BARTONELLA–HOST-CELL INTERACTIONS AND VASCULAR TUMOUR FORMATION

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Abstract | Bartonellae are arthropod-borne bacterial pathogens that typically cause persistent infection of erythrocytes and endothelial cells in their mammalian hosts. In human infection, these host-cell interactions result in a broad range of clinical manifestations. Most remarkably, bartonellae can trigger massive proliferation of endothelial cells, leading to vascular tumour formation. The recent availability of infection models and bacterial molecular genetic techniques has fostered research on the pathogenesis of the bartonellae and has advanced our understanding of the virulence mechanisms that underlie the host-cell tropism, the subversion of host-cell functions during bacterial persistence, as well as the formation of vascular tumours by these intriguing pathogens.

VASOPROLIFERATIVE
Capacity to stimulate the growth of blood vessels.

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The bartonellae are Gram-negative facultative intracellular pathogens that cause a long-lasting intra-erythrocytic bacteraemia in their mammalian reservoir host(s), thereby favouring their transmission by blood-sucking arthropods (TABLE 1). The infection of erythrocytes is limited to the species-specific reservoir host(s), which include humans, cats, dogs, ruminants and rodents, whereas endothelial cells are an important target cell type in probably all mammals, including humans incidentally infected by a zoonotic species[1]. Bartonellae subvert many functions of human endothelial cells, including the proinflammatory phenotype, cytoskeletal dynamics and the control of apoptosis and proliferation[2], and this subversion often culminates in the growth of VASOPROLIFERATIVE tumours, particularly in immunocompromised patients. *Bartonella*-triggered tumorigenesis has attracted considerable interest from both clinicians and basic scientists interested in the fields of infection and tumour biology, and it represents a paradigm for pathogen-triggered tumour formation[2]. Following a brief summary of the clinical associations of the bartonellae, this review summarizes the current understanding of *Bartonella*–host-cell interactions and vascular tumour formation.

Bartonella clinical associations

Bartonella bacilliformis is a deadly pathogen that causes Carrión's disease in endemic areas of the Andes. This biphasic disease is transmitted by the sandfly *Phlebotomus verrucarum*. The acute phase, called Oroya fever, is characterized by an intra-erythrocytic bacteraemia that results in an often-fatal haemolytic anaemia. The subsequent chronic phase, known as verruga peruana, manifests in vascular tumours that result from the proliferation of colonized endothelial cells[3]. A similar tropism for human erythrocytes and endothelial cells is observed for *Bartonella quintana*. This pathogen is transmitted by the human body louse and caused large epidemics of trench fever (an intra-erythrocytic bacteraemia) in Europe during the First and Second World Wars[3]. Recently, *B. quintana* has reemerged as a cause of vascular tumours in a disease manifestation known as bacillary angiomatosis in AIDS patients and other immunocompromised individuals. Humans serve as the exclusive reservoir host for *B. bacilliformis* and *B. quintana*, and for a long time these species were the only bartonellae that were known to be pathogenic to humans. The widespread occurrence of other bartonellae in various mammalian

Table 1 | ***Bartonella* hosts, vectors and associated human diseases**

Species	Reservoir(s)	Vector	Human disease(s)
Human-specific			
Bartonella bacilliformis	Human	Sandfly	Carrión's disease: Oroya fever and verruga peruana
Bartonella quintana	Human	Body louse	Trench fever, persistent bacteraemia, endocarditis, bacillary angiomatosis
Zoonotic			
Bartonella clarridgeiae	Cat	Cat flea	Cat-scratch disease
Bartonella elizabethae	Rat	?	Endocarditis, neuroretinitis
Bartonella grahamii	Mouse, vole	?	Neuroretinitis
Bartonella henselae	Cat	Cat flea	Cat-scratch disease, endocarditis, bacillary angiomatosis, bacillary peliosis, neuroretinitis, bacteraemia with fever
Bartonella koehlerae	Cat	?	Endocarditis
Bartonella vinsonii subsp. *arupensis*	Mouse	Tick	Bacteraemia with fever
Bartonella vinsonii subsp. *berkhoffii*	Dog	Tick	Endocarditis
Bartonella washoensis	Ground squirrel	?	Myocarditis
Animal-specific			
Bartonella alsatica	Rabbit	?	?
Bartonella birtlesii	Mouse	?	?
Bartonella bovis	Cattle, cat	?	?
Bartonella capreoli	Roe deer	?	?
Bartonella chomelii	Cattle	?	?
Bartonella doshiae	Vole	?	?
Bartonella peromysci	Deer, mouse	?	?
Bartonella phoceensis	Rat	?	?
Bartonella rattimassiliensis	Rat	?	?
Bartonella schoenbuchensis	Roe deer	Deer ked	?
Bartonella talpae	Mole	?	?
Bartonella taylorii	Mouse, vole	?	?
Bartonella tribocorum	Rat	?	?
Bartonella vinsonii subsp. *vinsonii*	Vole	Vole ear mite	?

reservoirs and their importance as zoonotic pathogens has only recently begun to be appreciated (TABLE 1). FIGURE 1 depicts the epidemiology and disease manifestations of the zoonotic species *Bartonella henselae*, the causative agent of cat-scratch disease, bacillary angiomatosis and bacillary peliosis, which accounts for most cases of human bartonellosis[4].

Intra-erythrocytic bacteraemia

The central theme of *Bartonella* ecology is a long-lasting intra-erythrocytic bacteraemia in the mammalian reservoir in conjunction with transmission by blood-sucking arthropods. The establishment of an intra-erythrocytic infection is a highly dynamic process that has not been reproduced in cell culture so far. However, appropriate animal models of intra-erythrocytic infection have been established for several bartonellae[5–9]. From the available data, the characteristics and course of intra-erythrocytic bacteraemia are similar for all these models, with the most detailed information being available for the rat model of *Bartonella tribocorum* infection[7] (FIG. 2).

Following intravenous inoculation with plate-grown *B. tribocorum*, bacteria are rapidly cleared from circulating blood, reestablishing sterility of the blood for about four days. The niche that supports bacterial replication during this time has not been identified experimentally; however, the marked tropism of *Bartonella* spp. for endothelial cells and their proximity to the bloodstream suggest that endothelial cells are an important constituent of this primary niche[1]. Typically, on day five post-infection, large numbers of bacteria are released from the primary niche into the bloodstream. Further episodes of synchronous release of bacteria follow at intervals of approximately five days, probably as

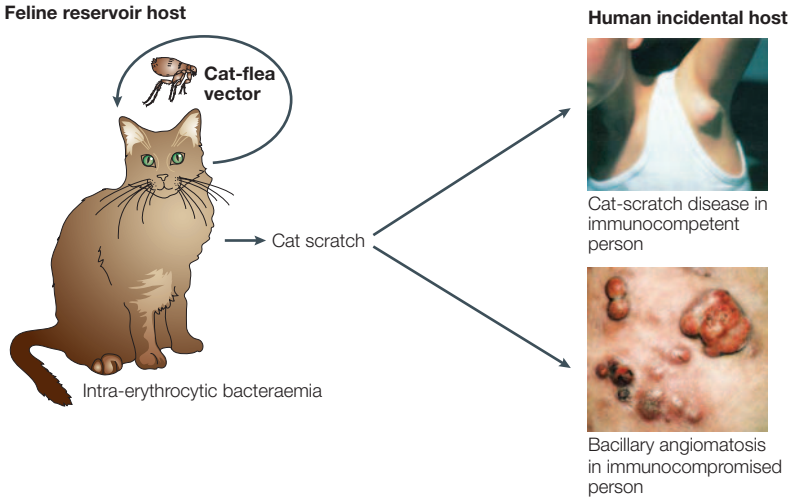

Figure 1 | ***Bartonella henselae* infection.** *Bartonella henselae* causes a sub-clinical intra-erythrocytic bacteraemia in its feline reservoir host. Through the bite of an infected cat flea or through direct contact/trauma (cat scratch), the pathogen is transmitted from cat to cat or from cat to human, respectively. Depending on the immune status of the human host, *B. henselae* can lead to different clinical manifestations, including cat-scratch disease (a febrile lymphadenopathy) in immunocompetent individuals, or bacillary angiomatosis and peliosis in immunocompromised patients (for further details see main text). Bacillary angiomatosis panel reproduced with permission from REF. 68 © (1992) Massachusetts Medical Society. Cat-scratch disease panel reproduced with permission from REF. 70.

a result of the five-day infection cycle that is triggered by re-infection of the primary niche by bacteria released at the end of each cycle. The approximately five-day periodicity of human trench fever caused by *B. quintana* could reflect these bursts of synchronous bacterial release into the bloodstream[10].

When *B. tribocorum* is released into the bloodstream, these bacteria efficiently adhere to mature erythrocytes, indicating that they become competent for erythrocyte interaction during colonization of the primary niche. Following adhesion to erythrocytes, bacteria invade and replicate intracellularly within a membrane-bound compartment until a critical density is reached. Thereafter, the number of intracellular bacteria remains static for the remaining lifespan of the infected erythrocytes, which is indistinguishable from that of uninfected erythrocytes[7]. *B. tribocorum* can therefore persist for several weeks in circulating blood in an immunologically privileged intracellular niche without causing undue harm to the infected rat. This haemotropic infection strategy is probably a specific adaptation to the transmission by blood-sucking arthropod vectors and is presumably shared by most *Bartonella* species. The exception to this rule is *B. bacilliformis*, which triggers massive haemolysis of colonized human erythrocytes, giving rise to an often fatal haemolytic anaemia. Interestingly, this pathogen can also cause an essentially asymptomatic intra-erythrocytic infection in humans that is similar to that caused by other *Bartonella* species in their respective mammalian reservoirs. The bacterial and/or host factors that are responsible for these different outcomes of *B. bacilliformis* infection are presently unknown.

The intra-erythrocytic bacteraemia caused by *B. tribocorum* in rats subsides spontaneously after approximately 10 weeks[7], and a similar duration of bacteraemia is observed in other experimental models of *Bartonella* infection[10]. This late clearance is associated with immunity against re-infection by the homologous strain. A *Bartonella grahamii* model of mouse infection has shown an important role for antibodies in controlling intra-erythrocytic infection[8]. However, antibodies are unlikely to function against infected erythrocytes, as the lifespan of these cells is similar to that of uninfected erythrocytes[7,8]. Instead, antibodies might neutralize bacteria that are released from the primary niche and thereby abrogate the infection of additional erythrocytes as well as re-infection of the primary niche.

Bartonella-triggered vasoproliferation

Endothelial cells are an important target cell type for bartonellae during infection of their mammalian reservoir host, but they also become infected in incidental hosts in which the subsequent step of erythrocyte infection does not take place. In humans, infection of the endothelium with either *B. bacilliformis*, *B. quintana* or *B. henselae* can lead to marked vasoproliferation, which manifests clinically in the formation of vascular tumours[2]. Other pathogenic bacteria have been associated with the formation of various malignant and benign tumours[11,12], however, the bartonellae are unique in the bacterial kingdom in causing vasoproliferation. Whether *Bartonella*-triggered vasoproliferation represents a dedicated bacterial pathogenicity strategy to expand a specific host-cell habitat[13] or instead reflects a 'biological accident' is presently unknown.

Bartonella-triggered vasoproliferative lesions have a multifaceted appearance. In Carrión's disease caused by *B. bacilliformis*, the partial state of immunosuppression that develops at the end of Oroya fever is considered to favour the eruption of vasoproliferative lesions, which marks the beginning of the verruga peruana phase[3]. The verruga nodules arise on the skin and can persist for months to years. Immunosuppression is also a prerequisite for vascular tumour formation by *B. henselae* and *B. quintana*. Bacillary angiomatosis caused by both species is characterized by vasoproliferative skin lesions, which resemble Kaposi's sarcoma caused by human herpes virus 8 in AIDS patients[14]. *B. henselae* can also trigger the formation of vasoproliferative lesions in inner organs (that is, in liver and spleen), which is known as bacillary peliosis[15].

Bartonella-triggered vascular tumour formation is reminiscent of tumour angiogenesis, the pathological process of blood-vessel formation in growing tumours. Angiogenesis comprises a complex sequence of morphogenetic events that result in blood-vessel formation by sprouting of pre-existing vessels. The sequential action of different pro-angiogenic factors (for example, vascular endothelial growth factor (VEGF) and angiopoietin-1) is required for this complex and highly orchestrated

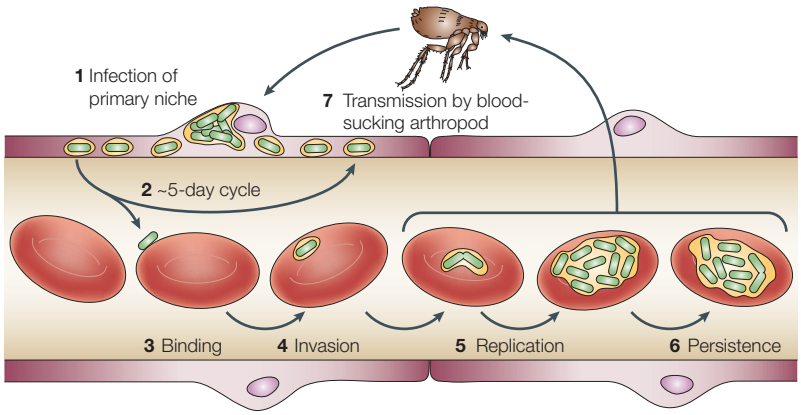

Figure 2 | **Model of the course of *Bartonella tribocorum* infection in the mammalian reservoir host.** (1) The primary niche of bacterial colonization is still poorly defined but is considered to include the vascular endothelium as a major constituent. (2) At five-day intervals, bacteria are released from the primary niche into the bloodstream, from where they can reinfect the primary niche to start another infection cycle, or (3) where they bind to erythrocytes, (4) invade, (5) replicate in an intracellular membrane-bound compartment, and (6) finally persist in a non-replicative intra-erythrocytic state for several weeks. This strategy is considered to be a specific adaptation to transmission by blood-sucking arthropods.

morphogenetic process[16]. However, in tumour angiogenesis as well as in *Bartonella*-triggered angiogenesis, the newly formed vessels do not mature properly, as deprivation of the angiogenic factors that are secreted by tumour tissues or eradication of the bacteria that colonize the vasoproliferative lesion by antibiotic treatment results in vessel regression[17]. Hence, *Bartonella*-triggered vascular tumours are benign and their growth depends on the continuous presence of bacteria in the tumour lesion[2], as is typically observed in chronically infected immunosuppressed patients.

The molecular basis of *Bartonella*-triggered angiogenesis is poorly defined and somewhat controversial. However, it seems that this process is multifactorial and involves various levels of host-cell interaction (FIG. 3). These *Bartonella*-triggered processes are described in more detail in the following sections. It should be noted that they have been exclusively studied *in vitro*, for example, by using human umbilical-vein endothelial cells (HUVECs) in a cell-culture model of angiogenesis. The development of an animal model will be essential to define the role of these cellular processes in *Bartonella*-triggered vascular tumour formation *in vivo*.

Vascular colonization. Each of the three vasoproliferative *Bartonella* species adheres to and enters HUVECs within a few hours by an actin-dependent process[18–20]. Bacteria that are internalized by this phagocytic process typically end up in small clusters within membrane-bound compartments that are mostly found in a perinuclear location. For *B. bacilliformis*, the uptake process was shown to depend on the small GTPases Rho, Rac and Cdc42, which are key regulators of actin reorganization[20–22]. These characteristics of bacterial uptake, which are presumably shared by all vasoproliferative bartonellae, are reminiscent of the bacterium-directed phagocytosis that has been described in more detail for other intracellular pathogens[23]. In competition with this conventional uptake process, *B. henselae* also enters HUVECs by a second route, which has slower kinetics (lasting about 24 h). Initially, migrating endothelial cells make contact with bacteria that are loosely attached to the substratum, followed by the formation of a bacterial aggregate on their surface. This aggregate is subsequently engulfed and internalized by an actin-dependent mechanism[19]. The characteristic invasion structure that is formed in this process (which comprises massive rearrangements of the actin cytoskeleton) is called the invasome. The *in vivo* relevance of invasome-mediated uptake is unclear, however, the bacterial aggregates that are formed in this process could correspond to the clumps of bacteria that are found in close association with proliferating endothelial cells within bacillary-angiomatosis lesions[24].

NF-κB-dependent proinflammatory activation. Nuclear factor κB (NF-κB) is the key regulator of the proinflammatory response, which mediates the recruitment of lymphocytes from circulating blood to the site of tissue infection. NF-κB is therefore a target for pathogens that either actively provoke inflammation (for example, *Shigella*, which trigger inflammatory destruction of epithelial barriers to enhance tissue invasion)[25] or specifically inhibit the inflammatory response (for example, *Yersinia*, which survive in the hostile environment of lymph nodes)[26]. In endothelial cells, the NF-κB-mediated proinflammatory response is characterized by surface expression of the cell-adhesion molecules E-selectin (which mediates rolling of lymphocytes on endothelial cell surfaces) and intercellular adhesion molecule-1 (ICAM-1, which mediates firm adhesion of lymphocytes through binding to activated CD11/CD18 surface receptors), and the release of the chemoattractant interleukin-8 (IL-8), which triggers trans-endothelial migration of lymphocytes[27]. These characteristics are seen in HUVECs infected by *B. henselae*[28,29]. Although the role of the induced inflammatory response in *Bartonella* infection is unclear, the bacteria might profit from the influx of monocytes/lymphocytes that are competent for the secretion of pro-angiogenic factors to further support vasoproliferative growth (see below).

Inhibition of endothelial-cell apoptosis. The capacity to modulate the apoptotic programme of infected cells by either the host or the pathogen is often a crucial factor for the outcome of an infection. *B. henselae* specifically inhibits apoptosis of HUVECs by blocking early (activation of effector caspases), intermediate (breakdown of the phospholipid asymmetry of the cytoplasmic membrane) and late (DNA fragmentation) events in apoptosis[29,30]. Interestingly, *B. quintana* infection of HUVECs also results in protection from apoptosis, whereas infection with *Bartonella vinsonii* and *Bartonella elizabethae*, which have not been associated with vasoproliferative lesions in humans,

NF-κB
A family of transcription factors important for proinflammatory and anti-apoptotic responses. They are activated by the phosphorylation and subsequent proteolytic degradation of the inhibitor molecule of κB (IκB).

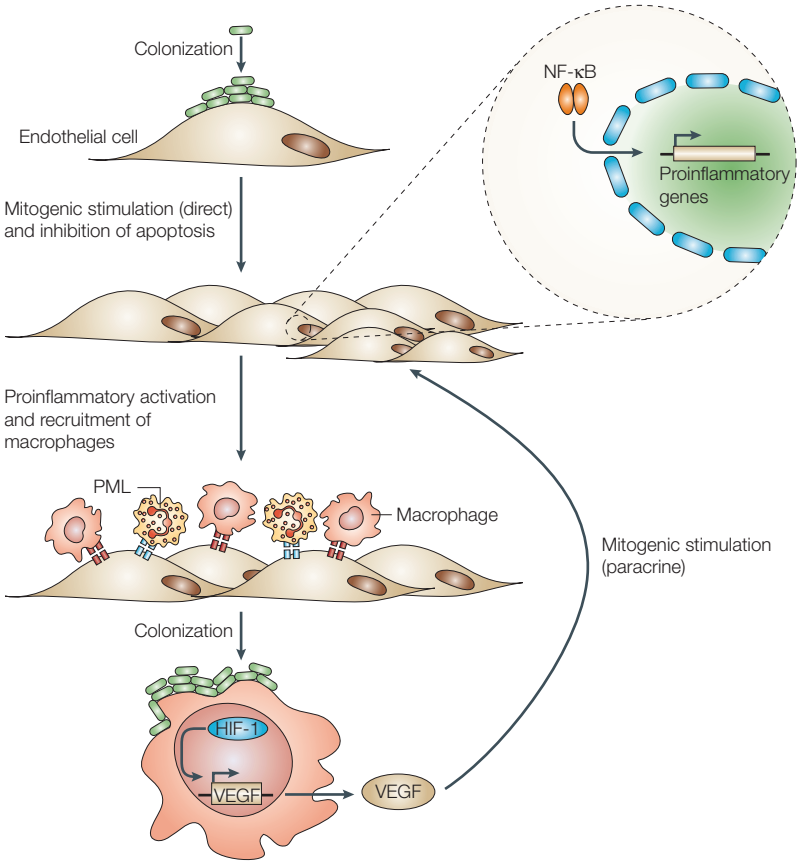

Figure 3 | **Model of *Bartonella*-triggered vascular tumour formation.** The vasoproliferative bartonellae adhere to and invade endothelial cells, which results in the direct stimulation of endothelial cell proliferation and inhibition of apoptosis. Bacteria also trigger a nuclear factor κB (NF-κB)-dependent proinflammatory phenotype that leads to the recruitment of macrophages and other lymphocytes. Bacterial colonization of macrophages results in hypoxic conditions that lead to activation of hypoxia-inducible factor 1 (HIF-1) and the subsequent upregulation of vascular endothelial growth factor (VEGF) expression. The release of VEGF by macrophages results in paracrine stimulation of endothelial cell proliferation. Together, these events are thought to mediate vascular tumour formation. PML, polymorphonuclear leukocyte.

are not anti-apoptotic in this model[30]. The capacity of bartonellae to prevent endothelial-cell apoptosis therefore correlates with their capacity to trigger vasoproliferative lesions. The protection of infected, proliferating endothelial cells from apoptotic cell death, triggered by the host immune response, might therefore be a crucial factor for the induction and maintenance of vasoproliferative tumour growth[1,2,29,30]. The cellular signalling pathway by which bartonellae trigger the anti-apoptotic response is presently unknown. However, the obligate intracellular pathogen *Rickettsia* was shown to protect its cellular habitat from apoptosis by activating NF-κB[31]. The activation of NF-κB by *Bartonella* might therefore not only trigger the pro-inflammatory response as described above, but could also mediate protection from apoptosis.

Direct stimulation of endothelial-cell proliferation and differentiation. Angiogenic-vessel formation is studied *in vitro* by testing for the proliferation, migration and capillary (or tube) formation of primary endothelial cells cultured under specific conditions. All three vasoproliferative *Bartonella* species can stimulate the proliferation of HUVECs[29,32–35]. *B. quintana* and *B. henselae* can also stimulate HUVEC migration through a porous membrane[32]. A recent study further showed increased tube formation of collagen-embedded HUVECs in response to infection with *B. henselae*[36]. Therefore, at least *B. henselae* displays pro-angiogenic activity in all these *in vitro* assays. To what extent *Bartonella*-triggered vasoproliferation is caused by this direct stimulation of endothelial cell proliferation and differentiation by interacting bacteria is presently a matter of discussion (see below).

The paracrine loop model of* Bartonella*-triggered vasoproliferation. The mixed inflammatory infiltrate that is typically found in *Bartonella*-triggered vasoproliferative lesions contains cells of the mononuclear phagocyte lineage such as macrophages. Upon activation, these phagocytes secrete potent pro-angiogenic factors such as VEGF[37]. This PARACRINE loop model of vasoproliferation is supported by several recent studies[38–40]. In response to infection by *B. henselae*, human macrophage and epithelial cell lines were shown to release VEGF at levels that support endothelial-cell proliferation[38–41]. VEGF expression was shown to be induced by HYPOXIA as a consequence of increased oxygen consumption by either bacteria or the infected and metabolically activated host cell[38]. The hypoxic conditions activate the transcription factor hypoxia-inducible factor 1 (HIF-1), which in turn activates expression of *VEGF* and other target genes[38,41]. By releasing pro-angiogenic substances in this paracrine fashion, infected macrophages could be important effector cells in vasoproliferation. However, this paracrine loop alone is unlikely to be sufficient to cause the vasoproliferative lesions triggered by *Bartonella* infections. Many bacterial infections cause a similar inflammatory response to that observed in *Bartonella*-triggered vasoproliferation, and several of these infections result in local anaerobic conditions that might activate HIF-1 (for example, in mycobacterial infections), yet vasoproliferative phenomena or vascular tumours are conspicuously absent. Instead, the paracrine loop of the release of pro-angiogenic factors from monocytic cells in the inflammatory infiltrate might act synergistically with the *Bartonella*-triggered proliferation of endothelial cells and inhibition of apoptosis to stimulate vasoproliferative-tumour growth (FIG. 3).

Bartonella virulence factors

Early work on *Bartonella* pathogenesis established appropriate *in vitro*[19,29,32,36,39,40] and *in vivo*[5,7,8] infection models, but few virulence factors were described. The recent advent of *Bartonella* molecular genetics[42–45] in combination with mutant testing in the established infection models[29,41,44–48] enabled the identification of genetically defined virulence factors (TABLE 2), which has significantly advanced our understanding of the

PARACRINE
Form of signalling in which the target cell is close to the signal-releasing cell.

HYPOXIA
Reduction of the oxygen supply to a tissue to below physiological levels.

Table 2 | **Genetically defined virulence determinants of *Bartonella* spp.**

Determinant(s)	Description/putative role in pathogenesis	References
BadA/Vomp	Non-fimbrial adhesins that mediate bacterial autoaggregation as well as adhesion to extracellular matrix proteins, resulting in cell adhesion and induction of a paracrine pro-angiogenic response	5,41
BepA–BepG	Translocated by the VirB/VirD4 T4SS into endothelial cells; mediate subversion of endothelial-cell function	45,66
Fla	Flagella; mediate motility and enhance binding to erythrocytes	47
IalB	Enhances association with erythrocytes	48,67
Trw	T4SS that is closely related to the Trw bacterial conjugation system of *Escherichia coli* plasmid R388; expansion of the locus by gene duplication in tandem; establishes erythrocyte infection by an unknown mechanism	1,46
VirB/VirD4	T4SS that is closely related to a bacterial conjugation system of *Agrobacterium tumefaciens* (AvhB/TraG); mediates subversion of endothelial cell functions by translocating BepA–BepG	29,44,47

BadA, *Bartonella* adhesin A; Bep, *Bartonella*-translocated effector protein; Ial, invasion-associated locus; T4SS, type IV secretion system; Vomp, variably expressed outer-membrane protein(s).

molecular mechanisms that govern *Bartonella*–host-cell interactions[1]. In addition to the main genetically defined virulence factors, I also discuss lipopolysaccharide (LPS) as a biochemically defined virulence factor of the bartonellae[49]. Flagella and IalA/IalB (invasion-associated locus A/B), as well as bacterial factors that have only been loosely implicated in *Bartonella* virulence (that is, Omp43, HbpA–D/Pap31, deformin and GroEL), are not covered — their putative role in *Bartonella* virulence has recently been reviewed elsewhere[1].

Low-toxicity LPS as a prerequisite for chronic vascular infection. The endotoxic activity of the LPS of most Gram-negative bacteria causes serious damage to vascular endothelial cells and is an important mediator of septic-shock syndrome[50]. The remarkable non-toxic interaction of bartonellae with endothelial cells, which eventually leads to vasoproliferation, and the apparent lack of septic shock in response to bacteraemia indicate that the LPS of bartonellae has reduced endotoxic activity. In fact, the endotoxic activity of purified LPS from *B. henselae* was shown to be 1,000–10,000-fold lower than that of enterobacterial LPS[49]. Structural analysis of *B. henselae* LPS revealed several unusual features, including a rare LIPID A core structure (2,3-diamino-2,3-dideoxy-glucose disaccharide bisphosphate), penta-acylation of lipid A, an uncommon long-chain fatty acid (25-hydroxyhexacosanoic or 27-hydroxyoctacosanoic acid) and a short carbohydrate region (glucose attached to 3-deoxy-D-*manno*-oct-2-ulosonic acid disaccharide)[49]. In contrast to the high-endotoxic LPS of enterobacteria such as *Escherichia coli*, some of the structural features of *B. henselae* LPS are shared by the low-endotoxic LPS of other intracellular bacteria that cause chronic infections — penta-acylation of lipid A of *Chlamydia trachomatis* and the presence of a long-chain fatty acid in the lipid A of *Legionella pneumophila*[49]. These features might provide the structural basis for the low endotoxic potency of these LPS structures, which in the case of *B. henselae* could be a prerequisite for chronic interaction with endothelial cells.

Non-fimbrial adhesins. Non-fimbrial adhesins (for example, YadA of enteropathogenic *Yersinia* species and NadA of *Neisseria meningitidis*) are an important class of proteobacterial adhesins that mediate binding to host cells and EXTRACELLULAR MATRIX (ECM) components[51,52]. Recent studies with *B. henselae* and *B. quintana* have identified several new members of this adhesin family, including BadA (*Bartonella* adhesin A) and VompA–D (variably expressed outer-membrane proteins A–D), respectively[5,41].

The 340-kDa BadA protein of *B. henselae* is the largest bacterial protein known to date. Based on phenotypic and functional properties (for example, adhesion to host cells and auto-agglutination), the corresponding filamentous surface structures visualized by transmission electron microscopy were initially called TYPE IV-LIKE PILI[53] (FIG. 4a). Related surface appendages of different lengths are expressed by several other bartonellae (for example, *Bartonella alsatica*[54], *B. tribocorum*[55] and *B. elizabethae*; FIG. 4b), indicating that they represent BadA homologues. BadA has a modular domain structure (FIG. 4c). The N terminus (encoding putative head repeats) and C terminus (encoding the putative membrane anchor) display sequence similarity to YadA[41]. Upon trimerization, the C-terminal membrane anchor is thought to form a 12-stranded pore that allows the autotransport of the adhesin across the outer membrane[56]. Similar to YadA[57], the N-terminal head repeats are proposed to fold as a left-handed β-helix that forms a globular head domain. The large central region of BadA is composed of 24 repeat units, each containing a putative coiled-coil segment and a separating neck sequence. Structurally, the fairly regular alternation of coiled-coil and globular sequences suggests an extended, rod-like shape with periodically recurring bulkier and thinner parts. This projection fits well with the estimated length of BadA (~100–300 nm) and its hair-like appearance in electron micrographs (FIG. 4a). Multiple passage of *B. henselae* on plates can result in irreversible loss of BadA expression, which has been shown to occur either by a large internal deletion or by frame-shift mutation of the *badA* gene[41]. The *B. henselae* genome encodes several truncated homologues of *badA*[58], indicating that genetic instability is a common trait of these paralogues. The dispensability of BadA during bacterial growth on plates and its repeat-rich modular domain structure (which on the DNA level is reflected by direct repeats that represent substrates for homologous recombination) might be responsible for the high rate of mutation of *badA* during passage on plates. However, expression of BadA in most low-passage clinical isolates of *B. henselae*[41,53] indicates an important role for this non-fimbrial adhesin during infection of the incidental human host. *In vitro*,

LIPID A
Component of lipopolysaccharide (LPS) that anchors the molecule to the cell surface by insertion into the outer membrane. The lipid A moiety is responsible for the endotoxic activity of LPS.

EXTRACELLULAR MATRIX
Secreted products of many different cell types that form an organized scaffold for cell support. Components of the extracellular matrix include collagen and laminin.

TYPE IV-LIKE PILUS
An elongated hair-like structure extending from the surface of Gram-negative cells that is independent of flagella, and which can retract and pull the cell forward.

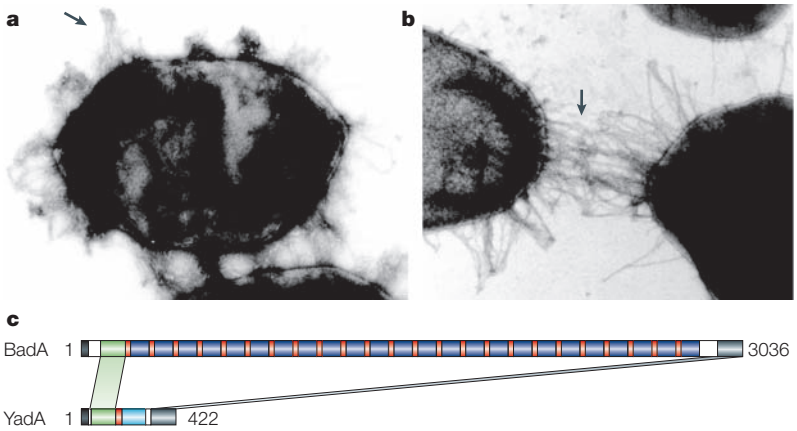

Figure 4 | ***Bartonella* adhesin A (BadA)-like non-fimbrial adhesins.** BadA adhesins are composed of a repeat-rich, modular domain structure that folds into an extended, rod-like stalk and a bulky head structure. BadA-like non-fimbrial adhesins visualized by transmission electron microscopy on the surface of (**a**) *Bartonella henselae* and (**b**) *Bartonella elizabethae*. **c** | Comparison of the modular domain structure of BadA from *B. henselae* and YadA from *Yersinia* species. The N-terminal head repeat domain (green) and the C-terminal membrane anchor domain (grey) are conserved. The inner region is composed of repeated coiled-coil domains (blue), each spaced by a neck domain (red). Modified with permission from REF. 41 © (2004) The Rockefeller University Press.

BadA mediates multiple effects that are crucial for *B. henselae*–host-cell interaction, including binding to various ECM proteins (for example, collagen I/III/IV, laminin and fibronectin), adhesion to epithelial cells and endothelial cells (possibly through β1-integrins), inhibition of phagocytosis by macrophages and activation of HIF-1, resulting in the secretion of pro-angiogenic factors such as VEGF[41]. Taken together, although BadA-mediated effects have so far been exclusively characterized *in vitro*, this non-fimbrial adhesin seems to be an important virulence factor for host-cell interaction by *B. henselae*, particularly with regard to the induction of vasoproliferative disorders.

Many of the features described for BadA are shared by the homologous Vomp proteins of *B. quintana*, for example, the genetic instability and functional properties of these adhesins, such as autoaggregation and binding to ECM components. Whether Vomps can also activate HIF-1 to trigger the release of VEGF in a paracrine fashion similar to that described for *B. henselae* BadA[41] is presently unknown.

The VirB/VirD4 system subverts endothelial cell function. Various bacterial pathogens use type IV secretion systems (T4SSs) to translocate bacterial effector molecules (proteins or DNA) into target host cells. These versatile transporters have evolved from bacterial CONJUGATION SYSTEMS[59]. The prototypic T4SS is the VirB/VirD4 apparatus of *Agrobacterium tumefaciens*, which mediates transfer of the tumorigenic T-DNA complex into infected plant cells. Mammalian pathogens have adapted T4SSs for the transfer of proteins directly into the host-cell cytosol (for example, the CagA protein of *Helicobacter pylori* is transported into gastric epithelial cells) or for the export of multi-subunit protein toxins to the extracellular medium (for example, pertussis toxin secreted by *Bordetella pertussis*)[59]. Bartonellae encode two distinct T4SSs, VirB/VirD4 and Trw, which are both key factors in mediating *Bartonella*–host-cell interactions[29,44,46].

The VirB/VirD4 T4SS is well conserved within the genus *Bartonella*[44,58,60]. Its closest relative is a genuine conjugation system, the AvhB/TraG system of the CRYPTIC PLASMID pATC58 of *A. tumefaciens*[44]. The *Bartonella* VirB/VirD4 system is encoded by an operon of 10 genes (*virB2–virB11*) and a downstream-located *virD4* gene (FIG. 5a). A YEAST TWO-HYBRID INTERACTION STUDY of the components of the *B. henselae* VirB system largely confirmed the protein interactions that were previously identified in other T4SSs studied in more detail[61]. In analogy to the model developed for the topology and function of these related T4SSs[59,62], the *Bartonella* VirB/VirD4 system is considered to encompass a VirB2 pilin and VirB5 minor pilus component, which form a pilus that can mediate contact with host cells; the components VirB3, VirB4 and VirB6–VirB11, which form a pore complex that spans both Gram-negative membranes and possibly also the host-cell membrane; and the T4SS coupling protein (T4CP) VirD4, an inner-membrane protein thought to function as an interface between the pore complex and the T4SS substrates.

The VirB/VirD4 system is an essential virulence factor for *Bartonella* infection in the mammalian reservoir. *B. tribocorum* mutants that carry deletions of either *virB4* or *virD4* are unable to establish an intra-erythrocytic infection in rats[44]. In this *in vivo* model, the VirB/VirD4 system is required for infection of the primary niche, but is not required for the subsequent step of erythrocyte infection[44]. Based on the theory that endothelial cells are a major constituent of the primary niche and an important target cell type for *Bartonella* in both reservoir and incidental hosts, it is reasonable to assume that the VirB/VirD4 system has an important role in mediating endothelial-cell interactions[1,44]. Consistent with this assumption is the observation that the *virB* operon of *B. henselae* is specifically induced during endothelial-cell infection *in vitro*[63]. Moreover, *B. henselae* that carry mutations in either *virB4* or *virD4* are deficient in inducing most of the known physiological changes that are associated with *B. henselae* infection of endothelial cells. These include invasome-mediated uptake, NF-κB-dependent proinflammatory activation and inhibition of apoptotic cell death[29,47]. All these physiological changes, but particularly the inhibition of apoptosis, are considered to contribute to vascular tumour formation *in vivo*. However, the direct mitogenic stimulation of endothelial cells by interacting bacteria is independent of VirB/VirD4 and might even be counteracted by VirB/VirD4-dependent cytostatic or cytotoxic effects elicited at high bacterial infection titres[29] (FIG. 6).

A recent report[47] showed that the VirB/VirD4-dependent cellular changes are mediated by translocated effector proteins. Seven *Bartonella*-translocated effector proteins (BepA–BepG) and the coupling protein

CONJUGATION SYSTEM
System that mediates the transfer of DNA between bacterial cells after cell–cell contact. Conjugation is mediated by mobile genetic elements (usually plasmids or transposons), and is unidirectional and conservative (a copy of the DNA remains in the donor strain).

T-DNA
A DNA segment of the tumour-inducing (Ti) plasmid of *Agrobacterium tumefaciens* that is transferred into the nucleus of infected plant cells, where it is then stably integrated into the host genome and transcribed, causing crown gall disease.

CRYPTIC PLASMID
Plasmid with unknown function.

YEAST TWO-HYBRID INTERACTION ASSAY
Assay in which one protein is fused to a transcriptional activation domain and the other to a DNA-binding domain, and both fusion proteins are introduced into yeast. Expression of a reporter gene with the appropriate DNA-binding sites upstream of the promoter indicates that the two proteins interact physically.

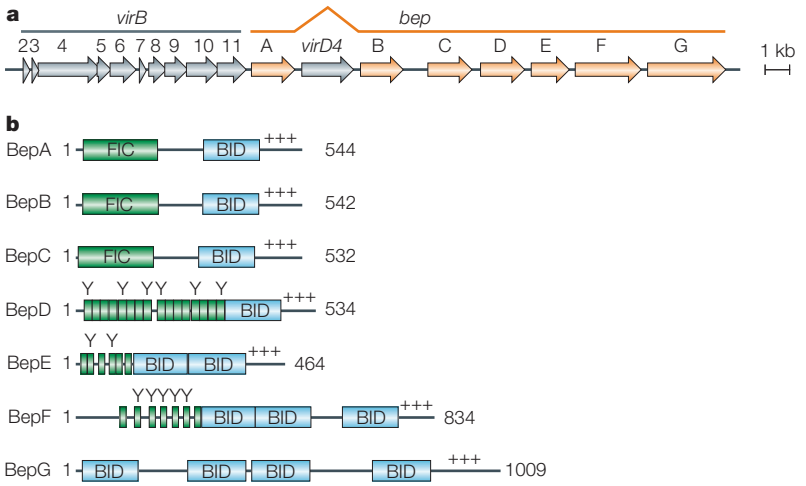

Figure 5 | ***Bartonella* VirB/VirD4 and Beps. a** | The genetic structure of the *virB/virD4/bep* pathogenicity island, which encodes 18 type IV secretion-related proteins. **b** | The domain structure of BepA–BepG. Bep, *Bartonella*-translocated effector protein; BID, Bep intracellular delivery; Fic, filamentation induced by cAMP; Y, tyrosine residue within a conserved phosphorylation motif.

VirD4 are encoded within 22 kb downstream of the *virB* operon[47] (FIG. 5a). The entire *virB/virD4/bep* region probably constitutes a PATHOGENICITY ISLAND (PAI) (FIG. 5a). The loss of all known VirB/VirD4-dependent changes in a mutant with deletions in the *bepA–bepG* genes indicates that BepA–BepG comprise the complete set of VirB/VirD4-translocated effector proteins. However, the specific contribution(s) of individual Beps to the complex VirB/VirD4-dependent phenotypic changes of endothelial cells is presently unknown.

The *virB/virD4/bep* PAI is present in both available genome sequences from the genus *Bartonella* (*B. henselae* and *B. quintana*)[58], but not in any other known genome sequence. However, in contrast to the highly conserved *virB/virD4* genes, the *bep* genes display a high degree of plasticity, including signatures of gene duplication and degradation as well as intragenic domain duplication and intragenic or intergenic domain reshuffling[47]. The Beps have a highly modular domain structure (FIG. 5b). The C terminus of each Bep harbours at least one copy of the novel Bep intracellular delivery (BID) domain and a short, positively charged tail sequence. This bipartite C terminus has been shown to constitute a transfer signal that is sufficient to mediate VirB/VirD4-dependent intracellular delivery of reporter fusion proteins[47]. After its discovery in the Beps, the BID domain was also found to be present in conjugative relaxases of α2-proteobacterial conjugation systems, indicating that conjugative relaxases represent the evolutionary origin of this signal for protein transfer by the *Bartonella* VirB/VirD4 system[47].

Whereas the C terminus of the Beps is required for protein transfer, their N-terminal region might be crucial for effector function within host cells. The N termini of BepA–BepC comprise the Fic (filamentation induced by cAMP) domain, which is conserved in many bacteria and was proposed to be involved in bacterial cell division[47]. The effector functions of the Fic domain within mammalian target cells are unknown. The N termini of BepD–BepF contain short repeated peptide sequences that contain conserved putative tyrosine-phosphorylation motifs (EPLYA), similar to the EPIYA motif of the T4SS substrate CagA of *H. pylori*, which, upon transfer into gastric epithelial cells, is phosphorylated by human Src-family kinases[64]. Likewise, BepD of *B. henselae* becomes tyrosine-phosphorylated upon T4SS-dependent transfer into endothelial cells[47]. Tyrosine-phosphorylated CagA has been shown to interfere with various signalling processes (for example, through binding to SHP-2 or CSK), resulting in physiological changes related to cytoskeletal dynamics, cell migration and malignant transformation[12]. Future studies might show how tyrosine-phosphorylated Beps contribute to the subversion of endothelial-cell function. Interestingly, BepD–BepG carry multiple copies of the BID domain, even though the domain nearest to the positively charged C terminus seems to be sufficient for protein transfer. The most extreme case is BepG, of which the putative N-terminal effector domain contains three additional copies of the BID domain (FIG. 5b). Whether multiple BID domains improve substrate transfer or whether this domain might also have effector functions within host cells remains to be elucidated.

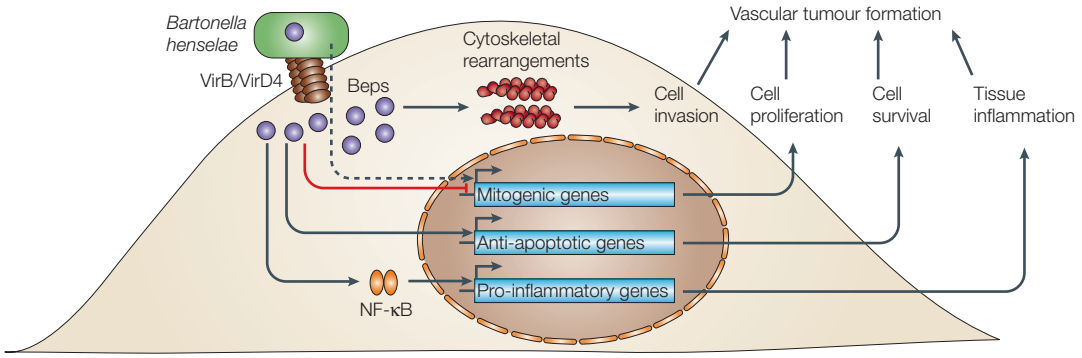

Figure 6 | ***Bartonella*-translocated effector proteins (Beps) subvert endothelial cell function.** The effects of Beps include nuclear factor κB (NF-κB)-dependent proinflammatory activation and inhibition of apoptosis, with the net result of vascular tumour formation.

PATHOGENICITY ISLAND
A contiguous block of genes acquired by horizontal transfer in which at least a subset of the genes code for virulence factors.

Box 1 | **Questions for future research**

- **Which (intra- and extra-)cellular habitats constitute the primary niche? During passage through the primary niche, how are bartonellae reprogrammed to become competent for the subsequent erythrocyte invasion?**
- **What is the mechanism that determines the host range for intra-erythrocytic infection?**
- **What are the molecular mechanisms of variable expression of non-fimbrial adhesins (BadA/Vomp) and what is their physiological role during infection?**
- **How do the individual *Bartonella*-translocated effector proteins (Beps) contribute to the subversion of endothelial cell function?**
- **Why does the *trw* locus encode multiple forms of variable pilus components? Are variable pili required for mediating adhesion to variable receptors on erythrocytes, that is, blood-group antigens? Which substrates (if any) are translocated by the Trw type IV secretion system (T4SS) into erythrocytes or other target cells?**
- **Based on the high similarities of Trw and VirB/VirD4 to bacterial conjugation systems, can any of these T4SSs mediate DNA transfer from *Bartonella* into host cells?**
- **What is the nature of the bacterial factor(s) that mediate pro-angiogenic activation of endothelial cells? In contrast to the paracrine route of pro-angiogenic factor release, what is the contribution of the direct route of pro-angiogenic activation to vascular proliferation?**

The Trw T4SS is required for erythrocyte infection. As for the VirB/VirD4 system, the Trw system of bartonellae is more closely related to bacterial conjugation systems than to any T4SS that has been implicated in bacterial pathogenesis. This system displays an exceptionally high level of sequence identity with the Trw conjugation machinery of the broad-host-range antibiotic-resistance plasmid R388 (up to 80% amino-acid sequence identity for individual T4SS components), and was therefore also termed Trw[46]. The loci that encode these conserved T4SSs are co-linear, except for a considerable expansion of the *Bartonella* locus by tandem repeats of *trwL* (encoding TrwL, the homologue of the pilin VirB2) and of the three-gene cluster *trwJIH* (encoding TrwJ, TrwI and TrwH, the homologues of the minor pilus-associated component VirB5, the inner-membrane protein VirB6 and the outer-membrane lipoprotein VirB7, respectively). Strikingly, the *trwL* and *trwJ* sequences are highly variable within the same *Bartonella* species, as well as between different species. By contrast, the various copies of the genes that encode the T4SS pore-complex components TrwH and TrwI are almost identical. The prominent difference in sequence conservation between PARALOGUES of the co-amplified *trwJIH* cluster indicates that, following tandem duplication, these genes were exposed to differential selective pressure. Negative selection against the accumulation of mutations in *trwI* or *trwH* paralogues might result from a deleterious dominant-negative effect of these mutations on the integrity and function of the T4SS, as has been described for homologous systems[65]. Positive selection for mutations in *trwJ* results in the generation of variable forms of TrwJ, which, together with the variably encoded TrwL pilins, might assemble highly variable pilus structures.

PARALOGUES
Homologous genes in the same organism that have evolved from a gene duplication and a subsequent divergence of function.

Interestingly, the co-linearity of the *Bartonella trw* locus with plasmid R388 is limited to the components that encode the T4SS (the *trwD–trwH* and *trwI–trwM* operons) and the upstream-located transcriptional regulatory system (*korA*/*B*), but does not extend to the downstream-located *trwB* gene of plasmid R388 that encodes the T4CP. Instead, a cryptic-phage-like integrase gene is encoded at the corresponding position of the *Bartonella* locus, indicating horizontal acquisition of the *trw* locus. The absence of a gene encoding a TrwB-like T4CP in the *trw* locus or elsewhere in the genome indicates that the *Bartonella* Trw system might be incapable of exporting substrates, unless VirD4 of the VirB/VirD4/Bep system can complement for the T4CP function. However, so far no substrate transported by the *Bartonella* Trw system has been identified. The role of the Trw system might therefore primarily be the formation of pili. The large number of genes that encode variant forms of the two pilus subunits might allow bacteria to synthesize particularly long pili and/or highly variable pili that might allow the interaction with different host-cell-surface structures (for example, different blood-group antigens on the erythrocyte surface), or could represent a general mechanism of immune evasion by antigenic variation.

It is important to note that all genes of the *trw* locus are co-regulated[46]. As in plasmid R388, the transcriptional regulation of the *Bartonella trw* locus is controlled by the heterodimeric repressor complex KorA/KorB, which binds to a conserved *kor* box that overlaps with all promoters in the *trw* PAI[46]. Similar to the *virB*/*virD4* locus[63], the *Bartonella trw* locus is not expressed during growth on solid media, but is induced in the course of endothelial cell infection[46]. Evidence for an essential role of the Trw system in infection of the mammalian reservoir host was obtained in the *B. tribocorum*–rat infection model. Unlike wild-type bacteria, a *trwE* deletion mutant was unable to establish an intra-erythrocytic bacteraemia in this model[46]. Preliminary evidence indicated that *trwE*-mutant bacteria can still colonize the primary niche, although, after seeding to the bloodstream, the mutant was rapidly cleared without establishing erythrocyte infection[1]. Collectively, these data indicate that the Trw T4SS is directly involved in mediating interaction with erythrocytes. Whether or not bartonellae establish erythrocyte interaction through Trw pili that are induced during passage through the primary niche remains an interesting question for future research.

Conclusions and perspectives

The recent advent of molecular genetics and suitable *in vitro* and *in vivo* infection models for the bartonellae, along with the availability of complete genome sequences, has greatly advanced our understanding of the virulence mechanisms that govern the complex interaction between bartonellae and endothelial cells and erythrocytes. This brought about the identification of *bona fide* virulence factors, for example, the

two T4SSs VirB/D4 and Trw and the YadA-like non-fimbrial adhesin BadA[41], which have indispensable functions in mediating bacterial interactions with endothelial cells and erythrocytes. However, there is an obvious imbalance between the *in vitro* and *in vivo* studies carried out on these major target cells of bartonellae. Erythrocyte interaction that leads to a long-lasting intra-erythrocytic bacteraemia has been extensively studied in animal models[7,8], but an appropriate *in vitro* model that allows the molecular basis of erythrocyte infection to be studied is not yet available. This is mostly owing to the fact that bacteria must passage through the primary infection niche outside the bloodstream to become competent for erythrocyte infection — a condition that cannot easily be mimicked *in vitro*. By contrast, the interaction of bartonellae with endothelial cells (at least with regard to vasoproliferative tumour formation) has been studied exclusively in cell-culture models but no suitable animal model is yet available. Hence, the precise role of the uncovered cellular and molecular interactions in vasoproliferative-tumour formation remains unclear. However, given the pace at which research on *Bartonella* pathogenesis is currently expanding, researchers are optimistic that many of these undecided issues will be resolved in due course. BOX 1 summarizes some of the most important questions for future research on *Bartonella* pathogenesis.

Note added in proof

It was recently shown that the chemokine monocyte chemoattractant protein 1 (MCP-1) is expressed in *B. henselae*-infected endothelial cells in an NF-κB-dependent manner, and that supernatants from *B. henselae*-infected endothelial cells were able to induce chemotaxis of the monocytic cell line THP-1. MCP-1 is therefore considered to be an important effector for the NF-κB-dependent recruitment of monocytes/macrophages to *B. henselae*-infected endothelial cells[69].

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Competing interests statement

The author declares no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

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Bartonella henselae | *Bartonella quintana* | *Bordetella pertussis* | *Chlamydia trachomatis* | *Escherichia coli* | *Helicobacter pylori* | *Legionella pneumophila* |*Neisseria meningitidis*

CDC Infectious Disease Information: http://www.cdc.gov/ncidod/diseases/index.htm

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