Infection of human CD34$^+$ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B henselae*

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Although there is evidence that endothelial cells are important targets for human pathogenic *Bartonella* species, 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). Fluorescence-activated cell sorting (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 day 13 after 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 suggest that HPCs might serve as a potential primary niche in *Bartonella* infections. (Blood. 2005;106:1215-1222)

Introduction

Hematopoietic proliferation and differentiation are sustained by hematopoietic progenitor cells (HPCs) and carry significant medical implications; for example, 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 8 distinct blood cell lineages including erythrocytes, granulocytes, monocytes, megacaryocytes, and lymphocytes.[1,2] 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 in vitro infection with *Listeria monocytogenes*, *Salmonella enteritica*, and *Yersinia enterocolitica*, but not when these cells were myeloid or monocytic differentiated.[3]

The genus *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] *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 *B quintana*, which is transmitted via body lice,[6] causes "trench fever" (also called "5-day fever"), characterized by periodic feverish relapses due to intraerythrocytic bacteremia.[7] Trench fever became known during World War I when more than one million soldiers suffered from the disease. Today, *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 *B bacilliformis* causing Carrion disease seems to be unique among *Bartonella* species,[5] prolonged periods of intracellular erythrocyte parasitism appear to be a crucial aspect of the pathogenicity of *Bartonella*.[11,12] Although it is known that *Bartonella* species invade erythrocytes in their animal reservoir hosts,[13] there is no evidence for a direct interaction of *B henselae* with human erythrocytes.[14]

An in vivo model with similarities to human trench fever has been described, in which rats were infected intravenously with *B tribocorum* leading to intraerythrocytic presence of bacteria during the course of infection.[4] Bacteria were first detectable in erythrocytes 5 days after challenge with a peak between 10 and 14 days after infection. Therefore, it may well be that *Bartonella* species are capable of colonizing an as-yet-unkown 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.[15] Such colonization of erythropoietic lineage would (1) protect *B quintana* from the host immune response and (2) 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

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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

The *B henselae* strain Marseille[16] 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% CO_2. For production of bacterial stock suspensions, bacteria were harvested after 5 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 (GFP_{mut2}) was used. The GFP_{mut2}-encoding plasmid pCD354 (kindly provided by C. Dehio, Basel, Switzerland)[17] was introduced into *B henselae* by electroporation,[18] and *B henselae* gfp_{mut2} 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 Tübingen. 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; 2 × 5 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 minutes 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). Cells were analyzed by flow cytometry for the expression of the HPC marker CD34 and the absence of the erthyroid marker glycophorin A (GPA). The purity of each HPC preperation was more than 95% as assessed by flow cytometry.

For experiments with human erythrocytes, whole blood of a healthy volunteer was diluted in phosphate-buffered saline–ethylenediaminetetraacetic acid (PBS-EDTA; 1 mM). Cells were counted in a Neubauer cell counting chamber (Superior, Lausa-Koenigshofen, Germany) and used at a concentration of 1×10^5 erythrocytes per milliliter.

Culture and infection of CD34+ cells

$CD34^+$ HPCs were cultured in 24-well plates (1×10^5 cells per well) at 37°C and 5% CO_2 for 13 to 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 Systems), and Epo (3 U/mL; Erypo FS10 000, Janssen-Cilag, Neuss, Germany) were added to the medium.

For infection experiments, bacterial stock solutions were thawed, washed in phosphate-buffered saline (PBS; containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4) and diluted in antibiotic-free cell culture media to obtain a multiplicity of infection (MOI) of 100 (100 000 HPCs and 1×10^7 bacteria per well). Bacteria were sedimented onto cultured HPCs by centrifugation at 400*g* for 5 minutes at RT. The actual MOI for each experiment was confirmed by plating serial dilutions of the infection inoculum and calculating the number of colony-forming units (CFUs).[19]

Culture and infection of endothelial cells

Human umbilical vein endothelial cell (HUVEC) culture (passages 2-6) was performed in EC growth medium containing EC growth supplement (PromoCell, Heidelberg, Germany). Infection experiments were performed in EC basal medium (PromoCell) as previously described.[19,20] Briefly, 1×10^5 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 in the previous section.

Flow cytometry

Flow cytometry was used to assess the differentiation of $CD34^+$ HPCs into GPA-expressing erythroid cells.[21] Usually, 1×10^5 to 5×10^5 cells resuspended in 100 μL PBS (pH 7.4) were incubated with fluorescein isothiocyanate (FITC)– and phycoerythrin (PE)–conjugated antihuman monoclonal antibodies for 30 minutes 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 immunglobulin 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 WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, San Diego, CA; 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 days 1, 9, and 16 of cultivation, and is expressed as the percentage of the total cell amount.

Invasion of *B henselae* into erythrocytes, ECs, and HPCs

Invasion (2 hours) and intracellular presence (erythrocytes and ECs: day 1, day 2; HPCs: day 1, day 6, day 13) were determined by gentamicin protection assays.[13,20] For this purpose, gentamicin (100 μg/mL) was added to the medium for 3 hours to kill extracellular bacteria. Cells were then washed extensively and lysed by the addition of 900 μL distilled water for 5 minutes, followed by equilibration with 100 μL of 10× PBS. Lysates were serially diluted, plated on CBA, and CFUs were counted after 3 weeks of incubation due to the slow growth of *B henselae*.[5]

Determination of VEGF and IL-8 secretion by HPCs

To quantify the secretion of vascular endothelial growth factor (VEGF) and IL-8 by *B henselae*–infected HPCs, cells were cultured without antibiotics to allow bacterial growth and without fetal calf serum (FCS) to avoid nonspecific VEGF secretion.[19] Supernatants were taken 5 days later, centrifuged to remove insoluble particles (10 minutes, 4°C, 20 000*g*), and frozen at −20°C. VEGF concentration was measured using a human $VEGF_{165}$–enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Quantikine; R&D Systems). IL-8 was determined by ELISA as previously described.[22]

Fluorescence in situ hybridization of *B henselae*

Fluorescence in situ hybridization (FISH) of bacteria on glass slides was performed as previously described.[20,23,24] The universal eubacterial oligonucleotide probe EUB-338 (GCT GCC TCC CGT AGG AGT)[25] 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 nonspecific binding of the probes (data not shown). Coverslips with ECs, or polysine-covered adhesion microscope slides (Multimed Wicker, Kirchheim/Teck, Germany) with 5×10^5 infected $CD34^+$ HPCs, were each analyzed by confocal laser scanning microscopy (CLSM).

Immunostaining and confocal microscopy

ECs (1×10^5) were seeded onto coverslips, and HPCs or erythroid differentiated cells were centrifuged onto glass slides. Infection (MOI 100)

with *B henselae* or *B henselae* gfp_{mut2} 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 minutes, rabbit polyclonal anti–*B henselae* Marseille antibodies[19,20] for 1 hour, FITC-conjugated goat anti–rabbit IgG (Dianova, Hamburg, Germany) for 1 hour, 0.1% Triton X-100 in PBS (ECs 15 minutes at RT, HPCs 5 minutes on ice), blocking solution for 15 minutes, rabbit anti–*B henselae* antibodies for 1 hour, indodicarbocyanine (Cy5)–conjugated goat anti–rabbit IgG (Dianova) for 1 hour, and tetramethylrhodamine-isothiocyanate (TRITC)–labeled phalloidin (Sigma, Taufkirchen, Germany) for 1 hour, 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) equipped with an HCX PL APO × 63/1.32-0.6 oil confocal scanning (CS) objective and a Leica TCS SP2 optical system. 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 minutes) was visualized on a fourth channel (light blue). Fluorescence images were acquired sequentially to avoid nonspecific channel interference. Images were taken using the Leica Confocal Software Package Version 2.5 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 and immunoelectronmicroscopy

Transmission electron microscopy (TEM) was performed as previously described.[20] For immunoelectronmicroscopy (IEM) of *B henselae*, postembedding 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 nickle grids and incubated with anti-BadA rabbit serum[22] followed by 10 nm gold-conjugated goat anti–rabbit IgG (Auroprobe EM; Amersham, Freiburg, Germany). 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 3 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 *t* test. A value of *P* less than .05 was considered statistically significant. The results of representative experiments are presented as mean values and SDs (error bars); determination was done in triplicate.

Results

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 hours and invasion was compared with results obtained from ECs at 2, 24, and 48 hours after 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 (Figure 1A). Similarly, data from gentamicin protection assays quantifying the number of intracellular *B henselae* revealed comparable results (Figure 1B). Based on our data, we conclude that, in contrast to ECs, *B henselae* does not invade human erythrocytes.

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 (Figure 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 (Figure 2B). Moreover, we investigated whether cocultivation

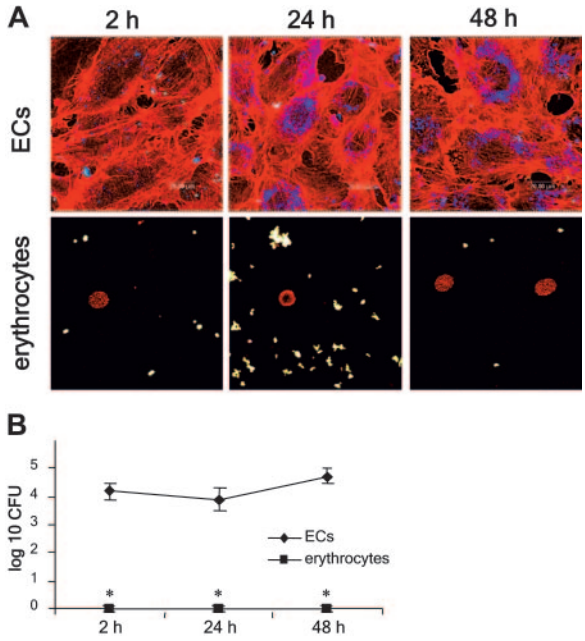

Figure 1. Interaction of *B henselae* with endothelial cells and erythrocytes. (A) CLSM of adherent and intracellular *B henselae* in endothelial cells (ECs, top row) and erythrocytes (bottom row; 100 000 cells each, respectively). Green signal indicates extracellular bacteria; blue signal, intracellular bacteria; and red signal, filamentous actin. (B) The number of intracellular bacteria was determined by gentamicin protection assays. *Significant difference compared with ECs ($P < .01$).

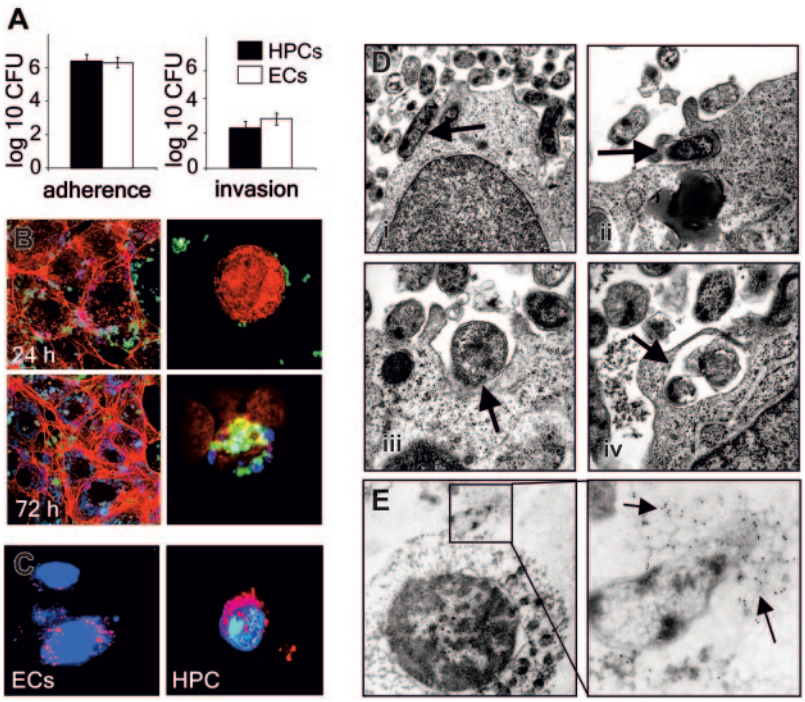

Figure 2. Interaction of *B henselae* with ECs and CD34⁺ HPCs. (A) Adherence and invasion rates of *B henselae* obtained with CD34⁺ HPCs and ECs (100 000 cells each, respectively). The number of adherent *B henselae* was assessed 30 minutes after infection and invasion of *B henselae* was quantified after 2 hours. (B) Detection of adherent and intracellular *B henselae* by CLSM 24 and 72 hours after infection (left: ECs; right: HPCs). Green signal indicates extracellular bacteria; blue signal, intracellular bacteria; and red signal, filamentous actin. (C) Detection of *B henselae* by FISH 24 hours 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 hours after infection with *B henselae* (arrows). Membrane ruffling (ii,iii) can be observed following adherence (i) of the bacteria to the host cells. Intracellular bacteria are located in vacuoles (iv). (E) Detection of *B henselae* by IEM using anti-BadA– and 10 nm gold-conjugated goat anti–rabbit IgG antibodies. The enlargement illustrates the interaction of *B henselae* with HPCs by immunogold staining of *B henselae* (see arrows).

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 hours after infection showed an increase in the production of *B henselae* rRNA (Figure 2C). Ultrastructurally, the infection process was analyzed by TEM and IEM (Figure 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 up-regulates 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 (Figure 3). These data suggest that beta-1 integrin expression is crucial for host cell infection by *B henselae*.

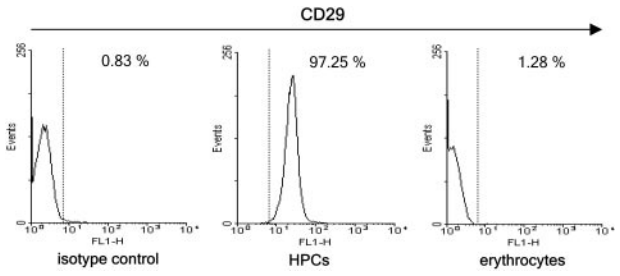

Figure 3. Beta-1 integrin (CD29) expression on HPCs and erythrocytes. CD29 expression of freshly isolated HPCs (middle) and erythrocytes (right) was determined by FACS analysis. 20 000 cells were analyzed and the number of CD29⁺ cells is given as the percentage of the total number of cells. All stainings were referred to isotype-matched control antibodies (left). Cells expressing CD29 are given right of the dotted line (upper fluorescence limit of isotype control).

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 days 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; Figure 4A). At later time points (days 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 days 1 and 9 after infection (Figure 4B-C). At day 1, approximately 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 (Figure 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 color of the cultured cell pellet from white (day 1) to red (day 9, 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.

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

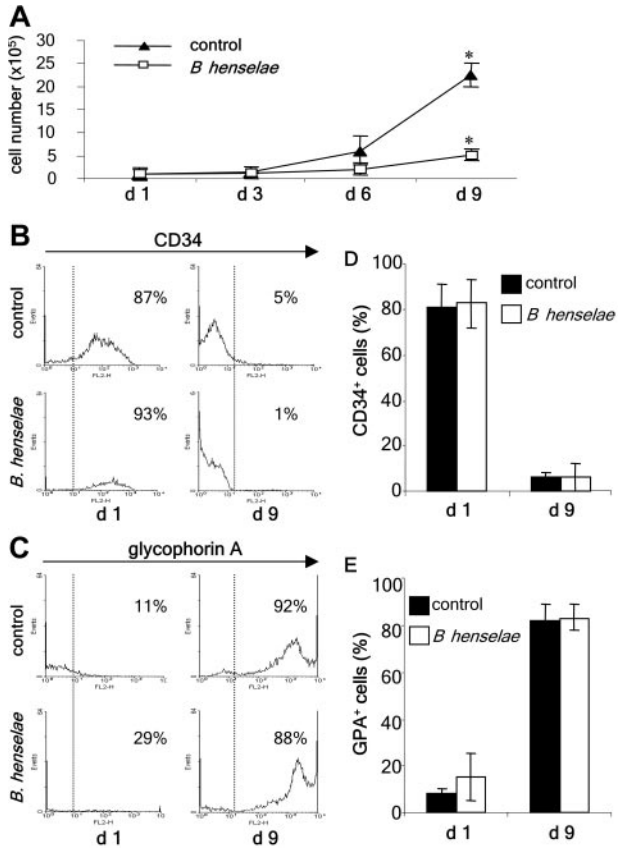

Figure 4. Proliferation and differentiation of B henselae–infected HPCs. (A) Proliferation of HPCs upon infection with *B henselae*. Cells were seeded in 24-well plates and total numbers of cells were counted at days 1, 3, 6, and 9. *Significant difference compared with day 1 ($P < .05$). (B,C) Flow 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 day 9 after infection. At each time point, 10 000 cells were analyzed. 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 "Materials and methods"). Cells expressing CD34 or GPA, respectively, are given right of the dotted line (upper fluorescence limit of isotype control). (D,E) CD34 and GPA expression of uninfected and *B henselae*–infected HPCs on day 1 and day 9 after infection. The graphs show the means and standard deviations of the percentage of CD34$^+$ (D) and GPA$^+$ (E) cells obtained from 3 independent experiments.

differentiating cells, HPCs were infected immediately after isolation, differentiated toward erythrocytes, and intracellular presence of *B henselae* was assessed by FACS analysis, CLSM, and TEM. At 9 or 16 days after infection, cells were analyzed by FACS for expression of GPA, revealing that approximately 80% had undergone erythroid differentiation (data not shown). Intracellular *B henselae* gfp_{mut2} (green) were detected via FACS (Figure 5A) in 25% (day 9) and 41% (day 16) of all cells. From GPA$^+$ cells, 13% (day 9) and 18% (day 16) contained intracellular *B henselae* gfp_{mut2} (data not shown). These data were consistent with those obtained by CLSM, showing a clear colocalization of GPA$^+$ cells and *B henselae* gfp_{mut2} (Figure 5B). Intracellular bacteria were also detectable by TEM (Figure 5C). From these data, we conclude that following initial infection of HPCs, *B henselae* persists intracellularly while these cells are differentiating toward erythrocytes.

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 reisolated bacteria. As demonstrated in Figure 6, the number of *B henselae* continuously increased (day 6: 5.4-fold; day 13: 23.1-fold; total intracellular bacteria at day 1: 337 ± 185; day 6: 1833 ± 388; day 13: 7790 ± 834), indicating that *B henselae* persists and replicates intracellularly during erythroid differentiation of HPCs.

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 hours after infection by TUNEL reaction and CLSM (Figure 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 "Materials 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 5 days after infection from *B henselae*–infected HPCs (Figure 7B) revealed that IL-8 secretion was strongly increased (11.3-fold) compared with 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 species cause several human diseases including trench fever, CSD, BA, BP, and other manifestations.[5] However, in all of these infections, the primary niche of the pathogens remains unclear, although *Bartonella* species are capable of infecting a wide variety of different host cells. In particular, it has been shown that *B henselae* infects endothelial cells,[20,22,26] epithelial cells,[19] and monocytes or macrophages,[29,30,32] and similar findings have been reported for *B quintana*.[33,34]

Intraerythrocytic presence of human pathogenic *Bartonella* species has been demonstrated several times in vitro by fluorescence mircroscopy using anti-*Bartonella* antibodies.[12,35] It has already been assumed that a potential primary niche in *Bartonella* infections might be represented by HPCs.[7] Consistent with this suggestion, there are currently 2 case reports describing immunofluorescence detection of *Bartonella* in erythroblasts from bone marrow aspirate.[15,35] In addition, a rat animal model of trench fever using *B tribocorum* demonstrated that the pathogens appear intraerythrocytically in the bloodstream 4 to 5 days upon infection,[4]

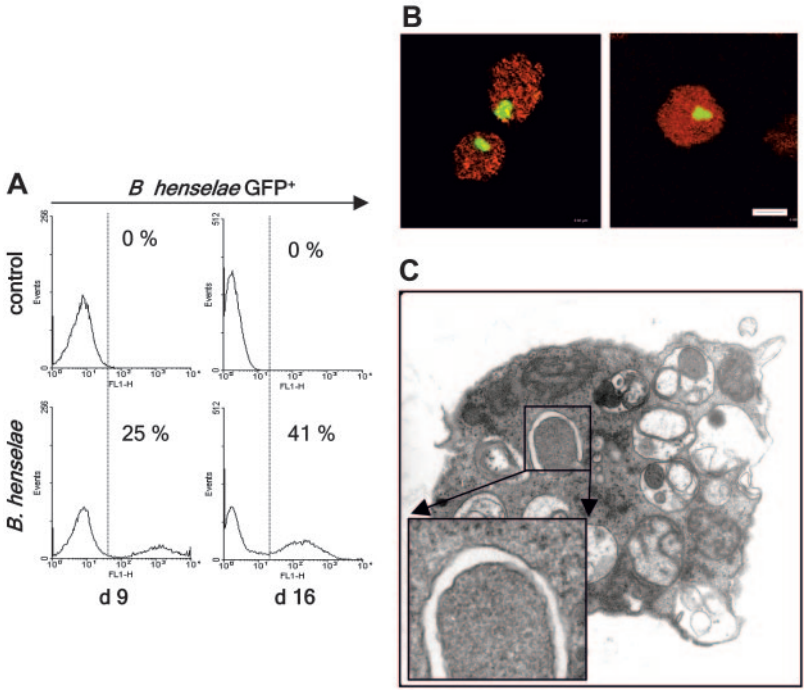

Figure 5. Detection of intracellular *B henselae* in differentiated erythroid cells. Freshly isolated HPCs were infected with *B henselae* gfp_{mut2} (MOI 100) and were subsequently cultivated for 16 days. (A) Flow cytometric detection of intracellular *B henselae* gfp_{mut2} (green fluorescence, x-scale) in erythroid cells 9 days (left) and 16 days (right) after infection (control: uninfected erythroid cells). Data for 50 000 cells per time point were analyzed. Percentages 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*. Cells harboring GFP-expressing *B henselae* are given right of the dotted line (upper fluorescence limit of uninfected control cells). (B) Detection of intracellular *B henselae* gfp_{mut2} (green) by CLSM 9 (left) and 16 (right) days after 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 illustrates that *B henselae* is located in a vacuolic compartment in differentiated erythroid cells.

and similar observations were made using a *B grahamii* mouse-infection model.[36] Moreover, *B henselae* and *B koehlerae* have been detected in erythrocytes of naturally infected cats.[37,38]

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: (1) *Bartonella* species remain undetectable in rat and mouse models for several days upon experimental infection,[4,36] (2) these pathogens are detected intraerythrocytically during the course of infection, and (3) *Bartonella* can be detected in HPCs in human infections.[15,35] These observations strengthen the hypothesis that hematopoietic stem cells are a possible niche for *Bartonella,* and it might be hypothesized that HPCs represent a sanctuary for chronic *Bartonella* infections responsible for recurrent intraerythrocytic bacteremia.[35] These suggestions are consistent with our observations that coculture of human erythrocytes did not result in intraerythrocytic presence of *B henselae* (Figure 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 are differentiated toward myeloid or monocytic cells.[3] Moreover, it was shown that infection with these bacteria accelerated the maturation of HPCs along the myeloid lineage.[39] Accordingly, human granulocytic ehrlichiosis (HGE), which is characterized mainly 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.[40]

We showed microscopically and via gentamicin protection assays that *B henselae* does not infect erythrocytes within the first 48 hours of infection (Figure 1). In contrast, *B henselae* was able to infect HPCs to a similar extent as ECs, which represent one of the most likely primary targets for *Bartonella* infections (Figures 1

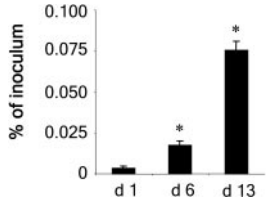

Figure 6. Quantification of intracellular *B henselae* in erythroid differentiating HPCs. Freshly isolated HPCs were infected with *B henselae* (MOI 100) and gentamicin protection assays were performed on days 1, 6, and 13 after infection. The number of intracellular bacteria (% of inoculum) was calculated by counting the CFUs on CBA after 3 weeks of incubation. *Significant difference compared with day 1 ($P < .01$).

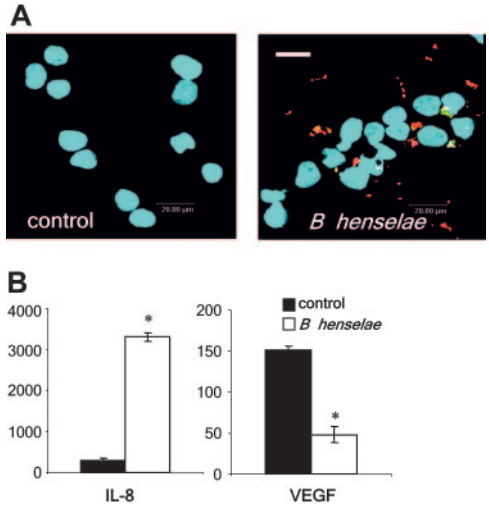

Figure 7. Resistance to apoptosis of HPCs upon *B henselae* infection and cytokine secretion. (A) Detection of apoptosis in HPCs 2 days after infection (MOI 100) with *B henselae* (red signal) by TUNEL reaction (green signal). Nuclei were stained with DAPI (light blue signal). Scale bar: 20 μm. (B) Determination of IL-8 and VEGF secretion by HPCs upon infection with *B henselae*. $CD34^+$ cells were infected (MOI 100) and supernatants taken 5 days later for ELISA. Values are given in pg/mL. *Significant difference compared with uninfected control cells ($P < .01$).

and 2). These data 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 (1) *B henselae* binds to host cells via beta-1 integrins[22] and (2) beta-1 integrins are expressed on HPCs but not on mature erythrocytes (Figure 3, Verfaillie et al,[41] and Brittain et al[42]).

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 (Figure 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 metabolic activity of *B henselae* and 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 (Figure 6). Taken together, these data strongly suggest that *B henselae* persists and replicates within the host progenitor cells during differentiation of these cells toward 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 (Figure 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 (Kempf et al[31] and Hanna Hartmann and V.A.J.K., unpublished data, November 2004). 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 hensleae*–infected HPCs with strong GPA expression in both cases (Figure 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 (Figure 7). IL-8 has a strong mobilizing effect on HPCs, leading to the spreading of these cells in the bloodstream.[45] 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 such as the endothelium. Therefore, it might be speculated that infected HPCs may function as a vehicle to carry *Bartonella* species 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,[31] 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, for example, toward erythrocytes. Our in vitro data provide evidence that infection of HPCs with human pathogenic bacteria (eg, *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.

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