

Molecular and cellular pathobiology of *Ehrlichia* infection: targets for new therapeutics and immunomodulation strategies

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Ehrlichia are small obligately intracellular bacteria in the order Rickettsiales that are transmitted by ticks and associated with emerging life-threatening human zoonoses. Vaccines are not available for human ehrlichiosis, and therapeutic options are limited to a single antibiotic class. New technologies for exploring host–pathogen interactions have yielded recent advances in understanding the molecular interactions between *Ehrlichia* and the eukaryotic host cell and identified new targets for therapeutic and vaccine development, including those that target pathogen virulence mechanisms or disrupt the processes associated with ehrlichial effector proteins. Animal models have also provided insight into immunopathological mechanisms that contribute significantly to understanding severe disease manifestations, which should lead to the development of immunomodulatory approaches for treating patients nearing or experiencing severe disease states. In this review, we discuss the recent advances in our understanding of molecular and cellular pathobiology and the immunobiology of *Ehrlichia* infection. We identify new molecular host–pathogen interactions that can be targets of new therapeutics, and discuss prospects for treating the immunological dysregulation during acute infection that leads to life-threatening complications.

Ehrlichia were first associated with veterinary disease in Africa in 1925 by Cowdry, who identified *Ehrlichia ruminantium* in cattle. A decade later Donatien and Lestoquard described *Ehrlichia canis* in Algerian dogs (Refs 1, 2). Ehrlichioses continue to be important veterinary diseases, but are now also associated with newly identified human tick-borne zoonoses.

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At the end of the 20th century, a new tick-borne disease, human monocytotropic ehrlichiosis, emerged in humans and a novel aetiological agent – *Ehrlichia chaffeensis* – was identified (Ref. 3). Twelve years later, *Ehrlichia ewingii*, a recognised canine pathogen that infects granulocytes, was detected in four immunocompromised patients with symptoms of ehrlichiosis (Refs 4, 5). Nearly a century after Cowdry's discovery, *Ehrlichia* are firmly established as zoonotic human pathogens of public health importance. Human monocytotropic ehrlichiosis is considered one of the most prevalent life-threatening tick-borne diseases in the USA, and ewingii ehrlichiosis is an important clinically indistinguishable disease in immunocompromised patients (Refs 4, 5).

The order Rickettsiales contains two families of arthropod-transmitted obligately intracellular bacteria that cause human diseases, including spotted fever rickettsiosis, typhus, scrub typhus, anaplasmosis and ehrlichiosis. The genus *Ehrlichia* is a member of the family Anaplasmataceae, which also includes the genera *Anaplasma*, *Wolbachia* and *Neorickettsia*. In addition, the family Rickettsiaceae and respective genera *Rickettsia* and *Orientia* are also members of Rickettsiales. The *Ehrlichia* genus consists of six formally named members (*E. canis*, *E. chaffeensis*, *E. muris*, *E. ruminantium*, *E. ewingii* and *E. ovis*). *E. chaffeensis* and *E. ewingii* are recognised as human zoonotic pathogens that also cause significant disease in the animal hosts (Refs 6, 7). *E. canis*, the aetiological agent of canine monocytic ehrlichiosis, is a globally distributed pathogen and has been recently associated with human infections (Refs 8, 9). *E. ruminantium* is a veterinary pathogen that causes a severe acute infection known as heartwater in domestic ruminants localised primarily to sub-Saharan Africa (Ref. 10).

Ehrlichia have small genomes, yet have evolved elaborate and complex molecular strategies that enable adaptation to distinct hosts (invertebrate and vertebrate) and intracellular survival in innate immune effector cells. Using *Ehrlichia* as a model to understand the molecular interactions between pathogen and eukaryotic host cell provides an attractive and manageable system to advance our knowledge of the molecular pathobiology of intracellular microbes as well as the molecular biology of

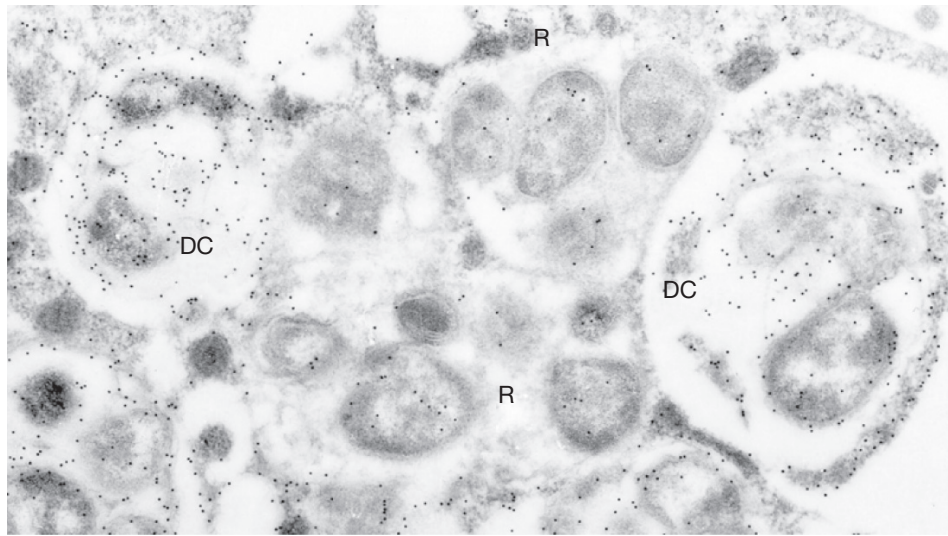
the eukaryotic cell. This review focuses on the molecular and cellular interactions of ehrlichiae and immunological responses that have clinical implications with regard to development of novel antimicrobial therapeutics or molecular countermeasures, and immunomodulatory approaches.

Physical characteristics and intracellular developmental biology

E. chaffeensis is confined to cytoplasmic membrane-bound vacuoles within monocytes/macrophages and dendritic cells (Ref. 11), replicating to form microcolonies called morulae that contain one to >400 organisms (Ref. 12). Morphologically, individual ehrlichiae are coccoid and coccobacillary and exhibit two ultrastructural cell types: a larger reticulate cell and a smaller dense-cored cell (Fig. 1). Morphological comparisons to other intracellular bacteria such as *Chlamydia* have suggested that *Ehrlichia* reticulate cells and dense-cored cells represent analogous replicating and infectious forms of the organism. This hypothesis was confirmed by a recent study of ehrlichial developmental biology that demonstrated that the dense-cored cell form of *E. chaffeensis* binds to the host cell surface where it is rapidly (<1 h) internalised and completes the developmental cycle within 72 h (Ref. 16) (Fig. 2).

The *Ehrlichia* genome: insight into host-pathogen interactions

The intracellular niche occupied by *Ehrlichia* has resulted in reductive evolutionary processes and a corresponding severe loss of genes associated with metabolic processes provided by the host cell. Hence the genome sizes (~1–1.5 Mb) of *Ehrlichia* are relatively small compared with those of extracellular bacteria. The genomes of three *Ehrlichia* species have been sequenced (Refs 17, 18, 19), and they show a high degree of genomic synteny, a low G+C content (~30%) and one of the smallest genome coding ratios, which is attributed to long noncoding regions and numerous long tandemly repeated sequences (Ref. 20). Long noncoding regions and low G+C content in other related Rickettsiales members are speculated to represent degraded genes in the final stages of elimination and excess GC-to-AT mutations (Refs 21, 22, 23). The large number of long tandemly repeated sequences that are a feature



Immunogold-labelled electron micrograph of *Ehrlichia chaffeensis* TRP47

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Figure 1. Immunogold-labelled electron micrograph of *Ehrlichia chaffeensis* TRP47. Within innate immune cells, ehrlichiae form membrane-bound microcolonies termed morulae. Individual ehrlichiae show two morphological forms: dense-cored cells (DC; 0.4–0.6 µm in diameter) and reticulate cell (R) forms (0.4–0.6 µm by 0.7–1.9 µm). Reticulate cells and dense-cored cells can be distinguished by the differential expression and secretion of two tandem-repeat-containing surface proteins – TRP47 and TRP120 – which are found only on dense-cored cells (Refs 13, 14). The intramorular space in some morulae contains a fibrillar matrix of ehrlichial origin (Ref. 15). Figure reproduced, with permission from American Society for Microbiology, from Ref. 14 (© 2006, American Society for Microbiology).

of *Ehrlichia* genomes have no phylogenetic relationships, suggesting that duplication occurred after diversification of the repeat-encoding DNA and divergence of the species (Ref. 24). The tandem repeats seem to be a result of locally occurring independent events, actively created and deleted through a mechanism compatible with DNA slippage (Ref. 24), and their generation might be a mechanism of adaptation to the host.

Features identified in *Ehrlichia* genomes associated with host–pathogen interactions include genes that encode tandem- and ankyrin-repeat-containing proteins, genes encoding actin polymerisation proteins, genes with poly(G-C) tracts encoding a family of proteins with short sequence repeats, and a multigene family encoding outer membrane proteins; in addition, there is an absence of genes for the biosynthesis of peptidoglycan and lipopolysaccharide (Ref. 18), the major pathogen-associated

molecular patterns recognised by the innate immune system. Tandem repeats are associated with regulation of gene expression and phase variation, and *Ehrlichia* species show two types of tandem repeats: small (12 bp) and large (100–300 bp) period repeats (Ref. 24). Secretion of effector proteins into the host cell requires secretion systems, and such delivery mechanisms have been identified, including many of the known type IV secretion system (T4SS) components (Refs 17, 18, 19). There is no evidence of a type III secretion system (T3SS), although other intracellular bacteria such as *Chlamydia* have this system and some secreted ehrlichial tandem repeat proteins (TRPs) are predicted to have an N-terminal type III secretion transport signal. The Sec-dependent and Sec-independent protein export pathways for secretion of proteins across the inner membrane as well as a putative type I secretion system (T1SS) have also been identified (Refs 17,

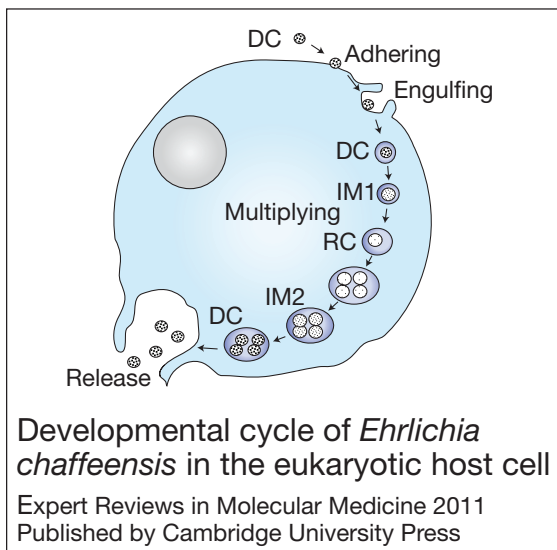


Figure 2. Developmental cycle of *Ehrlichia chaffeensis* in the eukaryotic host cell.

Infectious dense-cored (DC) ehrlichiae attach and enter the host cell through receptor-mediated endocytosis, and within 1 h after entry transform into the intermediate (IM) 1, and then into the reticulate cell (RC). During the next 48 h RC replicates, doubling every 8 h, and then transforms into IM2 and matures to DC ehrlichiae within 72 h after initial cell contact (Ref. 16). DC ehrlichiae obtained 72 h post-infection showed much higher infectivity than *E. chaffeensis* RCs obtained 24 h post-infection. RC and DC ehrlichiae can be distinguished by two differentially expressed tandem repeat proteins – TRP47 and TRP120 – that are found only on DC ehrlichiae, and the major outer membrane protein p28-19, which is expressed only on the RC (Ref. 16). However, intermediate, presumably transitional, forms that co-express these proteins have also been described (Ref. 16). Although ehrlichiae and chlamydiae have similar morphological forms and developmental cycles, homologous genes for the histone H1 homologue proteins (Hc1 and Hc2) involved in condensation and decondensation of the chlamydial nucleoid are not present in the *E. chaffeensis* genome (Ref. 16). Figure adapted, with permission from John Wiley and Sons (<http://www.interscience.wiley.com>), from Ref. 16 (© The Authors).

18, 19). The *Ehrlichia* genomes also have genes that encode three response regulator two-component systems (composed of a sensor histidine kinase and a response regulator), which allow bacteria to sense signals and respond to changes in their

environment through specific gene activation or repression (Ref. 17).

Proteins associated with host–pathogen interactions

Tandem repeat proteins

The presence of long-period tandem repeats distributed in intergenic regions of *Ehrlichia* is well recognised and is associated with expansion and contraction of these regions. Interestingly, long-period tandem repeats are also found in a small subset of proteins, many of which are strongly immunoreactive, suggesting that they are exposed on the surface or secreted. TRPs in pathogenic bacteria have been associated with host–pathogen interactions such as adhesion and internalisation (Refs 13, 25), actin nucleation (Ref. 26) and immune evasion (Ref. 27). Many of these proteins in *Ehrlichia* have been molecularly characterised, and major continuous species-specific antibody epitopes have been mapped to the acidic serine-rich tandem repeats of *E. chaffeensis* TRP120, TRP47 and TRP32 (Refs 14, 28, 29), and of the *E. canis* orthologues TRP140, TRP36 and TRP19, respectively (Refs 14, 29, 30). Immunoelectron microscopy has shown that these TRPs are secreted by the ehrlichiae and are associated with the morular fibrillar matrix and the morula membrane (Refs 13, 14, 28). Other bacteria have effector TRPs that are secreted by the T3SS; however, the absence of an identifiable T3SS in *Ehrlichia* suggests that another mechanism such as the T1SS is involved. In preliminary work, we have explored the possibility of these TRPs as T4SS substrates, but they were not secreted in the well-characterised model of the VirB/VirD4-dependent T4SS of *Agrobacterium tumefaciens* (Ref. 31).

The functional role of the tandem repeats in *E. chaffeensis* TRPs is not fully understood. However, homology between the tandem repeats and other functional protein domains and motifs of eukaryotic origin have been reported. The *E. chaffeensis* TRP47 contains seven 19-mer (ASVSEGDVVNAVVSQETPA) tandem repeats that dominate the C-terminal region of the protein, and approximately half of the TRP47 is represented by the tandem repeat domain (Ref. 32). The TRP47 tandem repeat region shows homology with eukaryotic proteins including the renin receptor (also known as ATP6AP2 and CAPER), the DNA polymerase III subunits gamma and tau-conserved domain, and

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ribonuclease E, suggesting similar functional characteristics (Ref. 32). TRP47 also has several N-terminal tyrosine and serine/threonine residues that are predicted sites of phosphorylation, and tyrosine phosphorylation has been detected on TRP47 with antiphosphotyrosine antibodies (Ref. 33). Other TRPs might be phosphorylated, such as TRP32, which has an unusually high frequency of tyrosine residues (20%) in the C-terminal tail (Ref. 28). The tandem repeat domains of TRP120 and TRP32 do not have homology with other conserved protein domains; however, recent studies have reported that the tandem repeat region of TRP120 directly binds host cell DNA (Ref. 34).

Ankyrin repeat proteins

The ankyrin repeat (Ank) is a ubiquitous eukaryotic motif that can occur in combination with other types of domains and can cooperatively fold into structures that mediate molecular recognition by protein–protein interactions. *Ehrlichia* species are among only a few prokaryotes that are known to have Ank-containing proteins. The most extensively studied Ank protein in *E. chaffeensis* is a 200 kDa protein (Ank200) that has a central domain containing 19 Anks flanked by acidic (pI 4–5) C- and N-terminal domains with a predominance of glutamate and aspartate residues (Ref. 35). In addition, like the TRPs, the *E. canis* and *E. chaffeensis* Ank200s have a high proportion of polar amino acids, including serine and threonine (Refs 35, 36). Several species-specific antibody epitopes have been mapped to acidic terminal domains of the Ank200s (Refs 35, 36). Our preliminary data suggest that *E. chaffeensis* Ank200 is not secreted by the T4SS (Ref. 31); however, *Anaplasma phagocytophilum* AnkA appears to be secreted by this mechanism (Ref. 37). Although *E. chaffeensis* and *A. phagocytophilum* are closely related, they are different in many aspects; for example, they show tropism for different cell types, residence in different cytoplasmic compartments (Refs 38, 39) and distinct immune evasion mechanisms (Refs 40, 41, 42). In addition, an *A. phagocytophilum* VirD4, the T4SS substrate-coupling protein, shows a higher identity with *A. tumefaciens* VirD4 than with that of *E. chaffeensis* (Refs 17, 41).

Major outer membrane proteins

A superfamily of immunoreactive outer membrane proteins that are members of the

Pfam PF01617 group of proteins (<http://pfam.sanger.ac.uk/>) has been identified in the family Anaplasmataceae. *E. chaffeensis* has a paralogous family of 22 major outer membrane proteins (OMP-1/p28) encoded at a single locus upstream from the *secA* gene and downstream from a hypothetical transcriptional regulator gene (Ref. 44). They were originally proposed to be involved in antigenic variation of the organism. Although recombination of the major outer membrane proteins of closely related *Anaplasma* species occurs to create antigenic diversity, there is no evidence that recombination of the *Ehrlichia* OMP-1 family occurs. Differential expression of the OMP-1 genes in ticks and animal hosts has been reported, suggesting that they have a role in host adaptation (Refs 45, 46). Expression of only one OMP-1 gene (OMP-1B) has been reported in ticks and tick cell lines and appears to involve a temperature-sensitive regulation mechanism (Refs 45, 47). By contrast, all OMP-1 family members are expressed in mammalian hosts and cells, and antibodies against all OMP-1 proteins have been detected in experimentally infected dogs (Refs 45, 48). Although the role of the outer membrane proteins in antigenic variation and immune evasion is still uncertain, other characteristics have been identified for the OMP-1 proteins, including transmembrane β -strand structural features and porin activity, suggesting that they might facilitate nutrient acquisition (Ref. 49).

Molecular and cellular biology of infection Entry and characteristics of the ehrlichial vacuole

Infection of the host cell involves dense-cored ehrlichiae that express TRP120 on the surface. TRP120 has an important role in the binding and entry process (Refs 13, 25), and a potential role has also been demonstrated for TRP47 in attachment to tick cells using the *E. ruminantium* orthologue (Erum1110) (Ref. 50). The stability of TRP120 and invasion of *E. chaffeensis* are regulated by the bacterial second messenger cyclic di-GMP and activity of ehrlichial surface serine protease HtrA (Ref. 25). Binding to the host cell occurs through receptors such as E- and L-selectin and other glycosylphosphatidylinositol (GPI)-anchored proteins located in caveolae (Refs 51, 52), triggering receptor-mediated endocytosis that

involves signalling events including transglutamination, tyrosine phosphorylation, phospholipase C γ 2 activation, production of inositol (1,4,5)-trisphosphate and increases in intracellular calcium (Ref. 53). Removal of surface-exposed GPI-anchored proteins by phosphatidylinositol-specific phospholipase C prevents the development of early inclusions (Ref. 52).

The vacuoles in which the organism enters contain caveolin-1, G_{M1} ganglioside and phospholipase C γ 2. Later (>3 h) after infection, vacuoles that contain replicating ehrlichiae show characteristics of early endosomes such as the presence of the small GTPase Rab5A (RAB5A), early endosomal antigen 1 (EEA1) and vacuolar (H⁺) ATPase, and they accumulate transferrin and transferrin receptor (Ref. 12). Replicative vacuoles of closely related *A. phagocytophilum* do not express these markers (Ref. 38). Other molecules found in *Ehrlichia* inclusions are vesicle-associated membrane protein 2, major histocompatibility class II and β_2 -microglobulin (Refs 12, 38). *Ehrlichia* vacuoles appear to be maintained in a caveolar trafficking system that interacts with recycling endosomal pathways (Ref. 52).

Modulation of host cell gene expression

E. chaffeensis appears to actively modulate host cell gene transcription and function through several mechanisms, including interactions with host chromatin and modulation of host signalling. One mechanism that has been identified involves the inhibition of host mitogen-activated protein (MAP) kinases by *E. chaffeensis*, leading to the downregulation of transcription factors and transcription of target genes related to host defence (Ref. 54). The discovery of DNA-binding proteins (TRP120 and Ank200) of *Ehrlichia* provides another mechanism by which host cell gene transcription can be modulated (Refs 34, 55).

The transcription levels of a relatively small percentage (5%) of host cell genes are altered significantly within the first 24 h post-infection (Ref. 56). This transcriptional profile has provided new information on host cell processes targeted by *Ehrlichia* and has revealed key themes in pathobiology and disease pathogenesis. Specific cellular processes that appear to be modulated are apoptosis, regulation of the cell cycle and differentiation, signal transduction, and the expression of

proinflammatory cytokines, biosynthetic and metabolic proteins, and membrane trafficking proteins. Host genes modulated during *E. chaffeensis* infection are distinct from those observed in infections by other intracellular bacteria, illustrating the complexity and diversity of intracellular pathogen–host interactions and survival strategies (Ref. 56).

Survival in mononuclear phagocytes requires the ability to evade innate and adaptive immune responses. *E. chaffeensis* represses the transcription of cytokines involved in the early innate immune response and cell-mediated immune response to intracellular microbes, including host cell cytokines that modulate innate and adaptive immunity to intracellular bacteria. Early in infection, genes for the proinflammatory cytokines interleukin (IL)-1 β (IL1B), IL-8 and tumour necrosis factor β (TNF- β ; also known as LTA) are upregulated, and others such as TNF- α (TNF) are not induced (Ref. 56). By contrast, most cytokines and receptors are downregulated, including IL-15, IL-18 and various chemokine receptors. These cytokines have fundamental roles in stimulating natural killer (NK) cells and T helper 1 (Th1) cells to produce interferon γ (IFN- γ ; IFNG), which then activates macrophages to kill phagocytosed bacteria. IL-12 and IL-15 also activate NK cells and cytotoxic T lymphocytes to kill cells infected with intracellular bacteria. Thus, active modulation of genes associated with the immune response appears to be essential to the survival of *E. chaffeensis*.

Modulation of genes associated with inhibition of apoptosis and cell cycle regulators is observed early during *E. chaffeensis* infection, and is probably necessary for delaying host cell death, as discussed further below. *E. chaffeensis* infection upregulates transcription of apoptosis inhibitors such as IER3, BIRC3 and BCL2, but inhibits apoptosis inducers such as BIK and BNIP3L during the early stage of infection, thus impairing host cell apoptosis and maintaining a prolonged growth opportunity for ehrlichiae (Ref. 56).

E. chaffeensis lives in an early endosome and inhibits the maturation of the endosome to evade destruction by lysosomal enzymes (Ref. 38). In an effort to modulate this process, *E. chaffeensis* inhibits the transcription of genes involved in membrane trafficking and lysosomal fusion: the production of Rab5, synaptosome-associated protein 23 (SNAP23) and syntaxin 16

(STX16) is repressed, most dramatically during the first hour of infection. Interestingly, Rab5 is associated with *E. chaffeensis* inclusions. Depletion of Rab5 inhibits the fusion of the phagosome containing *Listeria monocytogenes* with lysosomes (Ref. 57).

Acquisition of iron and cholesterol

Ehrlichia survival depends on an available supply of intracellular iron (Ref. 58), and this is also demonstrated by the fact that the antiehrlichial activity of IFN- γ is mediated by limiting the available cytoplasmic iron (Ref. 59). Interestingly, cytoplasmic vacuoles containing replicating ehrlichiae accumulate transferrin receptor, and transferrin receptor mRNA is upregulated during ehrlichial infection (Ref. 58), which might serve to counteract the reduction of surface transferrin receptor by IFN- γ . How *Ehrlichia* acquires iron from the host is not fully understood, but a conserved iron-acquisition mechanism has been identified in *Ehrlichia* involving the ATP-binding cassette transporter family, a mechanism shared by a diversity of bacterial species (Ref. 60). However, unlike most bacteria in which the genes encoding iron-acquisition proteins are found in an operon, *Ehrlichia* iron-acquisition genes are not part of a functional operon (Ref. 60). The iron-binding protein (Fbp) of *Ehrlichia* is conserved among other known iron-binding proteins in bacteria, and similarly it binds Fe(III). Notably, *Ehrlichia* Fbp has been observed outside the bacterium within the morular space containing dense-core cells, suggesting that iron is obtained from intracellular pools derived from transferrin and shuttled by Fbp to the bacterium (Ref. 60).

Although *Ehrlichia* lack structural membrane components such as peptidoglycan and lipopolysaccharide, they appear to use cholesterol as a structural substitute (Ref. 61). Ultrastructural changes are observed in *Ehrlichia* in the presence of cholesterol-extraction reagents. Cholesterol is incorporated as a component of the outer membrane, and *Ehrlichia* have an unidentified, but specific, direct uptake mechanism (Ref. 61). The structural integrity of the organism is dependent on cholesterol, and it is essential for maintaining an infectious state (Ref. 61).

Evading innate host defences

As indicated above, *Ehrlichia* have evolved strategies that allow them to survive in phagocytes and

evade innate host defence mechanisms. *Ehrlichia* manipulate innate immune defence mechanisms, including production of reactive oxygen species (ROS), apoptosis, lysosomal fusion and IFN- γ responsiveness. A major host defence mechanism is production of ROS that have strong antimicrobial effects. *E. chaffeensis* is highly sensitive to O $_2^-$ killing (Ref. 62) and lacks genes involved in the detoxification of ROS (Ref. 17). However, *E. chaffeensis* actively blocks O $_2^-$ generation in monocytes stimulated with phorbol myristate acetate and causes degradation of NADPH oxidase subunit p22phox in monocytes, but not neutrophils (Ref. 62). Degradation of p22phox appears to involve nonproteasomal proteolysis and heat-labile factors or proteins from *E. chaffeensis*. Degradation can be detected in isolated monocyte membrane fractions, which suggests that intracellular signalling events are not required (Ref. 62).

Apoptosis is an innate cellular defence mechanism against microbes that is modulated by many bacterial pathogens, and there is new information that indicates that *Ehrlichia* also modulate host cell death. In nature, *Ehrlichia* survive by persistently infecting vertebrate hosts, so delaying or preventing apoptosis could be a means of enhancing survival by preventing host cell death and subsequent immune recognition. For most intracellular pathogens, induction of apoptosis leads to pathogen killing and clearance of infection, and this is thought to be beneficial to the host and enhance the immune response to the infection. In the case of *Ehrlichia*, there appear to be several mechanisms involved in apoptosis modulation by *Ehrlichia*. As mentioned above, *E. chaffeensis* upregulates the transcription of genes related to antiapoptotic activity and the cell cycle, including cyclin-dependent kinase expression, in THP-1 cells (Ref. 56). *E. ewingii* delays apoptosis in neutrophils by stabilisation of host cell mitochondria (Ref. 63).

Phagolysosomes represent another important innate host defence mechanism against pathogens. *Ehrlichia* are able to inhibit fusion of the phagosome containing ehrlichiae with lysosomes and thus prevent their destruction by this defence mechanism (Ref. 12). Although very little is known about how *Ehrlichia* avoid this host defence mechanism, recent studies have demonstrated that functioning two-component systems have an important role in preventing

lysosomal fusion (Ref. 64). Two-component systems consist of a sensor with a histidine kinase that detects environmental signals, and a response regulator that has DNA-binding activity and regulates gene transcription (Ref. 65). *E. chaffeensis* has three two-component systems: PleC–PleD, NtrY–NtrX and CckA–CtrA (Ref. 66). Cells treated with closantel and incubated with *E. chaffeensis* have increased colocalisation between *E. chaffeensis* and lysosomal glycoprotein LAMP-1, and *E. chaffeensis* infection is completely inhibited by pretreatment with closantel (Ref. 66).

The macrophage-activating cytokine IFN- γ has an important role in innate and adaptive immune responses against intracellular pathogens. The activation of macrophages by IFN- γ induces several antimicrobial effector mechanisms, including regulation of iron homeostasis (Ref. 67), that are necessary for the production of antimicrobial effectors, including ROS and nitrogen radicals (Ref. 68). The ability to acquire iron is important for the survival of intracellular pathogens such as *Ehrlichia*, *Salmonella*, *Listeria* and *Mycobacterium* (Refs 68, 69). With respect to *E. chaffeensis*, macrophages that are stimulated with IFN- γ before infection or early in infection readily kill *E. chaffeensis* (Ref. 59). However, *E. chaffeensis* has developed strategies to circumvent the actions of IFN- γ . *E. chaffeensis* upregulates transferrin receptor expression to counteract the downregulation by the action of IFN- γ (Ref. 58). The inhibitory effects of IFN- γ on *E. chaffeensis* infection can be reversed by the addition of iron-saturated transferrin, and *E. chaffeensis* infection is inhibited by the intracellular iron chelator deferoxamine (Ref. 59), demonstrating the critical role of iron for *E. chaffeensis* infection and the role that IFN- γ has in regulating its availability. After *E. chaffeensis* infection is established, IFN- γ is no longer effective, possibly because of the ability of *E. chaffeensis* to upregulate host iron-acquisition mechanisms. The ability of *E. chaffeensis* to block the antimicrobial mechanisms of IFN- γ is mediated at least in part by inhibition of the JAK–STAT IFN- γ signal transduction pathway (Ref. 70). Binding of a protein component of *E. chaffeensis* to the host cell blocks tyrosine phosphorylation of JAK1 and STAT1 normally induced by IFN- γ (Ref. 70). JAK–STAT inhibition has been linked to the upregulation of protein kinase A by

E. chaffeensis (Ref. 70). Others have also suggested that avoiding IFN- γ activity might be influenced by transcriptional regulation of JAK–STAT expression mediated by the nuclear effector Ank200 (Ref. 55).

E. chaffeensis also appears to modulate the host innate immune response by influencing other cell signalling pathways. Antimicrobial activities of macrophages become progressively less responsive during *E. chaffeensis* infection in association with downregulation of pattern recognition receptors (PRR), including CD14, TLR2 and TLR4, and activity of the associated PRR transcription factor PU.1 (SPI1) (Ref. 54). Activation of PU.1 has been linked to a p38 mitogen-activated protein kinase (MAPK)-dependent pathway, and a p38-specific MAPK inhibitor shows similar effects on PU.1 and PRR activity and expression (Ref. 71). *Ehrlichia* also appear to target host tyrosine kinases and phosphatases (FYN and PTPN2; described in more detail below), but the downstream processes affected by these interactions are not known (Ref. 32).

***E. chaffeensis* effectors and molecular host interactions**

Significant progress has been made in the identification of host cell processes that are modulated by *Ehrlichia* in order to survive in phagocytes. However, the effector proteins involved in manipulating these host cell processes have been largely undetermined. Recent genome sequencing efforts and new biotechnology approaches to investigate molecular protein–protein interactions have focused attention on two small groups of *Ehrlichia*-encoded proteins: those containing tandem or ankryin repeats. These proteins appear to be effectors involved in novel, complex and multidimensional molecular strategies to reprogramme host cell defence mechanisms (Ref. 72). Two proteins, TRP47 and Ank200, have been the focus of recent studies demonstrating interactions with host cell DNA and molecular interactions with host proteins associated with distinct host cell processes.

***E. chaffeensis* TRP47**

Many of the small family of TRPs of *Ehrlichia* are strongly recognised by the host immune response, including TRP47 (Refs 14, 28, 29). TRP47 is differentially expressed on dense-cored

ehrlichiae and is found extracellularly, indicating that it is secreted (Fig. 1). A recent study to examine molecular interactions between TRP47 and the host identified several interactions with specific host cell targets, including polycomb group ring finger 5 (PCGF5), Src protein tyrosine kinase FYN, protein tyrosine phosphatase nonreceptor type 2 (PTPN2), adenylate cyclase-associated protein 1 (CAP1) and immunoglobulin lambda-like polypeptide 1 (IGLL1), with distinct cellular functions associated with signalling, transcriptional

regulation, vesicle trafficking, and cellular proliferation and differentiation (Ref. 32) (Fig. 3). Notably, none of these host targets had been identified by previous studies related to the cell biology of *Ehrlichia* infection. The interactions between TRP47 and the host cell illustrate the complexity and diversity of pathogen–host interactions that occur and will require additional research to fully comprehend. Although the relevance of these ehrlichiae–host molecular interactions in the context of ehrlichial pathobiology remains to be determined, the host

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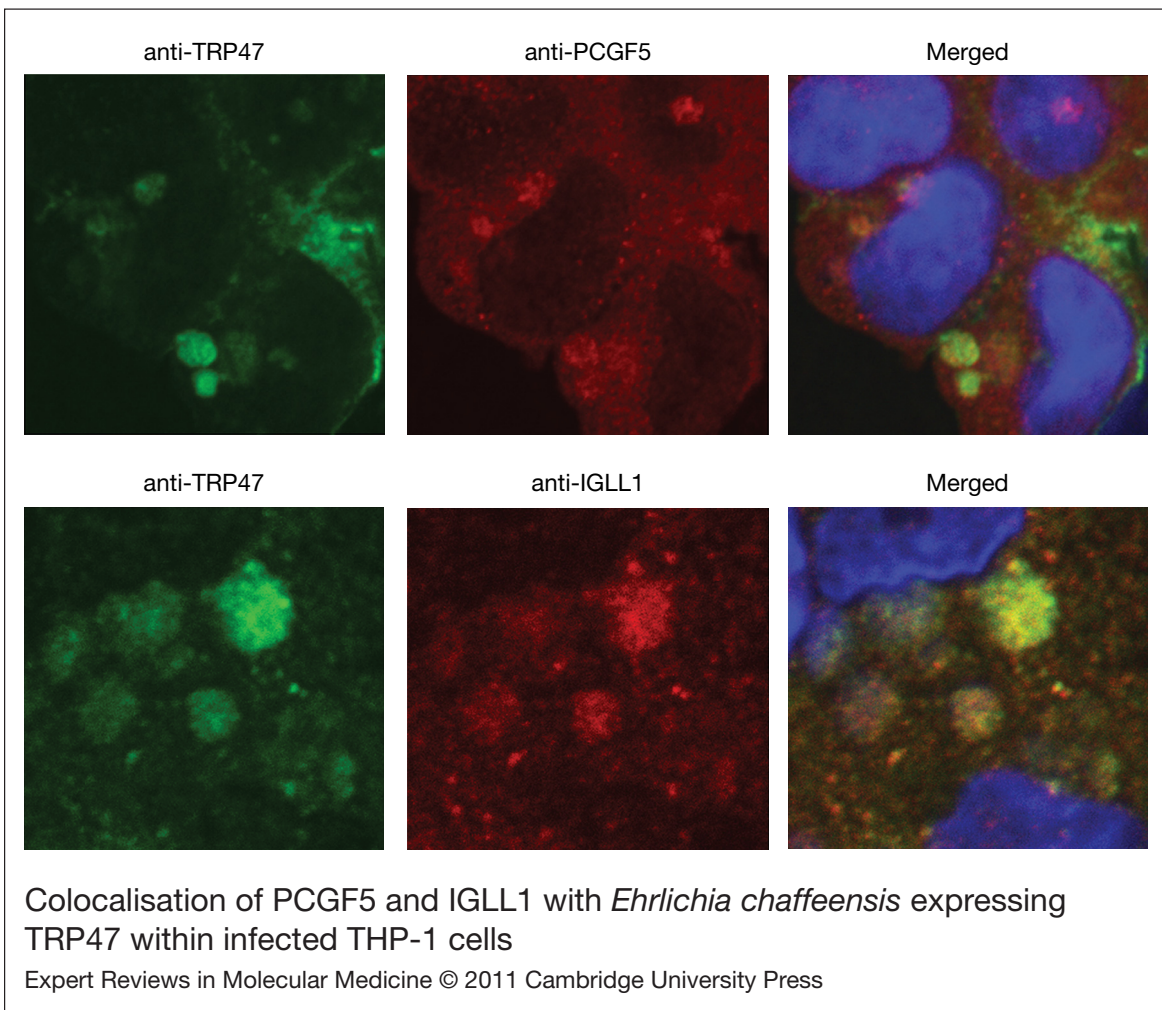


Figure 3. Colocalisation of PCGF5 and IGLL1 with *Ehrlichia chaffeensis* expressing TRP47 within infected THP-1 cells. *E. chaffeensis*-infected cells were dually labelled with anti-TRP47 (green) and PCGF5 or IGLL1 (red) and examined by confocal microscopy. PCGF5 and IgLL1 colocalise with *E. chaffeensis* TRP47-labelled morulae (yellow, right merged panels). Molecular interactions between PCGF5, IGLL1, FYN, CAP1 and PTPN2 were first reported in Ref. 32. Abbreviations: IGLL1, immunoglobulin lambda-like polypeptide 1; PCGF5, polycomb group ring finger 5; THP-1 cells, a human acute monocytic leukaemia cell line; TRP47, tandem repeat protein 47.

targets identified suggest that TRP47 is a multifunctional effector that has an important role in establishing bacterial infection and promoting intracellular survival (Fig. 4).

The strongest observed interaction between an *Ehrlichia* and host protein is between TRP47 and PCGF5 (Ref. 32) (Fig. 3). *E. chaffeensis* is known to modulate host cell gene transcription, and PCGF5 has been associated with DNA-dependent regulation of transcription, metal-ion binding and protein–protein interactions. PCGF5 is related to the polycomb group proteins (transcriptional repressors) Bmi-1 (PCGF4) and

Mel-18 (PCGF2), which have important roles in the regulation of *HOX* gene expression, X-chromosome inactivation, tumourigenesis, self-renewal, maintenance of pluripotency of stem cells and stimulation of E3 ubiquitin ligase activity. Thus, it appears that TRP47-expressing dense-cored ehrlichiae might interact with PCGF5 in order to modulate host cell gene expression to favour survival (Fig. 4).

Another interesting interaction that has been characterised is between TRP47 and IGLL1, the surrogate light chain associated with the pre-B-cell receptor (Ref. 73) (Fig. 3). The pre-B-cell

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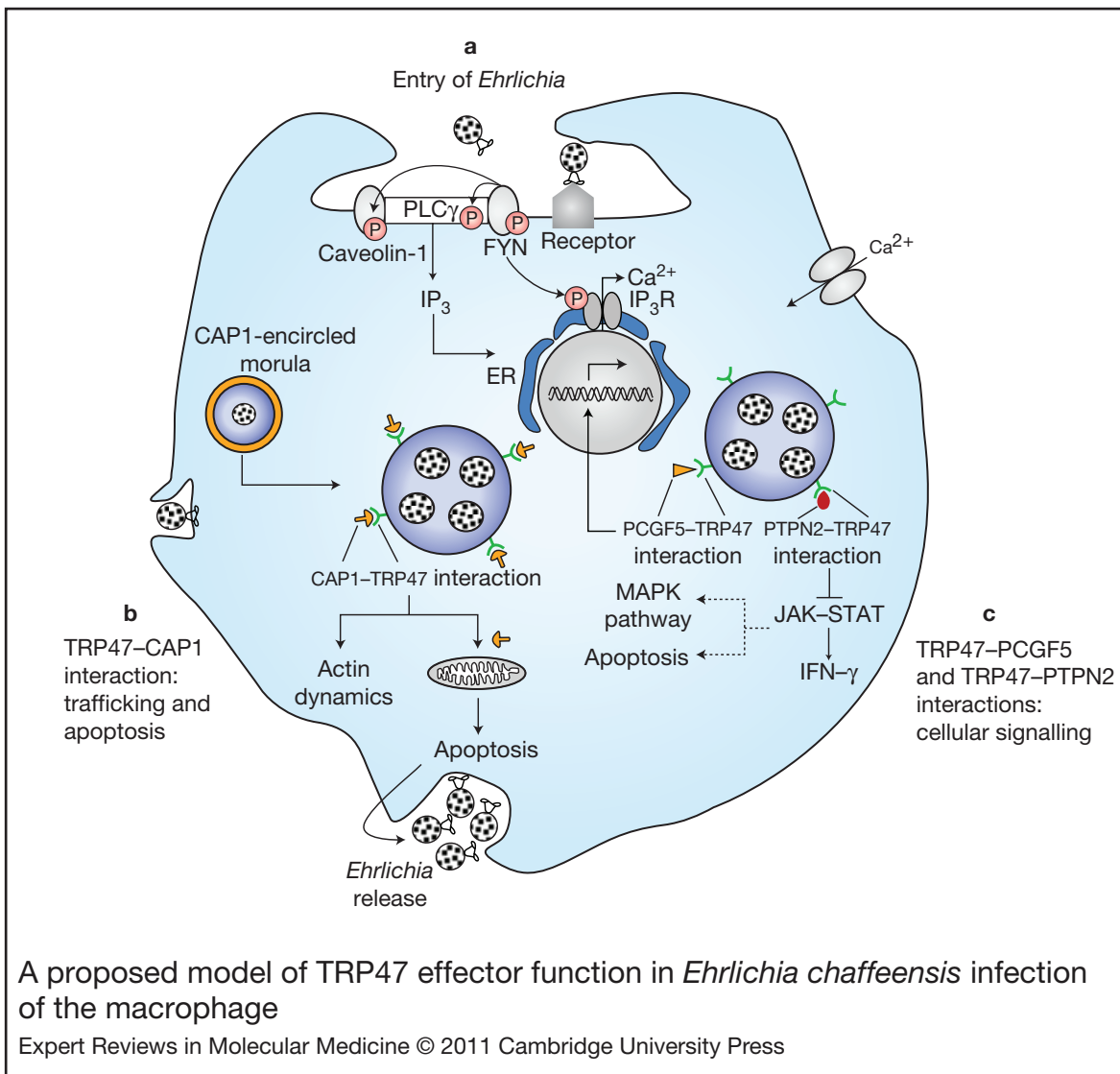


Figure 4. A proposed model of TRP47 effector function in *Ehrlichia chaffeensis* infection of the macrophage. (See next page for legend.)

Figure 4. A proposed model of TRP47 effector function in *Ehrlichia chaffeensis* infection of the macrophage. (See previous page for figure.) The schematic diagram is based on defined pathogen effector molecular interactions (Ref. 32) and previous reports of cellular events during infection (Refs 16, 52, 54, 56, 74). (a) Entry of *Ehrlichia*. Binding of *E. chaffeensis* to its receptor on the surface of an innate immune cell such as a monocyte directly or indirectly activates FYN. Activated (phosphorylated) FYN tyrosine kinase phosphorylates and activates PLC γ , which hydrolyses membrane phospholipid phosphatidylinositol (3,4)-bisphosphate (not shown), resulting in increased levels of inositol (1,4,5)-trisphosphate (IP $_3$) and thus release of Ca $^{2+}$ from intracellular stores and Ca $^{2+}$ influx. FYN might regulate the function of the IP $_3$ receptor by phosphorylation and promoting release of Ca $^{2+}$ from the endoplasmic reticulum. FYN also phosphorylates caveolin-1 involved in ehrlichial entry. (b) TRP47–CAP1 interactions. TRP47 is secreted by dense-cored *Ehrlichia* and associated with the morula surface, where it interacts with CAP1. This interaction might facilitate endocytosis and vesicle trafficking, because CAP1 promotes rapid actin dynamics in conjunction with ADF/cofilin and is required for cell morphology, migration and endocytosis; in addition, the TRP47–CAP1 interaction might promote apoptosis in the late stages of infection (which could have an important role in the release of dense-cored ehrlichiae), because mitochondrial shuttling of CAP1 promotes actin- and cofilin-dependent apoptosis. (c) TRP47–PCGF5 and TRP47–PTPN2 interactions. TRP47–PCGF5 interaction modulates gene transcription associated with cell signalling and remodelling of the cytoskeleton, facilitating and supporting intracellular survival of *E. chaffeensis*. TRP47–PTPN2 interaction might modulate cytokine signalling events by exerting negative feedback on the JAK–STAT pathway by dephosphorylation of JAKs and STATs involved in intracellular maintenance and survival of *E. chaffeensis*. Dashed lines indicate processes the JAK–STAT pathway might affect indirectly. Abbreviations: CAP1, adenylate cyclase-associated protein 1; ER, endoplasmic reticulum; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PCGF5, polycomb group ring finger 5; PLC γ , phospholipase C γ ; PTPN2, protein tyrosine phosphatase, non-receptor type 2; STAT, signal transducer and activator of transcription; TRP47, tandem repeat protein 47.

receptor is involved in transduction of signals for cellular proliferation, differentiation from the pro-B-cell to the pre-B-cell stage, allelic exclusion at the Ig heavy-chain gene locus and promotion of Ig light-chain gene rearrangements (Ref. 73). Thus, the significance of the interaction between TRP47 and IGLL1 might involve signalling and development, but suggests a novel role for IGLL1 in the macrophage and one that will require further study to understand.

The location of TRP47 on the surface of dense-cored ehrlichiae suggests that this protein might be involved in host cell attachment or entry. The association with FYN tyrosine kinase suggests that such a role is possible (Fig. 4). Tyrosine kinases are known to be involved in ehrlichial entry; however, the specific kinases involved have not been determined (Ref. 53). In addition, phosphorylation of host or bacterial proteins has been implicated in signalling pathways, triggering the entry of many intracellular pathogens. *Ehrlichia* enter through caveolae, and FYN is known to specifically phosphorylate caveolin-1 and is required for coxsackievirus internalisation and infection by caveolin-associated vesicles of polarised epithelial cells (Ref. 75). Evidence of tyrosine phosphorylation of TRP47 has been reported, suggesting that

there is a functional significance and that FYN might be responsible (Ref. 33).

TRP47 also interacts strongly with PTPN2, also known as T-cell PTP, which catalyses the dephosphorylation of phosphotyrosine peptides and regulates phosphotyrosine levels in signal transduction pathways. It is ubiquitously expressed with particularly high expression in haematopoietic tissues and appears to broadly influence haematopoietic cell development, but recent findings also demonstrate a role in several human illnesses, from autoimmune disease to cancer (Ref. 76). PTPN2 has several substrates, including CSF1R, EGFR, PDGFR, INSR, p52Shc (SHC1), JAK1, JAK3, STAT1, STAT3, STAT5A/B and STAT6 (Ref. 77). The JAK–STAT pathway is inhibited by monocytotropic *E. chaffeensis* (Ref. 74), supporting the possibility that TRP47 might be involved not only in the inhibition of IFN- γ -induced tyrosine phosphorylation of STAT1, JAK1 and JAK2 by interacting with PTPN2, but also in the regulation of cellular development (Fig. 4). *Ehrlichia* might favour PTPN2 upregulation because the loss of PTPN2 results in STAT5 hyperactivation, increased production of IFN- γ , TNF- α , IL-12 and inducible nitric oxide synthase, increased tyrosine phosphorylation, recruitment of a

complex of GRB2–GAB2–SHP2 (PTPN11) to the CSF1R and enhanced activation of ERK (MAPK1/3), and might affect transcription factor PU.1 signalling. Thus, TRP47 might influence a large number of important signal transduction pathways by interacting with PTPN2 (Fig. 4).

The membrane-bound vacuoles in which *Ehrlichia* reside in the host cell cytoplasm interact with endosomal recycling pathways. A specific interaction between TRP47 and the multifunctional protein CAP1 has been defined that appears to occur at the morula membrane interface, where CAP1 localises with the dense-core morulae adjacent to the morula boundaries (membrane) (Ref. 32). CAP1 is a highly conserved monomeric-actin-binding protein that contains binding domains for actin (C-terminal region), adenylate cyclase and cofilin (N-terminal region), and profilin (central region), and it has an active role in actin turnover (Ref. 78). Genetic studies in yeast have implicated CAP1 in vesicle trafficking and endocytosis. In mammalian cells, CAP1 is associated with the SH3-domain-dependent complex of actin-binding protein 1 and dynamin that is involved in receptor-mediated endocytosis (Ref. 79). Thus, in an effort to survive in the intracellular niche, *Ehrlichia* might manipulate cytoskeletal components of the mononuclear phagocyte such as actin by modulating CAP1 (Fig. 4). Interestingly, CAP1 has also been implicated in promoting apoptosis by functioning as an actin shuttle to mitochondria. Similar to cofilin, BAD and BAX, CAP1 rapidly translocates to mitochondria independently of caspase activation, where it promotes apoptosis (Ref. 80). Associations between ehrlichial morulae and mitochondria have been consistently observed (Ref. 15). Thus, the TRP47 and CAP1 interaction might be multifunctional by facilitating endocytosis and vesicle trafficking, and promoting apoptosis in the late stages of infection (Fig. 4).

E. chaffeensis Ank200 interaction with host Alu elements

There are four genes in the *E. chaffeensis* genome that encode proteins with Ank repeats. One of these proteins, Ank200, is a well-characterised major immunoreactive protein that has several species-specific antibody epitopes located primarily in acidic N- and C-terminal domains (Ref. 35). The *E. chaffeensis* Ank200 lacks a signal peptide, but is predicted to be secreted by a leaderless secretion system (Secretome 2.0), and

it has a T4SS motif (Ref. 81). Ank200 has recently been detected in host cell nuclei of *E. chaffeensis*-infected cells where it interacts with an adenine-rich motif in promoter and intronic Alu elements (Ref. 55). Alu elements are short, interspersed mobile DNA elements distributed in a nonrandom manner that comprise approximately 5–10% of the human genome and are thought to be involved in transcriptional regulation as carriers of cis-regulatory elements (Refs 82, 83). Alu elements have known binding sites for transcription factors including all MEF2 family members, as well as HNF1.03, OC.2, BARX2 and PAX4 (Ref. 84). The global analysis of binding sites of Ank200 demonstrates that this protein binds to several regions distributed on nearly every chromosome by direct DNA interaction or via other DNA-binding proteins.

The host cell processes targeted by Ank200 have been classified, and include genes associated with transcriptional regulation, apoptosis, ATPase activity and structural associations with the nucleus (Ref. 55) (Table 1). Interestingly, genes associated with host cell processes known to be altered during *E. chaffeensis* infection were found to be targets of Ank200. In addition, Ank200 appears to bind genes associated with transcription, and there is evidence that a large number of genes associated with transcription are modulated during *E. chaffeensis* infection (Ref. 56).

Several Ank200 target genes have been linked to pathogenesis and immune evasion, including TNF- α , JAK2 and CD48. TNF- α expression is not induced early in infection (<48 h) (Refs 85, 86), but expression is upregulated approximately 30-fold by day 5 post-infection (Ref. 55). *E. chaffeensis* Ank200 might contribute to the induction of TNF- α by binding directly to the promoter and upregulating gene transcription. Studies have demonstrated that overproduction or high serum concentration of TNF- α on day 7 post-infection is closely associated with fatality in severe monocytotropic ehrlichiosis (Refs 87, 88). One of the primary mechanisms by which *E. chaffeensis* survives in the host cell appears to be blocking macrophage responsiveness to IFN- γ (Ref. 59). Furthermore, JAK2 transcription appears to be silenced during *E. chaffeensis* infection, and JAK–STAT genes are also Ank200 targets, suggesting that *E. chaffeensis* uses several strategies, including directly modulating

Table 1. Selected *E. chaffeensis* Ank200 target genes identified by CHIP-on-chip^a

Gene	Chromosome	GeneBank (Accession no.)	Description
ZNF703	8	NM_025069	Zinc finger protein 703
ZNF513	2	NM_144631	Zinc finger protein 513
CD48	1	NM_001778	CD48 molecule
FGFRL1	4	NM_021923	Fibroblast growth factor receptor-like 1
PTK2B	8	NM_004103	Protein tyrosine kinase 2 beta
STAT1	2	NM_139266	Signal transducer and activator of transcription
PRCC	1	NM_199416	Papillary renal cell carcinoma
EFHC1	6	NM_018100	EF-hand domain (C-terminal) containing 1
TNF	6	NM_000594	Tumour necrosis factor α
JAK2	9	NM_004972	Janus kinase 2
DIO1	1	NM_000792	Deiodinase, iodothyronine, type I
ANKZF1	2	NM_018089	Ankyrin repeat and zinc finger domain containing 1
NDOR1	9	NM_014434	NADPH-dependent diflavin oxidoreductase 1
TDP1	14	NM_018319	Tyrosyl-DNA phosphodiesterase 1
USF1	1	NM_007122	Upstream transcription factor 1

^aInformation in the table is based on Ref. 55.
Abbreviation: CHIP-on-chip, chromatin immunoprecipitation and DNA microarray analysis.

genes associated with the JAK–STAT pathway (Ref. 56).

Immunopathological mechanisms and disease

During *E. chaffeensis* infection, there is a relatively low bacterial burden in the blood and tissues in nonimmunocompromised patients. However, the clinical manifestations, which include fever, multiorgan failure and adult respiratory distress syndrome, suggest that the pathogenesis of ehrlichiosis might involve immunopathological responses that are described as a toxic-shock-like syndrome (Refs 89, 90). Studies using a fatal murine model and a surrogate unnamed monocytic ehrlichia isolated from *Ixodes ovatus* ticks in Japan (IOE) suggest that such a mechanism is involved (Ref. 91). Mice inoculated with IOE develop histopathological lesions resembling those observed in human monocytotropic ehrlichiosis patients, and a similar disease course is observed in the IOE murine model. Lethal infections with IOE are accompanied by extremely high levels of serum TNF- α , a high frequency of splenic TNF- α -producing CD8⁺ T cells, decreased *Ehrlichia*-specific CD4⁺ T cell proliferation, low IL-12 levels in the spleen and a 40-fold decrease in the number of ehrlichial antigen-specific IFN- γ -

producing CD4⁺ Th1 cells (Refs 88, 92). Furthermore, mice lacking TNF receptors I/II are resistant to IOE-induced liver injury (an apparent effect of reduced immunopathology), but show higher bacterial burdens (indicating reduced protective immunity) (Ref. 93). Others have also demonstrated immunopathological responses linked to CD8⁺ T cells (Ref. 87). Interestingly, fatal memory responses against homologous but not heterologous challenge are associated with decreased bacterial burden, enhanced inflammatory response in the liver, decreased T cell responses and defective maintenance of IFN- γ -producing T cells (Ref. 94). CD1d-restricted NKT cells appear to be instrumental in the induction of immunopathological responses (Ref. 95).

Clinical implications and applications underlying

Ehrlichiosis manifests as an undifferentiated febrile illness that is difficult to diagnose, and delayed treatment can lead to serious complications and poor prognosis. There are no vaccines for human rickettsial diseases, including ehrlichiosis, and therapeutic options are limited. The rapid growth in antibiotic resistance among microbes suggests that

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antibiotics could become ineffective for ehrlichial and rickettsial infections in the future. The lack of broader therapeutic options is complicated by the fact that there are no effective vaccines available to prevent disease in susceptible populations. Recent advances in our understanding of molecular pathogen–host interactions, virulence mechanisms, effector proteins, the cellular basis of infection and the immunopathological basis of clinical manifestations have provided new targets for therapies that block pathogen virulence mechanisms, host–pathogen interactions or eukaryotic cell processes, and have identified additional approaches involving immunomodulatory therapies that can be considered.

Antibodies that block interactions between effector proteins and host targets can be used therapeutically. For example, passive transfer of *E. chaffeensis* TRP120-epitope-specific antibodies reduces ehrlichial burden and demonstrates the potential of therapeutic antibodies against ehrlichial effector proteins (Ref. 96). These studies also suggest that vaccines containing defined antigens are feasible and will be developed in the future. Additionally, drugs that target specific pathogen virulence mechanisms such as secretion systems and signal transduction systems or inhibit acquisition of essential components for pathogen structural integrity and function might be viable alternative therapies that can be used for patient treatment. Defining molecular host–pathogen interactions and ultimately the three-dimensional structures of effector molecules will allow the development of small-molecule inhibitors that can disrupt the ability of the pathogen to manipulate host cell processes resulting in pathogen clearance by the immune system.

Managing patients who are experiencing manifestations and complications associated with severe disease requires understanding of the underlying immune dysregulation, to develop rational strategies to alleviate its effects. The overproduction of inflammatory cytokines or chemokines is responsible for serious clinical manifestations that can be managed by targeted immunotherapies that block cytokine production or neutralise cytokine effects by blocking a specific cytokine–receptor interaction and its immunopathological consequences. Such patient management strategies can be

considered as the immune effectors are comprehensively defined.

Research in progress and outstanding research questions

The complexity of the pathogen–host relationship between *Ehrlichia* and the eukaryotic host cell is becoming better understood, and the mechanisms used by the organism to reprogramme cellular processes are becoming more defined. New pathogen–host interactions such as nuclear translocated proteins that bind host chromatin have been recently described, but much more research is needed to fully appreciate how these effector proteins modulate host cell transcription and whether they can be useful therapeutic targets. Other effector proteins and secretion mechanisms have been identified, but the mechanisms used by these effectors and how they modulate targeted host cell processes are areas of active research that will provide useful information for the development of therapeutics. Much research on the cellular processes that are affected during infection has been accomplished, and advancing knowledge regarding host cell processes that are targeted by the organism will be beneficial for designing therapeutic interventions for *Ehrlichia* as well as other rickettsial pathogens.

Development of immunomodulatory strategies for patient management will become more practical in the future, and research to understand the immunopathological mechanisms involved has taken large steps forward. Questions that need to be addressed are why some patients respond immunologically in a detrimental way and how we can accurately identify and manage these patients. More research needs to be devoted to the development of biomarkers for disease for diagnosis and development of rational strategies for patient management and therapeutic decisions.

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Further reading, resources and contacts

Rikihisa, Y. (2010) Molecular events involved in cellular invasion by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*. *Veterinary Parasitology* 167, 155-166

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Summarises recent advances in vaccine development.

Thomas, R.J., Dumler, J.S. and Carlyon, J.A. (2009) Current management of human granulocytic anaplasmosis, human monocytic ehrlichiosis and *Ehrlichia ewingii* ehrlichiosis. *Expert Review of Anti-Infective Therapy* 7, 709-722

Covers epidemiological and clinical aspects related to these diseases and suggestions for future research.

Features associated with this article

Figures

Figure 1. Immunogold-labelled electron micrograph of *Ehrlichia chaffeensis* TRP47.

Figure 2. Developmental cycle of *Ehrlichia chaffeensis* in the eukaryotic host cell.

Figure 3. Colocalisation of PCGF5 and IGLL1 with *Ehrlichia chaffeensis* expressing TRP47 within infected THP-1 cells.

Figure 4. A proposed model of TRP47 effector function in *Ehrlichia chaffeensis* infection of the macrophage.

Table

Table 1. Selected *E. chaffeensis* Ank200 target genes identified by ChIP-on-chip.

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