Anaplasma phagocytophilum and Ehrlichia ewingii

Exploit Host Signaling Pathways for Their Infection

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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ABSTRACT

Anaplasma phagocytophilum is an obligatory intracellular bacterium that infects granulocytes and causes human granulocytic anaplasmosis (HGA). This bacterium requires cholesterol for host cell infection in vitro and incorporates exogenous cholesterol into its membrane. However, the role of blood cholesterol in HGA in vivo and how A. phagocytophilum acquires cholesterol inside the host cells are still unknown.

To understand the role of host cholesterol in A. phagocytophilum infection in vivo, we analyzed the effects of a high-cholesterol diet and reduced apolipoprotein E (apo E) activity on A. phagocytophilum infection in mice. A high-cholesterol diet significantly increased A. phagocytophilum bacteria burden in the spleen, liver, and blood of apoE-deficient (apoE -/-) mice and also induced a significant elevation in the mRNA expression of MIP-2 and a MIP-2 receptor, CXCR2, in the spleen in apoE-/- mice fed a high-cholesterol diet. These results suggest that high blood cholesterol levels facilitate A. phagocytophilum infection and up-regulate a proinflammatory chemokine and its receptor, which may contribute to HGA pathogenesis.

In eukaryotes, intracellular cholesterol homeostasis and trafficking are tightly regulated. It is unknown how A. phagocytophilum, a cholesterol-dependent
bacterium, interacts with the host cell cholesterol regulatory pathway to acquire cholesterol. Here, we report that total host cell cholesterol increased >2-fold during *A. phagocytophilum* infection in HL-60 cells. Cellular free cholesterol was enriched in *A. phagocytophilum* inclusions. We determined that *A. phagocytophilum* requires cholesterol derived from low-density lipoprotein (LDL) uptake pathway, rather than de novo cholesterol biosynthesis. The uptake of fluorescence-labeled LDL was enhanced in infected cells. *A. phagocytophilum* infection up-regulated LDLR expression at both the mRNA and protein levels by stabilizing LDLR mRNA via the 3’ UTR region, but not through activation of the sterol regulatory element binding proteins. These data reveal that *A. phagocytophilum* exploits the host LDL uptake pathway and LDLR mRNA regulatory system to accumulate cholesterol in inclusions to facilitate its replication.

Extracellular signal–regulated kinase (ERK) was persistently activated by *A. phagocytophilum* infection in HL-60 cells, especially at middle-late infection stage, and the ERK activation is required for *A. phagocytophilum* infection. We demonstrate that Raf-1 is actively phosphorylated by *A. phagocytophilum* infection and this phosphorylation may activate the ERK. Up-regulation of LDLR mRNA by *A. phagocytophilum* was inhibited by a MEK inhibitor; however, it was unclear whether ERK activation is required for LDLR mRNA up-regulation by *A. phagocytophilum*. 
*Ehrlichia ewingii* is the most recently recognized human ehrlichiosis agent and is the only *Ehrlichia* sp. known to infect neutrophils. In the blood or in ex vivo culture, neutrophils generally have a short life span. In the present study, we investigated the effect of *E. ewingii* infection on spontaneous apoptosis of neutrophils. *E. ewingii* infection significantly delayed dog neutrophil apoptosis during ex vivo culture. By using the fluorescent mitochondrial dyes Mitotracker Red 580 and JC-1, we found that *E. ewingii* infection stabilized mitochondrial integrity by maintaining mitochondrial membrane potential in neutrophils. These results suggest that *E. ewingii* delays spontaneous apoptosis of neutrophils via stabilization of host cell mitochondria.
Dedicated to my lovely son Allen Xiong and my wife Linlin Xiao, my parents and mother-in-law, Xingshu Xiao for their generous support during my study
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CHAPTER 1

INTRODUCTION

Tick-borne human infections and human Ehrlichiosis agents

Since the agent of Rocky Mountain spotted fever (RMSF) was for the first time described in the United States in 1906, several clinically important tick-associated human infectious diseases have been identified (Table 1) (Bakken and Dumler, 2008). The case reports for some of these infections, especially human ehrlichioses and anaplasmoses, significantly increased during the last 20 years. Tick-borne infections frequently cause severe illness and occasionally death. While many patients with competent immune systems resolve their illnesses spontaneously, most patients benefit from specific antibiotic therapy. A severe outcome is usually associated with delayed diagnosis and therapy or misdiagnosis because early symptoms and signs of these infections are nonspecific, often mimicking a viral illness, and the limitation of available diagnostic tests. Thus, it may be difficult to arrive at a specific diagnosis early in the course of the illness when antibiotic therapy is most likely to be successful (Bakken and Dumler, 2008; Dumler, 2005a).
Table 1.1  Summary of important tick-borne human infections in the United States (Bakken and Dumler, 2008)

<table>
<thead>
<tr>
<th>Year of description</th>
<th>Clinical illness</th>
<th>Responsible agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1906</td>
<td>Rocky Mountain spotted fever</td>
<td><em>Rickettsia rickettsii</em></td>
</tr>
<tr>
<td>1915</td>
<td>Relapsing fever</td>
<td><em>Borrelia hermsii</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Borrelia turcicata</em></td>
</tr>
<tr>
<td>1976</td>
<td>Babesiosis</td>
<td><em>Babesia microti</em></td>
</tr>
<tr>
<td>1976</td>
<td>Lyme borreliosis</td>
<td><em>Borrelia burgdorferi</em></td>
</tr>
<tr>
<td>1987</td>
<td>Human monocytic ehrlichiosis</td>
<td><em>Ehrlichia chaffeensis</em></td>
</tr>
<tr>
<td>1994</td>
<td>Human granulocytic anaplasmosis</td>
<td><em>Anaplasma phagocytophilum</em></td>
</tr>
<tr>
<td>1999</td>
<td>Human granulocytic ewingii ehrlichiosis</td>
<td><em>Ehrlichia ewingii</em></td>
</tr>
</tbody>
</table>

Human ehrlichioses and anaplasmoses are emerging tick-borne infections in last two decades. The term ehrlichiosis has been broadly applied to a variety of diseases of humans and animals caused by pathogens formerly classified in the genus *Ehrlichia*. Since a proposed taxonomic reorganization has recommended the reclassifications of some former *Ehrlichia* species in 2001 (Dumler et al., 2001), currently, “Human Ehrlichiosis” is a generic name to describe infections with at least 5 separate obligate intracellular bacteria in 3 genera (*Neorickettsia, Ehrlichia, Anaplasma*) in the family *Anaplasmataceae* including *Ehrlichia chaffeensis, E. ewingii, E. canis, Anaplasma phagocytophilum, and Neorickettsia sennetsu* (Dumler, 2005a). Human monocytic ehrlichiosis (HME) caused by *E. chaffeensis*, a new monocytotropic ehrlichial species, was discovered in 1986 (Maeda et al., 1987), and human granulocytic anaplasmosis (HGA, formerly human granulocytic ehrlichiosis,
HGE) caused by *A. phagocytophilum* (formerly the HGE agent) was discovered in 1994 (Bakken *et al.*, 1994; Chen *et al.*, 1994). In 1999, another granulocytotropic ehrlichial species, *E. ewingii*, was recognized as a human pathogen (Buller *et al.*, 1999). The disease was proposed to be named as human ewingii ehrlichiosis (HEE) instead of human granulocytic ehrlichiosis, the previous name for HGA to avoid confusion (Dumler, 2005a). *Neorickettsia* (formerly *Ehrlichia* *sennetsu*, another monocytotropic sp., was the first human pathogen in the family *Anaplasmataceae* discovered in Japan in the 1950's, and also has been recently found in Malaysia (Rapmund, 1984) and Laos (Newton, 2008). In 1995 a monocytic ehrlichial species *E. canis* was isolated from a human patient in Venezuela (Perez *et al.*, 1996). Since 1986, *E. chaffeensis*, *A. phagocytophilum*, and *E. ewingii* are the causes of most human ehrlichioses in the United States, and among them only *E. chaffeensis* and *A. phagocytophilum* have been sufficiently investigated, since *E. ewingii* has not been isolated yet by any mammalian and tick cell culture system so far.

**The family *Anaplasmataceae***

*Anaplasmataceae* belongs to the class *α*-Proteobacteria, the order *Rickettsiales*. Members of the family *Anaplasmataceae* are small Gram-negative pleomorphic cocci that are obligate intracellular bacteria. They replicate in membrane-bound vacuoles in the cytoplasm of a specific type of host cell of hematopoietic origin and/or in invertebrates (Rikihisa, 2006a; Rikihisa, 1991). Under the light microscope, ehrlichial organisms are small cocci in the cytoplasm of the host cells that stain dark blue to purple with Romanowsky stain. Microcolonies of ehrlichiae in the host cytoplasm
may look like mulberries and thus are called morulae. On transmission electron microscopy, organisms generally are round but sometimes are highly pleomorphic and found in membrane-lined vacuoles called inclusions. These bacteria are vector-borne, that is, transmitted by ticks or trematodes. Infection of blood cells of domestic and wild animals, and humans with ehrlichial organisms may lead to a clinically apparent illness collectively called ehrlichiosis or anaplasmosis (or rickettsiosis), a febrile systemic illness often accompanied with hematological abnormalities, lymphadenopathy, and elevation of liver enzyme activity (Rikihisa, 2006a).

**Ecology of family Anaplasmataceae**

The agents of human ehrlichioses and anaplasmosis are tickborne. Genera *Ehrlichia* and *Anaplasma* require horizontal and bidirectional transmission alternating between mammals (or birds), and ticks. *Neorickettsia* spp. are 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*. Human and other animal infection with *Neorickettsia* takes place by accidental ingestion of the infected metacercaria stage of trematodes encysted in fish or aquatic insects (Rikihisa, 2006a; Rikihisa, 2003; Rikihisa, 1991).

*Anaplasma* and *Ehrlichia* spp. are maintained through an enzootic cycle between wild animals and bloodsucking ticks and can accidentally infect humans through the
bite of infected ticks. *A. phagocytophilum* has been found in ticks of *Ixodes* species, and wild rodents, such as white-footed mice (*Peromyscus leucopus*) and dusky-footed wood rats (*Neotoma fuscipes*), are believed to be the major reservoirs of *A. phagocytophilum* in the eastern and western United States, respectively. *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* (Rikihisa, 2006a; Rikihisa, 2003; Rikihisa, 1991). See table 2 for the comparison of 4 human ehrlichioses and anaplasmoses.

### Table 1.2 Epidemiology of human ehrlichioses and anaplasmoses (adapted from *Walker et al.*, 2008)

<table>
<thead>
<tr>
<th>Agent</th>
<th>Disease</th>
<th>Primary target cells</th>
<th>Tick Vector</th>
<th>Geographic Distribution</th>
<th>Vertebrate Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. chaffeensis</em></td>
<td>Human monocytotropic ehrlichiosis</td>
<td>Primarily mononuclear cells</td>
<td><em>Amblyomma americanum, Dermacentor variabilis, Ixodes pacificus</em></td>
<td>Southeastern and south-central United States, California</td>
<td>White-tailed deer, dogs, coyotes, goats</td>
</tr>
<tr>
<td><em>E. ewingii</em></td>
<td>Ehrlichiosis ewingii</td>
<td>Granulocytes</td>
<td><em>A. americanum</em></td>
<td>Southeastern and south-central United States, California</td>
<td>White-tailed deer, dogs</td>
</tr>
<tr>
<td><em>E. canis</em></td>
<td>Unnamed disease</td>
<td>Primarily mononuclear cells</td>
<td><em>Rhipicephalus sanguineus</em></td>
<td>Worldwide</td>
<td>Dogs, jackal, coyote, wild African dog, red and gray fox</td>
</tr>
<tr>
<td><em>A. phagocytophilum</em></td>
<td>Human granulocytotropic anaplasmosis</td>
<td>Granulocytes</td>
<td><em>I. scapularis</em></td>
<td>Northern United States</td>
<td>White-footed deer mouse, white-tailed deer, dogs, horses, squirrels, chipmunks, red-backed vole</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>I. pacificus</em></td>
<td>Pacific coastal United States</td>
<td>quirels, wood rats, elk, horses, llama, black-tailed deer, mice</td>
</tr>
</tbody>
</table>
**Human granulocytic anaplasmosis (HGA)**

Human granulocytic anaplasmosis was first recognized in 1990 in a Wisconsin patient who died with a severe febrile disease two weeks after a tick bite (Bakken et al., 1994; Chen et al., 1994). In 1994, the causative agent of HGA, *A.phagocytophilum* was identified using DNA sequencing in the United States(Chen et al., 1994) and subsequently reported worldwide in at least 14 European countries and perhaps in China, Siberian Russia, and Korea (Dumler, 2005a). Few patients have been shown to be coinfected with other tick-borne agents sharing the same *Ixodes* sp. tick vector such as Lyme borreliosis and babesiosis in Europe and the USA (Blanco and Oteo, 2002; Nadelman et al., 1997). The clinical signs for HGA include fever, headache, myalgia, malaise, absence of skin rash, leucopenia, thrombocytopenia, increased amounts of C-reactive protein and abnormal activities of hepatic transaminase. It can cause severe and potentially fatal disease in immunocompromised and elderly people. The case fatality rate for HGA is approximately 0.5%, whereas that of HME is 3% (Dumler, 2005a). Recent seroepidemiologic data suggest that as much as 15% to 36% of the population has

<table>
<thead>
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<th><em>N. sennetsu</em></th>
<th>Sennetsu ehrlichiosis/Sennetsu fever</th>
<th>Primarily mononuclear cells</th>
<th>Trematode in fish</th>
<th>Japan, Malaysia, Laos</th>
<th>unknown</th>
</tr>
</thead>
</table>

| *I. ricinus* | Europe | red deer, roe deer, fallow deer, horses, dogs, cattle, cats, sheep, bank voles, wood mice, yellow-necked mouse, common shrew |
been infected in endemic areas (Dumler, 2005a). The epidemiological data show that incidence of HGA increases with age, with the highest incidence among persons older than 60. And also there is increased severity of HGA in elder people (Bakken et al., 1996). However, the reason for this age-associated incidence and severity is unknown.

**Pathogenesis of Human granulocytic anaplasmosis**

Neutrophil is the first line of host defense system against invading pathogens. The niche for *A. phagocytophilum*, the neutrophil, indicates that the pathogen has unique adaptations and pathogenetic mechanisms. To create a sheltered niche permissive for replication, intracellular bacteria must bind to an appropriate host-cell receptor, which induces internalization of the bacteria without delivering to lysosomal traffic route or eliciting strong anti-microbial signaling. *A. phagocytophilum* have evolved remarkable strategies to exploit cellular systems that aid them in this role. Furthermore, these bacteria inhibit host leukocyte apoptosis to maximize their reproduction and dispersion (Rikihisa, 2006b). Intensive study has demonstrated interactions with host-cell signal transduction and possibly eukaryotic transcription. This interaction leads to permutations of neutrophil function and could permit immunopathologic changes, severe disease, and opportunistic infections.

[1] **Unusual cell wall: lack of lipopolysaccharide and peptidoglycan and presence of cholesterol**

Pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) or peptidoglycan can be recognized by host cells pattern recognition receptors,
such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-containing proteins, resulting in profound innate immune responses from these cells, which eliminate the invading microorganisms. Tick hemocytes also have a strong innate defensive mechanism responsive to PAMPs (Rikihisa, 2006b). Uniquely among Gram-negative bacteria, \textit{A. phagocytophilum} has lost all genes required for the biosynthesis of LPS, and most genes required for the biosynthesis of peptidoglycan (Lin and Rikihisa, 2003a). This loss must have facilitated the adaptation of anaplasma to leukocytes and cells of the tick vector. Interestingly, \textit{A. phagocytophilum} has acquired another unique feature: the ability to take up cholesterol from the host (Lin and Rikihisa, 2003a). Cholesterol has become indispensable for their survival and infection (Lin and Rikihisa, 2003a) and membrane cholesterol probably compensates for the reduction in membrane stability caused by the loss of LPS and peptidoglycan. Loss of peptidoglycan provides additional benefits, such as the flexibility to accommodate anaplasma in the limited intra-vacuolar space, which might further enhance compatibility with leukocytes and invertebrate hosts (Rikihisa, 2006b).

**[2] Internalization and creation of a replicative compartment that does not fuse with lysosomes**

Residence within host cells provides \textit{A. phagocytophilum} a sheltered niche to avoid several immune defense mechanisms of the host. Caveolae- or lipid raft-mediated endocytosis is utilized by a wide variety of pathogenic microorganisms to enter host cells in order to bypass phagolysosomal pathways (Lafont and van der Goot, 2005).
The entry and intracellular infection of *A. phagocytophilum* involves caveolae and glycosylphosphatidylinositol-anchored proteins (Lin and Rikihisa, 2003b). Caveolin-1 and tyrosine-phosphorylated proteins, including PLC-γ2, are colocalized with both early and late replicative inclusions of *A. phagocytophilum* (Lin et al., 2002). However, clathrin was not found in any inclusions throughout the infection. These data indicate that *A. phagocytophilum* may exploit a clathrin-independent lipid raft-mediated endocytosis for its entry to host cells. Also, these results suggest that signals from caveolae and transglutaminase (TG) converge to activate protein tyrosine kinase and PLC-γ2 during the internalization of *A. phagocytophilum*. A recent report described the transcriptional up-regulation of PLC-β1, TG3-like and Tec protein tyrosine kinase in *A. phagocytophilum*-infected HL-60 cells (de la Fuente et al., 2005). These data imply that *A. phagocytophilum* actively modify the host proteins required for both their entry and the establishment of replication-competent inclusions at the transcriptional and post-transcriptional levels.

Goodman and colleagues identified fucosylated platelet selectin glycoprotein ligand 1 (PSGL-1) as a receptor for *A. phagocytophilum* because the binding of *A. phagocytophilum* to HL-60 cells is dependent on the expression of both PSGL-1 and a α1–3-fucosyltransferase (Herron et al., 2000). Binding of *A. phagocytophilum* to murine neutrophils, however, requires expression of α1–3-fucosyltransferases, but not PSGL-1 (Carlyon et al., 2003). The molecular basis for this differential binding can be explained, at least in part, by the fact that *A. phagocytophilum* recognizes a short amino acid sequence found in the N-terminus of human, but not murine, PSGL-1.
(Yago et al., 2003). Thus, it appears that *A. phagocytophilum* uses at least two adhesins, which bind co-operatively to at least two ligands on both human and murine neutrophils. Studies by Park et al. suggest that a paralogue of the P44 (MSP2) family of outer surface proteins may facilitate *A. phagocytophilum* binding to human PSGL-1 acting as an adhesin (Park et al., 2003). *A. phagocytophilum* usage of PSGL-1 and α1,3-fucosylated, α2,3-sialylated glycans as a receptor-mediated pathway for cellular adhesion and entry explains not only its tropism for neutrophils but also one means by which it evades destruction by the normal neutrophil phagocytic pathway (Carlyon and Fikrig, 2003).

After docking to its host cell receptor(s), *A. phagocytophilum* enters and colonizes the hostile intracellular environment of the neutrophil. *A. phagocytophilum* evolved the ability to modulate vesicular trafficking, protecting them from delivery to lysosomes (Mott et al., 1999; Rikihisa, 1991). This is particularly important for these bacteria because they exclusively reside in professional phagocytes, which have abundant lysosomes. Co-localization studies using infected HL-60 cells revealed that none of lysosomal markers, including lysosome-associated membrane protein 1 (LAMP-1), CD63 (LAMP-3) and myeloperoxidase (MPO), associates with vacuoles containing *A. phagocytophilum* (Mott et al., 1999; Webster et al., 1998). *A. phagocytophilum* inclusion is not acidic because using acidic markers, neither the compound 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine nor acridine orange co-localizes within the same compartment as the bacterium (Mott et al., 1999; Webster et al., 1998). *A. phagocytophilum*-containing inclusions also do not label
with antibody directed against vacuole-type $\text{H}^+\text{-ATPase}$, which is located in early and late endosomes, lysosomes, and the Golgi apparatus and is required for acidification of these compartments (Mott et al., 1999), which is required for the acidification of vesicular compartments. However, in all these studies, lysosomes were observed accumulating in close proximity around the periphery of the \textit{A. phagocytophilum}-containing inclusion. Therefore, \textit{A. phagocytophilum} does not globally inhibit phagosome–lysosome fusion or lysosomal function but, instead, specifically blocks lysosome fusion with the protective phagosome in which it resides. This inhibition is dependent on bacterial protein synthesis, as oxytetracycline treatment results in maturation of the \textit{A. phagocytophilum}-containing vacuoles into phagolysosomes (Gokce et al., 1999).

Webster et al. also showed that a portion of the \textit{A. phagocytophilum}-containing vacuoles membranes incorporate endocytosed colloidal gold particles and co-localize with mannose-6-phosphate receptor (MPR), a marker of late endosomes, suggesting that these compartments are part of endocytic pathway (Webster et al., 1998). However, a study by Mott et al. demonstrated that \textit{A. phagocytophilum} inclusions do not label for MRP, and also not for transferrin receptor, Rab5 or early endosomal antigen I, each of which is a marker for early endosomes. Furthermore, they do not label for the general endocytic markers clathrin heavy chain, $\alpha$-adaptin or annexin I, II, IV or VI (Mott et al., 1999). Additionally, endogenously synthesized sphingomyelin in Golgi is not incorporated into inclusion. Brefeldin A, a fungal metabolite inhibiting antegrade (but not retrograde) transport of the Golgi complex,
did not affect the growth of *A. phagocytophilum* in HL-60 cells (Mott *et al.*, 1999).

Taken together, *A. phagocytophilum* inclusion avoids lysosomal fusion through excluding itself from host cell endocytic and exocytic vesicular traffic pathways (Rikihisa, 2006b). The characteristics of *A. phagocytophilum* inclusion and the intracellular lifestyle of *A. phagocytophilum* still remain mystery.

Recently Niu, *et al* reported that *A. phagocytophilum* inclusion has autophagosomes-like characteristics because several hallmarks of early autophagosomes were identified in *A. phagocytophilum* replicative inclusions, including a double-lipid bilayer membrane and colocalization with GFP-tagged LC3 and Beclin 1. Additionally, autophagy formation induced by rapamycin enhances *A. phagocytophilum* growth in the host cells. These data suggest *A. phagocytophilum* subverts host autophagy system to establish itself in an early autophagosome-like compartment segregated from lysosomes to facilitate its proliferation (Niu *et al.*, 2008).

Finally it is interesting to point out that, despite sharing the several common features with *E. chaffeensis*, the casative agent of HME with the primary target host cell as monocyte, *A. phagocytophilum* and *E. chaffeensis* inclusions are distinct from each other, and thus the two species never colocalize in the same inclusions even after coinfection of the same HL-60 cell (Mott *et al.*, 1999). The replicative inclusions of *E. chaffeensis* accumulate transferrin receptors (TfRs) and have several early endosomal markers, such as Rab5 and early endosomal antigen 1, whereas the inclusions of *A. phagocytophilum* are negative for these endosomal markers. These facts suggest that
the replicative inclusions of *E. chaffeensis* are early endosomes, whereas *A. phagocytophilum* replicative inclusions are unique and do not resemble any known endocytic or exocytic compartment. An additional difference is that *A. phagocytophilum* inclusions are often surrounded by lysosomes, whereas mitochondria are found near *E. chaffeensis* inclusions (Mott *et al.*, 1999).

**[3] Anaplasma phagocytophilum inhibits O2⁻ production**

Neutrophils have a powerful oxygen-dependent defense system that generates reactive oxygen intermediates (ROIs), such as superoxide, hydrogen peroxide and hydroxyl radicals, to kill the invading pathogens. Superoxide is produced by the tightly controlled, rapidly activatable NADPH oxidase complex including integral membrane proteins gp91phox and p22phox and several cytosolic components (p47phox, p67phox, p40phox and Rac2). *A. phagocytophilum* lacks genes encoding detoxifying enzymes, such as periplasmic Cu/Zn- superoxide dismutase (SOD), Mn-SOD and catalase, and oxygen-sensing two-component regulatory systems (oxyR or SoxRS). Thus, *A. phagocytophilum* generally does not induce NADPH oxidase activation in human and murine neutrophils (Rikihisa, 2006b), although one study showed that *A. phagocytophilum* induces a greater neutrophil respiratory burst than does LPS at the early time of infection (Choi and Dumler, 2003). *A. phagocytophilum* interferes with the assembly of the NADPH oxidase subunits in the inclusion membrane, and blocks subsequent activation of NADPH oxidase by phorbol myristic acetate, N-formylmethionyl-leucylphenylalanine or *Escherichia coli* (Mott *et al.*, 2002; Wang *et al.*, 2002; Mott and Rikihisa, 2000). In human neutrophils and HL-60 cells, *A.
Anaplasma phagocytophilum decreases the protein levels of p22\textsuperscript{phox}, but not other components of NADPH oxidase (gp91\textsuperscript{phox}, p47\textsuperscript{phox}, p67\textsuperscript{phox} and p40\textsuperscript{phox}) (Mott et al., 2002). Absence of colocalization of NADPH oxidase components with the inclusion further protects A. phagocytophila from oxidative damage (Mott et al., 2002). However, another study showed that the transcription of Rac1, Rac2 and gp91\textsuperscript {phox} is downregulated in infected HL-60 cells (Carlyon et al., 2002). Downregulation of the gp91\textsuperscript {phox} gene is associated with increased binding of the repressor CCAAT displacement protein to the promoter of the gp91\textsuperscript {phox} gene resulting from reduced interferon regulatory factor and PU.1 protein levels in infected HL-60 cells (Thomas et al., 2005). AnkA protein of A. phagocytophilum has been reported to be involved in downregulating expression of gp91\textsuperscript {phox} (Garcia-Garcia et al., 2009). Transcriptional downregulation of Rac1, Rac2, and gp91\textsuperscript {phox} was not, however, detected in recent microarray analyses using HL-60 cells, NB4 cells or human neutrophils infected with A. phagocytophilum (Borjesson et al., 2005; de la Fuente et al., 2005; Pedra et al., 2005). Despite several disparities in the functional responses of myeloid cell lines and neutrophils obtained with different assay methods, A. phagocytophilum and the neutrophil system provide an interesting opportunity to study the biology of NADPH oxidase downregulation (Rikihisa, 2006b).

[4] *Anaplasma phagocytophilum delays neutrophil apoptosis*

Apoptosis is an important mechanism for killing intracellular pathogens. The host cells induce apoptosis in the presence of several pathogens, whereas some pathogens are known to inhibit host cell apoptosis (DeLeo, 2004). *A. phagocytophilum* inhibits
spontaneous apoptosis of human neutrophils, allowing the bacterium sufficient time (>24 h post-infection) to develop intracellular microcolonies called morulae (Yoshiie et al., 2000). A. phagocytophilum prevents human neutrophils from spontaneously reducing the mRNA of the anti-apoptotic bcl-2 family member bfl-1, losing the mitochondrial membrane potential and activating caspase 3 (Ge et al., 2005). Recent microarray data using human neutrophils and NB4 cells confirmed these findings, showing that A. phagocytophilum infection upregulates expression of bfl-1 family members (Borjesson et al., 2005; Pedra et al., 2005). A. phagocytophilum also blocks anti-FAS (CD95/Apo-1)-induced programmed cell death of human neutrophils. The cleavage of pro-caspase 8, caspase 8 activation, and the cleavage of Bid in the extrinsic pathway of spontaneous neutrophil apoptosis are inhibited by A. phagocytophilum infection. Likewise, A. phagocytophilum infection inhibits the pro-apoptotic Bax translocation to mitochondria, activation of caspase 9, the initiator caspase in the intrinsic pathway, and the degradation of a potent caspase inhibitor, X-chromosome-linked inhibitor of apoptosis protein (XIAP), during spontaneous neutrophil apoptosis. Taken together, all these data suggest a novel multifacial inhibitory mechanism induced by A. phagocytophilum involving both extrinsic and intrinsic pathways to ensure to delay the apoptosis of host neutrophils (Ge and Rikihisa, 2006).

[5] Anaplasma phagocytophilum exploits the neutrophil chemokine response

Microbial infection stimulates the production of cytokines, which helps to control infection but may also contribute to microbial pathogenesis and disease pathology.
Chemokines, which are chemotactic cytokines predominantly secreted by inflammatory cells, are important mediators of leucocyte activation and chemotaxis. Their expression is induced by a variety of stimuli, including proinflammatory cytokines, lipopolysaccharide (LPS) and other bacterial products (Carlyon and Fikrig, 2003). Human anaplasmosis patient sera and supernatants from *A. phagocytophilum*-infected neutrophils display elevated levels of chemokines IL-8 (Akkoyunlu et al., 2001). And also, *A. phagocytophilum* infection of bone marrow progenitors, HL-60 cells and rHL-60( differentiated with retinoic acid) cells *in vitro* also results in the elevated expression of IL-8 and several other chemokines, such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α and -β and RANTES (Akkoyunlu et al., 2001; Klein et al., 2000).

Akkoyunlu et al. (2001) dissected the biological relevance of increased production of IL-8, a powerful neutrophil chemoattractant, during *A. phagocytophilum* infection(Akkoyunlu et al., 2001). The bacterium induces IL-8 secretion from rHL-60 cells in a dose- and time-dependent fashion. Furthermore, a recombinant P44 is responsible for the IL-8 production. IL-8 enhances neutrophil phagocytosis, as well as superoxide and granule release, which raises the question as to why *A. phagocytophilum* specifically induces its production. The answer paradoxically lies in the fact that increased IL-8 production effectively recruits naïve neutrophils to facilitate bacterial dissemination. Human neutrophils respond to IL-8 through the receptors CXCR1 and CXCR2. *A. phagocytophilum* -infected rHL-60 cells demonstrate elevated surface expression of CXCR2, but not CXCR1 (Akkoyunlu et
al., 2001). This hypothesis was further supported in murine model. Although the murine counterpart of IL-8 has yet to be identified, murine MIP-2 and KC do bind murine CXCR2 to facilitate neutrophil chemotaxis. Incubation of *A. phagocytophilum* with murine splenocytes results in significant increases in KC and MIP-2. CXCR2-induced neutrophil migration plays an important role in *A. phagocytophilum* dissemination during murine infection because CXCR2\(^{+/−}\) and wild-type mice pretreated with CXCR2 antiserum each exhibit considerable decreases in bacterial load compared with control background or mice pretreated with control serum respectively. Furthermore, wild-type mice pretreated with CXCR2 antiserum before *A. phagocytophilum* infection exhibit significant decreases in neutrophil chemotaxis into the peritoneal cavity compared with that of infected mice pretreated with control serum (Akkoyunlu *et al.*, 2001). As *A. phagocytophilum* is an obligate intracellular bacterium, successful establishment and maintenance of infection are dependent on its transfer to naïve neutrophils or neutrophil precursors. Thus, *A. phagocytophilum* exploits neutrophil IL-8 production as a mechanism of attracting neutrophils to sites of infection for further bacterial propagation (Carlyon and Fikrig, 2003).

**[6] Modulation of host cell Signaling**

*A. phagocytophilum* resides and proliferates in the professional killing host cells and disregulates many cell functions in order to benefit itself and establish the infection in the host. Not surprisingly, increasing bodies of evidence show that multiple signaling pathways are activated during intracellular *A. phagocytophilum*
infection which may be required to regulate cytokines response, to alter cell proliferation and survival, and to establish bacterial infection in the host cells.

Kim HY at al. studied the p38 mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways for induction of some pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 in vitro. Activation of p38 MAPK and NF-κB mRNAs in monocytes was detectable within 15 to 30 min after addition of *A. phagocytophila*. IL-1β mRNA expression by neutrophils was not dependent on p38 MAPK, and p38 MAPK was not activated in neutrophils incubated with *A. phagocytophila*. Neutrophil expression of IL-1β mRNA was dependent on transglutaminase, phospholipase C, and PTK, all of which are also required for internalization of *A. phagocytophila*. These results suggest that *A. phagocytophila* transduces different signals between its host neutrophils and monocytes for proinflammatory cytokine generation (Kim and Rikihisa, 2002; Kim and Rikihisa, 2000). Using NB4 as a human leukemic model, Erol Fikrig’s microarray data show that the expression of Toll-like receptors, MYD88, RNF36, IRF3, and TBK1 and inhibitors of the NF-kappaB gene was not altered by *A. phagocytophilum* infection. These results clearly indicate that *A. phagocytophilum* modulates NB4 cell machinery through transcription factors that subsequently affect multiple genes in downstream pathways (Pedra et al., 2005). Importantly, the protein level of the hematopoietic system-specific transcription factor PU.1, which acts specifically at the stage of promyelocyte differentiation into neutrophils, is down-regulated by *A. phagocytophilum in* HL-60 cells (Thomas et al., 2005). These results point to
different, but novel, mechanisms by which *A. phagocytophilum* survive: by inhibiting or delaying crucial signaling in host phagocyte activation and differentiation pathways.

Dumler *et al.* reported that an increased phosphorylation of p38 MAPK, rather than ERK and Akt, in *A. phagocytophilum* infected neutrophils and suggested that initial activation of the p38 MAPK pathway leads to *A. phagocytophilum*-delayed neutrophil apoptosis (Choi *et al.*, 2005). However, Goodman *et al.* firstly demonstrated that the ERK pathway, particularly ERK2, is activated in *A. phagocytophilum*-infected neutrophils even at 3h post infection (Lee and Goodman, 2006). And our data also showed ERK activation by *A. phagocytophilum* in HL-60 cells (Xiong *et al.*, 2009).

Thomas *et al.* identified a tyrosine phosphorylated Rho-associated, coiled-coil containing protein kinase (ROCK) 1 at *A. phagocytophilum* early infection in HL-60 cells and neutrophils and this pathogen-specific phosphorylation is associated with bacteria host cell receptor PSGL-1 and Syk. Moreover, knockdown of either Syk or ROCK1 markedly impaired *A. phagocytophilum* infection (Thomas and Fikrig, 2007). Lin *et al.* found that Abl-interactor 1 (Abi-1), an adaptor protein that interacts with Abl-1 tyrosine kinase, interacts with the first type IV secretion (T4S) system substrate, Ank by yeast two-hybrid and coimmunoprecipitation analyses. And AnkA is tyrosine phosphorylated by Abl-1 to facilitate infection (Lin *et al.*, 2007). Interestingly, another parallel study by Ijdo *et al.* showed that AnkA of *A. phagocytophilum* NCH-1 strain can be phosphorylated by Src kinase in COS cells cotransfected with Src and
AnkA of NCH-1 (Ijdo et al., 2007). Western blotting showed that a specific Src kinase inhibitor PP2 inhibited AnkA tyrosine phosphorylation, suggesting that Src is also involved in tyrosine phosphorylation of AnkA in A. phagocytophilum HZ strain. However, Src kinase inhibitor PP2 did not block HZ strain infection in HL-60 cells (Lin et al., 2007).

The studies of cellular biology of A. phagocytophilum infection, especially modulating of host cell signaling pathways, are still in infancy. Many fundamental questions await further investigation. For example, how those signaling pathways are triggered by A. phagocytophilum infection? Whether bacterial components involve in this process and if so, how these unknown components intersect with host cell signaling cascades? What are the downstream effects of activated signaling pathways by A. phagocytophilum? Or in another word, what are the benefits for A. phagocytophilum to activate these pathways? Several studies indicate the activated signaling may involve in the delayed neutrophils apoptosis; however, the real reason-and-cause relations between them are still elusive.

**Cytokines and immunopathology**

Ehrlichiosis is a febrile systemic illness accompanied by abnormal blood cell counts and liver enzyme activity. Since very few organisms are found in infected animals and humans and no endo- or exotoxin activities have been detected, host proinflammatory cytokines are considered to be generally responsible for pathologic changes and clinical signs of ehrlichiosis. And the discrepancy between bacterial and
histopathologic changes in HGA patients also suggests that disease relates to immune effectors that inadvertently damage tissues (Martin et al., 2001; Dumler et al., 2000; Martin et al., 2000).

Several studies have characterized the cytokine response associated with *A. phagocytophilum* infection, the majority of which overwhelmingly indicate a cytokine response weighted towards a TH1 phenotype. High levels of interferon-gamma and IL-10, but not TNF-α, IL-4, and IL-1β, are found in HGA human patients sera (Dumler et al., 2000). In contrast, Kim et al. found that *A. phagocytophilum* induces proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in human monocytes, but not in their host cells, neutrophils, *in vitro* (Kim and Rikihisa, 2000). TNF-α, IL-1β and IL-8 mRNA are also expressed by peripheral blood leukocytes (PBLs) from horses experimentally infected with *A. phagocytophilum* (Kim et al., 2002). The recombinant major outer membrane protein of *A. phagocytophilum*, rP44, can induce TNF-α, IL-1β, and IL-6 production by human PBLs and monocytes (Kim and Rikihisa, 2000). These results indicate that although *A. phagocytophilum* preferentially infects granulocytes, it also interacts with monocytes and induces different signals leading to the generation of different sets of proinflammatory cytokines (Kim and Rikihisa, 2002). Thus, in ehrlichiosis, temporal and spatial patterns of proinflammatory cytokine generation *in situ* may be complex, involving multiple cell types and also the influence of other humoral factors (Rikihisa, 2003).

It is well established that IFN-γ protects against infections by obligate intracellular bacteria and probably does so against *A. phagocytophilum*. Akkyunlu and Fikrig demonstrated that *A. phagocytophilum* also elicits a prominent IFN-gamma response.
in mice and that IFN-γ plays a critical role in the early eradication of \( A. \) phagocytophilum (Akkoynulu and Fikrig, 2000). The source and regulation of IFN-γ have been intensively investigated by Fikrig’s group. They reported that the inflammasome, forming by ASC and caspase-1, induces the activation of caspase-1, resulting in subsequent processing and secretion of IL-18 in response to \( A. \) phagocytophilum, which in turn is required for the IFN-γ-dependent phase of \( A. \) phagocytophilum clearance (Pedra et al., 2007a). And also, IFN-γ is regulated through an early IL-12/23p40-dependent mechanism. IL-12/23p40 is regulated in macrophages and dendritic cells after activation by microbial agonists and cytokines and constitutes a subunit of IL-12 and IL-23. IL-12/23p40-deficient mice displayed an increased \( A. \) phagocytophilum burden, accelerated thrombocytopenia and increased neutrophil numbers in the spleen at day 6 postinfection. Infection of MyD88- and mitogen-activated kinase kinase 3 (MKK3)-deficient mice suggested that the early susceptibility due to IL-12/23p40 deficiency was not dependent on signaling through MyD88 or MKK3. The lack of IL-12/23p40 reduced IFN-γ production in both CD4(+) and CD8(+) T cells although the effect was more pronounced in CD4(+) T cells. These data suggest that the immune response against \( A. \) phagocytophilum is a multifactorial and cooperative process. The IL-12/23p40 subunit drives the CD4(+) Th1 immune response in the early phase of infection and IL-12/23p40-independent mechanisms ultimately contribute to pathogen elimination from the host (Pedra et al., 2007b).

Interestingly, they also showed that c-Jun NH2-terminal kinase 2 (JNK2) inhibits IFN-γ production during \( A. \) phagocytophilum infection. Jnk2-null mice were more
refractory to infection with *A. phagocytophilum* and produced increased levels of IFN-γ after challenge with the pathogen. The resistance of jnk2-null mice to *A. phagocytophilum* infection was due to elevated levels of IFN-γ secreted by conventional and natural killer (NK) T cells. The administration of alpha-galactosylceramide, a strong NK T-cell agonist, increased IFN-γ release and protected mice from *A. phagocytophilum*, further demonstrating the inhibitory effect of JNK2 on IFN-γ production. Collectively, these findings demonstrated that JNK2 is an important regulatory protein for IFN-γ secretion upon challenge with *A. phagocytophilum* (Pedra et al., 2008).

*Anaplasma phagocytophilum* Type IV secretion systems (T4SS)

Type IV secretion systems (T4SS) are multi-protein complexes arranged in a needle-shaped structure that translocate bacterial factors across membranes. The T4SS of *Agrobacterium tumefaciens* has been studied in most detail and serves as the model T4SS (Christie, 2004). T4SS genes are conserved and have been identified in a number of intracellular pathogens, including *Helicobacter pylori*, *Brucella* spp., *Bordetella pertussis*, *Bartonella henselae*, *Legionella pneumophila*, *Rickettsia prowazekii*, *Rickettsia conorii*, *E. chaffeensis* and *A. phagocytophilum*. The number of known virulence factors translocated by these T4SS is growing rapidly and the diverse signaling pathways that these factors manipulate have been reviewed recently (Backert and Meyer, 2006). The *A. phagocytophilum* genome contains genes for a T4SS (Ohashi et al., 2002) and the T4S machinery is involved in the infection of *A. phagocytophilum* in host cells (Niu et al., 2006); however, to date only two T4SS-
translocated effector proteins ankA and Ats-1 have been identified and the detailed function of these two substrates for *A. phagocytophilum* infection are still remaining to be characterized.

**AnkA**

AnkA is a major tyrosine phosphorylated 160 kDa protein in *A. phagocytophilum*-infected cells and this protein is translocated into host cells by T4S system. By yeast two hybrid screening, Lin *et al.* further found that AnkA binds to human Abi-1, and recruits Abl-1 tyrosine kinase for AnkA phosphorylation. These data establish AnkA as the first proven T4S substrate of *A. phagocytophilum*, and even in members of obligate intracellular α-proteobacteria; furthermore, it demonstrated that AnkA plays an important role in facilitating intracellular infection by activating Abl-1 signaling pathway (Lin *et al.*, 2007). In a parallel independent study, Ijdo *et al.* reported that AnkA is phosphorylated by Src kinase and tyrosine phosphorylated AnkA can recruit protein tyrosine phosphatase SHP-1 perhaps through its SH2 domains (Ijdo *et al.*, 2007). Another interesting observation regarding AnkA is its nucleus localization: within the nucleus of neutrophils or HL-60 cells, AnkA binds nuclear protein and complexes to AT rich nuclear DNA that lack specific conserved sequences; and a direct pathogenetic role of AnkA in regulation of eukaryotic gene expression has been proposed (Park *et al.*, 2004). It was recently found that transcripts of gp91phox gene and other host defense genes are down-regulated by AnkA transfection in HL-60 cells (Garcia-Garcia *et al.*, 2009).
**Ehrlichial ewingii**

*E. ewingii* was first recognized as a canine granulocytic ehrlichiosis agent in a dog from Arkansas in 1971 and was considered a new strain of *E. canis* (Ewing et al., 1971). In 1992, it was identified as a separate *Ehrlichia* species based on 16S rRNA gene sequence differences from the two species most closely related, *E. canis* and *E. chaffeensis*, and named *E. ewingii* in honor of Dr. S. A. Ewing (Anderson et al., 1992). Since then, canine infection with *E. ewingii* has been detected by PCR based on the 16S rRNA gene sequence in Missouri, Oklahoma, North Carolina, and Virginia (Goodman et al., 2003; Liddell et al., 2003; Murphy et al., 1998; Dawson et al., 1996), and recently from Cameroon (Ndip et al., 2005). Canine granulocytic ehrlichiosis is characterized by fever and anorexia accompanied by mild thrombocytopenia and mild anemia (Liddell et al., 2003; Stockham et al., 1992). In 1999, *Ehrlichia ewingii* was recognized as a new human ehrlichiosis agent in the United States (Buller et al., 1999). Between 1996 and 2001, approximately 10 confirmed cases of human granulocytic ehrlichiosis caused by *E. ewingii* infection were identified in Missouri and Oklahoma (Gusa et al., 2001). Patients infected with *E. ewingii* experience symptoms such as fever, headache, myalgia, leukopenia, and thrombocytopenia. Many but not all of the individuals infected with *E. ewingii* were receiving immunosuppressive therapy at the time of infection (Buller et al., 1999). *E. ewingii* is transmitted by tick bite, with the white-tailed deer (*Odocoileus virginianus*) being an important reservoir; the dog is another possible reservoir (Liddell et al., 2003; Yabsley et al., 2002).
Objectives of my study

Objective 1

A large amount of cholesterol is found in *A. phagocytophilum* outer membrane and cholesterol is required for *A. phagocytophilum* infection in HL-60 cells (Lin and Rikihisa, 2003a), and also high incidence of HGA is associated with elder age of human patients (Bakken *et al.*, 1996). Considering blood cholesterol level is generally increased with age (Chung, 1992), in this study we test the hypothesis that high blood cholesterol level facilitates *A. phagocytophilum* infection in vivo and affect the severity of disease outcome. We used ApoE deficient mice plus high cholesterol diet to induce hypercholesterolemia mice model and compared the bacteria load in the blood and tissues of the mice and also we measured the several proinflammatory cytokines and chemokine which involve in the pathogenesis of HGA.

Objective 2

*A. phagocytophilum* has the unique capacity to take up exogenous cholesterol from environment (Lin and Rikihisa, 2003a). It is unknown how *A. phagocytophilum* acquires cholesterol in host cells and traps cholesterol into its inclusion. Basically, there are two resources of cholesterol for mammalian cells: uptake pathway via low-density lipoprotein receptor (LDLR)-mediated endocytothesis and de novo biosynthesis pathway. The objective of this study is to investigate the resources of cholesterol acquired by *A. phagocytophilum* and to further elucidate the mechanism of cholesterol regulation in *A. phagocytophilum* infected HL-60 cells.
Objective 3

Extracellular signal-regulated kinase (ERK) signaling pathway is activated in *A. phagocytophilum* infected host cells, including HL-60 and neutrophils (Xiong et al., 2009; Lee et al., 2008). It is unknown, however, how *A. phagocytophilum* triggers and activates this signaling cascade and what are downstream effects of the activation of ERK induced by *A. phagocytophilum* infection. The objective of this study is to investigate the detailed ERK signaling cascade involving in *A. phagocytophilum* infection in host cells.

Objective 4

*E. ewingii* has been recognized as a new human granulocytic ehrlichiosis agent in the United States (Buller et al., 1999). Its primary target host cell is neutrophil. Neutrophils typically undergo spontaneous apoptosis 6 – 12 h after their release from the bone marrow (Akgul et al., 2001). It is unknown how this obligate intracellular bacterium modulates neutrophil apoptosis to establish its infection in its host cell. Strikingly, we found that *E. ewingii* can survive in neutrophils derived from a patient dog severely infected by *E. ewingii* for over 5 days. The objective of this study is to investigate the mechanism of delayed neutrophils apoptosis using ex vivo dog neutrophils.
CHAPTER 2

High-Cholesterol Diet Facilitates *Anaplasma phagocytophilum* Infection and Up-regulates MIP-2 and CXCR2 Expression in Apolipoprotein E–Deficient Mice

2.1 Abstract

*Anaplasma phagocytophilum* is an obligatory intracellular bacterium that infects granulocytes and causes human granulocytic anaplasmosis (HGA). This bacterium requires cholesterol for host cell infection in vitro and incorporates exogenous cholesterol into its membrane. In order to understand the role of host cholesterol in *A. phagocytophilum* infection in vivo, we analyzed the effects of a high-cholesterol diet and reduced apolipoprotein E (apo E) activity on *A. phagocytophilum* infection in mice. We found that a high-cholesterol diet significantly facilitated *A. phagocytophilum* infection in the spleen, liver, and blood of apoE-deficient (apoE -/-) mice, compared with the level of infection in apoE -/- mice fed a normal-cholesterol diet or wild-type (WT) mice fed a high- or normal-cholesterol diet. *A. phagocytophilum* infection induced a significant elevation in the mRNA expression of MIP-2 and a MIP-2 receptor, CXCR2, in the spleen in apoE-/- mice fed a high-
cholesterol diet compared with other three groups. Our results suggest that high blood cholesterol levels resulting from an interaction between dietary and genetic factors facilitate *A. phagocytophilum* infection and upregulate a proinflammatory chemokine and its receptor, which may contribute to HGA pathogenesis.

**2.2 Introduction**

*Anaplasma phagocytophilum* is a tick-borne obligatory intracellular pathogen that causes acute and/or persistent infection of granulocytes and endothelial cells in various mammals (Herron *et al.*, 2005; Dumler *et al.*, 2001). In humans, *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), an acute febrile disease that is potentially fatal, especially in elderly or immunocompromised people (Bakken and Dumler, 2000).

*A. phagocytophilum* contains significant amounts of membrane cholesterol (Lin and Rikihisa, 2003a). The bacterium lacks genes for cholesterol biosynthesis or modification, but it directly takes up cholesterol and cholesterol esters from its host cells or the medium (Lin and Rikihisa, 2003a). Treatment of *A. phagocytophilum* with methyl-β-cyclodextrin, a cholesterol-extraction reagent, or NBD-cholesterol, a fluorescent cholesterol derivative, deprives the bacterium of the ability to infect leukocytes, thus killing it (Lin and Rikihisa, 2003a). The dependency of *A. phagocytophilum* on cholesterol for infection and survival in vitro suggests that increased blood cholesterol levels may enhance the severity of *A. phagocytophilum* infection in mammals. To our knowledge, this possibility has not been investigated either in a population-based study or in laboratory settings.
Blood cholesterol levels are influenced by both dietary and hereditary factors (van Ree et al., 1994). ApoE is a constituent of most cholesterol-carrying lipoproteins in the blood. It functions as a ligand for the receptor in the liver that clears chylomicrons and very-low-density lipoprotein (VLDL) remnants from the blood. ApoE deficiency increases serum cholesterol concentrations and causes sensitivity to a high-fat diet in mice (Bobkova et al., 2004). In humans, mutation of the ApoE gene can manifest as dysbetalipoproteinemia (type III hyperlipoproteinemia), which is characterized by the accumulation of \( \beta \text{VLDL} \) and an increase in total serum cholesterol (Brewer et al., 1983). Therefore, defects in cholesterol metabolism may play a role in the exacerbation of HGA, and interaction between dietary cholesterol and apoE may affect the risk of *Anaplasma* infection.

The involvement of various cytokines and chemokines in *A. phagocytophilum* infection and pathogenesis has been investigated in cultured cells, HGA patients, and experimentally infected animals (Kim et al., 2002; Akkoyunlu et al., 2001; Dumler et al., 2000; Kim and Rikihisa, 2000; Klein et al., 2000; Foley et al., 1999). Of the cytokines, the involvement of interleukin (IL)-8 and interferon (IFN)-\( \gamma \) in infection has been studied most. Patients with confirmed HGA have significantly higher concentrations of IL-8 in the serum than healthy controls (Akkoyunlu et al., 2001). CXCR2\(-/-\) mice, which lack CXCR2, the human IL-8 receptor homolog, have reduced amounts of *A. phagocytophilum* in the blood compared with control mice (Akkoyunlu et al., 2001). In one study, serum IFN-\( \gamma \) levels were significantly higher in C3H mice than control mice from day 2 through day 8 of infection. IFN-\( \gamma \)–deficient mice had a markedly higher bacterial burden in the blood on day 5 and 8.
postinfection than wild-type (WT) mice, suggesting that IFN-γ may facilitate bacterial clearance during the early stage of infection (Akkoyunlu and Fikrig, 2000).

In the present paper, we examined whether dietary cholesterol and ApoE determine the risk for *Anaplasma* infection using WT and apoE-/- mice on normal and high-cholesterol diets. Given the importance of cytokines and chemokines in the pathogenesis of HGA, we examined cytokine and chemokine profiles in these mice to examine the possibility of cross-talk between the 2 host pathogenesis factors, cholesterol and cytokines, in facilitating *Anaplasma* infection.

### 2.3 Materials and Methods

**Mice.** Five to six-week-old male apoE-/- C57BL/6 mice and congenic WT mice were purchased from Jackson ImmunoResearch Laboratory (Bar Harbor, ME). The animals were housed in a 12-h light/dark cycle and fed normal-cholesterol diet (Formulab diet 5008, PMI, Richmond, IN) or high-cholesterol diet (Teklad diet TD-88051, Harlan Teklad, Madison, WI) for 4 weeks. Once each week, a blood specimen (~100 µl) was collected from each mouse by venipuncture at the base of the mandible to track cholesterol concentrations over time using an Infinity™ Cholesterol Reagent Kit (Thermal Electron, Louisville, CO).

*A. phagocytophilum and mouse infection.*** *A. phagocytophilum* HZ strain was cultured in HL-60 cells as previously described (Rikihisa *et al.*, 1997). Five mice in each group were inoculated intraperitoneally with *A. phagocytophilum*-infected HL60 cells (>90% cells infected; 1 x10⁶ cells/mouse). Ten days after inoculation, all mice
in each group were sacrificed by CO₂ inhalation. Blood specimens were collected by cardiac puncture and the peripheral blood leukocytes (PBLs) were isolated as previously described (Kim and Rikihisa, 2000). Spleens and livers were harvested. These specimens were stored in RNAlater (QIAGEN, Valencia, CA) at -20°C prior to DNA and RNA analysis. The protocol for use of animals in this study was approved by the Institutional Laboratory Animal Care and Use Committee.

**Measurement of bacterial burden in blood and tissues.** Total DNA was extracted from blood, spleen, and liver specimens with a QIAamp blood kit (QIAGEN). To determine the number of *A. phagocytophilum* organisms, the *p44* competitive PCR (C-PCR) assay was performed to amplify *p44* paralogs using primer set 1 (Table 1) as previously described (Wang et al., 2004). Densitometric analysis of PCR products separated by agarose gel electrophoresis was performed as previously described (Kim and Rikihisa, 2000). To normalize the input of mouse DNA across samples, PCR amplification for the mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene was performed with primer set 2 (Table 1) over a linear range, as described previously (Kim and Rikihisa, 2000).

**Cytokine RT-PCR.** Total RNA was extracted from spleen, liver, and PBLs using an RNeasy kit (QIAGEN). The concentration and purity of the RNA were determined by measuring the A260 and the A260/A280 ratio with a GeneQuant II RNA and DNA calculator (Pharmacia Biotech Inc., Piscataway, NJ). The RNA was stored at -80°C until used. Total cellular RNA (2 µg) was reverse transcribed in accordance with the
manufacturer's instructions by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dT)12-18 primer (Invitrogen). The cDNA (2 µl) was amplified in a 25-µl reaction mixture containing 1X PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM deoxynucleoside triphosphates, and 0.4 µM (each) 3' and 5' primers (Table 1). RT-PCR conditions were as follows: after 5 min of denaturation, 28 cycles of denaturation at 94°C for 45 s, annealing at 60°C (62°C for MIP-2) for 1 min, and extension at 72°C for 1 min. The final extension was 7 min at 72°C. A mouse housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used to normalize the amount of input mRNA among different samples. As a positive control, splenocytes from uninfected mice were cultured in RPMI growth medium with 10% fetal bovine serum at 37 ºC in the presence of 1 µg/ml pokeweed mitogen (Sigma, St. Louis, MO) for 3 days. RNA was extracted from the splenocytes and used as a positive control for the cytokine and chemokine RT-PCR.

**Histopathology.** Spleen and liver specimens from euthanized mice were fixed in 10% formalin, embedded in paraffin, sectioned at 4-µm thickness, and stained with hematoxylin and eosin. Slide-mounted sections were observed under a microscope using appropriate magnifications.

**Statistical analyses.** Statistical analysis was carried out by Student \( t \) test or by one-way analysis of variance (ANOVA) with Tukey HSD test. \( P < 0.05 \) was considered to be significant.
2.4 Results

**Blood cholesterol level in apoE-/- and WT mice.** Four groups of five mice each were compared. After one week on high-cholesterol diet, the plasma cholesterol concentrations in both the apoE-/- group (AH) and the WT group (WH) increased over baseline; in the AH group, plasma cholesterol concentration increased 4-fold within one week. In contrast, plasma cholesterol concentrations were stable during the entire period of diet feeding (4 weeks) in apoE-/- and WT mice fed a normal diet (AN and WN, respectively). ANOVA analysis of the plasma cholesterol concentration over weeks 2-4 showed that cholesterol levels were significantly higher in AH group than in the other 3 groups (AH vs AN, \( P < 0.05 \); AH vs WH or WN, \( P < 0.01 \); Figure 1). These data are in agreement with a previous report (Bobkova et al., 2004) that apoE-/- mice are very sensitive to high-cholesterol diet.

**A. phagocytophilum burden in apoE-/- and WT mice fed formulated diets.** A previous study reported the presence of *A. phagocytophilum* in various tissues of experimentally infected mice (von Loewenich et al., 2004). In order to compare the *A. phagocytophilum* burden among the different groups of mice, we performed a competitive PCR in specimens of 3 different tissues to measure the amount of bacterial DNA relative to mouse tissue DNA. Our results showed that *A. phagocytophilum* burdens in the blood, spleen, and liver from AH mice were approximately 10-fold greater than in the respective tissues from the other 3 groups of mice (Figure 2). ANOVA analysis revealed no significant differences among the other 3 groups of mice for each tissue type, but we did find significant differences
among the tissues in the ratio of \textit{A. phagocytophilum} DNA to mouse DNA: the blood was the primary site of anaplasma burden, followed by the spleen, and the burden was lowest or undetectable in the liver (Figure 2).

\textbf{MIP-2, MIP-2 receptor, and cytokine mRNA levels in infected apoE-/- and WT mice fed formulated diets.} To explore alternative or concurrent mechanisms by which \textit{A. phagocytophilum} infection is increased in apoE-/- mice fed a high-cholesterol diet, we investigated the levels of several cytokines in the mouse tissue specimens. We examined the mRNA expression levels of mouse MIP-2, a functional analog of human IL-8, and the MIP-2 receptor, CXCR2, in vivo in the mouse specimens infected with \textit{A. phagocytophilum}. Our results showed that in infected mice, MIP-2 mRNA expression level was higher in the PBLs from the AH group than from the other 3 groups ($P < 0.05$), but no significant difference in CXCR2 mRNA was detected among the 4 groups (Figure 3A). MIP-2 mRNA concentrations in spleen tissues were more than 100-fold greater in the AH group than in the WH group, and were undetectable in both ApoE-/- and WT mice fed normal diet. CXCR2 mRNA was detected in the spleen in all 4 treatment groups infected with \textit{A. phagocytophilum}, and the expression level was approximately 2-fold greater in the AH mice than in the remaining 3 groups of mice ($P < 0.01$) (Figure 3B). In the livers of mice infected with \textit{A. phagocytophilum}, MIP-2 and CXCR2 mRNA were undetectable under the same PCR condition used for blood and spleen specimens (Data not shown). Chemokine KC mRNA was undetectable in all mouse groups under the same conditions used for MIP-2 and CXCR2.
Previous studies reported an association of hypercholesterolemia with elevated serum concentrations of CXC chemokines: KC and MIP-2 in the human apoE*3-Leiden transgenic mouse model (Murphy et al., 2002). Given this information, we examined MIP-2 and CXCR2 expression in specimens from 4 groups of uninfected mice also fed high- and normal-cholesterol diets. MIP-2 and CXCR2 mRNA were undetectable in blood or spleen specimens from all 4 groups of uninfected mice under the same PCR conditions used for infected tissues. These results suggest that the high MIP-2 and/or CXCR2 mRNA in the blood and spleen were induced by *A. phagocytophilum* infection.

IL-1β mRNA expression was detectable after 28 PCR cycles in the spleens of AH mice infected with *A. phagocytophilum* and was approximately 2-fold higher than in the other 3 groups of mice (*P* < 0.05, data not shown), whereas IL-6, TNF-α, IFN-γ, and inducible NOS mRNAs were undetectable by RT-PCR in spleens from all 4 mouse groups under the same conditions used for MIP-2 mRNA.

**Histopathologic observations.** Previous studies using the murine model of HGA have shown that host-mediated immunological injury contributes to HGA pathogenesis (Martin et al., 2001; Martin et al., 2000). Therefore, we examined the histopathology of the spleen and liver from 4 groups of mice infected with *A. phagocytophilum* and from 4 corresponding groups of uninfected control mice.

There was marked disorganization and lymphoid depletion in the lymphoid follicles and surrounding white pulp in spleens from AH and AN mice compared with those from WH and WN mice prior to *A. phagocytophilum* infection (Figure 4). *A.
*phagocytophilum* infection exacerbated the reduced cellularity and disorganization in lymphoid follicles and the surrounding white pulp with necrotic cells (AN, WH, and WN groups) or vacuolated cells (AH group) (Figure 4). *A. phagocytophilum* infection did not cause remarkable changes in the liver histopathology in the 4 groups of mice; differences seen among the 4 groups, including granulomatous infiltration, were associated with the high-cholesterol diet and apoE deficiency and were already present prior to infection (data not shown).

### 2.5 Discussion

The present study examined whether high blood cholesterol levels caused by dietary factors, such as high cholesterol intake, and genetic factors, such as apoE genotype, increase the risk for HGA. We have addressed this issue by studying the *A. phagocytophilum* burden in apoE-/- and WT mice fed a high- or normal-cholesterol diet. The apoE deficiency in apoE-/- mice is considered to be equivalent to the loss of function resulting from the presence of the apoE4 allele in humans (Gregg *et al.*, 1986). The high-cholesterol diet potently and invariably enhanced *A. phagocytophilum* infection in the blood, spleens, and livers of apoE-/- mice. In view of our observations, it is tempting to speculate that humans may be more susceptible to *A. phagocytophilum* infection if they consume a high-cholesterol diet and/or have an apoE deficiency.

Upon infection with *A. phagocytophilum*, apoE-/- mice on a high-cholesterol diet had significantly increased levels of mRNA for spleen and blood MIP-2, the mouse homolog of human IL-8, and the MIP-2 receptor CXCR2. IL-8 is produced by
neutrophils, monocytes, and other types of cells, and is the most studied chemokine (Hoffmann et al., 2002). Neutrophils are not only producers of IL-8, but are also primary targets for IL-8, responding to this mediator by chemotaxis, release of granule enzymes, respiratory burst activity, and up-regulation of adhesion to unstimulated endothelial cells (Baggiolini et al., 1994; Oppenheim et al., 1991). Akkoyunlu et al. (Akkoyunlu et al., 2001) proposed that IL-8 secretion by infected neutrophils recruits naïve neutrophils to enhance A. phagocytophilum infection. IL-8 is also a myelosuppressive chemokine (Cluitmans et al., 1997). Given these information, high blood cholesterol may not be the only reason for the higher bacterial burden in the tissues of Apo E-/- mice. It is possible that upregulation of IL-8 and IL-8 receptors would participate in the enhanced infection in apoE-/- mice on a high-cholesterol diet.

Several studies have suggested that apoE protein affects innate and acquired immune responses in vitro, as evidenced by its ability to suppress lymphocyte proliferation, generate cytolytic T cells, and stimulate cultured neutrophils (Laskowitz et al., 2000). Recently, human apoE or fragments containing the receptor-binding domain were reported to provide innate immunity to viral infection by direct disruption of viral particles and/or inhibition of viral attachment in cell culture (Dobson et al., 2006). An increasing body of evidence demonstrates that apoE-deficient animals have impaired immunity after challenge with bacteria, such as Listeria monocytogenes and Klebsiella pneumonia (de Bont et al., 1999; Roselaar and Daugherty, 1998). In agreement with these reports, A. phagocytophilum infection caused the histopathology of the spleen to show more severe loss of follicular cells
and architecture in apoE-/- mice regardless of the cholesterol content of the diet. However, since apoE-/- mice fed the normal-cholesterol diet had a much lower bacterial burden than apoE-/- mice fed the high-cholesterol diet, immunosuppression alone cannot be the reason for the much higher bacterial burden.

Bakken et al. reported that increased age is associated with the severity of HGA illness in patients (Bakken et al., 1996). A more recent study confirmed the unusually high median age of HGA patients (51 years old) compared with patients with other tick-borne diseases, such as Lyme disease (39 years old) and Rocky mountain spotted fever (38 years old) (Gardner et al., 2003). The difference is not due to different species of tick vectors or reservoir hosts, since Borrelia burgdorferi, which causes Lyme disease, and A. phagocytophilum are both transmitted by the Ixodes scapularis tick, and the reservoirs are white-footed mice (Telford et al., 1996). Generally immune functions are weakened (Linton and Dorshkind, 2004) and blood cholesterol levels are increased with advanced human age (Chung, 1992). Patients who are immunocompromised by natural disease processes or medications may develop severe HGA (Bakken et al., 1996). The present study showed in even genetically predisposed ApoE-/- mice, by giving low cholesterol diet, the bacterial burden was reduced to the level of WT mice. Therefore, lowering the plasma cholesterol level by dietary and pharmacological means may be beneficial in preventing and ameliorating severe HGA in elderly and immunocompromised patients in conjunction with prompt antibiotic therapy. Well-designed case-control studies on HGA patients are desired in this regard.
In summary, we have demonstrated that the combination of apoE deficiency and high cholesterol intake results in enhanced infection with *A. phagocytophilum* in vivo, perhaps facilitated in part by high blood cholesterol level, upregulation of IL-8 and IL-8 receptors, and immunosuppression. This influence of dietary and genetic factors could be relevant for the development of HGA in humans, and could inform the development of an improved supportive therapy to antibiotics and preventive measures for HGA.
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Table 2.1 Sequences of oligonucleotides used in competitive PCR and cytokine RT-PCR
Figure 2.1 Temporal plasma cholesterol concentrations in apoE-/- and wild-type mice fed a high- or normal-cholesterol diet. AH, apoE-/- mice fed a high-cholesterol diet; AN, apoE-/- mice fed a normal-cholesterol diet; WH, Wild-type mice fed a high-cholesterol diet; WN, Wild-type mice fed a normal-cholesterol diet. All values are means ± standard deviations of 5 mice each.

a indicates significant difference within the same group compared to week 0, prior to feeding with the formulated diet ($P < 0.05$, Student’s $t$ test). b indicates significant difference among groups within the same week ($P < 0.05$, ANOVA).
Figure 2.1

Cholesterol concentration (×10^3 mg/dL)

- AH
- AN
- WH
- WN

Week

0 1 2 3 4

ab ab ab ab
Figure 2.2 *A. phagocytophilum* burden in mouse blood, spleen, and liver
determined by competitive PCR. *A. phagocytophilum* DNA burden in blood, spleen, and liver specimens were determined 10 days postinfection in 4 groups of mice by competitive PCR with primers specific to the *A. phagocytophilum* p44 gene. The *A. phagocytophilum* chromosome equivalent was calculated according to previously acquired data (Wang et al., 2004). Each DNA sample was normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) DNA levels. AH, ApoE-/- mice fed a high-cholesterol diet; AN, ApoE-/- mice fed a normal-cholesterol diet; WH, Wild-type mice fed a high-cholesterol diet; WN, Wild-type mice fed a normal-cholesterol diet. All values are means ± standard deviations of 5 mice each. $^a,b,c$ indicate significant differences ($P < 0.05$) among the 4 groups in blood, spleen, and liver specimens, respectively, by ANOVA.
Figure 2.2

![Graph showing the comparison of A. phagocytophilum chromosome equivalent/mouse G3PDH in different sample types (Blood, Spleen, Liver) across AH, AN, WH, and WN mouse groups.](image-url)
Figure 2.3 MIP-2 and CXCR2 mRNA levels in peripheral blood leukocytes (A) and spleen specimens (B) from apoE-/- and WT mice fed formulated diets.

mRNA levels of MIP-2 and its receptor CXCR2 in the blood and spleen were determined by RT-PCR 10 days postinfection in 4 groups of mice. The input RNA sample was normalized to mouse hypoxanthine-guanine phosphoribosyltransferase (HRPT) mRNA levels. AH, apoE-/- mice fed a high-cholesterol diet; AN, apoE-/- mice fed a normal-cholesterol diet; WH, Wild-type mice fed a high-cholesterol diet; WN, Wild-type mice fed a normal-cholesterol diet. All values are means ± standard deviations of 5 mice each. a,b indicate significant differences ($P < 0.05$) among the 4 groups for MIP-2 and CXCR2 mRNA, respectively, by ANOVA.
Figure 2.3

A

PBLs

Mouse group

Relative MIP-2 and CXCR2 mRNA level

B

Spleen

Mouse group

Relative MIP-2 and CXCR2 mRNA level
Figure 2.4 Histopathology of the spleens of apoE-/- and WT mice fed formulated diets. Reduced cellularity was noted in the mantle regions (long white arrows in WH and WN), follicles, and white pulp in spleens from uninfected AH and AN mice compared with those from WH and WN mice prior to *A. phagocytophilum* infection. *A. phagocytophilum* infection exacerbated the reduced cellularity in splenic lymphoid follicles and the surrounding white pulp in all 4 groups of mice. Note necrotic cells with nuclear debris in the germinal center of AN, WH, and WN mice infected with *A. phagocytophilum* (white arrowheads and the inset at x 100 magnification in AN). The germinal center of AH mice had many cells with vacuoles (small white arrows in the inset at x 100 magnification in AH). H & E stained, original magnification × 20. AH, apoE-/- mice fed a high-cholesterol diet; AN, apoE-/- mice fed a normal-cholesterol diet; WH, Wild-type mice fed a high-cholesterol diet; WN, Wild-type mice fed a normal-cholesterol diet. A representative result from each group of mice is shown (Scale bar, 50µm).
CHAPTER 3

*Anaplasma phagocytophilum* Acquires Cholesterol through the Low-density Lipoprotein Uptake Pathway

3.1 Abstract

In eukaryotes, intracellular cholesterol homeostasis and trafficking are tightly regulated. Certain bacteria, such as *Anaplasma phagocytophilum*, also require cholesterol; it is unknown, however, how this cholesterol-dependent obligatory intracellular bacterium of granulocytes interacts with the host cell cholesterol regulatory pathway to acquire cholesterol. Here, we report that total host cell cholesterol increased >2-fold during *A. phagocytophilum* infection in a human promyelocytic leukemia cell line. Cellular free cholesterol was enriched in *A. phagocytophilum* inclusions as detected by filipin staining. We determined that *A. phagocytophilum* requires cholesterol derived from low-density lipoprotein (LDL), because its replication was significantly inhibited by depleting the growth medium of cholesterol-containing lipoproteins, by blocking LDL uptake with a monoclonal antibody against LDL receptor (LDLR), or by treating the host cells with inhibitors that block LDL-derived cholesterol egress from late endosomes or lysosomes.
However, de novo cholesterol biosynthesis is not required, since inhibition of the biosynthesis pathway did not inhibit *A. phagocytophilum* infection. The uptake of fluorescence-labeled LDL was enhanced in infected cells, and LDLR expression was up-regulated at both the mRNA and protein levels. *A. phagocytophilum* infection stabilized LDLR mRNA through the 3’ UTR region, but not through activation of the sterol regulatory element binding proteins. Extracellular signal–regulated kinase (ERK) was up-regulated by *A. phagocytophilum* infection, and inhibition of its upstream kinase, MEK by a specific inhibitor or siRNA knockdown reduced *A. phagocytophilum* infection. Up-regulation of LDLR mRNA by *A. phagocytophilum* was also inhibited by the MEK inhibitor; however, it was unclear whether ERK activation is required for LDLR mRNA up-regulation by *A. phagocytophilum*. These data reveal that *A. phagocytophilum* exploits the host LDL uptake pathway and LDLR mRNA regulatory system to accumulate cholesterol in inclusions to facilitate its replication.

### 3.2 Introduction

Cholesterol is an important component of biological membranes, and it is essential for many biological functions ranging from membrane trafficking to signal transduction in eukaryotic cells (Maxfield and Tabas, 2005). However, excess cholesterol must be avoided in cells as well as in the blood stream, because it alters intracellular vesicular trafficking, deregulates cellular signaling, and initiates atherosclerosis (Ikonen, 2008; Brown and Goldstein, 1997). The liver in large part regulates blood cholesterol levels by removing it from circulating blood. To maintain
cellular cholesterol levels within a specified range, cholesterol levels are constantly assessed and tightly regulated in a complex manner at the transcriptional, translational, and posttranslational levels (Brown and Goldstein, 1999). In recent years, cellular cholesterol has emerged as a significant factor, which influences outcome of infectious diseases from microbiological and cell biological studies. The cholesterol content of host cell membranes appears to be critical for microbial entry, intracellular localization, and exit by exocytosis (Goluszko and Nowicki, 2005). A growing body of evidence suggests that host cellular cholesterol levels affect the replication of intracellular microbial pathogens, such as *Salmonella, Mycobacterium, Brucella*, and *Coxiella* (Howe and Heinzen, 2006; Goluszko and Nowicki, 2005; Watarai et al., 2002), but how cholesterol influences replication of these pathogens are not completely understood. Among the above-mentioned pathogens, infection by *Salmonella* or *Coxiella* up-regulates cellular cholesterol levels, although the mechanisms of up-regulation are not clear (Howe and Heinzen, 2006; Garner et al., 2002). One of the common characteristics for these intracellular bacteria is that after internalization into their host cells the bacteria reside and proliferate in parasitophorous vacuoles. As such, cholesterol may play a role in nutrient acquisition by bacteria entrapped within vacuoles, or the accumulation of cholesterol may prevent phagolysosomal fusion (Goluszko and Nowicki, 2005).

*Anaplasma phagocytophilum* is a tick-borne obligatory intracellular bacterium that proliferates in membrane-bound inclusions in granulocytes and endothelial cells of various mammal species (Herron et al., 2005; Munderloh et al., 2004; Dumler et al., 2001). In humans, *A. phagocytophilum* causes an emerging and major tick-borne
disease called human granulocytic anaplasmosis, an acute febrile disease that is potentially fatal, especially in elderly or immunocompromised individuals (Bakken and Dumler, 2000). *A. phagocytophilum* is an atypical Gram-negative bacterium, because it contains substantial amounts of cholesterol in its outer membrane (Lin and Rikihisa, 2003a). The bacterium lacks genes for cholesterol biosynthesis or modification; rather, it directly acquires cholesterol from its host cells or the medium (Lin and Rikihisa, 2003a). Our previous data showed that cholesterol is required for *A. phagocytophilum* proliferation in host human promyelocytic leukemia HL-60 cells and that a high blood cholesterol level facilitates *A. phagocytophilum* infection in a mouse model (Xiong *et al.*, 2007; Lin and Rikihisa, 2003a). *A. phagocytophilum* enters host cells through caveolae or lipid rafts, and the inclusion membrane retains caveolin-1 throughout infection, suggesting continuous infusion of the lipid raft or caveosome into growing bacterial inclusions (Lin and Rikihisa, 2003b).

Considering cholesterol-dependence of *A. phagocytophilum* membrane integrity and the importance of cholesterol for the infection process, thus survival (Lin and Rikihisa, 2003a), we questioned how host cellular cholesterol uptake, trafficking, and regulatory systems are involved in *A. phagocytophilum* infection of human leukocytes. In this study, we present data on the intracellular cholesterol level and cholesterol distribution in *A. phagocytophilum*–infected HL-60 cells. We provide evidence that the source of increased level of cellular cholesterol required for *A. phagocytophilum* replication is extracellular low-density lipoprotein (LDL) rather than cholesterol synthesized by the host cells. Finally, we propose a mechanism by which the cellular LDL receptor (LDLR) level is increased in infected HL-60 cells to take up more LDL.
The data underscore an important evolutionary adaptation of *A. phagocytophilum* to hijack host cell cholesterol.

### 3.3 Materials and Methods

**Chemicals and antibodies**

Filipin, lovastatin, imipramine and U18666A were obtained from Sigma (St. Louis, MO). 25-Hydroxycholesterol (25-HC) was purchased from Steraloids, Inc. (Newport, RI). MAPK inhibitor U0126 was obtained from Biomol (Plymouth Meeting, PA). DiI-LDL and native LDL were purchased from Molecular Probes (Eugene, OR) and Intracel, Inc. (Frederick, MD), respectively.

The mouse mAb 5C11 recognizing the N-terminal conserved region of *A. phagocytophilum* major surface protein P44 has been described (Kim and Rikihisa, 1998). The anti-LDLR mAb was purified from the supernatant of hybridoma ATCC CRL-1691 (C7) grown in advanced MEM (ATCC, Manassas, VA) by affinity chromatography using HiTrap Protein G HP (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. The purity of the antibody was confirmed by SDS-PAGE followed by GelCode Blue staining (Pierce, Rockford, IL).

Other antibodies used include: mouse anti-SREBP-2 mAb (BD Parmingen, San Jose, CA), mouse anti-phospho-ERK1/2 mAb, rabbit anti-ERK1/2 antibody, mouse anti-MEK1/2 mAb (Cell Signaling, Danvers, MA), and mouse anti-α-tubulin mAb (Santa Cruz Biotechnology, Santa Cruz, CA). Peroxidase-conjugated secondary antibodies were obtained from KPL (Gaithersburg, MD). Normal mouse IgG were purchased from Santa Cruz Biotechnology.
Preparation of LPDS and lipoproteins

LPDS was prepared from fetal bovine serum (Mediatech, Inc., Herndon, VA) by gradient ultracentrifugation after density adjustment by solid KBr as described (Brown and Goldstein, 1974; Radding and Steinberg, 1960). LPs were used to supplement LPDS, as necessary for certain experiments. LPDS and LP fractions were dialyzed at least 36 h against buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. The volume of each fraction was adjusted to be equivalent to that of the original serum, and the cholesterol concentration of each fraction was measured by Infinity™ cholesterol reagent kit (Thermo Electron Corp., Louisville, CO).

A. phagocytophilum culture and inhibitor treatments

A. phagocytophilum HZ strain was cultivated in human promyelocytic leukemia cell line HL-60 as described (Rikihisa et al., 1997). Host cell-free A. phagocytophilum was prepared by sonicating highly infected HL-60 cells for 8 s twice 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 added to HL-60 or RF/6A cells. After 1 h incubation at 37°C, extracellular organisms were washed, fresh medium was added (this time point was considered 0 h p.i.), and continuously incubated at 37°C.

Inhibitors were added at indicated time points (0 h or 1 day p.i.), and the inhibitors were kept in the growth media throughout the incubation period or removed later as indicated. The highest final concentrations of inhibitors used were: lovastatin (5 µM),
imipramine (100 µM), U18666A (5 µM), and 25-HC (25 µM). Inhibitor treatments at these concentrations did not affect host cell integrity as assessed by light microscopy or by G3PDH mRNA level. For LPDS treatment, 10% LPDS or LP-reconstituted LPDS conditioned growth medium was added at 0 h p.i. in place of the growth medium containing 10% fetal bovine serum. To block LDLR function, HL-60 cells were pretreated with anti-LDLR (IgG2b; final concentrations:20 µg/ml) or IgG2b isotype control antibody at 4°C for 1 h followed by addition of host cell–free bacteria, and then culture was continued at 37°C for the indicated times.

The degree of bacterial infection in host cells was assessed by Diff-Quik staining (Baxter Scientific Products, Obetz, OH), and the number of *A. phagocytophilum* cells was estimated in 100 host cells in triplicate culture wells as described (Rikihisa *et al*., 1995).

**Cholesterol assay of infected cells**

Uninfected and *A. phagocytophilum*–infected HL-60 cells at the indicated time points (1 h, 1 day, 2 day and 3 day) were collected, and total cellular cholesterol levels were measured by an Amplex Red cholesterol assay kit (Molecular Probes) as described (Lin and Rikihisa, 2003a). The total cholesterol content was normalized by the total protein concentration as determined by bicinechinonic acid reagent (Pierce).

**DiI-LDL uptake assay**

DiI-LDL uptake by infected and uninfected HL-60 cells was measured by the modified method of Teupser *et al*. (Teupser *et al*., 1996). Briefly, uninfected and
approximately 40% infected HL-60 cells were incubated with LPDS-conditioned medium for 12 h to enhance LDLR expression. Then, increasing concentrations of Dil-LDL (2, 5, 10 µg protein/ml) with or without 30-fold excess of unlabeled LDL were added to HL-60 cells and incubated for 2 h at 37°C. The cells were thoroughly washed with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4) containing 0.4% bovine serum albumin, and lysed in the lysis reagent (0.1% SDS/0.1 N NaOH) for 1 h with gentle shaking. Cellular uptake of Dil-LDL was measured in a fraction (200 µl) of the lysate by fluorescence spectroscopy with excitation and emission wavelengths of 520 and 580 nm, respectively. The fluorometric data were normalized by the total protein content.

**Filipin staining and immunofluorescence microscopy**

Filipin staining was performed as described by Millard et al. (Millard et al., 2000). Cells were fixed in 4% paraformaldehyde at room temperature for 15 min and incubated with 50 µg/ml filipin in PBS/10% normal sheep serum for 30 min at room temperature. Then the cells were incubated with mouse anti-*A. phagocytophilum* antiserum in filipin/PBS/10% normal sheep serum for 60 min at 37°C followed by incubation with fluorescence-conjugated secondary antibodies for 30 min. Normal mouse antibodies were used as negative controls. Cells were then washed and observed under a Nikon Eclipse E400 fluorescence microscope with a xenon-mercury light source (Nikon Instruments, Melville, NY).
**Western blot analysis**

*A. phagocytophilum*–infected HL-60 and control HL-60 cells ($2 \times 10^6$) were washed and resuspended in 100 µl PBS containing freshly added protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II (Calbiochem, San Diego, CA), and lysed by mixing with 100 µl of 2× Laemmli sample buffer (4% SDS, 135 mM Tris-HCl [pH 6.8], 20% glycerol, and 10% β-mercaptoethanol). Samples were separated by SDS-PAGE with 7.5% or 10% polyacrylamide resolving gels and then transferred to a nitrocellulose membrane using a semidry blotter (WEP, Seattle, WA). The membrane was blocked using 5% (wt/vol) skim milk (Kroger, Cincinnati, OH) in Tris-buffered saline (150 mM NaCl and 50 mM Tris at pH 7.5) containing 0.1% Tween-20, incubated with primary antibodies (1:500 or 1:1,000 dilution) at 4°C for 12 h, and subsequently incubated with peroxidase-conjugated secondary antibodies at 1:1,000 dilution at room temperature for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence. To detect LDLR protein amount, the membrane fraction of cells was prepared according to Holla *et al.* (Holla *et al.*, 2007), and Western blotting was carried out as described (Beisiegel *et al.*, 1982).

**Quantitative RT-PCR**

Uninfected and *A. phagocytophilum*–infected HL-60 cells were harvested, and RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). Total RNA (2 µg) was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT)12-18 primer (Invitrogen). Quantitative PCR (20 µl total volume) was performed with 1 µl of cDNA (corresponding to 0.2-0.4 µg of total
RNA) and 0.25 µM of each primer using a SYBR Green PCR kit (Stratagene, La Jolla, CA) in a Mx3000P Real-time PCR system (Stratagene). All primers for cholesterol-related genes were described in Castoreno et al. (Castoreno et al., 2005) and G3PDH primers were described in Zhang et al. (Zhang et al., 2007).

Transfection of RF/6A cells and quantitative RT-PCR analysis of Luc transcript

The chimeric plasmid pLuc/LDLR 3’UTR-2 has been described (Kong et al., 2004). The constructs were sequenced, and individual clones were propagated to isolate plasmid DNA using the Endofree plasmid maxi kit (Qiagen). The plasmids were transfected into endothelial cells, RF/6A, using the FuGene transfection reagent (Roche, Indianapolis, IN). After 24 h, host cell–free A. phagocytophilum purified from highly infected HL-60 cells were inoculated into transfected RF/6A cells and incubated for additional 24-48 h. Samples were collected, and first-strand cDNA was synthesized as described above following DNase I (Invitrogen) treatment. The Luc transcripts were measured by quantitative real-time RT-PCR using specific primers: forward, TCCAACCCGGTAAGACACGACT, and reverse, TCAGCAGAGCGCAGATACCAAATA. Host cell G3PDH and the plasmid antibiotics gene zeocin (forward, GACGACGTGACCCTGTTCATCAGC; reverse, CACTCGGCGTACAGCTCGTCCAG) were used for normalization.

RNA interference

HL-60 cells were transfected with double-stranded siRNA (3 µg/2×10⁶ cells) using the Amaxa nucleofection system (kit V, program T-19; Lonza/Amaxa Inc,
Walkersville, MD) as described previously (Lin et al., 2007). Verified human-specific siRNAs targeting the genes encoding MEK1 (siRNA ID: s11167) and MEK2 (siRNA ID: s11170), or control siRNA (# 4390843) not targeting any known human genes were purchased from Ambion (Applied Biosystems/Ambion, Austin, TX). Two days after transfection, host cell-free *A. phagocytophilum* was added to cells and incubated for additional 2 days. Samples were then harvested and divided into two aliquots. One group of samples was lysed in M-PER lysis buffer (Pierce) supplemented with protease and phosphatase inhibitor cocktail (Calbiochem), and subjected to Western blotting using antibodies against MEK1/2, ERK1/2, phospho-ERK1/2 and *A. phagocytophilum* P44 outer membrane protein (Kim and Rikihisa, 1998). Images were then captured and densitometric analysis was performed using LAS3000 image documentation system (FUJIFILM Medical Systems USA, Stamford, CT). The other aliquots were stored in RNALater for further quantitative real-time RT-PCR analysis.

**Statistical analysis**

Statistical analyses were performed by unpaired, 2-tailed Student’s *t*-test. Two-way ANOVA was used to compare mRNA decay rates. *P* < 0.05 was considered to be significant.

### 3.4 Results

**Total cholesterol level is up-regulated in *A. phagocytophilum*–infected cells**

We previously measured the total cholesterol level of host cell–free *A. phagocytophilum* and found that the level of total cholesterol per milligram of
protein was higher than that of host cells (Lin and Rikihisa, 2003a). Here, we further measured the total cholesterol level in *A. phagocytophilum*–infected HL-60 cells following infection time course. The total cellular cholesterol level progressively increased at days 2 and 3 post-infection (p.i.), and the level was significantly greater than that at day 0 p.i. (after 1 h incubation at 37°C). The increase in cholesterol level in infected HL-60 cells correlated with bacterial growth (Figure 1). In contrast, the cholesterol level in uninfected HL-60 cells remained unchanged during the same observation period (data not shown).

**Free cholesterol is enriched in *A. phagocytophilum* inclusions**

Most of the free (unesterified) cholesterol in eukaryotic cells is located in the plasma membrane (Lange, 1991). Over-accumulation of free cholesterol in cells can be toxic due to the potential formation of solid crystals (Tabas, 2002). To determine the intracellular distribution of the observed increased cholesterol in *A. phagocytophilum*–infected HL-60 cells, we used a polyene antibiotic, filipin, which binds specifically to free cholesterol (Severs, 1997). A specific antibody against *A. phagocytophilum* was used to localize bacteria by double immunofluorescence microscopy. First, the microscopy analysis clearly showed the overall filipin signal was much stronger in *A. phagocytophilum*-infected HL-60 cells than that in uninfected HL-60, which supports the data shown in Figure 1 and further suggests the increased total cellular cholesterol might be free cholesterol, but not esterified cholesterol (Figure 2A). Second, most of the filipin signal was confined in *A. phagocytophilum*–containing vacuoles (“inclusions”) (Figure 2A). Uninfected host
cells showed weak filipin signal, which was mostly localized to the plasma membrane and some unknown compartments (assumed to be recycling endocytic compartments (Hao et al., 2002)). Notably, *A. phagocytophilum* inclusions outside of host cells also clearly displayed strong filipin signals (Figure 2B), suggesting that the inclusion has intrinsic ability to retain the cholesterol. Recently, it was shown that *Chlamydia* release from the infected host cells occurs by two mechanisms: lysis and extrusion (Hybiske and Stephens, 2007). How the *A. phagocytophilum* inclusion became extracellular remains to be studied. Taken together, these results indicate that *A. phagocytophilum* infection alters host intracellular cholesterol homeostasis and distribution and that free cholesterol is enriched in *A. phagocytophilum* inclusions.

*A. phagocytophilum* infection requires cholesterol derived from the host LDL uptake pathway rather than de novo biosynthesis

Mammalian cells acquire cholesterol from two sources: receptor-mediated uptake from exogenous lipoproteins and endogenous biosynthesis in the smooth ER (Brown and Goldstein, 1999). In leukocytes, LDL is the primary exogenous cholesterol source that is acquired via LDLR-mediated endocytosis (Brown and Goldstein, 1986). After hydrolysis of cholesterol esters in acidic late endosomes (enriched in acid lipases), the egress of free cholesterol occurs and free cholesterol is transported to the plasma membrane or delivered to the ER. Excess free cholesterol is catalyzed into cholesteryl esters by the resident ER acyl-CoA: cholesterol acyltransferase and stored as cytoplasmic lipid droplets (Ikonen, 2008; Chang et al., 2006).
Both undifferentiated and macrophage differentiated HL-60 cells express a regulated LDLR (Jouni and McNamara, 1991). Cholesterol is essential for *A. phagocytophilum* infection in HL-60 cells (Lin and Rikihisa, 2003a); thus, to better understand the source of free cholesterol required for *A. phagocytophilum* infection, we first examined the LDLR-mediated cholesterol uptake pathway using: 1) lipoprotein-deficient serum (LPDS), 2) anti-LDLR monoclonal antibody (mAb), and 3) pharmacological inhibitors of the LDLR-mediated cholesterol uptake pathway. LPDS was prepared from the fetal bovine serum by removing ~95% lipoproteins using potassium bromide gradient ultracentrifugation (data not shown). The fractionated lipoprotein (LP) was added back to LPDS in certain experiments. LPDS prevented the infection of host cells by *A. phagocytophilum*, and LPDS reconstituted with LP reversed this inhibition (Figure 3A). Moreover, the infection rate of LPDS-conditioned HL-60 cells was decreased on day 2 p.i. compared with that on day 1; and addition of LP rescued the growth on day 2 p.i. (Figure 3A), suggesting that cholesterol derived from LP is essential for *A. phagocytophilum* survival and proliferation in host cells. LDL enters host cells via LDLR-mediated endocytosis, which is blocked by a neutralizing antibody against LDLR (Brown and Goldstein, 1986). We found that the infection was also significantly blocked by the LDLR mAb (Figure 3B).

U18886A and imipramine are hydrophobic amines that accumulate in acidic cellular compartments, such as lysosomes, and block the post-lysosomal transport of cholesterol in the LDL uptake pathway (Lange *et al.*, 1997; Liscum and Faust, 1989). We found that both U18886A and imipramine significantly inhibited
A. phagocytophilum infection and replication in HL-60 cells in a dose-dependent manner. U18886A (5 µM) and imipramine (100 µM) added at 1 h p.i. almost completely blocked bacterial growth (Figure 3C). In contrast, lovastatin, an inhibitor of the rate-limiting enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase in the cholesterol biosynthetic pathway (Alberts et al., 1980), did not inhibit A. phagocytophilum infection, but rather significantly enhanced it in HL-60 cells in a dose-dependent manner (1-5 µM; Figures 3C and D). 25-Hydroxycholesterol (25-HC), which inhibits both LDL uptake and biosynthesis by acting as a negative feedback regulator of cholesterol metabolism (Lange and Steck, 1994), partially inhibited A. phagocytophilum infection (Figure 3C). Trypan blue and Diff-Quik staining followed by light microscopy showed that although infected host cells remained viable and did not show significant changes, the morphological characteristics of A. phagocytophilum inclusions were different: bacterial inclusions remained small in U18886A- or imipramine-treated cells compared with those in untreated cells (Figure 3D). The inhibitory effect of U18886A and imipramine, and the stimulatory effect of lovastatin, were observed at 1 and 2 day p.i. (Figure 3E). When inhibitors were added at 1 day p.i. (40% of HL-60 cells were infected), the bacterial proliferation was still significantly blocked in U18886A- and imipramine-treated cells at optimal concentrations of 5 and 100 µM, respectively (Figure 3F).

Taken together, these results indicate that not only A. phagocytophilum proliferation, but also survival of the bacterium in inclusions is dependent on cholesterol derived from the LDL uptake pathway, as bacterial numbers declined upon exposure to LPDS or imipramine. Furthermore, de novo cholesterol biosynthesis is not required, and
inhibition of this biosynthesis stimulated, rather than inhibited *A. phagocytophilum* infection in HL-60 cells. As *A. phagocytophilum* also infects endothelial cells (Niu et al., 2008; Munderloh et al., 2004), we used another cell line, monkey endothelial RF/6A to perform the inhibitor studies. Our data showed that the infection of *A. phagocytophilum* in RF/6A was also significantly inhibited by cholesterol transport inhibitors U18666A (5 μM) and imipramine (20 μM). However, no inhibitory effect was observed by lovastatin treatment (1 μM) (Figure S1). Taken together, these data suggest that LDLR-dependent cholesterol uptake pathway is critical for *A. phagocytophilum* infection in both leukocytes and endothelial cells.

**DiI-LDL uptake is enhanced by *A. phagocytophilum* infection in HL-60 cells**

Our data have shown that total cellular free cholesterol is increased in *A. phagocytophilum*–infected cells and that the LDL uptake pathway is required for *A. phagocytophilum* infection. We thus used LDL labeled with the fluorescent probe, 1, 1’-dioctadecyl-3, 3, 3’, 3’-tetramethyl indocarbocyanine (DiI–LDL) (Stephan and Yurachek, 1993), to compare the overall LDL uptake by *A. phagocytophilum*–infected and uninfected HL-60 cells. We found that LDL uptake was enhanced in *A. phagocytophilum*–infected cells (Figure 4C). After dissociation from LDLR in early sorting endosomes, LDL is directed to late endosomes for hydrolysis of cholesterol esters (Ikonen, 2008; Chang et al., 2006). By fluorescence microscopy, *A. phagocytophilum* inclusions were surrounded by DiI-LDL-containing small vesicles (Figures 4A and B), in agreement with our previous reports that lysosomes accumulate around *A. phagocytophilum* inclusions (Mott et al., 1999).
LDLR mRNA and protein levels are elevated in *A. phagocytophilum*-infected HL-60 cells

We next asked whether the increased cholesterol level and enhanced LDL uptake of host cells upon *A. phagocytophilum* infection may involve up-regulation of LDLR expression. First, we compared mRNA levels of LDLR and other genes involved in cholesterol and fatty acid biosynthesis, including HMG-CoA reductase, HMG-CoA synthase, and fatty acid synthase (Castoreno *et al.*, 2005) in infected and uninfected HL-60 cells by real-time RT-PCR. As shown in Figure 5A, LDLR mRNA level was significantly up-regulated at least 4-fold at day 2 p.i. However, the expression of cholesterol biosynthesis genes did not change significantly. The fatty acid synthase gene was significantly down-regulated upon *A. phagocytophilum* infection (Figure 5A). The pattern of LDLR mRNA normalized to host cell TATA-box binding protein mRNA levels was very similar to that normalized to G3PDH mRNA levels. Second, we examined the LDLR protein level by western blotting. As shown in Figure 5B, LDLR was markedly up-regulated upon *A. phagocytophilum* infection. To evaluate whether new protein synthesis or intracellular proliferation of *A. phagocytophilum* is required for LDLR mRNA up-regulation, 10 µg/ml of oxytetracycline was added to cell cultures at 1 h p.i. This treatment completely blocked bacterial proliferation as confirmed by Diff-Quik staining (data not shown) and western blotting using mAb 5C11 against the *A. phagocytophilum* major surface protein, P44 (Figure 5C inset). LDLR mRNA up-regulation at day 2 p.i. was abolished by oxytetracycline treatment (Figure 5C), suggesting that synthesis of new *A. phagocytophilum* proteins and/or intracellular proliferation is required to induce LDLR mRNA up-regulation in HL-60
cells. Notably, real-time RT-PCR analysis showed that LDLR mRNA levels in *A. phagocytophilum*–infected HL-60 cells were significantly increased by lovastatin treatment in a dose-dependent manner (Figure 5D). Western blotting also showed that the LDLR level was markedly higher in lovastatin-treated HL-60 cells at day 2 p.i. compared to DMSO–treated control cells (Figure 5E), which may explain the enhanced infection level in the lovastatin-treated sample as shown in Figure 3. Taken together, these results show that *A. phagocytophilum* infection enhances LDL uptake by up-regulating LDLR expression.

**SREBP cleavage is not involved in the up-regulation of LDLR mRNA in *A. phagocytophilum*–infected HL-60 cells**

Sterol regulatory element binding proteins (SREBPs) are key transcription factors for the cholesterol-mediated feedback regulation to maintain intracellular cholesterol homeostasis by regulating the LDLR gene as well as many cholesterol biosynthesis genes (Brown and Goldstein, 1997). Three SREBP isoforms have been characterized, namely SREBP-1a, SREBP-1c and SREBP-2 (Shimano, 2001). SREBP-1c, the predominant isoform in adult liver, preferentially activates genes required for fatty acid synthesis, whereas SREBP-2 preferentially activates the LDLR gene and various genes required for cholesterol synthesis, such as HMG-CoA reductase (Horton *et al.*, 2002). Therefore, we investigated whether the up-regulation of LDLR by *A. phagocytophilum* infection was due to activation of SREBP-2. SREBPs are activated by cleavage and translocation of the cleaved product from the cytoplasm to the nucleus (Brown and Goldstein, 1997). As shown in Figures 6, mature cleaved
SREBP-2 levels remained unchanged throughout *A. phagocytophilum* infection. As a positive control for SREBP-2 cleavage, uninfected cells were incubated with LPDS-conditioned medium overnight, as this is known to induce SREBP-2 cleavage in different cell lines including CHO and human monoblastic leukemia cell line U937 (Wang *et al.*, 1996). There was no significant difference between *A. phagocytophilum*–infected and uninfected HL-60 cells at any post-infection time point examined, suggesting that SREBP-2 activation is not involved in the up-regulation of LDLR mRNA upon *A. phagocytophilum* infection in HL-60 cells. In another word, the dramatic intracellular cholesterol homeostasis up-shift by *A. phagocytophilum* infection cannot be sensed by the host cell key regulatory factor SREBP-2.

**Stabilization of the LDLR mRNA during *A. phagocytophilum*–induced LDLR mRNA up-regulation**

LDLR is regulated not only at the transcriptional level but also at the posttranscriptional level via modulation of LDLR mRNA stability (Kong *et al.*, 2006). Therefore, we investigated LDLR mRNA stability in *A. phagocytophilum*–infected HL-60 cells after treatment with actinomycin D, a eukaryotic DNA-dependent RNA polymerase inhibitor. The half-life of LDLR mRNA in infected HL-60 cells was increased by almost 2-fold as compared to uninfected cells (Figure 7A). In contrast, the stability of HMG-CoA reductase mRNA did not change noticeably during *A. phagocytophilum* infection (Figure 7B). Human LDLR mRNA contains a 2.5-kb 3’UTR (Yamamoto *et al.*, 1984). The 3’ UTR of LDLR mRNA can be stabilized by
phorbol-12-myristate-13-acetate (PMA) and a Chinese herbal compound, berberine in
the human hepatic cell line HepG2 (Kong et al., 2004; Wilson et al., 1998). Three
AU-rich elements (AREs) are located in the 5' proximal region of the 3'UTR, which
have been shown to be responsible for the stabilization of LDLR mRNA by berberine,
but not by PMA (Kong et al., 2004). To investigate whether the LDLR 3’UTR
containing three AREs is involved in A. phagocytophilum–induced LDLR
stabilization, we transfected the luciferase fusion plasmid pLuc/LDLR 3’UTR-2,
containing three AREs of LDLR 3’UTR (nt 2,677-3,582) (Kong et al., 2004), into
RF/6A cells and measured the luciferase mRNA levels in A. phagocytophilum–
infected and control RF/6A cells. As shown in Figure 7C, luciferase mRNA levels
normalized to the antibiotic zeocin resistance gene (plasmid copy number) were
significantly increased in A. phagocytophilum–infected cells. Data normalized by
G3PDH (host cell number) showed a similar pattern (data not shown). These results
indicate that the LDLR 3’UTR containing three AREs may be involved in enhancing
LDLR mRNA stability in A. phagocytophilum–infected host cells.

ERK activation which is known to up-regulate LDLR expression, is required for
A. phagocytophilum infection

Accumulating evidence suggests that the extracellular signal–regulated kinase
(ERK) signaling cascade regulates the induction of LDLR expression in HepG2 cells
(Kong et al., 2006; Mehta, 2002). Moreover, berberine increases LDLR expression at
the posttranscriptional level via ERK-dependent stabilization of LDLR mRNA (Kong
et al., 2004; Wilson et al., 1998). Therefore, we asked whether and when ERK1/2
(p44/p42) is activated during *A. phagocytophilum* infection, whether ERK1/2 activation is required for *A. phagocytophilum* infection, and whether ERK1/2 activation up-regulates LDLR, constituting a positive feedback loop for *A. phagocytophilum* replication. First, we determined whether ERK1/2 is activated by *A. phagocytophilum* infection in HL-60 cells by western blotting using an antibody that only recognizes activated (phosphorylated) ERK1/2 but not inactive ERK1/2, as well as an antibody that recognizes both unphosphorylated and phosphorylated forms of ERK. We found that *A. phagocytophilum* activated ERK signaling especially during the exponential growth stage (day 2 p.i.) compared to day 1 p.i. or uninfected HL-60 cells (Figure 8A). Second, we examined the effect of U0126, the inhibitor of ERK upstream kinase MEK1/2, on ERK activation upon *A. phagocytophilum* infection. ERK activation by *A. phagocytophilum* infection and *A. phagocytophilum* replication in HL-60 cells were also almost completely inhibited by 10 µM U0126 (Figures 8A and B). This result was confirmed by western blotting for *A. phagocytophilum* P44 (Figure 8C). To confirm ERK activation is required for *A. phagocytophilum* infection, MEK1/2 proteins were knocked down by RNA interference with siRNAs targeting the MEK1 and MEK2 genes. Results showed that at 4 days post transfection, the protein amount of MEK1/2 was reduced by ~ 40% in MEK1/2 knockdown group, which resulted in the partial inhibition of the phosphorylation of ERK1/2 (~30%), as well as the infection of *A. phagocytophilum* (~50%)(Figure 8D). These data clearly demonstrate that ERK signaling is activated by and required for *A. phagocytophilum* infection in HL-60 cells.
To determine whether ERK activation is involved in up-regulation of LDLR upon *A. phagocytophilum* infection, we examined the LDLR mRNA levels in U0126-pretreated HL-60 cells by real-time RT-PCR. As shown in Figure 8E, the relative LDLR mRNA level upregulated by *A. phagocytophilum*–infected HL-60 cells was significantly reduced by U0126 treatment starting at a concentration of 0.5 µM at 2 day p.i. Although the expression of LDLR mRNA was slightly reduced in uninfected HL-60 cells by MEK1/2 siRNA knockdown, there was no significant reduction of LDLR mRNA in infected HL-60 cells (data not shown). Therefore, we could not draw the definitive conclusion whether MEK1/2 → ERK1/2 pathway is required for up-regulation of LDLR expression in the case of *A. phagocytophilum* infection of HL-60 cells.

### 3.5 Discussion

In this study, we present evidence that *A. phagocytophilum* is dependent on cholesterol derived from the LDLR-mediated uptake pathway of eukaryotic host cells. Several vacuole-occupying intracellular pathogens depend on host cholesterol stores or trafficking during their infection of cultured cells or mice (Goluszko and Nowicki, 2005), although mechanisms vary considerably. *Salmonella enterica* serovar Typhimurium requires nonsterol precursors of the cholesterol biosynthetic pathway for intracellular proliferation (Catron *et al.*, 2004), and cholesterol accumulates in *Salmonella*-containing vacuoles in a *Salmonella* pathogenicity island-2–dependent manner (Catron *et al.*, 2002; Garner *et al.*, 2002). The establishment of *Brucella abortus* infection in mice requires trafficking of plasma membrane cholesterol, which
is controlled by Niemann-Pick C1, an important cholesterol transport protein in late endosomes/lysosomes, as evidenced by resistance to *B. abortus* infection in Niemann-Pick C1 knockout mice (Watarai et al., 2002). *Chlamydia trachomatis* inclusions acquire cholesterol by selectively rerouting Golgi-derived vesicles (Carabeo et al., 2003) and multivesicular bodies (Beatty, 2006). Interestingly, cytoplasmic lipid droplets are translocated into the lumen of *Chlamydia* inclusions, which appears to be an alternate mechanism for acquisition of cholesterol (Cocchiaro et al., 2008).

*Coxiella burnetii* infection increases production of host cell cholesterol with concomitant up-regulation of host genes involved in cholesterol metabolism, including LDLR and several cholesterol biosynthesis genes (Howe and Heinzen, 2006). Unlike any of the above described intracellular pathogens, *A. phagocytophilum* acquires cholesterol preferentially from the LDL uptake pathway by up-regulating LDLR expression. *Toxoplasma gondii*, although a eukaryotic pathogen, cannot synthesize sterols via the mevalonate pathway and appropriates cholesterol to its parasitophorous vacuole exclusively from LDL uptake (Coppens et al., 2000).

Recently, an unexpected novel mechanism was demonstrated: *T. gondii* actively sequesters the host endocytic vesicles in its vacuolar spaces to provide its cholesterol needs (Coppens et al., 2006). Interestingly, *A. phagocytophilum* has an intracellular compartment somewhat similar to that of *T. gondii* (Mordue et al., 1999; Mott et al., 1999), which is segregated from both endocytic and exocytic pathways.

The present study raises an intriguing question: how does *A. phagocytophilum* acquire cholesterol derived from the host LDL endocytic pathway? Although *A.
*Phagocytophilum* inclusions do not fuse with lysosomes, our previous data clearly demonstrate that these inclusions are surrounded by host lysosomes (Mott et al., 1999). The physical proximity between the *A. phagocytophilum* inclusions and host lysosomes may facilitate cholesterol acquisition by *A. phagocytophilum*. Not only *A. phagocytophilum* enters host cells via caveolae-containing lipid rafts, but also the caveolar marker protein, caveolin-1, co-localizes with both early and replicative *A. phagocytophilum* inclusions (Lin and Rikihisa, 2003a). Caveolin-1 is a well-established cholesterol binding protein (Murata et al., 1995), and caveolae/caveosomes have been proposed to be a cholesterol transporter participating in the bidirectional shuttling of free cholesterol between the plasma membrane and various intracellular compartments, including the ER, Golgi and lipid droplets (Ikonen and Parton, 2000). Therefore, we hypothesize that the caveosome is involved in cholesterol transport to *A. phagocytophilum* inclusions. Another finding showed that some *A. phagocytophilum* inclusions co-localize with vesicle-associated membrane protein 2 (VAMP2) (Mott et al., 1999). In neutrophils, VAMP2 is believed to play a role in controlling vesicular targeting, docking, and fusion through interactions with other proteins, such as N-ethylmaleimide-sensitive factor and soluble N-ethylmaleimide-sensitive factor attachment protein (Borregaard et al., 2007). The presence of VAMP2 on *A. phagocytophilum* inclusions may provide a mechanism to acquire cholesterol for the replicating organism through regulated vesicle trafficking. A recent report showed that *A. phagocytophilum* modulates lipid metabolism by increasing perilipin mRNA and protein levels to facilitate infection of HL-60 cells (Manzano-Roman et al., 2008). Perilipin is a major adipocyte lipid droplet–associated
protein that plays a central role in lipolysis and cholesterol synthesis. It is unknown whether lipid droplets serve as an intermediate organelle for cholesterol acquisition by *A. phagocytophilum* after intracellular delivery via the LDLR pathway. Further work is required to determine the exact mechanism of cholesterol acquisition by *A. phagocytophilum*.

Our data demonstrate that *A. phagocytophilum* up-regulates LDLR expression in HL-60 cells by stabilizing the LDLR mRNA via a posttranscriptional mechanism. This is the first report on modulation of LDLR mRNA stability by an infectious agent, and to our knowledge this is also the first report regarding LDLR up-regulation on leukocytes. This aspect may have clinical relevance because increasing hepatic LDLR expression is currently one of the primary strategies for hypercholesterolemia therapy. Recent data suggest that mRNA stability is the major mode of posttranscriptional regulation of LDLR expression. The stability of LDLR mRNA is known to be modulated by only a few reagents, including gemfibrozil (Goto *et al.*, 1997), PMA, chenodeoxycholic acid and berberine (Kong *et al.*, 2006). Interestingly, the stabilization of LDLR mRNA via the 3’UTR by chenodeoxycholic acid (CDCA) and berberine requires activation of the ERK signaling pathway. However, it is so far unclear how the ERK pathway is linked to LDLR mRNA stabilization and whether any trans-acting RNA binding proteins are involved in the stabilization process. In addition, the activity of berberine to up-regulate LDLR expression is specific to hepatocytes, as the significant increase of LDLR was found only in HepG2 cells, but not in other non-hepatic cell lines, such as CHO, HEK293, or human primary
fibroblasts (Abidi et al., 2005). The present study shows that *A. phagocytophilum* potently activates the ERK signaling pathway, especially at the exponential growth stage when the bacterium requires substantial amounts of cholesterol for proliferation with concomitant expansion of inclusions. The result is somewhat consistent with the recent report that *A. phagocytophilum* activates ERK2 (p42) in host human neutrophils at 3 h p.i. (Lee et al., 2008a). For the first time, our MEK inhibitor and siRNA knock-down studies showed ERK activation is required for *A. phagocytophilum* infection. It has been reported that several other intracellular bacteria actively manipulate the host ERK signaling pathway to benefit microbial survival. Tapinos and Rambukkana reported a PKCε-dependent, but not MEK-dependent pathway for ERK1/2 activation by *Mycobacterium leprae* resulting in continuous proliferation of infected human Schwann cells, without inducing transformation (Tapinos and Rambukkana, 2005). Interestingly, activation of the host Ras-Raf-MEK-ERK-cPLA2 signaling cascade is required for chlamydial acquisition of host glycerophospholipids (Su et al., 2004). It remains to be elucidated why ERK activation is required for *A. phagocytophilum* infection or whether ERK involves in LDLR mRNA stabilization.

Recently, the use of cholesterol biosynthesis inhibitors, such as statins, was proposed to combat certain pathogen infections because these microbes utilize the host cholesterol biosynthesis pathway. For example, lovastatin and atorvastatin reduce *S. enterica* serovar Typhimurium proliferation in vitro and in BABL/C mice, respectively (Catron et al., 2004). Growth of *C. burnetii* can be also inhibited by...
lovastatin in vitro (Howe and Heinzen, 2006). It is important to point out that unlike *Salmonella* and *Coxiella*, lovastatin enhanced, at least, did not reduce *A. phagocytophilum* infection in both HL-60 promyelocytic and RF/6A endothelial cells. This result is not surprising, however, because our present data show that *A. phagocytophilum* acquires cholesterol derived from the LDL uptake pathway and not from the biosynthesis pathway. Additionally, lovastatin-treated *A. phagocytophilum*–infected HL-60 cells expressed higher levels of LDLR. Thus, the question then becomes: is statin treatment beneficial, if not detrimental for *A. phagocytophilum* infection in vivo? The answer to this question is very important because statin drugs are widely used in elderly patients to treat hypercholesterolemia, and *A. phagocytophilum* infection is more prevalent in this community. Our recent data showed that high blood cholesterol facilitates *A. phagocytophilum* infection in a mouse model (Xiong *et al*., 2007). However, our unpublished data suggest that there is a higher *A. phagocytophilum* level in the blood after Lipitor (atorvastatin) treatment of mice (Xueqi Wang and Yasuko Rikihisa, unpublished data). We had originally hypothesized that statin treatment lowers blood cholesterol levels and consequently results in lower bacterial burden in the mice. This paradox could be explained as follows: statins do not reduce overall plasma cholesterol levels in mice, as they do in humans, due to very low levels of LDLs in rodents, even though they do block mouse HMG-CoA reductase and the sterol biosynthetic pathway in mice (Krause and Princen, 1998). In fact, we have not found decreased blood cholesterol levels in statin-treated mice (Xueqi Wang and Yasuko Rikihisa, unpublished data). Similarly, there is no significant change in serum cholesterol levels in atorvastatin-treated mice.
compared with vehicle-treated mice, although statins reduce *S. enterica* serovar Typhimurium growth in vivo (Catron et al., 2004). Therefore, we speculate that, similar to our in vitro HL-60 cell culture model, up-regulation of LDLR in *A. phagocytophilum*–infected host leukocytes may result in a greater bacterial burden in statin-treated mice. Taken together, these results suggest that critical and careful consideration is required when treating granulocytic anaplasmosis patients, as statins are currently widely used to treat hypercholesterolemia in humans by lowering blood cholesterol levels.

The data presented here improve our understanding of how a cholesterol-dependent bacterium exploits eukaryotic cellular cholesterol trafficking and regulatory pathways and may provide insight regarding a new therapeutic target for the treatment of human granulocytic anaplasmosis. Most information on LDLR regulation is derived from studies using hepatocytes (Kong et al., 2006); however, as in the berberine’s case (Abidi et al., 2005), in non-hepatic cells, LDLR regulation by cholesterol modulating compounds may differ from those of hepatocytes. Our study enhances our understanding of the LDLR regulation pathway in leukocytes and perhaps endothelial cells (Herron et al., 2005; Munderloh et al., 2004), both of which are understudied, but important players in atherosclerosis.
Figure 3.1 Total cholesterol level is increased in *A. phagocytophilum*–infected HL-60 cells.

*A. phagocytophilum*–infected HL-60 cells were harvested on 0, 1, 2 and 3 day p.i., and total cellular cholesterol levels and bacterial burden were determined. Cholesterol concentrations are expressed as micrograms of total cholesterol (esterified and unesterified cholesterol) per milligram of total protein (black triangles). Bacterial burdens are expressed as bacterial numbers per 100 cells (black bars). Data are expressed as mean ± standard deviation (n = 3) and are representative of at least three independent experiments with similar results. *, $P < 0.05$ (unpaired two-tailed *t* test); **, $P < 0.01$ (unpaired two-tailed *t*-test).
Figure 3.1
Figure 3.2 Free cholesterol is enriched in *A. phagocytophilum* inclusions.

*A. phagocytophilum*–infected HL-60 cells (A) and *A. phagocytophilum* inclusions released from host cells (B) were fixed at 2 d p.i., stained with mouse anti-*A. phagocytophilum* (green) or normal mouse IgG and filipin (blue), and analyzed by fluorescence microscopy. The experiment shown is representative of at least three independent experiments. Bar, 5 μm. *Ap, A. phagocytophilum.*
Figure 3.2
Figure 3.3 *A. phagocytophilum* infection requires cholesterol derived from the host LDL uptake pathway rather than de novo biosynthesis.

(A) *A. phagocytophilum* growth in HL-60 cells cultured in the growth medium containing lipoprotein deficient serum (LPDS), LPDS reconstituted with lipoproteins (LP), and fetal bovine serum were determined on days 1 and 2 p.i.

(B) HL-60 cells were pre-treated with anti-LDLR mAb and isotype control mouse IgG and infected with host cell–free *A. phagocytophilum*. The number of bacteria was determined on days 3 and 4 p.i.

(C) Cholesterol transport and biosynthesis inhibitors U18886A, imipramine, 25-hydroxycholesterol (25-HC), and lovastatin were added at 1 h p.i. at different dosages, and numbers of bacteria were determined on day 2 p.i. Data are expressed as mean ± standard deviation (n = 3) and are representative of two independent experiments with similar results. *, P < 0.05 (unpaired two-tailed t test); **, P < 0.01 (unpaired two-tailed t-test).

(D) *A. phagocytophilum*-infected HL-60 cells at 1 h p.i. were treated with lovastatin (1 µM), U18886A (1 µM), imipramine (20 µM), and DMSO vehicle control, respectively. On day 1 p.i., infected cells were harvested and observed by light microscopy following Diff-Quik staining. Arrows indicate *A. phagocytophilum* inclusions. Note much smaller sizes of inclusions that contain much fewer numbers of bacteria in U18886A- or imipramine-treated cells than those in untreated or lovastatin-treated cells. N, nucleus. All figures are shown at the same magnification. Bar, 5 µm.
(E) Cultures were treated as in (C), and numbers of bacteria were determined on days 1 and 2 p.i. *, $P < 0.05$ (unpaired two-tailed $t$ test); **, $P < 0.01$ (unpaired two-tailed $t$-test).

(F) Lovastatin (1 µM), U18886A (5 µM), imipramine (100 µM), and vehicle control were added to A. phagocytophilum-infected HL-60 on day 1 p.i. (~40 % infected cells), and the number of bacteria was determined on day 2 p.i. Data are expressed as mean ± standard deviation (n = 3) and are representative of three independent experiments with similar results. *, $P < 0.05$ (unpaired two-tailed $t$ test); **, $P < 0.01$ (unpaired two-tailed $t$-test).
Figure 3.3
Figure 3.4 DiI-LDL uptake is enhanced upon *A. phagocytophilum* infection in HL-60 cells.

Uninfected and *A. phagocytophilum*-infected HL-60 cells were incubated with increasing concentrations of DiI-LDL at 37°C for 2 h. After DiI-LDL uptake, HL-60 cells were directly observed by fluorescence microscopy without fixation (A), or after being fixed and labeled with anti-*A. phagocytophilum* (B). Arrows indicate *A. phagocytophilum* inclusions. *Ap, A. phagocytophilum*. The amount of cell-associated DiI was determined by fluorometric assay. Data were normalized to total cellular protein, and specific association was determined by subtracting the fluorescence measured in the presence of a 30-fold excess concentration of unlabeled LDL from normalized cell-associated DiI fluorescence (C). Data are expressed as mean ± standard deviation (n = 3) and are representative of two independent experiments. *, *P* < 0.05 (unpaired two-tailed *t* test); **, *P* < 0.01 (unpaired two-tailed *t*-test).
Figure 3.4
Figure 3.5 LDLR is up-regulated in *A. phagocytophilum*-infected HL-60 at transcriptional and protein levels.

(A) At day 2 p.i., total RNA was extracted from uninfected and *A. phagocytophilum*-infected HL-60 cells. Analysis of mRNA amount was performed using quantitative RT-PCR with specific primers for each gene. Transcript levels were normalized to mRNA level of TATA-box binding protein or G3PDH in each sample. *Ap*, *A. phagocytophilum*.

(B) Cell samples were collected at the indicated time points, membrane fractions were isolated, and western blotting was performed to determine LDLR protein levels in uninfected HL-60 and *A. phagocytophilum*-infected HL-60 cells.

(C) Oxytetracycline (OTC, 10 µg/ml) was added to *A. phagocytophilum*-infected HL-60 cells at 1 h p.i., RNA was extracted from infected HL-60 cells on days 1 and 2 p.i., and LDLR mRNA was analyzed by quantitative RT-PCR. Transcript levels were normalized to mRNA level of TATA-box binding protein in each sample. Inset shows western blotting result using mAb 5C11 against *A. phagocytophilum* major surface protein P44.

(D) Relative LDLR mRNA in lovastatin-treated cell cultures on day 2 p.i. was analyzed by quantitative RT-PCR with primers specific for LDLR gene.

(E) LDLR protein levels in lovastatin- or mock (DMSO)-treated infected cells at day 2 p.i. were analyzed by western blotting using anti-LDLR mAb.

Data are expressed as mean ± standard deviation (n = 3) and are representative of two independent experiments. *, $P < 0.05$ (unpaired two-tailed $t$ test); **, $P < 0.01$ (unpaired two-tailed $t$-test).
Figure 3.5

A

Fold change of mRNA levels (Ap infected/uninfected) normalized by TATA-Box binding protein.

B

HL-60

Ap infected

1h 1d 2d 1h 1d 2d

~130

LDLR

C

OTC

1d 2d

1d +

2d +

P44

Relative LDLR mRNA

Days, p.i.

1 2

D

Relative LDLR mRNA

Lovastatin (μM)

0 1 5

E

Ap 2d

Lovastatin DMSO

~130

LDLR
Figure 3.6 SREBP activation is not up-regulated in *Anaplasma phagocytophilum*-infected HL-60 cells.

Uninfected and *A. phagocytophilum*-infected HL-60 cells were collected at the indicated time points, and western blotting was performed using anti-SREBP-2 mAb. α-Tubulin was used as the protein input control to normalize each sample. Positive control was set up by incubating uninfected cells with LPDS-conditioned medium overnight. Ratio of band intensities of mature form to precursor form of SREBP was calculated to show the SREBP-2 cleavage. Data are representative of three independent experiments. *Ap, A. phagocytophilum*. P, precursor form of SREBP; M, mature form of SREBP.
Figure 3.6
Figure 3.7 LDLR mRNA is stabilized in *A. phagocytophilum*–infected host cells.

Actinomycin D (5 µg/ml) was added to uninfected and *A. phagocytophilum*–infected HL-60 cells for different periods. Total RNA was isolated, and LDLR mRNA (A) and HMG-CoA reductase mRNA (B) were analyzed by quantitative RT-PCR. Data are expressed as mean ± standard deviation (n = 3) and are representative of two independent experiments. The decay rates of LDLR mRNA (A) are significantly different (*P* < 0.01), as tested by two-way ANOVA. (C) Chimeric pLuc/LDLR 3’UTR-2 was transfected into RF/6A cells using FuGene HD reagent, and host cell–free *A. phagocytophilum* was added to transfected RF/6A cells at 1 day post transfection. Luc mRNA was analyzed on day 2 p.i. by quantitative RT-PCR. Cell samples were normalized by the antibiotic gene zeocin mRNA level. Data are representative of two independent experiments. *Ap, A. phagocytophilum*. ND, not detectable. **, *P* < 0.01 (unpaired two-tailed *t*-test).
Figure 3.7

A

LDLR mRNA remaining (%)

T_{1/2} = 108 min

T_{1/2} = 57 min

**P = 0.0081

B

HMG-CoA reductase mRNA remaining (%)

T_{1/2} = 130 min

T_{1/2} = 101 min

P = 0.11

C

Relative Luc mRNA levels to zeocin

**

Ap

Control

pLuc/LDLR 3'UTR-2

pLuc/LDLR 3'UTR-2 + FuGene

ND ND
Figure 3.8 ERK signaling pathway is involved in the LDLR up-regulation upon *A. phagocytophilum* infection.

(A) Western blot analysis was performed using antibodies specific to either the phosphorylated or total ERK1/2. Uninfected and *A. phagocytophilum*–infected HL-60 cells with or without 10 μM ERK inhibitor U0126 treatment were collected at the indicated time points. α-Tubulin was used as the protein loading control to normalize each sample. The values under the bands show the ratios of band intensities of p-ERK and ERK. *Ap, A. phagocytophilum*.

(B) Bacterial numbers per 100 cells treated with the indicated concentrations of U0126 were determined on days 1 and 2 p.i.

(C) Western blot analysis of *A. phagocytophilum*–infected HL-60 cells on day 2 p.i. treated with the indicated concentrations of U0126 was performed using antibodies specific to phosphorylated or total ERK1/2, or *A. phagocytophilum* outer membrane protein P44. α-Tubulin was used as the protein input control to normalize each sample. The values under the bands show the ratios of band intensities of p-ERK and P44, normalized to total ERK or α-tubulin, respectively. Data are representative of three independent experiments.

(D) HL-60 cells were transfected with control (Ctl) or double-stranded siRNA specific targeting the genes encoding MEK1 and MEK2 (MEK1+2) (3 μg/2×10^6 cells) using the Amaxa Nucleofection system. Two days after transfection, host cell-free *A. phagocytophilum* was added to the cells and incubated for additional 2 days. One aliquot of samples were lysed and subjected to Western blotting using antibodies against MEK1/2, ERK1/2, phospho-ERK1/2, and *A. phagocytophilum* P44 protein.
The relative protein amount of MEK1/2, phospho-ERK1/2, and P44 were determined using total ERK1/2 as loading control by densitometry analysis. The values under the bands show the relative ratios of band intensities, with the ratios of those from samples nucleofected with control siRNA arbitrarily set as 1. Results are representative of three independent experiments.

(E) LDLR expression in *A. phagocytophilum*–infected HL-60 cells on day 2 p.i. treated with the indicated concentrations of U0126 was determined by quantitative RT-PCR. Data are expressed as mean ± standard deviation (n = 3) and are representative of two independent experiments. *, $P < 0.05$ (unpaired two-tailed $t$ test); **, $P < 0.01$ (unpaired two-tailed $t$-test).
CHAPTER 4

Anaplasma phagocytophilum Activates Extracellular Signal-regulated Kinase (ERK) 1/2 by Modulating Raf-1 Phosphorylation

4.1 Abstract

Anaplasma phagocytophilum, an obligatory intracellular bacterium of granulocytes, continuously activates extracellular signal-regulated kinase (ERK) 1/2 starting mid exponential growth stage in a human promyelocytic leukemia cell line HL-60. ERK1/2 activation was required for A. phagocytophilum infection. One of well known ERK1/2 activation pathways is Ras-Raf-1-MEK-ERK1/2. Therefore, in this study we investigated involvement of Ras and Raf-1 in ERK activation and A. phagocytophilum infection. A pharmacological inhibitor of Ras membrane translocation was found to inhibit both ERK activation and A. phagocytophilum infection. Raf-1 was highly phosphorylated including serine 259 and 338 of Raf-1 by A. phagocytophilum infection. Protein kinase C inhibitors had no effects on Raf-1 phosphorylation or A. phagocytophilum infection. Raf-1 and phosphorylated Raf-1 were found mostly in the cytosol of host cells, instead of being translocated to the membrane. These data indicate that Ras and Raf-1 activation is required for ERK1/2 activation and A. phagocytophilum infection. However, our result did not find direct
link between Raf-1 and Ras activation in inducing ERK1/2 activation by *A. phagocytophilum* infection.

**4.2 Introduction**

*Anaplasma phagocytophilum* is a tick-borne obligatory intracellular bacterium that proliferates in membrane-bound inclusions in granulocytes and endothelial cells of various mammal species (Herron *et al.*, 2005; Munderloh *et al.*, 2004; Dumler *et al.*, 2001). In humans, *A. phagocytophilum* causes an emerging and major tick-borne disease called human granulocytic anaplasmosis, an acute febrile disease that is potentially fatal, especially in elderly or immunocompromised individuals (Bakken and Dumler, 2000). *A. phagocytophilum* is an atypical Gram-negative bacterium, because it contains substantial amounts of cholesterol in its outer membrane (Lin and Rikihisa, 2003a). *A. phagocytophilum* resides and proliferates in the professional killing host cells and disregulates many cell functions in order to benefit itself and establish the infection in the host (Rikihisa, 2006b; Rikihisa, 2003).

Mitogen-activated protein kinase (MAPK) cascades are important signaling pathways that recognize and respond to extracellular signals. They regulate diverse cellular activities such as proliferation, differentiation, cell motility, survival, apoptosis, and immune response. To date, three distinct members of MAPKs have been well characterized in mammals: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun-N-terminal kinases (JNKs), and p38 kinases (p38). Although the ERK pathway, like the JNK and p38 MAPK pathways, participates in the regulation of a wide range of biological responses, it has been linked particularly to the control
of cell proliferation, differentiation and survival. The extracellular stimuli to activate ERK MAPK include phorbol ester (TPA), growth factors, and cytokines (Yoon and Seger, 2006; Roux and Blenis, 2004; Pearson et al., 2001). Interestingly, it has been reported that several intracellular bacteria can induce the activation of ERK signaling within host cells to modulate host cell proliferation or survival for bacterial beneficial (Rajalingam et al., 2008; Mimuro et al., 2007; Tapinos and Rambukkana, 2005; Su et al., 2004).

Goodman et al., demonstrated that the ERK pathway, particularly ERK2, is activated in A. phagocytophilum-infected neutrophils (Lee et al., 2008b), although Choi and Dumler et al., reported that an increased phosphorylation of p38 MAPK, rather than ERK, in A. phagocytophilum infected neutrophils at both 3 and 18 h p.i.(Choi et al., 2005). Our previous data showed ERK activation by A. phagocytophilum in HL-60 cells at mid exponential growth stage (Xiong et al., 2009) and this activation is required for infection. Therefore, in the present study, we examine whether upstream of ERK pathway is required for infection, and which upstream pathway is specifically activated by A. phagocytophilum infection.

4.3 Materials and Methods

Reagents and antibodies

Reagents used were: Oxytetracycline(OTC) (Sigma, St. Louis, MO). MEK1/2 inhibitor U0126 (Biomol, Plymouth Meeting, PA). Ras inhibitor Manumycin A, pan-PKC inhibitors Gö 6983, Bisindolylmaleimide I (Bis), broad range PTK inhibitor genistein, Src kinase inhibitor PP2 (Calbiochem, San Diego, CA), Raf-1 kinase
inhibitor BAY43-9006 (LC laboratory, Woburn, MA), and Abl-1 inhibitor Gleevec (Norvatis, Basel, Switzerland).

The mouse mAb 5C11 recognizing the N-terminal conserved region of *A. phagocytophilum* major surface protein P44 has been described (Kim and Rikihisa, 1998). Other antibodies used include: mouse anti-flotillin 1 mAb (BD Parmingen, San Jose, CA), rabbit anti-phospho-Raf-1(Ser259) antibody, rabbit anti-phospho-Raf-1(Ser338) mAb (56A6), mouse anti-phospho-ERK1/2 mAb(E10), rabbit anti-ERK1/2 antibody, mouse anti-MEK1/2 mAb (Cell Signaling, Danvers, MA), and rabbit anti-Raf-1(C-12) antibody, goat anti-phospho-Raf-1(Tyr 340/341) antibody, rabbit anti-Sos 1(C-23) antibody, mouse anti-α-tubulin mAb (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-phospho-Raf-1(Tyr 340/341) antibody, mouse anti-Ras mAb (clone RAS10) (Millipore, Temecula, CA). Peroxidase-conjugated secondary antibodies were obtained from KPL (Gaithersburg, MD). Normal mouse IgG were purchased from Santa Cruz Biotechnology.

*A. phagocytophilum* culture and inhibitor treatments

*A. phagocytophilum* HZ strain was cultivated in human promyelocytic leukemia cell line HL-60 as described (Rikihisa *et al.*, 1997). Host cell-free *A. phagocytophilum* was prepared by sonicking highly infected (>90% infected cells) HL-60 cells for 8 s twice 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 added to HL-60 or RF/6A cells. After 1 h incubation at 37°C, extracellular organisms were washed, fresh
medium was added (this time point was considered 0 h p.i.), and continuously incubated at 37°C.

Inhibitors were added at indicated time points (0 h or 1 day p.i.), and the inhibitors were kept in the growth media throughout the incubation period or removed later as indicated. The highest final concentrations of inhibitors used were: OTC (10µg/ml), U0126 (10µM), manumycin A (0.1 µM), Gö 6983 (1 µM), GW5074 (1 µM), Bis (1 µM), BAY43-9006 (20nM), genistein (40 µM), PP2 (10 µM), and Gleevec (20 µM). Inhibitor treatments at these concentrations did not affect host cell integrity as assessed by light microscopy or by G3PDH mRNA level.

The degree of bacterial infection in host cells was assessed by Diff-Quik staining (Baxter Scientific Products, Obetz, OH), and the number of *A. phagocytophilum* cells was estimated in 100 host cells in triplicate culture wells as described (Rikihisa *et al.*, 1995).

**Subcellular fractionation**

Subcellular fractionation procedure was performed as described by Salojin at al. (Salojin *et al.*, 2000) with some modification. 10⁷ HL-60 and infected cells were washed 2 times with ice-cold PBS and resuspended the pellet in 1 ml chilled hypotonic buffer A (10 mM Tris, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 2 mM EGTA) containing freshly added protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II (Calbiochem). The cells were sonicated for 2 min on ice (grade: 3, 5 sec by 6 times and repeat 4 times) followed by a brief centrifugation for 10 min to remove the nuclei and debris. The supernatant was centrifuged for 30 min at 100
100,000g at 4 °C to separate the membrane (pellet) and soluble cytoplasm (supernatant) fractions. The membrane fraction was washed with 0.5 ml buffer A and centrifuged at 100,000g for 10 min. The sedimented fraction was resuspended and solubilized by sonication (20 s) in 0.2 ml ice-cold buffer A supplemented with 150 mM NaCl and 1% Triton-100 plus 0.2% Nonidet 40. The protein concentrations were measured by BCA kit (Pierce, Rockford, IL) for normalization of samples.

**Western blot analysis**

*A. phagocytophilum*–infected HL-60 and control HL-60 cells (1 × 10⁶) were washed and resuspended in 200 µl ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) containing freshly added protease inhibitor cocktail set III and phosphatase inhibitor cocktail set II (Calbiochem), and lysed by mixing with 200 µl of 2× Laemmli sample buffer (4% SDS, 135 mM Tris-HCl [pH 6.8], 20% glycerol, and 10% β-mercaptoethanol). Samples were separated by SDS-PAGE with 7.5% or 10% polyacrylamide resolving gels and then transferred to a nitrocellulose membrane or PVDF membrane using a semidry blotter (WEP, Seattle, WA). The membrane was blocked using 5% (wt/vol) skim milk (BD, Sparks, MD) in Tris-buffered saline (150 mM NaCl and 50 mM Tris at pH 7.5) containing 0.1% Tween-20, incubated with primary antibodies (1:500 or 1:1,000 dilution) at 4°C for 12 h, and subsequently incubated with peroxidase-conjugated secondary antibodies at 1:1,000 dilution at room temperature for 1 h. Immunoreactive bands were visualized with enhanced chemiluminescence. The levels of GTP-bound Ras in HL-60 cells were detected by ras activation assay kit (Cell
Biolabs, Inc. San Diego, CA) by pulling down with glutathione-agarose beads bound with the Ras-binding domain of Raf-1 glutathione S-transferase fusion protein following the manufacturer’s instruction.

**Statistical analysis**

Statistical analyses were performed by unpaired, two-tailed Student’s *t*-test. *P* < 0.05 was considered to be significant.

**4.4 Results**

ERK1/2 is activated and up-regulated by *A. phagocytophilum* infection and this up-regulation is bacterial protein synthesis dependent.

Our previous data showed sustained ERK1/2 activation by *A. phagocytophilum* infection in HL-60 cells starting mid exponential growth stage and ERK1/2 upstream MEK1/2 kinase inhibitor U0126 and MEK1/2 siRNA reduce the bacterial infection in HL-60 and endothelial cell RF/6A, respectively (Xiong *et al.*, 2009). Here we further examined whether this ERK up-regulation requires *A. phagocytophilum* protein synthesis. OTC was added in *A. phagocytophilum* infected HL-60 at 1 d p.i. (around 40% infected cells) and kept in the culture for additional 2 days. *A. phagocytophilum* was almost cleared in HL-60 cells at day 2 and 3 p.i. indicated by western blot analysis using bacteria major outer membrane protein P44 monoclonal antibody (Figure 1). As shown in Figure 1, the increased phosphorylated ERK proteins at days 2 and 3 p.i. were effectively abrogated by OTC treatment, suggesting ERK activation by *A. phagocytophilum* infection is dependent on bacterial new protein synthesis.
Ras, Raf-1, and MEK1/2 inhibitors, rather PKC inhibitors block *A. phagocytophilum* infection in HL-60 cells

The ERK pathway is one of the most thoroughly studied cytoplasmic signaling pathways. In the conventional pathway, upon the stimulation of the extracellular mitogenic stimuli-activated receptor tyrosine kinases phosphorylate ERK1/2 through Grb2/Sos/Ras/Raf/MEK1/2 pathway. Initially, interaction of Ras GEF Sos with adapter protein Grb2 leads to Sos translocation to the plasma membrane where it stimulates the exchange of GDP for GTP on the membrane-bound Ras. Then activated Ras recruits the Raf family of kinases such as Raf-1 to the plasma membrane, a key step in a complex activation process (Ramos, 2008; Kolch, 2000). It has been reported that protein kinase C (PKC) is a key regulatory kinase involved in the regulation and cross talk with ERK cascade via Ras-dependent and Ras-independent mechanisms in some cell types under certain condition (Basu and Sivaprasad, 2007; Cacace *et al*., 1996; Kolch *et al*., 1993). To learn whether the upstream of ERK pathway is required for *A. phagocytophilum* infection of HL-60 cells, we examined *A. phagocytophilum* infection after the treatment of pan PKC inhibitors Go6983 (selective inhibitor of PKC isoforms, PKCα, β, γ, δ, and ζ) and Bis (selective inhibitor of PKC isoforms, PKCα, β, γ, δ, and ε), Ras prenylation inhibitor manumycin A and raf-1 inhibitor sorafenib (BAY43-9006). Both manumycin A and sorafenib significantly blocked *A. phagocytophilum* growth in a dose-dependent manner when added at 1 h p.i.; however, PKC inhibitors Go6983 or Bis did not have effect on *A. phagocytophilum* infection (Figure 2A). The Diff-Quik staining followed by light microscopy showed dramatic change of morulae after inhibitors treatment for
1 day at day 2 p.i.: smaller morulae with fewer bacteria inside and empty vacuoles with bacteria debris-like inside were found in manumycin A and sorafenib, repeatedly, treated HL-60 cells. In contrast large and structure-intact morulae were still maintained in Bis (Figure 2B) and Go6983 (Data not shown) treated cells. Taken together, these data suggest that Ras/Raf, not PKC activities is required for *A. phagocytophilum* infection in HL-60 cells.

**Raf-1 is actively phosphorylated on mutli-phosphrylation sites in *A. phagocytophilum* infected HL-60 cells**

Raf-1 phosphorylation and activation are required for sequential phosphorylation of MAPK kinase MEK1 and MEK2, which in turn activate the MAPKs ERK1 and ERK2 and further downstream effectors to elicit a variety of cellular responses. Activation of Raf-1 is a complex process involving association with GTP-bound form of Ras (GTP-Ras), membrane translocation through its Ras binding domain and both serine/threonine and tyrosine phosphorylation (Kolch, 2000; Morrison and Cutler, 1997). Raf-1 is shown to be phosphorylated on serine 338 by P21-activated serine/threonine protein kinase (PAK)-3, on tyrosine-341 by Src family, and on unknown sites by abl, and JAK (Janus kinase) family tyrosine kinases (Kolch, 2000; Morrison and Cutler, 1997). We found a clear “retard shift” of Raf-1 protein on the electrophoresis gel in *A. phagocytophilum* highly infected HL-60 compared with HL-60 cells (Figure 3A), indicating Raf-1 was highly phosphorylated by *A. phagocytophilum* infection as previously described (Li et al., 1991). This retard shift pattern was not observed before 36 h p.i. (Figure 3B), suggesting Raf-1 is only
actively phosphorylated in the mid-late exponential growth stage of *A. phagocytophilum* in HL-60 cells. This time-dependent phosphorylation pattern was correlated with ERK activation induced by *A. phagocytophilum* (Xiong et al., 2009). Additionally, the phosphorylation of Raf-1 was also abrogated by OTC treatment when added at 1 day p.i., similar to phosphorylated ERK1/2 (Figure 3C).

Raf-1 has multiple phosphorylation sites including both positive and negative regulatory sites, which play a key role in Raf-1 overall activation. Two sites whose phosphorylation have been shown to be necessary for activation are serine located at position 338 (S338) and the tyrosine located at position 341 (Y341). Another 3 serine residues (S43, S621, and S259) are negative regulatory phosphorylation sites for kinase activation (Kolch, 2000). Here we further examined the specific phosphorylation of Raf-1 in *A. phagocytophilum*-infected HL-60 cells using different phosphorylated Raf-1 antibodies. Our western blot analysis showed that one inhibitory phosphorylated site S259 and an active phosphorylated site S338 were highly phosphorylated by *A. phagocytophilum* infection, which may partially explain the “retard shift” of Raf-1 induced by *A. phagocytophilum* infection (Figure 3C). However, we could not see the phosphorylation of site Y340 in both *A. phagocytophilum* infected and control HL-60 cells.

* *A. phagocytophilum* infection does not alter the cellular localization of Sos 1 and Raf-1, and phosphorylated Raf-1 resides in the cytosol of host cells.*

To determine whether Ras activation is required for the ERK activation induced by *A. phagocytophilum*, we measured the levels of the active GTP-bound Ras
performing Ras-binding domain of Raf (RBD) protein beads pull-down assay and further investigated the membrane translocation of Sos 1, which is required for Ras activation, as well as Raf-1, another important player in ERK cascade activation, in A. phagocytophilum infected cells. However, we observed a slight increase (1.5-fold) of GTP-bound Ras in highly infected HL-60 cell (Figure 4A); there were no significant difference on the amount of total Ras proteins between A. phagocytophilum-infected and uninfected HL-60 cells, and the Ras was found in the membrane fraction in both groups indicated by western blot analysis after subcellular fractionation (Figure 4B and 4C). Unexpectedly, Sos 1 and Raf-1 were mostly found in the cytosol fraction of both infected and uninfected HL-60 cells (Figure 4B). Flotillin-1 and tubulin were used as membrane and cytosol fraction protein marker, respectively.

4.5 Discussion

ERK1/2 is activated by A. phagocytophilum in human peripheral blood neutrophils and HL-60 cells (Lee et al., 2008b; Xiong et al., 2009). However, it remains unknown how A. phagocytophilum modultes the ERK cascade signaling and what are the downstream of cellular responses. In this study, we for the first time showed that Raf-1 is actively phosphorylated in A. phagocytophilum-infected HL-60 cells and this phosphorylation may activate the ERK.

Raf/MEK/ERK signal transduction pathway is one of the mitogen-activated protein kinase (MAPK) pathways that govern cell proliferation, transformation, differentiation and cell survival. This pathway is employed by a wide variety of hormones, growth factors, as well as tumor-promoting substances (Yoon and Seger,
However, many DNA and RNA viruses are known to exploit this pathway for their replication (Pleschka, 2008). It has been reported that an increasing list of intracellular pathogens also induce ERK cellular signaling to benefit microbial survival (Rajalingam et al., 2008; Mimuro et al., 2007; Tapinos and Rambukkana, 2005; Su et al., 2004). Helicobacter pylori infection of the gastric epithelium increases anti-apoptotic protein MCL-1 expression via the MEK/ERK/SRE pathway to enhance cell survival and Helicobacter colonization (Mimuro et al., 2007). Tapinos and Rambukkana reported a PKCε-dependent, but not MEK-dependent pathway for ERK1/2 activation by Mycobacterium leprae resulting in continuous proliferation of infected human Schwann cells, without inducing transformation (Tapinos and Rambukkana, 2005).

Interestingly, activation of the host Ras-Raf-MEK-ERK-cPLA2 signaling cascade is required for chlamydial acquisition of host glycerophospholipids (Su et al., 2004). It remains to be elucidated why ERK activation is required for A. phagocytophilum infection. Our previous data demonstrated that A. phagocytophilum acquires cholesterol by up-regulating LDLR expression in the host cells and this increased LDLR transcript is abolished by MEK1/2 inhibitor U0126 (Xiong et al., 2009). However, whether ERK involves in LDLR up-regulation needs to be investigated. Moreover, A. phagocytophilum delays spontaneous neutrophil apoptosis by modulation of multiple apoptotic pathways (Ge and Rikihisa, 2006). Although one study showed that the initial activation of the p38 MAPK pathway is contributed to this delayed neutrophil apoptosis (Choi et al., 2005), the cell signaling mechanism on this remains to be clarified. Since a couple of apoptotic proteins and proteinases such
as MCL1, Bad, Bim, as well as caspase 9 are the known downstream substrates of ERK (Yoon and Seger, 2006), it is possible that ERK activation is involved in the delayed neutrophils apoptosis by *A. phagocytophilum*.

Another interesting issue is identification and characterization of bacterial components which triggers or intersects ERK signaling cascade. Although several intracellular bacteria are demonstrated to induce this ERK pathway, to our knowledge *H. pylori* CagA is the only known bacterial protein to induce ERK activation (Higashi *et al.*, 2004; Mimuro *et al.*, 2002). CagA is a type IV secretion system substrate of *H. pylori* and following translocation of CagA into gastric epithelial cells, CagA triggers cell morphological changes, which is important for the pathogenicity of *H. pylori* (Hatakeyama, 2006). Mimuro *et al.* showed that CagA can interact with Grb2 to activate Ras/MEK/ERK pathway and lead to cell scattering and proliferation (Mimuro *et al.*, 2002). Another study by Higashi *et al.* demonstrated that CagA binds to SHP-2 followed by tyrosine-phosphorylated by Src family kinases and this CagA-SHP-2 complex is involved in sustained ERK activation that is necessary for induction of cell morphological change by CagA, through the Rap1/B-Raf, but not Ras/Raf-1 pathway (Mimuro *et al.*, 2002). In both cases, CagA is regarded as a bacterial protein that mimics mammalian docking/scaffolding molecules, although it does not have any significant sequence homology with known mammalian proteins. Interestingly, similar to *H. pylori* CagA, AnkA, the first proven type IV secretion (T4S) system substrate of *A. phagocytophilum*, also contains EPIYA motifs at C-terminal and can be tyrosine-phosphorylated by host cell kinases upon translocation
into the host cell (JW et al., 2007; Lin et al., 2007). Ijdo et al. also showed that tyrosine-phosphorylated AnkA recruit SHP-1 during early infection of A. phagocytophilum (JW et al., 2007). AnkA is abundantly expressed in the host cells at mid-late infection stage and most secreted AnkA resides in cytosol of host cells (Lin et al., 2007). It is tempting to speculate that ankA is involved in the sustained ERK activation at middle-late infection stage induced by A. phagocytophilum. Another clue is possible recognition of AnkA with tyrosine 340 phosphorylated Raf-1 antibody and whether this phenomenon has biological meaning in ERK activation is under active investigation.
Figure 4.1 ERK activation by *A. phagocytophilum* infection is dependent on bacterial new protein synthesis

Oxytetracycline (OTC) was added in *A. phagocytophilum* infected HL-60 at 1 d p.i. (around 40% infected cells) and kept in the culture for additional 2 days. Cells were collected at day 2 and 3 p.i. and western blot analysis was performed using antibodies specific to either the phosphorylated or total ERK1/2, and *A. phagocytophilum* outer membrane protein P44. α-Tubulin was used as the protein loading control to normalize each sample. *Ap, A. phagocytophilum.*
Figure 4.1
Figure 4.2 *A. phagocytophilum* infection is blocked by Ras and Raf-1 inhibitors, but not by PKC inhibitors

(A) Pan PKC inhibitors Gö 6983 and bisindolylmaleimide I (Bis), Ras inhibitor manumycin A, and Raf-1 inhibitor sorafenib were added at 1 h p.i. at different dosages, and numbers of bacteria were determined on day 2 p.i. Data are expressed as mean±standard deviation (n = 3) and are representative of two independent experiments with similar results. *, P <0.05 (unpaired two-tailed t test); **, P <0.01 (unpaired two-tailed t-test). (B) *A. phagocytophilum*-infected HL-60 cells at 1 d p.i. were treated with bis (0.1 µM), manumycin A (1 µM), sorafenib (0.1 µM), and DMSO vehicle control, respectively. On day 2 p.i., infected cells were harvested and observed by light microscopy following Diff-Quik staining. Arrows indicate *A. phagocytophilum* inclusions. Note much smaller sizes of inclusions that contain much fewer numbers of bacteria and more vacuoles containing bacteria debris-like materials in manumycin A or sorafenib-treated cells, than those in untreated cells. All figures are shown at the same magnification. Bar, 5 µm. *Ap, A. phagocytophilum.*
Figure 4.2

A

![Graph showing data](image)

B

![Images showing cell morphology](image)
Figure 4.3 Raf-1 is highly phosphorylated by *A. phagocytophilum* infection at mid-late growth stage

(A) *A. phagocytophilum* highly infected (>95%) and uninfected HL-60 cells were collected and western blotting analysis was performed using anti-Raf-1 antibody. (B) Host cell-free *A. phagocytophilum* was inoculated into HL-60 cells and at indicated time points cells samples were harvested and subject to western blot analysis using anti-Raf-1 antibody. (C) Uninfected and highly infected HL-60 at day 3 p.i., as well as OTC treated HL-60 (as described in Figure 1 legend) cell lysates were prepared and western blotting was performed using anti-*A. phagocytophilum* P44, anti-Raf-1, anti-phospho-Raf-1(Ser259), anti-phospho-Raf-1(Ser338), anti-phospho-ERK1/2 antibodies. α-Tubulin was used as the protein loading control to normalize each sample. *Ap, A. phagocytophilum*. Ctl., control.
Figure 4.3

A

B

C

Ap - + - + +

Ctl. Ctl. OTC

Ap P44

Raf-1

Raf-1 pS259

Raf-1 pS338

pERK1/2

α-Tubulin
Figure 4.4 *A. phagocytophilum* infection does not alter the cellular localization of Sos 1 and Raf-1

(A) *A. phagocytophilum* highly infected (>95%) and uninfected HL-60 cells lysates were prepared and GTP-bound Ras was precipitated from the lysates with the agarose bead-immobilized GTP Ras-binding domain of Raf (RBD) and then subjected to western blotting analysis using anti-Ras antibody. The 293 cell lysate included in the kits serve as positive control and *A. phagocytophilum* highly infected HL-60 lysates loaded with GDP and GTPγS were used as loading negative and positive control, respectively. Lysates from *A. phagocytophilum* infected and uninfected HL-60 cells were subjected to western blotting analysis using anti-Ras, anti-*A. phagocytophilum* P44 and anti-α-Tubulin antibodies. (B) Cytosolic and membrane fractions in *A. phagocytophilum*-infected and uninfected HL-60 were prepared and western blotting were performed using anti-Ras, Sos 1, Raf-1, and anti-*A. phagocytophilum* P44. Flotillin-1 and α-Tubulin were used as membrane and cytosolic protein markers, respectively. Ap, *A. phagocytophilum*. Ctl., control.
Figure 4.4
CHAPTER 5

_Ehrlichia ewingii_ Infection Delays Spontaneous Neutrophil Apoptosis through Stabilization of Mitochondria

5.1 Abstract

The uncultivable obligate intracellular bacterium _Ehrlichia ewingii_, previously known only as a canine pathogen, is the most recently recognized human ehrlichiosis agent. _E. ewingii_ is the only _Ehrlichia_ sp. known to infect neutrophils. In the blood or in ex vivo culture, neutrophils generally have a short life span. In the present study, we investigated the effect of _E. ewingii_ infection on spontaneous apoptosis of neutrophils. _E. ewingii_ infection significantly delayed dog neutrophil apoptosis during ex vivo culture. The inhibitory effect on neutrophil apoptosis by _E. ewingii_ was reversible upon clearance of the organism. By using the fluorescent mitochondrial dyes Mitotracker Red 580 and JC-1, we found that _E. ewingii_ infection stabilized mitochondrial integrity by maintaining mitochondrial membrane potential in neutrophils. These results suggest that _E. ewingii_ delays spontaneous apoptosis of neutrophils via stabilization of host cell mitochondria.
5.2 Introduction

In 1999, *Ehrlichia ewingii* was recognized as a new human ehrlichiosis agent in the United States (Buller *et al.*, 1999). Between 1996 and 2001, approximately 10 confirmed cases of human granulocytic ehrlichiosis caused by *E. ewingii* infection were identified in Missouri and Oklahoma (Gusa *et al.*, 2001). Patients infected with *E. ewingii* experience symptoms such as fever, headache, myalgia, leukopenia, and thrombocytopenia. Many but not all of the individuals infected with *E. ewingii* were receiving immunosuppressive therapy at the time of infection (Buller *et al.*, 1999). Granulocyte-tropic Ehrlichia was first described in a dog from Arkansas in 1971, where it was considered a new strain of *Ehrlichia canis* (Ewing *et al.*, 1971). In 1992, it was identified as a separate *Ehrlichia* species based on 16S rRNA gene sequence differences from the two species most closely related, *E. canis* and *Ehrlichia chaffeensis*, and named *E. ewingii* in honor of Dr. S. A. Ewing (Anderson *et al.*, 1992). Since then, canine infection with *E. ewingii* has been detected by PCR based on the 16S rRNA gene sequence in Missouri, Oklahoma, North Carolina, and Virginia (Liddell *et al.*, 2003; Goldman *et al.*, 1998; Murphy *et al.*, 1998; Dawson *et al.*, 1996), and recently from Cameroon (Ndip *et al.*, 2005). Canine granulocytic ehrlichiosis is characterized by fever and anorexia accompanied by mild thrombocytopenia and mild anemia (Liddell *et al.*, 2003; Goldman *et al.*, 1998; Stockham *et al.*, 1990). Additionally, Stockham *et al.* (Stockham *et al.*, 1992) reported that arthritis or muscular stiffness is a common clinical sign in all dogs identified as having granulocytic ehrlichiosis based on the observation of morulae (intracytoplasmic inclusions of microcolonies of bacteria) in neutrophils and
eosinophils. *E. ewingii* is transmitted by tick bite, with the white-tailed deer (*Odocoileus virginianus*) being an important reservoir; the dog is another possible reservoir (Liddell *et al.*, 2003; Yabsley *et al.*, 2002).

Although related species such as *Anaplasma phagocytophilum* (formerly known as *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the human granulocytic ehrlichiosis agent), *E. chaffeensis*, and *E. canis* can be propagated in some mammalian and/or tick cell lines (Munderloh *et al.*, 2004; Munderloh *et al.*, 1999; Goodman *et al.*, 1996; Dawson *et al.*, 1991b; Dawson *et al.*, 1991a). To date, *E. ewingii* has not been successfully isolated and propagated in any cell culture systems. Consequently, very little is known about the *E. ewingii* bacterium and its pathogenesis to the host.

Neutrophils are essential for the innate immune response against invading microorganisms and are involved in initiation and execution of the acute inflammatory response (Kobayashi *et al.*, 2003). Neutrophils typically undergo apoptosis within 6-12 h after their release into the peripheral blood from the bone marrow (Akgul *et al.*, 2001). Although apoptosis is an intrinsic cell process, it is modulated by many environmental signals. Recently, an increasing number of microbial pathogens have been reported to modulate apoptosis of neutrophils and macrophages in vitro (DeLeo, 2004). Some microbial pathogens induce apoptosis of infected neutrophils and are subsequently removed by macrophages, to the host’s benefit (DeLeo, 2004). In contrast, inhibition of apoptosis of host cells in vitro by intracellular pathogens such as *A. phagocytophilum* (Scaife *et al.*, 2003; Yoshiie *et al.*, 2000), *Chlamydophila pneumoniae* (van Zandbergen *et al.*, 2004), and *Leishmania major* (Aga *et al.*, 2002) is generally advantageous to the pathogens by extending the
life of the host cells to create a favorable niche for the pathogen’s intracellular survival and proliferation. Among several bacteria that can delay apoptosis of neutrophils, only two bacteria, *A. phagocytophilum* and *C. pneumoniae*, have been shown to replicate within neutrophils (*DeLeo, 2004*). However, most studies on the effects of bacteria on neutrophil apoptosis were performed in cell culture systems, except for one study with *A. phagocytophilum*-infected sheep (*Scaife et al., 2003*). As such, whether in vivo infection with these bacteria delays apoptosis of host neutrophils remains largely unknown.

Here, we found that neutrophils taken from a dog naturally infected with *E. ewingii* were alive even after five days in cell culture. We thus hypothesized that *E. ewingii* infection delays or inhibits neutrophil apoptosis in vivo. The present study established a laboratory *E. ewingii* infection model in dogs, and investigated the effect of *E. ewingii* infection on apoptosis of canine neutrophils ex vivo. Given the importance of the mitochondrial pathway in neutrophil apoptosis (*Ge et al., 2005; Maianski et al., 2004; Fossati et al., 2003*), we measured the mitochondrial membrane potential of canine neutrophils, as well as expression levels of some anti- and pro-apoptotic genes, and the activity of caspase 3, the main downstream effector of the intracellular apoptosis signaling cascade.

### 5.3 Materials and Methods

*Infection of dogs with *E. ewingii*.* To identify dogs naturally infected with *E. ewingii*, 112 EDTA-treated canine blood samples shipped from the Sinclair Research Center animal laboratory (Hatton, MO), were screened by nested PCR using the
Anaplasmataceae family-specific primers ECC and ECB as the outside primers (Dawson *et al.*, 1994), and newly designed *E. ewingii* 16S rRNA gene-specific primers, EW1, 5’-cgaacaattcctaatgctctgc-3’, and EW2, 5’-cattatcttctctgtagtgaagc-3’, as inside primers. Dogs referred to the Ohio State University (OSU) College of Veterinary Medicine Teaching Hospital, Columbus, OH, for a suspected *E. ewingii* infection were also tested by PCR followed by sequencing of the PCR products. A single-step PCR for the *A. phagocytophilum* p44 gene (Zhi *et al.*, 1999) and a nested PCR specific for *E. chaffeensis* and *E. canis* using primers ECC and ECB as outside primers, and *E. chaffeensis*-specific HE-1 and HE-3 (Anderson *et al.*, 1992), and *E. canis*-specific ECA and HE-3 (Wen *et al.*, 1997) as inside primers were performed to exclude specimens infected with these ehrlichial agents. *E. ewingii*–positive dog blood samples were subsequently used for experimental laboratory dog inoculations.

*E. ewingii*–positive dog blood samples #21689, 22105, 22127, 22392, and 22393 were used for experimental dog inoculation. Specific pathogen-free (SPF) beagle dogs (Battelle, OH) were used as the recipients in the experimental inoculations and were first screened for *Ehrlichia* or *Anaplasma* sp. infection by PCR or nested PCR as described above before use in this study. Approximately 10 ml heparinized whole blood or buffy coat was inoculated intravenously into five recipient dogs: 2185 (female, 7.5 kg), 2119 (male, 9 kg), 2405 (male, 14 kg), DYI-5 (female, 9 kg) and CWTBK (female, 10 kg). On the same or one day prior to the inoculation date, all dogs received one treatment of cyclophosphamide (Cytoxan, Baxter Healthcare Corporation, IL), 10 mg/kg body weight, i.v. (Reardon and Pierce, 1981). The dogs were then monitored for clinical and hematological changes. After *E. ewingii*
infection was established for ~3 months, dog 2185 was treated with the antibiotic doxycycline orally, 50 mg/kg body weight, for one month. Blood samples (~3 ml) were taken into EDTA-coated tubes weekly to determine *E. ewingii* infection using nested PCR. After confirmation of infection, larger volumes (~20 ml) of blood specimens were collected at the time points indicated in the text and used to prepare neutrophils for apoptosis assays, as described below. Three other uninfected SPF dogs kept under the same conditions were used as controls. Animal experimentation protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Ohio State University.

**Assessment of apoptosis of peripheral blood neutrophils.** Neutrophils were isolated from the peripheral blood and cultured as described elsewhere (Yoshiie et al., 2000). Apoptotic cells were determined at 0, 4, 12, 16 and 24 h post-culture ex vivo based on their morphology, including densely condensed and homogeneous nuclei, and loss of connection between the lobules of nuclei as previously described (Yoshiie et al., 2000).

**Mitochondrial staining and flow cytometric analysis of mitochondrial membrane potential.** Neutrophils from *E. ewingii*-infected and control dogs cultured for 0 and 13 h were stained with Mitotracker Red 580 (Molecular Probes, Eugene, OR) as previously described (Ge et al., 2005). Neutrophils from *E. ewingii*-infected and control dogs cultured for 0 and 10 h were stained with 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolycarbocyanine iodide (JC-1) for flow cytometric analysis as described previously (Ge et al., 2005). As a control for the loss of mitochondrial membrane potential, carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP)
(Sigma, St. Louis, MO) was added to the freshly isolated neutrophils at 20 nM for 15 min at 37°C. Fluorescence emission was collected with a Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA) using a 525 nm band pass filter for JC-1 monomers (green fluorescence).

**Caspase 3 activity assay.** Caspase 3 activity was measured using the luminescent Caspase-Glo 3/7 Assay kit (Promega, Madison, WI) in accordance with the manufacturer’s instructions.

**RNA isolation and reverse transcription (RT)-PCR.** At 0, 4, 8, 12 h post-culture, a total of $1 \times 10^7$ neutrophils were harvested and stabilized in RNaLater™ (Qiagen, Valencia, CA). Total RNA was extracted and reverse transcribed as previously described (Xiong *et al.*, 2007). Selected anti-and pro-apoptotic genes were amplified using specific oligonucleotide primers (Table 1). The thermal cycling numbers for *mcl-1*, *bcl-xL*, *bcl-2*, *bax* and *g3pdh* were 25, 28, 35, 30 and 23, respectively within the linear amplification range. Canine glyceraldehyde-3-phosphate dehydrogenase (*g3pdh*) gene was used to normalize the amount of input mRNA among different samples.

**Statistical analysis.** Statistical analyses were performed by using analysis of variance or Student’s *t*-test. $P < 0.05$ was considered to be significant.

### 5.4 Results

**E. ewingii infection significantly extends neutrophil survival ex vivo.** An 8-week-old intact male German shepherd mixed breed puppy was hospitalized with fever and lameness at the OSU veterinary teaching hospital in May 2005. Morulae
were clearly observed in the cytoplasm of neutrophils from the blood and joint fluid under microscopic examination after Diff-Quik staining (Figure 1A and B). PCR and DNA sequencing confirmed that the dog was infected with *E. ewingii*. PCR tests for *E. canis, E. chaffeensis,* and *A. phagocytophilum* were negative. After 5 days of ex vivo culture, blood neutrophils containing *E. ewingii* morulae were still alive (Figure 1C), whereas neutrophils from uninfected dogs had died out through apoptosis by this time. This finding strongly suggests that in vivo infection with *E. ewingii* delays spontaneous apoptosis of dog neutrophils.

**Experimental infection of dogs with *E. ewingii***. To investigate the effect of *E. ewingii* on neutrophil apoptosis under controlled conditions, we first established a laboratory canine infection model. Stockhalm *et al.* (Stockham *et al.*, 1990) reported the transmission of canine granulocytic ehrlichial organisms from an infected dog to two male, 10-month-old dogs by an intravenous injection of whole blood, as evidenced by the transient observation of Ehrlichial morulae in blood neutrophils of recipient dogs after inoculation. We attempted to establish an *E. ewingii* infection in two laboratory dogs without prior immunosuppressive treatment with the drug cyclophosphamide, but were unable to do so as determined by nested PCR analysis up to 4 weeks post-inoculation. All five dogs treated once with cyclophosphamide were successfully infected with *E. ewingii* by 2 or 3 weeks post-inoculation as determined by PCR (Figure 2). Among the infected recipient dogs, CTWBKT, DYI-5 and 2119 were PCR positive for 21, 90 and 189 days, respectively, at which points the infection were spontaneously cleared. Dog 2185 was PCR positive for 98 days. Subsequent doxycycline treatment for one week starting at day 98 cleared the
infection. Dogs 2405 maintained the PCR-positive status for 105 days until the experiment was terminated. These data show that persistent *E. ewingii* infection can be established in laboratory dogs. However, no obvious clinical signs were observed in any of the dogs during the study. Furthermore, typical ehrlichiosis “morulae” in the cytoplasm of granulocytes were not detected in blood smears from these dogs.

*Morphological assessment of apoptosis.* The neutrophils analyzed in these studies were obtained from the dogs during the persistent infection stage, between 80-100 days after the blood specimen became *E. ewingii* PCR–positive. Neutrophils from both infected and control dogs underwent spontaneous apoptosis after ex vivo culture (Figure 3A). Apoptotic neutrophils had condensed nuclear lobules without connections. The percentage of apoptotic neutrophils by this criterion rapidly increased in samples from uninfected dogs after 8 h of incubation. In contrast, this apoptosis process was much slower in neutrophils derived from *E. ewingii*-infected dogs. The percentage of apoptotic neutrophils from infected dogs was significantly lower (*P* < 0.05) than that from uninfected dogs at 8, 12, and 16 h post-culture, suggesting that neutrophil apoptosis was delayed by in vivo *E. ewingii* infection (Figure 3B). Additionally, we compared the percentages of apoptotic neutrophils before and after *E. ewingii* infection was established, as well as between neutrophils collected during infection and after the bacteria were cleared spontaneously (dogs DYI-5 and 2119) or by doxycycline treatment (dog 2185). We found that inhibition of neutrophil apoptosis was eliminated in dogs DYI-5 and 2119 one week after *E. ewingii* was spontaneously cleared and in dog 2185 one week after *E. ewingii* was cleared by doxycycline (Figure 3C), indicating that the inhibition of neutrophil
apoptosis by in vivo *E. ewingii* infection is a reversible phenomenon. Clearance of *E. ewingii* were confirmed by weekly monitoring the blood specimens from these dogs by nested PCR up to three months.

**Neutrophils derived from *E. ewingii*-infected dogs maintain a high level of mitochondrial membrane potential.** The mitochondrial network regulates spontaneous neutrophil apoptosis via modulation of mitochondrial membrane integrity, which can be detected by measuring the membrane potential (Maianski *et al.*, 2004; Fossati *et al.*, 2003). Freshly isolated neutrophils from both control and infected dogs showed high mitochondrial membrane potential, as indicated by the strong granular red mitochondrial staining with Mitotracker Red 580 dye throughout the cytosol (Figure 4A). After 13 h incubation, neutrophils derived from uninfected dogs lost mitochondrial membrane potential, as shown by weak fluorescence staining (Figure 4B). In contrast, neutrophils derived from *E. ewingii*-infected dogs still maintained a high level of mitochondrial membrane potential at 13 h (Figure 4C). The substantial difference in Mitotracker Red 580 staining intensity between *E. ewingii*-infected and uninfected neutrophils suggests that in vivo *E. ewingii* infection inhibits the loss of neutrophil mitochondrial membrane potential associated with spontaneous apoptosis.

To confirm the data obtained with the Mitotracker Red 580 stain, we next treated the cells with JC-1, a fluorescent carbocyanine dye that is used as a mitochondrial membrane potential probe. When cells were treated with JC-1 (Reers *et al.*, 1991), mitochondria in freshly isolated neutrophils from both control and infected dogs were demonstrated to have high membrane potential (i.e. low green fluorescence;
Figure 5A and C). As a positive control, treatment with FCCP, a protonophore and uncoupler of oxidative phosphorylation in mitochondria, resulted in the loss of mitochondrial membrane potential (i.e. high green fluorescence; Figure 5E). After 10 h incubation, most of the neutrophils derived from the uninfected dogs showed a loss of mitochondrial membrane potential (Figure 5B), whereas neutrophils derived from *E. ewingii*-infected dogs maintained high levels of mitochondrial membrane potential (Figure 5D). Thus, both the microscopic data of mitochondrial staining using Mitotracker Red 580 and flow cytometric analysis using JC-1 indicated that in vivo *E. ewingii* infection delays the loss of mitochondrial membrane potential associated with spontaneous apoptosis in neutrophils.

**Caspase 3 activity and mRNA expression of selected members of the canine bcl-2 family.** Caspase 3 is the primary executioner caspase involved in apoptosis of human neutrophils (Pongracz *et al.*, 1999). Although there are few reports of canine caspases, canine caspase 3 was shown to have a similar function to human caspase 3 during apoptosis in neutrophils and in tumor tissues in dogs (Kumaraguruparan *et al.*, 2006; Sano *et al.*, 2004). Therefore, we examined the relationship between caspase 3 activity and inhibition of apoptosis of neutrophils from dogs infected with *E. ewingii*. Compared with neutrophils from uninfected dogs, caspase 3 activity in infected neutrophils was significantly lower (*P* < 0.05) beginning at 12 h post-culture (Figure 6).

The bcl-2 protein family regulates the downstream caspase cascade via modulation of mitochondrial membrane permeability. Some Bcl-2 family members are themselves regulated at the transcriptional level during apoptosis (Cory and Adams,
Inhibition of bfl-1 in human neutrophils infected with *A. phagocytophilum* in vitro has been reported (Ge *et al.*, 2005). So far only a few bcl-2 family members have been reported to be associated with neutrophil apoptosis in dogs (Oguma *et al.*, 2006; Oguma *et al.*, 2005; Sano *et al.*, 2005). Thus, using RT-PCR with primers specific for the dog bcl-2 family members *bcl-2, bcl-xL,* and *mcl-1* (anti-apoptotic), and *bax* (proapoptotic), we compared expression levels of these genes in neutrophils derived from *E. ewingii*-infected and control dogs after 0, 4, 8, and 12 h of culture and in neutrophils before and after *E. ewingii* infection. Transcripts of all four genes were detected in canine neutrophils during ex vivo culture. However, we could not detect any consistent differences between infected and uninfected dog neutrophils (data not shown). The result suggests these four bcl-2 family members are not involved, at least at the transcriptional level, in the inhibition of neutrophil apoptosis by *E. ewingii* infection.

**5.5 Discussion**

In this study we showed that in vivo infection of the obligatory intracellular bacterium *E. ewingii* delays spontaneous apoptosis in host canine neutrophils. While it is difficult to study apoptosis rates of human neutrophils infected in vivo with *E. ewingii*, it is expected that human neutrophils also survive longer upon infection with *E. ewingii*. Among the several known *Ehrlichia* spp, *E. ewingii* is the first *Ehrlichia* species reported to delay neutrophil apoptosis ex vivo. *E. ewingii* has been seen so far in blood smears in infected human patients and dogs. The present study showed that infected neutrophils can survive in cell culture up to 5 days and *E.*
Ewingii can continue to replicate in neutrophils. Altering neutrophil apoptosis has profound effects on the inflammatory response and resolution of infection (DeLeo, 2004). Similar to A. phagocytophilum’s effect in sheep (Scaife et al., 2003), this process of delayed neutrophil apoptosis following E. ewingii infection in vivo may an important pathological mechanism that prolongs infection and inflammation in the mammalian host. Other Ehrlichia species such as E. chaffeensis and E. canis infect monocytes/macrophages, and Ehrlichia ruminantium infects endothelial cells (Dumler, 2005b). While there was no report on apoptosis modulation of host cells by these Ehrlichia species, using human acute leukemia cell line THP-1 infected with E. chaffeensis in vitro, Zhang et al. (Zhang et al., 2004) reported that E. chaffeensis up-regulates apoptosis inhibitors, and differentially regulates cell cyclins and CDK mRNA expression. Although this mechanism is different from mitochondria-mediated inhibition of apoptosis in E. ewingii infection, it is possible for Ehrlichia species to inhibit respective host cell apoptosis through multiple signaling pathways as A. phagocytophilum does on human neutrophils (Ge and Rikihisa, 2006).

While the toxicities of cyclophosphamide is one of major concerns for its use in treatment of cancers and autoimmune diseases such as systemic lupus erythematosus, and rheumatoid arthritis (Marder and McCune, 2007), the effects of cyclophosphamide on neutrophil apoptosis is not known well. In our study, all dogs were treated only once with cyclophosphamide, and neutrophils were obtained from these dogs more than 3 months after the cyclophosphamide treatment. Importantly, the fact that the delayed apoptosis was reversible upon removal of E. ewingii by
doxycycline treatment or spontaneous clearance indicates the residual effects of this cyclophosphamide treatment was negligible.

In the first cell biological assessment of *E. ewingii*-infected host cells, we found that in vivo *E. ewingii* infection delays spontaneous apoptosis in host canine neutrophils by stabilizing mitochondrial membrane potential. Inhibitory mechanisms of neutrophil apoptosis by several bacteria are under intensive investigation but still remain poorly understood. Several factors produced and/or secreted by bacteria have been reported to delay host cell apoptosis, including lipopolysaccharide (LPS), N-formyl-methionyl-leucyl-phenylalanine (fMLP), CpG DNA (Jozsef et al., 2004), peptidoglycan (Into and Shibata, 2005), lipoprotein (Power et al., 2004), water-soluble proteins of *Helicobacter pylori* (Kim et al., 2001), and Wolbachia surface protein (Bazzocchi et al., 2007). *A. phagocytophilum* delays human neutrophil apoptosis during in vitro infection by modulating multiple apoptotic pathways, including stabilizing mitochondrial membrane potential, stabilizing *bfl-1* transcripts, and inhibiting spontaneous Fas clustering, Bax translocation to mitochondria, and activation of caspase 9 (Ge and Rikihisa, 2006; Ge et al., 2005); however, the detailed inhibitory mechanism still awaits investigation. Because *E. ewingii* has thus far been uncultivable, currently it is not feasible to identify bacterial component(s) that are involved in the inhibition of neutrophil apoptosis in *E. ewingii*-infected host cells. However, *A. phagocytophilum* and *E. ewingii* belong to the family *Anaplasmataceae* and their similarity is ~92.4% based on 16S rRNA sequence comparison (Anderson et al., 1992). Considering the similar inhibitory effects on host neutrophil apoptosis in both bacterial infections, comparative genomic and proteomic studies between
Anaplasma and Ehrlichia spp. may shed light on the inhibitory mechanism of E. ewingii infection in neutrophils.

Compared with other organisms, E. ewingii seems to have a very effective and powerful ability to delay dog neutrophil apoptosis as indicated by the fact that although the blood specimens were positive for E. ewingii by nested PCR, no organism was detected under the light microscope in neutrophils from the laboratory infected dogs during this whole study, suggesting a low level of neutrophil infection. However, our data show that the delay of dog neutrophil apoptosis is obviously dependent on active E. ewingii infection.

Similar to other immunosuppressive drugs, cyclophosphamide produces lymphopenia in human patients, making them susceptible to opportunistic microbial infections (Mackall et al., 1994). Although cyclophosphamide was reported not to affect E. canis infection of dogs (Reardon and Pierce, 1981), the present study showed that cyclophosphamide pretreatment was necessary in establishing E. ewingii infection lasting over approximately three months. E. ewingii infections in humans are often associated with immunosuppressive therapy (Gusa et al., 2001). Taken together, even transient immunosuppression may pose a risk for E. ewingii infection to dogs and humans. In agreement with the study by Stockham et al. (Stockham et al., 1990) that shows absence of physical or behavioral abnormalities other than a mild transient pyrexia, clinical signs were not evident in the experimentally infected dogs in the present study, suggesting that these E. ewingii strains are not highly pathogenic in dogs. As these strains were directly derived from naturally infected dogs with no associated clinical signs, subclinical infection of dogs with E. ewingii may be more
common than generally assumed (this study; (Stockham et al., 1990)). As even subclinical infection alters neutrophil apoptosis, it is possible that E. ewingii infection may influence or exacerbate the manifestation of other diseases in infected dogs. Thus the importance of E. ewingii infection in overall human and canine health and the potential risk to underlying or concurrent diseases in humans and dogs need to be evaluated.
<table>
<thead>
<tr>
<th>Target genes</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Orientation</th>
<th>Position</th>
<th>Target Size(bp)</th>
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<td>GCTGGGATGCTTTTGTGGAACTG</td>
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<td>297</td>
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<tr>
<td></td>
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<tr>
<td>Bcl-xl</td>
<td>TCACATCACCCCCAGGGACAGCATA</td>
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<td>741-764</td>
<td>239</td>
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<td></td>
<td>CAAAAGTATCCAGCCGCGTTCT</td>
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<tr>
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<td>977-955</td>
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NOTE. G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 5.1 Oligonucleotide primers used for reverse transcription polymerase chain reaction (RT-PCR) of canine Bcl-2 family members
Figure 5.1 Delayed apoptosis of neutrophils from a naturally occurring *E.

*ewingii*–infected dog. Typical morulae were clearly observed in the cytoplasm of neutrophils from freshly collected blood (A) and joint fluid (B) following Diff-Quik staining. After 5 days ex vivo culture, neutrophils containing *E. ewingii* morulae were still alive (C). Black arrows indicate *E. ewingii* morulae. The scale bar is 5 µm.
Figure 5.1
Figure 5.2 Detection of *E. ewingii* DNA by nested polymerase chain reaction (PCR) of total DNA from blood specimens of experimentally infected dogs. Lane M, 1 kb Plus DNA Ladder (Invitrogen). Lane 1, Positive control (DNA sequence confirmed sample shown in Figure1); lane 2, no DNA template (negative control); Lane 3, dog 2185 after 98 days PCR positive; lane 4, dog 2185 after 7 days doxycycline treatment; Lane 5, 2405 after 35 days PCR positive; Lane 6, 2119 after 130 days PCR positive; lane 7, dog DYI-5 after 70 days PCR positive; lane 8, dog CWTBKQT after 21 days PCR positive. The expected size of the PCR products is 383 bp.
Figure 5.3 Time course of the morphological indication of apoptosis of canine peripheral blood neutrophils. (A) Neutrophils were harvested at the indicated time points after incubation at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum. Peripheral blood neutrophils from control and *E. ewingii*-infected dogs were harvested by cytocentrifugation. The scale bar is 5 µm. (B) The numbers of apoptotic neutrophils from *E. ewingii*-infected and uninfected dogs were quantified and the data are shown as bar graphs. Data presented are representative of at least four independent experiments with 4 infected and 3 control dogs. (C) The percentage of apoptotic neutrophils after 12 h ex vivo culture were quantified for three control, and three *E. ewingii*-infected dogs (2119, DY1-5, and 2185) prior to clearance and one week after clearance spontaneously (dog 2119 and DY1-5) or by doxycycline treatment (dog 2185). Data are the means ± SD values from at least three different dogs in each group. *P < 0.05; ** P < 0.001; NS, not significant.
Figure 5.3

A

Control

Infected

B

C

Apoptotic neutrophils [%]

Apoptotic neutrophils [%]

Incubation time (h)

Control

Infected

Cleared infection

* p < 0.05

** p < 0.01

*** p < 0.001
Figure 5.4 Inhibition of the loss of mitochondrial Mitotracker Red 580 staining in canine neutrophils infected with *E. ewingii*. (A) Freshly isolated neutrophils from an uninfected dog showed strong mitochondrial staining. After 13 h culture ex vivo, (B) neutrophils from a control dog showed weak mitochondrial staining, whereas (C) mitochondria in neutrophils from an infected dog retained strong red fluorescence. Data presented are representative of three experiments. The scale bar is 5 µm.
Figure 5.4
Figure 5.5 Inhibition of the loss of mitochondrial membrane potential in *E. ewingii*-infected canine neutrophils measured by flow cytometry of 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) staining. Freshly isolated neutrophils from a control (A) and an infected (C) dog showed high mitochondrial membrane potential at 0 h culture. Freshly isolated neutrophils treated with carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) at 37°C for 15 min are shown as a positive control (E). Neutrophils from a control (uninfected) dog (B) and an infected dog (D) after 10 h of incubation. Data presented are representative of two independent experiments.
Figure 5.5

Loss of mitochondrial membrane potential (JC-1 green fluorescence)
Figure 5.6 Inhibition of caspase 3 activity in *E. ewingii*-infected canine neutrophils. Caspase 3 activity of neutrophils from the same dog before and after *E. ewingii* infection assayed at indicated time points in vitro (A). Caspase 3 activity of neutrophils from the control and *E. ewingii*-infected dogs assayed at the same time at indicated time points in vitro (B). The luminescence results using a Caspase-Glo 3/7 Assay kit are expressed in relative light units (RLUs). Data are the means ± SD values (N=3). *P < 0.05;** P < 0.001.
Figure 5.6

A

Caspase 3 activity (RLU)

Time (h)

Pre-infection
Post-infection

B

Caspase 3 activity (RLU)

Time (h)

Control
Infected

**
SUMMARY

*Anaplasma phagocytophilum* is an obligatory intracellular bacterium that infects granulocytes and causes human granulocytic anaplasmosis (HGA). One of unique characteristics for *A. phagocytophilum* is existence of cholesterol and lack of LPS and peptidoglycan. Our studies demonstrate that high blood cholesterol facilitates *A. phagocytophilum* infection in mice using Apo E genetic deficiency and high cholesterol diet intake. These data reveal a novel aspect of host requirement for *A. phagocytophilum* pathogenesis and also propose high blood cholesterol level as a risk factor related to older age-association of HGA.

In eukaryotes, intracellular cholesterol homeostasis and trafficking are tightly regulated. Our data suggest that *A. phagocytophilum* dramatically upshift and alter the cellular cholesterol homeostasis and distribution. Further, we demonstrate that *A. phagocytophilum* acquire requires cholesterol derived from low-density lipoprotein (LDL), rather de novo cholesterol biosynthesis. To do so, *A. phagocytophilum* greatly up-regates LDL receptor expression by stabilizing LDLR mRNA via its 3’ UTR. These studies improve our understanding of how intracellular bacteria exploit the host cholesterol homeostasis mechanism and regulatory pathway. To our knowledge, this is the first report on modulation of LDLR mRNA stability by infectious agent.
Our studies also have important implication for epidemiology and public health because we found that statin treatment enhances *A. phagocytophilum* growth in host cells. These results suggest that critical and careful consideration is required when treating granulocytic anaplasmosis patients, as statins are currently widely used to treat hypercholesterolemia in humans by lowering blood cholesterol levels.

*Ehrlichia ewingii* is the most recently recognized human ehrlichiosis agent in US and it is the only *Ehrlichia* sp. known to infect neutrophils. We established the canine *E. ewingii* laboratory infection model and found that *E. ewingii* delays dog neutrophil spontaneous apoptosis ex vivo through stabilization of host cell mitochondria. This is the first cell biological study for *E. ewingii*. 


