Interactions of *Bartonella henselae* with Myeloid Angiogenic Cells and Consequences for Pathological Angiogenesis

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1. Introduction

1.1 *Bartonella*

The bacterial genus *Bartonella* is comprised of slow-growing, facultative intracellular pathogens that infect mammalian hosts and are transferred by hematophagous arthropod vectors. Phylogenetically, these microaerophilic, pleomorphic, rod shaped, bacteria belong to the class of Gram-negative alphaproteobacteria and are most closely related to the bacterial genera *Brucella*, *Agrobacterium* und *Rhizobium* (Anderson and Neuman, 1997; Maurin *et al.*, 1997).

A common feature of *Bartonella* infection is the transition between an often asymptomatic intraerythrocytic phase and a secondary tissue niche which facilitates chronic bacterial persistence and a periodic reseeding of the bloodstream (Dehio, 2004).

The advent of polymerase chain reaction (PCR) technology has greatly expanded the diversity of the *Bartonella* genus. Samples from both wild and domestic animals have revealed a wide variety of *Bartonella* species specialized to various mammalian hosts and arthropod vectors (Kaiser *et al.*, 2011).

To date at least thirteen human pathogenic species of *Bartonella* have been identified (Kaiser *et al.*, 2011). Most clinically relevant *Bartonella* infections in humans are caused by three species: *Bartonella bacilliformis, Bartonella quintana* and *Bartonella henselae*.

Bartonella bacilliformis is believed to be the ancestral strain of human pathogenic *Bartonella* and causes the most severe form of disease (Engel *et al.*, 2011). *B. bacilliformis* is endemic to the South American Andes and is transmitted between human hosts via sand flies of the genus *Luzomyia*. Archeological evidence originating from pre-Inca ceramics and mummified remains indicate that *B. bacilliformis* has existed as a human pathogen for at least 2,000 years (Allison *et al.*, 1974). Infection first manifests as an intense fever (Oroya fever) which results from an anemia due to the massive colonization of erythrocytes and carries a high mortality rate. Individuals who survive Oroya fever may develop a secondary chronic tissue phase which can arise one to two months after the initial fever and involves the chronic growth of blood-filled, wart-like structures consisting of proliferating vessels on the skin of infected patients (Peruvian warts/ verruga peruana; Minnick *et al.*, 2014).

Bartonella quintana, emerged first as a significant human pathogen during the first and second world wars as it spread among soldiers in trenches and prisoner of war camps. More recently, cases of *B. quintana* infection have also been identified in urban homeless populations (Raoult *et al.*, 2001). Infection is transmitted by the human body louse, *Pediculus humanus,* and causes a recurring

dehabilitating fever (Trench fever) believed to be caused by the cyclic release of bacteria from a secondary niche into the blood stream and subsequent colonization of erythrocytes (Rolain *et al.*, 2002).

1.2 *Bartonella henselae*

Bartonella henselae emerged as a relevant human pathogen in the late 20th century. In contrast to the other major human pathogenic *Bartonella* species, *B. henselae* is a zoonotic pathogen specialized to the domestic cat as a reservoir host. Here it exists as an asymptomatic intraerythrocytic bacteremia and is transmitted between cats via the cat flea (*Ctenocephalides felis).* Humans are infected as incidental hosts through the contamination of cat bites or scratches with flea feces (Rolain *et al.*, 2001; Rolain *et al.*, 2003).

Depending on the immune status of the individual, *B. henselae* infection in humans can result in differing pathological outcomes.

In immune-competent individuals infection develops into a generally self limiting condition referred to as “cat scratch disease” which involves swelling of local lymph nodes and occasional granuloma formation (Anderson and Neuman, 1997).

In immune-compromised patients however, chronic infection leads to the development of a vaculoproliferative condition known as bacillary angiomatosis or peliosis hepatis which is characterized by the appearance of tumorous blood filled lesions on the skin and inner organs (Manders, 1996).

Before the advent of effective antiretroviral therapy, bacillary angiomatosis was most commonly observed in immunosuppressed patients suffering from AIDS, which lead to the first description of the condition in 1983 (Stoler *et al.*, 1983; Koehler and Tappero, 1993) and subsequent identification of the infectious agent by the newly developed 16S ribosomal DNA (rDNA) analysis in 1990 (Relman *et al.*, 1990).

1.3 Infection-associated pathological angiogenesis

A unique hallmark of *Bartonella* infections is the ability to induce angiogenic growth in human hosts. This pathogenic process is evident in the development of vascular lesions associated with chronic *Bartonella* infections: verruga peruana (*B. bacilliformis)* and bacillary angiomatosis (*B. henselae;* Dehio, 2005). Of the two, *B. henselae* induced pathological angiogenesis has been the most widely studied.

Ultrastructural examinations of *B. henselae* associated vascular lesions reveal a disorganized proliferation of immature, capillary-sized vessels, misshapen endothelial cells and myeloid cell infiltrate. Aggregates of rod shaped bacteria can be identified in and around the proliferating endothelial cells and complete regression of the angioproliferative structures can be achieved through antibiotic treatment (LeBoit *et al.*, 1989; Kostianovsky and Greco, 1994; Manders, 1996; Schwartz *et al.*, 1997).

The vascular architecture, myeloid cell infiltrate and proliferative growth of *B. henselae* induced vascular lesions bear a strong resemblance to the growth of malignant tumor vasculature suggesting that these neoplastic structures may develop via similar mechanisms (Cockerell and LeBoit, 1990; Koehler and Tappero, 1993; Manders, 1996).

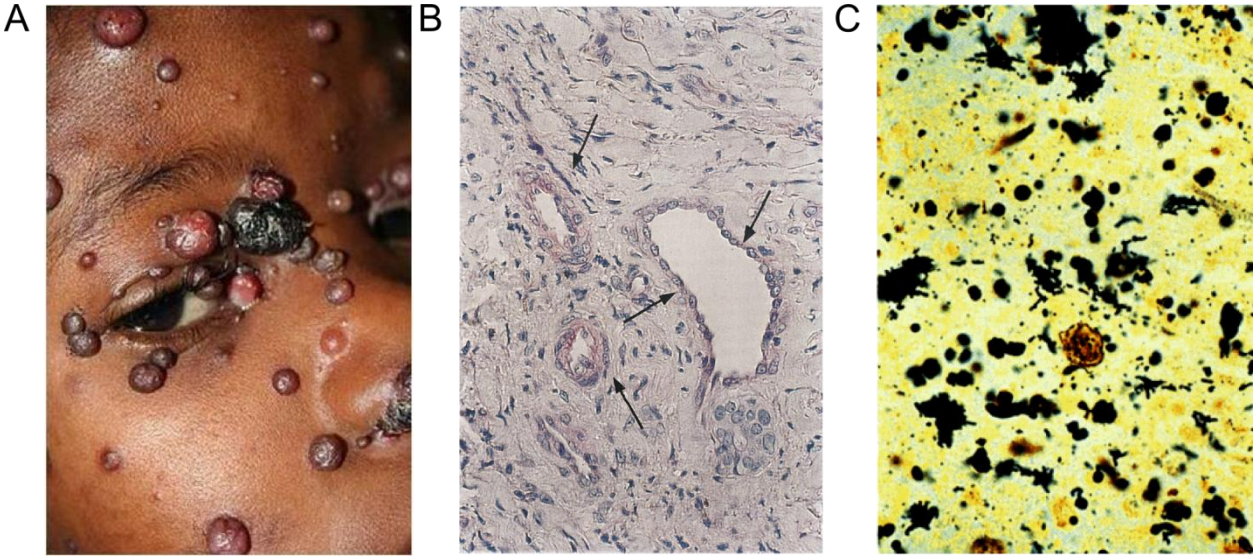


Figure 1.1 *B. henselae* associated pathological angiogenesis: Bacillary angiomatosis. (A) Bacillary angiomatosis is characterized by the occurrence of vascular tumors on the skin and inner organs of immune-suppressed patients (Beatty and Lukusa, October 24. 2014). **(B)** Histological section of bacillary angiomatosis lesion stained with Meyers hematoxlyin and anti-VEGF antibodies (red). Arrows indicate the presence of VEGF in the endothelium of pathologically formed vasculature (Kempf *et al.*, 2001). **(C)** Aggregates of rod shaped bacteria (black) can be identified within the vascular lesions via Warthin-Starry staining (Koehler, 1997).

Infection associated pathological angiogenesis has also been described in the context of several viral infections. Viruses such as human papilloma virus (HPV) and human herpes virus 8 (HHV8) have been associated with the development of neoplasia and increased angiogenic growth. Most notably, infection with HHV8 can lead to the development of Kaposi's sarcoma; a condition identified by the growth of vascular tumors on the skin of immunosuppressed patients (Flore *et al.*, 1998). These vascular lesions are characterized by the proliferation of endothelial cells, the formation of spindle shaped tumor cells and the dense vascularization of a localized area with chaotic and leaky blood

vessels. This appearance, combined with its particular occurrence in AIDS patients, makes it a common differential diagnosis to bacillary angiomatosis.

Increased angiogenesis in viral infections is often associated with cancer formation and therefore linked to the angiogenic qualities of viral oncogenic proteins such as the HHPV8 papillomavirus proteins E6 and E7 (López-Ocejo *et al.*, 2000).

In viral conditions specifically characterized by angiogenic pathologies, angiogenic growth is stimulated by the transcription of virally encoded proteins which mimic host regulators of angiogenesis. The Orf parapox virus, for example, is the agent of a vasculoproliferative condition in sheep and goats and encodes a viral homologue of vascular endothelial growth factor (VEGF), viral (v)VEGF-E which activates endothelial cell proliferation and vascular permeability at levels similar to human VEGF-A (Meyer *et al.*, 1999; Cébe-Suarez *et al.*, 2008). The HHV8 genome also encodes for several open reading frames (ORFs) with known homology to cellular proteins. The transcription of these virally encoded proteins such as viral C-C motif ligand v(CCL)-2, vCCL3 and vIL-6 modulates host immune responses, promotes angiogenesis, and dysregulates cell growth in Kaposi's sarcoma (Ensoli *et al.*, 2001; Gramolelli and Schulz, 2015).

In some cases, chronic bacterial infections have also been associated with increased angiogenic growth. Biopsies of gastric tissue from patients with *H. pylori* associated gastritis or gastric carcinoma demonstrate an increased capillary density (Pousa and Gisbert, 2006; Yeo, 2006). However, these effects are thought to be connected to the prolonged states of chronic inflammation associated with long-term bacterial infections rather than specific pathogenic activities.

Bartonella species, in contrast, are the only known bacteria with the ability to cause the concerted, proliferative growth of angiogenic vessels and the creation of localized vascular tumors in humans. The process by which *B. henselae* induces the development of vascular tumors presents a valuable topic for study due to the importance of angiogenesis in many pathological and regenerative processes and as an example of complex host-pathogen interactions.

1.4 *B. henselae* pathogenicity strategy

Evidence suggest that endothelial cells may represent an important cellular niche for *Bartonella* species *in vivo* (Harms and Dehio, 2012). It has been proposed that the induction of pathological angiogenesis by *B. henselae* infection may represent a pathogenicity strategy by which *B. henselae* induces the proliferation of its own nutrient rich, immune-privileged cellular niche (Kempf *et al.*, 2002).

In vitro experiments demonstrate that *B. henselae* readily invades endothelial cells and can persist intracellularlly (Kyme *et al.*, 2005). *B. henselae*, co-cultured with endothelial cells, show a rapid increase in rRNA production and a replication rate that is 100-fold higher than bacteria cultured in isolation (Kempf *et al.*, 2000). In another study, the proliferation of *B. henselae* infected endothelial cells in response to VEGF or conditioned medium was associated with a 75- and 150-fold increase in *B. henselae* growth rate respectively (Kempf *et al.*, 2001). The increased replication of intracellular *B. henselae* is inhibited when the synthesis of host cell proteins is blocked with cycloheximide, indicating that endothelial cells may provide intracellular bacteria with proteins to support growth (Kempf *et al.*, 2000).

Research into the mechanisms of *B. henselae* induced angiogenesis over the years has been limited by the lack of *in vivo* animal models. Although several models of *Bartonella* infection have been described in the literature, infected animals do not develop the pathological angiogenic growth characteristics of *B. henselae* induced bacillary angiomatosis (Regnath *et al.*, 1998; Arvand *et al.*, 2001; Koesling *et al.*, 2001; Schülein *et al.*, 2001; Velho, Paulo Eduardo Neves Ferreira *et al.*, 2002; Kabeya *et al.*, 2006; Chiaraviglio *et al.*, 2010). As a result, most studies examining *B. henselae* induced pathological angiogenesis have focused on *in vitro* primary-cell culture experiments, angiogenesis models and *ex vivo* immunohistochemistry

So far, *B. henselae* is believed to induce pathological angiogenesis through the synergistic combination of several mechanisms including i) direct stimulation of endothelial proliferation ii) inhibition of apoptosis iii) activation of the hypoxia-inducible factor (HIF)-1 dependent pro-angiogenic programs iv) activation of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NFκB) and release of angiogenic-inflammatory cytokines and e) immune evasion via a stealth-pathogen strategy.

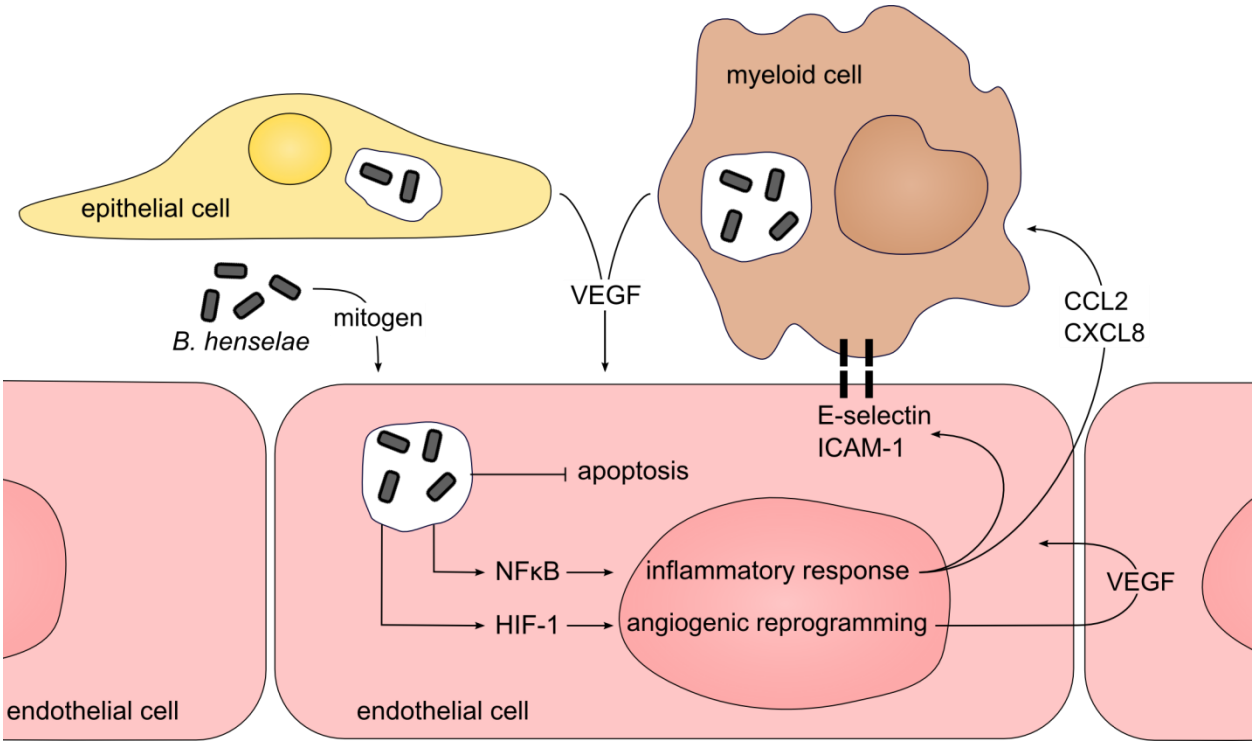


Figure 1.2 Several pathogenic mechanisms work synergistically to promote *B. henselae* associated pathological angiogenesis *in vivo*. *B. henselae* has been shown to release a mitogenic compound which can induce endothelial cell proliferation without host-cell contact. When *B. henselae* encounter endothelial cells they adhere and are taken up into intracellular vacuoles. Infection of endothelial cells results in inhibition of apoptosis and activation of the inflammatory and angiogenic transcription factors NFκB and HIF-1, respectively. Activation of HIF-1 leads to angiogenic re-programming including the secretion of the pro-angiogenic cytokine VEGF which stimulates angiogenesis in infected cells as well as the surrounding endothelium. Activation of NFκB results of inflammatory gene transcription. Infected endothelial cells show upregulation of cellular adhesion molecules ICAM-1 and E-selectin as well as secretion of the inflammatory angiogenic chemokines CCL2 and CXCL8 which induces infiltration of myeloid cells into the infectious microenvironment. Infection of accessory myeloid and epithelial cells with *B. henselae* also leads to secretion of VEGF, further promoting angiogenesis in the surrounding endothelium.

1.4.1 Mitogenic stimulus

Several early studies into *B. henselae* induced angiogenic growth identified a *B. henselae* secreted compound with the ability to increase endothelial cell proliferation in a manner that was independent of bacterial cell contact or invasion. Co-culture of *B. henselae* with endothelial cells separated by a filter membrane or the use of *B. henselae* conditioned cell culture medium was sufficient to induce endothelial cell proliferation (Maeno *et al.*, 1999). The nature of this bacterial factor or factors remains elusive, however, GroEL an endothelial mitogenic factor released by *B. bacilliformis* has been detected in *B. henselae* conditioned medium and was discussed as one of these mitogenic compounds (Minnick *et al.*, 2003).

1.4.2 Inhibtion of apoptosis

Apoptosis represents one of the most basic immune responses to intracellular bacterial infection. The programmed death of host cells acts to deprive bacteria of their immune privileged habitat and releases strong inflammatory alarm signals which trigger the immune response.

B. henselae has been demonstrated to inhibit apoptosis in both endothelial cells and monocyte-macrophage 6 (MM6) cells (Kirby, 2002; Kempf *et al.*, 2005b; Schmid *et al.*, 2006). The process by which *B. henselae* inhibits apoptosis is not well understood, however, studies have shown that *B. henselae* infection suppresses both early and late events in the apoptotic cascade. In MMP6 cells *B. henselae* infection was associated with inhibition of caspase three activity and induction of the anti-apoptotic molecules: cellular inhibitor of apoptosis proteins -1 and 2 (cIAP-1,-2; Kempf *et al.*, 2005b). In another study, inhibition of apoptosis in endothelial cells correlated with increased cytoplasmic concentrations of cyclic adenosine monophosphate (cAMP; Schmid *et al.*, 2006).

The ability of *B. henselae* to stimulate proliferation of endothelial cells has been shown to depend considerably on its ability to inhibit apoptosis (Kirby, 2002). Through the inhibition of programmed cell death, *B. henselae* circumvents a fundamental immune response mechanism and maintains the viability of its cellular niche. The inhibition of apoptosis is also an essential pre-requisite for further cellular reprogramming and the induction of angiogenic growth.

1.4.3 HIF/ VEGF

In addition to promoting angiogenic growth through direct stimulation of endothelial cell proliferation and inhibition of apoptosis, *B. henselae* infection activates autologous angiogenic response programs in infected cells. Central to this is the activation of HIF-1; a heterodimeric transcription factor that regulates responses to oxygen supply, metabolic demands and is the key regulator of the angiogenic response.

B. henselae infection has been shown to induce HIF-1 activation in several cell types including epithelial and endothelial cells (Kempf *et al.*, 2005a). The activation of HIF-1 in *B. henselae* infected cells leads to activation of pro-angiogenic response programs. In *B. henselae* infected epithelial and endothelial cells, infection is associated with up-regulation and release of angiogenic cytokines such as VEGF, adrenomedullin (ADM), and insulin-like growth factor-binding protein -1 (Kempf *et al.*, 2001; Kempf *et al.*, 2005a).

Among these cytokines, VEGF is probably the most important and has been found to play a significant role in *B. henselae* induced endothelial proliferation. This pro-angiogenic stimulation is

not only relevant to infected cells but is transmitted in a paracrine manner to the surrounding endothelium. Conditioned medium from *B. henselae* infected EA.hy 926 cells promoted the proliferation of endothelial cells and this pro-angiogenic effect was found to decrease 50% after treatment with VEGF neutralizing antibodies (Kempf *et al.*, 2001).

A role for HIF-1 activation and VEGF in *B. henselae* induced pathological angiogenesis is also supported by evidence from *ex vivo* bacillary angiomatosis samples showing strong HIF-1 and VEGF expression in and around vessels of infected vascular lesions (Kempf *et al.*, 2001; Kempf *et al.*, 2005a).

To date, the exact cellular mechanisms of *B. henselae* induced HIF-1 activation has not been elucidated. Although mitogen-activated protien kinases (MAPKs) are phosphorylated during *B. henselae* infection of epithelial cells, these signal transduction pathways appear to have little influence over *B. henselae* induced activation of HIF-1 dependent pro-angiogenic programs. Instead, *B. henselae* infection may activate HIF-1 by mimicking a cellular hypoxic state as infected cells display signs of cellular hypoxia, increased cellular oxygen consumption, decreased adenosintriphosphat (ATP) levels and a gene expression profile characteristic of the hypoxic response (Kempf *et al.*, 2005a).

It is known that HIF-1 activation in *B. henselae* infected cells is dependent on direct bacterial-host cell interaction, specifically the activity of the *B. henselae* outer membrane protein *Bartonella* adhesin A (BadA). HIF-1 activation decreases by 30% when cells are cultured with heat killed bacteria and a decrease of 90% was observed when cells were infected with *B. henselae* mutant strains lacking BadA protein expression (Riess *et al.*, 2004).

1.4.4 Myeloid cells and the inflammatory response

The interaction of *B. henselae* with components of the innate immune system is also believed to be an essential component of *B. henselae* induced pathological angiogenesis.

B. henselae infection clearly activates the inflammatory transcription factor NFκB in several cell types and is associated with the up-regulation and release of a range of NFκB dependent inflammatory cytokines such as C-X-C motif ligand (CXCL)8, CCL2, and granulocyte-macrophage colony-stimulating factor (GM-CSF; Resto-Ruiz *et al.*, 2002; Schmid *et al.*, 2004a; Kempf *et al.*, 2005a; Kempf *et al.*, 2005b; McCord *et al.*, 2005).

The induction of NFκB dependent inflammatory gene regulation and cytokine release is believed to play a role in activating the angiogenic response in infected cells.

Infection of endothelial cells with *B. henselae* was shown to result in secretion of the inflammatory-angiogenic cytokine CXCL8 and upregulation of the angiogenic chemokine receptor CXCR2. The secretion of CXCL8 from infected endothelial cells was found to be required for *B. henselae* induced cell proliferation, tube formation and apoptosis inhibition *in vitro* (McCord *et al.*, 2006).

NFκB regulated cytokines also act as chemokines to attract myeloid cells to the site of infection. NFκB dependent upregulation of cellular adhesion molecules intercellular adhesion molecule (ICAM)-1 and E-selectin in *B. henselae* infected endothelial cells resulted in increased polymorphonuclear leukocyte rolling and adhesion to infected endothelial monolayers. NFκB dependent release of CCL2 from infected human micro-vascular cells induced migration of THP-1 mononuclear cells *in vitro* (McCord *et al.*, 2005).

The attraction of myeloid cells to the site of *B. henselae* infection is hypothesized to contribute to pathological angiogenesis through the release of pro-angiogenic cytokines and the initiation of a pro-angiogenic paracrine loop of cell activation and cytokine secretion (Kempf *et al.*, 2002; Resto-Ruiz *et al.*, 2002). Infection of the J774 murine macrophage cell line with *B. henselae* induced increased secretion of tumor necrosis factor (TNF)α, IL-1β and IL-6 (Musso *et al.*, 2001). *B. henselae* infected THP-1 macrophages were found to secrete angiogenic cytokines VEGF and CXCL8 and conditioned medium from infected THP-1 cells induced increased proliferation of HMEC-1 cells *in vitro* (Resto-Ruiz *et al.*, 2002).

1.4.5 Immune evasion

Although *B. henselae* clearly activates important aspects of the inflammatory response in infected cells, *in vivo* infection is not associated with high levels of systematic inflammation. *B. henselae* employs what has been described as a “stealth pathogen” strategy of intercellular lifestyle and slow growth which requires simultaneous activation and suppression of the inflammatory responses to insure bacterial persistence and evasion of immunity (Pulliainen and Dehio, 2012).

To prevent recognition by host pattern recognition receptors, the *B. henselae* lipopolysaccaride (LPS) molecule contains structural modifications which prevent binding to toll-like receptor (TLR)4 and activation of the inflammatory response cascade. Purified LPS from *B. henselae* has been shown to be 1000-10,000 fold less potent at activating TLR4 signaling than *Salmonella enterica* LPS (Zähringer *et al.*, 2004).

Following infection of host cells, *B. henselae* can control the fate of its membrane-bound compartment. *B. henselae* containing vacuoles lack typical endocytic marker proteins and fails to acidify. In J774A.1 macrophages, fusion with the lysosome is delayed and in endothelial cells, fusion

can be inhibited completely. Viable bacteria are necessary to prevent lysosome fusion indicating that *B. henselae* actively subverts the normal endosomal trafficking pathways in the creation of its intracellular habitat (Kyme *et al.*, 2005).

Some evidence exists that *B. henselae* may also actively suppresses inflammatory responses as part of its pathogenicity strategy.

Within its natural host, the cat, *B. henselae* infection induces a Th2 immune response including a transient decrease in cluster of differentiation (CD)4^+ T cells (Kabeya *et al.*, 2006). In *B. henselae* infected mice and humans, infection is associated with an increased concentration of the anti-inflammatory cytokine IL-10 in blood plasma (Papadopoulos *et al.*, 2001; Kabeya *et al.*, 2007). *In vitro* examination of the dendritic cell response to *B. henselae* infection also revealed increased IL-10 secretion in *B. henselae* infected cells (Vermi *et al.*, 2006). Lastly, an early study found that *B. henselae* infection impairs the production of reactive oxygen species in polymorphonuclear leukocytes and thereby avoids the induction of oxidative burst (Fumarola *et al.*, 1996).

1.5 *B. henselae* pathogenicity factors

Two main *B. henselae* pathogenicity factors have been identified as being involved in the process of *B. henselae* induced pathological angiogenesis: the trimetric autotransporter adhesin BadA and the VirB/D4 type IV secretion system.

1.5.1 *Bartonella* Adhesin A (BadA)

Bartonella adhesin A (BadA) is a extremely long (ca. 240nm) fibril adhesion protein expressed on the outer membrane of *B. henselae*. BadA is a member of the trimeric autotranporter (TAA) family of proteins commonly found in animal and plant pathogenic proteobacteria and conserved within the *Bartonella* genus. The BadA structure displays the typical TAA modular construction of membrane anchor, multiple repeating neck and stalk components and a terminal head domain (O'Rourke *et al.*, 2011).

BadA is important for several pathogenicity functions. The head and stalk domains mediate bacterial autoagglutination and adhesion to several extracellular matrix proteins including fibronectin, laminin and various collagens (Kaiser *et al.*, 2008; Kaiser *et al.*, 2012). BadA also plays an essential role in effective bacterial adhesion and uptake into *Bartonella*-containing-vacuoles (BCV)s in various host cells including endothelial and epithelial cells as well as preventing phagocytic uptake by macrophages (Riess *et al.*, 2004; Kyme *et al.*, 2005). The role of BadA in adhesion to the extra cellular matrix and endothelial monolayers has been shown to be of particular significance under dynamic

flow conditions suggesting that this pathogenicity factor may be involved in colonization of the endothelial niche from the bloodstream (Müller *et al.*, 2011).

In addition to its roles in bacterial adhesion, BadA is essential in activation of the *B. henselae* dependent angiogenic response in infected cells. The expression of BadA on the surface of *B. henselae* is necessary to activate the angiogenic transcription factor HIF-1 and for the upregulation and release of the HIF-1 regulated angiogenic cytokine VEGF (Riess *et al.*, 2004). The mechanisms of this action are not yet clear but it has been hypothesized that the interactions of BadA with fibronectin and other extracellular matrix proteins may subvert intracellular signaling via interaction with β_1-integrins (Riess *et al.*, 2004).

Molecular studies employing various BadA mutant strains have found most BadA pathogenicity functions to be concentrated in the head domain including autoagglutination, binding to extracellular matrix and host cells as well as activation of the angiogenic response (Kaiser *et al.*, 2008). The significance of BadA's extreme length is not yet known. The length is determined by the number of neck-stalk sub-domain repetitions present in the stalk domain of the expressed protein and has been demonstrated to vary among isolates. Some regions of the BadA stalk domain have been shown to play a role in binding extracellular matrix proteins and are essential for binding to fibronectin (Kaiser *et al.*, 2012). The repetition of these domains along the length of the BadA stalk may allow for more efficient binding under the dynamic environment of the blood stream. It has also been suggested that the length of the BadA protein may have some physical function such as in extending the head domain at a defined distance from the bacterial cell surface (Kaiser *et al.*, 2012).

1.5.2 VirB/D4 Type IV Secretion System (T4SS)

Along with BadA, the VirB/D4 type IV secretion system (T4SS) represents an important pathogenicity factor in *B. henselae* induced pathological angiogenesis. T4SS are common structures among pathogenic bacteria and are constructed as needle-like macromolecular machines that translocate bacterial effector proteins into host cells.

The *B. henselae* T4SS is encoded by the VirB operon which encodes the individual components of a translocation channel spanning both inner and outer membranes and a longer external component that interacts with the host cell (Schröder and Dehio, 2005). Seven *Bartonella* effector proteins (Beps A-G) have been identified so far. Beps are modularly constructed, each containing an C-terminal secretion signal including a *Bartonella* intercellular delivery domain (BID) along with effector domains which are activated within the host cell (Schulein, 2005; Pulliainen and Dehio, 2009; Engel *et al.*, 2011).

T4SS activity has been shown to play an important role in several aspects of *B. henselae* pathogenicity. The most striking effect of T4SS on host cells is the formation of a specific invasion structure deemed the “invasome” in which stress fibers form an F-actin ring and create membrane protrusions which engulf and internalize large bacterial aggregates (Rhomberg *et al.*, 2009). The formation of the invasome has been shown to be dependent on the translocation of BepG and in part BepC and BepF. The interaction of Beps with host cell Rho guanosine-5'-triphosphate (GTP)ases induces massive F-actin reorganization and polymerization to form the invasome structures (Truttmann *et al.*, 2011a).

T4SS activity may also activate *B. henselae* specific NFκB response programs. In *B. henselae* infected endothelial cells the secretion of CXCL8 and expression of ICAM-1 was dependent on T4SS activity. NFκB transcriptional activity was shown to be significantly reduced in mutants lacking functional T4SS (Schmid *et al.*, 2004a).

The T4SS has also been shown to inhibit *B. henselae* mediated apoptosis. In particular the translocation of BepA to the cellular cytoplasm is associated with increased cAMP concentration in infected cells and inhibition of endothelial cell death (Schmid *et al.*, 2006). BepA translocation also appears to have a pro-angiogenic effect on vascular sprouting in a spheroid angiogenesis model; an effect which is believed to be connected to its anti-apoptotic qualities (Scheidegger *et al.*, 2009).

Interestingly, *B. henselae* T4SS has also been demonstrated to produce bacterial factors that inhibit angiogenic activity. Activities of the T4SS have been shown to interfere with VEGF-VEGFR2 downstream signaling in endothelial cells (Scheidegger *et al.*, 2011). The effector protein BepG, has been shown to be a potent anti-angiogenic factor inhibiting endothelial sprout formation in the spheroid model of sprouting angiogenesis (Scheidegger *et al.*, 2009). It has been speculated that these anti-angiogenic effects may coordinate with other pro-angiogenic factors to regulate *B. henselae* induced pathological angiogenesis or may be employed during a phase of infection in which angiogenic growth is unneeded or undesirable (Truttmann *et al.*, 2011b).

1.5.3 Interaction of BadA and T4SS

Although both BadA and T4SS clearly have significant functional roles in *B. henselae* infection, the interaction between these pathogenicity factors could not be examined until recently. Most studies describing *B. henselae* infections have been performed using *B. henselae* strains possessing either a functional T4SS but lacking expression of full length BadA proteins (strain *B. henselae* Houston-1) or strains with a full length BadA but only a weak ability to translocate bacterial effector proteins (strain *B. henselae* Marseille). It had been previously hypothesized that the two pathogenicity factors

could work synergistically when combined. However, a recent investigation revealed that strains carrying both pathogenicity factors were rare among available isolates. Further investigation utilizing a genetically manipulated *B. henselae* stain artificially expressing both pathogenicity factors revealed that BadA interferes with T4SS effector protein translocation into host cells. Bep translocation into host cells was found to be inversely correlated with the length of the BadA protein. The capacity of BadA to adhere to extra cellular matrix proteins, endothelial cells and to activate HIF-1 were not affected by the presence of the functional T4SS (Lu *et al.*, 2013). These results have led to the conclusion that BadA and T4SS have separate functions, possibly being expressed at differing time points over the course of *B. henselae* infection.

1.6 Angiogenesis

Angiogenesis; the system of blood vessel growth and expansion is a fundamental process in the development, growth and adaptive capacity of higher organisms. The regulation and dysregulation of vascular growth can have wide reaching consequences for many biological processes.

1.6.1 Development of the vascular network

Within the developing embryo, vasculature first develops from mesodermal hemangioblasts which act as a common progenitor for both endothelial and hematopoietic cell lineages. Hemangioblasts differentiate to angioblasts and finally endothelial cells which create a primitive vascular network in a process known as vasculogenesis (Coultas *et al.*, 2005).

Following initial vascular expansion newly formed vessels mature via the process of arteriogenesis in which vascular channels are stabilized by the secretion of a basement membrane and the establishment of intercellular junctions. A layer of pericytes is deposited and in larger vasculature, a concentric band of smooth muscle surrounds vessels to provide structural support and regulate vascular function (Jain, 2003).

In adult organisms new vessels are predominantly formed through the process of angiogenesis which is defined as the sprouting, branching and expansion of the vascular system from existing vasculature.

1.6.2 The angiogenic process

Once formed, adult blood vessels are maintained in quiescence though a dynamic balance of angiogenic and anti-angiogenic factors. An excess of angiogenic stimulus activates the so called “angiogenic switch” initiating the process of sprouting angiogenesis (Carmeliet, 2005; Herbert and Stainier, 2011).

Hypoxia represents one of the most important activators of angiogenesis in both healthy and pathogenic tissue growth. As tissue expands or when existing vasculature is damaged, diffusion can no longer adequately supply cells with oxygen creating a local hypoxic microenvironment. This triggers the activation of HIF-1, a transcription factor which acts upon hypoxic response elements in the promoters of angiogenic genes, activating angiogenic response programs and the release of pro-angiogenic signaling factors (Pugh and Ratcliffe, 2003).

The list of compounds with pro-angiogenic qualities includes specialized growth factors, cytokines, chemokines, lipid mediators, hormones and neuropeptides (Carmeliet and Jain, 2011). Among these, the most intensely studied is vascular endothelial growth factor (VEGF), an angiogenic growth factor which acts via binding to the vascular endothelial growth factor receptor 2 (VEGFR2) and activation of an angiogenic signaling cascade (Herbert and Stainier, 2011).

The activation of angiogenic signaling in endothelial cells initiates a multi-step angiogenic process which includes dissociation of the existing vascular barrier, vascular sprout extension, vessel stabilization and resolution.

In the first stage of angiogenic sprouting, activated endothelial cells initiate the dissolution of the existing vascular barrier via degradation of the extracellular matrix basement membrane, detachment of pericytes and dissociation of endothelial cellular junctions (Potente *et al.*, 2011). Next, the coordination of sprout formation is mediated by the differentiation of activated endothelial cells into migrating tip- and proliferating stalk-cells. Endothelial cells compete for tip-cell position through the secretion of inhibitory compounds which prevent the tip-cell differentiation in surrounding cells and induce a stalk cell differentiation by default. The selected tip cells then extend filopodia into the extracellular space and migrate towards the angiogenic stimulus (e.g. hypoxia) guided by a gradient of pro-angiogenic signaling molecules (e.g. VEGF). Stalk cells are guided by the migration of the tip cell and proliferate to extend sprout length in the direction of angiogenic stimulus (Carmeliet *et al.*, 2009).

Proteases such as matrix metalloproteinases (MMPs) and plasminogen activators (PAs) facilitate the invasion of vascular sprouts into surrounding tissue via directional degradation of the extracellular matrix (ECM). A support scaffold for the extending sprout is also provided by the secretion of provisional matrix proteins such as fibronectin and fibrin (Sottile, 2004).

As sprouts extend, stalk cells form lumens though the coordinated fusion of intracellular vacuoles and perfused branches are formed when neighboring tips cells encounter one another via filopodian interactions and fuse in a process of anastomosis.

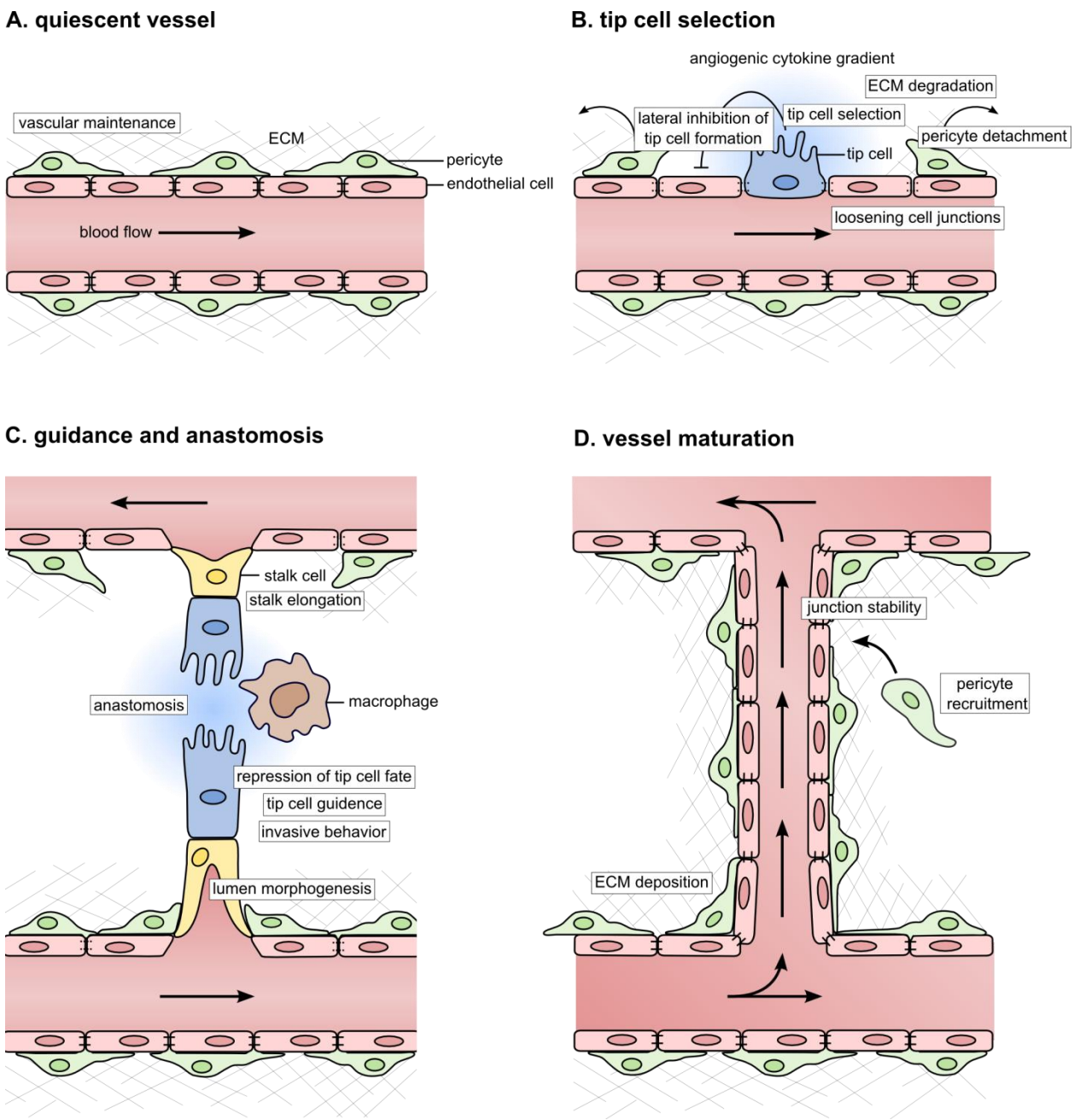


Figure 1.3 The angiogenic process. **(A)** In the absence of pro-angiogenic signalling, endothelial cells are maintained in a quiescent state**. (B)** In response to pro-angiogenic signals (e.g. VEGF) tip cells (blue) are selected for sprouting. Tip cell fate is inhibited in neighboring endothelial cells via Notch signaling. Vascular sprouting is facilitated via detachment of the pericytes layer (green), loosening of endothelia cell junctions and degradation of the extracellular matrix. **(C)** Invasive tip cell sprouting is guided by pro-angiogenic cytokine gradient and vascular sprouts elongated via the proliferation of stalk cells (yellow) followed by vascular lumen morphogenesis. Upon contact with other vessels, tip cell phenotype is repressed and vessels fuse via the process of anastomosis which is guided by accessory macrophages. **(D)** Successful perfusion of new vessels leads to a resolution phase of angiogenic growth in which pericytes are recruited, endothelial cell junctions are strengthen and the extracellular matrix re-established. Upon vessel maturation endothelial cells return to a quiescent phalanx cell state. ECM: extracellular matrix. Adapted from (Herbert and Stainier, 2011).

In healthy angiogenic growth, vascular perfusion also initiates a resolution stage. Endothelial cells take on a quiescent, phalanx cell phenotype increasing intercellular junctions, depositing a basement membrane, inducing recruitment and maturation of pericytes and reestablishing a smooth muscle layer.

Following successful angiogenesis, increased oxygen and tissue vascularization restores tissue homeostasis and reestablishes a balance of pro- and anti- angiogenic factors that maintain vessel quiescence. With the cessation of vascular stimulus, redundant or unperfused vessels regress in a process of vascular pruning (Potente *et al.*, 2011).

1.6.3 Angiogenesis in health and disease

Following embryonic development, angiogenesis occurs as a regular process during normal developmental tissue growth as well as in adulthood during the menstrual cycle and growth of the placenta. Expansion of the vascular network is also a component of normal adaptive tissue modification such as increased angiogenesis in muscle tissue in response to exercise and the vascularization of adipose tissue during weight gain (Wagner, 2001; Carmeliet, 2005; Ye, 2011).

The provision of damaged tissue with functional blood vessels is also an important factor in regenerative growth and is crucial to wound healing and tissue repair (Tonnesen *et al.*, 2000). The revascularization of damaged tissue after ischemic injury (myocardial infarction, thrombosis, stroke) has been shown to play a major role in the body's ability to recover (Liman and Endres, 2012; Silvestre, 2012; Ouma *et al.*, 2013).

Due to its comprehensive role in so many biological processes, dysregulation of angiogenesis can also precipitate or exacerbate a wide range of pathological conditions.

Excessive angiogenic growth is involved in the progression of cancer, psoriasis, arthritis and several retinopathies while a lack of sufficient angiogenic growth or vascular degradation are characteristic of cardiovascular conditions, diabetes, osteoporosis and neurodegenerative disorders such as Alzheimer's disease (Carmeliet, 2003).

The importance of angiogenesis in various fundamental biological processes has made the development of therapies to increase or prevent angiogenic growth a major focus of several fields of medical research. In particular, research into tumor angiogenesis has contributed much understanding to the process of pathological angiogenesis in general.

1.7 Tumor angiogenesis

The initiation of angiogenesis and the in-growth of new vessels to the tumor microenvironment is a decisive event in the progression of solid cancers.

As tumors develop, their metabolic requirements outstrip the existing supply of oxygen and metabolites, creating localized regions of hypoxia. The resulting release of proangiogenic cytokines from tumor cells and surrounding tissue activates the angiogenic switch and initiates angiogenic sprouting from neighboring vessels (Bergers *et al.*, 2000).

Reflecting the dysregulatory nature of tumor microenvironments, the architecture, phenotype and function of tumor vasculature differs greatly from that of healthy vascular networks. Tumor angiogenesis is associated with an uncoordinated barrage of pro-angiogenic factors (dominated in particular by a few growth factors e.g. VEGF) that continues indefinitely, resulting in the formation of a dense vascular network of constantly remodeling vessels (Nagy *et al.*, 2009). The vessels that form are irregularly shaped and vascular architecture is chaotic, tortuous and irregularly branched. Vessels are also often immature, lacking an effective pericyte layer. The overabundance of permeability cytokines, such as VEGF, makes vessels leaky and hemorrhagic. Tumor vessels also lack the defined hierarchy of arterious and venouls displaying mixed phenotypic characteristic of both. As a result, the circulation of blood through this network is inefficient. Blood flow is slow, and irregular, alternating in direction and pooling in the various “dead ends” created when disorganized vessel formation results in incomplete vascular connections (Goel *et al.*, 2011).

The access of tumors to the circulatory system facilitates the transport of nutrients to the growing tumor and increases opportunities for metastasis throughout the body. Microvessel density has been shown to correlate with disease prognosis and the rate of tumor progression in many cancers (Uzzan *et al.*, 2004; Yao *et al.*, 2005; Bremnes *et al.*, 2006; Des Guetz *et al.*, 2006; Rubatt *et al.*, 2009).

Elucidation of the distinct vascular phenotype associated with tumor angiogenesis is not only of relevance for understanding the mechanisms of tumor progression but also plays a role in effective cancer therapy and acts as a model for pathological angiogenesis in general (Jain, 2005).

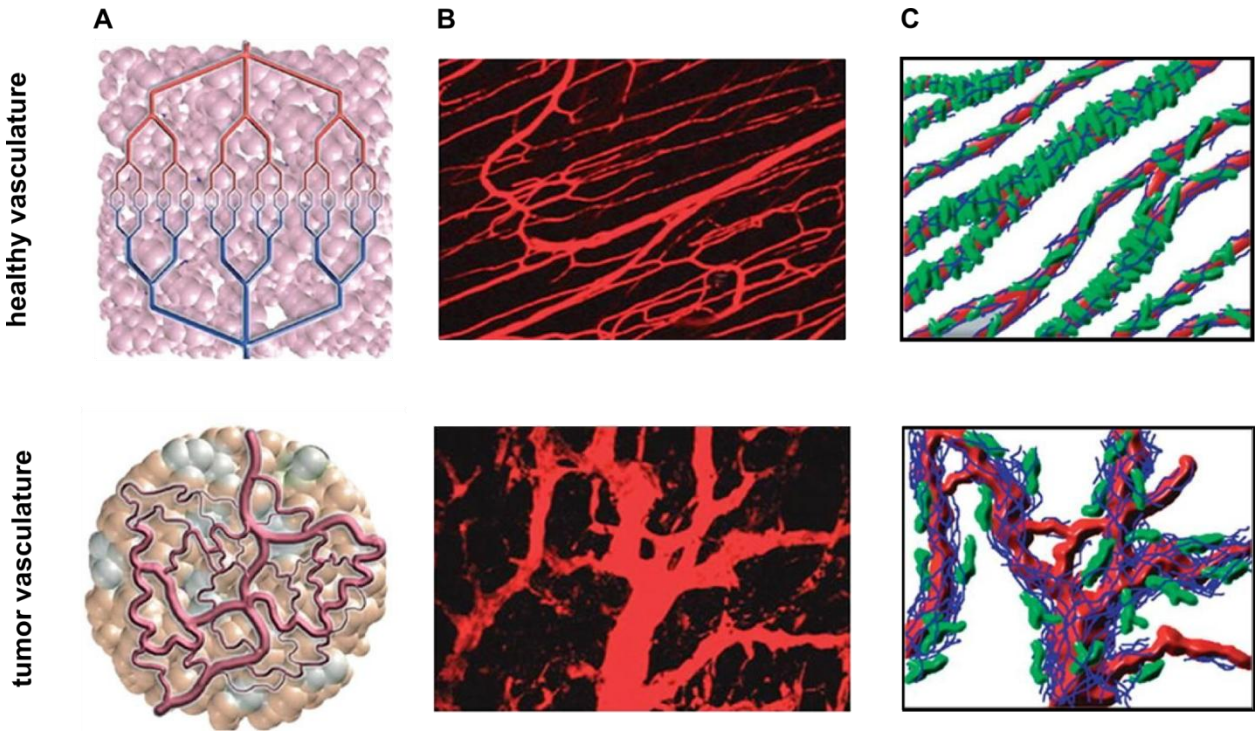


Figure 1.4 Tumor angiogenesis is characterized by dysfunctional vessels and a chaotic vascular architecture. Tumor angiogenesis is induced through an uncoordinated barrage of pro-angiogenic factors and thus results in immature vessels and a densely packed and disorganized vascular growth. Vasculature is dilated and leaky, lacking directional flow and ateriol/venouls differentiation characteristics. **(A)** Schematic representation of healthy vs. tumor vasculature **(B)** two-photon microscopy images showing vasculature in healthy skeletal muscle compared to human colon carcinoma in mice. **(C)** Diagram depicting the changes in pericytes (green) and basement membrane coverage between healthy and tumor vasculature. Modified from (Goel *et al.*, 2011).

1.8 Angiogenic progenitor cells

1.8.1 Endothelial Progenitor Cells (EPCs)

In the adult organism self renewing, multipotent populations of adult stem cells are distributed at specific sites throughout the body where they act as a source for new cells to replenish tissue and contribute to growth and repair. Derived from these are various sets of progenitor cells, with pre-defined differentiation spectrums and limited self renewal potential.

In 1997 a subset of bone marrow derived circulating cells were identified by Ashara *et. al.* as having the ability to take on endothelial characteristics in culture and to improve vascularization when applied in a mouse hind limb ischemia model (Asahara, 1997).

The discovery of these “endothelial progenitor cells” (EPCs) was welcomed as a prospective tool to improve therapeutic vascular regeneration and as a target in preventing pathological angiogenesis (Kocher *et al.*, 2001).

EPCs were hypothesized to be mobilized from the bone marrow niche to the circulation in response to vascular injury or hypoxia. The circulating progenitor cells were then believed to migrate towards sites of angiogenic growth and aid in neovascularization though differentiation into endothelial cells and formation of new vessels in a process of “post-natal vasculogenesis”.

In the following years several groups expanded the range of functional qualities attributed to endothelial progenitor cells. EPCs were found to improve angiogenesis and revascularization in several animal models including mouse models of hind limb ischaemia, myocardial infarction and wound healing (Kalka *et al.*, 2000; Kawamoto *et al.*, 2002; Bauer *et al.*, 2006).

In human subjects the importance of EPCs in the maintenance of vascular biology was underlined by studies demonstrating low levels and functional deficits of EPCs recovered from individuals with cardiovascular disorders. The level of EPCs in the blood was found to be a biomarker for atherosclerotic status and cardiovascular risk (Eizawa, 2004; Schmidt-Lucke *et al.*, 2005) and EPCs were identified as important players in cardiovascular disorders, tumor angiogenesis, and diabetes (Werner *et al.*, 2005; Gao and Mittal, 2009; Calzi *et al.*, 2010; Fadini *et al.*, 2012; La Puente *et al.*, 2013).

The regenerative qualities of EPCs in animal models encouraged the development of human therapeutic techniques. The isolation, culture and re-injection of patient autologous EPCs was developed as a technique to improve revascularization after myocardial infarction and thrombosis (Losordo and Dimmeler, 2004). These techniques were expanded and developed as far as randomized, double-blinded placebo controlled multicenter clinical trials (Lunde *et al.*, 2006; Mills and Rao, 2007). The results of these clinical studies however were mixed. While some were able to demonstrate improvement in revascularization, most advance stage clinical trials could not replicate the success seen in animal models (Fadini *et al.*, 2012).

1.8.2 Myeloid Angiogenic Cells (MACs)

Although the *in vivo* regenerative qualities of EPCs and their participation in angiogenic growth is well established, over the years debate has arisen about their identity and exact roles in angiogenesis (Richardson and Yoder, 2011).

To a large extent, this debate stems from the wide variety of techniques used to isolate and study EPCs. Due to the variation in isolation and cultivation techniques phenotypically and functionally distinct subsets of circulating progenitors cells have been studied under the umbrella term of “endothelial progenitor cells” (Seeger *et al.*, 2007).

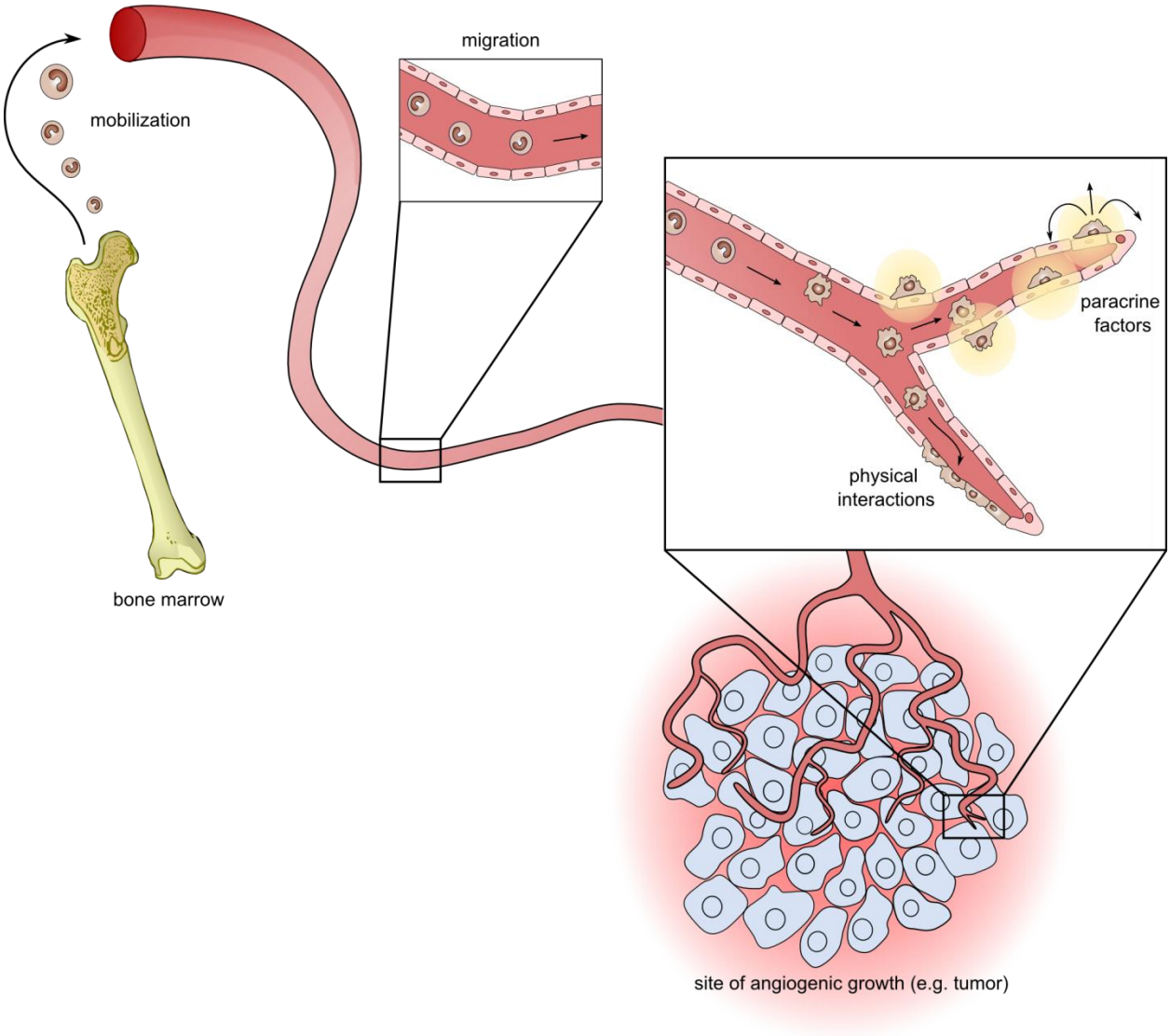


Figure 1.5 Myeloid Angiogenic Cells (MACs) are a subset of circulating myeloid progenitors that play an important role in pathological and regenerative angiogenesis and tumor vascularization. MACs originate in the bone marrow where they mature and are released into the blood stream. In response to angiogenic signals (e.g. hypoxia, inflammation) MACs migrate to the sites of angiogenic growth and contribute to increased angiogenesis in a paracrine manner as well as through physical interaction with the growing vessels.

Furthermore, the various combinations of endothelial markers used to isolate EPCs, identify EPCs *in vivo* and confirm the endothelial characteristic of isolated EPCs have since been shown to be unspecific, and to overlap with several other cell differentiation phenotypes including monocytes and other hematopoietic cells (Rohde *et al.*, 2007; Yoder, 2012).

Several studies examining the origin and phenotype of various EPC subtypes have revealed the majority of cells previously identified as EPCs to be of hematopoietic origin with myeloid, monocytic features. This includes the majority of EPC subtypes used in *in vivo* animal models, for enumeration

from human peripheral blood and in clinical trials (Rehman *et al.*, 2003; Sieveking *et al.*, 2008; Medina *et al.*, 2010b; Yoder, 2013b).

While some techniques used to culture isolated EPCs have been demonstrated to produce cells with true endothelial characteristics *in vitro*, controversy exists as to whether these cells represent a biologically relevant cellular subset or the product of progenitor phenotypic plasticity and differentiation pressure from culture conditions (Hur *et al.*, 2004; Rohde *et al.*, 2006; Rohde *et al.*, 2007).

Originally, EPCs were believed to simulate angiogenesis through the differentiation to mature endothelial cells and participation in the development of new vessels. Several groups reported EPCs as incorporating into the vascular wall, aiding in vascular regeneration via differentiation into endothelial cells and the formation of new vasculature (Lyden *et al.*, 2001; Aicher *et al.*, 2003). In one study utilizing an inducible "suicide gene" in infused cells, it was demonstrated that the incorporation of EPCs into newly formed vasculature was essential to maintaining vascular integrity in the weeks following myocardial infarction (Ziebart *et al.*, 2008). However, many studies have also disputed this mechanism as the source of EPC pro-angiogenic activity (Göthert *et al.*, 2004; Wickersheim *et al.*, 2009; Hagensen *et al.*, 2011; Hagensen *et al.*, 2012). More recently the majority of reports focus predominantly on paracrine effects (Rehman *et al.*, 2003; Kinnaird *et al.*, 2004; Urbich *et al.*, 2005; Yoon *et al.*, 2005; Purhonen *et al.*, 2008). Di Santo *et. al.* (2009) demonstrated that paracrine effects alone could account for the angiogenic regenerative qualities of EPCs in a rat model of chronic hind limb ischemia. Recent advances in three dimensional (3D) confocal microscopy have also localized "integrating" EPCs to the perivascular area of the vasculature but not to the endothelium itself (Göthert *et al.*, 2004; Rajantie *et al.*, 2004; Purhonen *et al.*, 2008; Wickersheim *et al.*, 2009) leading to the hypothesis that these cells may contribute to vascular stability by providing structural support and though the release of paracrine factors rather than through true endothelial differentiation (O'Neill *et al.*, 2005; Fadini *et al.*, 2009; Ieronimakis *et al.*, 2012).

To better reflect the new functional and phenotypic characterizations of these angiogenic progenitor cells, several authors have adopted a new name for this subset of circulating cells: "myeloid angiogenic cells" (MACs) to represent a circulating myeloid progenitor subset which responds to angiogenic signals and plays a role in stimulating regenerative and pathological angiogenesis (Medina *et al.*, 2010a).

The identification of many EPC subsets as being of myeloid differentiation is in line with the growing evidence from other fields demonstrating the importance of cells of the myeloid linage in angiogenic

processes. The monocyte- macrophage linage in particular has been attributed important roles in several aspects of pathological and regenerative angiogenesis.

1.9 Monocytes and macrophages

Cells of the myeloid lineage arise from hematopoietic stem cells in the adult bone marrow and include monocytes, neutrophils, basophils, eosinophils, mast cells and dendritic cells.

As they mature, monocytes are released into the circulation where they comprise approximately 5-10% of the peripheral blood mononuclear fraction. From there they migrate into tissues in response to physiological signals, differentiate into macrophages and develop distinct functional phenotypes depending on microenvironmental stimuli and their localization within the body (van Furth and Cohn, 1968; Gordon and Taylor, 2005; Geissmann *et al.*, 2010; Wynn *et al.*, 2013).

Phenotypic plasticity and motility are defining characteristics of the monocyte/macrophage linage. The ability of macrophages to transform local microenvironments through the secretion of diverse cytokines and growth factors as well as the ability to physically re-model surrounding tissue architecture through phagocytosis, the secretion of proteases and extracellular matrix proteins make them important functional players in many physiological processes (Wynn *et al.*, 2013).

1.9.1 Macrophage functions

Macrophages have diverse functional roles which range from innate immunity, to tissue regeneration, developmental growth, and regulation of metabolism.

In their most basic function both resident macrophages and circulating monocytes act as a rapid response mechanism to disruption of homeostasis such as infection, tissue damage, and other physiological disturbances including foreign materials, hypoxia and metabolic imbalances.

Inflammatory signals from the affected tissue stimulate the infiltration of resident tissue macrophages as well as extravasation and macrophage-differentiation of circulating monocytes. After migration to the site of disturbance, macrophages are activated by microenvironmental signals, identify and engulf microbial pathogens, infected and apoptotic cells, release antimicrobial compounds, inflammatory cytokines and play a role in antigen presentation to the adaptive immune system (Twigg, 2004).

Following the initial inflammatory response, macrophages are also involved in mediating a transition to the resolution phase of inflammation through the release of anti-inflammatory cytokines and participation in tissue regeneration processes (Porcheray *et al.*, 2005; Serhan and Savill, 2005).

For example, in wound healing, macrophages not only mediate the initial inflammatory response, but in later stages secrete growth factors and anti-inflammatory cytokines, clear debris and apoptotic cells and participate in fibrosis though matrix deposition and remodeling (Lucas *et al.*, 2010). The role of macrophages in regeneration of both peripheral and central nervous systems have been highlighted in recent research (Barrette *et al.*, 2008; Gensel *et al.*, 2012; Niemi *et al.*, 2013). Macrophages have also been attributed similar roles in regeneration of other tissues such as skeletal muscle, liver and kidney (Chazaud, 2014) and have recently been shown to be required for successful limb regeneration in adult salamanders (Godwin *et al.*, 2013).

In addition to their roles as rapid response mediators of inflammation and tissue regeneration macrophages also play important roles in several trophic processes and in maintenance of homeostasis. In developmental growth, macrophages have been shown to be particularly important in tissue patterning; guiding the development of branching morphologies in several tissue types including the nervous system, mammary glands, kidneys and pancreas (Pollard, 2009; Stefater *et al.*, 2011). Macrophages also facilitate maintenance and adaptive remodeling in tissues such as bone (Charles and Aliprantis, 2014), skeletal muscle (Bosurgi *et al.*, 2011) and adipose tissue (Sun *et al.*, 2011) as well as participating in the regulation of metabolic processes (Biswas and Mantovani, 2012). Some organs contain highly specialized populations of resident tissue macrophages that are responsible for maintaining tissue homeostasis in these microenvironments including Kupffer cells of the liver, alveolar macrophages in the lung, Langerhans cells in the skin, osteoclasts in bone, and microglia of the central nervous system (Pollard, 2009).

1.9.2 Macrophage polarization

The functional diversity of the monocyte/macrophage lineages is largely dependent on their phenotypic plasticity and ability to respond dynamically to microenvironmental signals (Stout and Suttles, 2004). Depending on the polarizing stimulus, macrophages can develop phenotypes that range from highly inflammatory cells that drive immune responses and eliminate infection to anti-inflammatory phenotypes that actively inhibit inflammation and facilitate regenerative tissue growth and immune suppression. The activation state of resident or infiltrating macrophages can have significant effects on their functional qualities and the terms M1 and M2 or classically and alternatively activated macrophages have been adopted to describe these inflammatory and anti-inflammatory phenotypes respectively.

M1 macrophages are attributed with activating and perpetuating the expansion of the inflammatory response and are classically found at sites of acute infection, tissue damage and hypoxia (Murray and Wynn, 2011). M1 macrophage polarization is induced *in vitro* through stimulation with

inflammatory cytokines such as IFNγ or treatment with LPS. Activated macrophages secrete large amounts of inflammatory cytokines including TNFα, IL-1β, IL-6, IL-12 as well as reactive oxygen species and other antimicrobial compounds. M1 macrophages also express higher levels of co-stimulatory molecules and enzymes including inducible nitric oxide sythase (iNOS; Mantovani *et al.*, 2004; Mosser and Edwards, 2008).

M2 macrophages have more multifaceted functions. They may be involved in inhibition or resolution of inflammatory responses, participate in tissue development, remodeling and repair such as angiogenic growth, and also play a role in the Th2 immune response to parasitic infections (Murray and Wynn, 2011). *In vitro* M2 macrophage polarization is stimulated by incubation with cytokines such as IL-10, IL-4 or IL-13. M2 activated macrophages in turn release anti-inflammatory cytokines such as IL-10, transforming growth factor (TGF)-β and IL-1 receptor antagonist (IL-1ra) and have been shown to express higher levels of molecules such as CD163 (scavenger receptor), CD206 (mannose receptor) and the enzyme arginase 1 (Arg1; Mantovani *et al.*, 2004; Mosser and Edwards, 2008).

Over the years a wide range of markers have been used to characterize M1 and M2 macrophages phenotypes. No set of markers, however, has been demonstrated to be reliably specific for one activation phenotype. Furthermore, several markers of macrophage polarization developed in mice do not apply to human macrophage phenotypes (e.g. Arg1 vs. iNOS expression and molecules F4/80 or Frizzels; (Murray and Wynn, 2011) making *in vivo* identification of macrophage activiation phenotype complicated. In reflection of these overlapping phenotypic identifiers, it has since been widely recognized that in a biological context the range of factors influencing macrophage polarization can be complex and often results in a spectrum of macrophage activation profiles, expressing elements of both M1 and M2 phenotypes simultaneously. *In vivo* these polarization phenotypes exist in a dynamic equilibrium that shifts in response to environmental changes (Stout *et al.*, 2005; Gratchev *et al.*, 2006).

Nevertheless, the overall temporal and spatial coordination of macrophage polarization *in vivo* has important functional implications and its dysregulation can determine the outcome of several conditions. Failure to transition to the M2 phenotype after an inflammatory response can lead to conditions of chronic inflammation and tissue damage whereas insufficient M1 activation or sustained M2 activation may suppress immunity through the inhibition of an effective inflammatory response (Kraakman *et al.*, 2014; van Overmeire *et al.*, 2014).

1.10 Macrophages and angiogenesis

During homeostasis macrophages maintain an intimate relationship with the vascular endothelium. Signals from the endothelium cause activation of circulating monocytes, macrophage differentiation and extraversion into the adjacent tissue. Similarly, activated macrophages also transmit signals to the endothelium regulating factors such as vascular tension and permeability (Sprague and Khalil, 2009). Macrophages have been shown to play important roles in multiple stages of angiogenic growth and exert their pro-angiogenic effects through several mechanisms including paracrine singles, matrix remodeling and direct cell-cell contacts.

In the developing organism, macrophages are essential to the formation of vascular architecture and are responsible for the vascular patterning of complex structures such as the hind brain and retina (Lobov *et al.*, 2005; Fantin *et al.*, 2010). In adult organisms, macrophages are involved in vascular development during tissue and organ re-modeling such as at the maternal-placenta interface during pregnancy (Hazan *et al.*, 2010), in adipose tissue (Pang *et al.*, 2008; Xu *et al.*, 2012), skeletal muscle (Rowe *et al.*, 2014) and remodeling of the vascular wall to accommodate increased blood flow under hemodynamic stress (Nuki *et al.*, 2008).

The importance of macrophages in regeneration has also been attributed in large part to their role in promoting the revascularization of newly formed or damaged tissue. In wound healing, macrophages initiate vascular sprouting at the early stages of granulomatous tissue formation and ensure stabilization and maturation of vasculature in the regenerated tissue (Lucas *et al.*, 2010; Okuno *et al.*, 2011). In several ischemic conditions such as myocardial infarction (Lambert *et al.*, 2008), limb ischemia (Hirose *et al.*, 2008; Jetten *et al.*, 2014; Yoo *et al.*, 2014) and stroke (Manoonkitiwongsa *et al.*, 2001) the infiltration of macrophages improves revascularization and plays a role in compensatory collateral artery remodeling (Fung and Helisch, 2012).

Several mechanisms have been proposed for the involvement of macrophages in angiogenic growth.

One major mechanism by which macrophages influence angiogenesis is through the release of diverse pro- angiogenic cytokines, chemokines and growth factors such as VEGF, CXCL8, CCL2, fibroblast growth factor (FGF)-2, platelet derived growth factor and TGF-β which activate and sustain the angiogenic program in surrounding tissue (Crowther *et al.*, 2001; Owen and Mohamadzadeh, 2013). Recently, macrophages have been shown to regulate angiogenesis in development and wound healing through the secretion of Wnt ligands and activation of the non-canonical Wnt-Flt signal transduction pathways (Stefater III *et al.*, 2011; Stefater *et al.*, 2013).

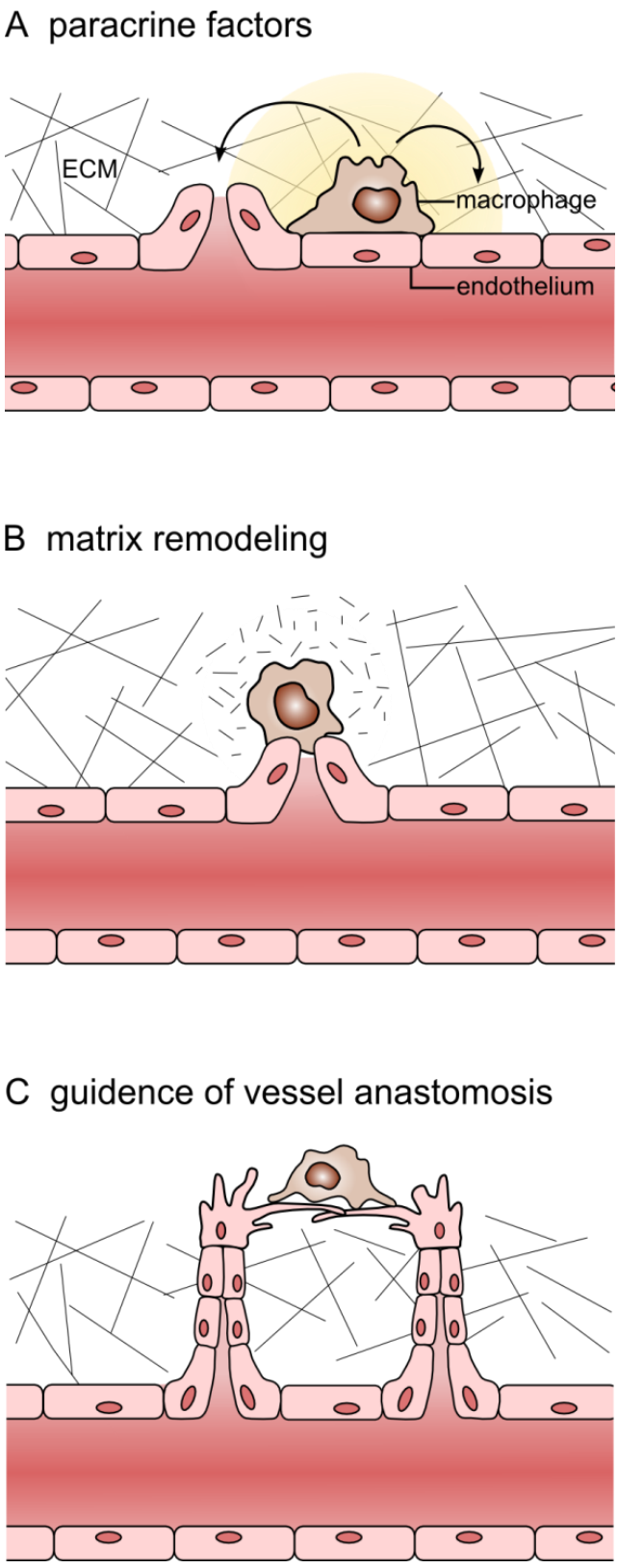


Figure 1.6 Macrophages play key roles in angiogenic growth. Macrophages have been shown to play several roles in promoting pathological and regenerative angiogenesis, most significantly via **(A)** paracrine mechanisms i.e. secretion of pro-angiogenic cytokines, **(B)** participation in matrix remodeling processes and secretion of proteolytic enzymes and **(C)** guidance of vessel anastomosis through physical interaction with neighboring tip cells. ECM: extracellular matrix.

Macrophages also secrete several proteases that degrade the extracellular matrix such as MMPs and urokinase-like plasminogen activator (uPA). These not only facilitate the invasion of vascular sprouts but also release matrix bound growth factors from the basement membrane. This, combined with the deposition of extracellular matrix proteins to form a provisionary matrix, stimulates and guides the growth of new vascular sprouts (Pepper, 2001; Arroyo and Iruela-Arispe, 2010).

Direct cell-cell contact between macrophages and endothelial cells also play a role in macrophage mediated vascular growth and maturation. In the developing mouse hindbrain macrophages have been shown to associate with sites of active vascular branching and assist vascular fusion by physically guiding vessel anastomosis in a VEGF independent manner (Fantin *et al.*, 2010). This phenomenon has also been observed in the developing mouse retina where macrophages were frequently found interacting with anastomosing tip cells. The presence of macrophages correlated with the number of filopodian-bearing vascular sprouts formed and the level of complexity in the mature retinal vascular network (Rymo *et al.*, 2011).

Some studies have reported monocyte/macrophages incorporating into newly formed vasculature and even displaying an extent of endothelial transdifferentiation (Fujiyama *et al.*, 2003; Li *et al.*, 2009; Al-Roubaie *et al.*, 2012). However, further studies have shown that while monocytes and macrophages associate very closely with the endothelium (i.e. within the perivascular space) and may incorporate into damaged vessels, they maintain their myeloid identity, suggesting therefore, that their contribution to vascular integrity may be to provide structural support or to maintain the physical barrier directly after vascular injury (Glod *et al.*, 2006).

1.10.1 Macrophages and pathological angiogenesis

In addition to their role in developmental and regenerative processes, macrophages can also be agents of pathological angiogenic growth. This is particularly true in conditions of chronic inflammation. In patients suffering from rheumatoid arthritis, macrophages are attracted to the inflamed synovial tissue where they release angiogenic inflammatory cytokines and stimulate the growth of pathological vasculature (Maruotti *et al.*, 2013). Similarly, in obesity, the inflammatory stimulus created by the death of engorged adipocytes also creates a chronic inflammatory environment that attracts macrophages and induces pathological angiogenesis of the adipose tissue (Pang *et al.*, 2008; Sun *et al.*, 2011; Xu *et al.*, 2012; Kraakman *et al.*, 2014).

1.11 Tumor Associated Macrophages (TAM)s

The best studied setting in which macrophages drive pathological angiogenic growth is that of the malignant tumor. As a reflection of macrophage phenotypic plasticity and involvement in diverse

physiological processes, the interaction of macropages with the developing tumor plays a role in multiple stages of cancer progression.

In recent years the connection between inflammation and the initiation of carcinogenesis has been firmly established. Conditions of chronic inflammation such as inflammatory bowel disease (colorectal cancer), persistent infections with bacteria or viruses such as *H. pylori* (gastric carcinoma) and hepatitis A and B (hepatocellular carcinoma) or chronic irritants such as smoking (lung carcinoma) and asbestos (mesothelioma lung carcinoma) all strongly pre-dispose individuals to carcinogenesis (Shacter and Weitzman, 2002). The role of macrophages as mediators of chronic inflammation makes them important players in this process. Through the release of inflammatory cytokines, macrophages participate in the perpetuation of the inflammatory response. The release of reactive oxygen and nitrogen species causes tissue damage and mutagenic stress which can inflict genetic instability and activate the oncogenic process.

Regardless of whether carcinogenesis is initiated by chronic inflammatory conditions or other factors, the early stages of tumor development are characterized by a state of chronic localized inflammation. Hypoxia, apoptosis, and nutrient starvation at the core of the developing tumor mass as well as oncogenic gene dysregulation result in the release of inflammatory signals such as colony stimulating factor 1 (CSF-1) and CCL2 and induce massive myeloid cell infiltration into the tumor microenvironment. A large proportion of this myeloid cell infiltrate is made up of macrophages.

Ideally, the function of infiltrating macrophages would be to recognize and eliminate malignant cells through phagocytosis and cellular cytotoxicity. Under the right conditions, activated macrophages can act as effective cytotoxic eliminators of cancer cells and form an important part of anti-tumor immune responses in cancer therapy (Chao *et al.*, 2010; Weiss *et al.*, 2010).

However, contrary to this, macrophage infiltration to the tumor microenvironment is generally associated with increased tumor angiogenesis, metastasis and overall poor prognosis in the majority of cancers (Hanada *et al.*, 2000; Leek *et al.*, 2000; Lissbrant *et al.*, 2000; Bingle *et al.*, 2002; Zhang *et al.*, 2012c). In malignant tumors, the nature of the tumor microenvironment acts to suppress macrophage anti-tumor immunity and instead reprograms infiltrating myeloid cells toward a pro-tumor macrophage phenotype (van Overmeire *et al.*, 2014).

Mouse studies investigating the depletion of macrophages in tumors via CSF-1 null mutations or liposome–encapsulated clodronate have identified tumor angiogenesis at the malignant transition and in late carcinogenic tumor progression as one of the most significant functional contributions of TAMs to tumor progression (Zeisberger *et al.*, 2006; Lin *et al.*, 2007). These studies are corroborated

by observations in human cancers demonstrating that macrophage density collates with increased tumor angiogenesis (Takanami *et al.*, 1999; Knowles *et al.*, 2004). It has been shown that the accumulation of TAM derived VEGF is the essential determining factor for the transition to the "angiogenic switch" in a murine breast cancer model (Lin *et al.*, 2006; Stockmann and Johnson, 2012). TAMs also secrete various other angiogenic factors such as TGFβ1, bFGF, ADM and CXCL8 into the tumor microenvironment and as well as proteases such as uPA and MMP9 which release matrix bound growth factors and degrade the extracellular matrix (Lewis *et al.*, 2000; Leek and Harris, 2002).

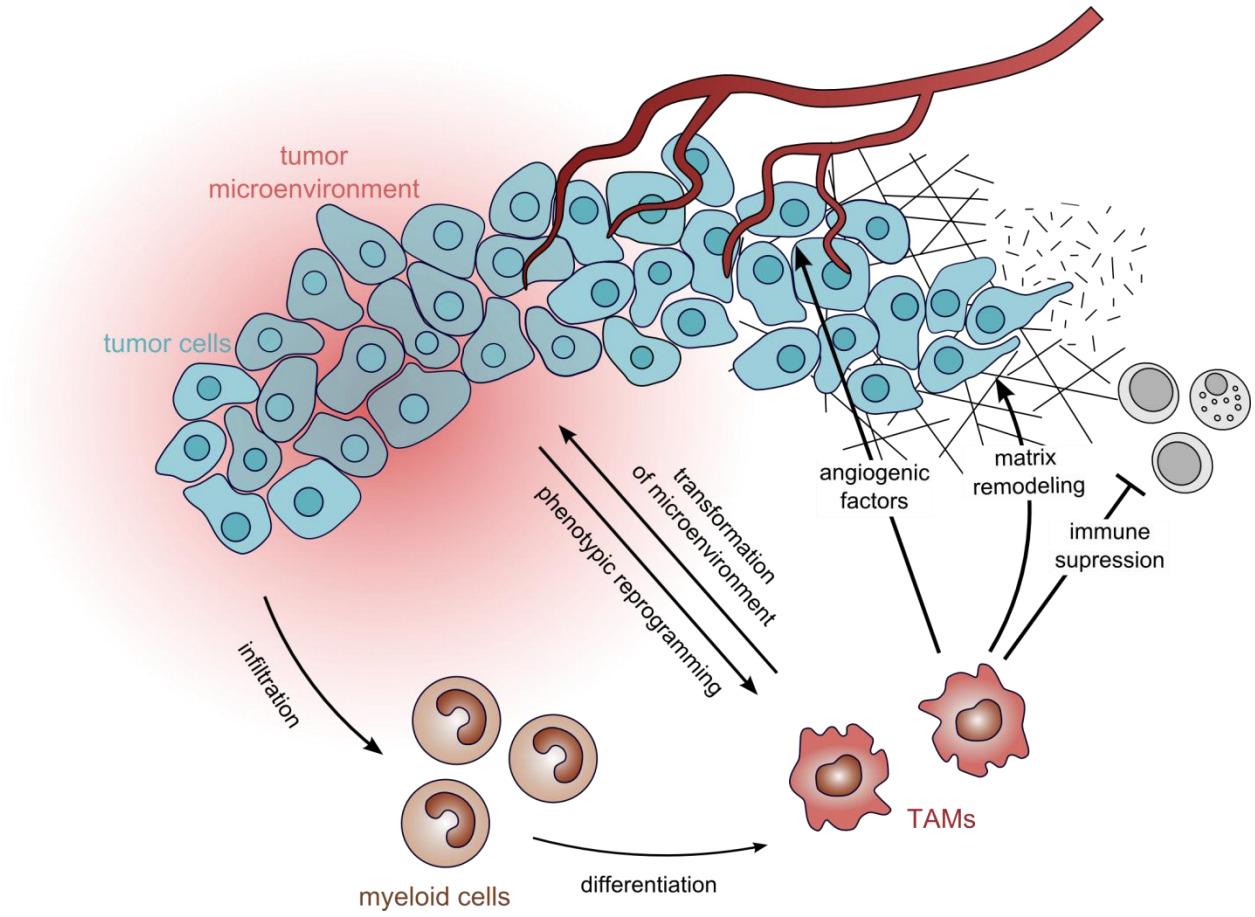


Figure 1.7 The infiltration of myeloid cells is a decisive factor in malignant tumor vascularization and progression. The microenvironment of hypoxia and chronic inflammation in the developing tumor cause the infiltration of myeloid cells which differentiate into tumor associated macrophages (TAMs). The tumor microenvironment re-programs TAMs to take on a pro-angiogenic, immune-suppressive and invasive phenotype. Through secretion of paracrine factors and participation in matrix re-modeling processes, TAMs transform the tumor microenvironment, drive tumor invasion, and vascularization, and suppress effective immune responses.

In addition to their angiogenic and immune suppressive properties, TAMs also play a key role in tumor cell invasion and migration and are found in high density at the leading edge of expanding tumors (Condeelis and Pollard, 2006). The invasive migration of TAMs and tumor cells may occur via a co-migratory CSF-1-EGF paracrine loop that leads to the infiltration of tumor cells into surrounding tissue (Wyckoff *et al.*, 2004). Other pathways, such as the non-canonical Wnt5a pathway normally employed by macrophages during developmental growth has also been associated with macrophage dependent tumor cell migration (Pukrop *et al.*, 2006; Yamamoto *et al.*, 2010). Macrophage participation in proteolytic degradation and matrix remodeling also establishes a migration pathway of least resistance for tumor cells into surrounding tissue and enables intravasion to blood vessel via the interaction with perivascular macrophages (Wyckoff *et al.*, 2004; Joyce and Pollard, 2008).

The combination of these pro-tumorogenic qualities makes the presence of TAMs in the tumor microenvironment a significant force in tumor progression. In some tumors TAMs can constitute up to 50% of the tumor mass (van Overmeire *et al.*, 2014). As a demonstration of this importance, the contribution of TAMs to tumor progression through the activation of angiogenesis, participation in invasion and immune suppression is so significant, that their activities can compensate for the malignant properties of the original tumor cells after irradiation or anti-angiogenic chemotherapy (DeNardo *et al.*, 2011; De Palma and Lewis, 2013).

1.12 Interaction of bacteria with stem and progenitor cells

Stem and progenitor cells have unique properties which make them important in many biological processes. However, the diverse differentiation potential and ability for self renewal, also make these cells susceptible targets for dysregulation. Despite the importance of stem and progenitor cells as mediators of homeostatic tissue renewal, regenerative tissue repair and immune function, little is known about the interactions of these cells with bacterial pathogens.

A major role of stem cells in the adult organism is in mediating wound healing and tissue regeneration. Mesenchymal stem cells (MSC) are important in these settings and participate in regeneration in part through their differentiation to fibroblasts. Examination of the MSCs in the cystic fibrosis lung found that quorum sensing molecules released by *Pseudomonas aeruginosa* interfere with MSC apoptotic signaling and cytokine secretion, hindering effective tissue regeneration (Holban *et al.*, 2014). Another study investigating the interaction of MSCs with bacteria found in wound infections (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus pyogenes*) revealed that bacteria can adhere to and invade cells. Bacterial antigens were also shown to increase MSC proliferation and influence their differentiation (Fiedler *et al.*, 2013).

Gut microbiota have also been found to have important effects on the functions of intestinal stem cells. Dysregulation of the gut microbiota brought on by high fat diets may impair the regenerative powers of intestinal stem cells and play a role in the development of metabolic syndrome (Serino *et al.*, 2014).

The differentiation potential of stem cells and their ability for self renewal also makes them susceptible to oncogenic dysregulation. The aberrant activation of stem cell activity can play an important role in cancer development. In *H. pylori* infection, the interaction of MSCs with *H. pylori* has been shown to induce their differentiation towards cancer-associated-fibroblast-like cells displaying an altered cytokine secretion profile. Secretion of inflammatory factors from transformed MSCs induces epithelial-mesenchymal transition in gastric epithelial cells which plays a key role in the development of adenocarcinoma and the progression of gastric cancer (Qian, 2013; Bessède *et al.*, 2014).

Hematopoietic stem and progenitor cells (HSCs; HPCs) in particular are intimately connected with bacterial infection as their expansion, differentiation and mobilization from the bone marrow forms the basis of the leukocyte based immune response.

HSCs have been shown to be resistant to infection with *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica*, a condition that is unique to their undifferentiated state (Kolb-Mäurer *et al.*, 2002). The infection of the more specialized HPCs with *Listeria monocytogenes* und *Yersinia enterocolitica*, on the other hand, was found to stimulate an accelerated differentiation toward myeloid cells and may lead to secretion of cytokines that can influence the hematopoietic stem cell differentiation capacity and immune response (Kolb-Mäurer *et al.*, 2004). In wound infections, the interaction of *S. aureus* with infiltrating hematopoietic progenitor cells induces a preferred differentiation towards granulocytes via interaction with the TLR2 signal transduction pathway and prostaglandin E2 signaling. (Granick *et al.*, 2013).

B. henselae has also been shown to interact with hematopoietic progenitor cells *in vitro*. *B. henselae* was found to invade, persist and proliferate within HSCs through their differentiation towards mature erythrocytes. The invasion and occupation of HSCs during the process of differentiation has been proposed as a mode of latent infection and dissemination via the blood stream to other sites throughout the body (Mändle *et al.*, 2005).

1.13 Interaction of *B. henselae* with MACs

Following the finding that *B. henselae* invades and persists in HSCs, the question arose as to whether other progenitor cell subsets may also play a role in *B. henselae* pathogenicity. The accumulation of MACs in sites of angiogenic growth and their roles in promoting pathological angiogenesis identified these cells as an important potential targets for *B. henselae* infection *in vivo*. Furthermore, as MACs were previously believed to differentiate into endothelial cells at sites of angiogenic growth the infection and persistence of *B. henselae* in circulating MACs was proposed as an immune privileged dispersion mechanism of *B. henselae* to sites of angiogenic growth (Mändle, 2005).

Initial investigations into the interaction of *B. henselae* with MACs were performed by Mändle (2005) [also published in (O'Rourke *et al.*, 2015)]. MACs were shown to be susceptible to *B. henselae* infection and as with endothelial cells, *B. henselae* infection inhibited apoptosis and induced the activation of the angiogenic transcription factor HIF-1 and inflammatory transcription factor NFκB. This was accompanied by the upregulation and release of angiogenic and inflammatory cytokines VEGF, ADM, CCL2, CXCL8, GM-CSF and ICAM-1. *B. henselae* infection was also shown to influence the rate of β-1 integrin expression on the MAC cell surface and increased migration along a stromal cell derived factor-1 (SDF-1) cytokine gradient.

Altogether, investigations by Mändle (2005) indicated that MACs represent a feasible infection target for *B. henselae in vivo* and that infection of MACs with *B. henselae* results in significant phenotypic changes. These initial results lay out a basic framework for the interaction between MACs and *B. henselae* and established a starting point for further investigations in this study.

2. Objectives

A unique quality of *B. henselae* infections is the ability to cause the development of vascular tumors in humans. The composition and development of *B. henselae* induced vascular tumors is in many ways similar to malignant tumor formation. In human cancers, the infiltration of myeloid cells to the tumor microenvironment contributes significantly to tumor angiogenesis and invasive phenotypes.

Myeloid angiogenic cells (MACs) are a subset of circulating myeloid progenitor cells associated with increased angiogenic growth and tumor vascularization. Their phenotypic plasticity and importance in pathological angiogenic processes makes MACs a particularly potent potential target for the induction of pathological angiogenesis in *B. henselae* infections.

To explore the possible role of MACs in the development of *B. henselae* associated vascular tumors, the main objective of this study was to investigate the interaction of *B. henselae* with MACs *in vitro* and to determine how this may affect their role in angiogenic processes. Specifically, the effect of *B. henselae* infection on MAC cell differentiation, angiogenic phenotype and functional angiogenic capacity were to be investigated. For this, *in vitro* angiogenesis assays, phenotypic functional assays, genomic and proteomic profiling of infected cells should be performed.

Furthermore, to examine parallels between infection associated pathological angiogenesis and cancer, a comparison is made between the possible role of MACs in *B. henselae* associated vascular tumor formation and the activity of myeloid cells in malignant tumors.

3. Materials and Methods

3.1 Laboratory equipment and analytical instruments

Table 3.1 Laboratory Equipment and Analytical Instruments

Instrument	Manufacturer
Analytical Scale, Kern 822-36	Kern & Sohn, Albstadt
Autoradiography Film Developer, Curix 60	AGFA, Köln
Bioanalyser 2100	Agilent, Santa Clara, CA, USA
Cell counter, CasyTT	OLS, OMNI Life Science, Bremen
Centrifuges: MiniSpin Plus	Eppendorf, Hamburg
Eppendorf 5417R	Eppendorf
Multifuge 3 S-R	Thermo Scientific, Waltham, MA, USA
Biofuge stratus	Thermo Scientific
Confocal Microscope: LSM 510 Axiovert 200M Software: Zen	Zeiss, Jena
Cytospin Centrifuge, Shandon	Thermo Scientific
ELISA-Reader, Tecan sunrise Software: Magellan 6	Tecan, Männedorf, CH
Eppendorf pipettes	Eppendorf
Multistepper	
Multicanalpipettes	
Flow Cytometer: FACS Canto Software: FACS Diva	Beckton Dickinson (BD), Franklin Lakes, NJ, USA
Fine-Balance, BP 110 S	Sartorius, Göttingen
Fluorescence Microscope for Digital Imaging: Axio Observer Z1 Software: Axiovision	Zeiss
GeneAmp PCR System 9700 Perkin Elmer	Applied Biosystems-life technologies, Darmstadt
Image Editing Software: Photoshop CS6	Adobe Systems, Mountain View, CA, USA
Incubator, BBD 6220	Thermo Scientific
Light Microscope for Cell Culture: Axiovert 4D CFL	Zeiss
Light Microscope for Digital Imaging: Z1 Cell Observer Software: AxioVision	Zeiss
Magnetic Stirrer, MR Hei Standard	Heidolph, Schwabach
Nanopdrop UV-Absorbance Spectrophotomerter	Thermo Scientific
Neubauer Cell-Counting Chamber	Labor Optik, Lancing, UK
pH-Meter 654	Methrom, Filderstadt-Plattenhardt
Photometer, Ultrospec 3100 pro	Amersham Biosciences, Freiburg
Pipetus	Hirschmann, Eberstadt
Power Supply for Gel Electrophoresis and Western Blotting, Power Pac Universal Power Supply	BioRad, München

Programmable Microwave, Jet-Chef Crisp	Wirlpool, Benton Harbour, MI, USA
Rocking Platform Shaker, Rocky	LTF Labortechnik, Wasserburg
Rotating Platform Shaker, Unimax 2010	Heidolph, Schwabach
SDS Gel Casting Chamber and Gel Cassettes	BioRad
SDS Gel Electrophoresis Running Cell, Mini Protean Tetra Cell	BioRad
Shaking Incubator, Novotron	Infors HT, Bottmingen, CH
Step One Plus, Real Time PCR System	Applied Biosystems
Sterile Workbench, Herasafe KS	Thermo Scientific
Thermomixer Comfort	Eppendorf
Ultra-Turrax T 10 Basic	Ika-Werke, Staufen
Vaccum System, VacuSafe Comfort	IBS, München
Vortexer	Heidolph
Waterbath	Memmert, Schwabach
Western-Blot Running Chamber and Blotting Cassettes	BioRad

3.2 Laboratory consumables

Table 3.2 Laboratory Consumables

Product	Manufacturer
Angiogenesis Micro-Slide	Ibidi, Martinsried
Autoradiography-Film (Kodak X-OMAT)	Sigma-Aldrich, St. Louis, MIS, USA
Poly-l-lysine Coated Glass Coverslips (ø12mm)	Marienfeld-Superior, Lauda-Königshofen
Cotton Swabs	WS Laborservice, Neu-Isenberg
Disposable Cuvettes	Sarstedt, Berlin
Eppendorf Tubes (0.5, 1.5 and 2ml)	Eppendorf
Eppendorf-pipette Tips	Star Lab, Hamburg
Falcon Polypropylene Test Tubes (15, 50ml)	Greiner Bio-One, Kremsmünster, AU
Filtered -Pipette Tips	Star Lab
Glass Coverslips	Menzel Gläser- Thermo Scientific
Glassware and Flasks	Schott Duran, Mainz, Germany
Hypodermic Needle, Sterican (0.40 x 20mm, 20 gauge)	Braun, Melsungen
Inoculation Loops	Greiner Bio-One
Parafilm	Pechiney Plastic Packaging, Menasha, WI, USA
PCR-Tubes	Fisher Scientific, Schwerte
Plastic Laboratory Spatula, Cell Lifter, Costar	Corning, Corning, USA
Polyvinylidene Difluoride (PVDF) Transfer Membrane	Millipore, Schwalbach
Serological Pipettes (5, 10, 25, 50 ml)	Greiner Bio-One
Sterile Filters, Minisart Single use Filter Units (0.2, 0.45µm)	Sartorius
Sterile, Square Petri Dish 595 x 395 x 370 mm	Greiner Bio-One

Super Frost Plus, Adhesive Immunohistochemistry Slides	Menzel Gläser- Thermo Scientific
Syringe, Luer-Lok^{TM} Tip, (1ml)	BD
TaqMan MicroAmp Adhesive Film	Applied Biosystems
TaqMan MicroAmp Optical 96 well Reaction Plate	Applied Biosystems
Tissue Culture Flasks (75cm^2)	Cell Star, Greiner Bio-One
Tissue Culture Plates (6, 12, 48, 96-well)	Cell Star, Greiner Bio-One
Whatman Paper	Schleicher & Schüll, Dassel

3.3 Chemicals and reagents

Table 3.3 Chemicals and Reagents

Chemicals/ Reagents	**Manufacturer**
123-base pair (bp)-Marker	Invitrogen, Karlsruhe
7-amino-actinomycin D (7-AAD) staining solution	eBiosciences
Acrylamide/Bis-acrylamide (37,5:1)	Roth, Karlsruhe
Ammonium persulfate (APS)	Applichem, Darmstadt
Biocoll-Seperating Solution (Density 1.077)	Biochrom, Berlin
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Bromphenol blue	Sigma-Aldrich
Cell Tracker (Red and Green)	Molecular Probes –life technologies
Citrate Buffer, 10mM pH 6.0, Target Retrieval Solution	Dako-Aglient Technologies, Santa Clara, CA USA
Complete Protease Inhibitor Cocktail	Roche
Dimethyl sulfoxid (DMSO)	Sigma-Aldrich
dNTPs	Roche
Dispase	BD
Dithiothreitol (DTT)	Invitrogen
DNAse	Qiagen, Venlo, NL
E. coli LPS (TLR grade Serotype R515)	Enzo Life Sciences, Farmingdale, NY, USA
Ethanol	AppliChem, Darmstadt
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Fetal Calf Serum (FCS)	Sigma-Aldrich
Fibronectin (from human plasma)	Sigma-Aldrich
Formalin , Roti-Histofix 4%	Roth
Glutaraldhyde	Electron Microscopy Science, Hatfield, PA, USA
Glycerin /Glycerol	Roth
Glycin	Sigma-Aldrich
Hydrogen peroxide (H_2O_2)	Roth
Hydrogen chloride (HCl)	Sigma-Aldrich
Isopropanol	AppliChem
M199 (10x)	Sigma-Aldrich

Matrigel	BD
Methanol	Merck, Darmstadt
Methylcellulose	Sigma-Aldrich
Mounting Medium, Aquatex	Merck
Paraformaldehyde (PFA)	Sigma-Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
Phosphate Buffered Saline (PBS; +$CaCl_2$/$MgCl_2$)	Gibco-life technologies, Karlsruhe
Phosphate Buffered Saline (without $CaCl_2$/$MgCl_2$)	Gibco
Ponceau S	AppliChem
Prestained Protein Ladder, Page Ruler Plus	Thermo Scientific-life sciences
Propidium Iodide Soultion (1mg/ml)	Sigma-Aldrich
Skim Milk Powder	Sucofin, Düsseldorf
Sodium Chloride (NaCl)	Merck
Sodium Hydroxide (NaOH)	AppliChem
Sodium dodecyl sulfate (SDS)	Roth
Sulphosalicylic Acid	AppliChem
Tetramethylenediamine (TEMED)	Sigma-Aldrich
Trichloroacetic Acid	Sigma-Aldrich
Tris(hydroxymethyl)-aminomethane (Tris)	AppliChem
Triton-X-100	Sigma-Aldrich
Trypsin-EDTA	Gibco-life technologies
Turk's Solution	Merck
Tween 20	Merck
Type-1 Collagen	BD
Urea	Roth

3.4 Kits

Table 3.4 Kits

Kit	**Manufacturer**
High Pure PCR Template Preparation Kit	Roche
Taq DNA Polymerase (with W -1) Kit, including: Taq DNA Polymerase 10x PCR Buffer 50mM $MgCl_2$	Invitrogen
Nested *Bartonella spp*. 16S rDNA PCR Primer Sets	TIBMOLBIOL, Berlin
Cytofix/Cytoperm™	BD
CompBeads	BD
RNeasy Mini and Micro Kits	Qiagen
DNase (RNase free DNase Set)	Qiagen
High Capacity RNA-to-cDNA Master Mix	Applied Biosystems
Fast SYBR Green Master Mix	Applied Biosystems
TaqMan Fast Universal PCR Master Mix, No AmpErase	Applied Biosystems

TaqMan Gene Expression Assays PolR2A VEGFR2 (KDR) VE-Cadherin	Applied Biosystems
Primer Set for RPLP0	Sigma-Aldrich
Primer Set for eNOS	Sigma-Aldrich
GeneChip Human Gene 1.0 ST Array	Affymetrix, Santa Clara, CA, USA
Ambion WT Expression Kit	Affymetrix
Human Angiogenesis Proteome Profiler	R&D Systems, Minneapolis, MN, USA
Human Cytokine Proteome Profiler	R&D Systems
Human CXCL10 Quantikine ELISA Kit	R&D Systems
Human TNFα Quantikine ELISA Kit	R&D Systems
Human IL-1ra Quantikine ELISA Kit	R&D Systems

3.5 Bacteria

3.5.1 Bacterial strains and growth conditions

For *in vitro* experiments the following bacterial strains were used : *B. henselae* strain Marseille $BadA^+$ (wild type) and $BadA^-$ [transposon mutant, deficient for BadA expression (Riess *et al.*, 2004)]. *Bartonella* were cultivated on Columbia blood agar plates, supplemented with 5% defibrinated sheep blood (CBA; BD) at 37°C, 5% CO_2, 95% humidity. For infection experiments frozen bacterial stocks were used. For control experiments, *Escherichia coli* (*E. coli*; ATCC 25922) and *Staphylococcus aureus* (*S. aureus*; ATCC 35556) cultivated on LB-agar at 37°C humidity were used.

3.5.2 Preparation of *Bartonella henselae* bacterial stocks

To minimize the occurrence of phase variation events, stocks were produced from original stocks of minimal passage. A small sample was taken from existing frozen stocks, streaked on CBA and incubated for 3-5 days at 37°C (5% CO_2, 95% humidity). Bacteria were harvested with cotton swabs and re-suspended in PBS. From this mixture bacteria were expanded onto 7 CBA plates. These were then harvested and expanded to 700 CBA plates 3-5 days later. Plates were incubated for a further 2 weeks after which they were harvested and re-suspended in PBS. Bacteria were centrifuged (10 min, 6000 rpm, 4°C) and uniformly resuspended in LB containing 20% glycerol. Bacterial stock-suspension were aliquoted in volumes of 0.5-1ml and frozen at -80°C. To determine bacterial concentration, a control sample of four aliquots was thawed after 24h. Aliquots were washed once in PBS and a ten-fold dilution series was plated on CBA. Within two weeks single colonies could be counted and an average bacterial concentration calculated.

Table 3.5 Bacterial Strains

Bacterial Strain	Reference
Bartonella henselae Marseille (BadA$^+$)	(Drancourt *et al.*, 1996)
Bartonella henselae Marseille BadA$^-$	(Riess *et al.*, 2004)
Escherichia coli	ATCC 25922
Staphylococcus aureus	ATCC 35556

Table 3.6 Bacterial Culture Media

Columbia Blood Agar Plates (5% defibrinated sheeps blood)	BD
Luria-Bertani (LB)-Medium	Roth, prepared mixture: 10g Tryptone 5g Yeast Extract 10g NaCl 1L H_2O, pH 7.0±0.2
LB-Agar	Roth, prepared mixture: 10g Tryptone 5g Yeast Extract 10g NaCl 15g Agar agar 1L H_2O, pH 7.0±0.2
Bacteria Freezing Medium	20% Glycerol in LB-Medium

3.6 Cell culture

Cell culture was performed under sterile conditions. Sterile materials and reagents were purchased from the manufacturer, autoclaved or sterile-filtered before use.

3.6.1 Ethics statement

Buffy coat samples from healthy donors were received from the German Red Cross blood donor bank (Deutsches Rotes Kreuz/Blutspendedienst) as residues from whole blood donation. Samples were pseudonymized to allow for tracking/tracing as required by law. Authors had no access to databases, nor was knowledge of any donor-related information (age, gender, etc.) relevant for the studies. Experiments were performed in accordance with ethical approval from the University Hospital Frankfurt Institutional Review Board (permit #329-10). All blood donors provided written consent to pseudonymized use of collected material for scientific purposes.

3.6.2 Human umbilical vein endothelial cells (HUVECs)

Single lot, pooled donor, HUVECs were purchased (Lonza) and cultured in 75cm^2 cell culture flasks coated with 50µg/ml fibronectin. Cells were maintained at 37°C, 5% CO_2, 95% humidity in Endothelial Growth Medium-2 (EGM, Lonza) with supplements, 10% fetal calf serum, penicillin (50 µg/ml) and streptomycin (50 µg/ml). Cells were detached with 0.05% trypsin-EDTA (Gibco, life technologies) and expanded to new flasks as needed. First to third passage HUVECs were used throughout. Cells were cultured in antibiotic-free medium for at least one passage before infection. The number of viable cells was determined using a CasyTT automatic cell counter (OLS OMNI Life Science).

3.6.3 Isolation and cultivation of Myeloid Angiogenic Cells (MACs)

Myeloid Angiogenic Cells (MACs) were cultivated from peripheral blood mononuclear cells (PBMCs) and isolated from buffy coats via gradient centrifugation as follows.

Buffy coats were diluted 1:3 in PBS without calcium and magnesium. 15ml Biocoll Seperating Solution (density 1.077) was added to prepared 50ml Falcon-polypropylene test tubes. 35ml of the diluted buffy coat was carefully pipetted onto the Biocoll Seperating Solution in each Falcon-tube to create a layered bi-phase composition. Falcon-tubes were centrifuged at 800g for 20 min without brake. After centrifugation a layered composition of erythrocyte pellet followed by Biocoll Seperating Solution, a thin layer of PBMCs and a top layer of plasma was formed. The PBMC layer was transferred to a fresh 50ml Falcon-tube using a 10ml pipette and remaining layers discarded. Falcon tubes containing PBMCs were filled to 50ml with PBS and centrifuged at 800g for 20 min with break. The supernatant was discarded and pellet resuspended in 50ml of PBS. The cell suspension was centrifuged for 10 min at 1800rpm and supernatant discarded as described. PBMCs were washed in this manner two following times and resuspended in 10ml of PBS. A small sample of cell suspension was used to determine viable cell number via 1:10 dilution in Turk's solution and a Neubauer cell-counting chamber.

Immediately after isolation (d0), cells were plated in 6 well cell culture dishes (2.0x10^7 cells/well), coated with 10µg/ml human fibronectin in PBS. Cells were maintained in EGM with supplements, 20% fetal calf serum and incubated at 37°C, 5% CO_2, 95% humidity. After three days of culture (d3), MACs are adherent to the fibronectin coated plates. Nonadherent cells were removed by washing with PBS (without calcium and magnesium). Cells were detached as needed via incubation with 1mM EDTA for 12min.

To determine viable cell number, a sample of 3 wells were detached and counted as described above to assess the average number of adherent MACs per well for each donor. This value varied from donor to donor but typically ranged between 0.3-1x 10^6 cells/well.

Antibiotic free medium was used throughout.

Table 3.7 Cells

Cell	Origin	Source
Human Umbilical Vein Endothelial Cells (HUVECs)	Purchased (Clonetics™), single lot, pooled donor, human primary cells	Lonza
Myeloid Angiogenic Cells (MACs)	human, Peripheral Blood Mononuclear Cell (PBMC) fraction, isolated from buffy coats	German Red Cross, blood donor bank, University Hospital Frankfurt

Table 3.8 Cell Culture Medium and Additives

Fetal Calf Serum (FCS) was complement-inactivated for 30min at 56°C before use. Media for cell culture was stored at 4°C.

Endothelial Cell Growth Medium- 2 (EGM), Lonza , Basal, CH	500 ml Endothelial Basal Medium- 2 (EBM) 10 or 20 % FCS included supplements: hEGF (0.5ml) VEGF (0.5ml) R3-IGF-1 (0.5ml) Ascorbic Acid (0.5 ml) Hydrocortisone (0.2ml) hFGF-β (2.0ml) Herapin (0.5ml)
Penicillin/Streptomycin	Gibco
Fibronectin (from human plasma)	Sigma Aldrich 10 or 50 µg/ml in PBS

3.7 Infection experiments

Infection experiments were performed four days after isolation (d4). Infections were done at a multiplicity of infection (MOI) of 100-300 (see below) according to the number of remaining adherent cells.

For infection with *B. henselae,* appropriate quantities of bacterial stocks were thawed and centrifuged at (1500rpm, 4°C for 5min). Bacteria were washed once in PBS and resuspended in the appropriate volume of cell culture medium according to the desired multiplicity of infection. Heat-killed *B. henselae* were generated by incubation of bacterial stock resuspended in EGM medium at 60°C for 30min in a water bath. Effective killing was confirmed by parallel long-term cultivation of heat killed sample stock on CBA.

For infection, with *S. aureus* and *E. coli,* bacteria were cultured in 15ml LB overnight on a shaking incubator at 37°C. To ensure exponential growth phase at the time of infection a secondary culture was inoculated 2h before infection and cultured under the same conditions. Bacterial concentration was measured via optical density (*E. coli* OD 1= $5x10^8$, *S. aureus* OD 1= $3x10^8$). The appropriate volume of bacterial culture was washed once in PBS (1500rpm, 4°C for 5min) and resuspended in cell culture medium according to the desired MOI.

MACs were washed once in PBS and culture medium replaced with fresh medium containing the appropriate bacterial suspension. Alternately, cell culture media was replaced with fresh cell culture media without bacteria as a negative control. Cell culture plates were centrifuged at 1000rpm for 5min to synchronize infection and incubated in a cell culture incubator at 37°C, 95% humidity and 5% CO_2.

3.8 Electron microscopy

For transmission electron microscopy (TEM) cells were grown in fibronectin coated cell culture plates as described above. For scanning electron microscopy (SEM) MACs were grown on ø12mm poly-l-lysine coated glass coverslips inserted in cell culture wells before fibronectin coating and cell seeding. For both processes, MACs were infected (MOI 300) for 30 and 60min, washed 3 times with PBS and fixated with a mixture of 4% formaldehyde and 2.5% glutaraldehyde in PBS. Samples were stored at 4°C until further processing.

For SEM, samples were post-fixed for 1h with 1% osmium tetroxide on ice, washed three times, dehydrated through a graded series of ethanol, and finally critical point dried with CO_2 as transitional fluid (E3000, Polaron, Quorum Technologies). Samples were mounted on a SEM sample holder and

sputter-coated with a 7nm layer of gold/palladium (MED010, Bal-Tec-Leica Microsystems). Samples were examined under vacuum with an S-800 field-emission SEM (Hitachi, Tokyo, Japan; Secondary electron detector and SEM Control Software DISS-5, Point Electronic GmbH) operated at 20kV acceleration voltage.

For TEM, samples were post-fixed for 1h with 1% osmium tetroxide on ice, washed three times, stained with 1% aqueous uranyl acetate for 1h. After washing three times, samples were dehydrated through a graded series of ethanol, infiltrated with increasing concentrations of epon/araldite epoxy resin and polymerized at 60°C for 48h. From the sample blocks, 70nm ultrathin sections were prepared and mounted on formvar-coated 400 mesh copper grids, post-stained with 1% aqueous uranyl acetate and lead citrate. Finally, the samples were examined with a Tecnai G2 TEM equipped with a 40µm aperture BioTwin objective (FEI) and operated at 120kV. Images were acquired with an Ultrascan 4000 digital camera and Digital Micrograph software (Gatan) at full resolution.

Electron microscopy was performed in co-operation with Matthias Flötenmeyer (Max-Planck-Institute for Developmental Biology, Department of Electronmicroscopy, Tübingen).

3.9 Nicoletti apoptosis assay

For determination of apoptosis, MACs were infected with *B. henselae* (MOI 100), *E. coli* and *S. aureus* (MOI 10). 6 and 24h after infection cell supernatant and adherent cells were harvested and pooled. Cells were fixed in Cytofix Cytoperm™ fixation and permabilization solution (BD) for 20min at 4°C and washed twice in Cytofix Cytoperm™ buffer. DNA content was stained with a 40µg/ml Propidium Iodid Solution (PI) and analyzed via flow cytometry according to the Nicoletti method (Riccardi and Nicoletti, 2006). The Nicoletti method is based on the principle that apoptotic cells are characterized by DNA fragmentation and a loss of nuclear DNA content. Under flow cytometry apoptotic hypodiploid nuclei are measured as a lower fluorescence signal and appear as a broad (sub G0) peak which is distinguishable from the narrow peak of normal diploid G0/G1 cells or the G2 peak of tetraploid cells shortly before division.

3.10 HIF-1 Western Blot

The activation of HIF-1 *in vivo* is dependent on the accumulation of the post-transcriptionally regulated HIF-1α protein subunit which is constitutively expressed but continually degraded under non-stimulatory (i.e. normoxic) conditions. To investigate the activation of HIF-1 in MACs in response to infection, the presence of HIF-1α in cell lysates was detected via Western Blot.

3.10.1 Cell stimulation and preparation of protein extracts

MACs were infected with *B. henselae* wild type, *B. henselae* BadA$^-$ (MOI 300), *E. coli* and *S. aureus* (MOI 10). Following 6h of incubation, media was removed and 250µl of extraction buffer was added to each well. Due to the susceptibility of HIF-1α to degradation, a specially formulated extraction buffer was used (Table 3.10). Adherent cells were scraped from the bottom of the cell culture well, transferred to an Eppendorf tube and homogenized using an Ultra-Turrax for 10s at full speed. Cell lysate was incubated at 70°C for 10min. Samples were stored at -80°C until further use. To prevent protein degradation reagents were incubated on ice throughout.

3.10.2 Sodium dodecyl sulfate- polyacrylamice gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate cellular proteins according to size. 8% SDS Resolving Gel was prepared and overlayed with a 5% Stacking Gel. Gel cassettes were placed in a stand, loaded into an electrophoresis running tank and overlayed with running buffer. 10µl of each sample was mixed with 2µl of 5x concentrated sample buffer and added the sample lanes of the prepared gels. For each gel, one lane was prepared with 5µl of Page Ruler, unstained protein ladder. Protein separation was performed at 30mA/gel for approximately 35min.

3.10.3 Western Blot

Following separation, the proteins were transferred to a PVDF-membrane via Western Blot. PVDF-membranes were briefly incubated in methanol (30s), washed for 2min in double distilled water and equilibrated in Blotting Buffer for several minutes. SDS gels were separated from their glass plates and the stacking gel removed. The equilibrated PVDF-membrane was layered over the gel and then sandwiched between two sheets of Whatman paper followed by two blotting sponges. Protein transfer was performed at 300mA for 1h. The blotted PVDF-membranes were removed from the blotting cassettes and briefly incubated in methanol (30s). Blots were air dried for approximately 15min.

To confirm successful transfer PVDF-membranes were incubated in a Ponceau Rouge solution for 10min and then washed with distilled water. Blots were then washed 3x in 20ml of Western Wash Buffer on a rotating platform shaker (10min, 50rpm).

To prevent unspecific antibody binding, membranes were incubated with 3% Blocking Buffer [1h, room temperature (RT)] on a rotating platform shaker. Blots were washed once as described above and incubated with primary mouse anti-HIF-1α antibodies (Abs; BD; 1:1000 in 3% Blocking Buffer, 4°C, over night) on a rocking platform shaker.

Following incubation with primary Abs, membranes were washed three times as described above and incubated with secondary horseradish peroxidase-conjugated, goat anti-mouse immunoglobulins (Dako; 1:1000 in 3% Blocking Buffer, 1h, RT) on a rotating platform shaker. Membranes were washed three times as described above.

Specific antibody binding to HIF-1α proteins was visualized with enhanced chemiluminescent reagent (ECL Plus, Amersham) and autoradiography films (X-OMAT, Kodak).

As a loading control, membranes were stripped and stained again for the presence of the reference protein β-Actin as follows. Membranes were rinsed briefly and washed for 5min in distilled water, then washed 3x in Western Wash as described above to remove remaining ECL solution and excess bound Abs. Membranes were incubated with monoclonal mouse anti-β-Actin primary antibody (Sigma-Aldrich; 1:10,000 in 3% Blocking Buffer, 1h, RT) on a rotating platform shaker. Secondary antibody staining and protein signal detection was performed as described above, using 5% Blocking Buffer and non-enhanced chemiluminescent reagent (ECL).

Table 3.9 Western Blot Antibodies and Substrates

		Manufacturer	**Dilution**
HIF-1α	Primary mouse anti-human HIF-1α	BD	1:1,000
β-Actin	Monoclonal mouse anti-human β-Actin	Sigma-Aldrich	1:10,000
Secondary antibody	Horseradish peroxidase (HRP)-conjugated goat anti-mouse	Dako	1:1,000
Developing Reagent	Enhanced and non-enhanced chemiluminescent reagent (ECL Plus/ECL)	Amersham- GE Healthcare Life Science, Chalfont St. Giles, UK	NA

Table 3.10 Buffer and Solution Compositions for Western Blot.

Cell Extraction Buffer	7M Urea 1% SDS 10% Glycerol 10mM Tris/HCl (pH 6.8) 1 Tablet Complete Protease Inhibitor™ Cocktail (Roche) 0.5mM PMSF 5mM DTT 50ml H_2O
SDS-Sample Buffer (5x) (Lämmli Buffer)	312.5mM Tris/ HCl (pH 6.8) 50% Glycerin 10% (w/v) SDS 250mM DTT 0.5% (w/v) Bromphenol Blue
SDS-Running Buffer	50mM Tris 384mM Glycine 0.1% SDS 1L H_2O
SDS-8 % Resolving Gel (2 Gels)	2.7ml 30 % Acrylamide-Stock solution 2.5ml 1M Tris (pH 8.8) 100µl 10% SDS 100µl 10% APS 6µl TEMED 4.6ml H_2O
SDS-Stacking Gel (2 Gels)	500µl 30% Acrylamide-Stock solution 380µl 1M Tris (pH 6.8) 30µl 10% SDS 30µl 10% APS 3µl TEMED 2.1ml H_2O
Blotting-Buffer (10x)	25mM Tris 192mM Glycine 1Lml H_2O
Western Wash (10x)	10mM Tris/HCl (pH7.4) 0.15mM NaCl 0.2% Tween 20 ad 2L H_2O

Blocking Buffer	3% -5% Skim Milk Powder in Western Wash
Ponceau-Rouge-Solution (10x)	2% Ponceau S 30% Trichloroacetic Acid 30% Sulphosalicylic Acid

3.11 Spheroid assay of sprouting angiogenesis

To examine the interaction of *B. henselae* infected MACs with growing endothelium and their functional effects on angiogenic growth, a co-culture spheroid assay of sprouting angiogenesis was developed.

For preparation of hanging drop spheroids a methylcellulose base solution was prepared as follows: 6g methylcellulose (Sigma) was measured in a 500ml glass bottle and autoclaved. 250ml of sterile, warmed (60°C) EBM was added and mixed with a magnetic stirrer until crystals were dissolved. A further 250ml of room temperature EBM was added and the mixture was allowed to cool at 4°C. When cool, the ethylcellulose base solution was aliquoted into 50ml Falcons and centrifuged at (200min, 4000rpm, 4°C) to sediment any remaining methylcellulose crystals. A 20% methylcellulose mixture was prepared by combining 10ml of methylcellulose basal solution with 40ml of warmed EBM.

To create endothelial spheroids, cells were detached from cell culture wells, resuspended in 1.5ml 20% methylcellulose and counted. A defined number of cells were resuspended in 20% methylcellulose according to the following calculation: Each 25µl hanging drop contains one spheroid of 400 cells. Each plate contains 125 drops. One plate is prepared for each experimental condition.

A 12 canal multi-pipette was used to pipette 125, 25µl drops onto the inner surface of a sterile, square plastic petridish. The petridish was flipped and hanging drops incubated overnight at 37°C. To observe for proper spheroid formation, cell culture plates were flipped again and droplets examined through the underside of the petri dish using an inverted light microscope. The spheroids were then washed from the plates using a cell culture pipette and 10ml PBS. Spheroids from each plate were pooled, centrifuged (3min at 1000rpm) and resuspend in 0.3ml warmed methylcellulose basal solution. For each plate 0.3ml collagen gel mixture (type 1 collagen; BD) was prepared on ice as follows: 0.3ml collagen was mixed with 42µl of 10x M199. 0.5M NaOH was added in small µl intervals until a colour change (red/fuchsia) was observed indicating a change in pH which catalyzes polymerization of the collagen gel (Phenol Red, red/fuchsia = pH ≥ 8) .Working quickly, collagen gel

and the methylcellulose-spheroid suspension were mixed and distributed between two wells of a 48-well cell culture plate. Spheroids were incubated briefly at 37°C to polymerize collagen gel and 200µl/well of EGM medium was added. Spheroids were incubated at 37°C for at least 12h and then fixated with 4% PFA.

Unless otherwise stated, spheroids were composed solely of HUVECs. For HUVEC-MAC co-culture spheroids, MACs were infected with *B. henselae* at increasing MOIs (200, 250, 300) or left uninfected for 24h prior to spheroid formation. MACs and HUVECs were mixed at a ratio of 1:3 before spheroid formation. Alternatively, MACs ($1.75x10^5$ cells/well infected or uninfected) were mixed with the collagen gel or seeded onto the spheroid gels with growth medium. Lastly, spheroid gels were cultured with 400µl conditioned EGM medium collected from MACs infected for 24h prior to spheroid formation. The rate of endothelial sprouting was assessed after 12h by computer assisted inverted light microscopy (Z1 Cell Observer, Zeiss).

For red-green co-culture spheroids, HUVECs and MACs were first stained with red and green Cell Tracker (Molecular Probes, life technologies) respectively according to manufacturer instructions. 30µl spheroid gel suspension and 30µl EGM was added to each well of an angiogenesis micro-slide (ibidi). The localization of MACs and HUVECs within the spheroids was analyzed using confocal laser scanning microscopy (LSM 510 Axiovert 200M, Zeiss). At least ten spheroids were analyzed for each condition.

3.12 Matrigel capillary formation assay

To further examine the angiogenic functional capacities of the *B. henselae* infected MACs, Matrigel capillary formation assays were performed. Cells were seeded on a 3D matrix gel which mimics the extracellular matrix and enables capillary-like vessel formation *in vitro*.

To prepare Matrigel coated plates, aliquots of Matrigel basement membrane matrix were thawed on ice. Using pre-chilled pipette tips, 12-well cell culture plates were coated with 200µl of Matrigel matrix/well. Cell culture plates were incubated at 37°C for 30min to allow polymerization of the matrix and 1ml of EGM medium without supplements or FCS was added to each well. Plates were further incubated at 37°C (5% CO_2, 95% humidity) overnight. At least three hours before seeding, medium on Matrigel coated plates was replaced with 1ml/well of fresh EGM without supplements or FCS.

MACs were infected with *B. henselae* wild type, $BadA^-$ or heat killed (MOI 300) for 24h. Cells were harvested and $2.0x10^5$ cells/well in 1.5 ml EGM medium without FCS were seeded onto Matrigel

coated plates. Matrigels with infected or uninfected MACs were incubated at 37°C (5% CO_2, 95% humidity). A partial medium change (500µl, EGM without FCS) was performed twice per week over a course of 41-77 days.

For fluorescence microscopy, chord structures were fixed with 3.75% PFA (15min at 4°C), washed with PBS, and permeabilized with 0.2% TritonX-100 (0.5ml, 30min, RT). To prevent unspecific binding of Abs, cells were blocked with 0.2% BSA blocking solution (0.5ml, 30min, RT). Structures were washed again and stained with tetramethylrhodamine (TRITC)-labeled phalloidin (1:500, 30min, RT, protected from light) and 4',6-Diamidin-2-phenylindol (DAPI; 1:500, 10min, 4°C, protected from light). Samples were analyzed using an Axio Observer Z1 (Zeiss). Images of complete capillary-like networks were produced by digitally stitching multiple individual images with 10% overlap (Axiovison software, Zeiss). The extent of capillary-like structure formation was quantified as relative pixel area of actin staining structures in full well images (ImageJ software).

To recover cells for fluorescence activated cell sorting (FACS) analysis, immunohistochemistry, RNA or DNA isolation, cultures were incubated with dispase (BD) for 2h at 37°C to dissolve the Matrigel matrix. Dispase digestion was stopped with 7mM EDTA and cells were washed and resuspended in PBS for further processing.

For analysis of intracellular *B. henselae* in Matrigel cultures, cells were isolated via dispase digestion, washed 3x in PBS and incubated in distilled H_2O for 4min to lyse cells. Lysis was stopped with 10x concentrated PBS and cell lysate cultured on blood-agar at 37°C and 5%CO_2 to observe the growth of colony forming units.

Table 3.11 Staining Reagents and Buffers for Florescent Labeling of Vascular Mimicry Structures.

Staining Agent	Manufacturer
Blocking Buffer	0.2% BSA in PBS
Tetramethylrhodamine (TRITC) -labeled Phalloidin	Sigma-Aldrich
DAPI (4',6-Diamidin-2-phenylindol)	Merck

3.13 16S rDNA PCR

To detect the presence of *Bartonella*-DNA in long term Matrigel cultures a 16S rDNA PCR was performed.

Cells were isolated from endpoint vascular mimicry structure via dispase digestion as described above. DNA isolation and a nested *Bartonella* spp. 16S rDNA PCR was performed in co-operation

with the PCR diagnostics department of the Institute for Medical Microbiology and Infection Control, University Hospital of the Goethe-University, Frankfurt am Main according to the accredited standard operating procedures of the institute and as described (Dauga *et al.*, 1996). In a nested PCR, two PCR reactions are performed in sequence with the PCR-product from the first amplification phase acting as the template for the second amplification phase. Primers for the second PCR amplification bind to sequences within the first PCR transcript (nested primers) providing increased amplification specificity and yield.

Briefly, DNA was isolated using the High Pure PCR Template Preparation Kit (Roche) according to manufacturer instructions. For the first amplification 5µl DNA was combined with 45µl master mix (containing forward and reverse primers, dNTPs, Taq-Polymerase, $MgCl_2$, buffer and H_2O) and amplified on a T3000 Thermocycler (Biometra) according to the cycling program given below. Two negative controls were included in the amplification reactions: an amplification control consisting of 5µl pure H_2O and an extraction control consisting of 5µl H_2O processed with samples through DNA extraction. A combined positive and inhibition control consisting of 2.5µl sample DNA and 2.5µl positive control was also used. For the second amplification phase 0.5µl of PCR product from the first reaction was combined with 49.5µl master mix and amplified according to the cycling program given below. The size of the final expected amplification product is 990bp.

15µl of each PCR amplification product along with two lanes containing 123-base pair ladder were loaded into a 1.5% agarose gel and separated via gel electrophoresis (35min, 170V). Gels were stained with ethidium bromide, visualized under UV light and documented using a gel documentation system (Biometra) and CU-5 UV-camera (Polaroid).

Table 3.12 *Bartonella* spp. 16S rDNA Primers

First Amplification Phase (forward and reverse)	5'-AGA GTT TGA TC(AC) TGG CTC AGA-3' and 5'-GTA GCA CGT GTG TAG CCC A-3'	1210bp
Second Amplification Phase (forward and reverse)	5´-CAC TCT TTT AGA GTG AGC GGC AA-3´ and 5´-CCC CCT AGA GTG CCC AAC CA-3´	990bp

Table 3.13 Master Mix Composition for 16S rDNA PCR

First Amplification Phase (45.0µl)	5µl 10x PCR Buffer 1.5µl $MgCl_2$ 8µl dNTPs 2µl forward primer 2µl reverse primer 26.25µl H_2O 0.25µl Taq-Polymerase
Second Amplification Phase (49.5µl)	5µl 10x PCR Buffer 1.5µl $MgCl_2$ 8µl dNTPs 2µl forward primer 2µl reverse primer 30.75µl H_2O 0.25µl Taq-Polymerase

Table 3.14 16S rDNA PCR Cycling Program, First Amplification Phase

2s at 94°C (1x)
Cycling Stage (35x)
Step 1: 1s at 94°C
Step 2: 1s at 62°C
Step 3: 1s at 72°C
5s at 72°C (1x)
Hold at 4°C

Table 3.15 16S rDNA PCR Cycling Program Second Amplification Phase

2s at 94°C (1x)
Cycling Stage (35x)
Step 1: 1s at 94°C
Step 2: 1s at 65°C
Step 3: 1s at 72°C
5s at 72°C (1x)
Hold at 4°C

3.14 Flow cytometry

Differentiation of MACs in response to *B. henselae* infection (MOI 300; d5-24h p.i. and d62 from Matrigel structures) was assessed via flow cytometry. To prepare for staining, detached cells were fixed in 1% PFA (15min, 4°C) washed 3 times with PBS and blocked with 10% FCS (30min, 4°C). Cells were incubated in staining buffer (PBS with 0.1% BSA) with the following monoclonal murine human-specific Abs for surface antigens: anti-CD14, anti-CD45, anti-human leukocyte antigen (HLA)-DR, anti-CD31 (BD) and anti-VEGF-R2 (R&D Systems; 30min, RT, protected from light). Cells were washed 3x in staining buffer. For detection of intracellular antigens cells were permeabilized with Cytofix Cytoperm (BD; 20min 4°C protected from light) washed 3x with Cytofix Cytoperm buffer and incubated with murine anti-CD68 (BD) in Cytofix Cytoperm buffer (30min, RT, protected from light).

Cells were washed 3x in Cytofix Cytoperm staining buffer and re-suspended in 1ml Cytofix Cytoperm staining buffer for analysis. In each experiment, control groups were stained with immunoglobulin-isotype control Abs [mouse IgG2aκ, IgG1κ, IgG2bκ (BD) and mouse IgG1 (R&D Systems)].

For assessment of cell viability, MACs isolated from Matrigel structures were incubated with 7-AAD staining solution (eBiosciences) and fixated with 1% PFA (15min, 4°C). Unstained cells were used as negative controls.

Cells were analyzed on a fluorescence-activated cell sorter (FACS Canto, BD) and results were analyzed using Flowing Software (Perttu Terho, 2012). Compensation settings were defined for polychromatic FACS analysis using individually labeled compensation beads (CompBeads; BD). Dead cells and artifacts were excluded according to their size and granularity.

Table 3.16 Antibodies and Reagents for FACS Analysis

Phycoerythrin (PE), Fluorescein isothiocyanate (FITC), Allophycocyanin (APC)

Target Antigen	Antibody	Conjugated Fluorophore	Manufacturer	Volume/ Test
NA	7-AAD staining solution	NA	eBiosciences-Affymetrix	NA
CD14	monoclonal, mouse IgG2aκ, anti-human	PE	BD	20µl
CD45	monoclonal, mouse IgG1κ, anti-human	FITC	BD	20µl
CD31	monoclonal, mouse IgG2aκ, anti-human	Alexa Fluor 488	BD	5µl
VEGFR-2	monoclonal, mouse IgG1, anti-human	PE	R&D Systems	10µl
CD68	monoclonal, mouse IgG2bκ, anti-human	Alexa Fluor 647	BD	5µl
CD11c	monoclonal, mouse IgG1κ, anti-human	FITC	BD	5µl
HLA-DR	monoclonal, mouse IgG2aκ, anti-human	APC	BD	20µl
Isotype Controls				
IgG2aκ	mouse, monoclonal	PE	BD	20µl
IgG2aκ	mouse, monoclonal	APC	BD	20µl

IgG2aκ	mouse, monoclonal	Alex Fluor 488	BD	5µl
Mouse IgG2bκ	mouse, monoclonal	Alexa Fluor 647	BD	5µl
Mouse IgG1κ	mouse, monoclonal	FITC	BD	20µl
Mouse IgG1κ	mouse polyclonal	PE	R&D Systems	10µl
CompBeads	Anti-mouse Ig, κ Negative Control Compensation Particles Set	NA	BD	NA

Table 3.17 Buffer Composition for FACS Analysis

Buffers	
Blocking Buffer	10% FCS in PBS
Staining Buffer	0.1% BSA in PBS

3.15 Immunohistochemistry

For further analysis of *B. henselae* infected MAC (d46, d51) cell differentiation phenotype, cells from Matrigel vascular mimicry structures were analyzed via immunohistochemistry. MACs were isolated from the Matrigel matrix via dispase digestion as described above. Samples in single cell suspensions (200µl, EGM) were centrifuged onto adhesive immunohistochemistry slides (Super Frost plus, Menzel Gläser-Thermo Scientific) using a Cytospin centrifuge (500rpm, 5min; Shandon-Thermo Scientific) and allowed to air-dry overnight.

The next day, specimens were fixed in 4% formalin for 5min and then rinsed under flowing tap water (1min) followed by a single washing step with distilled water. Antigen de-masking was performed via immersion in 10mM pH6.0 citrate buffer heated using a programmable microwave for 10min (jet-chef crisp, Wirlpool). Samples were cooled for 15min on ice and rinsed in distilled water.

When peroxide staining was used, blocking was performed with 3% H_2O_2 in methanol (10min) followed by rinsing under flowing tap water (1min) and distilled water as described above. Slides were briefly rinsed in Tris-buffered saline solution (TBS) and immersed in TBS for 5min. 100µl of blocking buffer (3% BSA in TBS) was pipetted onto each slide and incubated for 10min.

Slides were blotted dry with paper towel and 100µl/slide of the following primary Abs diluted in blocking buffer pipetted onto the area of cell deposition: anti-Vimentin, anti-CD34, anti-CD31, anti-

vWF (Dako) and anti-VEGFR2 (anti-Flk-1; Santa Cruz Biotechnology). Samples were covered with 21x26mm coverslips and incubated overnight (4°C, humid environment).

The next day, slides were rinsed and washed 3x via immersion in a TBS bath (5min) and blotted with paper towel. 100µl/slide of secondary antibody; rabbit anti-mouse biotinylated F(ab')2 fragment (Dako) was pipetted onto the area of cell deposition and incubated for 30min (RT, humid environment) then washed 3x in TBS as described above.

For slides to be developed with Permanent Red, 100µl/slide of the enzyme conjugate streptavidin-alkaline phosphatase (AP) diluted in blocking buffer was pipetted onto the area of cell deposition. For slides to be developed with peroxidase, streptavidin-HRP was used (30min, RT, humid environment).

Substrate chromogen solutions diaminobenzidine (DAB, Sigma-Aldrich) and Liquid Permanent Red™ (Dako) were prepared according to package instruction. Cells were washed 3x in TBS and incubated in 100µl/slide of DAB substrate solution or Liquid Permanent Red™. substrate solution. Enzyme reactions were allowed developed for 20min and then stopped by immersion in a TBS bath and rinsing in distilled water.

Counterstaining was performed via immersion in Mayer's hematoxylin (AppliChem; 1min) followed by rinsing under flowing tap water and then brief rinsing in distilled water. Specimens were sealed with glass coverslips and mounting medium (aquatex, Merck) for further analysis.

Control stains were performed omitting primary Abs.

Table 3.18 Antibodies and Staining Reagents for Immunohistochemistry.

Antibodies			
Target Antigen	**Antibody**	**Manufacturer**	**Dilution**
Vimentin	mouse anti-human monoclonal $IgG_1 \kappa$	Dako	1:500
CD34	mouse anti-human monoclonal $IgG_1 \kappa$	Dako	1:60
FactorVIII	mouse anti-human monoclonal $IgG_1 \kappa$	Dako	1:100
CD31	mouse anti-human monoclonal $IgG_1 \kappa$	Dako	1:100

Flk-1	mouse anti-human monoclonal $IgG_1 \kappa$	Santa Cruz Biotechnology, Dallas, TX, USA	1:100
Mouse IgG-Biotinylated Secondary Ab	anti-mouse immunoglobins /biotin rabbit F $(ab^1)_2$	Dako	1:200
Staining Reagents			
Strep-HRP- biotin binding enzyme conjugate	LSAB 2 Streptavidin-HRP	Dako	1:100
Strep-AP- biotin binding enzyme conjugate	Streptavidin - alkalische phosphotase	Dako	1:100
DAB-chromogenic substrate	Fast 3,3 Diaminobenzidine	Sigma- Aldrich	NA
Perm-Red-chromogenic substrate	Liquid Permanent Red	Dako	NA
Mayer's hematoxylin-nuclear stain		AppliChem	NA

Table 3.19 Buffers and Solutions for Immunohistochemistry.

Tris Buffered Saline Solution (TBS)	84g NaCl 12g Tris 10L H_2O set pH to 7.4 with 2M HCl	NA
Citrate Buffer	Target Retrieval Solution, 10mM pH 6.0	Dako
Peroxidase Blocking Buffer	3% H_2O_2 diluted in Methanol	NA
Blocking buffer	3% BSA in TBS	NA
Mounting Medium	aquatex	Merck

3.16 Quantitative real-time PCR (qRT-PCR)

3.16.1 Isolation of total RNA

For analysis of endothelial gene expression in freshly isolated MACs (d3) and infected MACs from vascular mimicry structures (d60), total RNA was extracted using the RNeasy Mini and Micro Kit (Qiagen), respectively according to manufacturer instructions.

Briefly, to harvest RNA from d3 MACs, medium was aspirated from cell cultures and 350µl of cell lysis buffer (RTL Buffer, RNeasy Kit) added to each well. Cells were scraped from the bottom of the cell culture plate with a plastic laboratory spatula. For isolation of RNA from vascular mimicry structures (d60), cells were first isolated from Matrigel culture via dispase digestion as described above. After centrifugation, the resulting cell pellet was resuspended in 350µl of cell lysis buffer (RTL Buffer, RNeasy kit). Cell lysates from both time points were passed through a 20 gauge hypodermic needle fitted to a sterile syringe at least 10x to insure adequate membrane disruption and homogenization.

RNA isolation from d3 MACs was performed using the RNeasy Mini Kit (Qiagen). To accommodate the expected low yield from the Matrigel capillary-like structures, RNA isolation was performed using the RNeasy Micro Kit. Both kits are based on a principle of specific RNA binding to silica based membranes, rinsing of accessory material by way of centrifugation and elution of the purified RNA into highly concentrated solution. To avoid contamination with genomic DNA an additional DNase treatment step (Qiagen) was performed during purification according to manufacturer instructions.

Concentration and purity of the isolated RNA was measured using the Nanodrop microvolume UV absorption spectrophotometer (Thermo Scientific).

3.16.2 Reverse Transcription PCR (RT-PCR)

To accommodate low RNA yield from the Matrigel structures, RT reactions were performed using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) which is designed to handle samples with a high variance in RNA abundance and specifically suited to transcribing samples with low total RNA.

For each sample 0.3µg of RNA was mixed with 4µl of High Capacity Mastermix (containing recombinant RNase inhibitor protein, reverse transcriptase, $MgCl_2$, Oligo(dT) primer, random primers, and stabilizers) and diluted to a total volume of 20µl with H_2O. The reverse transcription reaction was performed on a GeneAmp PCR System 9700, Perkin Elmer (Applied Biosystems) according to the following program.

Table 3.20 Cycling Program for RNA-cDNA Reverse Transcription.

Step 1: 5min at 25°C
Step 2: 30min at 42°C
Step 3: 5min at 85°C
Step 4: Hold at 4°C

3.16.3 Quantitative RT-PCR

To analyze the expression of endothelial marker genes in infected and uninfected MACs, quantitative RT-PCRs were performed using two fluorescent reporter systems, SYBR Green and Taqman.

The SYBR Green reporter system is based on the asymmetrical cyanine dye SYBR Green which intercalates with double stranded DNA. The resulting DNA-SYBR Green complex absorbs blue light (λ_{max} = 497nm) and emits green light (λ_{max} = 520 nm) a property used to dynamically measure the course of DNA amplification in qRT-PCR.

The TaqMan method of qRT-PCR makes use of oligonucleotide probes that are specific to target sequences. The probes bind to the single stranded DNA downstream of the PCR primer site and are equipped with a fluorophore covalently bound to the 5' end and a quencher molecule bound to the 3' end. Under normal conditions the fluorescence of the fluorophore is hindered through fluorescence resonance energy transfer by the close proximity of the quencher. However, as the Taq polymerase extends the primer and synthesizes the nascent strand, it also degrades the probe through its natural 5'-3' endonuclease activity. Through the degradation of the probe the fluorophore is released from the quencher molecule and a detectable fluorescence signal produced.

To analyze the expression of endothelial nitric oxide synthase (eNOS) in relation to the control gene ribosomal phosphoprotein P0 (RPLP0), qRT-PCRs were performed using SYBR Green as a fluorescent PCR reporter complex. 10µl of Fast SYBR Green Mastermix (2x) was combined with 8µl of complementary DNA (cDNA) from each sample and 2µl of specific primers.

For analysis of VE-Cadherin, VEGFR2, and the control gene DNA-directed RNA polymerase II subunit RPB1 (PolR2A) expression, TaqMan Gene Expression Assays were performed. 9µl of cDNA from each sample was combined with 10µl of Taqman Fast Universal PCR Master Mix (No AmpErase; 2x) and 1µl of specific TaqMan Gene Expression Assay (pre-formulated assay consisting of a pair of unlabeled PCR primers and a Taqman probe).

Samples where cycled and measured on a StepOne Plus, Real Time PCR System (Applied Biosystems) according to the programs below.

Table 3.21 SYBR Green (Fast) qPCR Cycling Program.

Holding Stage:
20s at 95°C
Cycling Stage (40x):
Step 1: 3s at 95°C
Step 2: 30s at 60°C
Melt Curve stage:
Step 1: 15s at 95°C
Step 2: 1s at 60°C
Step 3: +0.5°C, 15s at 95°C

Table 3.22 Taqman (Fast) qPCR Cycling Program.

Holding stage:
20s at 95°C
Cycling stage (40x):
Step 1: 1s at 95°C
Step 2: 20s at 60°C

Table 3.23 Primers

RPLP0 (forward and reverse)	5'-TCGACAATGGCAGCATCTAC-3'and 5'-ATCCGTCTCCACAGACAAGG-3'
eNOS (forward and reverse)	5´-CTGCACTATGGAGTCTGCTC-3´and 5´-AGCCCTTTGCTCTCAAG-3´

To prevent degradation, RNA samples were processed either chilled to 4°C or on ice at all times and only sterile, RNase free laboratory materials including filtered pipette tips were used throughout. Unless otherwise stated, all products from Applied Biosystems.

qRT-PCRs were performed in co-operation with Andrea Knau (Institute for Cardiovascular Regeneration, University of the Goethe University Frankfurt am Main).

3.17 Microarray gene expression profiling

To examine changes in overall gene expression profiles between freshly isolated MACs (d3) and infected MACs from vascular mimicry structures (d60) gene microarray transcriptional profiling was performed using GeneChip Human Gene 1.0 ST arrays (Affymetrix).

Cells were processed for RNA isolation using an RNeasy Mini and Micro Kit (Qiagen) and RNA quantity was measured using the Nanodrop UV-absorbance spectrophotometer (Thermo Scientific) as described above. To confirm RNA quantity and integrity, samples were also analyzed using the 2100 Bioanalyzer (Agilent) which utilizes a microfluidics chip system to measure the quantity and size distribution of fluorescently labeled RNA samples via gel electrophoresis. As RNA passes a detector, fluorescence signals can be measured and compared to standard RNA ladder probes to determine RNA concentration and degradation status. A RIN (RNA Integrity Number) ranging from 1

(most degraded) to 10 (most intact) is calculated for each sample. For this study, a RIN number of more that 7 was deemed acceptable for microarray analysis.

cDNA synthesis from RNA samples was performed using Ambion WT Expression Kit (Ambion) according to standardized protocols. Microarray hybridization, washing steps, and scanning of the microarrays were performed according to the Affymetrix protocol.

Statistical analysis was done with the statistical computing environment R version 2.12 (R Development Core Team, 2005). Additional software packages were taken from the Bioconductor project (Gentleman *et al.*, 2004).

Probe level normalization was conducted using the variance stabilization method by Huber *et al.*, (2002). Probeset summarization was calculated using the median polish method (Tukey, 1977) on the normalized data. For each probe set a robust additive model was fitted across the arrays, considering the different sensitivity of the probe sets via the probe effect.

Data was filtered with an intensity and variance filter. After the expression intensity filtering, p-values were calculated with the two sample t-test (variance=equal) to identify genes that are differentially expressed between infected and uninfected cells. For the multiple testing problems a False Discovery Rate (FDR) was used (Hochberg and Benjamini, 1990). Also, Fold Changes (FC) between the two groups were calculated for each gene. The lists of differentially expressed genes were filtered with FDR and FC criteria.

To examine the variation in gene expression between samples and compared to an endothelial control sample (HUVECs grown on Matrigel for 12h), unsupervised hierarchical clustering was performed for genes with a standard deviation ≥ 1.0 and ≥ 1.5 respectively, across all samples using the Manhattan distance and the average linkage method.

Detailed functional analysis of individual genes up-regulated 4.0 fold or higher and with a FDR <0.05 was performed via GeneCards (http://www.genecards.org/) and literature searches.

Functional annotation enrichment analysis was performed on a list of gene upregulated with a FC ≥ 2.0 and a FDR < 0.05 using the DAVID database functional annotation tool [http://david.abcc.ncifcrf.gov; (Huang *et al.*, 2008; Huang *et al.*, 2009a)]. Included in the search for enriched functional annotations were level 5 “molecular function” and “biological process” gene ontology terms as well as KEGG and BIOCARTA biological pathways. Terms with a minimum of 2 hits and a significant EASE score of < 0.01 were considered. Clustering of functional annotations was performed for terms with a similarity overlap of 3, similarity threshold of 0.05, initial group

membership of 3, final group membership of 3, and multiple linkage threshold of 0.05. The classification stringency was set at medium and the EASE significance score at < 0.01. A list of down-regulated genes with a FC ≤-2.0 and a FDR < 0.05 was similarly analyzed.

RNA processing, microarray chip analysis, data pre-processing, normalization, probe set summarization, calculation of differential expression values (FC and FDR) and hierarchical clustering analysis was performed in co-operation with Claudia Döring (Dr. Senckenberg Institute for Pathology, University Hospital Goethe University, Frankfurt am Main). Comparison of Microarray gene expression profiles with data set from the NCBI GEO Database was performed in co-operation with Mario Looso (Max Plank Institute for Heart and Lung Research, Bad Nauheim, Germany).

Data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE55170 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55170).

3.18 Secretome analysis

To determine what secreted compounds may be responsible for the increased angiogenesis associated with *B. henselae* infected MACs, secretome analysis was performed. Cell culture supernatants were collected from infected (MOI 200) and control MACs at d5-24h p.i. and at the midpoint (d25) and endpoint (d77) of long term Matrigel culture assays. Supernatants were centrifuged (5min, 4°C, 20,000g) to sediment debris, transferred to a new sample tube and stored at -20°C until further processing. Human Angiogenesis and Human Cytokine Proteome Profiler antibody arrays (R&D Systems) were incubated with supernatants from each condition (1ml) according to manufacturer instructions. Luminescence signals were developed using autoradiography (X-OMAT, Kodak).

3.19 Enzyme linked immunosorbent assays (ELISAs)

To compare the *B. henselae* induced cellular response to a general inflammatory response, cells were infected with *B. henselae* (MOI300) or treated with 1µg/ml *E. coli* LPS (TLR grade Serotype R515; Enzo Life Sciences). Supernatants were collected after 6h and 24h, centrifuged (5min, 4°C, 20,000g) to sediment debris, transferred to a new sample tube and stored at -20°C until further processing . TNFα, CXCL10 and IL-1ra concentrations were determined via ELISA (R&D systems) according to manufacturer instructions. Colorimetric signals were measured and cytokine concentrations calculated using the ELISA-Reader (Tecan) and Magellan 6 Software (Tecan). Samples were measured in triplicate.

3.20 Statistical analysis

All experiments were performed with cells from different donors and reproductions revealed comparable results. Differences between mean values of experimental and control groups were analyzed by the Student's *t* test. A value of $p < 0.05$ was considered statistically significant. Images were digitally processed as needed using Adobe Photoshop CS6.

4. Results

4.1 *B. henselae* invades MACs and resides in intracellular vacuoles

Preliminary investigations by Mändle (2005) found that *B. henselae* readily invades MACs and persists intercellularlly for up to seven days post infection (p.i.).

To further visualize the *B. henselae* adhesion and invasion process, MACs were fixated at different time points post exposure to *B. henselae*. When examined via scanning electron microscopy (SEM), uninfected cells appeared spherical or slightly flatten in shape with uneven surface protrusions often displaying many thin philopodian anchoring them to the surface or extending into space. In infected cultures large aggregates of bacteria were visible adhering to the surface of MACs after 30 min (Fig. 4.1A, enlargement). Transmission electron microscopy (TEM) of MACs revealed intracellular structures including mitochondria, endoplasmic reticulum and a large nucleus. In infected cells aggregates or single bacteria were observed interacting with MAC protrusions at 30 min (Fig. 4.1B, enlargement). After one hour, most bacteria appear internalized within intracellular vacuoles and can be identified as dense aggregates in intracellular compartments and by the double membrane structures of the individual bacteria (Fig. 4.1C, enlargement).

4.2 *B. henselae* infected MACs maintain high viability

B. henselae has been shown to inhibit apoptosis in infected endothelial and monocytic cells (Kempf *et al.*, 2005b; Schmid *et al.*, 2006). The ability of *B. henselae* to prevent apoptosis is considered an essential element of its pro-angiogenic re-programming of infected cells. In MACs, *B. henselae* infection has been shown to result in low basal cell death and significantly lower rates of cell death after stimulation with the cytostatic agent Mitomycin C. (Mändle *et al.*, 2005; O'Rourke *et al.*, 2015). To compare the apoptotic response in *B. henselae* infected MACs with other bacterial infections, MACs were infected with *B. henselae, E. coli* and *S. aureus* and the rate of apoptosis measured 6h and 24h p.i.. At both time points *B. henselae* infection caused very low rates of apoptosis, comparable to those measured in uninfected cells (~0%; Fig. 4.2). In contrast, *S. aureus* infection lead to apoptosis rates of over 70% after 6h which rose to over 80% after 24h. *E. coli* showed apoptosis rates of almost 20% after 6h rising to over 60% after 24h of infection. These findings confirm previous data indicating that *B. henselae* efficiently inhibits the apoptotic response in MACs and suggests this to be an important feature distinguishing *B. henselae* infection from a general inflammatory response.

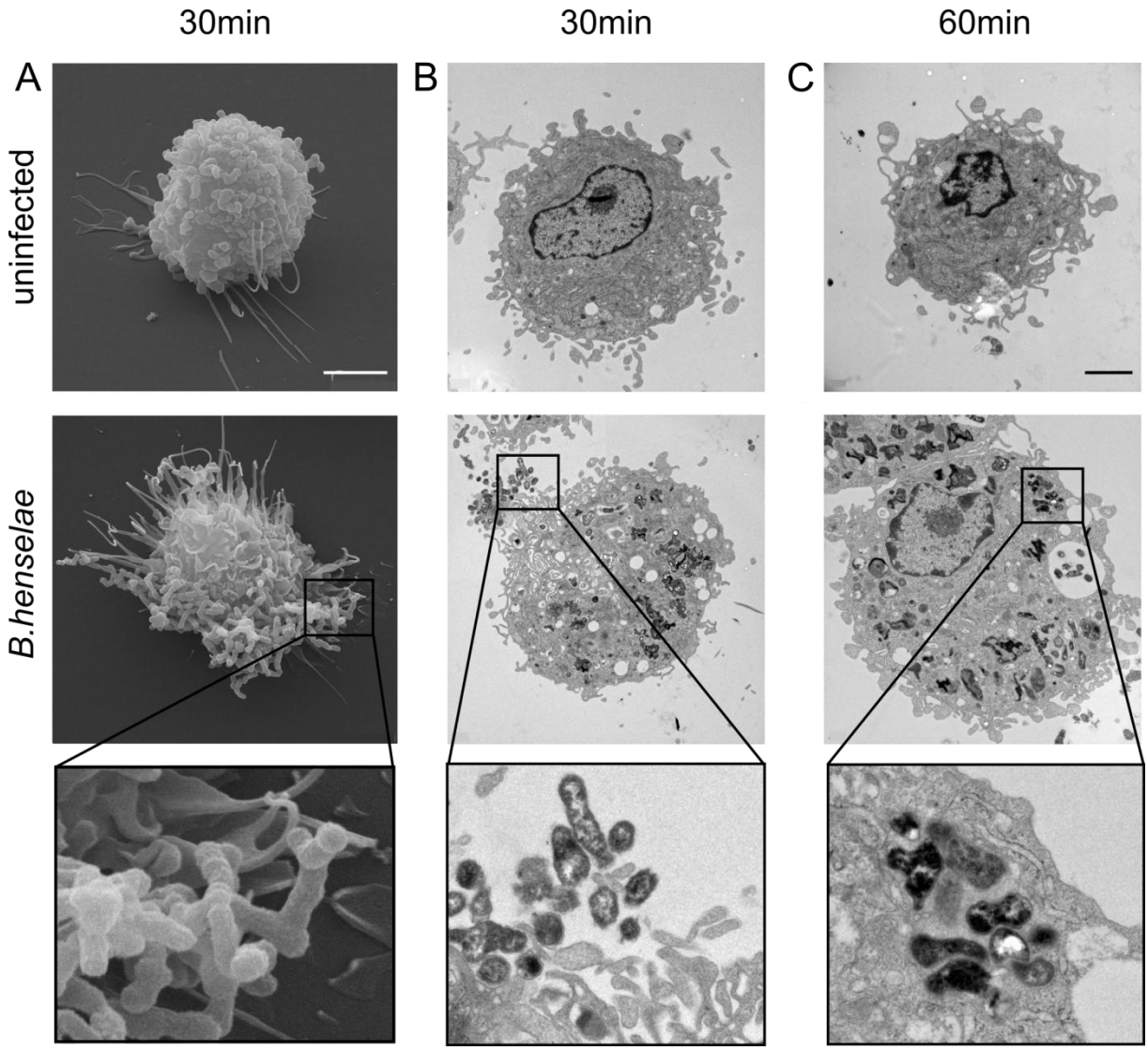


Figure 4.1 *B. henselae* invades MACs and resides in intracellular vacuoles. MACs were incubated with *B. henselae* (MOI 300). Aggregates of rod shaped bacteria are adherent to MACs after 30min as visible via (A) scanning electron microscopy (scale bar: 5µm) and (B) transmission electron microscopy (scale bar: 2µm). Enlargements show interaction of *B. henselae* with MAC membrane protrusions. Bacteria are internalized after 60min as visible via transmission electron microscopy (C). Vacuolic compartment harbouring *B. henselae* visible in enlargement.

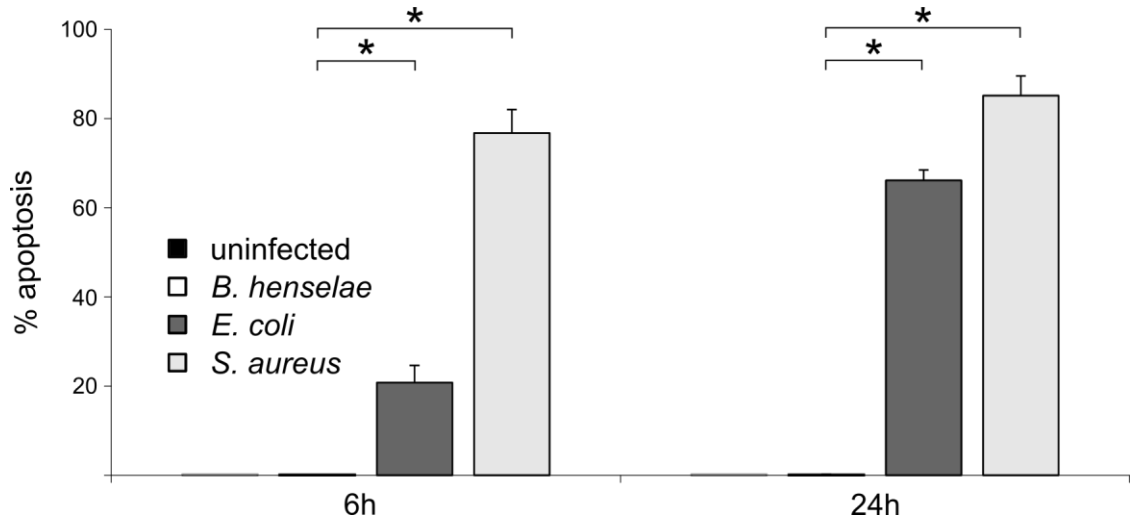


Figure 4.2 Apoptosis in MACs in response to *B. henselae, S. aureus* and *E. coli* infection. MACs were infected with *B. henselae* (MOI 300), *E. coli* (MOI 10) and *S. aureus* (MOI 10). Uninfected cells were used as a control. Rates of apoptosis were measured after 6h and 24h via Nicoletti assay. Figure shows representative results (mean and standard deviation, asterisk denotes $p<0.01$).

4.3 *B. henselae* infection of MACs induces BadA dependent HIF-1 activation

A major consequence of *B. henselae* infection in endothelial and epithelial cells is the activation of the angiogenic transcription factor HIF-1 (Riess *et al.*, 2004; Kempf *et al.*, 2005a). The activation of HIF-1 and the subsequent HIF-1 dependent pro-angiogenic response is assumed to be the dominant mechanism driving *B. henselae* induced angiogenic reprogramming in endothelial cells (Dehio, 2005). Mändle (2005) demonstrated that *B. henselae* infection of MACs also resulted in activation of HIF-1.

HIF-1 activation has been shown to be a general phenomenon in infections (Werth *et al.*, 2010). To assess the specificity of the *B. henselae* induced HIF-1 response in MACs, cells were infected with *B. henselae* or standard laboratory strains of *E. coli* and *S. aureus*. Results demonstrated that infection with not only *B. henselae*, but other pathogenic bacteria can induce HIF-1 activation in MACs (Fig. 4.3A).

In endothelial cells, the activation of HIF-1 in response to *B. henselae* infection has been shown to require the expression of the *B. henselae* pathogenicity factor BadA (Riess *et al.*, 2004). The HIF-1 response in *B. henselae* infected MACs was also found to be BadA dependent, as infection with the BadA$^-$ transposon mutant strain of *B. henselae* did not result in activation of HIF-1 (Fig. 4.3B). These results indicate that *B. henselae* infection induces HIF-1 activation in MACs not as part of a general inflammatory response but through the specific activity of its pathogenicity factor BadA.

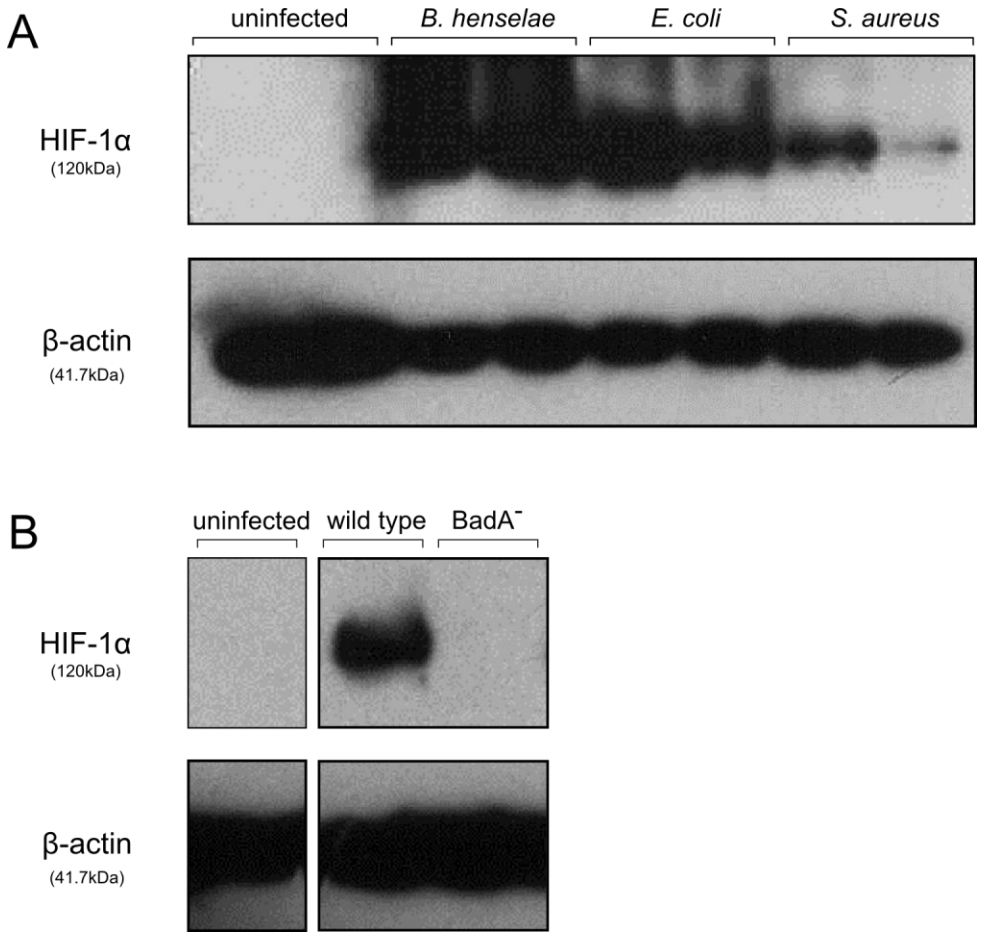


Figure 4.3 HIF-1 activation in MACs upon *B. henselae* infection. (A) HIF-1α protein (120 kDa) was analyzed via Western Blot (loading control: β-actin, 41.7 kDa) from *B. henselae* (MOI 250)*, E. coli* (MOI 10) and *S. aureus* (MOI 10) infected MACs in duplicate, 6h p.i. Uninfected cells were used as control. **(B)** BadA dependent HIF-1 activation upon *B. henselae* infection of MACs. HIF-1α protein was analyzed via Western Blot of *B. henselae* wild type and *B. henselae* BadA$^-$ infected MACs (MOI 250), 6h p.i.

4.4 *B. henselae* infected MACs incorporate into sprouting endothelium and increase the rate of angiogenic growth

To investigate the functional interaction of *B. henselae* infected MACs with mature endothelial cells a co-culture spheroid assay of sprouting angiogenesis was performed. Prior to spheroid formation, MACs were infected with *B. henselae* at increasing MOIs for 24h. HUVECs and MACs were stained with green and red cell tracker respectively and co-culture spheroids were composed of HUVECs and MACs at a ratio of 3:1. Both uninfected and infected MACs were found incorporated into the endothelial spheroids and associated with the sprouting vasculature (Fig 4.4). MACs were localized to both tip and stalk areas of the vascular sprouts. A significant increase in angiogenic sprouting was observed when MACs were infected with *B. henselae* indicating that the infection of MACs with *B. henselae* increases their functional angiogenic activity and does not interfere with their ability to home to and interact with angiogenic endothelium (Fig 4.5A).

4.5 Conditioned medium from *B. henselae* infected MACs is sufficient to increase sprouting angiogenesis in endothelial cells

To determine whether *B. henselae* infected MACs acted upon endothelial cells in a paracrine manner or through physical interactions, several variations on the spheroid co-culture assay were performed, placing the infected MACs at varying degrees of separation from the growing endothelium. Spheroids were created exclusively from HUVECs and suspended in collagen gel. MACs were infected for 24h and either mixed with the collagen gel or seeded onto the surface. In both conditions the rate of sprouting angiogenesis increased in a dose dependent manner when MACs were infected with *B. henselae* indicating that no physical contact was necessary to transmit the pro-angiogenic effects of infected MACs to the growing endothelium (Fig. 4.5B, C).

Finally, conditioned, filter-sterilized medium was collected from MACs infected with *B. henselae* for 24h at varying MOIs and cultured with spheroids composed solely of HUVECs. Here, *B. henselae* infection had a strong, dose dependent effect on the rate of sprouting angiogenesis induced by conditioned medium, indicating that paracrine effects are predominantly responsible for the pro-angiogenic activity of *B. henselae* infected MACs (Fig. 4.5D).

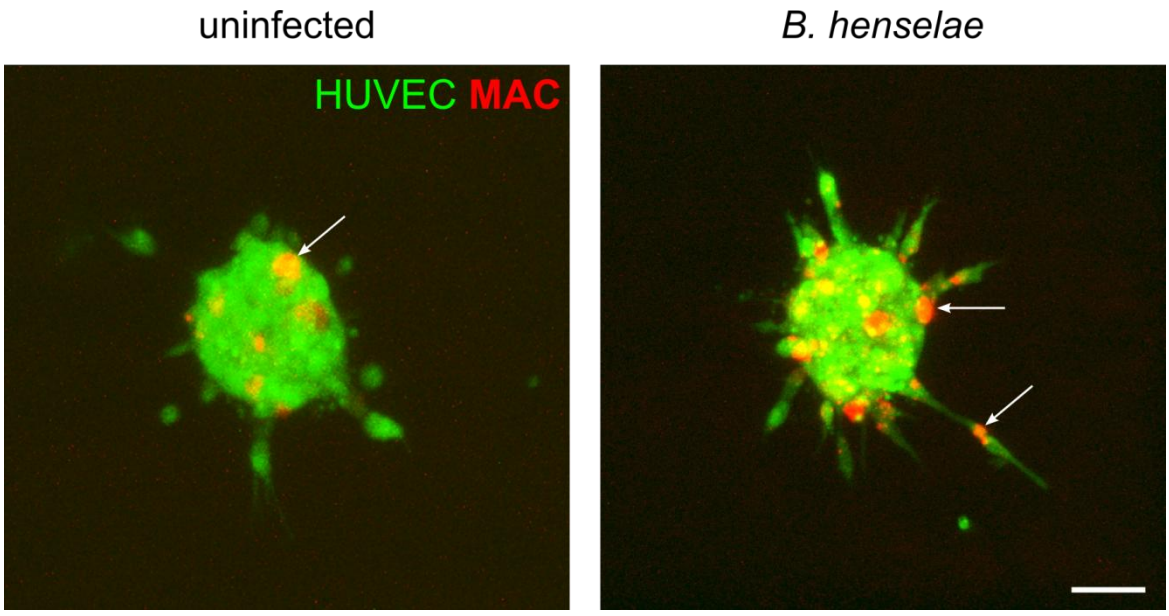


Figure 4.4 Incorporation of uninfected and *B. henselae* infected MACs into growing vascular sprouts. HUVECs and MACs were labeled green or red respectively with Cell-Tracker. MACs were infected with *B. henselae* for 24h. Co-culture spheroids were composed of HUVECs and MACs at a ratio of 3:1 and suspended in collagen gel. Localization of MACs within vascular sprouts was analyzed via confocal microscopy. Representative spheroids from 3 independent experiments are presented. Maximum intensity projections of 21-160µm/slice z-stacks are displayed. Scale bars: 50µm. Arrows indicate examples of individual MACs incorporating into the spheroid body and vascular sprouts.

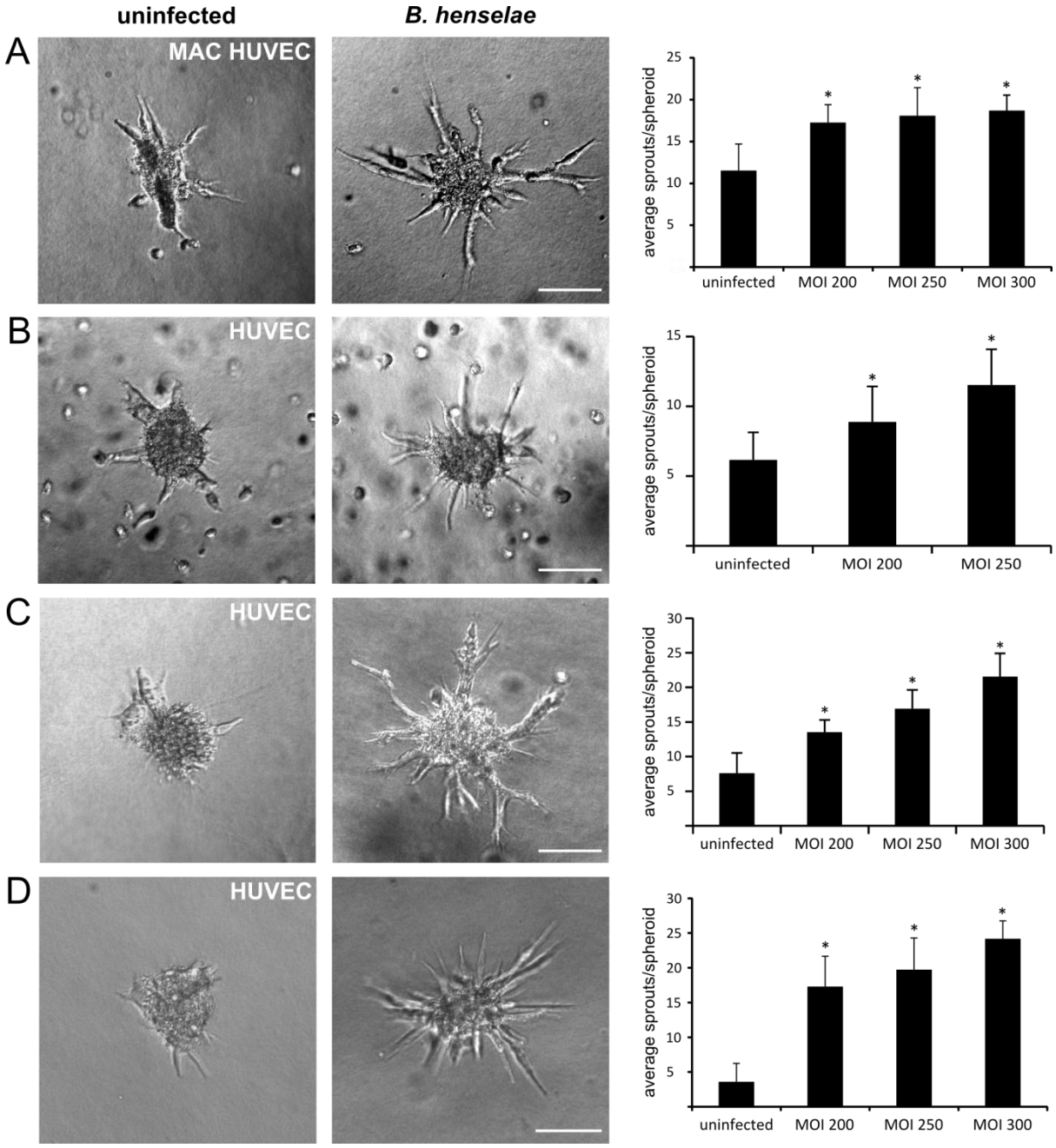


Figure 4.5 Infection of MACs with *B. henselae* results in increased angiogenic growth via paracrine mechanisms. MACs were infected with *B. henselae* for 24h. (A) Co-culture spheroids were composed of HUVECs and MACs at a ratio of 3:1 and suspended in collagen gel. (B-D) Spheroids were composed exclusively of HUVECs, suspended in collagen gel and grown in the presence of $1.75x10^5$ MACs (B) mixed with collagen gel, (C) seeded onto the gel with cell culture medium or (D) with conditioned medium from MACs infected with *B. henselae* for 24h. The rate of angiogenic sprouting was determined via light microscopy. Representative spheroids are presented, followed by means and standard deviations from representative experiments. A minimum of 10 spheroids was analyzed per condition in at least 3 independent experiments. Scale bars: 50µm, asterisk denotes $p<0.01$.

4.6 *B. henselae* infected MACs display a vascular mimicry phenotype over long term culture

To further examine the potential angiogenic capacity of *B. henselae* infected MACs, a modified Martrigel capillary forming assay was employed. Culture plates were coated with a thick layer of Matrigel basement membrane gel. MACs were infected for 24h with *B. henselae* and re-seeded onto Matrigel coated plates. Cells were maintained in long term culture (41-77 days). Initially, both infected and uninfected cells adhered to the matrix and maintained a flattened spherical morphology. Over time however, uninfected cells died and degraded completely in culture. Infected cells on the other hand, developed spindle shaped or spreading morphologies and extended long branched fillopodia (Fig. 4.6).

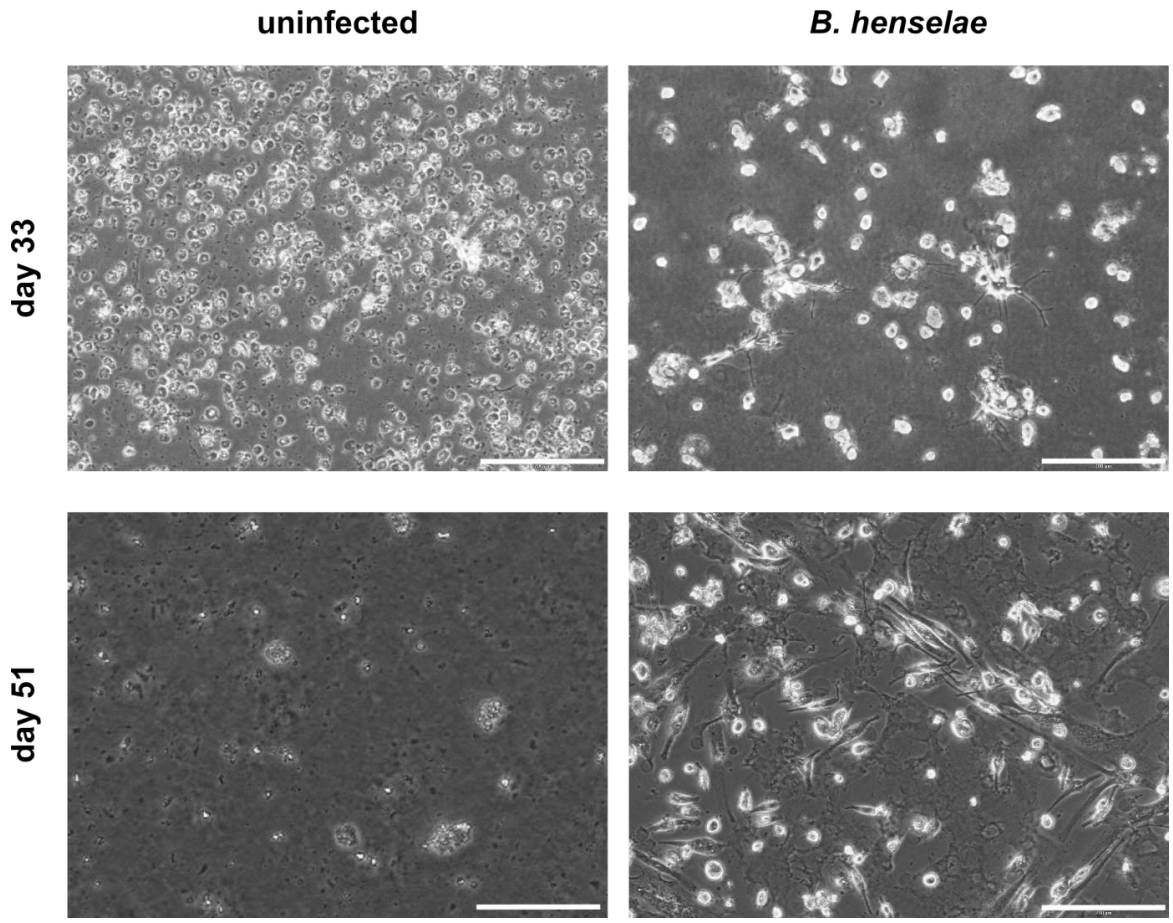


Figure 4.6 Detection of cell integrity and morphological changes of *B. henselae*-infected MACs in long-term Matrigel cultures. Phase contrast light-microscopy images of *B. henselae* infected (MOI 100) and uninfected Matrigel cultures at different time points. Infected cells extend long branching filopodia, develop spindle shaped or spreading morphologies and gradually assembled into complex two-dimensional chord structures of linearly aligned cells. Uninfected cells show no morphological changes and die over time. Note, that at d51 only cellular debris remains in uninfected Matrigel cultures whereas *B. henselae*-infected cells appear intact and display vascular mimicry structures. Scale bars: 200µm. Figures show representative images from two independent experiments.

Over the course of several weeks infected cells gradually assembled into long, intersecting, two dimensional chord structures, consisting of aligned cells , forming networks of approximately 2 cm in diameter (Fig. 4.7; enlargement). These complex networks resemble the “capillary net” formation displayed by endothelial cells cultured under similar conditions (Khoo *et al.*, 2011). The extent and morphology of the vascular mimicry phenotype was subject to donor variation but consistently associated only with *B. henselae* infection.

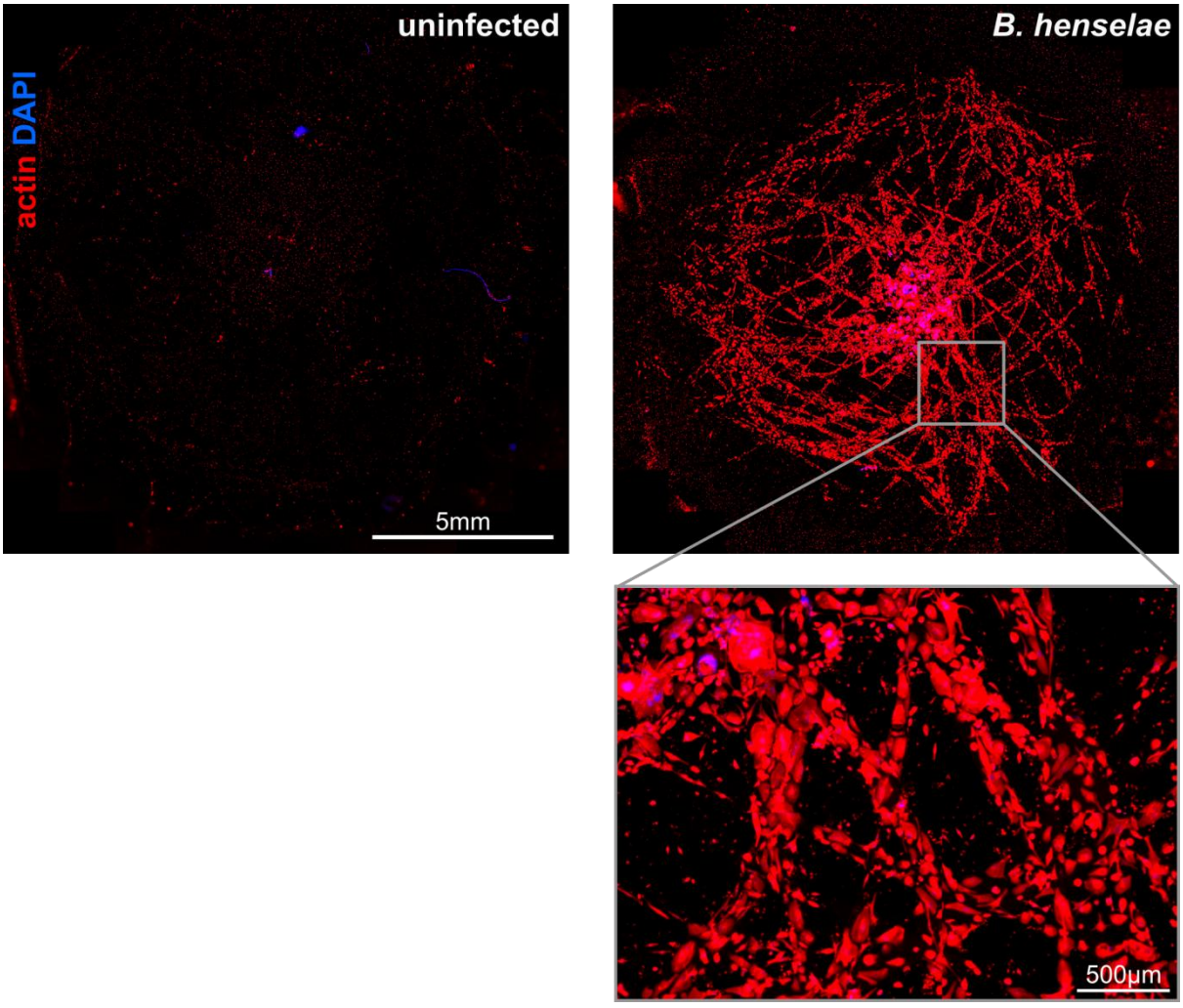


Figure 4.7 *B. henselae* infected MACs develop a vascular mimicry phenotype over long term culture. Full well composite images of uninfected and infected Matrigel cultures (d65) showing fully developed capillary-like networks (red: actin; scale bar: 5mm) and enlargement showing arrangement of individual cells within the chord structures (scale bar: 500µm). MACs were infected with *B. henselae* (MOI 300) for 24h and reseeded onto 3D Matrigel matrix. Cells were cultured for a further 60 days. Images display the extent of network formation over the full culture plate and enlargement as a composite picture of digitally assembled images

Almost 100% (96.72±4.43%) of cells isolated from fully developed vascular mimicry structures (d51) stained negative for the apoptosis marker 7-AAD in FACS analysis demonstrating sustained viability of *B. henselae* infected MACs in Matrigel culture (n=4, Fig. 4.8). *B. henselae* DNA was detectable via 16S rDNA PCR at the end of long term cultures, however, no viable bacteria could be cultured from vascular mimicry structures (n=3; Fig. 4.9 and data not shown) indicating that the sustained presence of viable *B. henselae* is not required for the full development of vascular mimicry structures..

4.7 The development of the *B. henselae*-induced vascular mimicry phenotype is BadA dependent

BadA is crucially involved in the HIF-1-dependent angiogenic reprogramming of *B. henselae*-infected cells (Riess *et al.*, 2004). Indeed, HIF-1 activation upon infection was also shown to be BadA dependent in MACs (see above; Fig. 4.3B). To further dissect the role of BadA in the *B. henselae*-induced vascular mimicry phenotype, MACs were infected with *B. henselae* wildtype ($BadA^+$), *B. henselae* $BadA^-$ and heat killed *B. henselae* and subsequently cultured on Matrigel as described above. Although some structure formation was observed in cultures infected with $BadA^-$ and heat killed *B. henselae*, results demonstrate that only BadA-expressing viable *B. henselae* were able to induces strong capillary-like structure formation in MACs (Fig. 4.10 A-E).

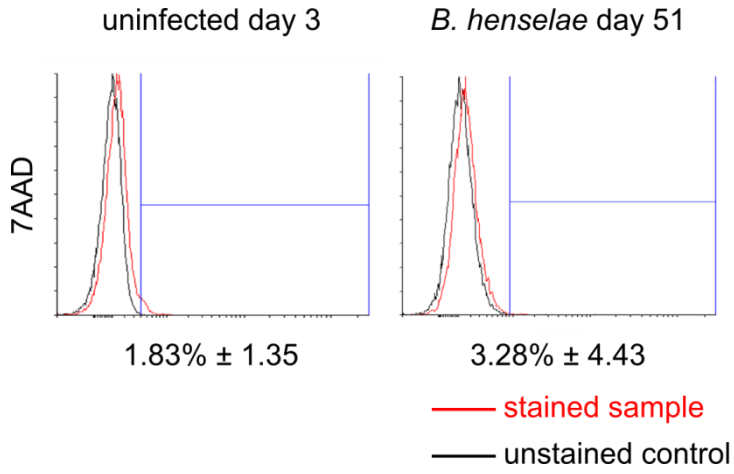


Figure 4.8 Viability of *B. henselae* infected MACs in long term Matrigel culture. FACS analysis of apoptosis marker 7-AAD [(red) compared to unstained negative controls (black)] on uninfected d3 MACs and *B. henselae* infected MACs (MOI300) from Matrigel vascular mimicry structures (day 51). Histograms display representative results [events vs. fluorescence intensity (log10)] as well as means and standard deviations of percent positive cells from at least three independent experiments.

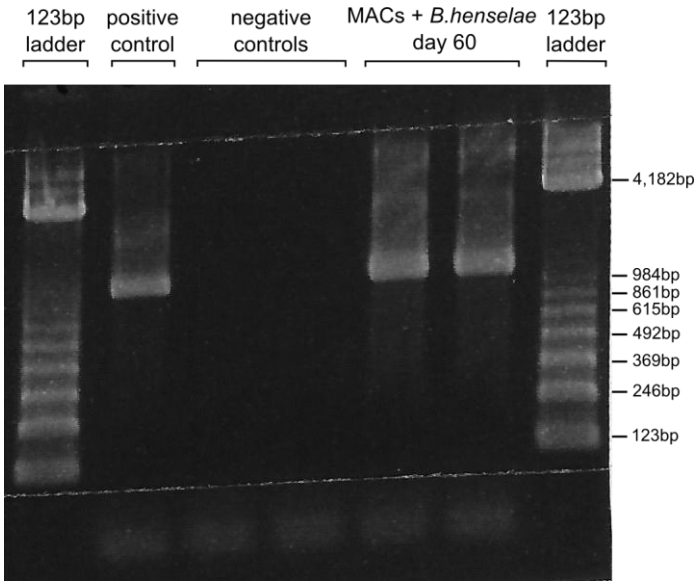


Figure 4.9 Presence of *B. henselae* DNA in fully formed vascular mimicry structures. MACs were isolated from Matrigel vascular mimicry structures at end point of culture (day 60) and analyzed via nested-16S rDNA PCR for the presence of *B. henselae* DNA. Expected amplification product: 990bp. Figure shows results in duplicate from one experiment.

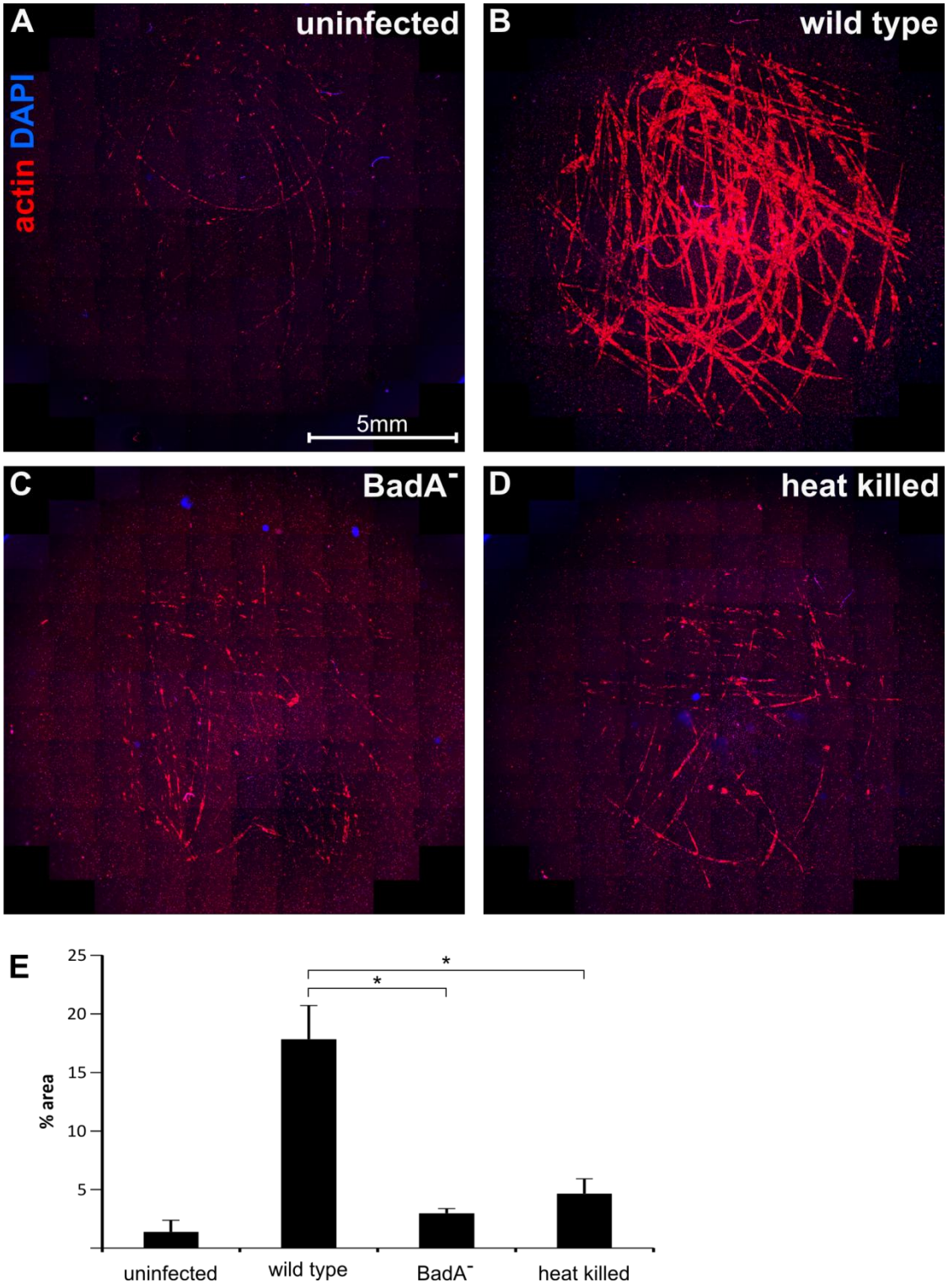


Figure 4.10 Development of the vascular mimicry structures is dependent on infection with viable BadA expressing *B. henselae*. MACs were infected with *B. henselae* (MOI 300) for 24h and reseeded onto 3D Matrigel matrix. Cells were cultured for a further 60 days. Representative images of capillary-like growth networks formed by (**A**) uninfected MACs, MACs infected with (**B**) *B. henselae*-wildtype, (**C**) *B. henselae*-BadA$^-$ or (**D**) heat-killed *B. henselae* (scale bar: 5mm). (**E**) Quantification of capillary-like growth in the presence of *B. henselae* wild type, *B. henselae* BadA$^-$ and heat-killed *B. henselae* as a measure of the relative actin-stained pixel area. Results show mean and standard deviation from three independent experiments. Asterisk denotes $p<0.01$.

4.8 MACs develop and maintain a macrophage phenotype after infection with *B. henselae*

To determine the differentiation phenotype of *B. henselae*-infected MACs, flow cytometric analysis was performed to test for the expression of cell surface markers at different time points [day 5 (24h p.i.) and day 62 from fully developed vascular mimicry structures; Fig. 4.11]. Endothelial markers antigens VEGFR2 and CD31 were absent or weakly expressed at all time points examined. Under all conditions, MACs showed moderate expression of the pan-leukocyte marker CD45 and no expression of the monocytic marker CD14. The marker of antigen presenting cells human leukocyte antigen (HLA)-DR and the macrophage marker CD68 were expressed on all cells. A strong increase in CD68 and HLA-DR occurred after infection with *B. henselae* and lasted over long term culture. In contrast, CD11c a marker of monocyte derived dendritic cells, was only moderately expressed on MACs and was completely absent on cells derived from the vascular mimicry structures.

Additionally, MACs were removed from Matrigel vascular mimicry structures by dispase-treatment and analyzed via cytospin-immunocytochemistry. Cells of the vascular mimicry structures stained positive for vimentin; a protein of the cytoskeleton expressed in cells of the mesenchymal lineage but also by hematopoietic cells such as macrophages. Staining for CD34, a stem cell marker also found on endothelial cells was found to be negative along with the endothelial markers CD31, von-Willebrand Factor (vWF) and vascular endothelial growth factor receptor-2 (VEGFR2; Fig. 4.12).

Finally, qRT-PCR analysis was preformed comparing the rate of endothelial marker gene transcription between uninfected MACs and infected cells of the vascular mimicry structures. Analysis revealed only a small and statistically insignificant fold increase in the transcription of key endothelial marker genes endothelial nitric oxide synthase (eNOS), vascular endothelial cadherin (VE Cadherin) and VEGFR-2 (Fig. 4.13).

Taken together, these results suggest that the phenotype induced in MACs in response to *B. henselae* infection and present in the vascular mimicry structures is of a myeloid cell phenotype with typical macrophage characteristics. Despite the pro-angiogenic phenotype of these cells and the formation of vessel-like structures, results did not demonstrate any evidence of the development of an endothelial cell phenotype in either gene transcription or protein expression of *B. henselae* infected MACs.

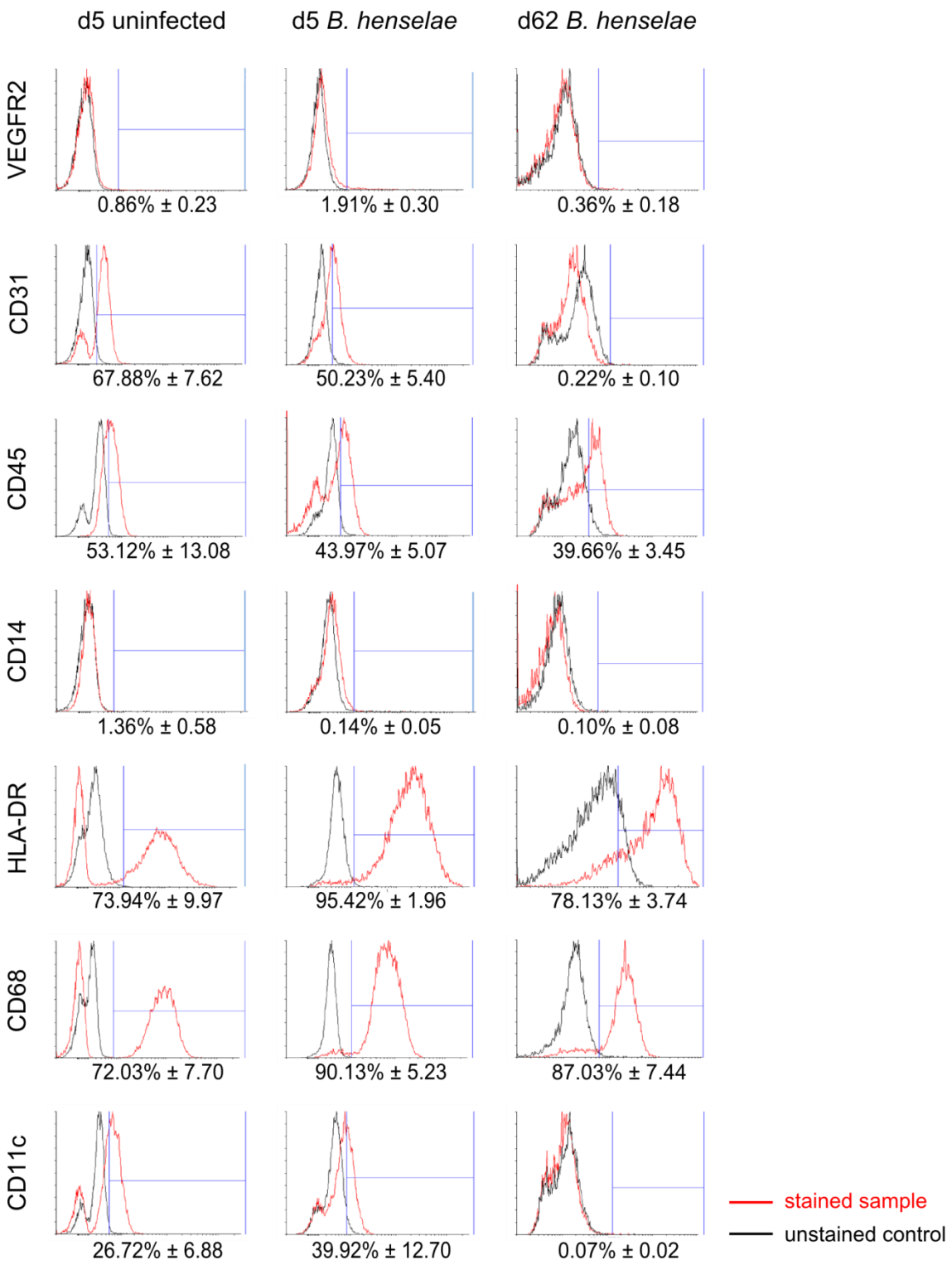


Figure 4.11 MACs develop and maintain a macrophage phenotype after infection with *B. henselae.* FACS analysis of endothelial cell markers VEGFR2, CD31, myeloid marker CD14, CD45, macrophage markers CD68, HLA-DR and dendritic cell marker CD11c expression [(red) compared to isotype-stained negative controls (black)] on uninfected and *B. henselae* infected MACs (MOI 300) d5-24h p.i. and d62 from vascular mimicry structures. Histograms display representative results [events vs. fluorescence intensity (log10)] as well as means and standard deviations of percent positive cells from three independent experiments.

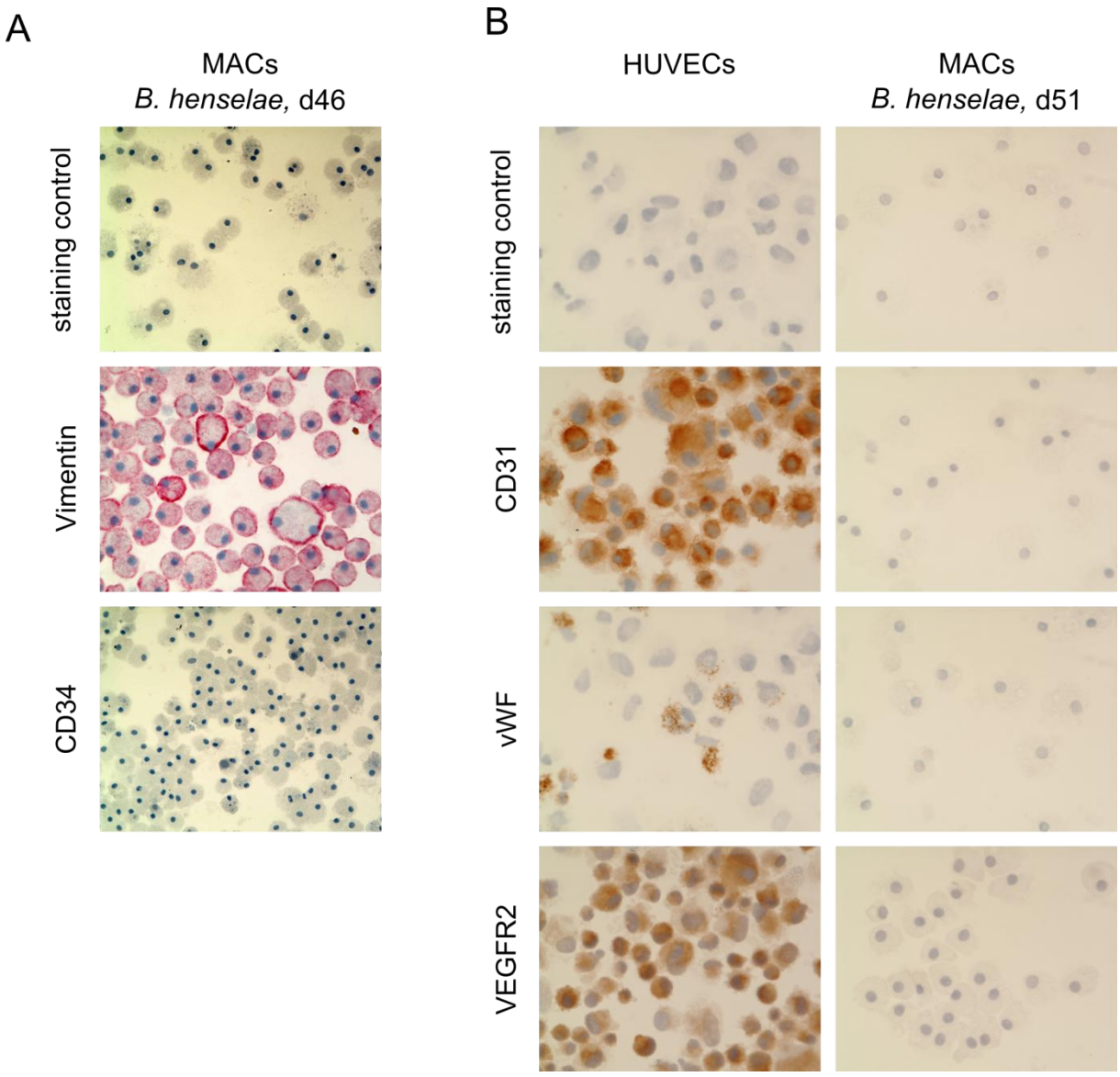


Figure 4.12 MACs maintain a non-endothelial phenotype after infection with *B. henselae* and formation of vascular mimicry structures. Infected MACs (MOI 300) were isolated from Matrigel vascular mimicry structures via dispase digestion and centrifuged onto glass slides for cytospin-immunohistochemistry. Cells were analyzed for the presence of (A) mesenchymal marker Vimentin, stem cell marker CD34 (d46) and (B) endothelial markers CD31, vWF and VEGFR2 (d51). For endothelial markers, HUVECs were used as internal positive control. Control stains were performed omitting primary antibodies.

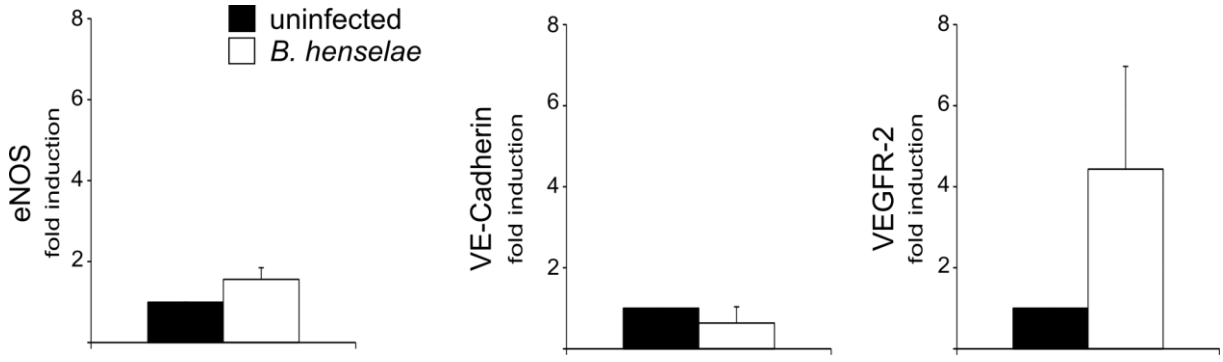


Figure 4.13 MAC angiogenic re-programming upon *B. henselae* infection does not involve upregulation of endothelial marker genes. Induction of endothelial marker genes eNOS, VE-Cadherin and VEGFR2 in *B. henselae* infected MACs (MOI 300) isolated at d60 from vascular mimicry structures was measured in relation to uninfected freshly isolated MACs at d3 via qRT-PCR. mRNA transcripts were quantified [ratio: eNOS/ RPLP0, VE-cadherin/ PolR2A and VEGFR2/ PolR2A]. Results show mean and standard deviation from three independent experiments.

Transcriptional profiling

To examine phenotypic changes induced in MACs in response to *B. henselae* infection, gene expression profiles were compared between uninfected MACs directly after isolation (d3) and *B. henselae* infected MACs cultured on Matrigel (d53) via gene expression microarray.

4.9 The infection of MACs with *B. henselae* is associated with broad phenotypic re-programming

To evaluate the general variation in overall gene expression between samples and between experimental conditions, unsupervised hierarchical clustering was performed for genes with a standard deviation ≥ 1.5 across all samples (Fig. 4.14 A-B).

All three donor samples isolated from d3 MACs clustered closely together [average person correlation coefficient (r)= 0.992; (max correlation r =1)] and were separate from the corresponding infected samples (d53; average r =0.989). In contrast, a high level of variance was displayed between the two experimental conditions (average r =0.963) indicating that variations in gene expression between donors is negligible in comparison to the broad phenotypic reprogramming induced in MACs in response to infection.

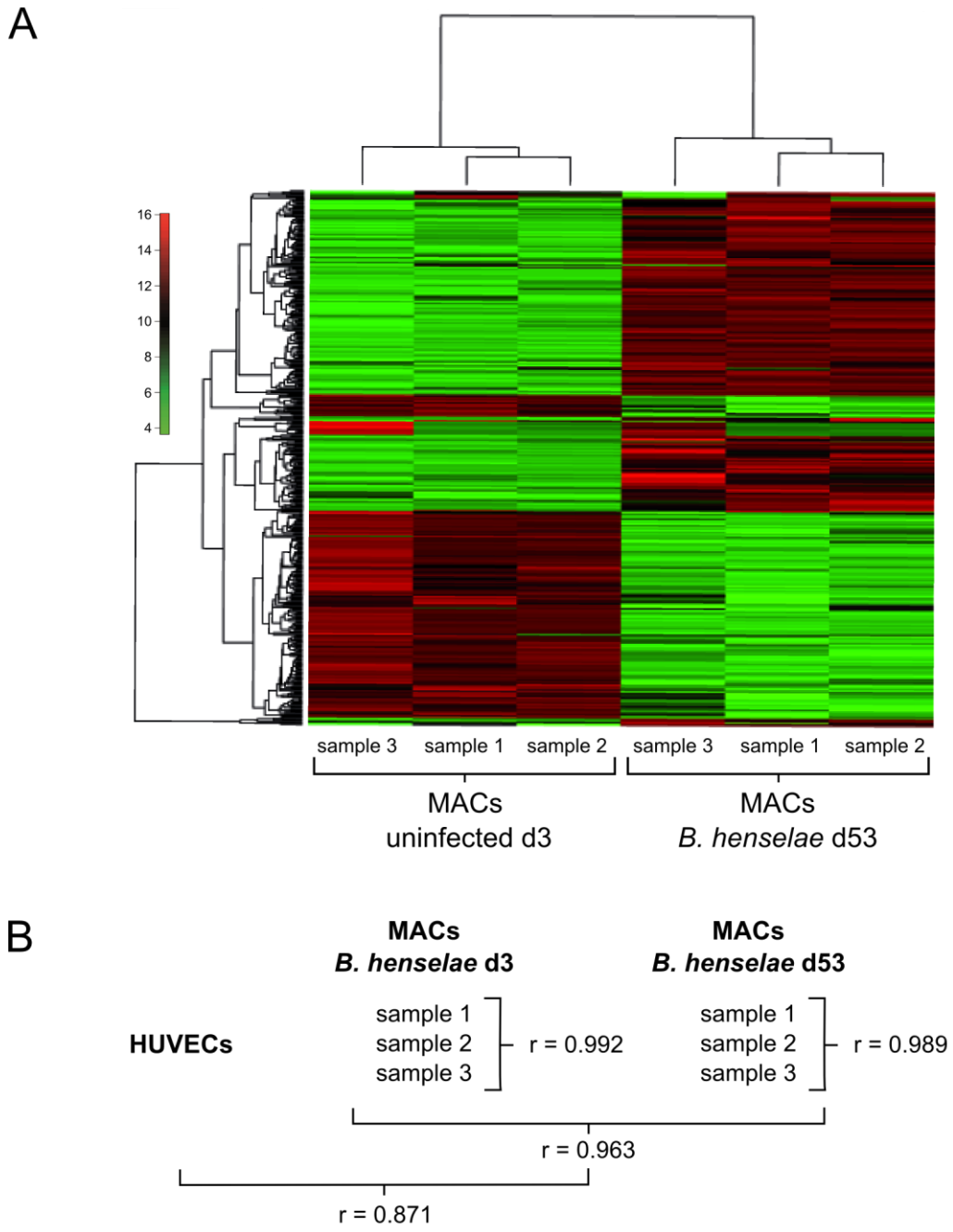


Figure 4.14 *B. henselae* infection of MACs leads to broad phenotypic re-programming. RNA was isolated from freshly isolated MACs (d3) and *B. henselae* infected, Matrigel cultured MACs (MOI 300; d53). mRNA gene expression profiles were analyzed via Affymetrix microarrays. Variance in gene expression profiles were compared between three biological replicates of uninfected MACs (d3) and *B. henselae* infected MACs (d53). **(A)** Unsupervised hierarchical clustering was performed for genes with a standard deviation ≥ 1.0 across all samples using the Manhattan distance and the average linkage method. Clustering arrangement of 399 genes of highest variance is shown. Absolute gene expression signals are represented in red (high) and green (low). Linkage between samples is represented by the length of the connecting branches displayed above the image. Connecting branches to the left display the linkage for expression variance of individual genes. **(B)** The average person correlation coefficient (r) is displayed as a measure of overall variance in gene expression among biological replicates and experimental conditions.

4.10 The gene expression profiles of *B. henselae* infected MACs are strongly divergent from endothelial cells and show most similarity to cells of the myeloid lineage

To compare gene expression profiles of infected and uninfected MACs to endothelial cells, an endothelial control sample (HUVECs grown on Matrigel for 12h) was included in the analysis as a control. A distinct clustering occurred, separating the HUVEC control sample from all other samples by a long linkage distance (Fig. 4.14B and Fig. 4.15; average r = 0.871). The pronounced disparity in gene expression between endothelial cells and both infected and uninfected MACs provides further evidence that the pro-angiogenic phenotype of *B. henselae* infected MACs is not associated with an endothelial phenotype.

As an additional measure to examine the cellular differentiation of *B. henselae* infected MACs, gene expression profiles were compared to datasets of know cell differentiation posted to the online database NCBI Gene Expression Omnibus (GEO).

In order to compare gene expression profiles of the *B. henselae* infected MACs to datasets found in the GEO databank, it was necessary to convert the relative gene expression values to an earlier version of the Affymetrix gene microarray platform (Affymetrix Human Genome U133 Plus 2.0 Array; GPL570). As conversion resulted in considerable statistical noise, direct comparison between datasets from the two platforms was not possible. Nevertheless, some general observations could be made.

Gene expression profiles form infected MACs (d53) showed the highest correlation with cells of the monocytic myeloid lineage; monocytes, macrophages and dendritic cells with calculated coefficients of determination (R^2) ranging from 0.530 to 0.380 (Table 4.1). These were followed by gene expression profiles from smooth muscle, fibroblasts, endothelial cells, other immune cells and finally embryonic stem cells for which the calculated R^2 was only 0.223.

The variation between infected and uninfected MACs (R^2=0.8742) was found to exceed the variation observed between monocytes and *in vitro* differentiated macrophages (GSE11430 R^2=0.909) as measured in one study documented in the GEO database. This suggests that *B. henselae* infection induces significant phenotypic changes which exceed the phenotypic transformation taking place during typical macrophage differentiation process and further supports the finding that *B. henselae* infection results in a comprehensive phenotypic re-programming in infected MACs (Table 4.2).

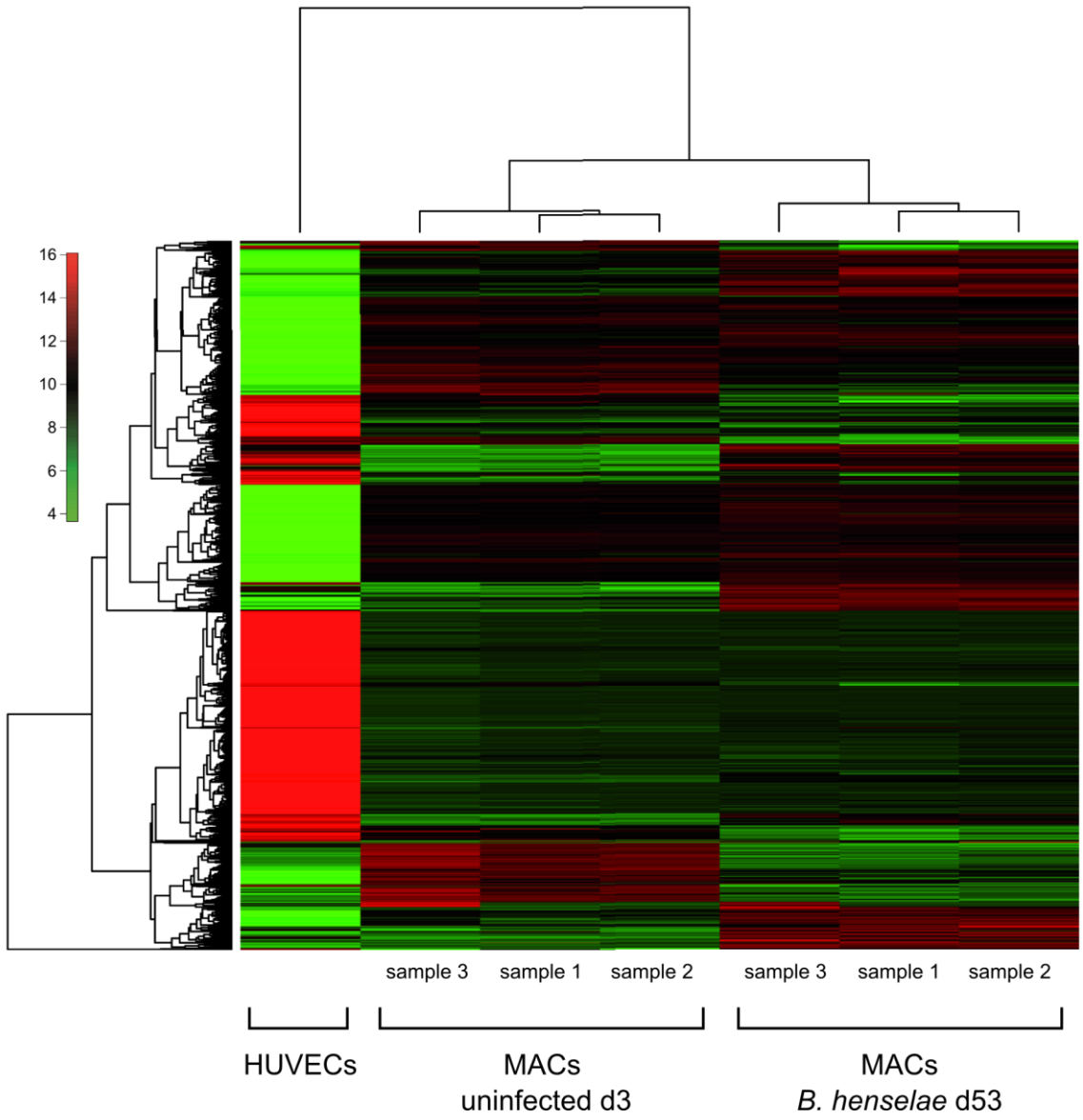


Figure 4.15 Comparison of gene expression profiles between MACs and mature endothelial cells. Variance in gene expression profiles were compared between three biological replicates of uninfected MACs (d3), *B. henselae* infected MACs (d53) and one HUVEC control. Unsupervised hierarchical clustering was performed for genes with a standard deviation ≥ 1 across all samples using the Manhattan distance and the average linkage method. Clustering arrangement of 1,423 genes of highest variance is shown. Absolute gene expression signals are represented in red (high) and green (low). Linkage between samples is represented by the length of the connecting branches displayed above the image. Connecting branches to the left display the linkage for expression variance of individual genes.

Table 4.1 Comparison of gene expression signals from *B. henselae* infected MACs to gene expression profiles of known cell differentiation. Gene expression profiles of *B. henselae* infected MACs (d53) were compared to selected reference gene expression profiles from cells of known differentiation. Reference gene expression profiles were retrieved from NCBI Gene Expression Omnibus (GEO) and expression signals converted between platforms for comparison. Similarity in gene expression patterns calculated as coefficient of determination (R^2).

Platform	Reference Series	Cell type	Description	Sample	R^2=
GPL6244	GSE55170	**MAC**	**MACs, uninfected, d3**		**0.874**
GPL570	GSE4984.	**dendritic**	monocyte derive dendritic cells	control	**0.530**
GPL570	GSE13670	**macrophage**	human monocyte-derived macrophages	control	**0.513**
GPL570	GSE16385.	**macrophage**	alternatively and classically activated macrophages	macrophage IL-4, 24h (alternatively activated)	**0.473**
GPL570	GSE20484	**macrophage**	monocyte derived macrophages stimulated with MCSF and CXCL4	MCSF	**0.478**
GPL570	GSE16385.	**macrophage**	alternatively and classically activated macrophages	control, 24h	**0.458**
GPL570	GSE6054	**monocyte**	monocytes	control	**0.449**
GPL570	GSE16385.	**macrophage**	alternatively and classically activated macrophages	macrophage INFγ+TNFα, 24h (classically activated)	**0.424**
GPL570	GSE12261	**smooth muscle**	human aortic smooth muscle cells	control	**0.340**
GPL570	GSE28491+ GSE28492	**B cell**	human immune cell subsets	B cells	**0.333**
GPL570	GSE14801	**endothelial**	human umbilical vein endothelial cells	control	**0.328**
GPL570	GSE11919	**fibroblast**	GM5659 skin fibroblasts	control	**0.321**
GPL570	GSE28491+ GSE28492	**eosinophils**	human immune cell subsets	eosinophils	**0.315**
GPL570	GSE28491+ GSE28492	**neutrophils**	human immune cell subsets	neutrophils	**0.315**
GPL570	GSE28491+ GSE28492	**CD8 T cells**	human immune cell subsets	CD8 T cells	**0.310**
GPL570	GSE28491+ GSE28492	**CD4 T cells**	human immune cell subsets	CD4 T cells	**0.309**
GPL570	GSE7896	**stem cell**	embryonic stem cells	control	**0.226**

Table 4.2 Comparison of *B. henselae* induced phenotypic re-programming in MACs to changes induced during *in vitro* monocyte to macrophage differentiation. The variance in gene expression profiles between uninfected MACs (d3) and *B. henselae* infected MACs (d53) was compared to changes in gene expression occurring between monocytes and *in vitro* cytokine differentiated monocyte-derived-macrophages. Reference expression patterns retrieved from NCBI Gene expression Omnibus (GEO). Samples evaluated within reference series and platforms. Similarity in gene expression patterns calculated as coefficient of determination (R^2).

Platform	Reference Series	Cell type	Description	R^2=
GPL6244	GSE55170	**MACs, uninfected, d3** **MACs, *B. henselae*, d53**		**0.874**
GPL570	GSE11430	**monocyte** **macrophage**	monocytes and macrophages	**0.909**

4.11 *B. henselae* infection of MACs induces regulation of genes involved in angiogenic and immune regulatory pathways.

To examine the nature of cellular re-programming in *B. henselae* infected MACs in more detail, absolute normalized expression values from the gene expression microarrays of infected and uninfected MACs [fold change (FC)] were compared. Overall, 1,400 genes or ca. 36% of the 3,872 messenger RNA (mRNA) transcripts evaluated, showed a change in expression in excess of 2.0 fold. 620 genes were upregulated and 780 genes downregulated at a rate of 2.0 fold or more. For genes upregulated 4.0 fold or higher, and with a false discovery rate (FDR) of less than 0.05, a detailed functional analysis was performed via GeneBank database and PubMed literature searches (Table 4.3). A functional annotation analysis using the DAVID database tool was performed on genes upregulated or downregulated with a fold change greater than or equal to 2.0 and a false discovery rate of less than 0.05.

Functional annotations in the upregulated gene sets enriched to statistical significance ($p<0.01$) were revealed in 157 annotation categories (S Table 10.1 and 10.2). These annotation categories fell into 20 broadly related functional groups (S Table 10.4 and 10.5). For downregulated genes, 52 enriched functional annotation categories could be identified in 6 broadly related functional groups. A selection of these highly enriched functional annotation categories from up- and downregulated transcripts are presented in (Fig. 4.16) and (Table 4.4 and 4.5).

Functional annotations relating to angiogenesis and vascular development were most highly enriched among the upregulated genes. Highly upregulated genes included some of the most important positive inducers of angiogenesis (*VEGFA, CXCL8, HIF1A, MMP9, TGFB*). These were followed by annotations relating to positive and negative regulation of apoptosis including upregulation of genes such as *IER3, CKS2, PPP1R15A, PHLDA1 CDKN1A* and *TNFAIP3*.

Enriched functional annotations were also found in categories for inter- and intracellular signaling pathways including cytokine production, receptor signaling, intracellular signaling cascades, MAPK pathways and transcriptional regulation. Genes for chemokines (*CCL2, CXCL8, CXCL1, CXCL2, CCL4L1, CC3L1/CCL3, CSF*) were particularly highly upregulated. Highly enriched functional categories also included annotations involved in structural organization such as those for cell migration and adhesion. Many of the highly upregulated genes also had functions related to interaction with the extracellular matrix such as matrix degrading metalloproteinases *MMP9, MMP2, MMP7, MMP12, ADAMDEC1, MME*, and other compounds involved in matrix remodeling *SERPINE1, CHI3L1, CHIT1 CTSK, HTRA1 TNFAIP6*. Furthermore, several extracellular matrix components such *FN1, SPARC, FBLN5, SPP1, THBS1* as well as several integrins *ITGB3, ITGA3, ITGB5, ITGA5, and ITGAV* were upregulated.

Other enriched functional groups were identified as being linked to the regulation of immune responses including those for myeloid cell differentiation, leukocyte activation, proliferation, and hematopoietic cell differentiation. This was reflected in strong upregulation of several genes involved in innate immune responses (*NFKB, IL1B , IL8, CSF, TLR2*). Along with the upregulation of pro-inflammatory gene clusters, upregulation of negative regulators of inflammation and NFκB mediated gene transcription was also observed (*NR4A2, PTGS2, AREG, IER3, IFRD1, NR4A1, ARG2, TGFB2, TNFAIP3, ZC3H12A, MMP12, IL1RA, SOCS3*).

Unexpectedly, functional groups representing sterol metabolism were also highly enriched. Several genes for cholesterol processing and metabolism *OLR1, LPL, LDLR, DHCR24, INSIG1, DHCR7*, general sterol biosynthesis, *CYP19A1 , SQLE*, and several members of the prostaglandin biosynthesis pathway *FADS1, PTGS2, ALOX5AP* were highly upregulated.

The functional annotation analysis of downregulated genes was less diverse and allocated functional annotations to three general categories. The majority of the down regulated genes were classified to regulation of gene transcription and RNA metabolic processes including numerous zinc finger proteins, 93 of which were downregulated 2.0 fold or more. Following this was a strong downregulation of genes involved in T-cell activation, differentiation and T-cell receptor signaling pathways such as the CD3-TCR complex components *CD3E, CD3D, CD3G, CD247, ZAP70*, and downstream pathway elements *LCK, FYN, STAT4, CD2, CD8A*. Annotations relating to positive regulation of innate immune responses were also enriched among the downregulated gene transcripts.

Table 4.3 Detailed functional annotation of upregulated genes in *B. henselae*-infected MACs at d53 (FC ≥ 4 and FDR < 0.05).

Gene Symbol	Fold Change	p value	False Discovery Rate	Gene Description	Function	Relevant Citations
NR4A2 (Nurr1)	80.5	9.13E-06	1.60E-03	nuclear receptor subfamily 4, group A, member 2	Nuclear receptor (NR) of orphan nuclear receptor subfamily 4A. NRs interact directly with DNA to influence gene transcription. NR4s are ligand-independently regulated. Induced in response to cAMP, growth factors, inflammatory signals and hormones. Associates with the p65 subunit of NFκB and attenuates inflammatory gene transcription. Attenuates the release of inflammatory cytokines in macrophages. Essential for VEGF mediated angiogenesis.	(Han, 2012) (Zhao and Bruemmer, 2010)
IL8 (CXCL8)	44.5	1.18E-06	1.29E-03	interleukin 8	Chemokine. Chemoattractant for neutrophils, basophils and T-cells. Neutrophil activation, inflammatory response, member of CXCL family of chemokines with ELR motif (glutamic acid-leucine-arginine sequence). Binds to CXCR1 and CXCR2. Potent angiogenic properties.	
OLR1 (LOX-1)	33.2	6.58E-04	4.72E-03	oxidized low density lipoprotein (lectin-like) receptor 1	Scavenger receptor that binds, internalizes and degrades oxidized low-density lipoprotein. This action activates NFκB and inflammation through the production of intracellular reactive oxygen. Marker of atherosclerosis. Also involved in immune response as a leukocyte-adhesion molecule and acts as a receptor for advanced glycation end products, activated platelets, monocytes, apoptotic cells, Gram-negative and Gram-positive bacteria.	(Campbell *et al.*, 2012) (Shimaoka *et al.*, 2001) (Zhu *et al.*, 2013)
GLDN	24.8	5.79E-04	4.48E-03	Gliomedin	Transmembrane protein that is characterized by olfactomedin and collagen domains in its extracellular region. Interaction with the axonodal cell adhesion molecules neurofascin and NrCAM induces the clustering of Na^+ channels at the nodes of Ranvier. Other functions have not been well described.	
SERPINE1 (PAI-1)	20.8	3.04E-04	3.77E-03	serpin peptidase inhibitor, clade E member 1, (plasminogen activator inhibitor type 1)	Inhibitor of the uPA which is responsible for plasmin formation, matrix degradation and proteolytic activation of extracellular growth factors and MMPs. Induced in response to inflammatory stimuli. NFκB and MAPK regulated.	
CCL20 (MIP-3ϐ)	18.8	7.44E-04	4.95E-03	chemokine (C-C motif) ligand 20	Chemokine. Attracts mainly immature dendritic cells (iDCs) and some leukocytes. Binds to CCR6. Induced in response to inflammatory stimulus and hypoxia. Associated with tumor associated macrophages.	
RGS1	16.6	1.82E-04	3.24E-03	regulator of G-protein signaling 1	Inhibits signal transduction by interacting with alpha subunits of G-proteins. Negatively regulates cytokine and growth factor induced signal transduction through G-protein coupled membrane receptors.	

PTGS2 (Cox-2)	16.0	1.96E-06	1.29E-03	prostaglandin-endoperoxide syntheses 2 (cyclooxygenase -2)	Enzyme responsible for conversion of arachidonic acid (AA) to prostaglandin H2. Induced under inflammatory conditions. Dysregulated in many cancers, e.g., gastric carcinoma. Involved in pro-inflammatory response, evasion of apoptosis, immunosuppression, sustained proliferation, angiogenesis, invasion and metastasis.	(Greenhough *et al.*, 2009) (Chen and Smyth, 2011) (Tjiu *et al.*, 2008) (Nakanishi *et al.*, 2011; Obermajer *et al.*, 2012)
LPL	15.8	1.13E-04	3.00E-03	lipoprotein lipase	Triglyceride hydrolase that converts very low density lipoprotien (vLDL) to low density lipoprotein (LDL) in blood plasma. Ligand bridging factor for receptor-mediated lipoprotein uptake.	
CD83	14.1	2.63E-06	1.29E-03	CD83 molecule	Common surface protein on mature dendritic cells. Preformed intracellularly in monocytes and macrophages. Rapidly exported to the surface in response to inflammatory stimuli. Function not well characterized. Immune modulation.	
CHI3L1 (YK-L40)	14.0	7.59E-04	5.03E-03	chitinase 3-like 1 (cartilage glycoprotein-39)	Secreted glycoprotein. Member of the glycosyl hydrolase 18 family but has lost chitanase activity. Involved in macrophage maturation, immune response, tissue remodeling, angiogenesis.	(Shao *et al.*, 2009) (Mizoguchi, 2006)
SLC16A10	12.7	1.46E-03	6.22E-03	solute carrier family 16, member 10 (aromatic amino acid transporter)	Plasma membrane amino acid transporter. Na(+)-independent transport of aromatic amino acids.	(Mariotta *et al.*, 2012) (Ramadan *et al.*, 2007)
CHIT1	12.6	4.18E-04	4.19E-03	chitinase 1 (chitotriosidase)	Secreted glycoprotein. Member of the glycosyl hydrolase 18 family. Chitanase activity. Host defense against chitinous pathogens, also involved in inflammation, infection and matrix remodeling.	(Cozzarini *et al.*, 2009) (Kanneganti *et al.*, 2012)
GPX3	12.4	1.04E-04	2.95E-03	glutathione peroxidase 3 (plasma)	Secreted protein. Catalyses the reduction of peroxides. Protection from oxidative stress.	(Falck *et al.*, 2010) (Peng *et al.*, 2012)
CYGB	12.4	2.30E-05	2.01E-03	cytoglobin	Member of the globin family of oxygen binding proteins. Protection from hypoxia. Reaction to oxidative stress.	(Emara *et al.*, 2010)
AREG	11.2	1.50E-03	6.31E-03	amphiregulin	Growth factor of the endothelial growth factor (EGF) family. Control of inflammation. Tissue growth and remodeling.	(Zaiss *et al.*, 2013)
CCL4L1	11.1	6.80E-04	4.79E-03	chemokine (C-C motif) ligand 4-like 1	Chemokine. Paralogous gene to CCL4. Encoded protein differs in three amino acid positions. Function appears to be redundant, see CCL4.	(Modi *et al.*, 2001) (Pedrosa *et al.*, 2011) (Howard, O M Zack *et al.*, 2004)
AQP9	10.1	1.94E-04	3.31E-03	aquaporin 9	Membrane transport channel with broad substrate selectivity.	(Calamita *et al.*, 2012) (Jelen *et al.*, 2011)
IL1B	10.0	1.68E-03	6.51E-03	interleukin 1, beta	Cytokine. Important mediator of the inflammatory response. Produced as a pro-protein in activated macrophages, which must be proteolytically processed to its active form by caspase 1	

R4A3	9.9	3.02E-05	2.06E-03	nuclear receptor subfamily 4, group A, member 3	Nuclear receptor of orphan nuclear receptor subfamily 4A. NRs interact directly with DNA to influence gene transcription. NR4s are ligand-independently regulated. Regulators of gene expression and metabolism. Induced by inflammatory stimulus. May be regulated by NFκB. Acts to negatively regulate inflammation (conflicting results). Induces M2 macrophage polarization.	
C3	9.9	1.74E-04	3.21E-03	complement component 3	Component of the complement cascade. Innate immune response.	
DHCR24	9.2	9.90E-04	5.37E-03	24-dehydrocholesterol reductase	Oxidoreductase enzyme involved in cholesterol biosynthesis.	
MMP9	9.2	1.14E-04	3.00E-03	matrix metallopeptidase 9	Matrix metallopeptidase. Matrix remodeling. Cytokine activation through cleavage. Angiogenesis.	(Jadhav *et al.*, 2004) (Morancho *et al.*, 2013) (van Hinsbergh and Koolwijk, 2008) (Zajac *et al.*, 2013)
CXCL1 (Gro-α)	9.1	2.69E-04	3.65E-03	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Chemokine. member of CXCL family of chemokines. Contains the ELR motif. Binds CXCR2. Chemoattractant for neutrophils. Inflammatory response. Pro-angiogenic. Oncogene.	
CXCL2 (Gro-β)	9.0	1.26E-04	3.00E-03	chemokine (C-X-C motif) ligand 2	Chemokine. Member of CXCL family of chemokines. Contains the ELR motif. Binds CXCR2. Chemoattractant for neutrophils. Inflammatory response. Pro-angiogenesis.	
CCL3	9.0	3.47E-04	3.95E-03	chemokine (C-C motif) ligand 3	Cytokine. Binds receptors CCR1, CCR4, CCR5. Acute inflammatory response. Immunomodulation. Chemoattractant. Recruitment and activation of monocytes/macrophages, neutrophils, lymphocytes. Shares 68% amino acid similarity with CCL4.	
CCL4	9.0	6.39E-04	4.66E-03	chemokine (C-C motif) ligand 4	Chemokine. Binds receptors CCR5 and CCR1. Chemoattractant forimmune cells such as natural killer cells and monocytes. Immunomodulation and inflammation.	
KAL1 (anosmin-1)	8.8	2.00E-04	3.34E-03	Kallmann syndrome 1 sequence	Extracellular matrix-associated glycoprotein. Can form part of active β1 integrin complex inducing downstream signaling pathways. Has been shown to facilitate tumor cell proliferation, migration, invasion, and survival.	
SLC7A11	8.7	4.63E-04	4.29E-03	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	Transmembrane transporter of amino acids. Highly specific for cysteine and glutamate.	
ITGB3	8.6	3.99E-03	9.35E-03	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	Beta integrin subunit (heterodimeric transmembrane extracellular matrix receptor).	(Feng *et al.*, 2013) (Steri *et al.*, 2014)

IER3	8.5	3.88E-05	2.25E-03	immediate early response 3	Anti-apoptotic. Interference with signaling pathways e.g. NFκB/MAPK.	(Arlt and Schäfer, 2011)
LDLR	8.5	4.18E-04	4.19E-03	low density lipoprotein receptor	Extracellular receptor that recognizes apoprotein B100, which is embedded in the outer phospholipid layer of LDL particles. Mediates the endocytosis of cholesterol-rich low density lipoprotein.	
CRIM1	8.4	3.00E-06	1.30E-03	cysteine rich transmembrane BMP regulator 1 (chordin-like)	Transmembrane protein with growth factor receptor domains. Control of cellular development including tube formation in the capillary development of VEGF induced angiogenesis.	(Fan *et al.*, 2013) (Glienke *et al.*, 2002)
FN1	8.4	2.61E-04	3.60E-03	fibronectin 1	Extracellular matrix glycoprotein. Important in cellular adhesion, migration and differentiation and involved in processes such as angiogenesis	
DUSP1	8.3	1.23E-04	3.00E-03	dual specificity phosphatase 1	Non-receptor-type protein-tyrosine phosphatase that interacts with members of signaling cascades e.g. MAPKs in response to cellular stress or growth factors.	(Echavarria and Hussain, 2013) (Patterson *et al.*, 2009)
IRAK2	8.3	1.56E-04	3.10E-03	interleukin-1 receptor-associated kinase 2	Associates with IL-1 receptor upon cytokine binding and transmits the signaling cascade. Involved in IL-1 associated NFκB activation.	
SPARC (osteonectin)	7.8	3.59E-05	2.24E-03	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular matrix glycoprotein with high affinity for calcium and collagen. Cell extracellular matrix protein interaction and growth factor response. Increases the production and activity of matrix metalloproteinases and angiogenesis.	(Rivera *et al.*, 2011)
INSIG1	7.7	2.28E-03	7.31E-03	insulin induced gene 1	Endoplasmic reticulum membrane protein involved in cholesterol metabolism.	
TM7SF4 (DCSTAMP)	7.3	4.72E-04	4.29E-03	transmembrane 7 superfamily member 4	Transmembrane protein highly expressed in dendritic cells. Involved in multinucleated osteoclast formation, myeloid cell differentiation and immune regulation.	(Chiu *et al.*, 2012) (Eleveld-Trancikova *et al.*, 2007) (Sawatani *et al.*, 2008)
CTSK	6.9	1.78E-03	6.66E-03	cathepsin K	Cysteine protease with potent endoprotease activity against fibrinogen at acid pH. Involved in bone re-absorption and matrix remodeling.	
HBEGF	6.8	4.67E-06	1.33E-03	heparin-binding EGF-like growth factor	Epidermal growth factor family member. Produced predominantly by monocytes and macrophages. Chemotactic and mitogenic effects. Cancer and wound healing.	
GK	6.8	4.01E-04	4.19E-03	glycerol kinase	Key enzyme in the regulation of glycerol uptake and metabolism.	
RGS2	6.6	7.24E-04	4.86E-03	regulator of G-protein signaling 2, 24kDa	Negative regulator of G-protein mediated signaling pathways. Involved in hypertension and other cardiovascular conditions	
MAMLD1	6.4	3.21E-03	8.42E-03	mastermind-like domain containing 1	Transcriptional regulator. Co-activator of Notch signaling.	

NAMPT (PBEF1, vistafin)	6.3	4.21E-05	2.25E-03	nicotinamide phosphoribosyltransferase	Inflammatory cytokine. Rate limiting enzyme in nicotinamide adenine dinucleotide (NAD)+ biosynthesis. Involved in macrophage differentiation and arteriosclerosis.	(Yun *et al.*, 2013)
SIGLEC8	6.1	3.03E-04	3.77E-03	sialic acid binding Ig-like lectin 8	Transmembrane receptor. Expressed predominantly in eosinophils. Involved in apoptosis when activated.	(Bochner, 2009) (Crocker *et al.*, 2012) (Kano *et al.*, 2013)
CCL3L1	6.1	1.13E-03	5.65E-03	chemokine (C-C motif) ligand 3-like 1	Paralogous gene to CCL3. 96% nucleotide and protein similarity. Functions appear to be redundant except that the encoded CCL3 isoform is 2-fold more efficient at chemoattracting human lymphocytes (see CCL3).	
GNG12	5.9	4.60E-05	2.32E-03	guanine nucleotide binding protein (G protein), gamma 12	G-protein modulator and transducer of transmembrane signaling pathways. May be involved in negative regulation of LPS induced inflammation.	(Larson *et al.*, 2010)
HTRA1	5.9	4.23E-04	4.19E-03	HtrA serine peptidase 1	Secreted serine peptidase. Degrades extracellular matrix proteins and cleaves growth factors notably insulin-like growth factors (IGFs) and TGF-β. May regulate cellular growth and matrix deposition.	
CKS2	5.9	2.00E-04	3.34E-03	CDC28 protein kinase regulatory subunit 2	Regulator of cyclin dependent kinases. Regulator of cell cycle and division.	
FOSL2	5.9	2.87E-05	2.06E-03	FOS-like antigen 2	FOS family protein. Can dimerize with members of the JUN family proteins to form the leucin zipper transcription factor activator protien (AP)-1 which binds to genes with 2-*O*-tetradecanoylphorbol-13-acetate (TPA) DNA response elements and regulate various processes such as cellular differentiation, proliferation and apoptosis.	
OTUD1	5.8	6.90E-05	2.71E-03	OTU domain containing 1	Deubiquitinating enzyme. Unknown function	
TNFAIP6	5.8	2.29E-02	2.96E-02	tumor necrosis factor, alpha-induced protein 6	Protein secreted in response to inflammatory stimulus. Contains a hyaluronan-binding domain that is involved in cell migration, extracellular matrix stability and protease activity.	
SEP3	5.8	3.46E-04	3.95E-03	septin 3	Filament forming GTP-binding protein. Expressed in neurons and may be involved in synaptic vesicle function.	(Macedo *et al.*, 2013)
IFRD1 (PC4, Tis7)	5.8	5.63E-05	2.58E-03	interferon-related developmental regulator 1	Negative regulator of NFκB. Involved in cell differentiation development and muscle regeneration.	
GPR183 (EB12)	5.7	1.27E-04	3.00E-03	G protein-coupled receptor 183	G-protein coupled membrane receptor for oxysterols. Directs immune cell migration. Particularly well characterized for B cell maturation and splenic DC positioning. Also a negative regulator of type I interferon responses in plasmacytoid DCs and CD11b+ myeloid cells through trans-regulation of the interferon regulatory factor 7.	(Chiang *et al.*, 2013) (Hannedouche *et al.*, 2011) (Yi and Cyster, 2013)

ATP1B1	5.7	6.68E-04	4.74E-03	ATPase, Na+/K+ transporting, beta 1 polypeptide	Regulatory β subunit of an integral membrane ion pump, responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane. These gradients are essential for osmoregulation, for sodium-coupled transport of a variety of organic and inorganic molecules, and for electrical excitability of nerve and muscle. Also acts as a cell adhesion molecule and signal transducer by interacting with small GTPases and MAPK cascades.	(Tokhtaeva *et al.*, 2012)
FOS *(c-FOS)*	5.6	2.76E-05	2.06E-03	FBJ murine osteosarcoma viral oncogene homolog	Dimerizes with members of the JUN family proteins to form transcription factor activator protien-1 (AP-1) which binds to genes with TPA DNA response elements and regulate various processes such as cellular differentiation, proliferation and apoptosis.	
NR4A1 *(Nurr-77)*	5.5	8.27E-05	2.77E-03	nuclear receptor subfamily 4, group A, member 1	Nuclear receptor of orphan nuclear receptor subfamily 4A. Involved in negative regulation of inflammation and macrophage polarization. Mediates VEGF induced angiogenesis	(Hanna *et al.*, 2012) (Lefebvre *et al.*, 2012) (Zeng *et al.*, 2006)
THBD	5.4	1.39E-03	6.09E-03	thrombomodulin	Integral membrane protein. Acts as a co-factor to thrombin. Anti-coagulative, anti-fibrolytic and anti-inflammatory.	(Conway, 2012) (Martin *et al.*, 2013)
HTR2B	5.4	3.29E-03	8.52E-03	5-hydroxytryptamine (serotonin) receptor 2B	G-protein coupled receptor. Serotonin receptor. Signal transduction through extracellular-signal regulated kinase (ERK)1/2. Preferentially expressed on M2 macrophages and tumor associated macrophages. Outside of nervous system serotonin also acts as a growth factor and anti-inflammatory agent through modulation of cytokine release by myeloid cells.	(las Casas-Engel *et al.*, 2013)
---	5.3	7.24E-04	4.86E-03	---		
SIGLEC10	5.3	2.46E-02	3.13E-02	sialic acid binding Ig-like lectin 10	Sialic-acid-binding transmembrane protein. SIGLEC-10 has been shown to associate with CD24 and negatively regulate inflammatory cascades via NFκB in distinguishing danger associated molecular patterns from pathogen associated molecular patterns. Has also been shown to be the leucocyte co-receptor for vascular adhesion protien -1 and is involved in leukocyte adhesion to endothelial cells and the inflammatory response.	(Chen *et al.*, 2009) (Crocker *et al.*, 2012) (Kivi *et al.*, 2009)

EGR2	5.3	6.82E-05	2.71E-03	early growth response 2	Transcription factor. Immediate early response gene that is important for the induction of cellular programs of differentiation, proliferation, and cell death in response to environmental stimuli, control of inflammation and antigen receptor-induced proliferation in a cell-intrinsic manner. Control of inflammatory autoimmunity, regulates AP-1, suppressor of cytokine signaling (SOCS) and signal transducer and activator of transcription (STAT).	(Li *et al.*, 2012) (Liu *et al.*, 2003) (Sumitomo *et al.*, 2013)
DUSP4	5.3	1.72E-03	6.55E-03	dual specificity phosphatase 4	Regulators of MAPK signaling. DUSP4 preferentially dephosphorylates ERK1/2, p38, and stress-activated protein kinase / c-Jun N-terminale Kinasen proteins.	(Balko *et al.*, 2013) (Caunt and Keyse, 2013) (Echavarria and Hussain, 2013) (Kao *et al.*, 2013)
NAMPT	5.3	3.55E-06	1.30E-03	nicotinamide phosphoribosyltransferase	Catalyses the rate limiting step in the NAD biosynthesis pathway. Also behaves as a cytokine with immunomodulating properties in its secreted form.	
EREG	5.3	2.19E-02	2.88E-02	epiregulin	Member of the epidermal growth factor family. Ligand to EGF receptor and avian erythroblastosis oncogene B 2 receptors. Involved in angiogenesis and cytokine production.	
LGI2	5.2	7.22E-04	4.86E-03	leucine-rich repeat LGI family, member 2	Not well characterized. Involved in synaptic development.	(Kegel *et al.*, 2013)
SOD2	5.0	2.65E-06	1.29E-03	superoxide dismutase 2, mitochondrial	Iron/manganese superoxide dismutase. Binds to the superoxide by products of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Active in the mitochondria where it is the primary antioxidant for cellular respiration.	
CYP19A1 (aromatase)	4.9	4.09E-04	4.19E-03	cytochrome P450, family 19, subfamily A, polypeptide 1	Monooxygenase localized to the endoplasmic reticulum. Catalyses a key step in biosynthesis of estrogens.	
SDC2	4.9	1.38E-03	6.08E-03	syndecan 2	Transmembrane protein. Heparin sulfate proteoglycan. Regulates cell proliferation, cell migration and cell-matrix interactions via its receptor for extracellular matrix proteins. May interact with integrins. Expressed highly in fibroblast and endothelial cells, involved in angiogenesis.	(Noguer *et al.*, 2009)
PDPN	4.9	4.32E-03	9.77E-03	podoplanin	Mucin-type transmembrane glycoprotein. Involved in actin cytoskeleton organization, filopodia formation and cell motility. Highly expressed in cancer where it mediates invasion, and metastasis. Expressed in lymphatic endothelial cells.	(Krishnan *et al.*, 2013) (Mei *et al.*, 2013)

BCL6	4.9	5.01E-06	1.35E-03	B-cell CLL/lymphoma 6	Zinc finger transcription factor. Transcriptional repressor. Suppression of cytokine production in macrophages. Involved in the development of B and T cells and germinal centers.	(Wagner *et al.*, 2011)
PDE3A	4.8	5.11E-04	4.38E-03	phosphodiesterase 3A, cGMP-inhibited	Enzyme that hydrolyzes both cAMP and cyclic guanosine monophosphate. Regulates amplitude and duration of intracellular cyclic nucleotide signals. Important in cell cycle, platelet aggregation and vascular smooth muscle contraction.	
RG2	4.8	1.86E-03	6.76E-03	arginase, type II	Enzyme that catalyses the hydrolysis of arginine. Anti-inflammatory as it competes with iNOS for the production of nitric oxide. Type II isoform expressed in the mitochondria.	(Lewis *et al.*, 2010) (Matsuo *et al.*, 2009) (Lee *et al.*, 2013) (Elms *et al.*, 2013)
SQLE	4.8	9.68E-05	2.95E-03	squalene epoxidase	Rate limiting enzyme in sterol biosynthesis. Catalyzes the first oxygenation step.	
GABARAPL1	4.8	1.37E-05	1.81E-03	GABA(A) receptor-associated protein like 1	Interacts with tubulin for intracellular transport of proteins and vesicles. Involved in late stages of autophagosome formation.	(Le Grand *et al.*, 2011) (Seguin-Py *et al.*, 2012)
TGFB2	4.7	9.33E-04	5.37E-03	transforming growth factor, beta 2	Cytokine. Signals through combinations of transmembrane type I and type II receptors (TGFBR1 and TGFBR2) and SMAD, ERK, JUN, p38, phosphoinositide 3-kinase pathways. Involved in many cellular processes including, proliferation, differentiation, migration, apoptosis and angiogenesis. Promotes M2 macrophage differentiation.	(Gong *et al.*, 2012) (Zhou *et al.*, 2012) (Worthington *et al.*, 2012)
FBLN5	4.7	5.99E-04	4.52E-03	fibulin 5	Extracellular matrix protein containing an Arginyl- glycyl- aspartic acid (RGD) motif. Promotes adhesion of endothelial cells through interaction with integrins. Anti-angiogenic.	(Albig and Schiemann, 2004)
SASH1	4.7	5.62E-04	4.46E-03	SAM and SH3 domain containing 1	Expressed in microvascular endothelial cells. Positively regulates TLR4 signaling and downstream activation and NFκB and MAPKs.	(Dauphinee *et al.*, 2013)
HSPA1B (HSP70-2)	4.7	1.33E-04	3.01E-03	heat shock 70kDa protein 1B	Molecular chaperones that accompany protein folding and prevent misfolding under conditions of stress such as hypoxia, heat, and oxidative stress. Upregulated in cancer.	(Huang *et al.*, 2009b)
TNFAIP3 (A20)	4.7	2.13E-05	1.95E-03	tumor necrosis factor, alpha-induced protein 3	Ubiquitin-editing enzyme. Induced in response to inflammatory stimulus. Terminates NFκB activation. Anti-apoptotic. Regulates Wnt signalling, interferon regulatory factor and autophagy.	(Catrysse *et al.*, 2014) (Vereecke *et al.*, 2011)
SCIN	4.6	3.05E-02	3.72E-02	scinderin	Ca(2+)-dependent actin-severing and -capping protein. Plays a role in actin network dynamics in exocytosis.	(Trifaró *et al.*, 2000)

VNN1	4.6	8.66E-03	1.49E-02	vanin 1	Response to oxidative stress and regulation of inflammation. Glycosylphosphatidylinositol (GPI)-anchored pantetheinase ectoenzyme. Recycles pantothenic acid (vitamin B5) to produce cysteamine, a potent anti-oxidant.	(Kaskow *et al.*, 2012) (Pitari *et al.*, 2000)
TIPARP	4.5	6.66E-05	2.71E-03	TCDD-inducible poly(ADP-ribose) polymerase	Nuclear mono- adenosindiphosphat (ADP) -ribosyltransferase which exhibits auto- and hetero-ribosylation activities. Repressor of aryl hydrocarbon receptor transactivation.	(MacPherson *et al.*, 2013)
SULT1C2	4.5	2.12E-02	2.80E-02	sulfotransferase family, cytosolic, 1C, member 2	Catalyzes the sulfate conjugation to endogenous and xenobiotic substances. Drug metabolism.	
SPP1 (osteopontin)	4.5	1.94E-03	6.93E-03	secreted phosphoprotein 1	Extracellular matrix protein. Bone mineralization and remodeling, matrix remodeling, pro-inflammatory, cytokine signaling. Regulates cell activation, cytokine production and anti-apoptosis and chemotaxis in immune cells.	
FOSB	4.5	9.02E-04	5.37E-03	FBJ murine osteosarcoma viral oncogene homolog B	Dimerizes with members of the JUN family proteins to form transcription factor AP-1 which binds to genes with TPA DNA response elements and regulate various processes such as cellular differentiation, proliferation and apoptosis (see *FOS* and *FOSL1*).	
CSF1 (M-CSF)	4.5	8.81E-07	1.29E-03	colony stimulating factor 1 (macrophage)	Cytokine. Involved in the differentiation, proliferation, cytokine secretion in monocytes and macrophages. Promotes phagocyte activity, pro-inflammatory cytokine release and chemotactic activity.	
THBS1	4.5	2.10E-02	2.78E-02	thrombospondin 1	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Binds fibrinogen, fibronectin, laminin, type V collagen and integrins alpha-V/beta-1. Involved in platelet aggregation, angiogenesis, and tumorigenesis.	
LRRC39 (myomasp)	4.5	2.47E-03	7.52E-03	leucine rich repeat containing 39	Not well characterized. Enriched in cardiac tissue where it is a component of the sacromeric M-band and may be involved in stretch sensing. Binds to C-terminal tail domain of myosin heavy chain.	(Will *et al.*, 2010)
CDKN1A	4.5	7.92E-05	2.76E-03	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	Inhibits the activity of cyclin- cyclin-dependent kinase (CDK)2 or -CDK4 complexes. Regulates cell cycle, apoptosis, response to DNA damage. Mediates the p53-dependent cell cycle G1 phase arrest in stress response.	
MMP2	4.4	5.97E-03	1.18E-02	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	Degrades type IV collagen, matrix degradation tissue remodeling, directed matrix degradation in angiogenesis. Vascular "tunneling", activation of cytokines and growth factors through cleavage, interacts with membrane-associated, type-I-MMP bound to tip cell and macrophage membranes for activation.	(He *et al.*, 2012)

PSD3	4.4	3.78E-03	9.07E-03	pleckstrin and Sec7 domain containing 3	Guanine nucleotide exchange factor. Regulates signal transduction by activating ADP-ribosylation factor 6.	
CES1	4.4	3.97E-03	9.34E-03	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	Member of the α, β-hydrolase family. Hydrolysis or transesterification of foreign and endogenous substrates. Drug and lipid metabolism.	(Zhang *et al.*, 2012a)
PHLDA1	4.4	2.85E-04	3.71E-03	pleckstrin homology-like domain, family A, member 1	Regulation of apoptosis.	
FADS1	4.4	1.08E-03	5.56E-03	fatty acid denaturize 1	Lipid metabolism. Biosynthesis of highly unsaturated fatty acids. Catalyzes the desaturation of dihomo-gamma-linoleic acid (DHGLA) to generate AA. AA is the preferred substrate of cyclooxygenase-2 COX-2 in the production of PGE2.	(Fan *et al.*, 2012) (Wang and DuBois, 2010)
MMP7 (matrilysin)	4.4	4.44E-02	5.11E-02	matrix metallopeptidase 7 (matrilysin, uterine)	Extra cellular matrix degradation. Degrades a broad range of extracellular matrix substrates, such as type IV collagen, laminin, gelatin, fibronectin, and entactin 1. Also cleaves additional substrates, such as osteopontin and cell-associated Fas ligands. Promotes the release of TNF-α, mediates E-cadherin ectodomain shedding; and activates other proteinases, such as urokinase plasminogen activator and pro–MMP-1, -2 and -9. Involved in cancer invasion and metastasis, cell proliferation and apoptosis, inflammation. Regulated by Wnt/β-catenin signaling.	(He *et al.*, 2012)
PPP1R15A (GADD34)	4.4	2.25E-04	3.43E-03	protein phosphatase 1, regulatory (inhibitor) subunit 15A	Regulation of mRNA translation and cell growth in response to stress or DNA damage. Apoptosis.	(Zhou *et al.*, 2013)
ALOX5AP	4.3	1.17E-02	1.81E-02	arachidonate 5-lipoxygenase-activating protein	Non-heme iron dioxygenase required for leukotriene synthesis from AA. Leukotrienes are eicosanoid inflammatory mediators which regulate immune responses through lipid signaling.	(Wang and DuBois, 2010)
GEM	4.2	1.90E-05	1.93E-03	GTP binding protein over expressed in skeletal muscle	Member of the RAD/GEM family of GTP-binding proteins. Is associated with the inner face of the plasma membrane and is speculated to play a role as a regulatory protein in receptor-mediated signal transduction.	
TM4SF19	4.2	5.33E-03	1.10E-02	transmembrane 4 L six family member 19	Member of the transmembrane 4 superfamily. Cell surface glycoprotein. Mediates signal transduction events that play a role in the regulation of cell development, activation, growth motility and proliferation.	
GPC4	4.2	3.38E-02	4.04E-02	glypican 4	Cell surface heparan sulfate proteoglycan. Involved in Wnt-β-catenin signaling.	(Sakane *et al.*, 2012)

ADAMDEC1 (decysin 1)	4.1	2.76E-04	3.66E-03	ADAM-like, decysin 1	Secreted disitegrin metalloproteinase. Selectively expressed in mature dendritic cells and macrophages.	(Lund *et al.*, 2013)
TBC1D30	4.1	6.12E-04	4.53E-03	TBC1 domain family, member 30	Membrane protein. Function not well understood. Putative small Rab GTPase.	
SLC5A3	4.1	1.92E-03	6.89E-03	solute carrier family 5 (sodium/myo-inositol co-transporter), member 3	Transmembrane co-transporter that maintains concentrations of myo-inositol (MI; an osmolyte) in cells via co-transport with two Na+ molecules. MI derivatives include MI polyphosphates, phosphatidylinositols, and phosphoinositides. These are involved in cell physiology processes e.g. cell signaling, cell adhesion, vesicular trafficking.	(Dai *et al.*, 2011)
HIVEP1	4.1	5.63E-04	4.46E-03	human immunodeficiency virus type I enhancer binding protein 1	ZAS family zinc finger transcription factor. Not well characterized. Binds specifically to the NFκB motifs and related sequences.	(Wu, 2002)
MME (neprilysin, CD10)	4.1	2.67E-02	3.34E-02	membrane metallo-endopeptidase	Integral membrane metalloproteinase. Catalyses peptide hydrolysis at the extracellular face. Involved in processing signaling peptides such as neuropeptides and angiotensin-1.	
VEGFA	4.0	3.27E-03	8.49E-03	vascular endothelial growth factor A	Growth factor. Key growth factor in angiogenesis. Vascular permeability.	
TNFSF9	4.0	5.10E-04	4.38E-03	tumor necrosis factor (ligand) super family, member 9	Cytokine. Positive regulation of T-cell activation and survival.	(Wang *et al.*, 2009)
ZC3H12A	4.0	1.60E-04	3.10E-03	zinc finger CCCH-type containing 12A	Down regulation of inflammatory responses. Degradation of cytokine mRNAs e.g. IL-6, inhibition of LPS- and IL-1-induced NFκB signaling pathway. Responsible for CCL2 induced angiogenesis.	(Jura *et al.*, 2012) (Matsushita *et al.*, 2009) (Niu *et al.*, 2008) (Uehata and Akira, 2013)
MMP12	4.0	7.62E-05	2.76E-03	matrix metallopeptidase 12 (macrophage elastase)	Matrix metalloproteinase with elastase activity. Predominantly secreted by macrophages. Matrix remodeling, inflammatory cytokine deactivation. Anti-inflammatory.	(Dean *et al.*, 2008)
CLN8	4.0	3.00E-05	2.06E-03	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	Transmembrane protein predominantly located to the endoplasmic reticulum. Function not well characterized. May be involved in synthesis and transport of lipids, vesicular/membrane trafficking, autophagy/mitophagy and apoptosis.	(Passantino *et al.*, 2013)
DHCR7	4.0	5.53E-06	1.41E-03	7-dehydrocholesterol reducates	Lipid metabolism, membrane-bound enzyme that catalyzes the final step of cholesterol biosynthesis.	
WNT5A	4.0	9.32E-04	5.37E-03	wingless-type MMTV integration site family, member 5A	Member of the Wnt family of secreted signaling proteins. Ligand for frizzeled-5 receptor and tyrosine kinase orphan receptor 2. Stem cell differentiation and inflammatory response.	
SLC19A2	4.0	8.52E-05	2.77E-03	solute carrier family 19 (thiamine transporter), member 2	Transmembrane thiamine transporter.	

CHMP1B	4.0	7.66E-05	2.76E-03	chromatin modifying protein 1B	Component of endosomal sorting complex required for transport III. A complex responsible for membrane deformations involved in the formation of endocytic multivesicular bodies which are responsible for recycling of receptor proteins out of the endocytic pathway.	
MXD1	4.0	2.71E-03	7.79E-03	MAX dimerization protein 1	Member of the MYC/MAX/MAD network of transcription factors. Antagonizes MYC-mediated transcriptional activation of target genes which are responsible for cellular processes such as proliferation, differentiation and apoptosis.	
MCOLN3 (TRPML3)	4.0	1.51E-04	3.10E-03	mucolipin 3	Organelle transmembrane Ca2+ permeable cation channel, membrane trafficking and autophagy.	(Choi and Kim, 2014)
---	4.0	2.12E-03	7.17E-03	---		

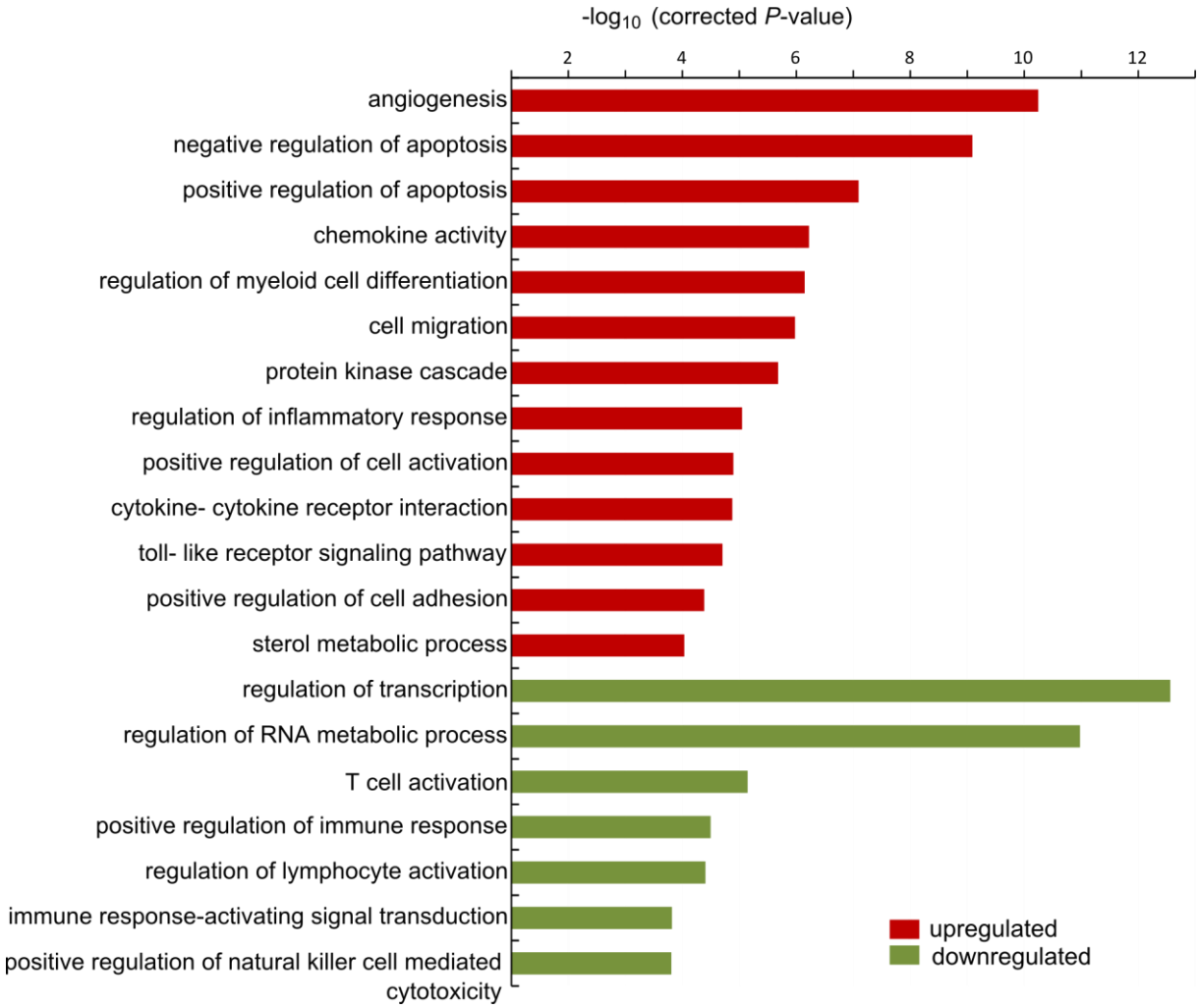


Figure 4.16 *B. henselae* infection of MACs leads to a broad phenotypic transformation characterized by upregulation of angiogenic and immune regulatory cellular programs. Gene expression profiles from uninfected MACs (d3) and *B. henselae* infected MACs (d53) were evaluated via gene microarray. Genes regulated with positive or negative fold change ≥ 2.0 (red and green respectively) and false discovery rate <0.05 were analyzed for enrichment of functional gene ontology annotations via the DAVID database analysis tool. Selected enriched functional categories are displayed (*P*-values represent a Bonferroni corrected EASE score).

Table 4.4 Selected functional annotation catagories enriched amoung genes upregulated (FC ≥ 2.0, FDR < 0.05) in MACs (d53) upon *B. henselae* infection.

Annotation Term	Fold Enrichment	*P*-Value	Upregulated Genes
angiogenesis	5.14	5.72E-10	*FLT1 , PLAU , JAG1 , THBS1, GNA13 , SHB, TNFRSF12A , JUN, NOTCH1, ZC3H12A , EPAS1 , B4GALT1, IL1B, IL8, CXCR4, MMP14, ARHGAP24, ANPEP, VEGFA, EREG, ENG, HIF1A, TGFB2*
negative regulation of apoptosis	3.17	8.16E-09	*RIPK2, HIPK2, IER3, IL1A, TNFAIP3, TGM2, BCL3, PIM2, CHST11, BCL6, CLN8, NR4A2, PDE3A, VNN1, DHCR24, CDKN1A, CEBPB, MCL1, SOD2, THBS1, APBB2, NFKB1, TNF, NOTCH1, UBC, PEA15, IL1B, HSPA1B, CLN8, HSPA1A, NFKBIA, PCSK6, VEGFA, PRLR, BCL2A1, SOCS3*
positive regulation of apoptosis	2.61	8.10E-07	*RIPK2, HIPK2, DDIT3, ARHGEF12, CADM1, JUN, DCUN1D3, FOXO3B , TGM2, BCL3, PMAIP1, DUSP1, BCL6, TNFRSF9, CDKN1A, MMP9, CEBPB, GAL, PTGS2, APBB2, TLR2, NOTCH1, TNF, UBC, B4GALT1, GCH1, IL1B, NUPR1, SCIN, P2RX7, PHLDA1, NR4A1, TNFRSF10B, TGFB2*
chemokine activity	7.39	6.01E-06	*IL8, CXCL2, CCL20, CCL18, CXCL3, CCL4, CCL3L1, CCL4L1, CCL3, CXCL1*
regulation of myeloid cell differentiation	5.66	7.15E-06	*RUNX1, SCIN, JAG1, CA2, NFKBIA, ZFP36, HIF1A, JUN, TNF, FOXO3B , CSF1, TOB2*
cell migration	2.87	1.06E-05	*FLT1 , PLAU , THBS1, APBB2, ICAM1, PDGFB, CXCL3, HBEGF, TNFRSF12A , TNF, B4GALT1, PPARD, IL1B, FN1, IL8, ---, CXCR4, NR4A2, MMP14, ARID5B, ENG, HIF1A, ITGA5, TGFB2*
protein kinase cascade	2.50	2.10E-05	*FLT1 , SPRED2, WNT5A, GNA13 , DUSP4, C5AR1, BCL3, OSM, TANK, RPS6KA2, THBS1, TLR2, NDST1, ZFP36, DUSP10, TNF, IL1B, MAP2K3, CXCR4, GNA12, P2RX7, NFKBIA, SPAG9, IRAK2, SIK1, PRLR, SOCS3, TNFRSF10B*
regulation of inflammatory response	4.78	8.99E-05	*SAA2, A2M, BCL6, PTGS2, C3, ZFP36, SERPING1, SERPINF1, CD276, TGM2, OSM*
positive regulation of cell activation	3.87	1.28E-04	*RIPK2, EBI3, THBS1, ICOSLG, PVRL2, CD276, CD83, IL1B, BCL6, VNN1, CDKN1A, CD80, PDPN*
cytokine-cytokine receptor interaction	2.33	1.34E-04	*FLT1 , CXCL2, CCL18, IL1A, TNFSF9, OSM, CXCL1, IL8, CCL20, CCL4, TNFRSF9, CCL3L1, CCL4L1, PDGFB, CXCL3, TNFRSF12A , TNF, CCL3, IL1B, CXCR4, VEGFA, CSF1, PRLR, TGFB2, TNFRSF10B*
toll-like receptor signaling pathway	3.39	1.98E-04	*TLR2, NFKB1, MAP3K8, TNF, JUN, CCL3, IL1B, MAP2K3, IL8, FOS, NFKBIA, CCL4, SPP1, CD80*
positive regulation of cell adhesion	4.96	4.14E-04	*THBS1, TNF, CSF1, TGM2, SPP1, IL1B, TGFB2*
sterol metabolic process	3.60	9.29E-04	*CLN8, CLN8, SC5DL, LDLR, FDFT1, DHCR7, SC4MOL, INSIG1, DHCR24, PPARD, SQLE, LSS*

Table 4.5 Selected functional annotation catagories enriched amoung genes downregulated (FC ≥ 2.0, FDR < 0.05) in MACs (d53) upon *B. henselae* infection.

Annotation Term	Fold Enrichment	*P*-Value	Downregulated Genes
T cell activation	3.59	7.17E-05	*FYN, CD2, FLT3, CD3E, GIMAP5, CD48, CD8A, GIMAP1, CD3D, LCK, RHOH, THEMIS, ZAP70, CD3G, IL7R*
regulation of transcription	1.70	2.75E-12	*IKZF3 , LBH, ZNF701, F2R, GABPA, STAT4 , ZNF382, ZNF551, ZNF507, GTF2E1, ZNF766, ZNF449, ZNF84, ZNF543, ZNF320, MDM4, ZNF564, ZNF43, ZNF808, ATF7IP2, ZNF671, ZEB1 , ZNF542, CASP8AP2, NCOA5, ZNF107, ZBTB3, ZNF765, ZNF577, ZNF304, SKAP1 , ZNF321, ZNF680, ZNF816A, PRKCQ, ZNF75A, ZNF619, ZNF101, 8180265, ZNF552, ZNF561, ZNF587, ZNF512, TFCP2, EZH1, RBL1, PPP1R3E, VPS25, ZNF429, ZNF548, ZNF93, ZNF75A, MYBL1, SUV420H1, ZNF253, ZNF28, TBP, RORA, ZNF525, ZNF776, MED6, TCF7, CHURC1, ZNF562, 8180200, STAT1, ZNF419, ZNF484, ZNF187, MSL3, ZNF426, ZNF595, SETDB1, GATA3, ZNF383, ING3, TCEANC, LEF1, ZNF845, ZNF90, MSL3L2, ZNF626, TRERF1, ZNF624, ZNF260, ZNF506, NRAS, ETS1, GTF2H2D, MED7, ZNF322A, ZNF302, MNDA, ZNF780A, ZNF772, FAM120B, ZNF66, ASF1A, ZNF518A, ZNF14, NME1, THAP1, ZNF559, ZNF235, ZNF226, MAP3K1, MTERF, ZNF813, ZNF594, ZNF234, MYC, ZNF600, ZNF486, ZNF347, ZNF780B, ZNF268, ZNF550, ZNF841, TAF7, JRKL, ZNF24, TIGD1, ZNF626, GTF2H2, ZNF721, ZFP14, ZNF518B, ZNF274, ZNF211, ZNF223, ZNF416, ZNF585B, ZNF616, CGGBP1, ZFP62, ZNF266, DMTF1, ZNF717, ZNF256, ZNF146, TRIM22, ZNF181, TADA2A, KLF12, ZNF154, RHOH, ZNF134, CDCA7L, ZNF583, ZNF763, ZNF83, ZFP3*
regulation of RNA metabolic process	1.83	1.06E-10	*IKZF3 , ZNF701, F2R, GABPA, STAT4 , ZNF382, ZNF551, ZNF766, ZNF449, ZNF543, ZNF84, ZNF320, MDM4, ZNF43, ZNF564, ZNF808, ZNF671, ZNF542, ZEB1 , ZNF765, ZNF577, SKAP1 , ZNF304, ZNF321, ZNF680, ZNF816A, ZNF619, ZNF75A, ZNF101, ZNF718, ZNF552, ZNF561, ZNF587, TFCP2, RBL1, PPP1R3E, ZNF429, ZNF548, ZNF93, MYBL1, ZNF75A, ZNF253, ZNF28, TBP, RORA, ZNF776, MED6, TCF7, ZNF562, ZNF493, ZNF419, STAT1, ZNF484, ZNF187, ZNF426, ZNF595, GATA3, ZNF383, TCEANC, LEF1, ZNF845, ZNF90, 8180217, TRERF1, ZNF624, ZNF506, ETS1, NRAS, 8057441, MED7, ZNF302, ZNF780A, ZNF772, ZNF66, ASF1A, ZNF14, THAP1, ZNF559, NME1, ZNF226, ZNF235, MAP3K1, ZNF813, MYC, ZNF234, ZNF486, ZNF347, ZNF780B, ZNF268, ZNF550, ZNF841, TAF7, ZNF24, ZNF626, ZFP14, ZNF274, ZNF223, ZNF211, ZNF416, ZNF585B, ZNF616, ZNF266, DMTF1, ZNF717 , ZNF256, ZNF146, TRIM22, ZNF181, ZNF154, KLF12, ZNF134, ZNF583, ZNF763, ZNF83*
T cell activation	3.59	7.17E-05	*FYN, CD2, FLT3, CD3E, GIMAP5, CD48, CD8A, GIMAP1, CD3D, LCK, RHOH, THEMIS, ZAP70, CD3G, IL7R*
positive regulation of immune response	3.12	3.21E-04	*FYN, KLRC4, CD3E, GIMAP5, CR1, GIMAP1, CD247, TNFSF13B, C5, SH2D1A , THEMIS, SKAP1, ZAP70, CD79A, CD226*
regulation of lymphocyte activation	3.05	3.96E-04	*CD2, KLRK1, ZEB1 , FLT3, CD3E, GIMAP5, CORO1A, GIMAP1, LCK, TNFSF13B, CD5, ZAP70, PRKCQ, CD27, IL7R*
immune response-activating signal transduction	4.63	0.001531	*FYN, KLRK1, CD3E, CD247, ZAP70, SKAP1 , THEMIS, CD79A*
positive regulation of natural killer cell mediated cytotoxicity	9.41	0.00157	*KLRK1, GIMAP5, SH2D1A , GIMAP1, CD226*

4.12 *B. henselae* infection of MACs induces a predominantly M2-alternativly activated macrophage phenotype

Depending on microenvironmental signals macrophages can take on activation profiles that range from classicaly activated M1-inflammatory to alternatively activated M2-anti-inflammatory phenotypes which have a strong influence on macrophage functional capacity and angiogenic activity.

To determine the activation profile induced in MACs by *B. henselae* infection, microarray data was used to examine absolute expression signals for typical M1 and M2 macrophage marker genes (Fig. 4.17). Typically, bacterial infections induce a highly polarized, highly inflammatory M1 macrophage activation state (Benoit *et al.*, 2008b). Contrary to this, the gene expression profiles of *B. henselae* infected MACs displayed a predominantly M2 activation phenotype. *B. henselae* infected MACs displayed particularly high expression of key M2 marker genes such as *MSR1, MRC1, CD163* and *TGFB1*. However, the M2 polarization of infected cells was not absolute. High expression of M1 inflammatory marker genes such as *IL-1B*, *NFκB*, *TNF* and *CCL3* was also observed.

4.13 The secretome of B. henselae infected MACs is dominated by angiogenic-inflammatory cytokines and matrix remodeling compounds

Macrophages are important accessory cells in many biological processes, secreting a diverse array of growth factors, cytokines and enzymes that transform local microenvironments and direct the actions of proximate cells (Wynn *et al.*, 2013). Results indicated that the pro-angiogenic effects of *B. henselae* infected MACs are paracrine in manner. To determine what compounds secreted by *B. henselae* infected MACs may be responsible for increased angiogenic activity, MAC-conditioned medium was analyzed via proteome profiler arrays at different time points day 5-24h p.i., from intermediate Matrigel cultures (day 25), and from fully formed Matrigel chord networks (d77; Fig. 4.18).

Infected MACs displayed a diverse cytokine secretion profile that was maintained over long term culture. Due to donor variation and the heterogeneity that developed in cell number and medium volume over long term culture, there were differences in the composition and intensity of the cytokine secretion profiles among samples. However, a consistent “core” of cytokines was found to be characteristic. In contrast, the cytokine signal from uninfected cells diminished over time and is almost completely absent at d77 reflecting the fact that no viable cells remained at this time point. The paracrine microenvironment created by *B. henselae* infected MACs was dominated by cytokines with inflammatory and angiogenic properties including CXCL1, CXCL8, CXCL16, CCL2 and macrophage migration inhibitory factor (MIF; Salcedo *et al.*, 2000; Strieter *et al.*, 2005; Bernhagen *et*

al., 2007). Angiogenin, a direct angiogenic activator and enhancer of angiogenic signals from other cytokines, appeared in the cytokine secretion profile late in culture (day 25; Kishimoto *et al.*, 2005).

Immune modulatory cytokines like IL-6, GM-CSF were detected along with CCL3, CCL4, CCL5, the anti-inflammatory cytokine interleukin-1 receptor antagonist (IL-1ra), soluble ICAM-1 and thrombospondin-1 (TSP-1). Anti-angiogenic CXCL4 and CXCL10 were also secreted in the early stages of infection (Strieter *et al.*, 2005).

Over time, many of the early inflammatory cytokines disappeared from the secretion profile and matrix remodeling elements became more prominent. *B. henselae*-infected MACs secreted MMP8, MMP9, urokinase-type plasminogen activator (uPA) as well as their respective inhibitors, tissue inhibitor of metalloproteinases-1 (TIMP-1) and plasminogen activator inhibitor -1 (PAI-1). The absence of the key inflammatory cytokines IL-1β, TNFα, interferon (INF)γ which were not secreted to detectable levels at any time point tested was also notable.

4.14 *B. henselae* induced cytokine secretion in MACs is distinct from general LPS mediated inflammation

To further characterize the activation profile of *B. henselae* infected MACs, the secretion of pro-inflammatory cytokine TNFα, angiogenesis-regulating chemokine CXCL10 and the anti-inflammatory cytokine IL-1ra by *B. henselae*-infected MACs was compared to MACs treated with 1µg/ml *E. coli* LPS. *B. henselae* infections resulted in significantly diminished TNFα and strongly increased CXCL10 and IL-1ra (Fig. 4.19) levels, demonstrating a *B. henselae* associated secretion profile distinct from a typical LPS-triggered inflammatory response.

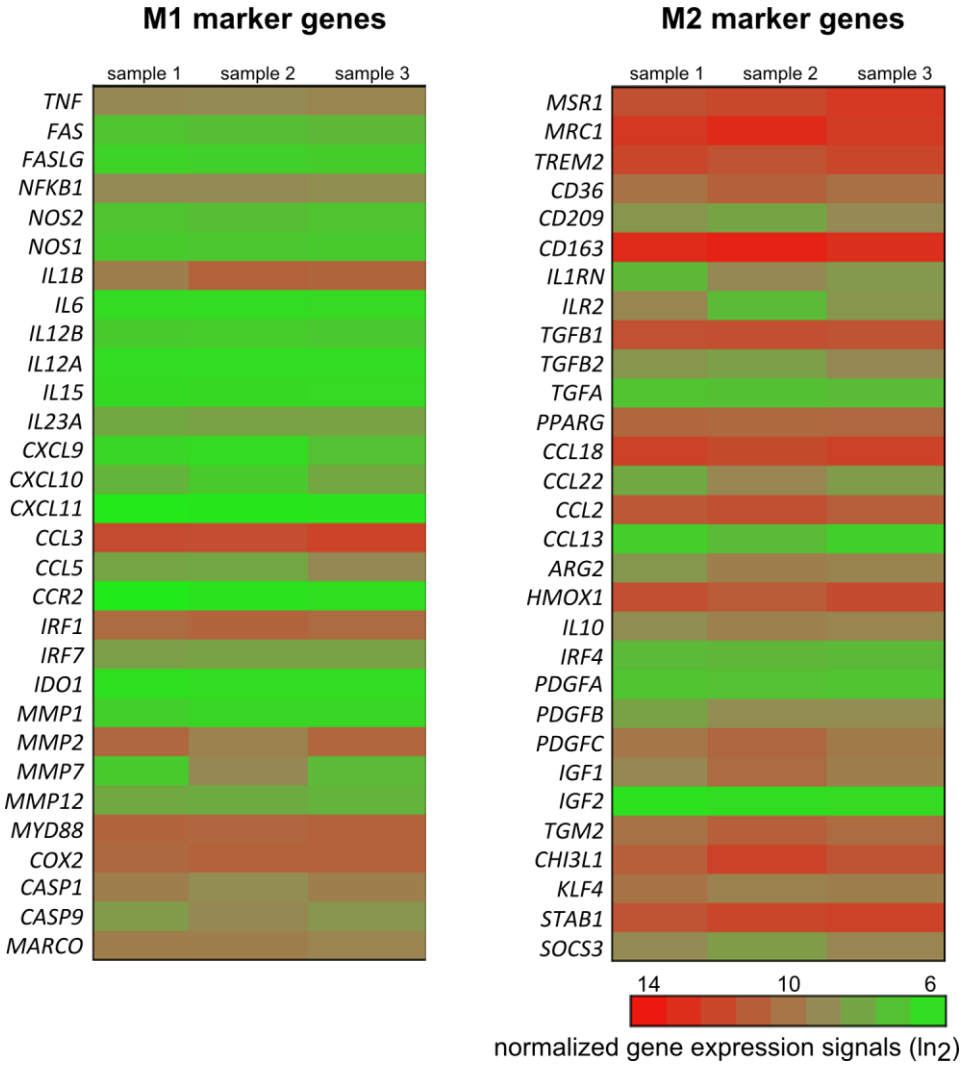


Figure 4.17 *B. henselae* infected MACs exhibit a predominantly M2 macrophage activation phenotype. Gene expression profiles of *B. henselae* infected MACs (d53) were evaluated via gene microarray. Absolute gene expression signals for typical human M1-classically activated and M2-alternatively activated macrophage marker genes were compared. Gene expression signals are displayed as ln2 logarithmic values. Heat map colour denominations range from red (high expression) to green (low expression) for individual genes. Results are displayed for three independent biological replicates.

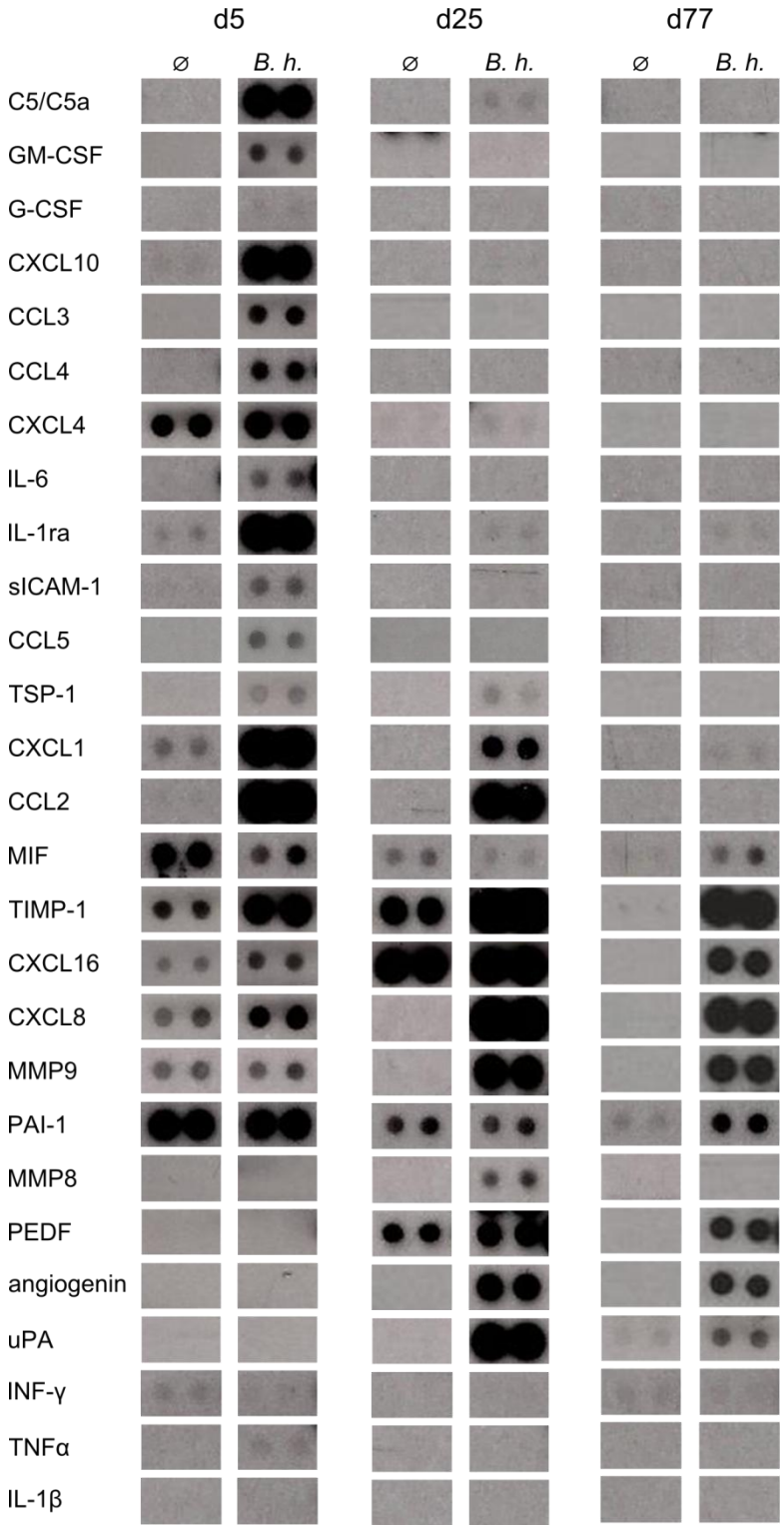


Figure 4.18 *B. henselae* infected MACs create a pro-angiogenic paracrine microenvironment over long term culture, dominated by angiogenic-inflammatory cytokines and matrix remodeling compounds. Conditioned medium was isolated from *B. henselae* infected (*B. h.*; MOI 200) and uninfected (ø) MACs d5-24h p.i. and from Matrigel cultures (d25, d77). The secretome was analyzed via human cytokine and angiogenesis proteome profiler arrays. Data show representative results for selected cytokines from three independent experiments.

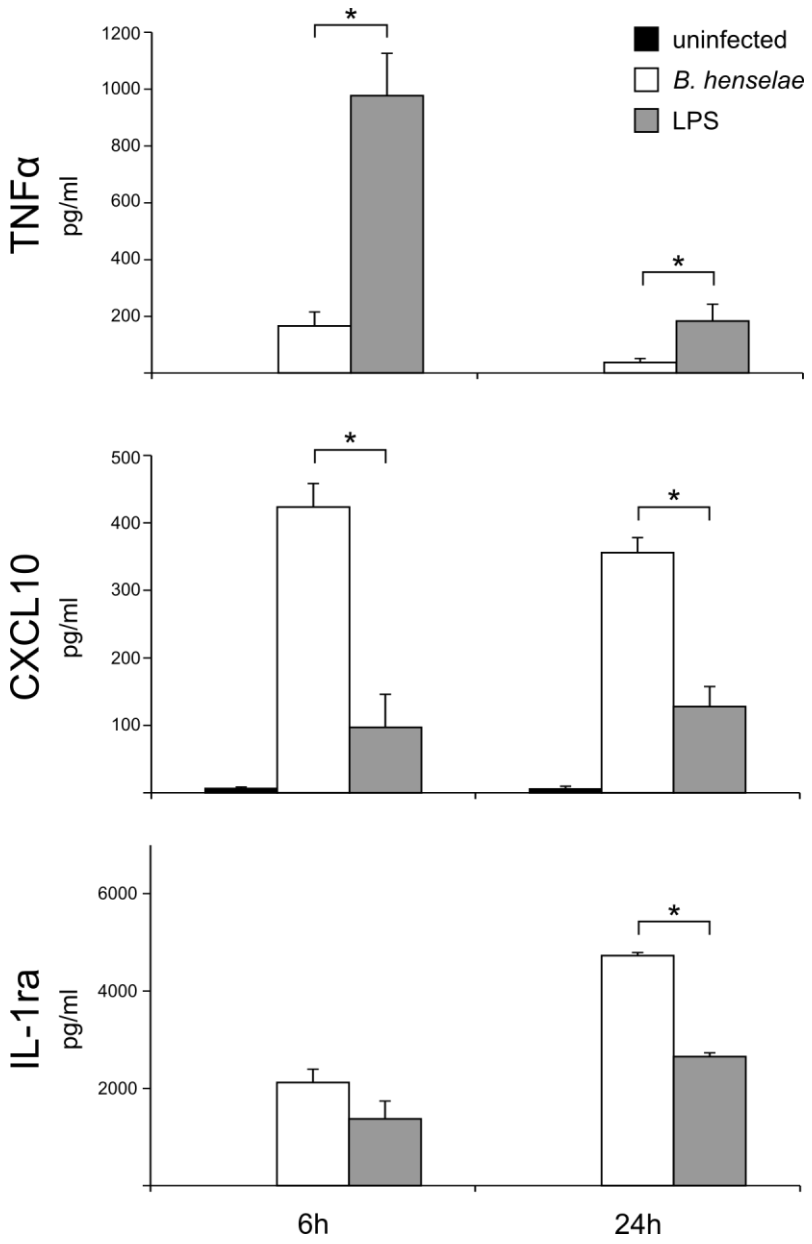


Figure 4.19 Inflammatory and anti-inflammatory cytokine secretion of MACs upon *B. henselae* infection or LPS treatment. MACs were infected with *B. henselae* (MOI 300) or treated with 1µg/ml *E. coli* LPS. Cytokine secretion was determined 6h and 24h p.i. via ELISA. Figure shows representative results (mean and standard deviation; asterisk denotes $p<0.01$).

5. Discussion

The ability to cause pathological angiogenesis in humans is a unique quality of the genus *Bartonella*. Angiogenesis plays a central role in a range of pathological and regenerative settings and the manipulation of this key biological process reflects a complex host pathogen interaction. In the case of *B. henselae*, the vascular architecture and cellular composition of the infection induced vascular lesions shows many similarities to malignant tumors suggesting that these two processes may share similar mechanisms (Koehler and Tappero, 1993). Further elucidation of the factors involved in *B. henselae* induced pathological angiogenesis and how these may relate to the formation of cancerous tumors may provide further insights into other pathological angiogenic conditions, cancer biology and bacterial pathogenicity.

Endothelial cells are suspected to be a key niche for chronic *B. henselae* infection and it has been suggested that the development of vascular tumors may represent a pathologically induced proliferation of the bacteria's own cellular habitat (Kempf *et al.*, 2002). Therefore, in the context of *B. henselae* associated pathological angiogenesis most research has focused on the interaction of *B. henselae* with endothelial cells directly.

In vivo angiogenesis however, is a multi-step process which can involve multiple cell types. In recent years there has been a growing interest in the contribution of progenitor cells to angiogenic growth. Myeloid angiogenic cells (MACs) are a heterogeneous group of circulating progenitor cells with key roles in regenerative and pathological angiogenic processes (Fadini *et al.*, 2012). MACs are known to accumulate in areas of angiogenic growth such as the tumor microenvironment and promote angiogenesis through the interaction with the endothelium and release of paracrine factors (Kalka *et al.*, 2000; La Puente *et al.*, 2013). Due to their key role as promoters of pathological angiogenesis, the interaction of *B. henselae* with MACs has the potential to significantly influence the development of *B. henselae* associated vascular tumors.

To gain further insight into what role progenitor cells play in the development of *B. henselae* associated pathological angiogenesis, this study aims to examine what effects the interaction of MACs with *B. henselae* has on the MAC phenotype and role in angiogenic processes.

5.1 Initial response of MACs to *B. henselae* infection

So far, *in vitro* cell culture studies have identified several characteristic aspects of *B. henselae* pathogenicity essential to the induction of pathological angiogenesis. Intracellular invasion, inhibition of host cell apoptosis and activation of angiogenic response programs are representative

features of *B. henselae* pathogenicity which lead to angiogenic re-programming of infected endothelial cells. To determine whether *B. henselae* infection of MACs results in similar cellular re-programming, the initial response of MACs to *B. henselae* infection was examined in detail.

Results from this study confirmed and expanded on preliminary findings of Mändle (2005) which establish a basic framework for the interaction of *B. henselae* with MACs. Initial investigations into the interaction between MACs and *B. henselae* by Mändle (2005) revealed that MACs are readily susceptible to *B. henselae* infection and that bacteria persist within cells for several days. In this study, scanning and transmission electron microscopy confirmed the adhesion of *B. henselae* and their invasion of MACs after 30min and 60min respectively. Transmission electron microscopy identified bacteria localized to intracellular vacuoles (Fig. 4.1).

B. henselae has been shown to inhibit apoptosis in several cell types including endothelial cells and MM6 monocytes/macrophages (Kempf *et al.*, 2005b; Schmid *et al.*, 2006). Mändle (2005) also found that *B. henselae* infection resulted in decreased caspase 3-like activity and inhibition of the apoptotic response in infected MACs. Results generated in the present study confirm the ability of *B. henselae* to prevent apoptosis in infected MACs. Infection of MACs with other pathogenic bacteria; *E. coli* and *S. aureus,* was shown to induce massive cell death within 24h as compared to the high viability of *B. henselae* infected cells (Fig. 4.2).

In examining the initial host-cell response of MACs to *B. henselae* Mändle (2005) found that infection with *B. henselae* resulted in the activation of angiogenic and inflammatory cellular activation programs similar to those associated with *B. henselae* infection of endothelial and epithelial cells. Activation of the key transcription factors regulating angiogenic and inflammatory responses; HIF-1 and NFκB were detected as well as the up regulation and release of HIF-1 regulated angiogenic cytokines VEGF, ADM and NFκB regulated cytokines CXCL8, CCL2 and ICAM-1.

In this study the activation of HIF-1 in *B. henselae* infected MACs was confirmed via Western Blot. HIF-1 activation is known to be a general phenomenon in infections (Werth *et al.*, 2010). Accordingly, infection of MACs with other pathogenic bacterial species *E. coli* and *S. aureus* was also found to result in HIF-1 activation (Fig. 4.3A). In contrast to *B. henselae,* these infections resulted in massive cell death (see above). Furthermore, whereas, the HIF-1 activation associated with *E. coli* and *S. aureus* may be associated with a general inflammatory response, *B. henselae* infection of MACs was found to be dependent on the expression of BadA (Fig. 4.3B).

Taken together, these results demonstrate that, as in endothelial cells, *B. henselae* infection of MACs results in an increase in angiogenic potential. Through the inhibition of apoptosis and activation of

basic angiogenic response programs, *B. henselae* infection establishes essential conditions for the induction of pathological angiogenic growth.

5.2 Functional effects of *B. henselae* infection on MAC pro-angiogenic activity

5.2.1 Spheroid assay of sprouting angiogenesis

As an intermediary between monolayer cell culture and *in vivo* assays, the endothelial spheroid assay of sprouting angiogenesis provides a reproducible and easily manipulated culture environment that more closely mimics the complexity of tissue *in vivo* (Pampaloni *et al.*, 2007). In the absence of suitable animal models, the spheroid assay is a reasonable setting to examine the effects of *B. henselae* infection on multiple molecular events and cellular interactions that make up *in vivo* angiogenic process.

In the spheroid assay cells form spherical aggregates and under angiogenic conditions develop radial sprouts which mimic the process of tip cell activation, tip-versus-stalk cell selection, matrix degradation, and chemotactic sprout extension. (Korff and Augustin, 1999). Importantly, the 3D spheroid culture environment provides realistic cell-cell and cell-matrix interactions which have strong effects on cellular phenotype and functional capacity (Fennema *et al.*, 2013).

The spheroid assay has previously been used to model *B. henselae* induced pathological angiogenesis in endothelial cells. Scheidegger *et. al.* (2009) demonstrated that infection of HUVECs with *B. henselae* prior to spheroid formation increased the rate of angiogenic sprouting significantly. Activities of the VirB/VirD T4SS translocated effector proteins were found to modulate the rate of infection associated angiogenic sprouting. BepA, which is strongly associated with *B. henselae* induced inhibition of apoptosis, was shown to increase angiogenic sprouting whereas BepG, which is associated with cytoskeletal rearrangement and invasome formation, was shown to decrease the rate of vascular sprouting.

To investigate how *B. henselae* infection may influence the interaction of MACs with the vascular endothelium, a co-culture spheroid model of HUVECs and MACs was developed for the first time.

The pro-angiogenic activities of MAC *in vivo* are dependent on their ability to home to sites of angiogenic activity and interact with the vascular endothelium. MACs are recruited to the site of neo-vascularization or repair in a multistep process involving maturation and release form the bone marrow, chemoattraction via cytokines (such as SDF-1 and CCL2), adhesion to the vascular wall (via

interaction with cellular adhesion molecules) and incorporation into the growing vessel or perivascular space (Urbich *et al.;* Shen *et al.*, 2011).

The ability of MACs to home to and integrate at the site of angiogenic growth would also be an essential prerequisite for their participation in the process of *B. henselae* induced pathological angiogenesis *in vivo*. To investigate the ability of *B. henselae* infected MACs to associate with growing endothelium, MACs and HUVECs were stained red and green respectively and their interaction observed in a co-culture spheroid assay.

When MACs and HUVECs were combined at a ratio of 1:3, MACs incorporated successfully into the endothelial spheroid. Both infected and uninfected MACs were found to associate with growing vascular sprouts. MACs were found localized to both, tip and stalk areas of sprouts, indicating that *B. henselae* infection does not affect their ability to integrate into and interact with the growing endothelium.

Some indications exist that *B. henselae* infection may in fact increase the affinity of host-cells to the endothelium. In endothelial and epithelial cells, *B. henselae* infection has been shown to increase the expression of cellular adhesion molecules E-selectin, ICAM-1 and myeloid chemokine CCL2 (Fuhrmann *et al.*, 2001; McCord *et al.*, 2005). Furthermore, Mändle (Mändle, 2005) demonstrated that the infection of MACs with *B. henselae* also increases their chemoatracttant migration along an SDF-1 cytokine gradient.

The retention of infected MACs to sites of angiogenic growth and their intimate interaction with the active endothelium is also important in facilitating the transfer of pro-angiogenic effects to adjacent endothelial cells and the surrounding microenvironment.

Initial experiments indicated that infection of MACs with *B. henselae* resulted in activation of endogenous angiogenic response programs, increasing their angiogenic potential. To determine whether *B. henselae* infection also resulted in increased functional angiogenic effects, the rate of angiogenic sprouting was compared between co-culture spheroids composed of HUVECs mixed with infected or uninfected MACs. Indeed, in spheroids seeded with *B. henselae* infected MACs, the rate of endothelial sprouting increased significantly. Infected MACs induced an average of up to 1.61 fold more angiogenic sprouting in endothelial spheroids than uninfected cells indicating that the infection of MACs with *B. henselae* increases their pro-angiogenic activity and has significant functional effects on angiogenic growth of adjacent endothelium (Fig. 4.5A).

In previous studies the infection of THP-1 macrophages, HeLas and EA hy. 926 cells with *B. henselae* was found to induce the secretion of vasculoproliferative cytokines such as VEGF and CXCL8 and

conditioned medium from *B. henselae* infected THP-1 macrophages increased endothelial cell proliferation *in vitro* (Kempf *et al.*, 2001; Resto-Ruiz *et al.*, 2002; McCord *et al.*, 2006). This has lead to the hypothesis that the infection of accessory cells such as myeloid and epithelial cells with *B. henselae in vivo* may contribute to pathological angiogenesis via the creation of a pro-angiogenic paracrine loop in which the release of pro-angiogenic cytokines stimulates proliferation of adjacent endothelium, attracts further myeloid cells and activates resident epithelial cells thus promoteing the progression of the *B. henselae* induced vascular tumor (Kempf *et al.*, 2001; Kempf *et al.*, 2002; Resto-Ruiz *et al.*, 2002).

A similar phenomenon is observed in malignant tumors where infiltrating myeloid cells (most prominently TAMs) are induced to secrete pro-angiogenic and immune regulatory cytokines, creating a pro-angiogenic tumor-microenvironment which stimulates the adjacent endothelium and contributes to tumor vascularization and invasion (Schmid and Varner, 2010).

To determine whether, the increased pro-angiogenic activity in *B. henselae* infected MACs was due to paracrine effects or physical interaction, further spheroid experiments were performed, placing infected MACs at varying degrees of separation from the endothelial spheroids. Spheroids were created purely from HUVECs and suspended in collagen gel. *B. henselae* infected MACs were either mixed with (Fig. 4.5B) or seeded onto the surface of collagen gel (Fig. 4.5C). In both conditions, the rate of sprouting angiogenesis increased dose-dependently when MACs were infected with *B. henselae,* indicating that no physical contact was necessary to transmit the pro-angiogenic effects of infected MACs.

Finally, conditioned medium from *B. henselae* infected MACs was used to culture HUVEC spheroids (Fig 4.5D). In this case, conditioned medium from *B. henselae* infected MACs showed a dose dependent effect and induced up to 6.71 fold more average sprouting than conditioned medium from uninfected MACs. These experiments indicate that paracrine effects are predominantly responsible for the pro-angiogenic activity of *B. henselae* infected MACs.

Overall, these results demonstrate that the infection of MACs with *B. henselae* not only results in the activation of angiogenic programs on a molecular level but increases their functional pro-angiogenic activity. Infection of cells did not inhibit their ability to home to and incorporate in growing vessels and had significant angiogenic effects on the surrounding endothelium.

In addition to demonstrating functional effects of *B. henselae* infection, results of the spheroid assay provides important clues about how *B. henselae* infection may affect the behavior of MACs *in vivo*. The co-culture spheroid assay reproduces the interaction of MACs with a 3D sprouting endothelium

in vito and provides a realistic representation of the role of MACs as accessory cells in the process of angiogenic growth.

When exposed to angiogenic endothelium, *B. henselae* infected MACs incorporate into growing vascular sprouts and significantly increase angiogenic growth of the adjacent endothelium via predominantly paracrine factors. In the context of *B. henselae* associated vascular tumor formation, the interaction of infected MACs with the activated endothelium and parallel contribution to angiogenic growth through the release of paracrine factors would make a significant contribution to pathological angiogenesis and vascular tumor progression.

5.2.2 Matrigel capillary formation assay

To further elucidate the effects of *B. henselae* infection on MAC pro-angiogenic activity a second *in vitro* assay of angiogenic growth, the Matrigel capillary formation assay, was employed.

In the Matrigel assay, cells are seeded onto a 3D proteinacious matrix composed of mostly laminin and collagen IV. Mature endothelial cells such as HUVECs adhere to the matrix and over the course of 2-4h arrange into evenly spaced cell cluster connected by a web of “tubules” consisting of elongated endothelial cell branches 2-3 cells in length (Khoo *et al.*, 2011).

In previous studies the infection of cultured endothelial cells with *B. henselae* has been shown to increases capillary structure formation in the Matrigel assay (McCord *et al.*, 2006; Berrich *et al.*, 2011).

In contrast to endothelial cells, MACs are not known to form spontaneous “capillary networks” in the Matrigel assay (Hur *et al.*, 2004; Yoder, 2013a). Accordingly, when uninfected MACs were seeded in Matrigel culture they displayed no morphological differentiation, maintained spherical phenotypes, died and degraded over time (Fig. 4.6). *B. henselae* infected MACs, on the other hand, adhered strongly to the Matrigel matrix, developed long branching philopodia and gradually assembled into a complex network of chord structures that at times extend over the surface of the entire well plate (Fig. 4.6 and 4.7).

Although they display a similar basic composition and morphology to endothelial capillary networks, the chord networks developed by infected MACs in Matrigel culture differ from endothelial structures in two major ways.

In contrast to endothelial capillary networks, which consist of a dense web of thin chord-like connections 2-3 cells long, the chords networks formed by the *B. henselae* infected MACs were thicker, usually consisting of many directionally elongated and aligned cells. The intersecting chords

of heterogeneous length formed a sparser network and left larger interim areas mostly void of other cells.

In addition to differences in morphology, the capillary structures formed by *B. henselae* infected MACs differed from endothelial cells in the rate of structure assembly. While endothelial cell networks form in Matrigel cultures within 2-12h and degrade again shortly after, the capillary-like networks formed by the *B. henselae* infected MACs assembled gradually over the course of several weeks and were viable over long periods of time (41-77days). It is unclear why *B. henselae* infected MACs require such lengthy incubation times to fully develop the capillary-like network structures, however, it is likely that chord formation by *B. henselae* infected MACs is a more complex process that requires different cellular events. *B. henselae* infected MACs may first have to undergo some differentiation process, cellular activation or angiogenic re-programming before functional effects such as complex structure formation become apparent. The extended development time and long-term stability of the capillary structures formed by *B. henselae* infected MACs also highlights the anti-apoptotic effect of *B. henselae* infection. Uninfected MACs generally died out and degraded in culture after approximately 25 days (a typical lifespan of untreated primary human myeloid cells in culture) wheras *B. henselae* infected cells in the capillary-like structures were shown to be still viable at the end point of Matrigel cultures (d51) (Fig. 4.6 and Fig. 4.8).

Despite the difference in capillary-like structure formation between *B. henselae* infected MACs and endothelial cells, the ability of infected MACs to self-organize into such complex structures requires coordination of complex inter- and intracellular signaling networks, cellular migration, changes in morphology, and possibly remodeling of the extracellular matrix as well as the ability to resist apoptosis over long culture periods. This activity further underscores previous results indicating a significant angiogenic re-programming in *B. henselae* MACs leading to increased functional pro-angiogenic activity in infected cells.

5.3 Differentiation phenotype of *B. henselae* infected MACs

MACs, previously known as endothelial progenitor cells (EPCs), were originally identified as circulating progenitors that participated in new vascular growth though differentiation into mature endothelial cells and formation of new vasculature (Asahara, 1997; Shi *et al.*, 1998). Many studies report endothelial phenotypic characteristics in MACs cultured *in vitro* and several *in vivo* studies describe the vascular incorporation and endothelial differentiation of MACs as the predominant mechanism of their pro-angiogenic effects (Lyden *et al.*, 2001; Aicher *et al.*, 2003).

This paradigm is conflicted by a growing body of evidence which indicates that circulating bone marrow derived angiogenic progenitor cells are of myeloid origin and contribute to angiogenic growth as accessory cells, either through paracrine effects, or by providing other structural and physiological support to growing vessels (Rehman *et al.*, 2003; Kinnaird *et al.*, 2004; O'Neill *et al.*, 2005; Medina *et al.*, 2010a).

Several of the pro-angiogenic qualities observed in *B. henselae* infected MACs could suggest that angiogenic re-programming induced by *B. henselae* infection may culminate in endothelial differentiation. Infection resulted in activation of angiogenic molecular programs including activation of HIF-1 and secretion of the pro-angiogenic cytokine VEGF. Infected cells incorporate into growing vascular sprouts and increase the rate of sprouting angiogenesis. Most strikingly, infected MACs were found to assemble into capillary-like chord networks similar to those formed by endothelial cells when cultured in a Matrigel capillary assay.

To investigate the differentiation phenotype of *B. henselae* infected MACs, several tests for endothelial gene expression and antigen expression were performed.

Results revealed that *B. henselae* does not induce upregulation of endothelial marker genes in infected MACs. (Fig. 4.12). Gene expression profiles from both infected and uninfected MACs showed little similarity to endothelial control samples (Fig. 4.14) and immunohistochemistry preformed on MACs derived from capillary-like structures proved negative for typical endothelial marker proteins (Fig. 4.11).

Instead, comparison of gene expression profiles from *B. henselae* infected MACs with samples of known cell differentiation revealed the most similarity with macrophages and other cells of the myeloid linage (Table 4.1).

Finally, a FACS analysis for various differentiation markers was performed on infected and uninfected MACs at different time points (d5, d62). Neither infected nor uninfected MACs displayed endothelial characteristics at any time-point tested. In contrast, markers for myeloid cells were displayed on both infected and uninfected cells. In particular, macrophage-specific differentiation markers were strongly expressed on all MACs and displayed upregulation after infection with *B. henselae*. This macrophage phenotype was maintained over long term culture and in the cells of the capillary-like structures (Fig. 4.10).

These results indicate that endothelial differentiation does not play a role in the development of the pro-angiogenic phenotype in infected MACs and confirms findings by other investigators demonstrating that MACs represent cells of a myeloid phenotype. Specifically, it can be concluded

that the pro-angiogenic cell phenotype induced in MACs by *B. henselae* infection and present in the capillary-like structures, is of macrophage differentiation.

Although macrophages cannot themselves form true vasculature, this cell type is well equipped to have a significant impact on the process of pathological angiogenesis. As accessory cells, macrophages are attracted by hypoxia, angiogenic and inflammatory signals to the site of angiogenesis and participate in a paracrine manner through the release of pro-angiogenic cytokines and growth factors into the cellular microenvironment (Chambers *et al.*, 2013). In addition to their paracrine effects macrophages are also known to participate structurally in the angiogenic process for example through matrix degradation and remodeling, aiding the process of tip cell fusion, capillary pruning in vascular patterning and facilitating vascular repair by adhering to the vessel wall at sites of vascular injury (Moldovan *et al.*, 2000; Fujiyama *et al.*, 2003; Fantin *et al.*, 2010; Stefater *et al.*, 2013).

5.4 Vascular mimicry

Among the endothelial-like behaviors of *B. henselae* infected MACs is the ability to form capillary-like chord networks over long term culture. Investigation into the differentiation phenotype expressed by cells of the capillary-like structures (discussed above) clearly demonstrates them to be of macrophage differentiation. This indicates that the phenomenon of capillary structure formation observed in *B. henselae* infected MACs reflects a vascular mimicry behavior rather than true capillary formation activity.

The formation of cellular networks and chord structures in Matrigel culture is not exclusive to endothelial cells. Fibroblasts and several cancer cell lines (breast cancer, prostate cancer and glioblastoma) have been described to form web-like networks when cultured on Matrigel (Donovan *et al.*, 2001).

Macrophages are known to associate closely with endothelial structures *in vivo* and play diverse roles in angiogenic processes (Baer *et al.*, 2013). Several examples of vascular mimicry behavior have been described in macrophages *in vivo* in conjunction with situations of intense angiogenic stimulus.

In some cases macrophages have been reported to participate in the formation of a provisory vascular barrier after vascular injury. Glod *et al.* (2006) found that circulating populations of $CD14^+$ monocytes associated with repairing vasculatureafter injury of the skin and brain, forming a vascular barrier and participating in vessel repair. $CD14^+$ peripheral blood monocytes were also found by

Fujiyama *et al.* (2003) to adhere to injured endothelium in a mouse carotid balloon injury model and inhibit the development of neointimal hyperplasia.

In several *in vitro* and *in vivo* studies a macrophage "tunneling" behavior has been described in which stimulated macrophages create tubes or chords via directed proteolytic degradation of the extracellular matrix (Anghelina *et al.*, 2002; Anghelina *et al.*, 2004; Anghelina *et al.*, 2006b; Anghelina *et al.*, 2006a). In an *in vivo* transgenic model of ischemic cardiomyopathy (Moldovan *et al.*, 2000) macrophages were observed to proteolytically degrade metalloproteinase-lined tunnels throughout the myocardial tissue. Macrophages are known to play a crucial role in the matrix degradation and remodeling that takes place during vessel sprouting (Pepper, 2001; Arroyo and Iruela-Arispe, 2010; Nucera *et al.*, 2011). Such macrophage "tunneling" behavior is believed to reflect this directed matrix remodeling processes which facilitate the invasion and extension of vascular sprouts into the surrounding tissue.

Vascular mimicry behavior is also a common feature of aggressive tumor phenotypes and has been observed in many cancers *in vivo.* Tumor cells assemble into vascular structures that are in some cases perfused and may even establish connections to endothelial vessels. This additional vascularization of tumor tissue contributes to cancer growth, progression and can even compensate for normal angiogenic growth in response to anti-angiogenic chemotherapy (Döme *et al.*, 2007). In cancerous tumors the development of vascular mimicry appears to be connected to the de-differentiation process that occurs in tumor cells when genetic deterioration leads to the breakdown of regulatory checkpoints and the aberrant activation of embryonic signaling pathways. Dysregulation of angiogenic pathways ,in particular the activation of VE-Cadherin, VEGFR-1, COX-2, metalloproteinases and hypoxic signaling via HIF transcription factors have also been shown to be important activating factors (Paulis *et al.*, 2010).

Recently, macrophages in some aggressive tumors have also been reported to participate in vascular mimicry. Macrophages from patients with aggressive multiple myeloma were found to participate in mosaic vessel formation within the bone marrow *in vivo*. Macrophages isolated from these patients form vascular mimicry structures and develop endothelial characteristics when stimulated with angiogenic growth factors *in vitro* whereas macrophages from patients with less aggressive melanoma and from control patients did not exhibit these qualities (Scavelli *et al.*, 2007). In anaplastic thyroid carcinoma, tumor associated macrophages have also been found to form a branching network radiating from endothelial vessels and may play a role in transporting nutrients to these poorly vascularized tumors (Caillou *et al.*, 2011).

It is unclear how exactly the *B. henselae* induced vascular mimicry phenotype in the Matrigel assay might translate to cellular behavior *in vivo.*

The capillary-like structure formation may reflect one of several structure building behaviors observed in pro-angiogenic macrophages, all of which would have functional angiogenic effects on the growth of *B. henselae* induced vascular tumors.

Vascular mimicry behaviors in malignant tumors can dramatically affect tumor growth and progression (Liang, 2013). The ability of *B. henselae* infected MACs to participate in the formation of such functional vascular structures would have meaningful implications for *B. henselae* induced pathological angiogenesis. Similarly, the coordinated "tunneling" degradation of extracellular matrix or the lining of growing vessels to provide physical support would also be advantageous for the invasion of new vessels and progression of the *B. henselae* associated vascular tumors *in vivo*.

In general, the vascular mimicry phenotype displayed by *B. henselae* infected MACs indicates a considerable phenotypic re-programming and angiogenic activity in infected cells. Cells demonstrate dramatic morphological and structural changes. The formation of complex cellular networks in the extracellular matrix also indicates an activated cell phenotype, as well as sophisticated proteolytic and cellular communication systems which would be significant to the role of MACs as accessory cells in the induction of pathological angiogenesis.

5.5 Phenotypic re-programming in *B. henselae* infected MACs

The myeloid cell linage represents one of the most functionally and phenotypically diverse cell types within the body. Myeloid cell phenotypic plasticity and ability to react rapidly to local microenvironmental conditions make them influential players in numerous biological processes (Pollard, 2009; Wynn *et al.*, 2013).

In this study it has been demonstrated that *B. henselae* infection leads to significant changes in MAC functional angiogenic capacity including the activation of angiogenic response programs, the ability to induce significantly increased angiogenic growth and the formation of vascular mimicry structures.

In order to examine further the phenotypic changes underlying these functional angiogenic effects, microarray transcriptional profiling was performed to compare the gene expression profiles of uninfected MACs (d3) and infected MACs (d53).

Infection with *B. henselae* was found to result in the regulation of 1400 genes in excess of 2.0 fold. This represents 36% of gene transcripts evaluated with 620 genes being upregulated and 780 down regulated in response to infection. Hierarchical clustering of evaluated samples revealed a high level of variance between sample groups (Fig. 4.13).The variance in gene expression induced in MACs in response to *B. henselae* infection was found to be considerably higher than changes induced in the course of a standard monocyte-to-macrophage differentiation process (Table 4.2). These results describe extensive changes in overall gene expression associated with *B. henselae* infection and make evident that the functional angiogenic effects observed in infected MACs are accompanied by a broad-scale phenotypic re-programming in infected cells.

Further analysis of individual genes via functional annotation and statistical analysis (DAVID analysis) revealed that changes in gene expression induced by *B. henselae* infection are associated with a broad range of cellular processes (Table 4.3-4.5, Fig. 15, S Table 9.1-9.4). *B. henselae* infection of MACs was found to alter gene expression patterns in four major areas of cellular function: angiogenesis, structural organization, apoptosis, sterol-metabolism and immune response.

5.5.1 Angiogenesis

Corresponding to the increase in angiogenic activity in *B. henselae* infected MACs, functional annotations relating directly to angiogenesis and vascular growth were found to be the most highly enriched among upregulated gene transcripts.

The gene *HIF1A* (encoding the HIF-1α protein subunit) was found to be highly upregulated in infected cells confirming that this angiogenic transcription factor is upregulated on both transcriptional and post-transcriptional levels in response to *B. henselae* infection.

Genes encoding angiogenic chemokines (*CCL2, CXCL8, CXCL1, CXCL2, CC3L1/CCL3, CSF*) and angiogenic growth factors (*VEGFA, TGFB*) were also particularly highly upregulated. Enriched functional annotations were also found in categories related to angiogenic growth including inter- and intracellular signaling (cytokine production, receptor signaling, intracellular signaling cascades, MAPK pathways and transcriptional regulation).

5.5.2 Structural organization

Highly enriched functional categories also included annotations involved in structural organization such as those for cell migration and adhesion. Many of the highly upregulated genes also had functions related to interaction with the extracellular matrix such as matrix degrading metalloproteinases (*MMP9, MMP2, MMP7, MMP12, ADAMDEC1, MME)*, and other compounds

involved in matrix remodeling (*SERPINE1, CHI3L1, CHIT1, CTSK, HTRA1, TNFAIP6)*. Furthermore, genes for several extracellular matrix components such as *FN1, SPARC, FBLN5, SPP1, THBS1* as well as several integrins (*ITGA1, ITGB5, ITGB3)*, were highly upregulated.

The upregulation of genetic programs involved in structural organization, cellular adhesion and matrix interaction may be important to structurally coordinate the formation of capillary-like networks and the interaction with vascular sprouts observed in Matrigel capillary formation and spheroid assays respectively.

These genetic activation patterns also indicate a pro-angiogenic re-programming in infected MACs as matrix remodeling represents an important function of macrophages in regulating angiogenic growth. *In vivo*, macrophage-mediated directional degradation of the extracellular matrix enables vascular sprouting into surrounding tissue. Deposition of matrix scaffolds supports vascular expansion and proteolytic processing of extracellular matrix proteins activates matrix bound pro-angiogenic growth factors (Pepper *et al.*, 1996; Arroyo and Iruela-Arispe, 2010).

5.5.3 Apoptosis

Following angiogenesis, functional annotations for negative, and to a lesser extent, positive regulation of apoptosis were most highly enriched among upregulated genes.

This reflects results generated in this study and by Mändle (2005) which demonstrated that *B. henselae* infection inhibits the apoptotic response in infected MACs (Fig. 4.2).

Although the mechanism by which *B. henselae* inhibits apoptosis is not yet clear, other authors have found this effect to be associated with upregulation of anti-apoptotic proteins, BCL2, BAX, BRIC-2 and -3 (cIAP-1,-2) as well as inhibition of pro-caspase preprocessing and -activity of the apoptotic executioner caspase-3 (Kempf *et al.*, 2005b; McCord *et al.*, 2006). Results of the transcriptome analysis did not show a significant increase in mRNA transcription for these anti-apoptotic proteins. However, BCL protein family members *BCL2A1*, *BCL3* and *BCL6*, also known to be important anti-apoptotic proteins, were highly upregulated. Furthermore, genes encoding caspase-3 and caspase 6 proteins (*CASP3, CASP6*) were downregulated indicating that caspase activity may be downregulated both transcriptionally and post-transcriptionally is response to *B. henselae* infection.

Given the anti-apoptotic nature of *B. henselae* infection, the overrepresentation of functional annotations for both negative and positive regulation of apoptosis to high statistical significance appears contradictory. However, this expression pattern may also reflect a state of dynamic equilibrium. Several apoptosis regulation pathways operate on a system of dynamic equilibrium with

the ratio of pro- versus. anti-apoptotic factors determining survival outcomes. The significance of over representation for "negative regulation of apoptosis" was considerably higher (P= 8.16 x 10^{-9}) than for "positive regulation of apoptosis" (P= 8.10x10^{-7}). Thus, although both pro- and anti-apoptotic factors are highly expressed, the activities of *B. henselae* and its pathogenicity factors may work to tip the balance of apoptotic factors toward anti-apoptosis, overriding the apoptotic response program and preventing cell death.

B. henselae inhibition of apoptosis represents a fundamental component of *B. henselae* pathogenicity and is an essential component of infection associated pathological angiogenesis (Kirby 2002; Schmid *et al.* 2006). Inhibiting cell death in *B. henselae* infected cells not only preserves the intracellular habitat but is also necessary to prolong the interaction of *B. henselae* with host-cells and allows for induced changes in phenotype to be translated into functional effects. The survival of *B. henselae* infected MACs also enables the development of long-term pathogenic consequences such as the creation of sustained paracrine microenvironments, the development of complex vascular mimicry structures and facilitates the overall chronic infection strategy which is characteristic of *Bartonella* pathogenicity.

5.5.4 Sterol metabolism

Along with functional annotations relating to cellular processes known to be associated with *B. henselae* infection such as angiogenesis, apoptosis inhibition and cytokine production, functional annotations for sterol metabolism were found to be highly enriched among upregulated gene transcripts.

11 genes with the functional annotations for "sterol metabolic process" were upregulated greater than or equal to 2.0 fold and overrepresentation in this category was attributed a high statistical significance (P = 9.29 x 10^{-4}). Several genes for low density lipoprotein (LDL) uptake, *ORL1*, *LPL* and *LDLR,* were very highly upregulated. Other genes for cholesterol biosynthetic processes such as *DHCR24*, *INSIG, SQLE*, and *DHCR7* were also highly upregulated along with two members of the eicosanoid pathway *PTGS2* and *ALOX5AP*.

No connection between *B. henselae* infection and sterol-metabolism has been described to date. It is possible that increased cholesterol-metabolism in *B. henselae* infected MACs represents a non-specific metabolic host-cell response to chronic infection. However, in several intracellular bacteria, host-cell sterol-metabolism has been shown to be essential for intracellular colonization and overall pathogenicity. Manipulation of sterol-metabolic pathways can be utilized by pathogenic bacteria for invasion, as an intracellular energy source or as a means of immune suppression.

Cholesterol is obtained by eukaryotic cells either through import of LDL or through endogenous cholesterol production in the endoplasmic reticulum (Ikonen, 2008). Cholesterol makes up a significant proportion of the eukaryotic plasma membrane lending structural stability and enriching lipid rafts (Simons and Sampaio, 2011). Cholesterol is also involved in regulating endosomal trafficking and serves as a precursor for steroids and vitamins (Ikonen, 2008).

In some cases the importance of host-cell cholesterol is limited to its role as a component of membrane lipid rafts. In several species, the organization of docking partners for bacterial secretion systems and outer membrane pathogenicity factors in lipid rafts has proved necessary for effective invasion and translocation of bacterial toxins and effector proteins (Orlandi and Fishman, 1998; Seveau *et al.*, 2004; Hayward *et al.*, 2005; Lai *et al.*, 2008).

There are also several examples of intracellular bacterial pathogens that actively hijack host-cell cholesterol-metabolism as part of their intracellular pathogenicity strategy. *B. henselae* infected MACs displayed an upregulation of several members of the LDL uptake pathway. This pathway in particular appears to be co-opted as part of the intracellular bacterial lifestyle.

Both *Chlamydia trachomatis* and *Anaplasma phagocytophilum,* obtain cholesterol from host-cell pathways to compensate for their inability to produce it endogenously. *A. phagocytophilum* upregulates host-cell LDL receptor expression leading to increased LDL uptake. LDL trafficking to the ER is intercepted and the transport of endocytic vesicles is redirected to the *Anaplasma* intracellular inclusion (Xiong and Rikihisa, 2012). *Chlamydia trachomatis* similarly redirects endocytic transport of cholesterol and sphingomyelin obtained from either LDL uptake or endogenous synthesis to the *Chlamydia* inclusion (Carabeo *et al.*, 2003; Moore *et al.*, 2008).

Within atherosclerotic lesions infiltrating macrophages engulf large amounts of LDL and oxidized LDL (oLDL) which induces a “foam-cell” phenotype (Shashkin *et al.*, 2005). *Mycobacterium tuberculosis* and *Chlamydia pneumoniae* infection has been shown to induce such foam-cell macrophage phenotypes independent of lipoprotein engulfment. As observed in *B. henselae* infected MACs, both *M. tuberculosis* and *C. pneumoniae,* induce the expression of host-cell cholesterol uptake proteins such as LDLR, ORL1 and LPL which leads to the accumulation of lipid bodies in infected cells (Azenabor *et al.*, 2004; Kim *et al.*, 2010; Lin *et al.*, 2011; Palanisamy *et al.*, 2012; Campbell *et al.*, 2013). In *M. tuberculosis* the fusion of these lipid bodies with bacteria-containing-vacuoles is believed to act as a carbon source for resident bacteria (Peyron *et al.*, 2008). Foam-cell differentiation of infected macrophages is also associated with an immune suppressed phenotype creating an immune privileged intracellular niche for bacterial persistence (Mahajan *et al.*, 2012). *C.*

pneumoniae dissemination from the lung via peripheral blood mononuclear cells and involvement in foam cell formation is now accepted as a potential, independent risk factor in the development of artherogenic disease (Sessa *et al.*, 2009).

Theoretically, interference in sterol-metabolism pathways to facilitate invasion, effector protein translocation, intracellular energy acquisition or immune evasion would be advantageous for *B. henselae* pathogenicity. However, it is still unclear whether the upregulation of sterol metabolic pathways observed in *B. henselae* infected MACs translates into a specific host response or any related functional effects.

5.6 Immune activation phenotypes of *B. henselae* infected MACs

In addition to gene expression patterns associated with angiogenesis, apoptosis, matrix-remodeling and sterol-metabolism, the gene expression profile of *B. henselae* infected MACs also displayed an overrepresentation of upregulated genes with roles in the regulation of immune responses.

Interaction with the innate immune system is a defining event in the infection process. The ability of pathogenic bacteria to alter regulation of immune responses can determine outcomes of bacterial infections and translate to important functional effects in infected host cells.

Interestingly, assessment of the MAC immune response to *B. henselae* infection revealed elements of both positive and negative regulation of inflammation and a distinct activation phenotype in infected cells. Although this activation profile appears contradictory, the combination of inflammatory and anti-inflammatory immune activation phenotypes may translate into an overall immune status that is particularly advantageous for the induction of pathological angiogenesis and overall *B. henselae* pathogenicity.

5.6.1 Inflammatory activation

Results of the gene microarray revealed functional annotations such as “immune response”, “myeloid cell differentiation” “leukocyte activation”, “leukocyte proliferation”, and “hematopoietic cell differentiation” to be highly enriched among upregulated transcripts induced in MACs in response to *B. henselae* infection. Several key genes involved in activation of the pro-inflammatory response (*NFκB, IL1B, IL8, CSF, TLR2)* also showed strong upregulation upon infection.

In past studies *B. henselae* has also been shown to actively stimulate the activation of inflammatory transcription factor NFκB in several cell types including MM6 monocytes/macrophages and endothelial cells (Fuhrmann *et al.*, 2001; Kempf *et al.*, 2005a; Kaiser *et al.*, 2008). The activation of NFκB signaling in *B. henselae* infected MACs and the upregulation and release of NFκB regulated

cytokines CXCL8, CCL2, GM-CSF, ICAM-1 was also demonstrated by Mändle (Mändle, 2005) via Electrophoretic Mobility Shift Assay (EMSA) and ELISA respectively.

Inflammation and NFκB activation can be powerful factors in the promotion of both pathological and physiological angiogenic growth. As a consequence, pathological angiogenesis is an inherent characteristic of chronically inflamed tissues and makes up a significant pathological component of chronic inflammatory conditions such as rheumatoid arthritis, psoriasis, diabetes, inflammatory bowel diseases, obesity and cancer (Walsh and Pearson, 2001; Szekanecz and Koch, 2007; Ye, 2011).

There is evidence that elements of the NFκB dependent inflammatory response also make up an important component of *B. henselae* induced angiogenic effects.

NFκB regulates the expression of key inflammatory-angiogenic compounds which contribute to the creation of a pro-angiogenic microenvironment at sites of chronic inflammation. In endothelial cells the NFκB dependent release of CXCL8 after *B. henselae* infection was found to be required to induce *in vitro* endothelial proliferation and capillary tube formation (Schmid *et al.*, 2004a; Kempf *et al.*, 2005a; McCord *et al.*, 2006). NFκB activation was also found to lead to the expression of cellular adhesion molecules on the surface of infected endothelial cells and the release of chemokine CCL2 which probably plays a role in the attracting myeloid cells to growing vascular lesions (Fuhrmann *et al.*, 2001; McCord *et al.*, 2005).

In this study, the upregulation of many NFκB-regulated pro-angiogenic factors was also observed in *B. henselae* infected MACs. Several NFκB regulated matrix remodeling proteases including *MMP9 MMP2*, *SERPINE1* and cellular adhesion molecule *ICAM1* were highly upregulated along with several important inflammatory angiogenic cytokines such as *CCL2, CXCL1*, and *CXCL8.* The secretion of such NFκB-regulated angiogenic compounds is likely to constitute a significant component of the *B. henselae* induced pro-angiogenic paracrine effects in MACs.

The interaction between inflammatory and angiogenic regulatory pathways may also play a role in *B. henselae* induced pro-angiogenic re-programming. Hypoxia represents a common stimulus for both inflammatory and angiogenic response programs and recent studies have revealed a high degree of interdependence between NFκB and the hypoxia-sensitive, pro-angiogenic transcription factor HIF-1. NFκB has been shown to transcriptionally regulates HIF-1α activity and basal NFκB activity was found to be essential for HIF-1α accumulation and induction of HIF-1 target genes (e.g. VEGF) (BelAiba *et al.*, 2007; Rius *et al.*, 2008; van Uden *et al.*, 2008). HIF-1 and NFκB also share overlapping downstream regulatory pathways with binding sites for both transcription factors located on a set of common genes (Fitzpatrick *et al.*, 2011; Bruning *et al.*, 2012). In this study

increased HIF-1 activity was observed on both transcriptional and post-transcriptional levels along with upregulation of several pro-angiogenic HIF-1 target genes. The activation of HIF-1 and related pro-angiogenic response programs is a key element of *B. henselae* angiogenic re-programming (Kempf *et al.*, 2005a). Thus, in *B. henselae* infected MACs the interaction of NFκB with the HIF-1 regulatory system may also play a role in increasing the angiogenic activity via its contribution to increased HIF-1α transcription and support of synergetic angiogenic gene expression.

5.6.2 Immune regulation

Despite its activation of fundamental elements of the inflammatory response, *B. henselae* remains a slow growing chronic infection *in vivo* and is not associated with rapid inflammatory reaction or septic shock (Pulliainen and Dehio, 2012). The importance of the immune response in *B. henselae* induced pathological angiogenesis is further highlighted by the fact that, in humans, the development of bacillary angiogenesis is seen almost exclusively in patients suffering from immune suppression such as acquired immune deficiency syndrome (AIDS) (Koehler and Tappero, 1993; Chian *et al.*, 2002).

Further analysis of gene expression profiles from *B. henselae* infected MACs reveal that along with several pro-inflammatory genes, many negative regulators of inflammation and NFκB mediated gene transcription were highly upregulated upon *B. henselae* infection (*NR4A2, PTGS2, AREG, IER3, IFRD1, NR4A1, ARG2, TGFB2, TNFAIP3, ZC3H12A, MMP12, IL1RA, SOCS3*). Furthermore, annotations such as “positive regulation of innate immune responses”, “T-cell activation”,”T-cell differentiation” and “T-cell receptor signaling” were highly enriched among down-regulated transcripts indicating that infection of MACs with *B. henselae* induces aspects of both positive and negative regulation of inflammation.

These results correspond to what previous authors have described as the *B. henselae* “stealth pathogen” strategy in which intracellular lifestyle and slow growth requires the simultaneous repression and activation of inflammatory responses at different points during the infection process (Pulliainen and Dehio, 2012).

Details of how *B. henselae* moderates inflammation in infected hosts as part of this “stealth” strategy is limited. The LPS of *Bartonella* is known to contain structural modifications hindering TLR activation (Zähringer *et al.*, 2004). This disguises bacteria from the immune system, preventing TLR4 dependent activation of NFκB, and subsequent pro-inflammatory cascade. NFκB activation observed in *B. henselae* infected cells must therefore be triggered by some alternate activation pathway. Evidence exists that the *B. henselae* VirB/VirD T4SS may, in part, be responsible for specific, TLR

independent, activation of NFκB in infected cells however, no further data exist in this area and there are no indications as to how NFκB signaling is activated in *B. henselae* strains lacking fully functional VirB/VirD T4SS (e.g. the strain Marseille) (Schmid *et al.*, 2004a).

Results of the microarray transcriptome analysis in this study indicate that not only NFκB but several of its inflammatory target genes are transcriptionally active in *B. henselae* infected MACs suggesting that any regulation of the inflammatory response induced by *B. henselae* infection must occur downstream of NFκB activation and selectively inhibit only some elements of the NFκB inflammatory response.

In *Shigella* the bacterial effector phosphates OspF acts downstream of NFκB activation to epigenetically restrict access of the transcription factor to a subset of inflammatory genes (Arbibe *et al.*, 2006). Several bacterial pathogens such as *Mycobacterium tuberculosis* also inhibit inflammasome activation via interaction with caspase 1, preventing the release of IL-1β (Master *et al.*, 2008). It is possible that *B. henselae* mediated attenuation of inflammation may function via similar mechanisms.

The gene expression profile of *B. henselae* infected MACs indicates upregulation of several genes with roles in regulation of NFκB mediated inflammatory signaling such as *NR4A2, PTGS2, AREG, IER3, IFRD1, NR4A1, ARG2, TGFB2, TNFAIP3, ZC3H12A, MMP12, IL1RA*, and *SOCS3*. The increased expression of these proteins may also be involved in regulating specific aspects of NFκB mediated gene translation and downstream inflammatory cytokine secretion.

The ability of *B. henselae* to prevent full activation of the inflammatory response in MACs is advantageous for *B. henselae* pathogenicity at multiple levels. Firstly, the attenuation of inflammation prevents the activation of immune signaling cascades and protects bacteria from effective elimination by the immune effectors. Secondly, the incomplete inhibition of inflammation allows for elements of low level inflammatory activation but prevents escalation of the immune response to a systematic scale. This maintains inflammatory stimulus to a chronic, localized level, permitting the prolonged secretion of angiogenic-inflammatory cytokines and matrix remodeling elements but avoiding the explosive release of cytotoxic molecules and indiscriminate tissue destructive forces associated with a short-lived acute immune reaction.

5.6.3 Alternative macrophage activation

Analysis of the cellular differentiation phenotype in this study revealed the pro-angiogenic cell phenotype induced in MAC in response to *B. henselae* infection to be of macrophage differentiation.

In the contrasting roles macrophages play as mediators of innate immunity but also regenerative and homeostatic processes, immune activation is one of the most important factors in determining macrophage phenotype and functional activities. In response to microenvironmental conditions macrophages can develop immune activation states ranging from highly inflammatory (M1) to highly anti-inflammatory (M2) phenotypes which have a significant impact on the nature of macrophage functional capacity and pro-angiogenic activity (Lawrence and Natoli, 2011).

Typically, bacterial infections induce a highly polarized, highly inflammatory M1 macrophage activation state that acts to eliminate infection (Nau *et al.*, 2002; Jenner and Young, 2005; Benoit *et al.*, 2008b). The manipulation of macrophage activation can thus be an advantageous pathogenicity strategy for immune evasion. Some *Yersinia* species, induce a complete reversal of macrophage polarization, not only inhibiting inflammatory macrophage activation but also triggering the release of anti-inflammatory cytokines that actively inhibit inflammatory signaling (Bliska *et al.*, 2013). In other bacterial species (e.g. *Coxiella burnetti, Salmonella enterocolitica* serovar Typhymurium and *Streptococcus pyogenes)* interference with selected aspects of macrophage activation results in an attenuated macrophage activation phenotype displaying elements of both M1 and M2 macrophage polarization (Goldmann *et al.*, 2007; Benoit *et al.*, 2008a; Kyrova *et al.*, 2012).

To examine the macrophage activation phenotype of *B. henselae* infected MACs, gene expression data from the transcriptome analysis was examined and absolute gene expression signals for typical M1 and M2 marker genes in *B. henselae* infected MACs were compared (Fig. 4.16).

Infected cells showed high absolute expression of some key inflammatory M1 marker genes such as *NFκB, IL1B* and *TNF* and inflammatory cytokines (e.g. CCL2, CCL3, CXCL8). However, infected cells also showed high absolute expression of key M2 macrophage markers such as *MRC1, CD163* and *TGFB1.* Overall gene expression profiles of *B. henselae* infected MACs displayed a predominantly M2 macrophage phenotype indicating that *B. henselae* infection can inhibit the development of inflammatory macrophage polarization.

Alternative macrophage activation in infected MACs may have a twofold effect on *B. henselae* pathogenicity. M2 macrophage polarization prevents expansion of a systematic inflammatory response ensuring bacterial persistence and enabling the *B. henselae* "stealth" pathogenicity strategy. In addition, alternatively activated macrophages play an important role in promoting angiogenic growth (Kodelja *et al.*, 1997; Medina and O'Neill, 2011; Jetten *et al.*, 2014). M2 macrophages have been shown to induce significantly greater rates of angiogenic growth and are found more frequently at sites of neoangiogenesis *in vivo* (Jetten *et al.*, 2013; Jetten *et al.*, 2014).

These effects have been associated with the secretion of pro-angiogenic cytokines such as, FGF-1, IGF-1, CCL2, PGF, CXCL8 and MMP9 into the pro-angiogenic cellular microenvironment and participation in regenerative processes.

Altogether, the distinct immune activation status of *B. henselae* infected MACs may contribute to the pro-angiogenic phenotype of infected cells via the interaction of both inflammatory and anti-inflammatory elements in the cellular response. Activation of inflammatory signaling in infected cells promotes angiogenesis through the co-stimulatory interaction of NFκB and HIF-1 regulatory pathways. The up-regulation of NFκB dependent response programs including the release of angiogenic-inflammatory cytokines and matrix degrading proteases also stimulates angiogenesis by contributing to the pro-angiogenic paracrine microenvironment. The attenuation of the inflammatory response, on the other hand, protects bacteria from immune elimination and maintains inflammation at a chronic inflammatory level conducive to angiogenic growth. The characterization of a predominantly M2-anti-inflammatory macrophage phenotype in *B. henselae* infected MACs further highlights the subversion of immune response associated with *B. henselae* infection and the pro-angiogenic phenotype induced in *B. henselae* infected MACs.

5.7 The Role of BadA in induction of a pro-angiogenic phenotype in *B. henselae* infected MACs

Of the pathogenicity factors possessed by *B. henselae*, the long outer membrane adhesin BadA is believed to be the most significant in inducing pro-angiogenic activity in infected cells (Riess *et al.*, 2004). BadA is an outer membrane adhesin of the trimeric autotransporter adhesin (TAA) family; a class of proteins found on many Gram-negative bacteria and often associated with pathogenicity (O'Rourke *et al.*, 2011). As with other TAAs the primary function of BadA is adherence to host-cells. It has been demonstrated to bind extracellular matrix proteins including fibronectin, laminin and collagen. BadA also mediates adhesion and invasion of host cells and participate in bacterial autoagglutination (Kaiser *et al.*, 2008; Kaiser *et al.*, 2012). In addition to its role in adherence, the expression of BadA is essential for *B. henselae* angiogenic re-programming. BadA expression was found to be required for induction of *B. henselae* associated HIF-1 activation and the release of VEGF from infected endothelial cells (Riess *et al.*, 2004).

To determine whether BadA expression also plays a role in the pro-angiogenic re-programming of MACs upon *B. henselae* infection, MACs were infected with the BadA$^-$ transposon mutant lacking the expression of the BadA protein.

Investigations revealed that *B. henselae* induce HIF-1 activation in MACs was also BadA dependent (Fig 4.3B). Furthermore, in the Matrigel capillary formation assay BadA expression was required to induce the full vascular mimicry phenotype in *B. henselae* infected MACs. MACs infected with transposon mutants lacking the expression of the BadA protein (BadA$^-$) and heat killed wild type *B. henselae* did not develop the extensive capillary like structures in Matrigel culture as observed in wild type infected cells (Fig. 4.10).

These results indicate that angiogenic re-programming in *B. henselae* infected MACs is a specific process dependent on a unique *B. henselae* pathogenicity factor and suggest a key function for BadA in the development of the *B. henselae* associated pro-angiogenic cell phenotypes.

Outer membrane proteins represent the first point of contact between host-cells and bacterial invaders. In several bacterial species the interaction of bacterial adhesins with cellular surface receptors not only function to mediate adhesion and bacterial invasion, but are also involved in subverting host-cell signaling and intracellular molecular pathways (Kline *et al.*, 2009). Recently, the *Fusobacterium nucleatum* adhesin FadA was found to be responsible for increased inflammation and carcinogenic growth in colorectal cancer cells through its interaction with E-cadherin and activation of beta-catinin signaling (Rubinstein *et al.*, 2013).

A common interaction partner for adhesins in such interactions are integrins; a class of heterodimeric bidirectional signaling molecules responsible for cell-cell and cell-matrix interactions (Hauck *et al.*, 2006). Each heterodimer consists of an alpha and beta subunit and the specific combination of subunits in an integrin pair determines the binding affinity with specific extracellular matrix molecules. Binding and clustering of integrin receptors initiates signal transduction cascades.

Several bacterial adhesins have been shown to manipulate host-cell signaling through direct or indirect interaction with integrins (Al-Okla *et al.*, 1999; Agerer *et al.*, 2003; Banerjee *et al.*, 2011; Hillman *et al.*, 2013). Two TAA homologues of BadA; YadA of *Yersinia spp.* and NadA of *Nesserium meningitidis* interact indirectly with β_1 integrins via binding to the extracellular matrix protein fibronectin (Eitel and Dersch, 2002; Unkmeir *et al.*, 2002; Nagele *et al.*, 2011). This interaction has been shown to result in cytoskeletal rearrangement, bacterial invasion and the release of inflammatory cytokine CXCL8 (Schmid *et al.*, 2004b).

Although the details of BadA molecular interaction with host-cells have not yet been elucidated, there is evidence to suggest that BadA may also interact indirectly with β_1 integrins through its binding to extracellular fibronectin. Riess *et. al.* (Riess *et al.*, 2004) found that adherence of

B. henselae was significantly decreased to cells lacking expression of β_1 integrins or when cells and bacteria were treated with anti-fibronectin antibodies.

Mändle (Mändle, 2005) demonstrated that infection of MACs with *B. henselae* resulted in increased surface β_1 integrin expression and that treatment of MACs with and a β_1 integrin inhibitory antibody reduced *B. henselae* adherence by approximately one third. In this study, microarray analysis revealed that genes encoding for various integrins (*ITGB3, ITGA3, ITGB5, ITGA5, ITGAV*) as well as many genes involved in downstream cellular signaling pathways, such as MAPK pathways were upregulated in response to *B. henselae* infection.

Integrin signaling can play an important role in regulating angiogenic growth and it is conceivable that interaction of BadA with integrins may be involved in transmitting angiogenic effects (Avraamides *et al.*, 2008). The interaction of integrin $\alpha_v\beta_3$ with extracellular matrix proteins plays an important role in regulating VEGF angiogenic signaling, endothelial cell survival and migration (Hodivala-Dilke, 2008). Due to its vital role in tumor angiogenesis and common over-expression in cancer cells $\alpha_v\beta_3$ is also an important target of anti-angiogenic cancer drugs (Kumar, 2003). The β_1 integrin family also plays multiple roles in tumor angiogenesis and is associated with resistance to anti-VEGF cancer therapies (Jahangiri *et al.*, 2014). The interaction of integrin $\alpha_5\beta_1$ with fibronectin, for example, was shown to be essential in mediating VEGF-independent angiogenic growth in tumors (Kim *et al.*, 2000). Integrins are also important in the function of myeloid cells. β_3 integrin activation has been implicated in macrophage polarization (Zhang *et al.*, 2012b). Integrin $\alpha_4\beta_1$ selectively promotes the homing of monocytes to tumors and is essential for the participation of myeloid cells in angiogenesis and tumor growth (Jin *et al.*, 2006). $\alpha_5\beta_1$–mediated interaction with fibronectin has been shown to contribute to pro-angiogenic phenotypes and secretion of pro-angiogenic cytokines in myeloid cells (White *et al.*, 2001).

It is also possible that BadA binding itself, may not be involved in angiogenic re-programming directly but only mediates the necessary events that enable downstream pro-angiogenic activities. In this study, infection of MACs with heat killed wild type *B. henselae* failed to induce significant vascular mimicry behavior, revealing that only viable BadA expressing bacteria can induce the vascular mimicry phenotype in infected cells. The fact that living bacteria are required suggests that the process of *B. henselae* induced pro-angiogenic re-programming in MACs may be more complex, requiring complementary downstream events for which bacterial viability is necessary. For example the activities of the VirB/VirD T4SS, includeing the inhibition of apoptosis in infected cells are known to play a key role in the *B. henselae* induced angiogenic response. The translocation of effector

proteins via this molecular structure requires ATP and would be inactivated in heat killed bacteria (Dehio, 2008).

In *Yersinia*, YadA, in addition to mediating adhesion, also acts as a docking system for T3SS injection of effector proteins (Yops) into host-cells. Yops are important manipulators of cellular behaviors and play a key role in *Yersinia* mediated immune suppression. Effective YadA, T3SS coordination is required for the delivery of Yops to the host-cell cytoplasm (Eitel and Dersch, 2002). Although such a synergistic interaction between BadA and the *B. henselae* effector protein transport system (VirB/VirD T4SS) have been recently ruled out, other such yet to be identified secondary aspects of *B. henselae* pathogenicity may be dependent on successful BadA activity to carry out their pro-angiogenic effects (Lu *et al.*, 2013).

5.8 The role of the paracrine microenvironment in the angiogenic activity of *B. henselae* infected MACs

It has been demonstrated in this study that *B. henselae* infected MACs cause a significant increase in the rate of angiogenic sprouting when co-cultured with endothelial cells. No-physical contact was found to be required to transmit these pro-angiogenic effects and conditioned medium from *B. henselae* infected MACs was sufficient to induce increased angiogenic sprouting. This indicates that the release of angiogenic compounds to the paracrine microenvironment must play a important role in mediating the pro-angiogenic effects of *B. henselae* infected MACs.

Myeloid cells are known to have important paracrine roles in angiogenic growth and secrete a large range of cytokines, chemokines, growth factors and proteases that contribute to the transformation of local microenvironments and the regulation of angiogenesis (Chambers *et al.*, 2013).

It has been suggested in the past that the infiltration of myeloid cells to *B. henselae* induced vascular lesions could contribute to the development of pathological angiogenesis. McCord *et. al.* (McCord *et al.*, 2005) demonstrated that the release of CCL2 from *B. henselae* infected endothelial cells causes migration of THP-1 monocytes and suggested that this may play a role in attracting myeloid cells to the developing vascular tumor *in vivo*. THP-1 cells have also been found to secrete angiogenic cytokines VEGF and CXCL8 when infected with *B. henselae* and conditioned medium from infected THP-1 macrophages promoted the proliferation of endothelial cells in culture (Resto-Ruiz *et al.*, 2002; McCord *et al.*, 2006).

To investigate the nature of the paracrine microenvironment created by *B. henselae* infected MACs, conditioned medium taken from infected and uninfected MACs was analyzed at three time points over long term culture (Fig. 4.18).

Secretome analysis revealed that infection of MACs with *B. henselae* induces a diverse cytokine secretion profile that includes growth factors, chemokines, immune modulatory cytokines and matrix remodeling compounds. Overall, *B. henselae* infected MACs create a pro-angiogenic microenvironment with the potential to promote pathological angiogenesis at multiple levels.

5.8.1 Inflammatory-angiogenic chemokines

In particular, the paracrine microenvironment created by *B. henselae* infected MACs was characterized by cytokines known to be dual mediators of both inflammatory and angiogenic processes. The secretome included several examples of inflammatory chemokines with potent pro-angiogenic properties including members of the CXCL and CCL chemokine families and MIF. These inflammatory angiogenic cytokines likely contribute to pathological angiogenesis through their direct stimulation of endothelial cells but also through the attraction of additional myeloid cells to sites of angiogenic proliferation.

The CXCL family of chemoattractant cytokines are well characterized as dual mediators of both inflammatory and angiogenic processes (Strieter *et al.*, 2005). The paracrine microenvironment created by *B. henselae* infected MACs included CXCL subfamily members CXCL1, CXCL4, CXCL8, CXCL10 and CXLC16. In response to inflammatory stimulus CXCLs act as chemokines, attracting mainly neutrophils in the early stages of the inflammatory response. CXCL chemokines containing the ELR motif (a glutamate-leucin-arginine amino acid sequence at the protein's N-terminus) such as CXCL1, CXCL8 and CXCL16 induce angiogenic growth in the absence of preceding inflammation through interaction with the endothelial receptor CXCR2 and CXCR1. In contrast CXCL chemokines lacking the ELR motif such as CXCL10 and CXCL4 act as anti-angiogenic agents through their interaction with the CXCR3 (Strieter *et al.*, 1995).

Along with the CXCL family, *B. henselae* infection of MACs also induced secretion of CCL chemokine family members CCL2, CCL3, CCL4 and low levels of CCL5. Whereas CCL3, CCL4 and CCL5 levels diminished after initial infection, CCL2 was found present in the conditioned medium of *B. henselae* infected MACs for an extend period of time and was still detectable at the midpoint of Matrigel culture (day 25). CCL chemokines function primarily as chemoattractants for monocytes, macrophages, polymorphonuclear leucocytes and natural killer cells. Like CXCL cytokines, they also play a role in promoting angiogenesis. CCL2 has been shown to have direct angiogenic properties

independent of its effect on leukocyte recruitment and acts via the endothelial CCR2 (Salcedo *et al.*, 2000; Stamatovic *et al.*, 2006).

MIF, a multi functional chemokine-like cytokine, was also detected in conditioned medium at high levels throughout long term culture. MIF acts as an inflammatory cytokine responsible for immune cell activation and recruitment but also displays direct angiogenic properties as a non-cognate ligand for endothelial surface receptors CXCR2 and CXCR4 (Calandra and Roger, 2003; Bernhagen *et al.*, 2007).

5.8.2 Immune regulatory cytokines

Along with inflammatory-angiogenic chemokines, *B. henselae* infected MACs also secreted GM-CSF, the key cytokine responsible for macrophage differentiation and mobilization, as part of the early paracrine microenvironment. GM-CSF may act to induce macrophage differentiation and maturation in MACs in response to *B. henselae* infection. *In vivo*, the presence of GM-CSF in the microenvironment could also promote the migration and macrophage differentiation of newly infiltrating myeloid cells. Additionally, GM-CSF has been attributed angiogenic properties and stimulates activation, proliferation and migration of endothelial cells (Bussolino *et al.*, 1989; Bussolino *et al.*, 1991).

Despite the presence of many inflammatory cytokines in the microenvironment of *B. henselae* infected MACs, some of the most important cytokines involved in amplification of the acute inflammatory response such as TNFα, IL-1β and INFγ were missing from the cytokine secretion profile. Although results from the gene microarray indicate up-regulation of NFκB regulated genes encoding the inflammatory mediators IL-1β (*IL1B*) and TNFα (*TNF*), these compounds are not represented in the paracrine microenvironment suggesting that their release is inhibited via some form of post-transcriptional regulation.

The early microenvironment of *B. henselae* infected MACs shows secretion of immunomodulatory cytokines IL-6 and IL-1ra. IL-1ra, a receptor antagonist, inhibits inflammatory signaling via binding to ILR1 and ILR2 and is typically elevated during the resolution phase of acute inflammation (Arend *et al.*, 1998). IL-6 similarly plays an important role in the transition from acute to chronic inflammation and has also been shown to act as an anti-inflammatory agent by inhibiting the activity of other inflammatory cytokines such as TNFα and IL-1β (Aderka *et al.*, 1989; Schindler *et al.*, 1990; Tilg *et al.*, 1994; Xing *et al.*, 1998; Gabay, 2006; Scheller *et al.*, 2011).

In particular, when compared to LPS stimulation, infection with *B. henselae* induced significantly reduced levels of TNFα and increased levels of IL-1ra and CXCL10 indicating that the activation

profile and paracrine microenvironment created by *B. henselae* infected MACs is distinct from a general inflammatory response (Fig. 4.19).

These results correlate with previous findings in this study examining the immune response of MACs to *B. henselae* infection (discussed above in section 5.6). Microarray data from this study as well as additional gene expression and protein secretion data from Mändle (Mändle, 2005) indicate that *B. henselae* infection of MACs activates elements of inflammatory signaling (activation of NFκB, upregulation and release of inflammatory cytokines) but demonstrates an attenuated inflammatory response overall (upregulation of immune regulatory gene expression, M2 macrophage activation). This activation pattern is reflected in the *B. henselae* infected MAC paracrine microenvironment which is characterized by inflammatory-angiogenic cytokines but also contains immune regulatory cytokines and lacks the secretion of key inflammatory mediators responsible for acute expansion of the inflammatory response. Thus, the *B. henselae* manipulation of the inflammatory response allows for the creation of a localized microenvironment of chronic pro-angiogenic stimulus but prevents elimination via effective immune responses. This distinctly balanced microenvironment of attenuated inflammation likely makes up an important component of *B. henselae* pathogenicity in MACs.

5.8.3 Matrix remodeling compounds

Over time, several of the early inflammatory cytokines disappear from the secretion profile of *B. henselae* infected MACs and matrix remodeling elements, including the metalloproteinases MMP8, MMP9, uPA and respective inhibitors TIMP-1 and PAI-1, became more prominent.

Matrix proteases systems play important roles in angiogenic processes by degrading the basement membrane, promoting cell migration, extra cellular matrix degradation and capillary lumen formation . As in many biological processes balance between proteolytic compounds and their inhibitors determines the net proteolytic activity. The balance of matrix remodeling factors drives endothelial cell invasion but preserves matrix scaffold stability and allows for matrix deposition and vessel stabilization (Pepper *et al.*, 1996).

The metalloproteinases MMP8 and MMP9 (zinc dependent collagenase and gelatinase respectively) are responsible for degrading various components of the extracellular matrix (Pepper, 2001). Of the secreted metalloproteinases, MMP9 in particular has been implicated as a key player in activation and regulation of angiogenic growth (Vu *et al.*, 1998; Jadhav *et al.*, 2004). MMP9 not only enables angiogenesis via matrix degradation and invasion, but also through proteolytic activation of matrix bound growth factors most notably VEGF and TGF-β (Bergers *et al.*, 2000; Yu and Stamenkovic, 2000;

van Hinsbergh and Koolwijk, 2008). TIMP-1 is considered an anti-angiogenic factor due to its inhibitory function on MMPs but upregulation has also been associated with increased angiogenic growth in some cases. This may be connected to the independent functions of TIMP-1 in preventing cell death and triggering cellular signaling (Hornebeck *et al.*, 2005; van Hinsbergh and Koolwijk, 2008).

The protease uPA cleaves plasminogen to create plasmin, a serine protease with multiple targets. uPA activity mediates matrix degradation and, as with MMPs, promotes angiogenesis through release and activation of matrix bound growth factors (Lyons *et al.*, 1990; Houck *et al.*, 1992). Additionally, through its interaction with the membrane receptor uPAR, uPA regulates cell migration, endothelial cell motility, invasion, proliferation and survival (Estreicher *et al.*, 1990; Smith and Marshall, 2010). The inhibitor of uPA, PAI-1, participates in angiogenesis through its regulation of uPA activity but has also been shown to have independent roles in pathological angiogenesis depending on its level of expression (Nykjaer *et al.*, 1997; Isogai *et al.*, 2001; Devy *et al.*, 2002; Bajou *et al.*, 2004).

5.8.4 Angiogenin

Angiogenin, an angiogenic ribonuclease also appeared in the *B. henselae* induced paracrine microenvironment at the mid-point of culture and was maintained over long term culture. Angiogenin is known to promote angiogenesis by several mechanisms. Following activation of endothelial cells, angiogenin is translocated to the nucleus where it facilitates and amplifies angiogenic signals through its regulation of ribosome biogenesis (Xu *et al.*, 2002; Kishimoto *et al.*, 2005). Angiogenin also binds endothelial cell receptors activating angiogenic signaling pathways directly and is involved in matrix degradation via it's interaction with actin and protease systems at the endothelial cell surface (Liu *et al.*, 2001; Kim *et al.*, 2007; Dutta *et al.*, 2014).

5.8.5 Angiogenic growth factors

The secretion of the angiogenic growth factor VEGF in response to *B. henselae* infection has been described in multiple cell types including monocytes, endothelial and epithelial cells and was understood to be one of the most important mechanism of *B. henselae* induced angiogenic effects to date (Kempf *et al.*, 2001). In this study we have found the gene encoding VEGF (*VEGFA*) to be strongly upregulated in *B. henselae* infected MACs. Increased levels of VEGF secretion in MACs due to *B. henselae* infection was also described by Mändle (2005) via ELISA. Unexpectedly, secretome analysis returned inconsistent results for the presence of VEGF across samples, time points and conditions. These contradictory results may be explained by the interference of supplementary

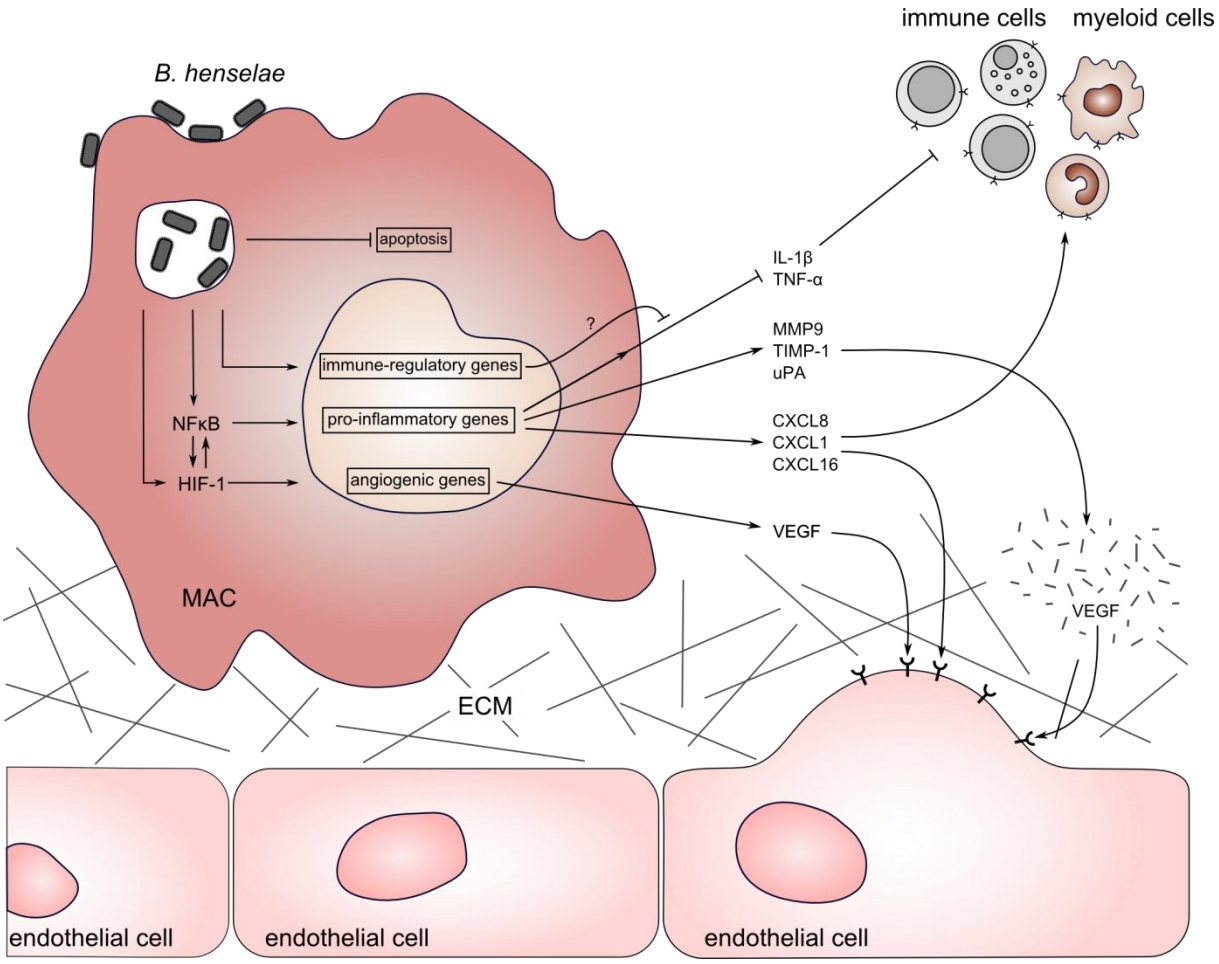


Figure 5.1 Infection of MACs with *B. henselae* results in a phenotypic transformation towards a macrophage with increased pro-angiogenic capacity. The infection of MACs with *B. henselae* induces broad phenotypic changes which combine to promote an increased pro-angiogenic phenotype. Infection of MACs with *B. henselae* inhibits apoptosis and results in activation of inflammatory and angiogenic transcription factors NFκB and HIF-1, respectively. The interaction of HIF-1 and NFκB regulatory pathways may also have co-stimulatory effects which amplify their combined transcriptional activity. Activation of HIF-1 in infected MACs results in the upregulation and secretion of angiogenic cytokines such as VEGF which stimulate angiogenesis in endothelial cells directly. The activation of NFκB results in the upregulation of various inflammatory regulated genetic programs which also play a role in the pro-angiogenic phenotype of *B. henselae* infected MACs. The upregulation and release of inflammatory-angiogenic cytokines such CXCL8, CXCL1 and CXCL16 may contribute to angiogenesis via their interaction with endothelial cells directly and via their role as chemoattractants for further myeloid cell infiltration. The upregulation and release of NFκB regulated matrix remodeling compounds such as MMP9, TIMP-1 and uPA may also play a pro-angiogenic role by facilitating the invasion of proliferating endothelial cells and via the activation of matrix bound angiogenic growth factors including VEGF. Despite the upregulation of inflammatory gene programs, *B. henselae* infected MACs also show upregulation of genes involved in regulation of the immune responses. These may be involved in mediating aspects of inflammatory activation. Accordingly, some of the most important inflammatory cytokines involved in expansion of the inflammatory response to the systematic level are absent from the *B. henselae* induced paracrine microenvironment although upregulated at the mRNA level. This regulation of the inflammatory response may further contribute to pathological angiogenesis by preventing an effective immune response and by establishing a microenvironment of chronic low level inflammation which is conducive to angiogenic growth.

growth factors with the accurate measurement of MAC generated cytokines. Recombinant angiogenic cytokines VEGF, FGF-B and EGF are present at unspecified concentrations as growth factor supplements in the endothelial cell culture medium (EBM, Lonza). The detection signals observed for FGF-B were similarly inconsistent across samples. EGF was observed at consistently high concentrations in the conditioned medium of both infected and uninfected MACs but oversaturation of the detection signal in all profiles prevented a relative comparison. As a result of these complications, no reliable conclusions could be made about the level of VEGF secretion induced by *B. henselae* infection via this particular assay.

Taken together, the examination of the cytokine secretion profile of *B. henselae* infected MACs has revealed the creation of a paracrine microenvironment characterized by a diverse range of secreted factors with the potential to promote pathological angiogenesis on multiple levels.

The release of inflammatory angiogenic cytokines such as CXCL8 and CCL2 promote angiogenesis through direct interaction with endothelial cells and by attracting myeloid cells to sites of angiogenic growth. Matrix remodeling compounds such as MMP9 allow invasion of angiogenic sprouts and activate matrix bound growth factors from the extracellular matrix. Angiogenin promotes angiogenesis by multiple mechanisms including the amplification of pro-angiogenic signals. The lack of acute inflammatory cytokines such as IL-1β and TNFα and the presence of immunomodulatory cytokines such as IL-1ra and IL-6 also reflect an overall attenuated inflammatory status which is conducive to angiogenic growth.

The results of the secretome array provides a broader picture of the diverse pro-angiogenic microenvironment created by *B. henselae* infected myeloid cells and expands the scope of cytokines known to be associated with *B. henselae* infection.

Angiogenesis is a multistep process requiring the coordinated expression of various cytokines, growth factors and proteases with the interaction of various factors resulting in additive and even synergistic effects (Ahlers, 2001; Gouwy *et al.*, 2005; Bartee and McFadden, 2013).

Although this assay does not provide evidence about the extent to which individual cytokines contribute to angiogenic effects, the delineation of the overall spectrum of factors secreted by *B. henselae* infected MACs and their dynamics over time provides a more detailed representation of the complex process of interactions involved in the development of *B. henselae* induced pathological angiogenesis.

It is likely that the diverse and distinctly balanced microenvironment created by *B. henselae* infected MACs, acting at multiple levels of the angiogenic process, contributes most significantly to the

development of *B. henselae* induced pathological angiogenesis as a whole. Further elucidation of the actions and interactions of individual cytokines in the complex paracrine microenvironment created by *B. henselae* infected MACs would however be a valuable topic for further study.

5.9 Parallels between *B. henselae* infected MACs and TAMs

The development of *B. henselae* associated vascular lesions has been shown to mirror malignant tumor progression in several aspects. Both *Bartonella* induced vascular proliferations and malignant tumors are dominated by prominent myeloid cell infiltrates (Cockerell and LeBoit, 1990; Manders, 1996), chronic inflammatory microenvironments and the chaotic growth of abnormal vasculature, (Koehler and Tappero, 1993) suggesting that these two processes may develop according to similar mechanisms.

As with chronic *B. henselae* infections, pathological angiogenesis is a defining characteristic of malignant tumors. Myeloid cells have been shown to be intimately involved in this process and the infiltration of myeloid cells to the tumor microenvironment has been linked to increased tumor angiogenesis, aggressive phenotype and poor prognosis (Murdoch *et al.*, 2008; Zhang *et al.*, 2012c).

In response to microenvironmental cues, infiltrating myeloid cells take on a specific tumor associated macrophage (TAM) phenotype characterized by a distinct pro- angiogenic, invasive and immune suppressive qualities (Allavena and Mantovani, 2012). Through the secretion of pro-angiogenic cytokines and participation in matrix remodeling processes TAMs transform the tumor microenvironment, activate the angiogenic switch and drive tumor vascularization and metastasis (Qian and Pollard, 2010; Quail and Joyce, 2013).

Examination of the interaction between *B. henselae* and MACs presented in this study revealed that infection induces a phenotypic transformation towards a distinct pro-angiogenic macrophage phenotype. Further examination of this phenotype revealed many functional and phenotypic parallels to tumor associated macrophages including increased pro-angiogenic, invasive and immune modulatory properties.

Similar to TAMs, *B. henselae* infected MACs display increased pro-angiogenic activity. Infected cells incorporate into growing vasculature and increase the rate of sprouting angiogenesis in a paracrine manner. *B. henselae* infection induced activation of the angiogenic transcription factor HIF-1 and transcriptome analysis revealed a strong up-regulation of genes involved in angiogenesis and related pathways such as signal transduction and cell migration.

The most significant contribution of TAMs to overall tumor growth and angiogenesis is the creation of a pro-angiogenic and immune-modulatory paracrine microenvironment. The cytokine secretion profile of *B. henselae* infected MACs also shows many similarities to the TAM-created paracrine microenvironment of malignant tumors including the release of inflammatory-angiogenic cytokines CXCL1, CXCL8, CXCL16, CCL2, CCL3, CCL4, MIF, IL-6 and the angiogenic ribonuclease angiogenin.

Pro-angiogenic cytokines of the CXCL family (e.g. CXCL1, CXCL8 and CXCL16) contribute significantly to tumor vascularization in several cancers and are common components of the tumor microenvironment (Chen, Jeremy J W *et al.*, 2003; Li *et al.*, 2004; Wang *et al.*, 2006; Han *et al.*, 2014). CXCL8 in particular is most commonly associated with tumor associated macrophages and promotes carcinogenesis through the activation and regulation of various cellular signaling pathways (Chen, Jeremy J W *et al.*, 2005; Waugh and Wilson, 2008). CCL chemokines in the tumor microenvironment primarily increase tumor angiogenesis by promoting the infiltration of myeloid cells to the tumor site but have also been attributed direct angiogenic effects (Salcedo *et al.*, 2000; Keeley *et al.*, 2011). CCL2 is a defining component of the tumor microenvironment and associated with increased tumor associated macrophage infiltration, pathological angiogenesis and progression in several cancers (Ueno *et al.*, 2000; Saji *et al.*, 2001; Bailey *et al.*, 2007). The presence of angiogenin in the tumor microenvironment is associated with the pro-angiogenic activity of tumor associated macrophages; promoting the invasion, survival and proliferation of cancer cells (Etoh *et al.*, 2000; Miyake *et al.*, 2014). Finally, the secretion of immune regulatory cytokines MIF and IL-6 to the tumor microenvironment have also been identified as drivers of tumor angiogenesis (Ren *et al.*, 2003; Huang *et al.*, 2004; Hira *et al.*, 2005; Nilsson *et al.*, 2005).

In accordance with the TAM-like qualities induced in MACs by *B. henselae*, the development of a vascular mimicry phenotype was also observed. The formation of non-endothelial vascular structures in aggressive tumors is associated with intense angiogenic pressure (Döme *et al.*, 2007; Kirschmann *et al.*, 2012). Tumor associated macrophages have also been reported to participate in mosaic pseudo-vessel formation in cooperation with other cell types, and also form independent vascular mimicry structures (Scavelli *et al.*, 2007; Caillou *et al.*, 2011).

The *B. henselae* infected MACs also mirror tumor associated macrophages in their tissue invasive qualities. Microarray analysis revealed an over-representation of upregulated genes involved in cell adhesion and migration as well as strong upregulation of genes for matrix components and matrix degrading proteases. In the paracrine microenvironment created by *B. henselae* infected MACs the secretion of compounds involved in the metalloproteinase and plasmin matrix remodeling systems (MMP9, MMP8, TIMP-1, uPA, PAI-1) was observed consistently over long culture periods.

Matrix remodeling compounds are a common component of TAM-created tumor microenvironments. In particular, members of the metalloproteinase and plasmin-plasminogen proteolytic systems such as MMP9, uPA and their inhibitors TIMP-1 and PAI-1 have been shown to be decisive factors in the angiogenic activity of tumor associated macrophages (Bajou *et al.*, 1998; Sidenius and Blasi, 2003; Hornebeck *et al.*, 2005; Zajac *et al.*, 2013). The activity of these proteolytic systems not only enables tumor angiogenesis via matrix degradation and invasion but also through interaction with cellular receptors and the proteolytic activation of matrix bound growth factors (Sidenius and Blasi, 2003; Kessenbrock *et al.*, 2010). The MMP9-dependent release of VEGF from the extracellular matrix, for example, was found to be the decisive factor leading to activation of the tumor angiogenic switch (Bergers *et al.*, 2000).

Tumor associated macrophages associated with the angiogenic and invasive stages of tumor progression are characterized by a distinct immune activation profile that is skewed towards an M2 anti-inflammatory phenotype (Movahedi *et al.*, 2010; Qian and Pollard, 2010). This characteristic anti-inflammatory macrophage activation maintains a chronic-inflammatory tumor microenvironments and prevents effective tumor immunity (Sica *et al.*, 2006; Movahedi *et al.*, 2010).

B. henselae infected MACs also exhibited a predominantly M2 macrophage activation profile and the paracrine microenvironment created by infected cells lacks several key inflammatory cytokines central to the acute inflammatory response (IL-1β, TNFα and IFNγ). Furthermore, the paracrine microenvironment of *B. henselae* infected MACs contained the characteristic tumor microenvironmental components CCL2, IL-6 and MIF which are responsible for TAM anti-inflammatory immune-conditioning (Gu *et al.*, 2000; Yaddanapudi *et al.*, 2013).

TAMs are highly effective drivers of pathological angiogenesis and tumor progression. *In vivo* the infiltration of TAMS to the tumor microenvironment correlates with increased tumor angiogenesis, progression and overall prognostic outcome (Takanami *et al.*, 1999; Knowles *et al.*, 2004; Tsutsui *et al.*, 2005; Lin *et al.*, 2006; Zhang *et al.*, 2012c). This activity is associated with transformation of tumor paracrine microenvironments, matrix degradation and immune modulation (Noy and Pollard, 2014).

B. henselae associated vascular lesion are also characterized by a prominent myeloid cell infiltrate and the induction of a parallel TAM-like phenotype in infiltrating MACs via *B. henselae* infection could conceivably have similarly dramatic effects on the development of *B. henselae* associated vascular tumors.

Mirroring the behavior of TAMs in the malignant tumor environment, *B. henselae* infected MACs were found to interact intimately with activated endothelium and formed vascular mimicry structures over long term culture. Infected cells create a tumor-like pro-angiogenic microenvironment of “smoldering” inflammation, pro-angiogenic cytokines and matrix remodeling compounds which extend significant angiogenic pressure to the surrounding endothelium.

Therefore, via its interaction with MACs and manipulation of myeloid cell phenotype, *B. henselae* can amplify its pathogenic activity. Through the powerful functional qualities of the TAM phenotype, *B. henselae* infected MACs extend pro-angiogenic pressure to surround microenvironment and induces pathological angiogenesis in a paracrine manner.

5.10 Possible mechanisms of *B henselae* induced TAM-like phenotypic differentiation in MACs

Within the tumor context, macrophages take on the specific TAM phenotype in response to microenvironmental signals from the developing malignancy (van Overmeire *et al.*, 2014). In the context of *B. henselae* infection, elements of *B. henselae* specific pathogenicity such as the BadA dependent activation of HIF-1 and the distinct immune activation phenotype may create similar conditions for the development of an analogous TAM-like phenotype in MACs.

5.10.1 Hypoxia and HIF-1

Hypoxia is a hallmark of the tumor microenvironment and the migration of macrophages into the hypoxic compartment is associated with the development of many aspects of the specific TAM phenotype (Movahedi *et al.*, 2010). Macrophages localized to hypoxic tumor areas develop an M2 macrophage activation, increased pro-angiogenic capacity, immune suppressive activity and are associated with a general increase in tumor progression and vascularization (Lewis and Murdoch, 2005; Movahedi *et al.*, 2010; Casazza *et al.*, 2013).

HIF-1, the most important regulator of the macrophage response to hypoxia is strongly activated in tumor associated macrophages. HIF-1α knockout studies have demonstrated that HIF-1 is required for the immune-suppressive activities and some of the angiogenic qualities of hypoxic TAMs (Doedens *et al.*, 2010; Werno *et al.*, 2010). Hypoxia is also an important activator of vascular mimicry phenotypes in aggressive tumor cells (Paulis *et al.*, 2010; Kirschmann *et al.*, 2012).

Results from this study indicate that *B. henselae* infection of MACs results in the up-regulation of HIF-1α on both transcriptional and post-transcriptional levels. Additionally, experiments revealed that HIF-1 activation in MACs and the development of the *B. henselae* induced vascular mimicry

phenotype was dependent on the expression of the *B. henselae* pathogenicity factor BadA. It is conceivable that BadA mediated HIF-1 activation may also play a role in inducing a TAM–like phenotype in *B. henselae* infected MACs.

5.10.2 Smoldering Inflammation

The ability of *B. henselae* to manipulate the inflammatory response may also be an important factor inducing the TAM-like phenotype in infected MACs.

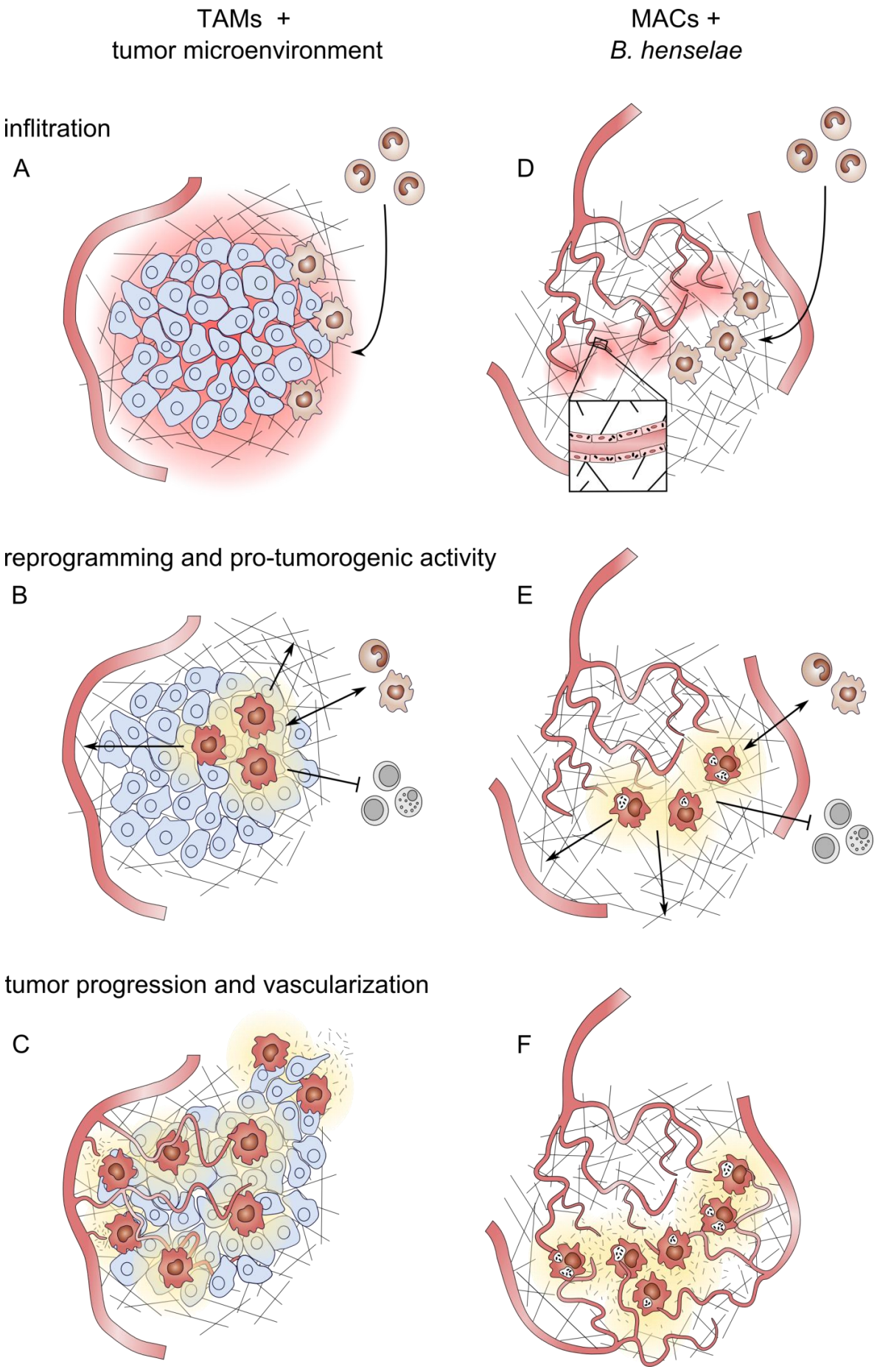
Tumors represent microenvironments of “smoldering” inflammation that contain a diverse array of angiogenic-inflammatory cytokines and matrix remodeling compounds. Simultaneously however, tumor-educated macrophages participate actively in immune suppression preventing the expansion of an effective immune response, thus creating a “perfect storm” of paracrine factors for the promotion of pathological tissue growth (Qian and Pollard, 2010).

Results of this study demonstrate that *B. henselae* infection of MACs also results in a duality of inflammatory and anti-inflammatory gene regulation. Infected cells show activation of the immune response but prevent escalation to an effective and systematic inflammatory reaction. Instead the *B. henselae* induced paracrine microenvironment is chronically dominated by secondary inflammatory cytokines with angiogenic properties. This ability to balance inflammation and immune suppression in *B. henselae* infection may contribute to the development of a TAM-like phenotype and tumor-like paracrine microenvironment in infected MACs.

5.11 Bacterial infection and pathological tissue growth

The intimate interaction of pathogenic bacteria with host-cells over the course of chronic infection requires the use of sophisticated cellular subversion strategies to ensure the survival and proliferation within the host-specific niche. The high evolutionary advantages attributed to manipulation of processes such as innate immunity, apoptosis and cellular differentiation has favored the ability of bacterial pathogens to intervene in host-cell pathways normally protected by layers of regulatory mechanisms. Inevitably, however, interference in such essential cellular systems can lead to fundamental dysregulation of overall cellular phenotypes.

Over the years, several bacterial species as well as interactions with the microbiome have been linked to pathological tissue growth and in particular the development of carcinogenesis (Martel *et al.*, 2012). Chronic *H. pylori* infection has been well established as a mediator of adenocarcinoma (Polk and Peek, 2010).


TAMs +
tumor microenvironment
MACs +
B. henselae
inflitration
A
D
reprogramming and pro-tumorogenic activity
B
E
tumor progression and vascularization
C
F

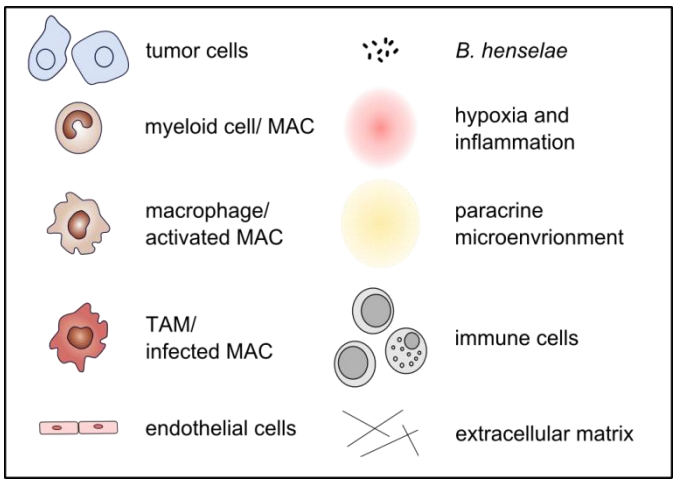


Figure 5.2 *B. henselae* infected MACs show many functional and phenotypic parallels to tumor associated macrophages. (A) The inflammatory and hypoxic microenvironment in early malignant tumors promotes myeloid cell infiltration and macrophage differentiation. **(B)** The tumour microenvironment reprograms tumour associated macrophages (TAMs) towards a pro-angiogenic, invasive and immune suppressive phenotype. TAMs secrete angiogenic factors, degrade the extracellular matrix and create an environment of "smouldering" inflammation which attracts further myeloid cell infiltrate but prevents an effective immune response.**(C)** The activities of TAMs transform the tumour microenvironment promoting tumour vascularization, invasive phenotypes and cancer progression. Similarly, in *B. henselae* associated vascular tumors, **(D)** the creation of a hypoxic microenvironment and secretion of chemoattractant cytokines by *B. henselae* infected endothelial cells causes the infiltration of myeloid cells such as MACs into the infectious microenvironment. **(E)** The infection of MACs with *B. henselae* results in phenotypic reprogramming towards a TAM-like cell with increased angiogenic, invasive and immune modulatory properties. Infected MACs secrete a range of paracrine factors which create a tumor-like microenvironment dominated by inflammatory–angiogenic cytokines and matrix remodeling factors. As in TAMs, *B. henselae* infected MACs show upregulation and secretion of inflammatory elements but also display a predominantly M2-anti-inflammatory activation resulting in a state of chronic low level inflammation. **(F)** Due to the important roles of myeloid cells in pathological angiogenesis and tissue growth, the ability of *B. henselae* to induce a TAM-like phenotype in MACs and the creation of a tumor-like microenvironment has the potential to contribute significantly to infection associated pathological angiogenesis and the development of *B. henselae* associate vascular tumors *in vivo*.

Other bacterial species *Salmonella enterica* serovar Typhi and *Chlamydia pneumonia* are associated with gallbladder cancer and lung cancer respectively (Dutta *et al.*, 2000; Zhan *et al.*, 2011) and dysregulation of gut microbial homeostasis has also been increasingly connected to colon carcinoma and may even have remote effects on cancer development in distant areas of the body (Iida *et al.*, 2013; Schwabe and Jobin, 2013).

As yet, mechanisms of bacterial associated carcinogenesis have focused on the acquisition of genetic instability resulting from the general genotoxic effects of chronic inflammation combined with the continuous dysregulation of oncogenic pathways via bacterial effectors [e.g., cytotoxin-associated antigen A (CagA) of *H. pylori* (Polk and Peek, 2010; Guidi *et al.*, 2013; Schwabe and Jobin, 2013)].

In the context of *B. henselae* induced re-programming of MACs, the continuous presence of viable bacteria did not appear to be necessary to induce the long term cellular effects such as the development of the vascular mimicry phenotype as no viable bacteria were isolated from the fully

formed structures at the end point of culture. This observation suggests a "*hit and run*" mechanism triggering a pro-angiogenic phenotype by initial host-cell re-programming. Similar phenomena have been observed in *H. pylori* CagA-initiated gastric carcinogenesis (Hatakeyama, 2014) or long term exposure to the cylomodulin family of bacterial toxins (Guerra *et al.*, 2011).

In the case of *B. henselae* infections *in vivo,* however, pathological tissue growth is transient and can be reversed by the elimination of infection suggesting an alternate mechanism for infection associated pathological tissue growth (Dehio, 2005). Through the manipulation of myeloid cell responses and the induction of a tumor associated macrophage-like phenotype; *B. henselae* infection induces a sustained stimulatory microenvironment that promotes pathological tissue growth via paracrine mechanisms.

For *B. henselae,* manipulation of myeloid cells and innate immunity in general may be an optimal strategy to ensure survival and persistence within its intracellular niche. The induction of a TAM-like macrophage phenotype in MACs may be due to the convergence of several evolutionarily advantageous *B. henselae* specific pathogenicity mechanisms (a) inhibition of cell death (Schmid *et al.*, 2006), (b) HIF-1 mediated angiogenic gene programming triggered by BadA (Riess *et al.*, 2004), (c) activation of NFκB and the release of inflammatory-angiogenic compounds (Schmid *et al.*, 2004a) and (d) the "stealth pathogen" strategy of *B. henselae* which prevent the expansion of a strong inflammatory response (Pulliainen and Dehio, 2012).

Myeloid cells, however, have a diverse functional spectrum and manipulation of their phenotypic qualities can translate into wide ranging effects to surrounding microenvironments. Due to the functional significance of MACs in conditions of pathological tissue growth, the re-programming of these cells and the resulting creation of a pro-angiogenic paracrine microenvironment has the potential to contribute disproportionately to the tumor-like growth of the *B. henselae* induced vascular lesions.

6. Summary

Vascular tumors associated with chronic *B. henselae* infections are unique examples of infection-associated pathological angiogenesis. The chaotic vascular architecture and prominent myeloid infiltrate of *B. henselae* induced vascular lesions show many similarities with malignant tumors.

In human cancers infiltrating myeloid cells play a decisive role in tumor progression and vascularization. In particular, tumor associated macrophages (TAMs) transform the tumor microenvironment, drive tumor invasion and vascularization through secretion of pro-angiogenic and immune modulatory cytokines and participation in matrix remodeling processes.

Myeloid angiogenic cells (MACs) are a subset of circulating myeloid progenitors with important roles in regenerative and pathological angiogenesis and a critical involvement in tumor vascularization. The phenotypic plasticity and importance of MACs in pathological angiogenic processes, position these cells as key potential players in *B. henselae* associated vascular tumor formation.

To investigate the possible role of MACs in *B henselae* induced pathological angiogenesis, the objective of this study was to examine the interaction of *B. henselae* with MACs and determine how this may affect their angiogenic capacity.

Building on previous work by Mändle (2005) this study has demonstrated that MACs are susceptible to infection with *B. henselae* and reside in intracellular vacuoles. As in endothelial cells, infection of MACs with *B. henselae* was associated with inhibition of apoptosis and activation of endogenous angiogenic programs including activation of the angiogenic transcription factor HIF-1.

In addition to angiogenic re-programming on a molecular level *B. henselae* infection increases MAC functional angiogenic capacity. *B. henselae* infected MACs were found to integrate into growing endothelium and increase the rate of angiogenic sprouting in a paracrine manner.

When cultured in a Matrigel capillary formation assay, infected MACs were also found to form networks of capillary-like structures that were stable over long periods of time. The *B. henselae* pathogenicity factor BadA was essential for the induction of this vascular mimicry phenotype as well as the activation of HIF-1 in infected MACs indicating that this factor may play an important role in MAC angiogenic re-programming.

Examination of infected MACs via FACS analysis, cytospin immunohistochemistry and qRT-PCR revealed that endothelial differentiation does not play a role in the *B. henselae* induced pro-angiogenic phenotype. Instead, MACs were shown to be myeloid in phenotype displaying typical

macrophage markers which were upregulated upon *B. henselae* infection and maintained over long-term culture.

The increased angiogenic activity of *B. henselae* infected MACs was found to be associated with a broad phenotypic reprogramming in infected cells. In particular, gene expression programs related to angiogenesis, structural organization, apoptosis, sterol metabolism and immune regulation, were upregulated. Further examination of microarray gene expression profiles revealed that *B. henselae* infected MACs display a predominantly M2 anti-inflammatory macrophage activation status.

Finally, examination of the paracrine microenvironment created by *B. henselae* infected MACs revealed a diverse cytokine secretion profile dominated by inflammatory-angiogenic cytokines and matrix remodeling elements and lacking expression of some of the most important cytokines involved in the expansion of the inflammatory response. This *B. henselae* induced activation status was demonstrated to be distinct from the general inflammatory response induced by *E. coli* LPS treatment.

Comparison of *B. henselae* infected MACs to TAMs revealed many parallels in functional and phenotypic characteristics. Both TAMs and *B. henselae* infected MACs demonstrate increased angiogenic capacity, invasive, and immune modulatory phenotypes and the ability to participate in the formation of vascular mimicry phenotypes under angiogenic pressure. Furthermore, the pro-angiogenic paracrine microenvironment created by *B. henselae* infected MACs shows many similarities to the TAM-created tumor-microenvironment.

In conclusion, these investigations have demonstrated that the infection of MACs with *B. henselae* results in the phenotypic re-programming towards TAM-like cells with increased pro-angiogenic, invasive and immune-modulatory qualities. The results of this study elucidate new aspects of *B. henselae* pathogenicity in myeloid cells and highlight the role of these cells as paracrine mediators of *B. henselae* induced vascular tumor formation. In addition, these findings demonstrate that manipulation of myeloid cells by pathogenic bacteria can contribute to microenvironmental regulation of pathological tissue growth and suggest parallels underlying bacterial infections and cancer.

7. Zusammenfassung

Bartonella ist die einzig bekannte Bakteriengattung, die beim Menschen im Zusammenhang mit pathologischer Angiogenese steht. Bei immunsupprimierten Patienten führt die chronische Infektion mit den Gram-negativen, fakultativ-intrazellulären *B. henselae* Bakterien zu der Entstehung vaskulärer Tumore in der Haut und den inneren Organen (bazilläre Angiomatose, Peliosis hepatis). Die Entstehung *B. henselae* assoziierter vaskuloproliferativer Läsionen ist ein einzigartiges Beispiel für eine bakterielle Angiogenese und ist auf komplexe Pathogen-Wirtszelle-Interaktionen zurückzuführen.

Die durch *B. henselae* verursachten vaskuloproliferativen Läsionen sind durch die Bildung unorganisierter, vaskulärer Strukturen und die Infiltration myeloide Zellen gekennzeichnet und weisen eine starke Ähnlichkeit zu der malignen Tumorvaskularisierung auf, was darauf hindeuten könnte, dass beide Prozesse einem ähnlichen Mechanismus unterliegen.

Bei der Tumorvaskularisierung und -progression spielt die Infiltration von myeloide Zellen eine entscheidende Rolle. Durch die Beteiligung an Matrix-modulierenden Prozessen und die Sekretion pro-angiogenetischer und immunregulatorischer Zytokine verändern Tumor-assoziierte Makrophagen (TAMs) das Mikromilieu des Tumors-und treiben die Vaskularisierung und Metastasierung voran.

Bisher wurde der durch *B. henselae* verursachte Mechanismus der pathologischen Angiogenese vorwiegend *in vitro* an Endothelzellen untersucht. Tatsächlich sind aber *in vivo* immer mehrere Zellarten an diesem Prozess beteiligt, wobei hier sich die Rolle von Stammzellen zunehmend als entscheidend herausstellt.

Myeloide angiogenetische Zellen (MACs) sind eine Unterklasse zirkulierender Vorläuferzellen, die eine entscheidende Funktion bei regenerativen und pathologisch-angiogenetischen Prozessen sowie bei der Tumorvaskularisierung übernehmen. MACs entstehen im Knochenmark und werden in den Blutkreislauf abgegeben, wo sie molekularen Signalen zum Ort des angiogenetischen Geschehens folgen. Dort begünstigen sie durch die Interaktion mit dem Gefäßendothel und die parakrine Sekretion pro-angiogenetischer Faktoren die Neoangiogenese.

Aufgrund ihrer phänotypischen Plastizität und Bedeutung bei pathologisch-angiogenetischen Prozessen könnten MACs auch eine wichtige Rolle bei der Entstehung und Progression der durch *B. henselae* verursachten vaskularen Läsionen spielen. Das Ziel dieser Arbeit war die Analyse der Interaktion von *B. henselae* und MACs und die Untersuchung einer möglichen Rolle dieser Zellen bei der Entwicklung *B. henselae*-assoziierter vaskulärer Tumore.

Vorangegangene Arbeiten an Endothelzellen haben die durch *B. henselae* verursachte pathologische Angiogenese auf ein Zusammenspiel mehrerer Pathogenitätsmechanismen zurückgeführt: Die Inhibierung der Apoptose in der infizierten Wirtszelle, die angiogenetische Reprogrammierung durch Aktivierung des Transkriptionsfaktors HIF-1, die Sekretion pro-angiogenetischer Zytokine (z.B. VEGF) und die selektive Aktivierung von NFκB regulierten (z.B. anti-apoptotisch) Gene. *Bartonella* Adhäsin A (BadA) gilt als wichtigster Pathogenitätsfaktor für die pathologische Angiogenese bei *B. henselae* Infektionen. BadA vermittelt die Adhäsion an die endotheliale Wirtszelle und ist für die Aktivierung von HIF-1 und der dadurch initiierten Sekretion von VEGF verantwortlich.

Initiale Untersuchungen dieser Arbeit hatten gezeigt, dass MACs auch für die Infektion mit *B. henselae* empfänglich sind, die dann intrazellulär in Vakuolen persistieren. Wie bei Endothelzellen induziert die Infektion von MACs mit *B. henselae* eine Inhibition der Apoptose sowie die Aktivierung von zellulären, angiogenetischen Programmen, wie die Aktivierung von HIF-1.

Neben der angiogenetischen Aktivierung von MACs auf molekularer Ebene verstärkt die Infektion mit *B. henselae* auch die angiogenetische Kapazität von MACs auf funktioneller Ebene. Um die Interaktion von MACs mit Endothelzellen zu untersuchen, wurde ein 3-dimensionales Ko-Kultur Sphäroid Angiogenese Model einwickelt. Hier wurde gezeigt, dass sich *B. henselae* infizierte MACs in wachsende Endothelzellsprosse integrieren und durch parakrine Faktoren das angiogenetische Wachstum (Sprossung) signifikant erhöhen.

Weiterhin führte die Kultivierung von *B. henselae* infizierten MACs in einem *in vitro* „*capillary-formation assay*“ über längere Zeiträume zu der Bildung stabiler, komplexer, 2-dimensionaler, kapillarer Netzwerke. Diese Formation kapillarer Netzwerke bei Zellen nicht endothelialen Ursprungs („*vascular mimicry*“) kann auch *in vivo* bei Makrophagen und Tumorzellen, die unter starkem angiogenetischen Druck stehen, beobachtet werden.

Der Pathogenitätsfaktor BadA wurde als essentieller Faktor für die angiogenetische Wirkung von *B. henselae* auf MACs und für das „*vascular mimicry*“ Verhalten identifiziert. Nur die Infektion mit dem BadA exprimierenden *B. henselae* Wildtyp-Stamm konnte HIF-1 in MACs aktivieren und die Bildung kapillarer Netzwerke induzieren, während die Infektion von MACs mit BadA defizienten *B. henselae* Mutanten weder zur HIF-1 Aktivierung noch zur Bildung kapillarer Netzwerke führte.

Bei der phänotypischen Charakterisierung von *B. henselae* infizierten MACs mittels FACS-Analyse, Immunzytochemie und qRT-PCR wurde die Expression endothelialer Merkmale ausgeschlossen. Stattdessen, wurden MACs als myeloide Zellen mit charakteristischen Eigenschaften von Makrophagen identifiziert.

Um die durch *B. henselae* erzeugten pro-angiogenetischen Veränderungen in MACs besser zu verstehen, wurden Gen-Expressionsprofile erstellt. Untersuchungen infizierter und uninfizierter MACs mittels Transkriptom- Analyse haben ergeben, dass die erhöhte pro-angiogenetische Aktivität von infizierten Zellen mit einer massiven phänotypischen Reprogrammierung zusammenhängt. Es zeigte sich, dass insbesondere Gene, die an der Regulation der Angiogenese und des Sterol-Metabolismus beteiligt sind, sowie immun- und Matrix-modulierende Gene durch *B. henselae* beeinflusst werden. Des Weiteren zeigte sich, dass *B. henselae* infizierte MACs trotz Expression Elemente eines inflammatorischen Genmusters, vorwiegend einen anti-inflammatorischen M2-Makrophagen Phänotyp aufweisen.

Um die parakrine, pro-angiogenetische Wirkung *B. henselae* infizierter MACs weiter zu untersuchen, wurde eine Sekretomanalyse durchgeführt. Dazu wurden Zellkulturüberstände an mehreren Infektionszeitpunkten einer Proteom-Profiler-Mikroarray-Analyse unterzogen. Die Ergebnisse zeigten, dass durch die Infektion mit *B. henselae* ein Mikromilieu erzeugt wird, in dem inflammatorisch-angiogenetische, sowie matrix-modulierende Zytokine dominieren, wobei die Anwesenheit von Zytokinen, die eine Ausbreitung der inflammatorischen Antwort bewirken, fehlen. Zudem sezernieren *B. henselae* infizierte MACs im Vergleich zu *E. coli*- LPS behandelten MACs signifikant geringere Konzentrationen des inflammatorischen Zytokins TNFα und höhere Konzentrationen des anti-inflammatorischen Zytokins IL-1ra sowie des Angiogenese-modulierenden Zytokins CXCL10. Diese Ergebnisse zeigen, dass die Infektion mit *B. henselae* einen Aktivierungzustand hervorruft, der sich deutlich von dem einer unspezifischen Entzündungsreaktion unterscheidet und sowohl inflammatorische als auch anti-inflammatorische Elemente besitzt.

Weitere Untersuchungen des pro-angiogenetischen Phänotyps *B. henselae* infizierter MACs im Vergleich zu tumor-assoziierten Makrophagen (TAMs) haben viele phänotypische und funktionelle Ähnlichkeiten gezeigt. Sowohl TAMs als auch *B. henselae* infizierte MACs weisen eine erhöhte angiogenetische Kapazität sowie einen invasiven, immunsuppremierenden Phänotyp auf und haben die Fähigkeit unter angiogenetischem Druck blutgefäßartige Strukturen zu bilden. Weiterhin weist das durch infizierte MACs erzeugte pro-angiogenetische, parakrine Mikromilieu viele Ähnlichkeiten mit dem des durch TAMs erzeugten Mikromilieus im Tumor auf. Beide Zelltypen tragen durch die Sekretion von angiogenetischen, sowie Matrix- und immun-modulatorischen Zytokinen zur Förderung der pathologischen Angiogenese und der allgemeinen Tumorprogression bei.

Zusammengenommen zeigen diese Untersuchungen, dass die Infektion von MACs mit *B. henselae* zu einer phänotypischen Reprogrammierung zu TAM-artigen Zellen mit erhöhten pro-angiogenetischen, invasiven und immun-modulatorischen Eigenschaften und zu der Bildung eines

tumor-ähnlichen Mikromilieus, das aus inflammatorisch-angiogenetischen und matrix-modulierende Substanzen besteht, führt. Diese Eigenschaften sind sowohl für die Entstehung maligner Tumore, sowie der *B. henselae* assoziierter vaskulärer Tumore von großer funktioneller Bedeutung und unterstreichen eine mögliche Rolle von MACs in der *B. henselae*-assoziierten pathologischen Angiogenese *in vivo*.

In der vorliegenden Arbeit konnten neue Erkenntnisse über die Rolle myeloider Zellen bei der Entstehung *B. henselae* induzierter, vaskulärer Tumore gewonnen werden. Darüber hinaus zeigen die Ergebnisse Parallelen zwischen Tumor assoziierten myeloiden Zellen und den durch *B. henselae* manipulierte myeloiden Zellen. Beide beeinflussen das Mikromilieus dahingehend, dass die Tumorprogression und -metastasierung vorangetrieben wird. Damit erschließen sich bisher unbekannte Parallelen zwischen bakteriellen Infektionen und Tumorgenese.

8. References

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9. Abbreviations

ʎ	wavelength
ø	diameter
3D	three dimensional
7-AAD	7-amino-actinomycin D
AA	arachidonic acid
Abs	antibodies
ADM	adrenomedullin
ADP	adenosindiphosphat
AIDS	acquired immune deficiency syndrome
AP	alkaline phosphatase
AP-1	activator protien -1
APS	ammonium persulfate
Arg1	arginase 1
ATP	adenosintriphosphat
B. bacilliformis	*Bartonella bacilliformis*
B. h.	*Bartonella henselae*
B. henselae	*Bartonella henselae*
BadA	Bartonella adhesin A
Beps	Bartonella effector proteins
BID	Bartonella intracellular delivery domain
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBA	columbia blood agar
CCL (as in CCL2)	C-C motif ligand (chemokine)
CD (as in CD45)	cluster of differentiation
CDK	cyclin-dependent kinase
cDNA	complementary DNA
cIAP	cellular inhibitor of apoptosis protien
cm^2	square centimeters
CO2	carbon dioxide
CSF-1	colony stimulating factor 1
CXCL	C-X-C motif ligand (chemokine)
d (as in d5)	day
DAB	diaminobenzidine
DAPI	4’,6-Diamidin-2-phenylindol
DC	dendritic cell
°C	degrees celcius
DMSO	Dimethyl sulfoxid
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	Dithiothreitol
e.g.	*exempli gratia* (for example)
EBM	endothelial basal medium
ECL	enhanced chemiluminescent reagent

ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	endothelial growth factor
EGM	endothelial growth medium
ELISA	enzyme linked immunosorbent assay
ELR motif	glutamic acid-leucine-arginine sequence
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ERK	extracellular-signal regulated kinase
etc.	*et cetera* (and others)
FACS	fluorescence activated cell sorting
FC	fold change
FCS	fetal calf serum
FDR	false discovery rate
FGF	fibroblast growth factor
Fig.	figure
g	gravitational force
GEO	NCBI Gene Expression Omnibus
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GTP	guanosine-5'-triphosphate
h	hours
H. pylori	*Helicobacter pylori*
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HHV8	human herpes virus 8
HIF-1	hypoxia-inducible factor-1
HLA-DR	human leukocyte antigen
HPC	hematopoetic progenitor cell
HPV	human papilloma virus
HRP	horseradish peroxidase
HSC	hematopoetic stem cell
HUVECs	human umbilical vein endothelial cells
i.e.	*id est* (that is)
ICAM	intercellular adhesion molecule
IFN	interferons
IGF	insulin-like growth factor
IL	interleukin
IL-1ra	IL-1 receptor antagonist
iNOS	inducible nitric oxide sythase
L	liter
LB	Luria-Bertani medium
LDL	low density lipoprotien
LPS	lipopolysaccharide
M	molar concentration
mA	milliampere

MAC	myeloid angiogenic cells
MAPK	mitogen-activated protien kinase
MI	myo-inositol
MIF	macrophage migration inhibitory factor
min	minute
ml	milliliter
MM6	monocyte-macrophage 6
MMP	matrix metalloproteinases
MOI	multiplicity of infection
mRNA	messenger RNA
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydoxide
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nm	nanometer
OD	optical density
oLDL	oxidized low density lipoprotien
ORF	openreading frame
PBMC	perifieral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PDGF	platlet derived growth factor
PFA	paraformaldehyde
PGE2	prostaglandin E2
PI	propidium iodid
PMSF	phenylmethylsulfonyl fluoride
PolR2A	DNA-directed RNA polymerase II subunit RPB1
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real-time PCR
r	person correlation coefficient
R (as in VEGFR2)	receptor
R^2	coefficients of determination
rDNA	ribosomal DNA
RIN	RNA Integrity Number
RNA	ribonucleic acid
RPLP0	ribosomal phosphoprotein P0
rpm	revolutions per minute
RT	room temperatur
RT-PCR	reverse transcription-PCR
s	second
S. aureus	*Staphylococcus aureus*
SDF-1	stromal cell derived factor-1
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate- polyacrylamice gel electrophoresis
SEM	scanning electron microscopy
SOCS	suppressor of cytokine signaling

T4SS	type four secretion system
TAA	trimeric autotransporter
TBS	tris-buffered saline solution
TEM	transmission electron microscopy
TEMED	tetramethylenediamine
TGF	transforming growth factor
TIMP-1	tissue inhibitor of metalloproteinases-1
TLR	toll-like receptor
TNF	tumor necrosis factor
TPA	2-O-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)- aminomethane
TRITC	tetramethylrhodamine
TSP-1	thrombospondin
µg	micro gram
µl	microliters
uPA	urokinase-like plasminogen activator
UV	ultraviolet
V	Volt
v (as in vVEGF)	viral
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
w t	wild type

10. Supplementary Data

S Table 10.1 DAVID analysis of upregulated transcripts in *B. henselae*-infected MACs at d53. List of enriched functional annotations (*P*<0.01).

Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0001944~ vasculature development	31	6.008	1.09E-10	4.081	1.82E-07
GOTERM_BP_5	GO:0048514~ blood vessel morphogenesis	28	5.426	2.04E-10	4.385	3.43E-07
GOTERM_BP_5	GO:0001568~ blood vessel development	30	5.814	2.82E-10	4.046	4.74E-07
GOTERM_BP_5	GO:0001525~ angiogenesis	23	4.457	5.72E-10	5.135	9.62E-07
GOTERM_BP_5	GO:0042981~ regulation of apoptosis	58	11.240	1.06E-09	2.384	1.78E-06
GOTERM_BP_5	GO:0043067~ regulation of programmed cell death	58	11.240	1.56E-09	2.360	2.63E-06
GOTERM_BP_5	GO:0060548~ negative regulation of cell death	35	6.783	3.42E-09	3.213	5.75E-06
GOTERM_BP_5	GO:0043066~ negative regulation of apoptosis	34	6.589	8.16E-09	3.174	1.37E-05
GOTERM_BP_5	GO:0043069~ negative regulation of programmed cell death	34	6.589	1.15E-08	3.129	1.94E-05
GOTERM_BP_5	GO:0009891~ positive regulation of biosynthetic process	49	9.496	5.87E-08	2.330	9.87E-05
GOTERM_BP_5	GO:0010557~ positive regulation of macromolecule biosynthetic process	47	9.109	6.78E-08	2.375	1.14E-04
GOTERM_BP_5	GO:0006935~ chemotaxis	21	4.070	7.14E-08	4.337	1.20E-04
GOTERM_BP_5	GO:0031328~ positive regulation of cellular biosynthetic process	48	9.302	9.98E-08	2.315	1.68E-04
GOTERM_BP_5	GO:0045597~ positive regulation of cell differentiation	25	4.845	1.09E-07	3.607	1.83E-04
GOTERM_BP_5	GO:0008285~ negative regulation of cell proliferation	32	6.202	1.55E-07	2.929	2.60E-04
GOTERM_BP_5	GO:0006916~ anti-apoptosis	23	4.457	2.68E-07	3.689	4.51E-04
GOTERM_BP_5	GO:0043065~ positive regulation of apoptosis	34	6.589	8.10E-07	2.613	0.001363
GOTERM_BP_5	GO:0043068~ positive regulation of programmed cell death	34	6.589	9.45E-07	2.595	0.00159
GOTERM_BP_5	GO:0010942~ positive regulation of cell death	34	6.589	1.04E-06	2.583	0.001756
GOTERM_BP_5	GO:0031347~ regulation of defense response	18	3.488	1.41E-06	4.159	0.002373
GOTERM_BP_5	GO:0051173~ positive regulation of nitrogen compound metabolic process	43	8.333	1.94E-06	2.206	0.003258
GOTERM_BP_5	GO:0048661~ positive regulation of smooth muscle cell proliferation	9	1.744	2.74E-06	9.593	0.004609
GOTERM_BP_5	GO:0010628~ positive regulation of gene expression	39	7.558	5.79E-06	2.218	0.00974
GOTERM_MF_5	GO:0008009~ chemokine activity	10	1.938	6.01E-06	7.388	0.007474
GOTERM_BP_5	GO:0045637~ regulation of myeloid cell differentiation	12	2.326	7.15E-06	5.665	0.012019
GOTERM_BP_5	GO:0010647~ positive regulation of cell communication	27	5.233	7.34E-06	2.712	0.012347
GOTERM_BP_5	GO:0048660~ regulation of smooth muscle cell proliferation	10	1.938	7.73E-06	7.183	0.013001
GOTERM_BP_5	GO:0008284~ positive regulation of cell proliferation	31	6.008	8.24E-06	2.474	0.013859

GOTERM_BP_5	GO:0009966~ regulation of signal transduction	51	9.884	9.37E-06	1.919	0.015753
GOTERM_BP_5	GO:0031325~ positive regulation of cellular metabolic process	51	9.884	1.00E-05	1.915	0.016833
GOTERM_BP_5	GO:0009967~ positive regulation of signal transduction	25	4.845	1.02E-05	2.800	0.017182
GOTERM_MF_5	GO:0042379~ chemokine receptor binding	10	1.938	1.04E-05	6.936	0.012902
GOTERM_BP_5	GO:0010604~ positive regulation of macromolecule metabolic process	50	9.690	1.05E-05	1.928	0.017661
GOTERM_BP_5	GO:0016477~ cell migration	24	4.651	1.06E-05	2.873	0.017807
GOTERM_BP_5	GO:0045935~ positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	40	7.752	1.25E-05	2.118	0.020986
GOTERM_BP_5	GO:0045941~ positive regulation of transcription	37	7.171	1.75E-05	2.168	0.029383
GOTERM_BP_5	GO:0045893~ positive regulation of transcription, DNA-dependent	33	6.395	1.98E-05	2.286	0.033202
GOTERM_BP_5	GO:0007243~ protein kinase cascade	28	5.426	2.10E-05	2.501	0.035299
GOTERM_BP_5	GO:0051254~ positive regulation of RNA metabolic process	33	6.395	2.33E-05	2.267	0.03918
KEGG_PATHWAY	hsa00100: Steroid biosynthesis	7	1.357	3.60E-05	10.066	0.042681
GOTERM_BP_5	GO:0051272~ positive regulation of cell motion	13	2.519	3.75E-05	4.383	0.063032
GOTERM_BP_5	GO:0045639~ positive regulation of myeloid cell differentiation	8	1.550	4.67E-05	8.010	0.078554
GOTERM_BP_5	GO:0030334~ regulation of cell migration	17	3.295	5.21E-05	3.324	0.087573
GOTERM_BP_5	GO:0002237~ response to molecule of bacterial origin	12	2.326	5.25E-05	4.611	0.088226
GOTERM_BP_5	GO:0042035~ regulation of cytokine biosynthetic process	11	2.132	7.14E-05	4.912	0.119995
GOTERM_BP_5	GO:0030335~ positive regulation of cell migration	12	2.326	7.23E-05	4.455	0.121464
GOTERM_BP_5	GO:0032768~ regulation of monooxygenase activity	7	1.357	7.90E-05	9.252	0.132767
GOTERM_BP_5	GO:0001819~ positive regulation of cytokine production	12	2.326	8.02E-05	4.406	0.134693
GOTERM_BP_5	GO:0042108~ positive regulation of cytokine biosynthetic process	9	1.744	8.37E-05	6.196	0.1407
GOTERM_BP_5	GO:0007584~ response to nutrient	15	2.907	8.44E-05	3.540	0.141834
GOTERM_BP_5	GO:0050727~ regulation of inflammatory response	11	2.132	8.99E-05	4.783	0.151106
GOTERM_BP_5	GO:0060325~ face morphogenesis	5	0.969	9.12E-05	18.357	0.153272
GOTERM_BP_5	GO:0032496~ response to lipopolysaccharide	11	2.132	1.01E-04	4.720	0.169062
GOTERM_BP_5	GO:0050867~ positive regulation of cell activation	13	2.519	1.28E-04	3.870	0.215144
KEGG_PATHWAY	hsa04060: Cytokine-cytokine receptor interaction	25	4.845	1.34E-04	2.333	0.158395
GOTERM_BP_5	GO:0002526~ acute inflammatory response	12	2.326	1.74E-04	4.046	0.292316
GOTERM_BP_5	GO:0045088~ regulation of innate immune response	9	1.744	1.97E-04	5.507	0.331072
KEGG_PATHWAY	hsa04620: Toll-like receptor signaling pathway	14	2.713	1.98E-04	3.389	0.235164
GOTERM_BP_5	GO:0060323~ head morphogenesis	5	0.969	2.28E-04	15.020	0.382159
GOTERM_BP_5	GO:0007596~ blood coagulation	12	2.326	2.49E-04	3.887	0.417199

GOTERM_BP_5	GO:0070665~ positive regulation of leukocyte proliferation	9	1.744	2.55E-04	5.310	0.42869
GOTERM_BP_5	GO:0032946~ positive regulation of mononuclear cell proliferation	9	1.744	2.55E-04	5.310	0.42869
GOTERM_BP_5	GO:0050900~ leukocyte migration	9	1.744	2.90E-04	5.217	0.485642
GOTERM_BP_5	GO:0031349~ positive regulation of defense response	10	1.938	3.33E-04	4.526	0.557776
GOTERM_BP_5	GO:0070304~ positive regulation of stress-activated protein kinase signaling pathway	6	1.163	3.33E-04	9.441	0.558
GOTERM_BP_5	GO:0002696~ positive regulation of leukocyte activation	12	2.326	3.48E-04	3.741	0.584291
BIOCARTA	h_il1rPathway: Signal transduction through IL1R	9	1.744	3.67E-04	4.645	0.443176
GOTERM_BP_5	GO:0045785~ positive regulation of cell adhesion	9	1.744	4.14E-04	4.956	0.694436
GOTERM_BP_5	GO:0002544~ chronic inflammatory response	4	0.775	5.10E-04	22.029	0.85352
GOTERM_BP_5	GO:0010740~ positive regulation of protein kinase cascade	15	2.907	5.35E-04	2.968	0.895818
GOTERM_BP_5	GO:0030278~ regulation of ossification	10	1.938	5.47E-04	4.236	0.916447
GOTERM_BP_5	GO:0048145~ regulation of fibroblast proliferation	7	1.357	5.61E-04	6.609	0.939517
GOTERM_BP_5	GO:0016126~ sterol biosynthetic process	7	1.357	5.61E-04	6.609	0.939517
BIOCARTA	h_nthiPathway: NFkB activation by Nontypeable Hemophilus influenzae	8	1.550	6.05E-04	4.990	0.728716
GOTERM_BP_5	GO:0007242~ intracellular signaling cascade	59	11.434	6.35E-04	1.552	1.062298
GOTERM_BP_5	GO:0032675~ regulation of interleukin-6 production	7	1.357	6.57E-04	6.425	1.098312
GOTERM_BP_5	GO:0030097~ hemopoiesis	18	3.488	8.21E-04	2.520	1.371521
GOTERM_BP_5	GO:0001893~ maternal placenta development	5	0.969	8.56E-04	11.014	1.429058
GOTERM_BP_5	GO:0048534~ hemopoietic or lymphoid organ development	19	3.682	9.24E-04	2.415	1.542063
GOTERM_BP_5	GO:0016125~ sterol metabolic process	11	2.132	9.29E-04	3.599	1.551325
GOTERM_BP_5	GO:0002697~ regulation of immune effector process	11	2.132	9.29E-04	3.599	1.551325
GOTERM_BP_5	GO:0032944~ regulation of mononuclear cell proliferation	10	1.938	9.43E-04	3.934	1.574111
GOTERM_BP_5	GO:0070663~ regulation of leukocyte proliferation	10	1.938	9.43E-04	3.934	1.574111
GOTERM_BP_5	GO:0051781~ positive regulation of cell division	7	1.357	0.001019	5.931	1.699989
GOTERM_BP_5	GO:0060326~ cell chemotaxis	7	1.357	0.001019	5.931	1.699989
GOTERM_BP_5	GO:0043627~ response to estrogen stimulus	11	2.132	0.001255	3.462	2.089198
GOTERM_BP_5	GO:0050671~ positive regulation of lymphocyte proliferation	8	1.550	0.001256	4.806	2.091096
GOTERM_BP_5	GO:0050714~ positive regulation of protein secretion	7	1.357	0.001335	5.641	2.221531
GOTERM_BP_5	GO:0007167~ enzyme linked receptor protein signaling pathway	22	4.264	0.001632	2.126	2.70881
GOTERM_BP_5	GO:0019220~ regulation of phosphate metabolic process	28	5.426	0.001673	1.908	2.775937

GOTERM_BP_5	GO:0051174~ regulation of phosphorus metabolic process	28	5.426	0.001673	1.908	2.775937
GOTERM_BP_5	GO:0050708~ regulation of protein secretion	8	1.550	0.001723	4.558	2.857548
KEGG_PATHWAY	hsa04610: Complement and coagulation cascades	10	1.938	0.001747	3.543	2.054861
GOTERM_BP_5	GO:0030593~ neutrophil chemotaxis	5	0.969	0.001786	9.179	2.960209
GOTERM_BP_5	GO:0050707~ regulation of cytokine secretion	6	1.163	0.001864	6.609	3.08897
GOTERM_BP_5	GO:0032663~ regulation of interleukin-2 production	6	1.163	0.001864	6.609	3.08897
GOTERM_BP_5	GO:0030099~ myeloid cell differentiation	10	1.938	0.001944	3.553	3.219375
GOTERM_BP_5	GO:0032874~ positive regulation of stress-activated MAPK cascade	4	0.775	0.002001	14.686	3.310843
GOTERM_BP_5	GO:0001890~ placenta development	8	1.550	0.002102	4.406	3.476077
GOTERM_BP_5	GO:0032755~ positive regulation of interleukin-6 production	5	0.969	0.002209	8.696	3.649076
GOTERM_BP_5	GO:0051223~ regulation of protein transport	11	2.132	0.002332	3.188	3.849594
GOTERM_BP_5	GO:0045089~ positive regulation of innate immune response	7	1.357	0.002451	5.028	4.042571
GOTERM_BP_5	GO:0001936~ regulation of endothelial cell proliferation	6	1.163	0.002508	6.196	4.133849
GOTERM_BP_5	GO:0051347~ positive regulation of transferase activity	17	3.295	0.002564	2.341	4.224849
GOTERM_BP_5	GO:0051251~ positive regulation of lymphocyte activation	10	1.938	0.002601	3.406	4.284066
GOTERM_BP_5	GO:0032570~ response to progesterone stimulus	5	0.969	0.002696	8.261	4.436943
GOTERM_BP_5	GO:0043549~ regulation of kinase activity	22	4.264	0.002783	2.036	4.577638
GOTERM_BP_5	GO:0045765~ regulation of angiogenesis	8	1.550	0.002788	4.196	4.585715
GOTERM_BP_5	GO:0032872~ regulation of stress-activated MAPK cascade	4	0.775	0.002794	13.217	4.595702
GOTERM_BP_5	GO:0030324~ lung development	10	1.938	0.002989	3.338	4.908618
GOTERM_BP_5	GO:0001569~ patterning of blood vessels	5	0.969	0.003252	7.867	5.328721
GOTERM_BP_5	GO:0000060~ protein import into nucleus, translocation	6	1.163	0.003299	5.831	5.403701
GOTERM_BP_5	GO:0048754~ branching morphogenesis of a tube	8	1.550	0.003333	4.067	5.458359
KEGG_PATHWAY	hsa04621: NOD-like receptor signaling pathway	9	1.744	0.003351	3.549	3.906517
GOTERM_BP_5	GO:0050670~ regulation of lymphocyte proliferation	9	1.744	0.003507	3.583	5.735185
GOTERM_BP_5	GO:0070201~ regulation of establishment of protein localization	11	2.132	0.003603	3.004	5.887738
GOTERM_BP_5	GO:0030323~ respiratory tube development	10	1.938	0.003656	3.240	5.972066
KEGG_PATHWAY	hsa04062: Chemokine signaling pathway	17	3.295	0.003657	2.222	4.256035
GOTERM_BP_5	GO:0010627~ regulation of protein kinase cascade	17	3.295	0.003698	2.256	6.038774
GOTERM_BP_5	GO:0051222~ positive regulation of protein transport	8	1.550	0.003955	3.945	6.446175
GOTERM_BP_5	GO:0031960~ response to corticosteroid stimulus	9	1.744	0.004062	3.499	6.61473
GOTERM_BP_5	GO:0006694~ steroid biosynthetic process	9	1.744	0.004062	3.499	6.61473

BIOCARTA	h_eponfkbPathway: Erythropoietin mediated neuroprotection through NF-kB	5	0.969	0.00413	6.804	4.879543
GOTERM_BP_5	GO:0031348~ negative regulation of defense response	6	1.163	0.004255	5.507	6.917593
GOTERM_BP_5	GO:0050778~ positive regulation of immune response	12	2.326	0.004476	2.735	7.265422
GOTERM_BP_5	GO:0002694~ regulation of leukocyte activation	13	2.519	0.004507	2.588	7.312761
GOTERM_BP_5	GO:0050715~ positive regulation of cytokine secretion	5	0.969	0.004588	7.183	7.439813
GOTERM_BP_5	GO:0030595~ leukocyte chemotaxis	6	1.163	0.0048	5.358	7.77107
GOTERM_BP_5	GO:0030162~ regulation of proteolysis	7	1.357	0.005037	4.364	8.139443
GOTERM_BP_5	GO:0045778~ positive regulation of ossification	5	0.969	0.005376	6.884	8.664881
GOTERM_BP_5	GO:0001892~ embryonic placenta development	5	0.969	0.005376	6.884	8.664881
GOTERM_BP_5	GO:0008360~ regulation of cell shape	7	1.357	0.005526	4.283	8.895555
KEGG_PATHWAY	hsa04010:MAPK signaling pathway	21	4.070	0.005633	1.923	6.486206
GOTERM_BP_5	GO:0017015~ regulation of transforming growth factor beta receptor signaling pathway	6	1.163	0.006036	5.084	9.678816
GOTERM_BP_5	GO:0048820~ hair follicle maturation	4	0.775	0.006226	10.167	9.968298
GOTERM_BP_5	GO:0046697~ decidualization	4	0.775	0.006226	10.167	9.968298
GOTERM_BP_5	GO:0042542~ response to hydrogen peroxide	7	1.357	0.006607	4.130	10.54598
GOTERM_BP_5	GO:0006631~ fatty acid metabolic process	14	2.713	0.007101	2.336	11.29158
GOTERM_BP_5	GO:0051353~ positive regulation of oxidoreductase activity	5	0.969	0.007214	6.354	11.46137
GOTERM_BP_5	GO:0048146~ positive regulation of fibroblast proliferation	5	0.969	0.007214	6.354	11.46137
GOTERM_BP_5	GO:0045766~ positive regulation of angiogenesis	5	0.969	0.007214	6.354	11.46137
GOTERM_BP_5	GO:0009968~ negative regulation of signal transduction	15	2.907	0.007235	2.243	11.49204
GOTERM_BP_5	GO:0001570~ vasculogenesis	6	1.163	0.007481	4.836	11.86103
GOTERM_BP_5	GO:0002761~ regulation of myeloid leukocyte differentiation	6	1.163	0.007481	4.836	11.86103
KEGG_PATHWAY	hsa04640: Hematopoietic cell lineage	10	1.938	0.007814	2.843	8.892868
GOTERM_BP_5	GO:0050870~ positive regulation of T cell activation	8	1.550	0.007901	3.478	12.4854
BIOCARTA	h_cdMacPathway: Cadmium induces DNA synthesis and proliferation in macrophages	5	0.969	0.008074	5.757	9.334089
GOTERM_BP_5	GO:0045428~ regulation of nitric oxide biosynthetic process	5	0.969	0.008271	6.119	13.03268
GOTERM_BP_5	GO:0033189~ response to vitamin A	6	1.163	0.008287	4.720	13.05632
GOTERM_BP_5	GO:0010648~ negative regulation of cell communication	16	3.101	0.008303	2.132	13.07958
GOTERM_BP_5	GO:0051046~ regulation of secretion	14	2.713	0.00834	2.290	13.13376
GOTERM_BP_5	GO:0045124~ regulation of bone resorption	4	0.775	0.009472	8.811	14.787
GOTERM_BP_5	GO:0032642~ regulation of chemokine production	4	0.775	0.009472	8.811	14.787
GOTERM_BP_5	GO:0046850~ regulation of bone remodeling	4	0.775	0.009472	8.811	14.787
GOTERM_BP_5	GO:0006633~ fatty acid biosynthetic process	8	1.550	0.009706	3.346	15.12374
BIOCARTA	h_eicosanoidPathway: Eicosanoid Metabolism	6	1.163	0.009903	4.277	11.33502

S Table 10.2 DAVID analysis of downregulated transcripts in *B. henselae*-infected MACs at d53. List of enriched functional annotations (*P*<0.01).

Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0045449~ regulation of transcription	147	22.755	2.75E-12	1.703	4.34E-09
GOTERM_BP_5	GO:0010468~ regulation of gene expression	156	24.149	6.08E-12	1.646	9.58E-09
GOTERM_BP_5	GO:0010556~ regulation of macromolecule biosynthetic process	154	23.839	1.27E-11	1.639	2.00E-08
GOTERM_BP_5	GO:0019219~ regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	153	23.684	1.67E-11	1.638	2.64E-08
GOTERM_BP_5	GO:0031326~ regulation of cellular biosynthetic process	158	24.458	1.87E-11	1.615	2.95E-08
GOTERM_BP_5	GO:0006355~ regulation of transcription, DNA-dependent	109	16.873	6.04E-11	1.852	9.52E-08
GOTERM_BP_5	GO:0051252~ regulation of RNA metabolic process	110	17.028	1.06E-10	1.828	1.67E-07
GOTERM_MF_5	GO:0046914~ transition metal ion binding	145	22.446	2.56E-10	1.576	3.02E-07
BIOCARTA	h_tcraPathway: Lck and Fyn tyrosine kinases in initiation of TCR activation	7	1.084	3.78E-06	13.064	0.00451
BIOCARTA	h_tcrPathway: T Cell Receptor Signaling Pathway	10	1.548	1.61E-05	6.038	0.01923
GOTERM_BP_5	GO:0002696~ positive regulation of leukocyte activation	14	2.167	4.62E-05	3.979	0.07274
GOTERM_BP_5	GO:0030217~ T cell differentiation	11	1.703	4.98E-05	5.098	0.07849
KEGG_PATHWAY	hsa04650: Natural killer cell mediated cytotoxicity	15	2.322	6.72E-05	3.540	0.07803
KEGG_PATHWAY	hsa04640: Hematopoietic cell lineage	12	1.858	7.14E-05	4.380	0.0829
GOTERM_BP_5	GO:0042110~ T cell activation	15	2.322	7.17E-05	3.587	0.11297
GOTERM_BP_5	GO:0050867~ positive regulation of cell activation	14	2.167	7.50E-05	3.800	0.11818
GOTERM_BP_5	GO:0002694~ regulation of leukocyte activation	17	2.632	1.25E-04	3.085	0.19649
BIOCARTA	h_tcytotoxicPathway: T Cytotoxic Cell Surface Molecules	6	0.929	1.35E-04	10.264	0.16066
GOTERM_BP_5	GO:0030098~ lymphocyte differentiation	13	2.012	1.48E-04	3.802	0.23362
GOTERM_BP_5	GO:0016070~ RNA metabolic process	53	8.204	1.56E-04	1.702	0.24566
GOTERM_BP_5	GO:0050870~ positive regulation of T cell activation	11	1.703	1.92E-04	4.361	0.30245
GOTERM_BP_5	GO:0050778~ positive regulation of immune response	15	2.322	3.21E-04	3.117	0.50462
GOTERM_BP_5	GO:0051251~ positive regulation of lymphocyte activation	12	1.858	3.53E-04	3.727	0.55481
GOTERM_BP_5	GO:0051249~ regulation of lymphocyte activation	15	2.322	3.96E-04	3.053	0.62252
GOTERM_BP_5	GO:0002521~ leukocyte differentiation	14	2.167	3.99E-04	3.220	0.62756

BIOCARTA	h_tcrmolecule: T Cell Receptor and CD3 Complex	4	0.619	4.10E-04	20.529	0.4885
GOTERM_BP_5	GO:0002768~ immune response-regulating cell surface receptor signaling pathway	8	1.238	4.10E-04	5.739	0.64457
BIOCARTA	h_il17Pathway: IL 17 Signaling Pathway	6	0.929	4.56E-04	8.211	0.54293
GOTERM_BP_5	GO:0050863~ regulation of T cell activation	13	2.012	4.91E-04	3.347	0.77067
KEGG_PATHWAY	hsa04660: T cell receptor signaling pathway	12	1.858	5.52E-04	3.488	0.63899
GOTERM_BP_5	GO:0050851~ antigen receptor-mediated signaling pathway	7	1.084	6.58E-04	6.391	1.03269
KEGG_PATHWAY	hsa05340: Primary immunodeficiency	7	1.084	6.95E-04	6.278	0.80431
BIOCARTA	h_CSKPathway: Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	6	0.929	8.71E-04	7.245	1.0351
GOTERM_BP_5	GO:0001910~ regulation of leukocyte mediated cytotoxicity	6	0.929	0.001193	7.231	1.86369
GOTERM_BP_5	GO:0006396~ RNA processing	33	5.108	0.001293	1.818	2.01886
GOTERM_MF_5	GO:0004540~ ribonuclease activity	9	1.393	0.001383	4.127	1.61736
BIOCARTA	h_ctla4Pathway: The Co-Stimulatory Signal During T-cell Activation	6	0.929	0.001518	6.483	1.79754
GOTERM_BP_5	GO:0002757~ immune response-activating signal transduction	8	1.238	0.001531	4.635	2.38663
GOTERM_BP_5	GO:0045954~ positive regulation of natural killer cell mediated cytotoxicity	5	0.774	0.00157	9.415	2.44733
GOTERM_BP_5	GO:0002717~ positive regulation of natural killer cell mediated immunity	5	0.774	0.00157	9.415	2.44733
BIOCARTA	h_thelperPathway: T Helper Cell Surface Molecules	5	0.774	0.001801	8.554	2.13014
GOTERM_BP_5	GO:0042269~ regulation of natural killer cell mediated cytotoxicity	5	0.774	0.002	8.861	3.10764
GOTERM_BP_5	GO:0002715~ regulation of natural killer cell mediated immunity	5	0.774	0.002	8.861	3.10764
GOTERM_BP_5	GO:0002764~ immune response-regulating signal transduction	8	1.238	0.00237	4.304	3.67197
GOTERM_BP_5	GO:0034470~ ncRNA processing	15	2.322	0.003749	2.417	5.75022
GOTERM_BP_5	GO:0002253~ activation of immune response	10	1.548	0.003885	3.205	5.95246
BIOCARTA	h_ctlPathway: CTL mediated immune response against target cells	5	0.774	0.004451	6.843	5.18769
GOTERM_BP_5	GO:0001912~ positive regulation of leukocyte mediated cytotoxicity	5	0.774	0.004531	7.173	6.90898
GOTERM_BP_5	GO:0045619~ regulation of lymphocyte differentiation	8	1.238	0.004642	3.826	7.07205
GOTERM_BP_5	GO:0006935~ chemotaxis	13	2.012	0.006931	2.448	10.3857
BIOCARTA	h_tcapoptosisPathway: HIV Induced T Cell Apoptosis	4	0.619	0.00723	9.124	8.30051

S Table 10.3 DAVID analysis of upregulated transcripts in *B. henselae*-infected MACs at d53. Clustering of enriched, functional annotations to related functional groups. Minimum similarity overlap > 3 (*P* < 0.01).

Annotation Cluster 1	**Enrichment Score: 9.612**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0001944~ vasculature development	31	6.008	1.09E-10	4.081	1.82E-07
GOTERM_BP_5	GO:0048514~ blood vessel morphogenesis	28	5.426	2.04E-10	4.385	3.43E-07
GOTERM_BP_5	GO:0001568~ blood vessel development	30	5.814	2.82E-10	4.046	4.74E-07
GOTERM_BP_5	GO:0001525~ angiogenesis	23	4.457	5.72E-10	5.135	9.62E-07
Annotation Cluster 2	**Enrichment Score: 8.141**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0042981~ regulation of apoptosis	58	11.240	1.06E-09	2.384	1.78E-06
GOTERM_BP_5	GO:0043067~ regulation of programmed cell death	58	11.240	1.56E-09	2.360	2.63E-06
GOTERM_BP_5	GO:0060548~ negative regulation of cell death	35	6.783	3.42E-09	3.213	5.75E-06
GOTERM_BP_5	GO:0043066~ negative regulation of apoptosis	34	6.589	8.16E-09	3.174	1.37E-05
GOTERM_BP_5	GO:0043069~ negative regulation of programmed cell death	34	6.589	1.15E-08	3.129	1.94E-05
GOTERM_BP_5	GO:0006916~ anti-apoptosis	23	4.457	2.68E-07	3.689	4.51E-04
Annotation Cluster 3	**Enrichment Score: 7.176**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0042981~ regulation of apoptosis	58	11.240	1.06E-09	2.384	1.78E-06
GOTERM_BP_5	GO:0043067~ regulation of programmed cell death	58	11.240	1.56E-09	2.360	2.63E-06
GOTERM_BP_5	GO:0043065~ positive regulation of apoptosis	34	6.589	8.10E-07	2.613	0.00136
GOTERM_BP_5	GO:0043068~ positive regulation of programmed cell death	34	6.589	9.45E-07	2.595	0.00159
GOTERM_BP_5	GO:0010942~ positive regulation of cell death	34	6.589	1.04E-06	2.583	0.00176
Annotation Cluster 4	**Enrichment Score: 5.575**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0009891~ positive regulation of biosynthetic process	49	9.496	5.87E-08	2.330	9.87E-05
GOTERM_BP_5	GO:0010557~ positive regulation of macromolecule biosynthetic process	47	9.109	6.78E-08	2.375	1.14E-04
GOTERM_BP_5	GO:0031328~ positive regulation of cellular biosynthetic process	48	9.302	9.98E-08	2.315	1.68E-04
GOTERM_BP_5	GO:0051173~ positive regulation of nitrogen compound metabolic process	43	8.333	1.94E-06	2.206	0.00326
GOTERM_BP_5	GO:0010628~ positive regulation of gene expression	39	7.558	5.79E-06	2.218	0.00974
GOTERM_BP_5	GO:0031325~ positive regulation of cellular metabolic process	51	9.884	1.00E-05	1.915	0.01683
GOTERM_BP_5	GO:0010604~ positive regulation of macromolecule metabolic process	50	9.690	1.05E-05	1.928	0.01766
GOTERM_BP_5	GO:0045935~ positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	40	7.752	1.25E-05	2.118	0.02099
GOTERM_BP_5	GO:0045941~ positive regulation of	37	7.171	1.75E-05	2.168	0.02938

	transcription					
GOTERM_BP_5	GO:0045893~ positive regulation of transcription, DNA-dependent	33	6.395	1.98E-05	2.286	0.0332
GOTERM_BP_5	GO:0051254~ positive regulation of RNA metabolic process	33	6.395	2.33E-05	2.267	0.03918
Annotation Cluster 5	**Enrichment Score: 4.732**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0006935~ chemotaxis	21	4.070	7.14E-08	4.337	1.20E-04
GOTERM_MF_5	GO:0008009~ chemokine activity	10	1.938	6.01E-06	7.388	0.00747
GOTERM_MF_5	GO:0042379~ chemokine receptor binding	10	1.938	1.04E-05	6.936	0.0129
KEGG_PATHWAY	hsa04060: Cytokine-cytokine receptor interaction	25	4.845	1.34E-04	2.333	0.1584
KEGG_PATHWAY	hsa04062:C hemokine signaling pathway	17	3.295	0.00366	2.222	4.25603
Annotation Cluster 6	**Enrichment Score: 4.283**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0051272~ positive regulation of cell motion	13	2.519	3.75E-05	4.383	0.06303
GOTERM_BP_5	GO:0030334~ regulation of cell migration	17	3.295	5.21E-05	3.324	0.08757
GOTERM_BP_5	GO:0030335~ positive regulation of cell migration	12	2.326	7.23E-05	4.455	0.12146
Annotation Cluster 7	**Enrichment Score: 4.171**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0010647~ positive regulation of cell communication	27	5.233	7.34E-06	2.712	0.01235
GOTERM_BP_5	GO:0009966~ regulation of signal transduction	51	9.884	9.37E-06	1.919	0.01575
GOTERM_BP_5	GO:0009967~ positive regulation of signal transduction	25	4.845	1.02E-05	2.800	0.01718
GOTERM_BP_5	GO:0010740~ positive regulation of protein kinase cascade	15	2.907	5.35E-04	2.968	0.89582
GOTERM_BP_5	GO:0010627~ regulation of protein kinase cascade	17	3.295	0.0037	2.256	6.03877
Annotation Cluster 8	**Enrichment Score: 3.867**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0045637~ regulation of myeloid cell differentiation	12	2.326	7.15E-06	5.665	0.01202
GOTERM_BP_5	GO:0045639~ positive regulation of myeloid cell differentiation	8	1.550	4.67E-05	8.010	0.07855
GOTERM_BP_5	GO:0002761~ regulation of myeloid leukocyte differentiation	6	1.163	0.00748	4.836	11.861
Annotation Cluster 9	**Enrichment Score: 3.599**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0031347~ regulation of defense response	18	3.488	1.41E-06	4.159	0.00237
GOTERM_BP_5	GO:0045088~ regulation of innate immune response	9	1.744	1.97E-04	5.507	0.33107
GOTERM_BP_5	GO:0031349~ positive regulation of defense response	10	1.938	3.33E-04	4.526	0.55778
GOTERM_BP_5	GO:0045089~ positive regulation of innate immune response	7	1.357	0.00245	5.028	4.04257

GOTERM_BP_5	GO:0050778~ positive regulation of immune response	12	2.326	0.00448	2.735	7.26542
Annotation Cluster 10	**Enrichment Score: 3.279**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
KEGG_PATHWAY	hsa00100: Steroid biosynthesis	7	1.357	3.60E-05	10.066	0.04268
GOTERM_BP_5	GO:0016126~ sterol biosynthetic process	7	1.357	5.61E-04	6.609	0.93952
GOTERM_BP_5	GO:0016125~ sterol metabolic process	11	2.132	9.29E-04	3.599	1.55132
GOTERM_BP_5	GO:0006694~ steroid biosynthetic process	9	1.744	0.00406	3.499	6.61473
Annotation Cluster 11	**Enrichment Score: 3.112**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
KEGG_PATHWAY	hsa04620: Toll-like receptor signaling pathway	14	2.713	1.98E-04	3.389	0.23516
BIOCARTA	h_il1rPathway: Signal transduction through IL1R	9	1.744	3.67E-04	4.645	0.44318
BIOCARTA	h_nthiPathway: NFkB activation by Nontypeable Hemophilus influenzae	8	1.550	6.05E-04	4.990	0.72872
BIOCARTA	h_cdMacPathway: Cadmium induces DNA synthesis and proliferation in macrophages	5	0.969	0.00807	5.757	9.33409
Annotation Cluster 12	**Enrichment Score: 2.975**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0050867~ positive regulation of cell activation	13	2.519	1.28E-04	3.870	0.21514
GOTERM_BP_5	GO:0032946~ positive regulation of mononuclear cell proliferation	9	1.744	2.55E-04	5.310	0.42869
GOTERM_BP_5	GO:0070665~ positive regulation of leukocyte proliferation	9	1.744	2.55E-04	5.310	0.42869
GOTERM_BP_5	GO:0002696~ positive regulation of leukocyte activation	12	2.326	3.48E-04	3.741	0.58429
GOTERM_BP_5	GO:0032944~ regulation of mononuclear cell proliferation	10	1.938	9.43E-04	3.934	1.57411
GOTERM_BP_5	GO:0070663~ regulation of leukocyte proliferation	10	1.938	9.43E-04	3.934	1.57411
GOTERM_BP_5	GO:0050671~ positive regulation of lymphocyte proliferation	8	1.550	0.00126	4.806	2.0911
GOTERM_BP_5	GO:0032663~ regulation of interleukin-2 production	6	1.163	0.00186	6.609	3.08897
GOTERM_BP_5	GO:0051251~ positive regulation of lymphocyte activation	10	1.938	0.0026	3.406	4.28407
GOTERM_BP_5	GO:0050670~ regulation of lymphocyte proliferation	9	1.744	0.00351	3.583	5.73519
GOTERM_BP_5	GO:0002694~ regulation of leukocyte activation	13	2.519	0.00451	2.588	7.31276
GOTERM_BP_5	GO:0050870~ positive regulation of T cell activation	8	1.550	0.0079	3.478	12.4854
Annotation Cluster 13	**Enrichment Score: 2.944**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0030097~ hemopoiesis	18	3.488	8.21E-04	2.520	1.37152
GOTERM_BP_5	GO:0048534~ hemopoietic or lymphoid organ development	19	3.682	9.24E-04	2.415	1.54206
GOTERM_BP_5	GO:0030099~ myeloid cell differentiation	10	1.938	0.00194	3.553	3.21937
Annotation Cluster 14	**Enrichment Score: 2.910**					

Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0070304~ positive regulation of stress-activated protein kinase signaling pathway	6	1.163	3.33E-04	9.441	0.558
GOTERM_BP_5	GO:0032874~ positive regulation of stress-activated MAPK cascade	4	0.775	0.002	14.686	3.31084
GOTERM_BP_5	GO:0032872~ regulation of stress-activated MAPK cascade	4	0.775	0.00279	13.217	4.5957
Annotation Cluster 15	**Enrichment Score: 2.899**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0050900~ leukocyte migration	9	1.744	2.90E-04	5.217	0.48564
GOTERM_BP_5	GO:0060326~ cell chemotaxis	7	1.357	0.00102	5.931	1.69999
GOTERM_BP_5	GO:0030593~ neutrophil chemotaxis	5	0.969	0.00179	9.179	2.96021
GOTERM_BP_5	GO:0030595~ leukocyte chemotaxis	6	1.163	0.0048	5.358	7.77107
Annotation Cluster 16	**Enrichment Score: 2.756**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0032768~ regulation of monooxygenase activity	7	1.357	7.90E-05	9.252	0.13277
GOTERM_BP_5	GO:0051353~ positive regulation of oxidoreductase activity	5	0.969	0.00721	6.354	11.4614
GOTERM_BP_5	GO:0032642~ regulation of chemokine production	4	0.775	0.00947	8.811	14.787
Annotation Cluster 17	**Enrichment Score: 2.675**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0019220~ regulation of phosphate metabolic process	28	5.426	0.00167	1.908	2.77594
GOTERM_BP_5	GO:0051174~ regulation of phosphorus metabolic process	28	5.426	0.00167	1.908	2.77594
GOTERM_BP_5	GO:0051347~ positive regulation of transferase activity	17	3.295	0.00256	2.341	4.22485
GOTERM_BP_5	GO:0043549~ regulation of kinase activity	22	4.264	0.00278	2.036	4.57764
Annotation Cluster 18	**Enrichment Score: 2.650**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0001893~ maternal placenta development	5	0.969	8.56E-04	11.014	1.42906
GOTERM_BP_5	GO:0001890~ placenta development	8	1.550	0.0021	4.406	3.47608
GOTERM_BP_5	GO:0046697~ decidualization	4	0.775	0.00623	10.167	9.9683
Annotation Cluster 19	**Enrichment Score: 2.621**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0032675~ regulation of interleukin-6 production	7	1.357	6.57E-04	6.425	1.09831
GOTERM_BP_5	GO:0032755~ positive regulation of interleukin-6 production	5	0.969	0.00221	8.696	3.64908
GOTERM_BP_5	GO:0032642~ regulation of chemokine production	4	0.775	0.00947	8.811	14.787
Annotation Cluster 20	**Enrichment Score: 2.533**					

Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0050714~ positive regulation of protein secretion	7	1.357	0.00134	5.641	2.22153
GOTERM_BP_5	GO:0050708~ regulation of protein secretion	8	1.550	0.00172	4.558	2.85755
GOTERM_BP_5	GO:0050707~ regulation of cytokine secretion	6	1.163	0.00186	6.609	3.08897
GOTERM_BP_5	GO:0051223~ regulation of protein transport	11	2.132	0.00233	3.188	3.84959
GOTERM_BP_5	GO:0070201~ regulation of establishment of protein localization	11	2.132	0.0036	3.004	5.88774
GOTERM_BP_5	GO:0051222~ positive regulation of protein transport	8	1.550	0.00396	3.945	6.44618
GOTERM_BP_5	GO:0050715~ positive regulation of cytokine secretion	5	0.969	0.00459	7.183	7.43981
GOTERM_BP_5	GO:0051046~ regulation of secretion	14	2.713	0.00834	2.290	13.1338

S Table 10.4 DAVID analysis of downregulated transcripts in *B. henselae*-infected MACs at d53. Clustering of enriched, functional annotations to related functional groups. Minimum similarity overlap > 3 (*P* < 0.01).

Annotation Cluster 1	**Enrichment Score: 10.621**					
Category	Term	Count	%	*P* Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0045449~ regulation of transcription	147	22.755	2.75E-12	1.703	4.34E-09
GOTERM_BP_5	GO:0010468~ regulation of gene expression	156	24.149	6.08E-12	1.646	9.58E-09
GOTERM_BP_5	GO:0010556~ regulation of macromolecule biosynthetic process	154	23.839	1.27E-11	1.639	2.00E-08
GOTERM_BP_5	GO:0019219~ regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	153	23.684	1.67E-11	1.638	2.64E-08
GOTERM_BP_5	GO:0031326~ regulation of cellular biosynthetic process	158	24.458	1.87E-11	1.615	2.95E-08
GOTERM_BP_5	GO:0006355~ regulation of transcription, DNA-dependent	109	16.873	6.04E-11	1.852	9.52E-08
GOTERM_BP_5	GO:0051252~ regulation of RNA metabolic process	110	17.028	1.06E-10	1.828	1.67E-07
GOTERM_MF_5	GO:0046914~ transition metal ion binding	145	22.446	2.56E-10	1.576	3.02E-07
Annotation Cluster 2	**Enrichment Score: 3.864**					
Category	Term	Count	%	P Value	Fold Enrichment	FDR
BIOCARTA	h_tcraPathway: Lck and Fyn tyrosine kinases in initiation of TCR Activation	7	1.084	3.78E-06	13.064	0.004509
GOTERM_BP_5	GO:0030217~ T cell differentiation	11	1.703	4.98E-05	5.098	0.07849
GOTERM_BP_5	GO:0042110~ T cell activation	15	2.322	7.17E-05	3.587	0.112972
GOTERM_BP_5	GO:0030098~ lymphocyte differentiation	13	2.012	1.48E-04	3.802	0.233619
GOTERM_BP_5	GO:0051249~ regulation of lymphocyte activation	15	2.322	3.96E-04	3.053	0.622524
GOTERM_BP_5	GO:0002521~ leukocyte differentiation	14	2.167	3.99E-04	3.220	0.627556
KEGG_PATHWAY	hsa04660: T cell receptor signaling pathway	12	1.858	5.52E-04	3.488	0.638988
KEGG_PATHWAY	hsa05340: Primary immunodeficiency	7	1.084	6.95E-04	6.278	0.804308

Annotation Cluster 3	**Enrichment Score: 3.653**					
Category	Term	Count	%	P Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0002696~ positive regulation of leukocyte activation	14	2.167	4.62E-05	3.979	0.072736
GOTERM_BP_5	GO:0030217~ T cell differentiation	11	1.703	4.98E-05	5.098	0.07849
GOTERM_BP_5	GO:0050867~ positive regulation of cell activation	14	2.167	7.50E-05	3.800	0.118177
GOTERM_BP_5	GO:0002694~ regulation of leukocyte activation	17	2.632	1.25E-04	3.085	0.196494
GOTERM_BP_5	GO:0050870~ positive regulation of T cell activation	11	1.703	1.92E-04	4.361	0.302447
GOTERM_BP_5	GO:0051251~ positive regulation of lymphocyte activation	12	1.858	3.53E-04	3.727	0.554805
GOTERM_BP_5	GO:0051249~ regulation of lymphocyte activation	15	2.322	3.96E-04	3.053	0.622524
GOTERM_BP_5	GO:0050863~ regulation of T cell activation	13	2.012	4.91E-04	3.347	0.77067
GOTERM_BP_5	GO:0045619~ regulation of lymphocyte differentiation	8	1.238	0.00464	3.826	7.072051
Annotation Cluster 4	**Enrichment Score: 3.445**					
Category	Term	Count	%	P Value	Fold Enrichment	FDR
BIOCARTA	h_tcraPathway: Lck and Fyn tyrosine kinases in initiation of TCR Activation	7	1.084	3.78E-06	13.064	0.004509
BIOCARTA	h_tcrPathway: T Cell Receptor Signaling Pathway	10	1.548	1.61E-05	6.038	0.019234
KEGG_PATHWAY	hsa04640: Hematopoietic cell lineage	12	1.858	7.14E-05	4.380	0.082903
BIOCARTA	h_tcytotoxicPathway: T Cytotoxic Cell Surface Molecules	6	0.929	1.35E-04	10.264	0.160658
BIOCARTA	h_tcrmolecule: T Cell Receptor and CD3 Complex	4	0.619	4.10E-04	20.529	0.4885
BIOCARTA	h_il17Pathway: IL 17 Signaling Pathway	6	0.929	4.56E-04	8.211	0.542926
KEGG_PATHWAY	hsa04660: T cell receptor signaling pathway	12	1.858	5.52E-04	3.488	0.638988
BIOCARTA	h_CSKPathway: Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor	6	0.929	8.71E-04	7.245	1.035101
BIOCARTA	h_ctla4Pathway: The Co-Stimulatory Signal During T-cell Activation	6	0.929	0.00152	6.483	1.797537
BIOCARTA	h_thelperPathway: T Helper Cell Surface Molecules	5	0.774	0.0018	8.554	2.130141
BIOCARTA	h_ctlPathway: CTL mediated immune response against target cells	5	0.774	0.00445	6.843	5.187692
BIOCARTA	h_tcapoptosisPathway: HIV Induced T Cell Apoptosis	4	0.619	0.00723	9.124	8.300512
Annotation Cluster 5	**Enrichment Score: 3.334**					
Category	Term	Count	%	P Value	Fold Enrichment	FDR
BIOCARTA	h_tcraPathway: Lck and Fyn tyrosine kinases in initiation of TCR Activation	7	1.084	3.78E-06	13.064	0.004509
GOTERM_BP_5	GO:0050778~ positive regulation of immune response	15	2.322	3.21E-04	3.117	0.504624
GOTERM_BP_5	GO:0002768~ immune response-regulating cell surface receptor signaling pathway	8	1.238	4.10E-04	5.739	0.644569

GOTERM_BP_5	GO:0050851~ antigen receptor-mediated signaling pathway	7	1.084	6.58E-04	6.391	1.032685
GOTERM_BP_5	GO:0002757~ immune response-activating signal transduction	8	1.238	0.00153	4.635	2.38663
GOTERM_BP_5	GO:0002764~ immune response-regulating signal transduction	8	1.238	0.00237	4.304	3.671966
GOTERM_BP_5	GO:0002253~ activation of immune response	10	1.548	0.00389	3.205	5.952457
Annotation Cluster 6	**Enrichment Score: 2.712**					
Category	Term	Count	%	P Value	Fold Enrichment	FDR
GOTERM_BP_5	GO:0001910~ regulation of leukocyte mediated cytotoxicity	6	0.929	0.00119	7.231	1.863695
GOTERM_BP_5	GO:0002717~ positive regulation of natural killer cell mediated immunity	5	0.774	0.00157	9.415	2.447332
GOTERM_BP_5	GO:0045954~ positive regulation of natural killer cell mediated cytotoxicity	5	0.774	0.00157	9.415	2.447332
GOTERM_BP_5	GO:0002715~ regulation of natural killer cell mediated immunity	5	0.774	0.002	8.861	3.10764
GOTERM_BP_5	GO:0042269~ regulation of natural killer cell mediated cytotoxicity	5	0.774	0.002	8.861	3.10764
GOTERM_BP_5	GO:0001912~ positive regulation of leukocyte mediated cytotoxicity	5	0.774	0.00453	7.173	6.908978

11. Acknowledgements

12. Peer Reviewed Publications

12.1 Scientific publications

F. O'Rourke, T. Mändle, C. Urbich , S. Dimmeler, U. R. Michaelis, R. P. Brandes, M. Flötenmeyer, C. Döring, M.-L. Hansmann, K. Lauber, W. Ballhorn, V. A.J. Kempf. (2015) Reprogramming of Myeloid Angiogenic Cells by *Bartonella henselae* leads to microenvironmental regulation of pathological angiogenesis. *Cellular Microbiology.* e-pub ahead of print

P. O. Kaiser, T. Riess, **F. O'Rourke**, D. Linke, V. A.J. Kempf. (2011) *Bartonella* spp.: Throwing light on uncommon human infections. *Internation Journal of Medical Microbiology*, **301** (1): 7-15

F. O'Rourke, T. Schmidgen, P. O. Kaiser, D. Linke, V. A.J. Kempf: Adhesins of *Bartonella* spp. (2011); Chapter in: "Bacterial Adhesion: Biology, Chemistry, and Physics". Springer Verlag (Book series "*Advances in Experimental Biology and Medicine*"). **715** : 51-70

C. Beerlage, **F. O'Rourke**, V. A.J. Kempf. (2011) Wenn Bartonellen ihre Anker auswerfen- *Bartonella henselae* verbindet Infektionsforschung mit Blutgefäßwachstum. *Forschung Frankfurt.* **2**: 26-29

12.2 Oral presentations

F. O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, U. R. Michaelis, R. P. Brandes, M. Flötenmeyer, C. Döring, M.-L. Hansmann, K. Lauber W. Ballhorn, V. A.J. Kempf: Reprogramming of myeloid angiogenic cells by *Bartonella henselae* leads to microenvironmental regulation of pathological angiogenesis, 66th annual Meeting of the German Society of Microbiology and Infection Control (DGHM), 05.-08.10.2014, Dresden

F. O'Rourke: Interactions of the intercellular pathogen *Bartonella henselae* with myeloid angiogenic cells; implications for infection associated angiogenesis, Invited Speaker, Sick Kids Hospital, Inflammatory Bowel Disease (IBD) Center, 06.01.2014, Toronto Canada

F. O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, U. R. Michaelis, R. P. Brandes, K. Lauber, V. A.J. Kempf: Infection of myeloid angiogenic cells with *Bartonella henselae* results in increased pro-angiogenic potential, 5th Congress of European Microbiologists FEMS, 7-11.06.2013, Leipzig

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, V. A.J. Kempf : Infection of myeloid angiogenic cells with *Bartonella henselae* induces vessel-like growth *in vitro*, 64th annual Meeting of the German Society of Microbiology and Infection Control (DGHM), 30.09.-03.10.2012, Hamburg

F. O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, U. R. Michaelis, R. P. Brandes, K. Lauber, V. A.J. Kempf: Infection of myeloid angiogenic cells with *Bartonella henselae* induces vessel-like growth *in vitro*, Symposium of the DGHM-Focus Group Microbial Pathogenicity, 18-20.06.2012, Bad Urach

12.3 Poster presentations

F. O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, U. R. Michaelis, R. P. Brandes, M. Flötenmeyer, C. Döring, M.-L. Hansmann, K. Lauber, V. A.J. Kempf: Infection of myeloid angiogenic cells with *Bartonella henselae* results in increased pro-angiogenic effects, 65th annual Meeting of the German Society of Microbiology and Infection Control (DGHM), 22-25.09.2013, Rostock

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, V. A.J. Kempf : Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel-like growth *in vitro,* IVBM 2012, 17th International Vascular Biology Meeting, 2-5.06.2012, Wiesbaden

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, V. A.J. Kempf : Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel-like growth *in vitro,* annual Meeting of the Association for General and Applied Microbiology (VAAM), 18.-21.03.2012, Tübingen

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, M. Schaller, V. A.J. Kempf : Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel growth *in vitro*, 9th annual Meeting of the International Society for Stem Cell Research, 15-18.06.2011, Toronto, Canada

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, V. A.J. Kempf : Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel growth *in vitro*, 2nd International Symposium of the SFB 766 Bacterial Cell Envelope, 23-25.05.2011, Kaufbeuren

F. O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, U. R. Michaelis, R. P. Brandes, K. Lauber, V. A.J. Kempf: Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel growth *in vitro,* 63th annual Meeting of the German Society of Microbiology and Infection Control (DGHM), 25-28.09.2011, Essen

F O'Rourke, T. Mändle, C. Urbich, S. Dimmeler, K. Lauber, M. Schaller, V. A.J. Kempf : Infection of human endothelial progenitor cells with *Bartonella henselae* induces vessel growth *in vitro,* 2011 Joint Meeting of the European Society of Microcirculation and the German Society of Microcirculation and Vascular Biology (GfMVB), 13-16.10.2011, Munich

13. Curriculum Vitae