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***Anaplasma phagocytophilum*: deceptively simple or simply deceptive?**

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Abstract

Anaplasma phagocytophilum is an obligate intracellular rickettsial pathogen transmitted by ixodid ticks. This bacterium colonizes myeloid and nonmyeloid cells and causes human granulocytic anaplasmosis – an important immunopathological vector-borne disease in the USA, Europe and Asia. Recent studies uncovered novel insights into the mechanisms of *A. phagocytophilum* pathogenesis and immunity. Here, we provide an overview of the underlying events by which the immune system responds to *A. phagocytophilum* infection, how this pathogen counteracts host immunity and the contribution of the tick vector for microbial transmission. We also discuss current scientific gaps in the knowledge of *A. phagocytophilum* biology for the purpose of exchanging research perspectives.

***Anaplasma phagocytophilum*: life on the inside**

From the Greek *an*, which means ‘without’, and *plasma*, ‘anything formed or molded’, the bacterium *Anaplasma phagocytophilum* represents a rickettsial pathogen of both veterinary and medical interest that is still unfamiliar to many microbiologists. This bacterium was first described in the early 1930s as *Rickettsia phagocytophila* infecting sheep. Later, it was renamed as *Cytoecetes phagocytophila*, *Ehrlichia phagocytophila*, *Ehrlichia equi* and the human granulocytic ehrlichiosis agent. Approximately a decade ago, its scientific name was once again changed to *A. phagocytophilum* after careful molecular phylogenetic analysis [1]. Currently, *A. phagocytophilum* belongs to the order Rickettsiales and the family Anaplasmataceae. This group of bacteria is confined within host membrane-bound compartments and includes both pathogenic and nonpathogenic obligate intracellular bacteria, such as *Anaplasma* spp., *Ehrlichia* spp., *Neorickettsia* spp. and *Wolbachia* spp. (Figure 1).

A. phagocytophilum is a small Gram-negative bacterium approximately 0.4–1.3 µm in size. *A. phagocytophilum* colonizes neutrophils when infecting mammals; however, it may also infect other cells of myeloid and nonmyeloid origin [1]. Inside ixodid ticks, it is known to

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survive in salivary glands and midgut cells. Two *A. phagocytophilum* morphotypes have been identified using electron microscopy: reticulate and dense core (DC) [2]. Proteomics studies have shown that these morphotypes differ by more than 20% when infecting human promyelocytic leukemia cells (HL-60) [3]. Experimentation indicates that the reticulate morphotype is the noninfectious replicative form of the *A. phagocytophilum* developmental cycle, whereas the DC morphotype has a dense nucleoid, is resistant to environmental changes and infects mammalian cells [3].

In the USA, *Ixodes scapularis* is the most important tick species transmitting *A. phagocytophilum* to humans. Humans are mere *A. phagocytophilum* accidental, or 'dead-end', hosts, and infection by this pathogen leads to the development of a disease called human granulocytic anaplasmosis (HGA) – the third most common tick-borne disease in the USA and Europe and an emerging infectious disease in Asia [4]. HGA clinical and laboratory abnormalities include, but are not limited to: fever, myalgia, headache, malaise, thrombocytopenia, leukopenia, anemia and mild-to-moderate hepatic injury leading to increased aspartate and alanine aminotransferase activity in the serum. The severity of the symptoms varies from asymptomatic to death [4]. Severe complications include septic shock-like syndromes, acute respiratory distress syndrome and opportunistic infections. While most patients have mild clinical signs and symptoms, infection results in hospitalization for 36% of patients, and 7% of clinical cases lead to intensive care unit admission; 0.6% are fatal. Treatment relies on the use of the broad-spectrum antibiotic doxycycline, but illness can evolve to severe and potentially fatal conditions in immunocompromised patients. The underlying causes of these fatal episodes, however, are unknown and misdiagnosis remains a common occurrence.

A. phagocytophilum also infects other mammalian hosts. Dogs, horses and sheep have been considered good animal models to understand HGA, as they show clinical symptoms similar to humans. Mice have also been widely studied, and have permitted researchers to properly identify and better understand how *A. phagocytophilum* successfully invades and proliferates inside host cells, causing a systemic disease. Mouse studies have also helped to uncover immunological processes during infection and how this unusual pathogen colonizes and is transmitted by ticks in nature. However, mice do not develop clinical signs of disease, despite mimicking inflammatory histopathological lesions similar to those observed in humans [4]. This review will primarily focus on the molecular and cellular events that lead to *A. phagocytophilum* pathogenesis, immunity and microbial transmission by ixodid ticks.

***A. phagocytophilum* host tropism**

There is abundant evidence suggesting that *A. phagocytophilum* strains are ecologically distinct and have diverse host tropisms [5,6]. Clinical hosts such as humans, sheep, horses and dogs may be acutely infected by some *A. phagocytophilum* strains, whereas cattle fail to induce disease when infected with a strain from equines [7]. Furthermore, distinct subpopulations of *A. phagocytophilum* coexist in separate enzootic cycles [8] and chronic disease occurs in some rodents and sheep, whereas humans and horses develop an acute self-limited infection [6]. The mechanisms that enable host tropisms during *A. phagocytophilum* infection remain mostly elusive. It has been shown that the *A. phagocytophilum* variant-1 strain (Ap-V1) differs from a human strain (Ap-ha) by a two-base pair substitution in the 16S rRNA sequence [9]. Phylogenetic analysis has also grouped clinical and nonclinical isolates in distinct clades based on *p44ESup1*, 23S-5S rRNA intergenic spacer, *ank* and *groESL* gene divergence [5,10].

A. phagocytophilum host tropism may also be associated with immune evasion via the *p44/msp2* gene family. The *A. phagocytophilum* genome possesses 113 *p44/msp2* loci with

truncated or short 5' or 3' fragments, several of which appear to function as donor sequences for conversion at the dominant expression locus. The *p44/msp2* family is composed of outer membrane glyco-proteins that may be used for a wide range of biological processes, such as antigenic variation, host adaptation, bacterial adhesion, structural integrity and porin activity [1]. The *p44/msp2 A. phagocytophilum* family lacks the RecBCD recombination pathway and uses the RecF pathway at a single expression locus for homologous recombination. The selection pressure affecting antigen variation in the *A. phagocytophilum p44/msp2* gene family most likely is due to random genetic drift [11]. This feature allows *A. phagocytophilum* to avoid the host immune response, contributing to its persistence within the intracellular environment [12]. Regulation of *A. phagocytophilum p44/msp2* genes is mostly unknown. However, the *A. phagocytophilum* gene encoding the DNA-binding protein ApxR is autoregulated and transactivates the promoter regions of the *p44E* locus [13].

***A. phagocytophilum* genomics & host regulation**

The *A. phagocytophilum* HZ strain has a genome size of 1.47 Mb, comprising approximately 12% of repetitive sequences. The *A. phagocytophilum* genome contains approximately 1300 open reading frames, most of which encode housekeeping genes [14]. Although this bacterium does not carry ATP/ADP translocase or cytochrome d-type oxidase genes, it does contain a partial glycolysis pathway. It is also capable of synthesizing all nucleotides and most vitamins and cofactors, but only four amino acids [14]. Interestingly, *A. phagocytophilum* lacks genes necessary for the synthesis of lipopolysaccharide (LPS) or peptidoglycans, which makes this pathogen very susceptible to mechanical stresses, such as sonication, freezing, thawing and osmolarity changes [1]. *A. phagocytophilum* does not produce cholesterol. Instead, cholesterol from the mammalian host is 'hijacked' to promote membrane stability, growth and survival. Treatment of *A. phagocytophilum* with methyl- β -cyclodextrin, a cholesterol extraction reagent, causes bacterial ultrastructural changes and inhibits infection of leukocytes [1]. Consistent with these findings, a high-cholesterol diet facilitates *A. phagocytophilum* infection [15], and perilipin, a phosphoprotein that plays a central role in lipolysis and cholesterol synthesis, is important for *A. phagocytophilum* colonization of mammalian cells [16].

Cholesterol is acquired by *A. phagocytophilum* from the low-density lipoprotein-mediated uptake pathway and not by *de novo* synthesis [17]. Proteins known as sterol regulatory element-binding proteins are transcription factors involved in regulating cholesterol-mediated feedback to maintain appropriate cholesterol homeostasis. Sterol regulatory element-binding proteins do not respond to the increase in cholesterol during *A. phagocytophilum* infection. Rather, there is a post-transcriptional mechanism that regulates low-density lipoprotein receptor expression in human HL-60 cells. This causes cholesterol to accumulate in the vertebrate host, which in turn facilitates *A. phagocytophilum* replication inside cells. Recently, Rikihisa and Xiong demonstrated the involvement of the NPC1 pathway in *A. phagocytophilum* cholesterol capture and membrane generation [18]. NPC1 is an endosomal transmembrane protein involved in the cellular transport of cholesterol. NPC1 is sequestered to *A. phagocytophilum* inclusions and siRNA studies point to the requirement of NPC1 for membrane inclusion homeostasis during pathogen infection.

A. phagocytophilum uses a type IV secretion system (T4SS), which is an ATP-dependent system to secrete proteins or DNA from the bacteria to the eukaryotic cell. Expression of the T4SS in *A. phagocytophilum* is tightly regulated to allow secretion of specific substrates that affect the host cell metabolism. *A. phagocytophilum* T4SS is composed of *virB* genes and this pathogen has up to eight distinct copies [1]. The *A. phagocytophilum*-infected ISE6 and HL-60 cells have been shown to have differential transcription of *virB2* homologs [19].

To date, only two T4SS effector molecules have been identified: AnkA and Ats-1. AnkA binds to a variety of molecules within the cell, including, but not limited to, genes that encode proteins with ATPase, tyrosine phosphatase and NADH dehydrogenase-like functions [20,21]. *A. phagocytophilum* infection stimulates phosphorylation of AnkA tyrosines, which then interact with the host tyrosine phosphatase, SHP-1, possibly by binding to DNA or protein after nuclear translocation [22]. AnkA phosphorylation is mediated by two host interacting proteins: Abi-1 and the Abl-1 tyrosine kinase. AnkA and Abl-1 are crucial for *A. phagocytophilum* infection, as depletion of AnkA enzymatic activity by antibody binding or silencing of Abl-1 inhibits pathogen colonization of mammalian cells [23]. Conversely, Ats-1 was identified in a targeted screening for effectors of the *A. phagocytophilum* T4SS [24]. Ats-1 translocates five membranes in a receptor-dependent manner to reach the mitochondria. Ats-1 inhibits etoposide-induced cytochrome c release and PARP cleavage – two features often associated with apoptosis [24].

***A. phagocytophilum* binding & colonization**

During the tick bite, *A. phagocytophilum* gains access to the bloodstream and soon reaches the intracellular environment necessary for its replication and host colonization. Besides infecting circulating leukocytes, the presence of *A. phagocytophilum* has also been linked to endothelial cells [25], and it has been speculated that infecting the endothelium may serve as an initial step after *A. phagocytophilum* transmission and before granulocyte infection. *In vitro* studies using human microvascular epithelial cells (HMECs) demonstrated that *A. phagocytophilum* can invade and grow within HMEC-1 cells and transfer from these cells to neutrophils when cocultivation is allowed. This model has been suggested because *A. phagocytophilum* upregulates the protein ICAM-1 in infected HMEC-1 cells, which is involved in leukocyte adhesion [26]. ICAM-1 also binds to ligands used by granulocytes to roll on inflamed endothelium, such as PSGL-1 [27]. Additionally, this pathogen induces the release of IL-8 from human neutrophils. This chemokine recruits neutrophils to the site of infection, which can be targets of microbial invasion and further propagation [28]. *A. phagocytophilum* binding also decreases neutrophil migration and diapedesis on inflamed endothelium [29], which may, in turn, inhibit inflammation signaling and facilitate the establishment of *A. phagocytophilum* inside a mammalian host.

A. phagocytophilum binding to HL-60 cells results in activation of the PSGL-1 signaling pathway, leading to phosphorylation of ROCK1 by Syk (Figure 2) [30]. ROCK1 is a serine/threonine kinase that regulates actin organization. Therefore, it has been speculated that actin reorganization through ROCK1 activation could facilitate *A. phagocytophilum* invasion of these cells. Moreover, *A. phagocytophilum* entry requires signaling platforms, such as lipid rafts and caveolin-1. These molecular structures colocalize with early inclusions of *A. phagocytophilum* in HL-60 cells [31]. Their role in entry and infection, however, is elusive. Clathrin is dispensable for *A. phagocytophilum* internalization, whereas glycosylphosphatidylinositol-anchored proteins and flotillin 1 have been found to be necessary for *A. phagocytophilum* binding to mammalian host cells [31]. The signaling cascades triggered downstream of these events remain poorly understood.

Different research groups have illustrated the complexity of *A. phagocytophilum* binding and colonization by employing different mammalian model systems. An example is the use of tetrasaccharide sialyl Lewis^x (sLe^x) present on PSGL-1 by *A. phagocytophilum*, which is required for human neutrophil infection [32,33]. Similarly, *A. phagocytophilum* infection of a megakaryocytic human cell line (MEG-01) depends on sialylated ligands and PSGL-1 [34]. Conversely, *A. phagocytophilum* uses α -1,3-fucosylation but not PSGL-1 for infection of murine neutrophils [35]. These differences in PSGL-1 requirements for humans and mice are likely due to a short amino acid sequence found in the N-terminal region of human but

not murine PSGL-1 [36]. Similarly, sialylated glucans are not required for endothelial cell infection [26], and *A. phagocytophilum* strains may use PSGL-1-dependent and -independent routes to infect myeloid cells [37]. Carlyon and colleagues have reported the enrichment for *A. phagocytophilum* organisms that do not rely on sialic acid for cellular adhesion and entry. They have shown that the selected bacteria exhibit decreased dependency on PSGL-1 and α 1,3-fucose structures [37,38]. They also showed that PSGL-1-independent infection by *A. phagocytophilum* does not require the protein Syk and leads to less efficient AnkA delivery in mammalian cells [39]. By adding antibodies to the culture medium, Mastronunzio *et al.* suggested that the DC morphotype protein APH_1235 is also involved in *A. phagocytophilum* infection but the precise role of this molecule remains unknown [40].

The vacuole where *A. phagocytophilum* is found is not entirely isolated from the host cell; instead, *A. phagocytophilum* recruits molecules associated with membrane trafficking in order to camouflage and attain the nutrition required for pathogen survival. In fact, *A. phagocytophilum* itself has 41 genes with functions associated with protein binding and transport [14]. Moreover, human proteins associated with cytoskeleton, trafficking, signaling and energy metabolism were shown to be upregulated in HL-60 cells infected with *A. phagocytophilum* when compared with noninfected cells [31], indicating that this microorganism interferes with host vesicular trafficking to persist in vacuoles. These membrane-bound inclusions lack major endosomal or lysosomal markers and Rikihisa and colleagues have demonstrated that approximately 80% of *A. phagocytophilum* organisms that expressed the VirB9 protein in HL-60 cells colocalized with LAMP-1. On the other hand, bacteria that did not express VirB9 did not colocalize with LAMP-1 [41].

A. phagocytophilum morulae or microcommunities have several hallmarks of autophagosomes, including a double lipid bilayer and colocalization with LC3 and Beclin-1, ATG8 and ATG6 [42]. *A. phagocytophilum* employs Rab GTPases associated with recycling endosomes that appear to facilitate pathogen survival [43]. Recently, proteins that associate with the *A. phagocytophilum*-occupied vacuolar membrane were described. The protein APH_1387 accumulates on the *A. phagocytophilum*-occupied vacuolar membrane during mammalian and tick cell colonization [44]. Similarly, the protein APH_0032 localizes to the vacuolar membrane but it is not secreted by the *A. phagocytophilum* T4SS [45]. Interestingly, monoubiquitinated proteins decorate the occupied vacuolar membrane during *A. phagocytophilum* infection of mammalian and tick cells [46]. These findings suggest that *A. phagocytophilum* actively modulates the host cell-derived vacuolar membrane.

***A. phagocytophilum* & humans: just an accident**

How does HGA happen?

Human outdoor activities, especially during the warmer spring and summer months, may lead to tick exposure and *A. phagocytophilum* infection. If tick feeding and pathogen transmission take place successfully, symptoms such as fever, chills, headache and muscle aches may occur [4]. The underlying events that lead to disease manifestation are not well understood. A remarkable feature of HGA is that this illness does not result from direct pathogen load, but from host-derived immunopathology. Furthermore, neutrophils do not seem to play a major role in *A. phagocytophilum* immunity, as these cells are not efficient in clearing *A. phagocytophilum* infection, have their signaling pathways modulated by *A. phagocytophilum*, and are significantly decreased during infection. Monocytes and macrophages, on the other hand, play a critical role in combating *A. phagocytophilum* infection. Dumler and colleagues have described that *A. phagocytophilum* triggers proinflammatory responses in macrophages via NF- κ B in primary murine macrophages

through the TLR2 [47]. Consistent with this, Rikihisa and Kim have also shown that *A. phagocytophilum* transduces different signals between neutrophils and monocytes for cytokine generation [48].

Decreased bone marrow function and changes in hematopoietic progenitor and peripheral blood cells in the spleen have been described in acute infection with *A. phagocytophilum* [49]. This has been associated with aberrant CXCL12/CXCR4 signaling and hematopoietic stem cell mobilization [50]. Studies characterizing cytokine response to *A. phagocytophilum* infection indicate that the response favors the Th1 phenotype. In addition, cytokine secretion varies according to the type of cell investigated, the sensitivity of the technique adopted during experimentation, and the approach used during pathogen infection. For example, Klein *et al.* did not report any measurable IL-1, IL-6 or TNF- α secretion in culture supernatants from HL-60 cells or cells enriched for marrow progenitors at any of the time points assayed [51]. Conversely, Johns *et al.* reported TNF- α and IL-6 secretion after *A. phagocytophilum* stimulation of bone marrow cells in an *ex vivo* model system [49].

In the early phase of infection, IL-12/23p40 regulates CD4⁺ T cells, while IL-12/23p40-independent mechanisms contribute to pathogen elimination from the host [52]. IL-18 produced by the inflammasome, a protein scaffold associated with the inflammatory process, also regulates CD4⁺ T-cell responses [53]. Mice vaccinated with *A. phagocytophilum* or protected by passive immunization also become refractory to *A. phagocytophilum* infection, suggesting that antibodies may participate in *A. phagocytophilum* immunity [54]. Expression of Toll-like receptors or *myd88* remains unaltered during *A. phagocytophilum* infection of neutrophils [55], and JNK2 inhibits production of IFN- γ by NK T cells upon *A. phagocytophilum* challenge in mice [56]. IFN- γ seems to play a role in controlling *A. phagocytophilum* infection and immunopathology. In IFN- γ -knockout mice, bacterial levels in the tissues are increased in the early phase of infection, but tissue damage is absent and bacteria are eventually eliminated. The same study described increased lesions in IL-10-knockout mice, which showed normal levels of IFN- γ [57].

Hepatic histopathology upon infection was dependent on IFN- γ produced by NK, and to a lesser extent, NK T cells [58]. Corroborating these findings, lipoprotein or glycolipid components of *A. phagocytophilum* membranes stimulate innate immune cell proliferation [59]. In addition, *cd1d*-knockout mice had a higher pathogen load at day 2 than the wild-type animals. Injection of α -galactosylceramide, a strong NK T-cell agonist, also increased IFN- γ release and protected mice from *A. phagocytophilum* [56]. Mice deficient in TLR2, TLR4, iNOS, MyD88, TNF and NADPH oxidase have been studied, and they are all capable of clearing *A. phagocytophilum* infection [60]. However, these innate immune molecules seem important for host-derived immunopathology [61]. CD11b and CD18, on the other hand, are crucial for bacterial clearance because infection of CD11b/CD18-knockout mice leads to an increase in bacterial load when compared with wild type mice [62]. Furthermore, the NLRC4, but not the NLRP3 inflammasome, is partially required for *A. phagocytophilum* host response *in vivo* [53]. Taken together, a concerted effort by several research groups have illustrated that *A. phagocytophilum* immunity is complex and multifactorial.

Downregulation of oxidative & inflammatory responses

Microarray analysis in neutrophils, together with proteomic analysis in HL-60 cells, indicated that genes and proteins involved in innate immunity and inflammation have their expression modulated by *A. phagocytophilum* infection [1]. Neutrophils are the most abundant type of phagocyte and the major mediator of the respiratory burst activated upon exposure to pathogens. *A. phagocytophilum* does not carry genes involved in detoxification and does not induce reactive oxygen species when infecting murine or human neutrophils

[1]. Moreover, this pathogen inhibits mRNA expression of gp91^{phox} (also known as NOX2) and decreases p22^{phox} protein levels, while leaving other components of this system unaffected in human neutrophils [1]. Once infected with *A. phagocytophilum*, neutrophils become refractory to stimuli such as LPS and phorbol myristate acetate [63,64], but this active inhibition is not seen in human monocytes [65]. In fact, *A. phagocytophilum* is easily killed when exposed to reactive oxygen species and this may explain why this pathogen does not infect circulating monocytes. In HL-60 cells, *A. phagocytophilum* prevents the assembly of NADPH oxidase subunits, and also downregulates NOX2 and surface protein levels [1]. Downregulation of NOX2 has been associated with the production of AnxA by *A. phagocytophilum*, which has been shown to bind to the CYBB/NOX2 locus [20,21]. Activation of nuclear cathepsin L and enhanced binding of CDP have also been described during *A. phagocytophilum* infection of neutrophils [66]. Furthermore, *A. phagocytophilum* minimizes the release of proinflammatory cytokines in human peripheral blood and HL-60 cells [51]. Inhibition of TNF- α , IL-6 and IL-13 was reported in *A. phagocytophilum*-infected mast cells [67], suggesting that mitigation of mast cell activation can also contribute to *A. phagocytophilum* subversion of host defenses. Chromatin modifications within the host cell have been linked to host gene transcription during *A. phagocytophilum* infection, and gene expression can also be regulated through histone acetylation. Histone modifying enzymes, such as histone deacetylases, maintain histone modification patterns. The upregulation of histone deacetylases together with the epigenetic silencing of host cell defense genes have been described as a requirement for *A. phagocytophilum* infection of THP1 cells [68].

Subversion of host apoptosis & autophagy

Neutrophils generally have a very short half-life, which makes it surprising that *A. phagocytophilum* would find them suitable to inhabit. To survive in a hostile environment, such as inside neutrophils, intracellular pathogens such as *A. phagocytophilum* are prompted to interfere with host cell apoptosis. *A. phagocytophilum* inhibits neutrophil apoptosis long enough to develop the morula [1]. *A. phagocytophilum* infection upregulates expression of anti-apoptotic *bcl-2* genes, blocks cell surface Fas clustering during spontaneous neutrophil apoptosis and inhibits cleavage of procaspase 8 and caspase 8 activation [1]. Inhibition of Bax translocation into the mitochondria, in addition to activation of caspase 9 and degradation of XIAP, a caspase inhibitor, have also been reported [1]. As previously mentioned, Ats-1 is secreted by the *A. phagocytophilum* T4SS and prevents mitochondria from responding to apoptotic signals. Autophagy works in synchrony with the host immune response owing to its role in clearing intracellular infections. *A. phagocytophilum* inclusions display a range of autophagosome markers and do not mature into autolysosomes. Indeed, *A. phagocytophilum* infection is favored by treatment with rapamycin, an autophagy inducer, but treatment with 3-methyladenine, which inhibits autophagy, reversibly arrests *A. phagocytophilum* growth without preventing pathogen internalization. This indicates that *A. phagocytophilum* infection is aided by subverting autophagy [42]. Taken together, *A. phagocytophilum* manipulates host cell machinery to induce autophagy and cytoplasmic recycling for its own development.

Activation of protein kinases

The function of one *A. phagocytophilum* sensor kinase (PleC) and one response regulator (PleD) have been recently described during cellular infection [69]. The expression of PleC and PleD was increased during *A. phagocytophilum* exponential growth and was inhibited prior to extra-cellular release. Recombinant PleD has diguanylate cyclase activity and generates c-di-GMP. c-di-GMP is essential for *A. phagocytophilum* colonization of mammalian cells, as the c-di-GMP derivative, 2'-O-di(tertbutyldimethylsilyl)-c-di-GMP, inhibited *A. phagocytophilum* infection in HL-60 cells [69]. This work was the first

description of a functional c-di-GMP signaling pathway in an obligate intracellular pathogen and it suggests that c-di-GMP may be important not only for *A. phagocytophilum* morulae formation, but also human infection. *A. phagocytophilum* infection also activates protein kinase pathways in the mammalian host. The p38 MAP kinase, the PKC, PKA and PTK were deemed important for proinflammatory cytokine secretion in monocytes during *A. phagocytophilum* stimulation [48]. Conversely, the p38 MAP kinase seems important for apoptosis inhibition during *A. phagocytophilum* infection of neutrophils, as a p38 MAP kinase antagonist increased neutrophil apoptosis during *A. phagocytophilum* infection [70].

Neutrophil apoptosis is also regulated by the PI3K/AKT signaling pathway. Sarkar *et al.* showed that *A. phagocytophilum* activates the PI3K/AKT pathway, which in turn regulates the expression of the anti-apoptotic protein Mcl-1 [71]. The PI3K/AKT pathway also triggers NF- κ B activation during pathogen infection of neutrophils and promotes IL-8 secretion in an autocrine manner. This process seems dependent on the E3 ubiquitin ligase cIAP2, as cIAP2 overexpression was observed during *A. phagocytophilum* infection of neutrophils. Finally, the ERK pathway is activated by *A. phagocytophilum* in neutrophils. Fikrig and colleagues used a yeast surrogate model and determined that the virulence molecule named AptA activates Erk1/2 phosphorylation and colocalizes with the intermediate filament protein vimentin [72]. The importance of Erk1/2 phosphorylation for *A. phagocytophilum* infection was independently confirmed by another group. Xiong and Rikihisa showed that the prenylation inhibitor manumycin A inhibited *A. phagocytophilum* infection of mammalian cells. Furthermore, manumycin A treatment reduced ERK activation in *A. phagocytophilum*-infected host cells [73].

The tick interface: so close but yet so far

Tick-borne pathogens have evolved an intimate relationship with their vectors. Nonetheless, the precise coevolutionary history of *Ixodes* spp. and *A. phagocytophilum* remains unclear. Once acquired via blood meal, *A. phagocytophilum* reaches the gut and later migrates to the salivary glands allowing transmission and continuity of its life cycle. This is only possible owing to an orchestrated pattern of gene expression regulating pathogen development and vector physiology. In order to survive and perpetuate this cycle, *A. phagocytophilum* not only has to control expression of its own genes, but it must also alter gene expression in ticks. Transcription profiling of *A. phagocytophilum* during tick infection shows a possible role for *virB2* genes. These genes code for a surface-exposed pilus and are part of the *A. phagocytophilum* T4SS. By using tiling arrays, Munderloh and colleagues have found that *virB2* genes show human or tick cell-dependent differential transcription [19]. Moreover, *A. phagocytophilum* has human- and tick-specific operons and paralogs, such as for the major surface proteins *p44/msp2* [19].

A recent postgenomic approach revealed that *A. phagocytophilum* transcription and translation are more active than replication during tick transmission [40]. Cell surface proteins and the virulence factors AnkA and AptA also appear to be highly induced in tick salivary glands during *A. phagocytophilum* transmission [40]. Another study analyzed the *A. phagocytophilum* expression profile during infection of ISE6 cells and found that this pathogen clearly modulates tick gene expression [74]. *A. phagocytophilum* morulae can be individually detected in HL-60, but not in ISE6 cells, in which *A. phagocytophilum* appears enlarged and pleomorphic [74]. These findings underscore the existence of specific adaptations to divergent hosts and suggest that this bacterium uses different strategies to colonize tick and mammalian cells. The P11 protein was recently shown to be required for *A. phagocytophilum* migration from hemocytes to the salivary glands in ticks (Figure 3) [75]. Another molecule affected by *A. phagocytophilum* infection of ticks is the salivary gland protein SALP16. *A. phagocytophilum* upregulates *salp16* to survive within the tick

vector [76] and alters the monomeric:filamentous actin ratio leading to translocation of phosphorylated/G-actin to the nucleus [77]. This selectively regulates *salp16* gene transcription in association with RNAPII and the TATA-binding protein. Strikingly, *A. phagocytophilum* failed to induce actin phosphorylation in primary cultures of human neutrophils, suggesting that this phenomenon is specific for the arthropod vector.

When in nature, ticks often have to survive extreme conditions, such as low humidity and temperatures. Fikrig and colleagues demonstrated that *A. phagocytophilum* appears to increase the ability of *I. scapularis* to survive in cold temperatures by upregulating an antifreeze glycoprotein [78]. α 1,3-fucosyltransferases are also upregulated in ticks during *A. phagocytophilum* infection. When α 1,3-fucosyltransferases are silenced *in vivo*, *A. phagocytophilum* is less efficient at colonizing ticks as demonstrated by a decrease in pathogen acquisition during feeding. However, pathogen transmission was unaffected, indicating that *A. phagocytophilum* uses α 1,3-fucose specifically upon acquisition [79]. On the other hand, the tick salivary protein subolesin was downregulated during *A. phagocytophilum* infection of *I. scapularis* nymphs, but the same was not observed in ISE6 cells. Additionally, vaccination against subolesin was protective against tick infection [80]. The mechanism by which subolesin contributes to *A. phagocytophilum* pathogenesis is still unresolved.

Conclusion

A. phagocytophilum has emerged as an important public health tick-borne pathogen in the USA, Europe and Asia. *A. phagocytophilum* is a rickettsial pathogen that has evolved a remarkable ability to colonize and replicate inside tick and mammalian cells. *A. phagocytophilum* survival has been facilitated by the deletion of LPS and peptidoglycan genes, acquisition of a cholesterol uptake pathway to support membrane integrity, expansion of outer-membrane proteins and the presence of a T4SS. When transmitted to humans by a tick bite, this bacterium may cause HGA. While adaptive immune responses direct pathogen clearance, innate immunity contributes to HGA pathology. The underlying mechanisms that control this dichotomy are still elusive and controversies among different groups call for further experimentation. In the mammalian host, *A. phagocytophilum* subverts neutrophil apoptosis and autophagy, and it also inhibits oxidative and inflammatory responses to promote its persistence. With the same intent, *A. phagocytophilum* modulates gene expression in the ixodid tick vector, thus increasing tick survival in cold temperatures. This leads to the principle that complex and distinct mechanisms are used by this pathogen to perpetuate its cycle despite having a small genome size and an obligate intracellular life.

Future perspective

An extensive characterization of *A. phagocytophilum* biology has been made in the past 10 years. This is primarily due to a combined effort made by rickettsiologists, physicians, epidemiologists and entomologists. Yet, significant challenges lie ahead to fully understand how *A. phagocytophilum* triggers pathogenesis and immunity. For example, it is mostly unclear which immune cells contribute to *A. phagocytophilum* immunopathology. It is known that neutrophils are the main site of pathogen colonization. However, other immune cells such as macrophages and monocytes seem to play a larger role in the onset of disease. How *A. phagocytophilum* manages to colonize mammalian cells and arthropod vectors remains poorly understood. Genetic manipulation of *A. phagocytophilum* associated with noninvasive, real-time imaging technology will offer new insights into pathogen trafficking. Likewise, the use of *A. phagocytophilum* mutants should uncover novel biochemical pathways necessary for *A. phagocytophilum* survival in both the arthropod vector and the mammalian host. In addition, the role of host microbiota, chromatin dynamics, autophagy,

noncoding RNAs, ubiquitination and intermediary metabolism during *A. phagocytophilum* transmission and acquisition remains mostly unknown. Finally, how the tick immune system responds to *A. phagocytophilum* infection has not been thoroughly studied. Considering that the tick immune system manages vector competence, considerable effort should be applied to this research area.

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Website

101. Phylogenetic Web Repeater. <http://power.nhri.org.tw/power/home.htm>

Executive summary

***Anaplasma phagocytophilum*: life on the inside**

- *Anaplasma phagocytophilum* is an intracellular bacterium that replicates in membrane-bound vacuoles and causes a disease called human granulocytic anaplasmosis.
- *A. phagocytophilum* uses a type IV secretion system and AnkA and Ats-1 are the only type IV secretion system substrates identified to date.
- *A. phagocytophilum* strains have host tropism.
- Cholesterol biosynthesis, trafficking and signaling are regulated during infection with *A. phagocytophilum* and promote colonization of human cells.

***A. phagocytophilum* & humans: just an accident**

- Neutrophils are not efficient in clearing *A. phagocytophilum* infection, and innate immune responses mediate human granulocytic anaplasmosis pathogenesis.
- Adaptive immunity represented mostly by CD4⁺ T-cell activation and IFN- γ production controls pathogen eradication.
- To overcome innate immunity, *A. phagocytophilum* downregulates oxidative responses and subverts apoptosis and autophagy.

***A. phagocytophilum*–tick interface: so close but yet so far**

- *A. phagocytophilum* infection of ticks requires P11, SALP16 and α 1,3-fucosylated structures.
- A tick antifreeze glycoprotein is upregulated by *A. phagocytophilum* and increases *Ixodes scapularis* survivorship in the cold.

Conclusion

- Complex and distinct mechanisms are used by *A. phagocytophilum* to colonize ticks and mammalian cells.

Future perspective

- How immunopathology occurs during *A. phagocytophilum* infection remains poorly understood.
- *A. phagocytophilum* virulence machinery needs to be further explored in ticks and mammals.
- The role of host microbiota, chromatin dynamics, autophagy, noncoding RNAs, ubiquitination and intermediary metabolism, in relation to *A. phagocytophilum* pathogenesis and immunity, remains unknown.
- How the tick immune system responds to *A. phagocytophilum* infection should be thoroughly studied.

Figure 1. Anaplasmataceae phylogenetic tree

The order Rickettsiales, family Anaplasmataceae includes bacteria such as *Anaplasma* spp., *Ehrlichia* spp., *Wolbachia* spp. and *Neorickettsia* spp. The Anaplasmataceae phylogenetic tree was built according to a maximum likelihood based on SEQBOOT alignment of 16S rRNA gene sequences utilizing POWER [101]. Accession numbers were obtained from GenBank [81].

Figure 2. *Anaplasma phagocytophilum* modulates the host machinery

Anaplasma phagocytophilum infection of human cells causes IL-8 secretion, which leads to the recruitment of neutrophils. Neutrophil apoptosis is inhibited through degradation of XIAP and dampening of apoptotic caspase function, such as CASP3 and CASP8. The p38 MAP kinase and the PI3K/AKT signaling pathways are involved in this process. ROS production is inhibited by modulating NADPH oxidase assembly and/or regulation of gene expression. The ERK pathway is also affected by this pathogen. PSGL-1 signaling is activated during infection leading to actin reorganization via the molecules Syk and ROCK1. *A. phagocytophilum* entry also requires lipid rafts, caveolin-1, GPI-GAP and flotillin 1. Recently, Ub was shown to decorate the *A. phagocytophilum* vacuole. GPI-GAP: Glycoinositol phospholipid anchored proteins; ROS: Reactive oxygen species; Ub: Monoubiquitination.

Figure 3. *Anaplasma phagocytophilum* manipulates the tick vector for its own benefit

The tick *Ixodes scapularis* pierces the skin using its hypostome. During feeding, *Anaplasma phagocytophilum* alters *I. scapularis* gene expression for colonization, enters the midgut and migrates to the salivary glands via hemocytes. Bioactive molecules, such as P11, bind to *A. phagocytophilum* during hemocyte colonization and facilitate pathogen trafficking to the salivary glands. *A. phagocytophilum* inhibits tick subolesin and modulates the expression of a tick salivary protein named SALP16 for its own survival. *A. phagocytophilum* also induces actin phosphorylation leading to the translocation of phosphorylated G-actin to the nucleus. Upregulation of antifreeze proteins favors tick survival in cold temperatures. When α1,3-fucosyltransferases are silenced by siRNA, *I. scapularis* acquisition of *A. phagocytophilum* is decreased, suggesting that α1,3-fucosylated structures are critical for pathogen colonization.