



Neutrophils as Trojan Horse Vehicles for *Brucella abortus* Macrophage Infection

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Brucella abortus is a stealthy intracellular bacterial pathogen of animals and humans. This bacterium promotes the premature cell death of neutrophils (PMN) and resists the killing action of these leukocytes. *B. abortus*-infected PMNs presented phosphatidylserine (PS) as “eat me” signal on the cell surface. This signal promoted direct contacts between PMNs and macrophages (Mφs) and favored the phagocytosis of the infected dying PMNs. Once inside Mφs, *B. abortus* replicated within Mφs at significantly higher numbers than when Mφs were infected with bacteria alone. The high levels of the regulatory IL-10 and the lower levels of proinflammatory TNF-α released by the *B. abortus*-PMN infected Mφs, at the initial stages of the infection, suggested a non-phlogistic phagocytosis mechanism. Thereafter, the levels of proinflammatory cytokines increased in the *B. abortus*-PMN-infected Mφs. Still, the efficient bacterial replication proceeded, regardless of the cytokine levels and Mφ type. Blockage of PS with Annexin V on the surface of *B. abortus*-infected PMNs hindered their contact with Mφs and hampered the association, internalization, and replication of *B. abortus* within these cells. We propose that *B. abortus* infected PMNs serve as “Trojan horse” vehicles for the efficient dispersion and replication of the bacterium within the host.

Keywords: *Brucella*, neutrophils, macrophages, Trojan horse, phosphatidylserine

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are the first line of defense of the innate immune system against bacterial pathogens (1–3). Upon contacts with invading bacteria, PMNs activate their killing mechanisms, release cytokines, and may generate PMN extracellular traps (3–5). Although PMNs kill most of the microorganisms they interact with, there are some pathogens capable to resist the microbicidal actions of these leukocytes (6).

Brucella abortus is a Gram-negative bacteria that cause disease in bovines and humans (7). After host invasion, PMNs are the first immune cells to encounter and phagocytize *Brucella* organisms (8, 9). However, *Brucella*-infected-PMNs release negligible amounts of proinflammatory cytokines, generate low levels of reactive oxygen species and seldom show degranulation (10–12). Moreover, *Brucella* pathogens survive inside PMNs for a protracted period of time (10) and induce the premature death of these cells (12, 13). Although the dying *Brucella*-infected PMNs display phosphatidylserine (PS) on the cell surface, they do not show chromatin condensation or signs

of necrosis or oncosis (12). Nevertheless, the exposure of PS on the *B. abortus*-infected PMNs resembles that of apoptotic PMNs. As demonstrated (14), non-infected apoptotic PMNs presenting PS on the surface are removed by macrophages (M ϕ s) in a non-phlogistic manner (14). Indeed, the removal of apoptotic PMNs is first established by the release of “find me” signals required for recruitment of mononuclear phagocytes. Then, the recognition of PS on the surface of the apoptotic PMNs constitutes an “eat me” signal, which in course induces the regulated suppression of M ϕ s activating mechanisms (14, 15).

We have proposed that the premature PMN cell death induced by *Brucella* organisms may promote the selective non-phlogistic removal of these infected cells by the mononuclear phagocytic system (12, 13). In course, *Brucella* infected PMNs may serve as “Trojan horse” vehicles for efficient bacterial dispersion, intracellular replication and establishing chronic infections, as suggested for other pathogens (16). Here we demonstrate that *Brucella*-infected PMNs are readily phagocytosed by murine M ϕ s in a non-phlogistic manner, and that bacteria delivered through PMNs, extensively replicate inside M ϕ s. The experiments shown here, are a proof of concept for the “Trojan horse” proposal, which states that *Brucella*-infected PMNs serve as vehicles for M ϕ infection and subsequent dispersion throughout the organism.

MATERIALS AND METHODS

Bacteria and Mouse Strains

B. abortus 2308 expressing constitutive red fluorescent protein from *Discosoma coral* (*B. abortus*-RFP), provided by Jean-Jacques Letesson (Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium), was used in all experiments. Female BALB/c mice (18–21 g) were supplied by the Escuela de Medicina Veterinaria, Universidad Nacional, Costa Rica, and Instituto Clodomiro Picado, Universidad de Costa Rica, Costa Rica.

Ethics

Bone marrow (BM) was obtained from mice following the consent and guidelines established by the “Comité Institucional para el Cuido y Uso de los Animales de la Universidad de Costa Rica” (CICUA- 47-12) and in accordance with the corresponding law, Ley de Bienestar de los Animales, of Costa Rica (law 9458 on animal welfare). All animals were kept in cages with food and water *ad libitum* under biosafety containment conditions.

Infection Protocols

PMNs were obtained from BM and infected *ex vivo* in the presence of anti-*Brucella* antibodies, following previous protocols (13, 17). Briefly, BM cells were isolated from tibia and femur of mice by flushing bones with HBSS (no calcium, no magnesium) or RPMI medium. Then, BM cells were infected with *B. abortus*-RFP (MOI 50) at 37°C for 1.5 h, washed with PBS, suspended in HBSS, and examined by fluorescent microscopy. The composition and proportion of the infected BM cells have been determined in previous work (17). Under the fluorescent microscope, the estimation of infected murine PMNs is a straightforward process due to the unique donut shape

of their nuclei. The proportion of infected and non-infected cells were counted by following a meaningful statistical sampling method (18). *B. abortus* PMN infections were confirmed by flow cytometry using *B. abortus*-RFP and PE anti-Ly6G (RB6-8C5) from eBioscience as previously described (17).

Peritoneal M ϕ s were harvested and cultured as previously described (19). *B. abortus*-RFP infection (MOI 50) of 2×10^5 RAW 264.7 or peritoneal M ϕ monolayers was performed by using the gentamicin protection assay to avoid extracellular bacteria (20). Additionally, RAW 264.7 or peritoneal M ϕ s were infected by co-cultivating with *B. abortus*-infected PMNs as follows. *B. abortus*-infected PMN were washed with PBS to remove extracellular bacteria. Then, *B. abortus*-infected PMNs were suspended in DMEM without gentamicin and added to the M ϕ monolayers at a rate of 1:1 and incubated for one hour at 37°C. After this period, gentamicin was added. Then, cells were cultivated for up to 48 h and CFU counts determined at 3, 7, 24-, and 48-h post-infection. Alternatively, *B. abortus*-infected PMN were pre-treated with 5 μ g/cell of Annexin V (Invitrogen) for 15 min (15) before co-cultivation with RAW 264.7 cells. The CFU counts within *B. abortus*-infected PMN added to RAW 264.7 and peritoneal M ϕ s monolayers were calculated retroactively by lysing the PMNs and counting bacteria in agar plates. Controls of co-cultivated non-infected PMN with M ϕ monolayers (at rate 1:1) were run in parallel.

Immunofluorescence

The percentage of cell association (direct cell-cell contact) between *B. abortus*-infected PMN and non-infected PMNs with M ϕ s was estimated by fluorescent microscopy at different time points. Infected and non-infected PMNs were fixed with 3.5% paraformaldehyde, centrifuged in a Cytospin 2 (Shandon), mounted with ProLong Gold Antifade reagent with DAPI (Thermo Fisher Scientific), and observed under the fluorescence microscope (Nikon ECLIPSE 80i). M ϕ monolayers co-infected with *B. abortus*-infected PMN were stained with DAPI and FITC-phalloidin (Sigma), fixed and mounted with MOWIOL for analysis as described (12). Controls of non-infected PMNs were used along with the corresponding assays. At least 200 PMNs were counted per slide. Cells were photographed under the fluorescence microscope (Nikon ECLIPSE 80i) using the appropriate color filter channel. Images were cut from microscope field, contrasted and saturated using Hue tool to obtain suitable color separation. Images were merged using Adobe Photoshop 8 software. Internalization of *B. abortus*-infected PMN and non-infected PMNs was documented by live-imaging using Cytation 5 Cell Imaging reader.

Cytokine Determination

For the quantitative determination of TNF- α and IL-10, the supernatants of the infected M ϕ s monolayers were collected at different time points and the concentration of cytokines measured by ELISA according to the manufacturer's specifications (Invitrogen).

PMNs Cell Death Determination

For cell death analysis, PMNs were stained with Alexa Fluor 488 Annexin V (Invitrogen) and PE anti-Ly6G (RB6-8C5) and APC Cy7 anti-CD16/32 antibodies (from eBioscience and BD Bioscience respectively). *B. abortus*-infected PMN cells were analyzed by flow cytometry using a Guava easyCyte (Millipore) and data analyzed using the FlowJo software, version 10.0.7 (Tree Star, Inc.) (13, 21). Evaluation of PMNs cell death assay was carried out as described before (13). Briefly, aliquots of BM were mixed with *B. abortus*-RFP (MOI 50), supplemented with anti-*Brucella* murine serum for opsonization, and incubated under mild agitation at 37°C for up to 4 h. Cells were then suspended in Annexin-binding buffer (Invitrogen) and Annexin V added and incubated for 30 min on ice in the dark. Cells were washed with ice-cold PBS, fixed with 3.2% paraformaldehyde and subjected to flow cytometry analysis.

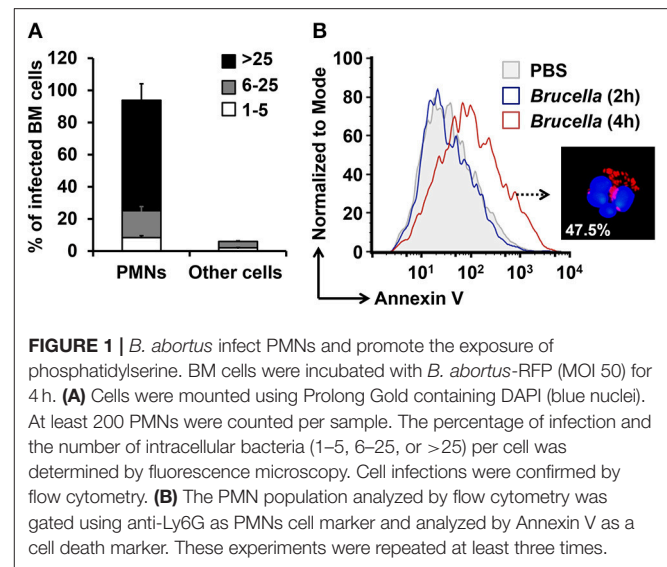
Statistics

The Wilcoxon signed-rank test was used to compare the proportion of association between non-infected PMNs and *Brucella*-infected PMNs to Mφs. Analysis of covariance (ANCOVA) was used to determine the effects of time and treatments on statistical the Log₁₀ CFU. Two-way analysis of variance (ANOVA) was used to measure the effect of time and treatments on the percentage of Mφ infection. Shapiro-Wilks test was applied to assess the normal distribution of data obtained in each experiment, and the Kolmogorov-Smirnov test was applied to data that did not adjust to normality. JMP (<https://www.jmp.com>) and GraphPad Prism software (<https://www.graphpad.com>) was used for statistical analysis. Data were processed in Microsoft Office Excel 2016 and GraphPad Prism software. For a meaningful counting number of infected cells, a probability index was followed, according to the total number of PMNs and infected PMNs (18).

RESULTS

The limited volume of mouse blood and the low number of PMNs in this fluid, preclude the isolation of a sizeable number of these leukocytes for functional studies. In addition, the extensive manipulation during purification procedures accelerates the cell death of PMNs. In contrast, the number of PMNs in the BM is rather high, comprising between 40 and 50% of all nucleated cells (22). In agreement with previous results (17), close to 94% of the *ex vivo* *B. abortus* BM-infected cells corresponded to PMNs (Figure 1A). We have previously shown that the remaining infected cells are monocytes or progenitor stem cells (17). The distinction between mononuclear infected cells and infected PMNs is straightforward due to the donut shape of the nuclei of the latter cells. Following this, we then tested if *B. abortus* were capable to induce the premature cell death of BM PMNs, as shown before for blood PMNs (13), up to 47.5% of the *B. abortus*-infected PMN were positive for Annexin V at 4 h post-infection (Figure 1B).

Then, we explored the association of *Brucella*-PMNs to Mφs by co-cultivating these two cells *in vitro*. As compared to the



non-infected PMN controls, a higher proportion of *Brucella*-infected-PMNs associated with RAW and peritoneal Mφs was detected (Figure 2A). Thereafter, the association between *Brucella*-infected-PMNs and Mφs, led to the infection of the latter (Figure 2B). This phenomenon was completed before 7 h and was specific since non-infected PMNs were not phagocytized by Mφs (Figure 3). However, a strict kinetic analysis was precluded, since Mφ phagocytosis and the concomitant digestion of PMNs was very fast an uneven event over time.

Then, we tested the rate of bacterial replication after internalization of Mφs by *Brucella*-infected-PMNs at 1 and 48 h post-infection. As shown in Figure 4A, *B. abortus* organisms infected Mφs at higher rates through phagocytosis of *Brucella*-infected PMNs than when infected with bacteria alone. Moreover, the higher efficiency of Mφ bacterial infection mediated by *Brucella*-infected-PMNs was evident by the different MOIs delivered in each case. Indeed, in the case of *Brucella*-infected PMNs the number of delivered bacteria corresponded to an MOI of 5; that is, ten times lower than the MOI of 50 used to infect Mφs with bacteria alone. The efficient internalization process promoted higher kinetics of *B. abortus* replication in Mφs incubated with *Brucella*-infected-PMNs (Figure 4B). In spite of this, the kinetics between RAW and peritoneal Mφs were different. For instance, RAW Mφs infected with *B. abortus* alone displayed an initial decline in CFUs at early times of infection, a phenomenon that has been reported before (23). However, after infection of these cells with *Brucella*-infected-PMNs, the initial decline was unnoticeable in these Mφs; instead, a steady increase in the number of CFUs was observed. In contrast, the kinetic profiles were similar in both, the *Brucella*-PMN infected peritoneal Mφs and in the controls; though, the number of CFU was always higher in the former infected cells.

The different bacterial replication kinetic observed between the RAW and the peritoneal Mφs, seemed related to the distinct profiles of cytokines produced during the infection process (Figure 5). Except for the regulatory IL-10, which was already

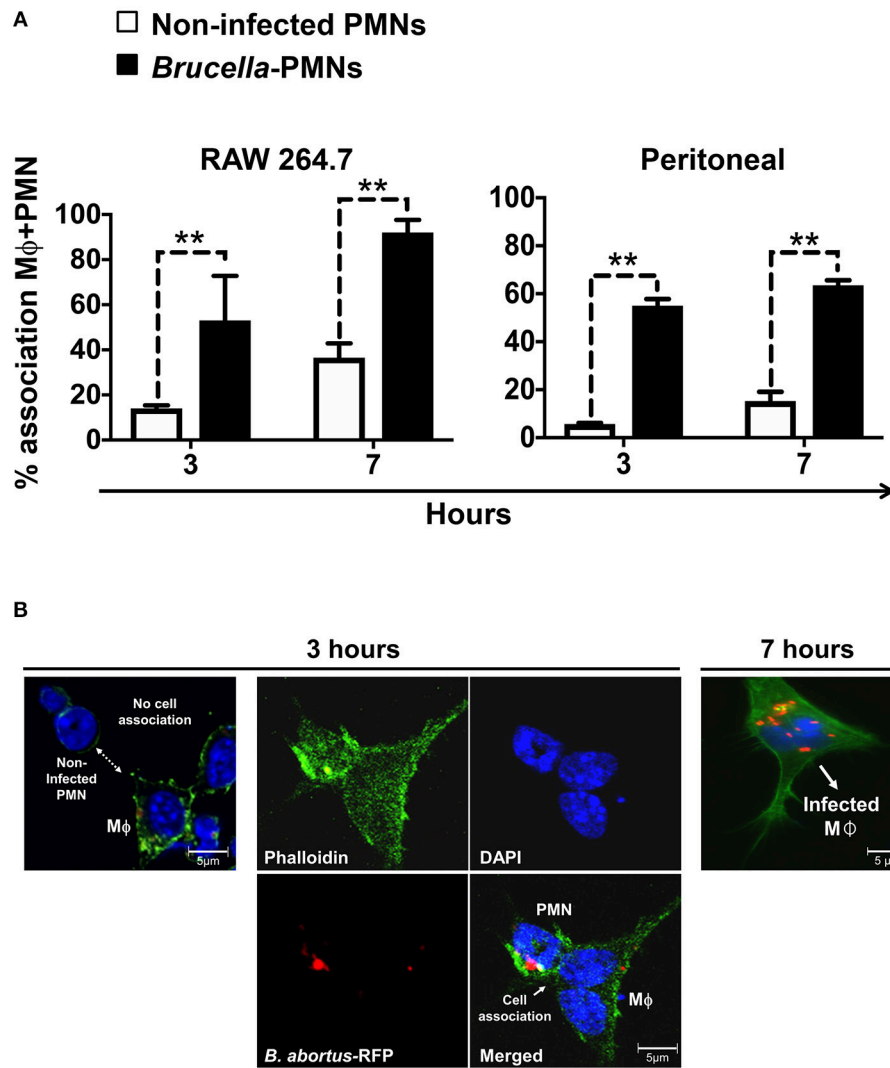


FIGURE 2 | *B. abortus*-infection increase the association of PMN with M ϕ s. **(A)** Non-infected or *B. abortus*-RFP-infected PMN were incubated with RAW or peritoneal M ϕ s (1:1) at different time points and PMN-M ϕ interactions were quantified. Cells were stained (DAPI, for nuclei; phalloidin-FITC for actin filaments), fixed and mounted with MOWIOL. At least 200 PMNs were counted and the percentage of PMN-M ϕ cell association determined. Values of $p < 0.01$ (**) are indicated in relation to M ϕ s incubated with non-infected PMNs. **(B)** RAW M ϕ in the process of association and ingestion *B. abortus*-infected PMN. Infected PMNs are distinguished from other cells by the “donut” shape of their nuclei. Images were photographed under the microscope using the appropriate color filter channel. These experiments were repeated at least three times.

high (>100 μ g), the quantities of the TNF- α were under background levels, at early times of the *Brucella*-PMN infected RAW cells. It is worth noting that RAW M ϕ s are TNF- α hyperproducers (24). Therefore, it was expected that at later times, once bacteria reached high numbers, the TNF- α increased to very high levels in the *Brucella*-PMNs infected RAW cells, as compared to the controls. Still, the higher amounts of TNF- α at later times of the RAW infected cells did not hamper bacterial replication. Likewise, at early times of *Brucella*-PMN infection of peritoneal M ϕ s, the production of TNF- α was low with significant high amounts of the regulatory cytokine IL-10. These differences in cytokine profiles may explain the differences observed between *Brucella*-PMN-infected RAW and peritoneal

M ϕ s in the replication kinetics. In any case, in both experiments, *Brucella*-PMN-infected M ϕ s reached much higher CFU values than the controls infected with bare bacteria alone.

In agreement with our previous reports (13, 21) *Brucella*-infected-PMNs displayed PS on the cell surface (**Figure 1B**). Since this phospholipid is commonly recognized as an “eat me” signal (14), we decided to explore the role of PS in the uptake of *Brucella*-infected PMNs by M ϕ s. For this, we used Annexin V to hinder the PS exposed on the *Brucella*-PMN surface. After treatment with Annexin V, the proportion of *Brucella*-PMNs associated with M ϕ s significantly diminished (**Figure 6A**). Moreover, bacterial replication was reduced in RAW M ϕ s at all-time points (**Figure 6B**), displaying the profile

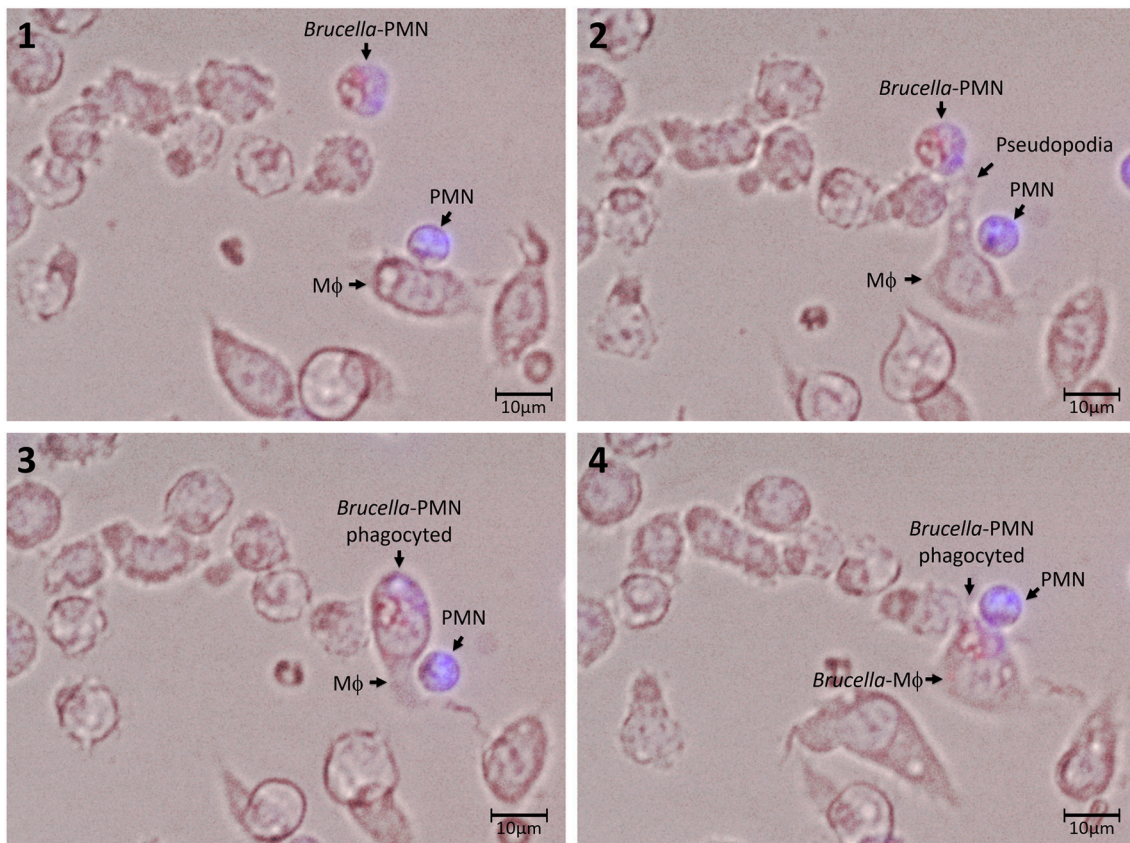


FIGURE 3 | Association and uptake of *B. abortus*-infected PMN by Mφs. PMNs were incubated with *B. abortus*-RFP (red) (MOI of 50) for 1.5 h; then, cells were pelleted and washed with PBS to remove extracellular bacteria. *Brucella*-infected-PMNs were suspended in DMEM without gentamicin and added to RAW Mφs monolayers (5×10^3) at a rate of 1:1 and incubated for 10 min at 37°C. After this period, the infected Mφ monolayers were washed and suspended in DMEM and incubated for up to 5 h. Infected PMNs were stained with Hoescht (blue). Cells were photographed and analyzed under Cytation 3 Cell Imaging Multi-Mode Reader (BioTek) using the appropriate color filter channel. Numbers 1 to 4 correspond to the order in the which images were capture very every 20 min. These experiments were repeated at least three times.

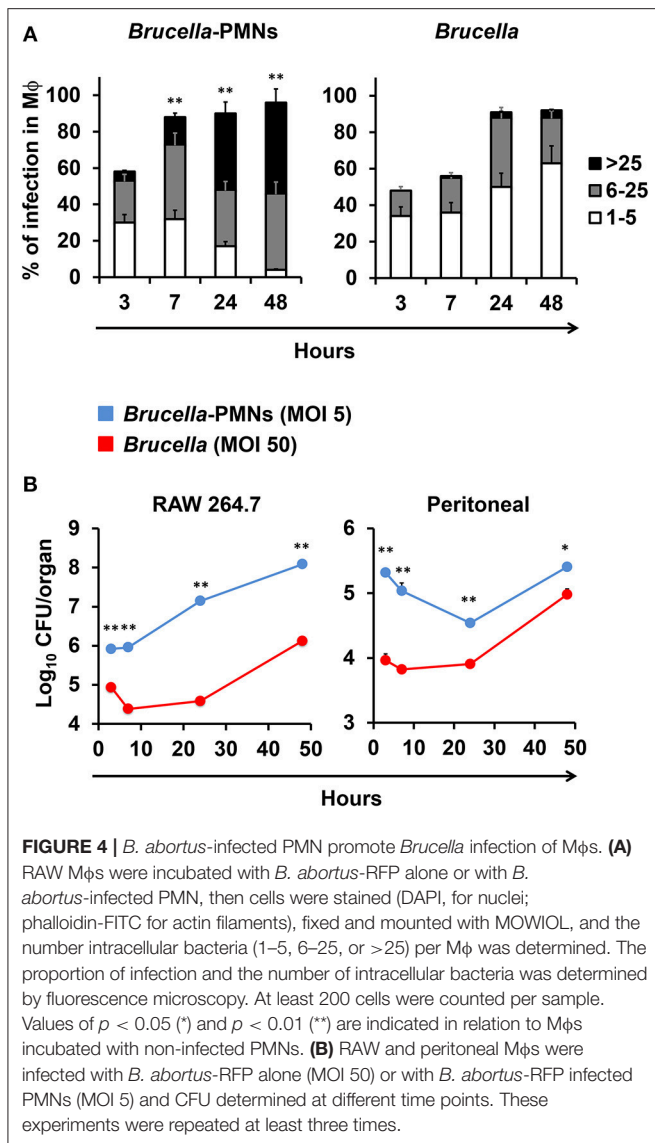
observed after infection with bacteria alone (compare profile with **Figure 4A**). Likewise, the proportion of infected Mφs was significantly reduced in the *Brucella*-PMNs treated with Annexin V (**Figure 6C**). Thus, PS on the *Brucella*-infected-PMNs surface acted as an “eat me” signal for Mφs.

DISCUSSION

There are various intracellular pathogens, such as *Chlamydia pneumoniae* and *Leishmania major*, capable to survive within PMNs, kill these cells and use them as vehicles for infecting and colonizing Mφs (25). This strategy, generally known as the “Trojan horse,” serves as a mechanism for microbial dispersion within the host (15). It seems, therefore, that *B. abortus* also follows a Trojan horse strategy by using infected PMNs as vehicles for the dispersion throughout the host mononuclear phagocytic system. A similar strategy to traverse microvascular endothelial cells of the central nervous system via *B. abortus*-infected-monocytes has been proposed (26).

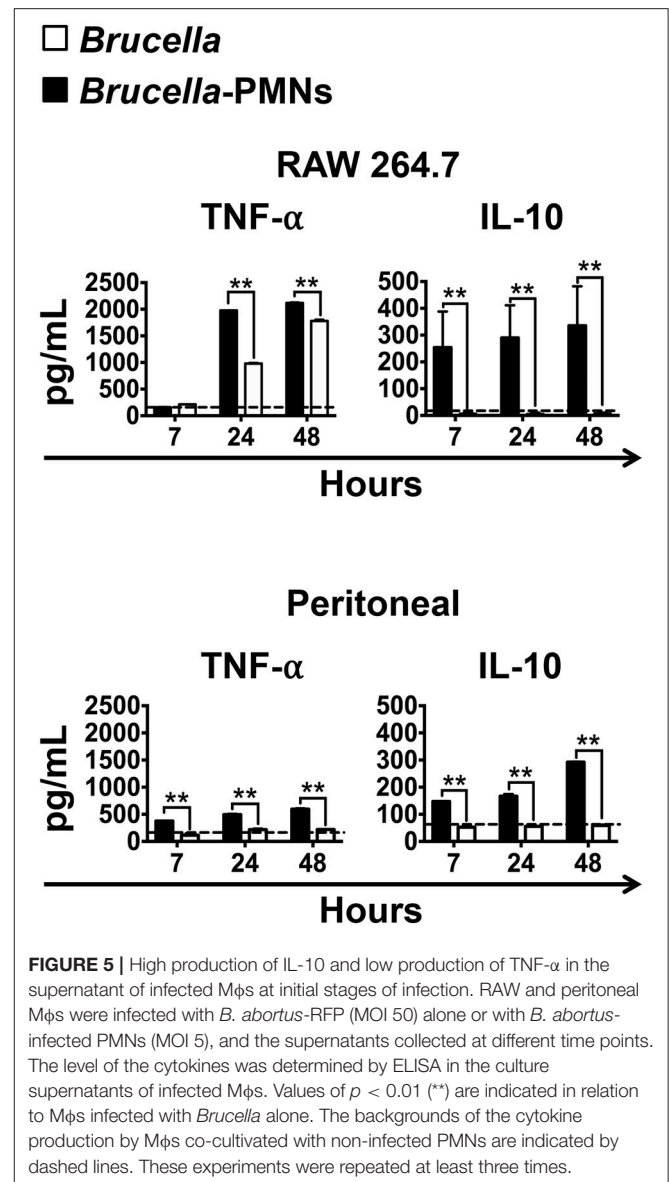
Infecting naïve Mφs monolayers (such as bone marrow) with bare *Brucella* grown in a bacteriological medium is highly inefficient (23). Infection protocols in cultured Mφs require high bacterial MOIs (>50) to obtain low numbers (<5 bacteria/cell) of intracellular bacteria. Moreover, a large proportion of these invading bacteria are killed by Mφs after a few hours (23). Following this, we propose that the common physiological infection of the phagocytic mononuclear system primarily occurs via *Brucella*-infected-PMNs.

There are at least two other pieces of evidence that support this proposal. First, it has been demonstrated that mice depleted of PMNs, eliminate *B. abortus* more readily than their “normal” infected counterparts (21). This is commensurate with the fact that Mφs kill bare “unprotected” *Brucella* cells more readily than those hidden within PMNs, as shown here. Second, the early internalization of *Brucella*-infected PMNs by Mφs, seems to occur in a non-phlogistic manner, displaying significant amounts of regulatory IL-10 and low quantities of proinflammatory cytokines, such as TNF- α at early stages of the infection. It is known, that the uptake of apoptotic PMNs by Mφs,



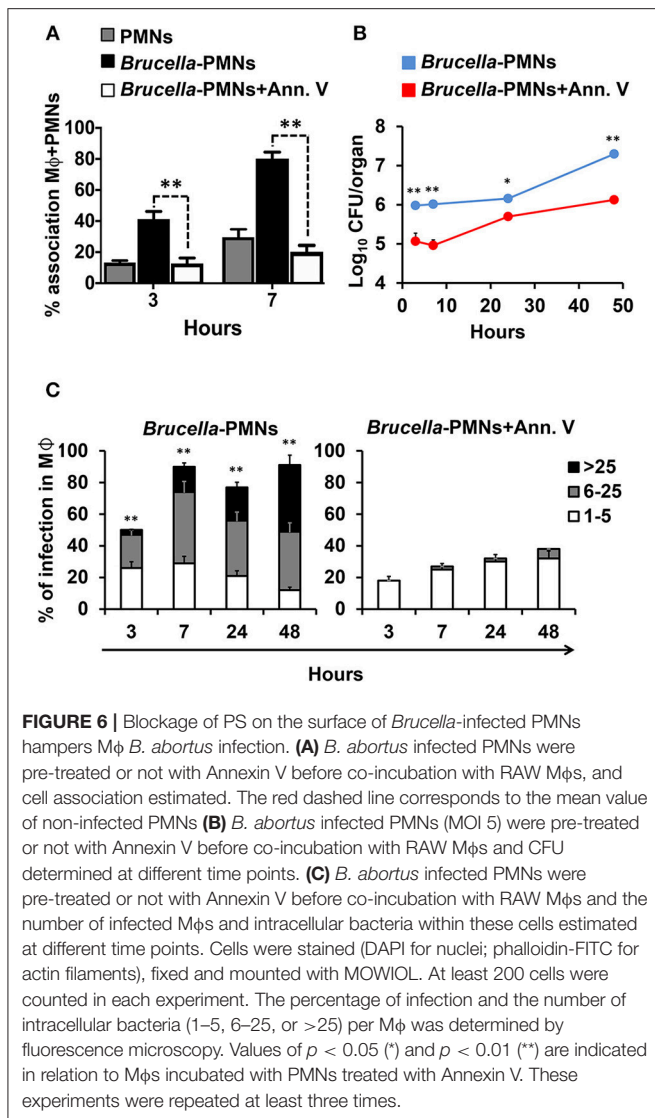
increases the secretion of anti-inflammatory IL-10 cytokine (27). This is relevant since the first 8 h after cell invasion are crucial for pathogenic *Brucella* to redirect its trafficking to its replicating niche within non-activated cells (23). Indeed, previously activated Mφs display high brucellicidal activity. However, if Mφs become activated (e.g., through TNF- α or lipopolysaccharide) after 8–24 h of infection, the intracellular bacteria are still capable to replicate extensively (11). The obvious explanation is that at this infection stage, *Brucella* are hidden within vacuoles of the early phagocytic compartment and then protected from Mφs microbicidal mechanisms. It is worth noting that the overall activation of the immune system in neutropenic *Brucella* infected mice is considerably higher than in the “normal” infected counterparts indicating that PMNs dampen the adaptive immunity in brucellosis (21, 28).

During the early stages of physiological cell death, PS translocates from the cytoplasmic to the extracellular side of



the cell membrane (29). The correct redistribution of PS on the outer surface of the plasmatic membrane is a key element for the recognition of dying cells and corresponds to a molecular “eat me” signal that indicates that these dying cells should be engulfed (30). But PS is also a “forget me” signal for the regulated suppression of Mφs activating mechanisms (14, 15, 31). Within this context, it seems that ingestion of *Brucella*-infected PMNs by Mφs follows a similar mechanism used to phagocytize apoptotic PMNs. In any case, it is becoming clearer that through evolution *Brucella* organisms are stealth pathogens that have evolved to hamper the activation of the first stages of innate immunity and to establish chronic infections.

In conclusion, the ability of *Brucella* to circumvent the immune response and to replicate within Mφs are key elements for the pathogen survival and for the establishing long-lasting infections. Here, we showed that *Brucella*-infected PMNs



promoted the internalization and replication of *Brucella* within Mφs using a “Trojan horse” strategy. To reinforce or reject our hypothesis *in vivo* experiments would be necessary.

In this work our main findings are: (i) *Brucella abortus* infected up to 96% of BM-PMNs, inducing a premature death of these cells; (ii) the *Brucella*-infected PMNs displayed PS

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as “eat me” signal, promoting the association with Mφs and favoring the bacterial replication within these mononuclear phagocytes; (iii) This phenomenon was specific, since non-infected PMNs were not phagocytized by Mφs and blockage of PS with Annexin V diminished the Mφs association and phagocytosis of *Brucella*-infected PMNs; (iv) the low production of proinflammatory cytokines and the high production of the anti-inflammatory IL-10 at the initial stages of infection, correlated with the non-phlogistic Mφ *Brucella*-PMN uptake and subsequent bacterial replication.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

EB-C, EM, and CG-J designed the experiments. CG-J, RM-C, and PA-S performed the experiments. CG-J, RM-C, PA-S, EB-C, EC-O, and EM analyzed the data. CC-D, EC-O, EB-C, and EM contributed reagents, materials, analysis tools. EB-C, EM, and CG-J wrote the paper. All authors revised and approved the manuscript.

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

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