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Cells within cells: Rickettsiales and the obligate intracellular bacterial lifestyle

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Abstract | The Rickettsiales are a group of obligate intracellular vector-borne Gram-negative bacteria that include many organisms of clinical and agricultural importance, including *Anaplasma* spp., *Ehrlichia chaffeensis*, *Wolbachia*, *Rickettsia* spp. and *Orientia tsutsugamushi*. This Review provides an overview of the current state of knowledge of the biology of these bacteria and their interactions with host cells, with a focus on pathogenic species or those that are otherwise important for human health. This includes a description of rickettsial genomics, bacterial cell biology, the intracellular lifestyles of Rickettsiales and the mechanisms by which they induce and evade the innate immune response.

Chlamydiales
An order of obligate intracellular bacteria that live in animals, insects and protozoa and includes species that are pathogenic to humans.

Endosymbiont
An organism that lives within another organism, either intracellularly or extracellularly, usually but not always in a mutually beneficial symbiosis.

Obligate intracellular bacteria are a group of highly specialized organisms that have become so effective at living within eukaryotic cells that they have lost the genes that would enable them to live outside their cellular hosts. There are two major groups of obligate intracellular bacteria that cause human disease: the Chlamydiales and the Rickettsiales. The Gram-negative alphaproteobacteria Rickettsiales are an order of vector-borne species that include non-pathogens, animal pathogens and human pathogens. Seven major genera and at least 87 species have been described within the order, and they exhibit a high degree of diversity in cell structure, vector preference, host cell type preference, pathogenicity and infection cycle (FIG. 1; TABLE 1). This natural diversity makes them a powerful group of organisms for the comparative study of factors that determine host–pathogen interactions, and their reduced genomes make them valuable model systems for understanding minimal requirements for complex machineries involved in bacterial growth and division.

The order Rickettsiales comprises two families (FIG. 1): the Anaplasmataceae, which includes species *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Neorickettsia sennetsu* and *Wolbachia*, and the Rickettsiaceae, which includes species *Orientia tsutsugamushi* and Rickettsia spp. The genus *Wolbachia* contains a single species, *Wolbachia pipientis*, classified into supergroups A–H and named after a key species in each supergroup, such as *Drosophila melanogaster* (*w*Mel) or *Brugia malayi* (*w*Bm)[1]. Bacteria in the genus *Rickettsia* are classified as being in the spotted fever group (SFG; *Rickettsia rickettsii*, *Rickettsia conorii*, *Rickettsia africae* and *Rickettsia parkeri*), the typhus group (TG; *Rickettsia prowazekii* and *Rickettsia typhi*), the transitional group (*Rickettsia felis* and *Rickettsia akari*) and the non-pathogenic ancestral group (*Rickettsia canadensis* and *Rickettsia bellii*)[2,3].

The relationship between Rickettsiales and their invertebrate hosts can be parasitic, symbiotic or commensal depending on whether the infection confers a fitness cost or benefit. The infection can be vertically transmitted from mother to offspring, or horizontally acquired through feeding on infected animals during the larval or nymph stage[4]. *Wolbachia* is a widespread endosymbiont of insects and arthropods that can be transmitted both vertically and horizontally in the case of reproductive parasite strains such as *w*Mel, or strictly vertically transmitted in the case of the non-parasitic mutualist *w*Bm[5]. *O. tsutsugamushi* is found in the ovaries and salivary glands of mites and is primarily vertically transmitted[6]. Horizontal acquisition from other mites or infected animals can occur through co-feeding, but bacteria acquired in this way are not efficiently transmitted to the next generation[7]. *Rickettsia* spp. can be vertically and horizontally transmitted[8]. *A. phagocytophilum*, *A. marginale* and *E. chaffeensis* are all maintained in animal reservoirs and transferred to ticks through horizontal transmission[9].

Rickettsiales species cause disease in humans or animals when the preferred invertebrate host feeds on mammals and transmits the pathogens, and the interaction between replicating bacteria and the mammalian immune system results in bacterial growth and tissue damage. With the exception of *R. prowazekii*, humans are 'dead-end' hosts and the bacterium does not return to the environmental reservoir. Rickettsial pathogens are inoculated into the blood or skin of humans through the saliva or faeces of feeding arthropods and insects, and are disseminated through the body via the blood and/or lymphatic system. The tropism to mammalian cells varies between species and includes endothelial cells (*Rickettsia* spp., *O. tsutsugamushi*)[6,10,11], dendritic cells (*O. tsutsugamushi*)[12], monocytes and

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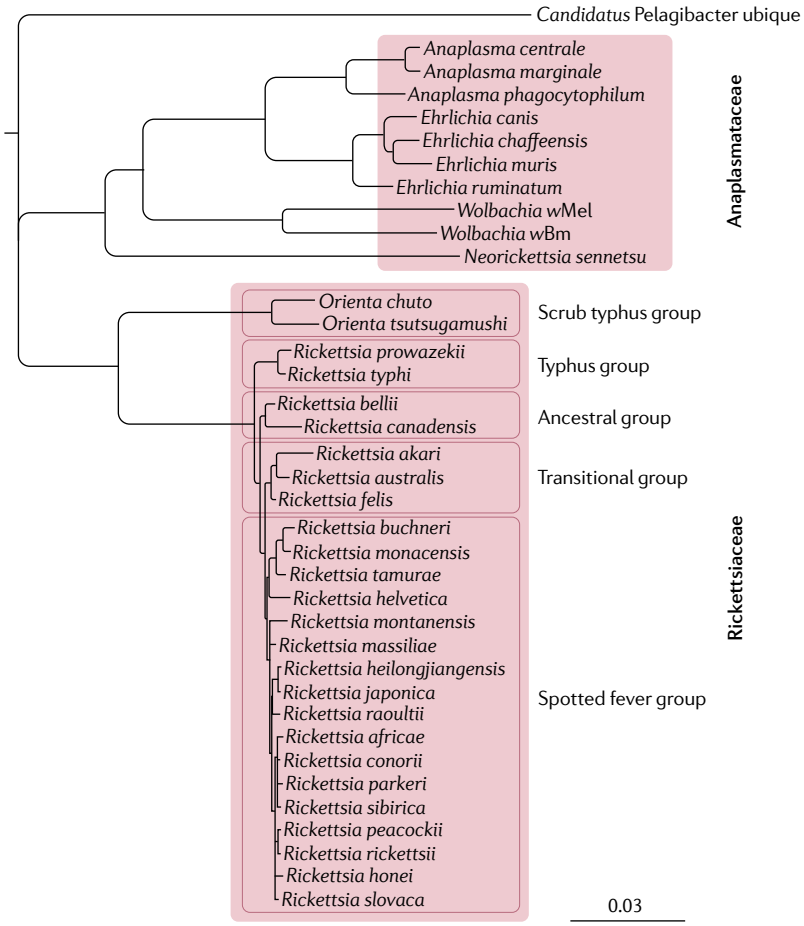


Fig. 1 | **Phylogenetic tree showing relationship between selected Rickettsiales species as determined by 16S sequences.** Sequences were aligned using a global alignment with free-end gaps, and the tree was built using the Tamura–Nei neighbour-joining method, using geneious software. *Candidatus* Pelagibacter ubique was used as an outgroup. Scale represents the number of substitutions between sequences.

macrophages (*O. tsutsugamushi* and *E. chaffeensis*)[12,13] and neutrophils (*A. phagocytophilum*)[14]. Symptoms begin 5–14 days post inoculation, and rickettsial diseases are characterized by headache, fever, myalgia and sometimes a rash, as well as an eschar in scrub typhus and some SFG rickettsial diseases[15]. Rickettsial species are not susceptible to many classes of antibiotics, and doxycycline is the preferred drug for treatment of rickettsial diseases[6]. Although disease severity is variable, all of the infections can be life threatening and there are currently no approved vaccines for any human rickettsial disease[16–18].

The intracellular life cycles of Anaplasmataceae and Rickettsiaceae are fundamentally different from each other (FIG. 2). Anaplasmataceae reside within a membrane-bound vacuole in the infected host cell, and *A. marginale*, *A. phagocytophilum* and *E. chaffeensis* differentiate between a replicative intracellular form called reticulate cells and an infectious extracellular form called dense-core cells. By contrast, Rickettsiaceae escape from the endolysosomal pathway shortly after entry and complete their growth and replication directly in the host cell cytoplasm. Following replication, *O. tsutsugamushi* buds out of infected cells prior to infecting new cells, whereas *Rickettsia* spp. move directly into adjacent cells within a monolayer (SFG) or lyse heavily infected cells (TG). Distinct developmental forms during the course of the cellular infection cycle, equivalent to the reticulate and dense-core forms in Anaplasmataceae, have not been described for any Rickettsiaceae species.

In this Review, I describe the key features of the best-studied Rickettsiales species that are pathogenic to humans or animals, or otherwise important for human health (*A. phagocytophilum*, *A. marginale*, *E. chaffeensis*, *Wolbachia*, *O. tsutsugamushi*, SFG and TG *Rickettsia* spp.) and the molecular basis of their interactions with mammalian host cells. In the case of *Wolbachia*, which does not directly infect mammalian cells, the interactions with invertebrate cells are described. Excellent recent reviews on specific organisms have previously been published[4,11,13,19–25]. This Review does not discuss the interactions between Rickettsiales and their invertebrate hosts, and readers are referred to the other reviews on this topic[5,26–28].

Rickettsial genomes

As obligate intracellular bacteria, Rickettsiales genomes are physically isolated from other bacteria and experience small effective population sizes. As a consequence, novel genes are rarely introduced into the population, and mutations lead to gene loss and pseudogenization[29]. This reductive genome evolution has resulted in small Rickettsiales genomes, with sizes ranging from 0.8 to 2.5 Mbp (TABLE 1), and a predicted core genome across the Rickettsiales of ~420 genes[30] and a GC content of 27–50% (Supplementary Table 1). Differences in genome size reflect differences in the number of repeats, non-coding DNA and pseudogenes, with 0.3% of the 1.11 Mbp genome of *R. prowazekii* being composed of repetitive elements compared with 49% in the 2.5 Mbp genome of *O. tsutsugamushi* (strain Karp).

The Rickettsiales include symbionts and pathogens, making them ideal models for studying pathogenicity in bacteria using comparative genomics approaches (reviewed in REFS[4,31,32]). Reductive evolution is positively associated with pathogenicity in Rickettsiales, with more virulent species generally having smaller genomes than avirulent relatives[33,34] as seen in *R. prowazekii*, *R. typhi*, *A. marginale* and *E. chaffeensis*. As an example, this has been shown by comparing highly virulent *R. prowazekii* with less virulent strains, including *R. conorii*[35] and *R. africae*[36]. This can be explained by a loss-of-control mechanism that regulates host cell entry, replication and transmission, which leads to increased host cell damage[32,36,37]. However, reductive evolution is not the sole driver of virulence, and Rickettsiales also encode genes that are positively associated with virulence. For example, the comparison of virulent *R. rickettsii* with less virulent *R. rickettsii* strains and an avirulent relative (*Rickettsia peacockii*)[33,34] led to the identification of potential virulence factors and, moreover, the identification of four genes specific to TG rickettsiae, which have the smallest genomes in the Rickettsiaceae, suggested a specific role for these genes in virulence[3,38].

The evolution of *Wolbachia* genomes has been shaped by the presence of bacteriophages and insertion

Eschar
A small mark on the skin that resembles a cigarette burn and that forms at the site of infection by *Orientia tsutsugamushi* and some spotted fever group rickettsiae.

Pseudogenization
An evolutionary process in which a gene loses its function through mutation or disruption of the coding sequence.

Table 1 | **Overview of pathogenic Rickettsiales species or those otherwise important for human health**

Family and group	Organism	Disease (human unless otherwise stated)	Host cells in mammals	Reservoir	Vector	Evidence of transovarian transmission	Currently known geographic distribution	Genome size (Mbp)
Anaplasmataceae								
–	*Anaplasma marginale* subsp. *centrale*	Bovine anaplasmosis	Erythrocytes	Cattle, wild ruminants	*Rhicephalus* spp., *Amblyomma variegatum* (ticks)	No	South America, Africa, Middle East	1.20
	A. marginale	Bovine anaplasmosis	Erythrocytes	Cattle, wild ruminants	*Rhicephalus microplus*, *Dermacentor* (ticks)	No	Worldwide in tropical/ subtropical regions	1.20
	Anaplasma phagocytophilum	Human granulocytotropic anaplasmosis	Neutrophils	Deer, rodents, ruminants, horses, dogs, cats	*Ixodes scapularis*, *Ixodes pacificus*, *Ixodes ricinus* (ticks)	No	North America, Europe, Asia	1.48
	Candidatus Neoehrlichia mikurensis	Neoehrlichiosis	n.d.	Mammals, birds	*I. ricinus* (ticks)	No	Europe	–
	Ehrlichia canis	Canine monocytic ehrlichiosis	Monocytes, macrophages	Dogs	*Rhipicephalus sanguineus* (ticks)	No	Worldwide	1.31
	Ehrlichia chaffeensis	Human monocytotropic ehrlichiosis	Monocytes, macrophages	Deer, goats, dogs	*Amblyomma americanum* (ticks)	No	North America, Africa, Asia	1.18
	Ehrlichia ewingii	Canine and human granulocytotropic ehrlichiosis	Neutrophils	Dogs	*A. americanum* (ticks)	No	Worldwide	–
	Ehrlichia muris eauclairensis	Human ehrlichiosis	n.d.	Unknown	*I. scapularis*, *Peromyscus leucopus* (ticks)	No	Worldwide	–
	Ehrlichia muris muris	Human ehrlichiosis	n.d.	Unknown	*I. scapularis*, *P. leucopus* (ticks)	No	North America, possibly elsewhere	1.20
	Ehrlichia ruminantium	Bovine heartwater or cowdriosis	Endothelial cells	Ruminants	*Amblyomma* spp. (ticks)	No	Africa, Caribbean	1.51
	Neorickettsia sennetsu	Sennetsu fever, glandular fever	Monocytes, macrophages	Fish	Unknown	Yes	Asia	0.86
	*Wolbachia pipientis w*Mel	None known	NA	Arthropods	NA	Yes	Worldwide	1.27
	Wolbachia sp. *w*Bm	River blindness (via filarial nematode)	NA	Nematode *Brugia malayi*	NA	Yes	Worldwide in tropical/ subtropical regions	1.08
Rickettsiaceae								
Spotted fever group	*Rickettsia africae*	African tick bite fever	Endothelial cells	*Amblyomma* ticks	*Amblyomma hebraeum*, *A. variegatum* (ticks)	Yes	Africa, West Indies	1.28
	Rickettsia buchneri	None known	n.d.	*I. scapularis*	*I. scapularis* (ticks)	Yes	North America	1.82
	Rickettsia conorii	Boutonneuse fever	Endothelial cells, macrophages	Dogs	*R. sanguineus*, *Rhipicephalus pumilio* (ticks)	Yes	Southern Europe, Africa, Southern Asia	1.27
	Rickettsia heilongjiangensis	Far eastern spotted fever	Endothelial cells	Rodents	*Dysmicoccus sylvarum* (ticks)	No	Asia	1.28
	Rickettsia helvetica	Aneruptive fever[a]	n.d.	Rodents	*Dermacentor reticulatus*, *Ixodes* spp. (ticks)	Yes	Asia	1.37

Table 1 (cont.) | **Overview of pathogenic Rickettsiales species or those otherwise important for human health**

Family and group	Organism	Disease (human unless otherwise stated)	Host cells in mammals	Reservoir	Vector	Evidence of transovarian transmission	Currently known geographic distribution	Genome size (Mbp)
Rickettsiaceae (cont.)								
Spotted fever group (cont.)	*Rickettsia honei*	Flinders Island spotted fever, Thai tick typhus	n.d.	Rodents, reptiles	*Bothriocroton hydrosauri*, *Ixodes* spp. *Haemaphysalis. novaeguineae* (ticks)	Yes	Australia, Asia	1.27
	Rickettsia japonica	Japanese spotted fever	n.d.	Rodents	*Haemophilus* spp., *Ixodes ovatus*, *Dermacentor taiwanensis* (ticks)	Yes	Asia	1.28
	Rickettsia massiliae	Mediterranean spotted fever-like disease	Endothelial cells	Unknown	*Rhipicephalus* spp., *I. ricinus*, *Haemaphysalis paraleachi* (ticks)	Yes	Europe, Africa, USA	1.28
	Rickettsia monacensis	Spotted fever	n.d.	Unknown	*Ixodes* spp. (ticks)	Yes	Europe, North Africa	1.35
	Rickettsia montanensis	Limited reports of pathogenicity[a]	n.d.	Lizards, possibly birds	*Dermacentor* spp., *A. americanum* (ticks)	Yes	North and Central America	1.28
	Rickettsia parkeri	Maculatum disease	Endothelial cells, macrophages	Dogs, cattle, *Amblyomma* ticks	*Amblyomma maculatum*, *Amblyomma triste*, *Dermacentor variabilis* (ticks)	Yes	North and South America	1.30
	Rickettsia peacockii	None known	NA	Unknown	*Dermacentor andersoni* (ticks)	Yes	North and Central America	1.29
	Rickettsia raoultii	Tick-borne lymphadenopathy (SENLAT)	n.d.	Unknown	*Dermacentor* spp., *I. ricinus*, *Haemaphysalis* spp., *Amblyomma testudinarium* (ticks)	Yes	Europe, Asia, North Africa	1.34
	Rickettsia rickettsii	Rocky Mountain spotted fever	Endothelial cells	Rodents	*Dermacentor variables*, *D. andersoni*, *R. sanguineus*, *Amblyomma cajennense*, *Amblyomma aureolatum* (ticks)	Yes	North and South America	1.3
	Rickettsia sibirica	North Asian tick typhus, Siberian tick typhus	n.d.	Rodents	*Dermacentor* spp., *R. sanguineus*, *Ixodes persulcatus*, *Hyalomma* spp., *Rhizomucor pusillus* (ticks)	No	Europe, Africa, Asia	1.25
	Rickettsia slovaca	Tick-borne lymphadenopathy (SENLAT)	n.d.	Unknown	*Dermacentor* spp. (ticks)	Yes	Europe, Asia, North Africa	1.28
	Rickettsia tamurae	Spotted fever	n.d.	Unknown	*Ixodes* spp. (ticks)	No	Asia	1.45
Typhus group	*Rickettsia prowazekii*	Epidemic typhus/ Brill–Zinsser disease	Endothelial cells	Humans, flying squirrels	*Pediculus humanus humanus* (louse), flying squirrel ectoparasites	No	Worldwide	1.11
	Rickettsia typhi	Mouse typhus	Endothelial cells	Rats	*Xenopsylla cheopis*, *Ctenocephalides felis* (fleas)	No	Worldwide	1.11
Transitional group	*Rickettsia akari*	Rickettsialpox	Endothelial cells, macrophages	Mice	*Liponyssoides sanguinus* (mites)	Yes	North America, Asia, Europe	1.23

Table 1 (cont.) | **Overview of pathogenic Rickettsiales species or those otherwise important for human health**

Family and group	Organism	Disease (human unless otherwise stated)	Host cells in mammals	Reservoir	Vector	Evidence of transovarian transmission	Currently known geographic distribution	Genome size (Mbp)
Rickettsiaceae (cont.)								
Transitional group (cont.)	*Rickettsia australis*	Queensland tick typhus	Endothelial cells	Unknown	*Ixodes holocyclus*, *Ixodes tasmani*, *Ixodes cornuatus* (ticks)	No	Australia	1.30
	Rickettsia felis	Flea-borne spotted fever[a]	n.d.	Fleas	*C. felis* (flea)	Yes	Worldwide	1.59
Ancestral group	*Rickettsia bellii*	None known	NA	Unknown	*Amblyomma* spp., *Dermacentor* spp. (ticks), *Haemophysalis* spp. (ticks)	Yes	North and South America	1.53
	Rickettsia canadensis	None known	NA	Unknown	*Haemaphysalis leporispalustris* (ticks)	No	North and Central America	1.16
Scrub typhus group	*Orientia tsutsugamushi*	Scrub typhus	Endothelial cells, dendritic cells, monocytes/ macrophages	Trombiculid mites	*Leptotrombidium* spp. (mites)	Yes	Asia, Oceania, Northern Australia	2.5
	Candidatus Orientia chiloensis	Scrub typhus	n.d.	Unknown	n.d.	n.d.	Chilé	–
	Candidatus Orientia chuto	Scrub typhus	n.d.	Unknown	n.d.	n.d.	United Arab Emirates	–

See Supplementary Table 1 for information on genome repetitiveness, percentage of genome that is coding, plasmids, predicted coding DNA sequence (CDS), hypothetical protein and GC content. The information provided in this table relates to the following strains: *Anaplasma centrale* Str. Israel, *Anaplasma marginale* str. St. Maries, *Anaplasma phagocytophilum* str. HZ, *Ehrlichia canis* str. Jake, *Ehrlichia chaffeensis* str. Arkansas, *Ehrlichia muris muris* str. AS145, *Ehrlichia muris eauclairensis* Wisconsin, *Ehrlichia ruminatum* str. Welgevonden, *Neorickettsia sennetsu* str. Miyayama, *Wolbachia pipientis* wMel endosymbiont of *Drosophila melanogaster*, *Wolbachia* sp. wBm endosymbiont of *Brugia malayi* isolate TRS, *Rickettsia africae* str. ESF-5, *Rickettsia buchneri* str. REIS, *Rickettsia conorii* str. Malish 7, *Rickettsia heilongjiangensis* str. 054, *Rickettsia helvetica* str. C9P9, *Rickettsia honei* str. RB, *Rickettsia japonica* str. YH, *Rickettsia massiliae* str. AZT80, *Rickettsia monacensis* str. IrR/Munich, *Rickettsia montanensis* str. OSU 85-930, *Rickettsia parkeri* str. Portsmouth, *Rickettsia peacockii* str Rustic, *Rickettsia raoultii* str. Khabarovsk, *Rickettsia rickettsii* str. Iowa, *Rickettsia sibirica* str. 246, *Rickettsia slovaca* str. 13-B, *Rickettsia tamurae* str. AT-1, *Rickettsia prowazekii* str. Madrid E, *Rickettsia typhi* str. Wilmington, *Rickettsia akari* str. Hartford, *Rickettsia australis* str. Cutlack, *Rickettsia felis* str. URRWXCal2, *Rickettsia bellii* str. RML369-C, *Rickettsia canadensis* str. McKiel, *Orientia tsutsugamushi* str. Karp, *Candidatus* Orientia *chuto* str. Dubai. NA, not applicable; n.d., not determined; SENLAT, scalp eschar and neck lymphadenopathy after tick bite. Table based on REFS[4,10,11,37,178–181]. [a]Pathogenicity in humans not confirmed.

sequence elements[39], whereas conjugative transfer elements are found in Rickettsiaceae[33,40]. One particular conjugative transfer element, the Rickettsiales amplified genetic element (RAGE), is widespread in some Rickettsiaceae genomes, including those of *O. tsutsugamushi* and *Rickettsia buchneri* (formerly *Rickettsia* endosymbiont of *Ixodes scapularis*)[40,41], and present in single and/or degraded copies in other rickettsial genomes[40]. Mobile genetic elements are not present in *Anaplasma* or *Ehrlichia* spp. Extrachromosomal plasmids have been described in some *Rickettsia* spp., and these have facilitated the development of genetic tools in Rickettsiales[2,42,43] (see BOX 1).

Bacterial cell biology

Cell membrane and cell wall. Gram-negative bacteria typically have a cell wall composed of peptidoglycan and an outer membrane in which the outer leaflet comprises lipopolysaccharide (LPS). However, many Rickettsiales species have been shown to reduce or lack these normally essential components of the cell wall and cell membrane. Although LPS has been found in species from the *Rickettsia* genus, genes for LPS biosynthesis are absent in *O. tsutsugamushi* and Anaplasmataceae species[44,45]. *Rickettsia* spp. are also the only Rickettsiales predicted to synthesize a complete, classic peptidoglycan cell wall[46]. *O. tsutsugamushi, Wolbachia* and *A. marginale* possess a subset of peptidoglycan biosynthesis genes and may build a minimal peptidoglycan-like structure[44,47,48], whereas *A. phagocytophilum* and *Ehrlichia* spp. lack genes for peptidoglycan biosynthesis[44]. *E. chaffeensis* and *A. phagocytophilum* are unusual among bacteria as they have cholesterol-rich membranes, which probably confer some rigidity in the absence of peptidoglycan and LPS. These bacteria lack the biosynthetic capability to synthesize cholesterol and scavenge it from host cells[49,50]. The osmotically protective intracellular niche is likely to enable survival in the absence of these otherwise essential bacterial components, but questions remain as to the differential selective pressures that led to these differences in cell wall and cell membrane structures across the Rickettsiales[44].

Bacterial surface proteins. Proteins embedded on the outer surface of the outer membrane of Rickettsiales bacteria have an important role in host–pathogen interactions because these structures are in direct contact

Peptidoglycan
A highly cross-linked polymer composed of a polysaccharide backbone and short peptide sidechains that encases bacterial cells and confers osmotic protection and rigidity.

Lipopolysaccharide
(LPS). A large, negatively charged, lipid-anchored polysaccharide that comprises the outer leaflet of the outer membrane of most Gram-negative bacteria.

RecF-mediated gene conversion
A pathway of homologous recombination that repairs double-stranded breaks in bacteria.

with the outside environment, and this is particularly important in organisms that lack LPS, which normally shields the outer membrane surfaces of bacteria. Outer membrane proteins have important roles in adhesion to host cells, in interactions with cytoplasmic components of host cells (in cytoplasm-dwelling Rickettsiaceae) and in stimulating and evading an immune response. The immunogenic nature of many Rickettsiales surface proteins has made them strong potential vaccine candidates. However, they are under selective pressure to evade immune recognition, and thus some surface proteins exhibit substantial variation either between strains and/or during the course of an infection (Supplementary Table 2), which limits their suitability for developing widely effective vaccines.

The most abundant and immunogenic outer membrane proteins in *E. chaffeensis*, *A. marginale*, *A. phagocytophilum* and *Wolbachia* are porin proteins from the OMP1 superfamily (alternatively called MSP2, MSP3, MSP4, P28 and P44 in different species) (pfam01617)[25]. These are composed of eight transmembrane β-barrel sheets, which are generally highly conserved, and numerous surface exposed loops containing hypervariable domains. Variations in the hypervariable and immunogenic domains enable immune evasion, and Anaplasmataceae have evolved different strategies to introduce variation in these hypervariable domains. In *Anaplasma* spp., the hypervariable regions in MSP2 undergo recombination during the course of an infection, contributing to immune evasion[25]. In *A. phagocytophilum* there are ~100 *msp2* (also called *p44*) paralogues (strain HZ) and one expression site, and the bacterium uses RecF-mediated gene conversion to vary gene expression[25]. *A. marginale* similarly uses recombination to generate antigenic variation of MSP2, but the number of donor pseudogenes is much smaller (five to seven in strain St. Maries), and segmental recombination is used to generate a mosaic gene, thus increasing variation[51]. *A. marginale* also switches expression between MSP2 and another OMP1–MSP2–P44 family protein, termed MSP3, which is also polymorphic, conferring further antigenic variation. The MSP2 homologue in *E. chaffeensis* is P28. *Ehrlichia* spp. encode a tandem series of 17–22 *p28* genes, mostly with functional promoters. Antigenic variation is achieved through differential expression of intact gene copies[52]. The MSP2 homologue in *Wolbachia* is *Wolbachia* surface protein (WSP), and the hypervariable regions of WSP exhibit strain to strain variation driven by mutation and intragenic and intergenic recombination[53]. Antigenic variation is generally thought to be driven in response to adaptive immunity of the host, and as this is lacking in invertebrates, the presence of antigenic variation in WSP suggests a different selective pressure during adaptation to specific invertebrate hosts.

The Anaplasmataceae encode other major surface antigens, including tandem repeat protein 32 (TRP32),

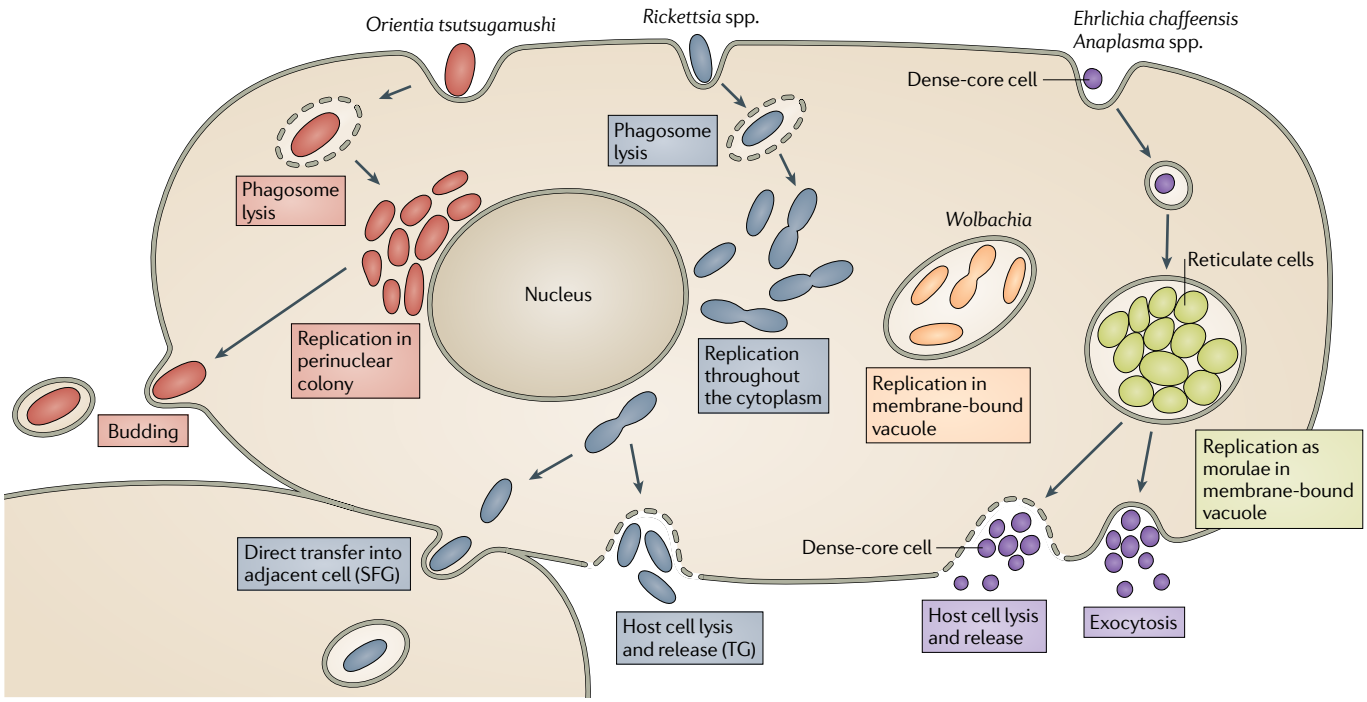


Fig. 2 | **Overview of the intracellular life cycles of Rickettsiales.** *Orientia tsutsugamushi*, *Rickettsia* spp., *Anaplasma* spp. and *Ehrlichia chaffeensis* all enter host cells using a 'zipper-like' mechanism of induced endocytosis. *O. tsutsugamushi* and *Rickettsia* spp. exit the endolysosomal pathway shortly after entry, whereas *Wolbachia*, *Anaplasma* spp. and *E. chaffeensis* replicate within dedicated pathogen-containing vacuoles. *O. tsutsugamushi* replicates in a microcolony adjacent to the host nucleus whereas *Rickettsia* spp. are distributed through the cytoplasm. *O. tsutsugamushi* exits infected cells using a virus-like budding mechanism. *Rickettsia* spp. move directly into adjacent cells or exit through host cell lysis. *E. chaffeensis* and *Anaplasma* spp. exit through exocytosis and host cell lysis. *E. chaffeensis* and *Anaplasma* spp. exhibit a biphasic life cycle, alternating between a small, infectious dense-core cell form and an enlarged, replicative reticulate cell form. Similar developmental differentiation through the intracellular infection cycle has not been described for *Wolbachia*, *Rickettsia* spp. or *O. tsutsugamushi*. SFG, spotted fever group; TG, typhus group.

Box 1 | **Tools to study Rickettsiales**

Genetic tools for working with Rickettsiales are limited in availability compared with other obligate intracellular bacteria, such as *Chlamydia trachomatis* and *Coxiella burnetii*, although important improvements have been made over the past decade. These are summarized in a recent comprehensive review[182]. Obligate intracellular bacteria are difficult to genetically modify because they generally need to be isolated from host cells for transformation, which risks damaging the bacteria. In addition, the intracellular life cycle means that it is not possible to generate mutations in genes required for internalization or intracellular growth. In spite of these challenges, genetic modification has been successful in *Rickettsia* spp., *Anaplasma* spp. and *Ehrlichia chaffeensis*. There are no reports of genetic modification in *Orientia tsutsugamushi* or *Wolbachia*. Transformation has been achieved using electroporation[183–185] and polyamidoamine dendrimers[186]. Protein overexpression has been achieved in *Rickettsia* spp. using shuttle vectors derived from plasmids from *Rickettsia amblyommatis*[43]. Random mutagenesis has been achieved in *Rickettsia* spp., *Anaplasma phagocytophilum*, *Anaplasma marginale* and *E. chaffeensis* using a *Himar 1* mariner transposon system[114,183,184,187], and recently in *Rickettsia conorii* using a *kkaebi* mini transposon system[188], and targeted mutagenesis has been achieved in *E. chaffeensis* and *Rickettsia* spp. using allelic exchange[184,189] and mobile group II introns[184,190]. Transient knockdowns using peptide nucleic acid technology has also been described in *Rickettsia* spp. and *A. marginale*[177]. Overexpression of fluorescence proteins has been used for imaging intracellular Rickettsiales in cultured cells and animals[191,192]. Despite these advances, fluorescent protein overexpression is not straightforward in most species, and alternative approaches for fluorescence microscopy are required. Giemsa staining is traditionally used to image bacteria in tissue sections and clinical samples[193], whereas immunofluorescence microscopy is widely used to study fixed samples of Rickettsiales in cultured cells and animal models, although this suffers the limitation that antibodies need to be custom-generated for particular organisms and strains. A clickable methionine probe that specifically labels bacteria that are undergoing protein synthesis has been used to label intracellular Rickettsiales species and identify individual cells that are metabolically active[194]. Commercially available fluorescent probes have been used to carry out live cell imaging of *O. tsutsugamushi*[195], *Wolbachia*[196] and *E. chaffeensis*[197], whereas fluorescence in situ hybridization has been used to label *Wolbachia* and *Rickettsia* spp. in fixed cells and tissues[198,199].

TRP47, TRP120, OmpA and EtpE in *E. chaffeensis*, MSP1a–MSP1b, MSP4, MSP5 and OmpA in *A. marginale* and MSP4, HSP70, AipA, OmpA and Asp14 in *A. phagocytophilum* (Supplementary Table 2). Some of these have a role in host cell attachment and entry (see below). In contrast to the hypervariable surface proteins described above, these proteins tend to have highly conserved domains to preserve the protein–protein binding site.

The Rickettsiaceae lack OMP1–MSP2–P44 homologues, but express multiple surface cell antigen (Sca) autotransporter family proteins (type V secretion systems) in their outer membrane. *Rickettsia* spp. encode a broadly conserved set of five autotransporter proteins, Sca0 (rOmpA), Sca1, Sca2, Sca4 and Sca5 (rOmpB), although TG rickettsiae lack Sca0, and a further 12 less conserved families of autotransporters across the *Rickettsia*[4]. *O. tsutsugamushi* encodes four conserved autotransporter proteins (ScaA, ScaC, ScaD and ScaE)[54], which are unrelated to the Sca proteins of *Rickettsia* spp., and an additional two autotransporters (ScaB and ScaF) that are not widely conserved across strains[55]. Rickettsiaceae autotransporter proteins are involved in host cell attachment and entry, and, in the case of some *Rickettsia* spp., actin polymerization[56]. In addition to Sca family proteins, the chaperones GroEL and DnaK are found at high levels on the surface of different Rickettsiales[57–61], although their roles in host cell interactions are not well described.

The major surface antigen of *O. tsutsugamushi* is TSA56, a strongly immunogenic protein that exhibits substantial variation in its four hypervariable regions that is driven by high mutation rates and intragenic recombination[62]. Recombination between strains may occur in mites co-infected with multiple genotypes or during co-feeding of multiple infected mites on one rodent, and the process is likely to be mediated by the conjugative transfer systems as well as the recombination and repair machinery encoded by *O. tsutsugamushi*, although this has not been shown experimentally. TSA22 and TSA47 are the other major immunogenic surface proteins of *O. tsutsugamushi*[63]. TSA22 has no known function or homologue. TSA47 is highly conserved between strains, contains serine protease and PDZ domains, and is involved in budding out of host cells[64].

Bacterial gene regulation. Gene expression in Rickettsiales species needs to be regulated to reflect the different growth conditions between mammals and invertebrates, and to support the differentiation between reticulate and dense-core cells in Anaplasmataceae. Two-component systems are used by bacteria to sense and respond to changes in their environment. *E. chaffeensis* and *A. phagocytophilum* each encode three predicted histidine kinase–response regulator pairs[65], and a similar number are found in the genomes of other Rickettsiales. Developmental differentiation in Anaplasmataceae resembles that of *Chlamydiae* and *Coxiella* spp., but the bacterial homologues of eukaryotic histone H1 used by those organisms are not found in Anaplasmataceae[66]. Rickettsiales encode only two predicted sigma factors, the housekeeping RpoD (sigma70) and alternate heat shock sigma factor RpoH (sigma32)[67], which may be important in regulating the response to temperature changes between ectothermic arthropods and warm-blooded mammals. Fewer than 10 transcription factors are predicted, although complete Rickettsiales genomes encode between 88 and 536 hypothetical proteins (TABLE 1), some of which may encode currently undiscovered transcription factors or other regulatory elements. The transcription factor CtrA, which is a well-described regulator of bacterial differentiation in the free-living alphaproteobacterium *Caulobacter crescentus*, is found across the Rickettsiales and has been characterized in *E. chaffeensis*[68]. The (p)ppGpp synthetase–hydrolase SpoT regulates dormancy and stress response in many bacteria, but whereas *Rickettsia* spp. possess multiple partially degraded paralogues within their genomes, *O. tsutsugamushi* is the only Rickettsiales known to encode a full-length *spoT* gene. Toxin–antitoxin systems are used by bacteria to regulate the stress response, and although a number of these have been identified in genomes from the *Rickettsia* genus, it is unknown whether they are functional[69]. Transcription of small RNAs has been demonstrated in *Rickettsia* spp.[70] and *O. tsutsugamushi*[71], and in *Wolbachia* small RNAs regulate the expression of both bacteria and host genes[72]. Gene expression is negatively regulated by antisense transcription in the RAGE element of *O. tsutsugamushi*[71].

Autotransporter family proteins
A family of outer membrane proteins that are widespread in pathogenic bacteria and are composed of a transmembrane β-barrel domain and a soluble extracellular passenger domain, which is sometimes cleaved and secreted into the extracellular space.

PDZ domains
Common structural domains of 80–90 amino acids found in proteins in both prokaryotes and eukaryotes.

Two-component systems
Signalling systems common in bacteria, typically comprising a histidine kinase sensor protein and a corresponding response regulator.

Endocytosis
A process by which eukaryotic cells internalize extracellular material that includes phagocytosis, pinocytosis and receptor-mediated endocytosis.

The intracellular life cycle

Binding and invasion. Rickettsiales enter mammalian host cells using a 'zipper-like' process of induced endocytosis, in those organisms in which this process has been studied. This process occurs within minutes of host cell contact and involves one or several adhesins on the bacterial surface interacting with one or several receptors on the host cells (FIG. 3). This triggers a signalling cascade within the host cells that leads to clathrin-mediated and/or caveolin-mediated internalization that is dependent on actin remodelling. Type III secretion systems that are used for the 'trigger-like' internalization

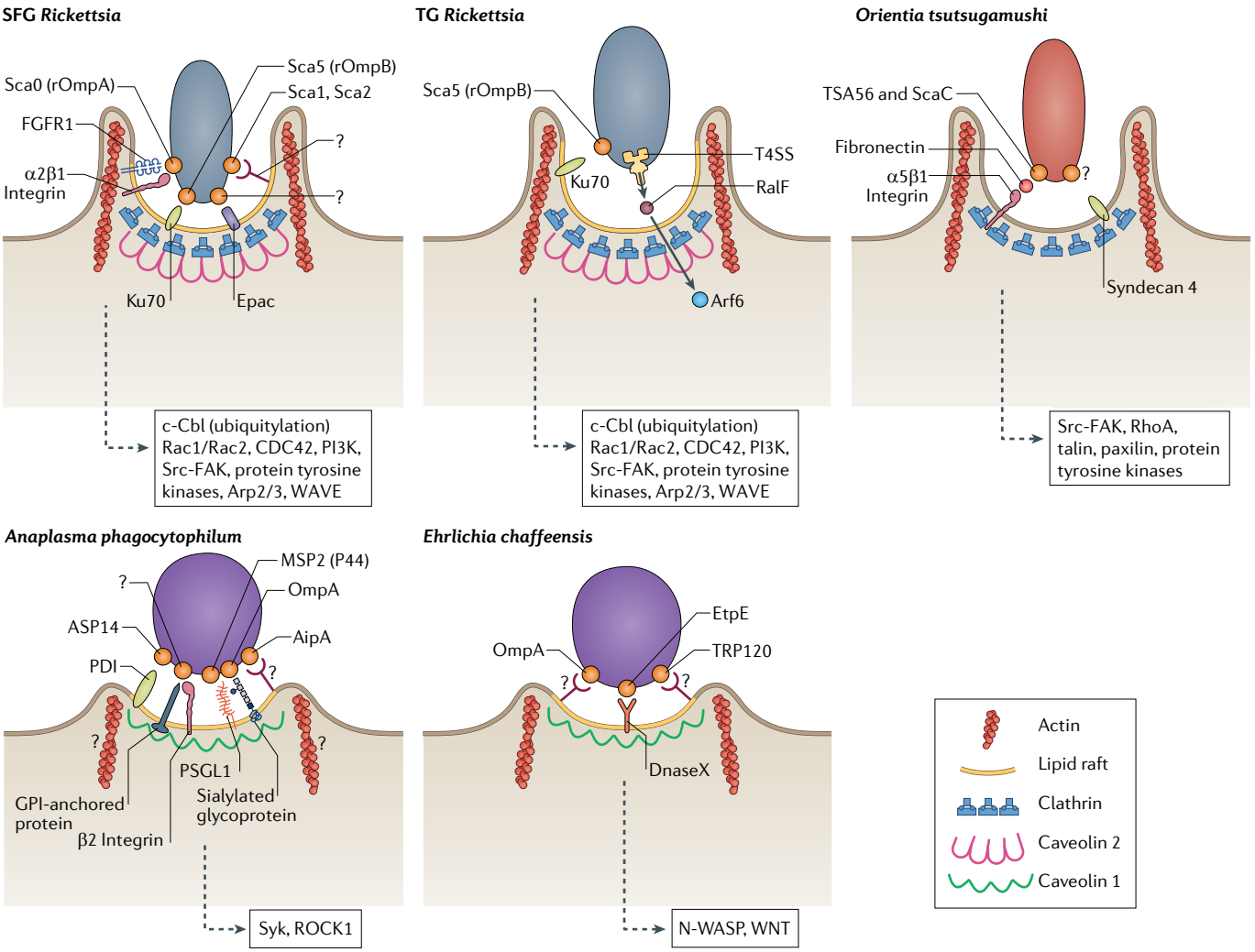


Fig. 3 | **Attachment and invasion of Rickettsiales species into mammalian cells.** Overview of the known surface adhesins and invasins and host cell receptors of spotted fever group (SFG) *Rickettsia* spp., typhus group (TG) *Rickettsia* spp., *Orientia tsutsugamushi*, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*. Downstream signalling pathways, where known, are shown. SFG and TG *Rickettsia*: surface cell antigen Sca0 (rOmpA) is a bacterial surface protein that binds to Ku70 in lipid rafts on the host cell membrane and induces bacterial entry in an actin, clathrin and caveolin 2-dependent manner. Host cell signalling pathways involved in this process include the E3 ubiquitin ligase c-Cbl, Arp2/3, the WAVE complex, Rho family GTPases Rac1 and Rac2, Src-FAK, Cdc42, phosphoinositide (PI) 3-kinase (PI3K) and protein tyrosine kinases. SFG *Rickettsia*: Sca0 (rOmpA) is an additional adhesin that interacts with α2β1 integrin and fibroblast growth factor receptor. Sca1 acts as an additional adhesin and Sca2 as an adhesin and invasin in *Rickettsia conorii*. Exchange protein directly activated by cyclic AMP (Epac) plays a role in internalization in *Rickettsia australis*. TG *Rickettsia*: type IV secretion system (T4SS) secreted effector RalF, a guanine nucleotide exchange factor of ADP-ribosylation factors (Arf-GEF), induces Arf6, and this leads to actin remodelling and bacteria internalization. *O. tsutsugamushi*: the abundant surface protein TSA56 functions as an adhesin and invasin, and the autotransporter ScaC also mediates attachment but not invasion. Fibronectin and syndecan 4 function as host receptors, and binding triggers an integrin-mediated signalling cascade that includes focal adhesion kinase (FAK), protein tyrosine kinases including Src kinase, RhoA GTPase and signal adapters talin and paxillin, and results in actin remodelling and clathrin-mediated internalization. *A. phagocytophilum*: bacterial surface proteins OmpA, AipA, Asp14 and MSP2 (P44) are involved in entry. OmpA interacts with α-2,3-sialic acid and α-1,3-fucose residues on sialyl Lewis x-capped P-selectin glycoprotein ligand 1 (PSGL1) and other undetermined sialylated glycoproteins. Asp14 interacts with protein disulfide isomerase. GPI-anchored proteins and β2 integrin are involved in bacterial entry but the bacterial ligands are unknown. Bacteria enter host cells at the site of lipid rafts and co-localize with caveolin 1, and Syk and ROCK1-dependent signalling cascades are involved in inducing endocytosis. *E chaffeensis*: EtpE is the best-characterized adhesin and interacts with GPI-anchored DNAse X, whereas tandem repeat protein 120 (TRP120) and OmpA are also reported to have a role in entry. Bacterial entry requires lipid rafts and GPI-anchored proteins, co-localizes with caveolin 1, and involves activation of Wnt and N-WASP that leads to actin remodelling and bacterial entry. FGFR1, fibroblast growth factor receptor 1; PDI, protein disulphide isomerase; WASP, Wiskott–Aldrich syndrome protein.

of many other intracellular bacteria are not found in Rickettsiales.

In *R. conorii* and *Rickettsia japonica*, the surface protein Sca5 (rOmpB) binds to Ku70 within cholesterol-rich lipid rafts and induces bacterial entry in an actin, c-Cbl, clathrin and caveolin 2-dependent mechanism[73–76]. Given the conservation of Sca5 (rOmpB), the use of these pathways is likely to be conserved in both SFG and TG Rickettsiaceae. In SFG rickettsiae, Sca0 (rOmpA) is an additional adhesin that interacts with α2β1 integrin and fibroblast growth factor receptor[77], and Sca1 and Sca2 have been shown to have a role in adhesion (Sca1) and adhesion and invasion (Sca2) of *R. conorii* to host cells[78,79]. Host cell exchange protein directly activated by cyclic AMP (Epac) has been shown to play a crucial part in internalization and pathogenicity of SFG *Rickettsia australis*, although the exact mechanism by which Epac facilitates bacterial entry is unknown[80]. Actin remodelling plays an important role in bacterial entry, and this is driven by the bacteria-induced activation of host cell signalling pathways. In *R. conorii*, these have been shown to include the E3 ubiquitin ligase c-Cbl, Cdc42, phosphoinositide (PI) 3-kinase, protein tyrosine kinases and Src-FAK[73,74]. An RNAi screen of entry of *R. parkeri* into *D. melanogaster* S2R$^+$ cells identified a role for Arp2/3, the WAVE complex and Rho family GTPases Rac1 and Rac2 (REF. [81]). Involvement of these different pathways in vertebrate cells depended on the cell type but the requirement for the Arp2/3 complex was conserved[81]. In SFG *Rickettsia*, the secreted Wiskott–Aldrich syndrome protein (WASP) RickA, which activates Arp2/3 during actin-based motility[82], may also contribute to actin remodelling during entry, but this has not been demonstrated. TG rickettsiae secrete a type IV secretion system (T4SS) effector RalF[83], which is a guanine nucleotide exchange factor of ADP-ribosylation factors (Arf-GEF), that induces Arf6 activation resulting in actin remodelling and bacterial internalization[83].

In *O. tsutsugamushi*, the abundant surface protein TSA56 functions as an adhesin and invasin[84], and the autotransporter ScaC also mediates attachment but not invasion[54]. Fibronectin and the heparan sulfate proteoglycan syndecan 4 function as host receptors, and binding triggers an integrin-mediated signalling cascade that includes focal adhesion kinase (FAK), protein tyrosine kinases including Src kinase, RhoA GTPase and signal adapters talin and paxillin, and results in actin remodelling and clathrin-mediated internalization[23,85,86].

In *A. phagocytophilum*, numerous adhesins have been described, including OmpA, AipA and Asp14, with OmpA interacting with α-2,3-sialic acid and α-1,3-fucose residues on sialyl Lewis x-capped P-selectin glycoprotein ligand 1 (PSGL1) and other undetermined sialylated glycoproteins and Asp14 interacting with protein disulfide isomerase[87]. GPI-anchored proteins and β2 integrin are receptors with unknown bacterial ligands, whereas *A. phagocytophilum* Msp2 (P44) may also interact with PSGL1 (REF. [88]). Entry of *A. phagocytophilum* is dependent on lipid rafts and flotillin 1, co-localizes with caveolin 1 (REF. [89]), and involves a Syk and ROCK1-dependent signalling cascade[88]. Clathrin is not required. OmpA is also an adhesin in *A. marginale*[90], although the mechanisms of entry are less well understood.

Entry of *E. chaffeensis* into the human acute leukaemia cell line (THP-1) is dependent on host cell lipid rafts and GPI-anchored proteins, and co-localizes with caveolin 1 (REF. [89]). EtpE, the best-characterized adhesin in *E. chaffeensis*, interacts with GPI-anchored DNAse X and induces bacterial entry via N-WASP-dependent actin polymerization[91]. TRP120 (glycoprotein 120) is also an *E. chaffeensis* adhesin that induces bacterial entry via activation of Wnt signalling[92], and data suggest that OmpA may have a role in entry[93].

Wolbachia differs from other Rickettsiales because it can remain in host cells through the host cell division cycle, rather than exiting infected cells and then infecting naive host cells[94]. However, *Wolbachia* has been shown to survive outside host cells[95], and entry of free *Wolbachia* into new host cells in a clathrin and dynamin-dependent manner was recently demonstrated[96], both of which are required for effective horizontal transfer to new hosts.

Cytoplasmic-dwelling rickettsiae. Rickettsiaceae escape from the endolysosomal pathway less than 30 min after attachment and internalization, and undergo replication directly in the host cell cytoplasm. *Rickettsia* spp. and *O. tsutsugamushi* can be cultured in a range of endothelial, epithelial, fibroblast and macrophage cells, and commonly used host cells include monkey epithelial Vero cells, human epithelial HeLa cells, mouse fibroblast L929 cells and baby hamster kidney (BHK-21) cells. Disruption of the endosomal membrane is required for rickettsiae to enter the host cytosol, and this is likely achieved by the activity of phospholipase D that is present in *Rickettsia* spp. and *O. tsutsugamushi*[97], with the possible involvement of haemolysin C in *Rickettsia* spp. Phospholipase D enzymatic activity has been demonstrated in *R. typhi*[98]. Rickettsiales generally lack flagella (although these have been described in the *Midichloria*, a non-pathogenic divergent lineage of the Rickettsiales[99,100]), and motility of cytoplasmic bacteria is dependent on the host cell cytoskeleton. Actin-based motility has been observed in SFG rickettsiae *R. rickettsii*, *R. conorii* and *R. parkeri*[101]. Two forms of actin-based motility are used: one driven by RickA on the bacterial surface that activates the host Arp2/3 complex and drives actin polymerization into short, curved tails; and another driven by Sca2 that functionally mimics host formins and drives the formation of long, straight actin tails[56,82]. *R. prowazekii* does not exhibit actin-based motility, and *R. typhi* generates much smaller actin tails than SFG rickettsiae that confer limited motility[101]. In contrast to the *Rickettsia* spp., *O. tsutsugamushi* uses minus-end directed dynein-dependent microtubule motility to move to the perinuclear region following entry into the cytoplasm, although the bacterial effectors that mediate this process are unknown[102].

Unlike the Anaplasmataceae, Rickettsiaceae do not generally differentiate into distinct forms during infection of a single cell. However, SFG rickettsiae transition into a dormant, non-infectious state in starving ticks, and these are reactivated upon tick feeding prior to or during infection of a susceptible host[103].

This reactivation is associated with changes in gene expression, in particular T4SS genes and OmpB[104], both of which are associated with entry into and manipulation of mammalian host cells. *R. felis* has also been shown to differentiate into elongated and short forms[105]. Those observations indicate that the Rickettsiaceae may modulate gene expression to exist in distinct subpopulations, although underlying mechanisms are not as well described as the developmental differentiation observed in Anaplasmataceae.

Bacterial differentiation and remodelling the parasitic vacuole. All studied Anaplasmataceae species remain within membrane-bound vacuoles during their intracellular life cycle[14,25]. With the exception of *Wolbachia*, this involves a differentiation from the infecting dense-core form in early endosomes to the replicative reticulate cell form within the mature parasitic vacuole[14,25] (FIG. 2). Parasitic vacuoles containing multiple reticulate cell bacteria are called morulae because of the resemblance to mulberries[17]. Parasitic vacuole maturation involves blocking lysosome fusion and redirecting host nutrients to the parasitic vacuole, and these processes are directed by secreted bacterial effector proteins[14,25]. Vacuole biogenesis and expansion imposes a substantial burden on host cells through increased requirement for lipid membranes[50].

A. phagocytophilum has mostly been studied in human promyelocytic HL-60 cells, *I. scapularis* embryonic ISE6 cells and primate RF/6A cells[14,88]. The *A. phagocytophilum* vacuole (ApV) does not acidify and exploits the autophagy machinery, the induction of which promotes bacterial growth[106]. Similar to autophagosomes, the ApV has a double lipid bilayer and associates with LC3, Beclin-1, ATG8 and ATG6, but these autophagosome-like vacuoles do not fuse with lysosomes. The ApV also recruits Rab GTPases typical of recycling endosomes and those that mediate endoplasmic reticulum (ER) to Golgi trafficking[107]. The ApV associates with the ER and Golgi apparatus[108,109], enabling it to scavenge amino acids and lipids for nutrition. *A. marginale* is typically grown in ISE6 and RF/6A cells, and its vacuole (AmV) also does not acidify, does not fuse with lysosomes and interacts with the Golgi and ER[108,110,111]. However, the AmV does not exploit autophagy and retains markers of the early endosome (Rab5), late endosome and recycling endosome[108,112]. *E. chaffeensis* is most commonly cultured in dog macrophage DH82 cells, although it can also be propagated in HL-60 cells, RF/6A cells and various tick cell lines[113–115]. The *E. chaffeensis* vacuole (EcV) has characteristics of both early[113] and late endosomes, and is acidified to pH 5.2 (REF. [115]), which suggests a different mechanism of vacuolar survival from *Anaplasma* spp. The *Wolbachia* vacuole (WbV) is associated with the ER[116] and Golgi-derived vesicles[117].

Nutrient acquisition and metabolism. Obligate intracellular bacteria are nutritional parasites that depend on host cells for carbon sources and metabolic intermediates. They have highly reduced metabolic pathways and an expanded repertoire of importers to take up nutrients from host cells. Chlamydiales and Rickettsiales (except Anaplasmataceae) are unique among bacteria in encoding an ATP–ADP symporter that enables them to scavenge ATP directly from host cells, although Rickettsiales can also generate their own ATP via oxidative phosphorylation. Analysis of the genomes of Rickettsiales shows variation in the extent of reduction of different metabolic pathways[30,118,119], with the Rickettsiaceae having generally more reduced pathways than the Anaplasmataceae, perhaps reflecting greater access to host cell metabolites in the cytoplasm compared with the vacuole. Rickettsiales lack the ability to carry out glycolysis or gluconeogenesis and have a limited ability to synthesize amino acids. Rickettsiaceae further lack an ability to synthesize nucleotides or carry out the pentose phosphate pathway, whereas these pathways are generally present in Anaplasmataceae. The tricarboxylic acid (TCA) cycle is complete in Anaplasmataceae and *Rickettsia* spp., but *O. tsutsugamushi* lacks full-length copies of the first three enzymes, including a functional pyruvate dehydrogenase complex. Pathways for de novo synthesis of vitamins and cofactors are largely absent in Rickettsiaceae, but partially present in Anaplasmataceae. Both Rickettsiaceae and Anaplasmataceae have largely intact lipid biosynthesis pathways, but may rely on host-derived lipids or lipid intermediates to make up for deficiencies in the biosynthesis pathways, as has been shown for *E. chaffeensis*[50]. Rickettsiales probably primarily utilize host-derived amino acids and TCA intermediates as carbon sources[30,118,119]. In support of this, *Wolbachia* has been shown to require an intact ER-associated protein degradation pathway as a source of amino acids[120], whereas *O. tsutsugamushi* actively manipulates the activity of this pathway via a secreted Ank4 effector protein[121]. Similarly, *A. marginale* and *A. phagocytophilum* interact closely with the ER[108], and *R. rickettsii* targets the ER via the secreted effector protein RARP2 (REF. [122]), although a direct role in nutrient scavenging has not yet been shown in these organisms.

Bacterial replication and host cell exit. Rickettsiales replicate free in the cytoplasm or in vacuoles by binary fission and then use a range of strategies to exit infected host cells (FIG. 2). SFG rickettsiae replicate to about 30–50 bacteria dispersed throughout the cytoplasm, and occasionally in the nucleus, and this level of replication causes substantial damage to the host cell ultrastructure beginning around 48 h post infection[123]. Bacteria move directly into adjacent cells using actin-mediated polymerization, in a process facilitated by Sca4 altering the force dynamics of the cell membrane[124], or they exit through eventual lysis of damaged host cells[123]. By contrast, TG rickettsiae replicate to 100 bacteria per cell or more but do not cause substantial damage to host cells. At very late time points (96–120 h post infection) host cell lysis occurs, releasing the intracellular bacteria[125]. *O. tsutsugamushi* moves to the perinuclear region after infection and replicates as a microcolony beginning around 24–48 h post infection. Bacteria can replicate to a level of 50 bacteria or more per cell, depending on the bacterial strain, and host cells can survive intact up to 7 days or longer. Bacterial exit begins around 4 days

post infection, with individual bacteria moving to the edge of infected cells and budding out as enveloped bacteria in a lipid raft-dependent process[64]. *E. chaffeensis*, *A. marginale* and *A. phagocytophilum* transition from the dense-core form to the reticulate cell form several hours post infection, and bacterial replication then occurs over around 48 h up to a level of several hundred bacteria per vacuole. Unlike some other vacuole-residing bacteria, individual vacuoles of Anaplasmataceae do not fuse with one another. Reticulate cell bacteria transition back to mature dense-core forms, completing the cycle around 72 h post infection[19]. *E. chaffeensis* uses two mechanisms to exit cells. At early stages of infection, bacteria traffic directly between adjacent cells in an actin-dependent mechanism via filopodia[126]. At later stages of infection, host cell rupture adjacent to morulae causes extrusion of bacterial cells[126]. In the case of *Anaplasma* spp., release of membrane-bound bacterial vacuoles by exocytosis has been described[127], although lysis of heavily infected cells also causes bacterial release.

Manipulating the host response

Secretion systems and effector proteins. A major strategy that intracellular bacteria use to manipulate host cell activity is the secretion of effector proteins that bind to host cell machinery and modulate its function to benefit the bacterium. Mechanisms of action for rickettsial secreted effectors include binding to host cell DNA or chromatin and modulating gene expression (nucleomodulins), binding to host proteins and interfering with signalling pathways, modifying the activity of host proteins through post-translational modifications, such as ubiquitylation, and interacting with phosphatidylinositol signalling through lipid binding[122,128–130]. Effector proteins typically interact with host cell proteins by structurally mimicking protein–protein interactions, and thus they can be identified by the presence of domains normally found in eukaryotic proteins. Ankyrin repeat-containing proteins (Ank proteins) are widely distributed among the Rickettsiales. A range of mechanisms has been ascribed to Ank proteins in *A. phagocytophilum* (AnkA), *E. chaffeensis* (Ank200), *Wolbachia* (pk1 and pk2), *Rickettsia* spp. (RARP-1 and RARP-2) and *O. tsutsugamushi* (Ank1, Ank4, Ank6 and Ank9), including disrupting organelles, modifying host gene expression, inhibiting apoptosis, subverting autophagy and interfering with vesicular trafficking[122,128,131–133]. Tetratricopeptide repeat proteins (TPR) are also well-known domains of effector proteins[134], and they have been found in *R. felis* and *O. tsutsugamushi* in which they have been shown to inhibit eukaryotic translation[135]. *E. chaffeensis* encodes numerous TRPs that, in addition to their roles as surface proteins described above, are secreted into host cells and interact with a range of host pathways[129,130]. TRP120 is the best-characterized TRP in *E. chaffeensis* and induces host SUMOylation[136] and ubiquitylation activity[137] to modify host and bacterial proteins, including Polycomb group proteins[138] and tumour suppressor FBW7 protein[139]. This activity and that of other secreted effectors leads to changes in host gene expression, through both interacting with signalling pathways and direct modification of host epigenetic machinery, and these changes in host cell gene expression promote intracellular survival of *E. chaffeensis*. *E. chaffeensis* also secretes ehrlichial translocated factor 1 (Etf1), Etf2 and Etf3. Etf1 and Etf2 bind Rab5 and modulate endosomal and autophagy pathways, whereas Etf1 also traffics to the mitochondria and modulates reactive oxygen species (ROS) and apoptosis[131]. Other secreted effector proteins include the TG *Rickettsia* Arf-GEF RalF, which is involved in host cell entry, and phoshatidylinositol 3-kinase effector Risk1, which is involved in autophagy evasion. A deubiquitylase from *O. tsutsugamushi*, otDUB, has been characterized in vitro but its role during infection is unknown[140]. Two effector proteins have been characterized in *Wolbachia*, TomO[141] and WalE[142], and numerous others have been predicted using bioinformatics pipelines and exogenous expression approaches[143]. In addition to the characterized effector proteins, there are likely to be many additional secreted effectors in the Rickettsiales. These can be predicted in silico using dedicated algorithms for the identification of putative new effectors[144] or identified using bacterial two-hybrid screens using components of the secretion apparatus as bait.

Rickettsiales encode five known protein secretion systems that transfer cytoplasmic proteins to the cell surface or extracellular space[69]. There are at least three known classes of T4SS, and a system called *rvh* that encodes 12 scaffold genes (*virB1–virB11* and *virD4*) homologous to the archetypal *vir* system encoded by the pTi plasmid of *Agrobacterium tumefaciens*[118] is widespread amongst the Rickettsiales, although the minor pilin subunit *virB5* that is required for pilus formation is lacking[69]. In addition to *rvh*, the RAGE mobile genetic element that is found in *O. tsutsugamushi* and some *Rickettsia* spp.[40,41] contains a second T4SS that is homologous to the conjugative F plasmid T4SS of *Escherichia coli*. The RAGE T4SS encodes 15 *tra–trb* genes, the protein products of which form a T4SS scaffold, but lacks components involved in the regulation of conjugation. Numerous *rvh* substrates, including Sec7 domain-containing proteins, a phosphatidylinositol 3-kinase effector and some Ank proteins, have been identified in *R. rickettsii*[122], *R. typhi*[83,122,145], *Wolbachia*[143], *A. marginale*[146], *A. phagocytophilum*[131] and *E. chaffeensis*[131].

In addition to the T4SS, Rickettsiales encode T1SS (TolC), twin-arginine translocation (Tat) systems, the Sec–TolC pathway and T5SS (autotransporter proteins). T1SS and the Sec–TolC pathway have been shown to be involved in the secretion of Ank proteins in *R. typhi*[132] and *O. tsutsugamushi*[147], and of TRPs in *E. chaffeensis*[148]. Although Tat systems have been identified across Rickettsiales, very few predicted substrates have been identified, which suggests that this is not a major system in these organisms[69]. Finally, the T5SS encodes Sca autotransporter proteins in Rickettsiaceae as described above.

Immune recognition and subversion of innate immunity. Pathogenic Rickettsiales cause vascular infections in humans, targeting primarily the endothelium (Rickettsiaceae) and circulating leucocytes (Anaplasmataceae). Interaction with these target cells leads to

Filopodia
Thin, actin-rich membrane protrusions that extend from the edge of eukaryotic cells and play a role in sensing the environment, cell migration and cell–cell interactions.

Ankyrin repeat-containing proteins
(Ank proteins). A large family of mostly eukaryotic proteins that contain 1–34 copies of an Ankyrin repeat of ~33 amino acids, present in pathogenic bacteria and viruses.

Tetratricopeptide repeat proteins
(TPR). A family of proteins containing a repeat of 34 amino acids arrayed in a tandem of 3–16 motifs, present in both prokaryotic and eukaryotic organisms.

the secretion of pro-inflammatory chemokines and cytokines, which results in endothelial activation and the infiltration of $CD4^+$ and/or $CD8^+$ T cells, natural killer cells as well as monocytes and/or macrophages. Overactivation of this immune response can cause tissue damage, and this is an important driver of pathogenicity in rickettsial diseases[10]. The interplay between the invading pathogen and the cellular innate immune system of the target cell determines whether the pathogen is rapidly cleared, or whether the bacterium replicates and disseminates causing damage to host cells and inducing a stronger and sometimes damaging inflammatory response (FIG. 4).

Intracellular Rickettsiales are sensed by cytosolic and membrane-bound pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). These include the Toll-like receptors TLR2 across Rickettsiales[149–152] and TLR4 in some species but not *A. phagocytophilum*[149–152]. The nucleotide-binding oligomerization domain proteins NOD1 and NOD2 recognize peptidoglycan fragments and have been shown to be activated in response to *O. tsutsugamushi*[153] and, possibly, *Wolbachia*[154,155] but this has not been tested in *Rickettsia* spp. In new hosts, *Wolbachia* (*w*AlbB) is recognized by Toll and immune deficiency (IMD) protein and also induces expression of peptidoglycan recognition

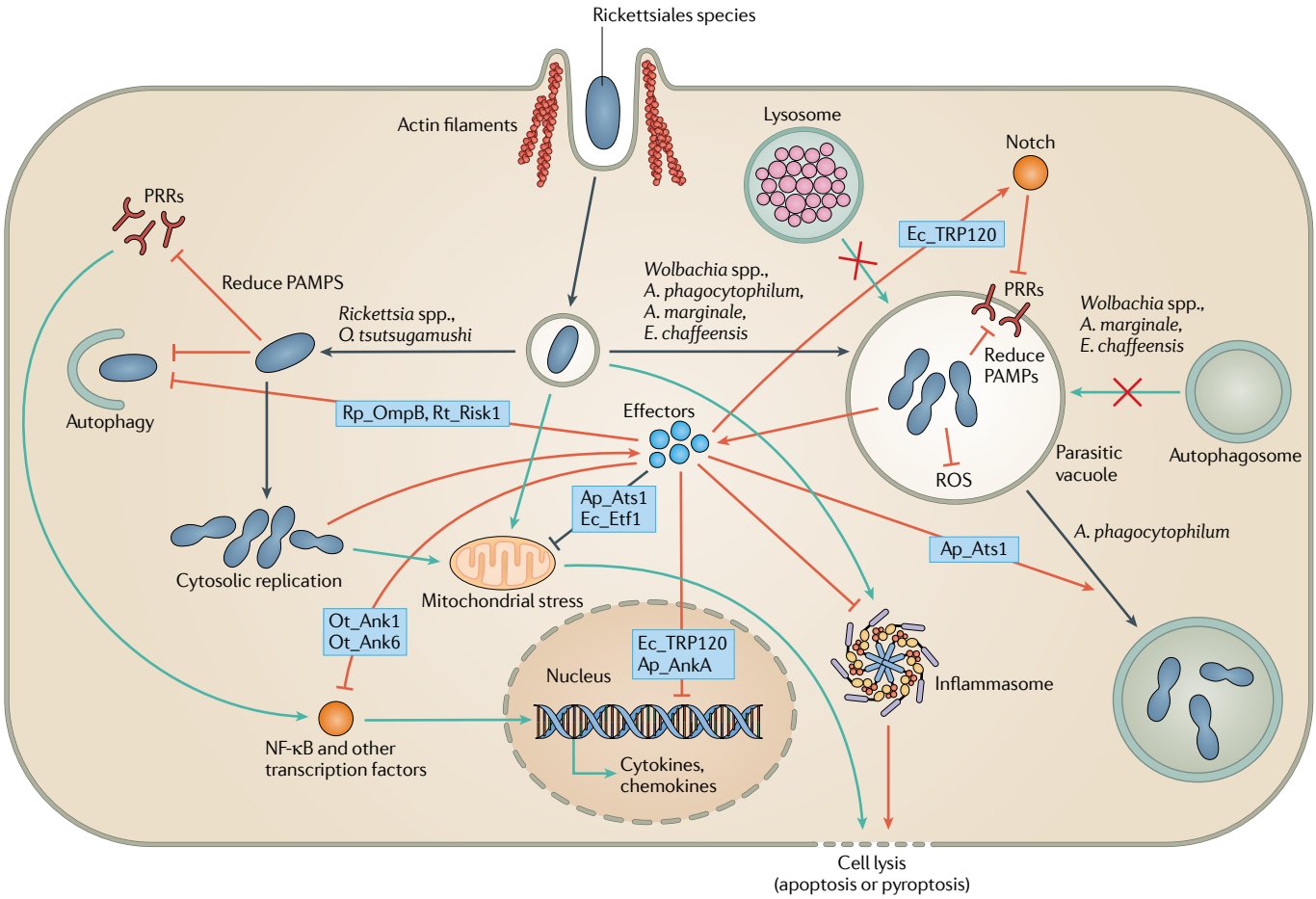


Fig. 4 | **Evasion of cellular innate immunity by Rickettsiales.** Overview of the mechanisms whereby Rickettsiales evade host innate immune mechanisms. Green lines indicate host activated pathways, and red lines indicate bacterial mechanisms. Specific bacteria using certain pathways are indicated. Following entry by induced endocytosis, *Rickettsia* spp. and *Orientia tsutsugamushi* move into the cytoplasm whereas *Wolbachia*, *Anaplasma phagocytophilum*, *Anaplasma marginale* and *Ehrlichia chaffeensis* block lysosome fusion and remodel the parasitic vacuole to support bacterial replication. *A. phagocytophilum* induces autophagy and uses this machinery to build a parasitic vacuole. All Rickettsiales secrete effector proteins into the host cell that interfere with innate immune mechanisms designed to inhibit bacterial growth. Some of the best-characterized effectors are shown. Autophagy is a cellular response that is typically induced in response to cytosolic pathogens, and both *O. tsutsugamushi* and *Rickettsia* spp. have evolved mechanisms to exploit or evade this, whereas *A. phagocytophilum* uses Ats1 to use the autophagy machinery to build its parasitic vacuole. Pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) on cytoplasmic and vacuolar pathogens, and activate signalling pathways leading to pathogen clearance; *O. tsutsugamushi* and Anaplasmataceae have reduced or eliminated the immunogenic PAMPs peptidoglycan and lipopolysaccharide (LPS) in their cell walls. *E. chaffeensis* secretes an effector protein (tandem repeat protein 120 (TRP120)) that leads to the downregulation of the PRRs Toll-like receptor 2 (TLR2) and TLR4 via the Notch pathway, whereas *O. tsutsugamushi* interferes with downstream NF-κB signalling using secreted Ankyrin repeat-containing proteins (Ank proteins) Ank1 and Ank6. Host gene expression is also directly modulated by the action of secreted effector proteins in the nucleus, including AnkA in *A. phagocytophilum* and TRP120 in *E. chaffeensis*. Reactive oxygen species (ROS) are induced in response to pathogens residing in the cytosol or vacuole, and *A. phagocytophilum* and *E. chaffeensis* directly inhibit the induction of ROS. Apoptotic or pyroptotic cell death is induced in cells following mitochondrial stress and inflammasome activation, and these processes are targeted by rickettsial effector proteins including *E. chaffeensis* Etf1 and *A. phagocytophilum* Ats1.

protein (PGRP)-LE[156]. However, in co-evolved associations, *Wolbachia* does not induce an immune response, including the release of antimicrobial peptides, reflecting adaptation by the bacterium and/or host[157]. TLR3 recognizes cytosolic double-stranded RNA and was recently found to be upregulated during infection with *O. tsutsugamushi*, which suggests a possible role in bacterial detection[71]. Cyclic GMP–AMP synthase–stimulator of interferon genes (cGAS–STING) recognizes cytosolic DNA in *R. parkeri*[158] infections, and this may be involved in other Rickettsiales. Finally, antibody-coated *E. chaffeensis* is recognized by the cytosolic receptor TRIM21, which targets the bacteria for degradation[159]. Rickettsiales have evolved mechanisms to reduce detection by PRRs. This includes reducing the level of PAMPs, as shown by reduced or absent LPS and peptidoglycan in Rickettsiales other than *Rickettsia* spp.[44], and reducing the level of PRRs whereby *E. chaffeensis* uses secreted TRP120 to activate Notch, which leads to the downregulation of TLR2 and TLR4 (REF.[160]) (FIG. 4).

Rickettsiales-activated PRRs induce the expression of interferons and pro-inflammatory cytokines and chemokines that trigger activation of the immune system. PRR activation also leads to the activation of the inflammasome that induces IL-1β and IL-18 as well as apoptotic and pyroptotic cell death. Rickettsial infection triggers an antiviral type I pro-inflammatory response, although the exact signalling pathways and cytokine profiles vary by pathogen and cell type. Rickettsiales have evolved to tolerate this response by repressing cytokine expression[161,162] and modulating inflammasome activation[158,163,164]. Secreted effector proteins play an important role in this process. In *O. tsutsugamushi*, Ank1 and Ank6 modulate NF-κB signalling[128], whereas in *E. chaffeensis* and *A. phagocytophilum* the effector proteins TRP120 and AnkA, respectively, are transferred to the nucleus to directly modulate host gene expression[130,131] (FIG. 4).

The inflammatory response leads to oxidative stress through the generation of ROS and reactive nitrogen species (RNS), and this is highly toxic to intracellular bacteria as well as leading to activation of antibacterial pathways including apoptosis, pro-inflammatory signalling and autophagy. The effect of oxidative stress is particularly pronounced in those bacteria that live inside macrophages and neutrophils, and both *A. phagocytophilum* and *E. chaffeensis* actively inhibit the formation of ROS in host cells[131,165,166]. However, endothelial cells infected with rickettsiae also induce a bactericidal RNS response[167]. In addition to having antimicrobial activity, oxidative stress can contribute to pathogenesis through causing tissue damage, and the antioxidant α-lipoic acid has been shown to be protective in *R. rickettsii* and *R. conorii* infections[167]. *Wolbachia* can induce both ROS and antioxidative mechanisms, and this may contribute to redox regulation in co-evolved associations between endosymbiont and host[168].

Intracellular bacteria are vulnerable to detection and destruction by autophagy, and Rickettsiales have evolved mechanisms to evade or exploit this system. Autophagy was shown to promote the growth of SFG *R. australis* in macrophages[169] and *R. typhi* in HeLa cells[145]. In the case of *R. typhi*, the bacteria induce autophagosome induction but inhibit its maturation, and this process is mediated by the secreted effector Risk1 (REF.[145]). Conversely, OmpB enables *R. parkeri* to evade autophagy in macrophages by shielding bacteria from the ubiquitylation machinery[170]. *O. tsutsugamushi* has been shown to induce autophagy upon internalization, but then evade it by an unknown mechanism[171,172]. *A. phagocytophilum* repurposes the autophagy machinery to build its vacuole, as described above, and this process is mediated by the type IV secreted effector Ats1 (REF.[131]). *E. chaffeensis* also exploits autophagy to provide nutrients for bacterial growth in the EcV, and this process is induced by Etf1 (REF.[131]). By contrast, the levels of intracellular *Wolbachia* seem to be controlled by autophagy, as the presence of autophagy inhibitor increased bacterial growth. This difference with *E. chaffeensis* and *A. phagocytophilum* could be explained by the mutualistic relationship of most *Wolbachia* species with their host and the dependence for survival on a healthy host that requires an intact autophagy system[173].

Cells that have been infected by pathogens frequently induce apoptosis as a means of pathogen clearance. Rickettsial pathogens have evolved to inhibit apoptosis in a range of cell types; for example, *Rickettsia* spp. in endothelial cells[174], *E. chaffeensis* in monocytes[131] and *E. ewingii* and *A. phagocytophilum* in neutrophils[175,176]. The latter is particularly important because neutrophils naturally have a very short lifespan, and the anti-apoptotic activity is required to allow time for bacterial replication. Anti-apoptotic activity is mediated by effector proteins Ats1 in *A. phagocytophilum* and Etf1 in *E. chaffeensis*. *O. tsutsugamushi* has been shown to have both pro-apoptotic and anti-apoptotic activity depending on the bacterial strain and host cell type as well as the stage of infection[23]. Similarly, *Wolbachia* has been shown to both inhibit and induce apoptosis in hosts, with protective inhibition of apoptosis seen in obligate mutualistic relationships and damaging induction of apoptosis observed in pathogenic relationships[173].

Conclusions and future directions

Studying the interactions between Rickettsiales and their host cells helps us to better understand these medically and agriculturally important organisms and, thus, to develop appropriate interventions; this is crucial as, currently, therapeutic treatment options for rickettsial infections are limited, and no licensed human vaccines are available. In addition, such studies will help elucidate how these cellular pathogens exploit host cell machinery, and provide insights into the mechanisms of that host cell machinery, for example actin polymerization and autophagy. Furthermore, obligate intracellular bacteria have reduced and streamlined genomes, retaining only those genes essential for their infection and growth. A study of those minimal machineries reveals the core requirements for systems that are more complex in other bacterial species. For example, a study of the peptidoglycan biosynthesis machinery in obligate intracellular bacteria revealed the minimal gene set required to build a peptidoglycan-like structure[44]. The field of rickettsial cell biology has made great advances since the first rickettsial genome was sequenced[177], although mechanistic analysis

of potential virulence factors as well as the characterization of functions of secreted effector proteins have been hampered by the limited availability of genetic tools. Future advances in the development of genetic technology as well as other cell biology tools, such as fluorescence microscopy (BOX 1), will support a deeper understanding of the biology of these important and fascinating bacterial species.

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