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Host-Dependent Expression, Transcriptional Regulation and Protection of *Ehrlichia chaffeensis* Tandem Repeat Proteins

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Host-Dependent Expression, Transcriptional Regulation and Protection of *Ehrlichia chaffeensis* Tandem Repeat Proteins

by

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Dedication

To my parents

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Host-Dependent Expression, Transcriptional Regulation and Protection of *Ehrlichia chaffeensis* Tandem Repeat Proteins

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Ehrlichia chaffeensis is an obligately intracellular bacterium that is the causative agent of human monocytotropic ehrlichiosis, an emerging life-threatening zoonosis. *E. chaffeensis* is transmitted by the lone star tick, *Amblyomma americanum*, and replicates in mononuclear phagocytes in mammalian hosts. The major immunoreactive proteins of *E. chaffeensis* include a group of tandem repeat proteins (TRPs) that are involved in various molecular strategies to reprogram the host cell during infection. The objectives of this dissertation were to examine host-specific expression of *E. chaffeensis* TRPs, the role of CtrA as a gene expression regulon and the protection mediated by antibodies directed at the molecularly characterized epitopes within the TRs of TRP32, 47 and 120. Our results revealed three novel characteristics of these ehrlichial proteins. First, we demonstrated differential expression of TRP transcripts and host-specific post-transcriptional regulation of TRP32 and TRP47 mRNA. Second, the transcriptional binding motif of the two-component system (TCS), response regulator, CtrA was identified, and using a histidine kinase inhibitor, we demonstrated transcriptional regulation of TRP by TCSs. Finally, using *in vitro* and *in vivo* models, we demonstrated protection during *E. chaffeensis* infection with antibodies directed against linear species-specific epitopes of TRP32, 47 and 120 of the IgG1 isotype through an extracellular and intracellular antibody-mediated mechanism. Collectively, these studies have improved our understanding of the pathogenesis of ehrlichiosis and have identified novel targets and mechanism for development of vaccines and therapies for ehrlichial diseases.

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aa	Amino acid
Ank	Ankyrin
bp	Base pairs
c-di-GMP	Cyclic dimeric GMP
COGs	Clusters of orthologous groups
CtrA	Cell cycle transcriptional regulator A
DC	Dense-cored cell
EMSA	Electrophoretic mobility shift assay
HK	Histidine kinase
HME	Human monocytotropic ehrlichiosis
Mbp	Million base pairs
ORF	Open reading frame
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RC	Reticulate cell
RR	Response regulator
SCID	Severe combined immunodeficiency
TCS	Two-component system
TR	Tandem repeat
TRP	Tandem repeat protein

Chapter 1

General Introduction

Ehrlichia *and ehrlichiosis*

PHYLOGENY

Ehrlichia chaffeensis is an obligately intracellular α-proteobacterium that belongs to the order Rickettsiales in the family Anaplasmataceae. The family Anaplasmataceae shares a common ancestor with *Rickettsia*, *Orientia* and mitochondria. The members of this family include the genera *Ehrlichia*, *Anaplasma*, *Wolbachia* and *Neorickettsia* that occupy a membrane-bound intravacuolar compartment within infected host cells. The genus *Ehrlichia* consists of five formally named species that share 97.7% similarity in the 16S rRNA gene sequence, including *E. canis*, *E. chaffeensis*, *E. muris*, *E. ruminantium* (formerly *Cowdria ruminantium*) and *E. ewingii* (1). All of these *Ehrlichia* species have tick vectors that transmit the bacteria to multiple vertebrate hosts.

E. CHAFFEENSIS TRANSMISSION CYCLE

E. chaffeensis has a life cycle that involves both vertebrate and invertebrate hosts (Fig. 1.1). The tick vector of *E. chaffeensis* is *Amblyomma americanum* (2), commonly known as the lone star tick for silvery white spot on its dorsal surface. *A. americanum* is a three-host, hard body tick, and all three life stages (larva, nymph, adult) feed on humans. *E. chaffeensis* multiplies in the midgut and salivary glands of the tick (3) and is transmitted during blood meals. The tick maintains the ehrlichiae transstadially, but not transovarially. *E. chaffeensis* DNA has been detected in other tick species including

Dermacentor variabilis, Ixodes pacificus, Ixodes ricinus; however, the roles of these ticks as efficient vectors for transmission of *E. chaffeensis* have not been definitively established (4). Little is known about the vector-pathogen relationship between *A. americanum* and *E. chaffeensis*.

A. americanum feed on a wide range of mammalian and avian species with the larvae and nymphs infesting primarily ground feeding birds, and all three stages feed on medium to large mammals. The white-tailed deer (*Odocoileus virginianus*) has been identified as the sole vertebrate reservoir host for maintaining the transmission cycle of *E. chaffeensis* (5). Other potential reservoir hosts include goats, domestic dogs and coyotes. Humans are accidental hosts and are not involved in maintaining the life cycle in nature. In humans, *E. chaffeensis* causes the life-threatening emerging zoonosis, human monocytotropic ehrlichiosis (HME).

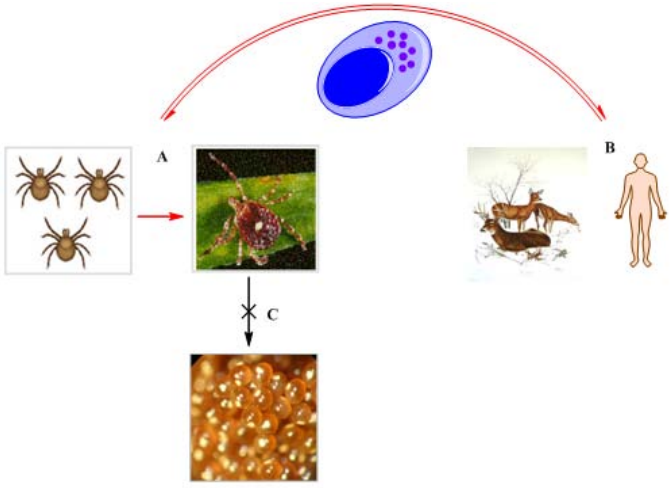

Figure 1.1 *E. chaffeensis* transmission cycle. Noninfected ticks obtain *E. chaffeensis* infection from vertebrate reservoir hosts (white-tailed) deer and the infection is transmitted transstadially (A). The bacteria are transmitted by the ticks to humans or other hosts (B); however, *E. chaffeensis* is not transovarially transmitted (C).

HUMAN MONOCYTOTROPIC EHRLICHIOSIS

The etiologic agent of canine ehrlichiosis, *E. canis*, was first recognized in Algeria in 1935 (6). In the United States, the first report of a human ehrlichial infection was in 1986 in a man who acquired the infection from a tick bite while traveling in Arkansas (7). The patient had antibodies reactive with *E. canis*, which was thought to be the causative agent. In 1991, a new ehrlichial species was isolated by cell culture from a military recruit that exhibited fever and headache after a tick bite (8), and *E. chaffeensis* was thus named after the military base where the patient was stationed (Fort Chaffee, Arkansas). Over 500 cases of HME were diagnosed from 1986 to 1999, predominantly in the southcentral and southeastern United States, and since 1999 human ehrlichioses has become nationally reportable (9). The number of cases of HME reported to the CDC had steadily increased since it became reportable, from 200 cases in 2000, to 961 cases in 2008 (Fig. 1.2A) (10). Globally, evidence of HME has been reported in Europe, Mexico, Argentina, Cameroon, Mali, Israel, and Thailand. There are multiple demographic and ecologic factors that have contributed to the emergence of HME. Among these factors include an increase in the tick vector, *A. americanum*, density and expanded geographic distribution (11). Additionally, an increase in the reservoir host (white-tailed deer) population of *E. chaffeensis* has also contributed to the emergence of HME (9). An increase in human contact with natural foci of infection through recreational and occupational activities, and an increased size, longevity and immunocompromised status of the human population has also contributed to the emergence of this tick-transmitted disease (9).

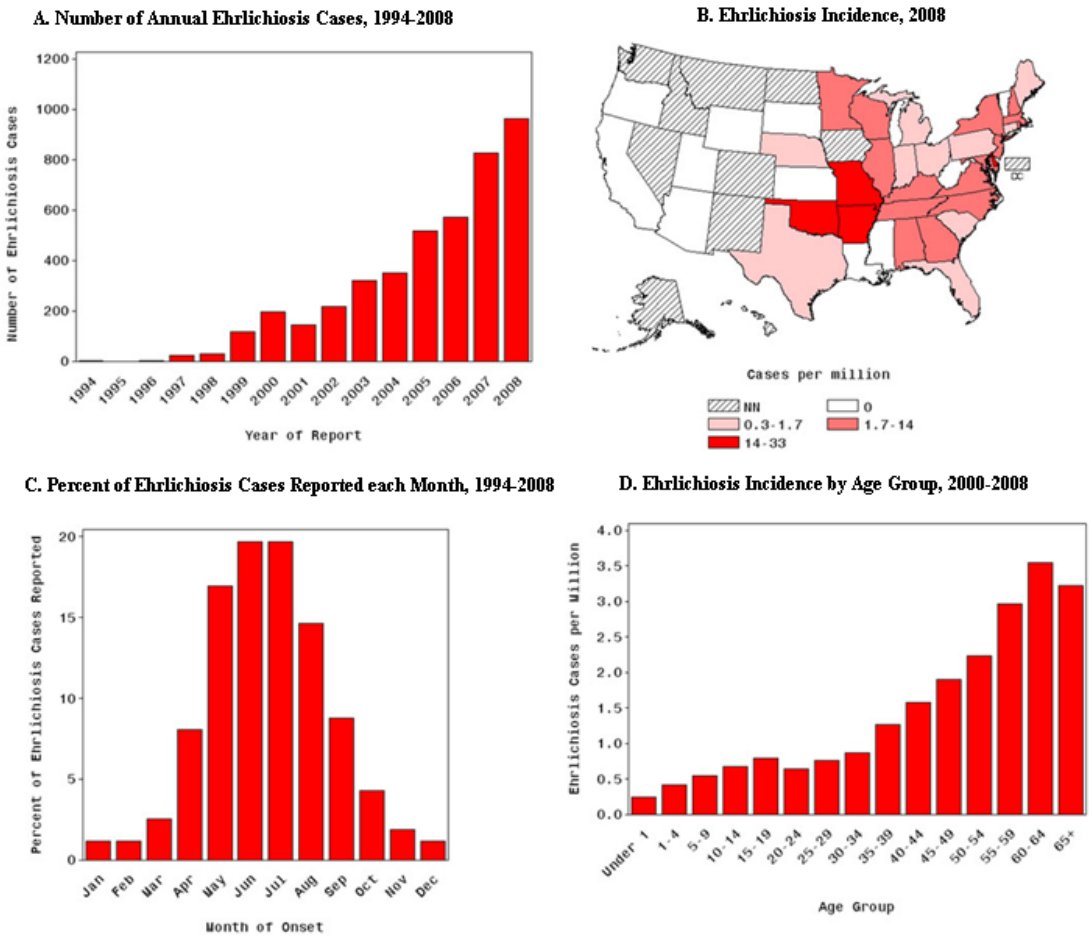

Figure 1.2 Epidemiology of ehrlichiosis in the United States. (A) Number of *E. chaffeensis* cases in the United States reported to CDC from 1994 to 2008, (B) Reported incidence (per million population) for *E. chaffeensis* in 2008, (C) Proportion of *E. chaffeensis* cases reported to CDC by month of onset, 1994-2008, (D) Annual incidence by age group from 2000-2008 (10).

The disease is often unreported or under-diagnosed due to the non-specific symptoms associated with the onset and has resulted in underestimation of the actual disease incidence. Active surveillance in endemic area suggested HME rates of 100 to 200 per population of 100,000 (12). The median age of patients is approximately 50 years with a slightly higher (57-61%) incidence in males (13). HME infection results in

hospitalization in 43-62% of cases, 17% with life-threatening complications, and approximately a 3% case fatality rate (14,15). The disease is more severe in elderly (>50 years) and immunocompromised patients (Fig. 1.2C).

The clinical signs of HME include fever as the universal symptom (97%), followed by malaise (82%), headache (80%) and myalgia (57%) (15). Additionally, gastrointestinal and respiratory systems are affected, and central nervous system infection is present in 20% of patients (16). Although clinical manifestations are nonspecific, laboratory findings can be helpful in patients with suggestive clinical histories that include tick exposure or bites. These include leucopenia, thrombocytopenia, anemia, and elevated serum transaminase levels, which are found in 83% of cases suggesting mild to moderate liver injury (17).

The diagnosis of HME is based primarily on evaluation of clinical and laboratory tests due to the limited availability of molecular tests and the absence of detectable serum antibodies at the time of clinical presentation (9). Approaches used for the laboratory confirmation of HME include examination of peripheral blood smears, molecular detection of ehrlichial DNA by PCR, *in vitro* cultivation and serodiagnosis (9). Serological test for *E. chaffeensis* specific IgG and IgM using indirect immunofluorescence assay (IFA) is considered the "gold standard" that is used as a confirmatory test for HME, detection of seroconversion or 4-fold change in antibody titer during convalescent phase (15,17). Since HME is rapidly progressive and fatal, antibiotics need to be initiated once an empirical clinical diagnosis has been rendered

(15). Tetracyclines have been shown to be effective and doxycycline is the drug of choice. Other classes of antibiotics are not effective against *Ehrlichia* (9).

Host-pathogen interactions

E. chaffeensis is a small, Gram-negative bacterium that replicates in membrane-bound vacuoles within the host cell cytoplasm. The intracytoplasmic vacuolar microcolony of replicating bacterial cells is called morula, derived from the Latin word "morus" for their mulberry-like appearance. Individual ehrlichiae within the morula are coccoid and coccobacillary (approximately 0.4-1.5 μm in length) and exist in two morphologically distinct cell types, dense-cored cells (DC) and reticulate cells (RC) (18). The DCs are the smaller (0.4-0.6 μm) of the two cell types, have electron-dense chromatin and are the infectious form that attach and enter the host cell through receptor-mediated endocytosis (18). By 48 hours post entry into the host cell, the larger (0.4-0.6 μm by 0.7-1.9 μm) RCs replicate every 8 hours by binary fusion (19). Within 72 hours post infection, RCs mature into infectious DCs (19). The mechanism involved in the exit of DCs from the host cell is not known; however, it has been proposed that they exit by host cell lysis or translocated to adjacent cells through host cell filopodia (20).

E. chaffeensis has a relatively small genome (1.18 Mbp) compared to the 4.6 Mbp of *Escherichia coli* (21,22). The 78.9% of the coding sequences of the genome encodes for 1, 115 open reading frames (ORFs) with a low G+C content (~30%) (Fig. 1.3) (21). *E. chaffeensis* and other members of the family Anaplasmataceae are capable of making all major vitamins, cofactors and nucleotides, which are suggested to play a beneficial

role in invertebrate and vertebrate hosts (21). Features of the *E. chaffeensis* genome associated with host-pathogen interactions include genes that encode tandem and ankyrin repeat-containing proteins, actin polymerization proteins and a group of proteins containing poly (G-C) tracts and a multigene family encoding outer membrane proteins (21,23). The genome also encodes for both Sec-dependent and Sec-independent protein export pathways for secretion of proteins across the inner membrane (21). In addition, *E. chaffeensis* genome also has genes that encode for type I and IV secretion system components (21,24).

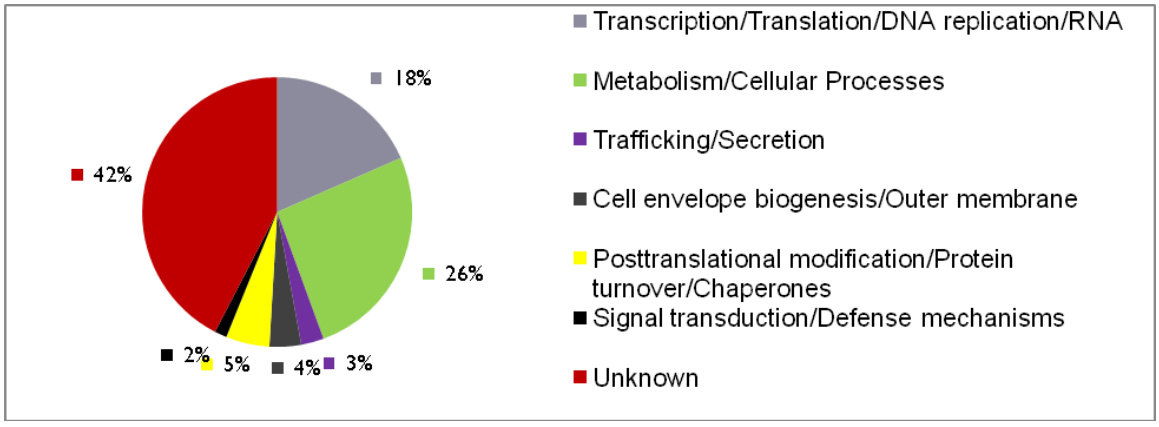

Figure 1.3 Categorization of the *E. chaffeensis* genes based on cluster of orthologous genes.

Ehrlichiae are pleomorphic with a thin outer membrane and no capsule layer (25). *E. chaffeensis* has a characteristic Gram-negative cell wall structure, but lacks important cell membrane components including lipopolysaccharide and peptidoglycan (26). However, ehrlichiae acquire cholesterol from the host cell and incorporate it into their membrane, which is important for its survival and entry into the mammalian host (26). The entry of *E. chaffeensis* into the mammalian host involves activation of

glycosylphosphatidylinositol-anchored proteins, protein tyrosine kinase, host caveolae, resulting in clathrin-independent, caveolae-mediated endocytosis (26-28). After internalization by mammalian monocytes, *E. chaffeensis* subverts innate host defenses, and resides in an endosome-like compartment that expresses early endosomal markers such as Rab5 and early endosome antigen 1 (EEA1) that does not fuse with lysosomes (29,30). Functional two-component systems (TCS) are essential for preventing lysosomal fusion with *E. chaffeensis* inclusions (31). Three pairs of TCSs have been identified in *E. chaffeensis* including PleC-PleD, NtrY-NtrX and CckA-CtrA (31,32). Sensor kinase (SK), PleC, and response regulator (RR), PleD, are upregulated synchronously during exponential growth of *E. chaffeensis*, and PleD is activated by phosphorylation and has diguanylate cyclase activity to produce c-di-GMP (33,34). Cyclic di-GMP signaling is required for bacterial invasion, intracellular aggregation and growth, and is involved in stabilization of *E. chaffeensis* surface exposed proteins, including TRP120 by preventing degradation by serine proteases (33,34).

Transcriptional profiling of human monocytes (THP-1) cells revealed that a wide range of host cell genes are regulated in response to *E. chaffeensis* infection (35). Among the genes induced by *E. chaffeensis* include apoptosis inhibitors, protein regulating cell differentiation, signal transduction, proinflammatory cytokines, biosynthesis and metabolic proteins and membrane trafficking proteins (35).

E. chaffeensis proteins associated with host-pathogen interactions

IMMUNOREACTIVE PROTEINS

The immunoreactive proteins of *E. canis* and *E. chaffeensis* have been identified and include proteins that range in mass from 19-200 kDa. Many of these proteins have been identified and molecularly characterized, including the outer membrane family of proteins (OMP-1), p19/TRP32, TRP36/47, TRP140/120 and Ank200 (36-39).

OUTER MEMBRANE PROTEIN-1 FAMILY

The *E. chaffeensis* genome has 49 genes (6.6%) predicted to encode envelope proteins (21). The most studied of these proteins are members of the OMP-1 family encoded by a multigene locus. The polymorphic multigene OMP-1 family consists of 22 paralogous genes that are clustered in a 29 kb gene locus flanked by a transcriptional regulator (*tr1*) and a preprotein translocase (*secA*) (40,41). The paralogs have conserved regions and three hypervariable regions (41,42). These genes are differentially expressed at the transcriptional level in the mammalian and arthropod hosts (43,44). Several (19 out of the 22) of the OMP-1 proteins were identified by mass spectrometery in outer membrane extracts of mammalian cell cultured *E. chaffeensis* (45). Although transcripts have been detected for several OMP-1 genes in tick cell cultured *E. chaffeensis,* only OMP-1B (p28-14) has been detected by proteomics (46). Monoclonal antibodies against immunogenic OMP-1g (p28-19) are protective in SCID mice from fatal *E. chaffeensis* infection (47). Recently, two of the OMP-1/P28 family of proteins (OMP-1G and 1F)

were found to have structural characteristics of Gram-negative bacterial porins with β-barrel comprised of 12 transmembrane β-strands and porin activity (48).

TANDEM REPEAT PROTEINS

TRs are adjacent repetitive sequences distributed widely among prokaryotes (49,50). TRPs are involved in a variety of host-pathogen interactions including adhesion, immune evasion and regulation of host cell signaling pathways. The alpha C protein of the group B *Streptococcus* is a surface-expressed TRP with opsonic and protective antibody epitopes, and variable TR numbers are associated with immune evasion (51-53). The most frequent form of the protein consists of nine identical 82 aa TRs flanked by a conserved amino terminal domain and a carboxy terminal with a consensus sequence that is associated with protein attachment to the cell wall (54). Rib and Esp are other group B streptococcal proteins with tandem repeats (55). TRs in the M proteins of group A *Streptococcus* are extensively studied and are virulence determinants (56). The *Staphylococcus aureus* extracellular adherence protein (Eap) is a multifunctional TRP and a broad spectrum adhesin that binds to several host matrix and plasma proteins (57). More than one of the Eap-TRs are required for aggregation, adherence and host cell invasion (57). Similarly, the *Mycoplasma hyorhinis* TR protein Vlp variants are involved in immune evasion and have antibody epitopes (58). Plant pathogens from the genera *Xanthomonas* and *Ralstonia* encode for TRPs that function as transcriptional activators (59,60).

The Gram-negative bacterium *Helicobacter pylori*, has a family of genes with repetitive DNA that encode for surface exposed TRPs (61). The TRs on membrane glycoprotein MUC1 of *H. pylori* are involved in adhesion of the bacterium to gastric epithelial cells (62). *Neisseria meningitidis* have three TRPs; PilQ which is pilin pore, a lipoprotein (Lip) and an outer membrane protein (DcaC) (63). The *Chlamydia trachomatis* translocated actin-recruiting phosphoprotein (TARP) is a type III-secreted effector protein and is an immunodominant TRP similar to the *E. chaffeensis* TRPs.

Immunodominant proteins of *E. canis* and *E. chaffeensis* include TRP ortholog pairs that have been molecularly characterized (39,64-67). These include TRP32/TRP19, TRP47/TRP36, and TRP120/TRP140 that have major species specific epitopes mapped to the highly acidic tandem repeat regions for these proteins (65,66,68). Other immunoreactive ehrlichial proteins include Ank200 and the recently characterized orthologs TRP75/TRP95 (68,69). However, unlike the other TRP orthologs of *Ehrlichia*, TRP75/TRP95 have a major cross reactive continuous epitope in the TR region (69). Common characteristics of these TRPs include acidic TR regions that are serine/threonine-rich, and they have a major molecularly distinct continuous antibody epitope (~20 amino acids) located within the tandem repeat regions (Fig. 1.4) (65,66,70). TRP47 and TRP120 are differentially expressed on the surface of infectious DC ehrlichiae (45,70). TRP32 is extracellularly associated with the morular fibrillar matrix, and the morula membrane and is expressed on both DCs and RCs (65). Until recently, little was known about the roles of these TRPs in ehrlichial pathobiology.

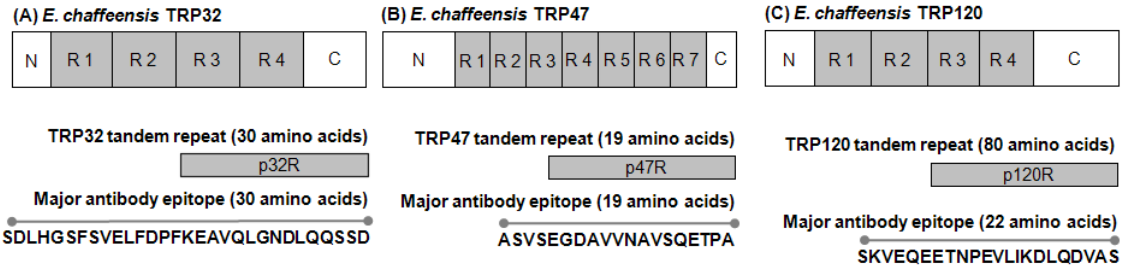

Figure 1.4 Schematic diagrams of *E. chaffeensis* immunoreactive (A) TRP32, (B) TRP47 and (C) TRP120 domains, tandem repeats and antibody epitopes.

TRP47 has carboxy-terminal TRs that vary in number and amino acid sequence among different isolates. The Arkansas strain of *E. chaffeensis* contains seven 19-mer tandem repeats (71). Among the recently identified proteins that interact with TRP47 on dense-core cells is Fyn, a member of the Src family of tyrosine kinases that is involved in adhesion-mediated signaling and is enriched in caveolae (72-74). TRP47 also interacts with cyclase-associated protein CAP1, which is associated with SH3 domain dependent mAbp1-dynamin complex involved in receptor-mediated endocytosis (74,75). Recently, molecular interactions have also been reported between TRP32 and TRP120 and host-cell proteins with distinct cellular functions including signaling, transcriptional regulation, vesicle trafficking and cellular proliferation and differentiation (76,77). Surface exposed TRP120 is involved in the adhesion of *E. chaffeensis*, and its degradation by endogenous serine protease HtrA has been linked to reduced bacterial aggregation and internalization into mammalian cells (33,34,45,78). TRP120 is also a novel DNA binding protein that binds to G+C-rich DNA motif and functions as a mammalian transcriptional regulator during *E. chaffeensis* infection (79). TRP120 and Ank200 are two *E. chaffeensis* effector

proteins that have been shown to be translocated to the host cell nucleus. Ank200 interacts with Alu-Sx element motifs located in promoters and introns of various host cell genes (80). Additionally, recently, TRPs 32, 47, 120 and Ank200 were identified as type 1 secretion system substrates (24).

IMMUNITY TO *E. CHAFFEENSIS*

Murine models have shown that both cellular and humoral immune mechanisms play a role in immunity to *Ehrlichia* (81). The SCID mouse model recapitulates several features of clinical signs and histopathology of the human disease including wasting, tissue and cell tropism, extensive tissue inflammation, splenomegaly, lymphadenopathy, liver granulomas and necroses (82). Immunocompetent inbred mice (C57 BL/6) are more resistant to *E. chaffeensis* and clear the infection within three weeks of infection with a peak in infection at around 7-10 days post-infection. In contrast, immunocompromised SCID mice are susceptible to *E. chaffeensis* resulting in a fatal infection. To study HME in immunocompentent mice, murine models have been developed with *E. muris* and *Ixodes ovatus Ehrlichia* (IOE) (83-86).

E. chaffeensis induces expression of proinflammatory cytokines and chemokines including IL-1β, IL-8, IL-10 and TNF-α upon contact with the mammalian host cells (35,87). Pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan and flagella are capable of inducing cytokines and chemokines and are recognized by pattern-recognition receptors such as Toll-like receptors (TLRs) (88). However, the *E.*

chaffeensis genome does not encode for these PAMPs, and recently none of the TLRs were shown to mediate proinflammatory cytokines induction, suggesting induction via other PAMPs or signaling pathways (21,89). Previous studies in our laboratory have demonstrated that *E. chaffeensis* proteins with ankyrin and tandem repeats are involved in inducing chemokines and cytokines during infection. Nuclear translocated *E. chaffeensis* effector protein, Ank200, binds to the promoter region of several mammalian host cell genes including TNF-α, which is upregulated at later stages of infection *in vitro* (80). Recombinant TRP120 induces a strong chemokine response including IL-8 and several members of the CC family of chemokines (90). Moreover, binding sites of nuclear transported TRP120 have been identified in the upstream regions of the CC family of chemokines (79). Additionally, TRP120 and TRP47 interact with several signaling proteins that have been suggested to induce cytokine and chemokine expression (74,77).

Several studies have demonstrated a critical role for IFN-γ production by $CD4^+$ and $CD8^+$ T cells during ehrlichial infection (83,91,92). However, *E. chaffeensis* also blocks the phosphorylation of the Jak/Stat pathway, inhibiting IFN-γ mediated antiehrlichial activity (93). Although several studies have focused on the role of cellular immunity during ehrlichial infection, antibodies have been shown to play an important role during immunity. Additionally, in a recent study, T-cell-independent humoral immunity (B cells and antibodies) was shown to be sufficient for protection against ehrlichial infection (47,94,95).

Antibodies have been shown to play an important role in immunity to several intracellular bacteria including, *Mycobacterium, Listeria, Salmonella, Francisella, Rickettsia* and *Ehrlichia* (96-103). Transfer of immune sera obtained from immunocompetent mice to SCID mice provided significant protection from *E. chaffeensis* infection and continued serum administration prolonged the survival of SCID mice (103). Antibodies were then shown to mediate clearance of *E. chaffeensis* in the absence of T or B lymphocytes (47). Monoclonal antibodies of the IgG2a and IgG3 isotypes against an outer membrane protein (OMP-1g) were identified as being effective during infection (47,104). However, characterization of the antibodies that mediate protection have been limited to the outer membrane family of proteins until the work discussed in chapter 4 for this dissertation. In these studies, antibodies against TRPs 32, 47 and 120 were shown to be protective during *E. chaffeensis* infection. Although Fc receptors have shown to be required for host defense during ehrlichial infection, the mechanism of antibody-mediated immunity is largely unknown.

OBJECTIVES OF DISSERTATION

TRPs are involved in an array of functions in several bacterial species ranging from immune evasion to actin relocation in the host cells. In the recent years, the role of *E. chaffeensis* immunodominant TRPs in pathobiology has also been emerging. The objectives of this dissertation were to examine host-specific expression of *E. chaffeensis* TRPs, the role of CtrA as a gene expression regulon and the protection mediated by antibodies directed at the molecularly characterized epitopes within the TRs of TRP32, 47 and 120.

Host-specific gene expression is a well established mechanism of adaptation utilized by several bacterial species that alternate between vertebrate and invertebrate hosts. Studies on differential gene expression in response to host cell environments and the role of immunodominant proteins in immunity to *E. chaffeensis* have been limited to the outer membrane family of proteins. However, little is known about the host-pathogen-associated genes involved in the adaptation and survival of *E. chaffeensis* within these distinct hosts, the molecular control mechanisms involved and the role of other major immunoreactive proteins in immunity. This gap in knowledge regarding essential host-specific genes, the mechanisms of transcriptional regulation and the components of the antibody mediated immunity is a hindrance to defining *E. chaffeensis* phenotypes, the molecular survival strategies within the distinct hosts, and for understanding the molecular mechanisms involved in host adaptation and immunity.

In chapter 2, the *E. chaffeensis* genes differentially expressed in the mammalian and tick host cells are identified. *E. chaffeensis* host-pathogen associated genes are host-specifically expressed, and TRPs genes are post-transcriptionally regulated. In chapter 3, the regulation of TRPs gene expression by the TCSs is demonstrated, and the transcriptional regulator, CtrA of the CckA-CtrA two-component system is characterized. The protection mediated by linear epitopes within the TRs of differentially expressed TRPs 32, 47 and120 are examined in chapter 4. We demonstrated that antibodies against linear epitopes in TRP32, TRP47 and TRP120 are protective against *E. chaffeensis* infection and the protection appears to involve extracellular and intracellular antibody-mediated mechanisms.

Chapter 2

Ehrlichia chaffeensis Transcriptome in Mammalian and Arthropod Hosts Reveals Differential Gene Expression and Post Transcriptional Regulation[1]

Introduction

Human monocytotropic ehrlichiosisis is a life-threatening emerging tick-borne zoonosis caused by obligately intracellular bacterium, *E. chaffeensis* (9). HME is a systemic disease characterized by clinical presentation that includes fever, headache, myalgia, anorexia, chills and laboratory abnormalities including leucopenia, thrombocytopenia, anemia and elevation of serum hepatic aminotransferases (9). The severity of the disease varies from asymptomatic seroconversion to a fatal multisystem failure (13). *E. chaffeensis* is transmitted by the lone star tick, *A. americanum,* and maintained in nature by persistent infection of mammalian hosts (9). In the mammalian host, *E. chaffeensis* replicates primarily within mononuclear phagocytes forming membrane-bound cytoplasmic microcolonies called morulae that are resistant to innate immune destruction (105).

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Bacterial pathogens survive by expressing genes necessary for transmission, invasion and persistence, and evasion of innate and adaptive defenses (106). Among these include surface proteins of *Borrelia burgdorferi* and *Yersinia pestis*, secreted effectors of *Shigella flexneri* and transcriptional regulator of *Bordetella pertussis* (107-109). Moreover, host-specific gene expression by *A. phagocytophilum* has been reported in human and tick cells (110), and the *E. chaffeensis* p28 outer membrane proteins encoded by the OMP-1 multigene locus are differentially expressed in human and tick cells (43,46,111). Furthermore, it is recognized that *E. chaffeensis* propagated in tick cells has a distinct antigen expression profile from that of mammalian phagocyte grown ehrlichiae (112).

E. chaffeensis has a relatively small genome (1.18 Mbp) (21), but has evolved within mammalian and arthropod hosts and developed mechanisms to subvert host immune defenses. There are numerous *Ehrlichia* genes that are associated with host-pathogen interactions (26), including tandem repeat (TRPs) and ankyrin repeat proteins (Anks), actin polymerization proteins, poly (G-C) tracts, Type IV secretion (T4S) system and a multigene family encoding the outer membrane proteins (OMP-1) that exhibit porin activity (23,48). TRPs (TRP120, TRP47 and TRP32) and Anks (Ank200) elicit strong antibody responses in the mammalian host and have major continuous species-specific antibody epitopes in acidic domains that include the serine-rich tandem repeats (65,68,70). The TRPs are secreted, and TRP47 and TRP120 are differentially expressed on the surface of dense-cored (infectious) ehrlichiae (65,66,70).

Molecular interactions between TRP47 and the mammalian host identified numerous host cell targets with distinct cellular functions associated with signaling, transcriptional regulation, vesicle trafficking and cellular proliferation and differentiation (74). TRP120 has been shown to play an important role in binding and internalization (78), and its expression is regulated by the second messenger cyclic di-GMP and protease HtrA (33). It is also associated with novel molecular protein-protein, protein-DNA interactions suggesting that it is involved in modulating host cell processes and gene transcription (77,79). *E. chaffeensis* Ank200 was recently detected in the mammalian host cell nuclei and interacts with an adenine-rich motif in promoter and *Alu* elements (80).

The macrophage transcriptome during *E. chaffeensis* infection has been previously determined (35); however, investigation of *E. chaffeensis* gene expression in distinct hosts has been limited to genes encoding the OMP-1 multigene family. In this study, we analyzed the *E. chaffeensis* transcriptome in human monocytes (THP-1), tick cells from the known arthropod vector (*A. americanum*; AAE2 cells) and the vector of *A. phagocytophilum* (*Ixodes scapularis*; ISE6 cells) and determined that well characterized ehrlichial proteins involved in host-pathogen interactions were differentially expressed.

Methods

CELL CULTURE AND CULTIVATION OF *E. CHAFFEENSIS*

E. chaffeensis (Arkansas strain) was cultivated in THP-1 cells, a human monocytic leukemia cell line (ATCC# TIB-202, Manassas, VA) and tick cells (AAE2

and ISE6). THP-1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% HEPES buffer (Sigma, St. Louis, MO), 1% sodium pyruvate (Sigma) at 37°C in a humidified 5% CO_2 atmosphere. Uninfected AAE2 and ISE6 cells and *E. chaffeensis*-infected tick cells were obtained from Dr. Ulrike Munderloh (University of Minnesota) and were maintained in L15B300 medium supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN), 10% tryptose phosphate broth (BD, Sparks, MD) and 1% bovine lipoprotein cholesterol concentrate (MP Biomedicals, Irvine, CA) at 34°C as previously described (113,114). The pH of the L15B300 medium was adjusted to 7.5 with 1M NaOH. The cells were cultured in 34°C, and the medium was changed once a week and cells were split 1:3 every two weeks. Infected cells were cultured in complete L15B300 supplemented with 7.5 % sodium bicarbonate (Cellgro) and 2.5% 1 HEPES buffer (Cellgro). Uninfected cells were propogated in T-150 flasks, and *E. chaffeensis* infection was maintained in the cells by subculturing with infected cells (10%) to uninfected cells. The level of ehrlichial infection was assessed by modified Giemsa stained (HEMA 3, Fisher Scientific) cytocentrifuged cells (Fig. 2.1).

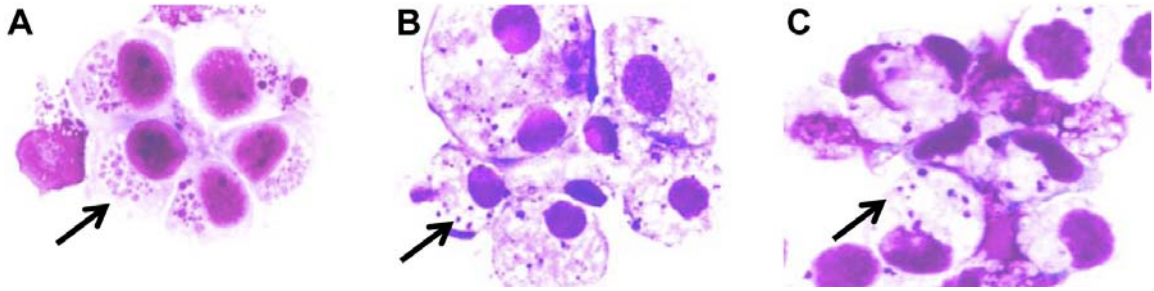

Figure 2.1 *E. chaffeensis* morulae in infected human and tick cells. Morulae in (A) THP-1, (B) AAE2, and (C) ISE6 cells stained with Giemsa stain (100x; identified by arrows).

RNA EXTRACTION

Total RNA was purified from uninfected and *E. chaffeensis*-infected (90% infected) THP-1, AAE2 and ISE6 cells (10^7cells per sample) using Tri reagent (Ambion, Austin, TX). Genomic DNA was eliminated by treatment with Turbo DNA-free (Ambion) according to the manufacturer's protocol. Polyadenylated host mRNA was removed using oligo (dT) columns (Oligotex, Qiagen, Valencia, CA) as previously described (115), and bacterial RNA was enriched using Terminator (Epicenter Biotechnologies, Madison, WI) that selectively digests RNAs with 5'-monophosphates that are present only on ribosomal RNA. RNA concentration was determined by NanoDrop (Thermo Scientific, Wilmington, DE) and quality confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) at the UTMB Genomics Core Facility (Fig. 2.2). The RNA quality requirements were established as: A260/A280 ≥ 1.8, A260/A230 ≥ 1.8 and concentrations ≥ 1μg/ul.

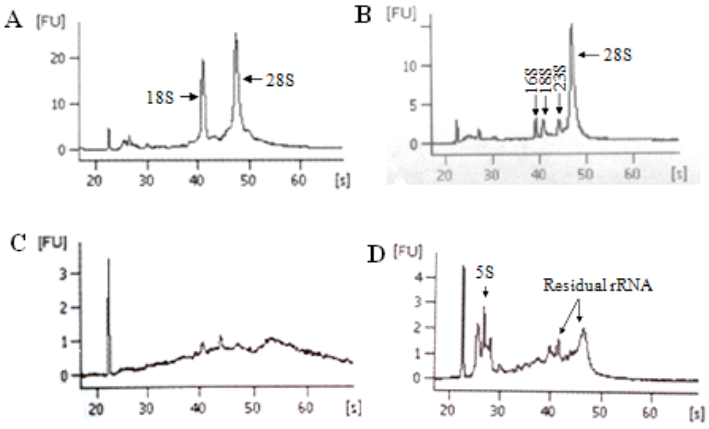

Figure 2.2 Bioanalyzer RNA profiles. (A) Total RNA from *E. chaffeensis* infected cells, only host cell ribosomal RNAs (18S and 28S) are visible. (B) Enriched microbial RNA with bacterial ribosomal RNA (16S and 23S). (C) Purified mRNA. (D) Enriched microbial mRNA after digestion of ribosomal RNA with Terminator.

MICROARRAY DESIGN

The genome sequence of *E. chaffeensis* (GenBank accession no. CP000236) (21) was submitted to Roche NimbleGen Systems (Madison, WI) for custom 4-plex microarray design. The arrays were manufactured using maskless, digital micromirror technology. Five replicates of the genome were included per chip, with an average of 12 different 60-base oligonucleotides (60-mer probes) representing each open reading frame in the *E. chaffeensis* genome. Unique probes were designed for 1031 of the 1158 ORFs (1.18 Mbp genome). Three biological replicates were included for *E. chaffeensis* cultivated in each cell line (THP-1, AAE2 and ISE6). Additionally, for each cell line, RNA was extracted from uninfected cells (negative controls) and was processed similarly, and these samples were used to establish background subtraction thresholds.

HYBRIDIZATION

Enriched *E. chaffeensis* RNA (10 μg) was used for cDNA synthesis using random hexamer primers and the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) according to the NimbleGen Arrays User's Guide (Gene Expression Analysis v3.2). Labeling and hybridization were done at the MD Anderson Cancer Center, Genomics Facility (Houston, TX). Briefly, double-stranded cDNA was random-prime labeled with Cy3-nonamers and hybridized to the microarray for 16 hrs at 42°C. The arrays were washed, dried and scanned using a GenePix 400B microarray scanner (Molecular Devices, Sunnyvale, CA).

MICROARRAY DATA ANALYSIS

Data were extracted from the scanned array images using NimbleScan software (Roche NimbleGen). Quantile normalization was performed across replicates within the 4-plex arrays, and RMA (Robust Multichip Average) analysis was performed to generate gene expression values (116). The genes expressed were determined by subtracting expression values obtained from uninfected cells from those of infected cells from the same cell line. Analysis and visualization of the expression data were performed using ArrayStar4 software (DNASTAR Inc., Madison, WI), using mean $\log_2$ expression values for the three biological replicates for each cell line. F-test (ANOVA) was used to compare the mean gene expression values for replicates (within same cell line) and groups of replicates (between cell lines) for a given gene. The microarray data generated in this study have been deposited in NCBI's Gene expression Omnibus (117). The data are accessible through GEO series accession number GSE29109 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29109).

REALTIME QUANTITATIVE PCR

Real time PCR of selected *E. chaffeensis* genes was performed with gene specific primers designed using Lasergene 8 (DNASTAR) (Table 2.1). RNA (1 μg) was used as template for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. qPCR was performed using iQ SYBR Green supermix (Bio-Rad), gene-specific primers and thermal cycling protocol that consisted of an initial denaturation step of 95°C for 2 min, and 40 cycles of 95° for

10 s, 55°C for 30 s, and 65°C for 30 s using a Mastercycler EP Realplex^2 S (Eppendorf). DNA from infected cells was used as positive control. Samples lacking cDNA and cDNA from uninfected cells were used as negative controls. qPCR data were converted by subtracting the Ct value from the number of cycles (40 cycles) to obtain values.

Table 2.1 Primers for qRT-PCR

Gene	SEQ_ID	Primers (5'-3')	Amplicon Size (bp)
Suc CoA	ECH_0979	F –ATTAGGCGAAGTTGATGGT R-TCTTTTGAGGTCTGATGAGTAAT	186
RpsL	ECH_0963	F-CGCGTGTAAAAATTGCTGGTT R-CGCGCCTTCTTCCTATTTTG	186
Omp1B	ECH_1136	F-AACGACAGCAGAGAAGGC R-AACAGGACAGATCCAGCC	183
Omp1N	ECH_1121	F-TCATGTTTAGGATTTGGAGTA R-GTGGCTTGCTGTTTGGT	220
TRP120	ECH_0039	F-TGGATATTGATAATAGTAACATAAGTAC R-TGTGTCATCTTCTTGCTCTTG	162
TRP32	ECH_0170	F- TGATTCACATGAGCCTTCTC R-GGACCAGGTAACTCAACAAA	303
TRP47	ECH_0166	F-CGTGGTGTACAAGCTGAAA R-CTCTTCCGTGTGCATCAT	202

WESTERN IMMUNOBLOTTING

Whole cell lysates (1µg) from uninfected and *E. chaffeensis*-infected (90% infected) THP-1, AAE2 and ISE6 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and western immunoblotting was performed as previously described (39) using rabbit anti-TRP32, anti-TRP47, or anti-TRP120 antibodies (65,66,70). Bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG (H+L) secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and

visualized after incubation with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium) substrate.

Results

DIFFERENTIALLY EXPRESSED *E. CHAFFEENSIS* GENES IN THP-1, AAE2 AND ISE6 CELLS

The transcriptome of *E. chaffeensis* in THP-1 consisted of 79% of all genes (n=1031). Similar expression levels were observed in AAE2 (76%) and ISE6 (81%). *E. chaffeensis* genes were differentially expressed in THP-1 compared to AAE2 and ISE6 cells. Minor differences in *E. chaffeensis* gene expression between the tick cell lines were observed (Fig. 2.3). There were 405 *E. chaffeensis* genes (39%) differentially expressed (greater than 2 fold change; $p<0.005$) between THP-1 and ISE6 cells, 371 genes (36%, $p<0.005$) differentially expressed between THP-1 and AAE2, and 351 were similarly expressed in the tick cell lines (Fig. 2.4A).

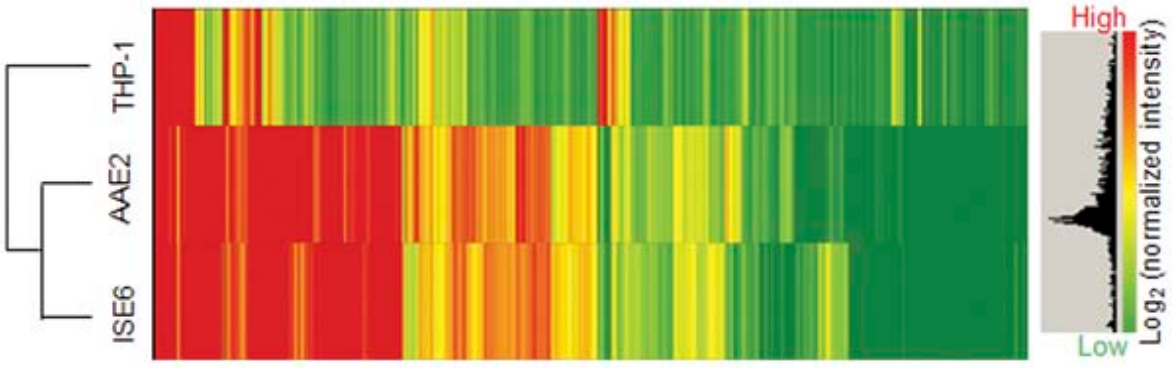

Figure 2.3 Microarray data of gene expression profiles from *E. chaffeensis*-infected human and tick cell lines. Heat map with gene expression in THP-1, AAE2 and ISE6 cells, coloring: red, up-regulated; yellow, normal; green, down-regulated.

E. CHAFFEENSIS GENES UPREGULATED IN THE HUMAN MONOCYTES

There were 50 *E. chaffeensis* genes upregulated (>2 fold; $p<0.05$) in the THP-1 cells compared to both AAE2 and ISE6 cells (Table 2.2), and 19 additional genes upregulated in THP-1 compared to AAE2 cells. In contrast, only five additional genes were upregulated in THP-1 compared to ISE6 cells. When classifying the genes according to the Clusters of Orthologous Groups (COGs) (118), the *E. chaffeensis* genes upregulated in the THP-1 cells were grouped into the metabolic and cellular process (C, G, P, Q, D); transcription, translation and DNA repair (J, K, L); cell envelope biogenesis and outer membrane (M); posttranslational modifications (O); general function predicted or unknown (R,S); trafficking and secretion (U) (Fig. 2.4B, black bars). The majority of these genes (54%) were classified as hypothetical with unknown functions.

E. CHAFFEENSIS GENES UPREGULATED IN THE TICK CELLS

There were 193 *E. chaffeensis* genes upregulated (>2 fold; $p<0.05$) in tick cells compared to human cells (Table 2.3). The largest proportion (32%) belonged to the COG with general function predicted or unknown (R, S), 30% were involved in metabolism and cellular process, and 7% of the genes were associated with translation (J) (Fig. 2.4B, grey bars). The remaining *E. chaffeensis* genes (31%) were distributed in the other COGs.

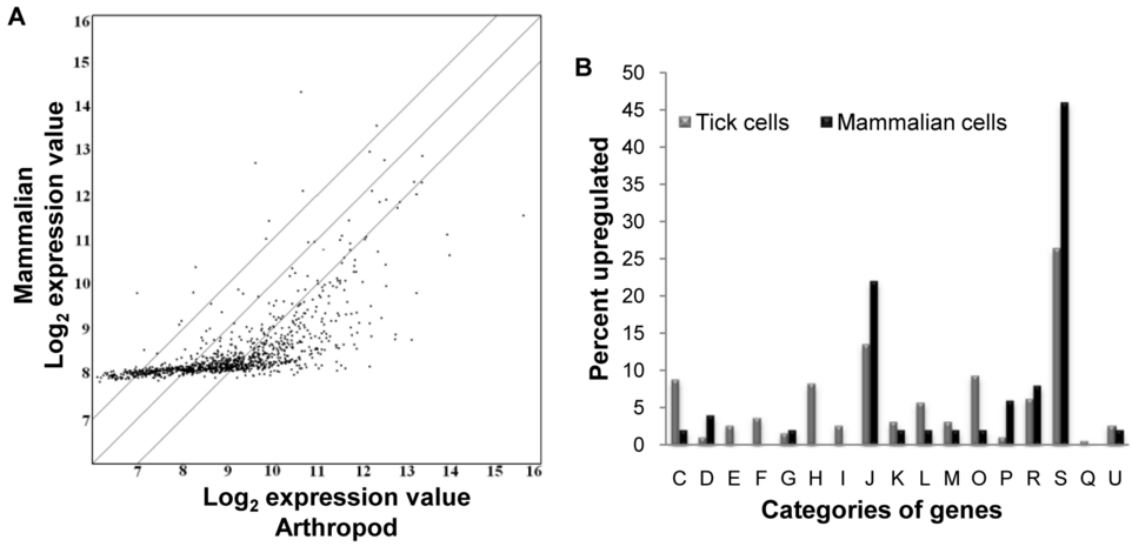

Figure 2.4 Genes upregulated by *E. chaffeensis* in human and tick cells. (A) Scatter plot of expression in mammalian (THP-1) vs. arthropod (AAE2 and ISE6) cells, center line represents equivalence and outer lines indicate two fold difference. Images generated using ArrayStar®. (B) Distribution of significantly upregulated *E. chaffeensis* genes in mammalian cells (black bars) and tick cells (grey bars) classified to Clusters of Orthologous Groups (COGs). C: Energy production and conversion, D: Cell cycle control and mitosis, E: Amino acid metabolism and transport, F: Nucleotide metabolism and transport, G: Carbohydrate metabolism and transport, H: Coenzyme metabolism, I: Lipid metabolism, J: Translation, K: Transcription, L: Replication and repair, M: Cell wall/membrane/envelope biogenesis, O: Post-translational modification, protein turnover, chaperone functions, P: Inorganic ion transport and metabolism, R: General functional prediction only, S: Function unknown, Q: Secondary structure, U: Intracellular trafficking and secretion.

Table 2.2 *E. chaffeensis* genes upregulated in THP-1 cells compared to AAE2 and ISE6 cells.

Gene Function	Gene ID	Fold Change THP-1 vs (AAE2, ISE6)>2fold; $p<0.05$
Function Unknown or Predicted		
1. Conserved hypothetical protein	ECH_0147	5.3, 4.8
2. Conserved hypothetical protein	ECH_0619	5.6, 5.3
3. Conserved hypothetical protein	ECH_0790	2.6, 2.6
4. Conserved hypothetical protein	ECH_1059	2.3, 2.4
5. Conserved hypothetical protein	ECH_1122	7.4, 6.7
6. Hypothetical protein	ECH_0034	2.6, 2.4
7. Hypothetical protein	ECH_0078	3.2, 3.1
8. Hypothetical protein	ECH_0253	3.2, 2.2
9. Hypothetical protein	ECH_0254	3.7, 2.2
10. Hypothetical protein	ECH_0265	3.3, 2.0
11. Hypothetical protein	ECH_0682	2.1, 2.5
12. Hypothetical protein	ECH_0685	2.6, 2.7
13. Hypothetical protein	ECH_0833	2.9, 3.1
14. Hypothetical protein	ECH_0834	3.1, 2.6
15. Hypothetical protein	ECH_0887	3.9, 2.0
16. Hypothetical protein	ECH_0909	2.8, 2.1
17. Hypothetical protein	ECH_0921	2.3, 2.2
18. Hypothetical protein	ECH_0965	2.2, 2.2
19. Hypothetical protein	ECH_1049	3.7, 4.1
20. Hypothetical protein	ECH_1056	4.8, 2.6
21. Hypothetical protein	ECH_1102	4.5, 2.5
22. Conserved hypothetical protein (TRP47)	ECH_0166	11.4, 14.1
23. Variable length PCR target protein (TRP32)	ECH_0170	9.4, 7.7
24. HAD-superfamily hydrolase, subfamily IA, variant 1	ECH_0332	2.2, 2.2
25. Putative flavin reductase	ECH_0442	2.5, 2.8
26. Putative NADH dehydrogenase I, J subunit, truncation	ECH_0550	3.1, 2.7
27. Rhodanese domain protein	ECH_0896	2.2, 2.0
Cell envelope biogenesis/Outer membrane		
28. Major outer membrane protein P28	ECH_1143	2.2, 2.2
Trafficking/Secretion		
29. Preprotein translocase, SecG subunit	ECH_0172	4.2, 4.3
Posttranslational modification/Protein turnover/Chaperones		
30. Trigger factor	ECH_0902	2.3, 2.0
Transcription/Translation/DNA replication/RNA		
31. Cold shock protein, CSD family	ECH_0298	2.9, 2.7
32. DNA-binding protein HU	ECH_0804	2.1, 2.6
33. Putative ribosomal protein S18	ECH_0309	3.2, 2.9
34. Ribosomal protein L13	ECH_1019	3.5, 3.4
35. Ribosomal protein L15	ECH_0427	2.3, 2.0
36. Ribosomal protein L20	ECH_0197	2.5, 2.7
37. Ribosomal protein L34	ECH_0440	2.8, 2.5
38. Ribosomal protein L35	ECH_0198	5.7, 5.1
39. Ribosomal protein S13	ECH_0430	2.2, 2.3
40. Ribosomal protein S15	ECH_0727	4.6, 4.0
41. Ribosomal protein S19	ECH_0413	2.2, 2.5
42. Ribosomal protein S6	ECH_0308	2.6, 2.4
43. Ribosomal protein S8	ECH_0423	2.2, 2.1
Metabolism/Cellular Processes		
44. ATP synthase F1, delta subunit	ECH_0131	2.4, 2.5
45. Cell division protein FtsA	ECH_1090	2.2, 2.1
46. Monovalent cation/proton antiporter, MrpF/PhaF subunit family	ECH_0466	2.7, 2.3
47. Na(+)/H(+) antiporter subunit C	ECH_0469	2.7, 2.2
48. Ribose 5-phosphate isomerase B	ECH_0638	3.4, 2.9
49. Superoxide dismutase, Fe	ECH_0493	2.6, 2.6
50. YGGT family protein	ECH_0891	3.1, 3.3

Table 2.3 *E. chaffeensis* genes upregulated in AAE2 and ISE6 compared to THP-1 cells.

Gene Function	Gene ID	Fold Change AAE2, ISE6 vs THP-1>2fold; $p<0.05$
Function Unknown or Predicted		
1. Conserved domain protein	ECH_0526	2.1, 2.3
2. Conserved domain protein	ECH_0894	3.2, 2.0
3. Conserved hypothetical protein	ECH_0397	5.3, 4.2
4. Conserved hypothetical protein	ECH_0477	2.7, 3.3
5. Conserved hypothetical protein	ECH_0540	2.0, 2.8
6. Conserved hypothetical protein	ECH_0663	3.0, 2.6
7. Conserved hypothetical protein	ECH_0706	2.8, 3.2
8. Conserved hypothetical protein	ECH_0725	3.6, 3.9
9. Conserved hypothetical protein	ECH_0767	2.3, 2.5
10. Conserved hypothetical protein	ECH_0836	2.1, 2.5
11. Conserved hypothetical protein	ECH_0854	3.0, 2.1
12. Conserved hypothetical protein	ECH_0988	2.6, 2.6
13. Conserved hypothetical protein	ECH_1148	2.2, 2.1
14. Hypothetical protein	ECH_0059	2.1, 2.3
15. Hypothetical protein	ECH_0114	5.7, 5.8
16. Hypothetical protein	ECH_0117	2.8, 4.7
17. Hypothetical protein	ECH_0150	4.3, 4.2
18. Hypothetical protein	ECH_0158	4.5, 3.1
19. Hypothetical protein	ECH_0159	3.7, 3.0
20. Hypothetical protein	ECH_0191	4.2, 4.0
21. Hypothetical protein	ECH_0205	5.3, 7.4
22. Hypothetical protein	ECH_0242	5.2, 8.9
23. Hypothetical protein	ECH_0244	4.4, 4.8
24. Hypothetical protein	ECH_0258	5.8, 5.8
25. Hypothetical protein	ECH_0270	3.8, 3.9
26. Hypothetical protein	ECH_0271	4.1, 3.4
27. Hypothetical protein	ECH_0272	2.7, 2.3
28. Hypothetical protein	ECH_0284	3.3, 2.6
29. Hypothetical protein	ECH_0285	5.4, 5.5
30. Hypothetical protein	ECH_0288	3.2, 4.1
31. Hypothetical protein	ECH_0329	2.8, 3.2
32. Hypothetical protein	ECH_0349	2.5, 2.0
33. Hypothetical protein	ECH_0388	3.0, 3.9
34. Hypothetical protein	ECH_0531	4.8, 3.7
35. Hypothetical protein	ECH_0535	2.6, 2.1
36. Hypothetical protein	ECH_0722	2.5, 2.0
37. Hypothetical protein	ECH_0723	3.6, 2.6
38. Hypothetical protein	ECH_0765	2.1, 2.1
39. Hypothetical protein	ECH_0825	2.3, 2.0
40. Hypothetical protein	ECH_0829	2.0, 2.0
41. Hypothetical protein	ECH_0878	5.1, 4.7
42. Hypothetical protein	ECH_0916	2.7, 3.1
43. Hypothetical protein	ECH_0925	2.0, 2.1
44. Hypothetical protein	ECH_0927	2.0, 2.2
45. Hypothetical protein	ECH_1048	6.0, 5.6
46. Hypothetical protein	ECH_1092	3.7, 4.5
47. Hypothetical protein	ECH_1104	2.8, 3.1
48. Peptidase, M16 family	ECH_0235	4.7, 2.5

49.	Phage portal protein, HK97 family	ECH_0033	2.6, 3.4
50.	Phospholipase/carboxylesterase family protein	ECH_0935	2.2, 2.4
51.	P-loop hydrolase family protein	ECH_0008	2.6, 2.7
52.	Putative flavoprotein	ECH_0061	2.0, 2.2
53.	Putative membrane protein, TIGR00023	ECH_0027	2.6, 2.8
54.	Putative osmotically inducible protein	ECH_0335	3.6, 2.4
55.	Putative oxidoreductase	ECH_0213	4.1, 6.1
56.	Putative phosphatidate cytidylyltransferase	ECH_0269	2.9, 2.3
57.	Putative surface protein	ECH_0188	2.3, 2.3
58.	Smr domain protein	ECH_0797	2.4, 4.1
59.	Tim44-like domain protein	ECH_0232	8.4, 13.1
60.	Acid phosphatase SurE	ECH_0791	3.8, 2.2
61.	Competence/damage-inducible protein CinA C-terminal domain	ECH_0005	3.7, 5.1
62.	Exopolysaccharide synthesis protein	ECH_0769	2.6, 2.8
63.	Hemolysin	ECH_0031	4.2, 4.0
	Cell envelope biogenesis/Outer membrane		
64.	Major outer membrane protein OMP-1B	ECH_1136	8.3, 8.2
65.	Major outer membrane protein OMP-1F	ECH_1142	3.1, 2.9
66.	Major outer membrane protein Omp-1N	ECH_1121	2.1, 2.1
67.	Outer membrane protein, OmpH family	ECH_1072	3.0, 3.1
68.	Putative membrane-associated zinc metalloprotease	ECH_1070	3.8, 2.1
69.	D-alanyl-D-alanine carboxypeptidase family protein	ECH_1067	2.8, 3.0
	Trafficking/Secretion		
70.	Protein-export membrane protein SecF	ECH_0095	3.0, 2.8
71.	Sec-independent protein translocase protein TatC	ECH_0560	2.7, 4.9
72.	Twin-arginine translocation protein, TatA	ECH_0844	3.4, 2.5
73.	Type IV secretion system protein, VirB6 family	ECH_0497	2.2, 2.2
74.	Type IV secretion system protein,VirB6 family	ECH_0498	2.5, 3.8
	Posttranslational modification/Protein turnover/Chaperones		
75.	ATP-dependent Clp protease, ATP-binding subunit ClpB	ECH_0367	2.0, 2.3
76.	ATP-dependent Clp protease, ATP-binding subunit ClpX	ECH_0900	2.2, 2.5
77.	ATP-dependent metalloprotease FtsH	ECH_1098	3.0, 2.6
78.	ATP-dependent protease HslV	ECH_0996	3.4, 2.6
79.	ATP-dependent protease La	ECH_0899	2.4, 2.3
80.	Chaperone protein HtpG	ECH_0853	2.3, 2.1
81.	Chaperonin, 10 kDa	ECH_0364	3.4, 5.5
82.	Co-chaperone GrpE	ECH_0168	2.5, 4.0
83.	Glutaredoxin 3	ECH_1062	4.3, 4.1
84.	Glycoprotease family protein	ECH_0730	5.2, 5.3
85.	Heat shock protein HslVU, ATPase subunit HslU	ECH_0997	3.9, 3.8
86.	Heme exporter protein CcmC	ECH_0321	4.8, 2.8
87.	HflC protein	ECH_1051	2.2, 2.1
88.	NifU domain protein	ECH_0202	4.9, 6.8
89.	Rotamase family protein	ECH_0731	2.9, 2.3
90.	Signal peptide peptidase SppA	ECH_0401	2.1, 2.4
91.	Thioredoxin-disulfide reductase	ECH_0735	4.5, 3.9
92.	Zinc finger-like domain protein	ECH_0057	2.7, 2.3
	Transcription/Translation/DNA replication/RNA		
93.	Ankyrin repeat protein	ECH_0877	2.1, 2.1
94.	Ankyrin repeat protein	ECH_0653	5.5, 2.9
95.	Aspartyl-tRNA synthetase	ECH_0334	2.3, 2.3
96.	Conserved hypothetical protein	ECH_0803	2.8, 4.4

97. Cysteinyl-tRNA synthetase	ECH_0768	3.1, 4.0
98. DNA gyrase, A subunit	ECH_0858	2.5, 2.3
99. DNA-directed RNA polymerase, beta subunit	ECH_0952	2.6, 2.2
100. DNA-directed RNA polymerase, beta' subunit	ECH_0951	2.3, 2.5
101. Glutamyl-tRNA synthetase	ECH_0605	2.3, 2.0
102. GTP-binding protein YchF	ECH_0154	6.3, 4.1
103. Holliday junction DNA helicase RuvB	ECH_0319	2.6, 2.5
104. Integration host factor, alpha subunit	ECH_0162	8.2, 10.0
105. Leucyl-tRNA synthetase	ECH_0794	2.0, 2.2
106. Lysyl-tRNA synthetase	ECH_0626	2.9, 2.0
107. Methionyl-tRNA formyltransferase	ECH_0897	3.3, 2.2
108. Methionyl-tRNA synthetase	ECH_1000	2.0, 2.0
109. N utilization substance protein A	ECH_0562	3.6, 2.5
110. Peptide deformylase	ECH_0073	2.6, 2.3
111. Phenylalanyl-tRNA synthetase, beta subunit	ECH_0434	2.4, 2.7
112. PolyA polymerase/tRNA nucleotidyltransferase family protein	ECH_1116	2.8, 3.0
113. Polyribonucleotide nucleotidyltransferase	ECH_0726	4.6, 2.9
114. Primosomal protein N'	ECH_0483	2.8, 2.6
115. Putative methyltransferase	ECH_0211	2.5, 4.0
116. Replicative DNA helicase	ECH_0451	2.0, 2.3
117. Ribonuclease III	ECH_1054	2.5, 2.7
118. Ribosomal protein L1	ECH_0955	2.1, 2.8
119. Ribosomal protein L14	ECH_0419	2.8, 3.0
120. Ribosomal protein L17	ECH_0433	3.2, 2.4
121. Ribosomal protein L21	ECH_0545	3.7, 4.0
122. Ribosomal protein L24	ECH_0420	4.6, 5.9
123. Ribosomal protein L3	ECH_0409	2.8, 4.0
124. Ribosomal protein L4	ECH_0410	2.4, 3.2
125. Ribosomal protein L7/L12	ECH_0953	3.2, 5.1
126. Ribosomal protein S5	ECH_0426	2.3, 4.6
127. Ribosomal protein S9	ECH_1018	8.3, 11.8
128. Ribosomal RNA large subunit methyltransferase J	ECH_0533	3.5, 2.1
129. RNA methyltransferase, TrmH family, group 3	ECH_0404	2.1, 2.5
130. RNA polymerase sigma factor RpoD	ECH_0760	3.1, 2.6
131. Single-strand binding protein	ECH_0815	4.0, 2.3
132. Single-stranded-DNA-specific exonuclease RecJ	ECH_1115	2.6, 2.3
133. Site-specific recombinase, phage integrase family	ECH_0341	2.6, 2.6
134. Sua5/YciO/YrdC/YwlC family protein	ECH_0802	2.3, 2.3
135. tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	ECH_0872	2.1, 2.2
Metabolism/Cellular Processes		
136. 2-amino-4-hydroxy-6- hydroxymethyldihydropteridine-pyrophosphokinase	ECH_0350	3.3, 2.0
137. 3-demethylubiquinone-9 3-methyltransferase	ECH_0637	3.3, 2.0
138. 3-oxoacyl-(acyl-carrier-protein) synthase II	ECH_0882	5.1, 3.5
139. 3-oxoacyl-(acyl-carrier-protein) synthase III	ECH_0448	4.4, 2.4
140. Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	ECH_0666	3.7, 2.9
141. Adenylosuccinate synthetase	ECH_0461	4.5, 2.6
142. Amidophosphoribosyltransferase	ECH_0139	3.8, 3.4
143. Argininosuccinate lyase	ECH_0937	2.3, 2.4
144. ATP synthase F0, B chain	ECH_1089	3.1, 2.5
145. Biotin--acetyl-CoA-carboxylase ligase	ECH_0848	2.4, 2.3
146. Chromosome partitioning ATPase, ParA family	ECH_1156	2.1, 2.1
147. Coproporphyrinogen III oxidase, aerobic, truncation	ECH_0592	5.2, 3.2
148. Cytochrome C, membrane-bound	ECH_0327	4.6, 3.6

149.	Deoxyuridine 5'triphosphate nucleotidohydrolase	ECH_0501	2.3, 2.1
150.	Diaminopimelate epimerase	ECH_0050	4.4, 5.3
151.	Dihydrolipoamide dehydrogenase	ECH_0509	3.2, 2.6
152.	Dioxygenase family protein	ECH_0368	6.1, 5.7
153.	Divalent ion tolerance protein CutA1	ECH_0756	2.3, 2.3
154.	DNA / pantothenate metabolism flavoprotein family protein	ECH_0374	2.6, 2.4
155.	Fatty acid/phospholipid synthesis protein PlsX	ECH_0447	4.7, 3.4
156.	Ferredoxin A	ECH_0038	6.5, 8.8
157.	Ferrochelatase	ECH_0395	7.2, 7.2
158.	FeS cluster assembly scaffold IscU	ECH_0630	4.9, 4.4
159.	FolD bifunctional protein	ECH_0324	2.5, 2.2
160.	Folylpolyglutamate synthase	ECH_0702	3.2, 3.2
161.	Fumarate hydratase, class II	ECH_0376	3.0, 2.6
162.	Glucose inhibited division protein A	ECH_0359	3.3, 3.2
163.	GMP synthase	ECH_0123	5.2, 3.0
164.	HIT family protein	ECH_0826	4.1, 4.5
165.	Inorganic pyrophosphatase	ECH_1014	9.6. 6.1
166.	Major facilitator family transporter	ECH_0816	2.2, 2.3
167.	NADH dehydrogenase I, B subunit	ECH_0787	5.7, 5.0
168.	NADH dehydrogenase I, H subunit	ECH_0617	2.6, 2.8
169.	NADH dehydrogenase I, I subunit	ECH_0691	6.0, 3.7
170.	NADH dehydrogenase I, K subunit	ECH_0553	2.2, 2.0
171.	NADH dehydrogenase I, L subunit	ECH_0554	2.0, 2.0
172.	NADH:ubiquinone oxidoreductase family protein	ECH_0184	2.3, 3.6
173.	NADH-ubiquinone/plastoquinone oxidoreductase family protein	ECH_0328	3.6, 3.8
174.	Ornithine carbamoyltransferase	ECH_0077	6.1, 8.0
175.	Phosphomethylpyrimidine kinase	ECH_0914	2.0, 2.0
176.	Phosphoribosylamine--glycine ligase	ECH_1006	3.2, 2.1
177.	Porphobilinogen deaminase	ECH_0701	2.2, 2.2
178.	Propionyl-CoA carboxylase, alpha subunit	ECH_0487	2.6, 2.1
179.	Propionyl-CoA carboxylase, beta subunit	ECH_0599	3.5, 2.1
180.	Putative phosphoribosylformylglycinamidine synthase I	ECH_0362	2.4, 2.3
181.	Putative pyruvate dehydrogenase complex, E1 component, beta subunit	ECH_0149	3.7, 2.3
182.	Pyridoxamine 5'-phosphate oxidase	ECH_0931	3.3, 3.0
183.	Riboflavin biosynthesis protein RibD	ECH_0169	2.5, 2.7
184.	Riboflavin synthase, alpha subunit	ECH_0239	4.3, 2.6
185.	Ribulose-phosphate 3-epimerase	ECH_0082	6.5, 6.9
186.	Serine hydroxymethyltransferase	ECH_0311	4.2, 3.1
187.	Succinate dehydrogenase, flavoprotein subunit	ECH_0315	3.2, 2.6
188.	Succinyl-CoA synthetase, alpha subunit	ECH_0980	3.6, 2.6
189.	Succinyl-diaminopimelate desuccinylase	ECH_0144	2.1, 2.0
190.	Thiamin biosynthesis protein ThiC	ECH_0798	3.5, 5.0
191.	Thiamin biosynthesis ThiG	ECH_0206	6.6, 3.3
192.	Transketolase	ECH_0465	4.1, 4.3
193.	Uroporphyrinogen decarboxylase	ECH_0030	3.4, 5.8

HYPER-EXPRESSED EHRLICHIAL GENES

There were ten *E. chaffeensis* genes expressed in the THP-1 cells with expression levels 10-15 times higher (hyper-expressed) than other genes identified as highly expressed. These genes included TRP47 (the highest expressed gene), TRP32, ribosomal proteins, malonyl CoA-acyl carrier protein transacylase, and hypothetical proteins (ECH_0166, ECH_1059, ECH_0570, ECH_0253).

EXPRESSION OF GENES ASSOCIATED WITH HOST-PATHOGEN INTERACTIONS

For the two *E. chaffeensis* proteins recently shown to bind mammalian host cell DNA, transcripts for TRP120 and Ank200 genes were detected in human and tick cells. The *E. chaffeensis* genome encodes for a polymorphic multigene family composed of 22 paralogues that are clustered in a 29 kb gene locus that is downstream of the transcriptional regulator gene *tr1* (41). Transcriptional regulator *tr1* (ECH_1118) of *E. chaffeensis* was expressed in human and tick cells. With respect to the OMP-1 (p28) family genes, transcripts were not detected for OMP-1H (p28-11) and OMP-1W (p28-7) in human cells and OMP-1P (p28-3), OMP-1U (p28-5) and OMP-1H (p28-11) were not detected in tick cells, but OMP-1B (p28-14) and OMP-1N (p28-1) were hyper-expressed. Notably, OMP-1B (p28-14) was up-regulated in tick cells, but OMP-1N (p28-1) was also highly expressed in human cells. OMP-1F (p28-18) and OMP-1D (p28-16) transcripts were also upregulated in tick cells compared to human cells, and P28 (p28-19) was among the most highly expressed OMP-1 genes in human cells.

E. chaffeensis genes associated with protein trafficking and secretion were expressed in tick and human cells; however, several of these genes were upregulated in the tick cells compared to human cells, including SecF, TatC, TatA and members of the type IV secretion systems. Additionally, in the tick cells, several of the genes associated with posttranslational modification, and protein turnover were upregulated including several ATP-dependent proteases and chaperones.

EXPRESSION OF HYPOTHETICAL GENES

A large percentage (42%) of annotated *E. chaffeensis* genes encode hypothetical proteins with unknown functions (21). In this study, we determined that most of these genes were differentially expressed in human and tick cell lines. There were 27 hypothetical genes (COG; R, S) that were highly expressed in the THP-1 cells, including TRP32 and TRP47 (Table 2.4). However, 11 of these 27 genes were not expressed by *E. chaffeensis* in the AAE2 and ISE6 cells. The genes in this group included ECH_0034, ECH_0078, ECH_0353, ECH_0790, ECH_0833, ECH_0834, ECH_0921, ECH_0965, ECH_1026, ECH_1056 and ECH_1113. Most of these genes (9/11) encode peptides (30-70 amino acids) that do not have orthologs. Host-specific expression of these genes suggested that they are required exclusively for adaptation and survival within the mammalian host.

There was a larger number of *E. chaffeensis* genes that were differentially expressed in the tick cells, including 63 genes categorized as hypothetical (COG; R, S). Some of these genes (n=18) were expressed only in the tick cells (Table 2.5). Of these genes, ECH_0114, ECH_0249, ECH_0258, ECH_0889, ECH_1030, ECH_1048 were

highly expressed (expression values 3-5 times greater than the average expression value of all genes). Of the 18 genes differentially expressed in the tick cells, seven did not have orthologs and six were peptides (30-80 aa). ECH_0114 was predicted to be a secreted protein, and ECH_0526 and ECH_1038 were predicted as outer membrane proteins (CELLO subcellular localization predictor) (119). In a previous study, ECH_0526 protein expression was detected in both AAE2 and ISE6 tick cell lines (120).

Table 2.4 Hypothetical genes expressed only in human (THP-1) cells.

Gene Function	SEQ_ID	Length (Amino Acids)	Predicted Cellular Location
Hypothetical protein	ECH_0790	44	Cytoplasmic
Hypothetical protein	ECH_0034	31	Cytoplasmic
Hypothetical protein*	ECH_0078	56	Cytoplasmic
Hypothetical protein	ECH_0353	73	Cytoplasmic
Hypothetical protein	ECH_0833	50	Cytoplasmic
Hypothetical protein	ECH_0834	49	Cytoplasmic
Hypothetical protein	ECH_0921	52	Cytoplasmic
Hypothetical protein	ECH_0965	203	Inner Membrane
Hypothetical protein*	ECH_1056	47	Cytoplasmic
Hypothetical protein	ECH_1113	48	Cytoplasmic
Hypothetical protein*	ECH_0887	39	Cytoplasmic

*Expression values 3-5 times greater than average expression values of all genes

Table 2.5 Hypothetical genes expressed only in tick (AAE2 and ISE6) cells.

Gene Function	SEQ_ID	Length (Amino Acids)	Predicted Cellular Location
Conserved hypothetical protein	ECH_0516	120	Cytoplasmic
Conserved hypothetical protein	ECH_0767	621	Cytoplasmic
Conserved hypothetical protein	ECH_0988	208	Cytoplasmic
Conserved hypothetical protein	ECH_1154	135	Cytoplasmic
Conserved domain protein	ECH_0526	495	Outer Membrane
Hypothetical protein	ECH_0059	49	Cytoplasmic
Hypothetical protein	ECH_0099	42	Cytoplasmic
Hypothetical protein*	ECH_0114	122	Extracellular
Hypothetical protein*	ECH_0249	46	Cytoplasmic
Hypothetical protein	ECH_0258	35	Cytoplasmic
Hypothetical protein	ECH_0635	357	Cytoplasmic
Hypothetical protein	ECH_0765	79	Cytoplasmic
Hypothetical protein	ECH_0868	31	Cytoplasmic
Hypothetical protein*	ECH_0889	38	Cytoplasmic
Hypothetical protein*	ECH_1030	62	Cytoplasmic
Hypothetical protein	ECH_1038	1963	Outer Membrane
Hypothetical protein*	ECH_1048	64	Cytoplasmic
Hypothetical protein	ECH_1077	68	Cytoplasmic

*Expression values 3-5 times greater than average expression values of all genes

VALIDATION OF MICROARRAY DATA

Real-time quantitative RT-PCR was used to verify the microarray results of a subset of ehrlichial genes. Eight *E. chaffeensis* genes assayed for relative transcript abundance by qRT-PCR included: Suc CoA (ECH_0979), RpsL (ECH_0963), OMP-1B (ECH_1136), OMP-1N (ECH_1121), TRP120 (ECH_0039), TRP32 (ECH_0170), TRP47 (ECH_0166). The relative transcripts levels for the selected genes within and between cell lines confirmed expression levels determined by microarray (Fig. 2.5).

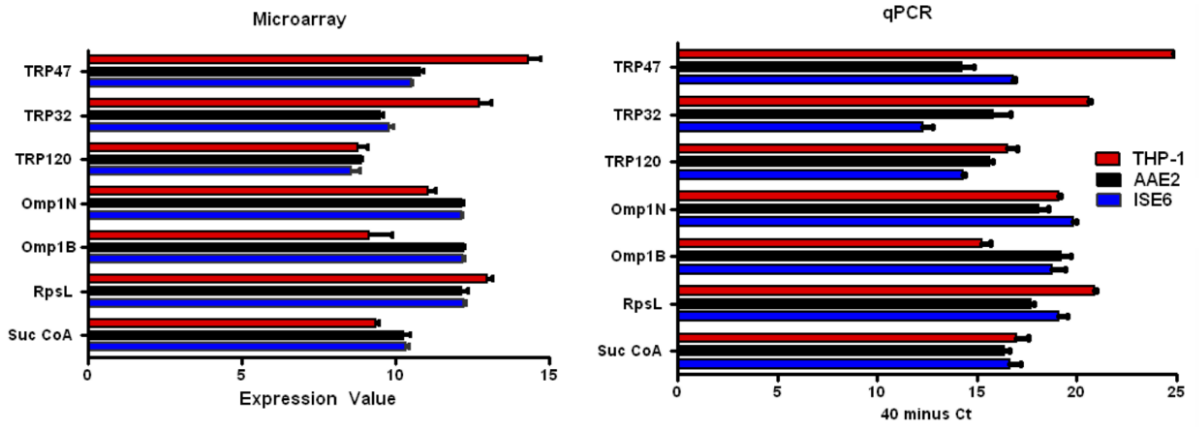

Figure 2.5 Comparison of microarray (gene expression value) and qPCR (40 minus threshold cycle) analysis of selected *E. chaffeensis* transcript levels.

EXPRESSION OF TANDEM REPEAT PROTEINS

Expression of three tandem repeat proteins; TRP32, TRP47 and TRP120 was analyzed in AAE2, ISE6 and THP-1 cells. TRP120 transcript was detected in all three cell lines, and the protein was also expressed in human monocytes and tick cells (Fig. 2.6). Interestingly, transcripts for hyper-expressed genes TRP32 and TRP47 were detected in tick cells by microarray and qRT-PCR; however, these proteins were not detected by western immunoblot in *E. chaffeensis*-infected AAE2 and ISE6 cell lysates (Fig. 2.6).

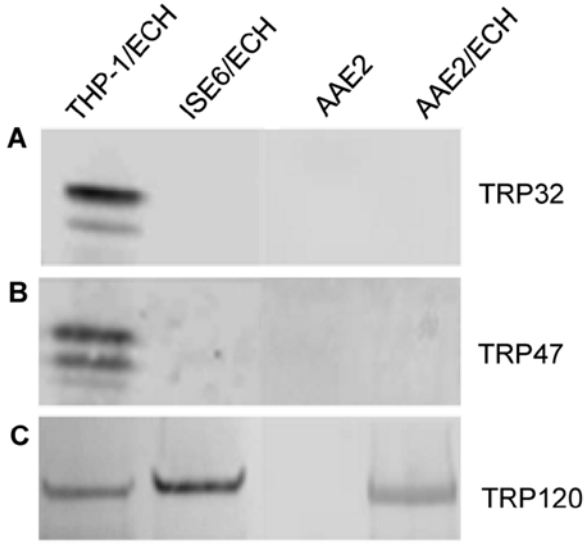

Figure 2.6 Expression of *E. chaffeensis* TRPs in human (THP-1) and tick (ISE6 and AAE2) cell lysates. Western immunoblots were probed with anti-TRP32 (A), anti-TRP47 (B) and anti-TRP120 (C) antibodies. TRP120 was detected in *E. chaffeensis*-infected human and tick cell lysates, and TRP32 and TRP47 were detected only in *E. chaffeensis*-infected THP-1 cells.

Discussion

Defining the dynamic changes in pathogen gene and protein expression that occur in infected hosts is essential to understanding pathobiology and having a rational basis for vaccine development. This investigation was conducted because of our lack of knowledge regarding the relative *E. chaffeensis* gene expression in mammalian and

arthropod hosts, which is a major impediment to understanding which genes are essential for ehrlichial adaptation. In this study, we demonstrated that the expression of many *E. chaffeensis* genes was influenced by the host environment. In addition, we examined *E. chaffeensis* gene expression in tick cell lines from the established vector species and another common tick that is not a vector. Significant differences in *E. chaffeensis* gene expression were not observed between the two tick cell lines, and similar expression patterns were observed in *E. chaffeensis* genes involved in metabolic and cellular processes between human and tick cells. Differentially expressed genes identified were primarily hypothetical genes and genes associated with translation and posttranslational modification. Furthermore, we have also found evidence of post-transcriptional regulation of select ehrlichial genes involved in host-pathogen interactions in mammalian and arthropod hosts.

In mammalian cells, the *Ehrlichia* developmental cycle occurs within 72 hrs and is characterized by entry of the dense-cored form, replication of the reticulate cells and transformation to infectious dense-cored ehrlichiae (19). In this study, enriched bacterial RNA was extracted from *E. chaffeensis* infected THP-1, AAE2 and ISE6 cells when 90% of the cells were infected. Although the infection was not synchronized to evaluate a specific phase of the developmental cycle, cells were harvested when 90% of the cells had *E. chaffeensis* morulae within their cytoplasm; therefore, the data presented in this study likely include genes expressed during all phases of development, but may be more representative of ehrlichial transcription during the later developmental stages that is dominated by dense-cored ehrlichiae (19).

The *I. scapularis* cell line, ISE6, has been routinely used in previous investigations involving arthropod-borne pathogens including *Ehrlichia*, *Rickettsia*, *Anaplasma* and *Borrelia* (121); however, *I. scapularis* is not a natural vector of *E. chaffeensis*. Recently, Munderloh *et. al*, developed the *A. americanum*, AAE2 cell line from tick embryos (Munderloh, U. and Davidson, W.R., unpublished data), and *E. chaffeensis* protein expression in AAE2 tick cells has been investigated (120), but a comprehensive analysis of *E. chaffeensis* gene expression has not been determined in the AAE2 cell line. Therefore, we investigated gene expression in both cell lines to determine if significant differences existed. Notably, we did not identify any significant differences in *E. chaffeensis* gene expression; hence, it appears that either cell line could be used for vector-pathogen studies for *E. chaffeensis*. However, the AAE2 cells grew more rapidly and appeared to support more robust growth of ehrlichiae, which are also important considerations.

Transcripts were detected for ~80% of the *E. chaffeensis* genes in human monocytes and tick cells. This level of transcript detection is slightly higher (~10%) than that previously reported for *A. phagocytophilum* in human (HL-60) and tick (ISE6) cells (110). This difference may be related to the fact that we utilized pathogen-enriched RNA rather than total RNA. Most *E. chaffeensis* genes (81-95%) involved in metabolic and cellular process, transcription, translation, DNA repair, cell envelope biogenesis, outer membrane proteins, posttranslational modifications, general function predicted or unknown, trafficking and secretion were expressed in all three cell lines. There were 77 genes for which transcripts were not detected in any of the three cell lines, and the

function of the majority of these genes is unknown. It is possible that these genes are required during stages of infection not depicted in this study such as transmission of the pathogen from one host to the other, reactivation of the pathogen after a blood meal in the tick, or in the presence of tick saliva.

Although similar numbers of genes were expressed by *E. chaffeensis* in the human and tick cells, the most striking discovery is that 38% of the *E. chaffeensis* genes were differentially expressed. When compared to human cells, *E. chaffeensis* was transcriptionally more active in the tick cells, and there were a larger number of genes with high expression levels in the tick cells. The functions of these genes were associated with protein modification, energy production and conversion and nutrient transport. Similar genes were upregulated in *Rickettsia conorii* and *R. rickettsii* under conditions of nutrient limitations and lower temperatures, when the metabolism of the host cells slows (122,123). In contrast, the majority of the genes had a moderate expression level in human cells. Additionally, there were several genes involved in metabolism, cellular process, and translation that were upregulated in tick cells. The upregulation of these genes in the arthropod host suggests that *Ehrlichia* has higher metabolic activity in the tick. The number of genes differentially expressed by *E. chaffeensis* between the two host cells was similar to that observed for *A. phagocytophilum* (110). However, only minimal differences were observed in gene expression when *R. rickettsii* grown in ISE6 was compared to *R. rickettsii* grown in Vero cells (123). Although all three organisms have evolved to adapt within both arthropod and mammalian cells, there appear to be

significant differences between *Rickettsia* compared to *Ehrlichia* and *Anaplasma* suggesting that they have different adaptation mechanisms and pathobiology.

In contrast to the total expression level (~80%) of genes from the other functional groups, the 437 *E. chaffeensis* genes with unknown function were expressed at a lower level (~67%) in each cell line and the majority were differentially expressed. We identified 11 genes highly expressed in the human cells that were not expressed in the tick cells and identified 18 genes exclusively expressed in the tick cells, and the majority of these hypothetical genes encoded peptides, 30-80 amino acids in length. Additionally, these peptides are *E. chaffeensis*-specific and do not have orthologs in other ehrlichial species. Nearly half of the genes (n=243) annotated as hypothetical proteins in the *E. chaffeensis* genome contain fewer than 100 amino acids, and recently peptides were detected for 66% of these proteins during infection in THP-1 cells (124). Host-induced expression of these *E. chaffeensis* peptides suggests that they are required exclusively for adaptation and survival within the mammalian host. Further studies are needed to characterize these genes and their role in host-specific adaption and survival.

In human cells, there were ten ehrlichial genes that were hyper-expressed, defined as genes with expression values 10-15 times greater than other highly expressed genes. Among these genes were two major immunoreactive tandem repeat proteins, TRP47 and TRP32. TRP47, the most highly expressed *E. chaffeensis* gene in human cells, contains seven 19-mer tandem repeats that dominate the C-terminal region and several N-terminal tyrosine phosphorylation sites (74,125). A recent study to examine molecular

interactions between TRP47 and the host identified several interactions with specific host cell proteins that have distinct cellular functions associated with signalling, transcriptional regulation, vesicle trafficking, and cellular proliferation and differentiation (74). The hyper-expression of TRP47 in human cells, the absence of TRP47 in tick cells, and our recent findings regarding molecular host-pathogen interactions, suggests that TRP47 is a multifunctional effector that is required for ehrlichial intracellular survival within the mammalian host. Unlike TRP47 which is differentially expressed by the dense-cored form of *E. chaffeensis*, TRP32 is extracellularly associated with the morular fibrillar matrix and the morula membrane, indicating that this protein is secreted. TRP32 does not have homology with other known proteins (65); however, we have recently demonstrated that TRP32 interacts with proteins with functions similar to those that interact with TRP47 (76). In the tick cells, although transcripts were detected for the TRP47 and TRP32, the proteins were not detected suggesting that they are regulated posttranslationally.

E. chaffeensis TRP120 is a well characterized protein that is differentially expressed on the surface of the dense-cored *E. chaffeensis*. Similar levels of TRP120 transcripts were detected in human and tick cells, and the protein was detected in both cell lysates. Our findings regarding TRP120 expression were in contrast to a previous study that examined macrophage- and tick cell-derived proteins of *E. chaffeensis*, in which TRP120 was not detected in macrophages, but was detected in tick cell lysates (120). However, numerous other studies have reported TRP120 expression in ehrlichiae cultivated in human cells (33,66,78). TRP120 has known functional properties including

binding and internalization, and its surface expression is regulated by second messenger cyclic di-GMP and interacts with host cell proteins associated with biological processes similar to TRP47 (33,77,78). Furthermore, we recently, demonstrated that TRP120 binds host cell DNA and targets genes associated with biological processes known to be altered during *E. chaffeensis* infection (79). Although TRP120 has important functions in the mammalian host related to pathobiology, the role of TRP120 in the arthropod host is unknown. The expression of TRP120 in the tick cells suggests that it may have similar functions in the arthropod host.

The OMP-1/P28 multigene family of *E. chaffeensis* has been well studied, and host cell-specific expression of these genes has been previously reported (43,46). The function of these immunoreactive outer membrane proteins has been associated with protection; however, Rikihisa *et. al.* recently demonstrated porin activity for OMP-1F and P28 (47,48,104), suggesting an important functional role in nutrient acquisition. Consistent with previous studies, we determined that Omp-1B and p28 were expressed in human and tick cells. The upregulation of p28 (p28-19) in human cells and the high expression of OMP-1B (p28-14) in tick cells were also consistent with previous *in vitro* studies (43,112), and expression of OMP-1B (p28-14) transcript has been detected in all three developmental stages of the tick vector, *A. americanum* (43). However, our finding that OMP-1N (p28-1) was upregulated in tick cells has not been previously reported. Transcripts were not detected for OMP-1H (p28-11) and OMP-1W (p28-7) in the human cells. The absence of OMP-1W (p28-7) expression was also consistent with the fact that it could not be detected in dogs experimentally infected with *E. chaffeensis* (43).

However, OMP-1H (p28-11) was detected in experimentally infected dogs and DH82 cells (canine cell line) (43,44), but not in the human and tick cells suggesting that there are other host factors that contribute to expression of OMP-1H. Similarly, although transcripts for OMP-1 family members have been detected in several studies, OMP-1B is the only OMP-1 paralogue detected by proteomics in *E. chaffeensis* cultured in ISE6 cells (120). The role of host-specific OMP expression is not clear, but our findings suggest that vaccines targeting ehrlichial OMPs expressed in the tick should include OMP-1N. Host cell specific expression of these genes could be related to adaptation to different host environments and for nutrient acquisition.

It is generally recognized that regulation of bacterial gene expression is controlled by transcriptional and posttranscriptional mechanisms (126-128). Several recent studies have investigated the mechanisms involved in the mRNA and protein stability and translational regulation in prokaryotes, and their dependence on environmental conditions and growth phase, especially with virulence factors (129,130). Bacterial protein expression is not only dependent on levels of mRNAs but also on other RNA species. Regulatory RNAs such as small RNAs (sRNA) controlling virulence and pathogenesis have been demonstrated in other Gram-negative bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Chlamydia trachomatis* (131,132). In this investigation, transcripts were detected for TRP47 and TRP32 in the tick cells, yet the proteins were not detected suggesting that their expression is, in part, controlled by posttranslational mechanisms in response to host cell environments, potentially by regulatory RNAs. Similarly, OMP-1B (P28-14) transcripts have been

routinely detected in mammalian cells, yet the protein has not been detected in numerous proteomic studies (43,45,111,120). Therefore, there is evidence that posttranslational mechanisms are involved in TRP expression and could also be involved in regulating OMP expression. Further investigation of posttranslational regulation mechanisms in *Ehrlichia* survival in mammalian and arthropod hosts is needed to understand how ehrlichial protein expression is regulated and its role in host adaptation.

Understanding the molecular survival strategies within the distinct hosts and the mechanisms involved in host adaptation will lead to novel prophylactic and therapeutic targets to prevent transmission and infection. We determined that some TRPs, OMPs, and hypothetical proteins are differentially expressed and, thus, appear to be important for adaptation to each host. Additionally, the hyper-expression of the TRP32 and TRP47 genes in the human cells and absence of the expression of these proteins in the tick cells demonstrate their significance in the mammalian host. The subset of *E. chaffeensis* hypothetical genes identified exclusively in each of the host cells in this study should be examined and their functions determined.

Chapter 3

Transcriptional Regulation of *E. chaffeensis* Tandem Repeat Proteins by Two-Component Systems and Characterization of Response Regulator, CtrA

Introduction

Two component signal transduction systems enable bacteria to sense, respond and adapt to a wide range of environments, stressors, and growth conditions (133). Two-component systems (TCS) are integral in the ability of pathogenic bacteria to mount and establish a successful infection within the host and consequently have been recognized as targets for new anti-microbial agents (134,135). Additionally, TCSs are encoded in bacterial genomes and are not present in mammalian genomes (135). A prototypical TCS is composed of a membrane-located sensor with a histidine kinase (HK) that catalyzes its autophosphorylation in response to environmental stimuli. The phosphorylated HK subsequently transfers the phosphoryl group to an aspartic acid residue in the phosphor-receiver domain of the cognate response regulator (RR). This transfer activates the output domain of the RR, which can then effect changes in cellular physiology, usually through DNA binding activity to regulate gene expression or an enzymatic activity (136). Among the signals that have been shown to be sensed by the two component system are chemical and physical parameters such as different ions, temperature, pH, oxygen pressure, osmolarity, autoinducer compounds, the redox state of electron carriers and the interaction with host cells (137). Three pairs of TCSs (PleC-PleD, NtrY-NtrX and CckA-CtrA) have been identified in *E. chaffeensis* through

sequence and domain analysis of known homologues (31,32). Direct phosphotransfter from PleC to PleD, NtrY to NtrX and CckA to CtrA has also been demonstrated *in vitro* (31). Additionally, it has been suggested that these TCSs are essential for intracellular infection and survival of *E. chaffeensis* within mammalian cells (32).

The role of these TCSs during *E. chaffeensis* infection was not known until recently when the phosphorylation of the RR of the PleC-PleD TCS was shown to have diguanylate cyclase activity that produces c-di-GMP (33,138). Cyclic-di-GMP regulates the stability of surface expressed proteins and is therefore required for internalization of *E. chaffeensis* into the host cell (33). Signaling via c-di-GMP facilitates serine protease, HtrA dependent degradation of specific surface exposed proteins including TRP120, which is associated with bacterial internalization (33).

The CtrA (cell cycle transcriptional regulator A), the response regulator of the CckA-CtrA TCS has a DNA-binding domain and is found only in α-proteobacteria (139). The phosphorylated form of CtrA has been shown to act as a transcriptional factor that directly regulates expression of genes involved in cell division, DNA methylation, flagellar biogenesis, pili biogenesis and cell wall remodeling (140). In *Caulobacter crescentus*, CckA/CtrA is essential for bacterial viability, and CtrA is well studied as a spatially and temporally regulated master response regulator, which acts as a molecular switch that allows the coordination of cell cycle progression and morphogenesis by controlling the expression of over 100 genes (141,142). In addition to free-living bacterium *C. crescentus*, CtrA response regulator has been identified in other α-proteobacteria such as the plant symbionts *Mesorhizobium loti*, and *Sinorhizobium*

melitoti, the plant pathogen *Argobacterium tumefaciens*, facultative mammalian pathogens *Brucella* spp. and obligate intracellular mammalian pathogens such as *R. prowazekii*, *A. phagocytophilum* and *E. chaffeensis* (139). Previous studies have demonstrated that CtrA controls several cellular and developmental processes that are regulated through the control of either orthologous target genes or distinct target genes in the above mentioned α-proteobacteria species. The two DNA-binding sequence motifs that CtrA recognizes and binds were identified by chromatin immunoprecipitation and DNA microarray, and they are well characterized in *C. crescentus*. These consensus CtrA binding sequences have also been shown to be conserved in *R. prowazekii*, *Brucella* spp. and *A. tumefaciens* (139). The direct phosphotransfer from CckA to CtrA and the intracellular location of these TCS proteins have been previously studied in *E. chaffeensis* (31,32). In *C. crescentus*, CtrA is also phosphorylated by PleC (which is also present in *E. chaffeensis*); however, *in vitro* phosphorylation studies did not show activation of CtrA by PleC in *E. chaffeensis* (31,141). Additionally, the signals that activate the CckA histidine kinase and the genes that are regulated by CtrA in *E. chaffeensis* are not known. In this study, we demonstrated down regulation of TRP transcripts in the presence of TCS inhibitor and the binding of *E. chaffeensis* CtrA to the conserved binding sequence in other bacterial species.

Materials and Methods

GENERATION OF BIOTINYLATED PROBES

The conserved consensus CtrA binding 9-mer (P1- GGTTAATCTTTCTTTAAC AA) and 8-mer (P2-ATATTAACCATGATTGTATA) and complementary oligonucleotides were synthesized (Sigma-Genosys). The oligonucleotides were biotinylated using a biotin 3'-end DNA labeling kit (Pierce). The biotinylated DNA pairs were purified and denatured at 100°C for 10 min and slowly cooled to room temperature to allow for proper annealing. A random oligonucleotide sequence was also labeled as a negative control.

CLONING AND EXPRESSION OF RECOMBINANT CtrA

The full length CtrA protein (795 bp), the N-terminus (372 bp) and C-terminus (358 bp) fragments were amplified from *E. chaffeensis* (Arkansas) genomic DNA and cloned into the pMAL-c5X vector (New England Biolabs) and transformed and amplified in TOP10 *E. coli* (Invitrogen). Recombinant protein expression was induced with isopropylthiogalactoside (IPTG) for 2 h at 37°C. Bacteria were pelleted at 4,000 x g for 10 min, and recombinant proteins with 42 kDa MBP tag were purified using an amylose resin (New England Biolabs). Purified recombinant proteins eluted in 10mM maltose were dialyzed in phosphate buffered saline.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Purified recombinant CtrA (full protein, N-terminus, C-terminus) or bovine serum albumin (BSA-negative control) were incubated with biotinylated oligonucleotides for 30 minutes at 4°C in 20 µl of binding buffer (10 mM Tris-HCl [pH7], 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2.5% glycerol, 0.1% [wt/vol] NP-40, 50ng/µl of poly (dI-dC). As controls, recombinant proteins were also incubated with unlabeled probe. A 6% non-denaturing polyacrylamide gel in 0.5X Tris-borate-EDTA (TBE) was pre-run for 1 hour, and samples (probe+protein) were loaded and separated by electrophoresis at 100 V for 1.5 hours at 4°C. The biotinylated probes were transferred to a nylon membrane (Pierce) at 20 V for 45 min. The transferred DNA on the membrane was detected with the LightShift chemiluminescence EMSA kit (Pierce).

CLOSANTEL TREATMENT OF INFECTED CELLS

Closantel (*N*-[5-chloro-4-[(*R*,*S*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide) was purchased from Wako Pure Chemical Industries (Osaka, Japan). *E. chaffeensis*-infected THP-1 cells (80% infected) were treated with closantel (100 µM in DMSO) at 37°C for 15, 30, and 120 minutes. The cells were washed three times with ice-cold PBS to stop the reaction and remove the closantel, and as a negative control, infected cells were treated with DMSO (1% v/v).

EVALUATION OF TRANSCRIPTION OF TCS REGULATED GENES

Total RNA was extracted from closantel treated cells using Tri reagent (Ambion, Austin, TX). Genomic DNA was eliminated by treatment with Turbo DNA-free (Ambion) according to the manufacturer's protocol. Gene specific primers for TRP32,

TRP47, TRP120 from chapter 2 were used. Additionally genes known to be regulated by TCS, FtsK, FtsZ and CtrA, were used as controls with previously published primers (32).

Results

E. CHAFFEENSIS CtrA BINDS TO CONSERVED CONSENSUS SEQUENCE

The DNA-binding domains of CtrA (9-mer TTAA-N7-TTAAC (P1) and 8-mer TTAACCAT (P2)) are well described in *C. crescentus* (140). In order to demonstrate the binding of *E. chaffeensis* CtrA to the predicted motif, two oligonucleotide probes were designed with the consensus sequences. An EMSA was performed using labeled (biotinylated) oligonucleotides and recombinant CtrA full protein, N-terminal and C-terminal fragments (Fig. 3.1). Binding and significant shift in migration was observed when P1 and P2 were incubated with full length rCtrA (Fig. 3.2). No shift was observed with CtrA fragments, tag (MBP) or BSA (data not shown).

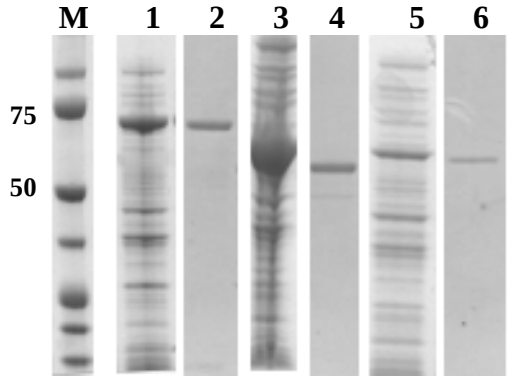

Figure 3.1 Expression of recombinant CtrA. SDS-PAGE and total protein staining of (1) full length CtrA, (3) N-terminal fragment and (5) C-terminal fragment expression. Recombinant proteins (2) full length CtrA (72 kDa), (4) N-terminus (55 kDa) and (6) C-terminus (59kDa) with the MBP tag (42 kDa) were purified from lysates using an amylose resin.

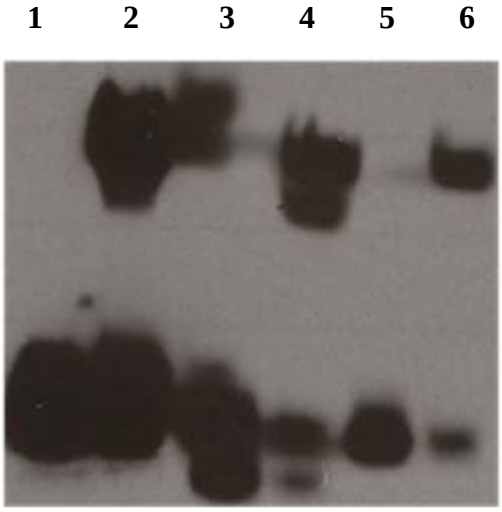

Figure 3.2 ***E. chaffeensis*** **CtrA binds to conserved consensus DNA binding motif.** EMSA with biotinylated oligonucleotides, P1 (TATTAAATGCTATTTAACTC) and P2 (ATATTAACCATGATTGTATA) in the absence (lanes 3 and 5) and presence of full length recombinant CtrA (lanes 4 and 6). Control probe (lane 1) with nuclear lysates (lane2).

TRP GENE EXPRESSION IS REGULATED BY TCSs

The histidine kinase inhibitor, closantel (143,144) was previously shown to inhibit autokinase activity of *E. chaffeensis* histidine kinases (32). Inhibition of the histidine kinases inhibited *E. chaffeensis* infection and downregulated the transcription of TCS genes and genes regulated by TCSs (32). Treatment of *E. chaffeensis* infected cells with closantel resulted in the reduction of mRNA levels of response regulator CtrA; transcript levels were normalized with 16S rRNA levels (Fig. 3.3). Additionally, a reduction in transcript levels for TRP32, 47 and 120 began to decrease after 15 minutes of closantel treatment. A reduction in transcripts was also observed after closantel treatment for FtsK and FtsZ (Fig. 3.3), genes regulated by CtrA in *C. crescentus* (145). A decrease in

transcript levels was observed as early as 15 minutes post-treatment and the continued to decrease over the time course. These results suggest that transcription of TRP genes is regulated by TCSs.

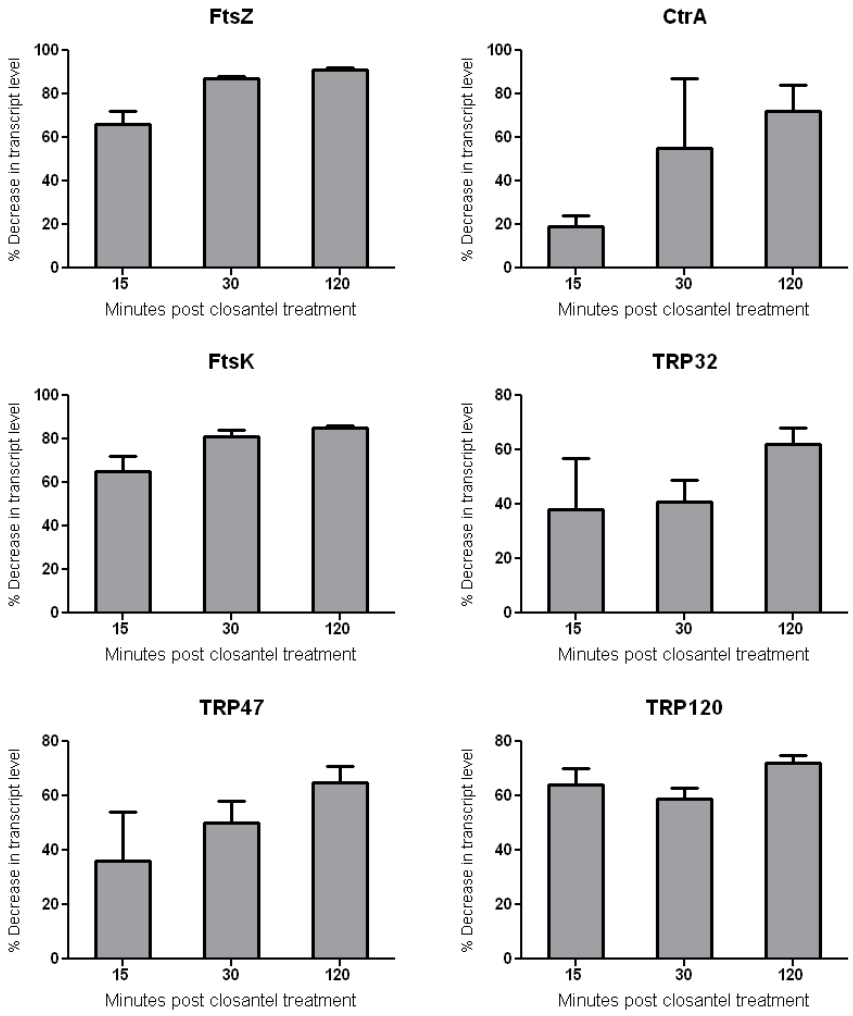

Figure 3.3 Closantel downregulates the transcription of TRPs. *E. chaffeensis* infected THP-1 cells were treated with closantel (100µM) for 15, 30 or 120 minutes or with DMSO (1% v/v) as control. The level of gene expression for each of the genes was determined by RT-PCR. The results are expressed as % decrease in transcript level in closantel treated cells compared to DMSO treated. Transcript levels were normalized to 16S rRNA expression.

Table 3.1 Genes with *E. chaffeensis* CtrA binding motif in the upstream intergenic region.

Consensus Sequence	Product Name
TTAACCAT	Glutamate--cysteine ligase
TTAACCAT	Type IV secretion system protein VirB8
TTAACCAT	Propionyl-CoA carboxylase, beta subunit
TTAACCAT	Hypothetical protein
TTAACCAT	Sua5/YciO/YrdC/YwlC family protein translation factor
TTAACCAT	Hypothetical protein
TTAACCAT	Succinate dehydrogenase, cytochrome b556 subunit
TTAACCAT	Hypothetical protein
TTAACCAT	Hypothetical protein
TTAATGATACATTAAC	Rrf2/aminotransferase, class V family protein
TTAATATAATTTTAAC	Hypothetical protein
TTAATCTTTCTTTAAC	Peptide chain release factor 2
TTAATTATCTTTTAAC	Hypothetical protein
TTAAGATTACTTTAAC	Hypothetical protein
TTAAAAAACCATTAAC	Putative bolA protein
TTAAATGCTATTTAAC	Protein-export membrane protein SecD

Discussion

E. chaffeensis genes are differentially expressed; however, how these genes are regulated is not known. Bacterial TCSs have been shown to regulate gene expression in response to environmental cues. Three pairs of TCSs have been identified in *E. chaffeensis*, PleC-PleD, NtrY-NtrX and CckA-CtrA (31). Recently, the PleC-PleD system was characterized and was shown to have diguanylate cyclase activity that produces c-di-GMP, which regulates the internalization of *E. chaffeensis* (33). However, the other two TCSs have not been characterized, and their role in *E. chaffeensis* infection is unknown.

CtrA is a well characterized transcriptional regulator in several α-proteobacteria species, and a conserved binding motif has been identified (141). It is the master cell cycle regulator in *C. crescentus* and an essential response regulator that directly activates the expression of about 70 genes and also regulates DNA replication by binding to and silencing the origin of replication (141). Progression through the *C. crescentus* cell cycle requires precise and controlled activity of CtrA. The phosporylation of the *C. crescentus* CtrA is not only dependent on CckA, but also other two component histidine kinases (146). Recently, CckA was reported to switch between a kinase state and a phosphatase state to help drive the changes in CtrA's activity that is crucial for proper cell cycle progression (147).

In this study, we identified the binding motif of *E. chaffeensis* CtrA. Several *E. chaffeensis* genes have been identified with this conserved consensus binding sequence in their upstream intergenic region (Table 3.1). Although homologs of CtrA have been identified in several α-proteobacteria, little is known about the function of these proteins. The N-terminal receiver domains with the aspartate residue and the C-terminal DNA binding domains are highly conserved (141). In this study, we demonstrate that the full protein of *E. chaffeensis* binds to DNA; however, the C-terminus fragment alone did not bind DNA, suggesting that the N-terminus fragment we used also had part of the DNA binding domain. The functional similarity between the *C. crescentus* CtrA and the *E. chaffeensis* protein can be studied using a *C. crescentus* CtrA mutant complemented with the *E. chaffeensis* CtrA.

The histidine kinase inhibitor, closantel, has been previously used to identify genes regulated by TCSs. FtsK and FtsZ are conserved in all members of the order *Rickettsiales* and were shown to be regulated by CtrA in *C. crescentus* (145). In a previous study, transcripts for *E. chaffeensis* CtrA, FtsK and FtsZ were reported to be downregulated in the presence of closantel. In this study, with closantel, we have demonstrated downregulation of transcripts of TRPs in the presence of closantel. Although this suggests regulation of TRPs gene expression by TCSs, it is not known which of the three TCs of *E. chaffeensis* is involved in the regulation.

The NtrB-NtrC system regulates genes involved in nitrogen fixation and metabolism, and its role in non-nitrogen fixing bacteria is not known (148). Therefore, the CckA-CtrA or the PleC-PleD is probably involved in the regulation of the TRPs. The surface expression of TRP120 has been previously shown to be regulated by c-di-GMP through the response regulator PleD (33). However, further studies need to be done to determine the effect of c-di-GMP antagonist on the expression of TRP47 and TRP32. The conserved binding sequences of CtrA were not identified in the 200 bp upstream of the start sites of the TRP genes. Further analysis of the upstream regulatory, exons, and downstream locations should be done to identify the CtrA binding motif as these regions can also influence transcriptional regulation (149).

Chapter 4

Molecular Basis of Antibody Mediated Immunity against *Ehrlichia chaffeensis* Involves Species-Specific Linear Epitopes in Tandem Repeat Proteins[2]

Introduction

Major immunoreactive proteins of *Ehrlichia chaffeensis* and *E. canis* include a small subset of proteins strongly recognized by antibody (39). Humoral immunity is essential for protection against ehrlichial infection, and antibodies against a linear epitope in one of the four hypervariable regions of the major outer membrane protein (OMP-1g) have been shown to provide protection against *E. chaffeensis* infection (47,103). Fc receptors have been identified as one of the mechanisms whereby antibodies mediate immunity to *E. chaffeensis* (103). However, the role of other major immunoreactive proteins in immunity to *E. chaffeensis* is unknown. The majority of the major immunoreactive proteins of *E. chaffeensis* and *E. canis* have been molecularly characterized, and many of these proteins contain tandem repeats. Molecularly defined tandem repeat protein (TRP) orthologs in *E. chaffeensis* and *E. canis* include TRP120/TRP140, TRP75/TRP95, TRP47/TRP36, and TRP32/TRP19 (65,66,68-70,150). Several TRP ortholog pairs have similar characteristics including the fact that they are secreted, are serine/threonine-rich and highly acidic, and have a major molecularly

[2] Submitted to *Infection and Immunity*, September 2011, with minor modifications.

distinct continuous antibody epitope (~20 amino acids) located within the tandem repeat regions (65,66,70).

The role of TRPs in pathogenesis is emerging, and it is well established that TRP47 and TRP120 are differentially expressed on the surface of dense-cored (infectious) ehrlichiae and TRP32 is extracellularly associated with the morular fibrillar matrix and the morula membrane and is expressed on both dense cored and reticulate cells (65-67). Additionally, TRP47 and TRP32 mRNA transcripts are hyper-expressed during infection of the macrophage (151). Recent studies have also demonstrated that *E. chaffeensis* TRPs are secreted effector proteins that interact with many host cell targets (74,76,152). Molecular interactions recently reported between host cell proteins and TRP47 and TRP120 include targets associated with distinct cellular functions including signaling, transcriptional regulation, vesicle trafficking and cellular proliferation and differentiation (74,152). The TRP120 is also involved in the binding and internalization of *E. chaffeensis*, and its expression is regulated by the second messenger cyclic di-GMP and protease HtrA (33,78). Moreover, the TRP120 is a DNA binding protein with transcriptional activator function that targets host genes associated with biological processes known to be altered during *E. chaffeensis* infection (153). Molecular host pathogen interactions between TRP32 and host targets associated with TRP47 and TRP120 have also been recently described (76).

Antibodies against a number of intracellular pathogens have been shown to mediate protection (99). Recently, protection against intracellular bacteria, *Mycobacteria*

and *Legionella*, was demonstrated by antibody mediated Fc receptor (FcR) signaling for lysosomal targeting (154). Prophylactic administration of immune serum or purified antibodies has been shown to reduce the severity and duration of disease caused by *Francisella tularensis* and *Mycobacterium tuberculosis* (96,102), and antibodies are required for protection, but not clearance of, *Salmonella typhimurium* (98). Antibodies against outer membrane proteins of *Rickettsia conorii* and secreted *Listeria monocytogenes* listeriolysin O (LLO) are also protective during infection (97,101), and in the case of *Listeria*, neutralization of LLO by antibodies occurs intracellularly (97).

Studies to determine protection provided by major immunoreactive proteins and experimental vaccine development for *Ehrlichia* spp. have focused primarily on the OMP family. The objective of this investigation was to determine the roles of major immunoreactive proteins TRP 32, TRP47 and TRP120 in immunity to *Ehrlichia*. In the present study, we demonstrate that antibodies directed at major linear epitopes of three secreted *E. chaffeensis* TRPs are protective through extracellular and intracellular antibody mediated mechanisms. In addition, we identified IgG1 as the predominant TRP epitope-specific antibody isotype in protective anti-TRP sera. Furthermore, an *in vitro* model was developed to test the immune protection stimulated by the major antibody epitopes of the TRPs that correlated with protection in the animal model.

Materials and Methods

ANTISERA AND ANTIBODY PURIFICATION

Mouse and rabbit (TRP32, TRP47, TRP120 and control) antisera were generated against the synthetic keyhole limpet hemocyanin-conjugated peptides located in the epitope-containing tandem repeat regions by a commercial vendor (Bio-Synthesis, Lewisville, TX). IgG was purified from the rabbit TRP32, TRP47, TRP120, control and pre-immune antisera using Melon Gel IgG purification kit (Thermo Fisher, Rockford, IL). A convalescent-phase anti-*E. chaffeensis* dog serum was derived from an experimentally infected dog (no. 2251).

E. CHAFFEENSIS AND CELL CULTURE

E. chaffeensis (Arkansas strain) was propagated in DH82 cells in DMEM as previously described (155). Cells were prophylactically treated with purified IgG (0.5µg/ml) for 2 hours prior to infection or therapeutically 2 and 24 hours post-infection in serum-free medium (1µg/ml). Host cell-free *E. chaffeensis* was purified by sonication and differential centrifugation. Briefly, infected DH82 cells were harvested and pelleted (5000 x g) for 15 min. The pellet was resuspended in sterile PBS and sonicated twice for 10 s on ice at 40W using an Ultrasonic Processor (Sonics, Newtown, CT). The suspension was centrifuged for 10 min (1500 x *g*) to pellet the cell debris. The supernatant containing the host cell-free ehrlichiae was pelleted for 15 min (10,000 x *g*). Monolayers of DH82 cells in serum-free medium were prophylactically treated with purified IgG (0.5µg/ml) for 2 hours prior to infection with host cell-free *E. chaffeensis.*

The recombinant TRP32 (rTRP32) and the TR domain (first two repeats) of TRP120 (rTRP120) were expressed and purified as previously described (65,156). In the competition experiments, recombinant proteins (1µg/ml) were added in addition to TRP IgG. The therapeutic effects of the anti-TRP IgG were tested by adding the IgG after internalization of *E. chaffeensis*. Cells were washed three times with sterile phosphate-buffered saline to remove extracellular *E. chaffeensis* and were treated with IgG (0.5µg/ml) 2 and 24 hours post-infection. Three control groups were used including IgG purified from sera generated against a control peptide, pre-immune sera and PBS. Monolayers were infected with same amount of host cell-free *E. chaffeensis* and were harvested 1, 2, and 3 days post infection.

WESTERN IMMUNOBLOTTING

E. chaffeensis whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and western immunoblotting was performed as previously described (39). The dog anti-*E. chaffeensis* serum was diluted 1:100, and mouse anti-TRP sera were diluted 1:500 (Fig. 1). Bound primary antibodies were detected with alkaline phosphatase-conjugated anti-rabbit IgG (H+L) secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and visualized after incubation with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium) substrate.

MICE AND INFECTION

C57BL/6-*scid* (B6.CB17-*Prkdc*^{scid}) and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed and cared for in the Animal Research

Center at the University of Texas Medical Branch in accordance with the Institutional Animal Care and Use Committee guidelines under whose review and approval the experiments were conducted. Six to 12-week-old, sex-matched mice were infected intraperitoneally with approximately 2×10^6 infected DH82 cells (>95% infected) as previously described (82). Immune serum was produced in C57BL/6 mice as previously described (103). Mice were intraperitoneally administered mouse anti-TRP (individually or in combination), *E. chaffeensis*-immune, or control sera (0.1 ml) on days 7 and 17 post-infection for the C57BL/6-*scid* mice and on day 3 for the C57BL/6 mice. Mice were sacrificed 21 days (C57BL/6-*scid*) or 5 days (C57BL/6) post-infection and the liver and spleen were collected. The control groups were administered sera generated against a control peptide, pre-immune sera or PBS. Three mice were used per group, and each experiment was repeated at least three times.

QUANTIFICATION OF *E. CHAFFEENSIS*

Total DNA was extracted from infected DH82 cells or homogenized mouse liver with DNeasy Blood and Tissue Kit (Qiagen). Recombinant plasmids were constructed by cloning the *E. chaffeensis dsb* gene using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The size of the plasmid (with the insert) was determined, and the concentration of the double-stranded DNA was calculated with a NanoDrop ND-1000 spectrometer (Wilmington, DE). The plasmid copy number for the standards was calculated using the following formula: plasmid copy/µl = [(plasmid concentration g/µl) / (plasmid length in basepairs x 660)] x 6.022 x 10^{23}. *E. chaffeensis* was amplified with

primers Dsb-321 forward, 5′ TTG CAA AAT GAT GTC TGA AGA TAT GAA ACA 3′, and Dsb-671 reverse, 5′ GCT GCT CCA CCA ATA AAT GTA TCT CCT A 3′ (157). The thermal cycling protocol consisted of an initial denaturation step of 95°C for 2 min and 40 cycles of 95° for 10 s, 55°C for 30 s, and 65°C for 30 s. The absolute *E. chaffeensis dsb* copy number in the cells (*in vitro*) and liver was determined by real-time qPCR and plotted against the standard curve.

ELISA

Plates (MaxiSorp; NUNC, Roskilde, Denmark) were coated with synthetic peptides (1 μg/well; 50 μl) suspended in phosphate-buffered saline (pH 7.4) as previously described (66). Plates were blocked with 100 μl of 10% equine serum (Sigma) in TBST for 1 h at room temperature with agitation and washed. Mouse immune sera or anti-TRP sera (1:100 or 1:6400) diluted in 5% equine serum-TBST were added to each well (50 μl) and incubated at room temperature for 1 h with gentle agitation. The plates were washed four times and incubated in 50 μl of rat anti-mouse IgG1, IgG2a, IgG2b or IgG3 (BD Biosciences, Sparks, MD) (1:1000) for 1 h at room temperature. The plates were washed and incubated in 50 μl of horseradish peroxidase-labeled goat anti-rat IgG antibody (Southern Biotech, Birmingham, AL) (1:2000) for 1 h at room temperature. Substrate (100 μl, TMB Liquid Substrate System, Sigma) was added to each well, and plates were incubated in the dark for 30 min with agitation. Optical density was determined on a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA) at A_{650} and data analyzed by SoftmaxPro v4.0 (Molecular Devices). Optical density (OD) readings

represent the mean OD for three wells (± standard deviations) after subtracting the OD value of the negative peptide control.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviations (SD), and the statistical significance was determined using a one-way analysis of variance followed by Tukey's multiple comparison tests were performed using Prism 5 software (GraphPad). *P* values of <0.05 were considered significant.

Results

ANTIBODIES AGAINST TRP32, TRP47 and TRP120

Major immunoreactive epitopes of tandem repeat proteins 32, 47, 120 have been previously determined (65,66,70). Mouse and rabbit antisera against these epitopes were generated and tested to confirm recognition of the ehrlichial proteins (Fig. 4.1, Table 4.1).

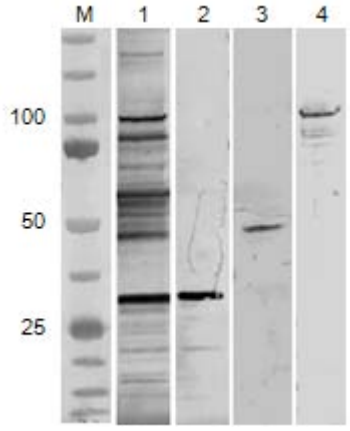

Figure 4.1 Identification of immunodominant *E. chaffeensis* TRPs with epitope-specific mouse antisera used for passive protection experiments. Western immunoblot of whole-cell lysate from *E. chaffeensis*-infected DH82 cells probed with anti-*E. chaffeensis* dog (no. 2251) serum (lane 1; positive control) and, mouse anti-TRP32 (lane 2), anti-TRP47 (lane 3) and anti-TRP120 (lane 4) sera. M, molecular mass marker (kilodaltons).

Table 4.1 TRP antibody titers in mouse and rabbit sera as determined by three immunoassays.

Sera	IFA		ELISA		Western Blotting	
	Mouse	**Rabbit**	**Mouse**	**Rabbit**	**Mouse**	**Rabbit**
Anti-TRP32	1:1000	1:500	1:6400	1:25600	nd	1:5000
Anti-TRP47	1:100	1:100	1: 12800	1:512000	nd	1:100
Anti-TRP120	1:1000	1:1000	1:12800	1: 6400	nd	1:1000
Immune Sera	1:1000		nd		nd	

nd, not determined.

PROPHYLACTIC PROTECTION BY TRP32 AND TRP120 ANTIBODY IN VITRO

To determine the effect of antibodies prior to ehrlichial entry, DH82 monolayers were treated with purified rabbit anti-TRP32 and anti-TRP120 IgG prior to infection. A significantly ($p < 0.05$) lower ehrlichial load was observed in anti-TRP32 and anti-TRP120 IgG treated cells compared to the controls over a period of three days (Fig. 4.2A). However, there was no difference observed with anti-TRP47 IgG. The reduction in bacterial load observed with anti-TRP32 and anti-TRP120 IgG was completely reversed by addition of recombinant TRP32 or TRP120 as a competitor (Fig. 4.2B). No significant difference in bacterial copy number was observed in the control peptide IgG, pre-immune IgG or PBS treated cells; thus, pre-immune sera or PBS were used in all subsequent experiments.

THERAPEUTIC PROTECTION OF TRP32 AND TRP120 ANTIBODY IN VITRO

To determine the effect of antibodies post ehrlichial entry, infected DH82 cells were treated with TRP32, TRP120 or control IgG 2 and 24 hrs post-infection. A significant ($p < 0.05$) reduction in bacterial load was observed when TRP120 IgG was added 2 hrs post-infection and TRP32 IgG was added 24 hrs post-infection (Fig. 4.2C).

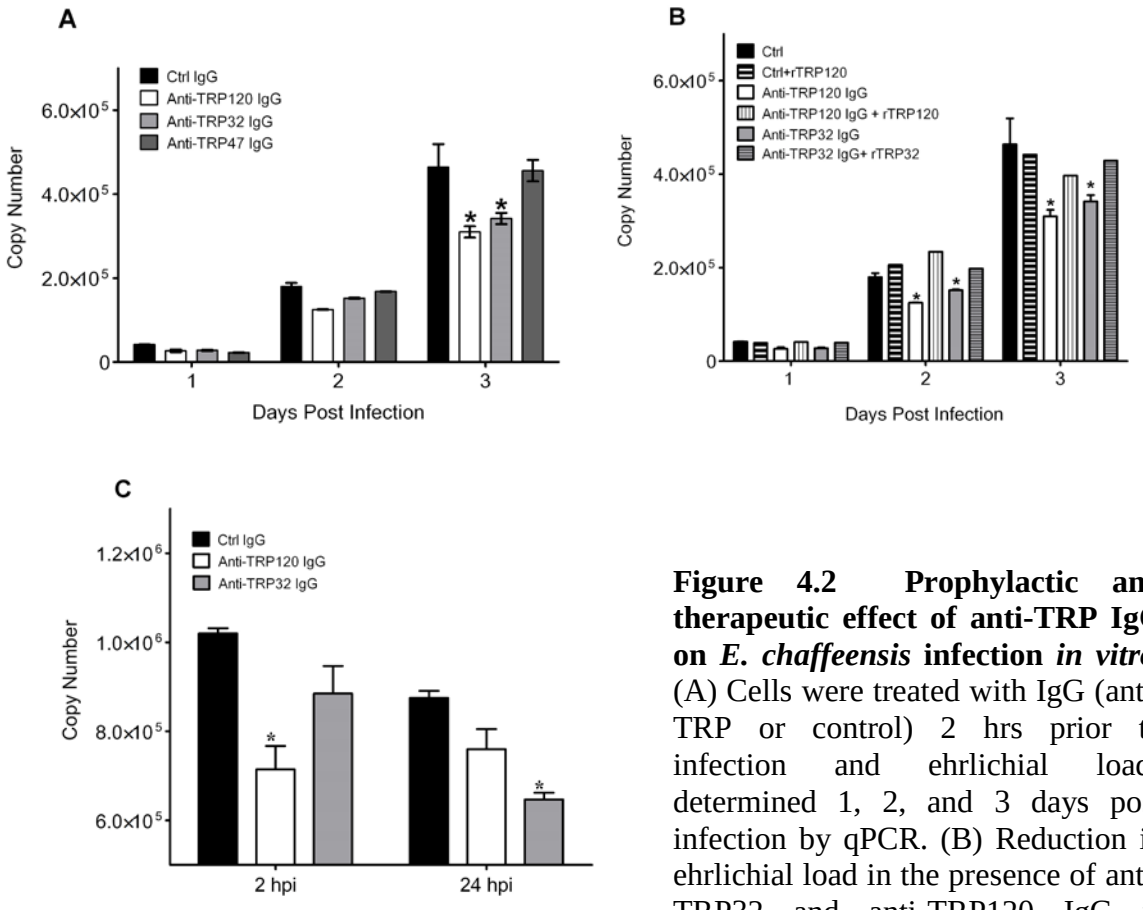

Figure 4.2 Prophylactic and therapeutic effect of anti-TRP IgG on *E. chaffeensis* infection *in vitro*. (A) Cells were treated with IgG (anti-TRP or control) 2 hrs prior to infection and ehrlichial loads determined 1, 2, and 3 days post infection by qPCR. (B) Reduction in ehrlichial load in the presence of anti-TRP32 and anti-TRP120 IgG is protein-specific. Cells were incubated with anti-TRP IgG alone or in combination with recombinant protein (rTRP32 and rTRP120) to neutralize the effect of specific antibody for 2 hrs prior to infection and ehrlichial loads determined by qPCR. (C) Infected cells were treated with IgG (anti-TRP or control) 2 and 24 hrs post-infection, and ehrlichial load determined 3 dpi by qPCR. Bar graphs represent means ± SD, (*p <0.05).

PASSIVE PROTECTION OF TRP ANTIBODIES INDIVIDUALLY IN VIVO

To determine the *in vivo* protection of anti-TRP sera, we used a previously developed murine model of *E. chaffeensis* infection (82). In several initial experiments, no significant differences were observed in control peptide sera, pre-immune sera or PBS-treated mice (data not shown); therefore, in subsequent experiments, pre-immune sera or PBS were used as controls. Immunocompetent C57BL/6 mice develop a transient infection and inflammation but clear ehrlichiae within two weeks, and immunocompromised SCID mice, lacking T and B lymphocytes develop persistent infection and disease and become moribund within three weeks after infection (82). Immunocompetent C57BL/6 mice and susceptible SCID mice were infected with *E. chaffeensis* and were treated with anti-TRP32, TRP47 or TRP120 sera to test their protective capacity. Ehrlichial burden in the liver, one of two principal sites of ehrlichial infection in mice (82), was determined by qPCR. Since *E. chaffeensis*-infected mice exhibit pronounced splenomegaly, the spleen weights of the anti-TRP sera-treated mice were compared to controls.

SCID mice were infected and treated with sera on days 7 and 17 post-infection, and the livers and spleens were harvested 21 days post-infection. A significant ($p < 0.005$) reduction in ehrlichial copy number was observed when TRP32, TRP47 or TRP120 antisera were administered to SCID mice during the course of the infection (Fig. 4.3A). Furthermore, anti-TRP antibody treated mice had significantly ($p < 0.05$) lower spleen weights than the controls (Fig. 4.3B).

To examine the anti-TRP protection in immunocompetent mice, C57BL/6 mice were infected and treated with TRP32, TRP47 or TRP120 antisera three days post infection, and organs were harvested five days post-infection. Similar to the SCID mice, significant reductions in the *E. chaffeensis* burden ($p < 0.005$) and spleen weights ($p < 0.05$) were observed in the immunocompetent mice compared to controls (Fig. 4.3C and D).

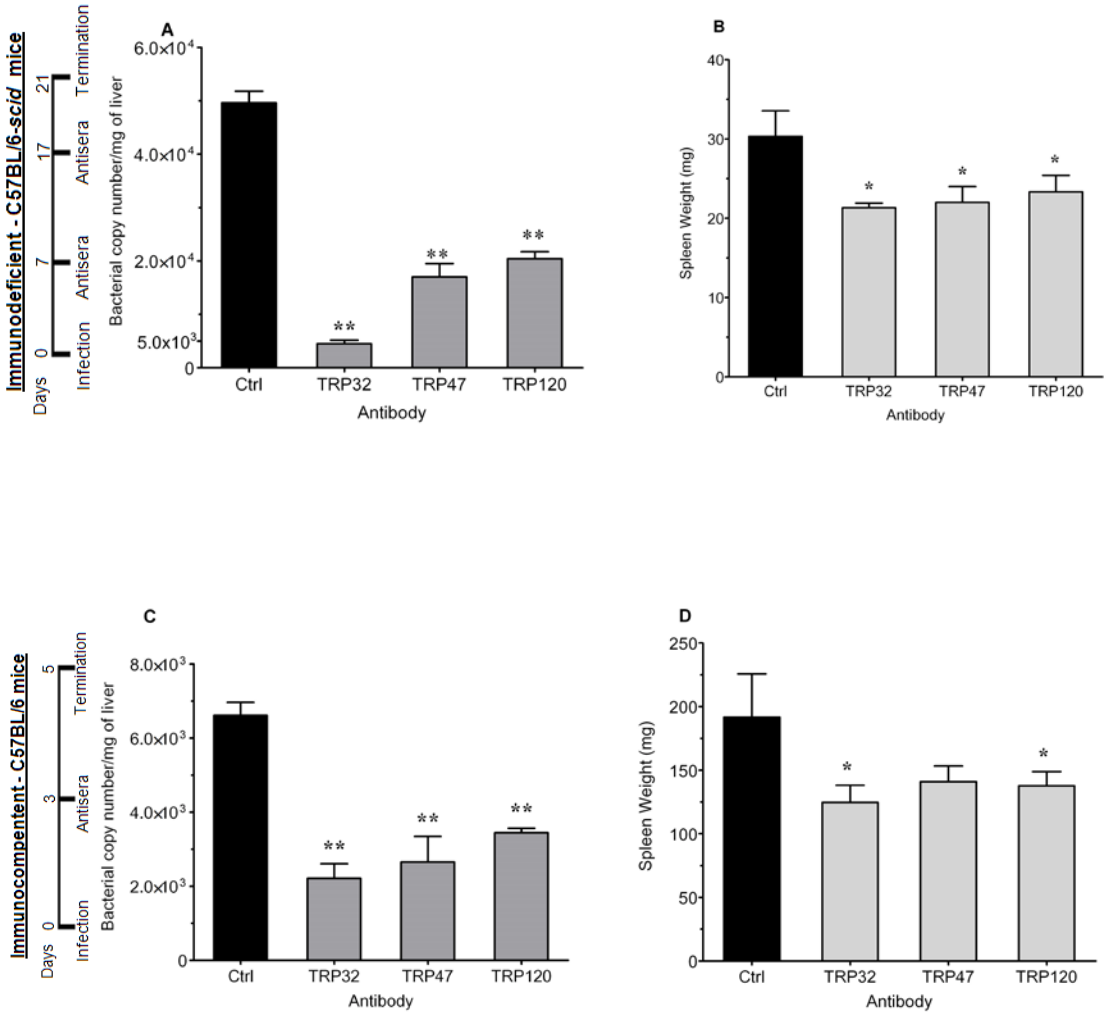

Figure 4.3 Reduction of ehrlichial burden in C57BL/6-*scid* and C57BL/6 mice treated with anti-TRP32, anti-TRP47 or anti-TRP120 sera. Mice (SCID and immunocompetent) were inoculated intraperitoneally with 2×10^6 *E. chaffeensis*-infected DH82 cells on day 0. SCID mice were administered anti-TRP32, anti-TRP47, anti-TRP120 or control sera on days 7, 17 and were sacrificed 21 days post-infection. Immunocompetent C57BL/6 mice were administered anti-TRP32, anti-TRP47, anti-TRP120 or control sera on day 3 and sacrificed 5 days post-infection. *E. chaffeensis dsb* copy number in the liver was determined by qPCR (A and C) and spleen weights (B and D) were compared to controls. Three mice were used per group and data are representative of one of three independent experiments. Graphs represent means ± SD, (**p* <0.05, ***p* <0.005).

PASSIVE PROTECTION WITH TRP ANTIBODIES AND *E. CHAFFEENSIS* IMMUNE SERA

In a previous study, significant protective effect was observed in SCID mice that received immune sera from immunocompetent mice during *E. chaffeensis* infection (103). To compare the protection mediated by antibodies directed at TRP epitopes compared to the protection previously observed with immune sera, immunocompetent and SCID mice were treated with a combination of anti- TRP32, TRP47 and TRP120 sera or *E. chaffeensis*-immune sera. SCID mice treated with the combination of TRP antisera and immune sera had a significantly ($p < 0.005$) lower ehrlichial burdens compared to the control group (Fig. 4.4A). Furthermore, the mice that received *E. chaffeensis* immune sera had fewer than 10 ehrlichiae/250 ng of total DNA. The spleen weights of mice that received immune sera were similar to the mice treated with the combination of anti-TRP sera, and both groups' spleen weights were significantly ($p < 0.05$) lower than the control group (Fig. 4.4B). In the immunocompetent mice, immune sera-treated mice and the mice treated with the combination of TRP antisera had significantly lower ehrlichial loads ($p < 0.005$) than controls. Most notable were the similar bacterial loads and spleen weights observed in mice treated with immune sera and combination of TRP antisera (Fig. 4.4C and D).

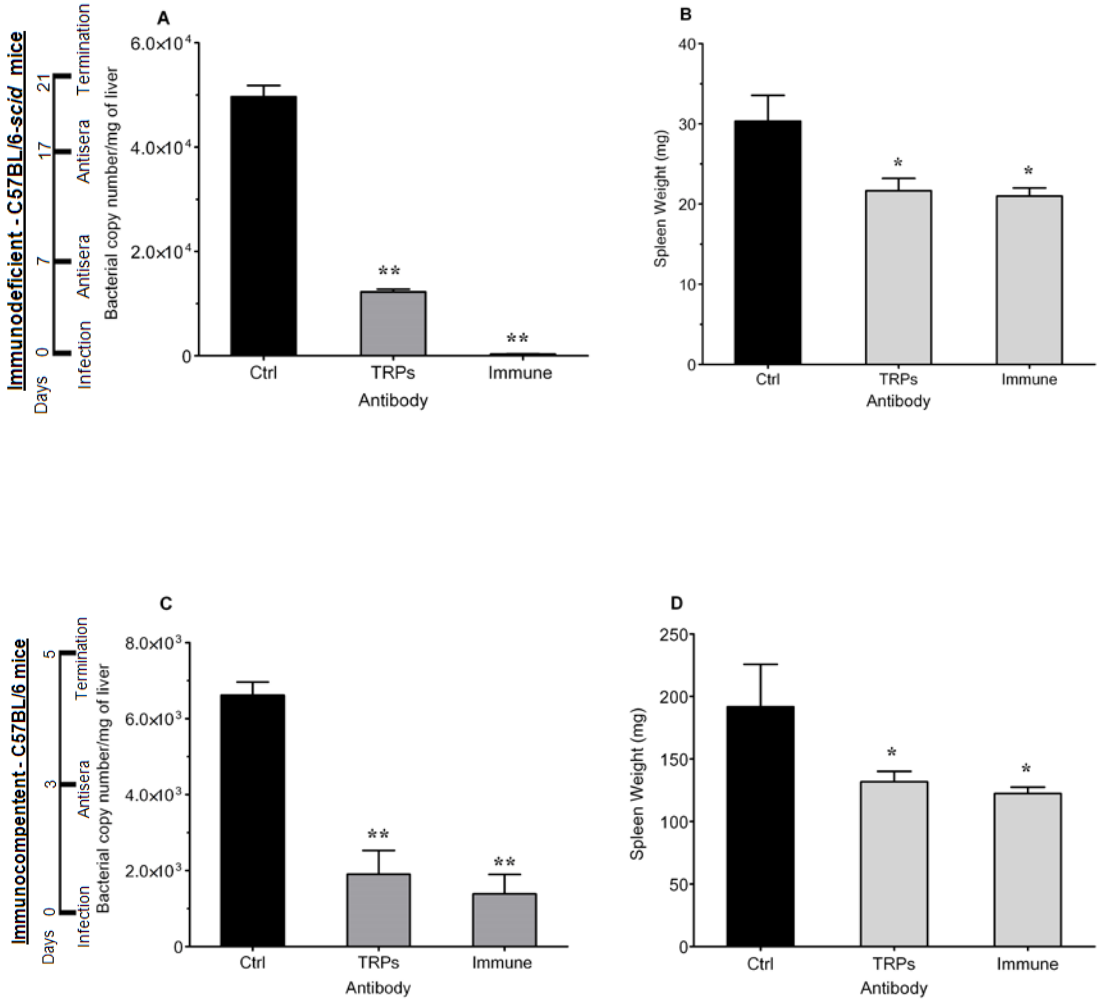

Figure 4.4 Reduction of ehrlichial burden in C57BL/6-*scid* and C57BL/6 mice after treatment with a combination of anti-TRP sera compared to immune sera. Mice were infected intraperitoneally with 2×10^6 *E. chaffeensis* infected DH82 cells on day 0. SCID mice were administered a combination of anti-TRP32, anti-TRP47 and anti-TRP120 sera (TRPs), immune or control sera on days 7, 17 and were sacrificed 21 days post-infection. Immunocompetent C57BL/6 mice were administered a combination of anti-TRP32, anti-TRP47 and anti-TRP120 sera (TRPs), immune or control sera on day 3 and sacrificed 5 days post-infection. *E. chaffeensis dsb* copy number in the liver was determined by quantitative real-time PCR (A and C) and spleen weights (B and D) were compared to control mice. Three mice were used per group, and data are representative of one of three independent experiments. Graphs represent means ± SD, (**p* <0.05, ***p* <0.005).

IgG ISOTYPES IN ANTI-TRP AND IMMUNE SERA

The TRP32, TRP47 and TRP120 epitope-specific IgG isotypes in the *E. chaffeensis*-immune sera were quantified by ELISA (Fig. 4.5A). Previously described protective OMP-1g (p28-19) epitope was also measured for comparison (47). In the *E. chaffeensis*-immune sera, all IgG isotypes (IgG1, 2a, 2b and 3) were detected against the three protective TRP epitopes, with TRP32 having the highest antibody levels of the three TRPs in immune serum obtained from *E. chaffeensis*-infected mice and used in passive transfer experiments. In hyperimmune mouse serum generated against peptides representing TRP epitopes, IgG1 was also the predominant isotype. The concentration the other isotypes (IgG2a, 2b, and 3) were detected in similar concentrations, but lower than IgG1 (Fig. 4.5B).

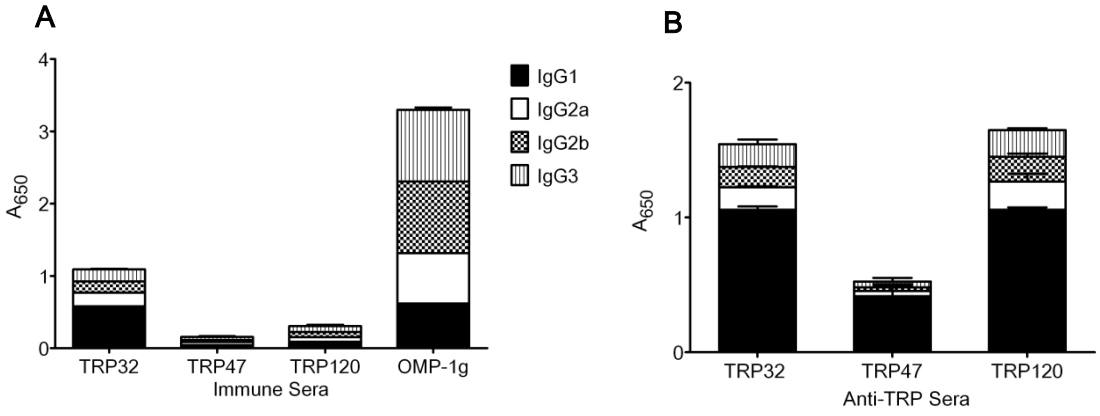

Figure 4.5 Quantification of antibody isotypes detected in *E. chaffeensis*-immune and TRP epitope-specific sera by ELISA. (A) Epitope-specific TRP32, TRP47, TRP120 and OMP-1g IgG isotypes in *E. chaffeensis*-immune sera and (B) mouse anti-TRP32, TRP47 and TRP120 serum.

Discussion

It is well established in that antibodies against the obligately intracellular pathogen, *E. chaffeensis*, are essential for immunity (103,104). However, during *E. chaffeensis* infection, many proteins elicit strong antibody responses, yet only antibodies against a linear epitope in the hypervariable region of OMP-1g protein primarily of the isotype IgG2a have been demonstrated to provide protection (47,103). This antibody mediated protection appears to involve Fc receptor-dependent mechanisms (95). The purpose of this study was to expand our knowledge of immunoprotective components of *Ehrlichia* and have a better understanding of the antibody-mediated immune mechanisms involved. We have previously identified and molecularly characterized a subset of immunodominant ehrlichial proteins that contain tandem repeats and determined that each of these TRPs contains a single major continuous species-specific antibody epitope within the TR region (65-67). In this study, we conclusively demonstrated antibody-mediated protection against *E. chaffeensis* infection by passively transferring TRP epitope-specific antibody against three *E. chaffeensis* TRPs (TRP32, TRP47 and TRP120).

Humoral immunity to bacteria has been associated with proteins localized on the cellular surface such as *B. burgdorferi* (OspA) and *Y. pestis* (PsaA) (158,159). In *A. marginale*, antibodies against the highly immunogenic multifamily major surface proteins (Msps) that are components of the outer membrane provide protection against infection, anemia and high-level bacteremia (160,161). In addition, the alpha C protein of the

group B *Streptococcus* is a surface-expressed TRP that elicits protective antibody-mediated immunity (51). Monoclonal antibodies to the alpha C protein induce opsonic killing of the bacterium and protect mice from lethal challenge, and altered tandem repeat numbers are associated with immune evasion (52,53,162). However, unlike the MSPs of *A. marginale*, many of the major immunoreactive proteins of *E. chaffeensis* are secreted TRPs that are involved in molecular interactions with the host cell. Secreted effector proteins of several pathogens have been shown to be immunogenic, such as the recently described *Chlamydia trachomatis* Tarp protein or LLO from *Listeria monocytogenes* (97,163). Although the *C. trachomatis* Tarp is a secreted TRP effector that is strongly recognized by antibodies in immune patient sera, its role in inducing protective antibodies is unknown (163,164). In contrast, antibodies against the secreted effector LLO have been demonstrated to provide protection (97).

In this investigation, the effect of *E. chaffeensis* TRPs linear epitope specific antibodies during infection was determined using an *in vitro* model which was validated and supported using immunocompetent and SCID murine models. In the *in vitro* model, anti-TRP120 IgG was protective when administered prophylactically and therapeutically at two hours post-infection, but not at 24 hours post infection. TRP120 is differentially expressed on the infectious dense-cored ehrlichiae and has been previously associated with ehrlichial binding and internalization (33,78). Moreover, it has recently been identified as a DNA binding protein and interacts with other defined host cell targets (152,153). The prophylactic efficiency of the anti-TRP120 IgG during the early stages of infection in the *in vitro* model when the antibody is in direct contact with the host cell-

free ehrlichiae suggests that the antibody is involved in neutralization or other extracellular antibody mediated mechanism. Recently, antibodies were shown to protect by altering the intracellular trafficking of *Legionella pneumophila* into lysosomal compartments via Fc receptor (FcR) mediated signaling in macrophages (165). Antibody mediated activation of the FcR blocks the functions of bacterial effector proteins secreted into the host cell cytoplasm which include avoiding fusion to the lysosome, making the host cell nonpermissive for intracellular replication, and this was observed both *in vitro* and *in vivo* (165). During ehrlichial infection of immunocompetent mice, FcR common γ chain and FcγRI have been previously demonstrated to be required for clearance of bacteria (95), consistent with the FcR mediated clearance of *L. pneumophila*.

A reduction in bacterial burden was observed when anti-TRP32 IgG was administered prophylactically and therapeutically at 24 hours post-infection, but not two hours post-infection. TRP32 is expressed on both dense-cored and replicating reticulate cells and is associated with morular fibrils and membrane (65). TRP32 was recently identified as a secreted ehrlichial effector protein that interacts with several host cell proteins involved in protein synthesis, iron acquisition, immune signaling and transcriptional regulation (76). The protection mediated by therapeutically administered anti-TRP32 IgG also likely involves extracellular neutralization, since both TRP32 and TRP120 are found on the surface of DC ehrlichiae. However, the therapeutic protection mediated by anti-TRP32 and anti-TRP120 antibodies two and 24 hours post-infection, after ehrlichiae have entered the host cell, suggests that intracellular antibody-mediated immune mechanisms are involved. In this case, the TRPs and associated functions may

be neutralized by cytoplasmic antibodies that block the effector functions of these proteins at different stages of infection. Antibody directly transfected into cells blocked interactions of type IV secreted effector protein, AnkA, resulting in reduction of the *A. phagocytophilum* burden (166). Moreover, antibodies against LLO, a secreted effector protein of *L. monocytogenes* provided protection through intracellular mechanisms post-internalization of the bacterium (97). Additionally, monoclonal antibodies against intracellular oncoproteins have been shown to enter host cells and inhibit tumor cell growth (167). Cytosolic IgG receptors such as the recently described TRIM21 could be involved in the intracellular antibody mediated immunity (168). TRIM21 is expressed on all cell types, binds all IgG isotypes, and mediates intracellular immunity by recruiting antibody-bound antigen for proteasomal degradation (169).

In the *in vivo* mouse model, the three TRP antisera were protective when therapeutically administered individually and in combination to immunocompetent and SCID mice after establishment of infection. In the SCID mice, anti-TRP32 was the most protective of the three TRP antisera. Interestingly, in the immunocompetent mice, when treated with the combination of the three antisera, mice had similar correlates of protection as immune sera treated mice. Similarly, Winslow *et.* al. previously reported that extremely low copy numbers of bacterial DNA was detected in immune sera-treated immunocompetent mice four and seven days post-infection (103). These results suggest that the TRP epitopes contribute to a significant fraction of antibody mediated protection in the immune sera in the immunocompetent mice in addition to previously characterized outer membrane protein epitopes.

The *in vivo* studies were supportive of the *in vitro* model data for TRP32 and TRP120 antibodies. The difference in protection mediated by TRP47 antibody *in vitro* and *in vivo* is potentially due to differences in antibody titer (as measured by IFA), or suggestive of a mechanistic deficiency that is reconstituted *in vivo*. Higher concentrations of anti-TRP47 antibody were not tested to determine if *in vitro* protection could be achieved, but based our *in vivo* findings, anti-TRP47 antibodies are protective. Results from the *in vitro* model of antibody protection correlated well with the *in vivo* model of protection, supporting the *in vitro* model as a valid screening alternative for defining immunoprotective proteins. During infection with *A. marginale*, an intracellular bacterium closely related to *E. chaffeensis*, a similar assay was used to demonstrate the inhibition of infection mediated by antibodies against immunogenic major surface proteins (Msp) of *A. marginale* during infection of tick cells (170).

Murine *E. chaffeensis*-specific mononclonal antibodies generated in a previous study were of the IgG2a, 2b and 3 isotypes (47), and protective antibodies identified were directed at the hypervariable region of OMP-1g and were of the IgG2a isotype (104). IgG2a is capable of activating complement and binding to Fc receptors. Thus, consistent with antibody mediated mechanisms associated with bacterial OMPs and protective ehrlichial OMP IgG2a, antibodies have been proposed to opsonize *E. chaffeensis* and involve FcγRI-mediated phagocytosis of antibody opsonized bacteria that either escaped from infected host cells or were released during cell lysis (95,171). In this study, we demonstrated that TRP-specific antibodies provide protection similar to OMP antibodies demonstrating that both TRP and OMP specific antibodies are important for protection.

However, the functional differences in these proteins and the cellular localization (membrane versus secreted) suggest that protective antibodies may confer protection through distinct mechanisms.

In the present study, IgG1 was the predominant isotype detected in the protective TRP-peptide antisera, but other antibody isotypes (IgG2a, 2b, and 3) were also present in substantially lower concentrations. Interestingly, although we detected similar antibody concentrations of all four isotypes in sera from mice infected with *E. chaffeensis*, monoclonal antibodies developed by Winslow *et*. al represented primarily IgG2a and 2b isotypes, and IgG1 monoclonal antibodies were not obtained (104). The predominance of the IgG1 isotype (which does not activate complement or bind Fc receptors) in the murine epitope-specific anti-TRP antisera, suggests that mechanisms other than complement activation and Fc receptor mediated killing could also be involved. Furthermore, higher concentrations of IgG1 antibody in anti-TRP peptide serum and immune serum (TRP32) from *E. chaffeensis*-infected mice suggest that antibodies to TRPs are more likely to be of the IgG1 isotype. Nevertheless, multiple antibody isotypes are likely to play a role in TRP specific antibody mediated immunity through different mechanisms. Characterization of antibody isotypes directed at TRPs and OMPs during *E. chaffeensis* human infections would also help define that antibody isotypes produced during infection and the antibody mediated mechanisms are associated with anti-TRPs and anti-OMP antibodies.

Our findings support the role of antibodies in host defense during ehrlichial infection. In this study we have demonstrated that antibodies against the linear epitopes of immunodominant TRPs (32, 47 and 120) are protective and appear to involve extracellular and intracellular antibody-mediated mechanisms. These epitopes should be included in development of vaccines and therapies for ehrlichial diseases and further studies need to be done to elucidate the mechanism of anti-TRP antibody-mediated protection during *E. chaffeensis* infection.

Chapter 5

Summary and Conclusions

The studies presented in this dissertation highlight the significance of three TRPs (32, 47 and 120) during *E. chaffeensis* infection of the phagocyte (Fig. 5.1). These proteins were initially identified based on their immunodominance in sera. Recent studies, mostly using the yeast two-hybrid system have identified these TRPs as proteins involved in an array of host cell processes that are regulated during ehrlichial infection of macrophages. A microarray experiment was performed to identify *E. chaffeensis* TRP genes expression in arthropod and mammalian hosts. We also compared *E. chaffeensis* arthropod host gene expression in the vector AAE2 cell line and the non-vector, ISE6 cell line, which has been frequently used for studies with *Ehrlichia* and other tick borne pathogens. In this study we demonstrate no significant difference in *E. chaffeensis* gene expression in these cell lines. However, with the availability of the vector cell line combined with the fact that the AAE2 cells grow more rapidly than the ISE6 cells and support robust ehrlichial growth, AAE2 cells are highly recommended for future studies for *E. chaffeensis* in the arthropod vector. Both cell lines were developed from embryos and therefore have a variety of cell types. The cell types that support higher ehrlichial burden have a large number of secretory vacuoles, suggesting they are possibly cells from the salivary gland.

Transcripts for TRPs were detected in both mammalian and arthropod host cells. Additionally we demonstrated that ehrlichial genes are differentially expressed,

especially genes that code for hypothetical proteins. Furthermore, from within the differentially expressed hypothetical genes, we identified a subset of genes that were expressed host-specifically. Many of these genes were bacterial peptides with sizes ranging from 30-70 amino acids in length. These peptides are *E. chaffeensis*-specific and do not have orthologs in other bacterial species. Although the expression of several of peptides has been verified in a previous proteomics study (124), the function of these peptides during ehrlichial infection is not known. Bacterial peptides are molecules that are small, unmodified, gene encoded and secreted by both Gram-positive and Gram-negative bacteria (172). These peptides are most commonly studied in bacterial cell-cell signaling (quorum sensing). However, there are other functions of bacterial peptides such as those that act as bacteriocins, peptides produced by bacteria that have antimicrobial activity and are important in microbial competition (173). Peptides secreted by bacterial species in mucosal surfaces have been shown to act as immunomodulators (174). Although the function of these peptides during *E. chaffeensis* infection is not known, the host induced expression suggests that they are required exclusively for the adaptation and survival within the host. The role of the host-specifically expressed *E. chaffeensis* peptides during infection in the arthropod host cells should be further studied as potential candidates for vaccines that could prevent transmission of the pathogen from the vector host.

Transcripts were not detected for a few hypothetical genes in either host cell. These are possibly genes that are expressed during stages of infection that are not depicted in this study such as during early stages of transmission of the pathogen from

the tick or reactivation of the pathogen after a blood meal or in the presence of tick salivary proteins. Although we did studies to test *E. chaffeensis* gene expression in tick salivary glands, the low yield of ehrlichial RNA purified from pooled salivary glands was a limiting factor. However, with the recent availability of techniques such as deep sequencing, gene transcripts can be quantified from very low amounts (1µg) of total RNA (175).

The post-transcriptional regulation of TRP32 and TRP47 mRNAs in the tick cells suggests that these proteins are essential for survival in the mammalian host and not in the arthropod host, in contrast to TRP120 which is expressed in both hosts. In this study, we also demonstrated regulation of TRP genes by the TCSs. Further studies need to be done to identify which of the three *E. chaffeensis* TCSs are involved in the regulation of these genes. The binding motif of the response regulator, CtrA was identified and the binding of recombinant CtrA to the motif was confirmed. The motif was identified in the upstream region of several *E. chaffeensis* genes. Although the motif was not identified in the upstream regulatory regions of TRPs, further analysis should be done in the exons and the downstream locations as these regions can also influence transcriptional regulation.

Protection mediated by antibodies against the TR epitopes of these immunodominant TRPs also suggests that these proteins play a significant role during infection of the mammalian host. Antibodies against these secreted and surface exposed effector proteins are protective during *E. chaffeensis* infection. In the *in vitro* model,

antibody-mediated protection was observed post-internalization of the ehrlichia into the host cell, suggesting an intracellular mechanism of protection in addition to extracellular neutralization. Previous studies have demonstrated that extracellular Fc receptors are required for the antibody-mediated protection during ehrlichial infection. However, several recent studies have demonstrated the presence of intracellular Fc receptors and functional intracellular antibodies. Further studies are warranted on the antibody mediated protection during ehrlichial infection to identify the mechanisms of immunity.

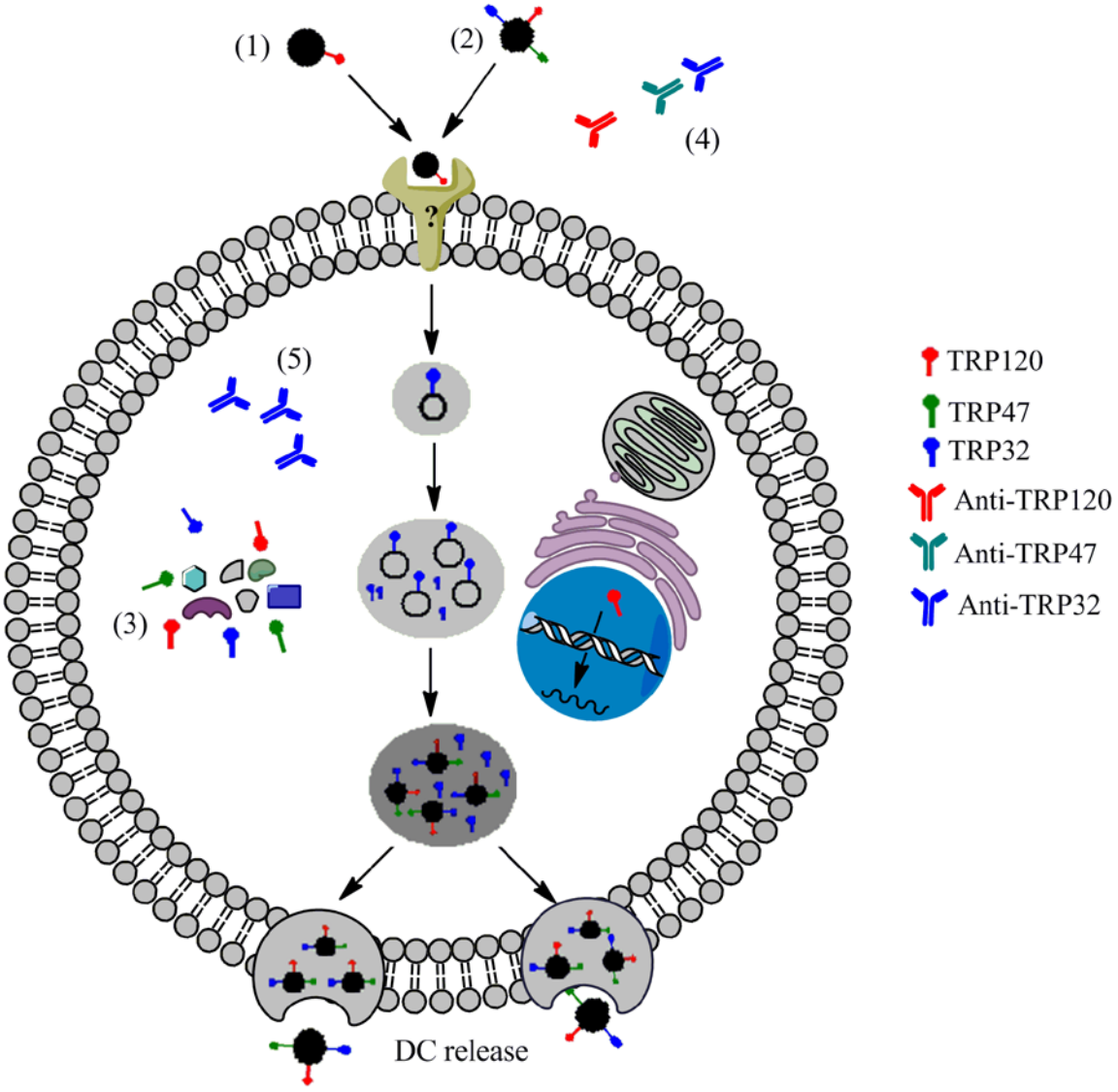

Figure 5.1 Proposed model for the role of *E. chaffeensis* TRPs during infection of the mammalian host cell. (1) DCs from tick cells expressing TRP120 enter the mammalian host cell as opposed to the (2) DCs that have previously been in mammalian cells expressing TRP32, 47 and 120. (3) Within the host-cell, TRPs interact with an array of proteins involved in ehrlichial pathogenesis. Antibodies directed against linear species-specific epitopes of TRPs regulate bacterial burden via extracellular (4) and intracellular (5) antibody-mediated mechanism.

CONCLUDING REMARKS

The present dissertation was aimed at improving the understanding of the functional role of TRPs in *E. chaffeensis* pathobiology. These proteins are post-trancriptionally regulated in the arthropod host and highly expressed in the mammalian host and their expression is regulated by the two-component system. Additionally, antibodies against these proteins are protective during infection. The results therefore support a significant role of TRPs during infection of the mammalian host.

Furthermore, the work presented in this dissertation has identified differential expression of ehrlichial genes essential for infection in the two hosts which includes a subset of bacterial peptides that are host-specific. Although this study and others are starting to identify roles of two-component system response regulators, little is still known about the factors that active the histidine kinases. Future studies should be focused on the analysis of host cell factors that influence differential expression of ehrlichial genes.

Antibodies in immune sera have been previously shown to be protective during infection by this intracellular pathogen; this dissertation work has demonstrated that a combination of the TRP antibodies have a similar level of protection as immune sera. Further studies need to be done to identify the TRP antibody mediated immunity. Collectively, these studies have improved our understanding of the pathogenesis of ehrlichiosis and have identified novel targets and mechanism for development of vaccines and therapies for ehrlichial diseases.

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VITA

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EDUCATION

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PUBLICATIONS

1. Kuriakose JA, Zhang X, Luo T, McBride JW (2011) Molecular basis of antibody mediated immunity against *Ehrlichia chaffeensis* is defined by linear epitopes in tandem repeat proteins. (Manuscript submitted to *Infection and Immunity*).

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ABSTRACTS

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3. Kuriakose JA, Miyashiro S, Luo T, Zhu, B, McBride JW (May 2011) Global transcriptomic analysis of *Ehrlichia chaffeensis* in the mammalian host and arthropod vector. ASM 110th General Meeting. New Orleans, LA.

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7. Luo T, Kuriakose JA, McBride JW (Jul 2010) *Ehrlichia chaffeensis* TRP120 interacts with a diverse group of host proteins involved in defined cellular processes. 24th Meeting of the American Society of Rickettsiology. Stevenson, WA.

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17. Kuriakose JA, Lyons P (Apr 2002). Characterization of soil fungi based on rDNA sequence variation. UHD-Student Research Conference. Houston, TX.

ORAL PRESENTATIONS

1. Kuriakose JA, Zhang X, Luo T, McBride JW (Jun 2011) Antibodies against *Ehrlichia chaffeensis* Tandem Repeat Proteins Linear Epitopes are Protective. 6th International Meeting on Rickettsiae and Rickettsial diseases. Crete, Greece.

2. Kuriakose JA, Miyashiro S, Luo T, McBride JW (Jul 2010) Transcriptomic analysis of *Ehrlichia chaffeensis* in the mammalian and arthropod hosts. 24rth Meeting of the American Society of Rickettsiology. Stevenson, WA.

3. Kuriakose JA, Zhu B, McBride JW (Aug 2009) Transcriptomic analysis of *Ehrlichia chaffeensis* in the mononuclear phagocyte reveals two tandem repeat proteins are hyper-expressed. 23rd Meeting of the American Society of Rickettsiology. Hilton Head, SC.