Detrimental effects of *Bartonella henselae* are counteracted by L-arginine and nitric oxide in human endothelial progenitor cells

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The recruitment of circulating endothelial progenitor cells (EPCs) might have a beneficial effect on the clinical course of several diseases. Endothelial damage and detachment of endothelial cells are known to occur in infection, tissue ischemia, and sepsis. These detrimental effects in EPCs are unknown. Here we elucidated whether human EPCs internalize *Bartonella henselae* constituting a circulating niche of the pathogen. *B. henselae* invades EPCs as shown by gentamicin protection assays and transmission electron microscopy (TEM). Dil-Ac-LDL/lectin double immunostaining and fluorescence-activated cell sorting (FACS) analysis of EPCs revealed EPC bioactivity after infection with *B. henselae*. Nitric oxide (NO) and its precursor L-arginine (L-arg) exert a plethora of beneficial effects on vascular function and modulation of immune response. Therefore, we tested also the hypothesis that L-arg (1–30 mM) would affect the infection of *B. henselae* or tumor necrosis factor (TNF) in EPCs. Our data provide evidence that L-arg counteracts detrimental effects induced by TNF or *Bartonella* infections via NO (confirmed by DETA-NO and L-NMMA experiments) and by modulation of p38 kinase phosphorylation. Microarray analysis indicated several genes involved in immune response were differentially expressed in *Bartonella*-infected EPCs, whereas these genes returned in steady state when cells were exposed to sustained doses of L-arg. This mechanism may have broad therapeutic applications in tissue ischemia, angiogenesis, immune response, and sepsis.

immune response | sepsis

Bacterial toxins and cytokines initiate a series of pathogenic events altering endothelial function, capillary leakage, and endothelial swelling (1). Physiologically, endothelial cells (ECs) are attached to the extracellular matrix; however, under pathologic conditions, ECs can be detached from the vasculature appearing in the bloodstream (2). The production of antiangiogenic factors is another cause for the release of ECs into the circulation (3). ECs have a low proliferative potential; thus, adequate vascular repair requires additional support. Growing studies established that vascular repair and neoangiogenesis are mediated, at least in part, by bone marrow stem cells (BMCs) (4–6) and endothelial progenitor cells (EPCs) (7, 8). BMCs (9) would be important targets for some human pathogens such as *Bartonella henselae*, a Gram-negative, facultative intracellular bacteria that cause a variety of human diseases (10). Mechanisms underlying vasculoproliferations in *B. henselae* infections include inhibition of EC apoptosis (11), activation of hypoxia-inducible factor 1 and vascular endothelial growth factor (12), and release of calcium from intracellular stores (13). Overall, *Bartonella* may also promote vascular tumor formation (14).

The interaction of human pathogen bacteria with EPCs is unknown. Indirectly, we can gain information by the evidence that precursor hematopoietic cells were resistant to *in vitro* infection with *Listeria monocytogenes*, *Salmonella enteritica*, and *Yersinia enterocolitica*, but not when these cells were differentiated (15). Interestingly, the genus *Bartonella* can cause long-lasting intraerythrocytic bacteremia and employ hematotropism (16). Moreover, *B. henselae* is the cause of cat-scratch disease and the vasculoproliferative bacillary angiomatosis and bacillary peliosis in humans (17). It has been proposed an essential role for NO in T lymphocyte activation and signal transduction (18, 19). Indeed, NO acts as a second messenger, activating soluble guanyl cyclase and modulating mitochondrial events involved in apoptosis and biogenesis in lymphocytes. Mechanisms for beneficial effects of L-arginine (L-arg), the precursor of NO, in sepsis include enhanced protein metabolism, improved microcirculation, and antibacterial effects (20, 21). Here we describe that *B. henselae* infects human EPCs. Moreover, L-arg modulates the EPC response induced by TNF or *Bartonella* infections.

Results

Effect of L-Arginine on Human EPC Number and Viability. Human EPCs cultured for 3 days resulted in an adherent population consisting of 23 ± 4% double-positive cells for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated LDL (Dil-Ac-LDL) uptake (red) and lectin binding (green) (Fig. 1*A*), as assessed by phase contrast fluorescent microscopy (22). By using a specific counting (22), we establish that EPC number was

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Conflict of interest statement: Louis J. Ignarro helped develop and has a financial interest in a commercially available dietary supplement that contains some of the amino acids studied in this article.

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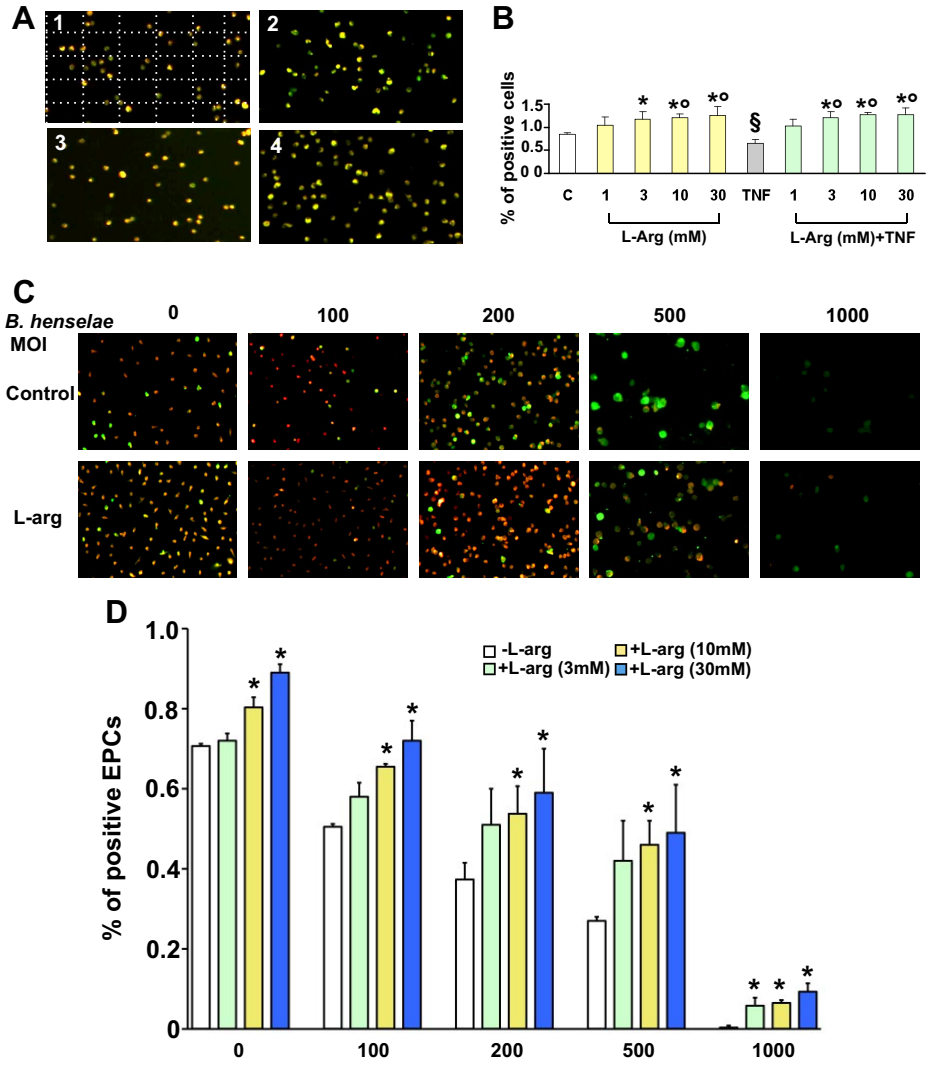

Fig. 1. Effects of L-arg in presence of TNF-α or *B. henselae* on EPCs. (*A* and *B*) Dil-Ac-LDL uptake and lectin binding staining of EPCs by microscopy. (*A*) Photomicrographs of merged double-positive Dil-Ac-LDL/Lectin cells (Magnification: $\times$100): untreated EPCs (*A*1), L-arg (10mM)-treated EPCs (*A*2), TNF-α-treated EPCs (*A*3), L-arg + TNF-α-treated cells (*A*4). (*B*) The bar graphs show EPC number calculated as described (22). Results are mean $\pm$ SD of 13 different experiments in duplicate. *, $P < 0.05$ vs. untreated controls; §, $P < 0.05$ vs. untreated control and L-arg; °, $P < 0.05$ vs. TNF-α. (*C* and *D*) Dil-Ac-LDL uptake and lectin binding staining of EPCs infected with *Bartonella* at different MOI, as described in *Results*. In *C* an image for each *B. henselae* infection point pretreated with or without L-arg at the indicated concentration is shown at $\times$100 magnification. In *D* the bar graphs indicate the percentage of EPC number. Results are representative of five different experiments in duplicate. *, $P < 0.05$ vs. L-arg untreated.

significantly ameliorated in the presence of L-arg (3–30 mM) both under basal conditions and in the presence of TNF-α (Fig. 1*A*). Treatment of EPCs with TNF-α impairs EPC functional activity and number (22, 23). Accordingly, when EPCs were incubated with TNF-α, the cell number consistently decreased compared with control cells (Fig. 1*B*). The presence of L-arg during treatment with TNF-α prevented the TNF-α-induced reduction of cell number ($P < 0.05$ vs. TNF-α).

EPCs (22) were then infected with *B. henselae* at different multiplicities of infection (MOI) in the presence or absence of L-arg. Interestingly, results indicated that EPC number and viability were ameliorated in the presence of L-arg at the MOI used for *Bartonella* infection (Fig. 1*C*). Indeed, infection of EPCs with *Bartonella* impaired EPC functional activity and reduced cell number, which consistently decreased compared with uninfected cells ($P < 0.05$ vs. untreated cells) (Fig. 1*D*). Pretreatment of EPCs with L-arg (10–30 mM) before infection significantly prevented the *Bartonella*-induced reduction of cell number ($P < 0.05$ vs. untreated cells). Infection with *B. henselae* was further investigated by fluorescence and confocal microscopy. Higher magnifications ($\times$630) indicate morphological major differences when EPCs are infected with increasing doses of *Bartonella* [supporting information (SI) Fig. S1]. These changes appear at higher doses when EPCs are pretreated with L-arg before infection as shown with a MOI of 500 suggesting a protective action of L-arg on EPCs infected with *B. henselae* (Fig. 2). Particularly, cell aggregates begin to appear at a MOI of 200 until to develop a syncytium at a MOI of 500. At a 500–1,000 MOI we observed cytoplasmatic disruption and loss of cellular membrane integrity; at 1,000 MOI no cells showed Dil-AcLDL uptake (Fig. S1)

***B. henselae* Adheres to and Invades Human EPCs.** We investigated the interaction of EPCs with *B. henselae* by transmission electron microscopy (TEM). Isolated EPCs were infected with *B. henselae* for up to 72 h and both cell morphology and bacteria internalization were evaluated on EPCs at 3 days after infection (Fig. 3*A–I*). The ultrastructural examination revealed that adhesion of *B. henselae* to cells was accompanied by the formation of cytoplasmic protrusions of cell membranes, followed by internalization of bacteria, detectable in vacuoles (Fig. 3 *H* and *I*). Invasion of *B. henselae* into EPCs resulted in two different intracellular patterns: diffuse perinuclear bacteria localization, similar to a distribution pattern already described (24), and intracellular accumulation of bacteria forming the so-called "invasomes" (25). Moreover, dividing intracellular bacteria were also frequently observed. The examination of untreated EPCs revealed normal morphology. EPCs infected with *B. henselae* at a MOI of 500 showed mitochondria disruption and expansion of endoplasmatic reticulum. In contrast, EPCs pretreated with L-arg before infection with *B. henselae*, even at a MOI of 500, presented a reduced number of intracellular invasomes, intact cytoplasmatic mitochondria, and a modest expansion of smooth endoplasmatic

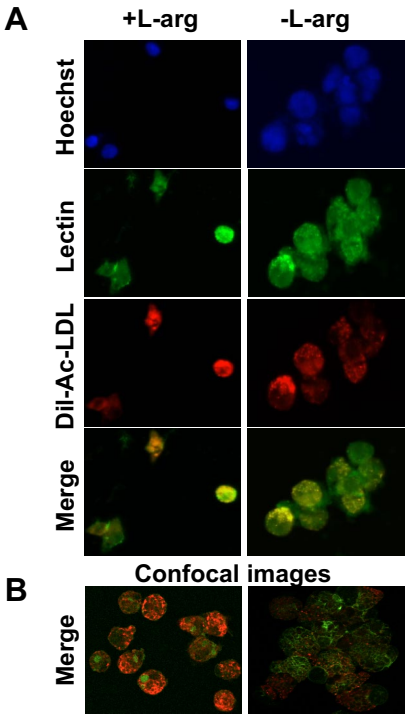

Fig. 2. *Bartonella* and L-arginine. EPCs infected with *Bartonella* at a MOI of 500 in the presence or absence of L-arg. (*A*) Representative images by immunofluorescence. (*B*) Selected merge images were acquired by confocal microscopy. (Magnification: all, ×630.)

reticulum compared with untreated cells. These data clearly show that *B. henselae* adheres to and invades human EPCs, also confirming the protective role of L-arg on EPCs infected with *B. henselae*. Furthermore, gentamicin protection assays quantifying the number of intracellular *B. henselae* revealed a dose-dependent increase (Fig. 3*L*).

Flow Cytometry Analysis. EPCs were incubated with Dil-Ac-LDL and FITC-labeled lectin from *Ulex europaeus* (see *Materials and Methods*). As indicated by representative four-quadrant FACS analysis of Dil-Ac-LDL/lectin double-positive EPCs with different treatments (Fig. 4*A*) and the corresponding bar graphs of EPC percent number (Fig. 4*B*), EPC number was significantly higher when cells were grown in the presence of L-arg. We considered the lymphocyte population for counting (see the inserted forward and sideward scatter plot image to the right of Fig. 4 *A* and *B*). We also investigated the effects of L-arg on *Bartonella*-infected EPCs at different MOI by FACS analysis. A representative forward and sideward scatter analysis for each indicated *Bartonella* MOI is shown (Fig. 4*C*). As shown in Fig. 4*C*, a novel cell population appeared in the blue area (named population 3), which essentially represents myelomonocytic cells CD13/CD15 positive (Fig. 4*C Lower Left*, similar to granulocytes). Bar graphs indicate the percentage of double-positive cell number (Fig. 4*D*) for different MOI. Considering the lymphocyte gate, EPC number was significantly reduced when infected with *B. henselae* at different MOI, with a protective action of L-arg ($P < 0.05$ vs. L-arg-untreated EPCs). Parallel experiments were carried out also in the presence of NO donor diethylamine-NO (DETA-NO) or NG-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis, which showed the involvement of NO in such mechanism.

p38 MAP Kinase Activation and NOx Release. The effect of TNF-α and *Bartonella* on EPC number was investigated in relation to p38 MAP kinase activation (22, 23). In our experimental conditions, we show that increased p38 phosphorylation after treatment with TNF-α was counteracted by L-arg ($P < 0.05$ vs. untreated control cells) (Fig. S2*A*). Such treatment did not influence significantly p38 after exposure to *Bartonella* indicating the involvement of other signaling events (Fig. S2*B*). Finally, NOx (nitrate and nitrite) release increased significantly after exposure of EPCs to L-arg in comparison to untreated cells (4.2 ± 0.5 vs. 2.2 ± 0.3 μM, $P < 0.01$) whereas it decreased after exposure to *Bartonella* (MOI 200, 1.6 ± 0.3 μM, $P < 0.05$ vs. untreated cells). Consistently, the cotreatment of *Bartonella*-infected cells with L-arg restored partially NOx levels (MOI 200, 2.1 ± 0.3 μM, $P < 0.05$ vs. *Bartonella* alone).

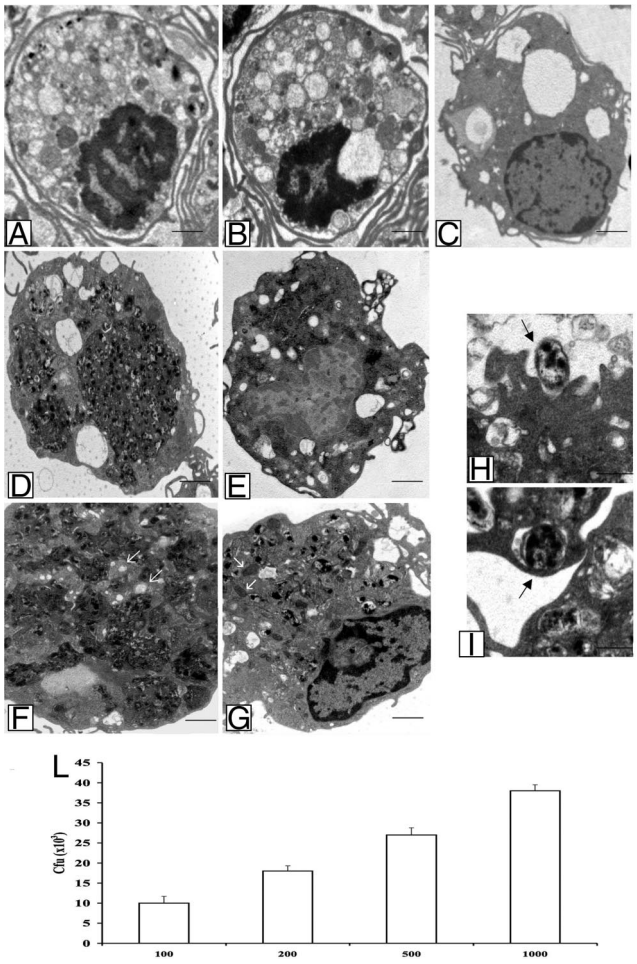

Fig. 3. The bacteria internalization was evaluated by TEM and gentamicin protection assay. Interaction of *B. henselae* with EPCs is shown. (*A*) Untreated EPCs. (Scale bar: 3 μm.) (*B*) EPCs treated with L-arg (10 mM). (Scale bar: 3 μm.) (*C*) EPCs after 1 h of infection with *S. aureus*. (Scale bar: 3 μm.) (*D* and *F*) Untreated human EPCs after 72 h of *B. henselae* infection with a MOI of 200 (*D*) and 500 (*F*). (Scale bars: 3 μm.) (*E* and *G*) L-arg-treated EPCs after 72 h of *B. henselae* infection with a MOI of 200 (*E*) and 500 (*G*). Adherence and invasion of *B. henselae* infection resulted in diffuse perinuclear accumulation of bacteria and large intracellular bacterial aggregates (invasomes). Black arrows indicate the formation of membrane ruffling and uptake of single bacteria by cellular protrusions (*H*), leading to internalization of *B. henselae* in cell vacuoles (*I*). (Scale bars 0.5 μm.) White arrows indicate the presence of mitochondria in *F* and *G*. In EPCs pretreated with L-arg, the mitochondria are intact (*G*). (*L*) Intracellular presence of *B. henselae* in EPCs determined by gentamicin protection assay at different MOI.

Modulation of EPC Gene Expression by *Bartonella* and L-Arginine. The cRNA generated from a pool of three different mRNA extractions ($n = 3$ in duplicate) for each condition (EPCs, both

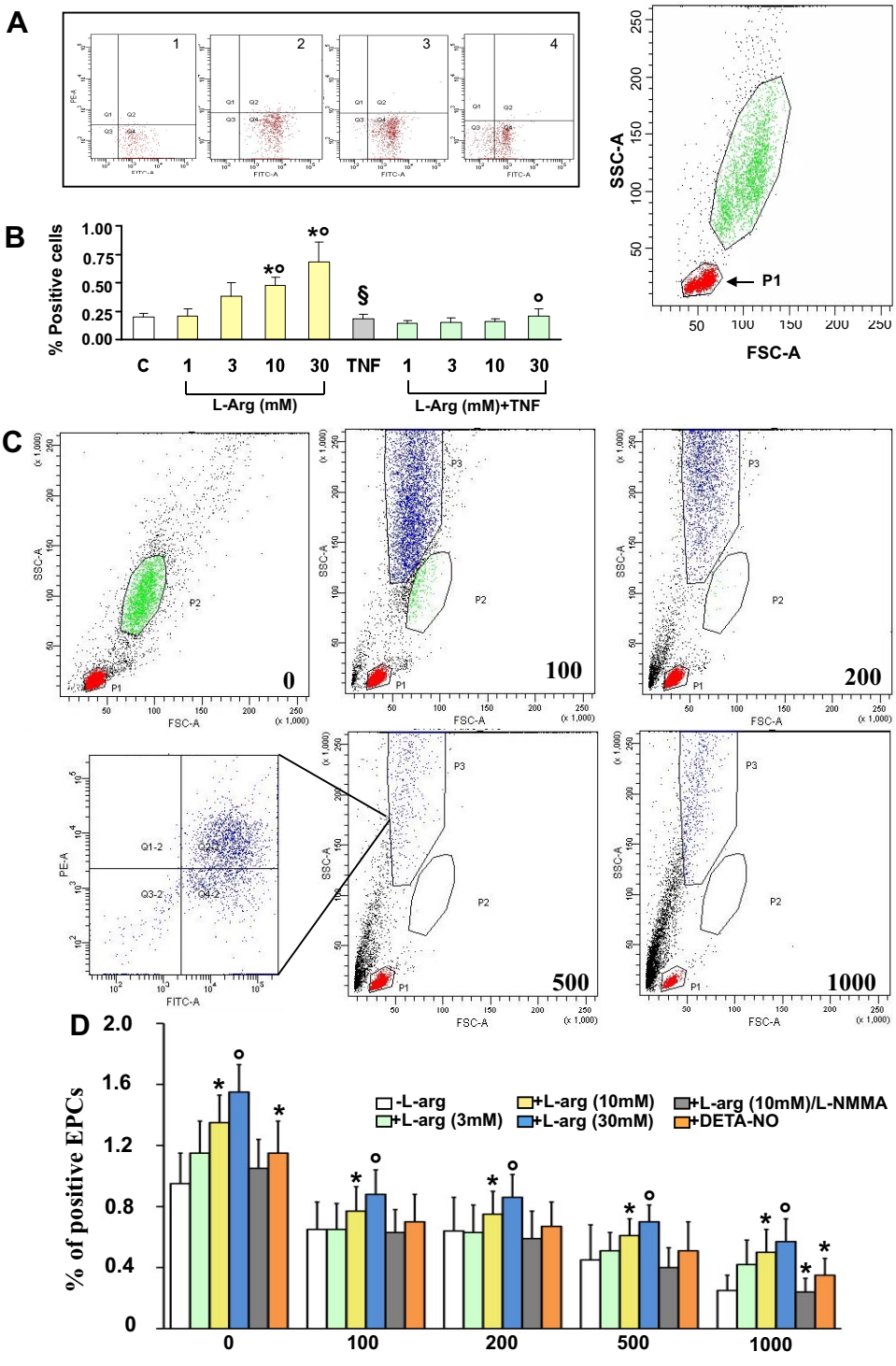

Fig. 4. Flow cytometry analysis. (*A*) Representative four-quadrant FACS analysis of DiI-Ac-LDL/lectin double-positive EPCs with different treatments: untreated EPCs (*A1*), L-arg (10 mM)-treated EPCs (*A2*), TNF-α-treated EPCs (*A3*), and L-arg + TNF-α-treated cells (*A4*). (*B*) Bar graphs showing EPCs percent number after treatments with L-arg (1–30 mM). The lymphocyte population was considered for counting as indicated by an arrow in the inserted forward and sideward scatter plot image on the right. (*C* and *D*) The effects of L-arg on *Bartonella*-infected EPCs by FACS analysis. Shown is FACS analysis of DiI-Ac-LDL/lectin double-positive EPCs, both uninfected and infected with *B. henselae* at different MOI, as described in *Results*. A representative forward and sideward scatter analysis for each indicated *Bartonella* MOI is shown (*C*). (*C Lower Left*) FACS analysis for CD13-PE/CD15-FITC antigens. (*D*) Bar graphs indicate the means and standard deviations of the percentage of double-positive cell number for the different MOI and in the presence of different treatments (L-arg (3–30 mM), L-arg 10 mM plus L-NMMA, DETA-NO). The lymphocyte population was considered for FACS counting. Results are representative of five different experiments in duplicate. *, $P < 0.05$ vs. L-arg untreated EPCs; °, $P < 0.05$ vs. both respective untreated or 10 mM L-arg EPCs.

untreated and treated with L-arg, infected and uninfected) and arrays containing 14,500 well characterized human genes (22,215 total genes comprehensive of EST sequences) were used to measure the effects of *Bartonella* infection on EPCs, untreated or pretreated with L-arg. Only genes with a threshold of at least 50 arbitrary detection units and a signal log ratio (SLR) value ≥1 and ≤−1, corresponding to an absolute fold change (FC) value of 2 [$FC = 2^{(SLR)}$] have been included in the lists. Major changes were observed for genes coding proteins with known biological functions, as listed in Table 1. The presence of several genes involved in immune response and cell death is noteworthy.

Discussion

We demonstrate that *B. henselae* can be effectively taken up into EPCs *in vitro*. Second, the treatment with L-arg can affect the response of EPCs to TNF-α and *B. henselae* via NO-dependent mechanisms. Intraerythrocytic presence of *Bartonella* has been demonstrated *in vitro* using anti-*Bartonella* antibodies (26). A potential primary niche in *Bartonella* infections could be represented by BMCs (9). Thus, mobilized EPCs could carry the pathogen to other organs and, more important, to endothelium of microcirculation. Thus, infected EPCs may reach endothelium where the vasculoproliferative disorders are initiated with the

Table 1. Modulation of EPC gene expression by *Bartonella* and L-arginine

Gene title	RefSeq transcript ID	Gene ontology (biological process)	Chromosomal location	L-arginine vs. EPC	*Bartonella* vs. EPC	L-arginine plus *Bartonella* vs. EPC
7-dehidrocoholesterol reductase (DHCR7)	NM_001360	Blood vessel development	chr11q13.2	3,0 I	NDE	NDE
RecQ protein like 4 (*RECQL4*)	NM_004260	DNA repair	chr8q24.3	2,0 I	NDE	NDE
Rho GDP dissociation inhibitor alfa (*ARHGDIA*)	NM_004309	Antiapoptosis	chr17q25.3	1,1 I	NDE	NDE
Talin (*TLN1*)	NM_006289	Cell motility	chr9p13	3,0 D	NDE	NDE
Programmed cell death 11 (*PDCD11*)	NM_014976	RNA processing	ch19p13.12	1,7 D	NDE	NDE
Hepcidin antimicrobial peptide (*HAMP*)	NM_021175	Response to pathogen	ch19p13.12	NDE	2,7 I	NDE
IL-1 receptor, type II (*IL1R2*)	NM_004633	Inflammatory and immune response	chr2q11.2	NDE	2,5 I	NDE
Wingless-type MMTV integration site family, member 5A (*WNT5A*)	NM_003392	Cell–cell signaling, signal transduction	chr3p14.3	NDE	2,4 I	NDE
Solute carrier family 7 member 11 (*SLCA11*)	NM_014331	Cationic amino acid transporter	chr4q28.3	NDE	2,4 I	NDE
Superoxide dismutase 2 (SOD2)	NM_00636	Cellular defense response	chr6q2513	NDE	1,2 I	NDE
IL-1 receptor, type I (IL1R1)	NM_000877	Inflammatory and immune response	chr2q12.1	NDE	1,2 I	NDE
Caspase 1, apoptosis-related cysteine peptidase (IL-1β, convertase) (*CASP1*)	NM_001223	Apoptosis	chr11q23	NDE	1,2 I	NDE
Chemokine (C-C motif) ligand 20 (*CCL20*)	NM_004591	Chemotaxis, defense response to bacteria	chr2q36.3	NDE	2,7 D	NDE
Plexin C1 (PLXNC1)	NM_005761	Cell adhesion	chr12q22	NDE	2,5 D	NDE
Laminin β4 (LAMB4)	NM_007356	Cell communication, focal adhesion	chr7q31.1	NDE	2,3 D	NDE
IL-1α (IL1A)	NM_000575	Inflammatory and immune response	chr2q14	NDE	1,3 D	NDE
Vascular endothelial growth factor B (*VEGFB*)	NM_003377	Regulation of progression through cell cycle	chr11q13	NDE	1,2 I	1,4 D
Upf3 regulator of nonsense transcripts homolog A (*UPF3A*)	NM_023011	mRNA catabolic process	chr13q34	1,1 D	1,6 I	1,2 D
Forkhead box P1 (*FOXP1*)	NM_00101250	Transcription regulator activity	chr3p14.1	1,1 D	1,2 I	1,1 D
Abhydrolase domain containing 8 (*ABHD8*)	NM_024527	Aromatic compound metabolism	chr19p13.11	1,1 D	3,8 I	2,6 D
Staphylococcal nuclease domain containing 1 (*SND1*)	NM_014390	Regulation of transription	chr7q32.1	1,3 D	1,1 I	1,3 D
Kinesin family member 5c (*KIF5C*)	NM_004522	Microtubule-based movement	chr2q23.1	1,4 D	1,4 I	1,1 D
Caspase recruitment domain family, member 11 (*CARD11*)	NM_032415	Positive regulation of T and B cell proliferation	chr7p22	1,1 D	1,4 I	1,5 D
Thrombospondin, type I, domain containing 3 (*THSD3*)	NM_182509	Immune response, involved in complement pathway	chr14q24.3	1,4 D	2,4 I	4,2 D
Serine/threonine kinase 4 (*STK4*)	NM_006282	Cell morphogenesis	chr20q11.2	1,1 I	1,4 D	1,6 I
Topoisomerase (DNA) I, mitochondrial (*TOP1MT*)	NM_052963	DNA topological change	chr8q24.3	1,1 I	1,4 D	1,1 I
IL-1β (*IL1B*)	NM_000576	Inflammatory and immune response	chr2q14	1,2 D	1,5 D	1,0 D

Results are expressed as both the signal log ratio and the direction of the change, i.e., an increase (I) or a decrease (D).

involvement of p38 kinase-dependent and -independent pathways. Until now, EPCs, which differentiate into angiogenic cells, have never been analyzed for their capacity to interact with human pathogens. Our *in vitro* data provide evidence that infection of EPCs with human pathogenic bacteria (e.g., *B. henselae*) results in the presence of bacteria in such cells.

Nutrients have traditionally been viewed as a means to provide basic calories to sustain homeostasis during sedentary style of life and physical exercise (27). However, critically ill, surgical, and trauma patients are in a constant dynamic state between systemic inflammatory response and compensatory antiinflammatory response (1–3). Results from ongoing research support the use of specific nutrients to modulate the immune and/or metabolic response (20, 21, 27). However, the postulate of using nutrients as therapeutic substances rather than just as "nutritional adjuvant support" would require a shift in the current belief. Among the most common nutrients found in currently available formulas are omega-3 fatty acids, antioxidants, nucleotides, glutamine, and L-arg. Multiple individual reports and at least five metaanalyses using combinations of immune-modulating nutrients have reported almost uniform beneficial results (20, 21, 27, 28). However, the wide use of L-arg in septic patients is difficult. Here, we support the adjuvant use of L-arg against the dissemination of infected EPCs in the bloodstream. This adverse event may compromise the tissue regeneration and neoangiogenesis afforded by EPCs. Notably, our microarray analysis revealed that several genes involved in immune

response were differentially expressed in *Bartonella*-infected EPCs, whereas these genes showed expression levels comparable to uninfected control cells when the EPCs were pretreated with L-arg. Recently, it was shown that the production of NO by *Helicobacter pylori*-stimulated RAW 264.7 cells was dependent on the L-arg concentration, and the ED_{50} for L-arg was 220 μM, which is above reported plasma L-arg levels (29). The inducible form of NO synthase protein increased in an L-arg-dependent manner, which resulted in the killing of *H. pylori*. The average baseline plasma concentration of L-arg in healthy subjects is 60-100 μM (30, 31). This value increases until 300 μM after a single oral administration of a 10-g dose with bioavailbility of ~20% and until 6.6 mM after intravenous administration of a 30-g dose (30, 31). In peripheral arterial disease patients, plasma L-arg concentration increased to a value of about 4.0 mM after 30-g dose infusion (32–34). Similar doses (about 15g) also have been administered to patients with chronic renal failure in a randomized controlled study (35). The administration of L-arg in sepsis is still debated. This condition is characterized by a reduction in plasma and tissue arginine levels compared with healthy individuals (20, 36). Thus, L-arg has been supplied in these patients by various forms, such as immunonutrition containing arginine with a range dose between 4.5 g and 18.7 g to intravenous administration of 30g (36, 37). However, a recent study performed on a small number of animals with septic shock (the well characterized canine model of *Escherichia coli* peritonitis) reported that parenteral L-arg, at dose above standard dietary practices, was markedly harmful (38). Despite the fact that our *in vitro* data is not a biodynamic model of L-arg bioavailability, the dose used in the present study is not much higher than doses reached in humans (30–37). Thus, L-arg and NO may exert multiple beneficial effects affecting tissue ischemia and its regeneration, immune response, sepsis, and angiogenesis.

Materials and Methods

EPC Isolation and Cultivation. Experiments with human EPCs were performed in agreement with the local ethics committee, and informed consent was provided according to the Declaration of Helsinki. EPCs were isolated from total peripheral blood mononuclear cells as previously described (22).

Cell Treatments and NOx Release. EPCs were incubated with L-arg (1–30 mM), TNF-α (22), NO donor diethylamine-NO (DETA-NO), or the NO inhibitor NG-monomethyl-L-arginine (L-NMMA), and infection was induced by *B. henselae* strain ATCC 49882 (LGC Promochem) or *Staphylococcus aureus* (*SI Materials and Methods*).

Dil-Ac-LDL/Lectin Staining and Cell Number Calculation. Cells were dual-stained with Dil-Ac-LDL and lectin from *U. europaeus* and counted as previously described (22) (*SI Materials and Methods*).

Flow Cytometry Analysis. Dual-stained cells were analyzed by flow cytometry (22) (*SI Materials and Methods*).

Western Blot Analysis. EPC extracts (20–50 μg per lane) were loaded onto SDS polyacrylamide gels, blotted onto membranes, and analyzed as described (22) (*SI Materials and Methods*).

Transmission Electron Microscopy. Ultrathin sections (80 nm) were stained with 2% uranyl acetate (Electron Microscopy Sciences) for 10 min at room temperature and 2.66% lead citrate (Electron Microscopy Sciences) for 8 min at room temperature. Grids were examined by using a Philips EM 208 S transmission electron microscope (Philips) operating at 80 kV at magnifications between $\times$2,500 and $\times$60,000 (*SI Materials and Methods*).

RNA Extraction and Microarray Analysis. Total RNAs were extracted from cultured EPCs, treated and untreated, using TRIzol solution (Invitrogen) according to the manufacturer's instructions. cRNA was used for hybridization onto the Affymetrix Human Genome U133 2.0 probe array cartridge (*SI Materials and Methods*).

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