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Glial Cell–Elicited Activation of Brain Microvasculature in Response to *Brucella abortus* Infection Requires ASC Inflammasome–Dependent IL-1β Production

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Blood–brain barrier activation and/or dysfunction are a common feature of human neurobrucellosis, but the underlying pathogenic mechanisms are largely unknown. In this article, we describe an immune mechanism for inflammatory activation of human brain microvascular endothelial cells (HBMEC) in response to infection with *Brucella abortus*. Infection of HBMEC with *B. abortus* induced the secretion of IL-6, IL-8, and MCP-1, and the upregulation of CD54 (ICAM-1), consistent with a state of activation. Culture supernatants (CS) from glial cells (astrocytes and microglia) infected with *B. abortus* also induced activation of HBMEC, but to a greater extent. Although *B. abortus*–infected glial cells secreted IL-1β and TNF-α, activation of HBMEC was dependent on IL-1β because CS from *B. abortus*–infected astrocytes and microglia deficient in caspase-1 and apoptosis-associated speck-like protein containing a CARD failed to induce HBMEC activation. Consistently, treatment of CS with neutralizing anti–IL-1β inhibited HBMEC activation. Both absent in melanoma 2 and Nod-like receptor containing a pyrin domain 3 are partially required for caspase-1 activation and IL-1β secretion, suggesting that multiple apoptosis-associated speck-like protein containing CARD–dependent inflammasomes contribute to IL-1β–induced activation of the brain microvasculature. Inflammasome-mediated IL-1β secretion in glial cells depends on TLR2 and MyD88 adapter-like/TIRAP. Finally, neutrophil and monocyte migration across HBMEC monolayers was increased by CS from *Brucella*-infected glial cells in an IL-1β–dependent fashion, and the infiltration of neutrophils into the brain parenchyma upon intracranial injection of *B. abortus* was diminished in the absence of Nod-like receptor containing a pyrin domain 3 and absent in melanoma 2. Our results indicate that innate immunity of the CNS set in motion by *B. abortus* contributes to the activation of the blood–brain barrier in neurobrucellosis and IL-1β mediates this phenomenon. ***The Journal of Immunology*****, 2016, 196: 000–000.**

Nervous system invasion by bacteria of the genus *Brucella* results in an inflammatory disorder called neurobrucellosis. It mostly affects the CNS, and it has an ominous prognosis (1). Neurobrucellosis may manifest as meningoencephalitis, meningovascular disease, brain abscesses, demyelinating syndromes, and myelitis (2–4). CNS involvement usually occurs by hematogenous dissemination, and lesions are believed to be produced by the presence of the bacterium within the brain parenchyma, the pathological action of inflammatory cytokines, and/or the demyelinating effect of an immunopathological response (5).

In vitro and in vivo models have been enlightening about the pathogenesis of bacterial meningitis and the CNS factors that contribute to inflammation and brain injury (6). Studies are also

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Abbreviations used in this article: AIM2, absent in melanoma 2; ASC, apoptosis-associated speck-like protein containing a CARD; BBB, blood–brain barrier; CASP-1, caspase-1; CS, culture supernatant; HBMEC, human brain microvascular endothelial cell; KO, knock-out; Mal, MyD88 adapter-like; MFI, mean fluorescence intensity; MOI, multiplicities of infection; NLR, Nod-like receptor; NLRP3, NLR containing a pyrin domain 3; WT, wild type.

arising about the specific contribution of the blood–brain barrier (BBB) endothelium to the initial threat of an invading pathogen. Several studies in animals and humans have shown that high-level bacteremia is required for the development of meningitis (7–9). Yet, for *Brucella* spp., the precise mechanism whereby the bacterium leaves the bloodstream and gains access to the CNS remains unclear. Regardless of mechanism, it is clear that once the bacterium reaches the CNS it induces a pathological proinflammatory response (5, 10).

Blood-borne brucellae must interact with cerebral endothelial cells and cross the BBB; subsequent bacterial replication within cells of the innate immunity of the CNS will then provoke the overwhelming host inflammatory response observed (10). It is likely that *Brucella* spp. tropism for human brain microvascular endothelial cells (HBMEC) might be a crucial step in the immunopathogenesis of neurobrucellosis, whereupon bacterial invasion, endothelial cell activation, and/or inflammatory responses modify the integrity of the BBB (5).

Pleocytosis (presence of leukocytes within the cerebrospinal fluid) is one of the pathognomonic signs of neurobrucellosis and, together with the specific production of intrathecal Abs, is used for the differential diagnosis of this form of the disease (3, 11, 12). Pleocytosis is incontrovertible clinical evidence that the integrity of the BBB is altered during neurobrucellosis and, together with our previous results that demonstrate that the presence of *B. abortus* within the brain parenchyma of mice stimulates an inflammatory infiltrate composed of polymorphonuclear neutrophils (10), is proof of concept that *Brucella* infection of CNS activates the BBB endothelium and modifies its integrity.

Although the clinical and diagnostic aspects of BBB alteration during neurobrucellosis have been widely described (3, 13–15), the pathogenic mechanisms involved in activation of the BBB caused by *Brucella* have not been investigated at the molecular and cellular levels. For this, we designed a minimal in vitro model of the interaction of *B. abortus* with cells of the BBB by using HBMEC (16). This model allowed us to elucidate, at the single-cell-type level, the ability of *B. abortus* to infect and replicate within these cells and the cytokine response to *B. abortus*. It also allowed us to investigate the response of HBMEC upon interaction with *Brucella*-infected astrocytes and microglia, the effector molecules involved, and the effect of these interactions, and thus of CNS innate immunity, on BBB activation and integrity. In this article, we present the results of this study.

Materials and Methods

HBMEC cultures

HBMEC were isolated from a brain biopsy of an adult female with epilepsy as previously described (17). These cells were positive for factor VIII–Rag, carbonic anhydrase IV, and *Ulex europaeus* agglutinin I. They took up fluorescently labeled low-density lipoprotein and expressed γ-glutamyl transpeptidase, thus demonstrating their brain endothelial cell properties (17). HBMEC were subsequently immortalized by transfection with SV40 large T Ag and maintained their morphological and functional characteristics for at least 30 passages (16). The cells are polarized and exhibit a transendothelial electric resistance of at least 100 ohms/cm^2 (8). HBMEC were cultured in 75-ml tissue cultures flasks in RPMI 1640 supplemented with heat-inactivated 10% FBS (Life Technologies, Grand Island, NY), 10% NuSerum IV (Becton Dickinson, Bedford, MA), 1% modified Eagle's medium nonessential amino acids (Life Technologies), sodium pyruvate (1 mM), L-glutamine (2 mM), MEM vitamin solution (Life Technologies), and penicillin-streptomycin. Cultures were incubated at 37˚C in a humid atmosphere of 5% CO_2.

Animals

For the primary cultures of astrocytes and microglia, 1- to 3 d-old MyD88 adapter-like (Mal)/TIRAP, TLR2, TLR4, TLR6, apoptosis-associated speck-like protein containing a CARD (ASC), Nod-like receptor (NLR) containing a pyrin domain 3 (NLRP3), absent in melanoma 2 (AIM2), and caspase-1 (CASP-1)/11 knock-out (KO) mice, as described previously (18–23), and C57BL/6 wild type (WT) mice (provided by Federal University of Minas Gerais, Belo Horizonte, Brazil, or University of La Plata, Argentina) were used. Mice were born from breeding pairs that were housed under controlled temperature (22 ± 2˚C) and artificial light under a 12-h cycle period. Mice were kept under specific pathogen-free conditions in positive-pressure cabinets and provided with sterile food and water ad libitum. All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institutes of Health. Animal experiments were approved by the Ethics Committees of the Federal University of Minas Gerais and Institute of Immunology, Genetics and Metabolism.

Primary glial culture

Highly pure astrocytes and microglia (>95%) as assessed by flow cytometry were established from primary mixed glial cultures obtained from the forebrain of 1- to 3-d-old mice following previously published procedures (10, 24).

Bacteria

B. abortus S2308 was grown overnight in 10 ml tryptic soy broth (Merck, Buenos Aires, Argentina) with constant agitation at 37˚C, harvested, and the inocula were prepared as described previously (24). All live *Brucella* manipulations were performed in biosafety level 3 facilities located at the Instituto de Investigaciones Biomédicas en retrovirus y SIDA (Buenos Aires, Argentina).

In vitro infection

HBMEC were infected with *B. abortus* S2308 at different multiplicities of infection (MOI); astrocytes and microglia were infected at a MOI of 100. Infection was carried out for 2 h in medium containing no antibiotics followed by extensive washing of cells to remove uninternalized bacteria. Infection was maintained for different times for HBMEC or for 24 h to obtain conditioned media from astrocytes and microglia in the presence of 100 μg/ml gentamicin and 50 μg/ml streptomycin to kill remaining extracellular bacteria. Cells were washed three times with PBS before processing. To monitor *Brucella* intracellular survival, we lysed infected HBMEC with 0.1% (v/v) Triton X-100 in H_2O after PBS washing, and serial dilutions of lysates were plated onto tryptone soya broth agar plates to enumerate CFU.

Stimulation with conditioned media

Culture supernatants (CS) from *Brucella*-infected glial cells (astrocytes or microglia) were harvested at 24 h postinfection, sterilized by filtration through a 0.22-μm nitrocellulose filter, and used to stimulate noninfected HBMEC. For stimulation, CS were diluted 1/2, 1/5, or 1/10 in complete HBMEC medium. Recombinant mouse IL-1β (500 pg/ml) and TNF-α (1 ng/ml) were used as controls. After 24 h, the supernatants from stimulated HBMEC cultures were harvested to measure cytokine levels and the cells to evaluate ICAM-1 (CD54) expression. Neutralization experiments were performed with anti–TNF-α (clone MP6-XT3; BD Biosciences, San Diego, CA), anti–IL-1β (clone B122; eBioscience, San Diego, CA) neutralizing Abs, or their respective isotype controls. CS from *Brucella*-infected glial cells (astrocytes or microglia) were preincubated with the corresponding neutralizing Abs (20 μg/ml for anti–TNF-α, 30 μg/ml for anti–IL-1β) or their isotype controls (used at the same concentrations) for 1 h at 37˚C before stimulating HBMEC.

Measurement of cytokine concentrations

Human IL-1β, IL-6, IL-8, MCP-1, and TNF-α were measured in CS from HBMEC infected with *B. abortus* or HBMEC stimulated with CS from *Brucella*-infected glial cells (astrocytes or microglia). Mouse IL-1β and TNF-α were measured in CS from *Brucella*-infected glial cells (astrocytes or microglia) used for stimulation of HBMEC. Sandwich ELISA were used to quantify cytokines using paired cytokine-specific mAbs, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA).

Determination of CD54 by flow cytometry

HBMEC were infected with *B. abortus* or stimulated with CS from *Brucella*-infected glial cells (astrocytes or microglia) as described earlier. Cells were then washed and stained with a PE-labeled Ab against human CD54 (ICAM-1) (clone HA58) or its isotype-matched control Ab (BD Pharmingen). Labeled cells were analyzed on a FACSCalibur flow

cytometer (BD Biosciences), and data were processed using CellQuest software (BD Biosciences). Results were expressed as mean fluorescence intensity (MFI) values representing the arithmetic mean ± SEM of the indicated experiments.

Neutrophil transendothelial migration assay

Human neutrophils were purified from healthy donors using a Ficoll-Paque PLUS gradient (GE Healthcare, Uppsala, Sweden), dextran sedimentation, and hypotonic lysis, as previously described (25). Human monocytes were purified as described previously (26). Cell purity was >95% as determined by flow cytometry. Polarized HBMEC monolayers were established from 2×10^4 cells per insert on Transwell plates of 6.5-mm diameter insert previously treated with rat tail collagen (50 μg/ml with 1% acetic acid; BD Biosciences) neutralized in ammonium hydroxide bell (Transwell Clear Polyester Membrane insert; Corning-Costar, Acton, MA) using a 3-μm pore size membrane. After 5 d, when cellular confluence was reached, monolayers were incubated overnight with cell-free CS obtained from astrocytes previously infected with *B. abortus*. CS from noninfected cells served as a control. Alternatively, HBMEC were infected with *B. abortus* at a MOI of 100 after which neutrophils or monocytes (1×10^5) were added to the upper chamber. Plates were incubated for 3 h at 37°C in 5% CO_2, after which transmigrated cells in the lower chamber were counted on a hemocytometer. In some experiments, CS were incubated with anti–IL-1β Ab (30 μg/ml for 1 h) before being added to the HBMEC monolayer.

Intracranial stereotaxic injections

For stereotaxic injections, 6- to 8-wk-old female mice ($n = 5$) were used. Animals were anesthetized with ketamine chlorohydrate (80 mg/kg body weight) and xylazine (8 mg/kg body weight) and then injected in the right striatum with heat-killed *B. abortus* (1×10^6 bacteria). The stereotaxic coordinates of the right striatum were: bregma +0.6 mm; lateral −1.7 mm; ventral −3.4 mm (27). Striatal injections of *B. abortus* (1 μl) were conducted with the help of a Hamilton 10-μl syringe over 5 min using an infusion pump with the needle kept in place for an additional 2 min before removal. A similar procedure was followed to inject vehicle (saline) in the left striatum. Mice were sacrificed 24 h after surgery.

Histology

Animals were deeply anesthetized and transcardially perfused with heparinized saline followed by cold 4% paraformaldehyde in 0.1 M PBS. The brains were removed and placed in paraformaldehyde overnight at 4°C. Brain tissues were cryoprotected by immersion in 30% sucrose, frozen in isopentane, and serially sectioned in a cryostat (30 μm) throughout the striatum in the coronal plane. Sections were stained with cresyl violet. Neutrophilic infiltration was determined and expressed as an infiltration score considering the amount of neutrophils present in a striatum section and the number of sections of the striatum that this infiltrate encompasses.

Statistical analysis

Statistical analysis was performed with one-way ANOVA, followed by Bonferroni posttest using GraphPad Prism 4.0 software. Data are represented as mean ± SEM.

Results

CS from B. abortus*–infected glial cells induce activation of HBMEC*

The activation of astrocytes and microglia, similar to the one that takes place during *B. abortus* infection (10, 24) may induce endothelial activation (28). Therefore, we decided to test whether factors secreted by *Brucella*-infected glial cells (astrocytes and

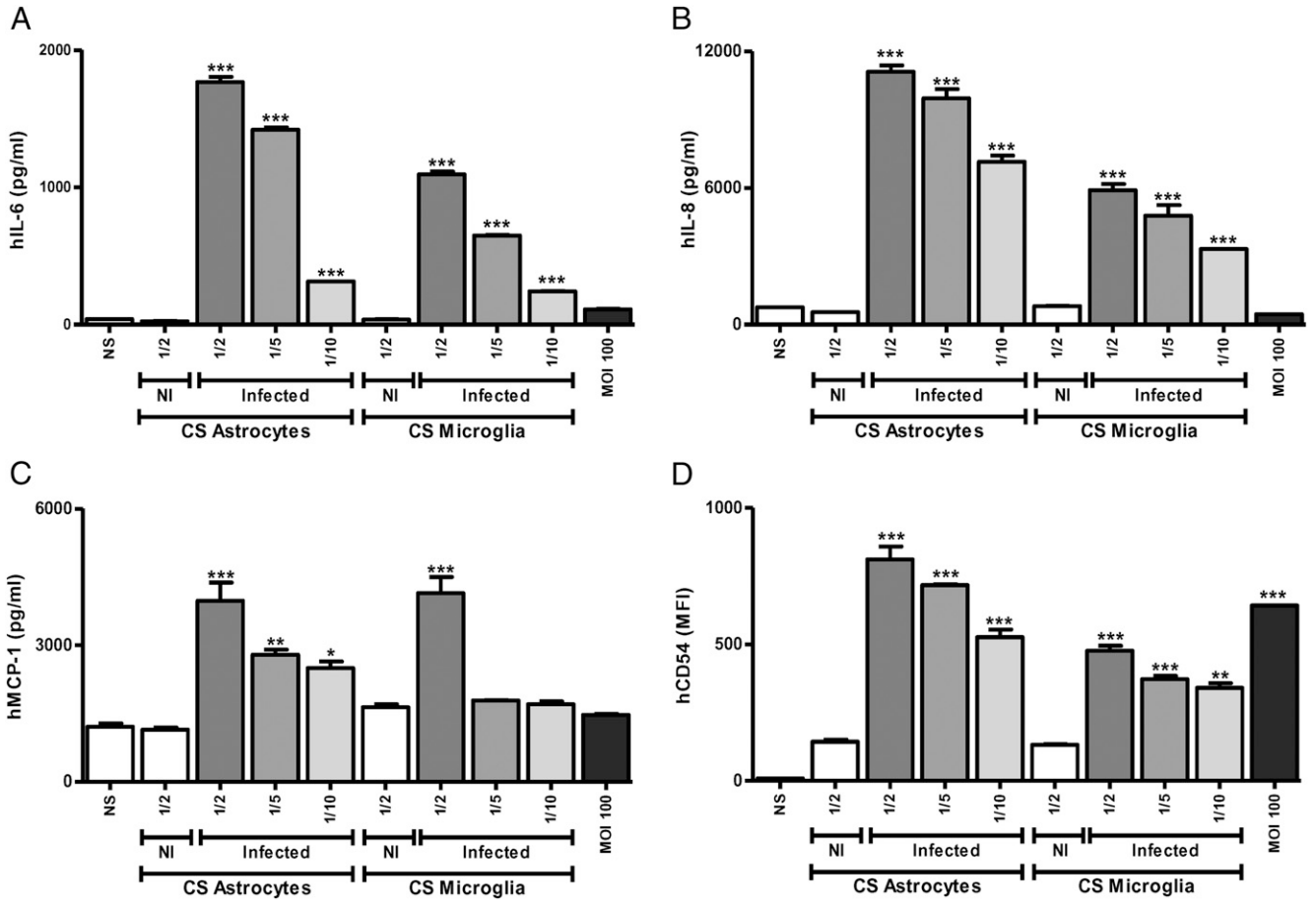

FIGURE 1. CS from *B. abortus*–infected glial cells induce the activation of HBMEC. HBMEC were stimulated for 24 h with CS from *B. abortus*–infected or noninfected (NI) astrocytes (CS Astrocytes) and microglia (CS Microglia) at the indicated dilutions. HBMEC were also infected with *B. abortus* (MOI 100) as a control. The secretion of human (h) IL-6 (**A**), hIL-8 (**B**), and hMCP-1 (**C**) was determined by ELISA. CD54 was determined on HBMEC by flow cytometry, and results were expressed as MFI (**D**). Bars represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$ versus the stimulation with the corresponding NI CS.

microglia) might induce activation of HBMEC. Activation was evaluated by the ability of the cells to secrete cytokines and chemokines, and the capacity to upregulate the adhesion integrin CD54 (ICAM-1). The addition of CS from *B. abortus*–infected astrocytes or microglia to uninfected HBMEC induced a significant secretion of IL-6, IL-8, MCP-1, and the upregulation of CD54 expression in a dose-dependent manner compared with unstimulated HBMEC or HBMEC stimulated with CS from uninfected glial cells ($p < 0.001$; Fig. 1). The amount of secreted factors present in culture of stimulated HBMEC corresponded exclusively to that secreted by these cells and not to that already present in the CS from infected glial cells, because Abs present in the ELISA kit did not cross-react between human and mouse cytokines/chemokines (data not shown). These results demonstrate that CS from *B. abortus*–infected glial cells activate HBMEC.

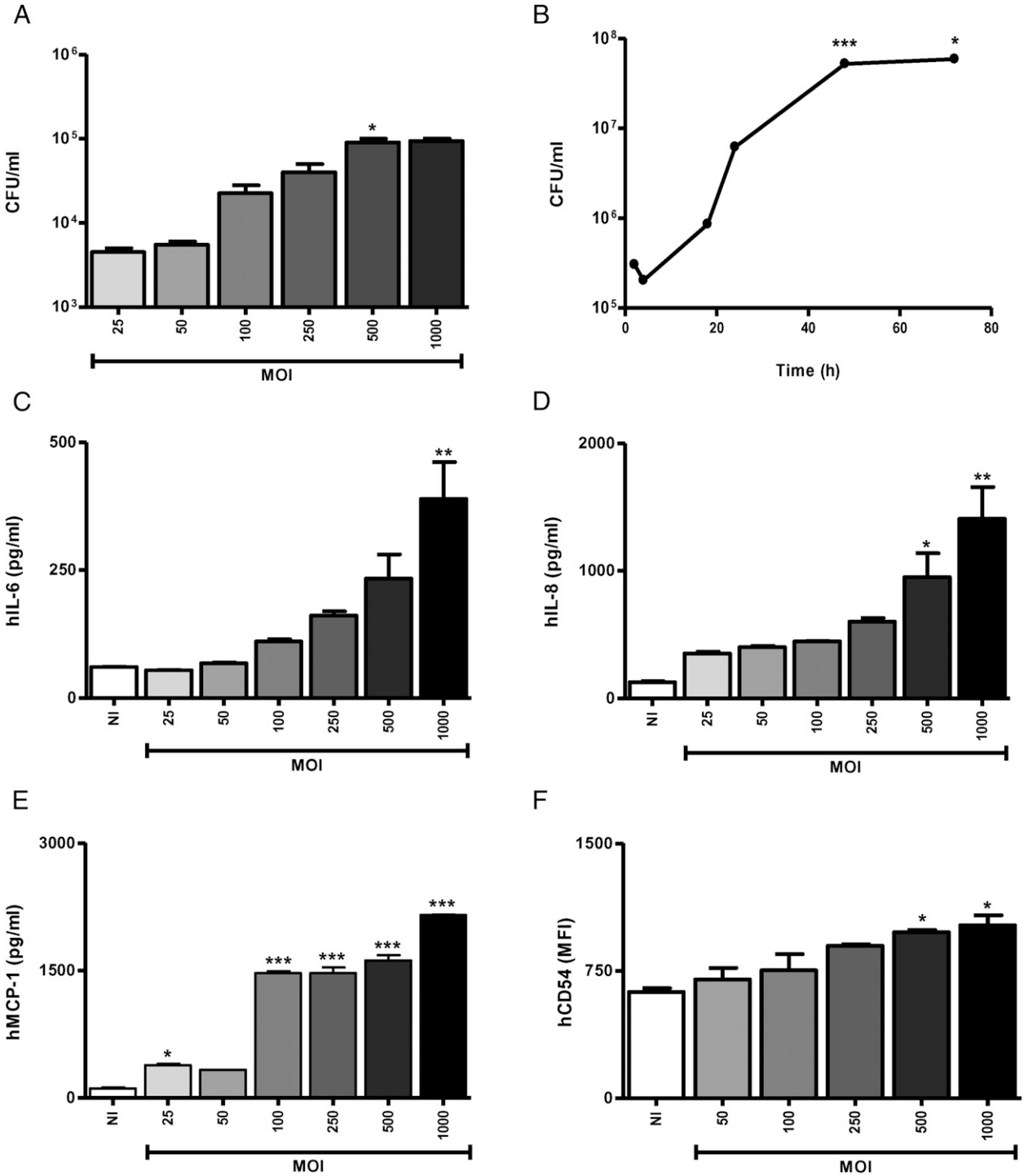

FIGURE 2. *B. abortus* infection induces the activation of HBMEC. HBMEC were infected with *B. abortus* at different MOI. Internalized bacteria were determined by enumerating the CFU within cells at 24 h postinfection (**A**), and bacterial replication was determined by counting CFU at different times at an MOI of 100 (**B**). CS were harvested at 24 h postinfection to assess the secretion of hIL-6, hIL-8, and hMCP-1 by ELISA (**C**–**E**). CD54 was determined on HBMEC by flow cytometry, and results were expressed as MFI (**F**). Bars and symbols represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$ versus immediate previous value (A and B) or versus noninfected (NI) HBMEC (C–F).

B. abortus *infection also induces the activation of HBMEC*

We next sought to investigate whether *B. abortus* could infect and activate brain microvascular cells. Infection experiments showed that *B. abortus* is internalized and can replicate in HBMEC in vitro (Fig. 2A, 2B). Infection resulted in significant ($p < 0.01$) secretion of IL-6, IL-8, and MCP-1, and the upregulation of CD54 expression in a MOI-dependent fashion (Fig. 2C–F). On the contrary, HBMEC were unable to secrete IL-1β and TNF-α upon infection (data not shown). The levels of IL-6, IL-8, and MCP-1 secreted by CS-stimulated HBMEC were significantly higher ($p < 0.01$) than those produced by HBMEC infected with *B. abortus* at an MOI as high as 100. In contrast, the upregulation of CD54 induced by CS from infected glial cells was similar to that induced by infection of HBMEC (Fig. 1). These results indicate that the effect of activating HBMEC with CS from *B. abortus*–infected glial cells is more potent than the direct infection. Differences in cytokine regulation between glial cells and HBMEC could potentially contribute to the observed differences in HBMEC activation between the two stimulating conditions.

Activation of HBMEC by glial cells is mediated by Brucella*-induced IL-1β*

Our results indicated that a soluble factor secreted by *Brucella*-infected glial cells could be involved in the activation of HBMEC. TNF-α and IL-1β have been shown to be major inducers of brain endothelium activation and responsible for the increase in endothelial permeability of the BBB (29–31). Because both cytokines are produced upon infection of astrocytes and microglia with *B. abortus* (10), we decided to investigate their role in the activation of HBMEC by *Brucella*-infected glial cells by first focusing on IL1-β. To assess the role of IL-1β on HBMEC activation, we used glial cells from mice deficient in CASP-1 and ASC. ASC is an adaptor protein for many inflammasomes, bridging microbial signals to CASP-1 activation and IL-1β production. IL-1β secretion induced by *B. abortus* infection was completely abolished

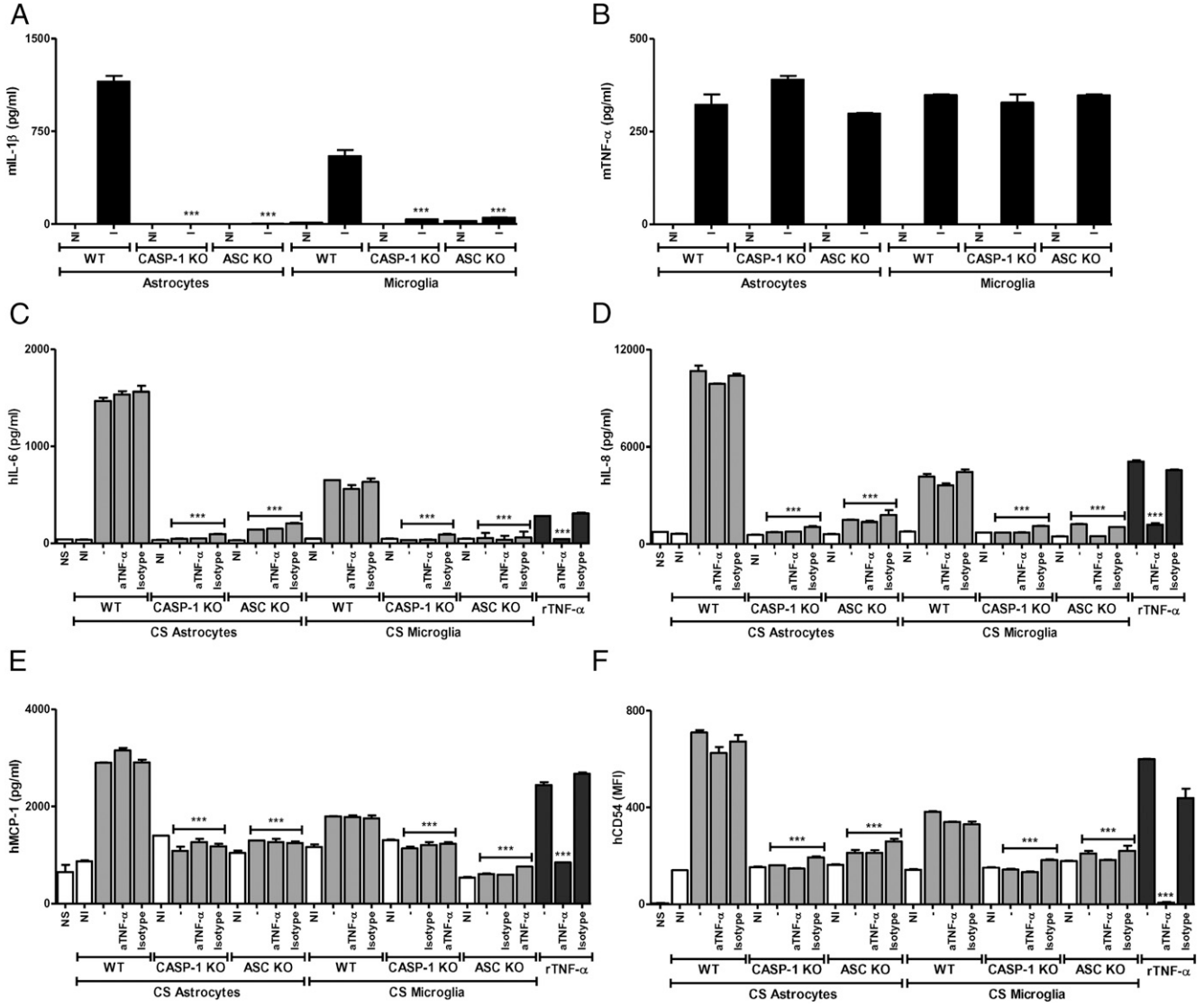

FIGURE 3. Activation of HBMEC requires CASP-1 and ASC. Astrocytes and microglia from WT, CASP-1, or ASC KO mice were infected with *B. abortus* (MOI 100), and the secretion of IL-1β (**A**) and TNF-α (**B**) was determined by ELISA after 24 h. HBMEC were stimulated for 24 h with CS from *B. abortus*–infected or noninfected (NI) astrocytes (CS Astrocytes) and microglia (CS Microglia) from WT, CASP-1, or ASC KO mice. Concomitantly, HBMEC were treated with CS of infected glial cells that were untreated (−) or were preincubated with anti–TNF-α neutralizing Ab (aTNF-α) or its isotype control (Isotype). rTNF-α (1 ng/ml) was used as control. After culture the secretion of hIL-6 (**C**), hIL-8 (**D**), and hMCP-1 (**E**) was determined by ELISA. CD54 was determined on HBMEC by flow cytometry, and results were expressed as MFI (**F**). Bars represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. ***$p < 0.001$ versus WT. NS, nonstimulated HBMEC.

in ASC- and CASP-1–deficient astrocytes and microglia when compared with WT glial cells, whereas TNF-α was not affected (Fig. 3A, 3B). Again, when HBMEC were stimulated with CS from *B. abortus*–infected astrocytes and microglia from WT mice, they induced the significant ($p < 0.001$) secretion of IL-6, IL-8, MCP-1, and the upregulation of CD54. On the contrary, CS from *B. abortus*–infected astrocytes and microglia from CASP-1 as well as ASC KO mice were unable to induce activation of HBMEC (Fig. 3C–F). To confirm the role of IL-1β in the activation of HBMEC by CS from *B. abortus*–infected glial cells, we preincubated CS with an IL-1β neutralizing Ab. Neutralization of IL-1β present in the CS from *B. abortus*–infected glial cells resulted in a significant reduction ($p < 0.001$) of the activation of HBMEC (Fig. 4). rIL-1β, used as control, induced a significant ($p < 0.001$) activation of HBMEC, and this was reduced ($p < 0.001$) in the presence of the neutralizing Ab (Fig. 4). In addition, CS were preincubated with a TNF-α neutralizing Ab to test the ability of this cytokine in the activation of HBMEC. Neither anti–TNF-α Ab nor its isotype control had any effect on the activation of HBMEC induced by CS from WT or KO glial cells (Fig. 3C–F). The neutralization capacity of the anti–TNF-α Ab was confirmed by blocking the activation induced by supraphysiological concentrations of rTNF-α (Fig. 3C–F). These results indicate that glial *B. abortus*–induced IL-1β is involved in the activation of the brain microvasculature.

Activation of HBMEC by glial-secreted IL-1β requires AIM2 and NLRP3

Our results suggested that ASC is essential for the CASP-1 response to *B. abortus* infection in glial cells and, therefore, a NLR could be involved in the production of IL-1β and the subsequent activation of HBMEC. Recently, it was demonstrated that the secretion of IL-1β by murine macrophages infected with *B. abortus* proceeds via AIM2 and NLRP3, independently of NLRC4 (32). To address whether NLRP3 and AIM2 are important for inflammasome activation and IL-1β secretion during infection, we infected astrocytes and microglia from C57BL/6, NLRP3, and AIM2 KO mice with *B. abortus*. IL-1β secretion induced by *B. abortus* infection was completely abolished in AIM2 and NLRP3 astrocytes and microglia when compared with WT glial cells, whereas TNF-α was not affected (Fig. 5A, 5B). Subsequently, CS from *B. abortus*–infected astrocytes and microglia from AIM2 as well as NLRP3 KO mice were incapable of activating HBMEC (Fig. 5C–F). These results demonstrate that in glial cells infected with *B. abortus*, the activation of ASC-dependent inflammasomes and the production of IL-1β are dependent on NLRP3 and AIM2. They also indicate that inflammasomes are critical for the glial activation of HBMEC by *B. abortus*.

TLR2 determines IL-1β production by glial cells and concomitant HBMEC activation in response to B. abortus *infection*

For the production of IL-1β, TLRs and NLRs function in concert. TLRs induce the expression of the precursor form of this cytokine (pro–IL-1β), after which NLR-dependent activation of CASP-1 regulates its proteolytic processing and release (33). Thus, to assess the requirements for the TLR signaling pathways in IL-1β production and subsequent HBMEC activation in response to *B. abortus* infection, we infected glial cells from C57BL/6 WT mice or from Mal/TIRAP, TLR2, TLR4, and TLR6 KO mice with *B. abortus* and assessed the production of IL-1β and TNF-α in the CS. IL-1β and TNF-α production by TLR2 and Mal/TIRAP KO

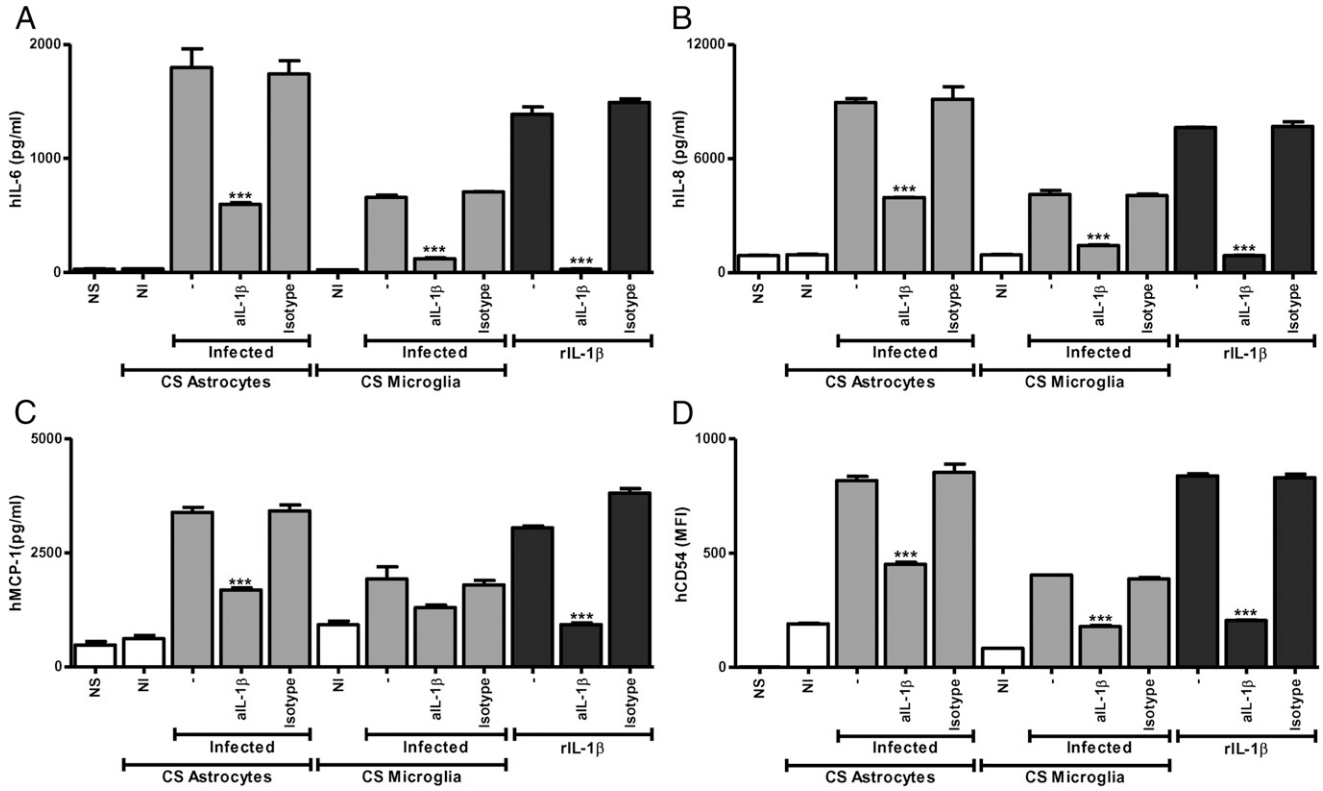

FIGURE 4. Activation of HBMEC by glial cells is mediated by *Brucella*-induced IL-1β. HBMEC were treated for 24 h with CS from noninfected (NI) glial cells or CS from *B. abortus*–infected glial cells that were untreated (−) or were preincubated with anti–IL-1β neutralizing Ab (aIL-1β) or its isotype control (Isotype). rIL-1β (500 pg/ml) was used as control. The secretion of hIL-6 (**A**), hIL-8 (**B**), and hMCP-1 (**C**) was determined by ELISA. CD54 was determined on HBMEC by flow cytometry, and results were expressed as MFI (**D**). Bars represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. ***$p < 0.001$ versus the stimulation with untreated CS. NS, nonstimulated HBMEC.

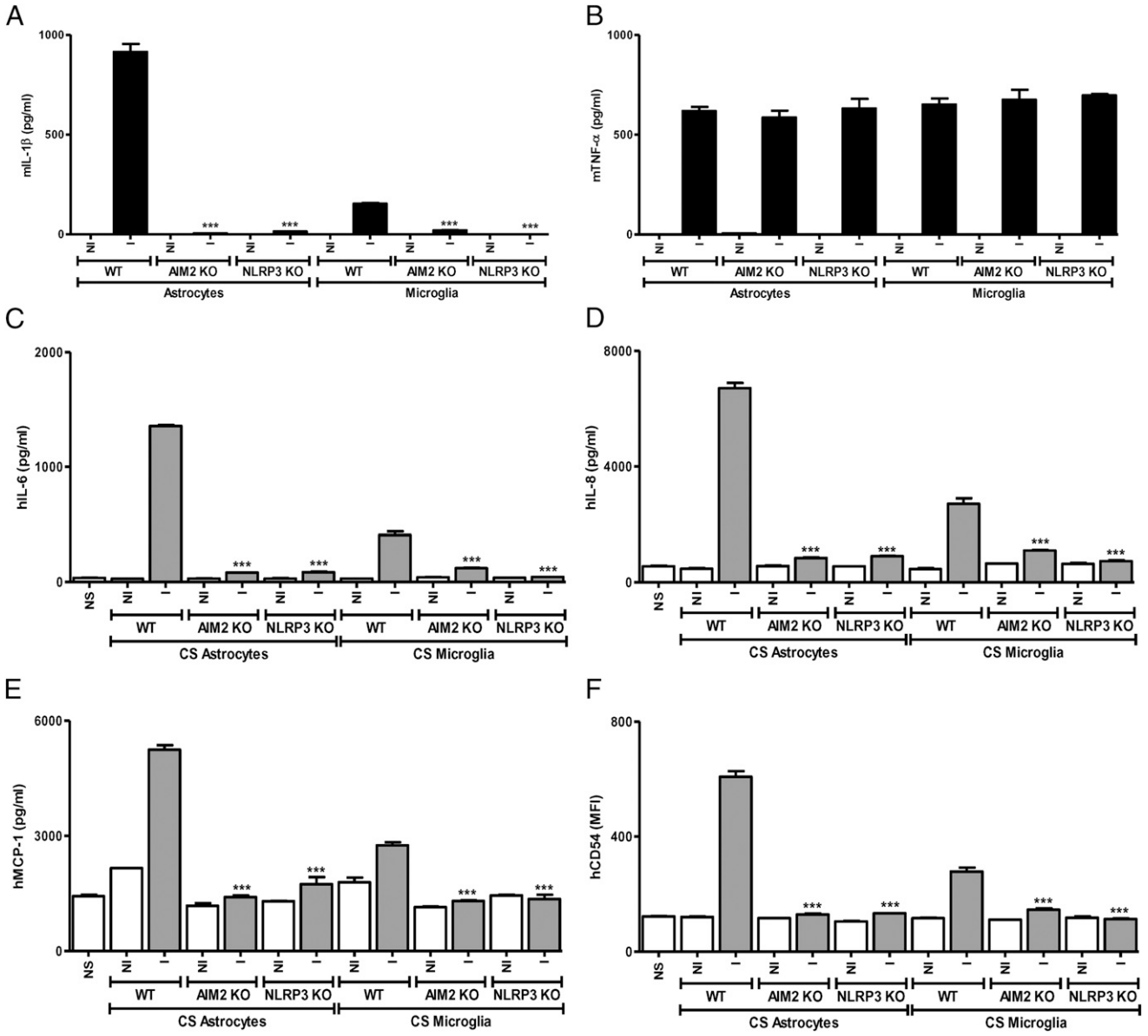

FIGURE 5. Activation of HBMEC by glial-secreted IL-1β requires AIM2 and NLRP3. Astrocytes and microglia from WT, AIM2, or NLRP3 KO mice were infected with *B. abortus* (MOI 100), and the secretion of IL-1β (**A**) and TNF-α (**B**) was determined by ELISA after 24 h. HBMEC were stimulated with CS from *B. abortus*–infected (I) or noninfected (NI) astrocytes (CS Astrocytes) and microglia (CS Microglia) from WT, AIM2, and NLRP3 KO mice for 24 h. After culture the secretion of hIL-6 (**C**), hIL-8 (**D**), and hMCP-1 (**E**) was determined by ELISA. CD54 was determined on HBMEC by flow cytometry and results were expressed as MFI (**F**). Bars represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. ***$p < 0.001$ versus WT. NS, nonstimulated HBMEC.

astrocytes and microglia infected with *B. abortus* was completely abrogated. On the contrary, glial cells from TLR4 and TLR6 KO mice produced similar amounts of IL-1β and TNF-α than those from WT mice (Fig. 6A, 6B). The higher amounts of IL-1β that TLR6 KO microglia produced could be because of cell-specific upregulation of other TLR on the surface of these cells that led to an overexpression of this cytokine. Other investigators have reported the upregulation of certain cytokines with specific TLRs KO (34, 35). Glial cells from Mal/TIRAP, TLR2, TLR4, and TLR6 KO mice did not produce IL-1β or TNF-α in response to their cognate ligands (Pam_3CyS, *E. coli* LPS, and Malp-2, respectively; data not shown). Then, CS from *B. abortus*–infected glial cells from C57BL/6, Mal/TIRAP, TLR2, TLR4, and TLR6 KO mice were used to stimulate HBMEC. A significant ($p < 0.001$) activation of HBMEC was induced by CS from WT, TLR4, and TLR6 KO glial cells infected with *B. abortus*. CS from Mal/TIRAP or TLR2 KO infected glial cells did not induce secretion of IL-6, IL-8, or MCP-1, or upregulation of CD54 on HBMEC (Fig. 6C–F). Collectively, these results indicate that inflammasome-mediated IL-1β secretion in glial cells depends on TLR2 and Mal/TIRAP. The results also confirm the crucial role of TLR2 for inflammatory responses induced by *B. abortus* (26, 36–38).

Glial-activated HBMEC promote transendothelial migration of human neutrophils and monocytes

The activation induced in HBMEC in response to *B. abortus*–infected glial cells could be the cause of the inflammatory infiltrate in the CNS that we (10) and others (12) have observed in neurobrucellosis. To test this possibility, we developed an in vitro functional assay of transendothelial migration. *B. abortus* infection of HBMEC did not induce the transmigration of neutrophils and monocytes (Fig. 7A, 7B), which is in agreement with the lower

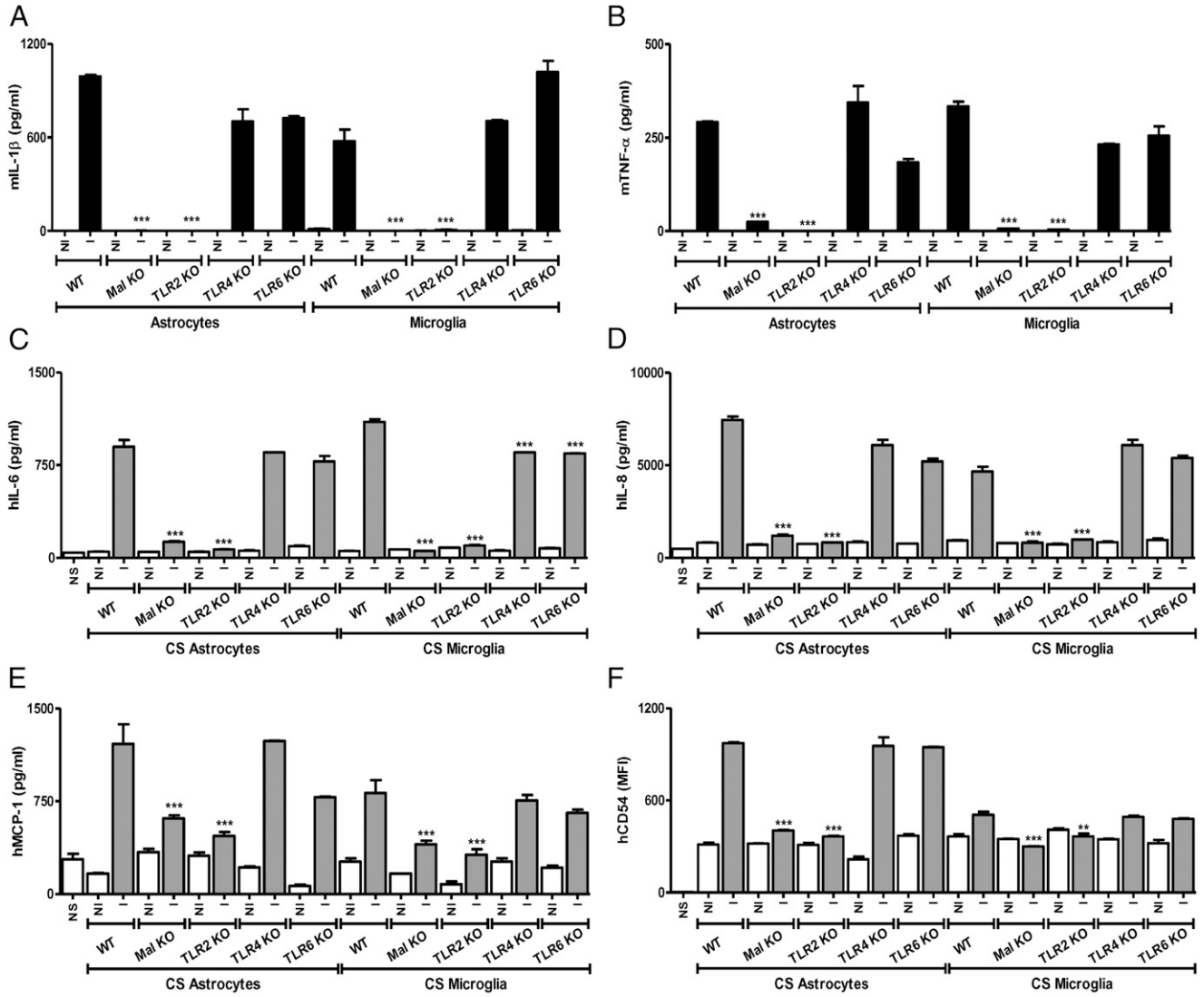

FIGURE 6. Inflammasome-mediated IL-1β secretion by glial cells depends on TLR2 and Mal/TIRAP. Astrocytes and microglia from WT, Mal/TIRAP (Mal), TLR2, TLR4, or TLR6 KO mice were infected with *B. abortus* (MOI 100), and the secretion of IL-1β (**A**) and TNF-α (**B**) was determined by ELISA after 24 h. HBMEC were stimulated with CS from *B. abortus*–infected (I) or noninfected (NI) astrocytes (CS Astrocytes) and microglia (CS Microglia) from WT, Mal, TLR2, TLR4, and TLR6 KO mice for 24 h. The secretion of hIL-6 (**C**), hIL-8 (**D**), and hMCP-1 (**E**) was determined by ELISA. CD54 was determined on HBMEC by flow cytometry and results were expressed as MFI (**F**). Bars represent the mean ± SEM of duplicates. Data shown are from a representative experiment of three performed. $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT. NS, nonstimulated HBMEC.

activation of HBMEC induced by infection as compared with HBMEC treatment with CS (Fig. 2). In contrast, stimulation of HBMEC with CS of *Brucella*-infected astrocytes significantly enhanced the transmigration of both neutrophils and monocytes, when compared with cells treated with noninfected CS (Fig. 7C, 7D). Notably, rIL-1β but not TNF-α or CS from CASP-1, ASC, NLP3, or AIM2 KO astrocytes induced transmigration of both cell types (Fig. 7C, 7D). IL-1β neutralizing Ab inhibited the transmigration of neutrophils induced by CS of *Brucella*-infected astrocytes or by rIL-1β, confirming the role of IL-1β in the transmigration of immune cells (Fig. 7E). These results indicate that activation of brain microvasculature by CS from *Brucella*-infected glial cells could induce transmigration of immune cells through the BBB and suggest that IL-1β mediates this phenomenon.

NLRP3 and AIM2 influence the infiltration of neutrophils into the brain parenchyma upon intracranial injection of B. abortus

Finally, we tested whether inflammasome-dependent IL-1β production influences migration of immune cells into the CNS in vivo. For this, *B. abortus* was injected in the striatum of mice (10), and 24 h later animals were sacrificed. Striatum sections were analyzed by microscopy after cresyl violet staining. An extensive and widespread inflammatory infiltrate, composed of polymorphonuclear neutrophils, was observed in the whole striatum of WT animals (Fig. 8). In contrast, NLRP3 and AIM2 KO animals presented a marked reduction in the neutrophilic infiltrate, albeit only significant in NLRP3 KO mice ($p < 0.1$) (Fig. 8). No neutrophilic infiltrates were observed in the contralateral hemispheres injected with saline (data not shown).

Discussion

The notion of the CNS as an immune-privileged organ has led to a common misconception that it is not an active immunological organ, protected from the environment by the BBB. Recent advances in this field clearly demonstrate that the CNS is a highly immunologically active organ, with complex immune responses

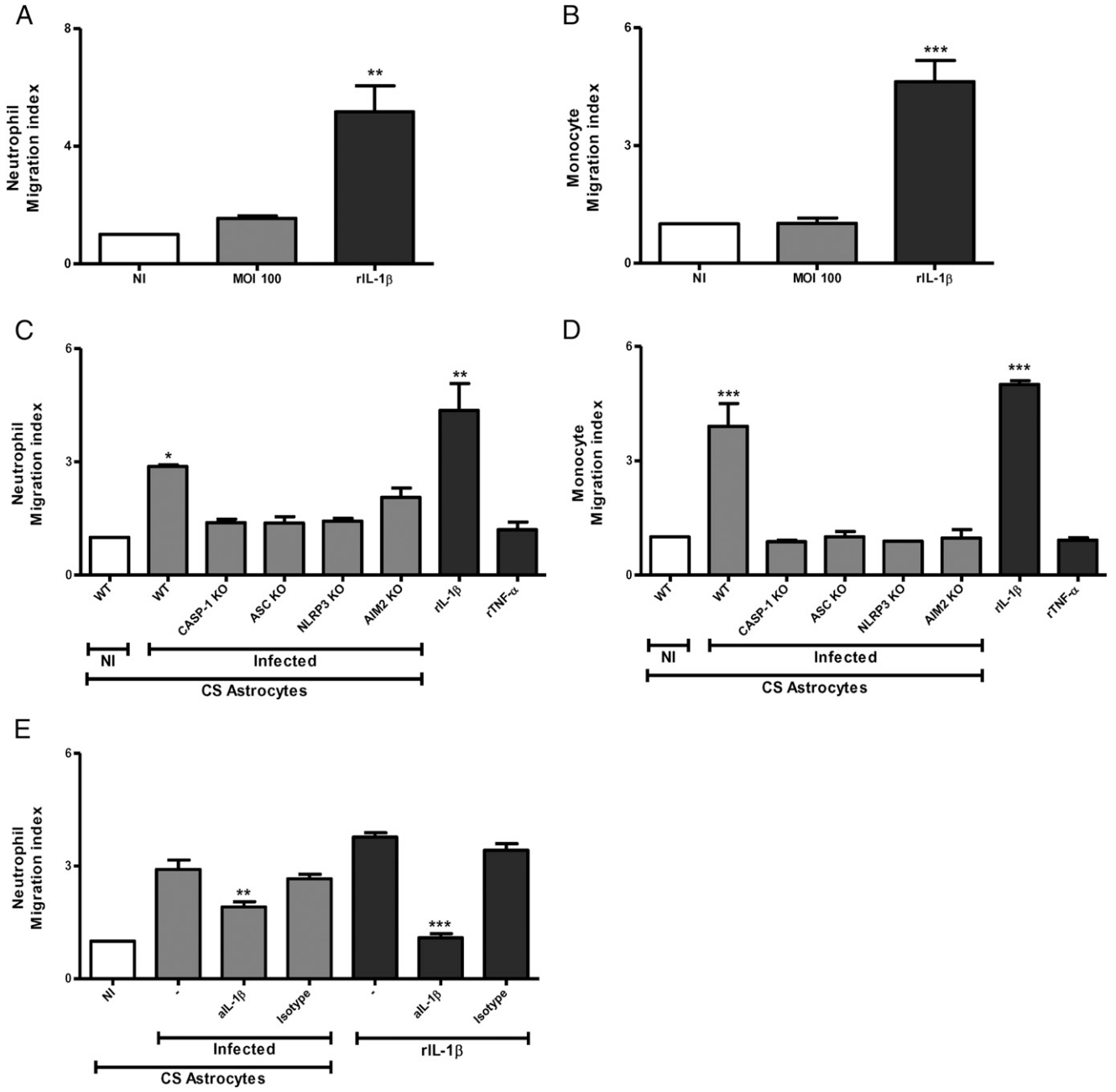

FIGURE 7. Glial-activated HBMEC promote transendothelial migration of human neutrophils and monocytes. Polar monolayers of HBMEC were established on Transwell inserts and were infected with *B. abortus* at MOI 100 (**A** and **B**) or stimulated with CS from *Brucella*-infected astrocytes from WT, CASP-1, ASC, NLRP3, and AIM2 KO mice (**C** and **D**). After that, neutrophils (A and C) or monocytes (B and D) were added to the upper chamber, and after 3 h of incubation the number of transmigrating cells was scored in the bottom well. In addition, CS from *Brucella*-infected WT astrocytes were incubated with IL-1β neutralizing Ab before stimulation of HBMEC. After that the number of transmigrating neutrophils was scored in the bottom well (**E**). Migration index was determined as the ratio of cells that migrated in response to infection in comparison with migration across the noninfected (NI) monolayer (A and B) or the ratio of cells that migrated in response to the presence of CS from *Brucella*-infected astrocytes in comparison with migration across a monolayer stimulated with CS from noninfected (NI) astrocytes (C–E). rIL-1β and rTNF-α were used as controls. Data shown are from a representative experiment of three performed. $*p < 0.1$, $**p < 0.01$, $***p < 0.001$ versus controls.

mostly based on innate immune processes (29). Such responses implicate a continuum of heterogeneous cell types inside the CNS, in the periphery, and at their interface: the BBB. Devoid of a lymphatic system, the CNS integrity is guarded exclusively through the innate immune system with the adaptive immunity present in only specific conditions (39). Paradoxically, the activation of this same innate immunity may also have detrimental effects on neurons, glial cells, and the brain microvascular endothelium (10, 29–31, 40).

Based on the knowledge acquired about infections by other bacteria (30, 41), it can be speculated that the activation of the brain endothelium in response to *Brucella* infection, with the consequent upregulation of adhesion molecules and the secretion of proinflammatory cytokines and chemokines, may be important for the BBB dysfunction observed in brucellosis patients (11, 12) and animal models (10). Previous studies from our laboratory have shown that *B. abortus* can induce an inflammatory profile in HUVECs (42), driving us to investigate the bacterium's interac-

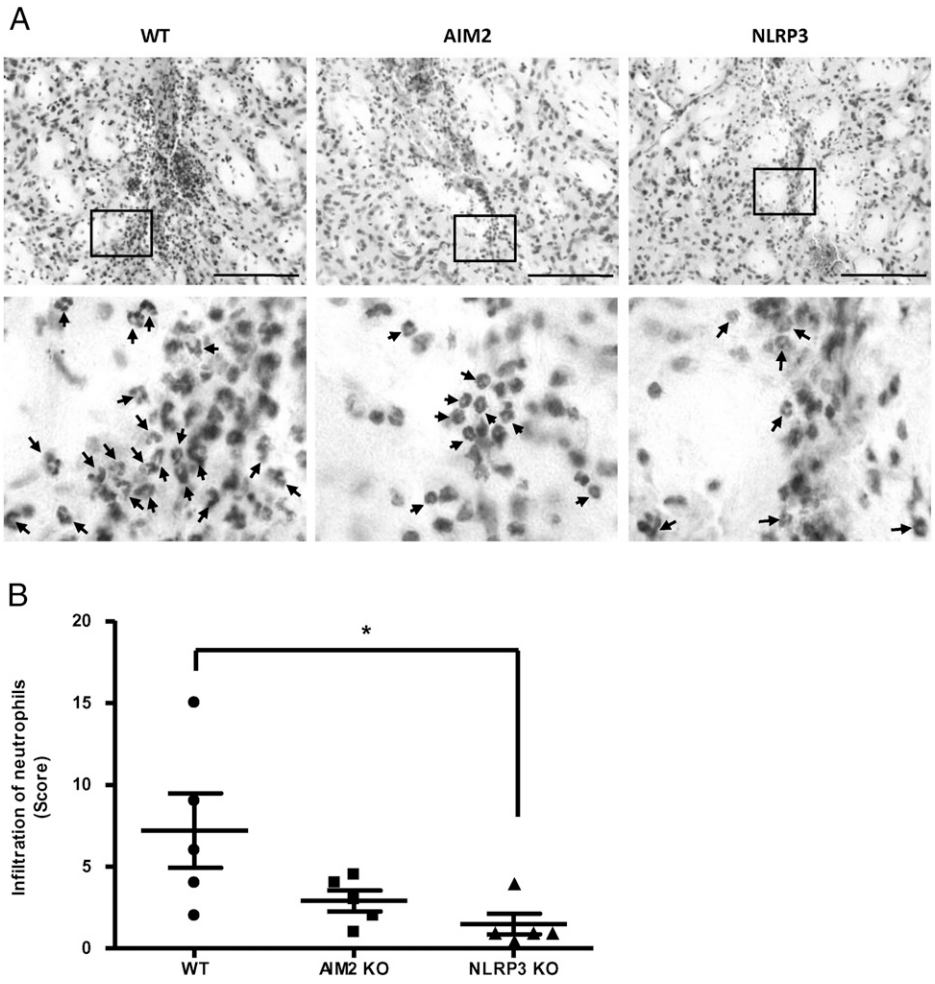

FIGURE 8. NLRP3 and AIM2 influence *Brucella*-induced infiltration of neutrophils into the brain parenchyma. Mice (five animals per group) received an intracranial injection of *B. abortus*. After 24 h animals were sacrificed and a histological evaluation was performed on striatum sections stained with cresyl violet using light microscopy. (**A**) *Top panels* show images taken at the original magnification (original magnification ×40; scale bars, 50 μm). *Bottom panels* show a detail of the amount of neutrophilic infiltrated. (**B**) Neutrophilic infiltration score of animals injected intracranially with *B. abortus*. Data shown are from a representative experiment of two performed. $*p < 0.1$ versus WT animals.

tion with the brain microvasculature as the possible cause of BBB dysfunction. Our study shows that *B. abortus* can infect and multiply within brain endothelial cells, adding new evidence to the ability of this bacterium to survive within nonphagocytic cell types such as epithelial enterocytes (43) that also form another specialized barrier that limits the entry of potential hazards to the organism: the gut immune barrier (44). HBMEC respond to *Brucella* infection with the secretion of chemokines (IL-8 and MCP-1) and proinflammatory cytokines (IL-6), and the upregulation of adhesion molecules (ICAM-1). Although these proinflammatory mediators could hypothetically play a role either directly or indirectly in host defense against *B. abortus*, they may also play an important role in the initiation, propagation, and regulation of inflammatory innate immune responses within the BBB milieu.

We contend that inflammation is a key contributor to the pathogenesis of neurobrucellosis (45, 46). As *Brucella* invades the CNS, inflammatory responses elicited by this organism upon its interaction with astrocytes and microglia (10) could also contribute to the activation of the brain microvascular endothelium. Astrocytes are the most abundant inflammatory cells in the brain (47, 48), surrounding the cerebral endothelium, and their interaction with endothelial cells determines the BBB phenotype and function (49–51). Although microglia, the macrophage-like cells of the brain, do not participate directly in maintaining the phenotype of the BBB, they have an important role in the maintenance of tissue homeostasis (52). Astrocytes and microglia are therefore in a critical position to influence the activation of the brain microvasculature and the BBB integrity once *B. abortus* reaches the brain parenchyma. Upon activation of the innate immune responses of the CNS, the proinflammatory cytokines TNF-α, IL-1β, and IL-6 have been shown to be important for activation of the brain endothelium (29, 53) and loss of the BBB integrity (54, 55). Our results indicate that *B. abortus*–mediated activation of HBMEC was induced by CS containing these cytokines (10), thus pointing to these mediators as potential effectors of the *B. abortus*–induced glial-elicited activation of HBMEC.

We have reported a major role of TNF-α and IL-6 in the immunopathogenesis of brucellosis (26, 38, 56, 57), including its neurological form (10, 24); yet the role of IL-1β in the pathogenesis of neurobrucellosis has never been investigated. IL-1β, and not TNF-α, is the effector cytokine involved in the glial-elicited activation of HBMEC induced by *B. abortus*. Although both IL-1β and TNF-α have been involved in the activation of the BBB (53, 55), it seems that TNF-α, at least in our model, is dispensable for the activation of the brain endothelial cells, as shown in the activation of brain endothelial cells in murine *Toxoplasma* encephalitis (58), whereas IL-1β is responsible for this phenomenon. The reasons for these discrepancies are not known, but they could be related to the concentrations used in the experiments in which TNF-α has been indicated as the main activator of the brain microvascular endothelium (30, 59, 60), which are at least two orders of magnitude higher than the range induced by *B. abortus*–infected glial cell in this article and our previous observations (10, 24). Thus, at least in our model, IL-1β signaling seems to have a major role in BBB activation.

Inflammasomes have emerged as critical signaling molecules of the innate immune system in the CNS, and the function of NLR in

neuroinflammation is a rather recent discovery (61). In this study, we investigated the mechanism of CASP-1 activation and IL-1β secretion in glial cells infected with *B. abortus*. Our results demonstrate that ASC inflammasomes are indispensable for inducing the activation of CASP-1 and the maturation and secretion of IL-1β upon infection of astrocytes and microglia with *Brucella*. Moreover, our results demonstrating that secretion of IL-1β by *Brucella*-infected glial cells depends on NLRP3 and AIM2 confirm and extend the previous work of Gomes et al. (32) on murine macrophages and underline the role of ASC-dependent inflammasomes, not only in recognition and host defense against *B. abortus* (32) but also in the immunopathology of neurobrucellosis.

The participation of TLR in the activation of the inflammasome and the secretion of IL-1β merits discussion. The activation and release of IL-1β requires two distinct signals. The first signal can be triggered by various pathogen-associated molecular patterns via TLR activation, which induces the synthesis of pro–IL-1β. The second signal is provided by the activation of the inflammasome and CASP-1 leading to IL-1β processing. Our results using knockout mice indicate that *B. abortus*–induced glial production of IL-1β that leads to activation of HBMEC is dependent on the adapter molecule Mal/TIRAP, a downstream signaling initiator shared by TLR1, TLR2, TLR4, and TLR6 (62). Furthermore, we showed that both phenomena are not TLR4 or TLR6, but TLR2 mediated. These findings are congruent with our previous observations indicating that TLR2/MyD88 signaling is crucial for inflammatory responses induced by *B. abortus* (38). Collectively, the data suggest, but do not definitively prove, that *Brucella* lipoproteins serve as the initial trigger for inflammasome activation and IL-1β production by glial cells via TLR2 signaling, consistent with findings in other cell types (26, 36–38). With respect to signal 2, it has been recently demonstrated that *Brucella* DNA is involved in CASP-1 activation via the AIM2 inflammasome and that NLRP3 inflammasome activation depends on mitochondrial reactive oxygen species induced by *Brucella* (32). It is unclear to us why the deletion of NLRP3 or AIM2 both resulted in the complete abrogation of IL-1β in response to infection. It is possible that NLRP3 and AIM2 are both components of the same multiprotein inflammasome complex formed in response to certain bacterial challenges. Alternatively, during *B. abortus* infection of glial cells, NLRP3 and AIM2 mutually require each other to induce the catalytic processing of bioactive IL-1β.

A defining characteristic of the cytokine-induced inflammatory response in the CNS is the destabilization of the BBB, which results in an increased vascular permeability. In this context, IL-1β signaling seems to have a major role, as demonstrated by its capacity to modulate the BBB physical permeability and enhance immune cell infiltration into the CNS (63). The activation of HBMEC by *B. abortus*–infected glial cells, but not HBMEC infection, correlates with an increased capacity of the microvascular endothelial cells to promote the transmigration of neutrophils and monocytes. This phenomenon depends on the secretion of IL-1β by glial cells because CS from CASP-1–, ASC-, NLRP3-, or AIM2-deficient astrocytes were unable to induce such transendothelial migration as also observed with IL-1β neutralization in CS from *Brucella*-infected astrocytes. This suggests that the interaction of *Brucella* with innate immunity in vivo may result in an increased transmigration of phagocytes to the brain parenchyma, which would explain the pleocytosis observed in neurobrucellosis patients (3, 12). Inflammasomes would dictate this cellular migration. Accordingly, the presence of *B. abortus* within the brain parenchyma of mice induced a neutrophilic infiltrate that was reduced in mice lacking AIM2 or NLRP3.

Finally, results presented in this article implicate a mechanism whereby *B. abortus* might activate CNS innate immunity and induce BBB dysfunction during neurobrucellosis. Because the physiological processes we describe in this work are not likely to be exclusively used in brucellosis, our findings may have relevance for other infectious neuroinflammatory disorders.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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