



REVIEW ARTICLE

Persistence of *Bartonella* spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation

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Abstract

Bartonella spp. are facultative intracellular bacteria that typically cause a long-lasting intraerythrocytic bacteremia in their mammalian reservoir hosts, thereby favoring transmission by blood-sucking arthropods. In most cases, natural reservoir host infections are subclinical and the relapsing intraerythrocytic bacteremia may last weeks, months, or even years. In this review, we will follow the infection cycle of *Bartonella* spp. in a reservoir host, which typically starts with an intradermal inoculation of bacteria that are superficially scratched into the skin from arthropod feces and terminates with the pathogen exit by the blood-sucking arthropod. The current knowledge of bacterial countermeasures against mammalian immune response will be presented for each critical step of the pathogenesis. The prevailing models of the still-enigmatic primary niche and the anatomical location where bacteria reside, persist, and are periodically seeded into the bloodstream to cause the typical relapsing *Bartonella* spp. bacteremia will also be critically discussed. The review will end up with a discussion of the ability of *Bartonella* spp., namely *Bartonella henselae*, *Bartonella quintana*, and *Bartonella bacilliformis*, to induce tumor-like vascular deformations in humans having compromised immune response such as in patients with AIDS.

Introduction

Bartonella spp. are Gram-negative facultative intracellular bacteria that taxonomically belong to the $\alpha 2$ -subgroup of the proteobacteria. *Bartonella* spp. have peculiar and demanding nutritional requirements such as the apparent inability to utilize glucose as the carbon source (Chenoweth *et al.*, 2004). Typically, the bacteria are grown *in vitro* on sheep blood or chocolate agar plates under 5% CO₂ atmosphere, and this may easily take several days or even weeks with primary isolates for visible colonies to appear. This creates challenges in clinical settings despite complementary serological and molecular approaches (Agan & Dolan, 2002). There have been attempts to establish liquid growth media and growth conditions (Schwartzman *et al.*, 1993; Wong *et al.*, 1995a; Chenoweth *et al.*, 2004; Maggi *et al.*, 2005; Riess *et al.*, 2008), which improve isolation and also have great value for basic research, for example, in the analysis of *Bartonella* spp. pathogenic mechanisms.

Figure 1 outlines the phylogeny of *Bartonella* spp., which is based on nucleotide sequence information of 478 core genome genes of the ten currently available *Bartonella* spp. genomes and four house-keeping genes (*rpoB*, *gltA*, *ribC*, and *groEL*) of nonsequenced *Bartonella* spp. (Engel *et al.*, 2011). Based on the work by Engel and co-workers, *Bartonella* spp. can be separated into four phylogenetic clades. First clade is represented by a single species, *Bartonella bacilliformis*, which is highly pathogenic in its human reservoir host. Clades 2, 3, and 4 contain species that cause more benign infections in their reservoir hosts as well as species such as *Bartonella henselae*, which represents a significant zoonotic threat to humans. At least one mammalian reservoir host is known for each of the described member of the genus *Bartonella*, that is, the bacterium has been cultivated from the blood of the corresponding mammal. *Bartonella* spp.–arthropod interactions remain poorly defined. However, *Bartonella* spp. have been detected from a number of blood-sucking arthropods either directly by molecular diagnostics or

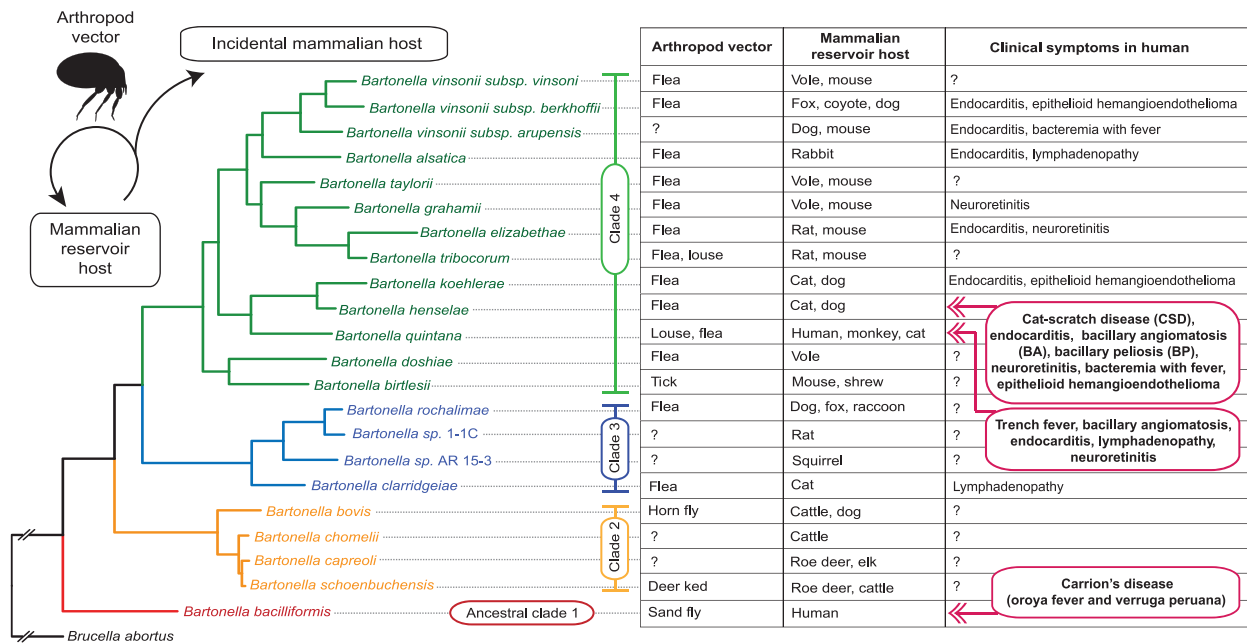


Fig. 1. Phylogeny and epidemiology of *Bartonella* spp. *Bartonella* spp. typically cause persistent and relapsing intraerythrocytic bacteremia in their mammalian reservoir hosts, thereby favoring transmission by blood-sucking arthropods. Incidental mammalian hosts may get infected via direct contact with the infected arthropod, with their feces, or with the infected animal. Phylogeny is modified from Engel *et al.* (2011), and it contains most of the currently known *Bartonella* species. Epidemiological data are based on (Engbaek & Lawson, 2004; Reeves *et al.*, 2007) *Bartonella vinsonii* ssp. *vinsoni*, (Breitschwerdt *et al.*, 1995; Chang *et al.*, 2000; Roux *et al.*, 2000; Breitschwerdt *et al.*, 2009; Schaefer *et al.*, 2011) *Bartonella vinsonii* ssp. *berkhoffii*, (Welch *et al.*, 1999; Fenollar *et al.*, 2005; Bai *et al.*, 2010, 2011b) *Bartonella vinsonii* ssp. *arupensis*, (Heller *et al.*, 1999; Raoult *et al.*, 2006; Angelakis *et al.*, 2008; Kernif *et al.*, 2010) *Bartonella alsatica*, (Birtles *et al.*, 1995; Bown *et al.*, 2004; Engbaek & Lawson, 2004; Marié *et al.*, 2006) *Bartonella taylorii*, (Birtles *et al.*, 1995; Kerkhoff *et al.*, 1999; Koesling *et al.*, 2001; Bown *et al.*, 2004; Engbaek & Lawson, 2004; Berglund *et al.*, 2010) *Bartonella grahamii*, (Daly *et al.*, 1993; O'Halloran *et al.*, 1998; Ellis *et al.*, 1999; Ying *et al.*, 2002; Inoue *et al.*, 2009; Tsai *et al.*, 2010) *Bartonella elizabethae*, (Heller *et al.*, 1998; Engbaek & Lawson, 2004; Li *et al.*, 2007; Reeves *et al.*, 2007; Tsai *et al.*, 2010; Billeter *et al.*, 2011) *Bartonella tribocorum*, (Droz *et al.*, 1999; Rolain *et al.*, 2003a; Avidor *et al.*, 2004; Marié *et al.*, 2006; Ohad *et al.*, 2010; Mascarelli *et al.*, 2011; Pérez *et al.*, 2011) *Bartonella koehlerae*, (English *et al.*, 1988; Welch *et al.*, 1992; Wong *et al.*, 1995b; Chomel *et al.*, 1996; Abbott *et al.*, 1997; Rolain *et al.*, 2003a; Ohad *et al.*, 2010; Mascarelli *et al.*, 2011; Pérez *et al.*, 2011; Regnery *et al.*, 1992; Tsai *et al.*, 2011) *Bartonella henselae*, (Fournier *et al.*, 2001; Foucault *et al.*, 2002; Safdar, 2002; George *et al.*, 2006; Marié *et al.*, 2006; Breitschwerdt *et al.*, 2007; Vitale *et al.*, 2009; Holmes *et al.*, 2011; Huang *et al.*, 2011; Yamada *et al.*, 2011) *Bartonella quintana*, (Birtles *et al.*, 1995; Marié *et al.*, 2006; Telfer *et al.*, 2007) *Bartonella doshiae*, (Bermond *et al.*, 2000; Engbaek & Lawson, 2004; Reis *et al.*, 2011) *Bartonella birtlesii*, (Chomel *et al.*, 2009b; Gabriel *et al.*, 2009; Henn *et al.*, 2009; Schaefer *et al.*, 2011) *Bartonella rochalimae*, (Lin *et al.*, 2008; Engel *et al.*, 2011) *Bartonella* sp. 1-1C, (Inoue *et al.*, 2009) *Bartonella* sp. AR15-3, (Heller *et al.*, 1997; Kordick *et al.*, 1997; Sander *et al.*, 2000b; Rolain *et al.*, 2003a; Tsai *et al.*, 2011) *Bartonella clarridgeiae*, (Bermond *et al.*, 2002; Chung *et al.*, 2004; Pérez *et al.*, 2011) *Bartonella bovis*, (Maillard *et al.*, 2004) *Bartonella chomelii*, (Bermond *et al.*, 2002; Bai *et al.*, 2011a) *Bartonella capreoli*, (Dehio *et al.*, 2001; Rolain *et al.*, 2003b; Dehio *et al.*, 2004) *Bartonella schoenbuchensis*, and (Schultz, 2010) *Bartonella bacilliformis*.

after conventional culture recovery (Fig. 1) (Chomel *et al.*, 2009b). Moreover, infection studies under laboratory conditions indicate that blood-sucking arthropods act as vectors of *Bartonella* spp. As an example, cat fleas (*Ctenocephalides felis*) that had been feeding on *B. henselae*-infected cats efficiently transmitted the bacterium into pathogen-free cats (Chomel *et al.*, 1996). Fleas (*Ctenophthalmus nobilis nobilis*) that were harvested from wild voles have also been reported to transmit *Bartonella grahamii* and *Bartonella taylorii* into pathogen-free voles under laboratory conditions (Bown *et al.*, 2004). Most likely flea feces and superficial scratching of the skin

mediate the actual transmission. It has been shown that intradermal inoculation of cats with *B. henselae*-containing flea feces causes bacteremia (Foil *et al.*, 1998).

The common theme in *Bartonella* spp. infection of the reservoir mammalian host is a chronic intraerythrocytic bacteremia (Abbott *et al.*, 1997; Schülein *et al.*, 2001), which appears to be a specific adaptation to the mode of transmission by blood-sucking arthropods. In incidental hosts, *Bartonella* spp. do not appear to establish the intraerythrocytic bacteremia. However, endothelial cells (ECs) appear to be targeted by *Bartonella* spp. both in the incidental host and in the reservoir host. Infections of the

reservoir hosts range from an apparently asymptomatic to subclinical (most animal-specific species), low morbidity to limited mortality (such as human-specific *Bartonella quintana* infections), and even to life-threatening, such as the severe hemolytic anemia associated with the human-specific infection by *B. bacilliformis* (Maguiña *et al.*, 2009). In most cases, infections of the reservoir hosts do not lead to severe disease symptoms (Chomel *et al.*, 1996; Regnery *et al.*, 1996; Abbott *et al.*, 1997; Guptill *et al.*, 1997; O'Reilly *et al.*, 1999; Pappalardo *et al.*, 2000; Boulouis *et al.*, 2001; Koesling *et al.*, 2001; Pappalardo *et al.*, 2001; Schülein *et al.*, 2001; Zhang *et al.*, 2004; Marignac *et al.*, 2010), suggesting a highly specific adaptation to the corresponding host and circumvention of its immune responses.

A significant progress in our understanding of the molecular and cellular basis of *Bartonella* spp. pathogenesis (Tables 1 and 2) has been achieved in recent years because of the establishment of bacterial genetics as well as animal and cell culture infection models. This review will focus on the current knowledge of mammalian host–*Bartonella* spp. interaction and excludes the arthropod host–*Bartonella* spp. interaction, which has recently been reviewed (Chomel *et al.*, 2009b). The main emphasis is laid on the description of *Bartonella* spp. tool-box to effi-

ciently circumvent and subvert host antimicrobial functions and to establish the typical chronic and relapsing infection. In the end, the most significant *Bartonella* spp. human infections and their vasoproliferative tumor-like manifestations bacillary angiomatosis (BA), bacillary peliosis (BP), and verruga peruana of the Carrion's disease will be discussed in light of the proposed molecular mechanisms of pathogenesis. *Bartonella* spp.-triggered tumorigenesis has attracted considerable interest from both clinicians and basic scientists in the fields of infection and cancer biology, and it represents a paradigm for pathogen-triggered tumorigenesis.

Progression of *Bartonella* spp. infection in the reservoir mammalian host

One of the strengths of the *Bartonella* spp. research field is the ability to conduct infection studies in natural reservoir hosts such as a mouse, cat, rat, or a dog, with the most detailed information available for the rat model of *Bartonella tribocorum* infection (Schülein *et al.*, 2001). The first reservoir host models, however, were established for *B. henselae* in domestic cats (Chomel *et al.*, 1996; Regnery *et al.*, 1996; Abbott *et al.*, 1997; Guptill *et al.*, 1997; O'Reilly *et al.*, 1999). *Bartonella henselae* infection

Table 1. Synopsis of the proposed pathogenicity factors of *Bartonella* spp.

Factor	Description with key reference(s)
LPS	Weak TLR4 agonist (Zähringer <i>et al.</i> , 2004)
Flagella	Weak TLR5 agonist, important in bacterial motility and possibly in bacterial adhesion to the erythrocytes (Andersen-Nissen <i>et al.</i> , 2005)
BadA, BrpA, VompA, VompB, VompC, VompD	TAAAs induce auto-aggregation, antiphagocytic properties, bind multiple ECM components, mediate cell adhesion, essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> (BadA), <i>B. quintana</i> (Vomps), and <i>B. birtlesii</i> (BrpA), required in colonization of the primary niche and/or in seeding of the bacteria from the primary niche into the bloodstream (Riess <i>et al.</i> , 2004; Zhang <i>et al.</i> , 2004; Gilmore <i>et al.</i> , 2005)
Pap31	Fibronectin-binding adhesin, also known as hemin-binding protein A (HbpA) (Dabo <i>et al.</i> , 2006a, b)
Hemolysin	Hemolysin of <i>B. bacilliformis</i> , proposed to mediate erythrocyte lysis in the end of Oroya fever (Hendrix, 2000)
VirB/VirD4-T4SS	Type IV secretion system that mediates by its BepB effector proteins (BepA–G) the invasome-mediated uptake of <i>B. henselae</i> into ECs and inhibition of EC apoptosis, essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> and <i>B. birtlesii</i> , required in the colonization of the primary niche and/or in seeding of the bacteria from the primary niche into the bloodstream (Schülein & Dehio, 2002)
Trw–T4SS	Type IV secretion system that mediates the erythrocyte adhesion of <i>Bartonella</i> spp., essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> and <i>B. birtlesii</i> (Seubert <i>et al.</i> , 2003)
Deformin	Induces deeply invaginated pits and trenches in the erythrocytes, presumably involved in the invasion of the erythrocytes, small molecule (~1.4 kDa) that binds albumin (Derrick & Ihler, 2001)
IalAB	Involved in the invasion of the erythrocytes, IalB is similar to Ail invasion of <i>Y. enterocolitica</i> , IalA is a nucleoside polyphosphate hydrolase of the MutT motif family, both proteins are required for the erythrocyte invasion, essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> and <i>B. birtlesii</i> (Mitchell & Minnick, 1995)
HbpB–HbpE	A family of hemin-binding proteins, some members are essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> and <i>B. birtlesii</i> (Carroll <i>et al.</i> , 2000; Minnick <i>et al.</i> , 2003b)
HutA	Hemin receptor, essential for intraerythrocytic bacteremia by <i>B. tribocorum</i> and <i>B. birtlesii</i> (Parrow <i>et al.</i> , 2009)
Omp43	Putative adhesin, recombinant Omp43 binds ECs (Burgess & Anderson, 1998)

Table 2. The most significant open questions of the *Bartonella* spp. research field

- (1) Adaptation to specific arthropod vectors and mammalian reservoir hosts seems to be a common strategy of *Bartonella* spp. pathogenesis. What determines the arthropod specificity and the mode of transmission, and what are the bacterial factors involved in the arthropod infection and transmission?
- (2) Which cellular or acellular habitat constitute the primary niche of the reservoir host? During passage through the primary niche, how are *Bartonella* spp. programmed to become competent for the subsequent erythrocyte invasion? What determines the peculiar periodicity of bacterial release from the primary niche into the bloodstream?
- (3) Immunosuppression appears as a prerequisite for vascular tumor formation by *B. henselae*, *B. quintana*, and *B. bacilliformis* in human. What does this commonly used phrase 'immunosuppression' actually mean at the molecular level at the interface of host-pathogen interaction?
- (4) What is the nature of bacterial factor(s) that mediate the angiogenic activation of ECs? In contrast to the autocrine and paracrine route of pro-angiogenic factor release, what is the contribution of the direct bacterial activation of EC proliferation in vascular deformations? Can we establish an animal model for *Bartonella* spp.-triggered vascular deformations?
- (5) What is the exact contribution of VirB/VirD4-T4SS in *Bartonella* spp.-triggered vascular deformations? What is the exact contribution of VirB/VirD4-T4SS in the reservoir host infections? How do the individual *Bartonella*-translocated effector proteins (Beps) contribute to the subversion of EC functions? Are there other cell types that are targeted by VirB/VirD4-T4SS and its effectors? How are Bep expression, timing of Bep translocation, quantities of the translocated Beps, and composition of the Bep pool that is received by a given cell type regulated?
- (6) Are *Bartonella* spp. infections an emerging zoonotic threat to humans? How well *Bartonella* spp. respond to commonly used antibiotics and can it be envisioned that the efficient capacity of these bacteria for conjugative spread of plasmids will create significant clinical problems in the future? How can we transfer the basic research findings into clinical practice?

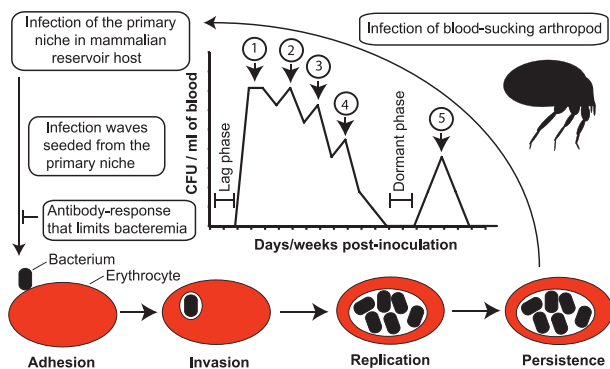


Fig. 2. Progression of the chronic and relapsing *Bartonella* spp. intraerythrocytic bacteremia in a mammalian reservoir host. After initial inoculation, for example, of bacteria in arthropod feces that are superficially scratched into the skin, the bacteria reside and persist in the still-enigmatic primary niche (lag phase). Bacteremia is initiated several days postinoculation by a rapid appearance of high numbers of bacteria in the bloodstream (arrow 1), with bacteria binding to and subsequently invading the erythrocytes. Intraerythrocytic bacteria replicate until reaching a steady number, which is maintained for the remaining life span of the infected erythrocytes. The primary niche is believed to seed additional erythrocyte infection waves at regular intervals (arrows 2–4) until a specific antibody response clears the infection by blocking the erythrocyte invasion. However, bacteremia may peak (arrow 5) after prolonged periods (weeks to months) of abacteremia (dormant phase) presumably by clonal expansion of antigenic and/or phase variants, which are seeded into the bloodstream from the primary niche. During the long-lasting intraerythrocytic bacteremia, efficient transmission of the intraerythrocytic pathogen to other susceptible hosts is mediated by blood-sucking arthropods, such as fleas and lice. Details are described in the text (see Progression of *Bartonella* spp. infection in the reservoir mammalian host).

in cats appears mainly subclinical, may last for several months or even years, and is manifested by the presence of bacteria in the blood within the erythrocytes (Kordick & Breitschwerdt, 1995). As an example, one naturally *B. henselae*-infected cat had > 10 000 CFU of *B. henselae* per milliliter of blood at day 0 of a longitudinal 2-year study of infection under laboratory conditions (Abbott *et al.*, 1997). The bacteremia gradually declined and the cat became culture negative 5 months later. However, bacteremia was again detected 2 months later. For the rest of the longitudinal infection study, the cat became cyclically culture negative at 2-month intervals with the abacteremic periods lasting, on average, for 2 months. The anti-*B. henselae* antibody titer of the cat remained stable throughout the whole 2-year follow-up (Abbott *et al.*, 1997). The cyclical nature of bacteremia appears common for *Bartonella* spp. infections in their natural reservoir hosts (Fig. 2). The location, designated as the primary niche in the *B. tribocorum*-rat infection model (Schüle *et al.*, 2001), where the bacteria reside during the nonbacteremic state remains unknown and is one of the most significant open questions in the field (see Identity of the primary niche). It has been shown that cats do not become bacteremic during housing with highly bacteremic cats, but fleas that have been feeding on highly bacteremic cats efficiently transmit *B. henselae* infection to uninfected cats (Chomel *et al.*, 1996; Abbott *et al.*, 1997). These findings clearly indicate the importance of arthropod vector for the transmission of *B. henselae*, and this most likely applies to most species of *Bartonella* spp. (Fig. 1). In support of the natural transmission route of *B. henselae*, five pathogen-free cats that were inoculated intradermally with 1×10^6 CFU of plate-grown *B. henselae*

became bacteremic at 9 days postinoculation, which was followed by seroconversion (Abbott *et al.*, 1997).

Similar to the *B. henselae*-cat infection model, *B. tribocorum* infection in its natural reservoir host rat (Heller *et al.*, 1998) appears mainly asymptomatic. Following intravenous inoculation with 3.5×10^7 CFU of agar plate-grown *B. tribocorum* (Schülein *et al.*, 2001), the bacteria appeared unable of entering the erythrocytes and instead were cleared from the circulation within hours postinoculation and remained below detectable levels (viability plating and flow cytometry/confocal microscopy-based detection) for about 4 days. The niche that supports replication of *B. tribocorum* during this time or with analogy the niche that supports replication of *B. henselae* during the abacteremic phases in cat (Abbott *et al.*, 1997) has not been identified experimentally. It has been speculated, mainly because of the marked tropism of *Bartonella* spp. for ECs that is especially prominent for example, in the Carrion's disease (Maguiña *et al.*, 2009) and their proximity to the bloodstream, that ECs are an important constituent of the primary niche (Dehio, 2005). However, the primary niche may also comprise other cell types that is, the migratory cells, such as dendritic cells (DCs), which might assist the passage of bacteria from the site of inoculation (e.g. bacteria in arthropod feces that are superficially scratched into the skin) into the circulation (see Proposal of DCs and the draining lymph nodes). Typically, on day 5 postinoculation, high numbers of bacteria ($\sim 10^6$ – 10^7 per milliliter of blood) are detectable in the blood (Schülein *et al.*, 2001). Presumably, this first wave of bacteremia represents the release of bacteria from the primary niche. The bacteria attach, invade, and replicate inside the erythrocytes until a plateau of approximately eight bacteria per infected erythrocyte is reached around day 14 postinoculation, although some infected erythrocytes may contain up to 15 intracellular bacteria. Thereafter, the number of intracellular bacteria remains static for the remaining life span of the infected erythrocytes. The life span of the infected erythrocytes is indistinguishable from that of uninfected erythrocytes. The intraerythrocytic bacteremia caused by *B. tribocorum* in rats drops below detectable levels after approximately 10 weeks (Schülein *et al.*, 2001). Prior to that, the numbers of viable bacteria in the blood decline, however, not in a steady manner. Instead, the decline is intercepted by peaks of bacteremia, that is, higher fraction of circulating erythrocytes that have been infected, which appear at intervals of 3–6 days (Fig. 2). It is currently believed that these waves are because of bacteria that have been synchronously released from the still-enigmatic primary niche. It remains unknown what determines this periodicity.

The main host immune surveillance mechanism that eventually clears the infection appears to be mediated by

antibodies, at least based on studies in the natural *B. grahamii* mouse infection model (Koesling *et al.*, 2001). In immunocompetent C57BL/6 mice, the *B. grahamii* bacteremia was transient with an average duration of 9 weeks and induced a strong antibody response. In contrast, bacteremia persisted in immunocompromised B-cell-deficient (C57BL/6-Igh^{-/-}) or B- and T-cell-deficient mice (C57BL/6-Rag1^{-/-}). Immune serum transfer beginning with day 6 postinfection from the immunocompetent mice to B-cell-deficient mice that are unable to produce immunoglobulins converted the persistent bacteremia to a transient course indistinguishable from that of immunocompetent animals (Koesling *et al.*, 2001). These data demonstrate an essential role of specific antibodies in abrogating the intraerythrocytic bacteremia of *B. grahamii* in mice, and this may also apply to other species of *Bartonella* spp. Indeed, it has been reported that cats that have cleared an earlier *B. henselae* infection caused by the same *B. henselae* strain do not become bacteremic (Abbott *et al.*, 1997).

Transmission from the arthropod vector

The long-lasting intraerythrocytic infection strategy as revealed by studies with several natural reservoir host infection models such as rhesus macaque-*B. quintana* (Zhang *et al.*, 2004), cat-*B. henselae* (Chomel *et al.*, 1996; Regnery *et al.*, 1996; Abbott *et al.*, 1997; Guptill *et al.*, 1997; O'Reilly *et al.*, 1999), rat-*B. tribocorum* (Schülein *et al.*, 2001), mouse-*B. grahamii* (Koesling *et al.*, 2001), dog-*Bartonella vinsonii* Berkhoffii (Pappalardo *et al.*, 2000, 2001), and mouse-*Bartonella birtlesii* (Boulouis *et al.*, 2001; Marignac *et al.*, 2010) is probably a specific adaptation mechanism to the transmission by blood-sucking arthropods and is presumably shared by most species of *Bartonella* spp. The only known exception to this rule is *B. bacilliformis*, which may trigger massive hemolysis of the colonized human erythrocytes, giving rise to an often-fatal hemolytic anemia (Maguiña *et al.*, 2009). When an infected arthropod comes into contact with an uninfected reservoir host, direct blood contact or intra-/subcutaneous inoculation through arthropod bite might take place, but the highest bacterial numbers are expected to be inoculated via arthropod feces (Chomel *et al.*, 2009b). Most likely, intra-/subcutaneous inoculation by feces takes place via superficial scratching and tissue trauma of the skin. It has been reported that viable *B. quintana* are present in feces of experimentally infected body lice up to the death of the lice (about 35 days) (Fournier *et al.*, 2001). In another study, viable *B. henselae* was detected in the feces of a cat flea 9 days after the flea had been fed with a concentration of 1×10^5 CFU mL⁻¹ of *B. henselae* in blood (Higgins *et al.*, 1996).

Molecular mechanisms of *Bartonella* spp. pathogenesis

Long-lasting bacteremic infections suggest a specific adaptation of *Bartonella* spp. to the corresponding reservoir host and circumvention of its immune responses. This chapter will follow the infection cycle of *Bartonella* spp. in a reservoir host, which starts with an intradermal inoculation (bacteria in arthropod feces that are superficially scratched into the skin) and ends up with the pathogen exit by the blood-sucking arthropod. The current knowledge of bacterial countermeasures against the mammalian immune response will be presented for each critical step of the pathogenesis (Fig. 3).

Evasion of innate immune responses

Strategies utilized by *Bartonella* spp. against professional phagocytes

Means to affect recognition by pattern recognition receptors (PRRs)

Professional phagocytes such as tissue-resident macrophages and DCs are both sentinels and the first line of defense against infection. Right after host entry and in the absence of an adaptive immune response, PRRs on the professional phagocytes are expected to play a major role in the recognition of *Bartonella* spp.. Lipopolysaccharide (LPS) is an essential outer membrane component in Gram-negative bacteria. LPS and in particular its lipid A portion are recognized by PRR-subgroup Toll-like recep-

tors (TLRs), mainly TLR4 together with CD14, which evokes the secretion of pro-inflammatory cytokines and subsequent recruitment of other inflammatory cells to the point of pathogen entry (Miller *et al.*, 2005) (Fig. 4). The apparent lack of LPS-associated septic shock in *Bartonella* spp. bacteremia in later stages of the infection indicates that the LPS of *Bartonella* spp. might be only weakly recognized by TLR4. Indeed, it has been reported that the purified LPS from *B. henselae* is 1000–10 000-fold less active than the purified LPS from *Salmonella enterica* sv. Friedenau in activating TLR4 signaling (Zähringer *et al.*, 2004). Structural analysis of *B. henselae* LPS revealed unusual features that might explain the weak TLR4 activation (Fig. 4), including a rare penta-acylated GlcN3N disaccharide bisphosphate as the lipid A, an uncommon hydroxylated long-chain fatty acid linked to the lipid A, and a small inner core composed of an α -(2→4)-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) disaccharide with one glucose residue attached (Zähringer *et al.*, 2004). It is known that enterobacterial lipid A with a reduced number of fatty acids (normally hexa-acylated), such as a penta-acylated lipid A lacking a secondary myristic acid (14:0) residue in *waaN* mutant of *Salmonella enterica* sv. Typhimurium, is significantly less toxic *in vivo* (Khan *et al.*, 1998). Moreover, similar to the LPS of *B. henselae*, LPS of *Legionella pneumophila* that causes chronic infections has an uncommon long-chain fatty acid linked to the lipid A, and it has been reported that this modification augments TLR4/CD14 activation owing to the lack of LPS–CD14 interaction (Neumeister *et al.*, 1998). It has also been reported that purified LPS of *B. quintana* does not induce the production of

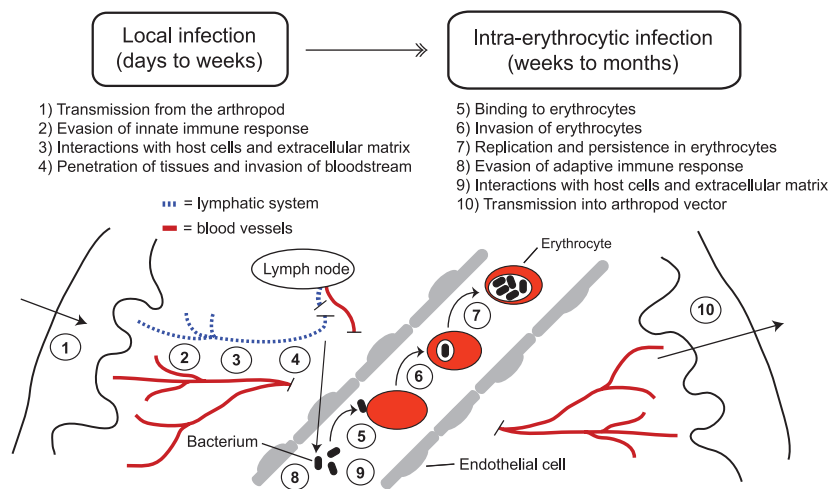


Fig. 3. Schematic representation of the critical steps of *Bartonella* spp. pathogenesis in a mammalian reservoir host. The infection cycle initiates typically by bacteria in arthropod feces that are superficially scratched into the skin and terminates by pathogen exit by the blood-sucking arthropod. Details are described in the text (see Molecular mechanisms of *Bartonella* spp. pathogenesis).

pro-inflammatory cytokines in human monocytes (Popa *et al.*, 2007). LPS of *B. quintana* even appears to be an efficient antagonist of TLR4 activation because it was able to inhibit *Escherichia coli* LPS-mediated release of tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1), and interleukin-6 (IL-6) by human monocytes at ratios ranging from 1000 : 1 to 10 : 1 (*B. quintana* LPS: *E. coli* LPS)

(Popa *et al.*, 2007). Because of this property, the LPS of *B. quintana* has been evaluated as a potential therapeutic tool to block TLR4 signaling in rheumatoid arthritis with promising results (Abdollahi-Roodsaz *et al.*, 2007). The chemically distinct form of LPS appears important for *Bartonella* spp. to establish the long-lasting bacteremia without symptoms of septic shock. Although not yet

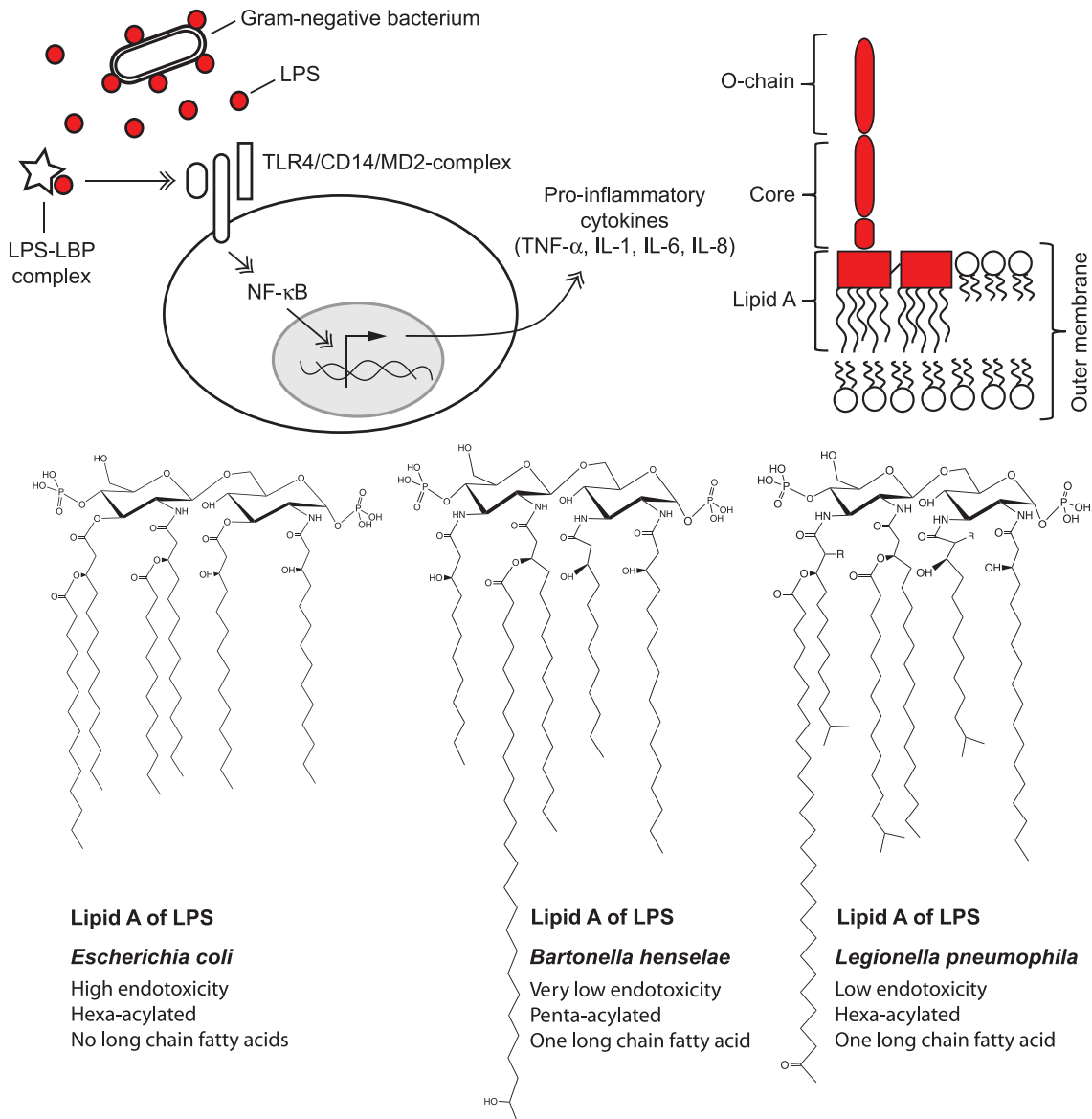


Fig. 4. *Bartonella henselae* produces a LPS of very low endotoxicity. Recognition of bacterial LPS by the innate immune system elicits strong pro-inflammatory responses. LPS-mediated activation of mammalian cells involves the interaction of LPS with LPS-binding protein (LBP) and subsequently with TLR4/CD14/MD2 complex. Proposed structure of the lipid A of *B. henselae* (LPS) and comparison to corresponding structures of *Escherichia coli* and *Legionella pneumophila* LPS. LPS of *B. henselae* has weak endotoxic activity, possibly due to the (1) penta-acylation of the GlcN3N disaccharide bisphosphate, (2) presence of an uncommon hydroxylated long-chain fatty acid linked to the lipid A, and (3) presence of a small inner core (not shown in the figure) composed of an α -(2 \rightarrow 4)-linked 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) disaccharide with one glucose residue attached. Details are described in the text [see Means to affect recognition by pattern recognition receptors (PRRs)].

experimentally proven, the lack of TLR4 activation might also be important right after the host entry to interfere with the pathogen recognition by professional phagocytes.

Some species of *Bartonella* spp. such as *Bartonella schoenbuchensis* (Dehio *et al.*, 2004), *B. bacilliformis* (Benson *et al.*, 1986; Scherer *et al.*, 1993), and *Bartonella clarridgeiae* (Kordick *et al.*, 1997; Sander *et al.*, 2000b) express flagella, polymeric rod-like structures that are important for bacterial motility. However, these structures may extend several micrometers from the bacterial outer membrane and thereby serve as ideal pathogen-associated molecular patterns for PRRs. TLR5 recognizes an evolutionarily conserved site on bacterial flagellin, the main constituent of flagella (Hayashi *et al.*, 2001; Smith *et al.*, 2003). It has been reported that the flagellated *B. bacilliformis* do not induce the typical flagellin-induced and TLR5-dependent activation of NF- κ B (Andersen-Nissen *et al.*, 2005). The flagellin of *B. bacilliformis* contains amino acid changes, which allow evasion of TLR5 recognition but at the same still preserve bacterial motility (Andersen-Nissen *et al.*, 2005). Although not yet experimentally proven, the high sequence similarity of *B. clarridgeiae* flagellin to *B. bacilliformis* flagellin (Andersen-Nissen *et al.*, 2005) indicates that also this member of *Bartonella* spp. might escape from TLR5-mediated flagellin recognition. Despite the efficient means to evade TLR4 and TLR5 recognition, it has been reported that infection of J774 mouse macrophages by *B. henselae* *in vitro* is followed by the release of high concentrations of IL-1 β and IL-6 in addition to TNF- α , a potent enhancer of macrophage bactericidal activities (Musso *et al.*, 2001). It remains to be studied how *Bartonella* spp. benefit *in vivo* from their apparently efficient means to evade from TLR4 and TLR5 recognition.

Means to affect effector functions of professional phagocytes

Macrophages. *Bartonella henselae* strain Marseille has been reported to delay its lysosomal targeting and destruction in J774A.1 mouse macrophages at least when compared in parallel with *Listeria innocua* (Kyme *et al.*, 2005). In another independent study, it was shown that *B. henselae* strain Houston-1 enters and stays viable at least for up to 8 h in J774 mouse macrophages (Musso *et al.*, 2001). Also, *B. quintana* has been reported to enter THP-1 human macrophages, although to a lesser extent than *B. henselae* (Schulte *et al.*, 2006). It appears that *B. henselae* enters the macrophage in a unique vacuolar compartment, *Bartonella*-containing vacuole (BCV), which lacks the typical early endocytic marker proteins such as transferrin receptor and early endosome antigen 1 (Kyme *et al.*, 2005). However, in later stages of infection (around

24 h), BCVs appear to fuse with lysosomes and bacteria get ultimately destroyed. The unusual trafficking and delayed lysosomal destruction of *B. henselae* is dependent on bacterial viability as heat-killed bacteria were detected in a LAMP1-positive compartment already 2 h postinfection, which typically took 24 h with viable bacteria (Kyme *et al.*, 2005). Based on this observation, the authors enriched transposon mutants that were located in a lysosome-fused compartment 2 h postinfection and report identification of four genes encoding for (1) putative virulence-associated protein VapA5, (2) putative heme-binding protein HbpD, (3) D-serine/D-alanine/glycine transport protein CycA, and (4) one protein without any known function. The detailed molecular functions of these proteins that possible regulate BCV trafficking have not been studied nor it is known whether the delayed lysosomal targeting and destruction could be beneficial for *Bartonella* spp. *in vivo*. In fact, it has been reported that *B. henselae* produces a *Bartonella* adhesin A (BadA), a trimeric autotransporter adhesin (TAA), which is a potent inhibitor of phagocytic uptake of *B. henselae* in J774A.1 mouse macrophages (Riess *et al.*, 2004).

Polymorphonuclear leukocytes. Polymorphonuclear leukocytes (PMNs) are actively recruited to the sites of microbial infection (Borregaard, 2010). *Bartonella* spp. have means to escape from professional phagocyte recognition [see Means to affect recognition by pattern recognition receptors (PRRs)], but the detected pro-inflammatory cytokine responses under *in vitro* conditions (Musso *et al.*, 2001) indicate that PMNs become a significant threat for *Bartonella* spp. right after the host entry. There exists one report, which indicates that *B. henselae* is able to inhibit the production of reactive oxygen species that is, the oxidative burst in PMNs, which is one of the most important antimicrobial effector mechanisms of PMNs (Fumarola *et al.*, 1994). This study has not been evaluated further nor was the bacterial factor(s) identified, but the inhibition of the oxidative burst potentially represents one additional survival strategy for *Bartonella* spp. in the reservoir host.

Dendritic cells. Upon exposure to microbial pathogens in peripheral tissues such as inflamed skin, DCs migrate to lymph nodes and undergo maturation into potent immunostimulatory cells, especially to evoke a clonal expansion of antibody-producing B cells (Martín-Fontecha *et al.*, 2009). In the context of an incidental host infection, it has been reported that immature human monocyte-derived DCs readily ingest *B. henselae* *in vitro* in the absence of opsonins (Vermi *et al.*, 2006). This appears as a strong stimulus for DC maturation because it induced the expression of CD83 and CCR7/CD197 and

the upregulation of HLA-DR and CD86 to levels comparable with those obtained with LPS of *S. enterica* sv Friedenau (Vermi *et al.*, 2006). Moreover, DCs that were matured by incubation with *B. henselae* were able to induce the proliferation of allogeneic T lymphocytes at levels that were comparable to those obtained with LPS (*S. enterica* sv Friedenau)-matured DCs (Vermi *et al.*, 2006). The infection also induced the production of high levels of CXCL13 (Vermi *et al.*, 2006), which is an extremely potent chemoattractant for B lymphocytes (Gunn *et al.*, 1998). In part, the data appear to explain the unusually high content of B lymphocytes in swollen lymph nodes of patients with cat scratch disease (CSD). Activation of DCs was shown to be dependent on TLR2 (Vermi *et al.*, 2006) and thereby most likely on the recognition of bacterial lipoproteins, which is in accordance with the data indicating that LPS and flagellin of *Bartonella* spp. are weak activators of TLR4 (Zähringer *et al.*, 2004) and TLR5 (Andersen-Nissen *et al.*, 2005), respectively [see Means to affect recognition by pattern recognition receptors (PRRs)]. Lipid modification of the N-terminal Cys residue (*N*-acyl-S-diacylglyceryl-Cys) is an essential, ubiquitous, and unique bacterial post-translational modification. Such a modification allows anchoring of even highly hydrophilic proteins to the membrane, which carry out a variety of functions important for bacteria, including pathogenesis (Kovacs-Simon *et al.*, 2011). TLR1, 2, and 4 are known to cooperate in the recognition of bacterial lipoproteins to elicit an antimicrobial response (Kawai & Akira, 2005). It has also been reported that *B. grahamii* induces the maturation of bone-marrow-derived conventional DCs isolated from its natural reservoir host mouse in a similar fashion to parallel-analyzed *B. henselae* (Kunz *et al.*, 2008). Bone-marrow-derived plasmacytoid DCs were also shown to get activated using interferon- α and interferon- β (IFN- α/β) secretion as the readout, but interestingly *B. grahamii* induced more IFN- α/β secretion than *B. henselae* (Kunz *et al.*, 2008). The authors substantiated this *in vitro* finding by analyzing the development of *Bartonella*-induced lymphadenopathy in type I interferon receptor (IFNAR1)-deficient mice. With wild-type background, it had already been observed that intradermal inoculation of *B. grahamii* induced only a mild and short-lived lymphadenopathy (as quantified by weighting of the lymph nodes) as compared to prolonged and enhanced swelling of lymph nodes with *B. henselae* inoculation (Vermi *et al.*, 2006). As expected, IFNAR1-deficient mice developed significantly larger lymph nodes after *B. grahamii* infection than the respective wild-type controls. Similar trend was observed with *B. henselae* infection, although the difference was not significant between the wild-type and IFNAR1-deficient mice (Kunz *et al.*, 2008). These findings are compatible with a model

where high IFN- α/β production induced by *B. grahamii* in its natural reservoir host mouse inhibits the development of lymphadenopathy, possibly via inhibition of the proliferation of B lymphocytes, which is typically pronounced in the swollen lymph nodes of patients with CSD (Vermi *et al.*, 2006). It can be speculated that in the context of a reservoir host infection, *B. grahamii* and possibly other species of *Bartonella* spp., selectively overactivate one cytokine response of DCs (Monroe *et al.*, 2010). The final outcome is the inhibition of B-cell response and thereby antibody response and overall attenuation of host immune surveillance, which in the end allow the establishment of a chronic intraerythrocytic bacteremia.

Means to affect complement activation

The complement system plays a major role in resistance against microbial infections both in the circulation and in the peripheral tissues either directly or via professional phagocytes. It can be activated (1) by antigen-antibody complexes (classical pathway), (2) by certain carbohydrates (lectin pathway), or (3) by a variety of pathogen surface structures (alternative pathway) (Rautemaa & Meri, 1999). Resistance to host complement appears to be an important pathogenic mechanism in bacteria that cause acute and fast progressing infections such as *Streptococcus pyogenes* (Akeson *et al.*, 1996) and also in bacteria that cause chronic and persistent infections such as *Borrelia burgdorferi* (Hellwage *et al.*, 2001). It has been reported that *B. quintana* is resistant to direct complement-mediated killing in a system where high-titered anti-*B. quintana* rabbit serum and nonimmune serum were studied with guinea pig complement (Myers & Wiseman, 1978). However, in another study, it was reported that *B. quintana* outer membrane extract induced the activation of rabbit serum complement (Hollingdale *et al.*, 1980). In this study (Hollingdale *et al.*, 1980), the authors did not address bacterial mechanisms to inhibit complement activation. One study has focused on complement resistance mechanisms of *B. henselae* (Rodriguez-Barradas *et al.*, 1995). The authors reported a concentration- and time-dependent bactericidal effect of nonimmune human serum to *B. henselae* Houston-1. In their study, 20% nonimmune human serum was sufficient to kill more than 99% of the organisms after 30-min incubation. The alternative pathway of the complement system appeared to be mainly involved, although the authors also detected the activation of classical pathway despite the apparent lack of opsonizing anti-*B. henselae* antibodies (Rodriguez-Barradas *et al.*, 1995). The importance and even the presence of complement resistance mechanisms in *Bartonella* spp. remain elusive, primarily by the lack of significant primary studies. It has been

argued that these studies should be conducted in the context of natural reservoir host infections (Vayssier-Taussat *et al.*, 2009) and potentially as one molecular mechanism of host specificity. This rationale arises from the observation that *B. burgdorferi* sensu lato differs in its sensitivity to complement from different vertebrate species (Kurtenbach *et al.*, 1998).

Penetration of tissues

Interactions with the extracellular matrix

In principle, tissue penetration from the primary site of inoculation could be achieved with analogy to secreted or surface-associated proteases such as SpeB of *S. pyogenes*, which directly degrades extracellular matrix components and also activates host matrix metalloproteinases (Rasmussen & Björck, 2002). However, there are no reports available that describe well-defined secreted or surface-associated proteases in *Bartonella* spp. Binding of extracellular matrix components *per se* appears to be an important feature of *Bartonella* spp. In some studies, it has been discussed in the context of open wound infections as a potent mechanism to circumvent cleansing (Dabo *et al.*, 2006a, b), although the major interest on extracellular matrix–*Bartonella* spp. interaction has focused on endothelial cell adhesion in later blood stages of the infection (Riess *et al.*, 2004; Müller *et al.*, 2011).

BadA of *B. henselae* has been reported to bind vitronectin, laminin, hyaluronic acid, fibronectin (both cellular and plasma forms), and collagens I, II, and IV (Riess *et al.*, 2004; Müller *et al.*, 2011). The expression of BadA varies in primary isolates of *B. henselae* and is sometimes absent indicating phase variation (Riess *et al.*, 2007). *Bartonella henselae* has also been reported to bind collagens IX and X (Dabo *et al.*, 2006a, b), and outer membrane protein (OMP) Pap31 appears to mediate BadA-independent binding to fibronectin (Dabo *et al.*, 2006a, b). *Bartonella quintana* variably express four different but highly conserved adhesins, VompA–D, where VompA and C were initially identified to bind collagen IV (Zhang *et al.*, 2004). The recent data indicate that the ligand repertoire of Vomps might also contain vitronectin, laminin, hyaluronic acid, fibronectin (both cellular and plasma forms), and collagens I and II (Müller *et al.*, 2011). In the context of *Bartonella* spp.–reservoir host interaction, it is significant that intradermally inoculated Vomp-null mutant was unable to establish bacteremia in a rhesus macaque animal model of *B. quintana* infection (Zhang *et al.*, 2004; MacKichan *et al.*, 2008). *Bartonella vinsonii* ssp. *arupensis* also appears to express a large OMP, BrpA, with similarity to Vomps and BadA (Gilmore *et al.*, 2005), but the molecular function of this

protein has remained elusive. *Bartonella bacilliformis* appears to also contain three variable but still functionally uncharacterized *brp* genes (Kaiser *et al.*, 2011). In conclusion, it appears that binding to ECM is a conserved molecular feature in *Bartonella* spp. and it may play an important role also in the early stages of infection. However, based on the current knowledge, it seems unlikely that the tissue penetration of *Bartonella* spp. would rely on ECM binding and subsequent degradation similar to, for example, *S. pyogenes* tissue invasion (Rasmussen & Björck, 2002).

Interactions with fibroblasts and epithelial cells

Skin is rich of dermal fibroblasts. They produce and organize the extracellular matrix (~ 70% of the dermis is collagen), actively communicate with each other and other cell types such as epithelial cells of the epidermis and cells of the hematopoietic origin of dermis/epidermis, and therefore play a crucial role in the regulation of skin physiology (Sorrell & Caplan, 2004). Early work on *B. bacilliformis* indicated that the bacterium is able to attach to and invade both human fibroblasts and epithelial cells (Hill *et al.*, 1992). The process appeared to require actin remodeling because cytochalasin D, a cell-permeable inhibitor of actin polymerization, inhibited the invasion (Hill *et al.*, 1992). Moreover, it was shown that genistein, a tyrosine kinase inhibitor, decreased the invasion of the epithelial cells by *B. bacilliformis* (Williams-Bouyer & Hill, 1999), which indicate that the cells were actively engaged in the invasion process. Interestingly, exposure of epithelial cell monolayers to anti- $\alpha 5$ and anti- $\beta 1$ integrin monoclonal antibodies decreased the invasion of the cells by *B. bacilliformis*, suggesting a possible role of $\alpha 5 \beta 1$ -integrin (fibronectin receptor) in the bacterial uptake (Williams-Bouyer & Hill, 1999). Integrins have also been implicated in the adhesion of *B. henselae* to fibroblast-like mouse embryonic stem cell line GD25 (Riess *et al.*, 2004). The bacteria adhered more strongly to GD25-derivative (GD25- $\beta 1 A$) cells that express $\beta 1$ -integrins than to the parental $\beta 1$ -integrin-deficient GD25 cells. The binding was inhibited by anti-fibronectin antibodies, which indicates that fibronectin is somehow involved in the process (Riess *et al.*, 2004). Integrins also appear important mediators of *B. henselae*–EC interaction (see Endothelial cell adhesion and subsequent invasion, *B. henselae*). *Bartonella henselae* can also invade epithelial cells (Batterman *et al.*, 1995; Zbinden *et al.*, 1995; Schulte *et al.*, 2006; Truttmann *et al.*, 2011a), and this process may lead into uptake of a large bacterial aggregate (Truttmann *et al.*, 2011a), which resembles the invasome-mediated uptake of *B. henselae* into human ECs (Dehio *et al.*, 1997). In conclusion, it appears that *B. bacilliformis* and *B. henselae* readily

invade fibroblasts and epithelial cells *in vitro*. There are no reports available that link this ability to *Bartonella* spp. pathophysiology *in vivo*. However, it appears impossible to invade and penetrate skin without encountering fibroblasts and epithelial cells. Perhaps these cells provide a transient and immunologically privileged niche for *Bartonella* spp. right after the host entry at the skin. Otherwise, *Bartonella* spp. should be able to subvert its potent capacity to invade fibroblasts and epithelial cells (Batterman *et al.*, 1995; Zbinden *et al.*, 1995; Schulte *et al.*, 2006; Truttmann *et al.*, 2011a), which could in principle be achieved by phase variation of surface adhesin(s) like in the case of BadA-mediated adhesion of *B. henselae* to human ECs (Riess *et al.*, 2007).

Invasion into the bloodstream

In previous chapters, we have outlined molecular mechanisms how *Bartonella* spp. may evade host innate immunity right after host entry. How *Bartonella* spp. are able to gain access to the bloodstream once intra-/subcutaneously inoculated and what is the identity of the primary niche? We assume that in nature the direct blood infection via arthropod bite is not a very realistic scenario and if so, the bacteria appear unable to infect erythrocytes based on the rat-*B. tribocorum* (Schülein *et al.*, 2001) and mouse-*B. grahamii* (Koesling *et al.*, 2001) natural reservoir host models. As an example, after intravenous inoculation of 3.5×10^7 CFU bacteria, *B. tribocorum* was rapidly cleared from the circulation, and only after several days, the bacteria reappeared in the blood from the still-enigmatic primary niche and invaded erythrocytes (Schülein *et al.*, 2001).

Identity of the primary niche

Several observations in natural reservoir hosts indicate that *Bartonella* spp. reside in a cellular or acellular compartment, persist, and are periodically seeded into the circulation (Fig. 2). As an example, one naturally *B. henselae*-infected cat had $> 10\,000$ CFU of *B. henselae* per milliliter of blood at day 0 of a longitudinal 2-year study of infection under laboratory conditions (Abbott *et al.*, 1997). The bacteremia gradually declined and the cat became culture negative 5 months later. However, bacteremia was again detected 2 months later. For the rest of the longitudinal infection study, the cat became cyclically culture negative at 2-month intervals with the abacteremic period lasting, on average, for 2 months. Experimental transmission of *B. henselae* to specific pathogen-free cats via fleas removed from bacteremic cattery cats largely reproduced the above data (Chomel *et al.*, 1996). It took on average 2 weeks until the bacteremia was detected, and when bacteria appeared in the blood, the numbers were immediately high (10^4 – 10^6 CFU mL⁻¹).

One kitten became culture negative after approximately 4 months of bacteremia, but had three relapses of bacteremia during the rest of the 1-year observation period (Chomel *et al.*, 1996). Similar relapsing bacteremia has been observed in the rhesus macaque-*B. quintana* model where bacteria were inoculated intradermally to mimic the natural infection route and it took 12 days until the bacteremia peaked (Zhang *et al.*, 2004). Indeed, the infection route appears to be of central importance to make physiologically significant conclusions. In the mouse-*B. birtlesii* model (Marignac *et al.*, 2010), bacteremia was detected as early as day 3 postinoculation when Balb/c mice were injected intradermally compared with day 7 postinoculation with the subcutaneous route. The peak of bacteremia for the intradermal group was reached at day 13 postinoculation and was roughly 10 times lower than the bacteremia peak for the subcutaneous group (day 23 postinoculation). The most significant finding of this study was, however, that the intradermally inoculated group entered the relapsing bacteremia phase and subcutaneously inoculated group did not, at least in the time frame of the reported experiment (Marignac *et al.*, 2010).

Hypothesis of the endothelial cells of the vascular wall

The most widely appreciated primary niche hypothesis is the vascular wall, in more particular an intravacuolar compartment in an EC (Dehio, 2005). This is primarily based on the disease pathologies of *B. quintana* and *B. bacilliformis* infections in their natural reservoir host human, in addition to the wealth of information available for *B. henselae*–human EC interaction. *Bartonella bacilliformis* is a deadly pathogen that causes a biphasic Carrion's disease in endemic areas of the Andes. The acute phase, called Oroya fever, is characterized by an intra-erythrocytic bacteremia that results in an often-fatal hemolytic anemia. The subsequent chronic phase, known as verruga peruana, manifests in vascular tumors that result from the proliferation of ECs where *B. bacilliformis* may form large cytoplasmic inclusions (Maguiña *et al.*, 2009). A similar tropism for human erythrocytes is observed for *B. quintana* (Rolain *et al.*, 2002; Rolain *et al.*, 2003c). However, the bacteria do not appear hemolytic, and the development of vascular deformations/tumors (BA) requires that the human host is immunocompromised as an example suffering from AIDS (Maguiña *et al.*, 2009). EC invasion by *B. bacilliformis* has been reproduced *in vitro* by several investigators (Garcia *et al.*, 1990, 1992; Hill *et al.*, 1992; Verma *et al.*, 2000, 2001; Verma & Ihler, 2002; Cerimele *et al.*, 2003). EC invasion by *B. quintana* appears more elusive *in vitro*, although the bacterium has been reported to adhere to and invade human ECs *in vitro* (Brouqui & Raoult, 1996;

Palmari *et al.*, 1996; Müller *et al.*, 2011) and potentially affect significant cellular processes such as apoptosis (Schmid *et al.*, 2006) and proliferation (Palmari *et al.*, 1996). Apart from *B. quintana* and *B. bacilliformis*, *in vitro* studies of *Bartonella* spp. interaction with their reservoir host ECs have largely been neglected nor has such interaction ever been described *in vivo*. Only significant exception is one recent *in vitro* study where effects of *B. henselae* on human and feline macro- and microvascular ECs were compared (Berrich *et al.*, 2011). The biological readouts with skin microvascular ECs clearly demonstrate that *B. henselae* increases the cell migration of human but not feline ECs in wound healing assays and that *B. henselae* strongly induces vascular endothelial growth factor (VEGF) secretion by human but not feline ECs (Berrich *et al.*, 2011). These results may explain the reduced pathogenic potential of *B. henselae* on cats as compared to humans. However, in the absence of direct cell adhesion and invasion data, the results appear inconclusive to answer the burning question if ECs truly constitute the primary niche of *B. henselae* in the cat. Further *in vitro* and *in vivo* studies in different mammals such as mouse or a rat with their respective *Bartonella* spp. colonizers are urgently needed. Conceptually, the EC primary niche hypothesis appears logical in the intravenous reservoir host models (Koesling *et al.*, 2001; Schülein *et al.*, 2001). However, can it be applied to the more natural intradermal infection routes? Onset of the intraerythrocytic bacteremia after intradermal inoculation is preceded by a significant lag (days–weeks) of abacteremia (Chomel *et al.*, 1996; Abbott *et al.*, 1997; Zhang *et al.*, 2004; Marignac *et al.*, 2010), which indicates that the bacteria should enter the ECs from the apical surface, replicate, persist, and be synchronously seeded into the bloodstream.

Hypothesis of the circulatory hematopoietic stem cells

ECs have a low proliferative potential, and therefore, vascular repair requires additional support. Vascular repair and neoangiogenesis are mediated, at least in part, by hematopoietic stem cells (HSCs) (Urbich & Dimmeler, 2004). It has been reported that *B. henselae* strain ATCC49882 adheres to and invades human HSCs, that is, CD133-positive cells that were enriched from peripheral blood mononuclear cells (Salvatore *et al.*, 2008). CD133 is expressed on HSCs but is absent on mature ECs (Urbich & Dimmeler, 2004). This is a significant finding because circulating HSCs appear inherently resistant to invasion by a variety of microbial pathogens (Kolb-Mäurer *et al.*, 2002). It was proposed that HSCs could carry *B. henselae* to peripheral tissues, in particular, to endothelium of microcirculation where vasoproliferative

disorders initiate (Salvatore *et al.*, 2008). Although this might be the cellular basis of *B. henselae*-induced BA/peliosis in the incidental host human, it has also been proposed that HSCs might constitute the primary niche of *Bartonella* spp. in their respective reservoir hosts (Mändle *et al.*, 2005). This argument was based on an observation that intracellular *B. henselae* were detected in human erythroid cells that were induced from HSCs, that is, CD34-positive cells that were enriched from peripheral blood (Mändle *et al.*, 2005), by the addition of interleukin-3 (IL-3), granulocyte-macrophage-colony-stimulating factor (GM-CSF), and erythropoietin (epo) (Mändle *et al.*, 2005). However, as *B. henselae* infection did not affect the differentiation of human HSCs into erythroid cells as judged by CD34 and glycophorin A cell surface markers, the results appear inconclusive given also the fact that *B. henselae* readily invade HSCs (Mändle *et al.*, 2005). It is also well documented, as an example in the intravenous *B. tribocorum*-rat reservoir host infection model, that after injection the bacteria are rapidly cleared from circulating blood within hours and that no bacteria can be detected in the blood for about 4 days until the bacteremia peaks (Schülein *et al.*, 2001). Also, the lag of several days post-inoculation until the bacteria are sharply detected in the blood with high numbers in the intradermal reservoir host models (Chomel *et al.*, 1996; Abbott *et al.*, 1997; Zhang *et al.*, 2004; Marignac *et al.*, 2010) does not support a circulatory nature of the primary niche.

Proposal of DCs and the draining lymph nodes

Upon exposure to microbial pathogens in peripheral tissues such as the inflamed skin, DCs migrate to lymph nodes and undergo maturation into potent immunostimulatory cells, especially to evoke a clonal expansion of antibody-producing B cells (Martín-Fontecha *et al.*, 2009). We have already described the current knowledge of *Bartonella* spp.–DC interaction in ‘Means to affect effector functions of professional phagocytes’. In short, *Bartonella* spp. or at least *B. grahamii* in its natural reservoir host mouse is not able to circumvent DC recognition and activation, which appears of central importance for several pathogenic bacteria such as *Brucella* spp. to cause chronic infections (Billard *et al.*, 2007). Actually, *B. grahamii* seems to overactivate cytokine responses of DCs, especially IFN- α/β secretion (Billard *et al.*, 2007). *Bartonella grahamii* infection causes a more severe regional lymphadenopathy in IFNAR1 (IFN- α/β receptor)-deficient mice compared with the wild-type mice (Kunz *et al.*, 2008). This indicates that IFN- α/β overproduction is limiting the B-cell recruitment and/or B-cell proliferation in the lymph nodes, the main cause of lymph node swelling/lymphadenopathy. The final outcome is the

inhibition of the antibody response and overall attenuation of host immune surveillance, which in the end allows the establishment of the chronic intraerythrocytic bacteremia. We would like to propose here that the draining lymph nodes are crucial anatomical sites first of all for the suppression of B-cell responses and thereby antibody production and secondly act as the anatomical site of the primary niche in reservoir hosts. The latter argument assumes that *Bartonella* spp. enter the draining lymph node within DCs that are migrating from the site of intradermal inoculation, although *Bartonella* spp. could also hypothetically gain access to the lumen of lymphatic vessels and eventually lymph nodes as a single bacterium and/or bacterial auto-aggregates, which are known to be formed by BadA of *B. henselae* (Kaiser *et al.*, 2008) and Vomps of *B. quintana* (Zhang *et al.*, 2004). Viability of the readily internalized *Bartonella* spp. in DCs (Vermi *et al.*, 2006) has not been reported, but it could be significant based on the intracellular survival studies in macrophages, which inherently have more potent bactericidal effects than DCs. Our proposal, like any other current hypothesis, cannot yet answer the peculiar periodicity of the bacterial appearance into the blood. Could it be envisioned that the bacterial replication to a certain density inside the lymph node-homed DCs would cause synchronous lysis of the infected DCs? One potential cytolytic bacterial factor is CAMP-like factor autotransporter Cfa, which was identified in *B. henselae* to induce hemolysis together with sphingomyelinase (Litwin & Johnson, 2005). The activity of this protein toward nucleated mammalian cells has not been reported, and it could also be functional in the arthropod host. Strong cytolytic activity of *B. henselae* has been identified toward tick *Amblyomma americanum* cell line (Billeter *et al.*, 2009). There also appears to be a contact-dependent hemolytic activity in *B. bacilliformis*, which is protease sensitive, suggesting that it corresponds to a surface-exposed protein (Hendrix, 2000). It remains to be studied whether this hemolysin is only present in *B. bacilliformis* and how important this potential hemolytic activity actually is in the presence of the extremely potent hemophagocytic activity of monocyte/macrophages (Silva-Herzog & Detweiler, 2008). The lymph node primary niche hypothesis could also explain the rapid clearance of bacteria from the circulating blood within hours postinoculation, with bacteremia peaking only after few days in the reservoir host intravenous infection models (Koesling *et al.*, 2001; Schülein *et al.*, 2001). It is difficult to envision that bacteria could be migrating from the blood-filtering lymph nodes into a more peripheral tissue localization of the primary niche and then come back few days later. In part, the lymph node primary niche hypothesis is supported by the CSD in humans that is,

B. henselae infection in the incidental human host. Usually, 2–3 weeks after a bite or a scratch of an infected cat, unusual lymphadenopathy of the lymph node(s) draining the area of the scratch or the bite develops, may suppurate and last for weeks (Klotz *et al.*, 2011).

Bacterial factors involved in the colonization of the primary niche

Several bacterial pathogens use type IV secretion systems (T4SSs) to translocate bacterial effector molecules (proteins or DNA) into the target host cells. These versatile transporters have evolved from bacterial conjugation systems (Seubert *et al.*, 2003; Christie *et al.*, 2005). The prototypic T4SS is the VirB/VirD4 apparatus of *Agrobacterium tumefaciens*, which mediates the transfer of the tumorigenic T-DNA complex into the 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 multisubunit protein toxins to the extracellular medium (for example, pertussis toxin secreted by *Bordetella pertussis*) (Christie *et al.*, 2005), although they may still be capable of translocating DNA (Schröder *et al.*, 2011). *Bartonella* spp. encode three distinct T4SSs, VirB/VirD4, Vbh, and Trw, which appear as key pathogenicity factors in mediating *Bartonella* spp.–host cell interactions (Schulein & Dehio, 2002; Seubert *et al.*, 2003; Schmid *et al.*, 2004; Vayssier-Taussat *et al.*, 2010; Engel *et al.*, 2011). The Trw–T4SS will be discussed in detail in ‘Adhesion to the erythrocytes’ and ‘Antigenic and phase variation of surface proteins’ in the context of erythrocyte adhesion and antigenic variation, respectively. The VirB/VirD4 T4SS has mainly been studied in *B. henselae* and *B. tribocorum*, but it appears to be well conserved also in other members of *Bartonella* spp., although it is absent in *B. bacilliformis* (Sweger *et al.*, 2000; Alsmark *et al.*, 2004; Saenz *et al.*, 2007; Engel & Dehio, 2009; Engel *et al.*, 2011). *Bartonella* spp. species of the clade 2 (Fig. 1), which apparently lack the VirB/VirD4–T4SS, contain a distinct T4SS that is homologous to VirB/VirD4–T4SS and is therefore designated as the *virB*-homolog–T4SS (Vbh–T4SS) (Saenz *et al.*, 2007). The Vbh–T4SS is absent in *B. bacilliformis* and several species of the clade 4 (Saenz *et al.*, 2007). Cellular functions of the Vbh–T4SS have not yet been studied in great detail (Engel *et al.*, 2011).

The VirB/VirD4–T4SS of *B. henselae* is encoded by an operon composed of 10 genes (*virB2*–*virB11*) and a downstream-located *virD4* gene (Schulein & Dehio, 2002; Schulein *et al.*, 2005) (Fig. 5). Its closest bacterial relative is a genuine conjugation system, the AvhB/TraG system of the pATC58 of *A. tumefaciens* (Schulein & Dehio,

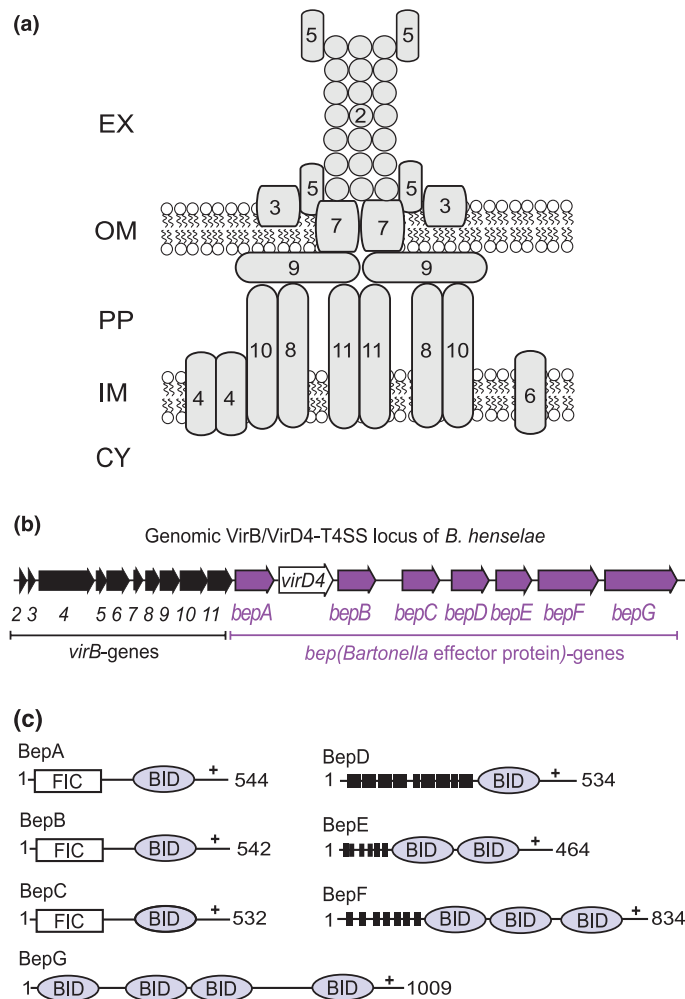


Fig. 5. Molecular characteristics of the *VirB/VirD4-T4SS* of *Bartonella henselae*. (a) A hypothetical model of the topology of *B. henselae* *VirB/VirD4-T4SS* based on studies on the archaetypical *VirB/VirD4 T-DNA* transfer system of plant pathogen *Agrobacterium tumefaciens* and yeast two-hybrid screen of protein–protein interactions between *VirB* proteins of *B. henselae* (Shamaei-Tousi *et al.*, 2004; Christie *et al.*, 2005). *VirB6* of *B. henselae* has not yet been shown to interact with other *VirB* proteins. The coupling protein *VirD4* is believed to recognize and target the effectors for translocation via the core *T4SS* machinery composed of *VirB2–VirB11*. CY, bacterial cytosol; IM, bacterial inner membrane; PP, bacterial periplasm; OM, bacterial outer membrane; EX, extracellular space. (b) Genetic organization of the genomic *VirB/VirD4-T4SS* locus. (c) Modular domain organization of Beps. The filamentous induced by cAMP (FIC) domain is characterized by a short amino acid motif (HPFXXGNG) of the catalytic center, which is highly conserved in *Fic* family proteins found in all domains of life and in viruses. Members of the *Fic* family of adenylyltransferases catalyze covalent addition of AMP moieties to target proteins, either at threonine or at tyrosine residues. The enzymatic activity was recently identified as the molecular basis how some pathogenic bacteria cause cytotoxicity through the modification of the switch I regions of Rho GTPases (Worby *et al.*, 2009; Yarbrough *et al.*, 2009; Palanivelu *et al.*, 2011), the key regulators of cellular actin dynamics. The cellular functions of *Bartonella* spp. *Fic* proteins remain elusive. The BID domain and the adjacent positively charged C-tail are necessary for any given Bep to be translocated via the *VirB/VirD4-T4SS* (Schulein *et al.*, 2005). Some of the Beps carry in their N-terminus short peptide motifs marked in black bars that resemble eukaryotic tyrosine phosphorylation motifs (e.g. EPLYA) (Selbach *et al.*, 2009).

2002). A yeast two-hybrid interaction study of the components of the *B. henselae* *VirB/VirD4-T4SS* has largely confirmed the protein interactions that were previously identified in other *T4SS*s (Shamaei-Tousi *et al.*, 2004; Christie *et al.*, 2005) (Fig. 5). In analogy to the model developed for the topology and function of these related *T4SS*s (Christie *et al.*, 2005), the *B. henselae* *VirB/VirD4-*

T4SS is considered to encompass a *VirB2* pilin and *VirB5* minor pilus components, which together form a pilus that apparently can mediate contact with the host cell; core components *VirB3*, *VirB4*, and *VirB6–VirB11*, which form a pore complex that spans both Gram-negative membranes; and the *T4SS* coupling protein *VirD4*, an inner membrane protein believed to function as an

interface between the pore complex and the T4SS protein substrates (Fig. 5).

The proposed tip adhesin VirB5 (Fig. 5) is expressed *in vivo* as an immunodominant protein of *B. henselae*, as shown both after experimental infection of mice with *B. henselae* (Padmalayam *et al.*, 2000) and by analyzing the sera of patients with CSD (Anderson *et al.*, 1995). The homologous T4SS in *B. tribocorum* is indispensable for the establishment of the intraerythrocytic bacteremia in an intravenous natural rat reservoir host model as evidenced by comparative analysis of parental and VirD4- or VirB4-deficient mutant bacteria (Schulein & Dehio, 2002). Interestingly, a high fraction (> 80%) of VirB4-deficient mutant bacteria, which had been genetically complemented by the introduction of a *virB4* copy in a replicative plasmid, were recovered from the blood without the complementation plasmid (Schulein & Dehio, 2002). The authors conclude that this finding indicates the functional importance of VirB/VirD4-T4SS for the initial abacteremic period of approximately 1 week during which the bacteria reside, replicate, and persist in the primary niche (Schülein *et al.*, 2001; Schulein & Dehio, 2002). Indeed, it has recently been reported that VirD4-deficient mutant of *B. birtlesii* is abacteremic in an intradermal natural mouse reservoir host model, although the bacteria display equal erythrocyte adherence and invasion rates *in vitro* as compared to the parental strain (Vayssier-Taussat *et al.*, 2010). At the moment, it remains unknown which of the VirB/VirD4-T4SS *Bartonella* spp. effector proteins, Beps (Fig. 5), are involved and how they are involved in the invasion and colonization of the primary niche and/or seeding of the bacteria from the primary niche into the bloodstream. However, BepD-deficient mutant has been identified in a signature-tagged mutagenesis (STM) screen as abacteremic (Saenz *et al.*, 2007). This effector becomes tyrosine-phosphorylated by the host cell-derived tyrosine kinases (Schulein *et al.*, 2005) and therefore may interfere with the host cell signaling processes. Tyrosine phosphorylation is a conserved feature in several effectors translocated by bacterial pathogens, as an example in the T4SS-effector CagA of *H. pylori* (Odenbreit *et al.*, 2000). Phosphorylation of CagA by Src family and Abl kinases recruits SH2-domain containing proteins such as tyrosine phosphatase Shp-2, tyrosine kinase Csk, and adaptor protein Crk, all having a defined role in the *H. pylori*-induced cytoskeletal rearrangements (Odenbreit *et al.*, 2000; Backert & Selbach, 2005). Recently, a nonbiased quantitative proteomics approach was utilized to identify cellular target proteins that bind to the tyrosine-phosphorylated peptides of BepD, BepE, and BepF (Selbach *et al.*, 2009). Several proteins such as Grb2, Grb7, Csk, Crk, Shp1, and Shp2 were identified; however, the biology behind these interactions remains to be studied.

BadA mediates bacterial binding to endothelial and epithelial cells and extracellular matrix components *in vitro* (Riess *et al.*, 2004, 2007; Kaiser *et al.*, 2008; Müller *et al.*, 2011). BadA-deficient mutants of *B. tribocorum* and *B. birtlesii* have been identified as abacteremic in STM screens (Saenz *et al.*, 2007; Vayssier-Taussat *et al.*, 2010). Strikingly, the BadA-deficient mutants isolated in the *B. birtlesii* STM screen displayed equal erythrocyte adherence and invasion rates *in vitro* as compared to the parental strain (Vayssier-Taussat *et al.*, 2010). This clearly indicates that in analogy to VirB/VirD4-T4SS, BadA is also required for the invasion and colonization of the primary niche and/or seeding of the bacteria from the primary niche into the bloodstream. Perhaps this also applies to Vombs of *B. quintana*, because Vomp-deficient mutant is abacteremic in an intradermal macaque model (MacKichan *et al.*, 2008). Several other bacterial factors with a possible role in the primary niche colonization, such as inducible *Bartonella* autotransporter (*iba*) and several ABC transporters, have been identified in the STM screens (Saenz *et al.*, 2007; Vayssier-Taussat *et al.*, 2010). However, their detailed molecular functions remain to be studied.

Adhesion to the erythrocytes

The hallmark of chronic *Bartonella* spp. infection in their reservoir hosts, but not in the incidental hosts, is a long-lasting intraerythrocytic bacteremia. This also applies to the human-specific *B. quintana* (Rolain *et al.*, 2002; Rolain *et al.*, 2003c), a louse-transmitted bacterium that was initially detected during World War I as the causative agent of trench fever (5-day fever), and *B. bacilliformis* (Walker & Winkler, 1981; Benson *et al.*, 1986), a sand fly-transmitted bacterium (Maguiña *et al.*, 2009). *Bartonella bacilliformis* is so far the only known species of *Bartonella* spp. that cause deleterious effects for the infected erythrocytes (Maguiña *et al.*, 2009).

Bacterial adhesion is the first step in the erythrocyte invasion process. *Bartonella bacilliformis* is highly motile owing to the expression of multiple unipolar flagella (Benson *et al.*, 1986; Scherer *et al.*, 1993), and it has been reported that antibodies raised against the flagellin subunit partially inhibit erythrocyte binding and almost completely abolish invasion (Scherer *et al.*, 1993). However, the direct role of flagella in erythrocyte adhesion *per se* remains elusive by the lack of knowledge of the erythrocyte ligand and genetic proof, that is, parallel analysis of wild-type and isogenic flagella-deficient mutant, although this mutant exists (Battisti & Minnick, 1999). The flagella-mediated motility could simply enhance bacteria-erythrocyte collisions, and other surface protein(s) might mediate the actual adhesion to the erythrocytes. This is in part supported by the fact that most *Bartonella* spp. are

nonflagellated but still capable of invading the erythrocytes. In a whole bacterium level, *B. bacilliformis* appears to interact with multiple surface-exposed membrane proteins of human erythrocytes, such as glycophorins A and B (Buckles & McGinnis Hill, 2000). In this study, the authors did not address the bacterial adhesin(s), but they observed complete abrogation of binding to some of the identified proteins following the exposure of erythrocytes to sodium metaperiodate oxidation (Buckles & McGinnis Hill, 2000). This indicates that carbohydrate moieties are involved in the interactions between *B. bacilliformis* and the human erythrocyte.

Recently, *in vitro* adhesion and invasion assays with isolated erythrocytes that reproduce the host specificity of erythrocyte infection as observed *in vivo* have been reported for mouse-specific *B. birtlesii*, human-specific *B. quintana*, cat-specific *B. henselae*, and rat-specific *B. tribocorum* (Vayssier-Taussat *et al.*, 2010). Moreover, STM in the intravenous *B. birtlesii*-mouse infection model allowed the identification of mutants that were impaired in their ability to establish intraerythrocytic bacteremia. Among 45 abacteremic mutants, five failed to adhere to and invade mouse erythrocytes *in vitro*. The corresponding genes encode components of the type IV secretion system (T4SS) Trw (Seubert *et al.*, 2003), demonstrating that this virulence factor is involved in the adherence of *B. birtlesii* to the erythrocytes. Moreover, ectopic expression of the Trw–T4SS of rat-specific *B. tribocorum* in cat-specific *B. henselae* or human-specific *B. quintana* expanded their host range for erythrocyte infection to rat, demonstrating that Trw mediates host-specific erythrocyte infection (Vayssier-Taussat *et al.*, 2010). Trw–T4SS had initially been identified in a differential fluorescence induction (DFI) screen as a *B. henselae* promoter that is significantly upregulated during the infection of ECs (Seubert *et al.*, 2003). In the *B. tribocorum*-rat model, mutant deleted for the *virB10*-like *trwE* gene was deficient in establishing the long-lasting intraerythrocytic bacteremia (Seubert *et al.*, 2003). Trw–T4SS and particularly its proposed pilus proteins TrwL and TrwJ could be significant adhesins for *Bartonella* spp. toward erythrocytes, because most of the species of *Bartonella* spp. have a genomic copy of Trw–T4SS (Engel & Dehio, 2009). Interestingly, *B. bacilliformis* and other flagellated bacteria appear to lack the Trw–T4SS, which might indicate functional redundancy of Trw–T4SS and flagella in erythrocyte adhesion and/or invasion (Dehio, 2008).

Invasion of the erythrocytes

The process of erythrocyte invasion by *Bartonella* spp. appears to be fundamentally different from invasion of

nucleated cells, as mature erythrocytes do not contain an active cytoskeleton that could be subverted by the bacterium to assist in its uptake. Consequently, bacteria have to enter erythrocytes by a process referred as forced endocytosis (Benson *et al.*, 1986) with most detailed knowledge available for *B. bacilliformis*. One striking feature of *B. bacilliformis* interaction with human erythrocytes is the production of deeply invaginated pits and trenches in the erythrocyte membrane (Benson *et al.*, 1986), which are considered to be the entry portals for invading bacteria. This phenomenon appears to be triggered by a secreted bacterial factor termed deforming factor or deformin (Mernaugh & Ihler, 1992; Derrick & Ihler, 2001). Originally reported to be a protein (Mernaugh & Ihler, 1992), later work by the same group has indicated that deformin is a small hydrophobic molecule with high affinity for albumin (Derrick & Ihler, 2001). Deformin can be extracted from albumin as a heat- and protease-resistant, water-soluble molecule with a molecular weight of ~ 1.4 kDa (Derrick & Ihler, 2001). It has been reported that deformation activity, which is similar to *B. bacilliformis* deformin, is also present in *B. henselae* (Iwaki-Egawa & Ihler, 1997).

The invasion-associated locus (*ial*), composed of *ialA* and *ialB*, is another putative virulence determinant implicated in the erythrocyte invasion by *B. bacilliformis*. The locus was identified in a heterologous expression system where the *ial* locus but not the individual *ialA* and *ialB* genes conferred an invasive phenotype to *E. coli* toward human erythrocytes (Mitchell & Minnick, 1995). The *ialA* gene encodes a 21-kDa protein, which has been reported to have a nucleoside polyphosphate hydrolase (MutT motif family) activity (Cartwright *et al.*, 1999; Conyers & Bessman, 1999). MutT motif family enzymes are believed to eliminate toxic nucleotide derivatives and to regulate the levels of important signaling nucleotides and their metabolites (Mildvan *et al.*, 2005). It remains to be studied if and how this enzymatic activity of IalA relates to the process of erythrocyte invasion. The *ialB* gene encodes an 18-kDa protein, which is well conserved (~ 60% amino acid similarity) with the 17-kDa Ail (adhesion and invasion locus) protein of *Yersinia enterocolitica*, which mediate epithelial cell binding and invasion (Leo & Skurnik, 2011). Indeed, the association of isogenic *IalB*-deficient mutant of *B. bacilliformis* with human erythrocytes is significantly reduced although not completely absent (Coleman & Minnick, 2001). This indicates that other bacterial factors are involved, possibly flagella (Benson *et al.*, 1986; Scherer *et al.*, 1993). The regulation of *IalB* expression by environmental cues has also been studied, and most strikingly, the low pH appeared as a strong stimulus for *IalB* expression and/or stability increase at the protein level (Coleman & Minnick, 2003). At the

moment, the functional implications of this observation can only be speculated. Low pH–IalB association might be significant outside the context of *B. bacilliformis*–erythrocyte interaction, perhaps in the survival of bacteria inside professional phagocytes. The IalB-mediated erythrocyte invasion might be a significant pathogenic strategy for *Bartonella* spp. in general because the *ialAB* locus is conserved in many species (Engel & Dehio, 2009). Indeed, STM in the intravenous *B. tribocorum*–rat infection model allowed the identification of 97 different protein-encoding genes that were important for the bacteria to establish the intraerythrocytic bacteremia and two independent insertional mutants mapped to the *ialB* gene (Saenz *et al.*, 2007). Moreover, intact *ialAB* locus was shown to be essential for *B. birtlesii* to establish an intraerythrocytic bacteremia in a natural mouse reservoir host infection (Vayssier-Taussat *et al.*, 2010). The abacteremic behavior of IalAB-deficient *B. birtlesii* correlated with defective erythrocyte invasion *in vitro*, although the adherence to the erythrocytes *per se* was not affected (Vayssier-Taussat *et al.*, 2010). The exact molecular mechanism [e.g. the putative cell surface ligand(s)] by which the IalAB contributes to the erythrocyte invasion remains elusive.

The STM screens on *B. birtlesii* and *B. tribocorum* allowed the identification of an IalAB-independent erythrocyte invasion locus, the *liv* locus (Saenz *et al.*, 2007; Vayssier-Taussat *et al.*, 2010). *Bartonella birtlesii* with an inactivating insertion in the *livG* gene and *B. tribocorum* with an inactivating insertion in the *livF* gene were identified. Moreover, the *B. birtlesii livG* mutant was shown to have a drastically decreased capacity to invade erythrocytes *in vitro*, although the adhesion to the erythrocytes *per se* was not significantly affected (Vayssier-Taussat *et al.*, 2010). In the *B. tribocorum* genomic sequence, the *livG* and *livF* genes have been annotated as ATPase components of a putative amino acid ABC transporter (Saenz *et al.*, 2007). These genes are also highly conserved in other members of *Bartonella* spp. It remains to be studied how the products of the novel *liv* locus mediate their apparent role in erythrocyte invasion. It is also possible that Liv system is required for the uptake of amino acid as nutrients for the invading bacteria inside the erythrocytes.

Metabolic adaptations to the host

Successful pathogen not only produces virulence factors ideally for a subclinical infection but also metabolically persists in the host with a minimal own input. A striking example of *Bartonella* spp. host-integrated metabolism is represented by heme, a crucial cofactor in many enzymatic processes. Early work demonstrated that *Brucella*

broth with 6–8% of Fildes solution and 250 µg of hemin per ml supported the *in vitro* growth of *B. henselae* up to OD_{600 nm} of 0.6 already on day 3 postinoculation as compared to virtually nongrowing culture up to day 9 postinoculation without the hemin supplement (Schwartzman *et al.*, 1993). More recently, it was reported that the *in vitro* growth-promoting effect of blood supplement for *B. henselae* on *Brucella* agar could be reproduced by the addition of hemin or hemoglobin (Sander *et al.*, 2000a). These findings indicate that growth of *B. henselae* is dependent on exogenous heme-containing compounds. Indeed, heme biosynthetic genes are largely missing in the genome of *B. henselae* (Alsmark *et al.*, 2004) and also in the genomes of other *Bartonella* spp. such as *B. quintana* (Alsmark *et al.*, 2004) and *B. tribocorum* (Saenz *et al.*, 2007). As an example, *gtrH* gene encoding for the HemH ferrochelatase that is responsible for the insertion of ferrous iron into protoporphyrin IX as the terminal step in proto-heme biosynthesis (Panek & O'Brian, 2002) is lacking. This is in accordance with the observation that addition of heme precursor protoporphyrin IX in combination with iron is not sufficient to rescue the *in vitro* growth defect of *B. henselae* on *Brucella* agar (Sander *et al.*, 2000a).

Heme is particularly abundant in the host niches colonized by *Bartonella* spp., that is, the erythrocytes and the mid-gut lumen of blood-sucking arthropods. Otherwise in the mammalian host, the free heme concentration is low because of the action of heme-binding plasma proteins (Baker *et al.*, 2003). In fact, even in the case of erythrocyte invasion in the blood of a reservoir host, vacuolar rupture inside the erythrocytes is expected for the bacterium to gain access to high amounts of heme. At the moment, no such activity is known except possibly for *B. bacilliformis* (Hendrix, 2000) and *B. henselae* (Litwin & Johnson, 2005), and therefore, efficient scavenging and uptake mechanisms are expected to be present in *Bartonella* spp. Indeed, *Bartonella* spp. have high abundance of genes encoding for proteins implicated in heme uptake (Alsmark *et al.*, 2004; Saenz *et al.*, 2007; Engel & Dehio, 2009). Experimentally, a surface-exposed hemin-binding ~25-kDa protein, designated as HbpA (Carroll *et al.*, 2000), has been identified in *B. quintana* with homologs present in *B. bacilliformis*, *B. clarridgeiae*, *Bartonella elizabethae*, *Bartonella doshaiae*, *B. vinsonii* Berkhoffii, and *B. henselae* where the protein is also known as the Pap31 (Bowers *et al.*, 1998; Zimmermann *et al.*, 2003; Maggi & Breitschwerdt, 2005). In *B. quintana*, four additional homologs, HbpB–HbpE, are present indicating the physiological importance of exogenous heme binding (Minnick *et al.*, 2003b), although direct evidence for bacterial surface localization is so far only available for HbpA (Carroll *et al.*, 2000) and HbpE (Boonjakuakul *et al.*, 2007). The five-member heme-

acquisition gene family (*hbpA–hbpE*) has a peculiar regulation pattern in *B. quintana* (Battisti *et al.*, 2006, 2007). At the level of mRNA, it has been shown that *hbpC* is significantly over-represented at 30 °C (louse-like) relative to 37 °C (human-like). High heme concentrations such as 5.0 mM (louse-like), close to the concentrations that become bactericidal, lead into significant over-representation of mRNA for *hbpB* and *hbpC* relative to 0.15 mM heme. On the other hand, low heme concentrations such as 0.05 mM (human-like), close to the concentrations that become growth limiting, lead into significant over-representation of mRNA for *hbpA*, *hbpD*, and *hbpE* relative to 0.15 mM heme (Battisti *et al.*, 2006, 2007). The authors propose that *B. quintana* utilize its HbpA–HbpE repertoire in a very distinct manner, that is, HbpA, HbpD, and HbpE are employed when heme is growth limiting such as in the circulation of the reservoir host human and HbpB and especially HbpC are employed when *B. quintana* encounter extremely high and toxic concentrations of heme such as in the mid-gut lumen of its arthropod host body louse (Battisti *et al.*, 2006). The data imply that HbpA–HbpE proteins might have differential subcellular localization, perhaps even extracellular and/or avidity to heme to fulfill their apparently diverse and putative functions in heme acquisition and detoxification. An additional heme utilization system has been identified in *B. quintana* (Parrow *et al.*, 2009). Genomic analyses revealed a locus encoding for a putative heme receptor (HutA), a TonB-like energy transducer (an ABC transport system comprised three proteins HutB, HutC, and HmuV), and a heme degradation/storage enzyme, HemS (Andrews *et al.*, 2003; Alsmark *et al.*, 2004; Parrow *et al.*, 2009). Direct functional evidence was provided for HutA heme receptor in a heterologous complementation analysis in *E. coli* (Parrow *et al.*, 2009). The importance of heme acquisition/detoxification for *Bartonella* spp. has also been observed in a natural reservoir host infection model. STM in the intravenous *B. bacilliformis*-rat infection model allowed the identification of 97 different protein-encoding genes that were important for the bacteria to establish the intraerythrocytic bacteremia, and 10 insertions were mapped to genes with putative functions in heme uptake such as *hbpB* and *hutA* (Saenz *et al.*, 2007). HutA was also identified as an essential protein in an STM screen for factors that are important for *B. birtlesii* to establish an intraerythrocytic bacteremia in a natural reservoir host mouse (Vayssier-Taussat *et al.*, 2010).

In conclusion, it appears that acquisition/detoxification of exogenous heme is of crucial importance for the metabolism of *B. henselae*, *B. quintana*, and *B. tribocorum* and most likely this applies to most, if not all, species of *Bartonella* spp.. In addition of being a crucial metabolite, heme might also elicit profound homeostatic functions

for *Bartonella* spp. as proposed by Battisti *et al.* (2006). *Bartonella* spp. lack peroxidases and heme-cofactored catalase but strikingly also Dps/Dpr proteins, which are indispensable for oxygen and H₂O₂ resistance of one other significant catalase-negative group of bacteria, streptococci (Yamamoto *et al.*, 2000; Pulliainen *et al.*, 2003, 2005). *Bartonella* spp. are members of the order *Rhizobiales*, along with some other important human (*Brucella* spp.) and plant (*Agrobacterium* spp.) pathogens. Many rhizobia form a symbiotic relationship with their host plant by fixing atmospheric nitrogen in root nodules. For nitrogen fixation to occur, a microaerophilic environment must be established for the bacteria. This is accomplished by the plant-generated leghemoglobin (a molecule similar to hemoglobin) binding to the rhizobial surface, effectively shielding the bacteria, and oxygen-labile nitrogenase, from oxygen (Ott *et al.*, 2005). Due to the fact that *Bartonella* spp. possess multiple surface-associated heme-binding proteins (Alsmark *et al.*, 2004; Battisti *et al.*, 2006; Saenz *et al.*, 2007; Parrow *et al.*, 2009), it has been speculated that heme binding could be a significant strategy to shield *B. quintana* from oxygen and thereby also from the partially reduced forms of oxygen; superoxide (·O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (·OH) (Battisti *et al.*, 2006). This interesting hypothesis of an evolutionary conserved strategy used by members of *Rhizobiales* to decrease oxygen in their environments remains to be studied.

One peculiar metabolic feature of *Bartonella* spp. is their apparent inability to use glucose as the primary carbon source, although glucose should be abundant in the host tissues and blood. When *B. henselae* was first isolated from the blood of patients infected with human immunodeficiency virus (HIV), preliminary characterization did not detect carbohydrate utilization (Regnery *et al.*, 1992; Welch *et al.*, 1992). In a similar fashion, *B. quintana* do not appear to metabolize glucose (Huang, 1967; Weiss *et al.*, 1978). More recently, it was observed in part of a study aimed to develop a robust liquid growth media for *Bartonella* spp. that *B. henselae* do not consume glucose (Chenoweth *et al.*, 2004). In contrast, *B. henselae* depleted amino acids and accumulated ammonia into the culture medium, an indicator of amino acid catabolism. Carbon dioxide was produced indicating that the amino acids were catabolized in a tricarboxylic acid cycle-dependent manner (Chenoweth *et al.*, 2004). What are the physiological implications of the amino acid-dependent catabolism in *Bartonella* spp.? At the moment, it can only be speculated that this type of metabolism might have evolved together with the host heme utilization. It is expected that proteins are actually more abundant than glucose in the host niches colonized by *Bartonella* spp., that is, the erythrocytes after heme

sequestration from hemoglobin by the bacteria and especially the mid-gut lumen of blood-sucking arthropods after digestive enzymes of the arthropod have processed the ingested erythrocytes.

Evasion of host adaptive immune response

Antigenic and phase variation of surface proteins

Intracellular localization of *Bartonella* spp. in reservoir host erythrocytes is undoubtedly one of the most efficient means to subvert both the humoral and the cellular immune responses. Owing to the lack of major histocompatibility molecules on their surfaces, erythrocytes are unable to present antigens of their invaders to the immune system. However, the host is eventually able to clear the infection by antibodies within the limits of natural life span of the erythrocytes (Fig. 2), at least based on studies in the natural *B. grahamii* mouse infection model (Koesling *et al.*, 2001). This is probably due to the opsonization of the bacteria that are periodically seeded from the primary niche into the bloodstream and thereby inhibition of the bacterial binding to the erythrocytes.

When the reservoir host produces antibodies targeted against the invading *Bartonella* spp., the strong selective pressure is expected to favor the appearance of clonal populations where the pathogen has altered the expressed surface-exposed antigens (antigenic variation), or no longer express the antigen on its surface because of phase variation or partial or total gene loss (Bayliss, 2009). The total gene loss has been directly witnessed *in vivo* in *B. quintana*-macaque reservoir host infection model (Fig. 6) (Zhang *et al.*, 2004). A clone was isolated from the blood 70 days after an intradermal inoculation, and it was shown that the genes encoding for two of its initial four surface-exposed TAAs, the Vomp adhesins, had been deleted (Zhang *et al.*, 2004). Vomps are important proteins for *B. quintana* to establish intraerythrocytic bacteremia after an intradermal inoculation in the macaque model (MacKichan *et al.*, 2008) and *in vitro* mediate bacterial adhesion to ECs (Müller *et al.*, 2011) and binding to ECM components such as collagen (Zhang *et al.*, 2004; Müller *et al.*, 2011). Moreover, nine independent human primary isolates of *B. quintana* were shown to have six different restriction fragment length polymorphism patterns as judged by Southern blotting with a probe annealing to a conserved 5'-region of VompA, VompB, and VompC (Zhang *et al.*, 2004). The data indicate that Vomp locus may be a subject to extensive genetic rearrangements *in vivo* including changes in the copy number of the genes. Strong selective pressure for alterations in Vomp expression and surface display is highlighted by

observation that Vomp-specific antibodies are the most frequently detected anti-*B. quintana* antibodies in the sera of human patients infected with *B. quintana* (Boonjakuakul *et al.*, 2007). The emergence of genetically distinct clones at various peaks of relapsing bacteremia has also been witnessed in a natural *B. henselae*-cat reservoir host infection model (Kabeya *et al.*, 2002).

BadA of *B. henselae*, which resembles Vomps, is variably surface-exposed in different strains resulting in some cases to complete lack of EC adhesion (Riess *et al.*, 2007). It remains to be shown whether BadA is subject of phase variation *in vivo*. At the moment, it is believed that the detected loss of surface localization in some strains *in vitro* is because of the loss of costly expression of the large ~ 300-kDa BadA upon extensive passaging of strains on blood agar plates (Kempf *et al.*, 2001; Riess *et al.*, 2007). BadA has a modular domain structure, that is, an N-terminal head region, a long and highly repetitive stalk and neck region, and a C-terminal membrane anchor domain (Riess *et al.*, 2004, 2007; Kaiser *et al.*, 2008) (Fig. 6). It has been proposed that the repetitive tandem DNA sequences encoding for the stalk domains of BadA increase the frequency of recombination and thereby the generation of variable surface-displayed adhesins with a net outcome of antigenic variation (Linke *et al.*, 2006). Indeed, it has been observed that of the nine analyzed *B. henselae* strains virtually, every strain had its own stalk length, whereas the length of the head and the membrane domains were constant (Riess *et al.*, 2007).

Extremely potent source of antigenic variability is displayed by Trw-T4SS, which is a crucial molecular determinant of erythrocyte adhesion and appears as an important mediator of reservoir host specificity (Seubert *et al.*, 2003; Vayssier-Taussat *et al.*, 2010) (Fig. 6). Trw-T4SS was initially identified in a DFI screen as a *B. henselae* promoter that is significantly upregulated during the infection of ECs (Seubert *et al.*, 2003). In the *B. tribocorum*-rat model, mutant deleted for the *virB10*-like *trwE* gene is deficient in establishing the long-lasting intraerythrocytic bacteremia (Seubert *et al.*, 2003). The most striking feature of Trw-T4SS is the remarkable locus expansion by tandem gene duplications (Fig. 6). In addition, the different copies of the major pilus components TrwL (seven tandem repeats of *trwL*) and of the minor pilus component TrwJ (five tandem repeats of the *trwJIIH* region) display a large degree of sequence variation. In contrast, the different copies of the lipoprotein TrwH, which is considered to link the pilus to the core complex (Krall *et al.*, 2002), and the inner membrane protein TrwI, known to be essential for the stabilization of TrwJ (Krall *et al.*, 2002), are basically identical. The tandem gene duplications of Trw-T4SS components are not restricted to *B. tribocorum* because both *B. henselae*

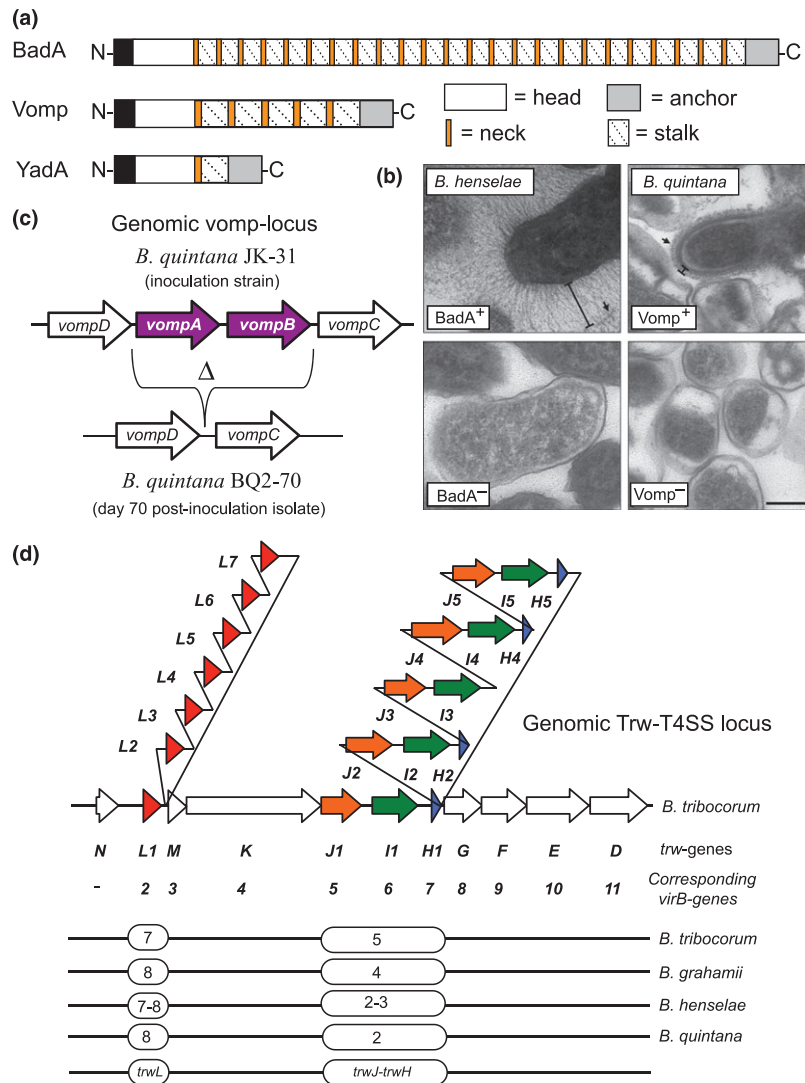


Fig. 6. Major adhesins of *Bartonella* spp. (a) Schematic representation of BadA of *Bartonella henselae* and variably expressed OMPs (Vomps) of *Bartonella quintana* and comparison to *Yersinia* adhesin A (YadA) of *Yersinia enterocolitica*. These TAAs are composed of an N-terminal signal peptide (black bar), the head sequence, variable neck and stalk repeats, and the C-terminal membrane anchor. It has been observed that virtually every *B. henselae* strain has its own neck/stalk length, whereas the length of the head and the membrane anchor domains are constant (Riess *et al.*, 2007). (b) Surface expression of BadA and Vomps detected by transmission electron microscopy (Müller *et al.*, 2011). Note the expression of long proteinaceous surface appendices (length, ~ 250 nm) on the surface of *B. henselae* wild-type (missing in the *B. henselae* BadA-deficient strain) and on the surface (length, ~ 40 nm) of *B. quintana* JK31 (missing in *B. quintana* BQ2-D70). Note the electron-dense globular structure on the top of the BadA and Vomps (marked by an arrow), presumably representing the head domain (scale bar 300 nm). (c) Schematic representation of the *Vomp* locus of *B. quintana* JK-31. As an indication of high antigenicity of Vomps and their possible temporal role in pathogenesis, total gene loss has been witnessed *in vivo* in a natural *B. quintana*-macaque reservoir host infection model. A clone (BQ2-D70) was isolated from the blood 70 days after an intradermal inoculation, and it was shown that the genes encoding for two of its initial four surface-exposed *Vomp* adhesins had been deleted (Zhang *et al.*, 2004). (d) *Trw*-T4SS mediates erythrocyte adhesion (Vayssier-Taussat *et al.*, 2010). Genetic organization of the chromosomal *trw* locus encoding for the *Trw*-T4SS of *Bartonella tribocorum* (Seubert *et al.*, 2003). The most striking feature of *Trw*-T4SS, also in other species (Nystedt *et al.*, 2008), is the partial locus expansion by gene multiplications. In addition, the different copies of the major pilus components *TrwL* and of the minor pilus component *TrwJ* display a large degree of sequence variation. Details are described in the text (see Antigenic and phase variation of surface proteins).

Houston-1 and *B. quintana* Toulouse harbor two copies of *trwJ* and eight copies of *trwL* (Alsmark *et al.*, 2004). The striking difference in sequence conservation between

paralogues of the co-amplified *trwJIIH* genes in *B. tribocorum* suggests that, after gene duplication, these genes have been exposed to differential selection pressure (Seubert

et al., 2003; Nystedt *et al.*, 2008). Diversifying selection for mutations in *trwJ* and/or *trwL* would generate variable pilus forms, which may allow the interaction with different host cell surface structures (e.g. different ligands on the erythrocyte surface) and eventually even lead into host switch or may represent a general mechanism of immune evasion by antigenic variation. In contrast, negative selection against the accumulation of mutations in *trwI* or *trwH* paralogues may result from a deleterious dominant-negative effect of those mutations on the integrity and function of the Trw-T4SS, as has been described for homologous systems (Dang *et al.*, 1999; Sagulenko *et al.*, 2001). Currently, it remains unknown whether pool of all the possible TrwJ, TrwL, TrwI, and TrwH proteins is expressed. Potentially, there could be a mechanism that allows selective isoform-specific expression and/or display. In conclusion, antigenic and phase variation appears as a common persistence strategy for *Bartonella* spp., at least based on the current *in vitro* and *in vivo* knowledge of Trw-T4SS, BadA, and Vmps. Several nonbiased screening studies have been conducted to identify immunogenic surface-exposed proteins in *Bartonella* spp. (Minnick, 1994; Rhomberg *et al.*, 2004; Boonjakuakul *et al.*, 2007; Eberhardt *et al.*, 2009) mainly for serodiagnostic purposes, but this information might become useful in the future to also understand how *Bartonella* spp. evade adaptive immunity and persist in the mammalian host.

Molecular basis of *Bartonella* spp.-triggered vascular tumorigenesis in human

Introduction to the *Bartonella* spp.-triggered vascular tumorigenesis

The role of viruses such as hepatitis B virus or human papilloma virus in carcinogenesis is widely accepted because of direct mechanistic effects of single viral gene products in cell transformation. Some bacteria, such as *H. pylori*, are also associated with malignant cell transformation (Lax & Thomas, 2002). *Bartonella* spp. and in particular *B. bacilliformis*, *B. quintana*, and *B. henselae* are unique in the bacterial kingdom in causing vasoproliferative tumor formation (Fig. 7). Human infection by *B. bacilliformis* can be divided into two distinct clinical phases. The initial acute phase of the disease (Oroya fever) is characterized by hemolytic anemia. The second phase occurs after clinical recovery from the acute phase and is characterized by the eruption of crops of nodular tumor-like skin lesions or ferruginous skin lesions, or both, predominantly on the head and distal extremities (Maguiña *et al.*, 2009). The partial state of immunosuppression that evidently develops at the end of the Oroya fever is considered to favor the formation

of vasoproliferative lesions (verruca peruana) (Dehio, 2005). Immunosuppression is also a prerequisite for vascular tumor formation by *B. henselae* and *B. quintana* (Maguiña *et al.*, 2009). BA caused by both of these species is characterized by vasoproliferative skin lesions, which resemble Kaposi's sarcoma caused by human herpes virus 8 infection in patients with AIDS (Tappero & Koehler, 1997; Relman *et al.*, 1999). BA was described in 1983 (Stoler *et al.*, 1983), and it has occurred most commonly in patients with severe immunosuppression caused by the HIV. BA has also occurred in immunocompetent individuals. However, in these cases, the patients have apparently experienced transient immunosuppression owing to organ transplantation or chemotherapy (Cockerell *et al.*, 1990; Myers *et al.*, 1992; Tappero *et al.*, 1993). *Bartonella henselae* can also trigger the formation of vasoproliferative lesions in inner organs such as in liver and spleen, a clinical state known as the BP (Welch *et al.*, 1992). Histologically, BA and BP lesions are composed of individual bacteria or clusters of bacteria, proliferated, and misshapen ECs, which may form capillary-like sprouts, and a mixed infiltrate of leukocytes such as monocyte/macrophages and PMNs (Kostianovsky & Greco, 1994). *Bartonella* spp.-triggered vascular tumorigenesis has been mainly studied *in vitro* using monolayers of primary human umbilical-vein ECs (HUVECs) with the most detailed knowledge available for *B. henselae*. Some studies have also been conducted with HUVECs or HUVEC spheroids that have been embedded in a three-dimensional matrix such as a collagen gel or Matrigel (Kirby, 2004; McCord *et al.*, 2006; Scheidegger *et al.*, 2009; Berrich *et al.*, 2011; Scheidegger *et al.*, 2011). Development of an animal model of BA/BP would be essential to define the molecular basis of *Bartonella* spp.-triggered vascular tumorigenesis.

Endothelial cell adhesion and subsequent invasion

Colonization of vascular endothelium and persistence therein is generally accepted as a crucial step in the establishment and maintenance of *Bartonella* spp.-triggered vascular deformations. Each of the three vasoproliferation-inducing *Bartonella* spp. species adheres to and invades ECs *in vitro* (Hill *et al.*, 1992; Brouqui & Raoult, 1996; Dehio *et al.*, 1997). The vascular pathologies could reflect a general tropism of *Bartonella* spp. for the vascular endothelium, that is, the ECs could be the still-enigmatic primary niche (see Identity of the primary niche).

Bartonella quintana and *B. bacilliformis*

In molecular terms, *B. quintana*-EC interaction is poorly understood. However, it appears that the bacterium is

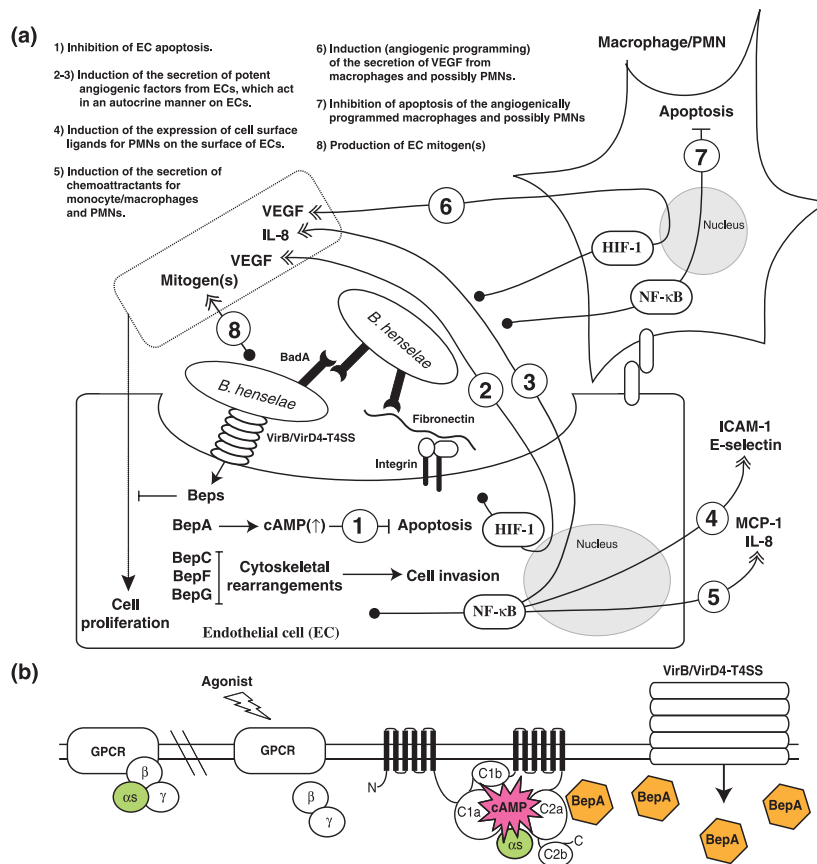


Fig. 7. Schematic representation of a model of *Bartonella henselae*-triggered vascular tumorigenesis. (a) *Bartonella henselae* displays marked tropism toward vascular ECs, resulting in bacterial adherence and invasion. BadA mediates the adhesion of *B. henselae* to host cells, possibly via a fibronectin bridge to integrins. BadA also mediates bacterial auto-aggregation (Riess *et al.*, 2004). A cocktail of different Beps is translocated from *B. henselae* via the VirB/VirD4-T4SS into the host cell (Pulliainen & Dehio, 2009). By their inherent or cell-modified properties, the effectors are targeted into different subcellular compartments to mediate their diverse functions. Cytoskeletal rearrangements mediated by BepC, BepG, and BepF result in the uptake of a large bacterial aggregate via the invasome structure, and this is believed to favor a long-term host cell colonization (Rhomberg *et al.*, 2009; Truttmann *et al.*, 2011a, b, c). (b) A schematic representation of the molecular basis of BepA-mediated subversion of host cell cAMP signaling. Translocation of BepA into ECs coincides with an elevation of cytosolic cAMP concentration (Schmid *et al.*, 2006). Recent data indicate that after the T4SS-mediated translocation, BepA directly binds host cell adenylyl cyclase to potentiate G α_s -dependent cAMP production (A.T. Pulliainen *et al.*, submitted). As opposed to the known microbial mechanisms such as ADP ribosylation of G-protein α -subunits by cholera and pertussis toxins, the fundamentally different BepA-mediated elevation of host cell cAMP concentration is subtle and strictly dependent on the release of G α_s from G-protein-coupled receptors by agonist stimulation. We propose that this mechanism is ideal for the chronic and stealthy persistence of *B. henselae* in the vascular endothelium.

able to adhere to and invade ECs *in vitro* (Brouqui & Raoult, 1996; Palmari *et al.*, 1996; Müller *et al.*, 2011). Actin rearrangements have been reported (Palmari *et al.*, 1996), which may be necessary for the bacterial uptake. Recently, it was shown that the large and variably expressed Vomp adhesins are important to mediate the adhesion of *B. quintana* to HUVECs (Müller *et al.*, 2011). In the case of *B. bacilliformis*, the invasion of ECs appears to require actin remodeling because cytochalasin D, a cell-permeable inhibitor of actin polymerization, inhibits bacterial uptake (Hill *et al.*, 1992). Based on an electron microscopy study, the bacteria invaded the EC within

1 h, forming a small membrane-bound inclusion, the BCV. By 12 h, a large membrane-bound inclusion, similar to the cytoplasmic inclusions of verruga peruana patients' ECs, containing numerous bacteria was present (Garcia *et al.*, 1992). Most likely, these inclusions represent bacteria that have been multiplying within a single BCV or a fusion vesicle of multiple BCVs. Pretreatment of the ECs with *Clostridium botulinum* C3 transferase, which inactivates one key regulator of actin reorganization, Rho-GTPase, via ADP ribosylation, strongly inhibited the bacterial internalization (Verma *et al.*, 2000). This is in accordance with the marked activation and plasma mem-

brane localization of Rho upon *B. bacilliformis* infection of ECs as judged by an affinity capture system to precipitate the activated form of Rho (Rho-GTP) with Rho effector protein, rhotekin (Verma *et al.*, 2000). Moreover, the other two major GTPases that are involved in actin remodeling, Rac and Cdc42, became activated, and this activation was associated with the formation of filopodia and lamellipodia together with bacterial clumping on the surface of the ECs (Verma & Ihler, 2002). In conclusion, it appears that *B. bacilliformis* and most likely also *B. quintana* actively subvert EC signaling to promote their internalization by a process resembling classical bacterium-forced phagocytic entry into normally nonphagocytic cells (Cossart & Sansonetti, 2004).

Bartonella henselae

Early work indicated that *B. henselae* infection induces morphological changes in human ECs. The infected cells became larger, more elongated, spindle shaped and displayed actin rearrangements (Palmari *et al.*, 1996). In 1997, it was shown that *B. henselae* actually enters HUVECs by two distinct routes (Dehio *et al.*, 1997). *Bartonella henselae* may first of all enter the cell as individual bacteria through a classical forced phagocytic pathway, which resemble the BCV-uptake mechanisms of *B. bacilliformis* (Garcia *et al.*, 1992). Alternatively, *B. henselae* may enter the cell as large bacterial aggregates that are formed on the cell surface, followed by their engulfment and internalization via the invasome structure in an actin-dependent manner. Typical appearance of the invasome is a solitary, globular structure of 5–15 μm in diameter, which may contain several hundreds of bacteria. The invasome-mediated cell entry has slower kinetics (days) when compared to the BCV-mediated uptake (hours) (Dehio *et al.*, 1997). The *in vivo* relevance of the invasome-mediated uptake is unclear; however, the bacterial aggregates that are formed in this process could correspond to the clumps of bacteria that are observed in histology sections of BA lesions (Tappero & Koehler, 1997; Relman *et al.*, 1999). Moreover, it appears that the invasomes do not acquire typical lysosomal markers such as LAMP-1 *in vitro* (Rhomberg *et al.*, 2009) within the same timeframes when BCVs have fused with the lysosomes (Kyme *et al.*, 2005; Rhomberg *et al.*, 2009). Invasome-mediated uptake could therefore represent an invasion strategy that is preferred over the BCV uptake and allows *B. henselae* to establish a chronic infection of the vasculature. In addition to the early work (Palmari *et al.*, 1996; Dehio *et al.*, 1997), several investigators have reported that *B. henselae* adheres to and efficiently invades primary ECs and also immortalized endothelial cell hybridomas such as Ea.hy926 (Kempf *et al.*, 2000; Resto-Ruiz *et al.*,

2000; Fuhrmann *et al.*, 2001; Kempf *et al.*, 2001; Schmiederer *et al.*, 2001; Riess *et al.*, 2004; Kyme *et al.*, 2005; Riess *et al.*, 2007; Kaiser *et al.*, 2008; Chang *et al.*, 2011; Müller *et al.*, 2011).

Major adhesin of *B. henselae* toward ECs is BadA (Riess *et al.*, 2004), although the EC invasion apparently takes place *in vitro* even in the absence of this TAA (Dehio *et al.*, 1997; Riess *et al.*, 2007). There are indications that the BadA ligand on EC surface is β 1-integrin via a fibronectin bridge (Riess *et al.*, 2004) (Fig. 7). The possible BadA–fibronectin–integrin triad in *B. henselae* cell entry would be analogous to the cell entry mechanism of Gram-positive pathogen *Staphylococcus aureus*. This bacterium recruits fibronectin to its surface, a process that is mediated by a direct interaction between fibronectin and the bacterial cell-wall-attached proteins FnBP-A and FnBP-B (Jönsson *et al.*, 1991). The interaction of the bacteria-bound fibronectin with its native receptor, integrin α 5 β 1, initiates integrin clustering and bacterial internalization into host cells (Agerer *et al.*, 2003). The importance of β 1 integrin both as the cellular ligand and an active signaling component in *B. henselae*-induced invasome formation has recently been identified in a HeLa cell-based RNA interference screen (Truttmann *et al.*, 2011a, b, c). Interestingly, these data were acquired with a *B. henselae* strain (Houston-1) that apparently lacks BadA expression. Accordingly, the Houston-1 strain did not significantly bind fibronectin, but at the same time it displayed a strong binding activity toward β 1 integrin. This indicates that *B. henselae* express direct adhesins for β 1 integrin. One possible candidate could be the 43-kDa OMP Omp43, which has been reported to bind ECs (Burgess & Anderson, 1998; Burgess *et al.*, 2000).

VirB/VirD4-T4SS

Expression of the *B. henselae* *virB* operon (Fig. 5) is induced upon EC interaction (Schmiederer *et al.*, 2001; Quebatte *et al.*, 2010). Moreover, it has been reported that the VirB4-deficient *B. henselae* strain is incapable of inducing its invasome-mediated uptake into HUVECs (Schmid *et al.*, 2004). Internalization of the mutant through the BCV invasion appeared equal or even more pronounced as compared to the parental strain (Schmid *et al.*, 2004). Recently, three of the total of seven currently known VirB/VirD4-T4SS effector proteins (Schulein *et al.*, 2005; Pulliainen & Dehio, 2009), that is, *Bartonella* effector proteins BepC, BepF, and BepG (Figs 5 and 7), were shown to be involved in the invasome-mediated bacterial uptake (Rhomberg *et al.*, 2009; Truttmann *et al.*, 2011a, b, c). Interestingly, there appears to be functional redundancy. First of all, expression of BepG in the Bep-deficient (Δ *bepA–G*) mutant restores the invasome-

mediated uptake. Likewise, ectopic expression of BepG in ECs also restores the invasome-mediated uptake of the Bep-deficient ($\Delta\text{BepA-G}$) mutant. Both of the above effects appear to inhibit the BCV route of cell entry. Indeed, ectopic expression of BepG in ECs also blocks heterologous endocytic processes such as the uptake of inert microspheres (Rhomberg *et al.*, 2009). The authors propose that BepG triggers the invasome-mediated bacterial uptake primarily by inhibiting bacterial endocytosis into BCVs. Bacteria accumulating at the cell surface, which could be assisted by BadA-mediated auto-aggregation (Riess *et al.*, 2004; Kaiser *et al.*, 2008) (Fig. 7), then induce locally the F-actin rearrangements characteristic for the invasome formation. These cytoskeletal changes include both the rearrangements of pre-existing F-actin fibers and the *de novo* polymerization of cortical F-actin in the periphery of the invasome by Rac1/Scar1/WAVE- and Cdc42/WASP-dependent pathways that involve the recruitment of the Arp2/3 complex (Rhomberg *et al.*, 2009). Secondly, co-infections of HUVECs with the Bep-deficient ($\Delta\text{BepA-G}$) mutant expressing either BepC or BepF restore invasome formation. Likewise, ectopic co-expression of BepC and BepF enables invasome-mediated uptake of the Bep-deficient ($\Delta\text{BepA-G}$) mutant strain. Furthermore, the combined action of BepC and BepF inhibits the endocytic uptake of inert microspheres. The invasome formation induced by the concerted action of BepC and BepF differs from BepG-triggered invasome formation in its requirement for cofilin-1, while the Rac1/Scar1/WAVE/Arp2/3 and Cdc42/WASP/Arp2/3 signaling pathways are required in both cases (Rhomberg *et al.*, 2009; Truttmann *et al.*, 2011a, b, c).

Direct *Bartonella* spp.-mediated effects on endothelial cell proliferation and migration

Bartonella bacilliformis

Early work on *B. bacilliformis* indicated that the bacterium produces a mitogenic factor, that is, factor present in centrifugation-cleared bacterial sonicates, which is heat sensitive, larger than 14 kDa in size and can be precipitated with 45% ammonium sulfate (Garcia *et al.*, 1990, 1992). Crude extracts of *B. bacilliformis* were also analyzed *in vivo*. Polyvinyl alcohol sponges were injected with the extract 3 days after the sponges had been implanted subcutaneously into a rat. Histological analysis of the sponges 7 days after implantation revealed a 2.5-fold increase in the number of blood vessels present in the sponges injected with 1 mg of *B. bacilliformis* extract (Garcia *et al.*, 1990). More recently, it was reported that the culture supernatants of *B. bacilliformis* contain a potent mitogen, which is heat sensitive and is inactivated with trypsin, therefore indicating its proteina-

ceous nature. The authors provided evidence that this mitogen and most probably the mitogen identified by Garcia *et al.* (1990) is the GroEL chaperon (Minnick *et al.*, 2003a). More studies are needed to define the GroEL function in detail and the angiogenic properties of *B. bacilliformis* in general because it has also been reported that *B. bacilliformis* counterintuitively reduces EC migration *in vitro* as judged by wound healing assays and single cell imaging (Verma *et al.*, 2001).

Apart from the possible bacterial proteinaceous mitogen(s), it has been proposed that the potent angiogenic factor, angiopoietin-2 (De Palma & Naldini, 2011), has an autocrine role in *B. bacilliformis*-induced EC proliferation (Cerimele *et al.*, 2003). It was observed *in vitro* that *B. bacilliformis*-infected ECs had higher amounts of angiopoietin-2 mRNA. Accordingly, *in situ* hybridization demonstrated that angiopoietin-2 is strongly expressed *in vivo* by ECs of verruga peruana lesions. Moreover, the major angiopoietin-2 receptor Tie2/Tek in addition to Tie1 was expressed in verruga peruana ECs as shown by immunohistochemistry (Cerimele *et al.*, 2003). It remains to be studied how *B. bacilliformis* induces the angiopoietin-2 expression and whether this is significant *in vivo* in the formation of verruga peruana lesions.

Bartonella quintana and *B. henselae*

Bartonella quintana and *B. henselae* appear to produce a mitogenic factor, which is trypsin sensitive, therefore indicating its proteinaceous nature (Conley *et al.*, 1994; Palmari *et al.*, 1996). It appears that direct *B. henselae*-EC interaction is not required to induce the proliferation because culture supernatants of *B. henselae* induce the proliferation of HUVECs (Maeno *et al.*, 1999; McCord *et al.*, 2007), live *B. henselae* that have been physically separated from ECs with a filter membrane induce the proliferation (Maeno *et al.*, 1999), and a nonpiliated spontaneous mutant strain of *B. henselae*, presumably BadA negative and therefore having reduced EC adhesion capacity (Riess *et al.*, 2004), possess the ability to stimulate the proliferation of co-cultivated HUVECs almost at the same level as the parental piliated strain (Maeno *et al.*, 1999). The identity of *B. henselae* and *B. quintana* mitogenic factor remains unknown and multiple factors could be involved. GroEL has been detected in the culture supernatants of *B. henselae*, and it has been proposed with analogy to *B. bacilliformis* that this factor could be mitogenic (Minnick *et al.*, 2003a; McCord *et al.*, 2007).

Apart from the possible bacterial proteinaceous mitogen(s), it has been proposed that interleukin-8 (IL-8), which is a potent mitogen (Waugh & Wilson, 2008), has an autocrine role in *B. henselae*-induced EC proliferation.

Bartonella henselae infection of HUVECs and human microvascular ECs (HMECs) induces the production of IL-8 (Schmid *et al.*, 2004; Kempf *et al.*, 2005b; McCord *et al.*, 2006), and the *B. henselae*-induced proliferation of HUVECs is inhibited by the addition of antibodies against IL-8 (McCord *et al.*, 2006). However, this mode of action apparently requires direct bacterium cell contact at least based on the lack of IL-8 secretion by HeLa cells with the BadA-negative strain (Riess *et al.*, 2004) and therefore can only partially explain the mitogenic activity of *B. henselae*. Recent report demonstrates that *B. henselae* infection of human skin microvascular endothelial cells (HskMECs) induces VEGF secretion under conditions where VEGF secretion by HUVECs was not detected (Berrich *et al.*, 2011). Hypoxia-inducible factor-1 (HIF-1) is one of the key transcription factors regulating angiogenesis as an example via the VEGF expression (Lu & Kang, 2010). Activation of HIF-1 has been detected in *B. henselae*-infected HeLa cells and HUVECs *in vitro* (Riess *et al.*, 2004; Kempf *et al.*, 2005b). HIF-1 is essential for *B. henselae*-induced secretion of VEGF (Kempf *et al.*, 2005b), which is extremely potent in HeLa cells (Kempf *et al.*, 2001, 2005b) and HskMECs (Berrich *et al.*, 2011) but has also been detected in HUVECs (Kempf *et al.*, 2005b) despite recent contradictory findings (Berrich *et al.*, 2011). Because the infection of HeLa cells with *B. henselae* resulted in an increased oxygen consumption and subsequent cellular hypoxia presumably because of intracellular bacterial replication (Kempf *et al.*, 2005b), the *B. henselae*-mediated HIF-1 activation appears to be oxygen dependent (Jaakkola *et al.*, 2001). The intracellular replication-mediated hypoxia is supported by the observation that the growth of *B. henselae* under *in vitro* liquid growth conditions consumes oxygen and the growth can be significantly increased by aeration of the cultures (Chenoweth *et al.*, 2004). The physiological importance of HIF-1 activation and VEGF secretion is supported by histology studies with intense staining patterns for VEGF and HIF-1 in BA/BP tissue lesions (Kempf *et al.*, 2001, 2005b).

VirB/VirD4-T4SS and direct mitogenic activities of *Bartonella* spp.

It has been reported that the secretion of IL-8 upon *B. henselae* infection of HUVECs is dependent on VirB/VirD4-T4SS (Schmid *et al.*, 2004). It was observed that for all infection doses tested (multiplicity of infection (MOI) = 30, 100, 300), the VirB4-deficient strain was attenuated in its capacity to induce the secretion of IL-8 as compared to the parental strain. The expression of IL-8 is primarily mediated by NF- κ B (Waugh & Wilson, 2008), and accordingly, the authors demonstrate that

NF- κ B is activated upon *B. henselae* infection primarily in a VirB/VirD4-T4SS-dependent manner (Schmid *et al.*, 2004). At the moment, it remains unknown which Beps (Fig. 5) are involved. VirB/VirD4-T4SS has also been implicated to negatively influence the mitogenic capacity of *B. henselae*, which under the experimental setup could have been mediated by bacterial mitogen(s) and/or possible autocrine stimuli (Schmid *et al.*, 2004). It was observed that for all infection doses tested (MOI = 10, 30 or 100), the VirB4-deficient strain strongly stimulated HUVEC proliferation resulting on day 5 to an eightfold increase in cell numbers, which even seemed to surpass the effect caused by VEGF. In contrast, infection with the wild-type *B. henselae* resulted only in a twofold increase in cell numbers at the lowest dose tested (MOI = 10) and the higher doses even appeared cytostatic (Schmid *et al.*, 2004). These data indicate that the potent mitogenic activity of *B. henselae* is VirB/VirD4 independent and that the VirB/VirD4-mediated cytostatic effects interfere with the activity of the VirB/VirD4-independent mitogen(s) in an infection dose-dependent manner. In the case of the autocrine VEGF stimulus (see *B. quintana* and *B. henselae*) or paracrine VEGF stimulus (see A paracrine loop of *Bartonella* spp.-mediated endothelial cell proliferation), it has recently been reported that *B. henselae* infection negatively influences the VEGF signaling in HUVECs (Scheidegger *et al.*, 2011). *B. henselae* infection abrogated VEGF-induced proliferation of HUVECs and wound closure of HUVEC monolayers as well as the capillary-like sprouting of EC spheroids. On the molecular level, *B. henselae* infection inhibited VEGF-stimulated phosphorylation of VEGF receptor 2 (VEGFR2) at tyrosine 1175 but it did not alter the VEGFR2 expression or cell surface localization. Inhibition of VEGFR2 signaling by *B. henselae* infection was strictly dependent on a functional VirB/VirD4-T4SS (Scheidegger *et al.*, 2011). In conclusion, VirB/VirD4-T4SS appears as an important regulator of direct mitogenic activities of *B. henselae*. On the one hand, it seems to be required to induce the secretion of one potent autocrine mitogen, IL-8, from the infected ECs. On the other hand, VirB/VirD4-T4SS counterintuitively inhibits other direct mitogenic activities of *B. henselae*, which may include the intrinsic bacterial mitogen(s) or autocrine cellular mitogen(s) such as VEGF.

Inhibition of endothelial cell apoptosis

Inhibition of host cell apoptosis appears essential for intracellular pathogens to establish chronic infections. Pathogen-triggered anti-apoptosis of infected host cells is expected to facilitate a slow microbial replication process and enables persistence in the infected host. For example, the obligate intracellular pathogens *Chlamydia pneumo-*

niae and *Chlamydia trachomatis* degrade pro-apoptotic BH3-only host cell proteins such as Bim, Puma, and Bad (Fischer *et al.*, 2004). *Chlamydia pneumoniae* has also been reported to protect invaded host cells from apoptosis by activating a nuclear factor kappa B (NF- κ B)-dependent survival pathway in a similar fashion to *Rickettsia rickettsii* (Clifton *et al.*, 1998; Paland *et al.*, 2006). In an early study, it was reported that *B. henselae* and *B. quintana* inhibit actinomycin D-induced apoptosis of human dermal microvascular ECs and HUVECs (Kirby & Nekorchuk, 2002). More recently, it was shown that the capacity of *B. henselae* to inhibit apoptosis of HUVECs, induced either artificially by the transcriptional blocker actinomycin D or more physiologically by cytotoxic T lymphocytes, is dependent on the VirB/VirD4-T4SS and its BepA effector (Schmid *et al.*, 2004, 2006). BepA of *B. henselae* has also been reported to promote capillary-like sprouting of EC spheroids in 3D collagen matrix (Scheidegger *et al.*, 2009), most likely via its potent capacity to promote cell survival. Translocation of BepA into ECs coincides with an increase in cellular cAMP concentration (Schmid *et al.*, 2006). Pharmacological elevation of cAMP by combined action of the adenylate cyclase activatory drug forskolin and the phosphodiesterase inhibitory drug 3-isobutyl-1-methylxanthine or by addition of the nonhydrolyzable and membrane-permeable cAMP analog dibutyryl-cAMP similarly protected ECs from apoptosis (Schmid *et al.*, 2006). This direct phenocopy effect indicates that the BepA-induced cAMP elevation is indeed the molecular basis of BepA-mediated anti-apoptosis. The molecular mechanism how BepA induces the cAMP elevation and the nature of the anti-apoptotic signaling events downstream of cAMP elevation are currently unknown. The observation that BepA mediates the protection of *B. henselae*-infected ECs against apoptosis triggered by cytotoxic T lymphocytes (Schmid *et al.*, 2006) suggests a physiologically relevant context in which the anti-apoptotic activity of BepA contributes to the vasoproliferative tumor formation in the chronically infected vascular endothelium.

A paracrine loop of *Bartonella* spp.-mediated endothelial cell proliferation

Monocyte/macrophages are capable of producing potent angiogenic factors (Qian & Pollard, 2010). Bacterial activation of the typical BA/BP-lesion monocyte/macrophage infiltrate (Kostianovsky & Greco, 1994) could therefore result in the release of angiogenic substances and thereby contribute to the process of vascular deformation. VEGF, one of the most potent mitogens of ECs and inducers of angiogenesis (Eilken & Adams, 2010), is released also from *B. henselae*-infected cells other than

HUVECs such as HeLa and Ea.hy926 cells (Kempf *et al.*, 2001; Riess *et al.*, 2004; Kempf *et al.*, 2005b). Accordingly, conditioned medium of the *B. henselae*-Ea.hy926 co-culture induced the proliferation of HUVECs, which was partially inhibited by the addition of anti-VEGF antibodies (Kempf *et al.*, 2001). These primary findings were later substantiated by an observation that phorbol 12-myristate 13-acetate (PMA)-differentiated or undifferentiated THP-1 human macrophages secrete VEGF upon *B. henselae* infection and that medium of the *B. henselae*-THP-1 co-culture induces the proliferation of HMECs (Resto-Ruiz *et al.*, 2002). Moreover, J774A.1 murine macrophages have been reported to release VEGF upon *B. henselae* infection (Kyme *et al.*, 2005) and THP-1 human macrophages upon *B. quintana* infection (Schulte *et al.*, 2006). These data are in favor of a paracrine loop of *Bartonella* spp.-mediated EC proliferation, with VEGF representing the major angioproliferative substance released by macrophages in response to *B. henselae* infection. Of note, *B. henselae* strain Marseille has been reported to enter J774A.1 mouse macrophages in the absence of opsonins and is capable of delaying its lysosomal targeting and destruction at least when compared in parallel with *L. innocua* (Kyme *et al.*, 2005). In an independent study, it was shown that *B. henselae* strain Houston-1 enters and stays viable at least for up to 8 h in J774 mouse macrophages (Musso *et al.*, 2001). Therefore, it appears that *B. henselae* persists inside the macrophages. Although these bacteria do not apparently gain anything *per se*, as the BCVs eventually fuse with the lysosomes (Kyme *et al.*, 2005), it can be speculated that the invaded bacteria program the paracrine angiogenic loop of macrophages to benefit the common good of the rest of the bacterial population that colonizes the vascular endothelium. Interestingly, it has been reported that *B. henselae* inhibits apoptosis of human macrophage-like MonoMac 6 cells in an NF- κ B-dependent manner (Kempf *et al.*, 2005a). This indicates that the bacterium might be able to prolong the lifespan of the angiogenically programmed macrophages. The mechanism how *Bartonella* spp. could activate VEGF production in macrophages remains elusive but most likely is analogous to the apparent oxygen-dependent activation of HIF-1 in infected HeLa cells (Kempf *et al.*, 2005b).

Leukocyte homing to the site of infection

Monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant for monocytes and macrophages to home to areas of bacterial inflammation (Melgarejo *et al.*, 2009). As an example, *E. coli* LPS is an extremely potent inducer of MCP-1 expression and secretion in HMECs, and this proceeds in an NF- κ B-dependent manner (McCord *et al.*, 2005). LPS of *B. henselae* does not activate MCP-1

expression in the same cells (McCord *et al.*, 2005), which is in accordance with the observation that the purified LPS from *B. henselae* is 1000–10 000-fold less active than the purified LPS from *S. enterica* sv Friedenau in activating TLR4 signaling (Zähringer *et al.*, 2004). However, intact *B. henselae*, in particular its OMPs, activate MCP-1 expression and secretion in an NF- κ B-dependent manner (Kempf *et al.*, 2005a, b; McCord *et al.*, 2005). The physiological significance of these findings in the *B. henselae*-triggered vascular deformations remains open because MCP-1 expression and secretion is expected to take place by any given bacterial infection (Melgarejo *et al.*, 2009). However, the unusual location of the chronic *Bartonella* spp. infections in vascular ECs could position the migrating monocytes and macrophages to favor the paracrine angiogenic loop mediated by these phagocytic cells.

PMNs are actively recruited to the site of microbial infection (Borregaard, 2010), which in the first place involves sequential establishment of receptor–ligand interactions between the activated ECs and circulating PMNs. ECs react to inflammatory cytokine stimulus and upregulate the expression of receptors such as E-selectin that is recognized by the constitutively expressed adhesins on PMNs resulting in PMN rolling on the surface of ECs. Induction of E-selectin expression has been observed in *B. henselae*-infected HUVECs in an NF- κ B-dependent manner (Fuhrmann *et al.*, 2001). The key molecules mediating subsequent firm adhesion of neutrophils to the activated endothelium are the β 2 integrins, present on PMNs, and their ligands, members of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2, present on the ECs. Induction of ICAM-1 expression has been observed in *B. henselae*-infected HUVECs in an NF- κ B-dependent manner (Fuhrmann *et al.*, 2001; Maeno *et al.*, 2002). Moreover, this upregulation appears to be mediated primarily by VirB/VirD4-T4SS (Schmid *et al.*, 2004) by a yet unknown Bep(s), although contribution of VirB/VirD4-T4SS independent factor(s), possibly OMPs as proposed by Fuhrmann *et al.* (2001), is clear (Schmid *et al.*, 2004). In accordance with the detected upregulation of ICAM-1 and E-selectin of *B. henselae*-infected ECs, it has been demonstrated that PMN rolling and adhesion (≥ 30 s of stable contact) to ECs is significantly increased upon *B. henselae* infection (Fuhrmann *et al.*, 2001). At first glance, PMN homing to the inflamed vasculature appears only detrimental for *B. henselae*. However, PMNs are a potent source of VEGF (Gaudry *et al.*, 1997), and VEGF secretion by PMNs has been detected upon *Streptococcus pneumoniae* infection (van Der Flier *et al.*, 2000) as well as it seems to be present in high concentrations in cerebrospinal fluid in bacterial meningitis (van Der Flier *et al.*, 2000; van der Flier *et al.*, 2001). Perhaps *B. henselae* triggers VEGF secretion also by PMNs and

engage an additional cell type to its paracrine angiogenic loop.

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