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Molecular Events Involved in Cellular Invasion by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*

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Abstract

Ehrlichia chaffeensis and *Anaplasma phagocytophilum* are obligatory intracellular bacteria that preferentially replicate inside leukocytes by utilizing biological compounds and processes of these primary host defensive cells. These bacteria incorporate cholesterol from the host for their survival. Upon interaction with host monocytes and granulocytes, respectively, these bacteria usurp the lipid raft domain containing GPI-anchored protein to induce a series of signaling events that result in internalization of the bacteria. Monocytes and neutrophils usually kill invading microorganisms by fusion of the phagosomes containing the bacteria with granules containing both antimicrobial peptides and lysosomal hydrolytic enzymes and/or through sequestering vital nutrients. However, *E. chaffeensis* and *A. phagocytophilum* alter vesicular traffic to create a unique intracellular membrane-bound compartment that allows their replication in seclusion from lysosomal killing. These bacteria are quite sensitive to reactive oxygen species (ROS), so in order to survive in host cells that are primary mediators of ROS-induced killing, they inhibit activation of NADPH oxidase and assembly of this enzyme in their inclusion compartments. Moreover, host phagocyte activation and differentiation, apoptosis, and IFN- γ signaling pathways are inhibited by these bacteria. Through reductive evolution, lipopolysaccharide and peptidoglycan that activate the innate immune response, have been eliminated from these gram-negative bacteria at the genomic level. Upon interaction with new host cells, bacterial genes encoding the type IV secretion apparatus and the two-component regulatory system are upregulated to sense and adapt to the host environment. Thus dynamic signal transduction events concurrently proceed both in the host cells and in the invading *E. chaffeensis* and *A. phagocytophilum* bacteria for successful establishment of intracellular infection. Several bacterial surface exposed proteins and porins are recently identified. Further functional studies on *Ehrlichia* and *Anaplasma* effector or ligand molecules and cognate host cell receptors will undoubtedly advance our understanding of the complex interplay between obligatory intracellular pathogens and their hosts. Such data can be applied towards treatment, diagnosis, and control of ehrlichiosis and anaplasmosis.

Keywords

Ehrlichia; Anaplasma; cellular invasion; signaling

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Conflict of interest

None declared.

1. Introduction

The family Anaplasmataceae contains pathogenic obligatory intracellular bacteria adept at targeting cells of hematopoietic origin in mammals and birds (Rikihisa, 2003a). The infection of humans causes human ehrlichiosis, a significant emerging infectious disease that was designated as a notifiable disease by the Centers for Disease Control and Prevention (CDC) in 1998 (Gardner et al., 2003). Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, a monocytotropic ehrlichia (Dawson et al., 1991), was discovered in 1986 (Maeda et al., 1987). Human granulocytic ehrlichiosis (HGE) was first reported in 1994 (Chen et al., 1994). The HGE agent initially isolated in 1995 (Goodman et al., 1996) was later reclassified with other related *Ehrlichia* spp. to *Anaplasma phagocytophilum* (Dumler et al., 2001). Therefore, a new name for the disease, human granulocytic anaplasmosis (HGA), has been adopted recently. Ehrlichiosis is an under-reported disease, because many physicians are still unaware of the disease and many states do not have a system for surveillance.

HME is a systemic disease characterized by fever, headache, myalgia, anorexia, and chills, and is frequently accompanied by leukopenia, thrombocytopenia, anemia, and elevations in serum hepatic aminotransferases. The severity of the disease varies from asymptomatic seroconversion to death, and severe morbidity is frequently documented (Paddock and Childs, 2003). HME cases are reported primarily in the southeastern and south central regions of the U.S.A. HME has also been reported in Europe, as well as in Mexico, Argentina, Mali, and Israel. *E. chaffeensis* has been most commonly identified in the Lone Star tick (*Amblyomma americanum*) (Anderson et al., 1993), and white-tailed deer are considered to be the major reservoir of *E. chaffeensis* (Ewing et al., 1995).

HGA is characterized by clinical signs and laboratory findings similar to those of HME (Dumler et al., 2007). Cases of HGA have been confirmed primarily in the upper Midwest, and northeastern and Pacific states in the U.S. HGA has been reported in Europe more frequently than HME (Oteo and Brouqui, 2005). *A. phagocytophilum* has been found in the black-legged tick (*Ixodes scapularis*) and other *Ixodes* sp. Wild rodents, such as white-footed mice (*Peromyscus leucopus*), are considered to be the major reservoir of the HGA agent in northeastern USA (Telford et al., 1996). No vaccines exist for any of the human ehrlichioses and the diagnoses are based on retrospective seroconversions or PCR analysis. Although doxycycline is generally effective in treating ehrlichioses, delayed initiation of therapy, the presence of underlying illness, advanced age, and immunosuppression often lead to severe complications or death.

This review primarily describes our current understanding of the molecular events during *E. chaffeensis* and *A. phagocytophilum* invasion of vertebrate host cells. In conjunction with these data, focused use of the *E. chaffeensis* and *A. phagocytophilum* whole genome sequence database will be discussed.

2. Unusual cell wall: lack of LPS and peptidoglycan

E. chaffeensis and *A. phagocytophilum* are gram-negative small cocci that infect monocytes-macrophages and granulocytes, respectively, cells equipped with powerful innate antimicrobial defenses (Rikihisa, 2003b). *A. phagocytophilum* also infects bone marrow progenitors and endothelial cells (Herron et al., 2005; Klein et al., 1997). Monocytes-macrophages and neutrophils express pattern recognition surface receptors, such as Toll-like receptors and nucleotide-binding oligomerization domain (NOD) containing intracellular protein receptors, that can recognize and bind to conserved pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) and peptidoglycan. Such binding elicits profound innate immune responses that generally eliminate most invading microorganisms from the body.

Several pathogens have developed strategies that avoid activation of pattern recognition receptors (Portnoy, 2005). *E. chaffeensis* and *A. phagocytophilum* have evolved a novel strategy: both have lost all genes for the biosynthesis of LPS and most genes for the biosynthesis of peptidoglycan (Lin and Rikihisa, 2003a) and thus these pathogens do not trigger an effective innate immune response. *Ehrlichia* and *Anaplasma* spp. survive and replicate inside cells of midgut and salivary gland of ixodid tick vectors. Cells of invertebrates also have a strong innate defensive mechanism responsive to PAMPs (Little et al., 2005). Therefore, loss of genes for the biosynthesis of LPS and peptidoglycan facilitated ehrlichial adaptation to cells of the tick vector as well. Loss of peptidoglycan provides additional benefits: ehrlichiae have the flexibility to survive in the limited intra-vacuolar space and the plasticity required for intravascular circulation of infected leukocytes. Lack of these genes explains the unusual ultrastructure of this group of bacteria noted previously (Rikihisa, 1991; Rikihisa et al., 1997): they are pleomorphic and enveloped within a rippled, thin outer membrane with a narrow periplasmic space, and has no sign of a capsule layer.

3. Hijacking host cholesterol for survival

E. chaffeensis and *A. phagocytophilum* have evolved another unique feature, the ability to take up cholesterol from the host (Lin and Rikihisa, 2003a). These bacteria were demonstrated to contain significant levels of membrane cholesterol by use of freeze-fracture techniques, fluorescence microscopy, and biochemical analysis (Lin and Rikihisa, 2003a). This study further showed that *E. chaffeensis* and *A. phagocytophilum* require cholesterol for survival. Treatment of the bacteria with a cholesterol-extraction reagent, methyl- β -cyclodextrin (M β CD), caused ultrastructural changes. Pretreatment of *E. chaffeensis* and *A. phagocytophilum* with M β CD or a fluorescent cholesterol derivative, NBD-cholesterol, rendered the bacteria incapable of infecting host cells, and thus killed them (Lin and Rikihisa, 2003a). The dependency of these bacteria on cholesterol for infection and survival in vitro suggests that increased blood cholesterol levels may enhance the severity of infection in mammals. In fact, 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 in mice (Xiong et al., 2007), which may contribute to HGA pathogenesis.

4. Molecular events that occur during internalization and creation of a replicative compartment

The binding of *E. chaffeensis* to host cells induces the following sequential signaling events required for the entry and reproduction in host cells: transglutamination, tyrosine phosphorylation, phospholipase C (PLC)- γ 2 activation, IP₃ production, and moderate increase in cytoplasmic [Ca²⁺] (Lin et al., 2002). The heat-sensitive component of viable *E. chaffeensis* is essential for these signaling events (Lin et al., 2002). Upon *E. chaffeensis* infection, several proteins in the infected cells are rapidly tyrosine phosphorylated. One of them is PLC- γ 2 and the delivery of a PLC- γ 2 antisense oligonucleotide into THP-1 cells significantly blocked ehrlichial infection (Lin et al., 2002).

Caveolae/lipid raft-mediated endocytosis is a vesicle trafficking system that bypasses phagolysosomal pathways, and is thus utilized by wide varieties of pathogenic microorganisms to enter the host cells (Lafont and van der Goot, 2005). *E. chaffeensis* and *A. phagocytophilum* entry and intracellular infection involve this system (Lin and Rikihisa, 2003b). Lipid rafts are specialized lipid microenvironments on the cell surface. They are enriched in cholesterol, glycosphingolipid, G_{M1} ganglioside, glycosylphosphatidylinositol-anchored proteins (GAPs), and several types of membrane proteins involved in signal transduction including receptors, signal transducers and membrane transporters (Simons and

Toomre, 2000). Caveolae are formed from lipid rafts when caveola-specific proteins, caveolins, accumulate (Anderson, 1998). Caveolin is a hairpin-like palmitoylated integral membrane protein that tightly binds to cholesterol. Caveolae form unique endocytic and exocytic invaginations at the surface of various cell types and can import molecules to specific locations within the cell or export molecules to extracellular spaces in a clathrin-independent manner. Caveolae are also implicated in compartmentalization of a variety of signaling activities (Anderson, 1998; Simons and Toomre, 2000).

GAPs are required for the internalization of *E. chaffeensis* and *A. phagocytophilum*. The removal of surface-exposed GAPs by phosphatidylinositol-specific phospholipase C (PI-PLC) pretreatment inhibited the development of early inclusions (<0.5 μm) of *E. chaffeensis* and *A. phagocytophilum* in their host cells. In contrast, clathrin, a protein involved in endocytosis, has never been found to be associated with the internalization of these bacteria (Barnewall et al., 1997; Lin and Rikihisa, 2003b). Caveolin-1 and tyrosine-phosphorylated proteins, including PLC- γ 2, are co-localized with both early (as early as 5 min post exposure) and late replicative inclusions of *E. chaffeensis* and *A. phagocytophilum* (Lin and Rikihisa, 2003b).

It is very likely that the binding of *E. chaffeensis* or *A. phagocytophilum* to unidentified receptors activates some (receptor) tyrosine kinases that in turn phosphorylate proteins located in caveolae. PLC- γ 2 is one of the proteins in *E. chaffeensis*-infected host cells that are rapidly tyrosine-phosphorylated (Lin et al., 2002). As the substrate of PLC- γ 2, phosphatidylinositol 4,5-bisphosphate (PIP₂), is also enriched in caveolae (Galbiati et al., 2001). Caveolae may facilitate PLC- γ 2 enzymatic action, leading to an increase in the intracellular Ca²⁺ level that is essential for bacterial infection (Lin et al., 2002). These results suggest the convergence of signals from caveolae and transglutamination result in activation of PTK and PLC- γ 2. These data demonstrated that *E. chaffeensis* and *A. phagocytophilum* actively modulate host proteins required for their entry and the establishment of replication-competent inclusions. These host proteins are retained through all bacterial replication stages suggesting that continuous localized activation and tethering mechanisms are involved, rather than transient signaling.

A recent report using microarray analysis described up-regulation of PLC- β 1, transglutaminase (TG) β 3-like, and Tec protein tyrosine kinase mRNA in *A. phagocytophilum*-infected HL-60 cells (de la Fuente et al., 2005). In *E. chaffeensis*-infected THP-1 cells, mRNAs of RAB5A, SNAP23, and STX16, which are genes encoding proteins involved in membrane trafficking, were reported to be transiently down-regulated (Zhang et al., 2004). Involvement of these gene products in internalization and infection of *A. phagocytophilum* and *E. chaffeensis*, respectively, is currently unknown.

P-selectin glycoprotein ligand-1 (PSGL-1) is the most studied host surface protein for *A. phagocytophilum* infection. Binding of *A. phagocytophilum* to cells of the human leukemia cell line HL-60 is dependent on the expression of both PSGL-1 and an α 1—3-fucosyltransferase (Herron et al., 2000). *A. phagocytophilum* binding to PSGL-1 causes the tyrosine phosphorylation of ROCK1, an effector kinase of the GTPase RhoA via a nonreceptor tyrosine kinase Syk, because PSGL-1 blocking antibodies and siRNA targeting Syk interfere with ROCK1 phosphorylation in *A. phagocytophilum*-infected cells (Thomas and Fikrig, 2007). Knockdown of either Syk or ROCK1 also markedly impaired *A. phagocytophilum* infection (Thomas and Fikrig, 2007). PSGL-1 is not found in the lipid raft fraction that is enriched with PLC- γ 2, caveolin-1, or bacterial antigenic components in *A. phagocytophilum*-infected HL-60 cells by western blot analysis (Lin and Rikihisa, 2003b). However, ROCK1 can localize to caveolae (Michaely et al., 1999). In addition, ROCK1 may associate directly with caveolin 1 (Rashid-Doubell et al., 2007). Recently a sialic acid- and PSGL-1-independent adhesion activity was reported in a sub-line of *A. phagocytophilum* NCH-1 strain, albeit that the alternative receptor was not described (Reneer et al., 2006). The entry of this subline does not

require Syk (Reneer et al., 2008). *A. phagocytophilum* can infect cultured vascular endothelial cells (Munderloh et al., 2004) which do not express PSGL-1. Binding of *A. phagocytophilum* to murine neutrophils, requires expression of $\alpha 1$ —3-fucosyltransferases, but not PSGL-1 (Carlyon et al., 2003). Thus additional host receptors are involved in *A. phagocytophilum* infection and signaling. It would be more advantageous for *A. phagocytophilum* to be able to utilize more than one receptor and signaling pathway to enter host cells.

Several studies suggest the involvement of major surface protein Msp2 (P44) in *A. phagocytophilum* binding and infection. A recombinant Msp2 protein and MSP mAb 20B4 inhibit *A. phagocytophilum* binding to HL-60 cells, and PSGL-1/FucT-VII-transfected BJAB cells (Park et al., 2003). mAb 5C11 and 3E65 are directed to different domains of P44 proteins, the N-terminal conserved region and P44-18 central hypervariable region, respectively. The two MAbs almost completely blocked the infection of the *A. phagocytophilum* population that predominantly expressed P44-18 in HL-60 cells by distinct mechanisms: mAb 5C11 blocked the binding, but mAb 3E65 did not block binding or internalization. Instead, mAb 3E65 inhibited internalized *A. phagocytophilum* to develop into microcolonies (Wang et al., 2006). *E. coli* expressing *E. chaffeensis* 120-kDa protein binds to canine monocytes cell line DH82 cells (Popov et al., 2000). Similarly, *E. coli* expressing *Anaplasma marginale* MSP1a was shown to bind to bovine erythrocytes and IDE8 tick cells as well as *Dermacentor variabilis* tick gut cells (de la Fuente et al., 2001). Furthermore, recombinant *A. marginale* MSP1a binds to tick IDE8 cell lysate proteins in glycosylation dependent manner (de la Fuente et al., 2004). *E. coli* expressing *Ehrlichia ruminantium* “mucin-like protein” binds to tick IDE8 cells and the recombinant “mucin-like protein” binds to tick IDE8 cell lysate proteins (de la Fuente et al., 2004). The host cell receptors for these adhesins and how they involve in invasion process are presently unknown.

Monocytes and neutrophils kill invading microorganisms by oxygen-independent mechanisms, such as fusion of the phagosomes containing bacteria with granules containing both antimicrobial peptides (e.g., defensins or lysozymes) and lysosomal hydrolytic enzymes or through sequestering vital nutrients (e.g., iron) (Cohen, 1994). *E. chaffeensis* and *A. phagocytophilum* modulate vesicular trafficking to avoid their delivery to lysosomes (Barnewall et al., 1997; Mott et al., 1999; Webster et al., 1998). This modulation is essential as these bacteria exclusively reside in professional phagocytes with abundant lysosomes. Furthermore, mere bacteria are quite susceptible to lysosomal enzymes and acidic pH. Curiously, despite sharing the common features described above, *E. chaffeensis* and *A. phagocytophilum* inclusions are distinct from each other. Data suggest that the replicative inclusions of *E. chaffeensis* are early endosomes that do not mature into late endosomes, whereas *A. phagocytophilum* replicative inclusions don't appear to be a part of the endosome pathway. 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* do not have these endosomal markers (Mott et al., 1999). Of note, TfRs are not present at the time of internalization of *E. chaffeensis*, but are progressively up-regulated and accumulate in the ehrlichial inclusions until eventually most cytoplasmic TfRs appear to be localized there (Barnewall et al., 1997; Lin and Rikihisa, 2003b). At least some *E. chaffeensis* inclusions also contain MHCI and MHCII (Barnewall et al., 1997; Mott et al., 1999). Recently, several hallmarks of early autophagosomes were detected in *A. phagocytophilum* replicative inclusions, including a double lipid bilayer membrane, and co-localization with GFP-tagged LC3 and Beclin 1, the human homologs of *Saccharomyces cerevisiae* autophagy-related proteins Atg8 and Atg6, respectively (Niu, 2008). Stimulation of autophagy by rapamycin favors *A. phagocytophilum* infection. Inhibition of the autophagosomal pathway by 3-methyladenine does not inhibit *A. phagocytophilum* internalization, but reversibly arrests its growth (Niu, 2008). An additional curious difference

is that *E. chaffeensis* inclusions are often surrounded by mitochondria, whereas lysosomes are found near *A. phagocytophilum* inclusions (Mott et al., 1999). These phenotypic similarities and differences are consistent with the recent comparative analysis of these two bacterial genomes: about 50% of genes in each genome are shared (Dunning Hotopp et al., 2006). Some of these molecular events involved in *E. chaffeensis* infection are illustrated in Figure 1.

5. Down-regulation of reactive oxygen species (ROS) generation

Neutrophils and monocytes/macrophages are primary mediators of an oxygen-dependent defense system that generates ROS (superoxide, hydrogen peroxide, and hydroxyl radicals) upon exposure to pathogens. Host cell-free *E. chaffeensis* is readily killed upon exposure to ROS (Barnewall and Rikihisa, 1994). Genes encoding detoxifying enzymes (e.g., periplasmic [Cu,Zn]-SOD, [Mn]-SOD, and catalase) and oxygen-sensing two-component regulatory systems (OxyR or SoxRS) are absent in *E. chaffeensis* and *A. phagocytophilum* genomes. Therefore, these bacteria have evolved remarkable strategies to prevent activation of NADPH oxidase that catalyzes the reduction of atmospheric oxygen to O_2^- using host cytoplasmic NADPH, the initial ROS that can be converted to series of other ROS. Unlike other bacteria, *A. phagocytophilum* does not induce O_2^- generation in human and murine neutrophils as observed by cytochrome C or nitroblue tetrazolium reduction, and chemoluminescence or fluorescence assays (Banerjee et al., 2000; Borjesson et al., 2005; IJdo and Mueller, 2004; Mott and Rikihisa, 2000; Wang et al., 2002). In addition, *A. phagocytophilum* blocks subsequent activation of NADPH oxidase by *E. coli*, PMA, fMLP, or Fc-Oxyburst immune complexes (Banerjee et al., 2000; Mott and Rikihisa, 2000; Mott et al., 2002; Wang et al., 2002), indicating active inhibition. However, more recent report from the same laboratory of the previous two reports (Banerjee et al., 2000; Wang et al., 2002) showed little or no inhibition of activation with PMA after *A. phagocytophilum* infection (Carlyon et al., 2004; IJdo and Mueller, 2004).

Generation of ROS by pre-activated or primed neutrophils in response to exogenous stimuli cannot be overridden by subsequent *A. phagocytophilum* exposure (Mott and Rikihisa, 2000). Thus, any conditions that elicit or pre-activate neutrophils, while providing more sensitive ROS assays with fewer neutrophils, should be not be used as these conditions preclude active inhibition of neutrophil O_2^- generation by *A. phagocytophilum*. The inhibition of O_2^- generation is specific to the host cell: *A. phagocytophilum* inhibits O_2^- generation in neutrophils but not in monocytes, whereas the converse is true for *E. chaffeensis* (Lin and Rikihisa, 2007; Mott and Rikihisa, 2000). A periodate oxidation and heat-sensitive component, rather than *Anaplasma* protein synthesis or viability, is required for the inhibition of O_2^- generation (Borjesson et al., 2005; Mott and Rikihisa, 2000; Mott et al., 2002). Cell contact with bacteria is presumably necessary in vitro, as this inhibitory factor does not diffuse through a 0.45- μ m filter (Mott and Rikihisa, 2000).

In resting neutrophils, the inactive NADPH oxidase components remain unassembled and segregated into membranes of secretory vesicles and specific granules and a cytosolic complex (Babior, 2004). Upon activation, secretory vesicles and specific granules rapidly fuse with plasma or phagosomal membrane and a complex of p47^{phox}, p67^{phox}, and possibly p40^{phox}, translocates and associates with cytochrome b₅₅₈ (DeLeo et al., 1999). A functional NADPH oxidase enzyme is assembled at the phagosomes and/or the plasma membrane allowing exertion of the lethal effects of O_2^- and its derivatives on extracellular or ingested bacteria in close proximity. The small GTP-binding protein, Rac2, the major Rac protein expressed in neutrophils, is required for oxidase activity through direct interaction with p67^{phox} and cytochrome b₅₅₈ (Heyworth et al., 1994). Cytosolic component p47^{phox} becomes phosphorylated on 8 or 9 serine residues in the C-terminal region, some of which are required for unmasking SH3 domain for binding to Pro-rich regions of p22^{phox} (Sumimoto et al.,

1994). *A. phagocytophilum* interferes with the assembly of the NADPH oxidase subunits in the inclusion membrane (Carlyon et al., 2004; Carlyon et al., 2005; IJdo and Mueller, 2004; Mott et al., 2002). In human neutrophils and HL-60 cells, *A. phagocytophilum* decreases the preexisting protein levels of p22^{phox}, but not other components of NADPH oxidase (gp91^{phox}, p47^{phox}, p67^{phox}, and p40^{phox}) within 1 h post exposure (Mott et al., 2002), raising the possibility that the rapid destabilization of the gp91^{phox} and p22^{phox} complex (cytochrome b₅₅₈) may be involved in the inhibition of O₂⁻ generation. At 5 days post infection the down regulation of gp91^{phox} mRNA and cytochrome b₅₅₈ surface protein levels occur in HL-60 cells (Banerjee et al., 2000). Two to five days after infection of HL-60 cells, the transcription of Rac1, Rac2, and gp91^{phox} is down-regulated (Carlyon et al., 2002). Down-regulation of the gp91^{phox} gene is associated with increased binding of the repressor CCAAT displacement protein to the promoter of the gp91^{phox} gene due to reduction of interferon regulatory factor and PU.1 protein level in infected HL-60 cells (Thomas et al., 2005). However, transcriptional down-regulation of Rac1, Rac2, or gp91^{phox} has not been detected in human neutrophils infected with *A. phagocytophilum* (Borjesson et al., 2005; Lee et al., 2008; Sukumaran et al., 2005).

Carlyon *et al.*, reported O₂⁻ generation by neutrophils upon addition of *A. phagocytophilum* and proposed that scavenging the exogenous O₂⁻ (that cannot readily diffuse through cytoplasmic membrane of intact bacteria) by *A. phagocytophilum* is one of primary mechanisms for its protection from ROS (Carlyon et al., 2004). *A. phagocytophilum* expresses an [Fe]-SOD encoded by the gene that lacks signal peptide sequence (Ohashi et al., 2002). This type of SOD generally scavenges bacterial cytoplasmic O₂⁻. Since catalase is absent in *A. phagocytophilum* at the genomic level, conversion of O₂⁻ to membrane permeable hydrogen peroxide by [Fe]-SOD, and to downstream ROS, is rather harmful to *A. phagocytophilum*. Recent genome sequence analysis revealed a gene that encodes a protein containing the 12 transmembrane segments and 6 conserved histidine residues consistent with members of the heme-copper oxidase family (Dunning Hotopp et al., 2006). Whether this protein is involved in detoxifying ROS, remains to be studied.

6. Activation of chemokines/cytokines

E. chaffeensis transiently causes rapid degradation of IκB-α and activates p38 MAPK, NF-κB within 1 h post infection. Proinflammatory chemokines/cytokines such as IL-8 and IL-1β, as well as immunosuppressive cytokine IL-10, are up-regulated in *E. chaffeensis*-infected THP-1 cells at mRNA and protein levels (Lee and Rikihisa, 1996, 1997; Lin and Rikihisa, 2004). Unlike LPS stimulation, anti-CD14 or polymixin B has no effect on IL-1β mRNA expression induced by *E. chaffeensis* (Lee and Rikihisa, 1996). Addition of *E. chaffeensis* preincubated with IgG of immune serum to THP-1 cells caused prolonged degradation of IκB-α and augmented activation of NF-κB as detected by electrophoretic mobility shift assays (EMSA), and induced IL-1β, TNF-α, and IL-6 mRNA and protein expression to levels equivalent to those observed with *E. coli* LPS stimulation (Lee and Rikihisa, 1997). In contrast, addition of *E. chaffeensis* preincubated with the Fab fragment of anti-*E. chaffeensis* IgG completely blocked generation of any of these cytokines. This result suggests that IL-1β, IL-8, and IL-10 generation requires initial engagement of bacterial ligand and the host cognate receptor, which can be blocked by the Fab fragment of immune IgG, and overridden by cross-linking of the Fc receptor. This may be one of reasons why immune serum is effective in inhibiting *E. chaffeensis* infection in vivo (Winslow et al., 2000).

In contrast to *E. chaffeensis*, activation of p38 MAPK or NF-κB is not observed in human neutrophils infected with *A. phagocytophilum* within 1 h post exposure, but activation of p38 MAPK and NF-κB in human monocytes occurs in response *A. phagocytophilum* (Kim and Rikihisa, 2002). The latter is consistent with IL-1β, TNF-α, and IL-6 mRNA and protein

expression by human peripheral blood leukocytes and monocytes in response to *A. phagocytophilum* at levels equivalent to that observed with *E. coli* LPS stimulation (Kim and Rikihisa, 2000). A recent microarray study reported up-regulation of genes in the inflammatory and innate immune pathways in buffy coat cells from sheep infected with *A. phagocytophilum* (Galindo et al., 2008). In human neutrophils, IL-1 β mRNA up-regulation is detected at 3 h and IL-1 β protein secretion is detected at 1 day post *A. phagocytophilum* infection (Kim and Rikihisa, 2002). TNF- α and IL-6 mRNA up-regulation was not detectable in human neutrophils after exposure to *A. phagocytophilum* in vitro within 1 day. Therefore, similar to inhibition of O₂⁻ generation, lack or delay of induction of proinflammatory cytokines (with the exception of IL-1 β) and lack or attenuation of p38 MAPK and NF- κ B activation are neutrophil-specific and require binding to the host cells. The results are consistent with effects of inhibitors specific to p38 MAPK and NF- κ B on cytokine generation by neutrophils and monocytes in response to *A. phagocytophilum* (Kim and Rikihisa, 2002).

The fact that *A. phagocytophilum* can induce NF- κ B, and P38 MAPK in human monocytes indicates that PAMPs other than LPS or peptidoglycan, such as lipoproteins and heat shock proteins of *A. phagocytophilum* can stimulate monocytes. These proinflammatory cytokines generated by monocytes are expected to contribute to HGA pathogenesis, bacterial clearance, and disease manifestation. Activation of NF- κ B in monocytes by *A. phagocytophilum* was confirmed using mouse macrophages and involves TLR2 (Choi et al., 2004). The lack of rapid activation in the host human neutrophils may be due to active inhibition by *A. phagocytophilum*, since human neutrophils have functional TLR2. TLR2 recognizes bacterial lipoproteins. Recently, proteomics analysis demonstrated all of 15 predicted lipoprotein expression by *E. chaffeensis* cultured in THP-1 cells and some of which were recognized by immune sera from dogs experimentally infected with *E. chaffeensis* (Huang et al., 2008).

Microarray analysis showed that several proinflammatory genes, such as IL-1 β , TNF- α , IL-6, CCL20, CXCL1, CXCL2, but not IL-8 are up-regulated after 6 h in neutrophils exposed to *A. phagocytophilum* (Borjesson et al., 2005). Several chemokines and cytokines, and their receptors are upregulated after 1 h infection in neutrophils and after 2 h infection HL-60 cells (Lee et al., 2008). But kinetics and patterns of transcription of these cytokine genes were distinct from those of neutrophils that had phagocytized *Staphylococcus aureus* (Borjesson et al., 2005). Increased IL-8 and CCL20 protein secretion by neutrophils upon incubation with *A. phagocytophilum* was detected at 4 and 7 h post infection (Akkoyunlu et al., 2001; Sukumaran et al., 2005). Although up-regulation of IL-8 transcription is relatively low in neutrophils (Sukumaran et al., 2005), at the protein level, IL-8 secretion is significantly increased. Therefore, it is important to confirm transcript analysis data at protein levels and by functional studies. It is clear that *E. chaffeensis* and *A. phagocytophilum* up-regulate limited sets of proinflammatory cytokine genes in respective host cells, that are distinct in composition and kinetics from those up-regulated in response to LPS stimulation (Kim and Rikihisa, 2000; Borjesson, et al., 2005; Lee and Rikihisa, 1996). These results are consistent with the absence of LPS in these bacteria (Lin and Rikihisa, 2003a). ERK2 is activated at 3 h p.i. and P38 MAPK activated at 1 and 3 h in neutrophils infected with *A. phagocytophilum* (Lee et al., 2008).

E. chaffeensis-infected human monocytes become progressively less responsive to exogenous stimuli, such as *E. coli* LPS, as evidenced in progressively reduced activation of ERK 1/2, p38 MAPK, and NF- κ B and impaired mobilization of ehrlichiaicidal activities (Lin and Rikihisa, 2004). This reduced response is likely related to down-regulated expression of the mRNA and protein of several pattern-recognition receptors, such as CD14, TLR2, and TLR4, observed 1 day post *E. chaffeensis* infection (Lin and Rikihisa, 2004). *A. phagocytophilum*, however, does not alter mRNA levels of genes encoding Toll-like receptors or MyD88 in NB4 promyelocytic leukemic cells at 4 h (Pedra et al., 2005) or weakly up-regulates TLR2 and TLR4, but not MyD88 in neutrophils at 4 h (Sukumaran et al., 2005). This effect has not been confirmed at

protein level. Pentraxins are soluble pattern-recognition receptors with a dual role: protection against extracellular microbes and protection against autoimmunity (Garlanda, 2005). Pentaxin3 transcription is up-regulated in neutrophils infected with *A. phagocytophilum* at 4 h (Sukumaran et al., 2005). Importantly, the hematopoietic system—specific transcription factor PU.1, that acts specifically at the stage of promyelocyte differentiation into neutrophils and monocytes, is down-regulated by *E. chaffeensis* and *A. phagocytophilum* infection: in binding activity as observed by EMSA (Lin and Rikihisa, 2004) and at the protein level (Thomas et al., 2005), respectively. These data point to slightly different, but similar mechanisms, by which *E. chaffeensis* and *A. phagocytophilum* survive by modulating or delaying critical signaling mechanisms in host phagocyte activation and in differentiation pathways.

7. Down-regulation of IFN-gamma activation of host cells

E. chaffeensis is readily killed in human monocytes by treatment with recombinant human IFN- γ , if this is given before exposure or at the early stage of infection in vitro (Barnewall and Rikihisa, 1994). *E. chaffeensis* is also inhibited in vitro by treatment with deferoxamine, a cell-permeable iron chelator and an actinomycete siderophore, suggesting that intracellularly, *E. chaffeensis* has access to the labile iron pool but does not produce siderophores with high iron binding affinity (Barnewall and Rikihisa, 1994). TfR-transferrin-mediated iron uptake is the major mechanism of iron acquisition by the host cells. In human monocytes, IFN- γ treatment drastically reduces the intracellular labile iron pool by reducing levels of surface TfRs. Iron-saturated (holo), but not iron-free (apo) Tf reverses IFN- γ -induced inhibition of *E. chaffeensis* (Barnewall and Rikihisa, 1994). In contrast, scavengers of ROS had no effects on IFN- γ -induced inhibition of *E. chaffeensis* (Barnewall and Rikihisa, 1994). Thus *E. chaffeensis* is likely killed in human monocytes by depletion of cytoplasmically available iron derived from Tf. When IFN- γ is added several hours after the establishment of infection, however, it is no longer effective (Barnewall and Rikihisa, 1994). This change in susceptibility may be related to the ability of *E. chaffeensis* to stabilize TfR mRNAs by activating iron responsive factor-1 (Barnewall et al., 1999), so that they can counteract the down-regulation of TfR induced with IFN- γ .

In addition, *E. chaffeensis*—more specifically the binding of its protein component to monocytes—blocks Jak-Stat signal transduction (Lee and Rikihisa, 1998). Tyrosine phosphorylation of Jak1 and Stat1 α are blocked when IFN- γ is added as early as 30 min after exposure, but not if added prior to infection. At least some of this inhibition can be attributed to increased host cell protein kinase A activity, because *E. chaffeensis* infection stimulates protein kinase A activity of the host cells by 25-fold within 30 min of infection, and addition of a protein kinase A inhibitor abrogates the inhibition of the Jak-Stat pathway by *E. chaffeensis* (Lee and Rikihisa, 1998). A defect in IFN- γ signaling was also reported in HL-60 cells infected with *A. phagocytophilum*; this defect does not inhibit Stat1 phosphorylation, but impairs binding of phosphorylated Stat1 to the promoter of interferon regulatory factor-1 (Thomas et al., 2005). Temporal analysis during progressive levels of infection showed that *A. phagocytophilum* infection does not change transcription of TfR or the activity of iron responsive factor in human myelocytic leukemia THP-1 cells (Barnewall et al., 1999). A microarray analysis found up-regulation of TfR and down-regulation of Tf in NB4 cells after 4 h of incubation with *A. phagocytophilum* (Pedra et al., 2005). Another study reported increased ferritin heavy chain mRNA expression and decreased ferritin protein levels in *A. phagocytophilum*-infected HL-60 cells and/or neutrophils (Carlyon et al., 2005). Up-regulation of ferritin heavy chain mRNA was not detected in recent microarray analysis in human neutrophils (Borjesson et al., 2005; Sukumaran et al., 2005). Our analysis of recent genome sequence data of *A. phagocytophilum* and *E. chaffeensis* did not reveal genes homologous to those of the known iron uptake system in other bacteria with the exception of the periplasmic iron binding protein. Overall, these studies suggest that *E. chaffeensis* and *A.*

phagocytophilum infections inhibit IFN- γ signaling and alter host cell iron metabolism, and there may be cross-talk between the two pathways.

8. Inhibition of host cell apoptosis

Apoptosis is an important mechanism for killing intracellular pathogens. A number of pathogens induce apoptosis of their host cells, whereas several others 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 (Ge et al., 2005; Yoshiie et al., 2000). ROS limits the life span of a neutrophil by activating death receptor signaling (Scheel-Toellner et al., 2004) and inhibition of apoptosis is consistent with the fact that *A. phagocytophilum* by itself does not induce NADPH oxidase activation in human and murine neutrophils (Banerjee et al., 2000; Borjesson et al., 2005; IJdo and Mueller, 2004).

A. phagocytophilum prevents human neutrophils from reducing the mRNA of the anti-apoptotic *bcl-2* family member *bfl-1* (*A1*), from losing the mitochondrial membrane potential, and from activating caspase 3 (Ge and Rikihisa, 2006; Ge et al., 2005). Several microarray data derived from human neutrophils and NB4 cells corroborate these data, showing that *A. phagocytophilum* infection up-regulates expression of anti-apoptotic *bcl-2* family members (Borjesson et al., 2005; Lee and Goodman, 2006; Pedra et al., 2005). *A. phagocytophilum* also blocks anti-FAS-induced programmed cell death of human neutrophils (Borjesson et al., 2005; Ge and Rikihisa, 2006). Furthermore, cell surface Fas clustering during spontaneous neutrophil apoptosis is blocked by *A. phagocytophilum* infection (Ge and Rikihisa, 2006). The cleavage of pro-caspase 8, caspase 8 activation and the cleavage of Bid, which links the intrinsic and extrinsic pathways, in the extrinsic pathway of spontaneous neutrophil apoptosis is also inhibited by *A. phagocytophilum* infection (Ge and Rikihisa, 2006). Likewise, *A. phagocytophilum* infection inhibited 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 (Ge and Rikihisa, 2006). In addition, transcription of many apoptosis and differentiation-related genes are modulated in human neutrophils infected with *A. phagocytophilum* (Borjesson et al., 2005; Lee and Goodman, 2006). Choi *et al.* proposed that *A. phagocytophilum* inhibits human neutrophil apoptosis through activation of p38 MAP kinase (Choi et al., 2005). These data point to a novel mechanism induced by *A. phagocytophilum* involving both extrinsic and intrinsic pathways to ensure to delay the apoptosis of host neutrophils. *E. chaffeensis* up-regulates NF- κ B and apoptosis inhibitors and differentially regulates cell cyclins and CDK expression in THP-1 cells (Zhang et al., 2004). *E. ewingii* delays spontaneous apoptosis of dog neutrophils via stabilization of host cell mitochondria (Xiong et al., 2008). Thus, *Ehrlichia* spp. also inhibit apoptosis of their host cells.

9. Events in the bacteria: Type IV secretion apparatus

Genes encoding the Type IV secretion (T4S) system, but not the Type III secretion system, have been identified in the order Rickettsiales (Niu et al., 2006; Ohashi et al., 2002). T4S apparatus transports macromolecules across the membrane in an ATP-dependent manner and is ancestrally related to the conjugation system of gram-negative bacteria. In the most extensively studied T4S system from *Agrobacterium tumefaciens*, the single *virB* operon, along with *virD4*, encodes 12 membrane-associated proteins that form a transmembrane channel complex (Christie, 1997). The split *virB/D* operons encoding the TFSS machinery have been found in the obligate intracellular parasites *Ehrlichia chaffeensis* and *A. phagocytophilum* (Ohashi et al., 2002), and analysis of recent whole-genome sequence databases indicates conservation of this split operon structure in other members of the order Rickettsiales.

(Andersson et al., 1998; Brayton et al., 2005; Collins et al., 2005; Malek et al., 2004). *A. phagocytophilum* *virB9* gene is transcribed in peripheral blood leukocytes from HGA patients and from experimentally infected animals (Ohashi et al., 2002). In a closely related monocyte-tropic obligatory intracellular bacterium, *Ehrlichia canis*, *virB9* is expressed in the blood from infected dogs, in the infected tick tissues, and infected canine monocyte cell culture (Felek et al., 2003). Transcription of *virB/D* loci were up-regulated during the exponential growth stage of *E. chaffeensis* synchronously cultured in THP-1 human monocytic leukemia cells and down-regulated prior to the release of *E. chaffeensis* from host THP-1 cells (Cheng et al., 2008). Proteomic analysis identified a unique DNA binding protein, EcxR that coordinately regulates all five *virB/D* loci of *E. chaffeensis* (Cheng et al., 2008). Similarly, both *virB9* and *virB6* of *A. phagocytophilum* are up-regulated at mRNA level and VirB9 at protein level during infection of human neutrophils in vitro and the majority of *A. phagocytophilum* spontaneously released from the infected host cells poorly expresses VirB9 (Niu et al., 2006). These results indicate developmental regulation of expression of components of the T4S system during the *A. phagocytophilum* intracellular life cycle (Niu et al., 2006). Genome sequence analysis predicted five putative proteins homologous to HGE-14 of *A. phagocytophilum* (Lodes et al., 2001) as type IV secretion substrates (Dunning Hotopp et al., 2006). Recently, we demonstrated that *A. phagocytophilum* HZ strain 160-kDa AnkA protein is delivered by a VirB/D-dependent manner into the host leukocyte cytoplasm and subsequently is tyrosine-phosphorylated (Lin et al., 2007). AnkA binds to Abl-interactor 1 that interacts with Abl-1 tyrosine kinase, thus mediating AnkA phosphorylation. AnkA and Abl-1 are critical for *A. phagocytophilum* infection (Lin et al., 2007). *A. phagocytophilum* NCH-1 strain infection specifically induces tyrosine phosphorylation of a 190 kDa AnkA (AnkA molecular size is bacterial strain-dependent) which then interact with the host cell tyrosine phosphatase SHP-1 (Ijdo et al., 2007). AnkA also localizes within nuclei of infected HL-60 cells and bind to the internucleosomal region of HL-60 cell chromosome, and DNA fragments in a cell-free system (Park et al., 2004).

10. Two-component system

The two-component system (TCS), a family of signal sensor, transduction, and response regulatory systems, enables bacteria to sense wide varieties of environmental signals and respond rapidly to changes in their environment through specific gene activation or repression (Dorman et al., 2001). TCSs are integral in the ability of certain pathogenic bacteria to mount and establish a successful infection within the hosts as emphasized by the many examples of attenuated virulence observed with pathogenic strains in which one or more TCSs have been deleted (Groisman, 2001). Through sequence and domain structure analysis, and due to overall similarities in protein structures, we identified three histidine kinases (homologs of NtrX, PleC, and CckA) and pairing response regulators (homologs of NtrY, PleD, and CtrA) in the genomes of *E. chaffeensis* and *A. phagocytophilum* (Chang et al., 2006; Cheng et al., 2006). All six genes and proteins from both *E. chaffeensis* and *A. phagocytophilum* are expressed when the bacteria infected human leukocyte culture (Chang et al., 2006; Cheng et al., 2006). As predicted, recombinant *E. chaffeensis* NtrY, PleC, and CckA proteins have specific His residue-dependent autokinase activity in vitro, and the specific Asp residue-dependent in vitro phosphotransfer from NtrY to NtrX, PleC to PleD, and CckA to CtrA, was demonstrated (Cheng et al., 2006).

Closantel (*N*-[5-chloro-4-[(*R,S*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide), an inhibitor of histidine kinases of several other bacteria (Stephenson et al., 2000) inhibits the recombinant histidine kinases from *E. chaffeensis* in vitro. Pretreatment of host cell-free *E. chaffeensis* or *A. phagocytophilum* with closantel, completely blocks the infection of host cells (Cheng et al., 2006). Treatment of infected cells one day post exposure with closantel clears infection in a dose-dependent manner. Lysosomal fusion with ehrlichial inclusions was shown to be involved in this process (Kumagai et al., 2006). More

than 12 genes from *E. chaffeensis*, including the three histidine kinases, were down regulated within 5–15 min post closantel treatment (Cheng et al., 2006). These results suggest that TCS plays an essential role in invasion and survival of *E. chaffeensis* and *A. phagocytophilum* in human leukocytes.

11. Genome sequence data

We now have much better tools than ever before for molecular analysis of *E. chaffeensis* and *A. phagocytophilum*, since several members of genus *Rickettsia*, family *Anaplasmataceae* have been sequenced. Complete genome sequences of *A. phagocytophilum* (1,471,282 base pairs) and *E. chaffeensis* (1,175,764 base pairs) were obtained and compared to each other and to the published *Rickettsiales* genome sequences. *E. chaffeensis* and *A. phagocytophilum* are syntenic. Detailed analyses of ORFs and proteins predicted to be encoded by these ORFs will help advance understanding cell biology and physiology of these bacteria in molecular terms. The two species share approximately 500 genes (Dunning Hotopp et al., 2006) and approximately 470–580 genes are unique to each species. These unique genes presumably are responsible for the important phenotypic differences between these two organisms. Comparative genome hybridization showed strain variation in genomic sequences, particularly genes predicted to encode cell envelope proteins (Dunning Hotopp et al., 2006; Miura and Rikihisa, 2007). Pathogenesis of *E. chaffeensis* strains also differs (Miura and Rikihisa, 2007). As more genetically defined strains are compared, we may identify bacterial virulence factors or genomic regions encoding virulence factors. Using the genome sequence data, recent proteomics analysis identified several ‘hypothetical proteins’ as *E. chaffeensis* and *A. phagocytophilum* surface exposed proteins (Ge and Rikihisa, 2007a, b). All of the *Anaplasmataceae* examined have a significantly lower coding capacity for biosynthesis and central intermediary metabolism than do free-living bacteria. Thus, *E. chaffeensis* and *A. phagocytophilum* have evolved to usurp and acquire various compounds from their hosts. Some of abundant expressed outer membrane proteins were shown to have porin activity (Huang et al., 2007; Kumagai et al., 2008). Using *A. phagocytophilum* genome tiled microarray, recently different bacterial gene expression pattern between tick and mammalian cells are reported (Nelson et al., 2008). The ongoing *Ixodes scapularis* tick genome sequence study will facilitate the analysis of tick cell stages of *E. chaffeensis* and *A. phagocytophilum*. Using RNA interference-mediated silencing it was shown that while *A. phagocytophilum* migrated normally from *A. phagocytophilum*-infected mice to the gut of engorging salp16-deficient ticks, up to 90% of the bacteria that entered the ticks are not able to successfully infect *I. scapularis* salivary glands (Sukumaran et al., 2006). Since tools for genetic manipulation are limited, obligatory intracellular bacteria are less well characterized than the facultative intracellular bacteria or free-living bacteria. The availability of the genome sequences will continue to facilitate understanding the ehrlichiosis agents and the signaling events between intracellular bacteria and host cells.

12. Future prospects

Although *E. chaffeensis* and *A. phagocytophilum* were discovered relatively recently, studies of these intracellular pathogens have already changed our paradigm of leukocyte-tropic bacteria. Instead of considering the traditional static pathogen-host relationship, we have learned that the interaction is mutually dynamic. Our eventual understanding of *E. chaffeensis* and *A. phagocytophilum* infections will entail the elucidation of the functions of a large number of genes. This work will require cloning and expressing recombinant proteins and studying their functions. It will be necessary to culture *E. chaffeensis* and *A. phagocytophilum* to identify and categorize ehrlichial proteins, and to analyze biological functions of naturally folded and modified proteins. It will also require understanding transcriptional and post transcriptional regulation of bacteria. Stable genetic manipulation of

obligatory intracellular bacteria is on the way, and this approach or the potentially more feasible use of surrogate bacterial or a yeast reporter system will allow testing of many hypotheses. Screening host genes required or inhibitory by using anti-sense library in insect cell or nematode culture system, and use of various mutant and transfected host cells and transgenic mice, are some of useful approaches for this group of bacteria. Future tick cell studies fill the important gaps in our knowledge on the *E. chaffeensis* and *A. phagocytophilum* life cycle. Along with these studies, natural or recombinant ehrlichial antigens and proteins offer a novel set of compounds that have diagnostic and therapeutic applications or be candidates for vaccine development.

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References

- Akkoyunlu M, Malawista SE, Anguita J, Fikrig E. Exploitation of interleukin-8-induced neutrophil chemotaxis by the agent of human granulocytic ehrlichiosis. *Infect Immun* 2001;69:5577–5588. [PubMed: 11500432]
- Anderson BE, Sims KG, Olson JG, Childs JE, Piesman JF, Happ CM, Maupin GO, Johnson BJ. *Amblyomma americanum*: a potential vector of human ehrlichiosis. *Am J Trop Med Hyg* 1993;49:239–244. [PubMed: 8357086]
- Anderson RG. The caveolae membrane system. *Annu. Rev. Biochem* 1998;67:199–225. [PubMed: 9759488]
- Andersson SG, Zomorodipour A, Andersson JO, Sicheritz-Ponten T, Alsmark UC, Podowski RM, Naslund AK, Eriksson AS, Winkler HH, Kurland CG. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* 1998;396:133–140. [PubMed: 9823893]
- Babior BM. NADPH oxidase. *Curr Opin Immunol* 2004;16:42–47. [PubMed: 14734109]
- Banerjee R, Anguita J, Roos D, Fikrig E. Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91^{phox}. *J. Immunol* 2000;164:3946–3949. [PubMed: 10754283]
- Barnewall RE, Ohashi N, Rikihisa Y. *Ehrlichia chaffeensis* and *E. sennetsu*, but not the human granulocytic ehrlichiosis agent, colocalize with transferrin receptor and up-regulate transferrin receptor mRNA by activating iron-responsive protein 1. *Infect. Immun* 1999;67:2258–2265. [PubMed: 10225882]
- Barnewall RE, Rikihisa Y. Abrogation of gamma interferon-induced inhibition of *Ehrlichia chaffeensis* infection in human monocytes with iron-transferrin. *Infect. Immun* 1994;62:4804–4810. [PubMed: 7927758]
- Barnewall RE, Rikihisa Y, Lee EH. *Ehrlichia chaffeensis* inclusions are early endosomes which selectively accumulate transferrin receptor. *Infect. Immun* 1997;65:1455–1461. [PubMed: 9119487]
- Borjesson DL, Kobayashi SD, Whitney AR, Voyich JM, Argue CM, Deleo FR. Insights into pathogen immune evasion mechanisms: *Anaplasma phagocytophilum* fails to induce an apoptosis differentiation program in human neutrophils. *J Immunol* 2005;174:6364–6372. [PubMed: 15879137]
- Brayton KA, Kappmeyer LS, Herndon DR, Dark MJ, Tibbals DL, Palmer GH, McGuire TC, Knowles DP Jr. Complete genome sequencing of *Anaplasma marginale* reveals that the surface is skewed to two superfamilies of outer membrane proteins. *Proc Natl Acad Sci U S A* 2005;102:844–849. [PubMed: 15618402]
- Carlyon JA, Abdel-Latif D, Pypaert M, Lacy P, Fikrig E. *Anaplasma phagocytophilum* utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. *Infect. Immun* 2004;72:4772–4783. [PubMed: 15271939]

- Carlyon JA, Akkoyunlu M, Xia L, Yago T, Wang T, Cummings RD, McEver RP, Fikrig E. Murine neutrophils require alpha1,3-fucosylation but not PSGL-1 for productive infection with *Anaplasma phagocytophilum*. *Blood* 2003;102:3387–3395. [PubMed: 12869507]
- Carlyon JA, Chan WT, Galan J, Roos D, Fikrig E. Repression of *rac2* mRNA expression by *Anaplasma phagocytophilum* is essential to the inhibition of superoxide production and bacterial proliferation. *J. Immunol* 2002;169:7009–7018. [PubMed: 12471136]
- Carlyon JA, Ryan D, Archer K, Fikrig E. Effects of *Anaplasma phagocytophilum* on host cell ferritin mRNA and protein levels. *Infect Immun* 2005;73:7629–7636. [PubMed: 16239567]
- Chang YF, Cheng CM, Chang LK, Jong YJ, Yuo CY. The F-box protein Fbxo7 interacts with human inhibitor of apoptosis protein cIAP1 and promotes cIAP1 ubiquitination. *Biochemical and biophysical research communications* 2006;342:1022–1026. [PubMed: 16510124]
- Chen SM, Dumler JS, Bakken JS, Walker DH. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. *J Clin Microbiol* 1994;32:589–595. [PubMed: 8195363]
- Cheng Z, Kumagai Y, Lin M, Zhang C, Rikihisa Y. Intra-leukocyte expression of two-component systems in *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* and effects of the histidine kinase inhibitor closantel. *Cellular microbiology* 2006;8:1241–1252. [PubMed: 16882029]
- Cheng Z, Wang X, Rikihisa Y. Regulation of type IV secretion apparatus genes during *Ehrlichia chaffeensis* intracellular development by a previously unidentified protein. *J Bacteriol* 2008;190:2096–2105. [PubMed: 18192398]
- Choi KS, Park JT, Dumler JS. *Anaplasma phagocytophilum* delay of neutrophil apoptosis through the p38 mitogen-activated protein kinase signal pathway. *Infect Immun* 2005;73:8209–8218. [PubMed: 16299317]
- Choi KS, Scorpio DG, Dumler JS. *Anaplasma phagocytophilum* ligation to toll-like receptor (TLR) 2, but not to TLR4, activates macrophages for nuclear factor-kappa B nuclear translocation. *J Infect Dis* 2004;189:1921–1925. [PubMed: 15122530]
- Christie PJ. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J Bacteriol* 1997;179:3085–3094. [PubMed: 9150199]
- Cohen MS. Molecular events in the activation of human neutrophils for microbial killing. *Clin Infect Dis* 1994;18:S170–S179. [PubMed: 8148462]
- Collins NE, Liebenberg J, de Villiers EP, Brayton KA, Louw E, Pretorius A, Faber FE, van Heerden H, Josemans A, van Kleef M, Steyn HC, van Strijp MF, Zweggarth E, Jongejan F, Maillard JC, Berthier D, Botha M, Joubert F, Corton CH, Thomson NR, Allsopp MT, Allsopp BA. The genome of the heartwater agent *Ehrlichia ruminantium* contains multiple tandem repeats of actively variable copy number. *Proc Natl Acad Sci U S A* 2005;102:838–843. [PubMed: 15637156]
- Dawson JE, Anderson BE, Fishbein DB, Sanchez JL, Goldsmith CS, Wilson KH, Duntley CW. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. *J. Clin. Microbiol* 1991;29:2741–2745. [PubMed: 1757543]
- de la Fuente J, Ayoubi P, Blouin EF, Almazan C, Naranjo V, Kocan KM. Gene expression profiling of human promyelocytic cells in response to infection with *Anaplasma phagocytophilum*. *Cellular microbiology* 2005;7:549–559. [PubMed: 15760455]
- de la Fuente J, Garcia-Garcia JC, Barbet AF, Blouin EF, Kocan KM. Adhesion of outer membrane proteins containing tandem repeats of *Anaplasma* and *Ehrlichia* species (Rickettsiales: Anaplasmataceae) to tick cells. *Vet Microbiol* 2004;98:313–322. [PubMed: 15036540]
- de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. *Int J Parasitol* 2001;31:145–153. [PubMed: 11239934]
- DeLeo FR. Modulation of phagocyte apoptosis by bacterial pathogens. *Apoptosis* 2004;9:399–413. [PubMed: 15192322]
- DeLeo FR, Allen LA, Apicella M, Nauseef WM. NADPH oxidase activation and assembly during phagocytosis. *J Immunol* 1999;163:6732–6740. [PubMed: 10586071]
- Dorman CJ, McKenna S, Beloin C. Regulation of virulence gene expression in *Shigella flexneri*, a facultative intracellular pathogen. *Int J Med Microbiol* 2001;291:89–96. [PubMed: 11437343]
- Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order

Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *Int. J. Syst. Evol. Microbiol* 2001;51:2145–2165. [PubMed: 11760958]

- Dumler JS, Madigan JE, Pusterla N, Bakken JS. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. *Clin Infect Dis* 2007;45:S45–S51. [PubMed: 17582569]
- Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen J, Seshadri R, Ren Q, Wu M, Utterback TR, Smith S, Lewis M, Khouri H, Zhang C, Hua N, Lin Q, Ohashi N, Zhi N, Nelson W, Brinkac LM, Dodson RJ, Rosovitz MJ, Sundaram J, Daugherty SC, Davidsen T, Durkin A, Gwinn M, Haft DH, Selengut JD, Sullivan SA, Zafar N, Zhou L, Benahmed F, Forberger H, Halpin R, Mulligan S, Robinson J, White O, Rikihisa Y, Tettelin H. Comparative Genomics of Emerging Human Ehrlichiosis Agents. *PLoS Genet* 2006;2:e21. [PubMed: 16482227]
- Dunning Hotopp JML, Madupu R, Crabtree J, Angiuoli S, Eisen J, Seshadri R, Ren Q, Utterback TR, Smith S, Lewis M, Khouri H, Zhang C, Hua N, Lin Q, Ohashi N, Zhi N, Nelson W, Brinkac LM, Dodson RJ, Rosovitz MJ, Sundaram J, Daugherty SC, Davidsen T, Durkin A, Gwinn M, Haft DH, Selengut JD, Sullivan SA, Zafar N, Zhou L, Benahmed F, Forberger H, Halpin R, Mulligan S, Robinson J, Rikihisa Y, Tettelin H. Comparative Genomics of Emerging Human Ehrlichiosis Agents. *PLoS Genetics*. 2005
- Ewing SA, Dawson JE, Kocan AA, Barker RW, Warner CK, Panciera RJ, Fox JC, Kocan KM, Blouin EF. Experimental transmission of *Ehrlichia chaffeensis* (*Rickettsiales:Ehrlichieae*) among white-tailed deer by *Amblyomma americanum* (Acari: Ixodidae). *J. Med. Entomol* 1995;32:368–374. [PubMed: 7616529]
- Felek S, Huang H, Rikihisa Y. Sequence and expression analysis of virB9 of the type IV secretion system of *Ehrlichia canis* strains in ticks, dogs, and cultured cells. *Infect Immun* 2003;71:6063–6067. [PubMed: 14500531]
- Galbiati F, Razani B, Lisanti MP. Emerging themes in lipid rafts and caveolae. *Cell* 2001;106:403–411. [PubMed: 11525727]
- Galindo RC, Ayoubi P, Garcia-Perez AL, Naranjo V, Kocan KM, Gortazar C, de la Fuente J. Differential expression of inflammatory and immune response genes in sheep infected with *Anaplasma phagocytophilum*. *Vet Immunol Immunopathol*. 2008
- Gardner SL, Holman RC, Krebs JW, Berkelman R, Childs JE. National surveillance for the human ehrlichioses in the United States, 1997–2001, and proposed methods for evaluation of data quality. *Ann N Y Acad Sci* 2003;990:80–89. [PubMed: 12860604]
- Garlanda CBB, Bastone A, Mantovani A. Pentraxins at the crossroads between innate immunity, inflammation, matrix deposition, and female fertility. *Annu Rev Immunol* 2005;23:337–366. [PubMed: 15771574]
- Ge Y, Rikihisa Y. *Anaplasma phagocytophilum* delays spontaneous human neutrophil apoptosis by modulation of multiple apoptotic pathways. *Cell Microbiol* 2006;8:1406–1416. [PubMed: 16922860]
- Ge Y, Rikihisa Y. Identification of novel surface proteins of *Anaplasma phagocytophilum* by affinity purification and proteomics. *J Bacteriol* 2007a;189:7819–7828. [PubMed: 17766422]
- Ge Y, Rikihisa Y. Surface-exposed Proteins of *Ehrlichia chaffeensis*. *Infect Immun*. 2007b
- Ge Y, Yoshiie K, Kuribayashi F, Lin M, Rikihisa Y. *Anaplasma phagocytophilum* inhibits human neutrophil apoptosis via upregulation of bfl-1, maintenance of mitochondrial membrane potential and prevention of caspase 3 activation. *Cell Microbiol* 2005;7:29–38. [PubMed: 15617521]
- Goodman JL, Nelson C, Vitale B, Madigan JE, Dumler JS, Kurti TJ, Munderloh UG. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. *N. Engl. J. Med* 1996;334:209–215. [PubMed: 8531996]
- Groisman EA. The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* 2001;183:1835–1842. [PubMed: 11222580]
- Herron MJ, Ericson ME, Kurti TJ, Munderloh UG. The interactions of *Anaplasma phagocytophilum*, endothelial cells, and human neutrophils. *Ann N Y Acad Sci* 2005;1063:374–382. [PubMed: 16481545]

- Herron MJ, Nelson CM, Larson J, Snapp KR, Kansas GS, Goodman JL. Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. *Science (New York, N.Y.)* 2000;288:1653–1656.
- Heyworth PG, Bohl BP, Bokoch GM, Curnutte JT. Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. *J Biol Chem* 1994;269:30749–30752. [PubMed: 7982999]
- Huang H, Lin M, Wang X, Kikuchi T, Mottaz H, Norbeck A, Rikihisa Y. Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infect Immun* 2008;76:3405–3414. [PubMed: 18490460]
- Huang H, Wang X, Kikuchi T, Kumagai Y, Rikihisa Y. Porin activity of *Anaplasma phagocytophilum* outer membrane fraction and purified P44. *J Bacteriol* 2007;189:1998–2006. [PubMed: 17172334]
- Ijdo J, Carlson AC, Kennedy EL. *Anaplasma phagocytophilum* AnkA is tyrosine-phosphorylated at EPIYA motifs and recruits SHP-1 during early infection. *Cell Microbiol* 2007;9:1284–1296. [PubMed: 17250594]
- Ijdo J, Mueller AC. Neutrophil NADPH oxidase is reduced at the *Anaplasma phagocytophilum* phagosome. *Infect Immun* 2004;72:5392–5401. [PubMed: 15322037]
- Kim HY, Rikihisa Y. Expression of interleukin-1beta, tumor necrosis factor alpha, and interleukin-6 in human peripheral blood leukocytes exposed to human granulocytic ehrlichiosis agent or recombinant major surface protein P44. *Infect. Immun* 2000;68:3394–3402. [PubMed: 10816490]
- Kim HY, Rikihisa Y. Roles of p38 mitogen-activated protein kinase, NF-kappaB, and protein kinase C in proinflammatory cytokine mRNA expression by human peripheral blood leukocytes, monocytes, and neutrophils in response to *Anaplasma phagocytophila*. *Infect. Immun* 2002;70:4132–4141. [PubMed: 12117921]
- Klein MB, Miller JS, Nelson CM, Goodman JL. Primary bone marrow progenitors of both granulocytic and monocytic lineages are susceptible to infection with the agent of human granulocytic ehrlichiosis. *J Infect Dis* 1997;176:1405–1409. [PubMed: 9359749]
- Kumagai Y, Cheng Z, Lin M, Rikihisa Y. Biochemical activities of three pairs of *Ehrlichia chaffeensis* two-component regulatory system proteins involved in inhibition of lysosomal fusion. *Infect Immun* 2006;74:5014–5022. [PubMed: 16926392]
- Kumagai Y, Huang H, Rikihisa Y. Expression and porin activity of P28 and OMP-1F during intracellular *Ehrlichia chaffeensis* development. *J Bacteriol* 2008;190:3597–3605. [PubMed: 18359808]
- Lafont F, van der Goot FG. Bacterial invasion via lipid rafts. *Cell Microbiol* 2005;7:613–620. [PubMed: 15839890]
- Lee EH, Rikihisa Y. Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1beta, IL-8, and IL-10 expression in human monocytes exposed to viable or killed *Ehrlichia chaffeensis*. *Infect. Immun* 1996;64:4211–4219. [PubMed: 8926090]
- Lee EH, Rikihisa Y. Anti-*Ehrlichia chaffeensis* antibody complexed with *E. chaffeensis* induces potent proinflammatory cytokine mRNA expression in human monocytes through sustained reduction of IkappaB-alpha and activation of NF-kappaB. *Infect. Immun* 1997;65:2890–2897. [PubMed: 9199464]
- Lee EH, Rikihisa Y. Protein kinase A-mediated inhibition of gamma interferon-induced tyrosine phosphorylation of Janus kinases and latent cytoplasmic transcription factors in human monocytes by *Ehrlichia chaffeensis*. *Infect. Immun* 1998;66:2514–2520. [PubMed: 9596710]
- Lee HC, Goodman JL. *Anaplasma phagocytophilum* causes global induction of antiapoptosis in human neutrophils. *Genomics* 2006;88:496–503. [PubMed: 16876385]
- Lee HC, Kioi M, Han J, Puri RK, Goodman JL. *Anaplasma phagocytophilum*-induced gene expression in both human neutrophils and HL-60 cells. *Genomics*. 2008
- Lin M, den Dulk-Ras A, Hooykaas PJ, Rikihisa Y. *Anaplasma phagocytophilum* AnkA secreted by type IV secretion system is tyrosine phosphorylated by Abl-1 to facilitate infection. *Cell Microbiol* 2007;9:2644–2657. [PubMed: 17587335]
- Lin M, Rikihisa Y. *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infect. Immun* 2003a;71:5324–5331. [PubMed: 12933880]

- Lin M, Rikihisa Y. Obligatory intracellular parasitism by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* involves caveolae and glycosylphosphatidylinositol-anchored proteins. *Cell. Microbiol* 2003b;5:809–820. [PubMed: 14531896]
- Lin M, Rikihisa Y. *Ehrlichia chaffeensis* downregulates surface Toll-like receptors 2/4, CD14 and transcription factors PU.1 and inhibits lipopolysaccharide activation of NF- κ B, ERK 1/2 and p38 MAPK in host monocytes. *Cell. Microbiol* 2004;6:175–186. [PubMed: 14706103]
- Lin M, Rikihisa Y. Degradation of p22phox and inhibition of superoxide generation by *Ehrlichia chaffeensis* in human monocytes. *Cell Microbiol* 2007;9:861–874. [PubMed: 17087735]
- Lin M, Zhu MX, Rikihisa Y. Rapid activation of protein tyrosine kinase and phospholipase C- γ and increase in cytosolic free calcium are required by *Ehrlichia chaffeensis* for internalization and growth in THP-1 cells. *Infect Immun* 2002;70:889–898. [PubMed: 11796624]
- Little TJ, Hultmark D, Read AF. Invertebrate immunity and the limits of mechanistic immunology. *Nature immunology* 2005;6:651–654. [PubMed: 15970937]
- Lodes MJ, Mohamath R, Reynolds LD, McNeill P, Kolbert CP, Bruinsma ES, Benson DR, Hofmeister E, Reed SG, Houghton RL, Persing DH. Serodiagnosis of human granulocytic ehrlichiosis by using novel combinations of immunoreactive recombinant proteins. *J Clin Microbiol* 2001;39:2466–2476. [PubMed: 11427556]
- Maeda K, Markowitz N, Hawley RC, Ristic M, Cox D, McDade JE. Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *N Engl J Med* 1987;316:853–856. [PubMed: 3029590]
- Malek JA, Wierzbowski JM, Tao W, Bosak SA, Saranga DJ, Doucette-Stamm L, Smith DR, McEwan PJ, McKernan KJ. Protein interaction mapping on a functional shotgun sequence of *Rickettsia sibirica*. *Nucleic Acids Res* 2004;32:1059–1064. [PubMed: 14872061]
- Michael PA, Mineo C, Ying YS, Anderson RG. Polarized distribution of endogenous Rac1 and RhoA at the cell surface. *J Biol Chem* 1999;274:21430–21436. [PubMed: 10409706]
- Miura K, Rikihisa Y. Virulence potential of *Ehrlichia chaffeensis* strains of distinct genome sequences. *Infect Immun* 2007;75:3604–3613. [PubMed: 17438035]
- Mott J, Barnewall RE, Rikihisa Y. Human granulocytic ehrlichiosis agent and *Ehrlichia chaffeensis* reside in different cytoplasmic compartments in HL-60 cells. *Infect. Immun* 1999;67:1368–1378. [PubMed: 10024584]
- Mott J, Rikihisa Y. Human granulocytic ehrlichiosis agent inhibits superoxide anion generation by human neutrophils. *Infect. Immun* 2000;68:6697–6703. [PubMed: 11083784]
- Mott J, Rikihisa Y, Tsunawaki S. Effects of *Anaplasma phagocytophilum* on NADPH oxidase components in human neutrophils and HL-60 cells. *Infect. Immun* 2002;70:1359–1366. [PubMed: 11854221]
- Munderloh UG, Lynch MJ, Herron MJ, Palmer AT, Kurtti TJ, Nelson RD, Goodman JL. Infection of endothelial cells with *Anaplasma marginale* and *A. phagocytophilum*. *Vet Microbiol* 2004;101:53–64. [PubMed: 15201033]
- Nelson CM, Herron MJ, Felsheim RF, Schloeder BR, Grindle SM, Chavez AO, Kurtti TJ, Munderloh UG. Whole genome transcription profiling of *Anaplasma phagocytophilum* in human and tick host cells by tiling array analysis. *BMC genomics* 2008;9:364. [PubMed: 18671858]
- Niu H, Yamaguchi M, Rikihisa Y. Subversion of cellular autophagy by *Anaplasma phagocytophilum*. *Cell Microbiol* 2008;10:593–605. [PubMed: 17979984]
- Niu H, Rikihisa Y, Yamaguchi M, Ohashi N. Differential expression of VirB9 and VirB6 during the life cycle of *Anaplasma phagocytophilum* in human leucocytes is associated with differential binding and avoidance of lysosome pathway. *Cell Microbiol* 2006;8:523–534. [PubMed: 16469062]
- Ohashi N, Zhi N, Lin Q, Rikihisa Y. Characterization and transcriptional analysis of gene clusters for a type IV secretion machinery in human granulocytic and monocytic ehrlichiosis agents. *Infect. Immun* 2002;70:2128–2138. [PubMed: 11895979]
- Oteo JA, Brouqui P. Ehrlichiosis and human anaplasmosis. *Enferm Infecc Microbiol Clin* 2005;23:375–380. [PubMed: 15970171]
- Paddock CD, Childs JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin. Microbiol. Rev* 2003;16:37–64. [PubMed: 12525424]
- Park J, Choi KS, Dumler JS. Major surface protein 2 of *Anaplasma phagocytophilum* facilitates adherence to granulocytes. *Infect Immun* 2003;71:4018–4025. [PubMed: 12819090]

- Park J, Kim KJ, Choi KS, Grab DJ, Dumler JS. *Anaplasma phagocytophilum* AnkA binds to granulocyte DNA and nuclear proteins. *Cellular microbiology* 2004;6:743–751. [PubMed: 15236641]
- Pedra JH, Sukumaran B, Carlyon JA, Berliner N, Fikrig E. Modulation of NB4 promyelocytic leukemic cell machinery by *Anaplasma phagocytophilum*. *Genomics* 2005;86:365–377. [PubMed: 16005178]
- Popov VL, Yu X, Walker DH. The 120 kDa outer membrane protein of *Ehrlichia chaffeensis*: preferential expression on dense-core cells and gene expression in *Escherichia coli* associated with attachment and entry. *Microb Pathog* 2000;28:71–80. [PubMed: 10644493]
- Portnoy DA. Manipulation of innate immunity by bacterial pathogens. *Curr Opin Immunol* 2005;17:25–28. [PubMed: 15653306]
- Rashid-Doubell F, Tannetta D, Redman CW, Sargent IL, Boyd CA, Linton EA. Caveolin-1 and lipid rafts in confluent BeWo trophoblasts: evidence for Rock-1 association with caveolin-1. *Placenta* 2007;28:139–151. [PubMed: 16480767]
- Reneer DV, Kearns SA, Yago T, Sims J, Cummings RD, McEver RP, Carlyon JA. Characterization of a sialic acid- and P-selectin glycoprotein ligand-1-independent adhesin activity in the granulocytotropic bacterium *Anaplasma phagocytophilum*. *Cell Microbiol*. 2006
- Reneer DV, Troese MJ, Huang B, Kearns SA, Carlyon JA. *Anaplasma phagocytophilum* PSGL-1-independent infection does not require Syk and leads to less-efficient AnkA delivery. *Cell Microbiol*. 2008
- Rikihisa Y. The tribe *Ehrlichieae* and ehrlichial diseases. *Clin. Microbiol. Rev* 1991;4:286–308. [PubMed: 1889044]
- Rikihisa Y. Mechanisms to create a safe haven by members of the family Anaplasmataceae. *Ann N Y Acad Sci* 2003a;990:548–555. [PubMed: 12860688]
- Rikihisa, Y. Mechanisms to create a safe haven by members of the family *Anaplasmataceae*. In: Hechemy, KE.; Zvsic-Zupanc, T.; Childs, JE.; Rault, DA., editors. *Ann. N. Y. Acad. Sci. N.Y. Acad. Sci.* New York, NY: 2003b. p. 548-555.
- Rikihisa Y, Zhi N, Wormser GP, Wen B, Horowitz HW, Hechemy KE. Ultrastructural and antigenic characterization of a granulocytic ehrlichiosis agent directly isolated and stably cultivated from a patient in New York state. *J. Infect. Dis* 1997;175:210–213. [PubMed: 8985223]
- Scheel-Toellner D, Wang K, Craddock R, Webb PR, McGettrick HM, Assi LK, Parkes N, Clough LE, Gulbins E, Salmon M, Lord JM. Reactive oxygen species limit neutrophil life span by activating death receptor signaling. *Blood* 2004;104:2557–2564. [PubMed: 15238425]
- Simons K, Toomre D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol* 2000;1:31–39. [PubMed: 11413487]
- Stephenson K, Yamaguchi Y, Hoch JA. The mechanism of action of inhibitors of bacterial two-component signal transduction systems. *J Biol Chem* 2000;275:38900–38904. [PubMed: 10978341]
- Sukumaran B, Carlyon JA, Cai JL, Berliner N, Fikrig E. Early transcriptional response of human neutrophils to *Anaplasma phagocytophilum* infection. *Infect Immun* 2005;73:8089–8099. [PubMed: 16299303]
- Sukumaran B, Narasimhan S, Anderson JF, DePonte K, Marcantonio N, Krishnan MN, Fish D, Telford SR, Kantor FS, Fikrig E. An Ixodes scapularis protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. *J Exp Med* 2006;203:1507–1517. [PubMed: 16717118]
- Sumimoto H, Kage Y, Nunoi H, Sasaki H, Nose T, Fukumaki Y, Ohno M, Minakami S, Takeshige K. Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. *Proc. Natl. Acad. Sci. USA* 1994;91:5345–5349. [PubMed: 8202490]
- Telford SR 3rd, Dawson JE, Katavolos P, Warner CK, Kolbert CP, Persing DH. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. *Proc. Natl. Acad. Sci. USA* 1996;93:6209–6214. [PubMed: 8650245]
- Thomas V, Fikrig E. *Anaplasma phagocytophilum* specifically induces tyrosine phosphorylation of ROCK1 during infection. *Cell Microbiol* 2007;9:1730–1737. [PubMed: 17346310]
- Thomas V, Samanta S, Wu C, Berliner N, Fikrig E. *Anaplasma phagocytophilum* Modulates gp91phox Gene Expression through Altered Interferon Regulatory Factor 1 and PU.1 Levels and Binding of CCAAT Displacement Protein. *Infect. Immun* 2005;73:208–218. [PubMed: 15618156]

- Wang T, Malawista SE, Pal U, Grey M, Meek J, Akkoyunlu M, Thomas V, Fikrig E. Superoxide anion production during *Anaplasma phagocytophila* infection. The Journal of infectious diseases 2002;186:274–280. [PubMed: 12134266]
- Wang X, Kikuchi T, Rikihisa Y. Two monoclonal antibodies with defined epitopes of P44 major surface proteins neutralize *Anaplasma phagocytophilum* by distinct mechanisms. Infect Immun 2006;74:1873–1882. [PubMed: 16495562]
- Webster P, IJdo JW, Chicoine LM, Fikrig E. The agent of Human Granulocytic Ehrlichiosis resides in an endosomal compartment. J. Clin. Invest 1998;101:1932–1941. [PubMed: 9576758]
- Winslow GM, Yager E, Shilo K, Volk E, Reilly A, Chu FK. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. Infect Immun 2000;68:2187–2195. [PubMed: 10722619]
- Xiong Q, Bao W, Ge Y, Rikihisa Y. *Ehrlichia ewingii* infection delays spontaneous neutrophil apoptosis through stabilization of mitochondria. J Infect Dis 2008;197:1110–1118. [PubMed: 18462160]
- Xiong Q, Wang X, Rikihisa Y. High-cholesterol diet facilitates *Anaplasma phagocytophilum* infection and up-regulates macrophage inflammatory protein-2 and CXCR2 expression in apolipoprotein E-deficient mice. J Infect Dis 2007;195:1497–1503. [PubMed: 17436230]
- Yoshiie K, Kim HY, Mott J, Rikihisa Y. Intracellular infection by the human granulocytic ehrlichiosis agent inhibits human neutrophil apoptosis. Infect. Immun 2000;68:1125–1133. [PubMed: 10678916]
- Zhang JZ, Sinha M, Luxon BA, Yu XJ. Survival strategy of obligately intracellular *Ehrlichia chaffeensis*: novel modulation of immune response and host cell cycles. Infect Immun 2004;72:498–507. [PubMed: 14688131]

Figure 1.

Raft-mediated entry, establishment, and maintenance of intracellular "niches" of *E. chaffeensis*, and requirement for infection and accompanying cellular changes. The vacuolar membrane fuses with Tf-TfR endosomes. At the same time lysosomal fusion with vacuolar membrane is inhibited. Blue lining represents the raft. Short vertical arrows in white represent up regulation or down regulation of molecules or events. This figure does not indicate that these proteins and events are present in the same cell at the same time.