Infection of human CD34⁺ progenitor cells with *Bartonella henselae*

results in intraerythrocytic presence of *B. henselae*

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Abstract

Although there is evidence that endothelial cells are important targets for human pathogenic Bartonella spp., the primary niche of infection is unknown. Here we elucidated whether human CD34+ hematopoietic progenitor cells (HPCs) internalize B. henselae and may serve as a potential niche of the pathogen. We showed that B. henselae does not adhere to or invade human erythrocytes. In contrast, B. henselae invades and persists in HPCs as shown by gentamicin protection assays, confocal laser scanning microscopy (CLSM) and electron microscopy (EM). FACS analysis of glycophorin A expression revealed that erythroid differentiation of HPCs was unaffected following infection with B. henselae. The number of intracellular B. henselae continuously increased over a 13-day period. When HPCs were infected with B. henselae immediately after isolation, intracellular bacteria were subsequently detectable in differentiated erythroid cells on day 9 and 13 post infection, as shown by CLSM, EM and FACS analysis. Our data provide for the first time evidence that a bacterial pathogen is able to infect and persist in differentiating HPCs, and suggests that HPCs might serve as a potential primary niche in Bartonella infections.
Introduction

Hematopoietic proliferation and differentiation is sustained by hematopoietic progenitor cells (HPCs) and carries significant medical implications, e.g., bone marrow transplantation upon myeloablative regimens. Human HPCs are defined as self-renewing pluripotent stem cells localized in the bone marrow with a capacity to differentiate into any of at least eight distinct blood cell lineages including erythrocytes, granulocytes, monocytes, megacaryocytes and lymphocytes\(^1\). Although it might be of enormous clinical relevance, not much is known about the interaction of human pathogenic bacteria with HPCs. It was recently shown that quiescent HPCs were resistant to \textit{in vitro} infection with \textit{Listeria monocytogenes}, \textit{Salmonella enteritica} and \textit{Yersinia enterocolitica}, but not when these cells were myeloid or monocytic differentiated\(^3\).

The genus \textit{Bartonella} comprises a unique group of emerging, Gram-negative, facultative intracellular bacteria which can cause long-lasting intraerythrocytic bacteremia and employ hematotropism as a likely parasitic strategy\(^4\). \textit{B. henselae} is the most common cause of cat-scratch disease (CSD) and the vasculoproliferative disorders bacillary angiomatosis (BA) and bacillary peliosis (BP) in humans\(^5\). The closely related species \textit{B. quintana}, which is transmitted via body lice\(^6\), causes “trench fever” (also called “five-day fever”) characterized by periodic feverish relapses due to intraerythrocytic bacteremia\(^7\), and it became known during World War I when more than one million soldiers suffered from this disease. Today, \textit{B. quintana} is a well known cause of fever, bacteremia, and endocarditis in HIV-seronegative, homeless, inner-city patients with chronic alcoholism\(^8,10\). While the hemolytic activity of \textit{B. bacilliformis} causing Carrion’s disease seems to be unique among \textit{Bartonella} spp.\(^5\), prolonged periods of intracellular erythrocyte parasitism appear to be a crucial aspect of the pathogenicity of \textit{Bartonella} spp.\(^11,12\). Although it is known that \textit{Bartonella} spp. invade erythrocytes in their animal reservoir hosts\(^13\), there is, however, no evidence for a direct interaction of \textit{B. henselae} with human erythrocytes\(^14\).

An \textit{in vivo} model with similarities to human “trench fever” has been described, in which rats were infected intravenously with \textit{B. tribocorum} leading to intraerythrocytic presence of bacteria during the
course of infection. Bacteria were first detectable in erythrocytes five days after challenge with a peak between 10 and 14 days post infection. Therefore, it may well be that *Bartonella* spp. are capable of colonizing an as yet unknown primary niche possibly represented by HPCs. This hypothesis is supported by a recent study in which cultivation and immunofluorescence detection of *B. quintana* in bone marrow-derived erythroblasts of homeless people suffering from *B. quintana* infections was reported. Such colonization of erythropoietic lineage would (i) protect *B. quintana* from the host immune response and (ii) explain the periodic bacteraemic relapses in the course of *B. quintana* infections.

Here we describe that *B. henselae* infects freshly isolated human CD34+ HPCs but not human erythrocytes. Infection of HPCs with *B. henselae* resulted in the subsequent detection of intracellular bacteria in differentiated erythroid cells. Our results are the first to demonstrate that a bacterial pathogen infects and persists in HPCs while these cells are differentiating to erythroid cells, and suggest that HPCs might serve as a primary niche in *Bartonella* infections.

**Materials and Methods**

**Bacterial strains and growth conditions**

*B. henselae* strain Marseille was grown on Columbia agar supplemented with 5% defibrinated sheep blood (CBA; Becton Dickinson, Heidelberg, Germany) in a humidified atmosphere at 37°C and 5% CO2. For production of bacterial stock suspensions, bacteria were harvested after five days of culture, resuspended in Luria-Bertani medium containing 20% glycerol and stored at -80°C.

In some experiments *B. henselae* constitutively expressing the green fluorescent protein (GFPmut2) was used. The GFPmut2-encoding plasmid pCD354 (kindly provided by C. Dehio, Basel, Switzerland) was introduced into *B. henselae* by electroporation, and *B. henselae* GFPmut2 was cultivated on CBA containing kanamycin (30 µg/ml), and used in infection experiments with addition of kanamycin (25 µg/µl) to the cell culture medium.
Isolation and purification of human CD34+ cells and erythrocytes

Experiments with human HPCs were performed in agreement with the ethics committee of the University of Tuebingen (ethics proposal 113/2002V of V.A.J.K.). Informed consent was provided according to the Declaration of Helsinki (ethics proposal 113/2002V of V.A.J.K.).

Cells were collected by apheresis following treatment with recombinant human granulocyte-colony-stimulating factor (G-CSF; 2x5 µg/kg per day) of healthy donors. A 4 ml cell suspension of the pilot aliquot was used for positive selection of CD34+ cells. Isolation of human CD34+ HPCs with the Dynal CD34 Progenitor Cell Selection System (Dynal Biotech, Hamburg, Germany) was performed according to the manufacturer’s instructions using a Dynal magnetic particle concentrator (Dynal MPC). To detach magnetic beads from the purified cells, HPCs were incubated with DETACHaBEAD CD34 (Dynal) for 45 min at room temperature (RT). After final washing, the CD34+-enriched cell pellet was resuspended in serum-free StemSpan™ SF Expansion Medium (CellSystems, St. Katharinen, Germany) supplemented with interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (Epo; see below). Cells were analyzed by flow cytometry for the expression of the HPC marker CD34 and the absence of the erythroid marker glycophorin A (GPA). The purity of each HPC preparation was > 95% as assessed by flow cytometry.

For experiments with human erythrocytes, whole blood of a healthy volunteer (V.K.) was diluted in PBS-EDTA (1 mM). Cells were counted in a Neubauer cell counting chamber (Superior, Lausa-Koenigshofen, Germany) and used at a concentration of 1x10^5 erythrocytes per ml.

Culture and infection of CD34+ cells

CD34+ HPCs were cultured in 24-well plates (1x10^5 cells per well) at 37°C and 5% CO2 for 13-16 days. For induction of erythroid differentiation, IL-3 (1 U/ml; R&D Systems, Wiesbaden, Germany), GM-CSF (0.05 U/ml; R&D, Wiesbaden, Germany) and Epo (3 U/ml; Erypo FS10000, Janssen-Cilag, Neuss, Germany) were added to the medium.

For infection experiments, bacterial stock solutions were thawed, washed in PBS (containing 1mM CaCl2 and 0.5 mM MgCl2, pH 7.4) and diluted in antibiotic-free cell culture media to obtain a multiplicity of infection (MOI) of 100 (100,000 HPCs and 1x10^7 bacteria per well). Bacteria were
sedimented onto cultured HPCs by centrifugation at 400 g for 5 min at RT. The actual MOI for each experiment was confirmed by plating serial dilutions of the infection inoculum and calculating the number of CFU.

**Culture and infection of endothelial cells (ECs)**

Human umbilical vein endothelial cell (HUVEC) culture (passages 2-6) was performed in EC growth medium containing EC growth supplement (Promo Cell, Heidelberg, Germany). Infection experiments were performed in EC basal medium (Promocell) as previously described. Briefly, 1x10^5 cells were seeded in 24-well plates (Nunc, Roskilde, Denmark) containing collagen-coated coverslips the day before the experiment and grown to confluency. For infection experiments, bacterial stock solutions were prepared as described above.

**Flow cytometry**

Flow cytometry was used to assess the differentiation of CD34^+ HPCs into glycophorin A (GPA)-expressing erythroid cells. Usually 1-5x10^5 cells resuspended in 100 µl PBS (pH 7.4) were incubated with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated anti-human monoclonal antibodies for 30 min at 4°C. The antibodies used were anti-CD34-PE, anti-CD34-FITC, anti-GPA-PE (BD Biosciences PharMingen, Heidelberg, Germany) and anti-CD29-FITC (Dako, Hamburg, Germany). In each experiment, control groups were stained with irrelevant immunoglobulin G (IgG)-PE and IgG-FITC antibodies for isotype control. After staining, cells were analyzed on a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, Heidelberg, Germany). Data were analyzed using the WinMDI 2.8 software (http://facs.scripps.edu/software.html). Data for 10,000 cells were collected in an appropriate gate to exclude dead cells or artifacts from analysis. The number of CD34^+ and GPA^+ cells was assessed on day 1, 9 and 16 of cultivation, and are expressed as the percentage of the total cell amount.

**Invasion of B. henselae into erythrocytes, ECs and HPCs**
Infection of human CD34+ cells with *B. henselae*

Invasion (2 h) and intracellular presence (erythrocytes and ECs: day 1, day 2; HPCs: day 1, day 6, day 13) were determined by gentamicin protection assays. For this purpose, gentamicin (100 µg/ml) was added to the medium for 3 h to kill extracellular bacteria. Cells were then washed extensively and lysed by the addition of 900 µl of distilled water for 5 min, followed by equilibration with 100 µl of 10x PBS. Lysates were serially diluted, plated on CBA, and colony forming units (CFU) were counted after three weeks of incubation due to the slow growth of *B. henselae*.

**Determination of VEGF and IL-8 secretion by HPCs**

To quantify the secretion of vasculoendothelial growth factor (VEGF) and interleukin (IL)-8 by *B. henselae*-infected HPCs, cells were cultured without antibiotics to allow bacterial growth and without FCS to avoid non-specific VEGF secretion. Supernatants were taken five days later, centrifuged to remove insoluble particles (10 min, 4°C, 20,000 g) and frozen at -20°C. VEGF concentration was measured using a human VEGF165-ELISA kit according to the manufacturer’s instructions (Quantikine, R&D Systems). IL-8 was determined by ELISA as previously described.

**Fluorescence in situ hybridization of *B. henselae***

Fluorescence in situ hybridization (FISH) of bacteria on glass slides was performed as previously described. The universal eubacterial oligonucleotide probe EUB-338 (GCT GCC TCC CGT AGG AGT) synthesized and 5'-labeled with the fluorochrome Cy-3 (Metabion, Munich, Germany; red signal) was used, and the complementary control probe NON-338 (CGA CGG AGG GCA TCC TCA) was implemented to exclude non-specific binding of the probes (data not shown). Coverslips with ECs, or polysine-covered adhesion microscope slides (Multimed Wicker, Kirchheim/Teck, Germany) with 5x10^5 infected CD34+ HPCs, were each analyzed by confocal laser scanning microscopy (CLSM).

**Immunostaining and confocal microscopy**

ECs (1x10^5) were seeded onto coverslips, HPCs or erythroid differentiated cells were centrifuged onto glass slides as described above. Infection (MOI 100) with *B. henselae* or *B. henselae gfp mutant* was
stopped by fixation in 3.75% PBS-buffered paraformaldehyde (PFA). For differential staining of intracellular and extracellular *B. henselae*, cells were sequentially incubated with blocking solution (0.1% BSA in PBS) for 15 min, rabbit polyclonal anti-*B. henselae* Marseille antibodies for 1 h, FITC-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) for 1 h, 0.1% Triton X-100 in PBS (ECs 15 min RT, HPCs 5 min on ice), blocking solution for 15 min, rabbit anti-*B. henselae* antibodies for 1 h, indodicarbocyanine (Cy5)-conjugated goat anti-rabbit IgG (Dianova) for 1 h and tetramethylrhodamine-isothiocyanate (TRITC)-labeled phalloidin (Sigma) for 1 h resulting in Cy5-labeled intracellular (blue) and FITC- and Cy5-labeled extracellular (green) bacteria. If *B. henselae* *gfp*mut2 was used, cells were not permeabilized, and extracellular bacteria were stained with rabbit anti-*B. henselae* antibodies and Cy5-conjugated goat anti-rabbit IgG, resulting in GFP-labeled intracellular (green) and GFP-and Cy5-labeled extracellular (blue) bacteria. GPA expression on the cell surface was stained using PE-labeled anti-GPA antibodies.

Samples stained for immunofluorescence were viewed with a Leica DM IRE 2 confocal laser scanning microscope (Leica, Bensheim, Germany). Three different fluorochromes could be detected representing the green (FITC or *gfp*), red (TRITC or PE) and blue (Cy5) channels. DAPI (4',6-diamidino-2-phenylindole) staining (1 µg/ml; 5 min) was visualized on a fourth channel (light blue). Fluorescence images were acquired sequentially to avoid non-specific channel interference. Images were digitally processed with Photoshop 7.0 (Adobe Systems, Mountain View, CA).

**TUNEL assay**

Nuclear changes associated with early apoptosis were detected by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) method using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany). Uninfected and infected (MOI 100) HPCs were centrifuged onto glass slides as described above. Cells were fixed in 3.75% PBS-buffered PFA and permeabilized with 0.1% Triton X-100 dissolved in 0.1% sodium citrate. Enzymatic incorporation of fluoresceinated nucleotides was performed according to the manufacturer’s instructions. Subsequent immunostaining of *B. henselae* was performed with rabbit anti-*B. henselae* antibodies and TRITC-conjugated goat anti-rabbit IgG (Dianova). Nuclei and bacteria were stained with DAPI. The
percentage of TUNEL-positive or *B. henselae*-infected cells was determined by counting 200 cells over at least 20 random microscopic fields.

**Transmission electron microscopy (TEM) and immunoelectronmicroscopy (IEM)**

TEM was performed as previously described. For IEM of *B. henselae*, post-embedding immunogold labeling was carried out. Infected cells were fixed and centrifuged, and the resulting pellet was embedded in 3% agarose at 37°C and cooled on ice. Small parts of the agarose blocks were embedded in Lowicryl (Polysciences, Eppelheim, Germany). Ultra-thin sections (50 nm) were mounted on Formvar-coated nickel grids and incubated with anti-BadA rabbit serum followed by 10 nm gold-conjugated goat anti-rabbit IgG (Auroprobe EM, Amersham). In control samples the primary antibodies were omitted. Samples were examined using a Zeiss EM 9 transmission electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV.

**Statistical analysis**

All experiments were performed at least three times with cells from different donors and revealed comparable results. Differences between mean values of experimental and control groups were analyzed by the paired Student's *t*-test. A value of *P* < 0.05 was considered statistically significant.

**Results**

1. *B. henselae does not adhere to or invade human erythrocytes*

First we wanted to analyze whether *B. henselae* is internalized by human erythrocytes. For this purpose, human erythrocytes were infected with *B. henselae* for up to 48 h and invasion was compared with results obtained from ECs at 2, 24 and 48 h post infection. By means of confocal microscopy, no bacterial adherence or invasion of erythrocytes by *B. henselae* was observed. In contrast, however, high numbers of *B. henselae* were located on the surface of ECs and intracellularly (Fig. 1A). Similarly, data from gentamicin protection assays quantifying the number of intracellular *B. henselae,*
revealed comparable results (Fig. 1B). Based on our data, we conclude that, in contrast to ECs, B. henselae does not invade human erythrocytes.

2. Infection of HPCs by B. henselae results in intracellular presence

We next investigated the interaction of HPCs with B. henselae. Human CD34+ HPCs and ECs were infected and the number of adherent and internalized bacteria was compared (Fig. 2A). Data revealed that B. henselae adheres to and invades HPCs to a similar extent as shown for ECs, which have already been described to be efficiently infected by B. henselae \(^{19,26}\). Consistent results were obtained by double-immunofluorescence staining and CLSM analysis showing intra- and extracellular B. henselae in both HPCs and ECs (Fig. 2B). Moreover, we investigated whether cocultivation of B. henselae with CD34+ HPCs resulted in increased levels of ribosomal RNA indicating bacterial growth, as this phenomenon has been described for B. henselae cocultivated with ECs \(^{20}\). For this purpose, B. henselae 16s-rRNA was detected via FISH upon cocultivation with ECs or HPCs. An intense red fluorescence 24 h after infection showed an increase in the production of B. henselae rRNA (Fig. 2C). Ultrastructurally, the infection process was by analyzed by TEM and IEM (Fig. 2D,E) revealing that adhesion was accompanied by membrane ruffling, and was followed by internalization of bacteria which were detectable in vacuoles. Taken together, these data show that B. henselae adheres to and invades HPCs, and upregulates production of 16S-rRNA when cocultivated with HPCs, indicating that HPCs are efficiently infected by B. henselae.

As it has recently been described that B. henselae binds to host cells via beta-1 integrins \(^{22}\), we wanted to elucidate the role of beta-1 integrins in the infection process of HPCs. Therefore, expression of CD29 on human CD34+ HPCs and erythrocytes was analyzed via flow cytometry revealing that CD29 is present on HPCs but not on erythrocytes (Fig. 3). These data suggest that beta-1 integrin expression is crucial for host cell infection by B. henselae.

3. B. henselae affects proliferation but not erythroid differentiation of HPCs
We analyzed the proliferation and erythroid differentiation of CD34+ HPCs upon infection with *B. henselae*. Proliferation of HPCs was investigated over a 9-day period by counting total cell numbers at day 1, 3, 6 and 9. The number of proliferating HPCs was increased (5.2-fold) in those infected by *B. henselae*, although it was significantly lower (~80% reduction) when compared with uninfected control cells (22.5-fold increase; Fig. 4A). At later time points (day 13 and 16) the number of viable cells decreased, indicating cell death in both infected and uninfected cells (data not shown).

To investigate whether the erythroid differentiation of HPCs is influenced by *B. henselae*, HPCs were infected and expression of the differentiation markers CD34 (HPCs) and GPA (erythroid cells) was analyzed by flow cytometry at day 1 and 9 post infection (Fig. 4B, C). At day 1, ~90% of the analyzed cells expressed CD34 indicating that the majority of the infected cells were HPCs. When incubated for 9 days, FACS analysis revealed that CD34 expression was significantly reduced in both uninfected (5%) and *B. henselae*-infected (1%) cells, but expression of GPA was strongly induced (uninfected: 92%; *B. henselae*-infected: 88%). These results were highly reproducible in all experiments performed (Fig. 4D, E). Similar results were obtained at day 16 of cultivation (data not shown). A further indication of erythroid differentiation was the shifting of the colour of the cultured cell pellet from white (d1) to red (d9, data not shown) in both uninfected controls and *B. henselae*-infected cells. We conclude, that although proliferation of HPCs is diminished when infected with *B. henselae*, bacterial infection does not affect erythroid differentiation of HPCs.

4. Infection of HPCs results in intracellular presence of *B. henselae* in erythroid differentiated cells

To investigate whether infection of naive HPCs with *B. henselae* results in the intracellular persistence of the pathogen in erythroid differentiating cells, HPCs were infected immediately after isolation, differentiated towards erythrocytes, and intracellular presence of *B. henselae* was assessed by FACS analysis, CLSM and TEM. Nine or sixteen days post infection, cells were analyzed by FACS for expression of GPA, revealing that ~80% had undergone erythroid differentiation (data not shown). Intracellular *B. henselae gfpmut2* (green) were detected via FACS (Fig. 5A) in 25% (d9) and 41% (d16) of all cells. From GPA+ cells, 13% (d9) and 18% (d16) contained intracellular *B. henselae gfpmut2* (data
not shown). These data were consistent with those obtained by CLSM showing a clear colocalization of GPA+ cells and *B. henselae gfp mut2* (**Fig. 5B**). Intracellular bacteria were also detectable via TEM (**Fig. 5C**). From these data, we conclude that following initial infection of HPCs, *B. henselae* persists intracellularly while these cells are differentiating towards erythrocytes.

5. **Infection of HPCs with *B. henselae* results in intracellular replication**

To analyze whether infection of HPCs with *B. henselae* leads to an increase of intracellular bacteria in erythroid differentiating cells, freshly isolated HPCs were infected and cultivated for 13 days. The amount of intracellular bacteria was quantified by gentamicin protection assays and was calculated as the relative amount of re-isolated bacteria. As demonstrated in **Fig. 6**, the number of *B. henselae* continuously increased (d6: 5.4-fold, d13: 23.1-fold; total intracellular bacteria at d1: 337±185, d6: 1833±388, d13: 7790±834) indicating that *B. henselae* persists and replicates intracellularly during erythroid differentiation of HPCs.

6. **Infection of HPCs with *B. henselae* influences cytokine secretion but not viability**

*B. henselae* inhibits apoptosis in ECs 27,28 and monocytes 29. Therefore, we investigated whether infection with *B. henselae* results in apoptosis of HPCs. HPCs were infected with *B. henselae* and the rate of apoptotic cell death was quantified 48 h after infection by TUNEL reaction and CLSM (**Fig. 7A**). Our data showed that infection of HPCs with *B. henselae* did not result in an increased rate of apoptotic cell death (uninfected: 1.3% apoptotic cells; *B. henselae*-infected: 0.5%; data calculated from counting 200 cells, for details see Material and Methods).

As it is known that *B. henselae* triggers secretion of VEGF and IL-8 upon infection of various host cells 19,28,30,31, we wanted to elucidate whether *B. henselae* would influence the secretion of these cytokines in HPCs. ELISA of culture supernatants taken five days post infection from *B. henselae*-infected HPCs (**Fig. 7B**) revealed that IL-8 secretion was strongly increased (11.3-fold) compared to uninfected control cells, whereas secretion of VEGF was significantly decreased (3.3-fold). Therefore, cytokine secretion of HPCs is modulated during *B. henselae* infection although cell viability is not affected.
Discussion

HPCs (erythroblasts, megacaryocytes and myeloid progenitors) located in the bone marrow are the source of erythrocytes, platelets and granulocytes. This highly important compartment of the body establishes the functional base for oxygen supply to the body, for clotting and for eradication of pathogens. The role of HPCs has been widely investigated in hematology where these cells are used for bone marrow transplantation. However, the interaction of bone-marrow derived human stem cells with human pathogens has only rarely been analyzed.

*Bartonella* spp. cause several human diseases including trench fever, CSD, BA and BP, and other manifestations. However, in all of these infections, the primary niche of the pathogens remains unclear, although *Bartonella* spp. are capable of infecting a wide variety of different host cells. In particular, it has been shown that *B. henselae* infects endothelial cells, epithelial cells and monocytes or macrophages, and similar findings have been reported for *B. quintana*.

Intraerythrocytic presence of human pathogenic *Bartonella* spp. has been demonstrated several times *in vitro* by fluorescence microscopy using anti-*Bartonella*-antibodies. It has already been assumed, that a potential primary niche in *Bartonella* infections might be represented by HPCs. Consistent with this suggestion, there are currently two case reports describing immunofluorescence detection of *Bartonella* in erythroblasts from bone marrow aspirate. In addition, a rat animal model of Trench fever using *B. tribocorum* demonstrated that the pathogens appear intraerythrocytically in the bloodstream four to five days upon infection, and similar observations were made using a *B. grahamii* mouse-infection model. Moreover, *B. henselae* and *B. koehlerae* have been detected in erythrocytes of naturally infected cats.

The primary intracellular niche in which the pathogen is present and suggested to replicate within, is however, still unknown. A possible habitat might be represented by hematopoietic stem cells for several reasons, including: (i) *Bartonella* spp. remain undetectable in rat and mouse models for several
days upon experimental infection, (ii) these pathogens are detected intraerythrocytically during the course of infection and (iii) *Bartonella* can be detected in HPCs in human infections. These observations strengthen the hypothesis that hematopoietic stem cells are a possible primary niche for *Bartonella*, and it might be hypothesized that HPCs represent a sanctuary for chronic *Bartonella* infections responsible for recurrent intraerythrocytic bacteremia. These suggestions are consistent with our observations that co-culture of human erythrocytes did not result in intraerythrocytic presence of *B. henselae* (Fig. 1).

The interaction of human pathogenic bacteria with HPCs has been described only rarely. It was shown that quiescent human HPCs are fully resistant to infections with *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica*, whereas these pathogens are taken up in a vacuolic compartment when HPCs were differentiated towards myeloid or monocytic cells. Moreover, it was shown that infection with these bacteria accelerated the maturation of HPCs along the myeloid lineage. Accordingly, human granulocytic ehrlichiosis (HGE) which is mainly characterized by cytopenia, also affects HPCs. CD34+ primary human bone marrow cells, stimulated to differentiate along myelomonocytic lineages, supported the replication of *Anaplasma phagocytophilum* (the agent of HGE), suggesting that HPCs represent potential target cells in this infection in vivo.

We showed microscopically and via gentamicin protection assays that *B. henselae* does not infect erythrocytes within the first 48 h of infection (Fig. 1). In contrast, *B. henselae* was able to infect HPCs to a similar extent as ECs, which represent one of the most likely primary target for *Bartonella* infections (Fig. 1, 2). These data strongly support the hypothesis that erythrocytes do not function as the primary target in *B. henselae* infections. These observations might be explained by the fact that (i) *B. henselae* binds to host cells via beta-1 integrins and (ii) beta-1 integrins are expressed on HPCs but not on mature erythrocytes (Fig. 3 and 4).

Moreover, we demonstrated by confocal and electron microscopy and flow cytometry that infection of HPCs with *B. henselae* results in bacterial presence in differentiated erythroid cells. It would seem
likely that a necessary prerequisite of *B. henselae* is to be able to avoid host cell death upon infection. In fact, we did not detect a significant number of apoptotic progenitor cells upon *B. henselae* infection as shown by TUNEL staining (Fig. 7). This is consistent with previous observations that *B. henselae* inhibits apoptosis of monocytes and ECs \(^{27,29}\), most likely via the virB type IV secretion system \(^{28}\).

Additionally, we found that the amount of *B. henselae* 16S-rRNA was clearly elevated indicating (i) metabolic activity of *B. henselae* and (ii) bacterial growth \(^{20,43}\). Consistent data were obtained from gentamicin protection assays, which indicate replication of *B. henselae* in erythroid differentiating HPCs over an incubation period of 13 days (Fig. 6). Taken together, these data strongly suggest that *B. henselae* persists and replicates within the host progenitor cells during differentiation of these cells towards erythrocytes.

The extent of proliferation of *B. henselae*-infected HPCs was, however, significantly lower compared with uninfected control cells. The mechanisms involved in such impaired cell proliferation of *B. henselae*-infected HPCs cannot be explained by apoptotic cell death since apoptosis was not induced upon infection (Fig. 7). It was described that adenosine triphosphate (ATP) induces proliferation of HPCs via P2 receptors \(^{44}\). Proliferation of differentiating HPCs might be affected by the facultative intracellular *B. henselae*, as bacterial presence is likely to result in increased metabolic demands of the host cells in order to cope with the bacterial infection. According to this suggestion, it has recently been shown that infection with *B. henselae* results in ATP deprivation in several host cells (\(^{31}\) and Hartmann & Kempf, unpublished data). Therefore, it can be speculated that such ATP depletion may be responsible for the impaired proliferation of HPCs when infected with *B. henselae*. Nevertheless, differentiation of HPCs into erythroid cells, quantified by FACS, was similar for uninfected and *B. henselae*-infected HPCs with strong GPA expression in both cases (Fig. 4).

Infection of several types of host cells with *B. henselae* results in the secretion of IL-8 and VEGF \(^{19,22,29-31}\). We observed that high amounts of IL-8 were secreted from HPCs when infected with *B. henselae*, but surprisingly, in contrast to our earlier reports, the secretion of VEGF was reduced in HPCs (Fig. 7). IL-8 has a strong mobilizing effect on HPCs, leading to the spreading of these cells in
the bloodstream. In terms of *Bartonella* pathogenicity, it might be suggested that IL-8 secretion leads to propagation and systemic spread of the latent infection from the bone marrow. The mobilized HPCs could carry the pathogen to other sites of the body leading to subsequent infections in other organs, e.g., the endothelium. Therefore, it might be speculated that infected HPCs may function as a vehicle to carry *Bartonella* spp. to the endothelial site where the vasculoproliferative disorders BA and PH are initiated. The fact that VEGF secretion is diminished in HPCs upon *B. henselae* infection, in contrast to most other cells, remains unclear.

HPCs, which differentiate into erythroid cells, have never been analyzed for their capacity to interact with human pathogens. Moreover, it has never been described that bacterial pathogens persist in stem cells while these cells are undergoing differentiation, e.g., towards erythrocytes. Our *in vitro* data provide evidence that infection of HPCs with human pathogenic bacteria (e.g., *B. henselae*) results in the presence of bacteria in differentiated cells (here: erythrocytes). These observations might suggest a newly described pathogenicity strategy of bacteria, in which the infection of human progenitor cells results in the spread of the bacteria via the differentiated cells.

5. Acknowledgements

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References


Figure legends

**Fig. 1: Interaction of *B. henselae* with endothelial cells and erythrocytes.** (A) CLSM of adherent and intracellular *B. henselae* in endothelial cells (ECs, upper panel) and erythrocytes (lower panel; each 100,000 cells, respectively). Extracellular bacteria (green signal), intracellular bacteria (blue signal) and filamentous actin (red signal). (B) The number of intracellular bacteria was determined by gentamicin protection assays. * Significant difference compared to ECs (*P* < 0.01).

**Fig. 2: Interaction of *B. henselae* with ECs and CD34+ HPCs.** (A) Adherence and invasion rates of *B. henselae* obtained with CD34+ HPCs and ECs (each 100,000 cells, respectively). The number of adherent *B. henselae* was assessed 30 min post infection and invasion of *B. henselae* was quantified after 2 h. (B) Detection of adherent and intracellular *B. henselae* by CLSM 24 and 72 h post infection (left: ECs; right: HPCs). Extracellular bacteria (green signal), intracellular bacteria (blue signal) and filamentous actin (red signal). (C) Detection of *B. henselae* by FISH 24 h after infection of ECs and HPCs. Overlay of FISH using a universal eubacterial oligonucleotide probe (EUB338-Cy-3, red signal) and DAPI staining (light blue) of the host cell nucleus. (D) TEM of HPCs 24 h after infection with *B. henselae* (arrows). Membrane ruffling (2,3) can be observed following adherence (1) of the bacteria to the host cells. Intracellular bacteria are located in vacuoles (4). (E) Detection of *B. henselae* by IEM using anti-BadA- and 10 nm gold-conjugated goat anti-rabbit IgG antibodies. The enlargement illustrate the interaction of *B. henselae* with HPCs by immunogold-staining of *B. henselae* (see arrows).

**Fig. 3:** Beta-1-integrin (CD29) expression on HPCs and erythrocytes. CD29 expression of freshly isolated HPCs (middle) and erythrocytes (right) was determined by FACS analysis. 20,000 cells were analyzed and the number of CD29+ cells is given as the percentage of the total number of cells. All stainings were referred to isotype-matched control antibodies (left).
Fig. 4: Proliferation and differentiation of *B. henselae*-infected HPCs. (A) Proliferation of HPCs upon infection with *B. henselae*. Cells were seeded in 24-well plates and total numbers of cells were counted at day 1, 3, 6 and 9. * Significant difference compared to day 1 (*P* < 0.05). (B, C) Flow cytometric analysis of erythroid HPC differentiation. CD34 and GPA expression of uninfected and *B. henselae*-infected HPCs (MOI 100) were determined by FACS analysis on day 1 and 9 post infection. 10,000 cells were analyzed per time point. The number of CD34+ (HPCs) and GPA+ (erythroid) cells is expressed as a percentage of the total number of cells. All stainings were referred to isotype-matched control antibodies (see Material and Methods). (D, E) CD34 and GPA expression of uninfected and *B. henselae*-infected HPCs on day 1 and 9 post infection. The graphs show the means and standard deviations of the percentage of CD34+ and GPA+ cells obtained from three independent experiments.

Fig. 5: Detection of intracellular *B. henselae* in differentiated erythroid cells. Freshly isolated HPCs were infected with *B. henselae* gfp mut2 (MOI 100) and subsequently cultivated for 16 days. (A) Flow cytometric detection of intracellular *B. henselae* gfp mut2 (green fluorescence, x-scale) in erythroid cells 9 days (left) and 16 days (right) post infection (control: uninfected erythroid cells). Data for 50,000 cells per time point were analyzed. Percentage shown in histogram analysis refer to *B. henselae* gfp mut2-infected cells. In total, 25% of all cells at day 9 and 41% at day 16 were positive for intracellular *B. henselae*. (B) Detection of intracellular *B. henselae* gfp mut2 (green) by CLSM 9 (left) and 16 (right) days post infection. Cells are counterstained by GPA (red signal). Scale bar: 20 µm. (C) TEM of an erythroid differentiated cell (day 9) containing intracellular *B. henselae*. The enlargement illustrate that *B. henselae* is located in a vacuolic compartment in differentiated erythroid cells.

Fig. 6: Quantification of intracellular *B. henselae* in erythroid differentiating HPCs. Freshly isolated HPCs were infected with *B. henselae* (MOI 100) and gentamicin protection assays were performed on day 1, 6 and 13 post infection. The number of intracellular bacteria (% of inoculum) was calculated by counting the CFU on CBA after three weeks of incubation. * Significant difference compared to day 1 (*P* < 0.01).
Fig. 7: Resistance to apoptosis of HPCs upon *B. henselae* infection and cytokine secretion. (A) Detection of apoptosis in HPCs two days after infection (MOI 100) with *B. henselae* (red signal) by TUNEL reaction (green signal). Nuclei were stained with DAPI (light blue signal). Scale bar: 20 µm.

(B) Determination of IL-8 and VEGF secretion by HPCs upon infection with *B. henselae*. CD34⁺ cells were infected (MOI 100) and supernatants taken five days later for ELISA. Values are given in pg/ml.

* Significant difference compared to uninfected control cells (*P* < 0.01).
Mändle et al., Fig. 1

A

ECs

erythrocytes

2 h 24 h 48 h

B

log 10 CFU

2 h 24 h 48 h

ECs

erythrocytes

*
Mändle et al., Fig. 2
0.83 % isotype control

97.25 % HPCs

1.28 % erythrocytes
A

B. henselae control

B

Mändle et al., Fig. 7
Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*

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