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RESEARCH ARTICLE

Brucella abortus-activated microglia induce neuronal death through primary phagocytosis

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Abstract

Inflammation has long been implicated as a contributor to pathogenesis in neurobrucellosis. Many of the associated neurocognitive symptoms of neurobrucellosis may be the result of neuronal dysfunction resulting from the inflammatory response induced by *Brucella abortus* infection in the central nervous system. In this manuscript, we describe an immune mechanism for inflammatory activation of microglia that leads to neuronal death upon *B. abortus* infection. *B. abortus* was unable to infect or harm primary cultures of mouse neurons. However, when neurons were co-cultured with microglia and infected with *B. abortus* significant neuronal loss occurred. This phenomenon was dependent on TLR2 activation by *Brucella* lipoproteins. Neuronal death was not due to apoptosis, but it was dependent on the microglial release of nitric oxide (NO). *B. abortus* infection stimulated microglial proliferation, phagocytic activity and engulfment of neurons. NO secreted by *B. abortus*-activated microglia induced neuronal exposure of the "eat-me" signal phosphatidylserine (PS). Blocking of PS-binding to protein milk fat globule epidermal growth factor-8 (MFG-E8) or microglial vitronectin receptor-MFG-E8 interaction was sufficient to prevent neuronal loss by inhibiting microglial phagocytosis without affecting their activation. Taken together, our results indicate that *B. abortus* is not directly toxic to neurons; rather, these cells become distressed and are killed by phagocytosis in the inflammatory surroundings generated by infected microglia. Neuronal loss induced by *B. abortus*-activated microglia may explain, in part, the neurological deficits observed during neurobrucellosis.

KEYWORDS
microglia activation, neurobrucellosis, neuroinflammation, neurotoxicity

1 | INTRODUCTION

Brucellosis is a worldwide zoonosis characterized by hepatomegaly, splenomegaly and peripheral lymphadenopathy. It is a chronic and debilitating infection caused by Gram-negative facultative intracellular bacteria that infect domestic and wild animals and can be transmitted to humans (Madkour, Al-moutaery, & Al-Deeb, 2001; Pappas, Akritidis, Bosilkovski, & Tsianos, 2005). *Brucella* organisms invade cells of the reticuloendothelial system at specific locations within the body, such as spleen, joints, liver, bone marrow, and central nervous system (CNS) (Young, 1983). CNS invasion by bacteria of the genus *Brucella* results in an inflammatory disorder called neurobrucellosis. Evidence of

neurological involvement occurs to varying degrees in both the central and peripheral nervous systems of patients with neurobrucellosis, and inflammation is often associated with the neurological manifestations that define this form of the disease (Bouza et al., 1987; Giambartolomei, Wallach, & Baldi, 2008). Upon gaining access to the CNS, *Brucella* induces pleocytosis, neuritis, central and peripheral demyelination as well as meningoencephalitis with neurocognitive abnormalities (Bouza et al., 1987; McLean, Russell, & Khan, 1992; Nalini et al., 2012; Shehata, Abdel-Baky, Rashed, & Elamin, 2010; Wallach, Baldi, & Fossati, 2002). This complex process of *Brucella*-induced pathology in the CNS has many aspects as yet to be deciphered, with reactive inflammation potentially being one of the principal contributors to neuronal dysfunction (Giambartolomei et al., 2008).

The most likely molecular basis of the inflammatory capacity of *Brucella abortus* has recently become apparent. Although *B. abortus* possesses lipopolysaccharide (LPS), the generic endotoxin of Gram-negative organisms, it may derive its inflammatory capacity from lipoproteins. Moreover, we have recently shown that the production of pro-inflammatory mediators by a variety of cells from the innate immunity is induced by *B. abortus* lipoproteins rather than LPS (Delpino et al., 2012; Giambartolomei et al., 2004; Scian et al., 2011; Zwerdling et al., 2008, 2009). As neuronal dysfunction is further analyzed in light of these findings, it becomes necessary to consider which cells of the CNS milieu can create an inflammatory environment that will contribute to the clearance of the pathogen, but could in the process, harm nearby neurons.

Recently, we have shown that the presence of *B. abortus* within the CNS elicits a powerful inflammatory response that leads to astrogliosis as well as cellular infiltrates and that, upon infection, astrocytes and microglia secrete pro-inflammatory cytokines, chemokines and metalloproteases (Garcia Samartino et al., 2010; Miraglia et al., 2013). Microglia, the resident macrophages in the brain, have been implicated as active contributors of neuron damage upon a variety of CNS insults, in which the over-activation and dysregulation of microglia might result in progressive neurotoxic consequences (Aloisi, 1999, 2001; Hoffmann et al., 2007; Lehnardt et al., 2006). The main reason of this is because microglia are the major source of pro-inflammatory cytokines, chemokines, and reactive oxygen species in the CNS. These mediators not only may recruit distinct immune effector cells within the brain parenchyma, but also can be directly involved in neuronal death (Lehnardt et al., 2006; Nguyen, O'Barr, & Anderson, 2007; Venters, Dantzer, & Kelley, 2000). Although astrocytes can secrete the same mediators, the repertoire and the concentration range is typically much lower than that of activated microglia (Bernardino, Myers, Alvarez, Hasegawa, & Philipp, 2008; Dong & Benveniste, 2001; Garcia Samartino et al., 2010; Myers, Kaushal, & Philipp, 2009). Moreover, being the professional phagocytes of the brain, microglia (at variance with astrocytes) are able to engulf and kill live neurons through a highly regulated process termed primary phagocytosis or phagoptosis (Brown & Neher, 2012, 2014; Neher, Neniskyte, & Brown, 2012). Although astrocytes are considered to be mainly supporting cells of the CNS, activated microglia can also secrete a variety of soluble agents that are potentially neuroprotective (Crotti & Ransohoff, 2016). However, the majority of soluble mediators produced by activated microglia are considered to be pro-inflammatory and neurotoxic (Aloisi, 1999; Crotti & Ransohoff, 2016). Thus, the microglial response to CNS infection with *B. abortus* could tilt the balance toward neuronal damage and death rather than survival in neurobrucellosis.

We have contended that the associated neurocognitive symptoms of neurobrucellosis may be the result of neuronal dysfunction resulting from the inflammatory response to infection of the CNS with *B. abortus* (Baldi & Giambartolomei, 2013a, 2013b; Garcia Samartino et al., 2010). In this study, we investigated the cause and effect relationships between the activation of glial cells, as induced by *B. abortus* infection, and neuronal damage. Our findings indicate that *B. abortus*-activated microglia cause neuronal demise by primary phagocytosis.

2 | MATERIALS AND METHODS

2.1 | Mice

Balb/c mice were born from breeding couples (provided by University of La Plata, Argentina) that were housed under controlled temperature (22°C ± 2°C) and artificial light under a 12 hr 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 Institute of Health, USA. MyD88 adapter-like (Mal)/TIRAP, toll-like receptor (TLR)2, and TLR4 knock out (KO) mice, as described previously (Miraglia et al., 2016), and C57BL/6 wild-type mice (provided by Federal University of Minas Gerais, Belo Horizonte, Brazil) were also used in some experiments. Animal experiments were approved by the Ethics Committee of the UFMG and by the Committee of Care and Use of laboratory animals of the School of Medicine, University of Buenos Aires (Permit Number: 358/2015).

2.2 | Primary cell cultures

Primary cultures of highly purified cortical neurons (>95%) were generated from forebrains of embryonic day 18 mice, as described previously (Franco, Rezaval, Caceres, Schinder, & Ceriani, 2010). Briefly, cortices dissected from mouse embryos were treated with trypsin (Gibco). Cells were dissociated by repeated pipetting and plated onto glass coverslips (12 mm in diameter) pre-treated with poly-l-lysine (1 mg/ml) (Sigma-Aldrich) at a density of 2×10^5 cells/well in 24-well plates (Jet Biofil). Cultures were maintained with DMEM-F12 (Gibco) plus 10% fetal bovine serum (FBS) (Gibco) for 3 hr to allow cell attachment. Then, the medium was replaced with Neurobasal medium (Gibco) supplemented with B27 and N2 (Gibco). Both media contained glucose, GlutaMAX (Gibco) and antibiotics (Gibco). Cultures were maintained in a humidified 37°C incubator with 5% CO_2 for 5–7 days before being used. Highly pure astrocytes and microglia cultures (>95%) were established from primary mixed glial cultures obtained from the forebrain of 1- to

3-day-old mice following previously published procedures (Garcia Samartino et al., 2010).

2.3 | Bacteria

B. abortus S2308 was grown overnight in 10 ml of tryptic soy broth (TSB) (Merck) with constant agitation at 37°C, harvested and the inocula were prepared as described previously (Giambartolomei et al., 2004). When indicated, *Brucella* organisms were washed in sterile phosphate-buffered saline, heat killed at 70°C for 20 min (HKBA), aliquoted and stored at −70°C until they were used. Absence of *B. abortus* viability after heat-killing was verified by the lack of bacterial growth on tryptose soy agar. All live *Brucella* manipulations were performed in biosafety level 3 facilities.

2.4 | Lipoproteins and LPS

B. abortus lipidated outer membrane protein 19 (L-Omp19) and unlipidated Omp19 (U-Omp19) were obtained as described (Giambartolomei et al., 2004). Both recombinant proteins contained less than 0.25 endotoxin U/μg of protein as assessed by Limulus Amebocyte Lysates (Associates of Cape Cod). *B. abortus* S2308 LPS was provided by I. Moriyon (University of Navarra, Pamplona, Spain). The synthetic lipohexapeptide (tripalmitoyl-S-glyceryl-Cys-Ser-Lys4-OH [Pam_3Cys]) was purchased from Boehringer Mannheim.

2.5 | Infection

Cells were infected with *B. abortus* S2308 at different multiplicities of infection (MOI) for 2 hr in medium containing no antibiotics. Then, cells were extensively washed to remove un-internalized bacteria and infection was maintained for different times in the presence of antibiotics (100 μg/ml gentamicin and 50 μg/ml streptomycin) to kill remaining extracellular bacteria. To monitor *Brucella* intracellular survival, infected cells were lysed with 0.1% (v/v) Triton X-100 in H_2O after phosphate-buffered saline washing; and serial dilutions of lysates were plated onto TSB agar plates to enumerate colony forming units (CFU).

2.6 | Culture treatment

Co-culture of neurons and glial cells were established by adding 1×10^5 microglia or astrocytes into neuron-containing wells. After 18 hr of culture to allow the glial cells to adhere, cultures were infected as indicated before. Additionally, co-cultures were stimulated for 48 hr with different concentrations of HKBA, L-Omp19, U-Omp19, *B. abortus LPS* (1,000 ng/ml), *Escherichia coli LPS* (100 ng/ml), or Pam_3Cys (50 ng/ml). In some experiments co-cultures were pre-incubated for 1 hr with aminoguanidine (AG) (200 μM) (Sigma-Aldrich) or nitro-L-arginine methyl ester (L-Name) (1 mM) (Sigma-Aldrich) and then infected for 48 hr in the presence of the inhibitors. Also, to inhibit microglial phagocytosis, cRGD (Cyclo(-Arg-Gly-Asp-D-Phe-Val)) and cRAD (Cyclo(-Arg-Ala-Asp-D-Phe-Val)) peptides (100 μM) (Bachem) or recombinant annexin V (100 nM) (eBioscience) were added together with infection inocula and left throughout the culture. For stimulation of neurons with conditioned media, culture supernatants from *Brucella*-infected microglia (MOI 100) were harvested at 24 hr post-infection, sterilized by filtration and used to stimulate noninfected neurons at several dilutions in complete Neurobasal medium for 48 hr. Recombinant mouse interleukin (IL)-1β (0.5 and 10 ng/ml) and tumor necrosis factor (TNF)-α (3 and 10 ng/ml), granulocyte monocytes colony stimulating factor (GM-CSF) (50 ng/ml) (BD Pharmingen) and diethylenetriamine (Deta-NO) (0.1 and 1 mM) (Sigma-Aldrich) were used where indicated. For transwell experiments, 1×10^5 microglia were plated onto transwell membrane inserts (pore size 0.4 μm) (Costar) and neurons onto glass coverslips on the bottom well of the transwell.

2.7 | Immunofluorescence microscopy and determination of neuronal densities

Co-culture cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.125% Triton X-100 and blocked with 5% FBS. Neurons were labeled with anti-βIII-Tubulin monoclonal antibody (mAb) (clone 2G10, Sigma-Aldrich) followed by Alexa 546-labeled anti-mouse IgG2a (Life Technologies Inc.). Microglia were stained with Isolectin-B4-Biotin (Vector Laboratories) followed by Cy™2 Streptavidin (Jackson ImmunoResearch). For nuclear counterstaining, 4, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes) or TO-PRO®-3 (Invitrogen) were used. Healthy vs. apoptotic neurons were recognized by their distinct nuclear morphology. Cell numbers were assessed using an Olympus microscope and counted using ImageJ software. Five microscopic fields per well (between 150 and 200 neurons per field in control wells) in 2 wells per condition were quantified for each experiment. Percentage of neuronal viability was calculated with respect to the nontreated/noninfected controls. To quantify the amount of phagocytosed neurons by *B. abortus*-infected microglia, bafilomycin A1 (80 nM) (Sigma-Aldrich) was added to co-cultures 24 hr after infection. Cultures were then cultured for 5 hr and the presence of phagocytized (not degraded) neurons inside activated microglia was enumerated by microscopy. Confocal images were analyzed using FV-1000 confocal microscope with an oil immersion Plan Apochromatic 60X NA1.42 objective (Olympus).

2.8 | Measurement of TNF-α and nitric oxide

Mouse TNF-α was measured in culture supernatants from different experiments, by sandwich ELISA, using paired cytokine-specific mAbs, according to the manufacturer's instructions (BD Pharmingen). NO concentration was measured by the colorimetric Griess reaction.

2.9 | Inducible NO synthase determination by flow cytometry

Microglia were harvested 24 hr post infection by trypsin-0.25% EDTA treatment and washed with PBS. Cells were fixed with 0.05% paraformaldehyde. Then, cells were treated with 0.25% Triton X-100 for 15 min, blocked with 3% bovine serum albumin (BSA) for 1 hr and stained with an anti-iNOS antibody (clone 6, BD Biosciences) for 30 min. An

isotype-matched antibody was assayed in parallel and fluorescence was determined by BD FACSCalibur on 20,000 events per each sample.

2.10 | Apoptosis assay

Apoptosis assay was conducted using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay using the Fluorescein-FragEL DNA Fragmentation Detection Kit (Calbiochem), following the instruction manual. Labeled cells were analyzed by fluorescence microscope using an Olympus Microscope.

2.11 | Phagocytosis assays

To determine the phagocytosis ability of microglia, the phagocytic uptake of *E. coli* DH5α (Invitrogen) was measured as described (Gaikwad & Agrawal-Rajput, 2015). Briefly, microglia were infected with *B. abortus* at different MOI; or treated with HKBA, L-Omp19, or U-Omp19 for 24 hr. Cells were washed twice and cultured in the presence of *E. coli* for 30 min at 37°C in 5% CO_2. Extracellular bacteria were washed and killed with gentamicin (100 μg/ml) for 30 min. Cells were washed, lysed with 0.1% (v/v) Triton X-100 in H_2O, and plated overnight on TSB agar and CFU were counted.

Activated microglia were incubated with FluoSpheres® Carboxylate-Modified Microspheres, 0.1 μm, red fluorescent (Molecular Probes) for 2 hr at 37°C in 5% CO_2. Then, medium was removed and cells were washed with ice-cold PBS to arrest bead uptake and fixed with 4% paraformaldehyde. Nuclei were labeled with DAPI and phagocytosis was evaluated by fluorescence microscopy using an Olympus microscope.

2.12 | Phosphatidylserine staining

Non fixed co-cultures of neurons and microglia were washed with Hank's solution and incubated with EGFP-tagged annexin V reagent (BioVision) (1:100) for 30 min at 37°C. Cells were washed and fixed with 4% paraformaldehyde and neurons were labeled as described above.

2.13 | Statistical analysis

Each experiment was performed at least three times with different culture preparations. Statistical analysis was performed with one-way analysis of variance, followed by the post hoc Bonferroni test using GraphPad Prism 5.0 software. Data are represented as means ± *SEM*.

3 | RESULTS

3.1 | *Brucella abortus* does not infect neurons or induces their death

There are no previous reports about the interaction of *B. abortus* with neurons. Thus, we first determined the ability of the bacterium to infect these cells. For this, primary murine neurons were cultured with *B. abortus* and intracellular bacteria were quantified at different times. As control, primary astrocytes and microglia were cultured in parallel. *B. abortus* was unable to infect and replicate inside of neurons. On the contrary, and as we have reported previously (Garcia Samartino et al., 2010), *B. abortus* was capable of replicating inside primary astrocytes and microglia (Figure 1a). Additionally, neurons did not secrete pro-inflammatory cytokines upon interaction with *B. abortus* (data not shown). Furthermore, different concentrations of *B. abortus* were unable to modify the viability of neurons, when compared with noninfected cells ($p > .05$), after 48 hr of culture (Figure 1b,c). These experiments demonstrate that *B. abortus* was unable to infect neurons or affect their viability.

3.2 | *Brucella*-activated microglia induce neuronal death

We have described that upon infection with *B. abortus* astrocytes and microglia are activated and secrete pro-inflammatory mediators (Garcia Samartino et al., 2010; Miraglia et al., 2013). Thus, to examine the fate of neurons under these inflammatory conditions, co-cultures of microglia or astrocytes and neurons were infected with *B. abortus*. Although infection induced the inflammatory activation of microglia (TNF-α secretion and proliferation) and astrocytes (TNF-α secretion) in the co-cultures (Figure 1e,f), only *B. abortus*-infected microglia induced a significant reduction ($p < .05$) in the number of neurons after 48 hr of culture in a MOI-dependent fashion (Figure 1b,c). This phenomenon did not depend on a particular neuronal or glial cell preparation since it was corroborated in six independent experiments using cells from different animals (Figure 1d). Neuronal loss was independent of bacterial viability since heat-killed *B. abortus* (HKBA)-activated microglia induced a significant ($p < .05$) reduction in the number of neurons in culture at both tested concentrations (Figure 2). We further evaluated the contribution of *B. abortus* LPS or its lipoproteins in inducing microglia-mediated neuronal death. For that, co-cultures of microglia and neurons were incubated with *B. abortus* LPS and *B. abortus* lipidated outer membrane protein 19 (L-Omp19), used as *Brucella* lipoprotein model (Giambartolomei et al., 2004). *E. coli* LPS, unlipidated (U)-Omp19 and Pam_3Cys were used as controls. L-Omp19, but not *B. abortus* LPS, recapitulated the neuronal loss induced by *B. abortus*-infected microglia and this phenomenon was dependent on the lipidation of L-Omp19, as U-Omp19 failed to induce microglia-mediated neuronal loss (Figure 2). Both, *E. coli* LPS- and Pam_3Cys-activated microglia induced neuronal death. Altogether, these results indicate that *Brucella*-activated microglia, but not astrocytes, induce neuronal demise and L-Omp19 is involved in this phenomenon.

3.3 | TLR2 determines microglia-mediated neuronal loss

Previously, we have reported that TLR2 signaling is crucial for inflammatory responses induced by *B. abortus* and L-Omp19 (Barrionuevo et al., 2008; Delpino et al., 2012; Giambartolomei et al., 2004; Miraglia et al., 2016). Thus, we assessed the requirements for the TLR signaling pathways in pro-inflammatory microglial activation

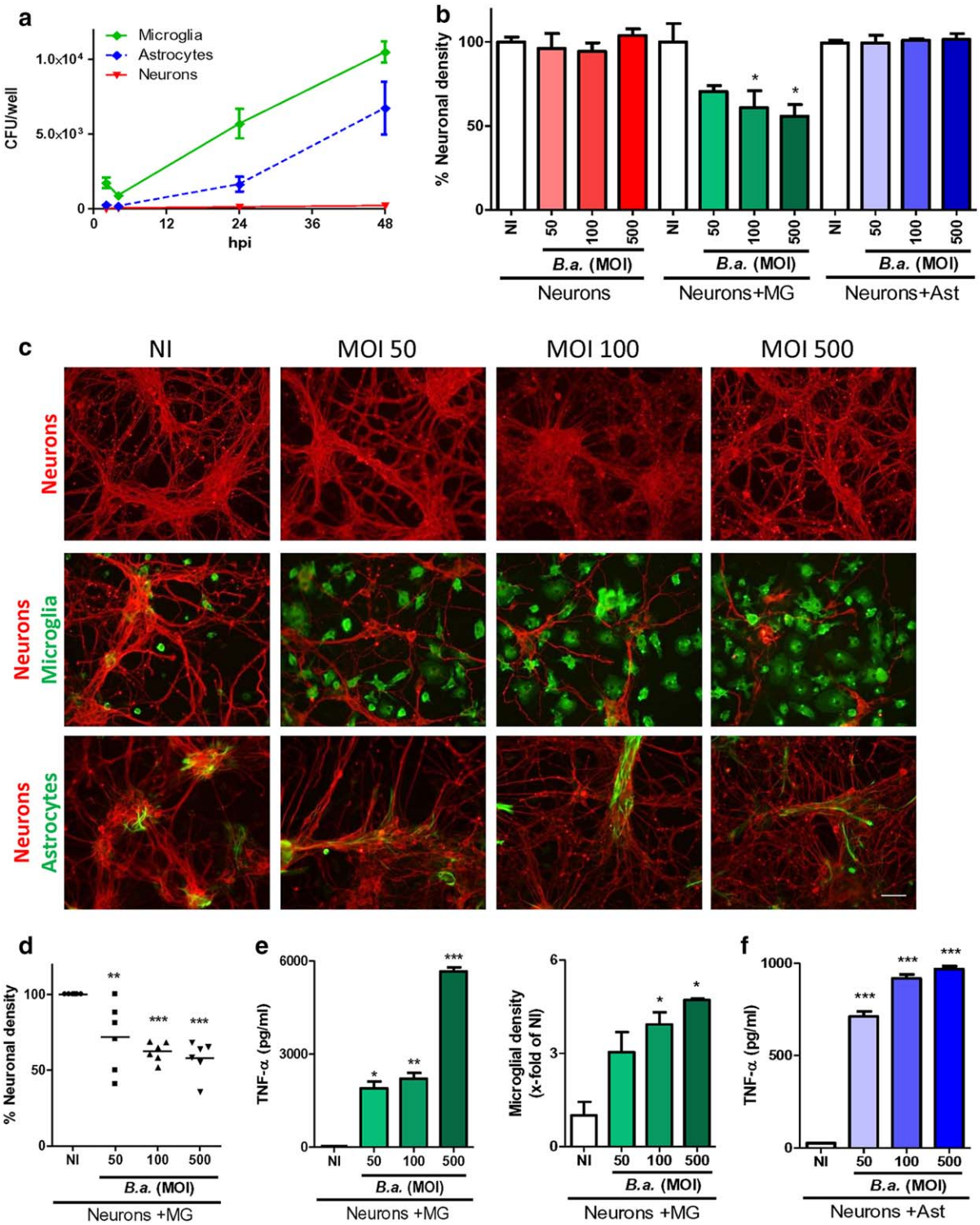

FIGURE 1 *B. abortus* infected-microglia mediate neuronal loss. Infection with *B. abortus* (*B.a.*) was performed at a MOI of 100, and CFU were determined after the indicated hr post-infection (hpi) in neurons (red), microglia (green), and astrocytes (blue) cultures (a). Cultures of neurons alone, neurons-microglia (MG) (2:1), or neurons-astrocytes (Ast) (2:1) were infected at indicated MOIs, and the viability of neurons was evaluated after 48 hpi by fluorescence microscopy. Percentage (%) of neuronal density was calculated vs. noninfected (NI) condition (b). Representative pictures of cultures of neurons alone, neurons-microglia or neurons-astrocytes are shown where neurons were stained whit anti-βIII-Tubuline (red), microglia were stained with isolectin-B4 (green), and astrocytes were stained with anti-GFAP (green). Scale bar: 50 μm (c). Percentage (%) of neuronal density representative of six independent experiments (d). TNF-α was measured in supernatants from *B. abortus*-infected cultures of neurons-microglia (e) and neurons-astrocytes (f) at 48 hpi. Microglial proliferation was evaluated by enumerating microglia in fluorescent microscopy pictures and represented as fold increase of NI cultures (e). Bars or symbols represent the media ± *SEM* of duplicates. Data shown are from a representative experiment of three performed, unless otherwise stated. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$ vs. NI condition

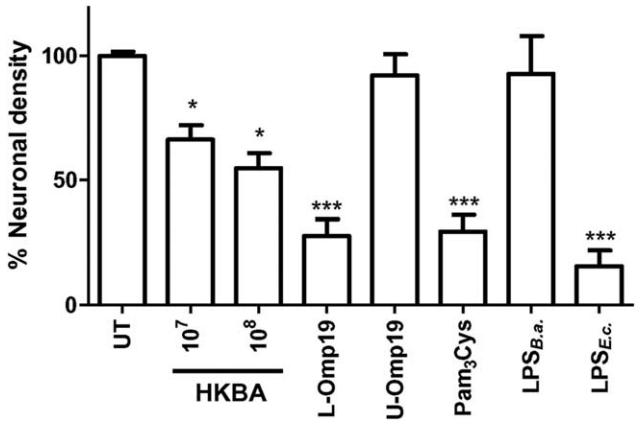

FIGURE 2 HKBA and L-Omp19 activated-microglia mediate neuronal death. Cultures of neurons-microglia were incubated with complete medium (untreated, UT), HKBA (1×10^7 or 1×10^8 bacteria/ml), L-Omp19 (500 ng/ml), U-Omp19 (500 ng/ml), Pam_3Cys (50 ng/ml), *B. abortus* LPS ($LPS_{B.a.}$, 1,000 ng/ml) and *E. coli* LPS ($LPS_{E.c.}$, 100 ng/ml), and viability of neurons was evaluated after 48 hr by fluorescent microscopy. Percentage (%) of neuronal density was calculated vs. UT condition. Bars express the mean ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. $^*p < .05$, $^{***}p < .0005$ vs. UT

and subsequent induction of neuronal death in response to *B. abortus* infection or L-Omp19 stimulation. Microglia from C57BL/6 wild type mice or from Mal/TIRAP, TLR2 and TLR4 KO mice were infected with *B. abortus* or treated with L-Omp19 to assess the production of TNF-α and determine microglial activation. TNF-α production by TLR2 and Mal/TIRAP KO microglia infected with *B. abortus* or stimulated with L-Omp19 was significantly reduced ($p < .0005$) when compared with wild type cells. On the contrary, microglia from TLR4 KO mice produced similar amounts of TNF-α than those from wild type mice. Microglia from Mal/TIRAP, TLR2, and TLR4 KO mice did not produce TNF-α in response to their cognate ligands (Pam_3Cys and *E. coli* LPS, respectively) (Figure 3a). Concomitantly, neuronal loss was only induced in co-cultures of neurons from wild type mice with microglia from wild type or TLR4 KO mice, either stimulated with L-Omp19 or infected with *B. abortus* ($p < .05$). Microglia from Mal/TIRAP or TLR2 KO mice did not induce neuronal loss (Figure 3b). U-Omp19-stimulated microglia from wild type or all KO mouse strains were unable to induce either TNF-α secretion or neuronal loss (Figure 3a,b). Collectively, these results indicate that Mal/TIRAP and TLR2 signaling mediate microglial activation induced by *B. abortus* and its lipoproteins and that this activation determines concomitant neuronal death.

3.4 | Nitric oxide is required for the neuronal death induced by activated microglia

Nitric oxide (NO) is an inflammatory mediator that was consistently implicated in neurodegeneration and microglial-induced neuronal death (Chao, Hu, Molitor, Shaskan, & Peterson, 1992; Lehnardt et al., 2006; Liu et al., 2002). Thus, we decided to investigate its role in the neuronal loss induced by *B. abortus*-activated microglia. First, we evaluated if microglia could produce and release NO in response to *B. abortus* infection or L-Omp19 treatment. Infected or L-Omp19-treated microglia released NO in a time- and MOI-dependent fashion. This phenomenon was dependent on TLR2, since microglia from TLR2 KO mice were unable to secrete NO upon infection or L-Omp19 treatment (Figure 4a, b). Consequently, we observed an intracellular increase of the inducible NO synthase (iNOS) expression in *B. abortus*-infected microglia, which was also dependent on the presence of TLR2 in microglia (Figure 4c). To determine whether NO was actually involved in *B. abortus*-induced neuronal loss, we used two inhibitors of NO production: aminoguanidine (AG), an inhibitor of both constitutive and iNOS (Joly, Ayres, Chelly, & Kilbourn, 1994), and nitro-L-arginine methyl ester (L-Name), an analog of arginine that inhibits iNOS in mouse cells (Furfine, Harmon, Paith, & Garvey, 1993). Again, when co-cultures of neurons and microglia were infected with *B. abortus*, neuronal viability diminished with respect to noninfected cultures ($p < .05$). However, when infection occurred in the presence of AG or L-Name there was no difference in the number of neurons with respect to noninfected controls ($p > .05$) (Figure 4d). Abrogation of NO, but not of TNF-α, secretion indicated the specific inhibition of NO release which did not affect the overall microglial activation (Figure 4e,f). These results demonstrate that the neurotoxic effect induced by *B. abortus* infected-microglia depends on NO.

3.5 | Neurons death is not due to apoptosis

It has been previously shown that NO may have neurotoxic effects by generating apoptosis on neurons (Brown, 2010; Lehnardt et al., 2006). Thus, to determine whether the observed neuronal death after infection of neuronal-microglial cultures with *B. abortus* was due to apoptosis, we performed the TUNEL assay and analyzed neurons after infection with *B. abortus* for 48 hr. Although infection of microglia significantly ($p < .05$) reduced the number of viable neurons after 48 hr of culture, the number of $TUNEL^+$ cells was always below 5% at any MOI tested (the same percentage of noninfected cultures), thus indicating that neurons were not dying due to apoptosis (Figure 5a and data not show). As apoptosis could have been taking place before the end of culture (48 hr), and neurons removed from culture by an alternative mechanism, we next evaluated the kinetic of TUNEL staining throughout the culture. Once more, $TUNEL^+$ cells remained marginal throughout the culture although neuronal loss induced by *B. abortus*-activated microglia significantly increased with culture time (Figure 5b). Of note, the kinetics of neuronal demise correlated with that of NO release (Figure 4a). These results indicate that although *B. abortus*-activated microglia induced neuronal death, apoptosis is not involved in the process.

3.6 | Inflammatory neuronal loss requires direct microglial-neuronal contact

Several inflammatory mediators induced by activated microglia such as reactive oxygen species, NO, TNF-α and even metalloproteases were shown to be neurotoxic (Block, Zecca, & Hong, 2007; Chao et al., 1992; Nguyen et al., 2007; Venters et al., 2000). *B. abortus*-infected

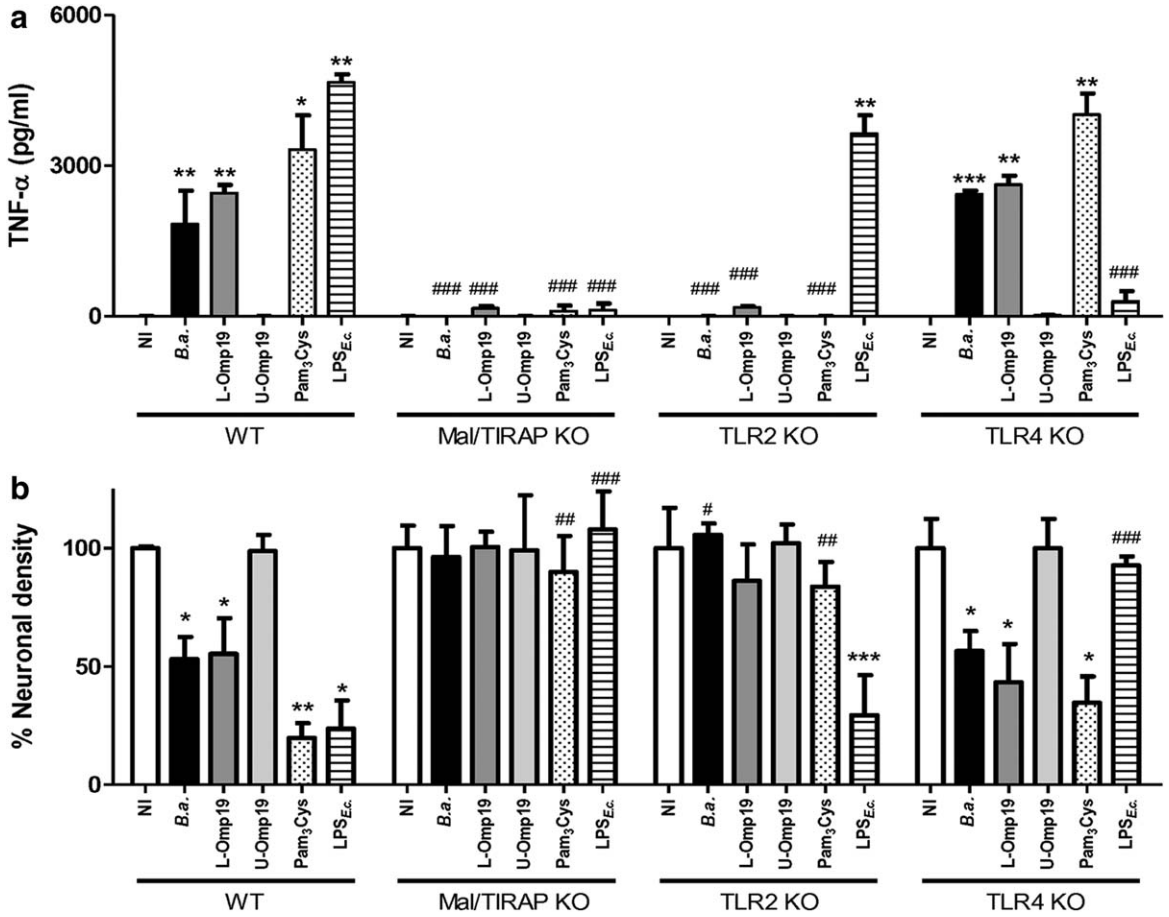

FIGURE 3 TLR2 determines microglia-mediated neuronal loss. Neurons from wild type (WT) mice were cultured with microglia from WT, Mal/TIRAP, TLR2, or TLR4 KO animals. Cells were infected with *B. abortus* (*B.a.*) at MOI 100 or stimulated with L-Omp19 (500 ng/ml), U-Omp19 (500 ng/ml), Pam_3Cys (50 ng/ml), or *E. coli* LPS ($LPS_{E.c.}$ 100 ng/ml) for 48 hr. TNF-α was measured in culture supernatants (a). Viability of neurons was evaluated by fluorescence microscopy. Percentage (%) of neuronal density was calculated vs. noninfected (NI) condition (b). Bars express the mean ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$ vs. NI. $^{\#}p < .05$, $^{\#\#}p < .005$, $^{\#\#\#}p < .0005$ vs. the same stimuli on WT microglia

microglia secrete most mediators implicated in neuronal injury (Garcia Samartino et al., 2010; Miraglia et al., 2013), even NO as described herein. However, neuronal demise can also occur via other mechanisms that require cell to cell contact between microglia and neurons (Myers et al., 2009; Neher et al., 2011). To test the requirement of direct microglial-neuronal interaction for the occurrence of neuronal death, microglia were cultured either directly or physically separated from neurons on transwell membrane inserts, followed by infection with *B. abortus*. In direct microglial-neuronal contact, *B. abortus* infection induced, as previously observed, about 50% of neuronal loss after 48 hr. However, when microglia were kept physically separated, there was no significant ($p < .05$) neuronal loss compared with uninfected co-cultures (Figure 6a). In addition, different dilutions of culture supernatants from *B. abortus*-infected microglial cells were unable to directly induce neuronal death (Figure 6b). Moreover, direct treatment of neurons with recombinant IL-1β, TNF-α (both used at concentration secreted by *B. abortus*-infected microglia) or diethylenetriamine (Deta-NO) (a NO donor used to produce a controlled release of NO in solution), used at a concentration that released NO in the same range as that of *B. abortus*-infected microglia (100 μM), were all unable to induce neuronal death without a visible increase in the number of apoptotic cells. However, and as described previously (Chen, Zhang, Zhang, Li, & Sun, 2016; Ye et al., 2013), higher concentrations of all mediators induced neuronal death with an increased number of apoptotic cells (Figure 6c). Altogether, these results indicated that, in the absence of direct microglial-neuronal interaction, soluble inflammatory mediators released by *B. abortus*-infected microglia were not sufficient to cause neuronal death. They also indicated that although secreted NO contributes to, it does not determine neuronal loss, as induced by *B. abortus*-infected microglia.

3.7 | *B. abortus* activated microglia phagocytose neurons

The above results have revealed that *B. abortus*-infected microglia can cause neuronal loss via a cell to cell contact mechanism that required NO. Microglia may play a crucial role in the phagocytosis of neurons during brain development, inflammation, ischemia and neurodegeneration.

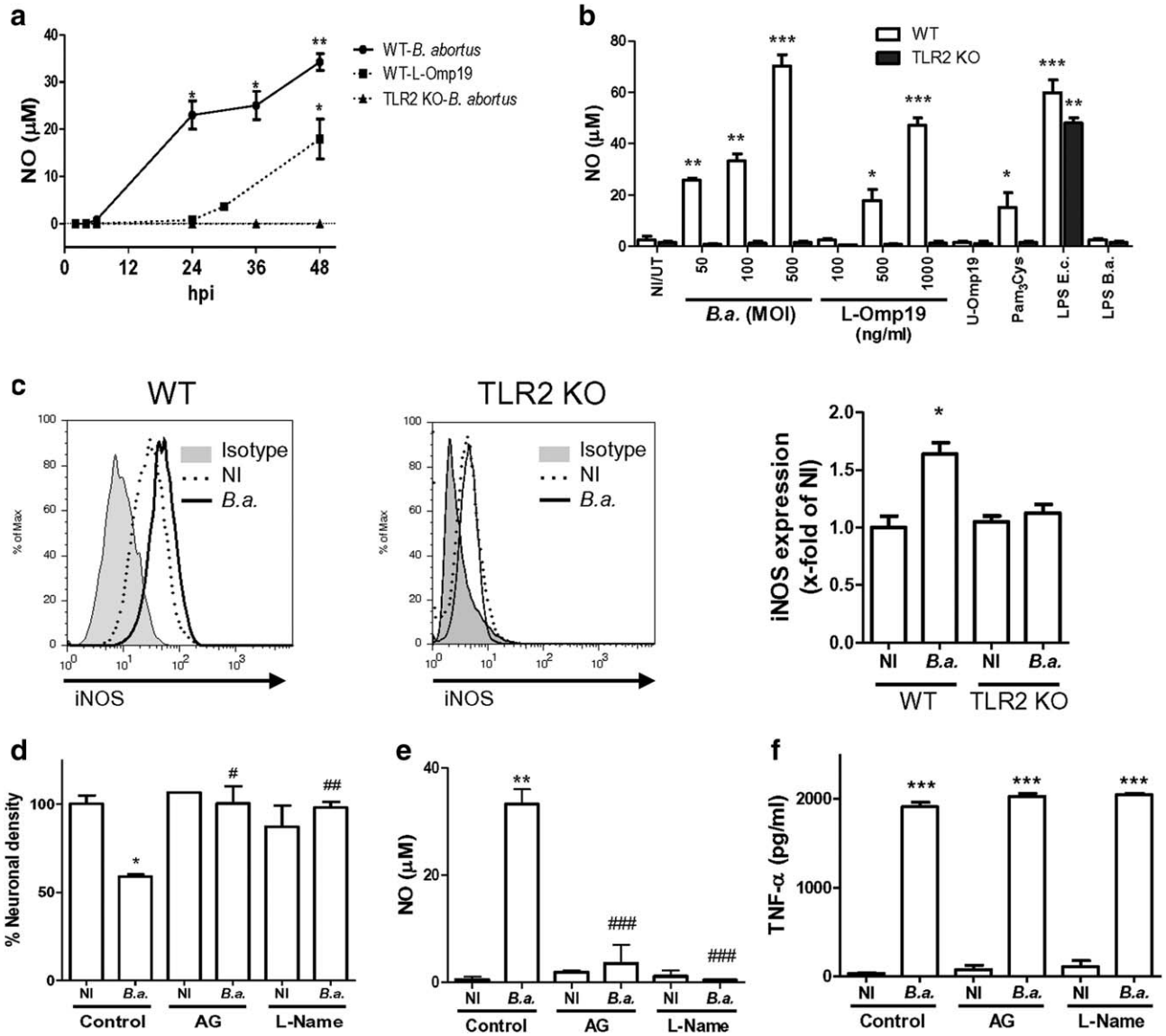

FIGURE 4 NO is required for *B. abortus*-induced microglial-mediated neurotoxicity. NO was measured by Griess reaction in supernatants from WT or TLR2 KO microglia infected with *B. abortus* (*B.a.*) at MOI 100 or stimulated with L-Omp19 (500 ng/ml) at the indicated hpi (a) or at 48 hr at the indicated MOIs of *B.a.* or concentrations of L-Omp19. U-Omp19 (1,000 ng/ml), LPS from *B.a.* (1,000 ng/ml) o from *E. coli* (100 ng/ml), or Pam_3Cys (50 ng/ml) were used as controls (b). WT or TLR2 KO microglia were infected at MOI 100 and 24 hpi the expression of iNOS was evaluated by flow cytometry (c). Cultures of neurons-microglia were pre-treated for 1 hr with 200 mM of AG or 1 mM of L-Name, infected at MOI 100 and cultured for 48 hr in the presence of the inhibitors, and viability of neurons was evaluated by fluorescence microscopy. Percentage (%) of neuronal density was calculated vs. noninfected (NI) control condition (d). NO (e) or TNF-α (f) were measured in culture supernatants. Bars or symbols represent the media ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. MFI, mean fluorescence intensity. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$ vs. NI. $^{\#}p < .05$, $^{\#\#}p < .005$, $^{\#\#\#}p < .0005$ vs. *B.a.* control condition

Several studies have demonstrated that this phenomenon involves signals on the target cell and receptors on the microglia cell surface (Brown & Neher, 2012, 2014; Neher, Neniskyte, Hornik, & Brown, 2014; Neher et al., 2012). To delineate the involvement of phagocytosis in neuronal loss induced by *B. abortus* infection, we first examined whether the phagocytic activity of microglia can be regulated by *B. abortus* infection. Thus, microglia were infected with *B. abortus* for 24 hr and then cultured with *E. coli* for 30 min. Unphagocytosed *E. coli* were killed by the addition of antibiotics and phagocytosed bacteria were measured by CFU counting. While uninfected cells ingested low numbers of bacteria, *B. abortus*-infected microglia significantly increased phagocytosed *E. coli* ($p < .05$) in a MOI-dependent fashion (Figure 7a). Same results were obtained when microglia was stimulated with HKBA or L-Omp19 (Figure 7b,c). Inflammatory activation of microglia caused by *B. abortus* infection, treatment with HKBA or L-Omp19 stimulation also strongly enhanced phagocytic uptake of negatively charged microbeads (Figure 7d). Next, we investigated the capacity of *B. abortus*-infected microglia to phagocytize neurons. For this co-cultures of microglia and neurons were infected

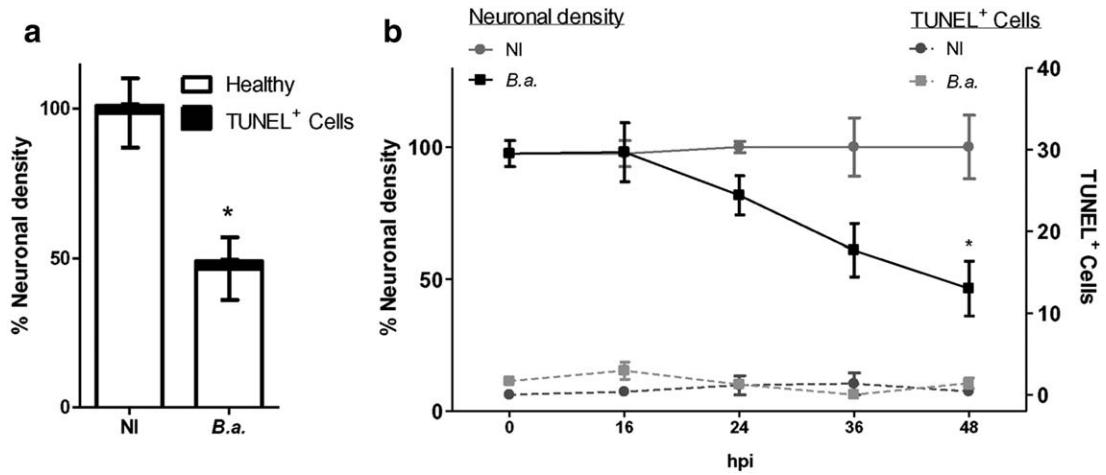

FIGURE 5 Neurons death is not due to apoptosis. Co-cultures of neurons and microglia were infected with *B. abortus* (*B.a.*) at MOI 100. Apoptosis (evaluated by TUNEL assay) and healthy neurons were evaluated by fluorescence microscopy after 48 hpi (a) or at 16, 24, 36, and 48 hpi (b). Percentage (%) of neuronal density was calculated vs. noninfected (NI) condition. Bars or symbols represent the media ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. $^*p < .05$ vs. NI

with *B. abortus* and analyzed by confocal microscopy. Confocal images revealed that neurons were being phagocytosed by *B. abortus*-infected microglia. Nuclear staining corroborated the cytoplasmic localization of phagocytosed neurons (Figure 7e). As no apoptotic cells were found at any evaluated time post-infection (Figure 5), these data suggest that *B. abortus* activated-microglia phagocytosed live neurons.

3.8 | Inhibition of primary phagocytosis prevents *B. abortus*-mediated neuronal loss

It has been described that NO-damaged neurons can expose phosphatidylserine (PS) on their surface (Neher et al., 2011); PS acts as an "eat me" signal for phagocytes. As such, we investigated if *B. abortus*-infected microglia could induce PS exposure on the membrane of neurons. Infection significantly increased PS exposure ($p < .05$) as determined by the binding of fluorescently labeled annexin V, with PS being localized mainly on neuronal processes. Interestingly, PS exposure was reduced to basal level when cultures were treated with the NO inhibitor L-Name (Figure 8a). Phagocytic recognition of externalized PS by microglia and other macrophages can be enhanced by soluble adaptor proteins, including milk fat globule epidermal growth factor-8 (MFG-E8, also known as lactadherin), which binds PS expressed on the surface of damaged cells and the vitronectin receptor on phagocytes, thus bridging phagocyte and target cell and improving phagocytic recognition (Akakura et al., 2004). This process is known as primary phagocytosis or phagoptosis (Brown & Neher, 2014). Accordingly, we tested whether blocking primary phagocytosis was sufficient to prevent neuronal death induced by *B. abortus*-activated microglia. Blocking primary phagocytosis by masking the exposed PS with recombinant annexin V completely prevented *B. abortus*-induced neuronal loss, indicating that blocking of exposed PS was sufficient to block neuronal loss (Figure 8b). MFG-E8 binds exposed PS through its C1 and C2 domains, and the macrophage vitronectin receptor through an RGD motif (Akakura et al., 2004). Infection of co-cultures in the presence of the peptide cRGD, an integrin antagonist specific for the MFG-E8 receptor (Haubner et al., 1996), also abrogated *B. abortus*-induced neuronal loss as compared with the control peptide cRAD which did not prevent neuronal loss induced by *B. abortus*-infected microglia (Figure 8c). Importantly, none of the blocking agents prevented inflammatory activation of microglia as assessed by TNF-α production (Figure 8d,e). In all cases (annexin V and cRGD), blocking microglial phagocytosis of neurons prevented neuronal loss without increasing the number of apoptotic neurons (Figure 8b,c), indicating that phagocytosis was the cause of death, rather than its consequence. Indeed, if microglia had killed neurons first and subsequently phagocytosed them, then inhibition of phagocytosis would have left dead (apoptotic) not live neurons.

Finally, microglial phagocytosis is known to be stimulated by GM-CSF (Giulian & Ingeman, 1988), which, however, does not increase microglial production of pro-inflammatory mediators such as TNF-α or IL-1β (Suzumura, Sawada, & Marunouchi, 1996). Although GM-CSF increased the phagocytosis of *E. coli* and negatively charged microbeads by microglia (Figure 8f), as well as the proliferation of microglial cells, without inducing TNF-α or NO secretion; it did not lead to neuronal loss (Figure 8g). This result suggests that the phagocytosis of neurons induced by *B. abortus*-infected microglia is not due to stimulation of microglial phagocytosis alone, but also requires inflammatory signaling, such as NO release. In summary, these results indicate that during *B. abortus*-induced neuroinflammation, activated microglia can phagocytose live neurons by the PS-MFG-E8-vitronectin receptor pathway.

4 | DISCUSSION

In most organ systems, the bystander injury caused by inflammation is usually reversible due to the inherent healing capacity of the cellular elements of that tissue. However, in the CNS the common consequence of bystander injury is irreversible neuronal loss and atrophy due to regenerative failure. Thus, although an efficient immune response is required for the defense against invading pathogens, in the

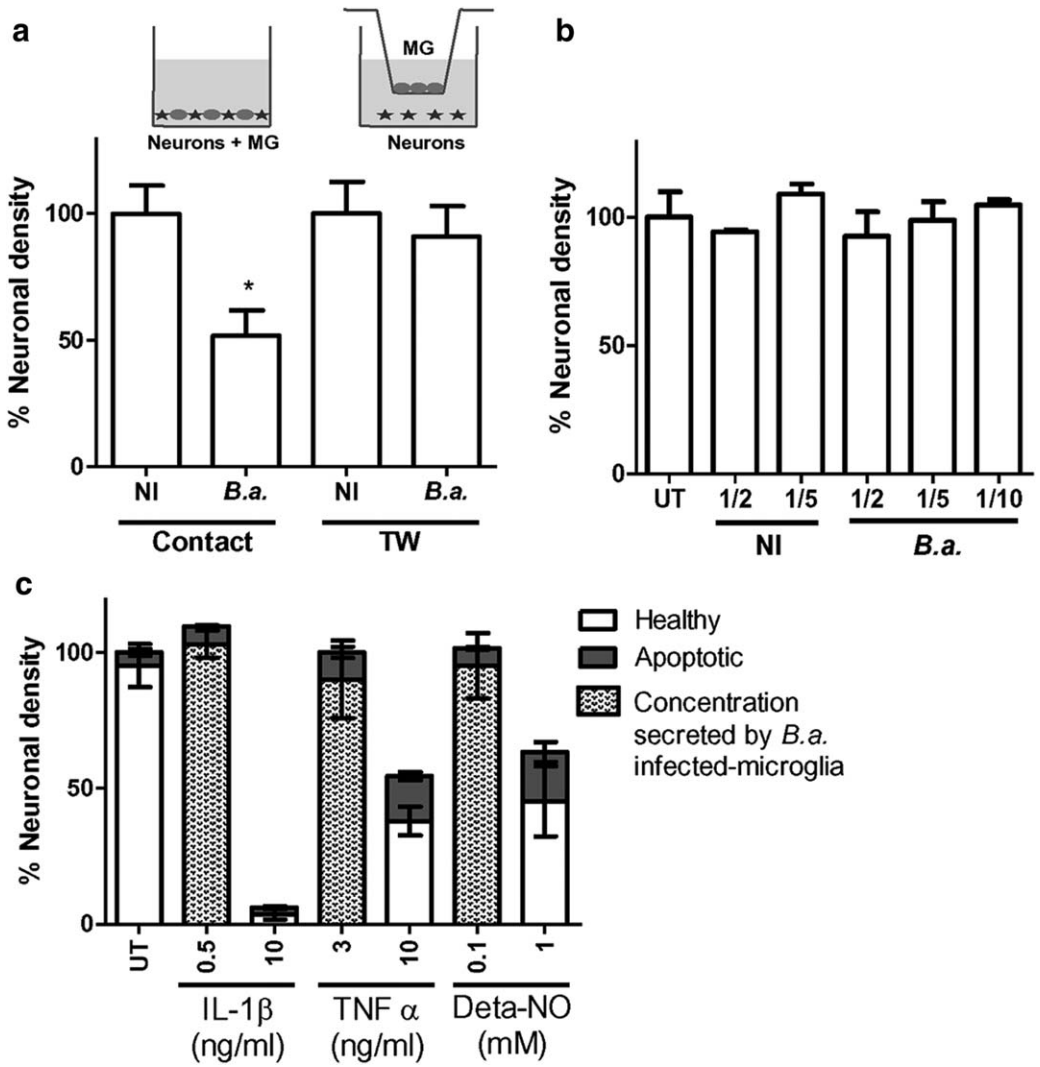

FIGURE 6 Inflammatory neuronal loss requires direct microglial-neuronal contact. Microglia (MG) and neurons were physically separated using transwell membrane inserts (TW) or kept in contact (contact). Cultures were infected with *B. abortus* (*B.a.*) MOI 100 and density of neurons was evaluated by fluorescence microscopy after 48 hpi. Percentage (%) of neuronal viability was calculated vs. noninfected (NI) (a). Cultures of neurons alone were stimulated or not (UT) with conditioned medium from *B.a.*-infected microglia (MOI 100 during 24 hr) or NI microglia at the indicated dilutions for 48 hr. Viability of neurons was evaluated by fluorescence microscopy after 48 hpi. Percentage (%) of neuronal density was calculated vs. UT condition (b). Cultures of neurons were stimulated or not (UT) with recombinant (*r*) IL-1β (0.5 and 10 ng/ml), rTNF-α (3 and 10 ng/ml), or diethylenetriamine (Deta-NO, 0.1 and 1 mM) during 48 hr (c). Viable or apoptotic neurons were evaluated by fluorescent microscopy. Percentage (%) of neuronal density was calculated vs. UT condition. Bars represent the mean ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. $^{*}p < .05$ vs. NI

CNS the concomitant inflammatory response elicited may also lead to tissue injury and neurodegeneration.

The mechanisms involved in the signs and symptoms of neurobrucellosis are not fully understood. Yet, it has been proposed that damage to the CNS can be attributed to the direct action of the bacterium or to an immunopathological process due to the inflammation triggered by innate immunity. No secreted proteases, toxins or lytic enzymes have been described so far in the bacterium, making a direct deleterious effect unlikely and pointing out to innate immune responses as the major cause of pathology (Baldi & Giambartolomei, 2013b; Giambartolomei et al., 2008; McLean et al., 1992). Indeed, our study shows that *B. abortus* was unable to directly harm neurons. It is *B. abortus*-induced activation of microglia, but not astrocytes, which causes neuronal death, which adds new evidence indicating that activation of innate immunity of CNS by *B. abortus* has detrimental effects not only on glial cells and the brain microvascular endothelium (Garcia Samartino et al., 2010; Miraglia et al., 2016), but also on neurons.

Although *B. abortus* infection of microglia induces a greater production of inflammatory mediators than astrocytes, as we have previously demonstrated (Garcia Samartino et al., 2010) and corroborated in this article; it is not the induction of these mediators which makes *B. abortus*-infected microglia kill neurons. Culture supernatants from *B. abortus*-infected microglial cells were unable to directly induce neuronal death, and it was necessary a direct cell to cell contact of neurons

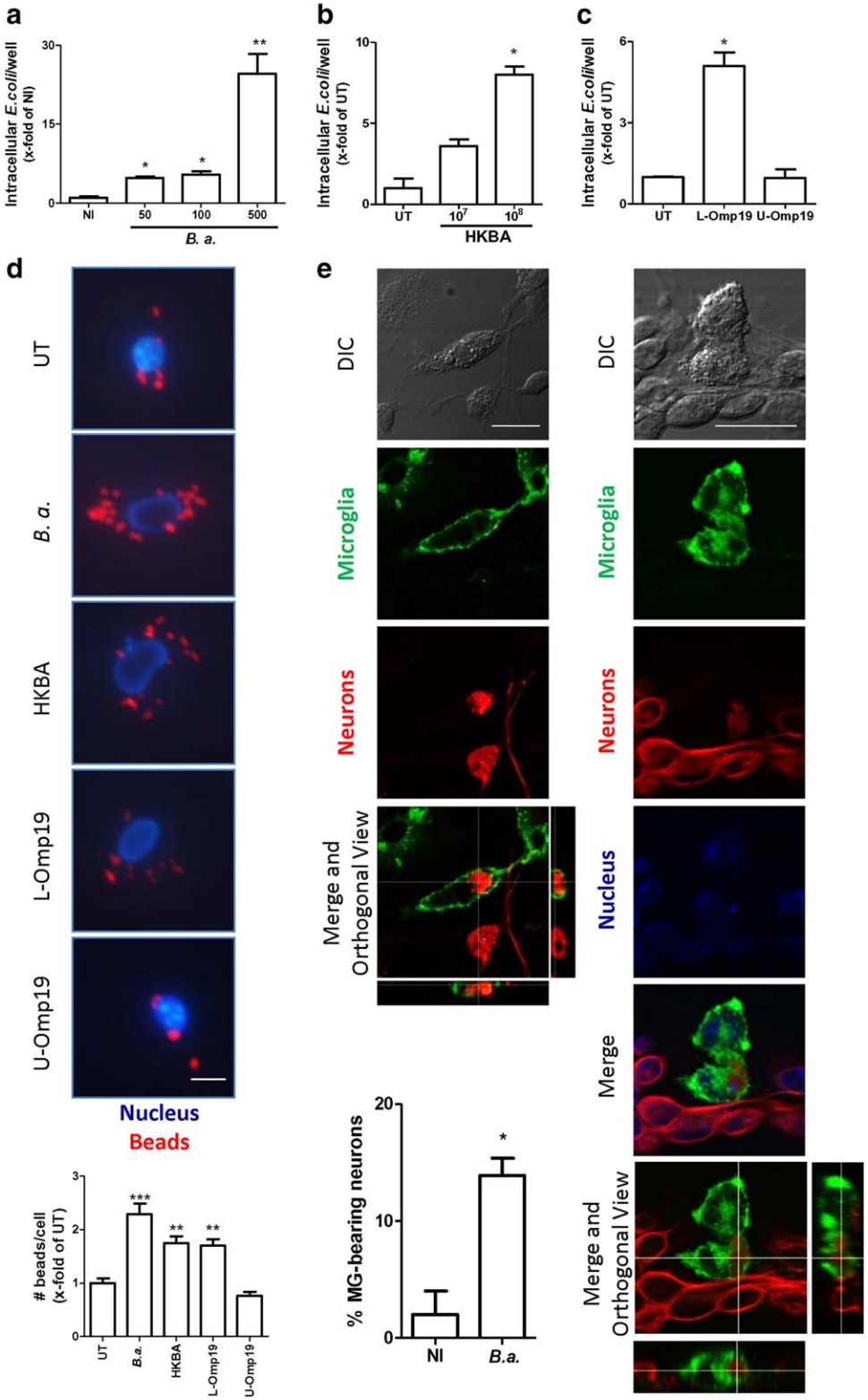

FIGURE 7 *B. abortus*-activated microglia phagocytose neurons. Microglia were infected with *B. abortus* (*B.a.*) at indicated MOIs or stimulated with HKBA (10^7 and 10^8 bacteria/ml) or L-Omp19 and U-Omp19 (500 ng/ml). After 24 hr, *E. coli* or fluorescent microbeads were added to the culture. Phagocytized *E. coli* were evaluated by intracellular CFU counting (a–c) and phagocytized beads (red) by fluorescent microscopy using DAPI to counter-stain nuclei (blue). Scale bar: 5 μm (d). Co-cultures of neurons-microglia were infected with *B.a.* and 24 hr later were fixed. Microglia were labeled with isolectin-B4 (green) and neurons were labeled with anti-βIII-Tubuline (red). Confocal z-stacks indicated by lines (*x* and *y*) are displayed below and to the right of the merged image. Scale bar: 20 μm. Percentage (%) of microglia (MG)-bearing neurons was calculated vs. NI condition on cultures treated with bafilomicin A1 (80 nM) (e). Bars represent the mean ± *SEM* of duplicates. Data shown are from a representative experiment of three performed. $^*p < .05$, $^{**}p < .005$ vs. NI or UT

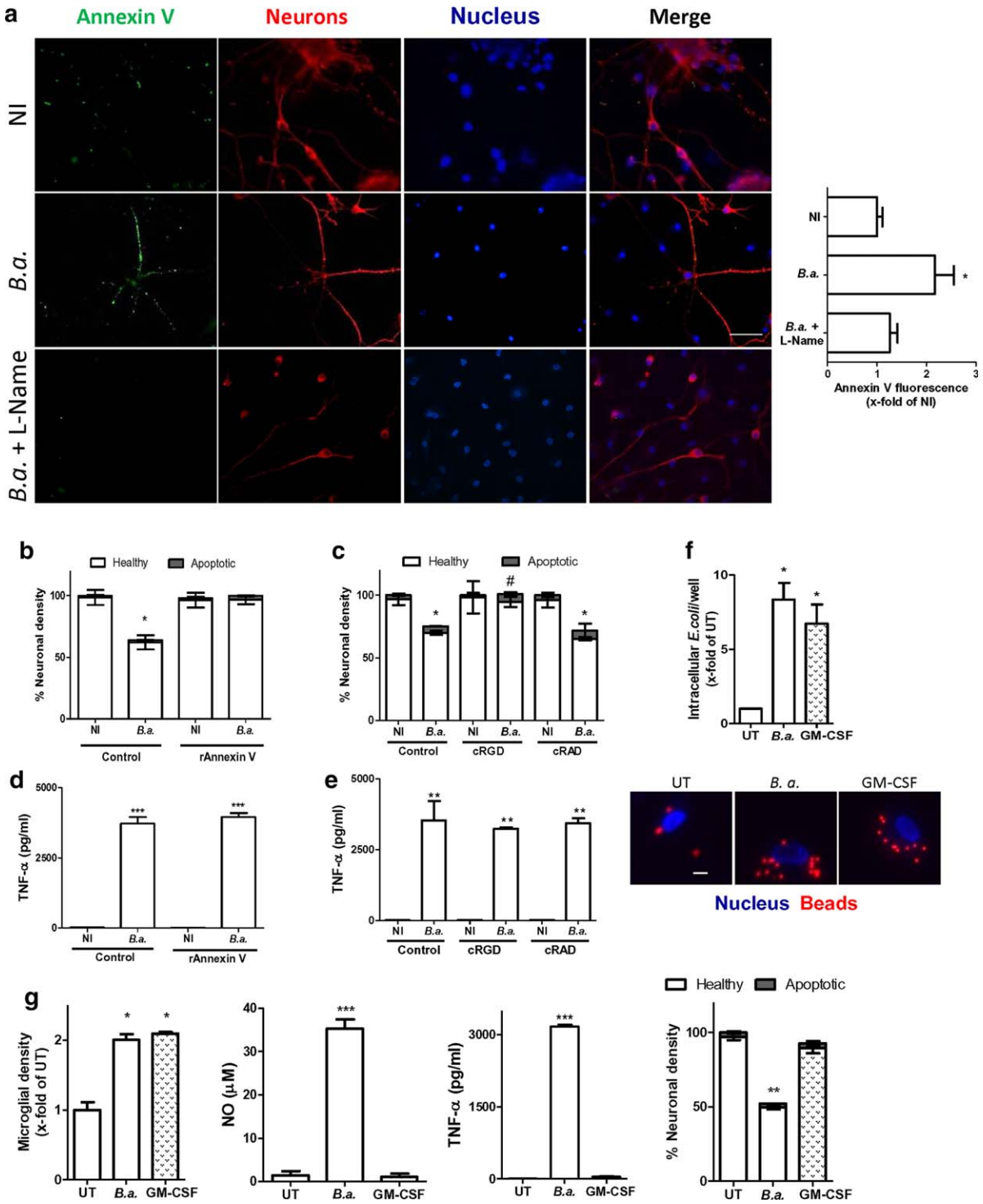

FIGURE 8 Inhibition of primary phagocytosis prevents *B. abortus*-mediated neuronal loss. Exposure of phosphatidylserine on neuron membranes was measured by annexin V-EGFP (green) binding on non fixed cultures of neurons-microglia infected with *B. abortus* (*B.a.*) or not (NI), or infected and treated with L-Name (*B.a.* ± L-Name) by 48 hr. Scale bar: 50 μm (a). Cultures of neurons-microglia were infected at MOI 100 with *B.a.* and cultured by 48 hr in presence or not of rAnnexin V (100 nM) (b) or cRGD or cRAD peptides (50 mM) (c). Healthy or apoptotic neurons were evaluated by fluorescence microscopy after 48 hpi and % of neuronal density was calculated vs. noninfected control condition. TNF-α was measured in supernatants (d and e). Microglia were treated with recombinant GM-CSF (50 ng/ml) or infected with *B. abortus* and after 24 hr, *E. coli* or fluorescent microbeads were added to the culture. Phagocytized *E. coli* were evaluated by intracellular CFU counting and phagocytized beads (red) by fluorescent microscopy using DAPI to counter-stain nuclei (blue). Scale bar: 5 μm (f). Cultures of microglia-neurons were treated with GM-CSF and after 48 hr microglial density and viability of neurons was evaluated by fluorescence microscopy. TNF-α and NO were measured in supernatants. Percentage (%) of healthy and apoptotic cells was calculated vs. un-treated (UT) condition (g). $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$ vs. NI or UT. $^{\#}p < .05$ vs. *B.a.* control condition

with microglia to trigger neuronal demise. *B. abortus*-induced neuronal loss is executed by microglial phagocytosis of live neurons. However, our results indicate that this process involves the inflammatory activation of microglia. In particular, neuronal loss seems to result from a sequence of events starting with microglial production of NO. Many studies have shown that neuronal loss depends on the inflammatory activation of microglia and have implicated NO as a common final mediator of inflammatory neurodegeneration (Chao et al., 1992; Lehnardt et al., 2006; Liu et al., 2002). We now show that NO secreted by *B. abortus*-activated microglia is necessary but not sufficient to induce neuronal loss. In contrast with the general assumption that NO causes direct apoptosis followed by phagocytic removal of apoptotic neurons (Brown, 2010; Lehnardt et al., 2006), we showed that neuronal demise is not due to apoptosis and that levels of NO produced by *Brucella*-activated microglia, mimicked by Deta-NO, are insufficient to cause neuronal death by themselves, yet inhibition of NO production rescued neurons from microglial-induced demise. This indicates that *B. abortus*-activated microglia would release sublethal levels of NO leading to neuronal "eat me" signal exposure, followed by the execution of neuronal death through microglial phagocytosis. Indeed, neurons exposed to *Brucella*-infected microglia display PS on their membranes. Conversely, although *B. abortus* infection increased the phagocytic activity of microglia, this event is not enough to kill neurons without a concomitant inflammatory response induced by the same cells, as indicated by the incapacity of GM-CSF-stimulated microglia to induce neuronal death, which are highly phagocytic but unable to secrete inflammatory mediators. Overall, these results suggest that the phagocytic removal of neurons observed in *B. abortus*-infected cultures is not due to stimulation of microglial phagocytosis alone, but also requires inflammatory signaling, specifically NO secretion.

Brucella-activated microglia execute neuronal death by primary phagocytosis, as opposed to phagocytosis secondary to neuronal apoptosis. We showed that when phagocytosis is blocked, without interfering with inflammation, the neurons survive rather than die, as would have been the case had phagocytosis occurred subsequent to cell death, where blocking of phagocytosis would have left apoptotic neurons in the cell culture, or at least a diminish number of viable cells. Neuronal loss by primary phagocytosis involves the PS-MFG-E8-vitronectin receptor pathway. MFG-E8 is one of several bridging molecules expressed by microglia (Milner & Campbell, 2003) that was demonstrated to enhance phagocytosis of neurons (Fuller & van Eldik, 2008). By blocking MFG-E8 interaction with neurons (by using annexin V to block PS recognition) or microglia (by using the cRGD peptide to block the vitronectin receptor recognition) we were able to completely inhibit inflammatory neuronal loss. Thus, although the contribution of other signaling pathways cannot be ruled out, this pathway seems to be nonredundant in phagocytosis-mediated neuronal loss during *Brucella* infection of microglia. While some reports have described primary phagocytosis of neurons by TLR ligand- or amyloid β-activated microglia (Neher et al., 2011; Neniskyte, Neher, & Brown, 2011), to the best of our knowledge, this study is the first to demonstrate that in the context of a bacterial infection, activated-microglia phagocytose live neurons through "eat me" signal recognition.

The inflammatory activation of microglia, the increment in their phagocytic capacity and their neurotoxic phenotype were not dependent on bacterial viability, since all phenomena were also induced by exposure to HKBA, suggesting that they were elicited by a structural bacterial component. We established that the structural element responsible for such response was not *B. abortus* LPS; on the contrary, *B. abortus* lipoproteins seem to be determinant. L-Omp19, a prototypical *B. abortus* lipoprotein (Giambartolomei et al., 2004), mimicked the phenotypic and functional microglial changes induced by *B. abortus*, which lead to neuronal loss. U-Omp19 was unable to induce any of these changes, confirming that the lipid moiety is required for inflammatory phagocytic removal of live neurons. Our results also indicate that the neuronal loss executed by activated microglia is dependent on the adapter molecule Mal/TIRAP, a downstream signaling initiator shared by TLR1, TLR2, TLR4 and TLR6 (Horng, Barton, Flavell, & Medzhitov, 2002). Furthermore, we showed that this phenomenon is not TLR4- but it is TLR2-mediated. These findings are congruent with our previous observations indicating that TLR2-Mal/TIRAP signaling is crucial for inflammatory responses induced by *B. abortus* on glial cells (Miraglia et al., 2016).

An important issue that merits discussion is why *B. abortus*-infected microglia execute neurons by primary phagocytosis instead of causing direct neurotoxicity or induce apoptosis. It has been postulated that phagoptosis may dominate in conditions in which TLRs are activated but pro-inflammatory cytokine levels are relatively low. This would lead to less severe neuronal insults resulting in phagoptosis, because the damage is sufficient to expose "eat me" signals without triggering apoptosis or necrosis (Brown & Neher, 2014). *Brucella* organisms provoke chronic infections characterized by an inflammatory response. However, inflammation seems to be milder than in infections caused by other bacteria, due in part, to molecular mechanisms exerted by the bacterium (Sengupta et al., 2010). Tissue damage is therefore caused by the persistence of viable bacteria in infected tissues that stimulate a persistent low degree of inflammation (Baldi & Giambartolomei, 2013a, 2013b). In this scenario, *B. abortus*-infected microglia would then cause a slow, progressive loss of neurons by primary phagocytosis in the CNS milieu. This damage could explain the neurological deficits observed during neurobrucellosis (Akcay, Kurtuncu, Celik, Gunduz, & Eraksoy, 2016; Giambartolomei et al., 2008; Nalini et al., 2012; Pappas et al., 2005). If, as we hypothesized, neuronal death is at the root of the neurocognitive symptoms observed in neurobrucellosis, then blocking microglial phagocytosis may be a significant therapeutic approach for the treatment of this form of the disease.

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