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Review

Ehrlichia's molecular tricks to manipulate their host cells

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Abstract

Ehrlichia is a large genus of obligate intracellular Gram-negative bacteria transmitted by ticks that cause several emerging infectious diseases in humans and are pathogenic on rodents, ruminants, and dogs. *Ehrlichia* spp. invade and replicate either in endothelial cells, white blood cells, or within midgut cells and salivary glands of their vector ticks. In this review, we discuss the insights that functional studies are providing on how this group of bacteria exploits their host by subverting host innate immunity and hijacking cellular processes.

Keywords: *Ehrlichia*; Bacterial-host interaction; Effectors; T4SS

1. Introduction

Obligate intracellular bacterial pathogens of the species *Ehrlichia* belonging to the *Anaplasmataceae* family in the order Rickttesiales parasitize a wide variety of mammalian reservoir hosts and are responsible for several emerging human infectious diseases [1]. Examples include human monocytic ehrlichiosis, and *Ehrlichia ewingii* ehrlichiosis caused by *Ehrlichia chaffeensis,* and *E. ewingii* respectively [2]. Reported primarily as veterinary pathogens, *Ehrlichia canis* and *Ehrlichia ruminantium* have also been documented in humans [3,4]. Infections with *Ehrlichia* spp. are responsible for a wide range of symptoms that can include fever, headache, myalgias and malaise. Other manifestations such as thrombocytopenia, leukopenia and anemia can occur [5]. These bacteria display the particularity of being able to replicate within two hosts, a mammalian host and a tick vector, and of orchestrating highly sophisticated and complex strategies to persist and then infect their natural hosts [6]. They provide a wealth of information about bacterial adaptation to various environments. Some species are associated with diverse host reservoirs, for example deers are considered important reservoir hosts of *E. chaffeensis* (Fig. 1) [7]. Transmission of these bacteria occurs through the bite of infected ticks [6]. All *Anaplasmataceae* share a similar biphasic developmental cycle involving two morphologically distinct forms (Figs. 2 and 3) [8]. First, the infectious extracellular forms (elementary bodies, EB, or dense cored cells, DC) attach to the surface of host target cells before entering by endocytosis (Fig. 2A and B). Inside the host cells, the bacteria develop within a membrane-bound vacuole where they create a niche for survival and replication. They differentiate into reticulate bodies (RB, or reticulate cells, RC) that divide by binary fission to form a large colony, called morula (Fig. 2C). After a few days, the bacteria redifferentiate into elementary bodies to be released outside the cell and to initiate a new infectious cycle (Fig. 2D) [2]. *Ehrlichia* and *Anaplasma* have evolved diverse genomic features. Concerning genome size, the complete genome of most representative species of these genera have been sequenced and revealed small contracted genomes with an average size of about 1.3 Mb and coding up to about 1200 proteins (Fig. 1) [9–13].

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In this review, we highlight the more recent advances in understanding the molecular mechanisms by which *Ehrlichia* spp. manipulate host cells to facilitate intracellular growth and spread infection, to gain full advantage of host cell properties for their own benefit, and to evade host immune response.

2. Advances in functional genomics

Functional genomic approaches have fostered our understanding of the molecular pathogenesis of *Anaplasmataceae,* despite their obligate intracellular nature. Global transcriptomic studies became powerful tools that provided us with a global view of pathogenic bacteria strategies in response to stimuli. However, a major limitation in gene expression analyses of obligate intracellular bacteria is the low amount of prokaryotic material isolated from host cells and the contamination with eukaryotic mRNA molecules. For *E. ruminantium*-infected host cells, Emboulé et al. developed a method called selective capture of transcribed sequences (SCOTS) for the capture of bacterial mRNAs [14]. Using SCOTS method, transcriptome analysis of *E. ruminantium* revealed overexpressed genes potentially involved in pathogenicity. In particular, a significant increase of expressed genes related to metabolism, nutrient exchange, and defense mechanisms was observed when bacteria are in the replicative vacuole, suggesting that the bacterium may undergo oxidative stress and nutrient starvation during early life cycle stages to develop [15]. Global proteomic studies in *Anaplasma phagocytophilum* and *E. chaffeensis* identified more than 1000 proteins for each species, giving a nice overview of bacterial proteins expressed in infected human cells and of the pathogenesis of these closely related obligate intracellular pathogenic bacteria [16]. Indeed, during growth in human leukocytes, important virulence factors, e.g., Type IV secretion system (T4SS) apparatus, regulatory systems including a diversity of transcriptional regulators, two-component systems were upregulated. Proteins required for central metabolism, protein synthesis and cell envelop were also predominant. This shows that *Ehrlichia* deploys a full arsenal of virulence proteins to replicate and infect host cells. Moreover, those authors

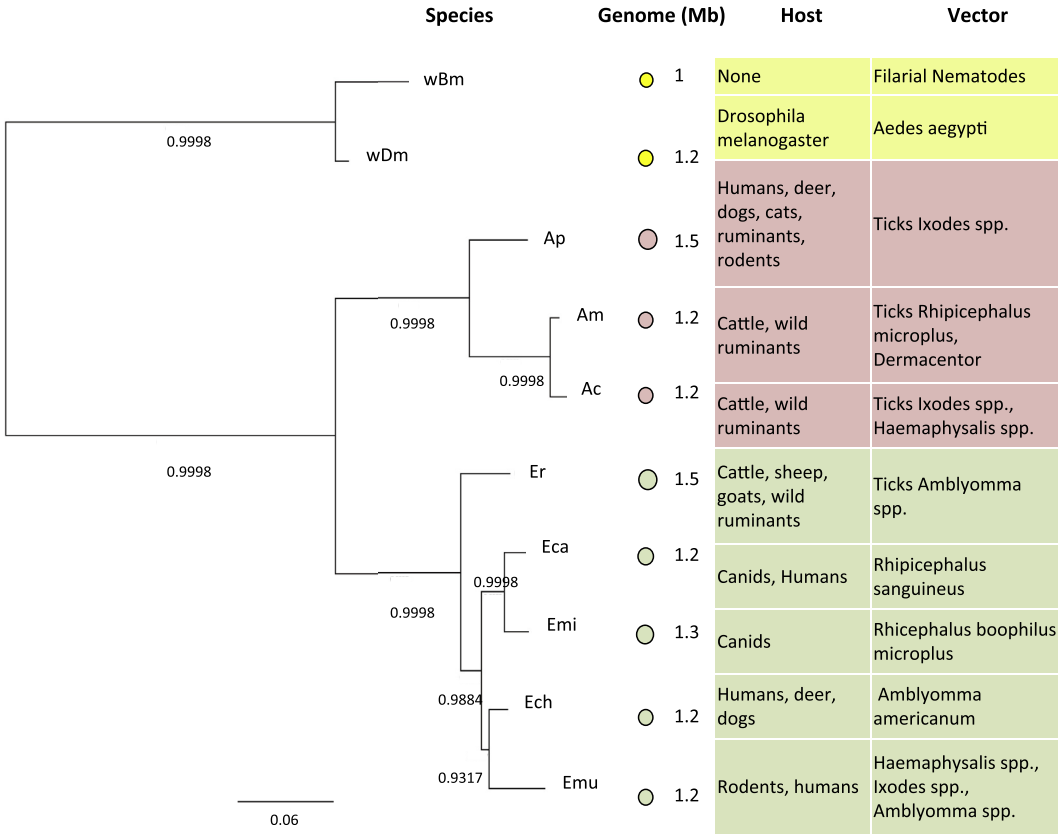

Fig. 1. Maximum likelihood reconstruction of the phylogeny of the most frequently studied strains of Anaplasmataceae based on their 16S rRNA sequences. Each branch is labeled with the species name followed by an icon representing its natural vertebrate host and vector. Abbreviations: Er, *E. ruminantium*; Eca, *E. canis;* Emi, *E. mineirensis*; Emu, *E. muris*; Ech, *E. chaffeensis*; Ap, *A. phagocytophilum*; Am, *A. marginale*; Ac, *A. centrale*; wBm, *Wolbachia from Brugia malayi*; wDm, *Wolbachia endosymbiont of Drosophila melanogaster.* In the "Genome" column, circles represent genomes and plasmids and are proportioned to show their relative sizes. The 16S rRNA sequences of several well studied *Anaplasmataceae* were aligned using the R-Coffee web server [1]. Phylogenetic reconstruction was performed using PhyML using the TPM3uf+I+G, chosen with jModeltest 2.15 [2,3]. [1] Moretti S, Wilm S, Higgins HG, Xenarios I, Notredame C. R-Coffee: a web server for accurately aligning noncoding RNA sequences. Nucleic Acids Res 2008;36: (Web Server issue):W10-3. doi: 10.1093/nar/gkn278. Epub 2008 May 15. [2] Guindon S, Gascuel O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. Systematic Biology 2003;52:696-704. [3] Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 2012;9:772. doi: 10.1038/nmeth.2109.

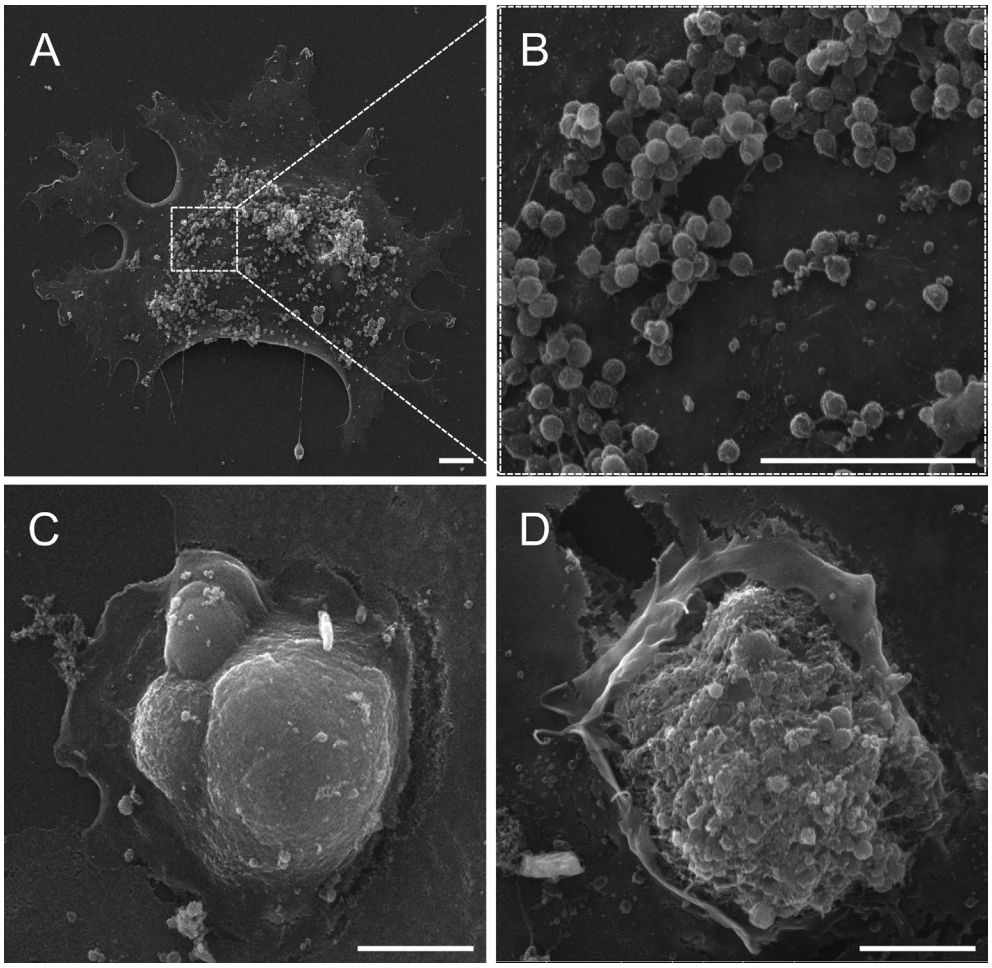

Fig. 2. Scanning electron micrographs of numerous *Ehrlichia ruminantium* infecting a bovine endothelial cell. (A–B) First, *E. ruminantium* elementary bodies attach to the host cell surface. A zoom image of *E. ruminantium* bacteria adhering to host endothelial cell is shown in upper right corner (B). (C) Then, the bacteria get internalized and multiply inside the cell. (D) Infectious elementary bodies are released from infected host cell by complete cell lysis. Scale bar, 5 μm.

were able to define the host protein expression profiles regulated by bacterial infection. A human proteome analysis suggested that *Ehrlichia* interferes with cellular processes including cytoskeleton rearrangement and intracellular trafficking to manipulate host cell gene expression [16]. Very recently, by combining bioinformatics predictions of protein subcellular localizations and proteome analysis of outer membrane fractions from *E. ruminantium* infectious elementary bodies, Moumene et al. identified 18 proteins very likely to be OMPs [17]. Among these 18 proteins, some contributes to cell membrane architecture (proteins of the BAM complex), some are involved in the interaction between bacteria and host cells (Map1 family proteins), and some are known to be virulence factors (T4SS components). In addition, 6 proteins are completely new OMPs and are therefore of importance as potential vaccine antigens [17]. Rapid advances in genetic manipulation of several members of the *Anaplasmataceae* family have facilitated the identification and the characterization of certain virulence factors. A random mutagenesis strategy using the *Himar1* system was successfully tested on *E. chaffeensis* [18]. Such an approach requires selecting appropriate antibiotics, constructing plasmid vectors, and optimizing bacterial transformation methods. The authors observed that mutations in certain hypothetical genes, including Ech_0660, inhibited infection and growth in deer, a natural reservoir of *E. chaffeensis,* identifying possible genes that may be important in the virulence of this organism [18]. This result showed, for the first time in *E. chaffeensis,* a connection between a mutation and its potential role during infection. Very recently, Nair et al. (2015) have also conducted a transposon mutagenesis within the genes Ech_0379 and Ech_0660 confering attenuation in *E. chaffeensis*. These attenuated mutants have been shown to reduce or eliminate the bacteria in animals and confer protection against wild-type infection challenge [19]. Taken together, these findings are promising for the development of vaccines against this pathogenic bacterium. In addition to lead to an attenuated growth in deer, the mutations within these genes and another one (Ech_0230) enhance or reduce gene expression of genes near the insertion sites. Interestingly, the attenuated growth phenotype was also observed in dogs (incendital host) but not in the tick vector. This study shed light on differential regulation of virulence factors essential for *Ehrlichia* pathogenesis [20]. The genetic manipulation of *Ehrlichia* will serve to

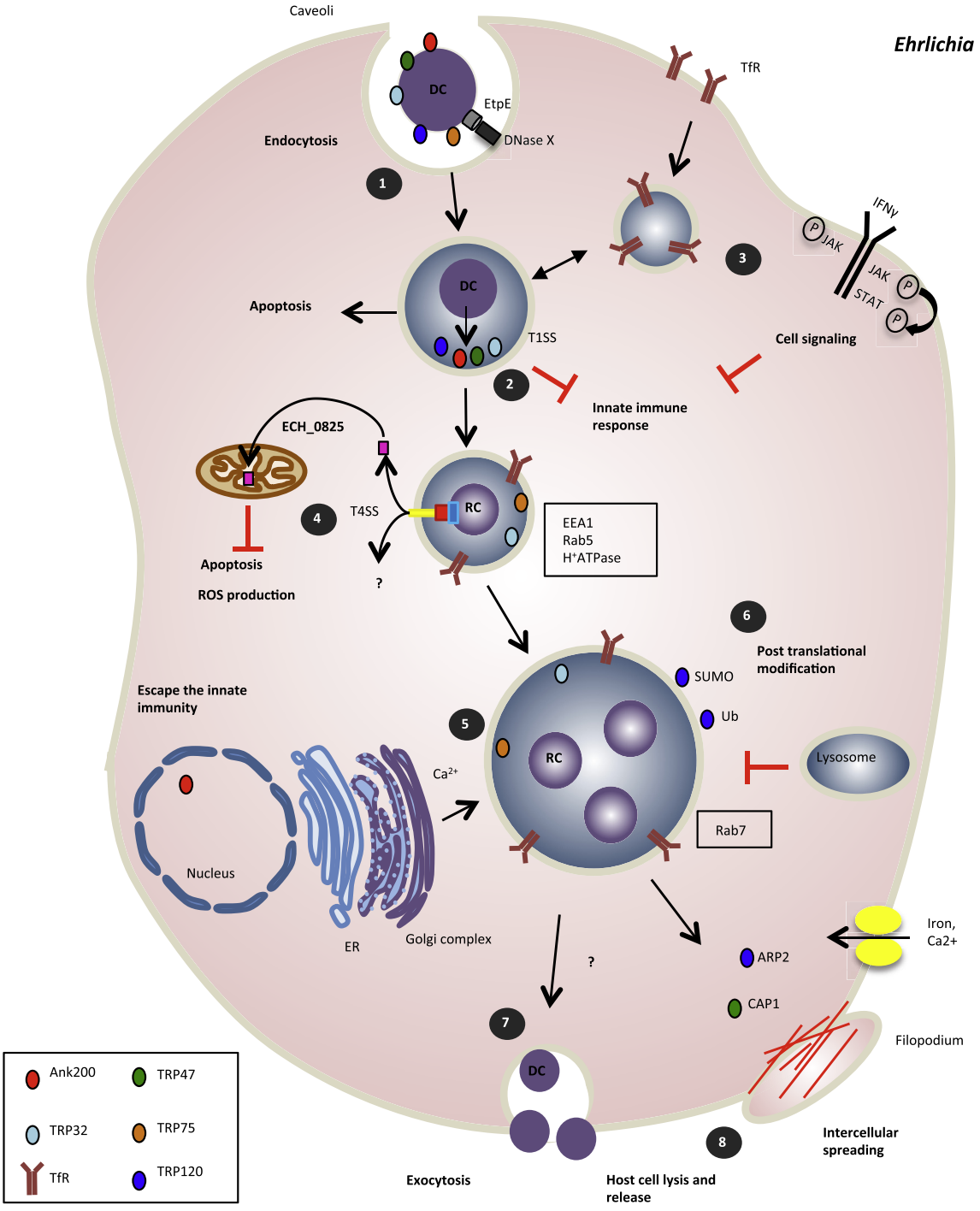

Fig. 3. *Ehrlichia* develops highly adaptative strategies inside host cell. (1) The DC enter and attach to mammalian cells by using EtpE that binds to GPI-anchored protein DNase X. (2) The bacteria replicate in an ECV (*Ehrlichia*-containing vacuole) that resembles early endosomes and secrete T1SS effector proteins (including TRP32, TRP47, TRP120, and Ank200) to escape host innate immune responses. (3) Then, the DC differentiate into RC. At this stage, the bacteria fuse with TfR endosome to acquire iron from the host cell and disrupt cell-signaling pathways like JAK/STAT to prevent innate immune response. (4) At the same time, *Ehrlichia* escapes the lysosomal pathway and secretes ECH_0825, a T4SS effector, to inhibit apoptosis and ROS production. (5) RC divide via binary fission to form microcolonies (morulae). (6) *Ehrlichia* exploits host SUMOylation pathways to mediate TRP120-host interactions to promote intracellular survival. (7) The DC are released by exocytosis or rupture of host cells. (8) *Ehrlichia* spreads to neighboring cells through the host cell filopodium. DC: Dense-cored cells; RC: Reticulate cells; ER: Endoplasmic reticulum; T1SS: Type I secretion system; T4SS: Type IV secretion system; SUMO: small ubiquitin-like modifier; Ub: ubiquitination; JAK: Janus kinase; STAT: signal transducers and activators of transcription; TfR: Transferrin-receptor; CAP1: Adenylate cyclase-associated protein 1; ARP2: Actin-related proteins 2; IFNγ: Interferon gamma; ROS: Reactive oxygen species.

define important genes for pathogenesis studies, to verify the functions of candidate virulence factor genes, and to open the way for other related emerging zoonotic pathogens.

Finally, the development of axenic growth, successful in *Coxiella burnetii* and *Chlamydia trachomatis*, should facilitate genetic analyses of these fascinating obligate intracellular pathogens and may lead to the development of vaccine against *Ehrlichia* diseases [21,22]. Indeed, Martinez et al. isolated 3000 *Coxiella* mutants and revealed a variety of bacterial factors involved in *Coxiella* infection [23]. The transposon mutagenesis screen revealed that mutation of 17 Dot/Icm effectors perturbs the replication of *Coxiella*, suggesting their importance for intracellular growth [23].

3. *Ehrlichia* subversion of host cell responses

Ehrlichia have evolved sophisticated mechanisms to invade and multiply in host tissues. Host recognition and elimination of invading pathogens are essential for the control of bacterial infections. However, *Anaplasmataceae* have developed strategies to subvert host cell processes ranging from host signaling, modulation of vesicular traffic, protection from oxidative burst, acquisition of nutrients, and control of innate immune activation.

3.1. Adhesion and invasion mechanisms

Anaplasmataceae family pathogens deploy a wide range of proteins, including adhesin and invasin, for entry into host cells. Adhesion and entry of *E. chaffeensis* into mammalian cells proceed via the invasin EtpE. This protein was found to be upregulated in DC and the authors observed that monoclonal antibodies raised against EtpE inhibited entry into the host cell, supporting the idea that EtpE may be the appropriate surface protein for adhesion. This outer membrane protein binds to glycosylphosphotidylinositol (GPI)-anchored protein DNase X within caveoli at the monocyte cell surface that signal host cytoskeletal rearrangement and filopodium formation promoting bacterial uptake (Fig. 3). Interestingly, immunization with EtpE has been shown to induce protection in infected mice making EtpE promising vaccine candidate [24]. After internalization, *E. chaffeensis* is contained in vacuoles that develop into early endosomes. They retain several hallmarks such as the early endosome antigen 1 (EEA1) and Rab5A protein, both of which are regulators of vesicular trafficking; the transferrin receptors (TfR) which accumulate during the development of early inclusions and contribute to acquire iron from host cells; and the vacuolar-type H+ ATPase [25,26]. Morever, the interaction between TRP47 and CAP1 participates in vesicle and endocytic trafficking that promotes *E. chaffeensis* infection. A recent study of the protein composition of ehrlichial vacuole revealed that *E. chaffeensis* vacuole exhibits a late endosome characteristic, protein Rab7 and is acidified at pH 5.2 in DH82 cells. This acidification may be necessary for intracellular survival and replication. Thus, *E. chaffeensis* is able to change its vacuolar membrane for efficient development. This process aims to escape fusion with lysosomes (Fig. 3) [27].

3.2. Manipulation of apoptosis

Ehrlichia spp. have evolved a myriad of virulence factors to evade host innate defenses, including apoptosis, to take advantage of the host cells. For instance, *E. ewingii* can inhibit spontaneous apoptosis in host canine neutrophils *in vivo* by stabilizing the mitochondrial membrane [28]. *Ehrlichia* morulae interact with mitochondria to deliver proteins permitting inhibition of mitochondrial activities [29]. Moreover, the type I secretion system substrates Ank200, TRP32, TRP120, and TRP47 play important roles in trafficking, apoptosis and signal transduction during *Ehrlichia* infection (Fig. 3) [30,31]. In particular, TRP47 interacts with CAP1, resulting in inhibition of apoptosis. These TRPs proteins are translocated into the host cell nucleus where they may modify host cell signaling pathways to escape the innate immunity mechanisms [32]. They are differently expressed during the developmental cycle of the bacterium. The expression of TRP32 and TRP75 is constitutive in DC and RC whereas TRP47 and TR120 are expressed only at the late stages of infection [33]. Interestingly, TRP orthologs p120/p140 of *E. chaffeensis* and *E. canis* elicit strong antibody responses, providing insight into the protective immune responses against these bacteria [34]. In addition, *E. chaffeensis* T4SS is used to inject bacterial effector protein into host mitochondria. Using bacterial two-hybrid screening, H. Liu *et al.* identified the first *Ehrlichia* effector, ECH0825. It is up-regulated in human monocytes during early infection when the intracellular activation of *virB* expression occurred. This effector is involved in the inhibition of apoptosis associated with intracellular infection (Fig. 3) [35].

3.3. Protection from oxidative stress and inhibition of immune responses

Ehrlichia is highly sensitive to reactive oxygen species and actively suppresses O_2^- production by T4E ECH0825 [35]. By upregulating a mitochondrial superoxide dismutase, ECH0825 seems to prevent ROS-induced cellular damage and apoptosis, allowing intracellular infection. In addition, *Ehrlichia* is able to perturb the JAK/STAT signaling pathway that have an essential role in a cytokine signaling and thus may be able to inhibit activation of macrophages by interferons and interleukins [36]. Moreover, during infection, *E. chaffeensis* circumvent immune responses by directly inhibiting transcription of cytokines involved in early immune response and cell-mediated immune response to intracellular bacteria such as IL-12, IL-15, and IL-18. These cytokines play a fundamental role in stimulating T_H1 and NK cells to produce IFN-γ, which then activates macrophages [37,38]. Thus, such suppression of cytokine production avoids intracellular killing of *Ehrlichia* by macrophages. Very recently, it was shown that *Ehrlichia* induced strong pro-inflammatory responses with the activation of inflammasomes involved in the cleavage of caspase-1, which in turn promotes the production of IL-1β, IL-1α, and type I interferon (IFN-I) leading to fatal ehrlichiosis [39]. The authors observed that resistance of knockout mice in

interferon receptor 1 (IFNAR1) to fatal infection was significantly higher than in wild-type mice. This finding is surprising because this inflammasome pathway is normally engaged against bacterial infection. The activation of IFN-I contributes to abolish the protective immunity by inhibiting induction of CD4(+) T and T-cell cytokines [39].

3.4. Manipulation of host cell signaling

A functional type IV secretion system (T4SS) and the related secretion of effectors play an important role in key steps of *Ehrlichia* infections. This macromolecular complex, encoded by *virBD* genes, serves to deliver substrates into the eukaryotic cells to promote invasion and pathogenesis of bacteria [40]. The first analysis of regulation of *virBD* genes expression in *Ehrlichia* revealed that this system is under the control of a transcriptional factor, EcxR, that regulates the activation of T4SS components during intracellular infection of the host cells [41]. Many other transcriptional regulators or environmental stimuli controlling expression of the VirBD system remain to be discovered, like the eclectic repertoire of transcription factors defined in *Brucella* [42–44]. Meyer et al. developed a bioinformatics algorithm for the prediction of T4SS effector proteins from the genomes of alpha- and gamma-proteobacteria [45]. This powerful tool will help in discovering new effectors involved in bacterial pathogenesis. Also, *E. chaffeensis* exploits host post-translational modification (PTM), SUMOylation to promote their intracellular survival (Fig. 3). During *Ehrlichia chaffeensis* infection, the type I secretion system effector TRP120 is SUMOylated on lysine residues that mediate interactions with host protein targets such as actin and myosin cytoskeleton component (Myo10) or GGA1 involved in vesicular trafficking [46]. TRP120 SUMOylation may control the interaction between the effector and host factors critical for the infection process. In host cells, this protein colocalizes with SUMO2/3 at the vacuole and in the cytosol and enhances recruitment of host proteins. Thus, *Ehrlichia* manipulates this PTM pathway as virulence mechanism to usurp host cell and establish its intracellular niche [46].

3.5. Exit mechanisms and spreading

The mechanisms underlying the release of *Ehrlichia* from cells are now better understood. At early stages of infection, *Ehrlichia* traffics between cells by the mean of actin-containing filopodia. These cellular protusions permit *Ehrlichia* to *exit* the infected cell and enter neighboring uninfected cells without entering the extracellular space and thus allow evasion of the host's immune system. Inhibition of filopodia formation by cytochalasin D prevents ehrlichial transport but the bacterium is then released by host cell membrane rupture adjacent to the morula during later stages of infection [47]. Similarly, inhibitory compounds affecting cytoskeleton rearrangement, protein kinases, calcium channels, or iron significantly reduce the number of *E. canis* in infected cells, indicating that these cellular processes are important for the proliferation of *E. canis* [48]. Alves et al. assessed the effect of several similar inhibitory drugs on spreading of *E. canis* in macrophages [49]. They showed that various host physiological processes like actin polymerization in the cytoskeleton and calcium and iron influx are required for full bacteremia and spreading in mammalian cells. They also showed that acid phosphatase, used to label lysosomes, rarely marked the inclusions of *E. canis*, suggesting that it escapes fusion with lysosome. Finally, TRP47 and TRP120 described above interact with host cytoskeletal proteins and with accessory proteins such as the ARP2/3 complex and CAP1 to facilitate exocytosis or filopodium formation [50].

4. Concluding statements

Considerable progress has been made in understanding the pathogenesis of *Ehrlichia* infection. Despite their obligate intracellular lifestyle, huge advances have been made in genetic manipulation of these bacteria. The recent development of targeted and random mutagenesis strategies, coupled with the tremendous evolution of sequencing technologies, omics approaches, and *in vivo* imaging, offers new perspectives for the molecular dissection of the unique lifestyles of these bacteria and the virulence factors involved in their pathogenesis. Although advances have been made in deciphering gene regulation of the intracellular growth and maturation of *Ehrlichia*, many regulatory pathways still need to be discovered. Moreover, questions remain on the ecology of these bacteria (e.g. life inside the vector).

The determinants of host-specificity are largely unexplored but they promise insightful knowledge on the biology of *Anaplasmataceae* and the adaptation to their host. Host-specificity is a fundamental concept that describes the nature of host-microbe associations, and most frequently gains attention for its impact on our understanding of pathogen virulence potential, zoonoses, and emerging and re-emerging infectious diseases. Thus, identifying key effector proteins involved in host-specificity, and more specifically in host-switching, could lead to the development of alternative therapeutic strategies to prevent future outbreaks of infectious diseases. Moreover, dissecting the repertoire of type IV effector proteins of *Ehrlichia* offers the unique possibility to genetically identify components of innate immunity. A full understanding of the molecular and genetic mechanisms by which *Ehrlichia* virulence factors induce differential regulation of host innate responses is thus of utmost importance. Testing the idea stating that bacterial virulence proteins may exploit host-signaling pathways to destabilize host innate response at early steps of infection could highlight some elegant examples of ultimate bacterial adaptation to their hosts.

Conflict of interest

The author have not provided a declaration of conflict of interest.

Acknowledgments

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References

[1] Montagna M, Sassera D, Epis S, Bazzocchi C, Vannini C, Lo N, et al. "Candidatus Midichloriaceae" fam. nov. (Rickettsiales), an ecologically widespread clade of intracellular alphaproteobacteria. Appl Environ Microbiol 2013;79:3241−8.

[2] Thomas RJ, Dumler JS, Carlyon JA. Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. Expert Rev Anti-Infect Ther 2009;7:709−22.

[3] Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Ann N Y Acad Sci 2006;1078:110−7.

[4] Allsopp MT, Louw M, Meyer EC. *Ehrlichia ruminantium*: an emerging human pathogen? Ann N Y Acad Sci 2005;1063:358−60.

[5] Bakken JS, Dumler JS, Chen SM, Eckman MR, Van Etta LL, Walker DH. Human granulocytic ehrlichiosis in the upper Midwest United States. A new species emerging? Jama 1994;272:212−8.

[6] Rikihisa Y. *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*: subversive manipulators of host cells. Nat Rev Microbiol 2010;8:328−39.

[7] Telford 3rd SR, 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 U S A 1996;93:6209−14.

[8] Zhang JZ, Popov VL, Gao S, Walker DH, Yu XJ. The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. Cell Microbiol 2007;9:610−8.

[9] Frutos R, Viari A, Ferraz C, Bensaid A, Morgat A, Boyer F, et al. Comparative genomics of three strains of *Ehrlichia ruminantium*: a review. Ann N Y Acad Sci 2006;1081:417−33.

[10] Herndon DR, Palmer GH, Shkap V, Knowles Jr DP, Brayton KA. Complete genome sequence of *Anaplasma marginale* subsp. centrale. J Bacteriol 2010;192:379−80.

[11] Thirumalapura NR, Qin X, Kuriakose JA, Walker DH. Complete genome sequence of *Ehrlichia muris* strain AS145T, a model monocytotropic *Ehrlichia* strain. Genome Announc 2014;2. 01234-13.

[12] Cabezas-Cruz A, Zweygarth E, Broniszweska M, Passos LM, Ribeiro MF, Manrique M, et al. Complete genome sequence of *Ehrlichia mineirensis*, a novel organism closely related to *Ehrlichia canis* with a new host association. Genome Announc 2015;3. 01450-14.

[13] Dunning Hotopp JC, Lin M, Madupu R, Crabtree J, Angiuoli SV, Eisen JA, et al. Comparative genomics of emerging human ehrlichiosis agents. PLoS Genet 2006;2:e21.

[14] Emboule L, Daigle F, Meyer DF, Mari B, Pinarello V, Sheikboudou C, et al. Innovative approach for transcriptomic analysis of obligate intracellular pathogen: selective capture of transcribed sequences of *Ehrlichia ruminantium*. BMC Mol Biol 2009;10:111.

[15] Pruneau L, Emboule L, Gely P, Marcelino I, Mari B, Pinarello V, et al. Global gene expression profiling of *Ehrlichia ruminantium* at different stages of development. FEMS Immunol Med Microbiol 2012;64:66−73.

[16] Lin M, Kikuchi T, Brewer HM, Norbeck AD, Rikihisa Y. Global proteomic analysis of two tick-borne emerging zoonotic agents: *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. Front Microbiol 2011;2:24.

[17] Moumene A, Marcelino I, Ventosa M, Gros O, Lefrancois T, Vachiery N, et al. Proteomic profiling of the outer membrane fraction of the obligate intracellular bacterial pathogen *Ehrlichia ruminantium*. PloS One 2015;10:e0116758.

[18] Cheng C, Nair AD, Indukuri VV, Gong S, Felsheim RF, Jaworski D, et al. Targeted and random mutagenesis of *Ehrlichia chaffeensis* for the identification of genes required for in vivo infection. PLoS Pathog 2013;9:e1003171.

[19] Nair AD, Cheng C, Jaworski DC, Ganta S, Sanderson MW, Ganta RR. Attenuated mutants of *Ehrlichia chaffeensis* induce protection against wild-type infection challenge in the reservoir host and in an incidental host. Infect Immun 2015;83:2827−35.

[20] Cheng C, Nair AD, Jaworski DC, Ganta RR. Mutations in *Ehrlichia chaffeensis* causing polar effects in gene expression and differential host specificities. PloS One 2015;10:e0132657.

[21] Omsland A, Cockrell DC, Howe D, Fischer ER, Virtaneva K, Sturdevant DE, et al. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. Proc Natl Acad Sci U S A 2009;106:4430−4.

[22] Omsland A. Axenic growth of *Coxiella burnetii*. Adv Exp Med Biol 2012;984:215−29.

[23] Martinez E, Cantet F, Fava L, Norville I, Bonazzi M. Identification of OmpA, a *Coxiella burnetii* protein involved in host cell invasion, by multi-phenotypic high-content screening. PLoS Pathog 2014;10:e1004013.

[24] Mohan Kumar D, Yamaguchi M, Miura K, Lin M, Los M, Coy JF, et al. *Ehrlichia chaffeensis* uses its surface protein EtpE to bind GPI-anchored protein DNAse X and trigger entry into mammalian cells. PLoS Pathog 2013;9:e1003666.

[25] Barnewall RE, Rikihisa Y, Lee EH. *Ehrlichia chaffeensis* inclusions are early endosomes which selectively accumulate transferrin receptor. Infect Immun 1997;65:1455−61.

[26] 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;67:1368−78.

[27] Cheng Y, Liu Y, Wu B, Zhang JZ, Gu J, Liao YL, et al. Proteomic analysis of the *Ehrlichia chaffeensis* phagosome in cultured DH82 cells. PloS One 2014;9:e88461.

[28] Xiong Q, Bao W, Ge Y, Rikihisa Y. *Ehrlichia ewingii* infection delays spontaneous neutrophil apoptosis through stabilization of mitochondria. J Infect Dis 2008;197:1110−8.

[29] Liu Y, Zhang Z, Jiang Y, Zhang L, Popov VL, Zhang J, et al. Obligate intracellular bacterium *Ehrlichia* inhibiting mitochondrial activity. Microbes Infect 2011;13:232−8.

[30] Wakeel A, Kuriakose JA, McBride JW. An *Ehrlichia chaffeensis* tandem repeat protein interacts with multiple host targets involved in cell signaling, transcriptional regulation, and vesicle trafficking. Infect Immun 2009;77:1734−45.

[31] Wakeel A, den Dulk-Ras A, Hooykaas PJ, McBride JW. *Ehrlichia chaffeensis* tandem repeat proteins and Ank200 are type 1 secretion system substrates related to the repeats-in-toxin exoprotein family. Front Cell Infect Microbiol 2011;1:22.

[32] Zhu B, Nethery KA, Kuriakose JA, Wakeel A, Zhang X, McBride JW. Nuclear translocated *Ehrlichia chaffeensis* ankyrin protein interacts with a specific adenine-rich motif of host promoter and intronic Alu elements. Infect Immun 2009;77:4243−55.

[33] McBride JW, Zhang X, Wakeel A, Kuriakose JA. Tyrosine-phosphorylated *Ehrlichia chaffeensis* and *Ehrlichia canis* tandem repeat orthologs contain a major continuous cross-reactive antibody epitope in lysine-rich repeats. Infect Immun 2011;79:3178−87.

[34] Luo T, Zhang X, McBride JW. Major species-specific antibody epitopes of the *Ehrlichia chaffeensis* p120 and *E. canis* p140 orthologs in surface-exposed tandem repeat regions. Clin Vacc Immunol CVI 2009;16:982−90.

[35] Liu H, Bao W, Lin M, Niu H, Rikihisa Y. *Ehrlichia* type IV secretion effector ECH0825 is translocated to mitochondria and curbs ROS and apoptosis by upregulating host MnSOD. Cell Microbiol 2012;14:1037−50.

[36] 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.

[37] Ismail N, Bloch KC, McBride JW. Human ehrlichiosis and anaplasmosis. Clin Lab Med 2010;30:261−92.

[38] Mansueto P, Vitale G, Cascio A, Seidita A, Pepe I, Carroccio A, et al. New insight into immunity and immunopathology of *Rickettsial* diseases. Clin Dev Immunol 2012;2012:967852.

[39] Yang Q, Stevenson HL, Scott MJ, Ismail N. Type I interferon contributes to noncanonical inflammasome activation, mediates immunopathology, and impairs protective immunity during fatal infection with lipopolysaccharide-negative ehrlichiae. Am J Pathol 2015;185:446−61.

[40] Gillespie JJ, Brayton KA, Williams KP, Diaz MA, Brown WC, Azad AF, et al. Phylogenomics reveals a diverse Rickettsiales type IV secretion system. Infect Immun 2010;78:1809−23.

[41] 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;190:2096−105.

[42] Delrue RM, Deschamps C, Leonard S, Nijskens C, Danese I, Schaus JM, et al. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. Cell Microbiol 2005;7:1151−61.

[43] Sieira R, Arocena GM, Bukata L, Comerci DJ, Ugalde RA. Metabolic control of virulence genes in *Brucella abortus*: HutC coordinates *virB* expression and the histidine utilization pathway by direct binding to both promoters. J Bacteriol 2010;192:217−24.

[44] Sieira R, Arocena GM, Zorreguieta A, Comerci DJ, Ugalde RA. A MarR-type regulator directly activates transcription from the *Brucella abortus virB* promoter by sharing a redundant role with HutC. J Bacteriol 2012;194:6431−40.

[45] Meyer DF, Noroy C, Moumene A, Raffaele S, Albina E, Vachiery N. Searching algorithm for type IV secretion system effectors 1.0: a tool for predicting type IV effectors and exploring their genomic context. Nucleic Acids Res 2013;41:9218−29.

[46] Dunphy PS, Luo T, McBride JW. *Ehrlichia chaffeensis* exploits host SUMOylation pathways to mediate effector-host interactions and promote intracellular survival. Infect Immun 2014;82:4154−68.

[47] Thomas S, Popov VL, Walker DH, Rikihisa Y. Exit mechanisms of the intracellular bacterium *Ehrlichia*. PLoS One 2010;5:e15775.

[48] Levenhagen MA, Alves RN, Rieck SE, Labruna MB, Beletti ME. The role of cytoskeleton, components of inositol phospholipid signaling pathway and iron in *Ehrlichia canis* in vitro proliferation. Veterinary Res Commun 2012;36:195−9.

[49] Alves RN, Levenhagen MA, Levenhagen MM, Rieck SE, Labruna MB, Beletti ME. The spreading process of *Ehrlichia canis* in macrophages is dependent on actin cytoskeleton, calcium and iron influx and lysosomal evasion. Vet Microbiol 2014;168:442−6.

[50] Dunphy PS, Luo T, McBride JW. *Ehrlichia* moonlighting effectors and interkingdom interactions with the mononuclear phagocyte. Microbes Infect 2013;15:1005−16.