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 α2-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. vinsonii, (Breitschwerdt et al., 1995; Chang et al., 2000; Roux et al., 2000; Breitschwerdt et al., 2009; Schaefer et al., 2011) Bartonella vinsonii ssp. berkhouffii, (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 abacata, (Birtles et al., 1995; Bown et al., 2004; Engbaek & Lawson, 2004; Marie et al., 2006) Bartonella taylorii, (Birtles et al., 1995; Kerkhoff et al., 1999; Koebling 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; Marie et al., 2006; 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; Marie 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; Marie et al., 2006; Telfer et al., 2007) Bartonella dushiae, (Bermond et al., 2000; Engbaek & Lawson, 2004; Reis et al., 2011) Bartonella birtlesii, (Chomel et al., 1999b; 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, (Bernard et al., 2002; Chung et al., 2004; Pérez et al., 2011) Bartonella bovis, (Maillard et al., 2004) Bartonella chomelli, (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.

<table>
<thead>
<tr>
<th>Arthropod vector</th>
<th>Mammalian reservoir host</th>
<th>Clinical symptoms in human</th>
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</thead>
<tbody>
<tr>
<td>Flea</td>
<td>Vole, mouse</td>
<td>Endocarditis, epiphlebitis hemangioendotheloma</td>
</tr>
<tr>
<td>Flea</td>
<td>Fox, coyote, dog</td>
<td>Endocarditis, bacteremia with fever</td>
</tr>
<tr>
<td>Flea</td>
<td>Dog, mouse</td>
<td>Endocarditis, bacteremia</td>
</tr>
<tr>
<td>Flea</td>
<td>Rabbit</td>
<td>Endocarditis, lymphadenopathy</td>
</tr>
<tr>
<td>Flea</td>
<td>Vole, mouse</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>Flea</td>
<td>Vole, mouse</td>
<td>Endocarditis, neuroretinitis</td>
</tr>
<tr>
<td>Flea, mouse</td>
<td>Rat, mouse</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>Flea, mouse</td>
<td>Rat, mouse</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>Flea, cat</td>
<td>Cat, dog</td>
<td>Endocarditis, epiphlebitis hemangioendotheloma</td>
</tr>
<tr>
<td>Flea</td>
<td>Cat, dog</td>
<td>Lymphadenopathy</td>
</tr>
<tr>
<td>Flea</td>
<td>Mouse, shrew</td>
<td>Torulosis</td>
</tr>
<tr>
<td>Flea</td>
<td>Dog, fox, raccoon</td>
<td>Carrion’s disease (oraya fever and verruga peruana)</td>
</tr>
</tbody>
</table>

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 (Ctenocephalides felis) 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

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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 (Maguiná 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; Guitiull et al., 1997; O’Reilly et al., 1999; Pappalardo et al., 2000; Bou-louis 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 efficiently 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; Guitiull et al., 1997; O’Reilly et al., 1999). Bartonella henselae infection

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

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description with key reference(s)</th>
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<tbody>
<tr>
<td>LPS</td>
<td>Weak TLR4 agonist (Zähringer et al., 2004)</td>
</tr>
<tr>
<td>Flagella</td>
<td>Weak TLR5 agonist, important in bacterial motility and possibly in bacterial adhesion to the erythrocytes (Andersen-Nissen et al., 2005)</td>
</tr>
<tr>
<td>BadA, BrpA, VompA, VompB, VompC, VompD</td>
<td>TAAs induce auto-aggregation, antiphagocytic properties, bind multiple ECM components, mediate cell adhesion, essential for intraerythocytic bacteremia by B. tribocorum (BadA), B. quintana (Vomps), and B. birtlesii (BrpA), required in colonization of the primary niche and/or in seeding of the bacteria from the primary niche into the bloodstream (Riess et al., 2004; Zhang et al., 2004; Gilmore et al., 2005)</td>
</tr>
<tr>
<td>Pap31</td>
<td>Fibronectin-binding adhesin, also known as hemin-binding protein A (HbpA) (Dabo et al., 2006a, b)</td>
</tr>
<tr>
<td>Hemolysin</td>
<td>Hemolysin of B. bacilliformis, proposed to mediate erythrocyte lysis in the end of Oroya fever (Hendrix, 2000)</td>
</tr>
<tr>
<td>VirB/VirD4–T4SS</td>
<td>Type IV secretion system that mediates by its BepB effector proteins (BepA-G) the invasome-mediate uptake of B. henselae into ECs and inhibition of EC apoptosis, essential for intraerythocytic bacteremia by B. tribocorum and B. birtlesii (Seubert et al., 2003)</td>
</tr>
<tr>
<td>Trw–T4SS</td>
<td>Type IV secretion system that mediates the erythrocyte adhesion of Bartonella spp., essential for intraerythocytic bacteremia by B. tribocorum and B. birtlesii (Seubert et al., 2003)</td>
</tr>
<tr>
<td>Deformin</td>
<td>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 &amp; Iler, 2001)</td>
</tr>
<tr>
<td>lalAB</td>
<td>Involved in the invasion of the erythrocytes, lalAB is similar to Ail invasion of Y. enterocolitica, lalAB is a nucleoside polyphosphate hydrolase of the MutT motif family, both proteins are required for the erythrocyte invasion, essential for intraerythrocytic bacteremia by B. tribocorum and B. birtlesii (Mitchell &amp; Minnick, 1995)</td>
</tr>
<tr>
<td>Hbp8–HbpE</td>
<td>A family of hemin-binding proteins, some members are essential for intraerythrocytic bacteremia by B. tribocorum and B. birtlesii (Carroll et al., 2000; Minnick et al., 2003b)</td>
</tr>
<tr>
<td>HuT1A</td>
<td>Hemin receptor, essential for intraerythrocytic bacteremia by B. tribocorum and B. birtlesii (Parrow et al., 2009)</td>
</tr>
<tr>
<td>Omp43</td>
<td>Putative adhesin, recombinant Omp43 binds ECs (Burgess &amp; Anderson, 1998)</td>
</tr>
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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. 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 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 (Schülein 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 × 10⁶ 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 $3.5 \times 10^7$ 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 (Maguin˜a 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-Rag$^{1/-}$). 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 birlesii* (Boulos 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 (Maguin˜a 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 $1 \times 10^5$ 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 receptors (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 *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-1β (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

![Diagram of Gram-negative bacterium with LPS, LPS-LBP complex, TLR4/CD14/MD2 complex, pro-inflammatory cytokines, NF-κB, O-chain, Core, Lipid A, and Outer membrane.]

**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→4)-linked 3-deoxy-c-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 claridgeiae (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-κB (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. claridgeiae 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-1β 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 (Schulke 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/glucine 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 (Martin-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
Mechanisms of Bartonella spp. stealth pathogen persistence

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-diacylglycerol-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 (Akkesson 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 & Wissman, 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-dependent 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 expresses a large OMP, BrpA, with similarity to Vomps and BadA (Gilmour 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-α5 and anti-β1 integrin monoclonal antibodies decreased the invasion of the cells by B. bacilliformis, suggesting a possible role of α5β1-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-β1A) cells that express β1-integrins than to the parental β1-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* (Schulzein et al., 2001) and mouse-*B. grahamii* (Koesling et al., 2001) natural reservoir host models. As an example, after intravenous inoculation of 3.5 × 10⁷ 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 (Schulzein 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 (10⁴–10⁶ 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 Carrión’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 (Maguïña 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 (Maguïña et al., 2009). EC invasion by *B. bacilliformis* has been reproduced *in vitro* by several investigators (García 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 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 glycoporphin 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 bacterial 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 (Billetter *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; Schulein *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,
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 T4SSs (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).
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 Vomps 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 loose-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 (Maguin˜a et al., 2009). Bartonella bacilliformis is so far the only known species of Bartonella spp. that cause deleterious effects for the infected erythrocytes (Maguin˜a 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, \textit{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 \textit{B. bacilliformis} and the human erythrocyte.

Recently, \textit{in vitro} adhesion and invasion assays with isolated erythrocytes that reproduce the host specificity of erythrocyte infection as observed \textit{in vivo} have been reported for mouse-specific \textit{B. birtlesii}, human-specific \textit{B. quintana}, and cat-specific \textit{B. tribocorum} (Vayssier-Taussat et al., 2010). Moreover, STM in the intravenous \textit{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 \textit{in vitro}. The corresponding genes encode components of the IV secretion system (T4SS) Trw (Seubert et al., 2003), demonstrating that this virulence factor is involved in the adherence of \textit{B. birtlesii} to the erythrocytes. Moreover, ectopic expression of the Trw–T4SS of rat-specific \textit{B. tribocorum} in cat-specific \textit{B. henselae} or human-specific \textit{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 \textit{B. henselae} promoter that is significantly upregulated during the infection of ECs (Seubert et al., 2003). In the \textit{B. tribocorum}-rat model, mutant deleted for the \textit{virB10}-like \textit{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 \textit{Bartonella} spp. toward erythrocytes, because most of the species of \textit{Bartonella} spp. have a genomic copy of Trw–T4SS (Engel & Dehio, 2009). Interestingly, \textit{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 \textit{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 \textit{B. bacilliformis}. One striking feature of \textit{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 \(\sim 1.4\) kDa (Derrick & Ihler, 2001). It has been reported that deformation activity, which is similar to \textit{B. bacilliformis} deformin, is also present in \textit{B. henselae} (Iwaki-Egawa & Ihler, 1997).

The invasion-associated locus (\textit{ial}), composed of \textit{ialA} and \textit{ialB}, is another putative virulence determinant implicated in the erythrocyte invasion by \textit{B. bacilliformis}. The locus was identified in a heterologous expression system where the \textit{ial} locus but not the individual \textit{ialA} and \textit{ialB} genes conferred an invasive phenotype to \textit{E. coli} toward human erythrocytes (Mitchell & Minnick, 1995). The \textit{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 \textit{ialB} gene encodes an 18-kDa protein, which is well conserved (\(\sim 60\%\) amino acid similarity) with the 17-kDa Ail (adhesion and invasion locus) protein of \textit{Yersinia enterocolitica}, which mediate epithelial cell binding and invasion (Leo & Skurnik, 2011). Indeed, the association of isogenic \textit{ialB}-deficient mutant of \textit{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} 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’Brien, 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. claridgeiae, Bartonella elizabethae, Bartonella doshiae, 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 hemin 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 intravascular bac teremia, 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 intravascular 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, strep tococci (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 (\(O_2\)), hydrogen peroxide \(\text{H}_2\text{O}_2\), and hydroxyl radical (\(^\cdot\text{OH}\)) (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 (Regney 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 naturalB. 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 TAs, the Vomp adhesins, had been deleted (Zhang et al., 2004). Vomps are important proteins for B. quintana to establish intraerythrocytic bactemia after an intradermal inoculation in the macaque model (MacKichan et al., 2008) and in vitro mediated 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 bacteraemia 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 intra-erythrocytic bacteraemia (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 trwJIH 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...
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 trwJH 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 \textit{trwl} and/or \textit{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 \textit{trwl} or \textit{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 \textit{Bartonella} spp., at least based on the current \textit{in vitro} and \textit{in vivo} knowledge of Trw-T4SS, BadA, and Vomps. Several unbiased screening studies have been conducted to identify immunogenic surface-exposed proteins in \textit{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 \textit{Bartonella} spp. evade adaptive immunity and persist in the mammalian host.

**Molecular basis of \textit{Bartonella} spp.-triggered vascular tumorigenesis in human**

**Introduction to the \textit{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 \textit{H. pylori}, are also associated with malignant cell transformation (Lax & Thomas, 2002). \textit{Bartonella} spp. and in particular \textit{B. bacilliformis}, \textit{B. quintana}, and \textit{B. henselae} are unique in the bacterial kingdom in causing vasoproliferative tumor formation (Fig. 7). Human infection by \textit{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 (Maguïña 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 \textit{B. henselae} and \textit{B. quintana} (Maguïña 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). \textit{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). \textit{Bartonella} spp.-triggered vascular tumorigenesis has been mainly studied \textit{in vitro} using monolayers of primary human umbilical-vein ECs (HUVECs) with the most detailed knowledge available for \textit{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; Berrick et al., 2011; Scheidegger et al., 2011). Development of an animal model of BA/BP would be essential to define the molecular basis of \textit{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 \textit{Bartonella} spp.-triggered vascular deformations. Each of the three vasoproliferation-inducing \textit{Bartonella} spp. species adheres to and invades ECs \textit{in vitro} (Hill et al., 1992; Brouqui & Raoult, 1996; Dehio et al., 1997). The vascular pathologies could reflect a general tropism of \textit{Bartonella} spp. for the vascular endothelium, that is, the ECs could be the still-enigmatic primary niche (see Identity of the primary niche).

**\textit{Bartonella quintana} and \textit{B. bacilliformis}**

In molecular terms, \textit{B. quintana}–EC interaction is poorly understood. However, it appears that the bacterium is
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-

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 Gαs-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 Gαs 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.
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 & Iler, 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; Kyne *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 β1-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 α5β1, initiates integrin clustering and bacterial internalization into host cells (Agerer *et al.*, 2003). The importance of β1 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 β1 integrin. This indicates that *B. henselae* express direct adhesins for β1 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 (ΔbepA–G) mutant restores the invasome-
mediated uptake. Likewise, ectopic expression of BepG in ECs also restores the invasome-mediated uptake of the Bep-deficient (ΔBepA–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 (ΔBepA–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 (ΔBepA–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/WAVE2/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 Tie2 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 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-κB (Waugh & Wilson, 2008), and accordingly, the authors demonstrate that NF-κB 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 pneu-
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-kB)-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 inhibitor 3-isobutyl-1-methylxanthine or by addition of the nonhydrolyzable and membrane-permeable cAMP analog dibuturyl-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 (Eiklen & 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-kB-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-kB-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 expres-
sion and secretion in an NF-κB-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 expres-
sion 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 in-
teractions between the activated ECs and circulating PMNs.
ECs react to inflammatory cytokine stimulus and upregu-
late 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-κB-dependent
manner (Fuhrmann et al., 2001). The key molecules medi-
sating subsequent firm adhesion of neutrophils to the acti-
vated endothelium are the β2 integrins, present on PMNs,
and their ligands, members of the immunoglobulin super-
family, intercellular adhesion molecule-1 (ICAM-1) and
ICAM-2, present on the ECs. Induction of ICAM-1 expres-
sion has been observed in B. henselae-infected HUVECs in
an NF-κB-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 contribu-
tion 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 signifi-
cantly increased upon B. henselae infection (Fuhrmann
et al., 2001). At first glance, PMN homing to the inflamed
vasculature appears only detrimental for B. henselae. How-
ever, 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 con-
centrations in cerebrospinal fluid in bacterial meningitis
(van der Flier et al., 2000; van der Flier et al., 2001). Per-
haps B. henselae triggers VEGF secretion also by PMNs and
engage an additional cell type to its paracrine angiogenic
loop.

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