INTERNALIZATION AND SURVIVAL MECHANISMS OF HUMAN EHRLICHIOSIS AGENTS EHRLICHIA CHAFFEENSIS AND ANAPLASMA PHAGOCYTOPHILUM IN HOST CELLS

DISSERTATION

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By

Mingqun Lin, M.S.

* * * * *

The Ohio State University
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Dissertation Committee:

Dr. Yasuko Rikihiisa, Adviser
Dr. Young C. Lin
Dr. James W. DeWille
Dr. William P. Lafuse

Approved by
Yasuko Rikihiisa
Adviser
Department of Veterinary Biosciences
ABSTRACT

Obligatory intracellular, human ehrlichiosis agents *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* have remarkable abilities to parasitize first-line immune defensive cells, monocytes/macrophages and granulocytes, respectively. As newly discovered pathogens, the bacterial or host components, the signaling pathways required for their internalization and proliferation, and how these bacteria inhibit the microbicidal activities in host cells remain vastly unknown. In this study, we found that the entry of *E. chaffeensis* into THP-1 cells rapidly induces the following essential signaling events: protein cross-linking by transglutaminase, tyrosine phosphorylation, phospholipase C (PLC)-γ2 activation, IP3 production, and an increase in cytosolic free calcium levels. The entry and proliferation of these pathogens involve caveolae-mediated endocytosis and glycosylphosphatidylinositol-anchored proteins. Furthermore, caveolar marker protein caveolin-1, tyrosine-phosphorylated proteins, and PLC-γ2 are colocalized in both early and replicative inclusions.

Because of the extremely fragile and pleomorphic nature of *E. chaffeensis* and *A. phagocytophilum*, we tested if cholesterol is also required for them as for mycoplasmas with similar physical characteristics. The genome of these bacteria lacks genes for
biosynthesis of lipid A and peptidoglycan. Although they lack genes for cholesterol biosynthesis or modification, host cell-free *E. chaffeensis* and *A. phagocytophilum* can directly incorporate significant amount of exogenous cholesterol into their membranes, which provides them physical strength and is essential for infecting host leukocytes.

We finally examine the mechanism whereby *E. chaffeensis* downregulates host microbicidal activities. Using lipopolysaccharides (LPS) as a model stimulant of monocytes, we demonstrate that *E. chaffeensis* infection disables human monocytes to kill *E. chaffeensis* in response to *E. coli* LPS. This is correlated with the downregulated expression of several pattern recognition receptors such as CD14, toll-like receptor (TLR) 2/4, and NF-κB activity from 1 d post infection (pi). This change is preceded by reduced phosphorylation of p38 MAPK/ERK in response to LPS from 3 h pi and decreased PU.1 activity, suggesting the role of p38 MAPK and PU.1 in downregulating expressions of TLR2/4 and CD14. Taken together, these results enhance our understanding of the entry and survival mechanisms of human ehrlichiosis agents, and may direct us to new approaches for prevention or treatment of these diseases.
Dedicated to my family
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VITA

April 5, 1973 .................................................. Born in Putian, Fujian, P.R.China
1990 ~ 1995 .................................................. B.S. of Microbiology,
                                             Fudan University, Shanghai, China
1995 ~ 1998 .................................................. M.S. of Cell Biology and Immunology,
                                             Shanghai Institute of Cell Biology, Chinese
                                             Academy of Sciences
1998 ~ present .............................................. Graduate Research Associate,
                                             The Ohio State University

PUBLICATIONS

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ABBREVIATIONS

HME, human monocytic ehrlichiosis
HGE, human granulocytic ehrlichiosis
TGase, transglutaminase
MDC, monodansylcadaverine
PLC, phospholipase C
PTK, protein tyrosine kinase
PIP₂, phosphatidylinositol 4,5-bisphosphate
IP₃, inositol 1,4,5-trisphosphate
[Ca²⁺], intracellular calcium level
GPI, glycosylphosphatidylinositol
PSGL-1, P-selectin glycoprotein ligand-1
TfR, transferrin receptor
MHC, major histocompatibility complex
EEA, early endosomal antigen
MβCD, methyl-β-cyclodextrin
NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3beta-ol
TEM, transmission electron microscopy
LPS, lipopolysaccharide
TLR, toll-like receptor
PRR, pattern recognition receptor
PAMP, pathogen-associated molecular pattern
MAPK, mitogen-activated protein kinases
ERK, extracellular signal-regulated kinase
JNK, c-Jun NH$_2$-terminal kinase
IL, interleukin
TNF, tumor necrosis factor
RT, Reverse transcription
PCR, polymerase chain reaction
EMSA, electrophoretic mobility shift assay
ECL, enhanced chemiluminescence
CHAPTER 1

INTRODUCTION

Human ehrlichiosis and human ehrlichiosis agents.

Once considered to be only veterinary pathogens, several *Ehrlichia* and *Anaplasma* spp in the order *Rickettsiales* have emerged as significant human pathogens during the last two decades. *Neorickettsia* (formerly *Ehrlichia* sennetsu, a monocytophagocytic sp., was the first human pathogen discovered in Japan in the 1950’s, and has also been found in Malaysia recently. *Ehrlichia chaffeensis*, a monocyte-specific species that causes human monocytic ehrlichiosis (HME) was discovered in 1986, and *Anaplasma phagocytophilum* (formerly the human granulocytic ehrlichiosis [HGE] agent), a granulocyte-specific species was discovered in 1994 (Dumler and Bakken, 1998; Goodman *et al.*, 1996; Dawson *et al.*, 1991; Rikihisa, 1991). Originally thought to be only canine pathogens, a granulocytic ehrlichial species *E. ewingii* was recognized as a human pathogen in 1998 (Buller *et al.*, 1999); and in 1995 a monocytic ehrlichial species *E. canis* was isolated from a human patient in Venezuela (Perez *et al.*, 1996).

Human ehrlichioses are systemic febrile illness characterized by fever, headache, myalgia, anorexia, and chills, and are frequently accompanied by leukopenia,
thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases. The severity of the disease varies from asymptomatic seroconversion to death, and severe morbidity is frequently documented with approximately 2 ~ 4% fatality, especially in immunocompromised patients (Paddock and Childs, 2003; Dumler and Bakken, 1998). From 1986 through 1997, 1,223 confirmed human ehrlichiosis cases (742 HME, 449 HGE, and 32 not ascribed to a specific ehrlichial agent) were reported by state health departments in the United States. However, the actual cases are much higher than this number (McQuiston et al., 1999). With increasing suburbanization and outdoor activities, human ehrlichioses pose significant public health problems in the United States and other parts of the world, and become one of the nationally recognized diseases since 1998 in the US (McQuiston et al., 1999).

**General characteristics of human ehrlichiosis agents.**

Four genera, *Ehrlichia*, *Anaplasma*, *Neorickettsia*, and *Wolbachia* are small gram-negative cocci and obligatory intracellular bacteria that are currently belong to the family *Anaplasmataceae* (Dumler et al., 2001). These bacteria are surrounded by thin outer and inner membranes. Unlike members of the genus *Rickettsia*, members of the genus *Ehrlichia* shows no thickening of either leaflet of the outer membrane. Outer membranes are more ruffled in *A. phagocytophilum* (HGE agent) than in *N. sennetsu* or *E. chaffeensis*. The ultrastructure of this group of bacteria is also distinct in several aspects when compared to *Rickettsia* that resides free in the cytosol (Rikihisa, 2003; Rikihisa, 1999; Rikihisa et al., 1997; Rikihisa, 1991; Rikihisa, 1990b; Rikihisa et al., 1985). First, they are highly pleomorphic. Second, the outer membrane is rippled and quite thin, and does
not show any thickening of either leaflet. Third, the periplasmic space is generally narrow and a peptidoglycan layer is undetectable. Lastly, there is no evidence of microcapsular layers in these bacteria. Furthermore, no physical or molecular evidences of lipopolysaccharide (LPS) have been found in the family Anaplasmataceae to which E. chaffeensis and A. phagocytophilum belong. In gram-negative bacteria, LPS and peptidoglycan have significant roles in providing strength to the outer membrane and maintaining overall structural integrity (Nikaido, 1996; Park, 1996). Consistent with absence or low levels of LPS and peptidoglycan, these bacteria are extremely fragile, and once extracellular, lose their infectivity within a few hours.

Members in the family Anaplasmataceae are obligatory aerobic and lack the glycolytic enzyme pathway. Metabolic activity of these bacteria is sensitive to acidic pH. Their genome size is relatively small, approximately 1 Mb (A. phagocytophilum, 1.5; E. chaffeensis, 1.2; N. sennetsu, 0.9) as determined by pulsed-field gel electrophoresis. The relatedness of 16S rRNA gene sequences of the genus Rickettsia to those of Ehrlichia, Cowdria, Anaplasma, and Wolbachia spp. is 83-84%. Members in the family Anaplasmataceae are as divergent among themselves as between these bacteria and Rickettsia spp. Based on 16S rRNA gene sequences, members in the family Anaplasmataceae are divided into four distinct genetic groups, with about 7-15% sequence divergence between the groups (Figure 1.1). Group 1 is genus Ehrlichia, which consists of E. canis, E. chaffeensis, E. muris, E. ewingii, and E. ruminantium (formerly Cowdria ruminantium). Group 2 is genus Anaplasma which consists of A. phagocytophilum (formerly E. equi, E. phagocytophila and HGE agent), and A. platys,
which infects platelets. *Anaplasma marginale*, which has a tropism for erythrocytes, is closest to Group 2 in 16S rRNA gene sequence comparison (97% similarity). Group 3 consists of *N. sennetsu*, *N. risticii*, *Stellantchasmus falcatus* agent (SF agent) isolated from the fluke *S. falcatus* (a fish parasite) in Japan, and *N. helminthoeca*. Members of the genus *Wolbachia* and several insect symbionts, which are not known to cause a disease in vertebrates, is another genus in family *Anaplasmataceae*.

![Phylogram of the Family Anaplasmataceae based on 16S rRNA](image)

New genus *Ehrlichia*, except for *E. ewingii* and *E. ruminantium*, infects monocytes and macrophages. *E. ewingii* infects granulocytes and *E. ruminantium* infects vascular endothelial cells and neutrophils. The genus *Anaplasma* group infects granulocytes,
platelets, and red blood cells. The Neorickettsia group mostly infects monocytes and macrophages. *N. risticii* in addition infects intestinal epithelial cells and mast cells.

**The transmission of human ehrlichiosis agents.**

Genera *Ehrlichia* and *Anaplasma* spp. are maintained through an enzootic cycle between animals and ticks, and can accidentally infect humans through the bite of infected ticks (Telford *et al.*, 1996; Ewing *et al.*, 1995). They require horizontal and bidirectional transmission alternating between mammals and ticks (Rikihisa, 2003). *E. chaffeensis* has been most commonly identified in the Lone Star tick *Amblyomma americanum*, and white-tailed deer are considered to be the major reservoir of *E. chaffeensis*. *A. phagocytophilum* has been found in ticks of *Ixodes* species, the same vector that transmits Lyme diseases, and wild rodents such as white-footed mice and dusky-footed wood rats are believe to be the major reservoirs of *A. phagocytophilum*. Human infection with *Neorickettsia* takes place by accidental ingestion of the infected matacercaria stage of trematodes encysted in fish or aquatic insects. *Neorickettsia* sp. is maintained through transovarial and transstadial passage in the trematode species specific to each *Neorickettsia* species. *Neorickettsia* can be horizontally transmitted unidirectionally from trematodes to mammals, but not from mammals to trematodes. Mammals are required for maintenance of the trematode lifecycle for some *Neorickettsia* species, but infection of mammals with *Neorickettsia* itself is not required for maintenance of *Neorickettsia*. 
**Internalization of the host cells by human ehrlichiosis agents.**

Members in the family *Anaplasmataceae* cannot survive extracellularly; therefore, it is essential for these bacteria upon binding to host cells, to trigger rapid and precise signals to spatially assemble host molecules for their internalization into a specific intracellular compartment: an early endosome conducive to their proliferation (Mott *et al.*, 1999; Barnewall *et al.*, 1997). Unlike their peer obligate intracellular bacteria *Rickettsia* or other obligate intracellular bacteria, several *Ehrlichia*, *Anaplasma* and *Neorickettsia* spp. have the remarkable ability to parasitize first-line immune defensive cells, monocytes/macrophages and neutrophils, as their exclusive survival sites. Studies have shown that most obligatory and facultative intracellular bacteria enter host cells by phagocytosis and actively exploit the actin cytoskeleton during infection, including *Orientia tsutsugamushi* (former name: *Rickettsia tsutsugamushi*) (Rikihisa and Ito, 1982), *Salmonella* (Finlay and Cossart, 1997), *Bartonella* (Guzman *et al.*, 1994), and *Listeria* (Ireton *et al.*, 1996).

Although the detailed entry route used by members in the family *Anaplasmataceae* is unknown, several studies have suggested that these bacteria enter host monocytes or neutrophils through an unconventional route: by clathrin-independent, probably receptor-mediated endocytosis, rather than by phagocytosis. For example, one study showed that the binding and internalization of *A. phagocytophilum* to HL-60 cells is dependent on P-selectin glycoprotein ligand (PSGL)-1 (Herron *et al.*, 2000). In another member of the family *Anaplasmataceae*, *Neorickettsia risticii*, which causes Potomac horse fever, the binding and internalization of this bacterium into the host macrophage were blocked by
the treatment of anti-\textit{N. risticii} Fab fragment, suggesting the involvement of a cognitive host cell receptor for the bacterial surface antigen in the bacterial entry (Messick and Rikihisa, 1994). Other studies showed that replicative inclusions of \textit{E. chaffeensis} and \textit{A. phagocytophilum} are not colocalized with clathrin (Mott et al., 1999; Barnewall et al., 1997). Furthermore, these bacteria consistently show higher sensitivity to monodansylcadaverine (MDC): an inhibitor of transglutaminase (TGase) that is related to receptor-mediated endocytosis (Abe et al., 2000; Davies et al., 1980; Levitzki et al., 1980); and taxol or colchicines, inhibitors of microtubules; rather than to cytochalasins, inhibitors of microfilament assembly (Rikihisa et al., 1994; Messick and Rikihisa, 1993).

The entry of these bacteria requires unique signaling pathways. For example, \textit{N. risticii} entry into host cells is highly sensitive to Ca$^{2+}$ channel blockers, calmodulin antagonists, Ca$^{2+}$ ionophores (Rikihisa et al., 1995), and protein tyrosine kinase (PTK) inhibitors (Zhang and Rikihisa, 1997). None of these inhibitors has direct inhibitory effects on CO$_2$ generation from L-glutamine by host cell-free ehrlichiae, the ATP producing pathway by members in the family \textit{Anaplasmataceae} (Rikihisa et al., 1994). Ca$^{2+}$ is required for internalization of \textit{Salmonella typhimurium} (Pace et al., 1993). PTK activities are required for internalization of the enteropathogenic strain of \textit{Escherichia coli} (EPEC) (Rosenshine et al., 1992b), \textit{Listeria} (Velge et al., 1994), \textit{Yersinia} (Rosenshine et al., 1992a), and \textit{Campylobacter jejuni} (Wooldridge et al., 1996). Except EPEC, identities of the tyrosine phosphorylated proteins, however, are largely unknown. \textit{N. risticii} is distinct from these bacteria since it consistently requires both Ca$^{2+}$ and PTK signals for internalization.
Replication of human ehrlichiosis agents in host cell cytosol.

After their entry, *E. chaffeensis* and *A. phagocytophilum* replicate in unique membrane-bound inclusions within host cytoplasm. The entire intracellular life stages of these bacteria in human professional phagocytes are confined inside membrane-bound inclusions (Rikihisa, 2003). These bacteria appear dark purple to blue color by Diff-Quik stain, and grow as microcolonies called morulae, which are membrane-bound inclusions in the cytoplasm of their eukaryotic host cells as visualized by transmission electron microscopy (Rikihisa, 2003; Rikihisa, 2000). The morphology of the inclusions of these bacteria is different. For example, *E. chaffeensis* inclusions are densely packed whereas *A. phagocytophilum* inclusions are more spacious (Mott et al., 1999).

Intracellular parasites have evolved diverse mechanisms to enhance their survival and replication within eukaryotic host cells. One widely used strategy to circumvent destruction in phagocytes is to simply avoid the phagocyte altogether and to gain access to cells that are far less hostile. Many pathogenic bacteria can enter epithelial cells, fibroblasts and other non-phagocytes, which is often achieved through induced adhesion and uptake (Pieters, 2001). In *Salmonella*, *Yersinia*, and *Shigella*, several proteins are injected into host cells through a type III secretion system, which assembled into a macromolecular structure resembling a needle spanning the bacterial envelope. These injected molecules promote their uptake into non-phagocytes. However, for those pathogens replicating in phagocytes, most of the mechanisms involve adaptations for survival in distinct intracellular compartments that permit the parasites to avoid lysosomal killing. For example, *Listeria*, *Shigella*, and *Rickettsia* escape into the
cytoplasm to avoid lysosomal digestion (Hackstadt, 2000). Phagosomal escape is best studied in *Listeria monocytogenes* in which a pore-forming cytolysin and two phospholipases are involved in the lysis of the phagosomal membrane (Goebel and Kuhn, 2000). A few bacteria, such as *Coxiella burnetii*, have adapted to survival within the harsh environment of the lysosome (Heinzen *et al.*, 1996). The majority of intracellular parasites remain within membrane bound vesicles that do not fuse with lysosomes. For example, *Mycobacterium* spp. reside in early endosomes (Sturgill-Koszycki *et al.*, 1996), whereas *Chlamydia* spp. seem to be replicated in exocytic vesicles (Hackstadt *et al.*, 1996).

The inclusions of *E. chaffeensis* and *A. phagocytophilum* also do not highly acidify or mature into phagolysosomes, since they lack lysosomal markers such as CD63 and the lysosomal membrane associated protein (LAMP-1) (Barnewall *et al.*, 1999; Mott *et al.*, 1999; Webster *et al.*, 1998; Barnewall *et al.*, 1997). Instead, they are colocalized with vesicle associated membrane protein 2 (VAMP-2, or synaptobrevin-2) and major histocompatibility complex (MHC) class I or class II surface antigens that are usually not found in early endosomes (Barnewall *et al.*, 1999; Mott *et al.*, 1999; Barnewall *et al.*, 1997; Brumell *et al.*, 1995). Notably, the replicative inclusions of *E. chaffeensis* and *N. sennetsu* accumulate transferrin receptor (TfR) and have several early endosomal markers, such as early endosomal antigen 1 (EEA1) and rab5; whereas the inclusions of *A. phagocytophilum* are negative for these endosomal markers (Barnewall *et al.*, 1999; Mott *et al.*, 1999; Barnewall *et al.*, 1997). Interestingly, the inclusions of *E. chaffeensis* and *A. phagocytophilum* do not contain some of the early-endosomal reversibly associated
proteins such as annexins, α-adaptin, clathrin, or protein markers of antegrade vesicle traffic from Golgi including beta-coatamer protein (COP) or C6-NBD-ceramide (Mott et al., 1999; Barnewall et al., 1997). These data suggest that the replicative inclusions of E. chaffeensis and A. phagocytophilum are different from any other intracellular compartments known to date.

**Downregulation of host microbicidal activities in human monocytes/macrophages and neutrophils by human ehrlichiosis agents.**

Innate immune response, the first line of defense system against microbial pathogens, consists of monocytes-macrophages and neutrophils. These cells can phagocytose and kill the pathogens through lysosomal fusion, the generation of microbicidal substances like reactive oxygen intermediates (ROI), nitric oxide intermediates (NOI), and coordination of additional host responses by producing a wide range of proinflammatory cytokines such as TNF-α, IL-1, IL-6. In order to discriminate a large amount of diverse pathogens from self, the innate immune system has developed “pattern recognition receptors” (PRRs) to recognize conserved motifs calls pathogen-associated molecular patterns (PAMPs). PAMPs are shared by large groups of microorganisms but not found in higher eukaryotes, which have essential roles in the biology of invading agents thus are evolutionally conserved (Kopp and Medzhitov, 1999).

In addition to escaping from the phago-lysosomal fusion pathways described above, intracellular bacteria in phagocytes must also develop strategies to avoid or delay the host immune responses and the microbicidal activities of monocytes (Rosenberger and Finlay, 2003). For example, *Yersinia enterocolitica* and *Mycobacterium ulcerans* disrupt NF-κB
activation, thus resulting in the decreased release of proinflammatory cytokines, such as TNF-α and IL-1 (Orth et al., 2000; Pahlewan et al., 1999). Similar effects on NF-κB activation can also be achieved by targeting signaling components that regulate NF-κB and AP-1 activity, such as MAPKs by YopJ from *Yersinia enterocolitica* (Orth et al., 2000). Other bacteria, such as *Mycobacterium avium*, deactivate macrophages by disruption of interferon signaling by blocking JAK-STAT activation, which leads to decreased production of ROI and blocks the ability of macrophages to coordinate antibacterial immunity through MHC expression and antigen presentation (Hussain et al., 1999).

In order to survive inside monocytes-macrophages, it is also essential for ehrlichiae not to induce bactericidal signals, but selectively induce ehrlichial entry signals which are independent from macrophage activation signals. Several PAMPs, such as LPS, peptidoglycan, bacterial flagella, heat shock proteins, and unmethylated CpG containing DNA, are potent activators of primary innate immune defensive cells. Although *E. chaffeensis* and *A. phagocytophilum* lack evidence of LPS and peptidoglycan in their outer membrane, they do contain other PAMPs including heat shock proteins and unmethylated CpG-containing DNA. Previous studies have shown that immunosuppression is commonly associated with infection by members in the family *Anaplasmataceae* (Dumler and Bakken, 1998; Dumler, 1997; Rikihisa, 1991). *A. phagocytophilum* strongly blocked the release of superoxide anion through the downregulation of a NADPH oxidase component p22phox (Mott et al., 2002; Banerjee et al., 2000; Mott and Rikihisa, 2000). In addition, several members in the family
Anaplasmataceae, including *E. chaffeensis*, *N. risticii* and *A. phagocytophilum* are able to delay and suppress the adaptive immune response. In the infected host monocytes/macrophages or neutrophils, major proinflammatory cytokines tumor necrosis factor (TNF-α) and interleukin (IL)-6 were not produced or even prevented in response to LPS (Kim and Rikihisa, 2002; van Heeckeren et al., 1993). Instead, immunosuppressive cytokine IL-10 was released (Lee and Rikihisa, 1996). In *N. risticii*-infected horses or mice, and *E. muris* infected mice there is generalized immunosuppression in spleen or peripheral blood lymphocytes responses (Kawahara et al., 1996; Rikihisa et al., 1988; Rikihisa et al., 1987). These results clearly demonstrate the powerful ability of members in the family *Anaplasmataceae* to downregulate microbicidal activities in host immune defensive cells.

**Objectives of this study.**

The primary goal of this study was to determine the signaling pathways utilized by *E. chaffeensis* and *A. phagocytophilum* to enter and proliferate in their host cells, the membrane components of these bacteria required for their entry and survival, and the mechanism utilized by these bacteria to downregulate host immune responses.

(1) Previous studies showed that protein tyrosine kinases (PTKs) and calcium are required for the internalization and infection of *N. risticii*. However, the detailed signaling pathways required for ehrlichial internalization is not known. Phospholipase C (PLC)-γ are known to be activated by PTKs and produce inositol 1,4,5-trisphosphate (IP₃) which mediates the increase in intracellular calcium level ([Ca²⁺]ᵢ). The objective of this
study was to characterize the role of PLC-γ isozymes in the increase in calcium levels and the role of PLC-γ in the infection and proliferation of *E. chaffeensis*.

(2) A novel clathrin-independent trafficking pathway has been found in several eukaryotic cells types, which involves a group of typically flask-shaped, but can be variously shaped invaginations at the cell surface called caveolae. Caveolae are formed from lipid rafts by polymerization of caveolae-specific proteins, caveolins (Duncan *et al.*, 2002; Anderson, 1998) and provide endocytic and exocytic trafficking pathways to import molecules to specific locations within the cell or export molecules to extracellular spaces. As a vesicle trafficking system that bypasses phagolysosomal pathways, lipid rafts or caveolae is utilized by several intracellular bacteria for entering the host cells to avoid their degradation in lysosomes (Jutras *et al.*, 2003; Duncan *et al.*, 2002; Norkin *et al.*, 2001; Shin and Abraham, 2001a). Studies have suggested that members of the family *Anaplasmataceae* enter their respective host cells through an unconventional clathrin-independent but receptor-mediated endocytosis, rather than by phagocytosis, but their entry routes are still unknown. The objective of this study was to characterize the involvement of caveolae and GPI-anchored proteins in the internalization and proliferation of *E. chaffeensis* and *A. phagocytophilum* in their host cells.

(3) The lack of peptidoglycan layer and LPS in bacteria of the family *Anaplasmataceae* and the unique physical characteristics suggest unusual cell wall compositions. These bacteria in the family *Anaplasmataceae* share some physical characteristics with mycoplasmas, such as being extremely fragile and pleomorphic; although mycoplasmas lack a cell wall and are extracellular parasitic bacteria. To date
mycoplasmas are only prokaryotes known to incorporate cholesterol or related sterols from hosts or environments to stabilize its cytoplasmic membrane (Dahl, 1993). The objective of this study was to examine if cholesterol exists in the membrane of *E. chaffeensis* or *A. phagocytophilum*, and test the role of bacteria-incorporated cholesterol in infecting their host cells.

(4) Monocytes and macrophages have strong bactericidal activities when activated by various cytokines or microbial components such as LPS. To assure their survival, bacteria in the family *Anaplasmataceae* have evolved powerful abilities to inhibit signaling events leading to clearance of bacterial infection, including the release of reactive oxygen intermediates (ROI), production of proinflammatory cytokines, and phago-lysosomal fusion. *E. chaffeensis* and other members in the family *Anaplasmataceae* have been shown to possess the ability to block the release ROI and proinflammatory cytokine. However, the mechanisms whereby these bacteria exert their inhibitory effects are unknown. The objective of this study was to investigate possible signaling pathways by which *E. chaffeensis* prevents activation of monocytes/macrophages by using LPS as a model stimulant for monocytes and macrophages.
CHAPTER 2

RAPID ACTIVATION OF PLC-γ2, INCREASE IN CYTOSOLIC FREE CALCIUM, RECRUITMENT OF PLC-γ2 AND TYROSINE PHOSPHORYLATED PROTEINS TO ITS INCLUSION BY *EHRLICHIAS CHAFFEENSIS* FOR INTERNALIZATION AND GROWTH IN THP-1 CELLS

2.1 Abstract

*Ehrlichia chaffeensis*, a bacterium that cannot survive outside of the eukaryotic cell, proliferates exclusively in human monocytes-macrophages. In this study, signaling events required for ehrlichial infection of a human monocytic cell line THP-1 were characterized. The entry and proliferation of *E. chaffeensis* in THP-1 cells were significantly blocked by various inhibitors that can regulate calcium signaling including TMB-8 and 2-APB (intracellular calcium mobilization inhibitors); verapamil and SKF-96365 (calcium channel inhibitors); neomycin and U-73122 (phospholipase C [PLC] inhibitors); monodansylcadaverine (transglutaminase [TGase] inhibitor); and genistein (protein tyrosine kinase [PTK] inhibitor). Addition of *E. chaffeensis* caused rapid increases in inositol 1,4,5-trisphosphate (IP₃) and cytosolic free calcium ([Ca²⁺]ᵢ) in THP-1 cells, which was prevented by pretreatment of THP-1 cells with inhibitors of TGase, PTK, or PLC. *E.*
induced rapid tyrosine-phosphorylation of PLC-\(\gamma\)2 and the delivery of a PLC-\(\gamma\)2 antisense oligonucleotide into THP-1 cells significantly blocked ehrlichial infection. Furthermore, tyrosine-phosphorylated proteins and PLC-\(\gamma\)2 were colocalized with ehrlichial inclusions as examined by double immunofluorescence labeling. The heat-sensitive component of viable \textit{E. chaffeensis} was essential for these signaling events. \textit{E. chaffeensis}, therefore, can recruit interacting signal transducing molecules to its inclusion and induce the following signaling events required for the establishment of infection in host cells: protein cross-linking by transglutaminase, tyrosine phosphorylation, PLC-\(\gamma\)2 activation, IP\(_3\) production, and an increase in [Ca\(^{2+}\)].

2.2 Introduction

Intracellular bacteria are known to usurp many preexisting host cell signaling and membrane sorting mechanisms for entry, survival, and proliferation. Recent findings of varieties of molecular mechanisms of intracellular parasitism, therefore, have been enlightening not only the microbial pathogenesis but also host cell biology (Duclos and Desjardins, 2000; Goebel and Kuhn, 2000; Hackstadt, 2000). \textit{Ehrlichia chaffeensis} is a recently discovered bacterium in the United States, which targets and multiplies only in primary host defensive cells, monocytes-macrophages. \textit{E. chaffeensis} causes a Rocky Mountain spotted fever-like illness called Human monocytic ehrlichiosis (HME) which can be fatal in immunocompromized patients or if inappropriately treated (Rikihisa, 1999). Unlike facultative intracellular bacteria such as \textit{Salmonella}, \textit{Yersinia}, \textit{Mycobacterium} etc, \textit{E. chaffeensis} cannot survive extracellularly. It is, therefore, essential
for *E. chaffeensis* upon binding to host cells, to trigger rapid and precise signals and to spatially assemble host molecules for their internalization to the specific intracellular compartment – an early endosome conducive to their proliferation (Mott *et al.*, 1999; Barnewall *et al.*, 1997). Majority of bacteria and various bacterial components such as LPS, peptidoglycan, heat shock proteins, or CpG containing DNA activate monocytes-macrophages through their pathogen-associated molecular patterns. In order to survive inside monocytes-macrophages, it is essential for ehrlichiae not to induce activation signals, but selectively induce ehrlichial entry signals which are independent from macrophage activation signals. Most obligatory and facultative intracellular bacteria such as *Orientia tsutsugamushi* (former name: *Rickettsia tsutsugamushi*) (Rikihisa and Ito, 1982), *Salmonella* (Finlay and Cossart, 1997), *Bartonella* (Guzman *et al.*, 1994), and *Listeria* (Ireton *et al.*, 1996) mobilize microfilaments and enter host cells by phagocytosis. However, we previously found that entry of *Neorickettsia risticii* (former name: *Ehrlichia risticii*) (Dumler *et al.*, 2001), a monocytic obligatory intracellular bacterium that causes Potomac horse fever (16S rRNA gene sequence identity between *N. risticii* and *E. chaffeensis* is 85%) (Rikihisa, 1999), into host cells is unusual, since it consistently shows higher sensitivity to monodansylcadaverine (MDC), an inhibitor of transglutaminase (TGase) related to receptor-mediated endocytosis (Abe *et al.*, 2000; Davies *et al.*, 1980; Levitzki *et al.*, 1980); and taxol or colchicines, inhibitors of microtubules, rather than to cytochalasins, inhibitors of microfilament assembly (Rikihisa *et al.*, 1994; Messick and Rikihisa, 1993). We recently found that the entry of *E. chaffeensis* and granulocytic obligatory intracellular bacterium, *Anaplasma phagocytophilum* (Human Granulocytic
Ehrlichiosis agent, 16S rRNA gene sequence identity between *A. phagocytophilum* and *E. chaffeensis* is 92.5%) are also sensitive to MDC (Dumler *et al.*, 2001; Mott and Rikihisa, 2000; Yoshiie *et al.*, 2000; Barnewall *et al.*, 1999; Rikihisa, 1999).

Furthermore, *N. risticii* entry into host cells is unique since it is highly sensitive to Ca\(^{2+}\) channel blockers, calmodulin antagonists (Rikihisa *et al.*, 1995), and protein tyrosine kinase (PTK) inhibitors (Zhang and Rikihisa, 1997). None of these inhibitors has direct inhibitory effects on CO\(_2\) generation from L-glutamine by host cell-free ehrlichiae (Rikihisa *et al.*, 1994). Ca\(^{2+}\) is required for internalization of *Salmonella typhimurium* (Pace *et al.*, 1993). PTK activities are required for internalization of the enteropathogenic strain of *Escherichia coli* (EPEC) (Rosenshine *et al.*, 1992b), *Listeria* (Velge *et al.*, 1994), *Yersinia* (Rosenshine *et al.*, 1992a), and *Campylobacter jejuni* (Wooldridge *et al.*, 1996). Except EPEC, identities of the tyrosine phosphorylated proteins, however, are largely unknown. *N. risticii* is distinct from these bacteria since it consistently requires both Ca\(^{2+}\) and PTK signals for internalization.

Since previous studies with *N. risticii* are limited to the use of inhibitors and the role of phospholipase C (PLC) was not examined and to test whether these calcium signals are also required for the infection of *E. chaffeensis*, in the present study, first, effects of inhibitors of PTK, phospholipase C (PLC), intracellular calcium mobilization, and calcium channel blockers on the internalization and proliferation of *E. chaffeensis* in THP-1 cells, a human monocytic cell line were examined. Second, the production of inositol 1,4,5-trisphosphate (IP\(_3\)), the level of [Ca\(^{2+}\)]\(_i\) in response to *E. chaffeensis*, and the effects of these inhibitors on IP\(_3\) generation and [Ca\(^{2+}\)]\(_i\) were determined. Lastly, the
Isozyme of PLC involved in IP₃ generation, its role in ehrlichial internalization, and localization of the PLC and tyrosine phosphorylated proteins were examined.

2.3 Materials and Methods

*Ehrlichia chaffeensis* and THP-1 Cells. *E. chaffeensis* Arkansas (Dawson et al., 1991) was propagated in THP-1 cells (ATCC, Rockville, MD), a human monocytic leukemia cell line (Tsuchiya et al., 1980) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2% L-glutamine at 37°C in 5% CO₂ and 95% air. No antibiotic was used throughout the study. Host cell-free *E. chaffeensis* was prepared by sonication for 8 s at an output setting of 2 with an ultrasonic processor W-380 (Heat Systems, Farmington, NY). After low speed centrifugation to remove nuclei and unbroken cells, the supernatant was centrifuged at 10,000 × g for 10 min, and the pellet enriched with host cell-free organisms was used to infect THP-1 cells. Based on the pilot experiments, multiplicity of infection (moi, the ratio of the number of bacteria to that of host cells) of 100 was chosen in this study, if not otherwise specified. For heat-treated *E. chaffeensis*, host-cell free *E. chaffeensis* was incubated in 42°C or 60°C water bath for 15 min.

Evaluation of the effect of various compounds on ehrlichial internalization or proliferation. The compounds (concentration) used were: genistein (100 μM, Sigma, St. Louis, MO); neomycin (50 μM, BioMol, Plymouth Meeting, PA), U-73122 [1-(6-([17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl)-1H-pyrrole-2,5-dione, 10 μM, BioMol], monodansylcadaverine (MDC, 250 μM, Sigma), TMB-8 [8-(Diethylamino)octyl-3, 4, 5-trimethoxybenzoate, 100 μM, BioMol], 2-APB (2-aminoethoxy-diphenyl borate, 75 μM,
Calbiochem, San Diego, CA), SKF-96365 (1-[β-(3-[4-Methoxyphenyl]propyl)-4-methoxyphenethyl]-1H-imidazole, 60 µM, Calbiochem), and verapamil (100 µM, BioMol).

To determine the effect on ehrlichial internalization, THP-1 cells (10^6 cells/well) were incubated in 12-well plates with various compounds 1 h prior to the addition of host cell-free *E. chaffeensis*. At 3 h post infection (pi), cells were centrifuged to remove extracellular ehrlichiae and treated with pronase E (2 mg/ml) for 3 min to remove bound but uninternalized *E. chaffeensis* as previously described (Rikihisa *et al.*, 1994). The cells were further cultured without inhibitors for 3 d, and the infectivity was determined by counting the number of *E. chaffeensis* organisms in 100 cells in triplicate assay wells (Rikihisa *et al.*, 1995). To determine the effects of compounds on ehrlichial proliferation, these compounds were added at 3 h pi to the mixture of THP-1 cells and host cell-free *E. chaffeensis*, and further cultured for 3 d and the infectivity was determined.

**Assay of IP3.** IP3 was determined by D-*myo*-Inositol 1,4,5-Trisphosphate (IP3) - [³H] assay system according to manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, THP-1 cells (1 × 10^7 cells/1ml) were aliquoted into 1.7 ml microcentrifuge tubes, and incubated at 37°C for 5 min. Prewarmed host-cell free *E. chaffeensis* at moi of 100 or as indicated in 0.5 ml volume was then added to THP-1 cells and mixed rapidly by inverting tubes for several times. At indicated time point, cells were spun down at 2,000 rpm at 4°C for 1 min and perchloric acid was added to the pellet. Times recorded were the time when the tube was taken out from the incubator. IP3 extracted in perchloric acid and ³H-labeled IP3 standards were coincubated with IP3-binding protein for 15 min on ice.
After washing unbound $[^3]H$-IP$_3$, the amount of $[^3]H$-IP$_3$ released from IP$_3$-binding protein by 0.15 N NaOH was determined using a scintillation counter.

**Intracellular Ca$^{2+}$ measurement by fluorometer.** THP-1 cells were loaded with 2 µM acetoxyethyl (AM) ester derivative of fluo-3 (Molecular Probes, Eugene, OR) at 37°C for 30 min on the rotating platform in a water bath. After washing twice, cells were resuspended in RPMI 1640 (with 5% FBS, without phenol red) and further incubated for 30 min to allow complete hydrolysis of intracellular AM esters. Cells were then stored on ice until assayed. Fluo-3-preloaded cells ($1.5 \times 10^6$) were incubated with or without compounds for 1 h at room temperature. After warming up to 30°C using a circulating water bath in the fluorometer, *E. chaffeensis* was added to the cells with brief mixing, and emission at 525 nm upon excitation at 488 nm was recorded every 5 sec for 10 min at 30°C. Intracellular calcium level was calculated using the formula: $[Ca^{2+}]_i = K_d \times (F - F_{\text{min}})/(F_{\text{max}} - F)$, and $K_d$ was determined by using a calcium calibration buffer kit (Molecular Probes).

**Intracellular Ca$^{2+}$ measurement by ratiometric imaging.** THP-1 cells were loaded with fura-2/AM (Molecular Probes) as for fluo-3/AM. Preloaded cells at $5 \times 10^5$ cells were incubated with or without compounds in 1 ml RMPI 1640 (with 5% FBS, without phenol red) for 1 h. Cells were then added to a poly-L-Lysine (Sigma)-coated round coverslip in a chamber, allowed to settle for 10 min, and gently washed twice to remove unattached cells. Changes in $[Ca^{2+}]_i$ in individual cells were monitored at room temperature by fluorescence video microscopy using a PTI ImageMaster system (Photon Technology International, Monmouth Junction, NJ) attached to a Nikon Eclipse TE200 inverted
microscope (Nikon, Melville, NY). Alternating excitation wavelengths of 340 nm and 380 nm were provided by a DeltaRAM monochromator at 1 s interval. Fields containing approximate 20 cells were selected and images at emission 520 nm were recorded for at least 5 min. After recording for 30 s to determine the basal level, equal volume of host-cell free *E. chaffeensis* at moi of 100:1 or as indicated was added to the chamber slowly and mixed by pipetting carefully not to disturb the image recording. Ratios of fluorescence (520 nm emission) intensities with excitation wavelengths of 340 nm vs. 380 nm for individual THP-1 cells were plotted against time.

**Immunoprecipitation and western blotting.** THP-1 cells (1 × 10^7) pretreated with compounds for 1 h were incubated with host cell-free *E. chaffeensis* as described previously in “Assay of IP₃” section. Cells were lysed in 1 ml of an ice-cold modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA), with freshly added protease and phosphatase inhibitors (1 µg/ml each of aprotinin, leupeptin, pepstatin A, and 1 mM each of PMSF, NaF, NaVO₃). After incubation on ice for 20 min, cells were sonicated and centrifuged at 10,000 × g for 10 min to collect the supernatants (cell lysates). For immunoprecipitation, cell lysates were incubated with anti-PLC-γ1 or -γ2 antibodies (0.2 µg/ml, Santa Cruz) overnight and immunocomplexes were captured by protein A-agarose beads (Santa Cruz). After washing 3 times with PBS, protein A-agarose beads were resuspended in 1 × SDS sample buffer and boiled for 5 min to dissociate the immunocomplexes from the beads. Samples were subjected to Western blot analysis, and detected with anti-phosphotyrosine antibody, anti-PLC-γ1, -γ2 antibodies, and
horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with enhanced chemiluminescence by incubating the membrane with LumiGLO™ chemiluminescent reagent (Cell Signaling, Beverly, MA), and exposing it to x-ray film.

**Double Immunofluorescence labeling.** *E. chaffeensis*-infected THP-1 cells (2 d pi) were cytocentrifuged onto glass slides, and the cells were fixed and permeabilized in Diff-Quik fixative containing methanol for 15 s (Mott *et al.*, 1999). For antibody labeling, cells were sequentially incubated with primary antibodies (rabbit anti-PLC-γ1, -γ2; mouse anti-phosphotyrosine, or dog anti-*E. chaffeensis* serum pre-absorbed with THP-1 cells); and secondary antibodies (lissamine rhodamine-conjugated anti-rabbit or mouse; FITC-conjugated anti-dog) in PBS. Labeled cells were observed under a Nikon microscope coupled to a BioRad MRC-1024 confocal laser scanning unit (BioRad, Hercules, CA).

**Effect of PLC-γ2 antisense oligonucleotides on *E. chaffeensis* infection.** Morpholino antisense oligos with properties of complete resistance to nucleases, predictable targeting, and reliable activity in cells (Summerton, 1999) were used. PLC-γ2 antisense Morpholino oligonucleotides (5'-AATCTACATTGACCCTGCGTGGACAT-3') were made based on the 5'-end first 25-nucleotide sequences in PLC-γ2 ORF (Gene Tools LLC, Corvallis, OR). Standard control Morpholino oligos (5'-CCTCTTACCTCAGTTACAATTTATA-3'), having no target and no significant biological activity in THP-1 cells, were supplied by the company. The Morpholino oligos were mixed with ethoxylated polyethyleneimine and added to THP-1 cells (2 × 10⁶) in triplicate wells in a 12-well plate. THP-1 cells were rinsed 3 h later and cultured in fresh medium for 3 days to reduce the level of previously synthesized protein. After changing the medium, cells were infected with host-cell free *E.
*E. chaffeensis* On 3 d pi, infectivity was determined as previously described, and western blot analysis was carried out to determine protein levels of PLC-γ1 and PLC-γ2.

### 2.4 Results

**Effects of various compounds on *E. chaffeensis* internalization into THP-1 cells.** Since it is difficult to visually count internalized ehrlichial organisms as they are so small and so few of them are internalized, various methods such as flow cytometry of immunofluorescence-labeled organisms and [³⁵S]-methionine-labeled *N. risticii* were previously devised to determine the internalization (Rikihisa *et al.*, 1995; Rikihisa *et al.*, 1994; Messick and Rikihisa, 1993). The internalization of the majority of host cell-free ehrlichiae takes place within 3 h incubation with host cells while extracellular ehrlichiae lose infectivity by 3h (Messick and Rikihisa, 1993; Park and Rikihisa, 1991). In the present study, THP-1 cells were incubated with host cell-free *E. chaffeensis* for 3 h in the presence and absence of inhibitors. Bound but uninternalized *E. chaffeensis* were removed by pronase E treatment. Cells were further incubated for 3 days in the absence of compounds and ehrlichial organisms in the cells were counted. Since all compounds are reversible inhibitors at the concentration used and only *E. chaffeensis* that had internalized during the initial 3 h incubation period will proliferate, this method provides a simple way to determine the effect of compounds on internalization of *E. chaffeensis* by amplifying the signal (similar to colony counting for determination of viable bacteria). The concentration of compounds used in this study was determined by dose response experiments and had no direct toxic effect on host cells or ehrlichiae.
The TGase inhibitor, MDC, inhibits clustering and internalization of the ligand-receptor complexes into clathrin-coated vesicles (Abe et al., 2000; Davies et al., 1980; Levitzki et al., 1980), and thus prevents the internalization of *N. risticii* into P388D1 cells (Rikihisa et al., 1994; Messick and Rikihisa, 1993), and that of human granulocytic ehrlichiosis (HGE) agent in HL-60 cells (Yoshiie et al., 2000), but not their bindings to the host cells (Rikihisa et al., 1994; Messick and Rikihisa, 1993). Internalization of *N. risticii* in P388D1 cells was also inhibited by the PTK inhibitor genistein (Zhang and Rikihisa, 1997), the Ca\(^{2+}\) channel blocker verapamil, a calmodulin antagonist W-7, and an inhibitor of intracellular Ca\(^{2+}\) mobilization TMB-8 (Rikihisa et al., 1995). These inhibitors do not have direct ehrlichicidal effects and are not toxic to the host cells at the concentrations used (Rikihisa et al., 1995; Rikihisa et al., 1994). Like *N. risticii* in P388D1 cells, MDC, genistein, and verapamil all prevented the internalization of *E. chaffeensis* into THP-1 cells (Table 1), therefore, the internalization, or the initial establishment of infection of *E. chaffeensis* in THP-1 cells requires PTK and TGase activities, and an increase in the intracellular Ca\(^{2+}\). The present study further showed that the inhibitor of PLC, neomycin, prevents ehrlichial internalization (Table 2.1). Since preincubation of host cell-free *E. chaffeensis* with neomycin for 30 min at 37°C did not reduce ehrlichial infectivity compared to *E. chaffeensis* preincubated with RPMI medium alone (negative control), the neomycin does not seem to have an inhibitory activity directly on *E. chaffeensis* (data not shown).

**Effects of various compounds on *E. chaffeensis* proliferation in THP-1 cells.** Since most internalization of ehrlichiae takes place within 3 h of incubation at 37°C (Messick and
Rikihisa, 1993; Park and Rikihisa, 1991), compounds were added at 3 h post incubation to assess their effects on ehrlichial proliferation, independent from the inhibitory effects on initial internalization. Proliferation of *N. risticii* in P388D1 was inhibited by genistein (Zhang and Rikihisa, 1997), MDC, verapamil, and TMB-8 (Rikihisa *et al.*, 1995). These compounds also inhibited the proliferation of *E. chaffeensis* in THP-1 cells (Table 2.2). Additional Ca\(^{2+}\) modulators used in the present study were 2-APB, a cell-permeable inhibitor of IP\(_3\)-induced Ca\(^{2+}\) release (Maruyama *et al.*, 1997), and SKF-96365, an inhibitor of receptor-mediated Ca\(^{2+}\) entry (Merritt *et al.*, 1990). Both compounds significantly inhibited the proliferation of *E. chaffeensis* at a concentration that was not toxic to THP-1 cells (Table 2.2). Inhibitors of PLC, neomycin and U-73122 (Smith *et al.*, 1990), also inhibited the proliferation (Table 2.2). The effect of PLC inhibitors was dependent on the dosage and the time of addition of inhibitors (Figure 2.1): the higher the concentration and the earlier the inhibitors were added, the greater the inhibitory effects were on ehrlichial proliferation. These results indicate that the activity of TGase, PTK, PI-PLC, and an increase in [Ca\(^{2+}\)], were required for the proliferation of *E. chaffeensis* in THP-1 cells.

**Effects of ehrlichiae and various compounds on the production of IP\(_3\) by THP-1 cells.**

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and produces two well-characterized second messengers, IP\(_3\) and diacylglycerol (DAG). Since ehrlichial entry and proliferation were prevented by PLC inhibitors, the levels of IP\(_3\) before and after infection were measured to evaluate PLC activity. DAG, an activator of PKC was not assayed, since our previous data had shown that PKC activity is not required for *N. risticii* infection of
P388D₁ cells (Rikihisa et al., 1995). *E. chaffeensis* induced a rapid release of IP₃ in THP-1 cells, which reached the peak at 2 min pi, and then decreased to near the basal level by 5 min (Figure 2.2A). Furthermore, inhibitors of PLC (neomycin and U-73122), PTK (genistein) and TGase (MDC) all blocked the increase of IP₃ level induced by ehrlichiae (Figure 2.2B), indicating that IP₃ was produced by PLC in a TGase and PTK-dependent manner. Since the activation of PLC-γ isozymes requires its tyrosine phosphorylation by PTK (Singer et al., 1997; Noh et al., 1995), these results suggest that *E. chaffeensis* induces the release of IP₃ by activating a PLC-γ isozyme through tyrosine phosphorylation.

**Effects of ehrlichiae and various inhibitors on intracellular calcium level ([Ca²⁺]ᵢ) in THP-1 cells.** IP₃ binds to IP₃ receptors located on endoplasmic reticulum membrane and causes the release of stored Ca²⁺. Since Ca²⁺ channel blockers and inhibitors of intracellular Ca²⁺ mobilization blocked ehrlichial infection, intracellular calcium level was determined in THP-1 cells infected with *E. chaffeensis*. In fluo-3 loaded THP-1 cells, a rapid rise in [Ca²⁺]ᵢ, up to 300 nM, was detected at 30 s after addition of host cell-free *E. chaffeensis*, which reached a maximum by 2 min. The increase in [Ca²⁺]ᵢ was greater with higher moi (Figure 2.3A). As a control, uninfected THP-1 cell lysate did not induce an increase in [Ca²⁺]ᵢ.

Increases in [Ca²⁺]ᵢ may result from a Ca²⁺ influx from extracellular space or Ca²⁺ release from internal stores. In order to determine the relative contributions of influx and release, [Ca²⁺]ᵢ was measured in THP-1 cells preincubated with a Ca²⁺ channel blocker verapamil, intracellular calcium release inhibitor TMB-8, or a PLC inhibitor neomycin, and stimulated with host cell-free *E. chaffeensis*. As shown in Figure 2.3B, both TMB-8 and verapamil significantly prevented the ehrlichia-induced increase of [Ca²⁺]ᵢ. However,
TMB-8 and verapamil had different effects on \([\text{Ca}^{2+}]_i\). It appears that \([\text{Ca}^{2+}]_i\) elevation occurs at two phases, a rapid elevation within 60 s pi followed by a sustained phase. Verapamil inhibited the initial peak and the sustained elevation similarly, indicating Ca\(^{2+}\) influx contributes to both portions (Figure 2.3B). Whereas, TMB-8 and neomycin completely blocked the rapid peak but a delayed \([\text{Ca}^{2+}]_i\) elevation, presumably representing Ca\(^{2+}\) influx, still occurred (Figure 2.3, B and C). Furthermore, PTK and TGase activities were required for the increase in \([\text{Ca}^{2+}]_i\), since genistein and MDC almost completely prevented \([\text{Ca}^{2+}]_i\) elevation with *E. chaffeensis* infection (Figure 2.3C).

The complete inhibition of the elevation of \([\text{Ca}^{2+}]_i\), by MDC or genistein suggests that they inhibit not only the release from intracellular calcium stores, but also the influx of extracellular Ca\(^{2+}\) through calcium channels.

Since \([\text{Ca}^{2+}]_i\) measurement by fluorometer was done for a population of 10\(^6\) cells, the kinetics of \([\text{Ca}^{2+}]_i\) changes shown in Figure 2.3 reflects that of the cumulative bacteria-host cell interactions. We thus studied the kinetics of \([\text{Ca}^{2+}]_i\) changes in individual cells by fluorescence microscopy video imaging. As shown in Figure 2.4A, the calcium level in individual cells rose within 20 s after addition of ehrlichia, and dropped to the basal level within 4 min. Also, to test whether active participation of *E. chaffeensis* is required for the increase in \([\text{Ca}^{2+}]_i\), *E. chaffeensis* was pretreated for 15 min at 42 or 60°C. Results showed that the increase in \([\text{Ca}^{2+}]_i\), did not take place when *E. chaffeensis* was heat-treated (Figure 2.4A). The calcium channel blocker SKF-96365 inhibited the initial peak and the sustained phase like verapamil in the fluorometric assay, whereas a cell permeable IP\(_3\)-receptor blocker 2-APB and PLC inhibitor neomycin were more effective in inhibiting
the initial rapid $[\text{Ca}^{2+}]_i$ rise. Genistein completely prevented $[\text{Ca}^{2+}]_i$ elevation by *E. chaffeensis* (Figs. 4B and C). Therefore, the two methods of $[\text{Ca}^{2+}]_i$ measurements gave similar results. However, data obtained were slightly different between these two methods. In Figure 2.3A, after the peak elevation caused by *E. chaffeensis* addition, $[\text{Ca}^{2+}]_i$ was sustained at a higher level than the basal level prior to the addition of ehrlichiae even at 6 min pi. In contrast, the $[\text{Ca}^{2+}]_i$ level was elevated more rapidly but decreased to the basal level within 4 min in Figure 2.4A. Because *E. chaffeensis* may bind to individual host cells at different time points, the rate of initial $[\text{Ca}^{2+}]_i$ rise in a population of cells may appear slower than that in a single cell due to averaging (Figure 2.3A, 4A). Alternatively, the apparent sustained high $[\text{Ca}^{2+}]_i$ level may be due to fluo-3 leakage. The video microscopic method is not affected by the leakage of the dye because the region of interest selected for calculating the ratios contains the cell but very little surrounding solution.

**Activation of PLC-γ2 by tyrosine phosphorylation was required for *E. chaffeensis* infection.** To our knowledge, three PLC isozymes are involved in eukaryotic calcium signaling. Unlike PLC-δ, PLC-β and -γ have been well characterized. The activity of PLC-β is regulated by heterotrimeric G proteins. PLC-γ contains two src-homologous domains, SH2 and SH3, activation of which is mediated by the interaction between its C-terminal SH2 domain and the cognate tyrosine-phosphorylated peptide. The binding of PLC-γ to tyrosine phosphorylated proteins (or receptors) via SH2 domain accounts for the initial translocation of the enzyme from cytosolic to particulate fractions and the rapid phosphorylation of PLC-γ by receptor tyrosine kinases or cellular tyrosine kinases.
coupled to activated receptors. Our results demonstrated that protein tyrosine kinases were required for PLC activation by *E. chaffeensis* infection, thus the role of PLC-γ was characterized on ehrlichial infection.

Western blot analysis revealed that THP-1 cells express both PLC-γ1 and γ2 isotypes, which have a similar molecular size of 145 kDa (data not shown). By western blot analysis using anti-phosphotyrosine mAb, a band of 145 kDa was found tyrosine phosphorylated in THP-1 cells as early as 1 min after addition of *E. chaffeensis*, and the phosphorylation of this protein was inhibited by genistein and MDC (Figure 2.5A), indicating that TGase is required for tyrosine phosphorylation of this protein. In addition, an approximately 200 kDa protein was also tyrosine phosphorylated by *E. chaffeensis* infection (data not shown).

The tyrosine-phosphorylated protein of 145 kDa was confirmed to be PLC-γ2 but not PLC-γ1 by immunoprecipitation with an anti-PLC-γ2 antibody and western blotting analysis with anti-phosphotyrosine antibody (Figure 2.5B) or anti-PLC-γ2 antibody (Figure 2.5C). Western blot analysis with anti-phosphotyrosine antibody showed no band from samples immunoprecipitated with anti-PLC-γ1 antibody (data not shown). PLC-γ2 remained tyrosine phosphorylated in *E. chaffeensis* infected THP-1 cells even at 2 d pi by western blot analysis. When *E. chaffeensis* was preincubated at 42°C or 60°C for 15 min, levels of tyrosine phosphorylation of PLC-γ2 was reduced (Figure 2.5D) suggesting that viable ehrlichia or native ehrlichial proteins are required for the activation of PLC-γ2.
Furthermore, confocal immunofluorescence microscopy revealed colocalization of tyrosine-phosphorylated proteins and PLC-γ2 but not PLC-γ1 with the inclusions of *E. chaffeensis* (Figure 2.6). Tyrosine-phosphorylated proteins and PLC-γ2 were randomly distributed in the cytoplasm of uninfected THP-1 cells (data not shown). Like *N. risticii* inclusions, most tyrosine-phosphorylated proteins were concentrated on *E. chaffeensis* inclusions (Zhang and Rikihisa, 1997). It is likely that PLC-γ2 and other tyrosine-phosphorylated proteins during *E. chaffeensis* infection, as observed by western blot analysis, are also concentrated on ehrlichial inclusions.

For further confirmation, the requirement of PLC-γ2 for *E. chaffeensis* infection was tested in THP-1 cells transfected with a PLC-γ2 antisense oligonucleotide. As shown in Figure 2.7, delivery of PLC-γ2 antisense oligos into THP-1 cells significantly decreased the protein level of PLC-γ2, but not PLC-γ1, and blocked the infection of *E. chaffeensis*. The control oligos showed no effect on the PLC-γ2 protein level or *E. chaffeensis* infection. These results demonstrate that *E. chaffeensis* induces rapid tyrosine phosphorylation of PLC-γ2 required for the internalization and proliferation of *E. chaffeensis* in a TGase-dependent manner, which catalyzes the formation of IP$_3$ and mediates the elevation in [Ca$^{2+}$]. Translocation and persistent colocalization of PLC-γ2 and other tyrosine phosphorylated proteins on *E. chaffeensis* inclusions suggests that ehrlichial inclusion can actively retain these proteins which play an important role to maintain the integrity of inclusions and to facilitate ehrlichial proliferation.
2.5 Discussion

The current study demonstrated that *E. chaffeensis* induces a rapid and modest (300 nM, 3 fold) rise in \([\text{Ca}^{2+}]_i\). The rate of \([\text{Ca}^{2+}]_i\) rise and the maximum level reached may be important for the internalization and proliferation of *E. chaffeensis* in THP-1 cells. Human monocytes incubated with opsonized zymosan particles, which induce superoxide generation in monocytes (Pearson *et al.*, 1987), exhibit a rapid rise in \([\text{Ca}^{2+}]_i\) from a basal level of 75 ± 11 nM to 676 ± 78 nM by 34 ± 5s (Kim *et al.*, 1992). Salmonella induces slow elevation of \([\text{Ca}^{2+}]_i\) in an intestinal epithelial cell line, Henle-407, over a 30 min incubation period from 100 to up to 800 nM, accompanied with membrane ruffling (Pace *et al.*, 1993). Salmonella, unlike ehrlichiae induces superoxide generation in monocytes (Mott and Rikihisa, 2000; Umezawa *et al.*, 1995). Fc-receptor-mediated phagocytosis, which induces rapid superoxide generation (Anderson *et al.*, 1990), is accompanied by the elevation of \([\text{Ca}^{2+}]_i\) to around 700 nM in monocytes (Edberg *et al.*, 1995). The relatively modest level of elevation in \([\text{Ca}^{2+}]_i\) by *E. chaffeensis* may keep THP-1 cells from being activated to kill ehrlichiae. As a matter of fact, the \(\text{Ca}^{2+}\) ionophore A23187, which causes a high elevation of \([\text{Ca}^{2+}]_i\) kills *N. risticii* and *E. chaffeensis* (M. Lin and Y. Rikihisa, unpublished data) (Rikihisa *et al.*, 1995), whereas, *Salmonella* invades more efficiently into Henle-407 cells when treated with A23187 (Pace *et al.*, 1993).

The increase in \([\text{Ca}^{2+}]_i\) appears to come from both internal stores and extracellular medium since inhibitors that block calcium mobilization from internal stores or extracellular space both prevented the elevation of \([\text{Ca}^{2+}]_i\). Although an increase or requirement of \([\text{Ca}^{2+}]_i\) for establishment of infection was reported for none of obligatory
intracellular bacteria, a few facultative intracellular bacteria such as EPEC and *Salmonella typhimurium* are known to raise \([\text{Ca}^{2+}]_i\) (Pace *et al.*, 1993; Baldwin *et al.*, 1991). In the case of *S. typhimurium*, an increase in \([\text{Ca}^{2+}]_i\) is required to induce membrane ruffling and internalization (Pace *et al.*, 1993), however, internalization of *Shigella flexneri* into HeLa cells occurs without an increase in \([\text{Ca}^{2+}]_i\) (Clerc *et al.*, 1989).

Using two different assays, we showed that infection of *E. chaffeensis* causes an increase in \([\text{Ca}^{2+}]_i\) in THP-1 cells. The PLC-induced \([\text{Ca}^{2+}]_i\) increase was composed of a release of \(\text{Ca}^{2+}\) from the internal stores via IP$_3$ receptors and a subsequent influx of extracellular \(\text{Ca}^{2+}\) through receptor/store-operated channels (Berridge, 1995). The relative contributions of \(\text{Ca}^{2+}\) release and \(\text{Ca}^{2+}\) influx to the rise in \([\text{Ca}^{2+}]_i\) in THP-1 cells were examined with the use of specific inhibitors for these pathways. Two non-selective \(\text{Ca}^{2+}\) channel blockers, verapamil and SKF-96365, significantly inhibited the initial rapid elevation and the sustained phase of \([\text{Ca}^{2+}]_i\) increase, suggesting that \(\text{Ca}^{2+}\) influx contributes to the bulk of \([\text{Ca}^{2+}]_i\) rise in response to the infection. These drugs are known to inhibit the activities of voltage-gated \(\text{Ca}^{2+}\) channels as well as receptor/store-operated channels, including those formed by transient receptor potential (TRP) proteins (Zhu *et al.*, 1998). Since monocytes do not express voltage-gated \(\text{Ca}^{2+}\) channels, it is likely that the influx is mediated by the receptor/store-operated channels. Because store-operated channels are activated only after the depletion of \(\text{Ca}^{2+}\) from internal stores, inhibition of intracellular \(\text{Ca}^{2+}\) release with the use of TMB-8, 2-APB, and neomycin should also block their activities. Therefore, the delayed increase in \([\text{Ca}^{2+}]_i\) in the TMB-8, 2-APB, or
neomycin treated cells may represent the activity of receptor-operated channels that are not activated by store depletion.

The present study showed that the elevation in \([\text{Ca}^{2+}]_i\) was mediated by protein cross-linking by TGase, translocation of PLC-\(\gamma 2\) to the ehrlichial inclusion, activation of PLC-\(\gamma 2\) by tyrosine phosphorylation, and the generation of IP3. Parts of these signals are somewhat similar to those induced by EPEC, *Yersinia* sp. and *Salmonella* sp., which internalize by inducing focal assembly of microfilaments at the site of attachment. The EPEC inoculates its receptor Tir by a type III secretion mechanism into the host cell, which is tyrosine phosphorylated upon insertion into the host cell membrane. Tir serves as the receptor for the outer membrane protein, intimin binding, as the focal site of actin polymerization, and induces additional signals which subsequently activate PLC-\(\gamma 1\). The cytoskeletal rearrangement by EPEC does not involve the small GTP-binding proteins Rho, Rac, or Cdc42 (DeVinney *et al.*, 1999). The effects of EPEC on \([\text{Ca}^{2+}]_i\) are, however, inconclusive. A recent report by Bain *et al.* showed an increase in \([\text{Ca}^{2+}]_i\) was not required for the lesion caused by EPEC (Bain *et al.*, 1998). In the case of *Yersinia pseudotuberculosis*, tyrosine phosphorylation of the focal adhesion protein CAS, subsequent formation of functional CAS-Crk complexes, and the activation of the small GTP-binding protein Rac1 led to actin cytoskeleton remodeling and yersinial uptake (Weidow *et al.*, 2000). Tir-mediated uptake of EPEC (Rosenshine *et al.*, 1992b), invasin-promoted uptake of *Yersinia* sp. (Rosenshine *et al.*, 1992a), but not *Salmonella typhimurium* uptake by HeLa cells is blocked by PTK inhibitors (Rosenshine *et al.*, 1994). *S. typhimurium* microfilament assembly is directed by a type III secretion system.
Delivered proteins include SopE which induces Rho GTPase activation, and SipA, an actin binding protein (Zhou et al., 1999; Hardt et al., 1998), neither of which requires tyrosine phosphorylation. In contrast, elevation of \([\text{Ca}^{2+}]_i\) and protein tyrosine phosphorylation signals don’t lead to polymerization of microfilaments in ehrlichial infection, rather microfilaments are disassembled during *N. risticii* infection (Rikihisa, 1990a), suggesting a mechanism distinct from those described above. Genes homologous to Type III secretion machinery have not been demonstrated in ehrlichiae or in the genome sequence of a related bacterium, *Rickettsia prowazeki* (Andersson et al., 1998). The identity of ehrlichial components required for induction of these signaling events leading to ehrlichial internalization is unknown. However, the present study revealed viable ehrlichiae or ehrlichial heat sensitive components (most likely proteins) are required for the induction of tyrosine phosphorylation of PLC-\(\gamma2\) and increases in \([\text{Ca}^{2+}]_i\).

It is unclear why \(\text{Ca}^{2+}\) channel blockers or inhibitors of intracellular \(\text{Ca}^{2+}\) release inhibit not only ehrlichial internalization, but also proliferation, since whole cell \([\text{Ca}^{2+}]_i\) was not continuously elevated during the course of ehrlichial infection. Paraphagosomal \([\text{Ca}^{2+}]_i\) was reported to be higher than surrounding cytosol in human monocytes, although whole cell \([\text{Ca}^{2+}]_i\) is not elevated compared to the control monocytes (Kim et al., 1992). Likely, local \([\text{Ca}^{2+}]_i\), surrounding ehrlichial inclusions may be higher than the remaining cytosol, and this local higher \([\text{Ca}^{2+}]_i\) may be required for ehrlichial proliferation.

Reasons why elevation of \([\text{Ca}^{2+}]_i\) is required for ehrlichial internalization and proliferation are unknown. Perhaps one reason is that ehrlichial internalization is dependent on a calcium-dependent enzyme, TGase, and microtubules (Rikihisa et al.,
1994). The second reason is that to accommodate proliferating ehrlichial organisms, new membranes should be added and iron and other nutrients should be supplied by fusion of ehrlichial inclusions with transferrin receptor endosomes (Barnewall et al., 1997), which are Ca$^{2+}$-calmodulin and TGase dependent process (Grasso et al., 1990; Hebbert and Morgan, 1985).

A striking finding of the present study is that PLC-$\gamma$2 and tyrosine-phosphorylated proteins were rapidly recruited and retained with ehrlichial inclusions even at 2 d pi, or probably within its entire intracellular life span. *E. chaffeensis* proliferates within membrane-bound vacuoles that display early endosomal markers (Mott et al., 1999; Barnewall et al., 1997) and do not fuse with lysosomes. The continued colocalization of PLC-$\gamma$2 and tyrosine phosphorylated proteins, the latter is also observed with *N. risticii* inclusions (Zhang and Rikihisa, 1997), may be required to maintain the inclusion status. This may explain why inhibitors of PTK and PLC inhibit both ehrlichial entry and proliferation in host cells. Moreover, the spreading of *E. chaffeensis* requires exocytosis of *E. chaffeensis* or lysis of host cell and subsequent endocytosis by another cell, which also requires the intracellular Ca$^{2+}$ mobilization and tyrosine phosphorylation, thus are inhibited by these inhibitors.

The inhibition of all of these signaling pathways as well as internalization and proliferation of *E. chaffeensis* by MDC indicates that activation of TGase may be the most upstream event known and is triggered by the binding of *E. chaffeensis*, although the mechanism of TGase activation is unclear. In our ongoing study, THP-1 cells showed a constitutive TGase activity, and its *in situ* activity rapidly increased as early as 30 sec pi.
upon addition of *E. chaffeensis* (M. Lin and Y. Rikihisa, unpublished data). TGase is required for ligand-receptor complex clustering and endocytosis of various ligands such as viruses, LDL, α₂-macroglobulin, epidermal growth factor, transferrin, and polypeptide hormones like insulin (Abe *et al.* 2000; Smethurst and Griffin, 1996; Davies *et al.*, 1980; Levitzki *et al.*, 1980; Maxfield *et al.*, 1979). The present results suggest that TGase has a more extensive role for *E. chaffeensis* in transducing signals for entry as well as sustaining its proliferation. Which proteins are transglutaminated and how these proteins induce tyrosine phosphorylation of PLC-γ2 remains to be studied. The *E. chaffeensis* inclusion is a part of the transferrin and transferrin receptor membrane recycling pathway as indicated by the uptake of FITC-conjugated iron-saturated transferrin by the inclusion (Barnewall and Rikihisa, 1994). Since the internalization and recycling of transferrin and transferrin receptor are dependent on TGase (Kohno and Tokunaga, 1985), this is another site where MDC can block the ehrlichial proliferation in host cells after its entry. Transferrin receptors can deliver iron to the cytoplasm through a continual cycle that shuttles the ligand transferrin between the endosomal compartments and the plasma membrane. Iron is essential for ehrlichial survival, since ehrlichiae are extremely sensitive to the intracytoplasmic iron chelator deferoxamine (Barnewall *et al*., 1997).

TGase is a calcium-dependent enzyme, and our current data showed that it is also able to indirectly regulate intracellular calcium by activation of tyrosine kinases-PLC-γ2 pathway. This implies that a positive feedback loop consisting of Ca²⁺-TGase may exist in facilitating ehrlichial entry and infection. As summarized in Figure 2.8, the present study demonstrated that *E. chaffeensis* activates the following signaling sequences
required for internalization: activation of TGase, protein tyrosine phosphorylation, PLC-γ2 activation, production of IP₃, increase in [Ca²⁺].
Table 2.1. Effect of various inhibitors on internalization of *Ehrlichia chaffeensis*.

Reagents were added to THP-1 cells at 1 h before infection. At 3 h pi, cells were centrifuged and treated with 2 mg/ml pronase E for 3 min to remove bound uninternalized *E. chaffeensis* and cells were continuously cultured. Internalized *E. chaffeensis* were determined by counting organisms at 3 d pi. Data were expressed as means ± standard deviations (n = 3).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ehrlichial organisms per 100 cells</th>
<th>% infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6030 ± 400</td>
<td>94.1 ± 2.2</td>
</tr>
</tbody>
</table>

**Inhibitor of Protein Tyrosine Kinases**
- Genistein (100 µM) 90 ± 20 7.9 ± 18

**Inhibitors of Phospholipase C (PLC)**
- Neomycin (10 µM) 75 ± 25 2.5 ± 0.9
- U73122 (10 µM) 485 ± 105 25.7 ± 3.6

**Inhibitors of Intracellular Ca^{2+} Release**
- TMB-8 (100 µM) 148 ± 30 18.8 ± 2.8
- 2-APB (75 µM) 475 ± 19 30.7 ± 4.6

**Inhibitors of Ca^{2+} Influx**
- Verapamil (100 µM) 12 ± 2 1.4 ± 0.4
- SKF-96365 (60 µM) 195 ± 60 8.6 ± 0.5

**Inhibitor of Transglutaminase**
- MDC (250 µM) 0 0

Table 2.2. Effect of various inhibitors on proliferation of *Ehrlichia chaffeensis*.

Reagents were added to THP-1 cells at 3 h pi and present throughout the incubation. Cells were infected with host cell-free *E. chaffeensis* at moi of 100, and infectivities were determined at 3 d pi. Data were expressed as means ± standard deviations (n = 3).
Figure 2.1. Effects of PLC inhibitors on infection of *Ehrlichia chaffeensis*.

PLC inhibitors neomycin and U73122 blocked the infection of *E. chaffeensis* in: (A) dose dependent (neomycin and U73122 were added 1 h prior to ehrlichial infection), or (B) time dependent manner (10 μg/ml neomycin was added to THP-1 cells before or after infection as indicated). Infectivity was determined at 3 days pi as described in “Materials and Methods”. Data are expressed as means ± standard deviations (n = 3), and are representative results from more than three independent experiments.
Figure 2.2. Effects of ehrlichial infection and inhibitors on the production of IP₃ in THP-1 cells. (A) The rapid release of IP₃ was induced by ehrlichial infection that reached the peak (approximately 3 fold) at 1.5 min pi and decreased to background level within 5 min; (B) the release of IP₃ induced by *E. chaffeensis* (EC) was prevented by pretreatment with inhibitors of PTK, PLC, and TGase.

Cells were pretreated with or without inhibitors at 1 h before ehrlichial infection, and IP₃ samples were extracted by perchloric acid at the time indicated (A) or at 1.5 min pi (peak release of IP₃) (B). The release of IP₃ was determined by measuring competitive binding inhibition of [³H]-labeled IP₃ to IP₃-binding protein. Data are expressed as means ± standard deviations (n = 3), and the result is a representative of three independent experiments.
Figure 2.3. Effect of ehrlichial infection and inhibitors on \([Ca^{2+}]_i\), in population of cells. 

(A) \([Ca^{2+}]_i\) was elevated rapidly and transiently in THP-1 cells by infection with \(E. chaffeensis\) (E.C.), but the increase was prevented by pretreatment with (B) inhibitors of calcium mobilization (verapamil and TMB-8), or (C) inhibitors of PLC (neomycin), PTK (genistein), and TGase (MDC).

Intracellular calcium levels were determined in fluo-3-loaded THP-1 cells using a fluorometer as described in “Materials and Methods”. Various inhibitors were added to cells 1 h before the assay, and host cell-free \(E. chaffeensis\) was added to THP-1 cells at the time point indicated by a vertical arrow (at approximate 50 s). Assays were carried out at 30°C in a circulating water bath. Fluorescence was measured at 525 nm at 5 s interval, and \(K_d\) was calibrated using calcium calibration kit from Molecular Probes. The result is representative from at least four independent experiments.
Figure 2.4. Effect of ehrlichial infection and inhibitors on \([Ca^{2+}]\), in an individual cell.

The rapid and transient elevation in intracellular calcium (as shown by the ratio of emission of Fura-2 at 520 nm under stimulation of 340/380 nm) was induced by \(E.\ \text{chaffeensis}\) infection in an individual THP-1 cell (A). The increase in \([Ca^{2+}]\), required the active participation of live \(E.\ \text{chaffeensis}\) since heat-treated (42°C or 60°C, 15 min) \(E.\ \text{chaffeensis}\) (E.C.) did not raise \([Ca^{2+}]\). However, the rise in \([Ca^{2+}]\), induced by ehrlichial infection was prevented by calcium mobilization inhibitors SKF-96365 and 2-APB (B), or by PLC inhibitor neomycin and PTK inhibitor genistein (C). THP-1 cells were loaded with fura-2/AM and treated with or without inhibitors for 1 h before measuring. Cells were added to poly-L-Lysine coated coverslip chambers, and images were recorded at emission 520 nm under 340/380 nm excitation at 1 s interval for at least 5 min. Host cell-free \(E.\ \text{chaffeensis}\) was added to the chambers after measuring for 30 s (a vertical arrow). The result is representative of at least three independent experiments with similar results measured in more than 20 cells for each experiment.
Figure 2.5. Tyrosine phosphorylation of PLC-\(\gamma\)2 in ehrlichial infection.

(A) A 145 kDa protein was rapidly tyrosine phosphorylated in THP-1 cells upon the binding of \textit{E. chaffeensis} (EC). This protein was confirmed to be PLC-\(\gamma\)2 by immunoprecipitation with anti-PLC-\(\gamma\)2 antibody and western blotting analysis by detection with (B) anti-phosphotyrosine (pTyr) antibody, or (C) anti PLC-\(\gamma\)2 antibody. Tyrosine phosphorylation of PLC-\(\gamma\)2 was reduced when \textit{E. chaffeensis} was pretreated at 42°C or 60°C for 15 min (D).

THP-1 cells were pretreated with genistein (Gen) or monodansylcadaverine (MDC) for 1 h prior to the addition of \textit{E. chaffeensis} (EC). At the indicated time point, cells were lysed in RIPA buffer. Whole cell lysates were immunoprecipitated with anti-PLC-\(\gamma\)2 antibody, and the immunoprecipitates or whole cell lysates were subjected to western blot using anti-phosphotyrosine antibody or anti-PLC-\(\gamma\)2 antibody. The result is representative of more than 4 independent experiments with similar results.
### Figure 2.5

<table>
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<th>Treatments:</th>
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<th>+MDC</th>
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<tbody>
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<td>0'</td>
<td>1'</td>
<td>2'</td>
<td>10'</td>
</tr>
<tr>
<td></td>
<td>2'</td>
<td>2'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**A.** WB: pTyr

- 145 kDa

**B.** IP: PLC-γ2

- WB: pTyr

- 145 kDa

**C.** IP: PLC-γ2

- WB: PLC-γ2

- PLC-γ2

**D.** WB: pTyr

- THP-1 EC 2' EC 5' 42°C treated EC 2' EC 5' 60°C treated EC 5'

- 145 kDa
Figure 2.6. Colocalization of *E. chaffeensis* inclusions with PLC-γ2 and tyrosine-phosphorylated proteins, but not with PLC-γ1.

*E. chaffeensis*-infected THP-1 cells (2 d pi) were double labeled as described and observed under confocal microscopy. Antibodies labeled are: *E. chaffeensis* (green, left panels); PLC-γ1, γ2 or phosphotyrosine (red, middle panels); and superimposed images under green and red filters (right panels). Note colocalization of *E. chaffeensis* with PLC-γ2 and phosphotyrosine (yellow). These results are representative from three independent labeling experiments. Bar indicates the length of 10 µm.
Figure 2.6
Figure 2.7. The effect of PLC-γ2 antisense oligonucleotides on *E. chaffeensis* infection.

(A) Delivery of PLC-γ2 antisense oligos into THP-1 cells significantly reduced the protein level of PLC-γ2 but not PLC-γ1, compared to the untransfected cells or cells transfected with standard control oligo as examined by western blotting. (B) Delivery of PLC-γ2 antisense oligos to THP-1 cells also significantly reduced *E. chaffeensis* infection > 50% (P < 0.05).

THP-1 cells were transfected with anti-PLC-γ2 oligos or standard oligos control as described in “Materials and Methods”. Three days after the delivery of antisense oligos, cells were infected with host cell-free *E. chaffeensis*. On 3 d pi, infectivity was determined and cells were extracted using RIPA buffer, and western blot analysis was carried out to determine the protein level of PLC-γ1 and PLC-γ2. Data are representative results of three independent experiments.
Figure 2.8. Model of signaling pathway induced by infection of *E. chaffeensis*.

The binding of *E. chaffeensis* to its receptor(s) on host monocytes triggers protein crosslinking by TGase, which activates the receptor- or nonreceptor- tyrosine kinase. PTK activation mediates the translocation and tyrosine phosphorylation of PLC-γ2 that produces IP₃. IP₃ in turn induces Ca²⁺ release from internal stores. The depletion of Ca²⁺ from internal stores activates store-operated calcium channels on the plasma membrane, and Ca²⁺ influx from external spaces contributes to the bulk of [Ca²⁺]ᵢ rise in response to the infection. Activation of TGase by Ca²⁺ constitutes a positive feedback loop and this may facilitate the entry or infection of ehrlichiae in host cells. Dotted lines indicate suggested pathways, whereas solid lines indicate proven pathways.
CHAPTER 3

OBLIGATORY INTRACELLULAR PARASITISM BY HUMAN MONOCYTIC AND GRANULOCYTIC EHRLICHIOSIS AGENTS INVOLVES CAVEOLAE AND GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEINS

3.1 Abstract

Obligatory intracellular, human ehrlichiosis agents *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* create unique replicative compartments devoid of lysosomal markers in monocytes/macrophages and granulocytes, respectively. The entry of these bacteria requires host phospholipase C (PLC)-γ2 and protein tyrosine kinases but their entry route is still unclear. Here, using specific inhibitors, double immunofluorescence labeling, and the separation of lipid rafts, we demonstrated that these bacterial entry and intracellular infection involves caveolae and glycosylphosphatidylinositol (GPI)-anchored proteins. Caveolae were found to be the site where tyrosine-phosphorylated proteins and PLC-γ2 are initially assembled. Caveolar marker protein caveolin-1 was colocalized in both early inclusions and replicative inclusions. In contrast, clathrin was not found in any inclusions of either bacterium. An early endosomal marker, transferrin receptor, was not present in the early inclusions of *E.*
*chaffeensis*, but was found in replicative inclusions of *E. chaffeensis*. The formation of bacteria-encapsulating caveolae, which assemble and retain signaling molecules essential for bacterial entry, and interact with the recycling endosome pathway, may ensure survival of these obligatory intracellular bacteria in primary host defensive cells.

### 3.2 Introduction

*Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, agents of human monocytic ehrlichiosis (HME) and human granulocytic ehrlichiosis (HGE), respectively, are recently discovered pathogens in the United States and other parts of the world. HME and HGE, collectively called human ehrlichioses, are emerging tick-borne zoonoses characterized by fever, headache, myalgia, anorexia and chills and are frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases (Paddock and Childs, 2003; Dumler and Bakken, 1998; Walker and Dumler, 1996). Human ehrlichioses frequently require hospitalization, and 2 to 4% of reported cases are fatal, especially in immunocompromised patients (Dumler and Bakken, 1998; Bakken *et al.*, 1996; Walker and Dumler, 1996). With increasing suburbanization and outdoor activities, human ehrlichioses pose significant health threats and become one of the nationally reportable diseases since 1998 (McQuiston *et al.*, 1999).

*E. chaffeensis* and *A. phagocytophilum* are gram-negative obligatory intracellular bacteria in the family *Anaplasmataceae* (Dumler *et al.*, 2001). *E. chaffeensis* and *A. phagocytophilum* exclusively parasitize monocytes-macrophage and neutrophils, respectively, and their entire intracellular life stages in human professional phagocytes are confined inside membrane-bound inclusions (Rikihisa, 2003; Webster *et al.*, 1998).
However, it is still unclear how members in the family *Anaplasmataceae* enter their host cells. One study showed that the binding and internalization of *A. phagocytophilum* to HL-60 cells is dependent on P-selectin glycoprotein ligand (PSGL)-1 (Herron *et al.*, 2000). In a related organism in the family *Anaplasmataceae*, *Neorickettsia risticii*, which causes Potomac horse fever, the binding and internalization of this bacterium into the host macrophage were blocked by the treatment of anti-*N. risticii* Fab fragment, suggesting the involvement of a cognitive host cell receptor for the bacterial surface antigen in the bacterial entry (Messick and Rikihisa, 1994). Other studies showed that replicative inclusions of *E. chaffeensis* and *A. phagocytophilum* are not colocalized with clathrin (Mott *et al.*, 1999; Barnewall *et al.*, 1997). Furthermore, these bacteria are highly sensitive to monodansylcadaverine, an inhibitor of transglutaminase involved in receptor-mediated endocytosis (Levitzki *et al.*, 1980), but are insensitive to cytochalasins that depolymerize microfilaments (Lin *et al.*, 2002; Yoshiie *et al.*, 2000; Barnewall *et al.*, 1999; Rikihisa *et al.*, 1994; Messick and Rikihisa, 1993). These studies suggested that these bacteria enter host monocytes or neutrophils through an unconventional route: by clathrin-independent, probably receptor-mediated endocytosis, rather than by phagocytosis.

Several unique signaling events, including protein tyrosine phosphorylation, phospholipase C (PLC)-γ2 activation, and calcium influx are triggered and required for the entry and proliferation of *E. chaffeensis*, *A. phagocytophilum*, and other members in the family *Anaplasmataceae* (Lin *et al.*, 2002; Mott *et al.*, 2002; Zhang and Rikihisa, 1997; Rikihisa *et al.*, 1995). Tyrosine phosphorylated proteins and PLC-γ2 colocalize
with the replicative inclusions of *E. chaffeensis*, indicating their role in the proliferation of these bacteria (Lin et al., 2002). However, bactericidal signals in their host monocytes-macrophages and neutrophils, such as the assembly of NADPH oxidase components in the inclusion, superoxide generation, and phagolysosomal fusions are not triggered or even actively blocked during their entry and subsequent growth (Rikihisa, 2003; Mott et al., 2002; Mott and Rikihisa, 2000).

After their entry, *E. chaffeensis* and *A. phagocytophilum* replicate in unique membrane-bound inclusions within host cytoplasm. These inclusions do not highly acidify or mature into phagolysosomes (Barnewall et al., 1999; Mott et al., 1999; Webster et al., 1998; Barnewall et al., 1997). Instead, they are colocalized with vesicle associated membrane protein 2 (VAMP-2, or synaptobrevin-2) and some major histocompatibility complex (MHC) class I or class II surface antigens and \(\beta_2\) microglobulin that are usually not found in early endosomes (Barnewall et al., 1999; Mott et al., 1999; Barnewall et al., 1997; Brumell et al., 1995). Notably, the replicative inclusions of *E. chaffeensis* and Neorickettsia sennetsu (the agent of human Sennetsu fever) in the family *Anaplasmataceae* accumulate transferrin receptor (TfR) and have several early endosomal markers, such as early endosomal antigen 1 (EEA1) and rab5; whereas the inclusions of *A. phagocytophilum* are negative for these endosomal markers (Barnewall et al., 1999; Mott et al., 1999; Barnewall et al., 1997). These data suggest that the replicative inclusions of *E. chaffeensis* and *A. phagocytophilum* are different from any other intracellular compartments known to date.
Lipid rafts are specialized lipid microenvironments on the cell surface characterized by their light buoyant density and resistance to nonionic detergents. They are enriched with cholesterol, glycosphingolipid G\textsubscript{M1} ganglioside, glycosylphosphatidylinositol (GPI)-anchored proteins, and several membrane proteins involved in signal transduction including receptors, signal transducers and membrane transporters (Simons and Toomre, 2000; Anderson, 1998). Caveolae, which are typically flask-shaped, but can be variously shaped invaginations at the cell surface, are formed from lipid rafts by polymerization of caveolae-specific proteins, caveolins (Duncan \textit{et al.}, 2002; Anderson, 1998). Caveolae provide endocytic and exocytic trafficking pathways to import molecules to specific locations within the cell or export molecules to extracellular spaces in a clathrin-independent manner. Caveolae are also implicated in compartmentalizing a variety of signaling activities (Simons and Toomre, 2000; Anderson, 1998). As it is a vesicle trafficking system that bypasses phagolysosomal pathways, several intracellular bacteria are found to utilize lipid rafts or caveolae to enter the host cells to avoid their degradation in lysosomes (Jutras \textit{et al.}, 2003; Duncan \textit{et al.}, 2002; Norkin \textit{et al.}, 2001; Shin and Abraham, 2001a).

In the present study, we characterized the involvement of caveolae and GPI-anchored proteins in the binding, internalization, and proliferation of \textit{E. chaffeensis} and \textit{A. phagocytophilum} in their host cells. In contrast to other bacteria that use caveolae for their entry, our results showed the involvement of caveolae throughout the intraleukocyte stage of life cycles of \textit{E. chaffeensis} and \textit{A. phagocytophilum} by retaining signaling molecules and by interacting with endosome recycling pathway.
3.3 Materials and Methods

**Cell lines and bacteria.** *E. chaffeensis* Arkansas strain and *A. phagocytophilum* HZ strain were cultivated in human leukemia cell lines: THP-1 cells and HL-60 cells, respectively (Rikihisa et al., 1997; Barnewall and Rikihisa, 1994). Cells and bacteria were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2% L-glutamine at 37°C in 5% CO\(_2\) and 95% air. No antibiotic was used throughout the study.

Host cell-free *E. chaffeensis* or *A. phagocytophilum* were prepared by sonication for 8 s with an ultrasonic processor W-380 (Heat Systems, Farmington, NY) at an output setting of 2. After low speed centrifugation to remove nuclei and unbroken cells, the supernatant was centrifuged at 10,000 \(\times\) g for 10 min, and the pellet enriched with host cell-free organisms was collected. Multiplicity of infection (moi) of 100 was used as determined previously (Lin et al., 2002).

**Antibodies.** *E. chaffeensis* and *A. phagocytophilum* were detected by antisera developed in our laboratory, including dog anti-*E. chaffeensis* serum preabsorbed with THP-1 cells and horse anti-*A. phagocytophilum* serum preabsorbed with HL-60 cells (Zhi et al., 1998; Barnewall et al., 1997). To detect components of caveolae or lipid rafts, commercially available agents were used, including rabbit anti-caveolin-1 antibody (Santa Cruz Biotech, Santa Cruz, CA) and biotin-conjugated cholera toxin B subunit (CTB, for specific binding of G\(_{M1}\) ganglioside) (Molecular Probes, Eugene, OR). Other antibodies used in this study were mouse anti-transferrin receptor (TfR) (Ancell, Bayport, MN), mouse anti-LAMP-1 (Transduction Laboratories, San Diego, CA), clathrin heavy chain (CHC) (Transduction Laboratories), rabbit anti-phospholipase C-\(\gamma\)2 (Santa Cruz), and mouse
anti-phosphotyrosine (PY99, Santa Cruz). Secondary antibodies used include Lissamine rhodamine-conjugated goat anti-dog and anti-horse antibodies, FITC-conjugated goat anti-rabbit and mouse antibodies (Jackson Immunoresearch, West Grove, PA). Alex Fluor 488-conjugated streptavidin (Molecular Probes) was used to detect biotin-conjugated CTB.

**Treatments of bacteria and evaluation of infection.** To determine the role of caveolae or lipid rafts on the bacterial infection, host cells were pretreated at 37°C for 30 min with 10 mM methyl-β cyclodextrin (MβCD) (Sigma, St. Louis, MO), 20 µg/ml water-soluble cholesterol (Sigma), 240 U/ml nystatin (Sigma), 40 µg/ml cholera toxin B subunit (Calbiochem, San Diego, CA), 1 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma), or 10 µg/ml 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) (Molecular Probes). Alternatively, cells were treated with the above reagents at varying concentrations or time points as indicated in the figure legends. Cells were washed with PBS prior to the addition of host cell-free bacteria. The infected cells were further cultured for 3 days in the absence of reagents, and the numbers of *E. chaffeensis* or *A. phagocytophilum* organisms were estimated in 100 cells each in triplicate culture wells as previously described (Lin *et al.*, 2002).

**Fluorescence labeling.** Infected and uninfected host cells (2 × 10⁶) were fixed in 3% paraformaldehyde at room temperature (RT) for 15 min, and incubated with primary antibodies in PBS (pH 7.4) containing 0.1% gelatin and 0.3% saponin for 1 h at RT with rotation. After washing twice with PBS, cells were incubated with fluorescence conjugated secondary antibodies for 30 min. Cells were then washed, cytocentrifuged
onto glass slides, and observed under a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microsystems, Germany). To test the binding and internalization of bacteria to host cells, THP-1 or HL-60 cells were pretreated with 10 mM MβCD or 1 U/ml PI-PLC for 30 min at 37°C and washed with PBS. Cells were then incubated with host cell-free *E. chaffeensis* or *A. phagocytophilum* at 37°C for 5 or 30 min. Cells were washed twice with PBS and fluorescence labeling was performed as described. Numbers of bound or internalized bacteria were counted under a Nikon Eclipse E400 fluorescence microscope (Nikon, Melville, NY).

**Isolation of lipid rafts and western blot analysis.** Based on their low-density and resistance to solubilization by Triton X-100 at 4°C, lipid rafts were prepared by an equilibrium sucrose density gradient centrifugation as previously described (Gatfield and Pieters, 2000; Sargiacomo *et al.*, 1993). Briefly, THP-1 cells (2 × 10^7) were incubated with or without host cell-free *E. chaffeensis* at 37°C for 1 h. Samples were then solubilized in 2 ml Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) containing 1% Triton X-100, and a cocktail of protease inhibitors (Calbiochem) on ice for 1 h without agitation. The cell lysates (2 ml) were mixed with an equal volume of 80% sucrose in MBS, overlayed with 2 ml each of 35, 25, 15, 5% sucrose in MBS, and centrifuged for 18 h at 39,000 rpm in a SW41Ti rotor (Beckman Instruments, Palo Alto, CA). Eleven equal fractions of 1 ml each were collected from the top of the gradient and western blot analysis was performed. Specific antibodies were used to detect host proteins, such as caveolin-1, clathrin heavy chain, transferrin receptor, and PLC-γ2, and bacterial proteins in these fractions (Lin *et al.*, 2002).
3.4 Results

Disruption of caveolae or lipid rafts prevented internalization and subsequent infection of leukocytes with *E. chaffeensis* and *A. phagocytophilum*. To study the role of the caveolae or lipid rafts on *E. chaffeensis* and *A. phagocytophilum* infection, we first examined the effects of disrupting the organization of lipid rafts or caveolae. Since cholesterol is an essential component of caveolae or lipid rafts, removal of cholesterol or the alteration in cholesterol structure results in the disruption of lipid rafts (Simons and Toomre, 2000). THP-1 and HL-60 cells were pretreated for 30 min with MβCD (which removes cholesterol), with nystatin (which binds cholesterol), or with NBD-cholesterol (a cholesterol derivative that can be incorporated in place for cholesterol) (Mukherjee *et al.*, 1998). After washing to remove extracellular reagents, *E. chaffeensis* or *A. phagocytophilum* were added to THP-1 or HL-60 cells, respectively, and the numbers of bacteria in the host cells were scored after incubation at 37°C after 3 d pi. Since these bacteria are obligatory intracellular bacteria, only viable bacteria that have internalized into the permissive compartments within a few hours of incubation can survive and further proliferate in their host cells. Extracellular bacteria failing to enter die out. Thus, to determine the role of cholesterol on ehrlichial infection, we first counted the number of bacteria at 3 d pi, which is proportional to the number of viable bacteria that have entered the permissive compartments as previously described (Lin *et al.*, 2002). This method is similar to a colony counting method used for quantitating viable bacteria that are not obligatory intracellular. As shown in Figure 3.1, the inhibition of bacterial infection by MβCD was dose dependent, and the maximum inhibitory effect on *E. chaffeensis* was
achieved at 10 mM (Figure 3.1A). The inhibitory effect was partially reversed by supplementation with water-soluble cholesterol during MβCD pretreatment (Figure 3.1B). Lipid raft disruption by nystatin (Figure 3.1C) or NBD-cholesterol (Figure 3.1D) also specifically blocked infection by these bacteria.

We also determined the effect of MβCD pretreatment on the internalization of *E. chaffeensis* and *A. phagocytophilum* at early internalization period. Since these bacteria are very small and only a few bacteria bind and internalize per leukocyte, immunofluorescent microscopy was required. Results showed that the binding and internalization of both organisms at 30 min pi were significantly blocked by the pretreatment of host cells with MβCD (Figure 3.1F). These results suggest that cholesterol is essential for the internalization and subsequent proliferation of *E. chaffeensis* and *A. phagocytophilum*.

To test the role of caveolae or lipid rafts in the internalization of these bacteria, we pretreated host cells with cholera toxin subunit B (CTB), which is known to bind GM1 ganglioside clustered within caveolae and triggers caveolae-mediated internalization of the toxin, thus depleting surface caveolae (Orlandi and Fishman, 1998; Montesano *et al.*, 1982; Heyningen, 1974). Pretreatment of host cells with 40 µg/ml of CTB resulted in up to 90% inhibition in the numbers of *E. chaffeensis* or *A. phagocytophilum* in host cells, suggesting that the depletion of surface caveolae prevented the entry of these bacteria and their subsequent infection of the host cells (Figure 3.1E).

**GPI-anchored proteins are required for the internalization of *E. chaffeensis* and *A. phagocytophilum***. GPI-anchored proteins are mainly located in lipid rafts or caveolae
(Anderson, 1998), and are implicated in the entry of a few facultative intracellular bacteria, such as FimH-expressing *Escherichia coli*, *Mycobacterium kansasii*, and *Brucella* spp. (Watarai et al., 2002; Shin and Abraham, 2001b; Peyron et al., 2000). We thus tested if GPI-anchored proteins are required for the internalization of *E. chaffeensis* and *A. phagocytophilum*. The removal of surface exposed GPI-anchored proteins by PI-PLC pretreatment effectively inhibited the internalization and thus the proliferation of *E. chaffeensis* and *A. phagocytophilum* in their host cells as determined at 3 d pi (Figure 3.2A). Immunofluorescence labeling showed that PI-PLC pretreatment also significantly reduced the number of *E. chaffeensis* and *A. phagocytophilum* that bound or internalized after 30 min incubation with the host cells (Figure 3.2B), indicating that GPI-anchored proteins are involved in the internalization of these bacteria.

**Caveolin-1, tyrosine phosphorylated proteins and PLC-γ2 were colocalized with early inclusions containing *E. chaffeensis* and *A. phagocytophilum**. We found that several predominant constituents of caveolae or lipid rafts, such as cholesterol and GPI-anchored proteins, were essential for the entry and subsequent infection of *E. chaffeensis* and *A. phagocytophilum*. To determine whether caveolae are involved in internalization, we examined if caveolin-1, the membrane protein found exclusively in caveolae, exists in the early intracellular inclusions of *E. chaffeensis* and *A. phagocytophilum*. Double immunofluorescence labeling revealed that in cells fixed at 5 or 30 min after incubation with bacteria at 37°C, caveolin-1 was colocalized with both *E. chaffeensis* and *A. phagocytophilum*-containing early inclusions (approximately 0.5 µm in diameter, and
contain only one organism) in the peripheral cytoplasm. Figure 3.3 shows micrographs after 30 min incubation. Similar results were obtained after 5 min incubation except that numbers of bound or internalized bacteria were less than those after 30 min incubation.

Our previous studies showed that protein tyrosine phosphorylation and the activation of PLC-γ2 are rapidly induced and required for the entry and infection of *E. chaffeensis* and *A. phagocytophilum* (Lin *et al.*, 2002; Mott *et al.*, 2002). Double immunofluorescence labeling revealed that both tyrosine-phosphorylated proteins (Figure 3.4A-F) and PLC-γ2 (Figure 3.4G-L) were colocalized the early inclusions of *E. chaffeensis* and *A. phagocytophilum* as early as 5 min pi. These results suggested that caveolae were the site where binding and signaling events take place for the internalization of *E. chaffeensis* and *A. phagocytophilum* prior to the formation of replicative inclusions in their host cells.

Several *E. chaffeensis* proteins and host protein PLC-γ2 were detected in the lipid raft fractions of infected cells. Because of their light buoyancies and resistance to detergent such as Triton X-100 at 4°C, lipid rafts can be separated from other membrane domains by fractionation of cell lysates (Anderson, 1998). By sucrose density gradient centrifugation, lipid rafts fractions are migrated to the region between 15 and 25% sucrose (Shin *et al.*, 2000). As shown in Figure 3.5, the lipid raft fractions from both uninfected and infected cells were located between 15 and 25% sucrose (fractions 4 and 5, Figure 3.5A-B) as identified by the presence of the caveolar marker, caveolin-1. Triton X-100 soluble fractions were located mainly in 40% sucrose (fractions 9 and 10), where
proteins associated with clathrin-dependent endocytosis, such as clathrin heavy chain and transferrin receptor were found (Figure 3.5E-H). Studies have shown that some intracellular bacteria, such as FimH-expressing *Escherichia coli* and the elementary body of *Chlamydia trachomatis* can even be cofractionated with lipid rafts or caveolae membrane complexes (Jutras *et al.*, 2003; Shin *et al.*, 2000). We thus tested whether bacterial proteins or some host molecules that are required for the entry of *E. chaffeensis* could be found in the lipid rafts isolated from the infected cells. In THP-1 cells infected with *E. chaffeensis* for 1 h at 37°C, *E. chaffeensis* antigenic proteins with approximate molecular sizes of 30, 45, 55, and 70 kDa were detected in the light-buoyant raft fractions 4 and 5 (Figure 3.5I-J). Notably, host protein PLC-γ2, which is essential for the entry and infection of *E. chaffeensis* (Lin *et al.*, 2002), also cofractionated to the light-buoyant raft fractions in *E. chaffeensis*-infected THP-1 cells, but not in uninfected cells (Figure 3.5C-D). In contrast, transferrin receptor and clathrin heavy chain remained in Triton X-100-soluble membrane fractions in *E. chaffeensis*-infected cells (Figure 3.5E-H).

**Caveolar components caveolin-1 and G_{M1} ganglioside, but not clathrin were colocalized with the replicative inclusions of *E. chaffeensis* and *A. phagocytophilum*.**

Since the replicative inclusions of *E. chaffeensis* retain PLC-γ2 and tyrosine phosphorylated proteins (Lin *et al.*, 2002), we examined whether caveolar components were present in the replicative inclusions of *E. chaffeensis* as well as *A. phagocytophilum*. Fluorescence labeling revealed that replicative inclusions of approximate 2 ~ 4 µm in diameter containing a number of *E. chaffeensis* or *A. phagocytophilum* organisms at 2 d pi, were colocalized with caveolar components such as caveolin-1 and G_{M1} ganglioside.
(Figure 3.6, arrows). At 2 d pi, we also found small inclusions of *E. chaffeensis* or *A. phagocytophilum* (approximately 0.5 µm in diameter) in the peripheral cytoplasm (arrowheads in Figure 3.6) that appeared to be newly internalized, suggesting that the second cycle of infection had begun. Similar to the initial internalization process at 30 min pi shown in Figure 3.3, caveolin-1 was also found in these small inclusions of *E. chaffeensis* and *A. phagocytophilum* at 2 d pi. Clathrin heavy chain was neither colocalized with the small inclusions of *E. chaffeensis* in the peripheral cytoplasm (Figure 3.7D-F, arrow) nor in larger replicative inclusions (Figure 3.7D-F). In agreement with immunoblotting of fractionations of *E. chaffeensis*-infected THP-1 lysates at 1 h pi (Figure 3.5), and as reported previously (Barnewall *et al.*, 1997), the early endosomal protein TfR was only found in larger replicative inclusions, but not in the small inclusions of *E. chaffeensis* near the plasma membrane (Figure 3.7A-C, arrow) or in any inclusions of *A. phagocytophilum* (data not shown). These findings indicated that the replicative inclusions of *E. chaffeensis* and *A. phagocytophilum* were actively maintained by the caveolar trafficking system. The existence of TfR in replicative inclusions of *E. chaffeensis* also indicated that the replicative inclusions could interact with recycling endosomal pathways.

### 3.5 Discussion

The present study demonstrates that members in the family *Anaplasmataceae* enter host cells by caveolae-mediated endocytosis, rather than by clathrin-mediated endocytosis. Caveolae are known to provide a platform for transmembrane signal transduction since a variety of receptors and signaling molecules are concentrated in
caveolae (Simons and Toomre, 2000; Anderson, 1998; Wu et al., 1997). However, the relationship between the caveolae-mediated microbial entry and the signals required for their entry has not been described. Our present study demonstrated that caveolae are the sites where these early signaling molecules (PLC-γ2 and tyrosine phosphorylated proteins) that we previously demonstrated essential for these bacterial entry were initially assembled (Lin et al., 2002). It is very likely that the binding of *E. chaffeensis* or *A. phagocytophilum* to unidentified receptors activate some (receptor) tyrosine kinases, which in turn phosphorylate proteins located in caveolae. PLC-γ2 is one of rapidly tyrosine phosphorylated proteins in *E. chaffeensis*-infected host cells (Lin et al., 2002). Since the substrate of PLC-γ2, phosphatidylinositol 4,5-bisphosphate (PIP₂) is also enriched in caveolae (Galbiati et al., 2001), caveolae may facilitate PLC-γ2 enzymatic action, leading to increase in intracellular Ca²⁺ level that is essential for *E. chaffeensis* infection (Lin et al., 2002). The present study demonstrated that *E. chaffeensis* and *A. phagocytophilum* assemble the same signaling molecules and enter by the same mechanism, thus their host cell type specificity (monocyte and granulocyte, respectively) is most likely dictated at the level of their binding receptors located in the caveolae.

The receptors used by these bacteria to enter their host cells are still under investigation. Although PSGL-1 was reported as the receptor for *A. phagocytophilum* (Herron et al., 2000), this alone does not explain the granulocyte specificity of this bacterium, since PSGL-1 is also expressed in monocytes and lymphocytes (Laszik et al., 1996). So far, there is no report on the association of PSGL-1 with lipid rafts or caveolae.
The current study revealed that some GPI-anchored proteins located in caveolae may be involved in the monocyte- or granulocyte-specific binding of these bacteria.

Caveolae or lipid raft-mediated endocytosis is exploited by several pathogens for entering various types of host cells without activating the host’s microbicidal pathways (Duncan et al., 2002; Pelkmans and Helenius, 2002; Shin and Abraham, 2001a). A partial list of pathogens that can utilize caveolae or lipid rafts includes viruses such as simian virus 40 (SV40) and polyoma virus, some facultative intracellular bacteria such as enteropathogenic *E. coli* strains, *Mycobacterium* spp. and *Campylobacter jejuni*, and an obligatory intracellular bacterium *Chlamydia trachomatis* (Jutras et al., 2003; Duncan et al., 2002; Norkin et al., 2001; Shin and Abraham, 2001a). However, unlike facultative intracellular bacteria in this list that can replicate outside host cells or *Chlamydia* spp. that can infect epithelial cells, *E. chaffeensis* and *A. phagocytophilum* can only survive and replicate inside the professional phagocytes. Since lysosomes are not known to be directed to caveosomes, entry through a caveolar trafficking system is essential for *E. chaffeensis* and *A. phagocytophilum* in order to sidestep the major obstacle utilizing phagocytes as their hosts: powerful lysosomal degradation (Duncan et al., 2002).

Caveolae-mediated entry leads to various intracellular destinations (Duncan et al., 2002). For example, SV40 and polyoma virus containing caveolae were transported to the endoplasmic reticulum after their entry (Pelkmans et al., 2001; Richterova et al., 2001); whereas *Chlamydia* sp. goes to exocytic compartments (Hackstadt et al., 1996) and retains caveolar marker caveolin-1 (Norkin et al., 2001). Likewise, despite sharing the same entry route and initial signaling events, *E. chaffeensis* and *A. phagocytophilum* are
directed to replicative inclusion compartments distinct from each other (Mott et al., 1999; Barnewall et al., 1997). Caveolae-mediated and clathrin-dependent endocytosis are known to be specialized in internalizing different types of molecules (Anderson, 1998). The interaction between these two intracellular trafficking systems has not been reported so far (Pelkmans and Helenius, 2002). According to our results, the presence of both caveolar marker caveolin-1 and some endosomal markers such as transferrin receptor and EEA-1 in the replicative inclusions of *E. chaffeensis* suggests that endosomes and caveosomes can interact with each other. However, the *A. phagocytophilum* caveosome is isolated from the endosome recycling pathway. It remains to be studied why the caveosomes of these closely related two organisms interact differently with the endosome recycling pathway.
Figure 3.1. Inhibition of the entry and intracellular infection of *E. chaffeensis* or *A. phagocytophilum* by lipid rafts or caveolae-disrupting and usurping agents.

A-E. THP-1 or HL-60 cells were pretreated with MβCD at indicated concentrations (A); 10 mM MβCD and 20 µg/ml water-soluble cholesterol (B); 240 U/ml nystatin (C); 10 µg/ml NBD-cholesterol (NBD-Cho) (D); or with 40 µg/ml cholera toxin subunit B (CTB) (E) at 37°C for 30 min. Cells were washed and then incubated with host cell-free bacteria at 100:1 moi in the absence of these reagents. Numbers of bacteria in host cells were determined after Diff-Quik staining at 3 d pi. Data are presented as means and standard deviations of triplicate samples and representative of three to four independent experiments.

F. THP-1 or HL-60 cells were pretreated with 10 mM MβCD at 37°C for 30 min. Numbers of bound or internalized *E. chaffeensis* (EC) or *A. phagocytophilum* (AP) were determined by immunofluorescence labeling and counting the bacteria associated with host cells at 30 min incubation at 37°C in the absence of reagents. Data are presented as means and standard deviations of triplicate samples and representative of three independent experiments.
Figure 3.1.
Figure 3.2. Inhibition of the entry and intracellular infection of *E. chaffeensis* and *A. phagocytophilum* by removal of GPI-anchored proteins with PI-PLC.

THP-1 or HL-60 cells were pretreated with 1 U/ml PI-PLC at 37°C for 30 min. Cells were washed and incubated with host cell-free bacteria without these reagents. Numbers of bacteria in host cells were determined after Diff-Quik staining at day 3 pi (A) or after immunofluorescence staining at 30 min pi as described in the Figure 3.1F legend (B). Data presented are means and standard deviations of triplicate samples and representative of three independent experiments.
Figure 3.3. Double immunofluorescent labeling for caveolin-1 and *E. chaffeensis* or *A. phagocytophilum* at 30 min pi.

Note colocalization of the caveolar marker protein, caveolin-1 with early inclusions (~0.5 µm in diameter, arrowheads) of *E. chaffeensis* (EC) (A-C) and *A. phagocytophilum* (AP) (D-F) in the peripheral cytoplasm of THP-1 and HL-60 cells, respectively. Scale bar: 5 µm.
Figure 3.4. Double immunofluorescent labeling for tyrosine-phosphorylated proteins or PLC-γ2, and *E. chaffeensis* or *A. phagocytophilum* at 5 min pi.

THP-1 or HL-60 cells were incubated with host cell-free *E. chaffeensis* (EC) (A-C and G-I) or *A. phagocytophilum* (AP) (D-F and J-L) at 37°C. Tyrosine-phosphorylated proteins (A-F) and PLC-γ2 (G-L) were colocalized with early bacteria-containing inclusions as indicated by arrowheads. Scale bar: 5 µm.
Figure 3.5. Cofractionation of caveolar membrane complexes with *E. chaffeensis* proteins and host PLC-γ2 in infected THP-1 cells at 1 h pi.

THP-1 cells were incubated with or without *E. chaffeensis* at 37°C. Cell lysates were fractionated by sucrose gradient centrifugation. Fractions 1-10 had increasing concentrations of sucrose: fraction 1-2: 5%; 3-4: 15%; 5-6: 25%; 7-8: 35%; 9-10: 40%. A portion of each fraction was subjected to SDS-polyacrylamide gel electrophoresis, and proteins were detected by Western blot analysis with antibodies against: caveolin-1 (A-B), PLC-γ2 (C-D), transferrin receptor (TfR) (E-F), and clathrin heavy chain (CHC) (G-H). The existence of *E. chaffeensis* proteins in lipid rafts fractions was determined by anti-*E. chaffeensis* dog serum preabsorbed with THP-1 cells (I-J). Host protein PLC-γ2 and *E. chaffeensis* antigenic proteins at approximately 30, 45, 55, and 70 kDa in size were cofractionated in light-density lipid raft fractions (fractions 4-5) with caveolin-1 in *E. chaffeensis*-infected THP-1 cells.
Figure 3.6. Double immunofluorescence labeling for caveolin-1 or G_{M1} ganglioside, and *E. chaffeensis* or *A. phagocytophilum* at 2 d pi.

Note that caveolin-1 and G_{M1} ganglioside were colocalized not only with the large replicative inclusions of *E. chaffeensis* (EC, panels A-F) and *A. phagocytophilum* (AP, panels G-I) (2-4 µm in diameter and containing multiple bacteria, arrows), but also with the small inclusions in the peripheral cytoplasm (~0.5 µm in diameter and near the plasma membrane, arrowheads). Scale bar: 5 µm.
Figure 3.7. Double immunofluorescence labeling for transferrin receptor or clathrin heavy chain and *E. chaffeensis* at 2 d pi.
Although the large replicative inclusions were colocalized with TfR (A-C), the small inclusions near the plasma membrane were not colocalized with TfR (C, arrow). Neither the large replicative inclusions nor the small inclusions were colocalized with clathrin heavy chain (D-F). Scale bar: 5 μm.
HUMAN MONOCYTIC AND GRANULOCYTIC EHRlichiosis AGENTS
INCORPORATE CHOLESTEROL FOR THEIR SURVIVAL

4.1 Abstract

*Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* are agents of human monocytic and granulocytic ehrlichioses, respectively. They are extremely fragile and pleomorphic gram-negative bacteria. For other fragile and pleomorphic bacteria mycoplasmas that lack a cell wall, membrane incorporation of cholesterol from the eukaryotic host is known to be essential. We, thus, tested if cholesterol is also required for *E. chaffeensis* and *A. phagocytophilum*. By freeze-fracture technique and biochemical analysis, these bacteria were found to contain significant levels of membrane cholesterol. The bacteria lack genes for cholesterol biosynthesis or modification. However, host cell-free bacteria had ability to take up directly exogenous cholesterol or NBD-cholesterol, a fluorescent cholesterol derivative. Treatment of the bacteria with cholesterol-extraction reagent methyl-β-cyclodextrin caused their ultrastructural changes. Furthermore, pretreatment of the bacteria with methyl-β-cyclodextrin or NBD-cholesterol deprived
these bacteria of ability to infect leukocytes, thus killed these obligate intracellular bacteria. Analysis of *E. chaffeensis* and *A. phagocytophilum* genome sequences revealed that these bacteria lack all genes for biosynthesis of lipid A and most genes for peptidoglycan that confer structural strength to gram-negative bacteria. Taken together, these results suggest that human ehrlichiosis agents became cholesterol dependent due to the loss of these genes. As the first report of gram-negative bacteria incorporating cholesterol for survival, these findings offer insight into the unique nature of their parasitism and imply importance of cholesterol in controlling human ehrlichioses.

### 4.2 Introduction

Ehrlichioses are emerging infectious diseases caused by gram-negative obligate intracellular bacteria in the family *Anaplasmataceae*. Five species in this family are known to infect humans (Rikihisa, 2003). Among them, the best documented are recently discovered *Ehrlichia chaffeensis*, the agent of human monocytic ehrlichiosis (HME) and *Anaplasma phagocytophilum*, the agent of human granulocytic ehrlichiosis (HGE). Human ehrlichioses are severe influenza-like febrile illnesses that frequently require hospitalization and 2 to 4% of reported cases were fatal (Dumler and Bakken, 1998; Bakken *et al.*, 1996; Walker and Dumler, 1996). Because of the disease’s potential, human ehrlichioses became one of the nationally reportable diseases in 1998 (McQuiston *et al.*, 1999).

Like Lyme disease and Rocky Mountain spotted fever, humans acquire these pathogens by the bite of infected ticks from wild animal reservoirs (Paddock and Childs, 2003; Dumler and Bakken, 1998; Bakken *et al.*, 1996; Walker and Dumler, 1996). Once
transmitted to humans, *E. chaffeensis* and *A. phagocytophilum* have the remarkable ability to parasitize first-line immune defensive cells, monocytes-macrophages and neutrophils, respectively, as their exclusive survival sites (Rikihisa, 2003). These bacteria then replicate in membrane-bound inclusions in the host cytoplasm secluded from the host immune surveillance and destruction by lysosomes and reactive oxygen intermediates (Rikihisa, 2003; Banerjee *et al.*, 2000; Rikihisa, 2000; Webster *et al.*, 1998).

Although several host cell signals required for these bacterial infection have been elucidated (Rikihisa, 2003; Lin *et al.*, 2002), the detailed mechanism by which these bacteria evade powerful microbicidal activity of the host remains to be discovered.

Unlike other gram-negative bacteria or even their closely related obligate intracellular bacteria *Rickettsia*, members in the family *Anaplasmataceae* display several unique characteristics. They are extremely fragile, highly pleomorphic, and enveloped with the rippled thin outer membrane which lacks thickening of inner or outer leaflet with no sign of peptidoglycan layer or lipopolysaccharide (LPS) as reported in previous studies (Rikihisa, 1999; Webster *et al.*, 1998; Rikihisa *et al.*, 1997; Rikihisa, 1991; Rikihisa, 1990b; Rikihisa *et al.*, 1985). These unique physical characteristics suggest the unusual cell wall compositions in bacteria of the family *Anaplasmataceae*. Mycoplasmas, although lacking a cell wall and being extracellular parasitic bacteria, share some physical characteristics with members of the family *Anaplasmataceae* such as being extremely fragile and pleomorphic. To date mycoplasmas are only prokaryotes known to incorporate cholesterol or related sterols from hosts or environments to stabilize its cytoplasmic membrane, while it lacks genes to synthesize or modify sterols (Dahl, 1993).
We, therefore, examined if *E. chaffeensis* and *A. phagocytophilum* can incorporate exogenous cholesterol into their membranes, and tested the role of bacteria-incorporated cholesterol in infecting their host cells. We also determined whether genes homologous to those required for the biosynthesis of LPS and peptidoglycan in most closely related bacteria *Rickettsia*, are present in human ehrlichiosis agents.

### 4.3 Materials and Methods

**Organisms.** *E. chaffeensis* Arkansas strain and *A. phagocytophilum* HZ strain were cultivated in human leukemia cell lines: THP-1 cells, and HL-60 cells, respectively in RPMI 1640 supplemented with 10% fetal bovine serum and 2% l-glutamine at 37°C in 5% CO₂ and 95% air (Rikihisa et al., 1997; Barnewall and Rikihisa, 1994). No antibiotic was used throughout the study.

To prepare host cell-free *E. chaffeensis* and *A. phagocytophilum* with minimum damage to the bacteria, infected host cells were homogenized in sucrose-potassium buffer (SPK: 0.2 M sucrose; 0.02 M potassium phosphate buffer, pH 7.4) for 20 strokes with a loose-fitted Dounce homogenizer. After removing nuclei and unbroken cells by low speed centrifugation, the host cell-free organisms in the supernatant were pelleted by centrifugation at 10,000 × g for 10 min.

**Filipin labeling and freeze fracture.** Host cell-free bacteria were fixed in 3% glutaraldehyde for 30 min and labeled with 50 µg/ml filipin III (Sigma, St. Louis, MO) for 1 hr at room temperature with rotation (Robinson and Karnovsky, 1980). The same volume of dimethyl sulfoxide (DMSO, 0.5% final concentration), the solvent of filipin,
was added to control groups. Bacteria were prepared for freeze fracture as described (Robinson and Karnovsky, 1980). Briefly, bacteria were washed three times in 0.1 M sodium cacodylate buffer and then cryoprotected with 30% glycerol-0.1 M cacodylate buffer for 1 hr on ice prior to freeze-fracturing. Samples were then gently pelleted, the thick slurry of the bacteria was mounted in a gold-plated specimen carrier, and the specimen was quickly frozen in liquid ethane cooled by liquid nitrogen. Freeze-fracture was performed in a Balzer 400T freeze-fracture apparatus, and samples were immediately shadowed with platinum at a 45° angle and coated with carbon. Replicas were cleaned with Clorox, and examined with a Philips CM12 transmission electron microscope operated at 60 kV.

**Cholesterol assay of purified bacteria.** The host cell-free bacteria were further purified by Percoll density gradient centrifugation at 61,900 × g for 30 min at 4°C with or without the addition of 20 µg/ml water-soluble cholesterol (Sigma) (Ohashi et al., 1998). The purified bacteria were washed three times in phosphate-buffer saline (PBS, pH 7.4), lysed in PBS containing 1% NP-40 and 0.1% SDS, and sonicated for 10 sec to shear the DNA. *Escherichia coli* or uninfected host cells cultured in the same medium and lysed by the same procedure were used as controls. The cholesterol contents were determined in a SpectraMax PLUS384 spectrophotometer (Molecular Devices, Sunnyvale, CA) using the Amplex Red cholesterol assay kit according to the instructions of the manufacturer (Molecular Probes, Eugene, OR). The total cholesterol content was normalized for total protein concentration determined by the BCA reagent (Pierce, Rockford, IL).
**Fluorescence labeling of bacteria.** For filipin labeling, host cell-free bacteria were fixed in 3% paraformaldehyde for 15 min, and incubated with 50 µg/ml filipin III in PBS for 1 hr at room temperature. To test the incorporation of 22-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3beta-ol (NBD-cholesterol, Molecular Probes), bacteria were incubated with 10 µg/ml NBD-cholesterol at 37°C for 30 min, washed twice with PBS, and fixed in 3% paraformaldehyde. Filipin or NBD-cholesterol labeled bacteria were then surface labeled with dog anti-*E. chaffeensis* serum pre-adsorbed with THP-1 cells or horse anti-*A. phagocytophilum* serum pre-adsorbed with HL-60 cells for 1 hr at room temperature. After washing twice with PBS, bacteria were incubated with Lissamine rhodamine-conjugated anti-dog or anti-horse secondary antibody (Jackson ImmunoResearch Lab, West Grove, PA) for 30 min. Fluorescence-labeled bacteria were observed under a Nikon Eclipse E400 fluorescent microscope with a xenon-mercury light source.

**Treatments of bacteria and examination of ultrastructure and infectivities.** Host cell-free bacteria were pre-incubated with 10 mM methyl-β-cyclodextrin (MβCD) (Sigma), 20 µg/ml water-soluble cholesterol, or 10 µg/ml NBD-cholesterol at 37°C for 5 to 30 min. After washing with PBS, the bacteria were added to their respective host cells, and the infection was determined after 3 days of culture by counting the bacterial numbers in 100 cells in triplicate wells (Lin et al., 2002). Transmission electron microscopy (TEM) of bacteria treated with MβCD was performed as previously described (Rikihisa et al., 1985). Briefly, bacteria were fixed in 3% glutaraldehyde, 2% formaldehyde, and 0.02% trinitrophenol in 0.1 M sodium cacodylate buffer, pH 7.4, stained at 4°C in reduced
osmium tetraoxide (1% OsO4 and 1% potassium ferrocyanide) for 1 hr, and rinsed 3 times with cold 0.1 M cacodylate buffer. After uranyl acetate block staining, the bacteria were then dehydrated with graded series of ethanol. Ultra-thin sections (60 nm) were stained with uranyl acetate and lead citrate and observed under a Philips EM 300 transmission electron microscope.

**Genes for the biosynthesis of LPS and peptidoglycan in the family Anaplasmataceae or other related bacteria.** Essential genes required for the biosyntheses of lipid A (the essential component of LPS), murein sacculus (the essential component of peptidoglycan), and diaminopimelate (amino acid unique to peptidoglycan) in *Rickettsia prowazekii* (Andersson *et al.*, 1998) were used to query GenBank microbial genome databases using tblastn with default settings as provided at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi) or the Institute for Genomic Research (TIGR) website (http://www.tigr.org/). Microbial genomes used including *E. chaffeensis*, *A. phagocytophilum*, other members in α-proteobacteria, and other obligate intracellular bacteria such as *Coxiella burnetii*, and *Chlamydia trachomatis*. Only genes with expect value (E-value) of $<10^{-5}$ were considered as significant homologous sequences.

### 4.4 Results

**E. chaffeensis and A. phagocytophilum have membrane cholesterol.** To determine if these bacteria contain cholesterol in their membranes, we first employed freeze fracture technique. If cholesterol is present in the membrane, when incubated with filipin, a polyene compound which binds specifically to cholesterol (Severs, 1997), filipin-
cholesterol complex can be visualized as intramembranous protuberances at diameters around 20-25 nm by this technique (Robinson and Karnovsky, 1980). Results showed that characteristics of filipin-cholesterol complexes were distributed evenly throughout the outer membranes of both *E. chaffeensis* and *A. phagocytophilum* (Figure 4.1, right panels). This protuberance was specific to filipin treatment, since without any treatment (Rikihisa, 1991) or controls treated with the same concentration of the solvent, DMSO, did not show such membrane protuberances, although membrane protein particles of much smaller sizes were visible (Figure 4.1, left panels).

To confirm this observation we examined the colocalization of cholesterol with bacterial surface antigen by the labeling of filipin-incubated host cell-free bacteria with anti-*E. chaffeensis* or anti-*A. phagocytophilum* antibody. Cholesterol-bound filipin exhibits a strong fluorescence at 480 nm when excited at 360 nm in low-polarity environments, allowing it detectable by fluorescence microscopy (Drabikowski *et al.*, 1973). We found the colocalization of cholesterol with bacterial surface antigen in both *E. chaffeensis* and *A. phagocytophilum* (Figure 4.2A). The binding of filipin was specific to these bacteria, as there was no binding of filipin to other gram-negative bacteria such as *E. coli* under the same incubation condition (Figure 4.2B).

The amounts of cholesterol were biochemically determined in bacteria purified by Percoll density-gradient centrifugation. This procedure generates highly purified bacteria free of membrane debris and host cell organelles as previously characterized by TEM (Ohashi *et al.*, 1998). As shown in Table 4.1, these two bacteria had higher total cholesterol contents per mg protein than those of their host cells (~130 µg total
cholesterol/mg protein in bacteria compared to approximately 80 µg total cholesterol/mg protein in host cells). Of note, the percentages of unesterified cholesterol in the total cholesterol of bacteria were very similar to those of their respective host cells (~91% in *E. chaffeensis* and its host THP-1 cells; ~82% in *A. phagocytophilum* and its host HL-60 cells), suggesting that these bacteria take up cholesterol or its derivatives directly from their host cells without modifications. In contrast, the cholesterol was undetectable in *E. coli* by this assay method (data not shown).

**E. chaffeensis and A. phagocytophilum can take up exogenous cholesterol.** By comparing the genome sequences of *E. chaffeensis* and *A. phagocytophilum* with all available protein sequence database using BLASTX with default settings as provided by NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/), there are no genes related to the biosynthesis or modification of cholesterol or related sterols in these bacteria (data not shown). This information and the identical percentages of unesterified cholesterol in the total cholesterol of bacteria and host cells (Table 4.1) suggested that cholesterol in these bacteria could be taken up from the environment. To test this hypothesis, water-soluble unesterified cholesterol was added to the Percoll-SPK buffer used during bacteria purification. Compared to those purified without cholesterol addition, the cholesterol amount in bacteria purified with cholesterol supplementation increased by three-fold (> 400 µg cholesterol/mg protein), and almost all the cholesterol content was unesterified cholesterol (98-99%) (Table 4.1). The uptake of exogenous cholesterol was specific to these bacteria, since under the same incubation condition there was no cholesterol uptake by other gram-negative bacteria such as *E. coli* (data not shown). To confirm this
observation, host cell-free *E. chaffeensis* and *A. phagocytophilum* were incubated with NBD-cholesterol, a fluorescent cholesterol derivative that can be incorporated in membranes of eukaryotic cells (Mukherjee *et al.*, 1998). Fluorescence microscopy showed that NBD-cholesterol was indeed localized in these bacteria (Figure 4.3). Taken together, these results demonstrated that *E. chaffeensis* and *A. phagocytophilum* could directly take up exogenous cholesterol or its derivatives, and there is no measurable modification of the incorporated cholesterol.

**Cholesterol in *Ehrlichia* sp. is required for maintaining their structural integrity.** Since mycoplasmas undergo irreversible lysis when treated with certain sterol binding agents such as digitonin and streptolysin-O (Bernheimer and Davidson, 1965; Razin and Argaman, 1963), we tested whether cholesterol is required for maintaining the structural integrity of *E. chaffeensis*. For this study, MβCD, a cage-like compound that is known to selectively extract cholesterol from the plasma membrane was used at the concentration that does not reduce eukaryotic cellular viability (Ohtani *et al.*, 1989). Host cell-free *E. chaffeensis* was treated with 10 mM MβCD at 37°C for 5 or 15 min and the changes in ultrastructure and the time period required to elicit a detectable effect were determined. In contrast to the tightly spaced inner and outer membranes characteristic of host cell-free *E. chaffeensis* in the control group; MβCD induced irregular dilations of the periplasmic space, which were filled with electron-dense materials presumably derived from the cytoplasm, as early as 5 min after the treatment (Figure 4.4, arrow). If the treatment of MβCD was extended to 15 min, the bacterial cytoplasmic content became sparse due to the exudation of ribosomes and other cytoplasmic contents to the periplasm through the
apparent discontinuity of the inner membrane of *E. chaffeensis* organisms (Figure 4.4, between two arrowheads). This result demonstrated that membrane cholesterol plays a critical role in maintaining the physical integrity of these bacteria.

**Cholesterol in *E. chaffeensis* and *A. phagocytophilum* is essential for infecting host leukocytes.** To assess the role of bacterial incorporated cholesterol in infecting host cells, *E. chaffeensis* and *A. phagocytophilum* were pretreated with MβCD for varying periods. After washing to prevent residual MβCD from removing cholesterol from host cells, bacteria were incubated with their host cells. Results showed that it required 30 min of MβCD pretreatment to effectively block these bacteria to infect their host cells (Figure 4.5A). At 15 min of MβCD treatment, ~50 % inhibition in the bacterial infection was observed (data not shown). Such inhibition could be partially reversed by supplementing *E. chaffeensis* and *A. phagocytophilum* with 20 µg/ml water-soluble cholesterol during preincubation (Figure 4.5A). This result suggests that cholesterol in the membrane of *E. chaffeensis* and *A. phagocytophilum* is essential for the infection of their host cells, thus for their survival.

Since secondary membrane damages caused by cholesterol extraction may inhibit the infection of *Ehrlichia* and *Anaplasma* spp., we next examined the role of cholesterol on the bacterial infection without extracting cholesterol. The cholesterol derivative NBD-cholesterol could be incorporated into the membrane in place of cholesterol in preservation of the intact membrane; however, due to the presence of a bulky and polar NBD group, it often fails to mimic cholesterol functions faithfully (Mukherjee *et al.*, 1998). As demonstrated in Figure 4.3, NBD-cholesterol could be incorporated into the
membrane of *E. chaffeensis* and *A. phagocytophilum* directly, but this incorporation completely rendered these bacteria unable to infect or survive in host cells (Figure 4.5B). In contrast, the preincubation of water-soluble cholesterol with host cell-free *E. chaffeensis* and *A. phagocytophilum* promoted the infection of these bacteria, suggesting a critical role of cholesterol in the bacterial membrane in infecting host cells (Figure 4.5B).

**E. chaffeensis** and **A. phagocytophilum** lack all genes for lipid A biosynthesis and most genes of murein sacculus biosynthesis. In gram-negative bacteria, LPS and peptidoglycan are their essential cell wall components, having significant roles in providing strength to the outer membrane and maintaining overall structural integrity (Nikaido, 1996; Park, 1996). The unique physical characteristics and the previous physical and molecular evidences (Rikihisa, 1999; Webster *et al.*, 1998; Rikihisa *et al.*, 1997; Rikihisa, 1991; Rikihisa, 1990b; Rikihisa *et al.*, 1985) suggest the absence of LPS and peptidoglycan in members of the family *Anaplasmataceae*. Upon the availability of draft genome sequences of *E. chaffeensis* and *A. phagocytophilum*, we thus examined if genes for the biosynthesis of lipid A (an essential component of LPS) and peptidoglycan were present in these bacteria.

The closest relative of the family *Anaplasmataceae*, *Rickettsia prowazekii* has all genes for biosynthesis of lipid A and peptidoglycan. We thus chose these genes to search for the homologous sequences in the family *Anaplasmataceae* and other related bacteria. All genes for the biosynthesis of lipid A and most genes for the biosynthesis of murein sacculus were not found in the genome sequences of *E. chaffeensis* and *A. 
Wolbachia spp., symbionts of invertebrates in the family Anaplasmataceae, also lacked lipid A biosynthesis genes but retained nearly all genes for the biosynthesis of diaminopimelate and murein sacculus (Table 4.2). Further analysis of all genes in the closed genome sequence of *E. chaffeensis* by comparing sequences to the database of clusters of orthologous groups of proteins (COG) as provided at NCBI website (http://www.ncbi.nlm.nih.gov/COG/) confirmed that there are no genes in functional categories related to the biosynthesis of lipid A or peptidoglycan (data not shown). In contrast, nearly all genes for the biosynthesis of lipid A and peptidoglycan homologous to those in *R. prowazekii* were found in other members of α-proteobacteria to which the family Anaplasmataceae belongs, including *Rickettsia conorii*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Mesorhizobium loti*, and in other obligate intracellular bacteria such as *Coxiella burnetii* and *Chlamydia trachomatis* (Table 4.2). The lack of cell wall components in *E. chaffeensis* and *A. phagocytophilum*, thus considered to be unique.

4.5 Discussion

The evidence presented here demonstrates for the first time that *Ehrlichia* and *Anaplasma* spp., are unique gram-negative bacteria that lack cell wall components LPS and peptidoglycan and can incorporate exogenous cholesterol into their membranes. Membrane cholesterol is required for the survival of these bacteria, since the depletion of membrane cholesterol or alteration in the structure of cholesterol renders them unable to infect their host cells.
In gram-negative bacteria, LPS and peptidoglycan are known to provide strength to the outer membrane and maintain overall structural integrity (Nikaido, 1996; Park, 1996). Studies showed that a conditional lipid A synthesis mutant of *E. coli* is extremely fragile (Vuorio and Vaara, 1992). In mycoplasma, virus or eukaryotes, cholesterol or related sterols provide stability and lipid bilayer fluidity to the cytoplasmic membrane (Dahl, 1993; Moore *et al.*, 1977). Thus, in order to compensate for the loss of mechanical strength provided by LPS and peptidoglycan, *Ehrlichia* and *Anaplasma* spp., may become cholesterol-dependent. The existence of cholesterol in place of LPS and peptidoglycan could also explain the unusual physical characteristics of these bacteria, such as extremely thin outer membrane, the highly pleomorphic nature, and unusual fragility as gram-negative bacteria.

The replacement of LPS and peptidoglycan with cholesterol in these bacteria may have been a critical event during the evolution of ancestors of *Ehrlichia* and *Anaplasma* spp., to become the present-day obligate intracellular bacteria of primary host defensive cells. Since the loss of LPS and peptidoglycan, eliminates their chance to trigger microbicidal activities in leukocytes (Beutler, 2000; Ulevitch and Tobias, 1994), it is expected to increase the chances of the intraleukocytic survival of *Ehrlichia* and *Anaplasma* spp. In agreement with this speculation our analysis showed that *Wolbachia* in the family *Anaplasmataceae*, that is not known to infect vertebrates, retained nearly all genes for the biosynthesis of peptidoglycan.

In mycoplasmas that lack a cell wall, cholesterol or other sterols are required to maintain the bacterial structural integrity, but more importantly, they have involved in
several membrane processes and cellular functions, such as ion transport, control of cell volume, activities of membrane bound enzymes, and distribution of membrane proteins through protein-sterol interactions (Dahl, 1993). It is possible that cholesterol might also play some of these roles in *E. chaffeensis* and *A. phagocytophilum*. The ultrastructure of *E. chaffeensis* treated with MβCD, showed the initial leakage in the inner membrane, followed by swelling and lysis of bacteria, suggesting that cholesterol is required for supporting inner membrane. These bacteria internalize into the host leukocytes by receptor-mediated endocytosis, but not by phagocytosis (Rikihisa, 2003; Herron *et al.*, 2000). Loss of infectivity by replacing cholesterol in *E. chaffeensis* and *A. phagocytophilum* with NBD-cholesterol, which has an additional polar group, suggests that cholesterol is not only providing the mechanical strength, but also it is involved in the binding of bacterial ligands or triggering the receptor-mediated endocytosis of these bacteria into the host cells.

Fishbein *et al.* and Bakken *et al.* originally pointed out more severe illness is associated with increased age in HME and HGE patients, respectively (Bakken *et al.*, 1996; Fishbein *et al.*, 1994). More recent study confirmed the unusually high median age of HME and HGE patients (53 and 51 years old, respectively) (Gardner and Childs, 2002). The dependency of *E. chaffeensis* and *A. phagocytophilum* on cholesterol for infection and survival may partially account for this age association of the illness, because increased cholesterol levels were reported in peoples with increased age (Chung, 1992). In other tick-borne infectious diseases, the patients’ median age is much younger. For example, in Lyme the patients’ median age is 39 years old; and in Rocky Mountain
spotted fever caused by *R. rickettsii*, the patients’ median age is 38 years old (Gardner and Childs, 2002). The difference is not due to different tick vectors, since *Borrelia burgdorferi* and *A. phagocytophilum* are transmitted by the same *Ixodes scapularis* tick. In summary, as the first report of gram-negative bacteria incorporating cholesterol for survival, these observations provide new perspectives for understanding the mechanism of obligate parasitism of human ehrlichiosis agents. The present observations also suggest that cholesterol lowering drugs may have a prophylactic and therapeutic value for human ehrlichioses.
<table>
<thead>
<tr>
<th>Bacteria or Host Cells</th>
<th>Total cholesterol content (µg/mg protein) (^a)</th>
<th>Percentage of unesterified cholesterol in total cholesterol (%) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60 cells</td>
<td>79.3 ± 5.7</td>
<td>82.3</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em></td>
<td>128.4 ± 2.9</td>
<td>81.8</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em> + cholesterol (^c)</td>
<td>464.3 ± 1.6</td>
<td>98.4</td>
</tr>
<tr>
<td>THP-1 cells</td>
<td>81.9 ± 2.9</td>
<td>91.2</td>
</tr>
<tr>
<td><em>E. chaffeensis</em></td>
<td>130.8 ± 0.5</td>
<td>91.4</td>
</tr>
<tr>
<td><em>E. chaffeensis</em> + cholesterol (^c)</td>
<td>549.8 ± 2.7</td>
<td>99.2</td>
</tr>
</tbody>
</table>

Table 4.1. Cholesterol contents of purified *E. chaffeensis* and *A. phagocytophilum*, and their respective hosts THP-1 and HL-60 cells.

\(^a\) Cholesterol contents were expressed as micrograms of total cholesterol (esterified and unesterified cholesterol) per milligram of total protein. Data were expressed as means ± standard deviations (n = 3) from representative of 3 independent experiments with similar results.

\(^b\) Unesterified cholesterol contents were determined in the Amplex Red cholesterol assay system without the addition of cholesterol esterase.

\(^c\) Exogenous water-soluble unesterified cholesterol was added to the Percoll-SPK buffer used during bacteria purification.
<table>
<thead>
<tr>
<th>Biosynthesis genes for cell wall components</th>
<th>α-Proteobacteria</th>
<th>Other obligate intracellular bacteria</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Family Anaplasmataceae</td>
<td>Rhizobiaceae group</td>
</tr>
<tr>
<td></td>
<td>E. chaffeensis and A. phagocytophilum</td>
<td>A. tumefaciens, S. meliloti, and M. loti</td>
</tr>
<tr>
<td></td>
<td>Wolbachia sp.</td>
<td>C. trachomatis, and C. burnetii</td>
</tr>
</tbody>
</table>

Component of lipopolysaccharide:

| Lipid A | - | - | + | + |

Components of peptidoglycan:

| Murein sacculus | -<sup>e</sup> | ±<sup>d</sup> | + | + |
| Diaminopimelate | ±<sup>e</sup> | + | + | + |

Table 4.2. Genes for the biosynthesis of lipid A and peptidoglycan in α-proteobacteria and other obligate intracellular bacteria

<sup>a</sup> Genes for the biosynthesis of LPS and peptidoglycan in Rickettsia prowazekii were used to blast sequence database for homologous genes. Only genes with E-value < 10⁻⁵ were considered as homologous genes. Database used include unfinished sequence of Coxiella burnetii and Wolbachia endosymbiont of Drosophila melanogaster as of 1/10/2003, completed genome sequences of Agrobacterium tumefaciens, Merorhizobium loti, Shinorhizobium meliloti, Chlamydia trachomatis from NCBI (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table cgi), and sequences of E. chaffeensis and A. phagocytophilum from The Institute for Genomic Research (http://www.tigr.org).
The gene names (GenBank Accession Number) for the biosynthesis genes of cell wall components from *R. prowazekii* are listed below:

Lipid A – *lpxA* (A71708), *lpxB* (C71688), *lpxC* (CAA14716), *lpxD* (CAA14482), *kdsA* (F71714), *kdsB* (D71695), *kdtA* (H71717) and *htrB* (E71631);

Diaminopimelate – *asd* (F71687), *dapA* (CAA14886), *dapB* (A71725), *dapD* (E71730), *dapE* (A71650), *dapF* (F71699), *lysC* (E71635);


Homologous gene of *dacF* was found in both *E. chaffeensis* and *A. phagocytophilum*, and *murB* was found in *A. phagocytophilum*.

*Wolbachia* endosymbiont of *Drosophila melanogaster* lacks genes *alr*, *mrcA*, *pbpE*, and *slt*.

*E. chaffeensis* has all homologous genes for diaminopimelate but *A. phagocytophilum* only has homologous genes for *asd* and *dapA*. 
Figure 4.1. Freeze-fracture of filipin-labeled host cell-free *E. chaffeensis* and *A. phagocytophilum*. Freeze-fractured replica of filipin-labeled *E. chaffeensis* (*EC*) and *A. phagocytophilum* (*AP*) were examined by TEM. Intramembranous protuberances of filipin-cholesterol complex in diameters of around 20-25 nm can be seen in the outer membrane of filipin-treated *EC* or *AP* (arrow 1), but are absent from the inner membrane of the bacteria (arrow 2), or in the cytosol (arrow 3). The bottom arrow indicates the direction of shadowing. Scale bar: 0.2 µm.
Figure 4.2. Fluorescence microscopy of filipin-labeled *E. chaffeensis* and *A. phagocytophilum* or *E. coli*.

(A) Fluorescence emitted by filipin-labeled *E. chaffeensis* (*EC*) or *A. phagocytophilum* (*AP*) was localized in the bacteria surface labeled with bacteria-specific antibodies. (B) Fluorescence emitted by filipin is undetectable in *E. coli* (*E. coli* was visualized by phase contrast). For an easier cognition, the blue fluorescence by filipin was converted to green pseudo-color in Adobe Photoshop. Scale bar: 5 µm.
Figure 4.3. NBD-cholesterol was directly incorporated into *E. chaffeensis* (*EC*) or *A. phagocytophilum* (*AP*).

Fluorescence microscopy showed the green fluorescence emitted by NBD-cholesterol was localized in the bacteria surface labeled with bacteria-specific antibodies, indicating the direct uptake of NBD-cholesterol by these bacteria. Scale bar: 5 µm.
Figure 4.4. Ultrastructure of *E. chaffeensis* was impaired by MβCD treatment.

Host cell-free *E. chaffeensis* was treated with 10 mM MβCD at 37°C for 5 or 15 min, and the control group was incubated with RPMI medium in the same conditions for 15 min. Arrow: irregular dilations of the periplasmic space in *E. chaffeensis* treated with MβCD for 5 min. Between two arrowheads: the discontinuity of the inner membrane in *E. chaffeensis* treated with MβCD for 15 min. Scale bar: 0.2 µm.
Figure 4.5. MβCD and NBD-cholesterol blocked the infection of *E. chaffeensis* and *A. phagocytophilum* in host cells.

Host cell-free *E. chaffeensis* (EC) or *A. phagocytophilum* (AP) was incubated with: (A) 10 mM MβCD, 20 µg/ml water soluble cholesterol (CHO), or (B) 10 µg/ml NBD-cholesterol (NBD-Cho) at 37°C for 30 min. Infectivities were determined at day 3 post infection. CTL1: bacteria treated with same volume of RPMI as the control groups for MβCD and cholesterol; CTL2: bacteria treated with same volume of methanol (0.05% final concentration in RPMI) as a control for NBD-cholesterol. One asterisk (*) indicates statistical difference (*P* < 0.05) from the control groups; two asterisks (**) indicates statistical difference (*P* < 0.05) from the MβCD treated groups as determined by Student’s *t* test. Data are representative of three experiments.
CHAPTER 5

**EHRLICHIA CHAFFEENSIS DOWN REGULATES SURFACE TOLL-LIKE RECEPTORS 2/4 AND CD14 AND TRANSCRIPTION FACTORS PU.1 AND NF-κB IN HOST MONOCYTES SUBSEQUENT TO INHIBITION OF P38 MAPK AND ERK 1/2**

5.1 Abstract

Microbial ligands, typically lipopolysaccharides (LPS) activate Toll-like receptors (TLRs) of mononuclear phagocytes, thereby activate a transcription factor NF-κB and induce antimicrobial activity. In the present study we found that *Ehrlichia chaffeensis*, an obligatory intracellular gram-negative bacterium, rendered human monocytes incapable of activating transcription factor NF-κB and mobilizing ehrlichiacidal activities in response to *E. coli* LPS. Reverse transcription-polymerase chain reaction analysis and flow cytometry revealed that the surface expression of several pattern recognition receptors, such as CD14, TLR2 and TLR4, and the activity of a transcription factor PU.1 were downregulated in human monocytes by the infection with *E. chaffeensis*. These changes were subsequent to inhibition of p38 MAPK and ERK 1/2, but not JNK
pathways. A p38 MAPK-specific inhibitor down regulated PU.1 activity and TLRs expression in human monocytes, suggesting that p38 MAPK inhibition led to suppression of several downstream signaling pathways. These data point to a novel mechanism that *E. chaffeensis* can survive in hostile human monocytes/macrophages by inhibiting critical signaling molecules in monocyte activation pathways.

5.2 Introduction

*Ehrlichia chaffeensis* exclusively infects and multiplies inside human monocytes or macrophages, and causes a febrile systemic disease called Human monocytic ehrlichiosis (HME). *E. chaffeensis* is an obligatory intracellular gram-negative bacterium in the family *Anaplasmataceae* (Rikihisa, 2003; Rikihisa, 1999). Unlike facultative intracellular bacteria, as *E. chaffeensis* can not survive extracellularly, it is essential for *E. chaffeensis* not to activate bactericidal signals in the macrophages. Previous studies have shown that host cells have ability to kill members in the family *Anaplasmataceae* when treated with various agents such as LPS, IFN-γ, deferoxamine, calcium ionophore, and calcium and calmodulin modulating agents (Lin *et al.*, 2002; Lee and Rikihisa, 1996; Barnewall and Rikihisa, 1994; Park and Rikihisa, 1991; Rikihisa, 1991). However, once infection is established these obligatory intracellular bacteria become refractory to these treatments. In addition, *Neorickettsia risticii* (the agent of Potomac horse fever) in the family *Anaplasmataceae* suppress immunostimulatory and proinflammatory cytokine and prostaglandin generation in response to *E. coli* lipopolysaccharide (LPS) in vitro (van Heeckeren *et al.*, 1993). However, signaling pathways whereby members in the family *Anaplasmataceae* downregulate host cell activation remained unknown.
Toll-like receptors (TLRs), a family of transmembrane proteins that contain a leucine-rich repeat motif in the external domain, are recently discovered pattern recognition receptors (PRRs) in mammals (Krutzik et al., 2001). Ten TLRs have been discovered to date, which recognize a diverse set of pathogen-associated molecular patterns and play a critical role in host defense (Ozato et al., 2002; Imler and Hoffmann, 2001). For example, TLR4, the best studied TLR, has a high affinity against bacterial LPS (Poltorak et al., 1998a; Poltorak et al., 1998b). The recognition of LPS by TLR4 requires several additional molecules. LPS binds to LPS-binding protein (LPB) in serum and the LPS-LBP complex then associates with CD14 that binds TLR4. The immune stimulatory activity of HSP60 has also been shown to be elicited through the recognition by TLR4 (Vabulas et al., 2001). TLR2 recognizes a wide variety of other microbial products including bacterial lipoproteins, the components of Gram-positive bacterial cell walls, as well as glycolipids (Hirschfeld et al., 1999; Means et al., 1999; Schwandner et al., 1999; Yoshimura et al., 1999). Mammalian TLRs represent a crucial link between innate immunity and the induction of adaptive immune responses (Medzhitov, 2001). Upon activation by microbial antigens, TLRs initiate multiple intracellular signaling events, predominantly the activation of NF-κB, which lead to the production of proinflammatory cytokines including interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)-α (Medzhitov et al., 1997). Several mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinase (ERK)-1/2, c-Jun NH2-terminal kinase (JNK), and p38 MAPK, are also activated by TLR4 upon LPS activation.
The regulation of TLR expression is still poorly understood. A recent study on the tlr4 promoter region suggested the role of PU.1 in the regulation of tlr4 gene expression (Rehli et al., 2000). The expression of the Ets/AP-1 transcription factor family PU.1 is specific to cells of the hematopoietic lineage (Macleod et al., 1992), and plays critical roles in regulating genes required for the development of myeloid cells and macrophages (Lloberas et al., 1999; Suzuki et al., 1998). Reports have suggested that PU.1 is regulated by MAPK pathways (Wang et al., 2003; Wasylyk et al., 1998). However, the regulation of PU.1 by bacterial infection has never been reported.

Using LPS as a model stimulant for monocytes and macrophages, in the present study, we have investigated signaling pathways by which E. chaffeensis prevents activation of monocytes/macrophages. The infection of E. chaffeensis down regulated the surface expression of TLRs, the activity of the MAPKs, and transcription factors NF-κB and PU.1 in human monocytes. To date, E. chaffeensis is the only bacterium known which can selectively down regulate PRRs in host monocytes/macrophages, thereby assuring their survival.

5.3 Materials and Methods

**E. chaffeensis and host cell-free bacteria preparation.** E. chaffeensis Arkansas was propagated in THP-1 cells (ATCC, Rockville, MD), a human monocytic leukemia cell line in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 2% l-glutamine at 37°C in 5% CO₂ and 95 % air (Dawson et al., 1991). No antibiotic was used throughout the study. Host cell-free E. chaffeensis was prepared by sonication of E. chaffeensis-infected THP-1 cells (with > 95% infectivities) for 8 s at an output setting of 2 with ultrasonic
processor W-380 (Heat Systems, Farmington, NY). After low speed centrifugation to remove nuclei and unbroken cells, the supernatant was centrifuged at 10,000 × g for 10 min, and the pellet enriched with host cell-free *E. chaffeensis* was used to infect host cells. Based on the pilot experiments, multiplicity of infection (moi) of 100 was chosen in this study.

**Isolation of human monocytes.** Human monocytes were isolated from buffy coats of healthy donors (American Red Cross, Columbus, OH). The buffy coat was overlaid onto Histopaque 1077 layer (Sigma, St. Louis, MO) and centrifuged at 700 × g for 30 min at room temperature. Monocellular cells at the interface between the plasma and Histopaque 1077 were collected and washed twice with PBS. Cells were then suspended in culture medium and seeded in 6-well plates. After culturing for 1 h at 37°C, unattached cells were removed. Adherent cells were mainly monocytes (> 90%) based on morphology after Diff-Quik staining and > 95% were alive by the trypan blue dye exclusion test.

**Evaluation of the effect of LPS on *E. chaffeensis* infection.** Human peripheral blood monocytes (10⁶ cells per group) as previously described were seeded in 6-well plates and infected with cell-free *E. chaffeensis* at moi of 100 (Barnewall and Rikihisa, 1994). At indicated time post infection (pi), *E. coli* LPS (*E. coli* 0111:B4, Sigma) at 100 ng/ml was added, and cells were further cultured in the presence of LPS. At 3 d pi the infectivity was determined by counting the number of *E. chaffeensis* organisms in 100 cells in triplicate assay wells as previously described (Lin et al., 2002).

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis.** Human monocytes or THP-1 cells were infected with host cell free *E. chaffeensis* at moi of 100:1. Alternatively, uninfected or infected-cells were treated with 20 µg/ml of actinomycin D
(ActD, Calbiochem, San Diego, CA) or 10 µM of SB 203580 (Calbiochem) as indicated. Total RNA was extracted from monocytes or THP-1 cells (5 × 10^6 cells) by TRIZol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. The concentration and purity of the RNA were determined by measuring the A_{260} and the A_{260}/A_{280} ratio with a GeneQuant II RNA and DNA calculator (Pharmacia, Piscataway, NJ). The RNA was stored at -80°C until used. For cDNA synthesis, total cellular RNA (2 µg) was reverse transcribed in a 30 µl reaction mixture containing 1× reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 0.5 mM each of dNTPs, 1 U of an RNase inhibitor, 1.5 µM oligo(dT) primer, and 10 U of Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h. The reaction was terminated by heating the reaction mixture at 70°C for 15 min. To assure the absence of DNA contamination in the RNA preparations, the assay was duplicated without reverse transcriptase.

For PCR reactions, 2 µl of cDNA samples were amplified in a 50-µl reaction mixture containing PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM each of dNTPs, 0.4 µM primers, and Taq DNA polymerase (2 U) in PE 8600 DNA thermal cycler (Perkin-Elmer, Norwalk, CT) for 25 cycles of 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min. PCR primer sequences are listed in Table 1. PCR reactions under this condition was within linear range as determined by measuring PCR band intensities of serial-diluted samples. The intensities of bands were quantitated by using a gel documentation system and analyzing the images with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA). Band intensities were normalized against G3PDH mRNA in the corresponding samples.
Flow Cytometry. Surface expression of CD14 and TLR4 in human monocytes was
determined using surface labeling with R-Phycoerythrin (R-PE) conjugated mouse
monoclonal IgG2a antibody against human CD14 (BD Pharmingen, Franklin Lakes, NJ),
and rabbit polyclonal antibody against TLR4 (Torrey Pines BioLabs, San Diego, CA)
which was detected with fluorescein isothiocyanate (FITC)-conjugated AffiPure goat
anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Isotype matched, R-
PE conjugated mouse IgG2a antibody served as control for CD14, and normal rabbit
serum served as control for TLR4 antibody. Samples were then fixed in 1%
paraformaldehyde and analyzed in Becton-Dickinson FACScan Immunocytometry
Systems (BD Biosciences, San Jose, CA).

Cell treatment and nuclear extract preparation. Human monocytes (2 × 10^6
cells/group) were plated in 6-well culture plates and infected with host cell-free E.
chaffeensis organisms. E. coli LPS at 100 ng/ml was added to the cultures at 3 h, 1 d, and
2 d pi as indicated. After stimulation for 1 h, cells were rinsed with 1 ml of Tris-buffered
saline (TBS: 25 mM Tris, pH 7.9, 0.14 M NaCl, 27 mM KCl). Nuclear extracts were
prepared as follows. Briefly, 400 µl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM
KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 µM aprotinin, 14
µM leupeptin, 1 µM pepstatin, 80 µg/ml benzamidine) was added to each well. After
being incubated on ice for 15 min, the cells were lysed by the addition of 24 µl of 10%
NP-40 (final concentration 0.6%). Cells were then transferred to microcentrifuge tubes,
and the lysis was completed by vigorous vortexing for 10 s. The homogenates were
centrifuged at 13,000 × g for 30 s at 4°C, and the nuclear pellet was suspended in 50 µl of
cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μM aprotinin, 14 μM leupeptin, 1 μM pepstatin, 80 μg/ml benzamidine). After centrifugation at 13,000 × g for 30 s, the resulting supernatant of nuclear extracts was stored in small aliquots at -80°C. Protein concentrations were determined by using the bicinchoninic acid protein assay reagents (Pierce, Rockford, IL).

**Electrophoretic mobility shift assay (EMSA).** Oligonucleotides used in gel shift analysis to determine the specific binding of transcription factors are listed in Table 1. NF-κB and PU.1 oligonucleotides were end labeled with [γ-32P]ATP with T4 polynucleotide kinase and biotin, respectively. NF-κB and PU.1 activities were detected by a gel shift assay system (Promega, Madison, WI) and LightShift™ chemiluminescent EMSA kit (Pierce), respectively according to manufacturer’s instructions. The reaction mixture to determine the interaction of NF-κB and its consensus oligonucleotides contained 2 μg of nuclear extract in 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.05 μg/μl poly(dI·dC) in 4% glycerol. The reaction mixture for PU.1-DNA interaction contained 2 μg of nuclear extract, 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 0.05 μg/ml poly(dI·dC), and 0.05% NP-40. The labeled DNA probes were added to the mixture and the final volume was adjusted to 25 μl. To confirm the specificity of probe binding, a 50-fold excess amount of unlabeled specific or non-specific oligonucleotides was added to the reaction mixture. Anti-human PU.1 antibody (Santa Cruz) was added after 15 min of incubation. After incubation at room temperature for 30 min, samples were loaded onto a
6% native polyacrylamide gel in 0.5 × Tris-borate-EDTA (TBE) that had been prerun for 1 h and were then electrophoresed at 100 V for 3 - 4 h.

**Western blot analysis.** Human monocytes (5 × 10^6 per group) were infected with host cell-free *E. chaffeensis*. At indicated time pi, LPS (100 ng/ml) was added and incubated for 30 min. After washing in PBS, cells were lysed in 1 ml of ice-cold RIPA (radioimmunoprecipitation) buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA), with freshly added protease and phosphatase inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 mM PMSF, 1 mM NaF and 1 mM NaVO₃). After incubation on ice for 20 min, cells were sonicated; and whole cell lysates were collected by centrifugation at 10,000 × g for 10 min. Cell lysates were mixed with equal volume of 2× SDS sample buffer and boiled for 5 min; and samples were subjected to Western blot analysis with appropriate antibodies (monoclonal Abs against p38 MAPK, phospho-p38 MAPK, phospho-JNK, phospho-ERK, Cell Signaling, Beverly, MA). Bands were visualized with enhanced chemiluminescence (ECL) by incubating membrane with LumiGLO™ chemiluminescent reagent (Cell Signaling), and exposing to x-ray film.

**5.4 Results**

*E. chaffeensis* was killed by host cells by LPS pretreatment, but became resistant to the killing in advanced infection. To determine the influence of ehrlichial infection on host innate bactericidal activity, we stimulated human peripheral blood monocytes with *E. coli* LPS. *E. coli* LPS is known to activate monocytes and macrophages, and enhance its
bactericidal activities through induction of various cytokines and generation of oxygen radicals (Guha and Mackman, 2001; Sweet and Hume, 1996; Inoue et al., 1995). Hence, we examined the effect of LPS treatment on *E. chaffeensis* infection in human monocytes at different infection stages. When added 1 h prior to *E. chaffeensis* infection (-1 h), LPS significantly reduced ehrlichial infection by around 6 folds (Figure 5.1). However, the ehrlichicidal effect of LPS was progressively reduced starting from 3 h pi to 2 d pi, which coincides with establishment of replicative inclusions of *E. chaffeensis* after 3 h pi. When LPS was added at 2 d pi, *E. chaffeensis* became almost completely refractory to LPS treatment (Figure 5.1).

**Decreased activities of NF-κB in response to LPS stimulation in human monocytes infected with *E. chaffeensis***. LPS activates a transcription factor NF-κB that regulates the expression of many proinflammatory cytokine genes (Irie et al., 2000; Kopp et al., 1999; Ninomiya-Tsuji et al., 1999). Our previous experiments showed that the activation of NF-κB in PMA-treated THP-1 cells by *E. coli* LPS was peaked at 1 h post-stimulation (data not shown), thus NF-κB activity in human monocytes was examined at 1 h after LPS addition (Lee and Rikihisa, 1997). As shown in Figure 5.2, NF-κB was transiently activated by *E. chaffeensis* infection at 3 h pi, but this activity was returned to the background level at 1 d and 2 d pi. The activity of NF-κB in response to LPS was significantly reduced in *E. chaffeensis*-infected monocytes at 1 d and 2 d pi compared with uninfected monocytes (Figure 5.2). In control uninfected monocytes, the DNA binding activities of NF-κB remained at similar activity levels during 3-day culture period (data not shown).
Decreased expressions of TLR2, TLR4, and CD14 mRNA and surface proteins in *E. chaffeensis*-infected monocytes. To investigate inhibitory mechanisms of LPS-induced killing and NF-κB activation in *E. chaffeensis*-infected monocytes in response to LPS, we examined the levels of LPS receptors on infected monocytes. LPS is known to activate NF-κB in monocytes through its receptors LBP-CD14 and TLR4 (Poltorak *et al.*, 1998a); and potentially through TLR2 since commercially purified LPS often contains lipoproteins which can activate monocytes by binding to TLR2 (Hirschfeld *et al.*, 2000; Hirschfeld *et al.*, 1999; Yang *et al.*, 1998). Therefore, mRNA and surface expressed protein levels of CD14, TLR4 and TLR2 were determined. Using RT-PCR, we found that TLR2, TLR4, and CD14 mRNA expression in human monocytes infected with *E. chaffeensis* was significantly reduced from 1 d pi, but there were no detectable changes at 3 h pi (Figure 5.3A-B). As *E. chaffeensis* does not possess genes required for lipid A (a core component in LPS) biosynthesis (Lin and Rikihisa, 2003), the downregulation in TLR4 was not due to the LPS-induced tolerance. In LPS-induced tolerance, the expression of TLR4 mRNA is significantly reduced at 3 h after LPS treatment but returns to the original level at 20 h after LPS stimulation (Nomura *et al.*, 2000). Furthermore, the down regulation in mRNA expression required new protein synthesis from *E. chaffeensis*, since the inhibitory effect by *E. chaffeensis* was completely abolished when infected monocytes were treated with antibiotic oxytetracycline starting at 4 h pi (Figure 5.3A-B). In human monocyte cell line THP-1 infected with *E. chaffeensis*, the mRNA expression of TLR-2/4 was also decreased after 1 d pi (data not shown).
The protein levels of TLR4 and CD14 on the surface of uninfected or *E. chaffeensis*-infected human monocytes were quantitated by flow cytometry analysis. In agreement with their mRNA levels, the surface protein levels of TLR4 and CD14 were significantly reduced by *E. chaffeensis* infection (Figure 5.3C-E). Flow cytometry data showed that the number of monocytes expressing both TLR4 and CD14 was decreased from 89.4% to 44.1% by *E. chaffeensis* infection at 1 dpi. In addition, the mean channel fluorescence of CD14 was reduced from 56.7 to 6.7 and that of TLR4 was reduced from 23.8 to 9.89.

**Decreased activity of transcription factor PU.1 in *E. chaffeensis*-infected monocytes.**

The downregulation in mRNA levels could be the consequence of either increased degradation rates of newly transcribed mRNAs, or the inhibition of active transcription of the mRNAs. We first examined if the infection of *E. chaffeensis* enhanced the TLR2, TLR4, and CD14 mRNA degradation in human monocytes by treating uninfected or *E. chaffeensis*-infected monocytes at 20 h pi with actinomycin D for 4 h. Actinomycin D inhibits transcription in eukaryotic, but not prokaryotic cells (Uzuka et al., 1981); therefore, the decrease in the mRNA levels in the presence of actinomycin D reflects the mRNA degradation in monocytes. TLRs and CD14 mRNA levels in the presence of actinomycin D were similar between uninfected and *E. chaffeensis*-infected monocytes (Figure 5.4), suggesting that reduced TLR2, TLR4, and CD14 mRNA levels were due to reduced transcription in *E. chaffeensis*-infected monocytes rather than due to increased degradation.

A recent study showed that PU.1 in Ets/AP-1 transcription factor family regulates the expression of TLR-4 (Rehli et al., 2000); we, hence, used EMSA to test PU.1 activities in
E. chaffeensis-infected monocytes. Results showed the strong PU.1 activity in uninfected human monocytes and THP-1 cells, which was specifically blocked by excess amount of unlabelled PU.1 consensus oligonucleotide or by anti-PU.1 polyclonal antibody (Figure 5.5). In contrast, PU.1 failed to bind PU.1M, which has a three-nucleotide mutation in the core binding site of PU.1 (TTCC → TAGT), and unlabelled PU.1M did not affect the binding of PU.1 to its labeled consensus sequence. The monocytes or THP-1 cells infected with E. chaffeensis for 1 d or 3 d, respectively had significantly reduced PU.1 activity (Figure 5.5).

Ets/AP-1 transcription factor family is mainly activated by MAPKs (Wasylyk et al., 1998). In Ba/F3 cells (mouse bone marrow-derived, IL-3-dependent pre-B cells), the activity of PU.1 is regulated by p38 MAPK (Wang et al., 2003). We, therefore, tested the activities of PU.1 in human monocytes in response to p38 MAPK specific inhibitor SB203580 (Cuenda et al., 1995). The inhibition of PU.1 activity by SB203580 indicated that PU.1 in human monocytes was regulated in a p38 MAPK dependent pathway (Figure 5.5).

**Inhibition of p38 MAPK and ERK activation in E. chaffeensis-infected human monocytes in response to LPS.** Activation of MAPKs by LPS is a critical step in activation of various transcription factors including PU.1 and NF-κB (Carter et al., 1999b; Carter et al., 1999a; Feng et al., 1999; DeFranco et al., 1998). We thus examined the activities of MAPKs in response to E. chaffeensis infection and LPS stimulation. Activation of MAPKs requires phosphorylation on Ser/Thr residues, and thus can be detected by using specific antibodies to the phosphorylated form of respective MAPKs. Western blot analysis showed that, upon the infection of monocytes with E. chaffeensis,
the phosphorylation of p38 MAPK, ERK 1/2, and JNKs, was slightly induced at 1 h pi, but the activation by *E. chaffeensis* was much weaker than that by LPS. These activations induced by *E. chaffeensis* were rapidly decreased from 3 h pi, and reduced to the background level at 1 d pi. Responsiveness of p38 MAPK and ERK 1/2 to LPS was also decreased in *E. chaffeensis*-infected monocytes from 3 h pi compared to uninfected monocytes, indicating down regulation (Figure 5.6). The activities of JNKs in response to LPS in *E. chaffeensis*-infected monocytes were not decreased significantly. The downregulation of MAPKs activities that began from 3 h pi was earlier than the down regulation of TLRs expression and PU.1 activity that began from 1 d pi, suggesting that MAPKs are the upstream signals inhibited by *E. chaffeensis* infection (Figure 5.6). When monocytes were treated with SB 203580, mRNA expression of TLR2, TLR4 and CD14 in monocytes was significantly reduced as compared to untreated groups, or even in *E. chaffeensis*-infected monocytes (Figure 5.7). These data supported that the down regulation of mRNA expression of TLR2, TLR4 and CD14 by *E. chaffeensis* infection in monocytes was mediated by p38 MAPK inhibition, likely through p38 MAPK inhibition of PU.1 activity as shown in Figure 5.5.

5.5 Discussion

As an obligatory intracellular bacterium that proliferates exclusively in human primary defensive cells, *E. chaffeensis* must prevent the activation of their bactericidal abilities. The present study demonstrated that *E. chaffeensis* exerts this inhibitory effects via a mechanisms that have so far not been identified in other pathogens. *E. chaffeensis* can prevent the ehrlichiacidal activities by downregulation of the expression of several PRRs
likely through its inhibition on MAPKs and PU.1, the only bacterium known to date that is capable of doing so. A recent report has shown that viable Francisella tularensis can inhibit LPS-induced activation of MAPK pathways and NF-κB in a mouse macrophage cell line J774A.1 (Telepnev et al., 2003). However, unlike E. chaffeensis, intracellular infection of F. tularensis neither down regulates TLR2/4 mRNA nor is inhibited by LPS treatment when added 24 h before infection or simultaneously with the bacterium. Furthermore, this inhibition does not require bacterial de novo protein synthesis. In agreement with our observation, although in wild-type mice, the infection of E. chaffeensis is cleared within two weeks, in tlr4 gene-deficient mice E. chaffeensis persists for more than 30 days (Ganta et al., 2002). Hence, E. chaffeensis induced down regulation of these PRR expression likely benefits its own survival and persistence in mammalian hosts.

In monocytes/macrophages infected with several facultative and obligatory intracellular bacteria, such as Mycobacterium, Listeria, Legionella, Salmonella, and Chlamydia species, various proinflammatory cytokines such as IL-1, TNF-α, and IL-6 are produced (Kuhn and Goebel, 1994; Yamamoto et al., 1994; Friedland, 1993). These cytokines can orchestrate various cell types in the immune system, leading to activation of macrophages and killing of the intracellular bacteria in infected patients. However, in human monocytes infected with E. chaffeensis, TNF-α, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were not produced, instead, a low level of IL-1α and an immunosuppressive cytokine IL-10 was released (Lee and Rikihisa, 1996). Since the expression of proinflammatory cytokine genes are known to be activated
by MAPK pathways and NF-κB (Naumann, 2000), disruption of these signaling pathways by *E. chaffeensis* may be one of the cause of weak or absent proinflammatory cytokine gene expression by monocytes infected with *E. chaffeensis*.

The present study showed that the inhibition of ERK and p38 MAPK activities occurred at 3 h pi, prior to the inhibition of TLR2/4 or CD14 expressions and the activity of NF-κB in response to LPS which occurred after 1 d pi. Thus the most upstream signals down regulated by *E. chaffeensis* in this study are MAPK family, p38 and ERK, which in turn further inhibited by down regulation of TLR2/4 and CD14 as illustrated in Figure 5.8. How *E. chaffeensis* down regulates p38 MAPK and ERK is currently unknown. Studies in other intracellular bacteria have suggested that different bacteria have evolved different strategies to perturb the activation of monocytes/macrophages by disrupting MAPK and NF-κB signaling (Rosenberger and Finlay, 2003). For examples, *Yersinia* sp. delivers a ubiquitin-like molecule YopJ through a type III secretion system to block not only the activation of MAPKs, but also the translocation, thus activation of NF-κB in host cells (Orth *et al.*, 2000). The sequenced *E. chaffeensis* genome does not contain any genes homologous to proteins in the type III secretion system, instead, gene clusters for a type IV secretion machinery is present (Ohashi *et al.*, 2002). Although no effector of type IV secretion systems has been found in *E. chaffeensis* yet, it may have a role in the downregulation of MAPKs and NF-κB. Alternatively, since the activation of ERK 1/2 or p38 MAPK is mainly mediated by Ras/Raf-1/MEK or MKK3/6, respectively, and this pathway can be inhibited by PKA-mediated phosphorylation of Raf-1 (Schorey and Cooper, 2003; Tamir *et al.*, 1996; Cook and McCormick, 1993), the rapid activation of
protein kinase A (PKA) by *E. chaffeensis* within 30 min pi (Lee and Rikihisa, 1998) suggested that PKA activation may be one of the upstream signals induced by *E. chaffeensis* leading to these monocyte activation suppressive signals.

Although *E. chaffeensis* overall disrupts these signal pathways, weak and transient activations of ERK 1/2, p38 MAPK, and transcription factor NF-κB were detected in infected monocytes at 1 h pi. Since *E. chaffeensis* does not possess typical LPS, the weak activation in these pathways are likely mediated by other conserved pathogen-associated molecular patterns in *E. chaffeensis*, such as bacterial heat shock proteins, or unmethylated CpG DNA (Hayashi *et al.*, 2001; Hemmi *et al.*, 2000). Alternatively, this may be due to the brief activation signals induced and required for their internalization, such as protein tyrosine phosphorylation, phospholipase-γ2 activation, and calcium influx (Lin *et al.*, 2002). These transient activations in MAPKs and NF-κB may lead to the expression of low levels of IL-1β and IL-8 in infected monocytes, which may contribute to the early clinical signs in ehrlichiosis patients prior to the antibody-mediated strong proinflammatory cytokine production (Lee and Rikihisa, 1996). The time when p38 and ERK began to be inhibited coincides with establishment of replicative inclusions of *E. chaffeensis* after 3 h pi, supporting that these down regulations are not required for ehrlichial internalization, but rather required for their survival and persistence. In agreement with this observation, new ehrlichial protein synthesis that likely occurs after formation of the replicative inclusions is required for this inhibition. From the view point of HME therapy, this indicates that these suppressive effects are reversible by treatment with antibiotic or other drugs that interfere with ehrlichial signaling pathways.
As summarized in Figure 5.8, the present study proposed a model how \textit{E. chaffeensis} can actively suppress mobilization of monocyte bactericidal effects and proinflammatory cytokine generation: likely through the positive feedback inhibition of critical cytoplasmic signaling molecules including MAPK family, expression of TLR2, TLR4, and CD14, and transcription factor PU.1 and NF-κB.
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<thead>
<tr>
<th>Oligonucleotides $^a$</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR primers:</strong> $^b$</td>
<td></td>
</tr>
<tr>
<td>CD14 primers</td>
<td>5'-ACTTATCGACCATGGAGC; and 5'-AGGCATGGTGCCGGTTA</td>
</tr>
<tr>
<td>TLR2 primers</td>
<td>5'-GCCAAAGTCTTGATTGATTGG and 5'-TTGAAGTTCTCCAGCTCCTG</td>
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<tr>
<td>TLR4 primers</td>
<td>5'-TGGATACGTTTCTTATAAG and 5'-GAAATGGAGGCACCCCTTC</td>
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<tr>
<td><strong>EMSA probes:</strong></td>
<td></td>
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<tr>
<td>NF-κB consensus sequence $^c$</td>
<td>5'-AGTTGAGGGGACTTTCCCAGGC</td>
</tr>
<tr>
<td>AP-2 consensus sequence $^c$</td>
<td>5'-GATCGAACTGACCGCCCGGCGGCGCGGT</td>
</tr>
<tr>
<td>PU.1 consensus sequence $^d$</td>
<td>5'-CTTCTTCTAACTTTCTCTCCTGTGAC</td>
</tr>
<tr>
<td>PU.1M (mutated PU.1 consensus sequence) $^d$</td>
<td>5'-CTTCTTCTAACTAGTTCTCCTGTGAC</td>
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</table>

Table 5.1. Sequences of oligonucleotides used in PCR and EMSA.

$^a$ Unless indicated otherwise, oligonucleotides used in this study were synthesized by Invitrogen.

$^b$ Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were obtained from Clontech (Palo Alto, CA).

$^c$ NF-κB and AP-2 consensus sequences were purchased from Santa Cruz Biotech.

$^d$ PU.1 consensus sequence was designed based on the promoter region of human TLR4 gene (underlined indicate the PU.1-binding consensus sequence) (Rehli et al., 2000).
PU.1M was used for the non-specific competition of PU.1 and three italicized nucleotides indicate mutated PU.1 consensus core sequence. To prepare double-stranded DNA oligos, synthesized complementary oligonucleotide pairs were annealed by mixing, boiling the oligos, and then slowly decreasing to room temperature.
Figure 5.1. *E. chaffeensis* became resistant to the killing by *E. coli* LPS in human monocytes when LPS was added at later stage of infection. Monocytes were infected with host cell-free *E. chaffeensis*, and LPS (100 ng/ml) were added to monocytes at indicated time prior to or post ehrlichial infection and present throughout the incubation. The percent infected cells (A) and organisms per 100 cells (B) were determined in 100 cells at 3 d pi in triplicate wells. Data were expressed as means ± standard deviations (n = 3).
Figure 5.2. Reduced activities of transcription factor NF-κB in *E. chaffeensis*-infected human monocytes in response to *E. coli* LPS.

Monocytes were infected with host cell-free *E. chaffeensis* (EC) at moi of 100:1. At indicated time point pi, cells were stimulated with 100 ng/ml *E. coli* LPS or medium for 1 h and nuclear extracts were prepared. Activities of NF-κB were assessed by EMSA according to manufacture’s instruction (Promega). N (negative control), without nuclear extract; P (positive control), HeLa nuclear extract plus NF-κB probe; S (specific competitor), HeLa nuclear extract plus NF-κB probe and a 50-fold excess of unlabeled NF-κB oligonucleotide; NS (nonspecific competitor), HeLa nuclear extracts plus NF-κB probe and a 50-fold excess amount of unlabeled AP-2 oligonucleotide. The autoradiogram is the representative of more than three independent experiments with similar results.
Figure 5.3. Decreased expression of TLR2, TLR4, and CD14 in human monocytes infected with \textit{E. chaffeensis}.

A. Human monocytes were infected with \textit{E. chaffeensis} (EC) for 3 h or 1 d. For antibiotic treatment, 10 µg/ml oxytetracycline (OTC) was added to infected monocytes at 4 h pi and cells were cultured in the presence of OTC for additional 20 h. At indicated time point, mRNA was extracted and RT-PCR was performed. Glyceraldehydes-3-phosphate dehydrogenase (G3PDH) mRNA was used to normalize RNA input across specimens. RT-: no reverse transcriptase; Control: uninfected human monocytes only. The number shown below each panel is a densitometric analysis of relative PCR band intensity. The ratio between TLR2/4 or CD14 and G3PDH cDNA levels for each sample is indicated below each panel. The ratio of the uninfected monocytes control was arbitrarily set as 1.0. The result was a representative from three independent experiments.

B-D. Flow cytometry analysis was used to detect the surface expression of TLR4 and CD14 in \textit{E. chaffeensis}-infected human monocytes. B. Isotype control: double immunofluorescence labeled with R-PE conjugated mouse mAb, and normal rabbit serum detected with FITC-conjugated goat anti-rabbit antibody. C-D. Double immunofluorescence labeled for R-PE conjugated anti-CD14 and rabbit anti TLR4. B-C. monocytes only; D. \textit{E. chaffeensis}-infected monocytes at 1 d pi. The result was a representative from three independent experiments.
Figure 5.3

A.

<table>
<thead>
<tr>
<th></th>
<th>RT-</th>
<th>Control</th>
<th>EC 3h</th>
<th>EC 1d</th>
<th>EC+OTC</th>
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<tbody>
<tr>
<td>TLR4</td>
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<td>TLR2</td>
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<tr>
<td>G3PDH</td>
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<td>0.1</td>
<td>0.01</td>
<td>0.1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

B. Isotype control

C. Monocytes

D. *E. chaffeensis* (1d pi)
Figure 5.4. Effect of actinomycin D on mRNA expression of TLR2, TLR4, and CD14 in *E. chaffeensis*-infected monocytes.

Uninfected or *E. chaffeensis* (EC)-infected human monocytes at 1 d pi were treated with 20 µg/ml of actinomycin D (Act. D) or untreated for 4 h. mRNA was then extracted and RT-PCR was performed. The ratio between TLR2/4 or CD14 and G3PDH cDNA levels for each sample is indicated below each panel. The ratio of Act. D-untreated group (both uninfected and infected monocytes) was arbitrarily set as 1.0. The result was a representative from three independent experiments.
Figure 5.5. Reduced activities of transcription factor PU.1 in *E. chaffeensis*-infected host cells. Human monocytes (mono) or THP-1 cells were infected with host cell-free *E. chaffeensis* (EC), or treated with 10 μM SB 203580 (SB). At indicated time point pi, nuclear extracts were prepared, and nucleotide binding activities of PU.1 were assessed by EMSA according to manufacture’s instruction (Pierce). Except for lane 1, which used mutated PU.1 consensus oligos (PU.1M) as probe, all other lanes (2 – 10) used the native PU.1 consensus binding oligonucleotide (PU.1, see Table 1) as probe. For specific competition (Spec. Comp.), a 50-fold excess amount of the unlabeled PU.1 oligonucleotide was used; and for nonspecific competition (Nonspec. Comp.), a 50-fold excess amount of unlabeled PU.1M (oligonucleotide mutated form) was used. To confirm the binding specificity of PU.1 to its consensus oligonucleotide, anti-PU.1 antibody was added to the reaction mixture after 15 min. Nuclear extracts used in lanes 1 – 8 were from monocytes and in lanes 9-10 were from THP-1 cells. This image is the representative of three independent experiments with similar results.
Figure 5.6. Reduced activities of ERK 1/2 and p38 MAPK, but not JNK in *E. chaffeensis*-infected human monocytes in response to *E. coli* LPS.

Cells were infected with host-cell free *E. chaffeensis* (EC). At indicated time point, medium (-) or 100 ng/ml LPS (+) was added and incubated for 30 min. Cellular extracts were prepared and MAPK activities were examined by Western blotting with antibodies specific for the respective phosphorylated forms of MAPKs (as indicated by prefix p-). Unphosphorylated p38 MAPK was served as a protein loading control. The result of a representative experiment (n = 3) is presented.
Figure 5.7. Effect of SB 203580 on mRNA expression of TLR2, TLR4, and CD14 in human monocytes.

Human monocytes were infected with *E. chaffeensis* (EC) or treated with 10 µM of SB 203580 (SB) for 1 d. mRNA was then extracted and RT-PCR was performed as described in Figure 5.3A legend. The ratio between TLR2/4 or CD14 and G3PDH cDNA levels for each sample is indicated below each panel. The ratio of the uninfected monocytes control was arbitrarily set as 1.0. The result was a representative from three independent experiments.
The binding of *E. chaffeensis* (EC) to its receptor(s) on host monocytes activates protein kinase A (PKA). PKA, or some unknown proteins released from or activated by *E. chaffeensis* prevents the activation of ERK 1/2 and p38 MAPK, which in turn downregulate the activity of transcription factor PU.1. Since several genes in monocytes like TLR4 are controlled by PU.1 transcription factor, the downregulation in PU.1 activity decreased the expression of these genes. This has positive feedback effects by further downregulating ERK 1/2 and p38 MAPK and thus the ehrlichiacidal activities of monocytes in response to LPS. Inhibition of NF-κB activation prevents proinflammatory cytokines generation; therefore, the activation of monocytes was significantly prevented by *E. chaffeensis* infection.
Figure 5.8

- **LPS**
- **CD14, TLR4, or TLR2**
- **ERK 1/2, p38 MAPK**
- **NF-κB**
- **EC**
- **PU.1**

**Monocytes Activation:**
- Inflammatory cytokines;
- Phagolysosomal fusion

**Signaling Pathway:**
- Activation
- Inhibition
References


phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* 163: 6403-6412.


Lee, E.H. and Rikihisa, Y. (1996) Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of
IL-1beta, IL-8, and IL-10 expression in human monocytes exposed to viable or killed Ehrlichia chaffeensis. *Infect. Immun.* 64: 4211-4219.


