TITLE

Bartonella henselae Persistence within Mesenchymal Stromal Cells Enhances Endothelial Cells Activation and Infectability Amplifying the Angiogenic Process.

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ABSTRACT

Some bacterial pathogens can manipulate the angiogenic response, suppressing or inducing it for their own ends. In humans, *B. henselae* is associated with cat-scratch disease and vasculoproliferative disorders such as bacillary angiomatosis and bacillary peliosis. Although endothelial cells (ECs) support the pathogenesis of *Bartonella*, the mechanisms by which *Bartonella* induces EC activation are not completely clear, as well as the possible contribution of other cells recruited at the site of infection. Mesenchymal stromal cells (MSCs) are endowed with angiogenic potential and play a dual role in infections exerting antimicrobial properties but also acting as a shelter for pathogens.

Here we delved into the role of MSCs as reservoir of *Bartonella* and modulator of EC functions. *B. henselae* readily infected MSCs and survived in perinuclear bound vacuoles for up to 8 days. Infection enhanced MSC proliferation and the expression of EGFR, TLR2 and NOD1, proteins that are involved in bacterial internalization and cytokine production. Secretome analysis revealed that infected MSCs secreted higher levels of the proangiogenic factors VEGF, FGF-7, MMP-9, PIGF, serpin E1, TSP-1, uPA, IL-6, PDGF-D, CCL5 and CXCL8. Supernatants from *B. henselae*-infected MSCs increased the susceptibility of ECs to *B. henselae* infection and enhanced EC proliferation, invasion and reorganization in tube-like structures.

Altogether, these results candidate MSCs as a still underestimated niche for *B. henselae* persistent infection and reveal a MSC-EC crosstalk that may contribute to exacerbate bacterial-induced angiogenesis and granuloma formation.

KEYWORDS

Mesemchymal stromal cells, *B. henselae*, angiogenesis, VEGF, CXCL8, EGFR, TLR, NOD

INTRODUCTION

Endemic among domestic cats, *B. henselae* is a fastidious gram-negative bacterium that, in humans, can cause subclinical intraerythrocytic bacteremia, mainly transmitted by cat fleas. In immunocompetent individuals, *B. henselae* infection can also lead to cat-scratch disease (CSD), characterized by lymphadenopathy with suppurative granulomas. Atypical clinical presentations of CSD, ranging from prolonged fever of unknown origin to hepatosplenic, ocular and neurological manifestations, have also been reported (1).

Individuals unable to mount an immune response against *B. henselae* tend to develop a tumor-like vascular proliferative response in the skin and/or internal organs, which can lead to bacillary angiomatosis (BA) or bacillary peliosis (BP) (2). After infection, *B. henselae* survives, stimulates the migration and the production of pro-angiogenic factors by human endothelial cells (ECs) (2–5). In addition, other cells types, such as monocytes/macrophages (6), recruited to the vasoproliferative lesions, stimulate EC proliferation in a paracrine manner through the production of VEGF and CXCL8 (7). Mononuclear phagocytes, $CD34^{+}$ progenitor cells and ECs can also function as a reservoir from which *B. henselae* periodically enters the bloodstream and disseminates within the host (8). Despite the clinical implications of protracted *Bartonella* infections, the underlying mechanism of intracellular *B. henselae* persistence is poorly understood, and the existence of different reservoirs still remains to be determined.

Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in various tissues, including the bone marrow and the adipose tissue, which have recently received much attention due to their regenerative potential and immunomodulatory properties (9). MSCs actively participate in angiogenesis through several mechanisms, including paracrine cytokines and exosomes and cell contact interactions with endothelial cells. (10, 11). A diverse and multitasking role of MSCs during bacterial infection has recently emerged (12, 13). MSCs can sense pathogens and mount an appropriate cytokine/chemokine response through the activation of Toll-like receptors (TLRs),

NOD-like receptors (NLRs) and the scavenger receptors MARCO and SR-B1 (12). Moreover, MSCs express EGFR, a (member of the ErbB receptor tyrosine kinase family), shown to enhance their proliferation and the release of angiogenic factors (14). However, despite the emerging role of MSCs in infectious diseases, the mechanisms regulating the interplay between MSCs and bacteria are yet to be defined. Recent evidence suggests that MSCs can have a double edge sword effect by playing a role in clearing infection but also promoting persistent bacterial infection. MSCs exert antimicrobial effects by secreting antimicrobial peptides and expressing indoleamine2,3-dioxygenase (IDO) and MSC administration reduce pathogen burden in animal models of antimicrobial sepsis (12). However, MSCs can also serve as a niche where *M. tuberculosis* can survive and persist during antimicrobial therapy. Indeed, viable *M. tuberculosis* was recovered from MSCs infiltrating TB granulomas in humans and in a tuberculosis mouse model (15, 16). It is likely that other chronic bacterial pathogens may exploit MSCs to favor their survival in the host and we hypothesized that *B. henselae* infects MSCs and that infected-MSCs contribute to the angiogenesis via interaction with endothelial cells that are one of *Bartonella* preferential target.

Here we show that *B. henselae* can invade and survive within human MSCs and demonstrate that TLR2, NOD1 and EGFR are implicated in bacterial recognition and cytokine production. Moreover, we provides evidence for a MSC-EC crosstalk involved in bacteria intracellular survival and activation of a proangiogenic program.

RESULTS

***B. henselae* invades and persists in MSCs**. To characterize the interaction of MSCs with *B. henselae*, adipose-derived MSCs were infected with MOI of 100:1 for 1, 2, 3, 4 and 8 days and then treated with gentamicin to kill all residual extracellular bacteria. Subsequently, the number of viable intracellular bacteria was measured by colony-forming unit (CFU) assay. The number of *B.*

henselae invading MSCs increased progressively over a 3-day period and the number of CFUs in MSCs remained unchanged up to 8 days ($P < 0.05$) (Fig. 1a). At day 8 post-infection (pi), the vast majority of MSCs contained *B. henselae*, as demonstrated by the strong cytoplasmic reactivity of an anti-*B. henselae* monoclonal antibody (anti-BH) (Fig. 1b, upper panel). The presence of internalized bacteria was confirmed by immunofluorescence (Fig. 1B, lower panel). To assess *B. henselae* intracellular survival after the initial infection and gentamicin treatment, MSCs were cultured in medium without gentamicin for four additional days. The number of viable intracellular bacteria recovered, which remained stable during the first 96 h, was significantly lower at day 8 compared to day 4 (Fig. 1c). The ability of *B. henselae* to invade MSCs was further assessed by comparing its infection efficiency in MSCs *vs* HUVECs, a known target of *B. henselae* infection. The number of intracellular bacteria recovered after 24 h of infection from MSCs was significantly higher than that recovered from HUVECs (Fig. 1d).

Next, we followed MSC infection by fluorescence microscopy. At day 1 pi, *B. henselae* stained with DAPI (Cyan) remained mainly anchored to the MSC membrane, with only a few bacteria present in the cytoplasm (Fig. 2a, upper right panel, arrowhead). From day 2 pi onward, the number of internalized bacteria increased, and most of *B. henselae* were enclosed in perinuclear vesicles (Fig. 2a, lower left and central panel, thin arrows). After 8 days pi, aggregates of bacteria colocalized with F-actin in globular structures called invasomes, first described in *Bartonella*-infected ECs (Fig. 2a, lower right panel, large arrow; and Fig. 2b) as attested by 3D immunofluorescence analysis.

Altogether, these findings indicate that *B. henselae* is internalized by MSCs, even more efficiently than HUVECs, where it can persist for a prolonged time.

***B. henselae* infection enhances MSC proliferation.** We next asked whether *B. henselae* infection would affect MSC survival. *B. henselae* infection did not induce cell death in MSCs as demonstrated by similar amounts of Annexin V positive cells found in uninfected *vs* infected MSCs

(Fig. 3a). This finding was further supported by the unaltered *Bcl-2* (antiapoptotic) /*Bax* (apoptotic) expression ratio observed in these cells (Fig. 3b). We then assessed the effect of infection on the proliferation rate of MSCs. Infected-MSCs grew significantly faster compared to their uninfected counterparts. Conversely, heat-inactivated *B. henselae* (HK *B. henselae*) failed to enhance MSC proliferation (Fig. 3c).

Role of TLR2, EGFR and NOD1 in MSC infection with *B. henselae*. TLRs and NODs play a key role in bacterial detection and their cooperation become relevant in the context of infections. Interaction between cell surface TLR2 and intracellular surveillance NOD1/2 are of relevance in the recognition of pathogens and in the induction of the inflammatory response (17). However a number of cell surface receptors, such EGFR, that signal through pathways not related to TLRs and NODs, are also used by pathogens and an interaction between TLRs and EGFR has been demonstrated (18, 19).

We therefore assessed the expression of these receptors in response to *B. henselae* infection. Interestingly, *B. henselae* infection led to a more than 6-fold increase in TLR2 expression at both mRNA and protein levels, while TLR4 expression remained basically unchanged (Fig. 4a and 4b). Furthermore, RT-PCR analysis showed a significant upregulation of NOD1 mRNA at day 2 and 4 pi (Fig. 4a). NOD2 gene expression was not detected in uninfected or infected MSCs. Lastly, *B. henselae* infection significantly increased EGFR mRNA and phosphorylation levels (Fig. 4a and 4c, respectively). Specifically, we detected increased phosphorylation as early as 30 min pi, which remained above basal levels up to 120 min pi (Figure 4c).

The involvement of these receptors was evaluated in the production of CXCL8, a cytokine shown to be triggered by *Bartonella* in different cell types (20), *Bartonella* infection of MSCs enhanced their ability to produce CXCL8, which was neutralized by incubation with an anti-TLR2 neutralizing antibody (Fig. 4d, upper panel). Similarly, treatment with the EGFR inhibitor gefitinib or with the selective RIP2K inhibitor GSK583 significantly reduced the release of CXCL8 in *B. henselae*-infected MSCs (Fig. 4d, lower panel), suggesting that the EGFR/NOD pathway may play

a role in CXCL8 transcriptional regulation. Finally, to address the role of bacterium-activated EGFR in *Bartonella* entry, we treated MSCs with the EGFR inhibitor gefitinib and a neutralizing anti-EGFR antibody, detecting a reduced bacterial internalization by about 70% and 50%, respectively, compared to untreated cells (Fig. 4e).

***B. henselae*-infected MSCs promote angiogenesis and infection of endothelial cells.** Since MSCs regulate vascular remodeling and angiogenesis (21), we assessed the proangiogenic activity of conditioned medium (CM) from *B. henselae*-infected MSCs. To this end, CM from uninfected or *B. henselae*-infected MSC cultures were tested in a scratch wound healing assay using HUVECs. CM from *B. henselae*-infected MSCs (CM-MSC *B. henselae*), induced a more rapid repair of HUVECs monolayer (Fig. 5a). In addition, the CM-MSC *B. henselae* was 9 fold more powerful then CM of uninfected MCS (CM-MSC CTRL) on aspheroid-based sprouting assay, which faithfully recapitulate the proliferation, invasion and reorganization in tube-like structure of ECs (Fig. 5b). In keeping with the proangiogenic activity of MSCs, the CM-MSC CTRL induced the formation of radial sprouts, similarly to what induced by spheroids stimulation with 30 ng/mL of VEGF-A (Fig. 5b, right panel). Importantly, CM-MSC *B. henselae* but not that from uninfected cells (CM-MSC CTRL) accelerated the morphogenesis of HUVECs when seeded on Cultrex Extracellular Matrix, as judged by the number of closed structures formed at 18 h pi (Fig. 5c).

Even though ECs and MSCs can crosstalk through soluble mediators (22), there is no data on the effects of MSC on the susceptibility of ECs to bacterial infection. We thus assessed the extent of *Bartonella* internalization, at day 1 pi, in HUVECs pretreated with CM from uninfected MSCs (CM-MSC CTRL) or *B. henselae*-infected MSCs (CM-MSC *B. henselae*). While there were no differences in the yield of bacteria between control HUVECs (Ctrl) and HUVECs pretreated with CM-MSC CTRL, a significantly higher number of bacteria was detected in HUVECs pretreated with CM-MSC *B. henselae* (Fig. 5d). After 1 day of culture we did not observe a significant increase in the proliferation of infected HUVECs pre-treated with CM-MSC *B. henselae* over that pre-treated with CM-MSC CTRL, or directly infected. The number of cells

harvested/number of cells seeded (mean ± SEM) obtained were 1.23±0.2 (unconditioned medium), 1.063±0.06 (*B. henselae* infected), 1.125±0.1 (CM-MSC CTRL) and 1.25±0.05 (CM-MSC *B. henselae*). In accord with our observation endothelial cell proliferation during *B. henselae* infection has been shown after 3 or 4 days of incubation (7, 23) . Our results indicate that the treatment with CM-MSC *B. henselae* makes HUVECs more infectable and the increase in intracellular CFU does not depend on HUVEC proliferation.

Angiogenic expression profile of *B. henselae*-infected MSCs. Finally, we assessed the impact of *B. henselae* infection on the ability of MSCs to modulate the expression of proinflammatory and proangiogenetic molecules. For this purpose, we probed an antibody angiogenesis array with CM from uninfected and 4-day-infected MSCs. Among the 55 proteins of the assay, 27 were detected in CM of both uninfected and infected MSCs. Densitometric analysis showed the upregulation of FGF-7, CXCL8, MMP-9, PIGF, Serpin E1, TSP-1, uPA and VEGF, in *B. henselae*-infected MSCs CM compared to those from uninfected MSCs (Fig. 6a and 6b). Intriguingly, activin A was the only growth factor downregulated in *B. henselae*-infected MSCs (Fig. 6a and 6b). Of note, the elevated expression of MCP-1, PTX3 and TIMP-1 was not modulated by infection (Fig. 6a, and 6b). The quantification by ELISA of the increased production of CXCL8 and VEGF in the supernatants of MSCs infected for 1, 4 and 7 days was in good agreement with the array data (Fig. 6c). Finally, other molecular factors known for their angiogenic activity, but not included in our array, such as IL-6, CCL5 and PDGF-D, were also induced following *B. henselae* infection (Fig. 6c).

DISCUSSION

Bartonella spp exploits several mechanisms to hide inside erythrocytes and ECs to evade immune responses and persist in both animal reservoir and human host. Numerous evidence indicate that the blood-stage phase is preceded by the infection of cellular niches that periodically release bacteria able to invade erythrocytes. ECs were the first cell types considered a primary niche as they support *Bartonella* replication and reside in proximity to the bloodstream (2, 24). However,

later studies identified additional *Bartonella* persistence sites including hematopoietic progenitor cells and dendritic cells (8, 25).

Here we show that once inside, *B. henselae* resides in MSCs without proliferating for several days. During this time, *Bartonella* localizes in numerous perinuclear membrane bound vacuoles, as previously shown in HUVECs and MonoMac cells (26, 27), or at late time points of infection, as aggregated bacteria enclosed into F actin-rich cell membrane protrusions identified as invasome structures (28).

MSCs sense microorganisms trough the expression of various PRR including Toll-like receptors (TLRs) and Nod-like receptors (NLRs). The engagement of such receptors modulate MSC functions and their abilities to secrete cytokines (29). Our studies revealed that TLR2, NOD1 and EGFR are involved in the recognition and responses to *Bartonella* by MSCs. Upon infection with *B. henselae*, MSCs secret large amounts of CXCL8, which is curbed by incubation with an anti-TLR2 antibody. A central role of TLR2 signaling during *Bartonella* infection is consistent with previous findings indicating that *B. henselae*, despite being Gram-negative, preferentially activates TLR2 (25). In infected cells, NOD1 and NOD2 recognize bacterial peptidoglycan derivatives released into the cytosol and, upon ligand association with the adaptor protein receptor-interacting-serine/threonine-protein kinase 2 (RIPK2 or RIP2), trigger proinflammatory signaling (30). In our experimental system, inhibition of the RIP2 with the highly RIPK2-specific compound GSK583(31) decreased CXCL8 release, indicating that NOD1 activation and signaling through RIP2 during MSC infection is, in part, responsible for inducing the inflammatory response to *B. henselae* infection. Consistent with our results, NOD1 mediates CXCL8 induction after recognition of *Helicobacter pylori*, *Escherichia coli* (32, 33) and *Chlamydia pneumoniae* (34). Importantly, gefitinib, an inhibitor of EGFR tyrosine kinase domain, used to treat various forms of cancer, can hamper *B. henselae*-mediated induction of CXCL8, suggesting a role of EGFR in this pathway. Gefitinib also exerts an off-target inhibitory activity on the expression of RIP2 (35), thus the inhibition of CXCL8 secretion may be due to blockage of NOD/RIP2 signaling alongside that of

EGFR. In support to this hypothesis, EGFR/NOD cooperation has been recently involved in cytokine production in dengue virus infected monocytes (36). Moreover, a growing body of literature highlights the importance of EGFR/ErbB in several bacterial and viral inflammatory responses (18, 37) and in pathogenic angiogenesis (38). In addition to stimulation of EGFR tyrosine phosphorylation, *Bartonella* enhanced EGFR mRNA expression suggesting that this upregulation could serve as a positive feedback system. A functional role of EGFR signaling in the immune response against *B. henselae* is further supported by the observation that treatment of MSCs with the kinase inhibitor gefitinib or an anti-EGFR antibody significantly decreases *Bartonella* internalization. In this regard, EGFR has been recently shown to act as a cofactor in mediating pathogen internalization in host cells (e.g., HBV, HCV, Chlamydia and Candida) (18). Our finding indicates an important role of EGFR activation in *Bartonella* invasion; however, as these EGFR inhibitors do not completely abrogate *Bartonella* uptake by MSCs, it is likely that other receptors, other than EGFR, may play a role in *Bartonella* infection. Moreover, it remains to be investigated whether EGFR activation is due to the direct interaction of *Bartonella* with the EGFR extracellular domain or by its transactivation by EGFR ligands (i.e., EGF, HBEGF, TGFα, BTC, AREG, EREG and EPGN) as shown for *H. pylori* and *Neisseria* spp. (39, 40). EGFR signaling pathways exert an antiapoptotic activity in *Pseudomonas*- and *Helicobacter*- infected cells (41, 42) suggesting that EGFR activation by *Bartonella* promotes the survival and proliferation of infected MSCs.

These effects may also be explained at least in part by the robust release of cytokine/growth factors caused by *Bartonella* infection. In addition to CXCL8, angiogenic factors upregulated in infected MSCs include FGF-7, MMP-9, PIGF, serpin E1, TSP-1, uPA, IL-6, CCL5 and VEGF, leading to the induction of a proangiogenic phenotype in ECs as well as an increased susceptibility of ECs to infection. Data reporting a role of MSCs in facilitating the infection of other cell types are sparse and concern mainly phagocytic cells. MSCs was shown to enhance bacterial uptake and clearance by PMNs (43), and to mediate the reactivation of HIV in monocytic cells (44). A secretome highly rich in inflammatory angiogenic cytokines and matrix remodeling factors was

previously described in *B. henselae* infected myeloid angiogenic cells (MACs). Similarly to our observation in MSCs conditioned medium from MACs increased angiogenic sprouting (45). In the past, infected ECs have been shown to upregulate the expression of VEGF and CXCL8 that directly lead to host cell proliferation and potentiate angiogenesis (23, 46); in parallel, *Bartonella* triggers the release of proinflammatory chemokines which recruit monocytes/macrophages in the vasoproliferative lesions and the production of angiogenic factors by phagocytic cells upon infection plays a central role in mediating angiogenesis-(7, 20, 45). Since at sites of infection/inflammation, MSCs localize in contact with ECs (22, 47), we propose that infected MSCs may support this angiogenic loop.

A role for MSCs can be envisioned in different scenarios of *Bartonella* infections. For instance, MSCs are recruited in tuberculosis around the lymph node granulomas to establish a persistent infection and likely to suppress T cell response (48). Moreover, MSCs are found in oral pyogenic granuloma tissues (49). Granulomatous lymphadenitis is the pathological hallmark of cat scratch disease whereby MSCs could also be hired in *Bartonella* granuloma to contribute to the immune pathogenesis. MSCs reside in the bone marrow (BM) interacting with other cellular components. We have previously shown the co-localization DCs and MSCs in human BM (50). The role of MSC-EC crosstalk has been characterized in the maintenance of the hematopoietic stem cell niche and in infection-induced emergency myelopoiesis (51, 52). Interestingly MSCs were shown to regulate proliferation and erythroid differentiation of $CD34^+$ stem cells (53). As *B. henselae* can infect $CD34^+$ BM progenitor cells, BM has been proposed as one of the potential niches. In this regard, multifocal BM involvement was shown in CSD (54, 55) and a contribution of *B. henselae* to ineffective erythropoiesis was suggested (56). *Bartonella*-infected MSCs, releasing soluble molecules, can recruit and activate ECs which in turn collaborate with MSCs in the fine regulation of the hematopoietic stem cell niche.

In conclusion, this study provides novel insights into the role of MSCs in serving as a reservoir during *B. henselae* infection and identifies TLR2, NOD1 and EGFR as the receptors

involved in the recognition of *B. henselae*. Infection of MSCs triggers a potent proangiogenic program, which activates and enhances EC susceptibility to bacterial infection. A better understanding of the involvement of MSCs in *Bartonella*-induced angiogenesis may allow the development of targeted therapeutic strategies for the treatment of vascular proliferative disorders.

MATERIALS AND METHODS

Cell culture. Human MSCs were isolated from adipose tissues as previously described (50). Human adipose tissues were collected by lipoaspiration from healthy donors after written consent and in compliance with the Declaration of Helsinki and the local Ethic Committee (Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - ASL TO1, No. 0009806). Subsequently, MSCs were analyzed by flow cytometry to verify their phenotype was positive for CD73, CD90 and CD105 and negative for CD11b, CD34 and CD45.

HUVECs were isolated from umbilical cords of healthy informed volunteers in compliance with the Declaration of Helsinki. HUVECs were used at early (I-IV) passages and grown on culture plates coated with porcine gelatin in M199 medium (Gibco Life Technologies, ThermoFisher Scientific Group) supplemented with 20% heat-inactivated fetal calf serum (FCS, Gibco Life Technologies), endothelial cell growth factor (ECGF) (10 µg/mL), and porcine heparin (100 µg/mL) (Sigma Aldrich) (100 µg/mL) or in complete EBM2 medium (Lonza Group Ltd Basel, Switzerland).

Bacterial cultures. *B. henselae* Houston I strain (ATCC 49882; Manassas, VA, USA) was grown on 5% sheep blood Columbia agar plates (BioMerieux, Lyon, France) under anaerobic conditions (i.e., candle jar) at 37°C for 10 days. Bacteria were harvested under a laminar-flow hood by gently scraping colonies off the agar surface. They were then suspended in MICROBANK™ cryopreservative solution and stored at -80°C in 1-mL aliquots. For biological assays, frozen bacteria were incubated in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% FBS, as described by Riess et al (57), at 37°C and 5% CO_2 for 6 days. Spectrophotometry was

performed to evaluate bacterial growth [optical density (OD600) 0.6, corresponding to $1x10^8$ bacteria/mL] and confirmed by plating serial dilutions on 5% sheep blood Columbia agar plates. Bacteria, washed 3 times with 1X PBS, were then added to cell cultures. Where indicated, *B. henselae* were killed by heating thawed bacteria at 56°C for 30 min.

Preparation of conditioned medium. MSCs, cultured into 12-well plates at a density of 0.5×10^5 cells/well in RPMI 10% FBS without antibiotics, were left untreated or infected for 96 h with *B. henselae*. Cells were then extensively washed to remove extracellular bacteria, and fresh RPMI was replaced for 72 h. Conditioned medium was collected, centrifuged at 4000 rpmi for 10 min and then filtered, aliquoted, and stored at -20°C.

Infection assay. *B. henselae* invasion of MSCs was assessed by GPA. Briefly, 12,500 cells/cm^2 MSCs were seeded for 24 h in RPMI supplemented with 10% FCS. To compare MSCs with HUVECs, infection was carried out with 60,000 cells *per* well seeded in DMEM 10% FCS or complete EBM2 medium (Lonza Group Ltd), respectively. The next day, cells were washed twice and cultured in RPMI supplemented with 10% FCS without antibiotics. *B. henselae* (MOI 100) was added to the cells, immediately centrifuged at 1200 g for 5 min to allow the association of bacteria with the cellular surface, and incubated for 1, 2, 3, 4 and 8 days. At the end of infection period, gentamicin sulfate (Sigma-Aldrich) (100 µg/mL) was added to the medium for 2 h to kill all extracellular bacteria. This assay was performed in triplicate, and control wells were left uninfected. Cells were then washed extensively and lysed by the addition of 200 µL of distilled water for 5 min and sonicated for 1.30 min. Lysates were serially diluted, plated on Columbia blood agar, and CFUs were counted after 1 week of incubation. To determine intracellular survival after 96 h of infection, extracellular bacteria were killed by gentamicin treatment for 2 h. Cells were further incubated in normal medium for the remaining time of the indicated infection period. When indicated, cells were pretreated for 6 h with the specific inhibitors gefitinib (10 µM) and GSK583 (1 µM) (both from MedChemExpress NJ, USA) or with a specific antibody against EGFR (mouse IgG1, clone LA1) or its corresponding isotype control antibody (both from EMD Millipore Corporation CA, USA) at 10

μg/ml. GPA was performed as described above after 1 or 2 days. In some experiments, HUVECs were cultured in the presence of CM from untreated and infected MSCs. Briefly cells seeded at 60,000 cells per well were pretreated overnight with the indicated CM and then infected with *B. henselae* (MOI 100) for 24 h. Cells were harvested and counted directly with an hemacytometer. Proliferation is reported as an index calculated as number of cells harvested/number of cells seeded. In parallel a GPA assay was performed.

Staining procedures. MSCs (1×10^4) were seeded on glass coverslips and infected with *B. henselae* at a MOI of 100. For immunohistochemical staining, cells were fixed in methanol, saturated with 0.1% BSA in PBS and incubated for 1h with an anti-*B henselae* mAb (anti-BH, dilution 1:50, mouse IgG2b/clone H2A10, Abcam , Cambridge, United Kingdom).The H2A10 clone reacts with a 43-kDa epitope present only in *B. henselae* strains and not in other Bartonella species (58). After washing an anti-mouse biotinylated Ab was added for 30 min. and the slides were then stained with horseradish peroxidase streptavidin (HRP Streptavidin) or with the chromogen DAB (3, 3'-diaminobenzidine) (ThermoFisher Scientific). For immunofluorescence analysis, the slides were incubated with anti-BH mAb, followed by goat anti-mouse Alexa Fluor® 594 (dilution 1:500, A21023, ThermoFisher Scientific). Nuclei were counterstained with DAPI (4',6-diamidin-2-fenilindolo) (ThermoFisher Scientific). To follow bacterial infection, MSCs were seeded at 0.25×10^4 on glass coverslips, infected with *B. henselae* (MOI 100) and incubated for 1, 2, 3, 4 and 8 days. At the end of infection period, cells were fixed with 4% paraformaldehyde (PFA) for 10', washed with PBS and permeabilized in PBS with 0.25% saponin. Samples were then saturated with blocking solution (PBS with 5% normal goat serum, and 2% BSA) for 1h at RT. After washes in PBS, samples were incubated with the wheat germ agglutinin-Alexa Fluor 594 or 488 conjugate, Alexa Fluor® 594 phalloidin (A12381) (1 h) and with DAPI (5 min) (all from ThermoFisher Scientific CA, USA) to stain cell membranes, actin and nuclei/bacteria respectively. Cells were analyzed under a Zeiss Observer.Z1 epifluorescence microscope equipped with a Plan-Apochromat 100×/1.4 NA oil objective and ApoTome2 imaging system for optical sectioning. Z-

stack images were elaborated through AxioVision 3D and Extended Focus modules.

Immunoblotting. Total cell lysates from cellsuntreated or treated for 30, 60 and 120 min with *B. henselae* (MOI 100) or with 50 ng/ml EGF (R&D System, MN, USA) for 15 min, were prepared in cold lysis buffer (1% Triton X-100, 1% NP-40 in PBS, pH 7.4) containing a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich). Samples (10-20 µg) were analyzed by 10% SDS-PAGE under denaturing conditions, followed by Western blotting, using the antibodies against EGFR (clone A10, sc-373746), pY1068-EGFR (sc-377547) and secondary antibodies HRP-conjugated (all from Santa Cruz Biotechnology, Inc.,Texas, USA). Chemiluminescent signal (Clarity Western ECL Substrate, Bio-Rad) was acquired by ChemiDoc™ Imaging System (BioRad).

Real-time PCR. Total MSC RNA isolated with the Qiagen RNeasy mini kit was treated with DNase I (Qiagen, Hilden, Germany) and retrotranscribed into cDNA by iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Gene specific primers were: TLR-2 (sense, 5'- CTCATTGTGCCCATTGCTCTT -3'; antisense, 5'- TCCAGTGCTTCAACCCACAAC -3'), TLR-4 (sense, 5'- GGCCATTGCTGCCAACAT -3'; antisense, 5'- CAACAATCACCTTTCGGCTTTT -3'), Bax (sense, 5'- AGAGGATGATTGCCGCCGT -3'; antisense, 5'- CAACCACCCTGGTCTTGGATC -3'), Bcl-2 (sense, 5'- TGCA.CCTGACGCCCTTCAC -3'; antisense, 5'- AGACAGCCAGGAGAAATCAAACAG -3'), HPRT (sense, 5'- TGACCTTGATTTATTTTGCATACC -3'; antisense, 5'- CGCTTTCCATGTGTGAGGTGATG -3'), RPL13A (sense, 5'-CATAGGAAGCTGGGAGCAAG-3'; antisense, 5'- GCCCTCCAATCAGTCTTCTG-3'). For EGFR, NOD1 and NOD2, validated primers from Bio-Rad were used (Unique Assay ID qHsaCID0007564, qHsaCED0005079 and qHsaCED0056944 respectively). For quantitative real-time PCR, the iQTM SYBR Green Supermix (Bio-Rad Laboratories Inc., Segrate, MI, Italy) was used according to the manufacturer's instructions. Reactions were run in duplicate on a CFX96 Real Time System and analyzed by BioRad CFX Maestro Software (Bio-Rad Laboratories Inc.). Gene expression was normalized to HPRT or

RPL13A mRNA content.

MTT assay. MSC cell viability was measured by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma Aldrich, MO, USA). Cells were seeded at a density of $2x10^3$/well in 96-well plates. After 24 h of incubation in RPMI 10% FBS without antibiotics, cells were infected with *B. henselae* (MOI 100). The medium was changed after 4 days to wash out all extracellular bacteria. When indicated, cells were treated with heat-killed *B. henselae*. Cells were then incubated for 3 h with 20 µl MTT (final concentration 0,5 mg/ml). Formazan crystals were solubilized for 10 min in 100 µl DMSO, and OD 570 nm was measured using a microplate reader (VICTOR3^{TM}, PerkinElmer, MA, USA). To determine the contribution of bacteria in MTT reduction to overall values of infected MSCs, a bacterial suspension with the same concentrations per milliliter of those recovered from cells was assessed in parallel and the obtained values subtracted to results from infected cells.

Annexin V Assay. MSCs untreated or infected for 96 h with *B. henselae* were stained with annexin V-FITC and PI (Sigma Aldrich) according to the manufacturer's instructions. Samples were analyzed by FACS Calibur (Becton Dickinson), and results were quantified using FlowLogic (Miltenyi Biotec, Bergisch Gladbach, Germany).

Flow cytometry. MSCs were collected at the indicated times after infection and preincubated for 30 min at 4°C in 1X PBS supplemented with 2% goat serum and 0.2% sodium azide, washed twice with 1% bovine serum albumin (BSA). Successively, cells were incubated for 30 min at 4°C with anti-human TLR-2 FITC (mouse IgG2a) and anti-human TLR-4 PE (mouse IgG2a) or respective isotype controls (all from BioLegend CA, USA). Flow cytometry analysis was performed using FACS Calibur and FlowLogic as described above.

Cytokine measurements. MSCs seeded in 24-well plates were infected with a MOI of 100 for the indicated times. For some experiments, cells werepretreated with the pharmacological inhibitors gefitinib and GSK583 or the neutralizing antibody anti-TLR2 (anti-human TLR2-IgA, clone B4H2) and the human IgA2 isotype control (both purchased from InvivoGen, CA, USA).

Cell-free supernatants were then harvested to measure human VEGF-A, CXCL8, IL-6 and CCL5 production by ELISA (R&D Systems, Minneapolis, MN, USA). To quantify human PDGF-D, a specific kit from Elabscience (Wuhan, Hubei, P.R.C) was employed.

Angiogenesis array. The human angiogenesis array (Proteome Profiler™ Array; R&D Systems) was used to assess the expression of 55 angiogenic-related proteins in MSCs uninfected or infected with *B. henselae* for 96 h. The array membranes were probed with pooled supernatants derived from three independent experiments according to manufacturer's instructions. Chemiluminescent signal was acquired by ChemiDoc™ Imaging System (BioRad).

The signal intensity of each antigen-specific antibody spot was quantified using Fiji-ImageJ (NIH) software. For comparison of the relative expression of proteins in uninfected *vs* infected cells, the mean pixel density of the pair of duplicate spots for each protein, after subtraction of the mean pixel density of the negative control spots of the respective array, was normalized to the mean pixel density of the positive control spots. Heat map analysis using the normalized data was performed by GraphPad PRISM 8.0 software.

Sprouting assay. Sprouting of HUVEC spheroids was assessed as described previously (59). Briefly, spheroids were prepared in 20% methylcellulose medium, embedded in a fibrin gel and stimulated with recombinant human VEGF-A_{165} (30 ng/mL) (R&D System, MN, USA) or with different concentrations of CM from uninfected or infected MSCs. The number of radially growing cell sprouts was counted after 24 h using an Axiovert 200M microscope equipped with LD A Plan 20X/0.30PH1 objective (Carl Zeiss) and expressed as relative increase over untreated spheroids.

Motility assay. HUVEC motility assay was based on "scratch" wounding of a confluent monolayer. Briefly, HUVECs (1×10^5) were seeded onto 0.1 % collagen type I (BD Biosciences, Italy)-coated six-well plates in complete medium until a confluent monolayer was formed. The cell monolayers were scratched using a pipette tip, washed with 1X PBS to remove the undetached cells and treated with MSC conditioned medium. After 24 h, cells were photographed under an Axiovert 200M microscope (Carl Zeiss) equipped with LD A Plan 20X/0.30PH1. The healed area was

quantified through computerized analysis by subtracting the wound area at 24 h from the initial area.

Tube formation assay. EC vessel formation was assessed by tube morphogenesis assay in a three-dimensional (3D) collagen matrix. To this end, HUVECs were seeded onto Reduced Growth Factor Basement Membrane Matrix Cultrex® (BME) (Trevigen, Italy)-coated μ-slide angiogenesis chamber (Ibidi, Martinsried, Germany) at a density of $4.0x10^4$ cells/cm^2 in the absence or presence of CM from untreated or infected MSCs. After 48 h, cells were photographed using an Axiovert 200M microscope, and the number of meshes/field was counted. **Statistical Analysis.** Statistical significance was determined by non-parametric Student's t-test and one-way analysis of variance followed by Tukey's multiple-comparison test. Results were analyzed by GraphPad PRISM 8.0 software (CA, USA).

Abbreviations

MSCs: mesenchymal stromal cells

ECs: endothelial cells

DCs: dendritic cells

TLRs: Toll-like receptors

NLRs: NOD-like receptors

PRRs: Pattern Recognition Receptors

CSD: cat scratch disease

BA: bacillary angiomatosis

BP: bacillary peliosis

GPA: gentamicin protection assay

CM: conditioned medium

MOI: multiplicity of infection

BM: bone marrow

ANOVA: analysis of variance

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DECLARATIONS

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethics Statement

This study was carried out in accordance with the recommendations of "Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino—A.O. Ordine Mauriziano—ASL TO1, number 0009806" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino—A.O. Ordine Mauriziano—ASL TO1."

Availability of data and materials

All data and materials are available upon request.

Author Contributions

SaS, SM, SiS, and TM participated in the design of the study.

SaS, RS, GP, EG, MB, VS, DA, and TS participated in data acquisition and analysis.

TM, SM and SaS wrote the manuscript.

SiS participated in data interpretation and manuscript revision.

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FIGURE LEGENDS

FIG. 1 *B. henselae* invades and persists in MSCs. (a) Invasion rates of *B. henselae* into MSCs were measured at day 1, 2, 3, 4 and 8 pi by gentamicin protection assay (GPA). After infection, cells were treated with gentamicin, and the number of intracellular bacteria was determined by CFU count. Data are expressed as mean ± SEM from two independent experiments carried out in triplicate (*$P < 0.05$ *vs* Log_{10} CFU at 1 day; unpaired t-test). (b) Uninfected (CTRL) or *B. henselae*-infected MSCs (8 days) were immunostained with an anti-*BH* antibody and counterstained with hematoxylin (upper panel 20X, lower panel 40X) or with goat anti-mouse Alexa Fluor® 594 conjugate and DAPI for immunofluorescence visualization (lower panel 100X). (c) To determine intracellular survival after 4 days of infection, extracellular bacteria were killed by gentamicin treatment and incubated in normal medium for the indicated times. Mean values ± SEM of four independent experiments performed in triplicate (*$P < 0.05$; unpaired t-test). (d) Invasion rates of *B. henselae* in MSCs or HUVECs (60,000 cells each, respectively). The number of intracellular

bacteria as Log_{10} CFU was quantified at 1 day pi. Mean ± SEM of three experiments (*$P < 0.05$ MSCs *vs* HUVECs; unpaired t-test).

FIG. 2 *B. henselae* localizes in invasome structures in MSCs. (a) Immunofluorescence of *B. henselae*-infected MSCs at 1, 2, 4 and 8 days pi and uninfected control MSCs (CTLR). *B. henselae* and cell membranes were stained with DAPI (cyan) and wheat germ agglutinin-Alexa Fluor 594 (red), respectively, and analyzed with an epifluorescence microscope. Bacteria anchored to the MSC membrane are indicated with arrowheads. The thin arrows (2 and 4 days) indicate internalized bacteria within membrane bound compartments in the perinuclear area, whereas the large arrows (8 days) highlight sizeable intracellular bacterial aggregates called invasomes. Each image also shows the basal portion of adherent MSC cells, with the orthogonal z reconstruction of the whole cell. (b) Representative image of an invasome. MSCs were infected with *B. henselae* for 8 days and then washed and fixed with PFA. Samples were stained for F-actin (red), wheat germ agglutinin (WGA) (green) and DAPI and analyzed as described in panel a (bar: 10 µm).

FIG. 3 *B. henselae* favors the proliferation of infected MSCs. (a) MSC death was evaluated by FACS analysis after 4 days of infection with *B. henselae*. Uninfected MSCs (left panel; CTRL) and infected MSCs (right panel; *B. henselae*) were double-stained with FITC-annexin V and PI. Counterstaining with PI allowed differentiation of necrotic cells (upper left quadrant of the dot plot), late apoptotic cells (upper right quadrant) and early apoptotic cells (lower right quadrant). The percentages of cells localizing to these quadrants are indicated in each quadrant. Data are representative of three independent experiments. (b) The Bcl-2/Bax expression ratio was analyzed in control and *B. henselae*-infected MSCs at 2 days pi by qPCR. Gene expression was normalized to HPRT. Data are expressed as mean ± SEM of four independent experiments (ns not significant; unpaired t-test). (c) Proliferation assay. MSCs were treated as indicated for 0, 2, 4, and 8 days and analyzed by MTT assay. Untreated MSCs (white circle); *B. henselae* infected MSC (black circle); and heat killed *B. henselae*-treated MSCs (HK *B.henselae*) (grey circle). Data are expressed as

mean ± SEM of three independent experiments performed in triplicate (*$P < 0.05$ *B. henselae vs* CTRL, unpaired t-test).

FIG. 4 Expression of TLR2, NOD1 and EGFR in *B. henselae*-infected MSCs. (a) mRNA expression levels of TLR2, TLR4, NOD1 and EGFR in uninfected (white bar) and *B. henselae*-infected MSCs (black bar) were determined by qPCR and normalized to RPL13A. Data are expressed as mean ± SEM of four independent experiments (* $P < 0.05$; unpaired t-test). (b) TLR2 and TLR4 protein expression levels on MSC membranes were analyzed by FACS in MSCs at 4 days pi. Cells were immunostained with anti-TLR2, anti-TLR4 or specific isotype control antibodies. The percentages of positive cells are indicated in each quadrant. Fluorescence minus one (FMO) controls for the antibodies are shown as well. Data are representative of three independent experiments (left panel) or as mean ± SEM (right panel). (c) Cell extracts from MSCs infected with *B. henselae* for 30, 60, and 120 min or with hEGF (50 ng/mL) for 15 min were subjected to immunoblotting using anti-EGFR pY1068 or anti-EGFR antibodies. (d) Analysis of CXCL8 in the supernatants from uninfected or *B. henselae*-infected MSCs pre-treated or not for 6 h with a neutralizing anti-TLR2 antibody (10 µg/mL) (upper panel, n=6 experiments) or with the EGFR inhibitor gefitinib (10 µM) or the RIP2K inhibitor GSK583 (1 µM) (lower panel, n= 4 experiments) and then stimulated for 96 h. Data are shown as percentage (means ± SEM) of CXCL8 production compared to specific isotype control antibody or DMSO respectively set as 100% (* $P < 0.05$ *vs B. henselae*-infected cells; unpaired t-test). (e) To evaluate *B. henselae* internalization, MSCs were pretreated for 6 h with the neutralizing anti-EGFR (10 µg/mL) (upper panel, n=3 independent experiments) or gefitinib (10 µM) (lower panel, n=4 independent experiments), and CFU values of intracellular bacteria, determined, after 1 and 2 days of incubation, are expressed as percentage relative to CFU of specific isotype control antibody or DMSO-treated cellsset as 100%. Data are shown as mean ± SEM; * $P < 0.05$ *vs* internalized bacteria in untreated cells; unpaired t-test.

721 **FIG. 5 Conditioned medium from *B. henselae*-infected MSCs curbs the infection rates and**
722 **angiogenic response of HUVECs.** The effects of conditioned medium (CM) from *B. henselae*-
723 infected MSCs were tested by means of different angiogenic assays. (a) HUVEC monolayers were
724 wounded with a 1.0-mm-wide rubber policeman and incubated in fresh medium supplemented with
725 5% FCS and 1:2 diluted CM from infected (black bar, CM-MSC CTRL) or uninfected (white bar,
726 CM-MSC *B. henselae*) MSCs. After 1 day, HUVECs invading the wound were quantified by
727 digital imaging to calculate the relative increment in cell-covered area induced by MSC-CM
728 compared to untreated HUVECs. Mean ± SEM of three independent experiments. *$P < 0.05$ *vs* Ctrl;
729 unpaired t-test. (b) Sprouting analysis of HUVEC spheroids. Spheroids were prepared in 20%
730 methylcellulose medium, embedded in fibrin gel and stimulated with 1:2 diluted CM obtained from
731 MSCs treated in the presence (black bar) or absence (white bar) of bacteria or with 30 ng/ml VEGF-
732 A (dashed bar). The number of growing cell sprouts was counted after 1 day. Data are expressed as
733 mean fold change vs Ctrl ± SEM of 20-40 spheroids/ experimental condition in three independent
734 experiments and indicated as fold increase in the number of sprouts/spheroid *vs* Ctrl. *$P < 0.05$*vs*
735 Ctrl; unpaired t-test. (c) The effect of CM from uninfected *vs B. henselae*-infected MSCs on
736 HUVEC morphogenesis was assessed by tube morphogenesis assay in three-dimensional (3D)
737 collagen matrix. HUVECs were seeded (40000 cells/cm^2) on Cultrex Extracellular Matrix in the
738 presence of 1:2 diluted CM from uninfected (white bar) or *B. henselae*-infected MSCs (black bar).
739 After 8 h, the formation of capillary-like structures was examined. Representative images are shown
740 in the left panels. Quantification (right panel) was performed to calculate the relative increment in
741 capillary-like structure induced by MSC-CM compared to untreated HUVECs. Data are expressed
742 as mean ± SEM relative to three independent experiments. * $P < 0.05$ *vs* Ctrl; unpaired t-test. (d)
743 Invasion rate of *B. henselae* in HUVECs (expressed as total CFUs) after 1 day of infection in the
744 absence (grey bar) or presence of 1:2 diluted CM-MSC CTRL (white bar) and CM-MSC *B.*
745 *henselae* (black bar). Mean ± SEM of three independent experiments * $P < 0.05$; unpaired t-test.

746 **FIG. 6 Angiogenic signature of *B. henselae*-infected MSCs.** (a) Human angiogenesis antibody
747 array analysis was performed using a pool of supernatants from 96 h uninfected MSC (CTRL) or *B.*
748 *henselae*-infected MSCs. Some of the most representative angiogenic factors are highlighted in
749 different colors. (b) Representative heat map (left panel) and relative gene expression shown as
750 normalized pixel density of the duplicated spots for each angiogenic-related protein in the array of
751 supernatants of MSCs and *B. henselae*-infected MSCs (right panel). * $P < 0.01$ ** $P < 0.001$ vs
752 CTRL; ANOVA followed by Tukey's multiple-comparison test. (c) Quantification of VEGF-A,
753 CXCL8, IL-6, CCL5 and PDGF-D production in uninfected (CTRL) and *B. henselae*-infected
754 MSCs. Data are expressed as mean ± SEM of three independent experiments. * $P < 0.05$ *vs* CTRL;
755 unpaired t-test. nd= not detectable.

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757

758

759 **Figure 1**

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