, 50°C for 15 sec and 60°C for 15 sec. Data are expressed as average copy number of rickettsial *gltA* per 10,000 copies of *β-actin*.

Statistics

Data are expressed as mean ± standard error of mean (SEM) or standard deviation (SD), and the significant differences between two series of results were determined using the Student's unpaired t test. Values of $p < 0.05$ were considered significant. For survival comparison between treatment groups, the Log-Rank Sum test was used.

CHAPTER 3: DENDRITIC CELLS MEDIATE PROTECTIVE IMMUNITY TO RICKETTSIAE

The role of dendritic cells (DC), initiators and orchestrators of the immune response, remains unclear in rickettsial infections. To investigate their importance in rickettsioses, we analyzed the responses of murine bone marrow-derived DC (BMDC) *in vitro* following rickettsial stimulation and their protective role *in vivo*. *Rickettsia conorii* stimulation of BMDC *in vitro* caused significant maturation as well as production of pro-inflammatory cytokines. Adoptive transfer of rickettsia-stimulated DC protected mice from lethal rickettsial challenge by limiting rickettsial proliferation *in vivo*; interestingly, partial protection was observed in mice receiving LPS-stimulated DC, indicating that Ag-specificity was not required to induce a protective phenotype. Immunity to *R. conorii* after adoptive transfer of DC was associated with upregulation of costimulatory molecules CD40, CD80, CD86, and MHC II, as well as production of IL-2, IL-12, and IL-23 and antigen-specific IFN-γ production in T-cells. Together, our data suggest that a vigorous pro-inflammatory response in DC is associated with protective immunity to rickettsiae, and that generation of antigen-specific immunity is crucial to achieve complete protection.

INTRODUCTION

Dendritic Cells: Modulators of Immunity

DC are paramount in initiation of both innate and adaptive immunity towards pathogens as well as tolerance to self-antigens (95). This importance has prompted many investigators to examine the role of DC responses to different infectious agents, and other important immunologic events such as autoimmunity and initiation of immunity to cancerous tumors in order to more fully understand the establishment of the immune response and the requirements for protective immunity.

Upon exposure to and uptake of foreign antigens, DC process and present foreign antigens and upregulate co-stimulatory molecules such as CD40, CD80, and CD86 (B7-1

and B7-2, respectively) (96;97). Antigen exposure also results in the polarization of DC towards a DC1 or DC2 phenotype, initiating their production of Th1- or Th2-driving cytokines (typically IL-12p70 and IL-10, respectively), crucial for initiation of an adaptive immune response (98-100). Moreover, DC migrate from the periphery (the site of antigen exposure) via draining lymphatic vessels to lymph nodes (LN) where they prime antigen-specific T-lymphocytes and induce their proliferation, effector functions, and Th-phenotype polarization through direct interaction and cytokine production (101-103)]. In experimental systems, ablation of $CD11c^+$ DC has been shown to inhibit the spontaneous recovery of grafted T-lymphocytes in lymphopenic host—definitively demonstrating the importance of DC in promoting T-lymphocyte proliferation (104).

Upregulation of co-stimulatory molecules is crucial for DC to act as professional antigen-presenting cells, and their ability to produce polarizing cytokines is critically important. Cytokine production by DC, in addition to being integral towards the polarization and activation of T-lymphocytes, may also play a significant role in the B-cell response and augmentation of innate immunity, particularly NK cells. Production of IL-2 by DC stimulated by microbial ligands causes NK cell activation in concert with IL-12p70 (105-107). Moreover, DC-derived IL-18 and IL-15 production may also play an important role in activating NK cells *in vivo* (108).

Dendritic cells in infectious diseases

The sentinel nature of DC in the periphery is ideally suited for recognition of infection in the body. As such, the importance of DC has been demonstrated in numerous infectious diseases—although the discrete responses and mechanisms of protection induced by DC vary depending on the type of pathogen encountered. In several infectious models, cytokine production by DCs is a critical determinant in disease outcome (90;109;110).

For intracellular protozoal pathogens such as leishmania and toxoplasma, DC production of IL-12, and subsequent initiation of a Th1 response, is critical in immunity and limiting pathogenesis; however, the mechanisms by which IL-12 production is initiated varies between these two protozoal pathogens.

In addition to protozoa, DC cell responses to bacteria are critical for immunity—although DC may also play a role in pathogenesis of infection. In the Gram-negative facultatively intracellular bacterium *Salmonella*, DC are important in both the pathogenesis as well as immunity. DC in the intestine may be productively infected by *Salmonella*, effectively allowing non-invasive *Salmonella* access to other areas of the body. Despite these data suggesting that DC may increase the probability of invasion and dissemination, the activation of DC towards a Th1 response, and subsequent initiation of protective immunity suggests that these cells are important in protective immunity.

DC are also integral in protection against Gram-positive bacteria. The intracellular bacterium, *Listeria monocytogenes,* can productively infect DC, leading to a protective Th1 response and potent cell-mediated immunity (111;112). Interestingly, heat-killed *Listeria* are unable to initiate similar responses despite similar upregulation of important costimulatory molecules (113); these data suggest that *Listeria* viability is important. Investigators subsequently observed that cytosolic invasion resulted in copious amounts of IFN-β production and that IFN-β production sensitized naïve T-lymphocytes for Ag-dependent activation.

Bacteria also possess mechanisms of inhibiting protective immune responses of DC. The phospholipase C (plc) possessed by *Bacillus anthracis*, but not that of *Listeria monocytogenes*, has been proposed to inhibit toll-like receptor-specific activation of DC by cleaving GPI-anchored proteins. Transfection of *Listeria monocytogenes* with a *B. anthracis* plc resulted in significant reduction of Ag-specific $CD4^+$ T-lymphocytes (114). Likewise, *Yersinia* outer membrane protein P (YopP) has been shown to significantly blunt $CD8^+$ T-lymphocyte responses, possibly through the induction of DC death and inhibition of DC maturation (115).

Together, the data regarding the role of DC in infectious diseases suggest that DC play a multitude of roles, both in induction of protective immunity, but also in contribution to or limiting pathogenesis. Additionally, it is clear that infectious agents have also evolved mechanisms which inhibit DC function and protective immunity.

Dendritic cells as cell-based vaccines

The ability of DC to induce protective immunity against different insults has prompted investigation into their use as cell-based vaccines (116-118). Pretreatment of DC with antigens or whole-pathogens, followed by transfer into animals, has the ability induce expansion of antigen-specific T-lymphocytes, leading to protection in some cases. Specifically, this methodology has proven successful in viral and bacterial infections. Pretreatment of DC with Hepatitis C virus NS3 protein and oligodeoxynucleotides containing CpG motifs prior to adoptive transfer led to NS3-specific protection *in vivo* (119). Moreover, targeted-delivery of HIV-Tat to DC *in vivo* through use of a detoxified *Bordetella pertussis* adenylate cyclase led to Th1-polarized and neutralizing antibody responses *in vivo* (120). Transfer of *L. monocytogenes*-infected DC also led to protection from challenge in mice (118).

Although this technique is not ideal for clinical treatment or protection against infectious disease, study of an animal model in which transfer of DC induces protective immunity will extend our understanding of the requirements to induce protective immunity with a component vaccine. In fact, a great deal of research has employed a reverse-technique in which investigators have begun analyzing the responses of DC to known vaccines and/or microbial components *in vitro* in order to more fully elucidate how protective immunity is triggered *in vivo* (121-123).

Dendritic cells in rickettsial disease

The importance of DC has been demonstrated in many infectious diseases; however, the role of DC in rickettsial diseases is unknown. The potential importance of DC in rickettsial disease is many fold. Firstly, DC in the skin may comprise an important initial target cell, providing a site for early rickettsial proliferation as well as providing a conduit for rickettsial access to their main target organ, the endothelium. Additionally, characterization of the response of DC upon encountering rickettsiae may provide information regarding how the immune response recognizes rickettsiae and the type of immune responses initiated.

RESULTS

Dendritic cells are targets of rickettsial infection *in vitro* and are capable of killing rickettsiae.

To define the interactions which occur between rickettsiae and DC *in vitro*, we completed an ultrastructural analysis of BMDC alone and BMDC following culture with *R. conorii*. As shown by transmission electron microscopy, these cells appear morphologically similar to DC (**Figure 3.1a**). Following co-culture with rickettsiae, we observed rickettsiae engulfed into phagosomes. Of note, we observed non-viable appearing rickettsiae residing inside phagosomes and being degraded, suggesting that DC may be capable of intracellular killing of rickettsiae (**Figure 3.1b**); however, it is also possible that DC may have engulfed dead rickettsiae. We also observed that *R. conorii* were capable of escaping the phagosome and replicating in the cytoplasm of DC (**Figure 3.1c**).

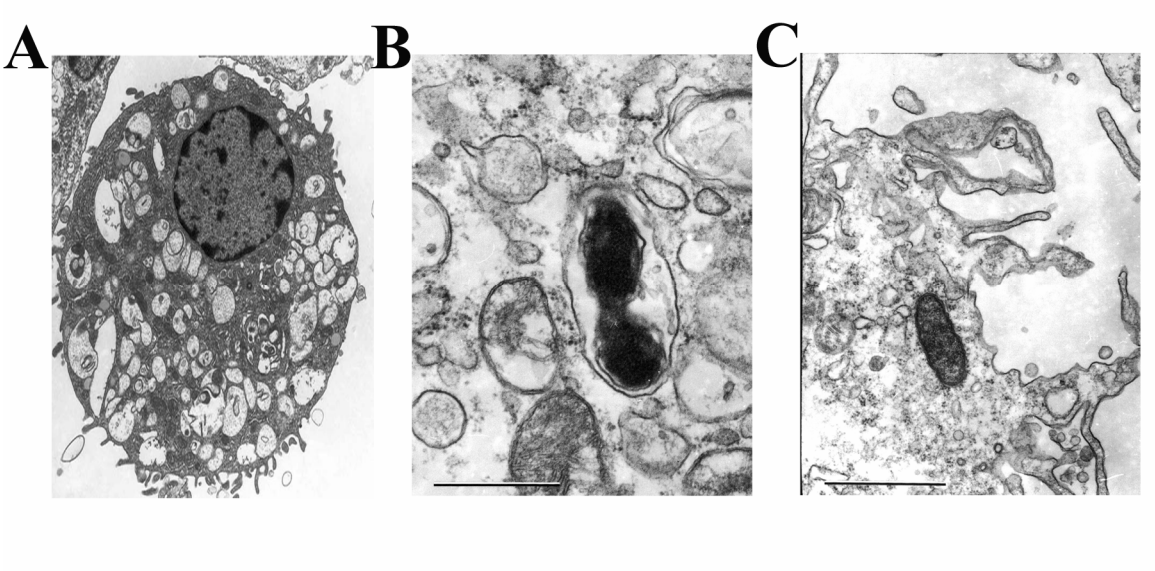


Figure 3.1. Ultrastructural analysis of rickettsial interactions with BMDC *in vitro*. (A) Morphologic characterization of BMDC after 10 day culture prior to infection with *R. conorii*. After infection of BMDC *in vitro* with renografin-purified *R. conorii*, non-viable appearing rickettsiae were visualized inside phagosomes (B, bar = 0.5 μm). Viable rickettsiae were also visualized inside the cytoplasm 24 h post-infection (C, bar = 1 μm).

Rickettsiae are visualized inside DC in draining lymph nodes after *R. conorii* intradermal inoculation

The results observed in figure 3.1 lend support to our hypothesis that rickettsiae can productively infect DC by escaping from the phagosome prior to lysosomal fusion. Furthermore, we attempted to identify if DC in the skin would become infected *in vivo*, facilitating transit to the lymphatics and access to the endothelium. Twenty-four hours after intradermal inoculation, we observed rickettsial antigen staining in draining lymph nodes by immunohistochemistry (**Figure 3.2a**). Moreover, we observed a marked influx of DC ($DEC205^+$ cells) into the skin after rickettsial injection (**Figure 3.2b**). It is well established that upon encountering antigen in the skin, DC migrate to the draining lymph nodes. Consistent with this concept, we co-localized rickettsiae inside DC of the draining (axillary) lymph nodes, visualized by confocal microscopy (**Figure 3.2c**). Together, these events suggest that dendritic cells may provide a mechanism whereby rickettsiae can access endothelium through infection and migration into draining lymphatics.

Rickettsial infection *in vitro* upregulated DC maturation markers and induced a DC1 phenotype

To investigate the responses of DC after rickettsial infection, phenotypically immature ($CD40^{dim}$MHC $II^{low}CD11b^+CD11c^+$) BMDC were stimulated *in vitro* with live *R. conorii* (moi = 5) or *E. coli* LPS (50 ng/ml) as a positive control. Rickettsial infection induced DC activation, characterized by the upregulation of CD40, CD80, CD86, and MHC-II (**Figure 3.3**). Expression of MHC-II was significantly upregulated in *R. conorii*-infected DC by a factor of nearly 8 compared to mock-infected DC (mean fluorescence intensity (MFI) = 3440 vs. 453, respectively). Rickettsiae-infected DC upregulated CD40 and CD80 expression nearly 5-fold over mock-infected DC (MFI = 25 vs. 6 and 150 vs. 27, respectively).

Rickettsial infection of BMDC also resulted in significant production of pro-inflammatory cytokines important in both innate and acquired immunity. Rickettsiae rapidly induced significant production of IL-2 and IL-12p40 within 4 h post-infection, kinetically similar to LPS-stimulation (**Figure 3.4a, b**). Production of IL-23 and the

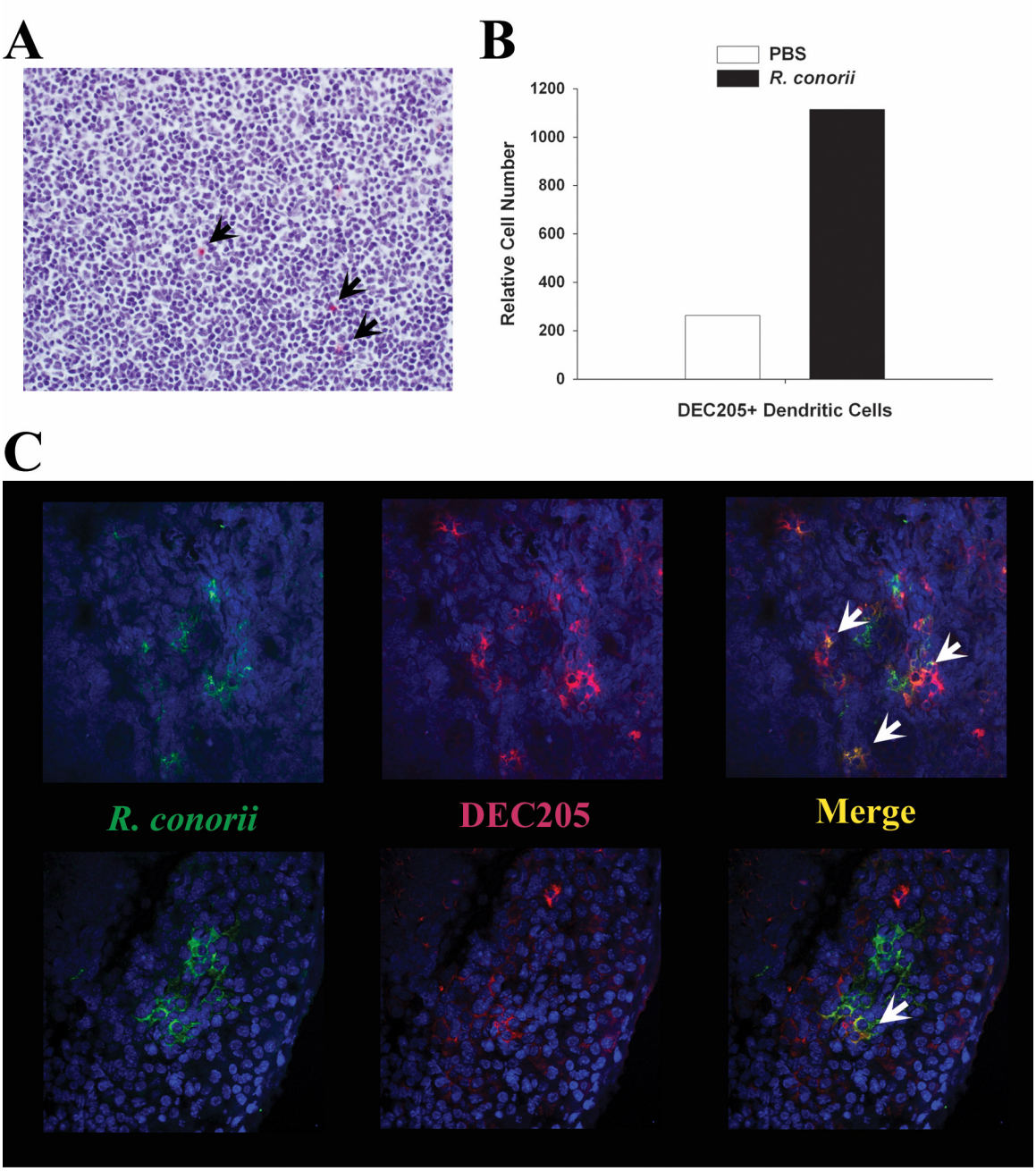
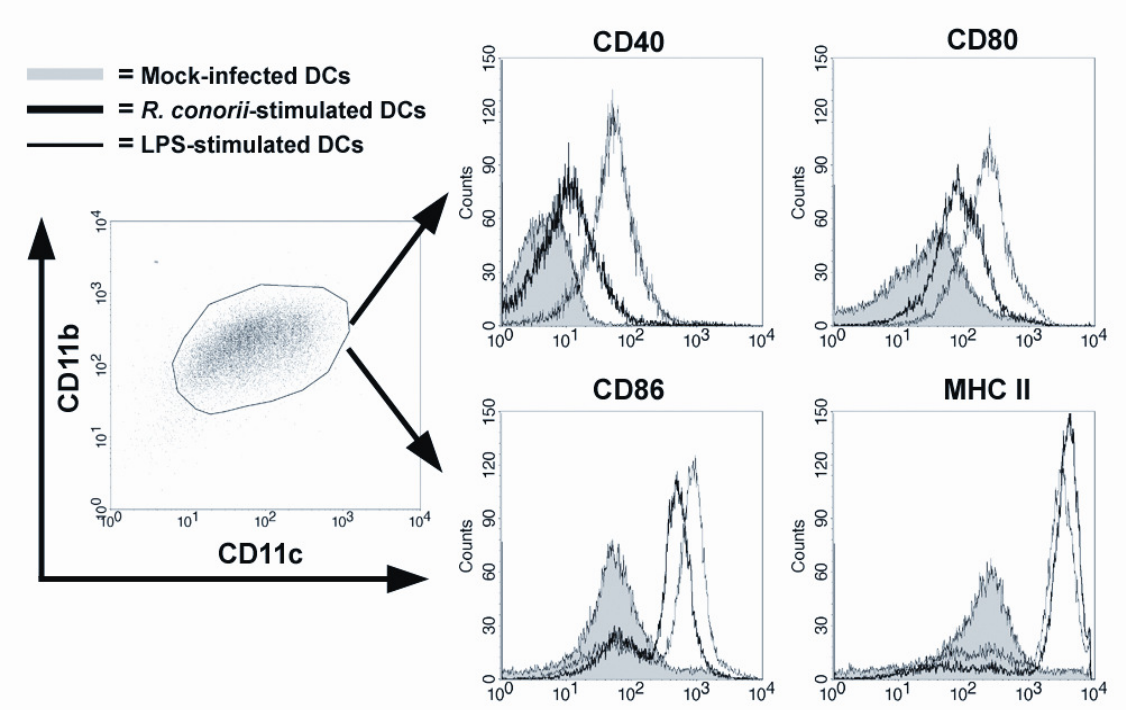


Figure 3.2. Rickettsiae are visualized inside DC after intradermal inoculation. (A) Twenty four h after intradermal inoculation with *R. conorii,* rickettsial antigen (appearing red) was visualized in draining (axillary) lymph node by immunohistochemistry. (B) Intradermal injection of renografin *R. conorii* resulted in a marked increase of DEC205^{+} DC in the skin 24 h after rickettsial infection, analyzed by flow cytometry. (C) Rickettsial antigen (green fluorescence) is co-localized with DEC205^{+} DC (red fluorescence) 24 h after *R. conorii* inoculation in axillary lymph nodes.



	Mock-infected DCs	*R. conorii*-stimulated DCs	LPS-stimulated DCs
CD40	6	25	74
CD80	27	150	340
CD86	43	466	750
MHC II	453	3440	2413

Figure 3.3. *Rickettsia conorii* induced activation and maturation of BMDC *in vitro*. The activation phenotype of BMDC was assessed by flow cytometry at 24 h following mock infection, *R. conorii* stimulation (moi=5), or stimulation with LPS (50 ng/ml). When possible, 10,000 $CD11b^{+}CD11c^{+}$ events were collected, and mean fluorescent intensity (MFI) of surface expression of activation and maturation markers (CD40, CD80, CD86, MHC II) was determined. Numbers in tables denote the MFI of surface expression of cell surface molecules. Results are representative of at least three independent experiments with similar results.

acute phase cytokine IL-6 occurred later; however, rickettsiae stimulated production of significant quantities compared with mock-infected DC within 24 h (**Figure 3.4c, d**). Thus, rickettsial infection of DC induced the activation toward a DC1 phenotype.

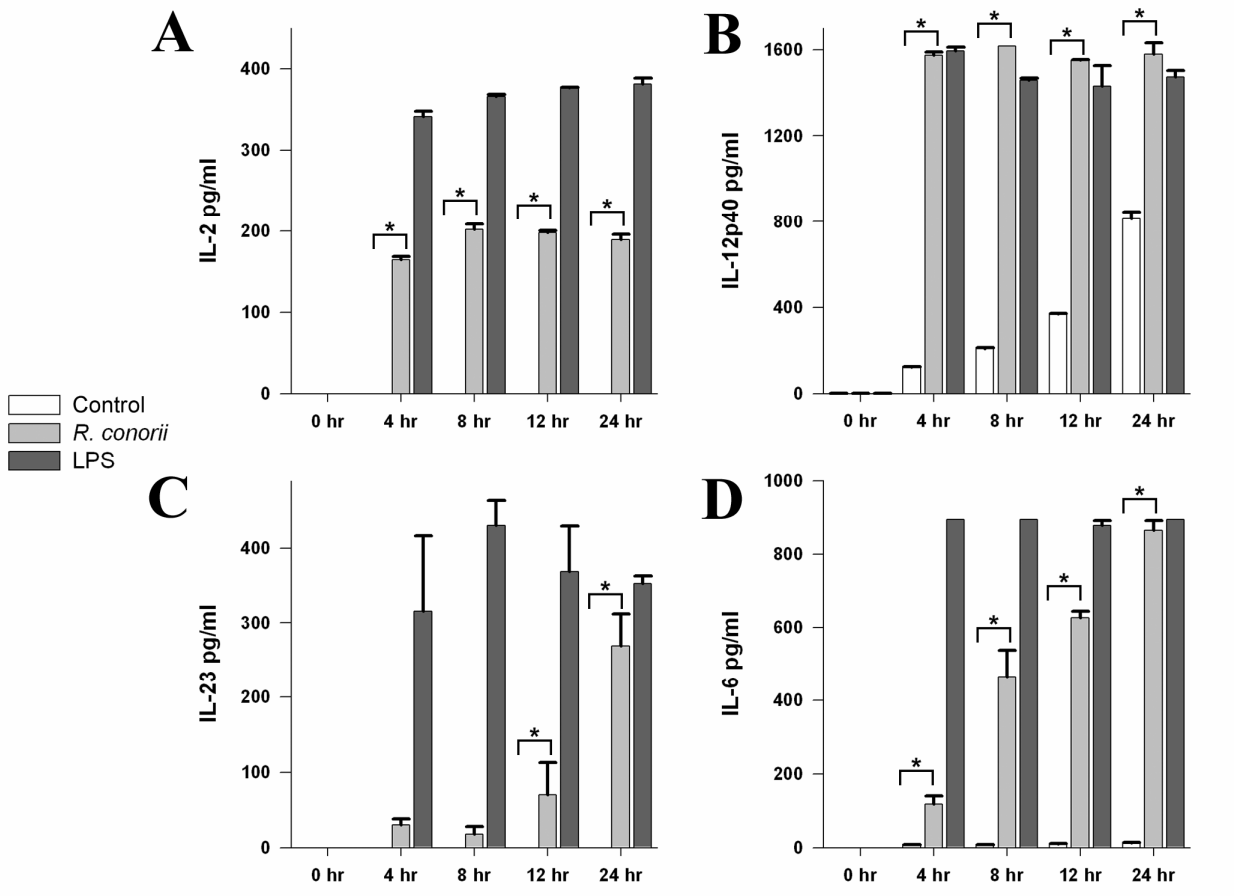


Figure 3.4. ***Rickettsia conorii*-stimulated DC secreted proinflammatory cytokines.** Supernatants from DC cultures stimulated with *R. conorii* (moi = 5), LPS (50 ng/ml) or control were collected at 4, 8, 12, and 24 h post-stimulation for measurement of (A) IL-2; (B) IL-12p40; (C) IL-23; and (D) IL-6. The mean ± SEM of triplicate results from one of three representative experiments are shown. * $p < 0.05$

Rickettsiae-stimulated BMDC migrate to draining lymph nodes following intradermal inoculation

To determine if protection correlated with migration of stimulated DC from the footpad to draining lymph nodes, inguinal lymph nodes were harvested 24 hrs following injection of CFSE-labeled DC. We demonstrated that rickettsiae-infected DC migrated to inguinal lymph nodes following footpad inoculation, although in our system we were unable to demonstrate a clear upregulation of CCR7 by flow cytometric analysis (data not shown). Laser scanning confocal microscopic examination revealed DC migration, primarily to the T-cell rich paracortex of the lymph node. It is clear that CFSE-labeled DC infected with rickettsiae were present in the lymph node (**Figures 3.5a, b**). Of note, close examination of a *R. conorii*-infected DC in the lymph node revealed CFSE- and DAPI-labeled rickettsiae residing in the cytoplasm. Previous data, indicating that CD8 T-lymphocytes are activated in the absence of CD4 help, suggest that rickettsiae-infected DC may induce a CTL response through a cross-presentation mechanism. A possible cross-presentation mechanism in rickettsial infection was further supported by the observation that fluorescent green vesicles were found in cells which were not otherwise green (**Figure 3.5c**). This finding suggests that transferred DC acted as donors to resident DC to endocytose cell particulates containing rickettsial antigen.

Rickettsia-infected DC induced activation of T-cells to a Th1 phenotype *in vitro*

To determine the role of the DC1 cytokines in T-cell activation, we co-cultured DC with naïve or immune T-lymphocytes ($CD4^+$ or $CD8^+$) and evaluated activation based on T-cell cytokine production. DC, when compared with macrophages, were more efficient inducers of T-cell activation, characterized by the production of significantly greater quantities of Th1 cytokines, IFN-γ and IL-2 (**Figure 3.6a, d**), presumably driven by the secretion of IL-12p70 by DC (**Figure 3.6b**) and upregulation of MHC II and costimulatory molecules (**Figure 3.3**). Immune $CD4^+$ T-cells produced IL-10 after exposure to infected DC (**Figure 3.6c**). Interestingly, in the absence of $CD4^+$ T-cell help, naïve $CD8^+$ T-cells were activated by *R. conorii*-infected DC (indicated by secretion of

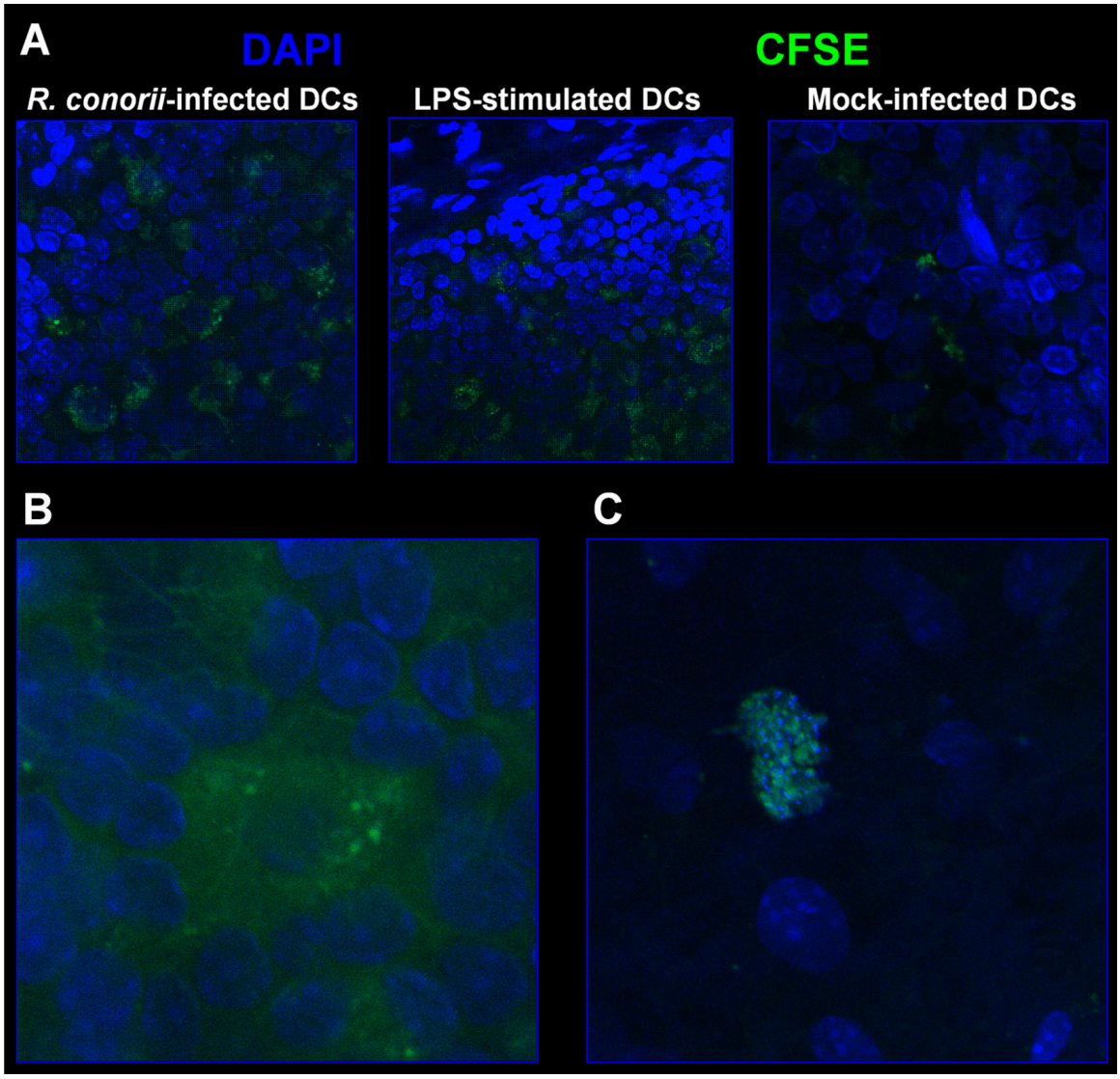


Figure 3.5. Activated DCs injected into the footpad of mice migrated to draining lymph node. 5 x 10^6 CFSE-labeled DC stimulated with mock infection, *R. conorii* infection (moi = 5), or LPS stimulation (50 ng/ml) were injected into the footpad of naïve mice. Twenty-four hours post-injection, inguinal lymph nodes were harvested, fixed in 10% paraformaldehyde, and processed for examination by laser scanning confocal microscopy (DAPI = blue; CFSE = green). (A), Analysis of lymph node paracortex sections from animals receiving *R. conorii*-infected DCs, LPS-stimulated DCs or mock-infected DCs. (B) High-magnification image of migrated DC from mouse receiving *R. conorii*-infected DCs with lymphocyte-appearing cells surrounding. (C) Rickettsiae are stained with DAPI inside green cytoplasm.

IFN-γ) (**Figure 3.6a**). These data suggest that rickettsia-infected DC can prime $CD4^+$ T-cells to become protective Th1 cells and that naïve $CD8^+$ T-cells may be activated in the absence of T-cell help.

Adoptive transfer of rickettsiae-stimulated BMDC induced protective immunity against a lethal *R. conorii* challenge

Having demonstrated that rickettsiae-infected DC induced a Th1-promoting response *in vitro*, we examined if rickettsia-infected DC were sufficient to initiate a protective response *in vivo*. We challenged mice with 3 LD_{50} of *R. conorii* 24 h after adoptive transfer of *R. conorii*-infected DC into the footpad of naïve mice. Transfer of *R. conorii*-infected DC induced significant protection ($p < 0.05$) against an ordinarily lethal challenge compared to animals receiving mock-infected DC (100% vs. 0% survival) (**Figure 3.7a**). Surprisingly, we observed partial protection in mice receiving LPS-stimulated DC compared to mock-infected DC (50% vs. 0%), although protection afforded by *R. conorii*-infected DC was significantly greater than LPS-stimulated DC, suggesting a critical role of Ag-specific

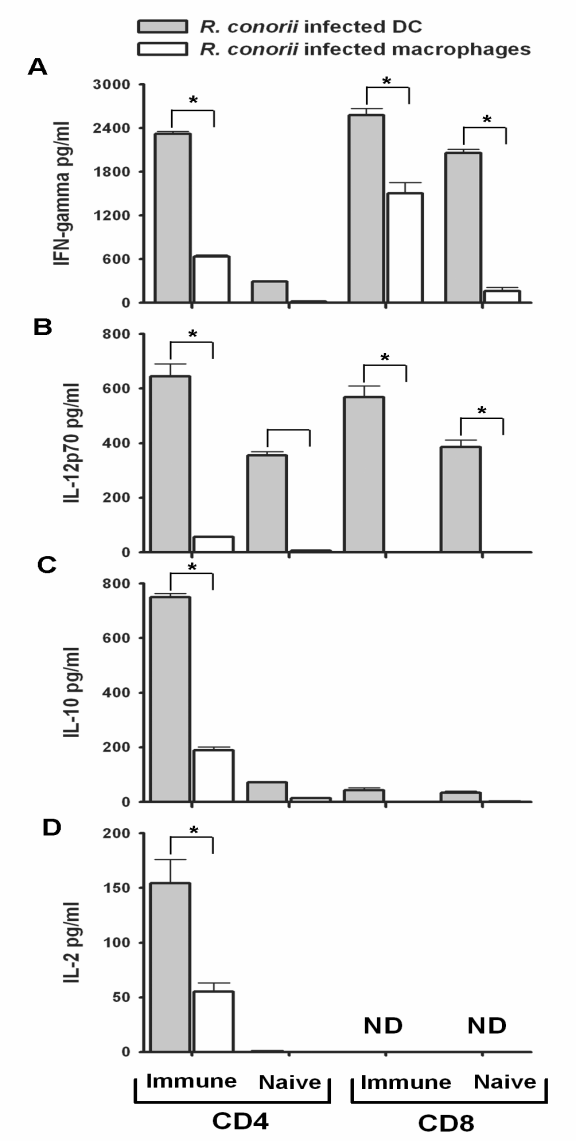


Figure 3.6. *R. conorii*-stimulated DC induced activation of T-lymphocytes to a Th1 phenotype. Rickettsiae-stimulated BMDC or macrophages were utilized as antigen presenting cells and co-cultured with T-cells obtained from either naïve or immune mice. Supernatants from co-cultures were obtained after 48 h of culture for the measurement of (A) IFN-γ; (B) IL-12p70; (C) IL-10; and (D) IL-2. The mean ± SEM of triplicate results from one of two representative experiments are shown. (ND), not determined. * $P < 0.05$

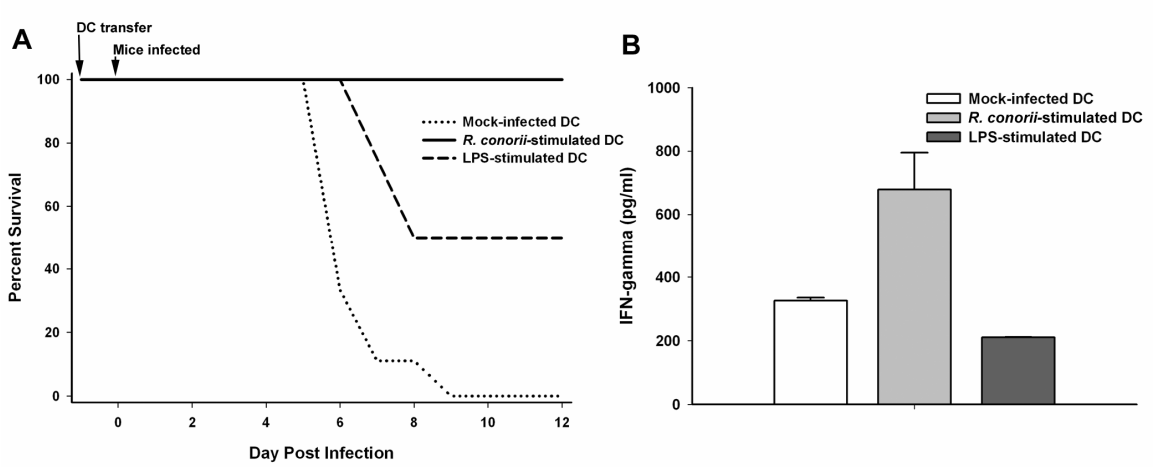


Figure 3.7. Transfer of *R. conorii*-stimulated DC protected mice from lethal challenge. (A) Stimulated DC were injected into the footpads of mice. Twenty four h thereafter mice were infected with an ordinarily lethal dose (3 LD50) of *R. conorii*. Survival graph depicts mortality following challenge. Shown is a representative survival curve of 3 independent studies (10 mice per group). (B) IFN-γ was quantitiated from sera collected from animals on d 6 post infection. The mean ± SD of triplicate results from one of three representative experiments are shown.

immunity. Of note, animals that received LPS-stimulated DC and succumbed to infection survived 1-2 days longer than most animals receiving mock-infected DC. Protection in C3H/HeN mice inoculated with *R. conorii*-infected DC correlated with production of IFN-γ. On day 6 post-infection, sera of mice receiving *R. conorii*-infected DC contained nearly two-fold higher quantities of IFN-γ than mice receiving mock-infected or LPS-stimulated DC (**Figure 3.7b**).

Transfer of *R. conorii*-infected DC to naïve animals limited rickettsial proliferation and induced IFN-γ production *in vivo*

To evaluate levels of rickettsial infection in primary pathologic organs and elucidate mechanism(s) underlying protection in animals receiving *R. conorii*-infected or LPS-stimulated DC, we performed serial time course studies. Rickettsial loads were significantly lower in animals receiving rickettsiae-infected DC than in those receiving mock-infected DC as early as day 4 post-infection, suggesting that transfer of rickettsiae-infected DC induced an anti-rickettsial phenotype limiting rickettsial proliferation in the brain, lung and spleen (**Figure 3.8a, b, c**). By day 6, rickettsiae were virtually

undetectable in brain and lung samples, the two most important organs relating to pathologic injury and death (**Figure 3.8a, b**). *R. conorii* Ag visualized by immunohistochemistry had decreased intensity and quantity of staining (**Figure 3.8d**). On day 6, mice receiving mock-infected DC had nearly 40- and 20-fold greater rickettsial levels in the brain and lung, respectively, when compared to recipients of *R. conorii*-infected DC. We expected to observe intermediate rickettsial titers in the lungs and brain of mice receiving LPS-stimulated DC compared with those receiving mock-infected and rickettsial-infected DC. The mechanisms underlying protection afforded mice injected with LPS-stimulated DC are not so clear-cut, however. Rickettsial titers in spleen and brain of mice that received LPS-stimulated DC prior to challenge were similar to mice that had received mock-infected DC (**Figure 3.8a, c**). On day 6 we observed a difference in rickettsial titers between mice receiving LPS-stimulated and mock-infected DC in the lungs, suggesting that lung titers may be a prognostic indicator of survival from rickettsial challenge.

Transfer of rickettsiae-stimulated DC induced expansion of T-cells and NK cells *in vivo*

Because protection from rickettsial challenge depends on vigorous CTL and NK responses, we determined relative and absolute numbers of lymphocytes in spleen and draining lymph node 48 h after DC transfer. To avoid confounding effects, mice were not infected intravenously with rickettsiae. There were no significant differences in immune cell percentages among spleen or lymph node cells assessed by flow cytometry (data not shown); however, transfer of either LPS- or *R. conorii*-stimulated DC induced significant expansion of absolute numbers of splenic lymphocytes and NK cells (**Figure 3.9a**). Mice receiving LPS- or *R. conorii*-stimulated DC had two-fold greater $CD3^+$ and $CD4^+$ T-cells in the spleen. Transfer of *R. conorii*- or LPS-stimulated DC induced a greater than two-fold increase in splenic NK cells. In addition, transfer of *R. conorii*-stimulated DC induced a significantly greater percentage of IFN-γ-producing $CD8^+$ T-cells 6 days post-transfer of stimulated DC (**Figure 3.9b**).

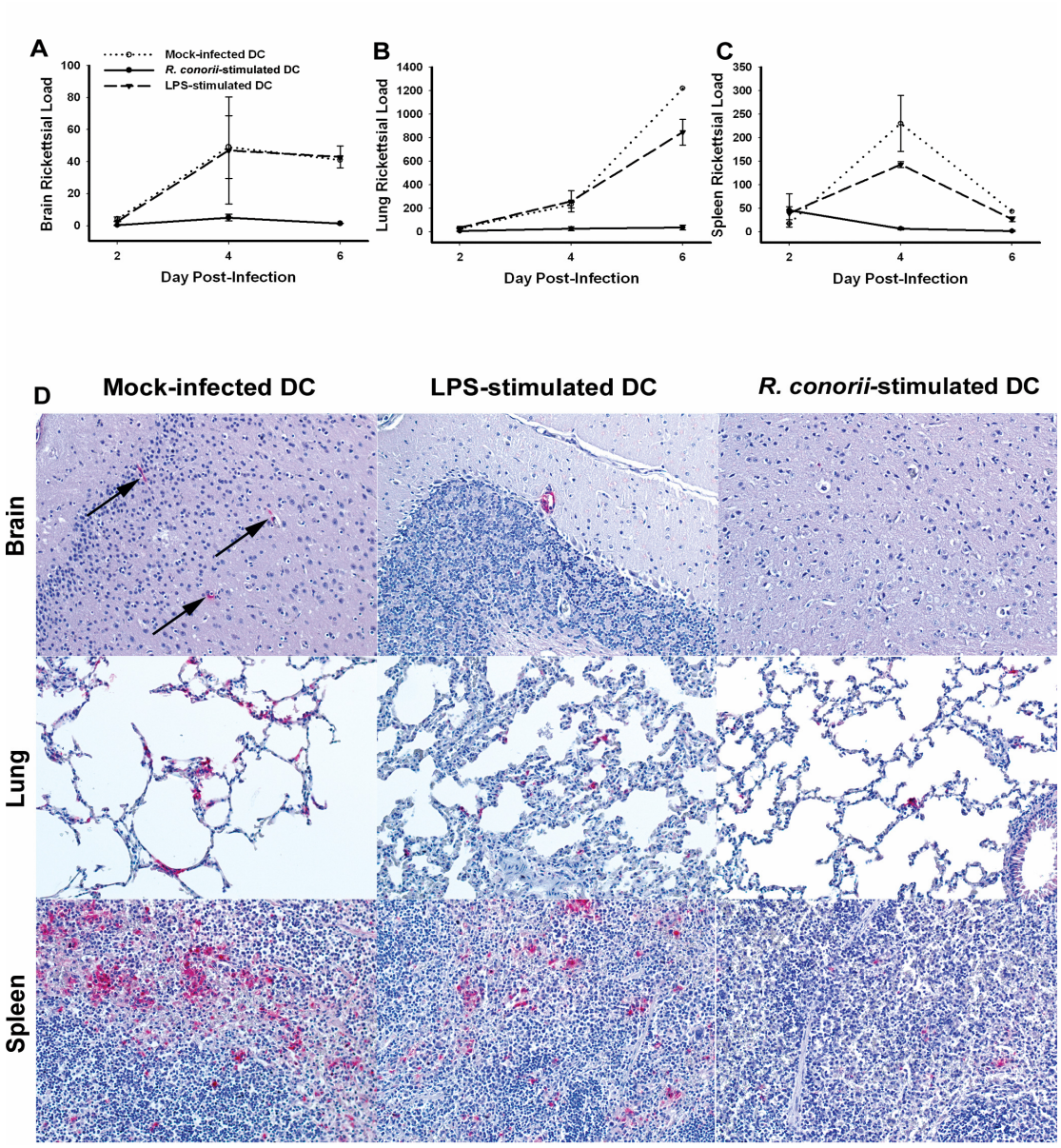


Figure 3.8. Transfer of *R. conorii*-stimulated DC resulted in significantly decreased rickettsial loads in pathologically relevant tissues. Animals were injected with 5 x 10^5 DC and challenged 24 h later with 3 LD50 of *R. conorii*. Two animals were sacrificed per treatment group (control DC, *R. conorii*-stimulated DC and LPS-stimulated DC) on d = 2, 4, and 6 post-challenge. Tissue loads of rickettsial gene copy levels were determined by quantitative real-time PCR and normalized to murine *β-actin* in (A) brain, (B) lung, and (C) spleen. Data are expressed as copies of rickettsial-specific citrate-synthase gene per 10,000 copies of murine *β-actin*. (D) Photomicrographs of brain, lung, and spleen samples collected on d 6 post-challenge and stained with *R. conorii*-specific polyclonal Ab. *R. conorii* antigen is stained red.

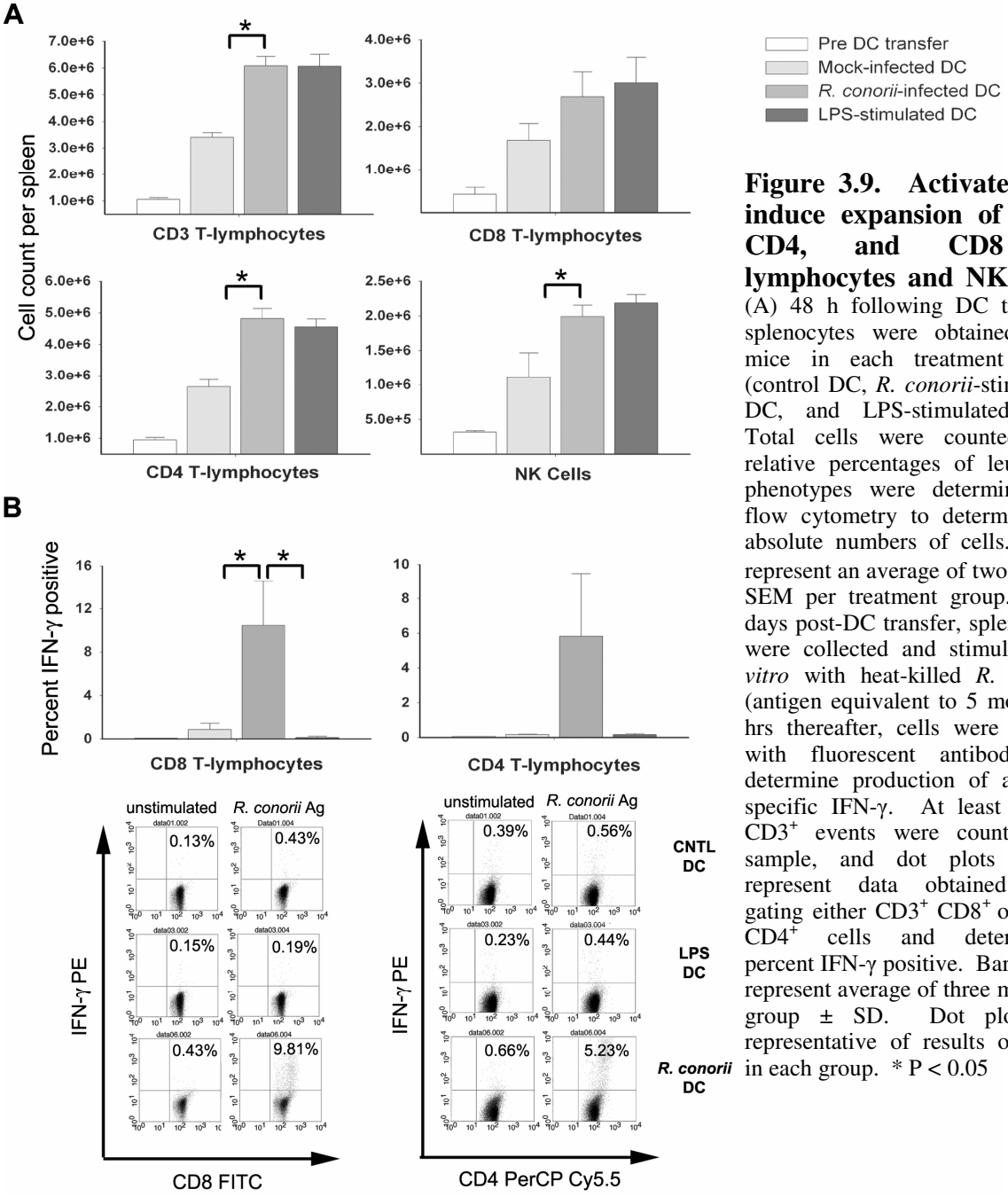


Figure 3.9. Activated DC induce expansion of CD3, CD4, and CD8 T-lymphocytes and NK cells. (A) 48 h following DC transfer, splenocytes were obtained from mice in each treatment group (control DC, *R. conorii*-stimulated DC, and LPS-stimulated DC). Total cells were counted, and relative percentages of leukocyte phenotypes were determined by flow cytometry to determine the absolute numbers of cells. Data represent an average of two mice ± SEM per treatment group. (B) 6 days post-DC transfer, splenocytes were collected and stimulated *in vitro* with heat-killed *R. conorii* (antigen equivalent to 5 moi). 24 hrs thereafter, cells were stained with fluorescent antibodies to determine production of antigen-specific IFN-γ. At least 50,000 $CD3^+$ events were counted per sample, and dot plots shown represent data obtained from gating either $CD3^+$ $CD8^+$ or $CD3^+$ $CD4^+$ cells and determining percent IFN-γ positive. Bar graphs represent average of three mice per group ± SD. Dot plots are representative of results obtained in each group. * $P < 0.05$

DISCUSSION

DC induce immunity to a variety of pathogens (124). In the present study, we characterized the responses of immature murine DC to rickettsiae and the protective role of DC. This model of protection, predicated on DC-based immune priming, allowed us to investigate the role of DC in rickettsial infection and to determine their initial responses following rickettsial infection.

Initial characterization of rickettsiae-DC interaction elucidated that *R. conorii* can productively infect DC *in vitro*; moreover, these DC, when transferred via footpad injection migrated to draining lymph nodes. These data support the hypothesis that DC in the skin may provide a mechanism for rickettsial dissemination via infection in the dermis followed by migration to draining lymphatics. Moreover, we have shown that upon rickettsial infection, DC become significantly activated; DC activation has been shown to lead to significant angiogenesis in lymph nodes. Specifically, endothelial cell proliferation in lymph nodes is dependent on $CD11c^+$ DC; moreover, injection of DC, as was accomplished in this study was sufficient to trigger endothelial growth and proliferation (125). As a result of these data, it is conceivable that in addition to rickettsia-infected DC disseminating infection through migration, DC may also enhance early infection by initiating angiogenesis in draining lymph nodes leading to a greater accumulation of endothelium—the main target organ. Nevertheless, it still seems clear that DC are important initiators of innate and adaptive immune responses, leading to a protective phenotype *in vivo*.

Significant protection from rickettsial infection was afforded by adoptive transfer of activated DC—either LPS- or *R. conorii*-stimulated DC. These DC produced significant amounts of pro-inflammatory cytokines (IL-2, IL-6, IL-12p40, and IL-23) and activated T-cells toward a Th1 phenotype *in vitro*. We therefore suggest that protection was likely initiated by augmenting the innate immune response through NK cell activation and proliferation and initiating an adaptive Th1 immune response (126-128).

Both $CD8^+$ T-lymphocytes and NK cells are important in protection against numerous intracellular infections, including rickettsioses, owing to their production of

IFN-γ and other immune effector functions (67;86;87;129). Upon transfer of *R. conorii*-infected DC, critical effector cells—specifically $CD8^+$ T-cells and NK cells—rapidly increased in the spleen and correlated with protection from lethal rickettsial challenge and significant blunting of rickettsial proliferation *in vivo*. Additionally, mice receiving *R. conorii*-infected DC produced significantly more IFN-γ than mice receiving mock-infected or LPS-stimulated DC. These data are consistent with previous observations from other investigators. Badovinac *et al*. demonstrated that vaccination with peptide-pulsed DC significantly increased the rate of effector cell (antigen-specific $CD8^+$ T-lymphocyte) expansion (130).

DC upregulate phenotypic activation markers after rickettsial uptake, similar to other intracellular bacterial or parasitic infections (90;110;131). The early responses of DC to rickettsiae *in vitro*, in the absence of T-cell interaction or CD40L, suggest that rickettsiae may induce activation of DC through as-of-yet undetermined Toll-like receptors (TLR) or other pathogen-associated molecular pattern receptors (132). Ligation of TLR is important in appropriately skewing and influencing the vigor of an immune response, and the importance of TLR ligands as components in vaccine formulations is becoming apparent (121).

Our observation that transfer of LPS-stimulated DC leads to partial protection against rickettsial challenge provides further evidence that TLR-specific activation of DC may boost innate immunity favoring protection. Both LPS- and rickettsia-stimulated DC produced IL-2 in a kinetically similar manner, implying that both may signal through the same TLR, presumably TLR4. Indeed, another rickettsial pathogen, *R. africae,* induces the activation of TLR4 on endothelial cells (133). Most likely, both stimuli augment NK cell activation prior to ordinarily lethal challenge through IL-2 and IL-12p70 production, thereby favoring protection (106;134). We have demonstrated previously that NK cells are important effectors of anti-rickettsial activity through the production of IFN-γ (67). Despite our observations indicating the importance of innate immunity, the lack of complete protection in animals receiving LPS-stimulated DC, nevertheless, indicates that Ag-specific immunity is crucial. Consistently, we observed that one main difference

between animals receiving rickettsiae-infected DC and LPS-stimulated DC was the generation of IFN-γ-producing $CD4^+$ and $CD8^+$ T-lymphocytes after antigenic stimulation *in vitro*.

Despite our observations, augmentation of innate immunity may not always lead to protection, however. For example, systemic activation of DC by *Plasmodium spp*. through TLR ligation leads to global maturation of DC *in vivo*, and inhibits Ag-specific immunity to viral infections through decreasing cross-presentation by DC (135). It is possible that in our system, we may actually increase cross-presentation through transfer of cell-associated Ag available for uptake by resident DC. This effect would not be surprising as we induced transient innate activation after injection of *R. conorii*-infected DC in contrast to continual systemic-activation leading to global DC maturity. Others have shown that ligation of TLR may induce cross-presentation by DC (136). Cross-presentation of microbial antigens has been described in numerous infectious diseases (137;138). Utilizing laser scanning confocal microscopy, we labeled *R. conorii*-infected DC with the vital dye CFSE and injected them similarly to previous DC-protection experiments. Twenty-four h after injection of CFSE-labeled DC, we observed green-fluorescent bodies inside otherwise unlabeled cells in the paracortex of inguinal lymph nodes in animals that received *R. conorii*-infected DC, suggesting that *R. conorii*-infected DC may act as donors of rickettsiae or rickettsial antigen to dermal DC or Langerhans cells following injection into the skin, or possibly migrate to draining lymph nodes to donate cell-associated Ag to follicular DC. This phenomenon has been demonstrated previously in *M. tuberculosis* infection (139).

Our results further support a cross-presentation mechanism by the observation that *in vitro R. conorii*-infected DC can activate $CD8^+$ T-lymphocytes in the absence of $CD4^+$ T-cell help, judged by their production of IFN-γ in DC-$CD8^+$ T-cell co-culture experiments. Activation of $CD8^+$ T-cells by DC in the absence of T cell help is correlated with the upregulation of CD70, and suggests that Ag is presented to DC through a cross-presentation mechanism (140;141). Additional support to the hypothesis that *R. conorii*-infected DC can activate $CD8^+$ T-cell responses in the absence of $CD4^+$ T-

cells *in vivo* includes demonstration that *R. conorii*-infected mice generate protective CTL responses despite $CD4^+$ T-cell depletion (86).

Here, we provide a model of protection which may facilitate design of post-exposure prophylaxis for rickettsial disease, as well as new vaccines. NK cells, which may be activated by DC production of IL-2 and IL-12p70, increased in number following transfer of LPS-stimulated and *R. conorii*-infected DC. Moreover, transfer of activated Ag-independent DC confers partial protection in a murine model. Therefore, augmenting the innate immune response through immunotherapy may provide some post-exposure protection. DC, when stimulated with rickettsiae, are capable of inducing a protective immune response against a typically fatal challenge of *R. conorii*, implying that a rickettsial vaccine which induces DC to direct a Th1 response and to induce NK cell activation may protect against human rickettsioses.

CHAPTER 4: MICE DEFICIENT IN TLR4 SIGNALING ARE PREDISPOSED TO OVERWHELMING RICKETTSIAL INFECTION

We have previously demonstrated that LPS signaling in DC leads to partial protection from an ordinarily lethal *R. conorii* infection in mice. These data suggest that LPS ligation of toll-like receptor 4 (TLR4) may play a role in protection from rickettsioses. The significance of TLR4 ligation in immunity to rickettsioses has not been elucidated, however. Here, we demonstrated that mice dysfunctional in TLR4 signaling were more susceptible to fatal *R. conorii* infection. The LD_{50} of mice with dysfunctional TLR4 signaling was nearly one log lower than those that possessed the ability to signal through TLR4. Moreover, we were able to titrate a dose that, after intravenous inoculation led to 100% fatality in mice with dysfunctional TLR4 signaling compared to 17% fatality in controls. Indicators of vigorous immunity were significantly greater in TLR4-competent mice. Specifically, TLR4-competent mice had a significantly greater expansion of effector cells, namely $CD8^+$ T-lymphocytes and NK cells, as well as significantly greater amounts of pro-inflammatory cytokines in the sera. We also observed that the apparent immunosuppression observed in TLR4 dysfunctional mice correlated with an expansion of regulatory T-cells. Together, this study indicates that absence of TLR4 signaling leads to a depressed anti-rickettsial pro-inflammatory response, correlated with decreases in Th1 cells and serum pro-inflammatory cytokines.

INTRODUCTION

Toll-like receptors and immunity

The immune system is activated by specialized receptors upon pathogen exposure by recognition of evolutionarily conserved structures in pathogens—pathogen-associated molecular patterns (PAMP)—that are not found in mammals. Toll-like receptors (TLR), named because of their homology to the *Drosophila* Toll protein, are a family of PAMP receptors that possess the ability to recognize a wide range of structural components of

microbial origin leading to innate and acquired immune responses (142;143). Many TLR have been characterized, and the microbial components important in ligation have been implicated; these include peptidoglycan and atypical-LPS (TLR2), double-stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), and CpG motifs (TLR9) (**Figure 4.1**) (144). TLR ligation generally leads to a signaling cascade culminating in NF-κB activation and translocation to the nucleus and subsequent production of important mediators of inflammation (145).

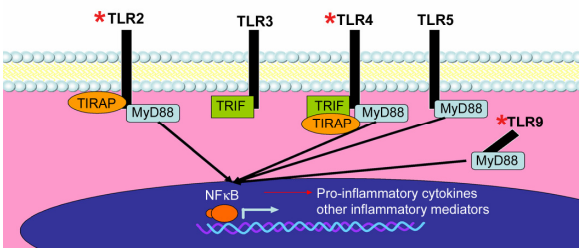


Figure 4.1. Toll-like receptor-mediated signaling. * = TLR likely important in rickettsial immunity. Adapted from Kaisho and Akira, (2004) Microbes. Inf.

Although TLR are generally considered important in initiation of innate immunity, they also play an important role in adaptive immunity. The importance of TLR in initiating and polarizing immune responses towards pathogens has been extensively chronicled in the literature (100;132;146-148). TLR signaling leads to maturation of DC and upregulation of co-stimulatory molecules critical in generating antigen-specific immunity. Moreover, the cytokines produced by TLR-activated DC also facilitate the polarization of the immune response (discussed in greater detail in Chapter 3) (149). TLR9 knockout (KO) mice have been shown to be defective in IL-12p40 and IFN-γ responses *in vivo*, leading to decreased protective Th1 responses to *Mycobacterium tuberculosis* challenges in mice (150). Similarly, Th1 cytokine responses following administration of heat-killed *Brucella abortus* have been shown to be dependent on TLR2 and TLR9 signaling in mice (151;152). Blander and Medzhitov have further elucidated the importance of TLR ligation in adaptive immunity by demonstrating that TLR dictate the selection of antigens and efficiency of antigen presentation in DC (153).

Significance of TLR4 in immunity

LPS has long been recognized as the prototypic activator of innate immunity and has been extensively studied as a key mediator of septic shock syndrome despite the fact

that how LPS was exerting its proinflammatory actions was unclear. The discovery of TLR4 as the natural ligand for LPS facilitated the understanding of the molecular mechanisms behind the physiologic reactions to LPS. Consequently, we now understand that TLR4 plays a multitude of roles both in terms of protection from Gram-negative bacterial infection but also in pathogenesis.

Mice that are either completely lacking TLR4 or incapable of signaling through TLR4 have been shown to be more susceptible to a variety of microbial organisms. Mice which lack TLR4 signaling capability have increased susceptibility to *Bordetella pertussis* infection, characterized by delayed clearance of bacteria and significant decreases in pro-inflammatory cytokines IL-1β and TNF-α (154). Additionally, TLR4-defective mice are overwhelmed by the spirochete *Leptospira interrogans* following infection leading to significantly higher leptospiral burdens, decreased cytokine responses and increased pathology (155)

The mechanism(s) behind the increased susceptibility of TLR4-deficient mice has begun to be clarified. For instance, TLR4 ligation is important in activating DC towards the initiation of Th1-type responses in mice and humans, although addition of another TLR signal to that of TLR4 was shown to 'boost' the observed response (156;157). Additionally, TLR4 stimulation is important in the initiation of another unique pro-inflammatory response; IL-17 producing T-lympohcytes (Th17), distinct from Th1 responses are characterized by IL-17 producing CD4 T-lymphocytes (158). TLR4 is necessary for the generation of IL-17-secreting T-lymphocytes in the lung during acute lung infection with *Klebsiella pneumoniae* as well as in response to whole-cell *Bordetella pertussis* vaccines (159;160).

While it appears clear that TLR4 signaling leads to a pro-inflammatory responses *in vivo*, it has also been shown that animals deficient in TLR4 signaling suppress immunity. Lack of TLR4 stimulation during antigen presentation leads to a disproportionate expansion of regulatory T-lymphocytes (T_{reg}) and subsequent suppression of immunity (161;162). Interestingly, TLR2 stimulation on T_{reg} cells has been shown to lead to cellular expansion concurrent with TCR stimulation (163).

Mouse models for elucidating TLR4 significance

Long before the significance of TLR4 was realized, several mouse strains were recognized to be "non-responsive" to LPS, notably C3H/HeJ and C57Bl/10ScCr mice. C3H/HeJ mice possess a missense mutation in the TLR4 gene, which leads to a single amino acid change in the cytoplasmic portion of TLR4, impeding signal transduction and leading to a phenotype similar to TLR4 KO (164-166). Therefore, C3H/HeJ mice are defective in TLR4 signaling and defective in responding to LPS ($TLR4^{(LPS\text{-}d)}$).

TLR4 and rickettsioses

Our laboratory has previously demonstrated that ligation of TLR may be important in initiating immunity to rickettsioses. We have shown that transfer of DC stimulated with *E. coli* LPS induces partial protection from a typically lethal rickettsial infection, suggesting that TLR4 ligation is important in protection (Chapter 3). These data led us to examine the importance of TLR4 signaling in initiating a protective immune response *in vivo*. To this end, we have exploited our well-established model of rickettsiosis in C3H/HeN mice, and compared it to the genetically related mouse strain, C3H/HeJ.

In our laboratory, we have been able to elucidate many of the important immune-effector mechanisms against rickettsiae using a *R. conorii*-infected C3H/HeN mouse model (41). Owing to the obligately intracellular lifestyle of rickettsiae, CTL and the production of NO in endothelium subsequent to IFN-γ and TNF-α stimulation are critical in immunity (66;86). Evidence also suggests that NK cell activity and early NK cell-derived IFN-γ production are also important (67). Nevertheless, the early triggers leading to protective immunity, particularly the significance of TLR-ligation, have not been evaluated.

Previous studies have demonstrated that spotted fever group rickettsiae, namely *R. africae,* and a phylogenetically-related organism, *Wolbachia*, are capable of inducing cellular activation through TLR2 and TLR4, for which the natural ligands are peptidoglycan and LPS, respectively (133;167). Despite these data, we currently do not understand the immunologic significance of TLR4 in rickettsioses.

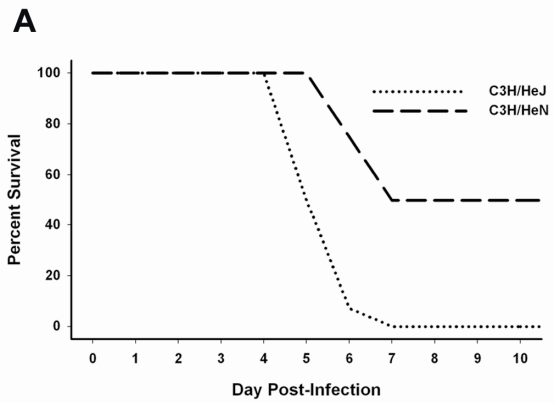
Using our model of human spotted fever rickettsioses in C3H/HeN mice and comparing the pathogenesis and immune responses to rickettsiae in the genetically related C3H/HeJ mouse, we present evidence that $TLR4^{(LPS\text{-}d)}$ mice are predisposed to overwhelming fatal rickettsial infection when given an inoculum that is non-fatal to TLR4-competent C3H/HeN mice . $TLR4^{(LPS\text{-}d)}$ mice had significantly greater rickettsial proliferation levels and decreased pro-inflammatory cytokine levels throughout infection. Specifically, mice which possess functional TLR4 responses have significantly greater levels of TNF-α, IL-6, IL-12p40, IL-12p70 and IL-17. Consistent with data suggesting a greater pro-inflammatory response in TLR4 competent mice, $TLR4^{(LPS\text{-}d)}$ mice had a significantly greater percentage of T_{reg} cells in the spleen during infection and significantly fewer activated $CD4^+$ and $CD8^+$ T-lymphocytes.

RESULTS

TLR4 dysfunction leads to increased susceptibility to severe *R. conorii* infection

In order to determine the significance of TLR4 signaling in immunity to spotted fever rickettsiae, we used our well-established murine model of spotted fever rickettsioses in the C3H/HeN mouse and compared it to the genetically-related mouse with defective TLR4 function, C3H/HeJ ($TLR4^{(LPS\text{-}d)}$). $TLR4^{(LPS\text{-}d)}$ mice that were given a *R. conorii* inoculum that is near the LD_{50} in C3H/HeN mice were all lethally infected (**Figure 4.2a**). Additionally, these mice succumbed to infection earlier than C3H/HeN mice. It is interesting to note also that lethally infected $TLR4^{(LPS\text{-}d)}$ mice appeared ill (characterized by hunched posture, lethargy, and ruffled hair) later than C3H/HeN mice.

These data led us to investigate the susceptibility of $TLR4^{(LPS\text{-}d)}$ mice more fully. While the typical LD_{50} dose of *R. conorii* in C3H/HeN mice is between 8 x 10^3 and 1 x 10^4 PFU, infecting $TLR4^{(LPS\text{-}d)}$ mice with doses as low as 7.5 x 10^2 PFU of *R. conorii* led to greater than 60% mortality while C3H/HeN mice all survived infection at this dose (**Figure 4.2b**).



B

R. conorii dose	C3H/HeN number died/number infected (% mortality)	C3H/HeJ ($TLR4^{(LPS\text{-}d)}$) number died/number infected (% mortality)
2.4 x 10^4 PFU	6/6 (100%)	6/6 (100%)
1.2 x 10^4 PFU	5/6 (83%)	6/6 (100%)
6 x 10^3 PFU	3/6 (50%)	6/6 (100%)
3 x 10^3 PFU	1/6 (17%)	6/6 (100%)
1.5 x 10^3 PFU	0/6 (0%)	4/6 (67%)
7.5 x 10^2 PFU	0/6 (0%)	4/6 (67%)
3.7 x 10^2 PFU	0/6 (0%)	3/6 (50%)

Figure 4.2. Deficiency in TLR4 leads to greater susceptibility to fatal *R. conorii* infection in mice. (A) C3H/HeJ ($TLR4^{(LPS\text{-}d)}$) and C3H/HeN mice were infected with 6 x 10^3 PFU *R. conorii* i.v. and monitored for survival. Curve is representative of one of three independent experiments. (B) $TLR4^{(LPS\text{-}d)}$ mice were infected with two-fold dilutions of *R. conorii* i.v. and monitored for survival. The established LD_{50} for this stock is approximately 8 x10^3 PFU in C3H/HeN mice.

Rickettsial loads in the organs are significantly greater in mice deficient in TLR4 function

To evaluate whether the enhanced susceptibility observed in TLR4$^{(LPS\text{-}d)}$ mice correlated with rickettsial bacterial burden differences *in vivo*, we determined the relative rickettsial loads throughout infection in the brain and lung, the main pathologic organs. TLR4$^{(LPS\text{-}d)}$ mice had significantly greater numbers of rickettsiae in the brain and lungs on days 3 and 5 post-infection (**Figure 4.3a, b**). It is notable that rickettsial loads were nearly undetectable in C3H/HeN mice, relative to TLR4$^{(LPS\text{-}d)}$ mice, suggesting significant immune pressure to limit rickettsial proliferation in TLR4-competent mice. Moreover, it is important to note that in both strains of mice, rickettsial loads in the lung were approximately one log greater than those observed in the brain, suggesting that following tail vein injection, many rickettsiae infect the endothelium of the alveolar capillaries, delaying spread to the brain until later in infection. The differences in

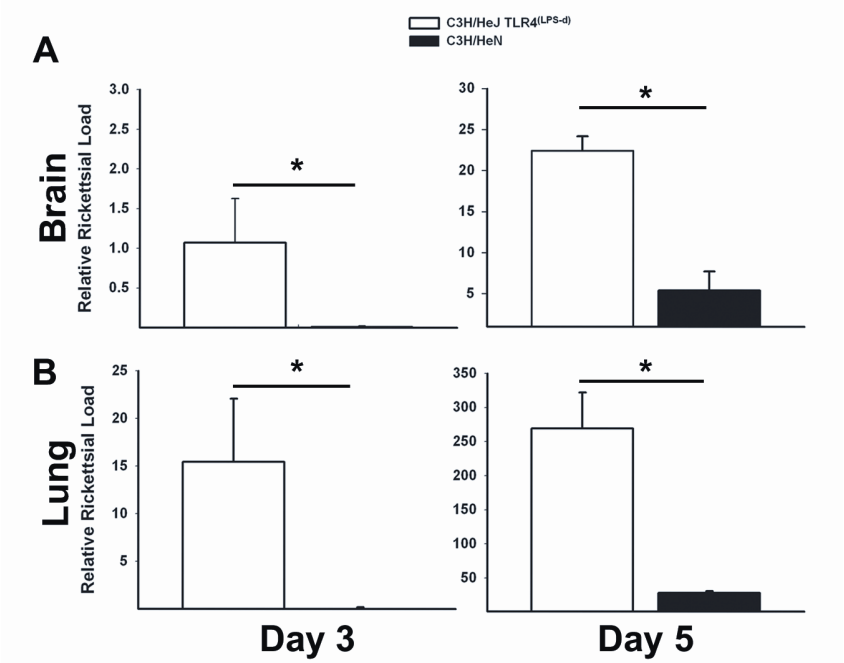


Figure 4.3. Increased susceptibility of TLR4(LPS-d) mice to *R. conorii* is due to an *in vivo* proliferation advantage. Rickettsial titers were quantitated in main pathologic organs (brain (A) and lung (B)) in mice on days 3 and 5 post-infection by quantitative real-time PCR. Data represent avg of 3 mice per treatment group per day ± SD and are representative of one of two experiments. * $p < 0.05$

rickettsial loads in brain and lung were also observed histologically. Localization of rickettsial antigen by immunohistochemistry revealed greater intensity and quantity of staining in mice lacking TLR4 function (**Figure 4.4**).

TLR4$^{(LPS\text{-}d)}$ mice have fewer splenic CD8 T-lymphocytes after rickettsial infection in association with increased T_{reg} cells in lymph nodes

In light of the observation that TLR4 ligation appears to significantly limit rickettsial proliferation *in vivo*, we focused on the evaluating the differences in cellular immunity after *R. conorii* infection. Necropsies performed on day five post-infection revealed marked differences in the size of the spleen in TLR4$^{(LPS\text{-}d)}$ mice, when compared to mice with competent TLR4 responses (data not shown). Consistent with a grossly enlarged spleen, mice with functional TLR4 responses had significantly greater splenic lymphocyte content on day 5 post-infection, although significant differences in lymphocyte quantities were not present in the peripheral lymph nodes (**Figure 4.5a, b**).

In addition to determining absolute lymphocyte populations, we also determined the percentages and absolute numbers of immune-effector cells in the spleen. TLR4$^{(LPS\text{-}d)}$ mice had significantly fewer activated ($CD69^+$) $CD4^+$ and $CD8^+$ T-lymphocytes in the spleen on day 5 of infection (**Figure 4.5c, d**). Despite these dramatic differences in absolute cell quantities, it is important to note that the percentages of the lymphocyte subsets did not differ significantly (data not shown); therefore, although TLR4 leads to significant augmentation of the cellular immune response, it does not alter the overall proportions of immune cells in response to rickettsial infection.

Our observation of possible immunosuppression in TLR4$^{(LPS\text{-}d)}$ mice, indicated by the suppression of immune cell proliferation and significantly fewer early-activated T-lymphocytes, also spurred investigation into the role of T_{reg} cells in these susceptible mice. TLR4$^{(LPS\text{-}d)}$ mice had a significantly greater percentage of T_{reg} cells ($FoxP3^+$ $CD25^+$ $CD4^+$ T-lymphocytes) in peripheral lymph nodes when compared to C3H/HeN mice (**Figure 4.5e**) on day 3 post-infection. TLR4$^{(LPS\text{-}d)}$ mice also had greater numbers of T_{reg} cells in the spleen (**Figure 4.5f**).

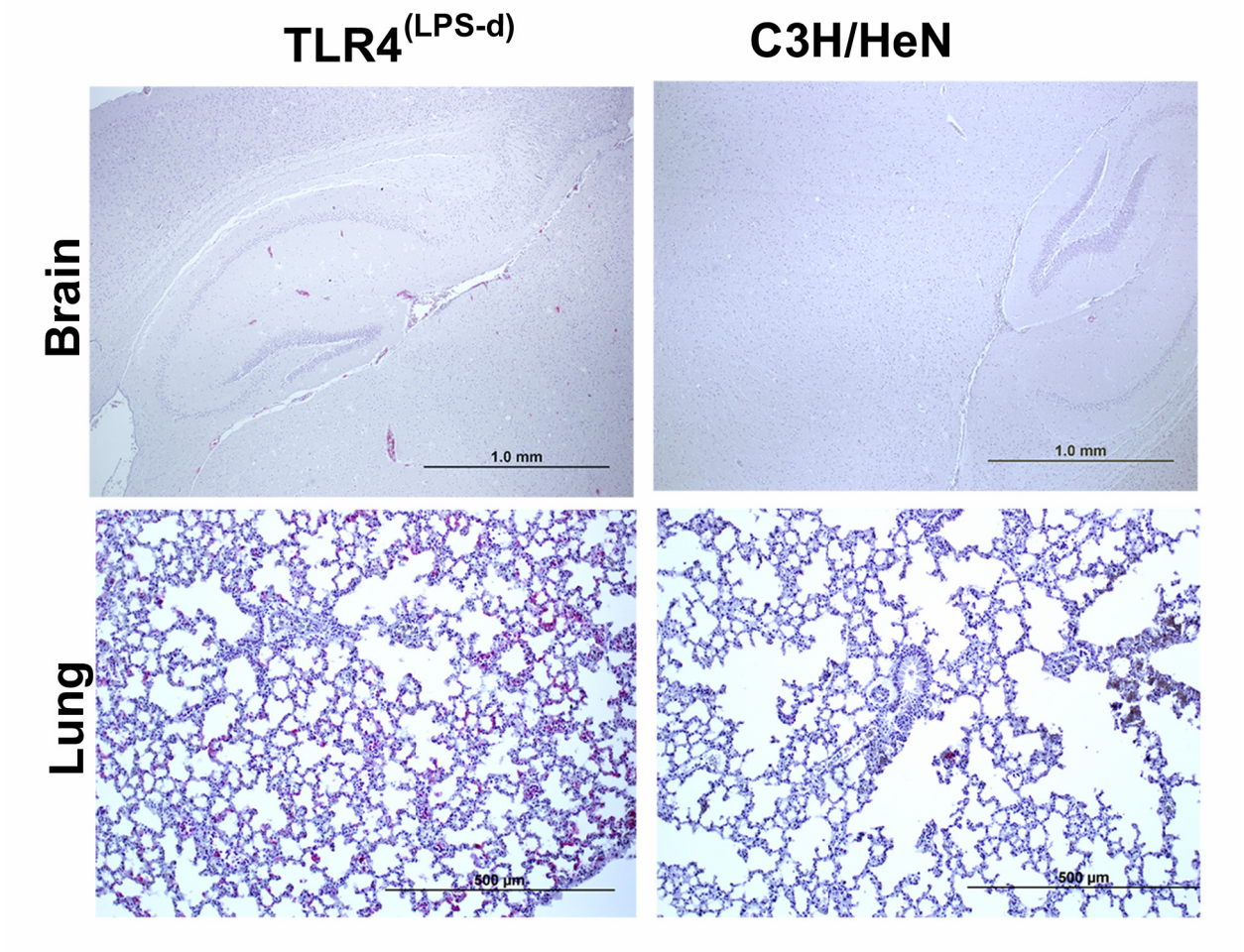


Figure 4.4. Immunohistochemical localization of rickettsial antigen. Organs were collected on day 5 post-infection, fixed and processed for immunohistochemical determination of rickettsial antigen

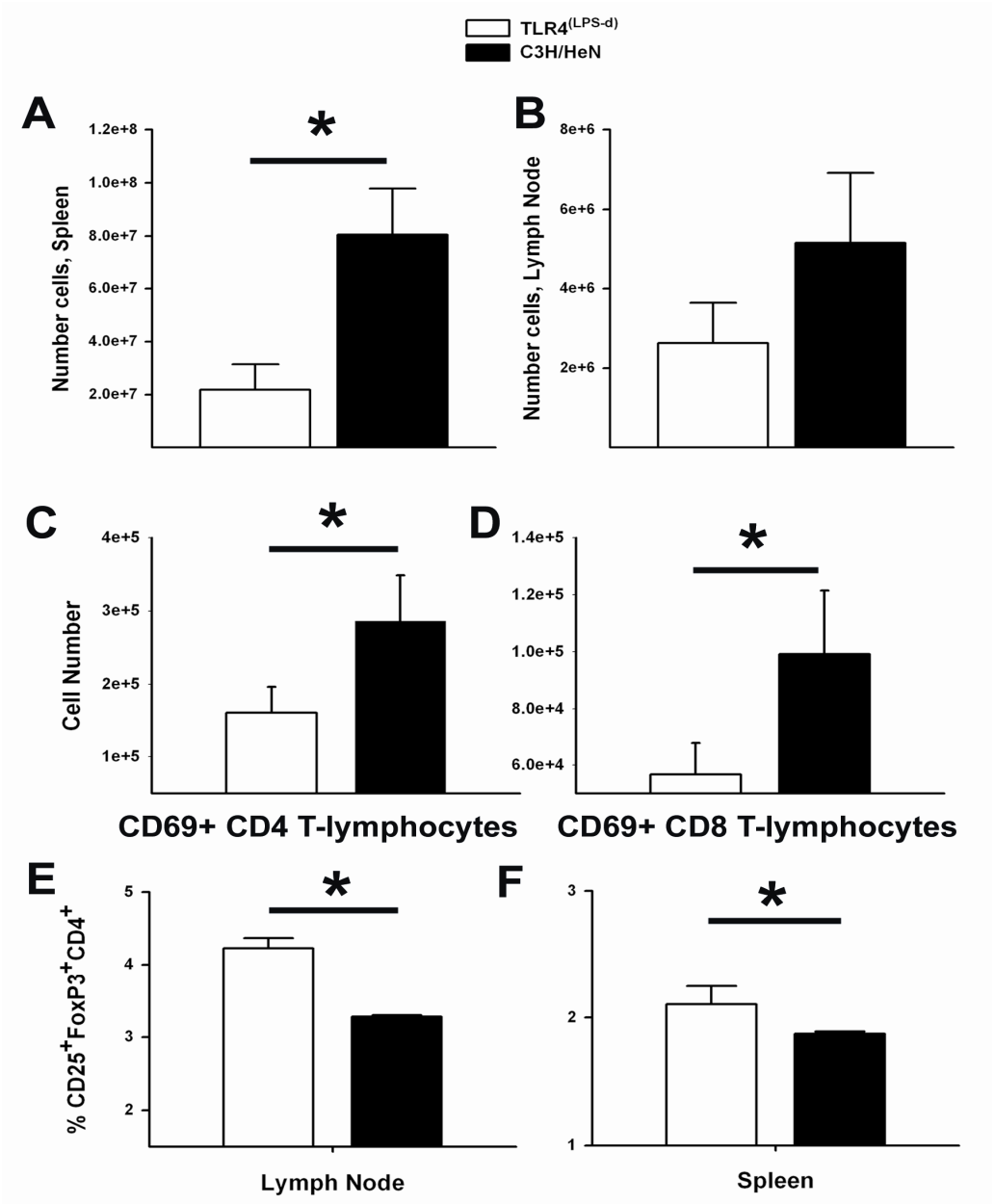


Figure 4.5. TLR4 ligation leads to significantly greater numbers of activated T-lymphocytes *in vivo* after *R. conorii* infection. Splenic (A) and lymph node (B) single cell suspensions were enumerated on day 5 post-infection to determine the total cellular numbers. Percentages of early-activated ($CD69^+$) $CD4^+$ (C) and $CD8^+$ (D) T-lymphocytes in the spleen were assessed by flow cytometry, and total numbers were determined by multiplying percentages obtained by numbers of cells recovered. Percentages of regulatory T-lymphocytes ($CD4^+$ $CD25^+$ $FoxP3^+$) were assessed by flow cytometry in peripheral lymph nodes (E) and spleen (F) on day 3 post-infection. Data represent mean of three mice per timepoint ± SD. * = $p < 0.05$.

Mice with competent TLR4 responses produced significantly greater levels of pro-inflammatory cytokines *in vivo*.

Our evaluation of the immune-effector cell response, specifically the expansion of immune effector subtypes, revealed that mice that possessed functional TLR4 responses had significantly greater numbers of immune effector cells (activated CD4 and CD8 T-lymphocytes), while TLR4$^{(LPS\text{-}d)}$ mice had a greater percentage of T_{reg} cells in lymph nodes and spleen. We, therefore, examined the cytokine concentrations in the sera after infection with *R. conorii* on days 3 and 5 post-infection to determine the effect that TLR4 ligation had on systemic cytokine production. Mice possessing functional TLR4 receptors had significantly higher levels of the prototypical Th1-inducing cytokines IL-12p40 and IL-12p70 three days post-infection, when compared to TLR4$^{(LPS\text{-}d)}$ mice (**Figure 4.6a**). TLR4-competent mice also had significantly higher levels of the leukocyte-chemotactic factor RANTES (CCL5) and IL-6, although two cytokines implicated in anti-rickettsial activity, TNF-α and IFN-γ were not significantly higher than TLR4$^{(LPS\text{-}d)}$ mice.

On day 5 post-infection, similar cytokine profiles prevailed; however, IL-12p40 and RANTES were no longer statistically different. However, IL-12p70 still remained significantly elevated in TLR4-competent mice compared to TLR4$^{(LPS\text{-}d)}$ mice, and TNF-α was also significantly elevated in TLR4-competent mice (**Figure 4.6b**). Of note, IL-12p70 was nearly undetectable in TLR4$^{(LPS\text{-}d)}$ mice, suggesting a significant failure in polarization towards a Th1 response.

Mice with functional TLR4 responses generated antigen-specific Th17 cells and had significantly greater levels of IFN-γ-producing CD8$^+$ and CD4$^+$ T-lymphocytes during acute rickettsial infection

Our data suggested that despite the perception of diminished pro-inflammatory cytokine responses and blunted recall responses, memory responses were still generated despite a lack of TLR4 signaling capability. We therefore sought to further characterize the differences in the immune response during acute infection. Previous research had suggested that in other bacterial infections TLR4 ligation was necessary for generation of IL-17-producing T-lymphocytes. To determine if rickettsial infection induced IL-17

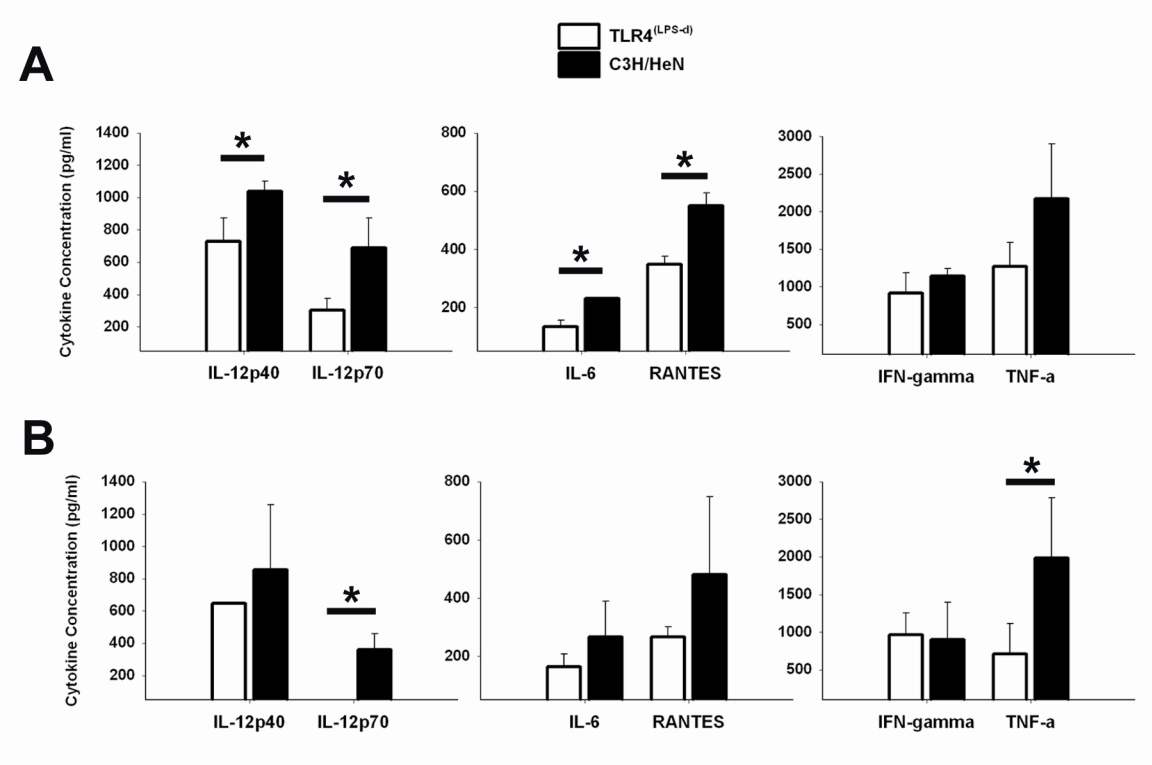


Figure 4.6. TLR4 dysfunction significantly decreases serum proinflammatory cytokine production *in vivo*. Sera was obtained from TLR4$^{(LPS\text{-}d)}$ and C3H/HeN mice on days 3 (A) and 5 (B) post-infection by cardiac puncture. Quantitation of serum cytokines was determined by BioPlex assay. Data indicate mean of 3 mice per time point ± SD. * $P < 0.05$.

production and the generation of IL-17 in response to TLR4 stimulation, we collected serum by cardiac puncture on days 3 and 5 post-infection and isolated splenocytes on day five and exposed them to rickettsial antigen for 24 h *in vitro* and determined the quantities of IL-17 and IFN-γ-secreting $CD4^+$ and $CD8^+$ T-lymphocytes. Mice with competent TLR4 responses had significantly higher IL-17 quantities in the serum on both days 3 and 5 post-infection than mice deficient in TLR4 signaling (**Figure 4.7a**). Moreover, we demonstrated that TLR4 competent mice had significantly more Th17 cells ($CD4^+$ IL-17 producing T-lymphocytes) on day 5 post-infection (**Figure 4.7b**). In addition to significantly higher levels of serum IL-17 and Th17 cells, TLR4 competent mice also had a greater number of antigen-specific Th1 cells ($CD4^+$ IFN-γ producing T-

lymphocytes) on day 5 post-infection; however, we were unable to demonstrate a significant difference in IFN-γ-producing $CD8^+$ T-lymphocytes (**Figure 4.7c, d**).

Functional TLR4 leads to greater quantity of Ag-specific IFN-γ producing T-lymphocytes after restimulation.

During acute rickettsial infection, mice possessing TLR4 have significantly greater Th1-type inducing cytokines, namely IL-12p40 and IL-12p70 and significantly greater effector cell numbers. However, we sought to determine whether TLR4 ligation lends an advantage to generation of memory T-lymphocytes. TLR4-competent and $TLR4^{(LPS\text{-}d)}$ mice were infected with a dose of rickettsiae that was sublethal for both strains (2×10^2 PFU *R. conorii*, although one $TLR4^{(LPS\text{-}d)}$ mouse did succumb to infection at this dose). Twenty-one days after infection, lymphocytes were obtained from spleens of mice and were restimulated with rickettsial antigen *in vitro*. TLR4-competent mice had a significantly greater percentage of IFN-γ-producing $CD4^+$ T-lymphocytes when compared to $TLR4^{(LPS\text{-}d)}$ mice (**Figure 4.8a, b**). The percentage of IFN-γ-producing $CD8^+$ T-lymphocytes was slightly higher in TLR4 competent mice, although this result was not significant (**Figure 4.8a, c**).

In contrast to data from acute rickettsial infection, absolute splenocyte numbers obtained were not significantly different between TLR4 competent and incompetent mice at this timepoint (data not shown). Despite fewer IFN-γ-producing $CD4^+$ and $CD8^+$ T-lymphocytes in $TLR4^{(LPS\text{-}d)}$ mice, they were solidly immune to rechallenge with a normally lethal dose, demonstrating that, although absence of TLR4 signaling leads to a blunted primary Th1 response and diminished recall response to antigenic stimulation *in vitro*, mice are still able to mount protective immunity to a secondary infection if they survive an initial challenge.

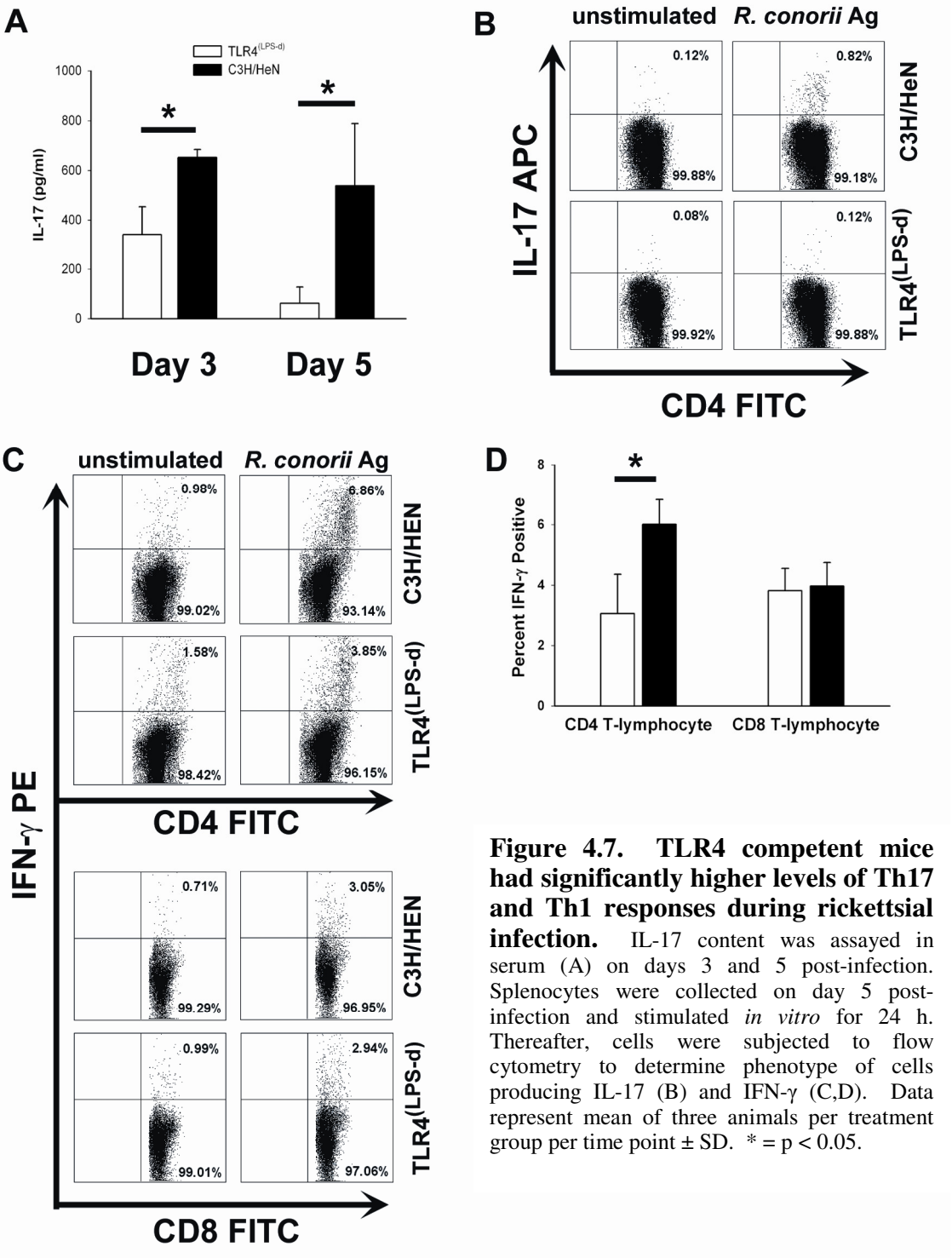


Figure 4.7. TLR4 competent mice had significantly higher levels of Th17 and Th1 responses during rickettsial infection. IL-17 content was assayed in serum (A) on days 3 and 5 post-infection. Splenocytes were collected on day 5 post-infection and stimulated *in vitro* for 24 h. Thereafter, cells were subjected to flow cytometry to determine phenotype of cells producing IL-17 (B) and IFN-γ (C,D). Data represent mean of three animals per treatment group per time point ± SD. * = $p < 0.05$.

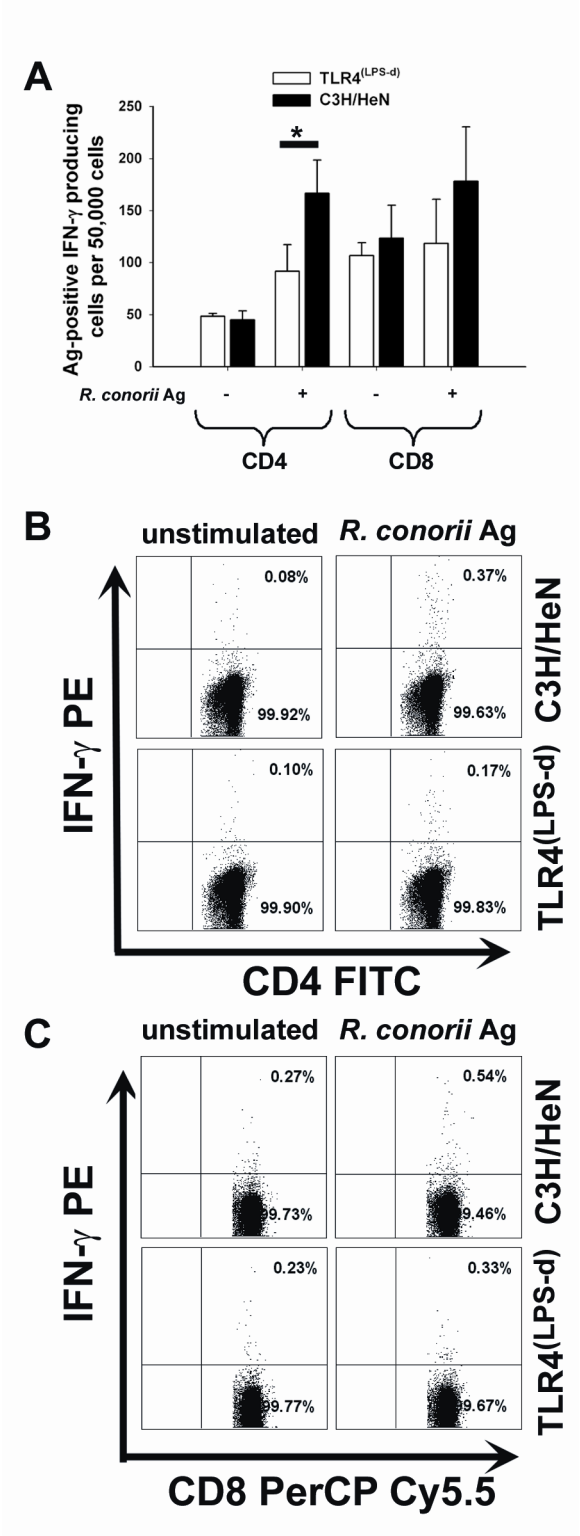


Figure 4.8. TLR4-competent mice generated greater CD4+ T-lymphocyte recall responses after antigenic stimulation *in vitro*. Twenty one days after recovery from non-lethal rickettsial infection, splenocytes were obtained and subjected to either no-stimulation or stimulation with heat-killed *R. conorii* antigen *in vitro*. 24 h thereafter cells were subjected to flow cytometric analysis to determine percentages of IFN-γ-producing $CD4^+$ or $CD8^+$ T-lymphocytes. Data are expressed as average number of Ag-positive cells per 50,000 ± SD (A). Dot plots representative of data collected for $CD4^+$ (B) and $CD8^+$ (C) events. * $p < 0.05$.

DISCUSSION

In the current study, we have demonstrated the importance of TLR4 signaling in a murine model of human rickettsioses. Specifically, we have determined that mice which lack the ability to signal through TLR4 were more susceptible to rickettsial infection than mice that possessed competent TLR4 signaling capability. Mice which possessed functional TLR4 capability had an increase in pro-inflammatory cytokines as well as increased Th1-polarized $CD4^+$ and $CD8^+$ T-lymphocytes. Moreover, we demonstrated that TLR4 was necessary for the generation of Th17 responses after rickettsial infection. This study corroborated previous findings of the importance of TLR4 stimulation in initiation of either Th1 or Th17 driven pro-inflammatory responses. TLR4 stimulation in DC causes polarization toward either Th1 or Th17 cellular differentiation, in part due to IL-12 or IL-23 production (160;168-170). Additionally, we further demonstrated that $TLR4^{(LPS\text{-}d)}$ mice had a significantly higher percentage of T_{reg} cells in the spleen and peripheral lymph nodes, similar to previous studies (162).

Increases in T_{reg} cells have been implicated in numerous infectious diseases as a mechanism for increased microbial burdens and diminished protective responses (171-175). We observed that $TLR4^{(LPS\text{-}d)}$ mice had a significantly greater percentage of T_{reg} cells in peripheral lymph nodes and spleen, which further correlated with decreases in quantities of early activated T-lymphocytes after rickettsial infection. T_{reg} ($CD4^+$ $CD25^+$ $FoxP3^+$) cells suppress pro-inflammatory responses through the surface expression of TGF-β and CTLA-4 (176). Additionally, T_{reg} cells limit the initiation of adaptive immunity by decreasing the number of effector cells during antigen-stimulation and suppressing CTL function (177;178). Mice which lack T_{reg} cells under some conditions eventually succumb to massive immune-mediated damage (179). Together, these data imply that T_{reg} cells play an important role in suppressing harmful auto-immunity; however, the data also indicate that deleterious expansion of T_{reg} cells during infection may limit protective immunity.

In this study, we demonstrated that $TLR4^{(LPS\text{-}d)}$ mice had significantly lower Th1-like immune responses possibly due to greater T_{reg} cell percentages. Specifically, we

demonstrated that mice capable of signaling through TLR4 had significantly greater amounts of early activated ($CD69^+$) $CD4^+$ and $CD8^+$ T-lymphocytes. Moreover, we have demonstrated that $TLR4^{(LPS\text{-}d)}$ mice given either a lethal or sublethal dose of *R. conorii* had a defect in the generation of Ag-specific T-lymphocyte responses. $CD4^+$ T-lymphocytes from $TLR4^{(LPS\text{-}d)}$ mice produced significantly less IFN-γ than mice with competent TLR4 responses after antigenic stimulation, suggesting that TLR4 signaling leads to a greater number of Ag-specific T-lymphocytes. However, we were unable to demonstrate significant differences in antigen-specific IFN-γ-producing $CD8^+$ T-lymphocytes. In terms of acute infection, splenocytes were obtained on day 5 post-infection. Due to the more-resistant phenotype in TLR4-competent mice, we believe that these mice generate a vigorous antigen-specific $CD8^+$ response which is more discernable after recovery from infection (as seen in Chapter 3).

It is possible that, at this time point, $CD8^+$ T-lymphocyte activation and proliferation had not progressed to a point which would allow the observation of significant differences. However, despite the importance of TLR-mediated signaling, protective immune responses may occur in their absence (180). In agreement, we have shown that despite the absence of TLR4, mice infected with a sublethal dose were able to mount protective immune responses in the face of a reinfection with an ordinarily lethal dose. Moreover, rickettsiae may also ligate other TLR (such as TLR2 and TLR9), which may play a role in initiating anti-rickettsial immunity.

This work also presents the first evidence that suggests the generation of Th17 cells is crucial to protection from rickettsiae, although the mechanism behind this protection is unclear. Th17 cells play a critical role in autoimmunity, yet have also been implicated in protective immune responses to bacterial pathogens (181). IL-17 contributes to protection from *Mycobacteria* infection in the lung, as IL-17-deficient mice produce less IFN-γ and have impaired granuloma formation (182). IL-17 also induces the production of anti-microbial peptides in cells (183). We observed a significantly higher amount of IL-17 in the sera of mice which had competent TLR4 responses compared to $TLR4^{(LPS\text{-}d)}$ mice throughout infection. Additionally, we demonstrated that

TLR4-competent mice generated significant Th17 responses after antigenic stimulation, whereas we were unable to detect significant quantities of Th17 cells in rickettsiae-infected TLR4$^{(LPS\text{-}d)}$ mice; therefore, it appears that TLR4 signaling is required for generation of antigen-specific Th17 cells during rickettsial infection.

Previous studies have demonstrated that TLR4 stimulation is important in DC production of IL-23 (184). IL-23 is composed of the common IL-12p40 chain and a distinct IL-23p19 chain, and promotes the expansion of a distinct population of Th17 cells (185;186). We also have demonstrated that DC produce significant quantities of IL-23 after stimulation with *R. conorii*, similar to LPS stimulation (Chapter 3). IL-23 has been shown to be essential for the production of IL-17 responses after infection (187). Therefore, we believe that IL-23 is produced in a TLR4-dependent manner by DC leading to the expansion of Th17 cells. Despite these data, we demonstrated that TLR4$^{(LPS\text{-}d)}$ mice were able to produce detectable amounts of IL-17 following *R. conorii* infection. Invariant NK-T cells and γδ T-cells have also been shown to produce IL-17 during infection (188;189). Therefore, the IL-17 observed in the sera of TLR4$^{(LPS\text{-}d)}$ mice may have come from a cell type other than Th17 cells.

In conclusion, the present study has established that TLR4 signaling plays a significant role the adaptive immune response against *R. conorii* infection *in vivo*. We established that TLR4 signaling was important in inducing vigorous Th1-like immune responses *in vivo*. In addition, this work represents the first demonstration of a Th17 response to rickettsial infection; moreover, we demonstrated that TLR4 signaling is necessary for the generation of Th17 cells *in vivo*.

CHAPTER 5: RICKETTSIAE STIMULATE DENDRITIC CELLS THROUGH TLR4, LEADING TO ENHANCED NK CELL ACTIVATION IN VIVO

The importance of DC in inducing innate immunity to rickettsiae is unclear. Previous data by our laboratory have suggested that transfer of DC activated by TLR4 into mice was capable of inducing partial protection from an ordinarily lethal challenge. Moreover, we have demonstrated that mice defective in TLR4 signaling have significantly blunted adaptive immune responses—specifically decreased pro-inflammatory Th1 and Th17 lymphocytes. To address the importance of TLR4 in immunity to rickettsiae, we determined the importance of TLR4 ligation in primary endothelial cells and determined the significance of TLR4 in early immunity to rickettsiae *in vivo*. Rickettsial growth in mice lacking TLR4 signaling ability proceeds logarithmically *in vivo,* whereas in TLR4-competent mice, rickettsiae experience a lag phase early in infection. We determined that differences in growth rates *in vivo* were not due to inherent differences in susceptibilities of primary microvascular endothelial cells. We did observe significant production of IL-1α and TNF-α production in endothelium as a TLR4-dependent mechanism; however we were unable to demonstrate rickettsia-induced NO production. Systemic cytokine production in TLR4-competent mice after *R. conorii* infection revealed significantly greater amounts of serum IFN-γ on day one post-infection. This IFN-γ production was associated with a significantly greater percentage of activated NK cells in the spleen. Moreover, we demonstrated that NK cells from TLR4-competent mice produced significantly more IFN-γ after rickettsial infection and have greater indices of cytotoxicity activity. Lastly, we demonstrated that activation of DC through TLR4 ligation could induce NK cell activation *in vivo*. Together, these data demonstrate an important role for DC in recognizing rickettsiae through TLR4, inducing early anti-rickettsial immunity.

INTRODUCTION

Toll-like receptors and innate immunity

The role of TLR in adaptive immunity has been discussed and, in the case of rickettsiae, demonstrated in the previous chapter. TLR are expressed on a wide variety of cell types throughout the body—including those not typically thought to play an integral role in the immune response; however, the local and systemic production of cytokines and chemokines by cells not typically part of the immune response may also assist in the protection of hosts against pathogens. Also, TLR signaling by DC may also serve to initiate innate immune responses.

Toll-like receptor-dependent signaling in endothelium

In the case of rickettsial diseases, TLR ligation in the primary target cells, the endothelium, may play an important role in host-defense or pathogenesis. Endothelium possesses many TLR, notably TLR2, TLR4, and TLR9, the three TLR likely important in recognition of rickettsiae (see Figure 4.1) (190-192). Endothelium predominantly expresses TLR4, and small amounts of TLR2 and TLR9; however, after TLR4 ligation endothelium upregulates the expression of TLR2 (193). Additionally, IFN-γ and TNF-α may also upregulate TLR2 in human endothelium (194).

Extensive research has focused on the role of TLR4 ligation in endothelium in the face of septic shock-like conditions, although the role of TLR4 in cooperation with the immune response is less clear. Similar to cells of the immune system, TLR4 ligation in endothelium leads to a MyD88-dependent signaling pathway culminating in the activation of NF-κB and mitogen-activated protein kinase (MAPK) (reviewed in reference 195). This signaling has been shown to be important in the production of pro-inflammatory cytokines which may be important in immunity to rickettsiae. Additionally, in models of sepsis, TLR4 ligation in endothelial cells upregulates iNOS expression in cooperation with IFN-γ and TNF-α leading to significant NO production, a known anti-rickettsial effector (196).

Stimulation of TLR on endothelium may also lead to pathology though. *Neisseria meningitidis* infection in the brain leads to extensive blood brain barrier (BBB) dysfunction and has been well-described in the literature. Extensive vascular leakage has been hypothesized to be due to massive iNOS activation in brain microvascular endothelial cells, culminating in endothelial cell death. Meningococcal lysates induce iNOS expression in cerebrovascular endothelial cells *in vitro*. Furthermore, pretreatment of endothelium with antibody blocking both TLR2 and TLR4 diminishes this effect *in vitro* (197). Coincidentally, rickettsiae have also been suggested to cause BBB dysfunction; however, the role of iNOS in BBB dysfunction is under investigation. It is possible that rickettsiae cause iNOS activation in endothelium in a similar manner; however, the implications toward protection or pathogenesis are unclear.

The above data suggest that TLR ligation during rickettsioses may play an important role either in pathogenesis of rickettsial infection or in the immune response. However, the significance of TLR ligation by rickettsiae in endothelium has not been previously established.

Augmentation of NK cell activity by dendritic cells

NK cells were traditionally thought of as a "first-line" of defense, with crucial importance in malignancies and viral infections (177). NK cells circulate in the blood, become activated by cytokines or by target cells expressing ligands for NK cell receptors and have been implicated as an important effector against numerous infectious diseases (198-200). Much of the interest in cytokine-activation of NK cells has traditionally centered on IL-12p70. In fact, IL-12p70 was originally described as an NK cell growth factor (134). However, multiple other cytokines have also recently been implicated including IL-2, IL-15, and IL-18 (106;201)

Early mobilization of NK cells may also play a role in protection from bacterial diseases. Specifically, NK cell-derived IFN-γ production has been suggested to play an important role in *Shigella flexneri* infection (202). Mice deficient in T, B, and NK cells demonstrated extreme susceptibility to *S. flexneri* infection; however, mice that were only

deficient in T and B cells had significantly greater production of IFN-γ, lower bacterial burden and increased survival. These data suggested that NK cell-derived IFN-γ could limit infection. Despite these observations, NK cells may also play a role in pathogenesis of bacterial infections. NK cell-depleted mice are less susceptible to *Streptococcus pyogenes* infection, exhibiting increased survival and slower disease progression (203).

The importance of NK cells in rickettsial disease, however, appears to be in protection as opposed to pathogenesis. Depletion of NK cells led to greater susceptibility to rickettsial infection in mice (67). Additionally, rickettsial antigens increase human NK cell cytotoxicity activity *in vitro* (204). The mechanism(s) leading to rickettsial activation of NK cells have not been elucidated, however.

Recent evidence suggests that DC can regulate important aspects of innate immunity, particularly NK cells, in addition to their role as initiators of adaptive immunity. Originally, Fernandez *et al.* demonstrated that DC directly triggers NK cell function *in vivo* (205). Consequently, other investigators have also demonstrated that TLR-matured DC may also lead to NK cell activation *in vivo* through cytokine production. DC-derived IL-12 and IL-2 have both been implicated in enhancing NK cell activity *in vivo* (106;107); IL-12 production by DC has been characterized by numerous investigators in response to TLR ligation. Granucci *et al*. demonstrated that IL-2 production by DC results from LPS stimulation (105;206). Moreover, we have demonstrated that stimulation of DC with LPS or rickettsiae leads to kinetically similar IL-2 production *in vitro* (Chapter 3). These results suggest that different TLR may initiate DC-NK cross-talk, leading to NK cell activation *in vivo*.

The Gram-negative bacterium *Actinobacillus actinomycetemcomitans*, strongly induces IL-12 production in DC. Moreover, co-culture of of stimulated DC with normal peripheral blood leads to significant NK cell derived IFN-γ (207). Stimulation of DC with either flagellin (the natural ligand for TLR5) or LPS leads to significant production of IL-12p40 *in vitro* and, upon transfer, NK cell mobilization in the draining lymph node. Importantly, flagellin or LPS-stimulated DC are capable of inducing NK cell proliferation *in vivo* (208). Despite these data, TLR ligation had not been definitively demonstrated to

be necessary for DC-induced NK cell activation *in vivo.* Recently, a role for TLR9 has been shown to be important in NK cell activation; mice unable to signal through TLR9 have reduced NK cell-derived IFN-γ, and TLR9 expression of DC is important in inducing this activity (209;210). Further investigation into the impact of TLR9 ligation in DC has revealed that TLR9 activation of DC can directly induce NK cell activity *in vivo* (211). This research also demonstrated that IL-12 was required for the activation of NK cells *in vivo*.

In conclusion, it appears that TLR ligation of DC may lead to NK cell activation *in vivo*; however, the importance of TLR4 ligation in DC-induced NK cell activation has not been conclusively demonstrated. Additionally, the mechanisms that rickettsiae utilize to induce NK cell activation has not been conclusively identified.

We have previously demonstrated that TLR4-defective mice are more susceptible to lethal *R. conorii* infection; moreover, transfer of LPS-stimulated DC are also capable of inducing partial protection from an ordinarily lethal challenge in an antigen-independent fashion. Together, these results suggest that TLR4 signaling may be important in the initiation of early innate immunity. Herein, we present evidence that the kinetics of rickettsial growth in mice deficient in TLR4 activity proceeded in a logarithmic fashion early in infection, whereas rickettsial growth experienced a "lag phase" in mice that retained TLR4 function. Endothelial responses suggested that TLR4 ligation by rickettsiae does not impede rickettsial proliferation *in vitro*, and rickettsiae did not induce NO production *in vitro*. We demonstrated that TLR4 ligation is important in augmenting NK cell activity *in vivo*, and that DC are likely important mediators of this activity.

RESULTS

Rickettsial growth increases at a logarithmic rate in mice deficient in TLR4 function

In order to examine the effect of innate immunity on rickettsial infection, we sought to determine the kinetics of rickettsial infection *in vivo*, as previous results suggested that TLR4-dysfunctional mice had significantly higher titers of rickettsiae in pathologically relevant organs. After i.v. inoculation, increases in rickettsial titers occurred logarithmically in the brain and lung of $TLR4^{(LPS\text{-}d)}$ mice, whereas mice that possessed functional TLR4 responses controlled the initial rickettsial proliferation during the first three days, resulting in a lag phase of rickettsial growth early in infection (**Figure 5.1a, b**). These data suggest that in addition to the differences observed in chapter 4, defective TLR4 signaling also results in a significant defect in innate immunity to rickettsiae.

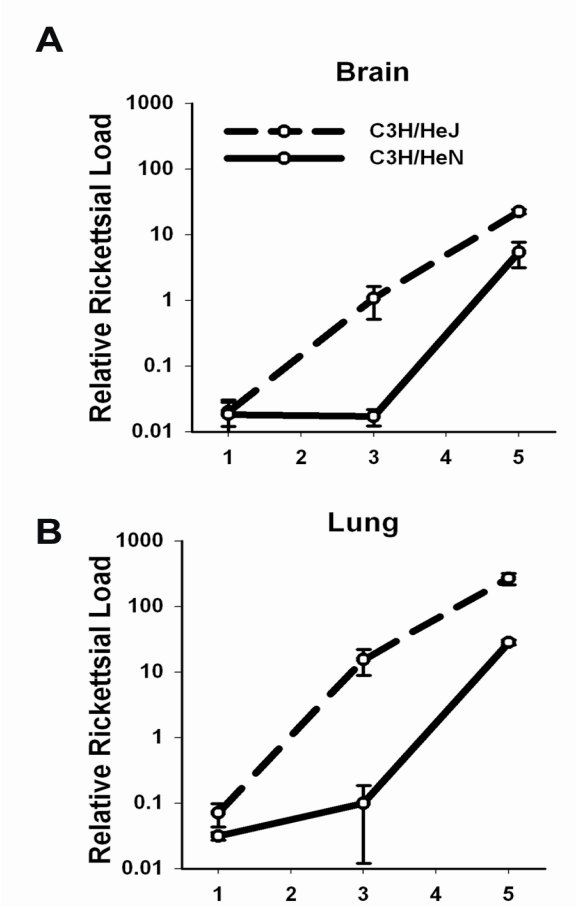


Figure 5.1. Early rickettsial proliferation *in vivo* is blunted through a TLR4-dependent mechanism. To determine the influence that TLR4 has on the kinetics of rickettsial proliferation *in vivo*, mice were infected and rickettsial titer was determined by quantitative real-time PCR on DNA obtained from brain (A) and lung (B). Data represent a mean of 3 mice per time point ± SD.

Enhanced resistance to rickettsial disease is not due to differences in rickettsial growth or cytokine-independent rickettsicidal NO production in endothelial cells *in vitro*

The kinetic differences in rickettsial proliferation *in vivo* led us to evaluate whether the differences in growth rate observed were due to TLR4-mediated activation of innate immune responses, or whether TLR4 ligation in the main rickettsial target cells, the vascular endothelium, led to an inherent anti-rickettsial effect. To determine if TLR4 ligation on vascular endothelium played a significant role in rickettsial proliferation, we cultured primary brain microvascular endothelial cells *in vitro* to determine the rate of proliferation of rickettsiae in the absence of immune pressure. The rickettsial growth rates *in vitro* were similar in endothelium obtained from $TLR4^{(LPS\text{-}d)}$ and TLR4-competent mice, although at 24 h post-infection endothelial cells derived from TLR4-competent mice had significantly greater loads than $TLR4^{(LPS\text{-}d)}$ mice ($p < 0.05$) (**Figure 5.2a**). Moreover, our results demonstrated that, similar to results obtained *in vivo* in $TLR4^{(LPS\text{-}d)}$ mice, rickettsial proliferation occurred in a logarithmic fashion in both sets of endothelial preparations. These data argued that proliferation differences observed *in vivo* were due to early initiation of innate immunity.

However, NO production is an important innate immune effector response against rickettsiae in endothelium. Previous research by other investigators has shown that TLR4 ligation is important in inducing NO production in macrophages, yet the role of TLR4 ligation in endothelium after rickettsial infection is unknown. We sought to investigate whether NO production in endothelium would be affected by the presence or absence of TLR4 signaling. *R. conorii*-infected primary endothelial cells obtained from $TLR4^{(LPS\text{-}d)}$ and TLR4-competent mice did not have significant differences in NO production (indicated by nitrite concentrations in the supernatant) after 48 h *in vitro* (**Figure 5.2b)**.

Despite our observations that *R. conorii* did not stimulate significant NO production by endothelial cells in a TLR4-dependent mechanism, we did determine that several cytokines and chemokines were produced by endothelium in a TLR4-dependent mechanism after rickettsial infection. Supernatants obtained from TLR4-competent, but not $TLR4^{(LPS\text{-}d)}$, murine primary brain microvascular endothelial cells 48 h after infection

with *R. conorii* revealed markedly elevated levels of IL-1α, MCP-1, MIP-1a, and RANTES (**Figure 5.2c**). Additionally, we were surprised to observe markedly elevated levels of TNF-α in the supernatant of TLR4-competent endothelial cells. These data demonstrate that although TLR4 ligation does not play a direct role in limiting rickettsial proliferation, TLR4-dependent production of cytokines and chemokines in endothelium may cooperate with other immune effectors in inducing anti-rickettsial immunity *in vivo*.

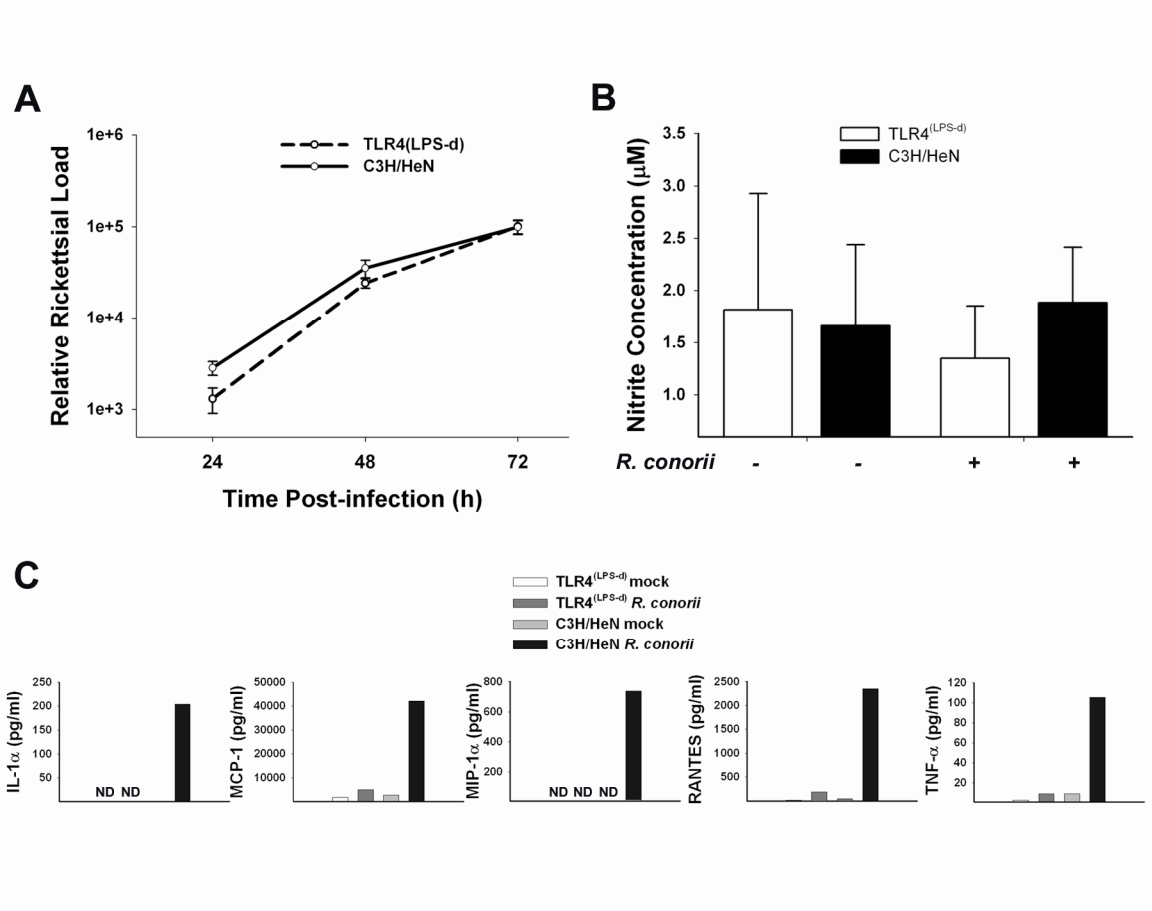


Figure 5.2. Role of TLR4 signaling in endothelial responses to *R. conorii* infection. Primary brain microvascular endothelial cells obtained from TLR4$^{(LPS-d)}$ and TLR4-competent mice were infected *in vitro* with renografin-purified *R. conorii,* and rickettsial content was quantitated by real-time PCR (A). NO production in endothelium following 48 h after *R. conorii* infection was determined by the Greiss assay (B), and chemokine and cytokine production (C) was determined by Bio-Plex assay. ND, not detected.

Significantly elevated levels of IFN-γ in the sera of TLR4-competent mice after *R. conorii* infection correlate with significantly greater numbers of activated splenic NK cells.

Given that we did not observe a difference in endothelial proliferation *in vitro* due to the presence or absence of TLR4, we hypothesized that TLR4-mediated activation of innate immune responses, and probable subsequent production of IFN-γ and TNF-α, might be responsible for the differences observed in rickettsial proliferation *in vivo*. Examination of serum cytokine concentrations in $TLR4^{(LPS\text{-}d)}$ and TLR4-competent mice on day one post-infection with *R. conorii* demonstrated significantly elevated levels of IFN-γ in TLR4-competent mice (**Figure 5.3a**). However, in contrast to data presented in chapter 4, on day one post-infection levels of IL-12p40 and IL-12p70 were not significantly elevated; although increased levels of IL-17 were nearly significantly different (P = 0.056) (**Figure 5.3a**).

Our laboratory has previously shown that early IFN-γ production is largely due to activated NK cells; moreover, the importance of IFN-γ and TNF-α in limiting rickettsial proliferation in endothelium has been discussed previously. In order to determine if increased IFN-γ on day one post-infection in TLR4-competent mice was due to increased NK cell proliferation and activation, we determined the percentage and absolute number of splenic NK cells during infection, and assessed the activation status of NK cells by flow cytometry. On day one post-infection, splenocytes obtained from TLR4-competent mice did not demonstrate a significant increase in splenic NK cell numbers or percentage of splenic NK cells (data not shown); however, in agreement with data demonstrating a significant increase in IFN-γ, NK cells obtained from TLR4-competent mice were activated to a greater extent than those obtained from $TLR4^{(LPS\text{-}d)}$ mice, based on $CD69^{+}$ phenotype (**Figure 5.3b**).

On day three post-infection, however, TLR4-competent mice did attain significantly greater numbers and percentages of splenic NK cells, suggesting that TLR4-competent mice are able to mobilize NK cell activity *in vivo* to a greater extent (**Figure 5.3c**).

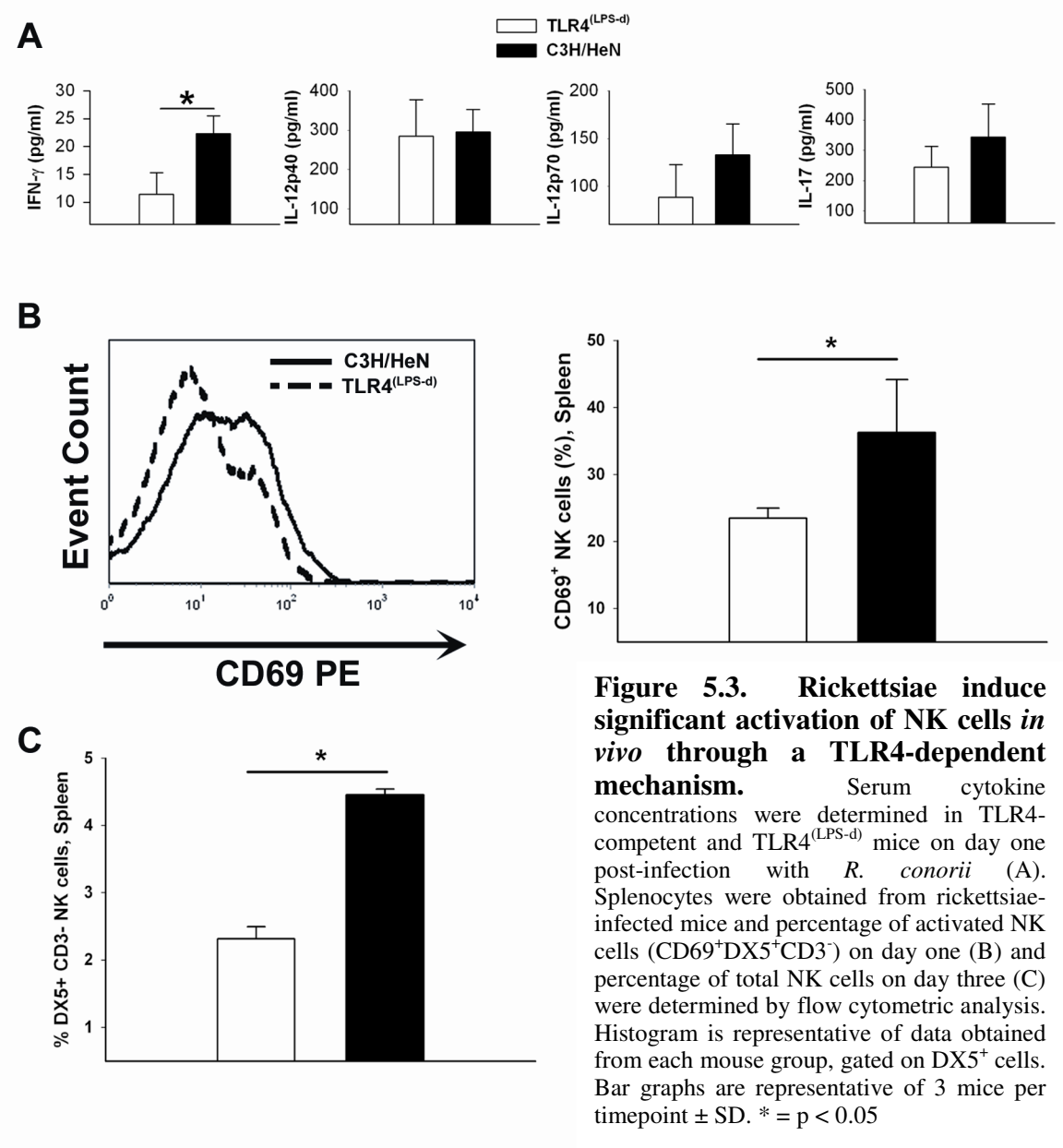


Figure 5.3. Rickettsiae induce significant activation of NK cells *in vivo* through a TLR4-dependent mechanism. Serum cytokine concentrations were determined in TLR4-competent and TLR4$^{(LPS-d)}$ mice on day one post-infection with *R. conorii* (A). Splenocytes were obtained from rickettsiae-infected mice and percentage of activated NK cells ($CD69^+DX5^+CD3^-$) on day one (B) and percentage of total NK cells on day three (C) were determined by flow cytometric analysis. Histogram is representative of data obtained from each mouse group, gated on $DX5^+$ cells. Bar graphs are representative of 3 mice per timepoint ± SD. * = $p < 0.05$

NK cells from TLR4-competent mice produce significantly greater levels of IFN-γ after stimulation *in vitro*

To further characterize these cells, we sought to determine the ability of NK cells to produce IFN-γ after stimulation with Yac-1 cells *in vitro*. NK cells from both TLR4-competent and TLR4$^{(LPS-d)}$ mice that were infected produced markedly more IFN-γ than

uninfected mice; however, NK cells obtained from TLR4-competent mice on day 3 post-infection produced significantly more IFN-γ than those obtained from TLR4$^{(LPS-d)}$ mice (**Figure 5.4**). Therefore, it is probable that the increase in IFN-γ observed in the TLR4-competent mice is due to activated NK cells.

Mice defective in TLR4 signaling have diminished NK cell cytotoxicity activity *in vivo*

We have demonstrated that TLR4-competent mice have a greater percentage and absolute number of activated NK cells in the spleen, and that the increased IFN-γ observed in the serum is likely attributed to these cells; however, we sought to examine their cytotoxicity activity as another criterion of NK cell activation. NK cells obtained from *R. conorii*-infected TLR4-competent mice had significantly greater percentages of Yac-1 cell cytotoxicity than mice that were incapable of signaling through TLR4 on days 1 and 3 post-infection (**Figure 5.5**). Therefore, mice which are more protected from rickettsial

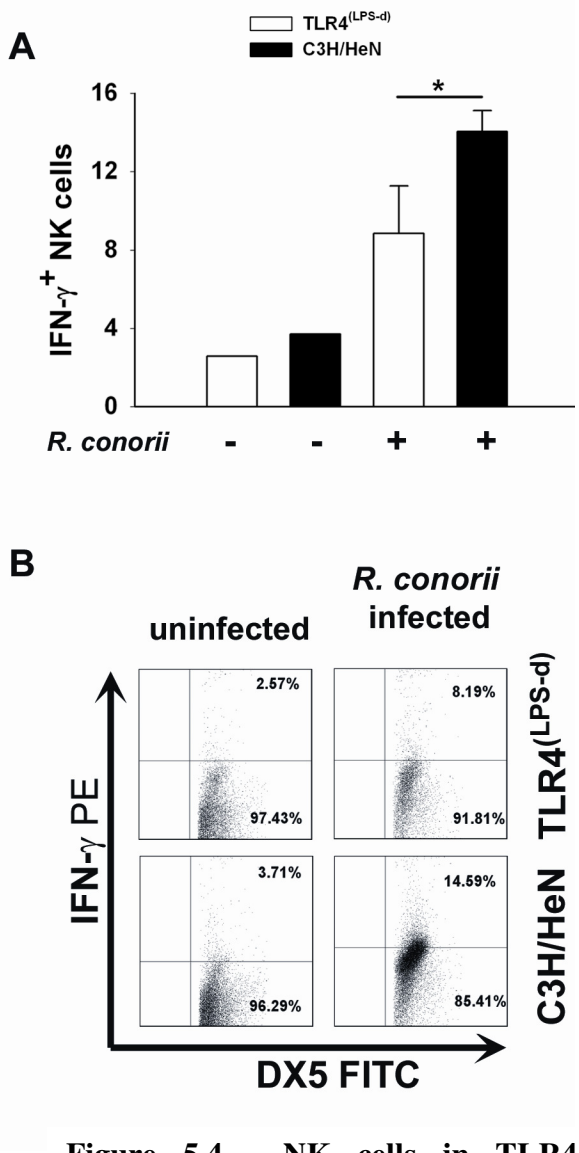


Figure 5.4. NK cells in TLR4-competent mice secrete significantly greater quantities of IFN-γ after stimulation. Splenocytes from TLR4$^{(LPS-d)}$ and TLR4-competent mice were obtained on day 3 post-infection. Splenocytes were stimulated *in vitro* with Yac-1 cells and IFN-γ production was assessed by flow cytometry. Bar graph represents percent IFN-γ positive NK cells ± SD. Dot plots are representative of data obtained. * = $p < 0.05$

infection have greater numbers of cytotoxic NK cells. Despite this observation, rickettsial infection appears to also induce NK-cell cytotoxic activity through a TLR4-independent mechanism as well. All *R. conorii*-infected mice, regardless of ability to signal through TLR4, had significantly greater NK cytotoxicity than uninfected controls.

Dendritic cells activate NK cells through a TLR4-dependent mechanism *in vivo*

We have demonstrated that TLR4-competent mice have significantly greater NK cell activities after *R. conorii* infection. Furthermore, we demonstrated in Chapter 3 that adoptive transfer of TLR4-stimulated DC could protect mice from an ordinarily lethal challenge. Given these data, we hypothesized that TLR4-mediated signaling in DC could lead to NK cell activation *in vivo*. Toward this end, BMDC were obtained from $TLR4^{(LPS\text{-}d)}$ and TLR4-competent mice and stimulated *in vitro* with *R. conorii.* Following a 24 h maturation

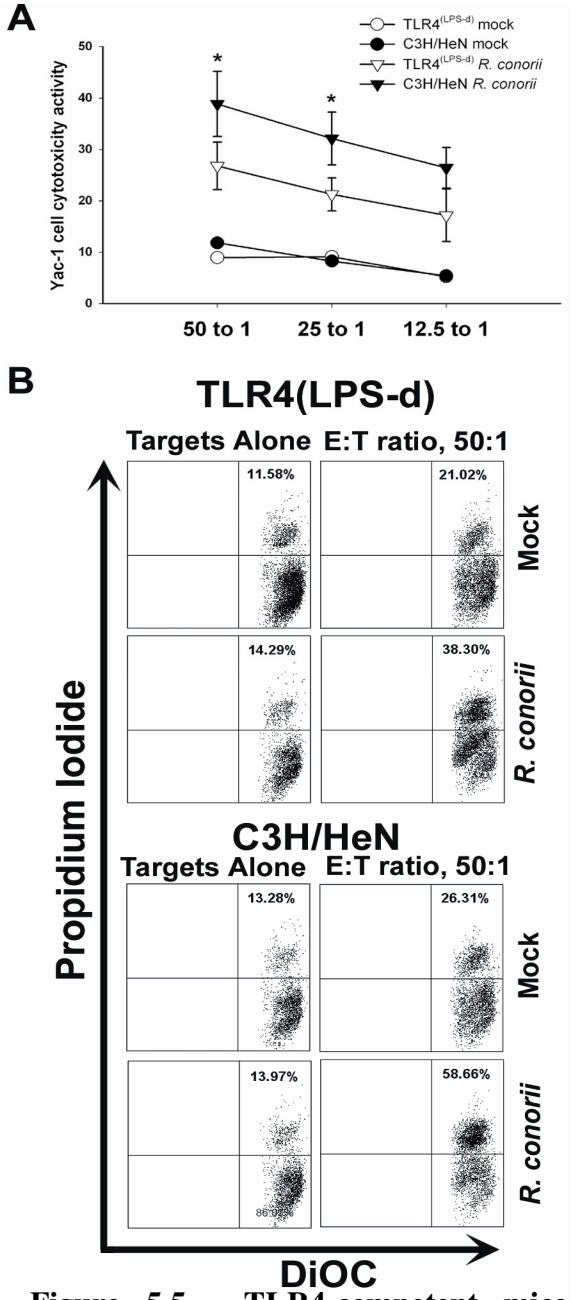


Figure 5.5. TLR4-competent mice have significantly greater NK cell cytotoxicity activity. Splenocytes were obtained from *R. conorii*-infected $TLR4^{(LPS\text{-}d)}$ or TLR4-competent mice or mock-infected controls. Splenocytes were co-cultured with DiOC-labeled Yac-1 cells and percent killing was determined by flow cytometry (A). Representative dot plots demonstrate percent killing (B). * = $p < 0.05$

process, DC were transferred into TLR4-competent mice by footpad injection, and splenocytes were harvested 24 h thereafter for determination of splenic Yac-1 cell cytotoxicity. DC capable of signaling through TLR4 induced significantly greater levels of NK cell cytotoxicity activity after *R. conorii* infection than DC obtained from $TLR4^{(LPS\text{-}d)}$ mice (**Figure 5.6**), demonstrating that DC are capable of inducing NK cell activity in a TLR4-dependent mechanism *in vivo*.

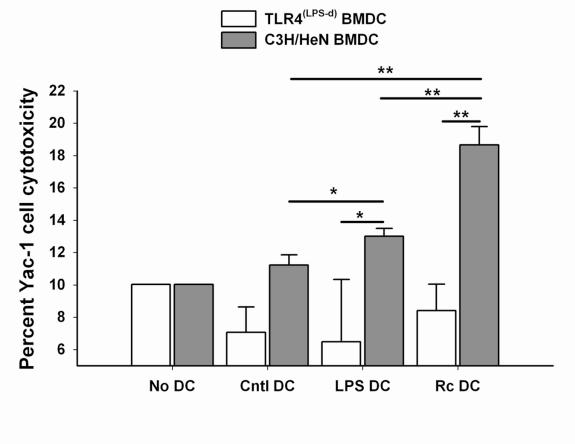


Figure 5.5. Ligation of TLR4 in DC induces NK cell activation *in vivo*. Bone marrow-derived DC were obtained from $TLR4^{(LPS\text{-}d)}$ and TLR4-competent mice and stimulated *in vitro* with *R. conorii* (moi = 5), LPS (50 ng/ml), or mock-infection for 24 h. DC were then injected into mice (5×10^5 per mouse) and splenocytes were harvested 24 h thereafter to determine Yac-1 cell cytotoxicity (E:T, 50:1). * = $p < 0.05$, ** = $p < 0.005$.

DISCUSSION

These data provide conclusive evidence that DC recognize rickettsiae through TLR4 and lead to the activation of NK cells *in vivo*. Additionally, we have demonstrated, for the first time, that TLR4 stimulation is important both in initiation of anti-rickettsial innate immunity, namely the expansion of NK-cells, and in the expansion of Th1 T-lymphocytes (Chapter 4). Our work and that of others have shown that TLR4-ligated DC induce recruitment of NK cells to draining lymph nodes (Chapter 3); furthermore, this recruitment and the production of NK cell-derived IFN-γ in draining lymph nodes are important in augmenting the Th1 immune response (212). We believe that NK cell production of IFN-γ in lymph nodes after rickettsial infection is critical in augmenting the Th1 immune response (213;214).

Our results also argue strongly for an important role of TLR4-induced innate immunity. After *R. conorii* infection in $TLR4^{(LPS\text{-}d)}$ mice, rickettsial proliferation proceeded immediately into logarithmic phase, as compared with the lag phase observed in mice possessing functional TLR4. These data suggested two possibilities: 1) that rickettsiae possess an inherent proliferation advantage inside their main target cell, the vascular endothelium, in $TLR4^{(LPS\text{-}d)}$ mice or 2) that early innate immunity, triggered by TLR4 ligation, blunted the rickettsial growth thereby making it appear that proliferation had a lag phase.

NO production, mediated by the synergistic actions of IFN-γ and TNF-α activating iNOS, is an important anti-rickettsial effector in both human and mouse endothelium (65;66). In experiments focused on the role of endothelium during septic shock, TLR4 signaling in endothelium results in early activation of NF-κB and MAP kinase through MyD88 as well as late-phase activation of NF-κB through the MyD88-independent pathway involving TRIF-related adaptor molecule (TRAM) (195). iNOS activation has been shown to be induced by NF-κB and MAP kinase in response to CpG-DNA—the ligand for TLR9—in cultured murine glial cells (215). Moreover, pro-inflammatory cytokine production by human endothelium proceeds through TLR4 via the

adaptor molecule TRAM (216). Together, these data suggested that TLR4 ligation in endothelium may blunt rickettsial proliferation owing to NO production. However, we demonstrated that *in vitro* rickettsial proliferation rates, determined by quantitative real-time PCR, did not differ in $TLR4^{(LPS\text{-}d)}$ or TLR4-competent primary brain microvascular endothelial cells. Moreover, we did not observe any difference in NO produced by endothelial cells after *R. conorii* infection. These data therefore suggest that the synergistic action of IFN-γ and TNF-α in inducing rickettsicidal NO in endothelium may be more important than TLR4-mediated NO production in rickettsiae-infected endothelial cells.

Importantly, we did observe that endothelial cells do produce significant levels of pro-inflammatory cytokines in a TLR4-dependent manner. Previous research had shown that endothelial cells did not produce significant amounts of IL-1α or TNF-α (see Chapter 1, (68)). However, these previous studies were undertaken with HUVEC cells, which do not contain significant levels of TLR4; therefore, these results suggest that endothelium may play a greater role in the immune response than previously thought. Specifically, TLR4-mediated endothelial cell production of TNF-α may play a role in NO production *in vivo*.

Of note, we did observe significant production of NO in cultured endothelium in response to the related *R. rickettsii* infection in a TLR4-dependent mechanism (data not shown). These results demonstrate that, although there are high levels of homology among spotted fever rickettsiae, different spotted fever group rickettsiae may differentially ligate TLR, leading to differences in immune responses and pathogenesis. Specifically, *R. rickettsii* is known to be more pathogenic than *R. conorii,* with significantly more human cases demonstrating severe complications such as cerebral edema. It is possible that *R. rickettsii* may cause ligation of TLR4 in cerebrovascular endothelium, leading to iNOS activity and BBB dysfunction, similar to the pathogenic manifestations seen in *N. meningitidis*.

Recent studies have demonstrated that TLR4 stimulation is also important in inducing NK cell activation *in vivo* (108). Consistent with these observations, we

demonstrated that the quantities and percentage of NK cells in spleen were consistently lower in TLR4$^{(LPS-d)}$ mice than in TLR4-competent mice after *R. conorii* infection. Additionally, splenocytes from TLR4$^{(LPS-d)}$ mice had decreased NK cell cytotoxic activity compared to TLR4-competent mice *in vitro*. Moreover, TLR4-competent mice also had significantly greater levels of IFN-γ in the sera early during infection; and subsequent investigation demonstrated that NK cells in TLR4-competent mice produced significantly greater amounts of IFN-γ after stimulation *in vitro*. Given these data, we further suggest that this IFN-γ production is important in inducing early NO production in infected endothelial cells. Additionally, production of TNF-α by *R. conorii*-infected endothelial cells may act in synergy with NK cell-derived IFN-γ to induce endothelial production of rickettsicidal NO. We propose that this NO production is an important factor leading to the lag phase of rickettsial growth observed in TLR4-competent mice. In sum, we have demonstrated that TLR4 ligation is an important mediator in limiting early rickettsial proliferation by activating NK cells leading to increased NK cell cytotoxicity and IFN-γ levels.

In conclusion, the present study has established that TLR4 signaling plays a significant role in both innate and adaptive protective immunity against *R. conorii* infection *in vivo*. We found that TLR4 is important in inducing NK cell proliferation and cytotoxic activity, presumably limiting rickettsial growth *in vivo*. Moreover, we demonstrated that TLR4 ligation does not play a direct role in rickettsial killing in endothelial cells.

CHAPTER 6: CONCLUSIONS AND DISCUSSION

INITIATION OF RICKETTSIAL IMMUNITY

Understanding the mechanisms by which a protective immune response is generated to rickettsiae is crucial towards the engineering of a protective vaccine. Currently, a protective vaccine against any rickettsial disease does not exist. In light of their extreme virulence, several rickettsial diseases, namely *R. prowazekii* and *R. rickettsii*, have recently been classified as Category B and C select agents by the CDC and NIH due to their potential for weaponization, highlighting their significance to society and the need to develop new vaccines and/or therapeutics.

Because of their importance in initiating immunity, this work focused on the role of DC in rickettsial infection and the significance of TLR4 ligation in immunity. Elucidation of the important aspects of DC recognition of pathogens and the characterization of responses which generate protective immunity are critical towards this effort. I believe that any vaccine developed must be tailored in such a way as to optimize DC responses *in vivo.* Toward this end, the work detailed in this dissertation details that DC are capable of transferring immunity to naïve animals; moreover, we also detail the importance of TLR4 in augmenting the protective Th1 response, as well as demonstrating the participation of a novel T-lymphocyte effector, the Th17 cell, in immunity to rickettsiae.

Vaccines and dendritic cells

As the significance of DC in generating protective immune responses were illuminated, researchers began to investigate how protective vaccines were developed by "reverse engineering", that being taking known efficacious vaccines and determining how DC recognize the vaccine and initiate protective immune responses *in vivo*. This technique has been demonstrated on one of the most-successful and longest-used vaccines, yellow fever 17D (YF17D).

Independently, two groups of investigators examined the effects that YF17D. Barba-Spaeth *et al.* demonstrated that YF17D is capable of infecting both mature and immature human DC (217). Also, infection of immature DC results in cell death, whereas TNF-α-mature DC are resistant to cytolysis. Moreover, they demonstrated that YF17D-infected DC are capable of processing and presenting antigen, inducing antigen-specific expansion of $CD8^+$ T-lymphocytes. Subsequently, Querec *et al.* demonstrated that YF17D activates multiple TLR after infection of DC; specifically, YF17D activates TLR2, 7, 8, and 9 leading to production of pro-inflammatory cytokines IL-12p40, IL-6 and IFN-α (121). Moreover, although dogma suggests that protective responses to viral diseases are generally Th1-directed, the resulting immune responses suggested a mixed Th1/Th2 response. Further characterization showed that $MyD88^{(-/-)}$ mice exhibit impaired Th1 responses, and $TLR2^{(-/-)}$ mice demonstrate enhanced Th1 and Tc1 responses. Overall, these data suggest that multiple TLR work cooperatively towards an optimized antiviral response.

The whole-cell *B. pertussis* vaccine has also been investigated (160). Investigators demonstrated that protective responses are inhibited in $TLR4^{(LPS\text{-}d)}$ mice, whereas in TLR4-competent mice, protective Th1 responses are generated. Additionally, and of particular importance and relevance to our results presented here, TLR4-competent mice also produce Th17 cells in response to whole-cell *B. pertussis* vaccine, while this response is completely deficient in $TLR4^{(LPS\text{-}d)}$ mice.

In addition to determining how DC recognize efficacious vaccines, investigators have also sought to look at the responses of DC to investigational vaccines or less-than-ideal vaccines in order to understand which characteristics may be lacking in order to improve upon the formulation. The anthrax and plague antibiowarfare vaccines are less than ideal; both vaccines require multiple inoculations in order to attain adequate protection, and in some cases, protection is not attained. Investigators sought to determine the responses of these vaccines. They demonstrated that while the plague vaccine induces DC maturation, a strong mixed-leukocyte reaction and Th-cell differentiation, anthrax vaccine formulations are poor inducers of DC maturation, with

DC demonstrating low levels of maturation markers after stimulation (122). Their results suggested that one potential problem with the anthrax vaccine is its sub-optimal maturation of DC; however, their results did not demonstrate a conclusive reason for less-than-ideal results with the plague vaccine formulation.

As discussed above, understanding the characteristics of protection induced by DC is imperative to understanding the necessary considerations for engineering a vaccine. The protection conferred by transfer of rickettsiae-infected DC is associated with upregulation of known rickettsial effectors, specifically Th1 responses, leading to expansion of effector $CD8^+$ cells and IFN-γ production. Moreover, we also demonstrated that rickettsiae-infected DC also produce significant levels of IL-23, in addition to IL-12p70. It has previously been shown that IL-23 production is important in polarization towards a Th17 response (218). Although we did not investigate the production of Th17 cells in Chapter 3, based on the data obtained in Chapter 4 and the IL-23 production seen in Chapter 3, we believe that transfer of rickettsiae-stimulated DC led to an expansion of Th17 cells.

An important consideration when developing a vaccine against rickettsioses is the ability to protect against multiple rickettsial diseases in a single forumulation. While previous data have shown a large degree of cross-reactivity within spotted fever group and typhus group rickettsiae (51;219), it has recently been demonstrated that T-lymphocytes specific for spotted fever group rickettsial antigens respond *in vitro* to typhus group rickettsial antigens. Additionally, cross-protection was also demonstrated in the murine epidemic typhus and RMSF models. Mice which were immune to *R. conorii* are resistant to lethal *R. typhi* challenge. Also, $CD8^+$ T-lymphocytes from human volunteers previously infected with *R. conorii* were shown to proliferate and secrete IFN-γ after stimulation with *R. typhi* antigen (94). These data suggest that developing a vaccine that protects against both spotted fever and typhus group rickettsiae may be a realistic goal. To this end, we believe that the characterization of the responses in DC which lead to a protective immunity against rickettsial challenge will help to define the characteristics necessary to incorporate into a vaccine.

Toll-like receptor ligands—natural adjuvants

In 1989, the late Charles Janeway, a preeminent immunologist and "father of innate immunity," observed that Freund's adjuvant had the ability to augment immunity, and theorized that "pattern recognition receptors" reacted to microbial components in Freund's adjuvant, leading to these augmented responses (220). He observed that adjuvants are necessary to induce long-lasting immunity, and mere differences in simple antigens are generally not enough to induce immunity. Further, he postulated that antigen presenting cells are constantly in an "off" state, and that it is necessary for pattern recognition receptors to bind to microbial components, leading to an "on" signal that would lead to efficacious immunity. In support of Janeway's theories, the data discussed above regarding YF17D demonstrate that recognition of TLR ligands in a vaccine formulation by DC is critical in initiating protective responses.

We have demonstrated that TLR4 is important in augmenting Th1 responses. Previously, our laboratory has demonstrated that Th1 responses are critical for protection against rickettsiae. However, despite the fact that Th1 responses are important, we also observed that TLR4 is crucial in the production of the Th17 subset. This is the first description suggesting an important role for Th17 cells in immunity against rickettsiae. Previous research has demonstrated an importance for Th17 cells in protection against *K. pneumoniae* in the lung (159). Additionally, IL-17 producing T-cells have been shown to be important in augmenting the IFN-γ response, leading to efficient granuloma formation in *Mycobacterium* infection (221). If Th17 cells augment the IFN-γ response in rickettsial infections, it is logical that this response may inhibit rickettsial spread through NO production in endothelium; however, possible other effector roles for the Th17 response may also be present, begging the need for further characterization of the role of this cell type in immunity to rickettsioses. As a result of these data presented, it is clear that TLR4 ligation in a vaccine would be appropriate to help polarize a protective response.

The contributions of TLR4 to induce protective innate immunity, despite not necessarily playing an important role in the design of a vaccine, cannot be overlooked.

Vaccines are traditionally thought of as inducers of protective adaptive immunity—that is protective Ab or T-lymphocyte responses. However, the cross-talk between 'innate' and 'adaptive' cells of the immune system clearly blurs this line. For instance, production of IFN-γ by activated NK cells in the lymph node has been shown to be important in polarizing immunity towards a Th1 response (214;222).

Although we demonstrated that TLR4 plays a significant role in both innate and adaptive immunity against rickettsiae, the overall contribution of TLR4 ligation in endothelial cells remains unknown. Despite our observation that TLR4 does not play a role in inhibiting rickettsial proliferation in endothelium *in vitro*, it is possible that the cytokine and chemokine responses of endothelial cells are important in the overall immunologic response.

Future Directions

Tick saliva as immunomodulators

In the data presented here, we demonstrate that transfer of DC pulsed with rickettsiae were capable of protecting mice from a lethal challenge. Additionally, we demonstrated that intradermal inoculation of rickettsiae (to replicate natural infection) led to the observation of rickettsial antigen-containing DC in the draining lymph node. We also observed an increase in DC in the skin upon rickettsial infection suggesting that DC migrate after inoculation. With the typical incubation period in humans being 7-10 days, these observations beg the question "why does DC infection in the skin not lead to protective immunity prior to widespread rickettsiosis?"

One important aspect absent in our experimental design was the introduction of tick saliva into the intradermal inoculum. The components of tick saliva have multiple pharmacologically active compounds including vasodilators, anticoagulants, inhibitors of platelet aggregation and immunomodulators (223). The immunomodulatory actions of tick saliva proteins include inhibition of complement and other innate-immune effector mechanisms; suppression and deviation of cytokine production; diminution of humoral immunity; suppression of T-lymphocyte proliferation and inhibition of migration; and impairment of antigen presenting cell function (224;225). Therefore, while the

importance of DC cannot be overlooked, understanding their role in the face of natural infection requires the addition of tick saliva.

Additional Toll-like receptors

Clearly this work has opened up several new important areas of investigation. As suggested above, although the importance of TLR4 ligation is clear, we also know that some, likely all, spotted fever group rickettsiae also contain TLR2 ligands. In addition TLR9 is another likely candidate important in initiation of immunity. TLR2 ligands have been suggested to complement TLR4 activation by tempering the pro-inflammatory response

Lastly, there exists a great deal of homology amongst typhus group and spotted fever group rickettsiae. Overall, both groups of rickettsiae require similar effector responses for clearance. However, comparison of DC responses is necessary to elucidate whether generation of protective responses occur in a similar fashion. In preliminary experiments, both typhus group rickettsiae (*R. typhi*) and spotted fever group rickettsiae (*R. conorii*) caused similar responses after DC stimulation (data not shown). It is necessary to further investigate the responses of DC to different pathogens—particularly, investigation of their ability to ligate TLR. We have already demonstrated that, although marked homology exists between *R. conorii* and *R. rickettsii*, they do appear to induce different responses in endothelium through TLR4 (discussed briefly in Chapter 5).

Role of Th17 cells in immunity against rickettsioses

While the production of Th17 cells occurred in TLR4-competent mice and were completely absent in $TLR4^{(LPS\text{-}d)}$ mice, the overall importance of this cell type in rickettsioses requires further clarification. We presume that, given the enhanced susceptibility observed in $TLR4^{(LPS\text{-}d)}$ mice, it is likely that Th17 cells play an important role. However, it is important to note that, while we observed an absence of Th17 cells in $TLR4^{(LPS\text{-}d)}$ mice, we did observe detectable levels of IL-17 in the sera of these mice, albeit less than TLR4-competent mice. Therefore, other cell types are able to produce IL-17 in a TLR4-independent mechanism.

CONCLUSIONS

The work presented here represents the first description of the protective role of DC in a murine model of spotted fever rickettsioses. We have demonstrated that TLR4 ligation, both by rickettsiae and also by LPS, led to significant protection from an ordinarily lethal challenge. Further, we showed that protection was due to an expansion of Th1-polarized T-lymphocytes. Although we did not demonstrate that LPS-stimulated DC led to antigen-specific expansion of T-lymphocytes, it is possible that LPS-stimulated DC did in fact induce greater protective T-lymphocyte responses than control DC due to a bystander effect (226).

However, it is also clear that the importance of TLR4 was multifactorial, and we are unable to define one predominant characteristic leading to the different susceptibilities. While we demonstrated that TLR4 ligation on DC could induce NK cell activation *in vivo*, we still observed activated NK cells—albeit to a lesser extent—in $TLR4^{(LPS\text{-}d)}$ mice. These results together demonstrate an important role for DC in inducing NK cell activation, yet also demonstrate that NK cells can be activated in the absence of TLR4 signaling.

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VITA

Jeffrey Jordan was born on December 22, 1976 to Michael and Judith Jordan. While at graduate school, Jeffrey received several honors. In 2004, Jeffrey was elected into the Phi Kappa Phi honorary society, awarded the Truman Graves Blocker Jr. Research Scholars award and won the Edward S. Reynolds award for mechanistic basic research by a graduate student. In 2006 he again received the award for mechanistic basic research as well as the Edward S. Reynolds Experimental Pathology Scholarship. In 2007, Jeffrey was won the M.D./Ph.D. combined degree program Abstract competition and was awarded a trainee travel award by the American Society for Investigative Pathology to attend Experimental Biology. Jeffrey's research was supported by a James W. McLaughlin Pre-Doctoral fellowship and the NIAID T32 Emerging and Tropical Infectious Diseases Pre-Doctoral Training Grant.

Jeffrey also contributed significantly in other endeavors while at the University of Texas Medical Branch. He participated in the Graduate Student Organization and Student Government Association as a Senator from 2002-04, representing the graduate Students at UTMB. He also participated in the National Student Research Forum at UTMB from 2003-2005, serving as Co-Director in 2004 and Sr. Co-Director in 2005.

Jeffrey can be contacted through his parents at 16260 SW Falcon Dr, Beaverton, OR 97007.

Education

B.S., May 2002, Washington State University, Pullman, WA.

Publications

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Abstracts

1. Valdez RA, TC McGuire, WC Brown, WC Davis, JM Jordan and DP Knowles. Selective in vivo depletion of bovine $CD4^+$ T lymphocytes with anti-CD4 monoclonal antibody during acute anaplasmosis. 6^{th} International Veterinary Immunology Symposium, Uppsala, Sweden, July 15-20, 2001. *Accepted, not presented.*

2. Jordan JM, G Wen, and JP Olano. Fluorescence analysis of permeability induced by spotted fever group rickettsiae in an in vitro model of the blood brain barrier. 2003 Gulf Coast Tropical Medicine Association Meeting, Galveston, Texas, April 25-26, 2003.

3. Jordan JM**,** HM Feng, and DH Walker. Live and heat-killed *Rickettsia conorii* induced maturation and cytokine production in bone-marrow derived dendritic cells *in vitro*. 2004 Federation for American Societies in Experimental Biology annual meeting, Washington DC, April 17-21, 2004

4. Jordan JM, ME Woods, L Soong, and DH Walker. Dendritic cell vaccination protects mice from lethal rickettsial challenge 2006 Bugs N Drugs Meeting, Galveston, TX, April 5-6, 2006.

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8. Jordan JM**,** ME Woods, and DH Walker. Mice lacking TLR4 function exhibit enhanced susceptibility to *Rickettsia conorii*, correlated with decreased proinflammatory cytokines and NK cell activity. 2007 Annual IHII/McLaughlin Colloquium, Galveston, TX. March 22, 2007

9. Jordan JM, ME Woods, and DH Walker. Early events after intradermal inoculation of rickettsiae; identifying initial target cells and elucidating the immunomodulatory effects of tick saliva. 2007 Federation for American Societies in Experimental Biology annual meeting, Washington DC, April 28-May 2 2007.

10. Jordan JM, ME Woods, and DH Walker. Mice lacking TLR4 function exhibit enhanced susceptibility to *Rickettsia conorii*, correlated with decreased NK cell activity. 2007 American Association of Immunology annual meeting, Miami, FL. May 18-22 2007.

Summary of Dissertation

Identifying the role(s) dendritic cells play in rickettsial infection is important in determining the characteristics which elicit protective immunity. Previous data imply that Th1 responses are essential for immunity to rickettsiae; however, they do not address mechanisms important in initiating early immunity, particularly those involving dendritic cells. Dendritic cells are instrumental in initiation and control of a strong Th1 response towards invading pathogens. I hypothesize that cutaneous dendritic cells comprise an important initial target for rickettsial infection. Additionally, activation and migration of rickettsiae-infected dendritic cells to draining lymph nodes may be critical to activation

of NK and CD8 T-cells. Lastly, preliminary data suggest that rickettsiae act as TLR4 agonists on dendritic cells, resulting in their activation. To elucidate the significance of dendritic cells in rickettsioses, I developed a model of dermal infection to demonstrate that cutaneous dendritic cells comprise an important initial target cell. Furthermore, I demonstrated that rickettsiae-infected dendritic cells were capable of inducing protective immunity in naïve mice to an ordinarily lethal challenge. This protection was correlated with significantly elevated levels of IFN-γ producing CD4 and CD8 T-cells as well as NK cells, indicating that rickettsiae-infected dendritic cells are capable of inducing protective Th1 responses and NK cell mobilization. Lastly, we demonstrated that mice defective in TLR4 signaling were more susceptible to lethal rickettsial infection. This susceptibility was correlated with a significant decrease in Th1 immune responses. Additionally, TLR4 was shown to be critical towards the generation of Th17 responses. TLR4 ligation in dendritic cells also appears important in augmenting NK cell activation *in vivo*.