UNIVERSIDAD DE COSTA RICA
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INTERACTION OF MURINE NEUTROPHILS WITH *Brucella abortus*

INTERACCIÓN DE NEUTROFILOS MURINOS CON *Brucella abortus*

Tesis sometida a la consideración de la Comisión del Programa de Doctorado en Ciencias para optar al grado de Doctor en Ciencias

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PRÓLOGO

El eje central de esta tesis es examinar el papel de los neutrófilos (PMNs) en las infecciones ocasionadas por *Brucella abortus.* Todos los manuscritos incluidos en este trabajo han sido publicados en revistas internacionales indexadas en modalidad de acceso libre. Debido a que el estado del conocimiento en ciencia se realiza en inglés, la mayoría de los escritos se redactaron en este idioma, con la excepción de este prólogo y el resumen.

La primera parte de este trabajo incluye una revisión que tiene como objetivo describir hallazgos en modelos de infección y de depleción de PMNs ante distintos patógenos bacterianos. Esta revisión sirve a manera de introducción para esta tesis que usó estas técnicas como ejes centrales en la mayoría de los trabajos. Seguidamente se adjuntan dos publicaciones que describen el rol protagónico de los PMNs murinos ante *B. abortus,* así como diferencias importantes en la interacción inicial de estos leucocitos murinos con respecto a los de otros hospederos. Posteriormente se adiciona una publicación en las que se demuestra que *B. abortus* induce una muerte no proinflamatoria de los PMNs. Finalmente se presentan dos otros manuscritos en los que se explora la persistencia de *Brucella* en medula ósea y se propone a los PMNs como vehículos o "caballos de Troya" para la dispersión y replicación de la bacteria. Las hipótesis, materiales, métodos, resultados, discusión, conclusiones derivadas de dichos trabajos están explicados dentro de cada manuscrito adjunto.

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INDICE

RESUMEN

La brucelosis es una enfermedad zoonótica con distribución mundial. La enfermedad se caracteriza por ocasionar fiebre ondulante en humanos y abortos en animales domésticos como vacas, ovejas, cabras, cerdos y perros. *Brucella abortus* ha evolucionado como un patógeno furtivo capaz de eludir las respuestas proinflamatorias, incluyendo a los neutrófilos polimorfonucleares (PMNs), que son las principales células efectoras del sistema inmune innato. El eje central de esta tesis explora la interacción de los PMNs con *Brucella abortus*.

Debido a que el modelo experimental central de esta tesis se centró en los modelos de infección bacteriana y depleción de PMNs murinos, la primera parte de esta tesis incluye una revisión sobre estos modelos y el uso de ratones neutropénicos en diferentes infecciones bacterianas. El primer artículo que publicamos contrasta la interacción de los PMNs de ratones con aquellos de humanos, perros y bovinos, mostrando que estos últimos no reconocen a las bacterias lisas de *B. abortus* en las primeras etapas de la infección. Los componentes del suero normal murino no opsonizan las cepas lisas de *B. abortus*, y la fagocitosis de PMNs se logra solo después de la aparición de anticuerpos. La falta de opsonización de suero murino y la ausencia de reconocimiento de PMN murino son específicos, y las moléculas responsables del camuflaje de *B. abortus* son los homopolisacáridos de superficie de N-formil-perosamina que incluye la cadena O del lipopolisacárido y los haptenos nativos. Posteriormente exploramos cómo los PMNs modulan la inmunidad adaptativa durante las etapas iniciales de la infección aguda en la brucelosis murina. En ratones, la depleción de los PMNs al inicio de la inmunidad adaptativa favorece la tasa de eliminación de *B. abortus*. También se demuestra que los PMNs modulan activamente el curso de la infección de *B. abortus,* aun cuando la respuesta adaptativa está desarrollada.

Bajo una línea de trabajo afín, mostramos que *Brucella* tiene la capacidad de inducir la muerte prematura de PMNs humanos, lo que sugiere que *Brucella* podría disminuir la presencia de PMNs infectados en los órganos diana y promover la neutropenia durante la brucelosis crónica. Esta muerte celular no proinflamatoria de los PMNs infectados por *B. abortus* concuerda con la propuesta de que estos leucocitos sirven como "caballos de Troya" ya que sirven como vehículos para infectar células fagocíticas profesionales sin inducir activación.

Finalmente, describimos la persistencia de *Brucella* en las células de la médula ósea de los ratones y proponemos que este tejido es esencial en el establecimiento de infecciones crónicas de larga duración. Así mismo, demostramos que los PMNs infectados con *Brucella* son fagocitados fácilmente por Mϕs murinos de una manera no proinflamatoria, y que las bacterias liberadas a través de PMNs se replican ampliamente dentro de Mϕs. Por lo tanto, se proporciona una prueba de concepto de que los PMNs sirven como "caballos de Troya" o vehículo para la dispersión y replicación de *B. abortus* en el hospedero. Esta tesis demuestra que los PMN tienen un papel importante en la patogénesis de *Brucella*, así como en la modulación de la respuesta inmune del hospedero.

ABSTRACT

Brucellosis is a zoonotic disease with a worldwide distribution, which causes undulant fever in humans and abortions in domestic animals, such as cows, sheep, goats, pigs, and dogs. *Brucella abortus* has evolved as a stealthy pathogen capable of circumventing pro-inflammatory responses, including PMNs, which are the main effector cells of the innate immune system. In this thesis, we explore the interaction of PMNs with *B. abortus*.

The first part of this thesis includes a review of the depletion model of murine PMNs and the use of neutropenic mice in various bacterial infections. The first published paper compares the interaction of naïve murine PMNs with human, dog, and bovine PMNs, showing that murine PMNs fail to recognize smooth *B. abortus* cells at early stages of infection. The murine normal serum components do not opsonize smooth *Brucella* strains, and neutrophil phagocytosis is achieved only after the appearance of antibodies. The lack of murine serum opsonization and absence of murine PMN recognition are specific, and the molecules responsible for the *Brucella* camouflage are N-formyl-perosamine surface homopolysaccharides of lipopolysaccharide and native haptens. Afterward, we explored how PMNs modulate adaptive immunity in the initial stages of the acute murine *Brucella* infection. The removal of PMNs influences adaptive immunity at the onset of *B. abortus* infection, enhancing bacterial elimination from the target organs of mice. We demonstrate that PMNs have an active role modulating the course of *B. abortus* infection after the adaptive immune response already developed.

In a related manuscript, we show that *B. abortus* can prematurely kill human PMNs suggesting *Brucella* may hamper the presence of infected PMNs in the target organs and promote neutropenia during chronic brucellosis. This non-phlogistic cell death of *Brucella* infected PMNs agrees with the proposal of these leukocytes function as "Trojan horse" vehicles for infecting phagocytic cells without promoting activation.

Finally, we describe the persistence of *B. abortus* in cells of the mice bone marrow and propose this tissue as essential in the establishment of long-lasting chronic infections. Here we demonstrate that murine Mϕs readily phagocyte *Brucella*-infected PMNs in a non-phlogistic mxr and that bacteria delivered through PMNs, extensively replicate inside

Mϕs. Following this we provide a proof of concept for the "Troyan horse" hypothesis that proposes that *B. abortus* infected PMNs function as vehicles for the dispersion and replication of the bacteria inside the host, modulating the host immune response.

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CHAPTER 1

In this Introduction section, we provide general insight into the role of PMNs on different bacterial murine models. We emphasize on the depletion of PMN by antibodies in the course of bacterial infections.

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are primary cells of defense of innate immune system (Mantovani et al., 2011) and determine the host's resistance against many microbial infections (Kumar & Sharma, 2010). PMNs are produced in large quantities in the bone marrow (BM) where they differentiate, following a maturation procedure that includes lysosomal granule formation. Mature PMNs migrate from bone marrow to the peripheral blood, to the reticuloendothelial mononuclear phagocyte system, and beneath the mucous membranes (Kennedy & Deleo, 2009). Once outside the BM, PMNs are commonly short-lived cells with a circulating half-life of 6-8 hours and hence produced at a rate of $5 \times 10_{10}$ -$10 \times 10_{10}$ cells per day (Summers et al., 2010). PMNs are capable of responding to chemotactic signals and migrate through endothelial membranes and exert their microbicidal actions, following various intracellular and extracellular mechanisms. PMNs homeostasis is maintained by a fine balance between granulopoiesis, bone marrow storage and release, intravascular margination, clearance, and destruction (Mantovani et al., 2011). Recruitment of PMNs at a site of infection is a key phenomenon in the innate immune response (Kobayashi et al., 2005). Chemoattractants influence leukocyte migration by activating a family of related chemoattractant G protein-coupled receptors. PMN chemoattractant factors include chemokines, cytokines, complement-derived peptides, lipid mediators such as leukotriene B4 and microbial components such as *N*-formylated peptides (Petri & Sanz, 2018).

PMNs are professional phagocytes capable of ingesting microbes and killing them intracellularly by activation of phagolysosomal hydrolytic enzymes and the production of reactive oxygen species (ROS). Alternatively, PMNs may degranulate releasing microbicidal substances in the surrounding or explode, generating extracellular traps (NETS) composed of sticky DNA, histones and other elements that trap and kill microorganisms (Brinkmann et al., 2004). PMNs also release many chemokines and cytokines to attract and activate more PMNs and other cells required for mounting an immune response (Tecchio et al., 2014).

Under certain circumstances, PMNs may live up to five days (Pillay et al., 2010). This longer life span may allow them to exert a variety of roles such as modulation of inflammation (Soehnlein et al., 2017; Uhl et al., 2016), and interaction with elements of

the adaptive immune response (Leliefeld et al., 2015). There is evidence showing that PMNs are also able to communicate with Mϕ (John & Hunter, 2008), DC (Schuster et al., 2013), natural killer (NK) (Jaeger et al., 2012) and B and T cells through cytokines (Puga et al., 2012; Tecchio et al., 2014). Also, PMNs may limit T cell responses after antigen stimulation by releasing lipid mediators such as thromboxane A2 (Yang & Unanue, 2013). PMNs interaction with elements of innate immunity has also been linked to the development of autoimmune diseases (Diana et al., 2013).

Neutropenic murine models have been extensively used to understand the role of PMNs in diseases (Barquero-Calvo et al., 2013; Gong & Koh, 2010; Huang et al., 2015; Mora-Cartín et al., 2019; Yang & Unanue, 2013). Chemotherapy treatment, such as cyclophosphamide (Zuluaga et al., 2006), was the first method used to generate mice depleted of PMNs. However, treatment with these chemical agents is not specific for PMNs since they display broad cytotoxic effects over other cell types (Zuluaga et al., 2006). Following this, antibody-mediated depletion of PMNs and mutant mice with defects in the production of mature PMNs have been the preferred models for studying the role of PMNs in microbial infections (Barquero-Calvo et al., 2013; Bruhn et al., 2016; Ordoñez-Rueda et al., 2012). Here we review the advantages and limitations of these two latter neutropenic murine models for dissecting the role of PMNs in bacterial infections *in vivo*.

Neutropenic murine models

Depletion employing anti-PMN antibodies

The depletion of mouse PMNs with antibodies against specific cell surface antigens has been broadly used to study the role of PMNs during bacterial infections. After PMN depletion, mice show an absence of PMNs in blood and lymphatic organs. A complete PMN depletion in the BM is not achieved, reaching close to 35 % removal (Mora-Cartín et al., 2019). After PMN depletion, no significant changes in other blood parameters are observed. Likewise, PMNs depleted mice do not show histological alteration of lymph nodes, spleen, lungs or kidneys and do not display pro-inflammatory responses, or elevation of cytokines and or chemokines (Barquero-Calvo et al., 2013; Casson et al.,

2017; Deniset et al., 2017; Verdrengh & Tarkowski, 1997). Although the liver parenchyma displays slight vacuolar degeneration, the liver blood vessel of antibody PMN depleted mice show an abundance of pyknotic PMNs phagocytized by Kupffer cells (Fig 1).

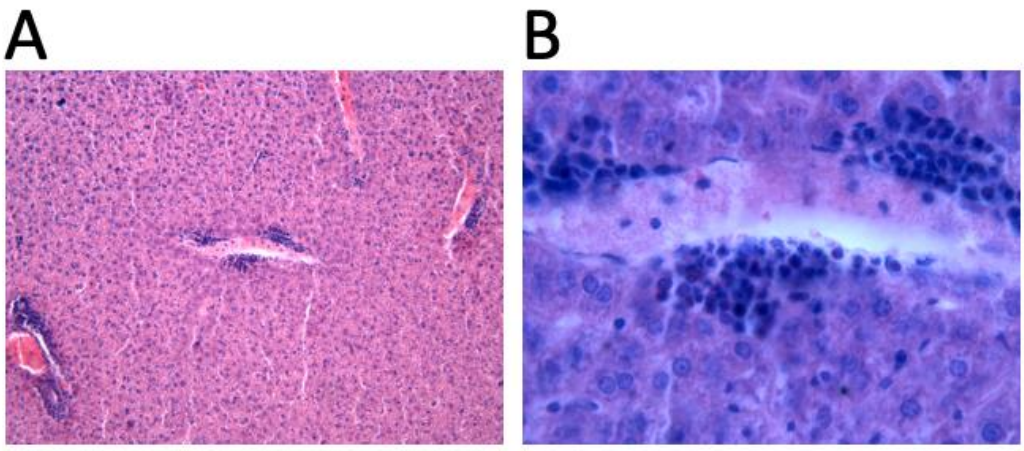

Figure 1. Histology of a liver venule in antibody PMN depleted mice showing the phagocytosis of PMNs by Kupffer Mϕs. The abundance of pyknotic PMNs, as well as fragmented segments, are taken up by Mϕ. Magnification of x20 (A) and magnification of x100 (B) are displayed.

The protocol for PMN antibody depletion *in vivo* is straight forward, and may be used at any time during the infection course (Fig 2; Mora-Cartín et al., 2019; Naglak et al., 2017). A significant constraint of this model is the limited time frame in keeping the neutropenic state (Barquero-Calvo et al., 2013), which usually lasts 78 hours (Fig 3). For this reason, repeated injections of anti-PMN antibodies are required every three days (Fig 2). Though, this brings another limitation, since the PMN depleted mice mount a quick antibody response against the foreign rat IgG monoclonal antibodies used for depletion (Fig 2). This response gives a window of no more than seven days, before the function of the anti-PMN IgGs is abrogated (Barquero-Calvo et al., 2013).

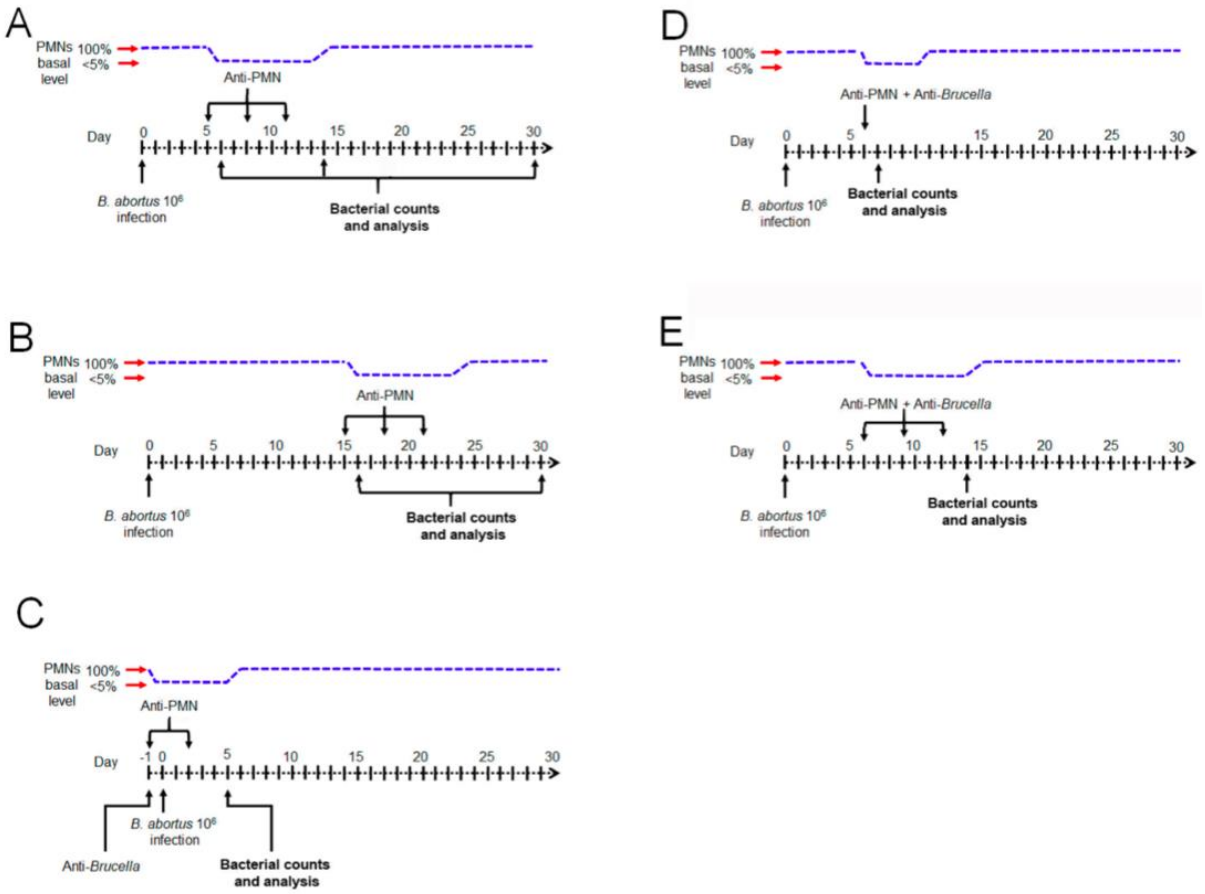

Figure 2. Examples of different depletion protocols to dissect the collaboration of PMNs at different time points in a murine bacterial infection. Top black arrows indicate the days at which a group of mice was treated with anti-RB6-8C5 or 1A8 antibodies for PMN depletion, or with non-immune rat IgG as mock-control. Bottom black arrows indicate the days of i. p. infection with 0.1 mL containing 10_6 CFUs *B. abortus* 2308W, determination of CFU counts, histopathology, and cytokines in the various groups of mice. Blue dashed line at the indicated times represent the PMNs basal level over time after treatment with anti-RB6-8C5. The red arrows indicate the proportion of PMNs at the indicated times (Taken from (Mora-Cartín et al., 2019)).

Two rat monoclonal antibodies against mouse PMNs have extensively used for depleting these leucocytes in mice: clone RB6-8C5 and clone 1A8. Both antibodies have their advantages and drawbacks. The first anti-PMN antibody developed was the RB6-8C5 clone directed against the membrane antigen called Gr-1 (Rogers & Unanue, 1993),

represented by the two surface molecules Ly-6G and Ly6C (Fleming et al., 1993). The RB6-8C5 antibody displays high affinity against PMNs, achieving a neutropenic blood-stage lasting from three to four days after a single intravenously or intraperitoneal injection of 60 to 100 μg of antibody/mouse (Fig 3). The RB6-8C5 antibody has the disadvantage that cross-reacts with Ly6C protein, mainly present in activated monocytes, which may dampen after administration in neutropenic models. The Ly6C is also present to lesser extent in some subpopulations of DC, NK and CD8 T cells (Asselin-Paturel et al., 2003; Jutila et al., 1994; Jutila et al., 1988; Kung et al., 1991; Nakano et al., 2001; Sato et al., 1996; Schlueter et al., 1997; Walunas et al., 1995); however, in the explored neutropenic models, there is no significant interference. Still, caution in the interpretation of the results using RB6-8C5 antibodies are required, since other cells targeted by the RB6-8C5 antibody may play a role in the system tested. For instance, in some cases, PMNs may work together with subpopulations of cells expressing Ly6C$^{hi;}$ (Casson et al., 2017). Still, in a few cases, the depletion of Ly6C^{hi} cells different from PMNs has been demonstrated to be the responsibility of the measured biological effect (Dunay et al., 2010; Shi et al., 2011; Wojtasiak et al., 2010).

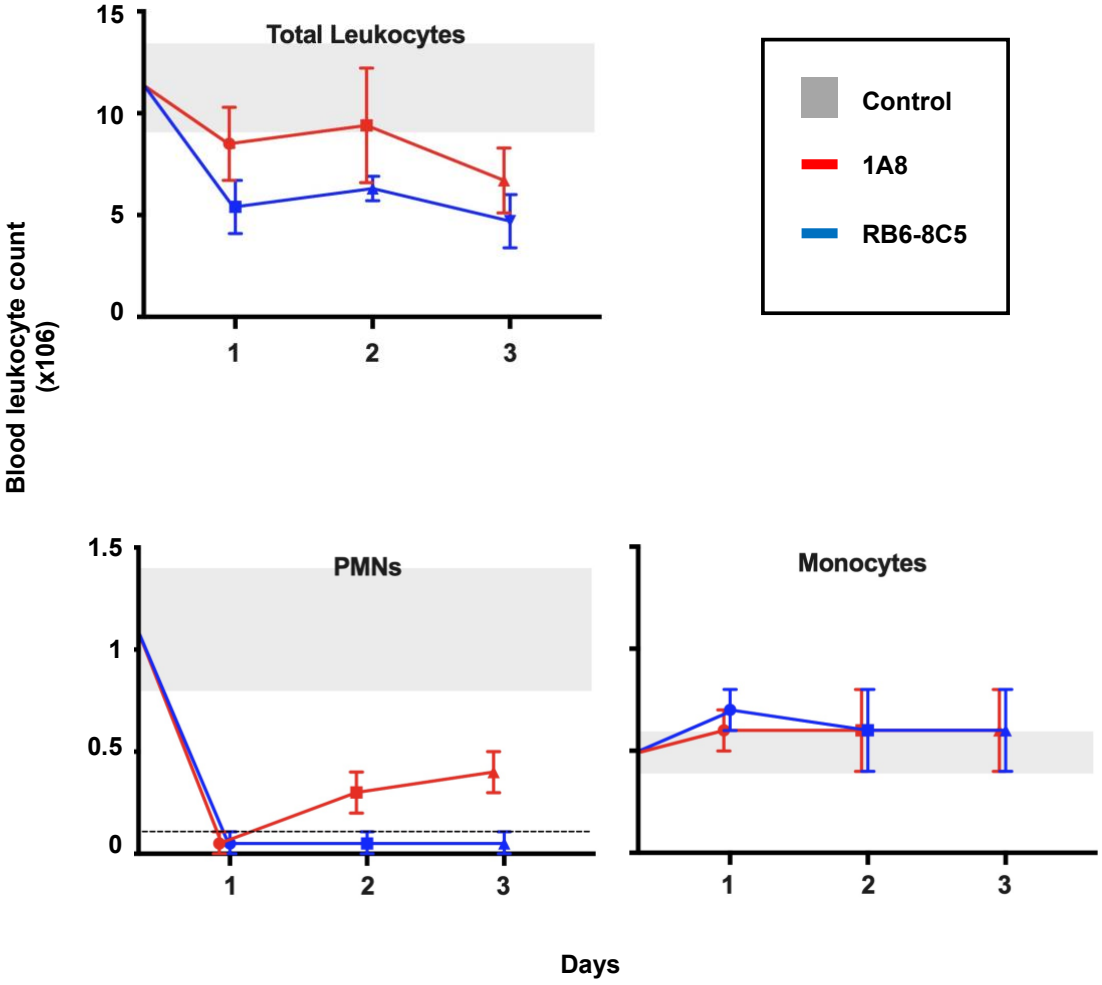

Figure 3. Effect of 1A8 or RB6-8C5 on blood leukocyte counts. Mice were i.p. treated with a single injection of 1000 µg of 1A8 or 500 µg of RB6-8C5 and the hematological parameters were determined at the indicated time (Adapted from Daley et al., 2008). Gray areas represent the standard deviations of the non-depleted control mice.

The 1A8 clone directed against an epitope of the Ly6G molecule, has the advantage of being more specific for mouse PMNs; however, it has the handicap that very high quantities of antibodies are required (between 500 to 1500 µg/mouse) (Boldock et al., 2018; Daley et al., 2008; Mora-Cartín et al., 2019). In addition, the blood of mice treated with 1A8 seldom reaches 100 %, PMN depletion (Daley et al., 2008), and the period of PMN removal does not go beyond three days (Daley et al., 2008; Fig 2). Incomplete and

less efficient depletion of circulating PMNs may be an issue that hampers the interpretation of the results. Moreover, in large and long-lasting experiments, the use of 1A8 clone becomes impractical, due to the high amounts of antibodies required (Boldock et al., 2018). Still, direct comparisons between RB6-8C5 and 1A8 antibodies have shown a good correlation on the performance of these two antibodies (Braverman & Stanley, 2017; Casson et al., 2017; Mora-Cartín et al., 2019; Steiner et al., 2017). In the case of 1A8 antibody, a recommended strategy is to run most experiments with RB6-8C5 antibody, and control some crucial points with the 1A8 clone (Agbayani et al., 2018; Carr et al., 2011; Dunay et al., 2010; Mora-Cartín et al., 2019; Shi et al., 2011; Wojtasiak et al., 2010).

Neutropenic mutant mice

Two models of neutropenic mutant mice have been used: homozygous *Gfi1* and Genista. The distinct advantage over the antibody PMN depleted mice in that the neutropenic stage is permanent in all organs, including the BM. The zinc-finger protein Growth Factor Independence 1 (*Gfi1*) knock out mutant mouse model was the first one to be developed. These mice are homozygous from a null allele *GFI1*$^{-/-}$, coding for a transcriptional zinc finger domain repressor protein involved in the regulation of many target genes in different hematopoietic lineages (Hock et al., 2003; Yücel et al., 2003; Yücel et al., 2004). In addition to severe neutropenia (Hock et al., 2003; Karsunky et al., 2002), the gene targeted *GFI1*$^{-/-}$ mice also display significant defects in the B- and T-cell lineage as well as in the hematopoietic stem cell fraction, since *Gfi1* is crucial for myeloid and lymphoid differentiation. Therefore, blurring the exact role of PMNs by dampening other cell lines; for this reason, the conclusions reached on the role of PMNs are limited.

Genista mutant mice harbor a point mutation in the *Gfi1* gene as the result of exposure to N-ethyl-N-nitrosourea (Ordoñez-Rueda et al., 2012). Genista mice differ from homozygous null allele of *GFI1*$^{-/-}$ mice, in that they display normal survival and body weight, a slight increase of monocytes, and impairment of NK maturation. The point mutation in the *Gfi1* gene has a limited impact on lymphopoiesis or T- and B-cell maturation and function (Ordoñez-Rueda et al., 2012). Genista point mutation blocks a terminal step in the granulopoiesis, after the metamyelocytic stage. This mutation results

in the generation of reduced numbers of atypical nonfunctional CD11b+ Ly-6Gint PMNs released from the BM to the circulation. These atypical PMNs may induce a mild form of immune complex-mediated lung alveolitis and autoantibody-induced arthritis later in the life of mice. In recent years this has been a valuable model to study the role of PMNs in different fields of study such as tumor biology, autoimmunity, protozoans and bacteria pathogenesis (Barquero-Calvo et al., 2013; Charmoy et al., 2016; Desnues et al., 2016; Mensurado et al., 2018).

Bacterial infections in neutropenic mice

Chlamydia

Chlamydia trachomatis is a Gram-negative, sexually transmitted obligate intracellular bacterial pathogen of epithelial cells of the genital tract (Hafner et al., 2008; WHO, 2011). The bacterium causes an inflammatory disease of the reproductive organs, characterized by infiltration of broad leukocyte populations, including PMNs, plasma and peri-glandular lymphoid follicles (Kiviat et al., 1990). In mice, the immunopathology is also characterized by the extravasation of leukocytes into the genital tract that can lead to occlusion of luminal spaces in ovaries and uterus (Lijek et al., 2018).

The preferred bacterial species in the mouse model is *Chlamydia muridarum* though *C. trachomatis* is also capable of infecting mice. There are differences in the pathogenesis caused by these two chlamydial species. *C. muridarum* murine infection is generally more aggressive than *C. trachomatis*, producing high numbers of lesions in the vagina, particularly at early times. Besides, *C. muridarum* causes intraluminal occlusion fibrosis, hydrosalpinx, and infertility. In comparison, C. *trachomatis* infections of susceptible mice are less aggressive, showing reduced pathogenicity and seldom causing oviduct occlusion or hydrosalpinx. Moreover, while *C. muridarum* readily colonized vaginal squamous epithelial cells, *C. trachomatis* does not (Yang et al., 2017).

Chlamydia-infected mice show a vaginal inflammatory response characterized by a large number of PMNs and B and T cells infiltrating submucosae (Yang et al., 2017). The abundance of PMNs in the murine urogenital *C. muridarum* infection model, promote

hydrosalpinx formation and cause infertility (Shah et al., 2005). In this site, PMNs are a source of matrix metalloproteinases that are involved in the proteolysis and resynthesizes of the extracellular matrix (Ramsey et al., 2005). Specific inhibition of PMN metalloproteinases has a protective effect over chronic chlamydiosis, demonstrating the role of these enzymes in the pathogenesis of the disease (Imtiaz et al., 2006).

Although PMNs efficiently internalize *Chlamydia*, a significant proportion of the ingested bacteria are capable of surviving inside these phagocytic cells (Register et al., 1986). *C. trachomatis* can prevent the activation of PMNs by releasing a protease-like activating factor (CPAF) that targets and release of a formyl peptide receptor 2 (FPR2) on the surface of PMNs. The cleavage of FPR2 dampens G-protein coupled receptor signaling, further preventing the downstream activation of PMNs. CPAF suppresses the oxidative burst and interferes with chemical-mediated activation of PMNs, including NETs formation and enabling pathogen survival inside PMNs for extended periods.

Both 1A8 and RB6-8C5 antibodies have been used to dissect the role of PMNs in experimental murine chlamydiosis (Barteneva et al., 1996; Frazer et al., 2011; Naglak et al., 2017; Rajeeve et al., 2018). The administration of progesterone before *Chlamydia* infection favors the bacterial colonization of the genital tract (Tuffrey & Taylor-Robinson, 1981). Therefore, in some of the protocols, mice are prepared by injecting progesterone before PMN depletion and infection (Frazer et al., 2011; Naglak et al., 2017; Rajeeve et al., 2018). In one experimental condition of the *C. muridarum* infection, PMN depletion (1A8 antibody) did not alter the course of the disease or influenced the pathological symptoms (Frazer et al., 2011). However, in other cases, using RB6-8C5 or 1A8 antibodies and *C. trachomatis* or *C. muridarum* as infection sources, the trend was that the absence of PMNs at the onset of the infection was linked to higher bacterial loads (Barteneva et al., 1996; Rajeeve et al., 2018). Likewise, neutropenia in re-challenge infection models (when the adaptive immune response is established), leads to a significant increase in bacterial burden, associated with the augmented bacterial shedding (Barteneva et al., 1996; Naglak et al., 2017; Rajeeve et al., 2018). It was also demonstrated in antibody PMN depleted pregnant mice that PMNs play an essential role in killing *Chlamydia* (Buendía et al., 1999).

The discrepancy in the results obtained between the 1A8 and RB6-8C5 antibodies may be due to the different amounts of the former antibody used in these protocols. As

stated before, depletion with 1A8 antibody seldom achieves complete depletion of blood PMNs; therefore, higher concentrations of this antibody are required (Daley et al., 2008). Moreover, 1A8 antibody does not deplete PMNs at the genital tract; therefore, caution in the interpretation of the results is needed when using 1A8 antibody to explore the role of PMNs during murine chlamydial infections (Frazer et al., 2011).

Francisella

Francisella tularensis is a Gram-negative facultative intracellular pathogenic bacterium, which is the causative agent of tularemia, a chronic zoonotic infection (Ellis et al., 2002). This intracellular pathogen is highly virulent (Sjöstedt, 2006) and considered a potential biological warfare agent (Maurin, 2015; Oyston et al., 2004). After invasion, *F. tularensis* distributes throughout the mononuclear phagocytic system and invades lungs, skin, and eyes successfully replicating inside Mϕs, DCs, and epithelial cells. *F. tularensis* dampens the bactericidal effect of human PMNs by evading the respiratory burst and the phagosome-granule fusion, leading to bacterial replication in the cytosol (McCaffrey & Allen, 2006; McCracken et al., 2016; Mohapatra et al., 2010). The bacterium also delays apoptosis increasing the lifespan of the infected PMNs (Schwartz et al., 2012).

PMNs play a significant role in the host defense against *F. tularensis* and the pathogenesis of the disease (Allen, 2013). Neutropenia increases susceptibility to infection in human patients (Sarria et al., 2003). Alternatively, an exacerbated PMNs response causes tissue damage in the lungs and other target organs (Allen, 2013). Conditions that lead to an increase of PMNs influx in the primary infection or secondary infection mouse models, mostly lead to detrimental outcomes, such as severe liver damage (Bosio & Elkins, 2001; Melillo et al., 2013).

RB6-8C5 antibody depleted mice prior to *F. tularensis* intradermal infection, render higher bacterial loads in the target organs and increased mortality (Sjöstedt et al., 1994), also indicating that PMNs play a significant role in controlling *F. tularensis* reinfection (Sjöstedt et al., 1994). Likewise, using the same anti-PMN antibody, it was demonstrated that PMNs were required for controlling the infection, even in the presence of anti-*F. tularensis* antibodies (Kirimanjeswara et al., 2007). Other works have supported

these findings, reporting increased *F. tularensis* loads in neutropenic mice (Conlan et al., 2002) as well as reduced survival, regardless of whether PMN depletion was accomplished with RB6-8C5 or 1A8 antibodies (Steiner et al., 2017).

In contrast to these findings, a study using intranasal infection of *F. tularensis* and lower doses of RB6-8C5 showed that PMNs did not play a significant role in controlling *F. tularensis* (KuoLee et al., 2011). This last result was somewhat supported by the survival of mice to a lethal dose of *F. tularensis,* after inhibiting the migration of PMN into the lungs (Malik et al., 2007). Others have suggested that neutrophilia conditions exacerbate the tularemia severity (Bosio & Elkins, 2001). The discrepancies between the results obtained with the RB6-8C5 antibody may be related to different concentrations of antibodies used, as well as the different protocols and routes of infection. When pondering the current evidence, it seems that PMNs besides being protective against *F. tularensis* at certain stages of infection may aggravate the disease at other stages.

Legionella

Legionella pneumophila is a facultative intracellular Gram-negative bacterium that lives in aquatic environments, where it establishes parasitic relationships with protozoans (Barker et al., 1995; Oliva et al., 2018). Commonly, immunocompetent individuals are capable of controlling *L. pneumophila* infection; however, in immunocompromised people, the infection may progress and cause death (Brown et al., 2017). *L. pneumophila* primarily infects lungs via inhalation of contaminated water aerosols (Oliva et al., 2018). Once *L. pneumophila* is in the lungs, a large number of professional phagocytes, NK cells and lymphocytes are recruited at the site of infection (Park et al., 2017). The bacterium primarily infects alveolar Mϕs (Finsel & Hilbi, 2015; Horwitz, 1983). Once inside these cells, the bacterium uses its type IV secretion system to inject close to 300 different effector proteins into the cytosol. These effectors block phagosomal maturation and hamper lysosomal fusion, preventing *L. pneumophila* degradation and favoring intracellular replication (Sherwood & Roy, 2016).

Mice are generally resistant to *L. pneumophila*. After airway infection, the bacterium is controlled rapidly by the innate immune system, paralleling the responses

observed in immunocompetent humans (Brown et al., 2013). In mice, pathogen recognition receptors (PRR) sense *L. pneumophila* pathogen-associated molecular patterns (PAMPs) influencing PMN lung infiltration (Archer & Roy, 2006; Berrington et al., 2010; Berrington et al., 2013; Frutuoso et al., 2010; Hawn et al., 2006; Mascarenhas et al., 2015). Some deficiencies in molecules such as MyD88, TLR-5, NOD1 and RIPK2 fail to promote early PMN influx; however, no significant differences in bacterial clearance is observed in relation to wild type mice (Frutuoso et al., 2010; Hawn et al., 2007). In order to kill *L. pneumophila,* host alveolar Mϕs require activation via the release of TNF-α by primed PMNs and monocytes (Ziltener et al., 2016).

L. pneumophila does not replicate inside PMNs, and upon phagocytosis, these leucocytes readily kill the bacterium, mainly through ROS microbicidal mechanisms (Ziltener et al., 2016). If PMNs recruitment is affected, the number of *L. pneumophila* increases in the lungs of infected mice (Barry et al., 2013; Casson et al., 2013; Mascarenhas et al., 2015). After PMN removal employing the RB6-8C5 antibody, the arrival of these leucocytes in the lungs of mice is abrogated promoting an increase of *L. pneumophila* loads after two days of infection (Tateda et al., 2001). Under these conditions, the PMN depleted infected mice experience lower levels of cytokines associated with a Th1 response such as IFN-γ and IL-12 and higher levels of cytokines associated with a Th2 response such as IL-4 and IL-10, revealing a shift in the immune response (Tateda et al., 2001).

Since the RB6-8C5 antibody may deplete a subpopulation of monocytes (Daley et al., 2008), 1A8 antibody was included in the PMN depletion protocol, to dissect the role of PMNs and other cells (Casson et al., 2017). Ly6C^{hi} inflammatory monocytes also play a role in shaping the key immune responses in *L. pneumophila* lung mouse infections. Following this, it was determined that both PMNs and Ly6C^{hi} monocytes play a relevant role in controlling *L. pneumophila* in the lungs of infected mice (Casson et al., 2017).

Listeria

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium (Pamer, 2004). Infection occurs primarily upon the oral intake of contaminated foods (Farber & Peterkin, 1991; Vázquez-Boland et al., 2001). Unlike other microorganisms, this

foodborne pathogen can persist at 4°C, low pH, and high salt conditions, complicating its control in the food industry (de Noordhout et al., 2014). Upon ingestion of contaminated foods, *L. monocytogenes* crosses the intestinal barrier and invades several organs of the mononuclear phagocytic system. If the immune system does not control the bacterium, then it may disperse and cause bacteremia, cross the blood-brain barrier, and cause meningitis.

The manifestations of clinical listeriosis vary according to the host immune status. In healthy people, *L. monocytogenes* organisms are commonly controlled, usually only causing self-limiting digestive symptoms (Dortet et al., 2009). In susceptible individuals such as immunocompromised people, pregnant women, and newborns, *L. monocytogenes* may cause severe illnesses such as septicemia, meningitis, brain infection and even death with an overall mortality rate of close 30% (Dortet et al., 2009). In pregnant women, *L. monocytogenes* is capable of crossing the placental barrier causing generalized infections in neonates and abortion (Radoshevich & Cossart, 2018).

The bacterium primarily infects the liver and spleen, but it is capable of infecting other organs like the heart, brain and the BM (Alonzo et al., 2011; Hardy et al., 2008; Join-Lambert et al., 2005). Once in the bloodstream, a variety of professional phagocytes can rapidly internalize this bacterium (Waite et al., 2011; Williams, et al., 2012). In the spleen of mice, *L. monocytogenes* can interact with a variety of resident myeloid cells in the marginal zone such as PMNs, DC, marginal, metallophilic and F4/80+ Mϕ (Williams et al., 2012). PMNs infiltrate the livers of *L. monocytogenes* infected mice, indicating that these cells are effectors of innate immunity during listeriosis. *L. monocytogenes* is capable of replicating within non-activated Mϕ and epithelial cells but not PMNs.

This intracellular bacterium displays several virulence factors, including listeriolysin O, two distinct phospholipases, a protein that interacts with actin (ActA), several internalins, among others. Upon cell infection, the bacterium is found within vacuoles of host cells. Within this site, the bacterium releases listeriolysin O and promotes its escape from the vacuole into the cytoplasm, avoiding lysosomal fusion. Within the cytosol, the microbe extensively replicates and uses the actin of the host cell to move intracellularly and to spread to other adjacent cells (Kortebi et al., 2017). Upon bacterial infection, and dispersion, PMNs infiltrate the target organs. Although *L. monocytogenes* can invade

human PMNs, these cells readily kill the bacterium, suggesting that they are relevant in the control of the bacterium (Czuprynski et al., 1983).

Both the innate and adaptive immune systems are required to eliminate *L. monocytogenes* from the host (Zenewicz & Shen, 2007). A Th1 adaptive immune response with the concomitant activation of Mϕ is required to control *L. monocytogenes* in mice. PMNs are recruited rapidly into the infectious site by IL-8 and chemotactic factors, and they, in turn, secrete chemokines such as CSF-1 and MCP-1, that signal to Mϕ to arrive at the site of infection. *L. monocytogenes* are readily internalized and killed by murine PMN, and several works have demonstrated the role of these leucocytes in controlling *L. monocytogenes* infection (Lieschke et al., 1994; Zhu et al., 2009). Pretreatment with serum significantly enhanced killing efficiency and triggered the intracellular production of ROS in a dose-dependent manner. However, PMNs from gp91phox-deficient mice can kill *L. monocytogenes*, suggesting the participation of nonoxidative killing mechanisms in the bactericidal activities against this bacterium (Pitts et al., 2018).

Several studies using the RB6-8C5 and 1A8 antibodies for PMN depletion have demonstrated the relevance of these leucocytes in controlling and killing *L. monocytogenes*, highlighting their participation in the innate immune response against this bacterium in mice (Agbayani et al., 2018; Carr et al., 2011; Conlan, 1997; Czuprynski et al., 1994a; Czuprynski et al., 1994b; Czuprynski et al., 1996; Edelson et al., 2011; Rakhmilevich, 1995; Rogers & Unanue, 1993). Despite the robust and broad evidence, there is one report claiming that PMN depletion using the 1A8 antibody did not affect the outcome *L. monocytogenes* infection (Shi et al., 2011). The 1A8 PMN depleted mice did have a slight increase in the mean of bacterial loads in the target organs; however, this difference was not statistically significant (Shi et al., 2011). It is important to notice that in this work the anti-PMN 1A8 antibody was administrated at a lower concentration and at the same time with the intravenous *L. monocytogenes*. It may be that the inefficient depletion of PMN, combined with the fast uptake of the bacterium by these cells after intravenous administration (Waite et al., 2011) influenced the outcome of the infection.

Mycobacterium

Mycobacterium tuberculosis is a Gram-positive facultative intracellular pathogen (Gengenbacher & Kaufmann, 2012). Tuberculosis remains a global health problem causing mortality in millions of individuals around the world (Dye & Williams, 2010). The outcome of *M. tuberculosis* infection ranges from active disease, latent infection, to a complete pathogen clearance (Gengenbacher & Kaufmann, 2012). Most cases are associated with the reactivation of dormant *M. tuberculosis* in infected individuals rather than new infections (Gengenbacher & Kaufmann, 2012).

The lungs are the most important target organs. Patients with pulmonary tuberculosis are the most important source of infection being aerosols the primary route of transmission (Gengenbacher & Kaufmann, 2012). A broad range of cells such as alveolar endothelial cells, type 1 and type 2 pneumocytes, DCs and Mϕs might become infected, being these last two cells the main targets (Ahmad, 2011; Blomgran & Ernst, 2011). *M. tuberculosis* subverts the killing action of alveolar Mϕ, and therefore, it can replicate and persist in these cells. The bacterium may also cross the alveolar epithelium causing systemic dissemination (Scordo et al., 2016) and replicate in different organs before an efficient adaptive response is mounted (Ahmad, 2011).

M. tuberculosis infections elicit a classical Th1 immune response as well as effector Th17 cells (Lyadova & Panteleev, 2015). Th1 cells provide an essential source of IFN-γ, stimulating the bactericidal mechanisms of Mϕs. In mice and humans, activation of Th17 cells depends upon pro-inflammatory cytokines such as IL-6, IL-1, and IL-23. DCs and Mϕs generate them in response to *M. tuberculosis*. The Th17 response has been associated with neutrophilic inflammation, and with the damage caused by the exacerbated response to *M. tuberculosis* (Lyadova & Panteleev, 2015).

Within a few hours of *Mycobacterium* infection, there is a fast recruitment of PMNs (Appelberg & Silva, 1989). Although PMNs, become infected with *M. tuberculosis,* there is no active replication of the bacterium in these cells. PMNs from the lungs of mice and humans can phagocytize *Mycobacterium* (Eruslanov et al., 2005; Ganbat et al., 2016), and these cells are the predominantly *Mycobacterium* infected phagocytic cells from bronchoalveolar lavage, sputum and cavity contents (Eum et al., 2010).

There are contradictory reports on the role of human PMNs against *M. tuberculosis*. A study shows that TNF-α is required for the activation of bactericidal mechanisms of human PMNs against *Mycobacterium* (Kisich et al., 2002). However, others have reported that human PMNs modestly kill *M. tuberculosis* (Jones et al., 1990; Kisich et al., 2002; Martineau et al., 2007), even after these cells are activated with TNF-α (Reyes-Ruvalcaba et al., 2008). After treatment with IFN-γ, human PMNs display activation markers; but still insignificant killing activity against *Mycobacterium* is detected (Corleis et al., 2012; Denis, 1991; Ellis & Beaman, 2004).

PMNs from different donors display variability regarding mycobactericidal ability (Kisich et al., 2002), suggesting that genetic, environmental factors or both are relevant in the outcome of the infection. Bacterial virulence also plays an important role since some attenuated *Mycobacterium* strains are more susceptible to the killing action of PMNs than others (Corleis et al., 2012). The physiological condition of PMNs also influence the host response against *M. tuberculosis*. For instance, necrotic PMNs seem to have a detrimental effect on the host response (Lowe et al., 2018). Likewise, the uptake of necrotic infected PMN by Mϕs promotes *M. tuberculosis* growth (Dallenga et al., 2017). Therefore, PMNs seem to play a role in immunity as well as in the pathogenesis (Lowe et al., 2013).

The experimental mouse model has shown limitations for studying the pathobiology of human *M. tuberculosis* infections, due to critical differences regarding granuloma formation, cellular composition, and kinetics of bacterial growth (Dallenga & Schaible, 2016). C57BL/6 and BALB/c mouse strains are highly resistance to *M. tuberculosis* infections, and the disease in these mice does not resemble the chronic presentation of tuberculosis in humans (Keller et al., 2006). Under certain conditions, highly susceptible C3HeB/FeJ and I/St mice strains against *M. tuberculosis* seem more relevant models (Harper et al., 2012; Yeremeev et al., 2015). A higher PMNs influx in the lungs is observed in *M. tuberculosis* susceptible mice (Eruslanov et al., 2005) and similar to humans, PMNs also play a significant role in lung pathogenesis (Yeremeev et al., 2015).

Several investigators have shown that early depletion of PMNs using RB6-8C5 antibodies induce an increase in *Mycobacterium* loads in neutropenic infected mice (Appelberg et al., 1995; Barrios-Payán et al., 2006; Feng et al., 2006; Fulton et al., 2002; Pedrosa et al., 2000; Petrofsky & Bermudez, 1999). However, this observation is not

entirely consistent among the scientific literature and might plausibly be influenced by *Mycobacterium* susceptibility, bacterial strain, route of infection, depletion method, and time of depletion in the course of infection, as explained below.

Following the RB6-8C5 depletion model, one research group (Seiler et al., 2000) highlighted the relevance of PMNs with the fast-growing *M. fortuitum* but not for other *Mycobacterium* pathogenic strains (Erdman, CDC 1551, BCG). Moreover, they claimed that PMNs are dispensable in the control of this *M. tuberculosis* (Seiler et al., 2000).

Likewise, following PMN depletion with the 1A8 antibody has indicated that these leukocytes do not play a significant role in controlling *Mycobacterium* in the lungs (Blomgran & Ernst, 2011; Mishra et al., 2017; Nandi & Behar, 2011). However, this antibody did not completely abrogate the PMNs presence in the lungs, but instead only mildly reduces the total counts of these leukocytes in this organ (Casson et al., 2017).

Depletion of PMNs in genetically susceptible mice, results in reduced lung tissue pathology, decreased bacterial count and increased survival time when challenged with *Mycobacterium* (Keller et al., 2006; Yeremeev et al., 2015). In contrast, neutropenia in resistant C57BL/6 mice yields no changes in survival and bacterial load in response to *Mycobacterium* (Keller et al., 2006; Yeremeev et al., 2015).

Due to the fact that PMNs antibody depletion at different times of *M. tuberculosis* invasion renders different outcomes, some investigators have suggested that PMNs might have different roles during the course of infection, while neutropenia usually causes an increase of bacterial loads, depletion of PMNs at chronic stages does not affect the bacterial loads in target organs (Pedrosa et al., 2000; Petrofsky & Bermudez, 1999). Moreover, in some cases slightly lower bacterial counts are observed in the antibody PMN depleted mice, suggesting a limited beneficial outcome exerted (Zhang et al., 2009).

When taking into consideration susceptible mice and the variety of recent literature on the new roles of PMNs, most evidence highlights that PMNs are important players in *Mycobacterium* infections.

Brucella

Brucella is a Gram-negative facultative intracellular-extracellular pathogenic bacteria (Moreno & Moriyón, 2006). This stealthy pathogen is responsible for the zoonotic

disease called brucellosis, characterized by a long incubation period that leads to a chronic infection. Brucellosis is a worldwide disease, causing significant animal and public health problems in low-income countries.

Infection occurs through direct contact with aborted fetuses, vaginal secretions, milk, or semen. Humans commonly become infected after the consumption of unpasteurized dairy products from *Brucella* infected animals. The disease in humans causes severe clinical symptoms such as undulant fever, arthritis, hepatomegaly, and splenomegaly. If the infection is not treated, the bacterium may invade the brain and heart and cause death. In domestic animals, such as cows, sheep, goats, pigs, and dogs, *Brucella* organisms cause abortion and in males orchiepididymitis, with the corresponding economic losses associated with the animal industry (Moreno, 2014; Moreno & Moriyón, 2006).

Brucella evades the initial innate immune response recognition, since several bacterial PAMPs, such as lipopolysaccharide (LPS), lipoproteins and flagellum are modified, hampering the recognition by various PRR (Barquero-Calvo et al., 2007; Weiss et al., 2005). In addition, the bacterium is capable of evading lysosomal fusion and resisting the bactericidal activity of phagocytic cells. This furtive strategy opens a window for the bacterium to invade and replicate within the cell before the Th1 adaptive immunity becomes activated (Martirosyan et al., 2011).

Brucella species replicate inside professional and non-professional phagocytic cells such as monocytes, DCs, Mϕs, and epithelial cells (Celli et al., 2003; Pizarro-Cerdá et al., 1998). In the case of pregnant animals, the bacterium invades placental trophoblasts, where it extensively replicates, causing abortion (Carvalho Neta et al., 2008; Meador & Deyoe, 1989). After cell invasion the bacterium avoids the constitutive lysosomal route through several virulent factors, being the most conspicuous the type IV secretion system (T4SS) VirB with and the corresponding effector proteins (Celli, 2019; O'Cellaghan et al., 1999). Another *Brucella* key element required for sensing the intracellular environment is the BvrR/BvrS two-component system. Through this system, *Brucella* senses the transition from the extracellular to the intracellular environment allowing this bacterium to adapt to its replicative niche (Altamirano-Silva et al., 2018).

In humans as in animals, *Brucella* can infect PMNs, but the bacterium does not replicate within these leucocytes. Instead, the intracellular bacterium can resist the bactericidal mechanisms (Barquero-Calvo et al., 2007, 2015; Mora-Cartín et al., 2016; Riley & Robertson, 1984). *Brucella* infected PMNs do not degranulate or undergo NET formation, and produce a modest ROS and induce meager quantities of pro-inflammatory cytokines (Barquero-Calvo et al., 2007, 2009, 2013). Instead, *Brucella* promotes premature cell death of PMNs through its LPS with a signaling pathway that involves CD14 and NADPH oxidase (Barquero-Calvo et al., 2015; Mora-Cartín et al., 2016). This event correlates with the neutropenia observed in about one-fourth of the patients (Crosby et al., 1984), and it is specific for PMNs since in Mϕ and DCs the bacterium prolongs the life of and their maturation, respectively (Celli, 2006; Salcedo et al., 2008). The *Brucella*-infected PMNs display increased levels of phosphatidylserine on the cell surface commonly recognized as an "eat me" signal for Mϕ*s* (Barquero-Calvo et al., 2015; Lauber et al., 2004). Mϕs phagocyte the *Brucella*-infected PMNs in a non-phlogistic manner (Gutiérrez-Jiménez et al., 2019). Moreover, when the bacterium gains access to Mϕs via the uptake of infected PMNs, the microorganisms replicate more efficiently than *Brucella* directly phagocytized by Mϕs (Gutiérrez-Jiménez et al., 2019). These results propose that infected PMNs function as "Trojan horse" vehicles, for the efficient dispersion of the bacterium into the host.

As with other bacterial pathogens, the most widely used animal model in brucellosis is the mouse (Grilló et al., 2012). However important differences in the interaction with *Brucella* organisms have been recorded between murine and human PMNs: while human PMNs readily ingest *Brucella* opsonized with complement alone, murine PMNs require the presence of antibodies (Mora-Cartín et al., 2016).

The use of monoclonal antibodies (RB6-8C5, 1A8) to deplete PMNs, and Genista mice showed that the absence of PMNs during brucellosis infections promoted the efficient elimination of *B. abortus* from the target organs such as spleen and BM. However, the role of PMNs is slightly different at the onset of *B. abortus* infection in comparison to the acute and chronic stages of infection, once the adaptive immune response has initiated. (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019). The early absence of PMNs in Genista or anti-PMN (RB6-8C5 or 1A8) treated-infected mice promotes a rapid increase

of bacterial after five days of infection. However, after this initial increase, it follows a more efficient bacterial clearance and bacterial elimination as compared to control infected mice with PMNs (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019), a phenomenon that is promoted by the efficient Th1 and INF-γ responses (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019). The absence of PMNs stimulates the recruitment of Mϕ/DCs, promote M1 Mϕ polarization, and increase the activity of B and T lymphocytes (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019). As the infection progresses, the levels of IFN-γ diminished while afterward IL-6, IL-10, and IL-12 become prevalent cytokines in the neutropenic mice (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019). Moreover, the efficient bacterial elimination is not due to an increase in the production of antibodies since the absence of PMN at the onset of the infection caused a lower antibody response against bacterial antigens (Mora-Cartín et al., 2019).

The augmented secretion of IFN-γ is a prevalent aspect in PMN-depleted mice at different time points, either when the depletion was induced at the onset of the infection or after the adaptive immunity had developed (Barquero-Calvo et al., 2013; Mora-Cartín et al., 2019). Still, the IFN-γ values are much higher in PMN-depleted mice once the adaptive immune response has started (Mora-Cartín et al., 2019).

Another aspect influenced by the absence of PMNs at different stages of *B. abortus* infection corresponds to the spleen histopathology. The depletion of PMNs at the onset of the infection is linked to an increase in Mϕ infiltration, granuloma formation, and lymphoid depletion (Barquero-Calvo et al., 2013). In contrast, PMN depletion, once the immune response has started, favors the premature resolution of the inflammation (Mora-Cartín et al., 2019).

Salmonella

Salmonella enterica is a flagellated Gram-negative facultative intracellular pathogen. Salmonellosis is a worldwide foodborne disease, more prevalent in economic burden groups in developed as well as middle- and low-income countries (Lee et al., 2015; Sánchez-Vargas et al., 2011). Most of the *Salmonella* strains that cause infection in humans and other mammals are assigned to *Salmonella enterica* species (Kurtz et al., 2017).

The clinical symptoms of salmonellosis vary widely, depending on the host susceptibility and the serotypes of *Salmonella* involved. The main manifestations are: enteric fever (typhoid and paratyphoid), diarrheal disease (colitis), invasive disease, and chronic asymptomatic carriers (Herrero-Fresno & Olsen, 2018). The more significant *Salmonella* infections induced in humans are: i) gastroenteritis caused by non-typhoidal *Salmonella*, such as the serotype Typhimurium and, ii) typhoid fever, caused by *Salmonella* Typhi and the various *Salmonella* Paratyphi pathovars (Gunn et al., 2014). The human restricted *S. enterica* serovar Typhi causes Typhoid fever, being one the pathogens to cause more deaths globally (Havelaar et al., 2015).

S. enterica serovar Typhimurium (*S.* Typhimurium) has been used as an experimental mouse model to study human Typhoid infections (Broz, Ohlson, & Monack, 2012). Inoculated mice with *S.* Typhimurium display symptoms compatible with a systemic disease, similar to typhoid fever in humans. However, caution is needed since *S.* Typhimurium and *S.* Typhi display different virulence factors (Santos et al., 2001).

In order to circumvent the systemic typhoid-like illness present in mice, and mimic the gastroenteritis seen in *Salmonella* infected humans; before infection, mice are pretreated frequently with aminoglycoside antibiotics (e.g., streptomycin) to suppress the microbiota (Barthel et al., 2003). This pretreatment allows *Salmonella* to overcome "colonization resistance", and to establish an efficient infection in the cecum and the colon of mice. Pretreatment with streptomycin causes an exacerbated increase in the PMNs influx and inflammation in the cecal mucosa (Diaz-Ochoa et al., 2016; Hapfelmeier et al., 2004; Maier et al., 2014; Müller et al., 2016).

Different mice strains vary in the levels of resistance to *Salmonella* infection. C57BL/6 strain is highly susceptible to *Salmonella* infection, and these mice usually succumb during the first week of infection. In contrast, 129S6/SvEvTac mice are highly resistant to *Salmonella* infections (Brown et al., 2013; Roy & Malo, 2002). These differences may resemble at least two states of the disease in humans. Indeed, after humans overcome the acute phase of the *S.* Typhi infection, they commonly become reservoirs. This persistent carrier state of the infection is mimicked in the 129Sv mice (Broz et al., 2012).

The virulence of *Salmonella* relies on its ability to establish a replicative niche within eukaryotic host cells named the *Salmonella*-containing vacuole (Malik-Kale et al., 2011). The pathogenesis of *Salmonella* is highly dependent on a set of effector virulence proteins encoded by the *Salmonella* pathogenicity islands. *Salmonella* thrives in a variety of eukaryotic cells, including Mϕs and DCs. These pathogenicity islands code for the type III secretion system (T3SS) translocation machinery, devoted to delivering a variety of proteins to the cytoplasm. These effectors control the *Salmonella* vacuole biogenesis, the evasion of the intracellular killing mechanisms, and intracellular survival (Larock et al., 2015).

The acute symptoms in human non-typhoidal salmonellosis have been associated with the early activation of the host innate immune response against *Salmonella,* triggered by recognition of PAMPs and phagocytosis of *Salmonella* (Gunn et al., 2014; Winter et al., 2009). The release of cytokines and chemokines in non-typhoidal salmonellosis induce the influx of PMNs and other phagocytic cells to the intestinal lumen (Dougan et al., 2011). Natural Killers (NK) are also involved in the host response, and they are a source of IFN-γ during the early phase of the *Salmonella* infection (Kupz et al., 2013).

During the early phases of non-typhoidal salmonellosis, monocytes and PMNs are recruited rapidly in Peyer's patches and mesenteric lymph nodes. In these sites, leukocytes produce IFN-γ, IL-12 and TNF-α and IL-1β, which are essential in the control and elimination of *Salmonella* (Nauciel & Espinasse-Maes, 1992). In contrast, IL-10 and IL-4 seem to dampen the bactericidal response against *Salmonella* (Arai et al., 1995; Everest et al., 1997; Lee et al., 2011). The sum of the pro-inflammatory reactions elicited in non-typhoidal salmonellosis in the intestine results in inflammatory diarrhea (Larock et al., 2015). Although phagocytes of the luminal intestine can phagocyte *Salmonella*, the bacterium preferentially induces its uptake by intestinal epithelial and M cells (Jones et al., 1994).

The Th1 response has a major role in the defense against *Salmonella* (Bao et al., 2000; Ravindran et al., 2005). The main cytokine IFN-γ and other IFN-γ-inducing cytokines such as IL-12, IL-18, and IL-15 are produced during salmonellosis and are relevant in the control of the infection (Mastroeni et al., 1999; Mizuno et al., 2003). It

seems that resident bacteria confer protection to *Salmonella* infection by priming host IFN-γ responses (Thiemann et al., 2017).

Th17 cells are also important in the host defense against salmonellosis (Raffatellu et al., 2008; Schulz et al., 2008) since *Salmonella* infection can lead to increased IL17 levels. Th17 cells can secrete IL-17 family cytokines 1L-17A and IL-17F as well as IL-22, IL-26, and granulocyte-macrophage colony-stimulating factor (Sandquist & Kolls, 2018). Despite this, the host's Th17-associated response against *Salmonella* seems dispensable in the presence of an adequate Th1 response (Schulz et al., 2014).

Although PMNs seems to have many functions in the pathogenesis of salmonellosis, there are some contradictory reports on the role of these cells during systemic and gut infections. *Ex vivo* experiments indicate PMNs are bactericidal toward *Salmonella* during the first two hours; afterward this bacterium seems to overcome the velocity of killing by fast replication in the media where PMNs stand (Baron & Proctor, 1984; Chiu & Ou, 1999; Shiloh et al., 1997). A similar phenomenon is observed in Mϕs (Buchmeier & Heffron, 1989). Still, *Salmonella* seems to evade PMN killing by hiding within Mϕs. Despite this, murine PMNs might serve to a certain degree as a *Salmonella* hidden niche, mainly in the gut. Indeed, in this site *Salmonella* partially resists the killing action of these PMNs (Geddes et al., 2007; Loetscher et al., 2012; Rydstrom & Wick, 2007). *Salmonella* is found within PMNs of mesenteric lymph nodes and Peyer's patches at four days after oral inoculation (Rydstrom & Wick, 2007). In the streptomycin mouse model, the luminal gut PMNs actively engulf *Salmonella* after twenty hours of infection (Loetscher et al., 2012). Despite the presence of luminal PMNs, the *Salmonella* loads increase during the first days of infection (Stecher et al., 2007), suggesting that luminal PMNs display a limited bactericidal effect toward *Salmonella*. Intracellular *Salmonella* inside PMNs induces the expression of virulence genes required for intracellular life such as those of *Salmonella* pathogenicity islands that encode various T3SS (Loetscher et al., 2012). Despite this, consensus shows that PMNs are necessary for the control of *Salmonella* at the early stages of the infection.

The role of PMNs has been widely supported in multiple studies of neutropenic mice with the RB6-8C5 clone (Cheminay et al., 2004; Conlan, 1996, 1997; Dejager et al., 2010; Seiler et al., 2000; Vassiloyanakopoulos et al., 1998) and with the 1A8 clone (Chen

et al., 2014; Diaz-Ochoa et al., 2016; Franchi et al., 2012; Spees et al., 2014) and Genista mouse (Ordoñez-Rueda et al., 2012). Following this, it has been determined that PMNs are essential for controlling the bacterial numbers in systemic salmonellosis in the target organs, including the gut (Diaz-Ochoa et al., 2016; Franchi et al., 2012; Maier et al., 2014).

The relevance of PMNs has been demonstrated further in the IL-8R knockout mice. These mice have fewer PMNs in the colon and become more susceptible to *S.* Typhimurium, showing higher *Salmonella* loads in the colon (Marchelletta et al., 2015). PMNs are an important source of IFN-γ and IL-1β cytokines during acute salmonellosis (Chen et al., 2014; Spees et al., 2014). The PMNs also play an essential role in mediating microbiota perturbations in *Salmonella* infections (Loetscher et al., 2012). They can dampen bacterial growth by several antimicrobial effects such as by the production of proteins that sequester nutrients essential to microbes (Diaz-Ochoa et al., 2016). Hence, PMNs are important in controlling *Salmonella* loads in the target organs as well in the cecal population (Maier et al., 2014).

Borrelia

Borrelia burgdorferi is the etiological agent of zoonotic Lyme disease, a persistent public health problem, and the most common tick-borne infectious disease in both North America and Europe (Mead, 2015). *B. burgdorferi* circulates between ticks and a broad range of small vertebrates such as various bird species, squirrels, hedgehogs, hares, and rabbits, among others. This spirochetal bacterium is a microaerophilic organism that requires special conditions to grow in the laboratory. Although this pathogen displays a double membrane, it lacks an LPS (Takayama et al., 1987) and contains a flat wave body (Aslam et al., 2017). *B. burgdorferi* is vector-borne pathogen maintained in nature through a vertebrate-arthropod infection cycle and transmitted to humans through Ixodes ticks (reviewed in (Radolf et al., 2012)). *B. burgdorferi* possesses a series of surface lipoproteins which are virulence factors (Kenedy et al., 2012). Outer surface protein OspC readily binds plasminogen, diminishing host-pathogen recognition, and hence, it is involved in pathogen dissemination (Önder et al., 2012). *B. burgdorferi* lipoproteins can bind to glycosaminoglycans, decorin, and fibronectin. Moreover, some lipoproteins provide

antigenic variation, diminish complement deposition, providing substantial evasion toward the host response (Kenedy et al., 2012).

Lyme disease may cause severe cardiac, arthritis, and neurological symptoms in humans. The initial clinical pathognomonic symptom is the appearance of erythema migrans, a characteristic skin expanding rash (Müllegger & Glatz, 2008). Within days or weeks, this pathogen may disseminate to different tissues, causing meningitis, neuritis, carditis; if untreated, the bacterium may lead to Lyme arthritis or even death (Gerold et al., 2012).

The interaction between the vertebrate host and *Borrelia* starts when a tick feeds on its hosts. Inflammatory cells infiltrate the injured tissues, and PMNs are the first responders to accumulate at the feeding site (Amosova, 1994). Human PMNs, DCs and Mϕs contribute to the inflammatory milieu against *B. burgdorferi* by producing cytokines, such as IL-1β, IL-6, IL-12, and TNF-α (Dennis et al., 2009; Suhonen et al., 2003). Many salivary tick molecules are involved in the modulation of host defense responses (Šimo et al., 2017), which are necessary for the *Borrelia* pathogenicity (Horká et al., 2009). For instance, tick saliva has substances that inhibit the action of complement system components (Ribeiro, 1987), anaphylatoxins (Ribeiro & Spielman, 1986), platelet aggregation (Ribeiro et al., 1985), and histamine binding (Paesen, et al., 1999). In addition, tick saliva can impair Mϕs (Kuthejlová et al., 2001) and PMN function; including ROS production during *Borrelia* invasion (Hartiala et al., 2008; Menten-Dedoyart et al., 2012; Ribeiro et al., 1990). Tick saliva also diminishes the expression of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1 and increments the production of anti-inflammatory cytokines, including IL-10 (Kopecký et al., 1999; Ramachandra & Wikel, 1992). Despite this, tick saliva does not affect NET formation against *Borrelia* (Menten-Dedoyart et al., 2012).

The Th1 immune response is important in controlling the *B. burgdorferi* infection. For example, the absence of Th1-type inflammatory cytokines, such as INF-γ in humans is associated with persisting symptoms even after treatment (Sjöwall et al., 2011). In mice, a weak Th1-type response at the beginning of the infection leads to a latter increase in spirochete loads associated with arthritis. In contrast, an initial strong Th1 response conducts to disease resistance, and an augmented eradication of the *Borrelia* (Kang et al.,

1997). Tick inoculation of *B. burgdorferi* also affects the adaptive host response resulting in a polarization toward a Th2 immune response, in contrast to experimental syringe inoculation, which results in a mixed Th1/Th2 response (Christe et al., 2000).

Borrelia infected human patients develop a Th17 response that may be involved in the early control of the disease and also in pathogenesis (Strle et al., 2017). Borrelial neutrophil-activating protein A can elicit a synovial fluid Th17 cell response with the secretion of IL-17 (Codolo et al., 2008, 2013). IL-17 may participate in the development of *Borrelia*-induced arthritis in the murine experimental model (Burchill et al., 2003; Hansen et al., 2013; Kuo et al., 2017). Alternatively, IL-17 has also been suggested to have a limited role in the development of Lyme arthritis, but it may contribute to the disease by interacting with IFN-γ (Kuo et al., 2016).

PMNs and Mϕ readily phagocytize *Borrelia* (Benach et al., 1984; Peterson et al., 1984). PMNs display a wide range of molecules with bactericidal activity toward *Borrelia* such as hydrogen peroxide, nitric oxide, and different microbicidal lysosomal elements (Lusitani et al., 2002). PMNs can also produce NETs to trap and kill *Borrelia* (Menten-Dedoyart et al., 2012). Even though PMNs can eliminate spirochetes by a variety of pathways, they readily ingest and kill more avidly opsonized spirochete (Montgomery et al., 2002).

Not all mice are equally susceptible to *Borrelia* infection. For instance, *Borrelia* infections in C3H/He, resemble arthritis occurring in human infections (Barthold et al., 1990). In contrast, BALB/c, DBA/2, and C57BL/6 strains only display mild arthritis (Barthold et al., 1991; Brown & Reiner, 1998; Ma et al., 1998). Regarding this, there are some contradictory reports on the role of PMNs against *Borrelia.* For instance, in a histopathological skin analysis from the site of infection, no major difference in the PMN influx from mice infected with WT or with an OspC mutant strain was observed (Antonara et al., 2010). A recent observation using the 1A8 clone antibody to deplete murine PMNs, show a limited role of PMNs at early times post-infection, displaying no statistically significant change in bacterial burden (Curtis et al., 2018). It has also been reported that the absence of PMNs does not favor the infectivity of attenuated *Borrelia* strains (Carrasco et al., 2015; Curtis et al., 2018). Despite this, consensus display PMNs play as important cells in the pathogenesis and *B. burgdorferi* control. For instance, an increased PMN influx

at the infection site diminishes *B. burgdorferi* infectivity (Xu et al., 2007). Dampening the recruitment of PMNs in the *Borrelia* infected joints of CXCR2-/- mice, reduced Lyme arthritis severity (Brown et al., 2003). Likewise, PMN depletion by the RB6-8C5 antibody increased the bacterial burden and arthritis pathogenicity in mice (Brown et al., 2004). PMN depleted (RB6-8C5) mice did not display a difference in IL-4, IL-6, IFN-γ, or IL-12 levels in the joints. Still, it leads to higher levels of MCP-1 (CCL2) and keratinocyte chemoattractant (a murine analog of IL-8) (Brown et al., 2004). These cytokines recruit more inflammatory cells, hence collaborating in the development of severe arthritis (Brown et al., 2004).

Acinetobacter

Acinetobacter baumannii is a non-motile, opportunistic Gram-negative bacillus (Howard et al., 2012) which is commonly associated with pneumonia, urinary tract infections, soft tissue infections and septicemia in immunocompromised individuals. *Acinetobacter* is commonly considered a low-grade pathogen; thus, it can live on or within the human body without causing clinical symptoms. In spite of this, this bacterium has gained attention as a nosocomial etiological agent (Asif et al., 2018), since it is capable of surviving for extended periods on inanimate objects in hospital environments including beds and medical equipment, among several (Asif et al., 2018). Moreover, this bacterium displays outstanding resistance to many antibiotics (Evans et al., 2013) compromising the ability to treat patients (Mihu & Martinez, 2011). *A. baumannii* also possesses genes for the resistance to several antiseptics such as quaternary ammonium compounds and chlorhexidine (Paulsen et al., 1993). These characteristics make *A. baumannii* a severe nosocomial infection (Asif et al., 2018).

The polysaccharide capsule is the main *A. baumannii* virulence factor. This capsule hampers the host immune response (Russo et al., 2010; Skerniškytė et al., 2019) and confers resistance against environmental factors such as disinfectants, and desiccation (Tipton et al., 2018). The outer membrane protein A is involved in many pathogenic mechanisms such as biofilm formation (Gaddy et al., 2009), host apoptosis (Choi et al., 2005) and invasion (Choi et al., 2008). Other critical membrane-bound molecule is the

LPS, involved in the modulation of the host innate immune response (Erridge et al., 2007; Kim et al., 2013). *A. baumannii* secretion systems type I, II, III, IV, V and VI are also involved in the pathogenesis and adaptation of the bacterium to the host environment (Harding et al., 2017; reviewed in (Weber et al., 2017). The type VI secretory system functions in many processes such as bacterial competition (Carruthers et al., 2013) and regulation of host response (Repizo et al., 2015). The outer membrane vesicles which may be important in antibiotic resistance process, are also involved in the bacterial horizontal gene transfer (Rumbo et al., 2011).

The exact role of a Th1/Th2 response in the host defense against *A. baumannii* infection has not been thoroughly described. Reports show that different *Acinetobacter* strains induce distinct cytokine host response (de Breij et al., 2012). Despite this, an effective Th2 immunity has been suggested to be the most relevant response against *A. baumannii* systemic infection (Lin et al., 2013). In the mouse pneumonia model, the severity of the infection was directly correlated with the augmented levels of pro-inflammatory Th1 cytokines IL-12p40 and IL-23 and with low levels of the anti-inflammatory IL-10 (de Breij et al., 2012). It has been described that *Acinetobacter* infection (Breslow et al., 2011) as well as the rOmpA vaccine (Lin et al., 2013) led to an increase in IL-17; still, antibody depletion of IL-17 or the use of an IL-17a -/- mice, show that IL17a is dispensable in the immune response against *Acinetobacter* (Breslow et al., 2011). Nonetheless, these findings do not dismiss the possibility of other IL-17 family members that share homology with the IL-17a, such as the IL-17f, to be involved in the host response (Yan et al., 2016). Immunization with a *Acinetobacter* rough LPS induces a decreased level of cytokines, related to the polarization towards a Th17 profile such as IL-1β and IL-6 in the immunized mice (García-Quintanilla et al., 2014). Therefore, it has been proposed that these elevated cytokines induced by immunization could benefit the Th17 response (Yan et al., 2016).

Immunocompetent mice are resistant to *A. baumannii* when inoculated by the intranasal route or intravenously. In order to bypass this resistance, different immunosuppression models have been used (Knapp et al., 2006; Luo et al., 2012; Renckens et al., 2006), together with different infection protocols (Breslow et al., 2011). A typical experimental murine model consists of inducing a transient neutropenic state

prior to infection (Table 2). *A. baumannii* has been described as one of the most commonly isolated Gram-negative bacteria from neutropenic pyretic patients in nosocomial environments, providing an initial indication of the importance of PMNs against this bacteria (Karim et al., 1991; Wu et al., 2017; Yadegarynia et al., 2013). Still, only a small proportion of *Acinetobacter* human infections display neutropenia, and the reduction of these leukocytes constitute a comparatively minor risk factor (Chopra et al., 2014; Freire et al., 2016).

Even though the neutropenic murine model has been valuable, different mice strains display diverse resistance towards *A. baumannii*. For instance, A/J mice show high susceptibility linked to a reduced early influx of PMNs in the lung and a reduced local pro-inflammatory response (lower IL-1β, MIP-2, and TNF-α levels) (Qiu et al., 2009). In another scenario, the loss of a tumor suppressor protein called Fus1 in mice causes an increased resistance to *A. baumannii* linked with an augmented lung PMNs influx and upregulation of IL-17. However, in this case, PMNs seem particularly crucial since the PMN depletion, abrogated the enhanced antibacterial of the Fus1$^{-/-}$ mice (Hood et al., 2013).

Upon *A baumannii* infection, PMNs are rapidly recruited to the infection site (van Faassen et al., 2007; Zhao et al., 2011). The infection with *A. baumannii* leads to an increase in critical pro-inflammatory cytokines and chemokines such as IL-1β, IL-6, KC, MIP-1α, MIP-2, MCP-1, IL-17 and TNF-α accompanied by an increase in the IL-10 expression (Breslow et al., 2011; Renckens et al., 2006; van Faassen et al., 2007).

The presence of PMNs is particularly important in controlling this pathogen since augmentation of the PMN influx (either by MIP-2 or by c-di-GMP) leads to enhanced host resistance to *A. baumannii* (van Faassen et al., 2007; Zhao et al., 2011). The granules content from PMNs display bactericidal effects against *Acinetobacter* (Loeffelholz & Modrzakowski, 1988). In agreement, a delay in the influx of PMN was associated with higher susceptibility to *A. baumannii* (Qiu et al., 2009).

Much information regarding PMN function in *Acinetobacter* has been accessed by using the immunosuppression agent cyclophosphamide (Bruhn et al., 2015; Joly-Guillou et al., 1997; Manepalli et al., 2013; Thompson et al., 2014). This agent affects myelopoiesis, therefore, inducing neutropenia in addition to other deficiencies (Zuluaga et

al., 2006). Even though this is a valuable immunosuppression model it has many offset effects (Ghiringhelli et al., 2004; Yasunami & Bach, 1988), therefore as previously stated this might not be the optimal approach to study the specific PMN function.

Depending on the infection model, PMN depletion allows *Acinetobacter* to colonize wounded tissue, generate a pneumonia infection, or display a septic state (Breslow et al., 2011; Grguric-Smith et al., 2015; van Faassen et al., 2007). Furthermore, the depletion of PMNs converts the *Acinetobacter* self-limiting infection into a lethal one, causing an augmented bacterial replication with the respective dissemination throughout the host (Breslow et al., 2011; van Faassen et al., 2007).

RB6-8C5 PMN-depleted mice infected with *A. baumannii* display clinical severe signs such as cachexia and weight loss, consequently most of them succumb to infection after a few days. The anti-PMN treated mice (RB6-8C5) show a transient increase in the expression of IL-6, IL-10, KC, MIP-2, and MCP-1 (van Faassen et al., 2007).

Even though the importance of PMNs in the control of this pathogen is evident, still many innate effector mechanisms against *Acinetobacter* have only partially been characterized (Parra-Millán et al., 2018). For example, many intracellular trafficking factors remain poorly understood. Another example is the current role of NETs in response to *A. baumannii* is controversial and recent studies show conflicting data (Kamoshida et al., 2015; Konstantinidis et al., 2016; Lázaro-Díez et al., 2017).

Staphylococcus

Staphylococcus aureus is a Gram-positive, facultative anaerobic bacterium. Despite its commensal status, it causes significant morbidity and mortality throughout the world (Coates, Moran, & Horsburgh, 2014; Laupland et al., 2013; Lowy, 1998), mainly causing skin and soft tissue infections. Under certain conditions, *S. aureus* may cause life-threatening infections, such as sepsis, pneumonia, osteomyelitis, meningitis, and endocarditis (Coates et al., 2014). Due to the emergence of methicillin-resistant strains, the frequency of complications has increased (Coates et al., 2014).

S. aureus has been considered an extracellular pathogen; however, there is evidence that this bacterium can invade various cell types (Löffler et al., 2014; Sendi &

Proctor, 2009). This intracellular lifestyle enhances the ability to persist and provides shelter against antibacterial immune elements and antimicrobial treatments (Löffler et al., 2014; Sendi & Proctor, 2009). *S. aureus* is also able to manipulate the innate host immunity by modulating cell receptors (reviewed in (Askarian et al., 2018)) and through the action of different proteases (reviewed in (Pietrocola et al., 2017)).

S. aureus infection commonly occurs through the traumatic breach of the skin barriers providing access to the underlying tissue. This invasion leads to an influx of immune cells to the site of infection. Infection can also occur at sites without apparent breaches (e.g., folliculitis) and the clinical manifestations could progress to a disseminated infection (Kobayashi et al., 2015; Thomer et al., 2016).

Defective phagocyte function is a significant risk factor against *S. aureus*. An impaired generation of ROS (such as that displayed in chronic granulomatous disease) causes susceptibility toward *S. aureus* (reviewed in Buvelot et al., 2017). Similar findings were observed in mice with defective phagocyte NOX2 activity, which also became more susceptible to *S. aureus* (Buvelot et al., 2017; Pizzolla et al., 2012).

DCs kill some strains of *S. aureus*; however, depending on the virulence factors harbored by *S. aureus,* the bacterium may lead to an efficient intracellular survival inside these cells (O'Keeffe et al., 2015). Even though, phagocytes have a bactericidal potential against *S. aureus*, still the bacterium is capable of thriving in the intracellular milieu of Mϕ (Flannagan et al., 2016; Greenlee-Wacker & Nauseef, 2017; Kubica et al., 2008).

It has been shown that protective Th1 cells are expanded in both immunocompetent humans and mice after *S. aureus* infections (Brown et al., 2015). The generation of IFN-γ enhances the Mϕ killing against *S. aureus* (Greenlee-Wacker & Nauseef, 2017; Kubica et al., 2008). The deficiency of IFN-γ enhanced the susceptibility of mice to *S. aureus* (Lin et al., 2009). The host produces both IFN-γ and IL-17 in response to a systemic infection with *S. aureus* with involvement of Th1 and Th17 subsets (Schmaler et al., 2011). Th17 response is protective against *S. aureus* and its role in skin and respiratory is relevant (Cho et al., 2010; Ma et al., 2008; Minegishi et al., 2009). Th17 cytokines are involved in the production of anti-staphylococcal factors, including PMN-recruiting chemokines and antimicrobial peptides (Minegishi et al., 2009). Th17 cells are also necessary in the immunity conferred by protective vaccines such as that induced by IsdB (Joshi et al., 2012)

or by the fibrinogen-binding domain of clumping factor A (Narita et al., 2010). Despite this, an exacerbated Th17 response against *S. aureus* may be detrimental to the host (Greenberg et al., 2018). It may be that the Th17 response is relative to the other plausible protective elements such as Treg, Th2, and Th1 responses (Greenberg et al., 2018).

C57BL/6 mice are usually more resistant than BALB/c and DBA/2 in different *S. aureus* infection models such as in a keratitis (Hume et al., 2005), or an intravenous injection (von Köckritz-Blickwede et al., 2008) and the resistance has been partially associated with higher influx of PMNs (Nippe et al., 2011). However, in a subcutaneous murine infection model, C57BL/6 mice are more susceptible than BALB/c and DBA/2 mice. Despite this, no major differences in the bactericidal activity of PMNs toward *S. aureus* are observed among different mice strains, such as C57BL/6, BALB/c, and DBA/2 (Nippe et al., 2011; von Köckritz-Blickwede et al., 2008).

PMNs are rapidly recruited at the local infection site and are critical players in the immune response against *S. aureus* (Liese et al., 2013; Nippe et al., 2011). Phagocytosis by PMNs commonly leads to *S. aureus* elimination (van Kesse et al., 2014). However, *S. aureus* displays a broad range of evasive strategies against phagocytosis, preventing recognition, binding to antibodies as well as the escape from phagosomes (reviewed in (Guerra et al., 2017). Among relevant virulence factors are capsules (O'Riordan & Lee, 2004; Vuong et al., 2004) adhesion molecules (Mack et al., 1996; Vuong, Voyich, et al., 2004), molecules to evade PMN such as superantigen-like protein and inhibitory proteins (Bestebroer et al., 2006; Postma et al., 2004), as well as resistance to survival inside PMNs (Gresham et al., 2000). Certain virulent *Staphylococcus* strains can kill PMNs through the release of toxins such as alpha-phenol soluble modulins (Surewaard et al., 2013) and leucocidins (DuMont et al., 2013). Additionally, *S. aureus* can produce enzymes able to degrade and detoxify the PMN ROS activity (Karavolos et al., 2003; Liu et al., 2005). Under some conditions, PMNs may function as "Troyan horse" providing a protected site from many antibiotics and for dispersion of the bacterium to distant sites (Thwaites & Gant, 2011).

Despite all this, the consensus is that PMNs contribute to the host resistance, and they provide crucial microbicidal activity towards *S. aureus* (reviewed in (van Kesse et al., 2014; reviewed in (Spaan et al., 2013)). *S. aureus* can induce NETs formation (Pilsczek et

al., 2010). However, *S. aureus* may release DNases to dissolve and escape from NETs (Berends et al., 2010). *S. aureus* can degrade these traps by converting the NETs to deoxyadenosine leading to Mϕ cytotoxicity (Thammavongsa et al., 2013).

PMN depletion in mice through administration of 1A8 or RB6-8C5 antibodies have demonstrated that PMNs are necessary to control *Staphylococcus* infections (Alonzo et al., 2012; Barin et al., 2016; Boldock et al., 2018; Mölne et al., 2000; Robertson et al., 2008; Short et al., 2012; Verdrengh & Tarkowski, 1997; Wilde et al., 2015). This has been shown in *Staphylococcus* experimental infection ranging from brain (Kielian et al., 2001), skin (Mölne et al., 2000), arthritis (Verdrengh & Tarkowski, 1997) bone (Wilde et al., 2015), and respiratory infections (Robertson et al., 2008). Likewise, in another model in which PMNs influx was limited (CXCR2 knockout mice), host control of bacteria was also dampened (Kielian et al., 2001).

S. aureus infected PMN-depleted mice experience a substantial increase of TNF-α, IL-6, and IFN-γ (Verdrengh & Tarkowski, 1997) and pulmonary MCP-1 (Robertson et al., 2008) which relate with cachexia and mortality (Verdrengh & Tarkowski, 1997). PMN depletion promotes higher bacterial loads, even in non-traditional target organs such as lungs (Robertson et al., 2008) and kidneys (Verdrengh & Tarkowski, 1997). Further evidence highlights the importance of PMNs, since the absence of these cells restores the infectivity of attenuated *S. aureus* strains (Alonzo et al., 2012; Wilde et al., 2015). The PMN depleted mice experience lower levels of DNA depositions and of circulating free DNA (Luo et al., 2014). The PMN depletion is also associated with a series of changes in the lymph nodes such as a boost in the early humoral response with increase antibody production (mainly IgG and IgM), B cells and an increased vascularization (Kamenyeva et al., 2015).

Streptococcus

Streptococcus pneumoniae is a Gram-positive, extracellular bacteria that can survive in both aerobic and anaerobic environments (Brooks & Mias, 2018). This opportunistic pathogen colonizes the human upper respiratory tract, characterized by a "carriage" state which is usually asymptomatic. If the bacterium reaches the bloodstream or other sites, it may cause disease such as otitis, pneumonia, sepsis, and meningitis

(Weiser et al., 2018). Immunosuppression or co-infection with other pathogens may aggravate symptoms causing a life-threatening disease (Klugman et al., 2009).

S. pneumoniae is prominently spread through airborne droplets by carrier individuals. The rate of *S. pneumoniae* carriage varies, and infection depends on several risk factors. An estimate of the carrier state corresponds to 10% of adults and up to 20-65% of healthy children. Infancy is an important *S. pneumoniae* infection risk factor that decreases with age. Crowded areas such as childcare centers, favor transmission (Loughran et al., 2019), which is more frequent on drier and colder months, associated with an increase in airway secretions. *S. pneumoniae* infections potentiate by co-infection with viral infections of the upper respiratory tract (Weiser et al., 2018).

The colonization induces a rapid influx of PMNs followed by increased infiltration of Mϕs (Zhang et al., 2009) followed by sustained increased levels of Mϕs for at least a few weeks post-infection. The resident alveolar Mϕs are the most prominent innate effector cells during the initial stages of infection, displaying high bactericidal potential that circumvents the mucosal barrier (Dockrell & Brown, 2015). Mϕs phagocytose and kill these bacterium (Jonsson et al., 1985). The interaction between the Mϕs and *S. pneumoniae* has a considerable influence on the outcome of the disease and a significant factor determining the outcome of the infection in the lungs (Dockrell & Brown, 2015; Dockrell et al., 2003). Mϕs in other target organs such as the spleen, are important in controlling *S. pneumoniae* (Brown et al., 1981; Gerlini et al., 2014) and the absence of Mϕs may enhance septicemia progression (Gerlini et al., 2014). Mϕs are involved in the host antimicrobial defense against *S. pneumoniae* and modulate inflammation in the lungs, through the elimination of apoptotic PMNs (Knapp et al., 2003).

S. pneumoniae displays several virulence factors including the pore-forming pneumolysin toxin, the activity of the peroxide-producing enzyme coined pyruvate oxidase (SpxB), adhesins, pili and exoglycosidases (Subramanian et al., 2019). The capsule of *S. pneumoniae* is a major virulence determinant and is involved in the high genetic diversity among the different *S. pneumoniae* strains (Weiser et al., 2018). The capsule has been linked to the resistance against PMNs due to its ability to inhibit antibody and complement deposition, hence reducing the opsonophagocytosis (Gordon et al., 1986; Melin et al.,

2009). Pneumolysin modulates the inflammatory response, T cell immunity (Zhang et al., 2007) as well as the PMN function (Kadioglu et al., 2000; van Rossum et al., 2005). The administration of pneumolysin is associated with a dose-dependent influx of PMNs (Rijneveld et al., 2002). However, after a few days of disease progression, this pore-forming toxin pneumolysin is associated with a lower persistence of PMNs (Matthias et al., 2008). The decrease in PMN levels is linked to the cytotoxic effector mechanisms of pneumolysin towards these leukocytes (Matthias et al., 2008; Zysk et al., 2000). Pneumolysin in low doses displays several modulatory effects over PMNs, such as the stimulation of migration (Moreland & Bailey, 2006), and ROS activation (Martner et al., 2008). Recent work shows that pneumolysin induces differential cytokine expression among distinct leukocytes (Subramanian et al., 2019). Pneumolysin induces higher pro-inflammatory cytokine expression in PMNs and Mϕ (characterized by higher TNF-α, IL-1β, IL-12) in contrast to the inhibitory response in human DCs and murine alveolar Mϕs (Subramanian et al., 2019). DCs and Mϕs possess an inhibitory receptor for pneumolysin, which binding leads to the upregulation of the cytokine suppressor SOCS1 (Subramanian et al., 2019).

Th1 and Th17 have also been implicated in the immune response against *S. pneumoniae* (Pido-Lopez et al., 2011). Pneumococcal infections are associated with the augmented trafficking of Th1 cells (Kemp et al., 2002). Mice lacking the Th1 signature cytokine-IFN-γ are more susceptible to *S. pneumoniae* (Rubins & Pomeroy, 1997), being the IFN-γ necessary to avoid local dissemination of streptococci (Hyland et al., 2009). Experiments in mice indicate that Th17 cells and the IL-17a mediate immunity and the clearance of pneumococcal colonization (Lu et al., 2008; Zhang et al., 2009). In humans, the role of Th17 has only been partially characterized; however, there is evidence suggesting that Th17 is also involved in the host response against *S. pneumoniae.* The protein domain of pneumolysin can elicit a memory Th17 in the human nasopharynx-associated lymphoid tissue (Gray et al., 2014). Colonization studies using *S. pneumoniae* show that the carriage state is sufficient to increase the IL17a-secreting CD4+ T cells (Th-17 cells) not only in mice but also in humans (Wright et al., 2013). In order for *S. pneumoniae* to colonize the nasopharynx without inducing significant damage, this pathogen promotes the production of TGF-β and T-regulatory cells. This suppressive

response has been suggested indispensable for the establishment and maintenance of the nasopharyngeal carrier state (Neill et al., 2014).

Some mice strains have a higher susceptibly toward *S. pneumonia* than others, suggesting that the lower resistance strains display a weaker PMN response (Gingles et al., 2001; Partida-Sánchez et al., 2001). Furthermore, humans with inherited neutropenia experience a high incidence of respiratory infections (Rezaei et al., 2005). Moreover, *S. pneumoniae* displays abundant strategies to evade the killing activity of PMNs (reviewed in (Lewis & Surewaard, 2018)) and the complement attack (reviewed in (Andre et al., 2017). *S. pneumoniae* is resistant to the bactericidal activity of NETs (Beiter et al., 2006). Its capsule reduces the trapping of NETs (Wartha et al., 2007). Moreover, this bacterium has an endonuclease called EndA that allows *S. pneumoniae* to free itself from NETs through the degradation of the DNA molecules (Beiter et al., 2006).

The luminal PMNs are effective at promoting pneumococcal degradation and enabling the generation and release of pneumococcal-specific antigen (Matthias et al., 2008). Despite this, the antibodies generated during this period and the PMNs, failed to exert significant bactericidal pressure against *S. pneumoniae* in the upper respiratory tract. The acute inflammatory response and PMNs influx are inadequate in eliminating the carrier state, since *S. pneumoniae* can endure on the epithelial surface, even after the resolution of neutrophilic infiltrate (Kadioglu et al., 2008).

Despite the bactericidal role of PMNs in the early colonization against *Streptococcus,* many factors influence their contribution, such as bacterial strain, route of infection, the period of the infection, and co-infection with other pathogens. Higher bacterial loads administered by the intranasal route in a 1A8 antibody PMN depleted mice are cleared at similar rates than in the non-depleted mice after a one day (Hergott et al., 2015) or in a three-days period (with RB6-8C5 treated) (Matthias et al., 2008). Antibody RB6-8C5 PMN depleted *Streptococcus* infected mice displayed higher levels of IL-6 in the lungs than infected control mice (Marks et al., 2007).

Concluding remarks

The absence of a mature PMN cell line and the difficulties in preserving these cells, impose a limitation on the investigation on the function of PMNs. Still, there are some bacterial infections, in which the role of PMNs in protecting against disseminating sepsis is clear cut, and *ex vivo* studies correlate well with the predicted function of PMNs. For instance, patients with neutropenia or inherited defects in PMN function have a predisposition toward certain bacterial infections with rapid disease progression, and unusual presentation of the illness (Andrews & Sullivan, 2003; Aprikyan & Dale, 2001; Bodey, 2000; Lima et al., 2013). The bacterial species that invade neutropenic patients are usually not randomly distributed; instead they are represented frequently by opportunistic microorganisms such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, Klebsiella pneumoniae,* among others (Chen et al., 2010; Lima et al., 2013; Yadegarynia et al., 2013). In immunocompetent patients, these opportunistic infections frequently course with neutrophilia and the proficiency of PMNs in killing these bacteria *ex vivo*, correlates well with their role in controlling the infections *in vivo* (Cruz et al., 2017; De et al., 2014).

In other bacterial infections, the activity displayed *ex vivo* by PMNs, do not necessarily correlate or reveal their function *in vivo* (Georgilis et al., 1991; Kroon et al., 2018; Lowe et al., 2012; Sugawara et al., 2004; Xu et al., 2007). This discrepancy may be due in part to the restrictions of the *ex vivo* procedures, once PMNs have been isolated from blood or tissues. Some limitations relate to the short life span of PMNs (Summers et al., 2010), the tendency of the isolated PMNs to activate, undergo physiological changes and to suffer apoptosis (Calzetti et al., 2017; Casson et al., 2017; Lowe et al., 2018). Moreover, PMN purification methods vary greatly, displaying distinct duration periods and yielding different degrees of PMNs purity. For instance, contamination with other leukocytes may result in different PMN cytokine expression (Calzetti et al., 2017), hence attributing roles of PMNs that might plausibly be caused by other cell lines or to the interaction between cells.

In addition to the limitations already stated in the Introduction section, there are other considerations, regarding the murine neutropenic models. Depending on the bacterial infection, residual PMNs in different organs, such as spleen (Deniset et al., 2017), tumor tissue (Moses et al., 2016), lungs (Casson et al., 2017), liver (Ma et al., 2012), upper genital

tract (Frazer et al., 2011), gut mucosa (Spees et al., 2014) and BM (Mora-Cartín et al., 2019; Ribechini et al., 2009) may remain after depletion of PMNs with antibodies. Therefore, it is mandatory to evaluate the anti-PMN clone, the interval of the injections, and the dose of anti-PMN to be used. These considerations are necessary to keep the neutropenia stage, and to control the number of PMNs at different stages.

As stated before, there are differences in the efficiency of PMN depletion between the RB6-8C5 and 1A8 clones have been observed. For instance, the PMN spleen repopulation kinetics after 1A8 administration display a decrease of Ly6G^{hi} PMNs; in contrast, Ly6G^{int} PMNs remain unaltered (Deniset et al., 2017). Therefore, The Ly6G^{hi} marker has been suggested to be associated with the mobilized PMNs and the immobilized or resident PMNs with the Ly6G^{int} PMNs. This supports the notion of phenotypically different PMN populations in the red pulp of the spleen consisting of at least these two groups: the Ly6G^{int} which tend to be immature PMNs comprised of band cells and the Ly6G^{hi} composed of the mature PMNs (Deniset et al., 2017). Likewise, the 1A8 anti-PMN treatment in a murine cancer model shows an incomplete PMN depletion, counteracted by the persistence of intratumoral cells, enhanced extramedullary granulopoiesis, and accelerated reappearance of immature cells (Moses et al., 2016). In the nasal turbinates, the treatment with the 1A8 anti-PMN leads to an approximately 90% depletion or reduction of PMNs (Short et al., 2012). In the cecal mucosa, the treatment with 1A8 leads to a significantly reduced presence of PMNs. In a *Salmonella* infection model, this depletion method leads to an approximate 50% decrease in the influx of PMNs to the cecal mucosa (Spees et al., 2014). Also, the use of 1A8 significantly reduced the total numbers of PMN in the lungs either in uninfected mice or in a *Legionella* infection model (Casson et al., 2017); a more efficient depletion was observed with the RB6-8C5 antibody clone (Casson et al., 2017). Despite the greater ability of RB6-8C5 to keep the neutropenic stage, still, this antibody cannot achieve 100% depletion of PMNs in the BM (Mora-Cartín et al., 2019; Ribechini et al., 2009). This resilience to the PMN antibody-mediated depletion is at least partially linked to the antiapoptotic MCL-1 expression (Ribechini et al., 2009) and the continuous production of PMNs from precursor cells.

The use of anti-PMN antibodies in mice is accompanied by an experimental control group of mice that receives PBS, saline, or rat IgG (mock control). However, these control

conditions may also introduce a variable among the various research groups. For instance, in murine listeriosis, control mice may be injected PBS (Carr et al., 2011), saline (Edelson et al., 2011) or high quantities of rat IgG (Czuprynski et al.,1994a; Czuprynski et al., 1996; Rakhmilevich, 1995). Although at first glance, the IgG mocked mice seem the appropriate group control, under certain conditions administration of high amounts of immunoglobulins (Igs) may have pleiotropic effects over the immune system, as demonstrated in humans (Schwab & Nimmerjahn, 2013). In mice, the administration of high quantities of Igs has been shown to modify host response to bacterial pathogens such as to *Mycobacterium* and *Listeria* (Coade et al., 2005; Park-Min et al., 2007). Likewise, these high quantities of "normal" Igs can shape autoimmune responses in mice (Hirose et al., 2015). It has even been suggested that the passive administration of Igs diminishes the infiltration of PMN in the skin (Hirose et al., 2015). One last consideration regarding the murine neutropenic models concerns the functional, phenotypic and genetic variance between the murine and humans PMNs, such as different expression of cytokine and chemokine receptors, complement receptors, cytokine production, granule composition, opsonization requirements, life span and killing activities (Döring et al., 2015; Eruslanov et al., 2017; Mestas & Hughes, 2004; Mora-Cartín et al., 2016).

The murine neutropenic systems remain the most valuable models to evaluate the *in vivo* function of PMNs in bacterial infections. The antibody PMN depleting strategies have the advantage that they may be combined with different strains of mice, including knock out constructs. Neutropenic murine models in bacterial infections have shown a cross-talk between PMNs and other cells of the immune system (Barquero-Calvo et al., 2013; Casson et al., 2017; Kamenyeva et al., 2015; Mora-Cartín et al., 2019) a phenomenon that is mostly precluded in *ex vivo* studies. Regarding tissue injury and repair, neutropenic models expose PMNs as mayor players in the lung pathology (Harper et al., 2012; Yeremeev et al., 2015), as well as in the tissue damage caused by massive influx of PMN in some bacterial infections, such as in tuberculosis (Dallenga et al., 2018, 2017). This phenomenon correlates with an increased risk of mortality (Lowe et al., 2013). Indirect evidence of murine neutropenic models also shows that the abrogation of the immunopathological PMN response can diminish inflammation and improve the outcome of infection in some bacterial diseases (Nandi & Behar, 2011; Vilaplana et al., 2013).

Neutropenic murine models have also contributed in dissecting the role of PMNs in other diseases of different etiology. For instance, in leishmaniasis, it has been demonstrated that PMNs are key regulators of the anti-*Leishmania* immune response and that the presence of these leukocytes has important implications on the outcome of infection (Carlsen et al., 2015). In candidiasis, the PMN depletion enhances survival (Han & Cutler, 1997). The absence of PMNs diminishes liver dysfunction in sepsis models (Hewett et al., 1992; Molnar et al., 1997) and hampers the LPS induced hepatocyte necrosis (Sato et al., 1993). With the use of neutropenic mice, it has been demonstrated that the harmful effect of PB1-F2 influenza protein is due to an excessive inflammation mediated by an increased PMN mobilization (Vidy et al., 2016). Finally, antibody PMN depletion improves wound healing (Dovi et al., 2003).

As demonstrated here, the *in vivo* neutropenic models are powerful methods that complement the *ex vivo* investigations for determining the role of PMNs in various bacterial diseases, mainly when concentrating on their function during a specific phase of disease progression. However, they should be used with caution. The understanding of their advantages and limitations is essential for the adequate interpretation of the results.

CHAPTER 2

In this chapter, we explore the interaction of naïve mouse PMNs with *Brucella abortus* and show these cells fail to recognize this bacterium in the absence of antibodies.

The mouse has been the preferred animal model in brucellosis research to test and evaluate different hypotheses. In this murine model, *Brucella abortus* is found within macrophages (Mϕs) and dendritic cells (DCs) at early times of infection but seldom in neutrophils. Based on this observation, we describe in the first published manuscript the interaction of mouse neutrophils with *B. abortus*. In contrast to human, dog, and bovine PMNs, naive mouse PMNs fail to recognize smooth *B. abortus* bacteria at the early stages of infection. Murine normal serum components do not opsonize smooth *Brucella* strains, and PMN phagocytosis is achieved only after the appearance of antibodies. Alternatively, normal mouse serum is capable of opsonizing rough *Brucella* mutants. Despite this, PMNs still fail to kill *Brucella*, and the bacterium induces cell death of murine leukocytes. In addition, mouse serum does not opsonize *Yersinia enterocolitica* O:9, a bacterium displaying the same surface polysaccharide antigen as smooth *B. abortus*. Therefore, the lack of murine serum opsonization and absence of murine PMN recognition are specific, and the molecules responsible for the *Brucella* camouflage are N-formyl-perosamine surface homopolysaccharides. This is significant since the outcome of brucellosis in a given animal species may be determined during the initial stages of the infection that influence the downstream events of the immune response. Although the mouse is a valuable model for understanding the immunobiology of brucellosis, direct extrapolation from one animal system to another has to be undertaken with caution.

In a second published manuscript, we describe how PMNs modulate adaptive immunity in the initial stages of the acute murine brucellosis. Removal of polymorphonuclear PMNs at the onset of adaptive immunity against *Brucella abortus* favored bacterial elimination in mice. This was associated with higher levels of interferon-gamma (IFN-γ) and a higher proportion of cells expressing interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS), compatible with M1 Mϕs, in PMN-depleted *B. abortus*-infected (PMNd-*Br*) mice. At later times in the acute infection phase, the amounts of IFN-γ fell while IL-6, IL- 10, and IL-12 became the predominant cytokines in PMNd-

Br mice. IL-4, IL-1β, and tumor necrosis factor-alpha (TNF-α) remained at background levels at all times of the infection. The depletion of PMNs at the acute stages of infection promoted the premature resolution of spleen inflammation. The efficient removal of bacteria in the PMNd-*Br* mice was not due to an increase of antibodies since the immunoglobulin isotype responses to *Brucella* antigens were dampened. Anti-*Brucella* antibodies abrogated the production of IL-6, IL-10, and IL-12 but did not affect the levels of IFN-γ at later stages of infection in PMNd-*Br* mice. These results demonstrate that PMNs have an active role in modulating the course of *B. abortus* infection after the adaptive immune response has already developed.

Annex. 2.1

Mora-Cartín, R., Chacón-Díaz, C., Gutiérrez-Jiménez, C., Gurdián-Murillo, S., Lomonte, B., Chaves-Olarte, E., … Moreno, E. (2016). N-Formyl-Perosamine Surface Homopolysaccharides Hinder the Recognition of Brucella abortus by Mouse Neutrophils. *Infection and Immunity*, *84*(6), 1712–1721. https://doi.org/10.1128/IAI.00137-16

Annex. 2.2

Mora-Cartín, R., Gutierrez-Jimenez, C., Alfaro-Alarcón, A., Chaves-Olarte, E., Chacón-Díaz, C., Barquero-Calvo, E., & Moreno, E. (2019). Neutrophils dampen adaptive immunity in brucellosis. *Infection and Immunity*, IAI.00118-19. https://doi.org/10.1128/IAI.00118-19

CHAPTER 3

In this section, we reveal a novel microbial-host cross-talk through which *B. abortus* can hinder and evade host innate PMN response and suggest a mechanism by which *Brucella* may hamper the presence of infected PMNs in the target organs and promote neutropenia during chronic brucellosis.

Most bacterial infections induce the activation of PMNs, enhance their microbicidal function, and promote the survival of these leukocytes for protracted periods. *Brucella abortus* is a stealthy pathogen that evades innate immunity, barely activates PMNs, and resists the killing mechanisms of these phagocytes. Intriguing clinical signs observed during brucellosis are the low numbers of *Brucella* infected PMNs in the target organs and neutropenia in a proportion of the patients; features that deserve further attention. In an attempt to improve our understanding of the mechanisms underlying the fate of PMNs during brucellosis, in this published paper we have explored the outcome of these leukocytes upon interaction with *Brucella abortus*. Here we demonstrate that *B. abortus* prematurely kills human PMNs in a dose-dependent and cell-specific manner. Death of PMNs is concomitant with the intracellular *Brucella* lipopolysaccharide (*Br*-LPS) release within vacuoles. This molecule and its lipid A reproduce the premature cell death of PMNs, a phenomenon associated with the low production of proinflammatory cytokines. Blocking of CD14 but not TLR4 prevents the *Br*- LPS-induced cell death. The PMNs cell death departs from necrosis, NETosis and classical apoptosis. The mechanism of PMN cell death is linked to the activation of NADPH-oxidase and a modest but steady increase of ROS mediators. These effectors generate DNA damage, recruitments of checkpoint kinase 1, caspases 5 and to minor extent of caspase 4, RIP1 and Ca^{++} release. The production of IL-1β by PMNs was barely stimulated by *B. abortus* infection or *Br*-LPS treatment. Likewise, inhibition of caspase 1 did not hamper the *Br*- LPS induced PMN cell death, suggesting that the inflammasome pathway was not involved. Although activation of caspases 8 and 9 was observed, they did not seem to participate in the initial triggering mechanisms, since inhibition of these caspases scarcely blocked PMN cell death. These findings suggest a mechanism for neutropenia in chronic brucellosis and reveal a novel *Brucella*-host cross-talk through which *B. abortus* can hinder the innate function of PMN.

Annex. 3.1.

Barquero-Calvo, E., Mora-Cartín, R., Arce-Gorvel, V., de Diego, J. L., Chacón-Díaz, C., Chaves-Olarte, E., … Moreno, E. (2015). Brucella abortus Induces the Premature Death of Human Neutrophils through the Action of Its Lipopolysaccharide. *PLoS Pathogens*, *11*(5), e1004853. https://doi.org/10.1371/journal.ppat.1004853

CHAPTER 4

In this chapter, we initially describe the persistence of *Brucella abortus* in cells of the mice Bone Marrow (BM) and propose that this tissue is essential for establishing long-lasting chronic infections.

Brucella infections may persist for long periods causing relapses in antibiotic-treated patients. The ability of *Brucella* to develop chronic infections is linked to its capacity to invade and replicate within the mononuclear phagocyte system, including the BM. Persistence of *Brucella* in the BM has been associated with hematological complications such as neutropenia, thrombocytopenia, anemia, and pancytopenia in human patients. In this publish paper we demonstrate in the mouse model that the number of *Brucella abortus* in the BM remained constant for up to 168 days of post-infection. This persistence was associated with histopathological changes, accompanied by augmented numbers of BM myeloid GMP progenitors, PMNs, and CD4+ lymphocytes during the acute phase (eight days) of the infection in the BM. Monocytes, PMNs, and GMP cells were identified as the cells harboring *Brucella* in the BM. We propose that the BM is an essential niche for the bacterium to establish long-lasting infections and that infected PMNs may serve as vehicles for dispersion of *Brucella* organisms, following the Trojan horse hypothesis. Monocytes are strong candidates for *Brucella* reservoirs in the BM.

In the second published paper of this chapter, we showed a proof of concept for the "Trojan horse" proposal. Here we demonstrate that *Brucella*-infected PMNs are readily phagocyted by murine Mϕs in a non-phlogistic manner and that bacteria delivered through PMNs, extensively replicate inside Mϕs.

Brucella abortus 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 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. Hence, we propose that *B. abortus* infected PMNs serve as "Trojan horse" vehicles for the efficient dispersion and replication of the bacterium within the host.

Annex. 4.1.

Gutiérrez-Jiménez, C., Hysenaj, L., Alfaro-Alarcón, A., Mora-Cartín, R., Arce-Gorvel, V., Moreno, E., … Barquero-Calvo, E. (2018). Persistence of Brucella abortus in the Bone Marrow of Infected Mice. *Journal of Immunology Research*, *2018*, 5370414. https://doi.org/10.1155/2018/5370414

Annex. 4.2.

Gutiérrez-Jiménez, C., Mora-Cartín, R., Altamirano-Silva, P., Chacón-Díaz, C., Chaves-Olarte, E., Moreno, E., & Barquero-Calvo, E. (2019). Neutrophils as Trojan Horse Vehicles for Brucella abortus Macrophage Infection. *Frontiers in Immunology*, *10*, 1012. https://doi.org/10.3389/fimmu.2019.01012

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Supplementary Tables

Table 1. Differences between mouse and human PMNs

	Mouse	Human	Reference
EC_{50} * for primary granule release by fMLF	0.71 µM	0.01 µM	(Barrowman, et al., 1986)
fMLP receptor affinity	Low	High	(Gao & Murphy, 1993)
PMN maturity in bone marrow	More mature and live longer than peripheral blood PMNs	Less mature	(Boxio et al., 2004)
Defensins	Absent	Present	(Eisenhauer & Lehrer, 1992)
Number of PMN in blood	10-25%	50–70%	(Mestas & Hughes, 2004)
Chemokine receptors CXCR1 and CXCR2	Absent	Present	(Mestas & Hughes, 2004)
CXCL8, CXCL7, CXCL11, CCL13. CCL14, CCL15, CCL18, CCL23, CCL24/CCL26	Absent	Present	(Eruslanov et al., 2017)
TLR10	Absent	Present	(Mestas & Hughes, 2004)
CD33 myeloid differentiation protein	Present	Absent	(Mestas & Hughes, 2004)
Caspase 10	Absent	Present	(Mestas & Hughes, 2004) (Mestas & Hughes, 2004)
Proteinase 3 and elastase substrates	Differences in substrate affinity mouse PR3 and mouse NE **	Differences in substrate affinity Human PR3 and human NE	(Wiesner et al., 2005_
Activation of ROS	Does not require phosphatidylinositol 3-	Requires phosphatidylinositol 3-	(Bagaitkar, et al., 2012)

	phosphate binding to p40phox for regulation of NADPH oxidase activity	phosphate binding to p40phox for regulation of NADPH oxidase activity	
L-selectin binding to E-selectin	Absent	Present	(Zöllner et al., 1997)
Expression of IL-6, IL-17A, IL-17F and IFN-γ, IL-10	High	Absent or low	(Mantovani et al., 2011)
Serine proteases	Different substrates	Different substrates	(Kalupov et al., 2009)
Morphology	Ring-like	Segmented	(Biermann et al., 1999)
Expression of Ly6G And Gr-1	Present	Absent	(Hickey, 2012)
Expression of bactericidal/permeability-increasing protein, myeloperoxidase, β-Glucuronidase, Lysozyme, Alkaline phosphatase, and Arginase-1	lower	Higher	(Rausch & Moore, 1975)
Intracellular localization of Arginase-1	Cytosolic	Azurophil Granules	(Munder et al., 2005)
Arginase 1 expression	Displayed upon stimulation	Constitutively expressed	(Munder et al., 2005)
FcαRI	Absent	Present	(Reljic, 2006)
FcγRI	Absent	Inducible expression	(Bruhns, 2012)
FcγRIIB	Present (inhibitory)	Very low	
FcγRIIIA	Present	Absent	

FcγRIIA	Absent	Present	(Van de Velde et al., 2010)
FcγRIIIB	Absent	Present	(Marois et al., 2011)
FcγRIII	Present	Absent	(Jönsson et al., 2012)
FcγRIV	Present	Absent	

*EC50. Half maximal effective concentration refers to the concentration of N-formyl methionyl peptide (fMLF) which induces a response halfway between the baseline and maximum after a specified exposure time.

Table 2. Antibody induced neutropenia in different bacterial mice models

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
RB6-8C5	*Acinetobacter baumannii*	$1x10_6$ - $4.3x10_6$	4502	Intraperitoneal	C3HeB/FeJ	N/A	Increased	25	(Breslow et al., 2011)
RB6-8C5	*Acinetobacter baumannii*	$1x10_7$	ATCC 17961	Intranasal	C57BL/6 BALB/c	Increased	Increased	25	(van Faassen et al., 2007)
1A8	*Acinetobacter baumannii*	$1x10_7$	0057, 1422, 1611, 2098, 2231, 3559, and 7405[i]	Directly in the wound	BALB/c	Increased	N/A	500	(Grguric-Smith et al., 2015)
RB6-8C5	*Acinetobacter baumannii*	$1x10_7$	ATCC 17961	Intranasal	C57BL/6	Increased	N/A	25	(Zhao et al., 2011)
RB6-8C5	*Acinetobacter baumannii*	$3x10_7$-$5\times10_8$	A112-II-a[i]	Intranasal	C57BL/6	Increased	Increased	250	(Tsuchiya et al., 2012)
RB6-8C5	*Acinetobacter baumannii*	$3.0\times10_7$	N/A	Intranasal	129sv Fus1-/-	Increased	N/A	250	(Hood et al., 2013)
RB6-8C5	*Borrelia burgdorferi*	$2.5x10_3$ - $2.5x10_6$	N40	Inoculation in both hind footpads	DBA/2J[ii] C3H/HeJ[iii]	Increased	N/A	200	(Brown et al., 2004)
1A8	*Borrelia burgdorferi*	$1x10_5$	B31 A3 Δp66	Subcutaneous [iv]	C3H/HeN	No difference	N/A	500	(Curtis et al., 2018)
1A8	*Borrelia burgdorferi*	$1x10_5$	B31-A3	Intradermal	C3H/HeN SCID	No difference	N/A	250	(Carrasco et al., 2015)
RB6-8C5	*Brucella abortus*	$1x10_6$	2308	Intraperitoneal	C57BL/6	Increased[v]	N/A	100	(Barquero-Calvo et al., 2013)
RB6-8C5 1A8	*Brucella abortus*	$1x10_6$	2308	Intraperitoneal	C57BL/6	Increased[vi] Reduced[vii]	N/A	100	(Mora-Cartín et al., 2019)
RB6-8C5	*Ochrobactrum anthropi*	$1x10_9$	LMG 3331	Intraperitoneal	BALB/c	N/A	Increased	100	(Barquero-Calvo et al., 2009)
RB6-8C5	*Brucella abortus*	$1x10_6$	2308	Intraperitoneal	BALB/c	No difference	N/A	100	(Barquero-Calvo et al., 2007)
RB6-8C5	*Salmonella enterica*	$1x10_5$	SL1344	Intraperitoneal	BALB/c	Increased	N/A	100	(Barquero-Calvo et al., 2007)
RB6-8C5	*Chlamydia trachomatis*	$5x10_6$	Nigg II	Intravaginal	BALB/c	Increased	N/A	200	(Barteneva et al., 1996)
1A8	*Chlamydia muridarum*	$1x10_5$	Nigg	Intravaginal	C3H/HeOuJ	No difference	N/A	300	(Frazer et al., 2011)
1A8	*Chlamydia trachomatis*	$1x10_7$	RST17 CPAF[viii]- mutant	Transcervical	C57BL/6	Increased	N/A	500	(Rajeeve et al., 2018)
RB6-8C5	*Chlamydia muridarum*	$5x10_4$	Weiss strain	Intravaginal	C57BL/6	Increased	N/A	500	(Naglak et al., 2017)

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
RB6-8C5	*Chlamydia abortus*	$1x10_6$	AB7	Intraperitoneal	Swiss OF1	Increased	Increased	500	(Buendía et al., 1999)
RB6-8C5	*Chlamydia abortus*	$1x10_6$	AB7	Intraperitoneal	Swiss OF1	N/A	N/A	500	(Montes de Oca et al., 2000)
RB6-8C5	*Francisella tularensis*	$1x10_2$ - $4x10_4$	LVS (ATCC 29684)	Intradermal	B6D2F1[ix]	Increased	Increased	250	(Sjöstedt et al., 1994)
RB6-8C5	*Francisella tularensis*	$5x10_3$ - $1x10_6$	LVS (ATCC 29684)	Intravenous	B6D2F1[ix]	Increased	Increased	N/A	(Sjöstedt et al., 1994)
RB6-8C5	*Francisella tularensis*	3 $x10_1$ - 3.18 $x10_3$	SCHU S4	Intranasal	C57BL/6	No difference	No difference	25	(KuoLee et al., 2011)
RB6-8C5 1A8	*Francisella tularensis*	$5x10_2$	LVS	Intranasal	BALB/c	N/A	Increased	100 500 [x]	(Steiner et al., 2017)
RB6-8C5	*Francisella tularensis*	$9.2x10_2$ - $1x10_4$	LVS (ATCC 29684)	Intradermal or aerosol	BALB/c	Increased	N/A	500	(Conlan et al., 2002)
RB6-8C5	*Francisella. tularensis*	$1x10_4$	LVS	Intranasal	BALB/c	N/A	Increased	N/A	(Kirimanjeswara et al., 2007)
RB6-8C5	*Legionella pneumophila*	$1.4x10_4$ - $9x10_7$	Suzuki strain (serogroup-1) [i]	Intratracheal	A/J	Increased	Increased	100	(Tateda et al., 2001)
RB6-8C5 1A8	*Legionella pneumophila*	$1x10_6$	JR32-derived	Intranasal	C57BL/6J	Increased	N/A	250	(Casson et al., 2017)
1A8	*Listeria monocytogenes*	$1x10_4$	EGD	Intravenous	129S6/SvEv 129S6/SvEv Batf3-/-	Increased	N/A	500	(Edelson et al., 2011)
RB6-8C5	*Listeria monocytogenes*	$2.5x10_4$	Fluorescent RFP [xi]*	Intravenous	LysM-EGFP	Increased[xii]	N/A	125	(Waite et al., 2011)
RB6-8C5	*Listeria monocytogenes*	$3x10_3$	N/A	Intraperitoneal	C.B-17 C.B-17 SCID	Increased	Increased	200	(Rogers & Unanue, 1993)
RB6-8C5	*Listeria monocytogenes*	$1x10_2$ - $1x10_7$	N/A	Intravenous	N/A	Increased	Increased	150 -200	(Czuprynski et al., 1994a)
RB6-8C5	*Listeria monocytogenes*	$1x10_1$ - $1x10_5$	EGD	Intravenous	BDF1	Increased	Increased	150	(Czuprynski et al., 1994b)
RB6-8C5	*Listeria monocytogenes*	$3x10_3$ - $1x10_6$	EGD	Intravenous	BALB/c	Increased	Increased	100	(Appelberg, et al., 1994)
RB6-8C5	*Listeria monocytogenes*	$1x10_4$ - $5x10_6$	EGD	Intravenous or Intraperitoneal	CB6/F1	Increased	N/A	250	(Conlan & North, 1994)

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
RB6-8C5	*Listeria monocytogenes*	2 x 10^3 - 5 x10^5	EGD	Intravenous	CB6F1	Increased	Increased	300	(Rakhmilevich, 1995)
RB6-8C5	*Listeria monocytogenes*	1x10^4 - 3.5 x10^9	N/A	Intragastric	CD1	Increased	Increased	150-250	(Czuprynski et al., 1996)
RB6-8C5 1A8	*Listeria monocytogenes*	1x10^3 - 1x10^6	10403S	Intravenous	C57BL/6	Increased	Increased	200 500[xiii]	(Carr et al., 2011)
RB6-8C5 1A8	*Listeria monocytogenes*	3x10^3 - 5x10^5	10403S	Intravenous	C57BL/6	No differences [xiv] Increased [xv]	No differences [xiv] Increased [xv]	250	(Shi et al., 2011)
RB6-8C5 1A8	*Listeria monocytogenes*	1x10^4 - 1x10^5	10403S	Intravenous	C57BL/6J FtD KO[xvi]	Increased[xvii]	Increased	100 200[xviii]	(Agbayani et al., 2018)
RB6-8C5	*Listeria monocytogenes*	5x10^2	10403S	Intravenous	CB6/F1	Increased	Increased	250	(Conlan, 1997)
RB6-8C5	*Salmonella enterica*	1x10^3	C5R	Intravenous	CB6/F1	Increased	Increased	250	(Conlan, 1997)
RB6-8C5	*Yersinia enterocolitica*	5x10^4	WA	Intravenous	CB6/F1	Increased	Increased	250	(Conlan, 1997)
RB6-8C5	*Listeria monocytogenes*	1x10^6	DP-L4056	Intraperitoneal	B7-H4 KO[xix]	Increased	N/A	150	(Zhu et al., 2009)
NimpR14	*Listeria monocytogenes*	1x10^3	10403S	Intravenous	C57BL/6	Increased	N/A	100	(Navarini et al., 2009)
RB6-8C5	*Mycobacterium tuberculosis*	1x10^6	H37Rv	Intratracheal	BALB/c	Increased	N/A	200	(Barrios-Payán et al., 2006)
RB6-8C5	*Mycobacterium avium*	1x10^6	2447	Intravenous	C57BL/6	Increased	N/A	N/A	(Appelberg et al., 1995)
RB6-8C5	*Mycobacterium avium*	1x10^7	101	Intravenous	C57BL/6	Increased	N/A	5 mg/kg	(Petrofsky & Bermudez, 1999)
RB6-8C5	*Mycobacterium bovis*	0.5x10^4 - 1x10^4	BCG	Intratracheal	C57BL/6	Increased	N/A	500	(Fulton et al., 2002)
1A8	*Mycobacterium tuberculosis*	1x10^2	H37Rv	Aerosol	C57BL/6	No difference (Lung)	N/A	300	(Blomgran & Ernst, 2011)
RB6-8C5 1A8	*Mycobacterium tuberculosis*	3x10^2	Erdman	Aerosol	C57BL/6	N/A	Increased	200	(Braverman & Stanley, 2017)
RB6-8C5	*Mycobacterium tuberculosis*	1x10^3	H37Rv	Aerosol	C57BL/6 BALB/c [xx] or CBA/J DBA/2[xxi]	N/A	Reduced [xxii] No change[xxiii]	100	(Keller et al., 2006)
RB6-8C5	*Mycobacterium tuberculosis*	4x10^2	H37Rv	Aerosol	Card9-/-	No difference	Reduced	200	(Dorhoi et al., 2010)

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
1A8	*Mycobacterium tuberculosis*	1-2x10_2	Erdman	Aerosol	IFN-γR1-/-	No difference[xxiv]	Reduced	200	(Nandi & Behar, 2011)
1A8	*Mycobacterium tuberculosis*	2x10_2	H37Rv	Aerosol	C57BL/6 Nos2-/- [xxv] C3HeB/FeJ [xxvi]	No difference[xxvii] Reduced[xxviii]	N/A	200	(Mishra et al., 2017)
RB6-8C5	*Mycobacterium tuberculosis*	1x10_5	Erdman	Intraperitoneal	BALB/c	Increased	N/A	200	(Pedrosa et al., 2000)
RB6-8C5	*Mycobacterium tuberculosis*	1x10_2	H37Rv	Aerosol	T deficient RAG-/-	Increased	N/A	500	(Feng et al., 2006)
NIMP-R14 1A8	*Mycobacterium tuberculosis* or *Mycobacterium bovis*	1x10_2 - 8x10_6	BCG H37Rv	Intranasal or Aerosol	C57BL/6 or IL10 -/- and BALB/c	No difference [xxix] Increased[xxx]	N/A	200	(Zhang et al., 2009)
Re	*Mycobacterium tuberculosis H37Rv.*	1-2x10_2	H37Rv	Aerosol	I/StSnEgYCit(I/St) C57BL/6JCit (B6)	Reduced [xxxi] No difference [xxxii]	Reduced [xxxi] No difference xxxii	150	(Yeremeev et al., 2015)
RB6-8C5	*Salmonella* Dublin *S. enterica*	5x10_3 - 9x10_3	LD842[i] 14028	Intravenous	BALB/c[xxxiii] Congenic BALB/c [xxxiv]	Increased	Increased	150 and 300	(Vassiloyan akopoulos et al., 1998)
RB6-8C5	*Salmonella enterica*	5x10_7 - 1x10_{11}	NCTC 12023 or P10H1 (purD::mTn5, Kan^r) [xxxv]	Oral	C57BL/6	Increased	N/A	300	(Cheminay et al., 2004)
RB6-8C5	*Salmonella enterica*	1x10_3	SL1344	Intravenous	C57BL/6	Increased	Increased	100	(Seiler et al., 2000)
RB6-8C5	*Listeria monocytogenes*	1x10_3	EGD Sv 1/2a	Intravenous	C57BL/6	Increased	Increased	100	(Seiler et al., 2000)
RB6-8C5	*Mycobacterium tuberculosis* *Mycobacterium bovis* BCG *Mycobacterium fortuitum*	1x10_6	Erdman CDC 1551 BCG fortuitum	Intravenous	C57BL/6	No difference[xxxvi] Increased[xxxvii]	N/A	100	(Seiler et al., 2000)

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
1A8	*Salmonella enterica*	$1x10^9$ - $1x10^{10}$	IR715	Intragastrical	C57BL/6	Increased	N/A	500	(Spees et al., 2014)
1A8	*Salmonella enterica*	1×10^9	IR715 (WT) And sitA mntH zupT sodA[xxxviii]	Intragastrical	C57BL/6	Increased	N/A	500	(Diaz-Ochoa et al., 2016)
1A8 Anti-G-CSF (clone 67604)	*Salmonella enterica*	$5x10^7$	SL1344 (SB300, SmR)	Intragastrical	C57BL/ 6Ptprc	No difference	N/A	0.4mg/kg[xxxix] 6mg/kg[xl]	(Müller et al., 2016)
NIMP-R14	*Salmonella enterica*	$5x10^7$	SB300[xli]	Oral	129 Sv/Ev	Increased	N/A	100	(Maier et al., 2014)
1A8	*Salmonella enterica*	$1x10^6$	SL1344	Intraperitoneal	C57BL/6	Increased	N/A	100	(Chen et al., 2014)
RB6-8C5	*Salmonella enterica*	$1x10^7$	LMG3264	Intraperitoneal	C3H/HeN SPRET/Ei [xlii]	Increased	Increased	300	(Dejager et al., 2010)
1A8	*Salmonella enterica*	$1x10^5$	N/A	Orogastric	BALB/c or C57BL/6	Increased	Increased	500	(Franchi et al., 2012)
RB6-8C5	*Salmonella enterica*	$1x10^4$ - $5x10^8$	C5R	Intravenous or Intragastric	CB6/F1	Increased	N/A	500	(Conlan, 1996)
RB6-8C5	*Salmonella enterica*	$1x10^6$	LT2 strain (ATCC 15277)	Intraperitoneal	BALB/c	N/A	Increased	600	(Lehner et al., 2001)
RB6-8C5	*Salmonella enterica*	0.5×10^6	12023[xliii] (ATCC 14028)	Oral	C57BL/6J	N/A	Increased	100	(Ordoñez-Rueda et al., 2012)
1A8	*Staphylococcus aureus*	$1x10^8$	Newman or DlukED	Intravenous[xliv]	ND4 Swiss Webster	N/A	Increased	300	(Alonzo et al., 2012)
1A8	*Staphylococcus aureus*	$1x10^6$	USA300 strain LAC (AH1263) Δ*srrA* mutant	Intraosseous	C57BL/6J	Increased	N/A	N/A	(Wilde et al., 2015)
1A8	*Staphylococcus aureus*	N/A	Isolated Strains [xlv]	Oral	IFN-γR1-/-[xlvi]	Increased[xlvii]	N/A	500	(Barin et al., 2016)
RB6-8C5	Sepsis	N/A	N/A	Cecal ligation and puncture	C57BL6	Increased[xlviii] Reduced[xlix]	Increased [xlviii] Reduced [xlix]	25	(Hoesel et al., 2005)
1A8	Sepsis	N/A	N/A	Cecal ligation and puncture	C57BL/6	N/A	N/A	2 mg/kg	(Luo et al., 2014)
Polyclonal Anti-mouse PMN	*Staphylococcus aureus*	$2\ x10^7$	313	Intratracheal	FVB/N	Increased	Increased	1ml	(Robertson et al., 2008)

Antibody maB	Pathogen	Bacterial Dose (CFU or IFU)	Bacterial Strain/isolate	Bacterial Route of Infection	Mice strain	Change in bacterial load	Mortality in depleted mice	Antibody Concentration (µg)/dose or (mg/kg)	Reference
RB6-8C5	*Staphylococcus aureus*	$1.5x10^6$ - $3x10^7$	LS-1	Intravenous	BALB/c	Increased	Increased	1000	(Verdrengh & Tarkowski, 1997)
RB6-8C5	*Staphylococcus aureus*	$1x10^7$ - $1x10^8$	LS-1	Intracutaneous	BALB/c	Increased	N/A	1000	(Mölne et al., 2000)
RB6-8C5	*Staphylococcus aureus*	$1x10^9$	RN6390	Intracerebral	AKR/J	Increased	N/A	100	(Kielian et al., 2001)
RB6-8C5	*Streptococcus pneumoniae*	$1x10^6$	Mixed infection with TIGR4, D39, DP1004, G54	Intravenous	BALB/c	Increased[l]	N/A	150	(Gerlini et al., 2014)
1A8	*Streptococcus pneumoniae*[i]	$2×10^3$	EF3030 strain (type 19F)	Intranasal	C57BL/6	Increased	N/A	300	(Short et al., 2012)
1A8	*Streptococcus pneumoniae*	$1x10^4$	D39-GFP strain	Intravenous	C57BL/6	Increased	N/A	500 200 [ii]	(Deniset et al., 2017)
RB6-8C5	*Streptococcus pneumoniae*	$1x10^7$	TIGR4 cps[iii]	Intranasal	C57BL/6	No change	N/A	150	(Nelson et al., 2007)
RB6-8C5	*Streptococcus pneumoniae*	$1x10^7$	P1121	Intranasal	BALB/c	No difference	Increased	100	(Matthias et al., 2008)
RB6-8C5	*Streptococcus pneumoniae*	$5x10^2$ - $5x10^3$	Type 8 [iv] (ATCC 6308)	Intranasal Intraperitoneal	BALB/c	Reduced	Reduced	25	(Marks et al., 2007)
1A8	*Streptococcus pneumoniae*	$1x10^7$	P1121[i] (Δpce)[lv]	Intranasal	C57BL/6	No difference[lvi] Increased[lvii]	N/A	250 [lviii]	(Hergott et al., 2015)

[i]Strain corresponding to a clinical isolate. [ii]Arthritis-resistant mice. [iii]Arthritis-susceptible mice. [iv]Injected subcutaneously between the scapulae. [v]Bacterial load is increased at 5 days post infection and at later time points no statistical difference is observed. [vi]An initial bacterial load increase is shown at 1-day post depletion with either RB6-8C5 and 1a8 ab. [vii]A decrease in bacterial load in bone barrow at latter stages of the infection when depleted with RB6-8C5 and 1A8 ab. [viii]CPAF=secreted Chlamydial protease-like activating factor. [ix]B6D2F1 mice= C57BL/6 x DBA/2. [x]Administered doses of RB6-8C5 and 1A8 respectively. [xi]*L. monocytogenes* strains were constructed in the DP-L4056 strain background. [xii]Bacterial growth increased, but remained in foci. [xiii]Doses of RB6-8C5 and 1A8 respectively. [xiv]No differences with 1A8. [xv]Increased with RB6-8C5. [xvi]Mice lacking the expression of fucosyltransferase-IV and -VII (Fucosyltransferase-IV and -VII double knockout). These mice exhibit deficient functionality of selectin-ligand interactions. [xvii]Except in 1A8 treated FtD KO mice. [xviii]Doses of RB6-8C5 and 1A8 respectively. [xix]B7-H4 is an immunoglobulin superfamily molecule, and it is inhibitory for T response. [xx]Higher resistant mice strains to *M. tuberculosis*. [xxi]Higher susceptible strains to *M. tuberculosis*. [xxii]Decreased only in susceptible strains. [xxiii]No change observed in resistant strains. [xxiv]Slight decrease without reaching statistical difference. [xxv]B6.129P2-Nos2tm1Lau/J. [xxvi]B6.SJL-Ptprca Pepcb/BoyJ. [xxvii]Slight decrease however no statistical difference in lungs of C57BL/6 mice (WT). [xxviii]Decrease in the lungs of 1A8 treated Nos2 Ko and in C3HeB/FeJ mice. [xxix]No change in the acute model. [xxx]Increased in chronic infection model. [xxxi]Reduced in I/St mice. [xxxii]No difference in B6 mice. [xxxiii]BALB/susceptible to *Salmonella* contains a point mutation in the macrophage-expressed gene Nramp1. [xxxiv]Resistant to *Salmonella* containing the wild-type Nramp1 gene that makes them resistant to *Salmonella.* [xxxv]*Salmonella* Pathogenicity Island 2 deficient mutant. [xxxvi]No difference with the use of Erdman, 1551, BCG. [xxxvii]Increased with M. *fortuitum*. [xxxviii]Both of these strains are Manganese transporter deficient strains. [xxxix]Concentration of Anti-G-CSF ab. [xl]Concentration of 1A8. [xli]Corresponds to a clone of *S.* Tm SL1344. [xlii]Corresponds to an inbred mouse

strain derived from *Mus spretus*, it is resistant to infection with *Salmonella* enterica. [xliii]sifA−: an attenuated strain. [xliv]Dose administered by the retro-orbital venous plexus. [xlv]These isolated strains used were Methicillin-susceptible and negative for mecA/C, USA300, luk-PV genes. [xlvi]These mice have BALB/c background. [xlvii]This value is increased without reaching statistical significance. [xlviii]Increased in the early depletion protocol. [xlix]Reduced in the delayed depletion protocol. [l]Observed in G54 strain. [li]Co-infection model with Influenza A virus. [lii]In acute infection and long-term infection respectively. [liii]Unencapsulated mutant in which a cps operon was deleted and made from a type 4 clinical isolate. [liv]Pneumolysin-deficient serotype 8 strain. [lv]Mutant lacking ChoP esterase. They exhibit a survival defect during acute infection of the airway. [lvi]No difference in *S. pneumoniae* strain P1121(WT) infected mice. [lvii]Δpce infected mice/attenuated strain.

PUBLISHED MANUSCRIPTS

Annex

Numeration of published manuscripts is according to chapters and not in chronological order of publication.

Annex 2

Annex. 2.1

Mora-Cartín, R., Chacón-Díaz, C., Gutiérrez-Jiménez, C., Gurdián-Murillo, S., Lomonte, B., Chaves-Olarte, E., … Moreno, E. (2016). N-Formyl-Perosamine Surface Homopolysaccharides Hinder the Recognition of *Brucella abortus* by Mouse Neutrophils. *Infection and Immunity*, *84*(6), 1712–1721. https://doi.org/10.1128/IAI.00137-16

Annex. 2.2

Mora-Cartín, R., Gutierrez-Jimenez, C., Alfaro-Alarcón, A., Chaves-Olarte, E., Chacón-Díaz, C., Barquero-Calvo, E., & Moreno, E. (2019). Neutrophils dampen adaptive immunity in brucellosis. *Infection and Immunity*, IAI.00118-19. https://doi.org/10.1128/IAI.00118-19**.**

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N-Formyl-Perosamine Surface Homopolysaccharides Hinder the Recognition of *Brucella abortus* by Mouse Neutrophils

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***Brucella abortus* is an intracellular pathogen of monocytes, macrophages, dendritic cells, and placental trophoblasts. This bacterium causes a chronic disease in bovines and in humans. In these hosts, the bacterium also invades neutrophils; however, it fails to replicate and just resists the killing action of these leukocytes without inducing significant activation or neutrophilia. Moreover, *B. abortus* causes the premature cell death of human neutrophils. In the murine model, the bacterium is found within macrophages and dendritic cells at early times of infection but seldom in neutrophils. Based on this observation, we explored the interaction of mouse neutrophils with *B. abortus*. In contrast to human, dog, and bovine neutrophils, naive mouse neutrophils fail to recognize smooth *B. abortus* bacteria at early stages of infection. Murine normal serum components do not opsonize smooth *Brucella* strains, and neutrophil phagocytosis is achieved only after the appearance of antibodies. Alternatively, mouse normal serum is capable of opsonizing rough *Brucella* mutants. Despite this, neutrophils still fail to kill *Brucella*, and the bacterium induces cell death of murine leukocytes. In addition, mouse serum does not opsonize *Yersinia enterocolitica* O:9, a bacterium displaying the same surface polysaccharide antigen as smooth *B. abortus*. Therefore, the lack of murine serum opsonization and absence of murine neutrophil recognition are specific, and the molecules responsible for the *Brucella* camouflage are *N*-formyl-perosamine surface homopolysaccharides. Although the mouse is a valuable model for understanding the immunobiology of brucellosis, direct extrapolation from one animal system to another has to be undertaken with caution.**

Polymorphonuclear neutrophil leukocytes (PMNs) are the first line of defense of the innate immune system. These cells detect microbial structures through various receptors, and the recognition of these structures influences their activation and fate, which are essential to promote inflammatory responses and host defense mechanisms.

Brucellosis is a chronic disease of domestic and wildlife mammals and a worldwide human zoonosis caused by *Brucella* species (1). Members of this genus are intracellular pathogens that invade monocytes (Mo), macrophages (Mϕ), and dendritic cells (DCs), as well as placental trophoblasts (1). Although in the natural host and in humans *Brucella* also invades PMNs (2–4), the bacterium fails to replicate in these cells and just resists their killing action without inducing significant activation (3–6). Indeed, *Brucella*-infected PMNs do not degranulate and induce low levels of reactive oxygen species (ROS) and cytokines, and the infection follows its course without significant neutrophilia (5, 7, 8). Likewise, the PMNs infiltration of the cervical lymph nodes after oral infection is very low, even in well-developed granulomas after 15 days of infection (9). Moreover, after 5 days of infection, the bacterium is found within Mϕ and DCs of mice but seldom inside PMNs in the target organs (10). In addition, the absence of PMNs during brucellosis promotes the activation of the Th1 adaptive immune response (11). More significantly, *Brucella* is capable of inducing the premature cell death of human PMNs by means of its lipopolysaccharide (*Br*-LPS) through a mechanism that involves CD14 and mild NADPH oxidase activation (6). This has led to the proposal that *Brucella*-infected PMNs function as "Trojan horses" after nonphlogistic phagocytosis by Mϕ and DCs. This would favor the spread of bacteria to different organs, fostering the chronicity of the disease (6).

The mouse has been the preferred animal model in brucellosis research to test and evaluate different hypotheses (12, 13). Here, we have explored the interaction of naive mouse PMNs with *Brucella abortus* and found that these cells fail to recognize this bacterium in the absence of antibodies. This is significant since the outcome of brucellosis in a given animal species may be determined during the initial stages of the infection that influence the downstream events of the immune response.

MATERIALS AND METHODS

Ethics. Experimentation in mice was conducted with the consent of and according to 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 7451 on Animal Welfare). Mice were accommodated in the animal building at the Veterinary Medicine School of the National University, Costa Rica. All animals were kept in cages with food and water *ad libitum* under biosafety containment conditions, previous to and during the experiment. Blood from roaming dogs kept at the shelter of the Hospital of the Veterinary Medicine School of the National

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University, Costa Rica, was taken for routine diagnostic purposes, according to the respective consent and approval (SIA 0434-14) and the guidelines established by the Ley de Bienestar de los Animales of Costa Rica (Law 7451 on Animal Welfare).

Human blood samples were collected from volunteer donors at the Tropical Disease Research Program (PIET), of the National University, Costa Rica, according to the corresponding institutional approval (SIA 0248-13). Accordingly, volunteers were carefully informed regarding the study and provided written consent. Samples were taken following the procedures dictated by the Costa Rican National Health system (Ley 9234, Costa Rica, La Gaceta 79, 2014) and by the World Medical Association (WMA) Declaration of Helsinki (59th WMA General Assembly, Seoul, October 2008), regarding the use of blood samples.

Experimental animals. Wild-type *Mus musculus* animals were captured from the grounds of the university campus of the National University, Costa Rica. C57BL/6, BALB/c, and CD-1 mice (18 to 21 g) were provided by the following animal facilities: Instituto Clodomiro Picado, University of Costa Rica; School of Veterinary Medicine, National University, Costa Rica; and Laboratorio de Ensayos Biológicos, University of Costa Rica.

Bacterial strains and *Br*-LPS preparations. Virulent *B. abortus* 2308, *B. abortus* 2308 Δ*wadC*, *B. abortus* 2308 Δ*per* (14), *B. abortus* 2308 expressing green fluorescent protein (*B. abortus* 2308-GFP) (15), transgenic *B. abortus* 2308 with an integrated chromosomal gene coding for the red fluorescent protein (*B. abortus* 2308-RFP) from *Discosoma* coral (provided by Jean-Jacques Letesson, Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium), *Yersinia enterocolitica* O:9 (16), *Staphylococcus aureus* (ATCC 25923), and *Escherichia coli* (ATCC 25922) were grown in tryptic soy broth as previously described (15). Purified *Br*-LPS suspensions were prepared from *B. abortus* 2308, as reported elsewhere (17).

Immunization and immune serum production against *B. abortus*. C57BL/6 and CD-1 mice were intraperitoneally infected with 0.1 ml of phosphate-buffered saline (PBS) containing 10^6 CFU of virulent *B. abortus* 2308 bacteria. Mice were bled at different times, and antibody titration was carried out by microagglutination in 96-well round-bottom plastic plates. Briefly, Rose Bengal antigen was diluted 1/20 in PBS and used as a bacterial suspension for agglutination. Volumes of 50 μl of serum dilution were added to 50 μl of antigen suspension. Samples were incubated at 4°C for 24 h, and bacterial agglutination was recorded in the bottom of the plate. Agglutination titers beyond 1/50 were considered positive.

For immune serum production, mice were infected as described above; after 30 days of infection mice were bled, and serum was separated by centrifugation and filtered through a 0.2-μm-pore-size membrane (Millipore). Serum was then stored at −20°C in aliquots. IgGs were purified from immune mouse serum as reported elsewhere (18). Western blotting of mouse immune serum revealed that most of the antibody recognition was directed against *Br*-LPS (16). For *ex vivo* opsonization experiments, subagglutinating doses of antibodies were added to each well. In all experiments nonimmune mouse immunoglobulins were used as controls and administered at the same concentrations as the specific antibodies.

Bone marrow-derived PMNs. Murine bone marrow cells were isolated essentially as described by Boxio et al. (19). Briefly, bone marrow was collected from the femurs and tibiae of BALB/c mice and suspended in 1 ml of Hanks' balanced salt solution (HBSS; no calcium and no magnesium) containing 2 mM EDTA and 2% inactivated fetal calf serum. After samples were washed once with 2 ml of HBSS, cell concentration was determined with a Neubauer chamber, and the PMN percentage was calculated by Giemsa staining after cytospin centrifugation (Shandon Cytospin 2) or by flow cytometry using Guava easyCyte (Millipore). Data were analyzed with FlowJo software, version 10.0.7 (Tree Star, Inc.), as described previously (6). For some experiments, direct observation of spleen cells from *B. abortus* 2308-GFP-infected mice was performed as described elsewhere (20).

PMN phagocytosis assay. Aliquots of 350 μl of human, canine, or murine fresh heparinized blood were incubated with bacteria or ~2-μm latex beads (Sigma-Aldrich) at 37°C for 1 h under mild agitation, at the multiplicity of infection (MOI) indicated in the figure legends. Alternatively, bone marrow-derived mouse PMNs suspended in 350 μl HBSS or mouse serum were incubated with bacteria at 37°C for 1 h under mild agitation, at the MOI indicated in the figure legends. Blood smears in three glass slides were fixed with methanol, centrifuged in a cytospin, mounted with ProLong Gold Antifade reagent with 4′,6′-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), and observed under a fluorescence microscope. Before cell staining on glass slides, bone marrow cell suspensions were fixed with BD fluorescence-activated cell sorter (FACS) lysing solution or 3.5% paraformaldehyde. At least 50 PMNs were counted per slide, and the number of intracellular fluorescent bacteria/PMN was determined.

***Ex vivo* bactericidal activity of serum and PMNs.** Bacteria (10^5 to 10^6 CFU) were incubated with 350 μl of normal, heat-inactivated (56°C for 30 min), and yeast-consumed and -inactivated (37°C for 1 h) serum or with immune mouse serum at 37°C at different times. After incubation, aliquots were dispersed on Trypticase soy agar plates and incubated at 37°C for 48 to 72 h, and CFU counts were determined. Aliquots of 350 μl of fresh mouse heparinized blood or bone marrow PMNs (suspended in HBSS or mouse serum) were mixed at an MOI of 2 or 5 bacteria/PMN under mild agitation at 37°C for up to 90 min. In some cases the blood was supplemented with 0.25% anti-*Brucella* murine immune serum. After incubation, cells were lysed with 0.2% Triton X-100 and 1,000 U/ml DNase (Sigma); aliquots were dispensed on Trypticase soy agar plates and incubated at 37°C for 48 to 72 h, and CFU counts were determined.

PMN cell death assays. Aliquots of 350 μl of fresh human or mouse heparinized blood were mixed with different concentrations of *B. abortus* or *Br*-LPS under mild agitation at 37°C for 2 h. After incubation, red blood cells were lysed by mixing 100 μl of heparinized blood with 1,500 μl of red blood cell lysis buffer (8.02 g of NH_4Cl, 0.84 g of $NaHCO_3$, and 0.37 g/liter EDTA, pH 7.2) for 5 min. Then the remaining leukocytes were washed with ice-cold PBS to remove cell debris and resuspended in 100 μl of annexin V binding buffer (Life Technologies). Volumes of 5 μl of annexin V and 2 μl of AquaDead (Invitrogen) diluted 1/20 in PBS were added and incubated for 30 min on ice in the dark. Cells were washed once with ice-cold PBS and resuspended in 500 μl of BD FACS lysing solution. Samples were then subjected within 1 h to flow cytometry analysis using Guava easyCyte (Millipore), and data were analyzed using FlowJo software, version 10.0.7 (Tree Star, Inc.), as described previously (6).

Adsorption of serum components by *B. abortus* and protein identification. For serum adsorption, 5×10^{10} CFU of the corresponding *B. abortus* strain was incubated with 3 ml of murine or human serum at 37°C for 45 min under mild agitation. Control bacteria were incubated with only PBS, pH 7.2. Bacteria were washed four times with PBS, pH 7.2. All bacterial preparations were treated with 0.1 M glycine-HCl (pH 2.7) to remove adsorbed proteins or with PBS for control purposes. The eluted supernatants were neutralized with 1 M Tris-HCl (pH 9) and precipitated with methanol-chloroform. Samples were subjected to 10% SDS-PAGE under reducing conditions. The Coomassie blue-stained protein bands were excised and subjected to reduction, alkylation, and in-gel tryptic digestion, followed by matrix-assisted laser desorption ionization–two-stage time of flight (MALDI-TOF-TOF) mass spectrometry analysis on a Proteomics Analyzer 4800 Plus mass spectrometer (Applied Biosystems), as described elsewhere (21). Resulting fragmentation spectra were searched against the corresponding mouse or human UniProt databases (www.uniprot.org) using ProteinPilot, version 4.0, and the Paragon algorithm (ABsciex) for protein identification at ≥95% confidence.

Complement and fibronectin detection. For Western blotting, samples were transferred to a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE. The membranes were blocked and incubated for the detection of murine complement C3 with 1:500 diluted goat anti-mouse C3 antibody (Thermo Scientific) and further with 1:1,000 protein G-per-

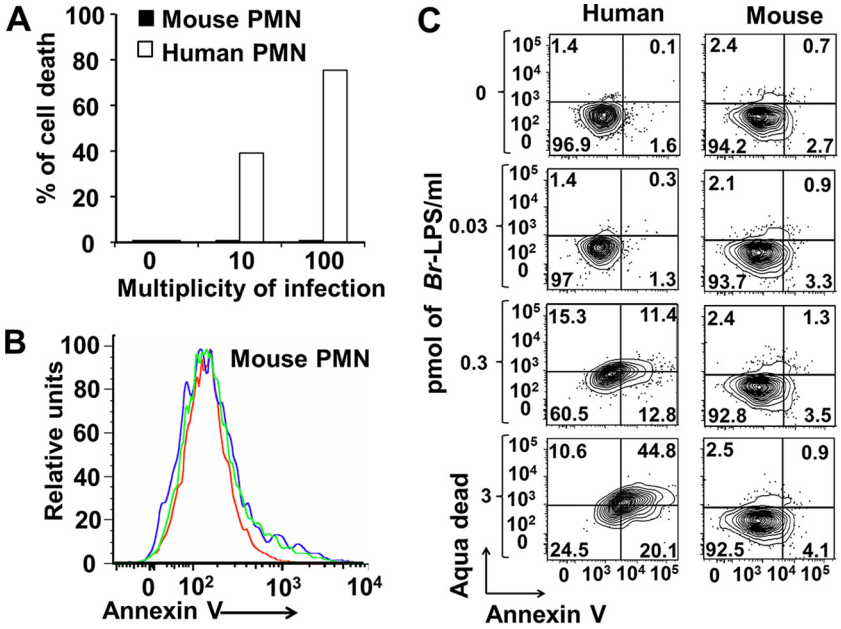

FIG 1 *B. abortus* and its LPS do not induce the cell death of mouse PMNs. (A) Heparinized blood of humans or C57BL/6 mice was incubated with *B. abortus* 2308-GFP at the indicated MOI for 2 h, and the PMN population was gated and analyzed by cytometry using annexin V as a cell death marker. (B) Heparinized blood of C57BL/6 mice was incubated with *B. abortus* 2308-GFP at an MOI of 10 (green line) or an MOI 100 (blue line) or with PBS (red line) for 2 h, and the PMN population was gated and analyzed by cytometry using annexin V as a cell death marker. (C) Heparinized blood of humans or C57BL/6 mice was incubated with *Br*-LPS at the indicated concentrations for 2 h, and the PMN population was gated and analyzed by cytometry for cell death markers using AquaDead and annexin V. The proportions of PMNs positive for the respective marker are presented with each section of the corresponding cytogram. While human PMNs acquire death cell markers, mouse PMNs remain unaffected. The figure represents one experiment of at least four repetitions.

oxidase (Life Technologies). For the detection of murine fibronectin, membranes were incubated with 1:500-diluted goat IgG anti-fibronectin antibodies (Sigma) and further with 1:1,000 protein G-peroxidase (Life Technologies). Proteins were detected with enhanced chemiluminescence (Roche).

Statistics. Analysis of variance (ANOVA) or Student's *t* test was used to determine statistical significance in the different assays (JASP Software, 2015 [https://jasp-stats.org/]). Data were processed in Microsoft Office Excel 2013.

RESULTS

***B. abortus* is not recognized by naive murine PMNs.** We have previously shown that *B. abortus* is phagocytized by human PMNs and induces the premature death of these leukocytes through the action of its LPS (6). This has led to the hypothesis that infected PMNs function as Trojan horses that spread the *Brucella* infection in different organs (6). Based on this, we asked whether *B. abortus* and its LPS could induce the same effect in murine PMNs. As shown in the experiment presented in Fig. 1, while human PMNs died after contact with *B. abortus* or its LPS, mouse PMNs did not show any signs of cell death after exposure to these components.

In order to understand this phenomenon, we then explored the early interaction of murine PMNs with *B. abortus* and compared it with that of PMNs of other animals. It has been shown that naive mouse PMNs readily ingest *S. aureus* and *E. coli* in the presence or absence of complement opsonization (22, 23). In agreement with this, we also observed phagocytosis of *S. aureus* and *E. coli* by PMNs of an outbred CD-1 strain and wild *Mus musculus* (Fig. 2A). Likewise, human and dog PMNs readily ingested these two bacterial species. In contrast to PMNs of humans and dogs, murine PMNs failed to ingest *B. abortus* (Fig. 2A). Phagocytosis of *Brucella* by murine PMNs was seldom detected even at a high MOI (~100). Moreover, at this concentration, some bacteria may remain on the cell surface and not be internalized (Fig. 2B). The absence of *Brucella* recognition was specific since murine PMNs were capable of ingesting not only other bacteria but also large numbers of latex beads (Fig. 2B). Similar observations were obtained with PMNs from inbred C57BL/6 (Fig. 2B) and BALB/c mice. Consistent with previous results (5), *B. abortus* was isolated from the blood of infected mice as early as 1 h after infection, and bacteremia persisted for at least 2 days. In spite of this, we did not detect *Brucella*-infected PMNs in blood or in target organs during the first 4 days of infection, corroborating previous results (10).

Altogether these results demonstrate that the interaction of murine PMNs with *B. abortus* significantly departs from that of PMNs from humans, guinea pigs, cows, goats, and dogs, which are capable of phagocytizing *Brucella* in the absence or presence of complement (3, 4, 24–26).

Mouse PMNs ingest *B. abortus* after the development of adaptive immunity. Antibodies are well-known opsonizing elements. We then explored the ability of murine PMNs to phagocytize *B. abortus* during development of the adaptive immune response. At the onset of infection (first 2 days) *Brucella* was not internalized by murine PMNs (Fig. 3A); however, at later times the bacterium was readily phagocytized by these leukocytes (Fig. 3A). This internalization correlated with a quick rise in murine antibodies against *Br*-LPS (the main *Brucella* antigen) (27). Both immune mouse serum and specific IgG anti-*Brucella* promoted the phagocytosis of *B. abortus* by murine PMNs (Fig. 3B and C). As indicated previously (5), under the microscope, these in-

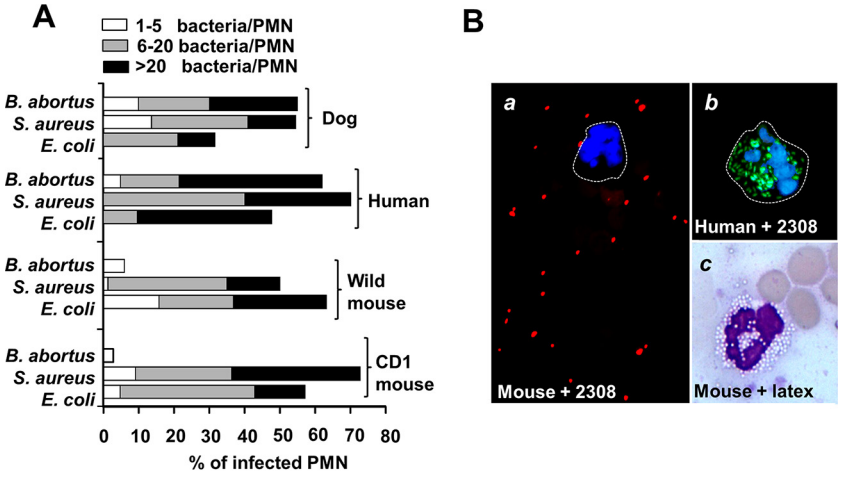

FIG 2 Naive murine PMNs do not phagocytize *B. abortus*. (A) Blood from the indicated species was incubated with *B. abortus*-GFP, *S. aureus*, or *E. coli* at an MOI of 20 for 1 h. Blood smears were then fixed and mounted with ProLong Gold Antifade reagent with DAPI. At least 50 PMNs were counted per sample, and the number of intracellular bacteria in each PMN and the proportion of phagocytosis were estimated under fluorescence microscopy. (B) Comparison between C57BL/6 mouse PMNs incubated with *B. abortus* 2308-RFP at an MOI of 100, human PMNs incubated with *B. abortus*-GFP at an MOI of 50, and Giemsa staining of C57BL/6 mouse PMNs incubated with latex beads at an MOI of 100, as indicated. Magnifications, ×200 (frame a) and ×400 (frames b and c). Images were cut from the microscope field, contrasted, and saturated using the hue tool to obtain suitable color separation. Similar results were obtained with BALB/c mice.

fected leukocytes did not show obvious signs of alterations at early times of infection (<1 h). These results indicate that phagocytosis of *B. abortus* by murine PMNs is efficiently mediated by opsonizing antibodies through Fc receptors at the surface of these leukocytes.

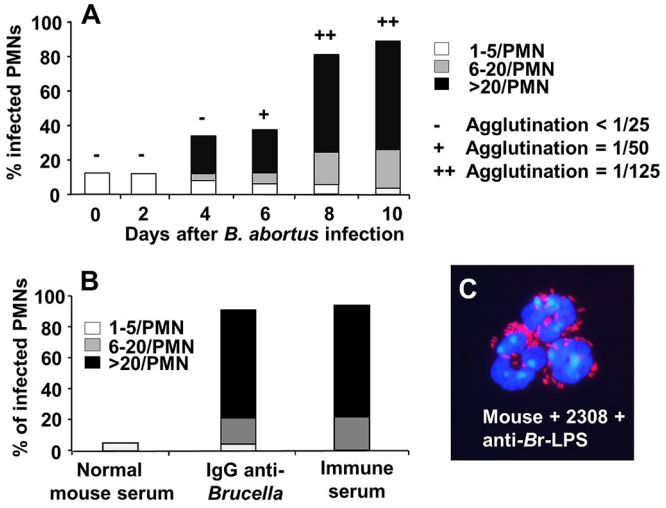

FIG 3 Mouse PMNs phagocytize *B. abortus* after antibodies are generated. CD-1 mice were infected intraperitoneally with 10^6 CFU; then, mouse blood was collected at different days of infection and incubated with *B. abortus* 2308-RFP (MOI of 50) for 1 h. Blood smears were then fixed and mounted with ProLong Gold Antifade reagent with DAPI. At least 50 PMNs were counted per sample, and the number of intracellular bacterial in each PMN was determined. The relative agglutination titer for the day evaluated after intraperitoneal injection with *B. abortus* is indicated at the top of the bars according to the legend on the figure. (B) Mouse PMNs of CD-1 mice were incubated with *B. abortus* 2308-RFP at an MOI of 50 for 2 h and under different conditions of opsonization. Then, the number of phagocytized bacteria was recorded by fluorescence microscopy. (C) CD-1 mouse PMNs incubated with *B. abortus* 2308-RFP and anti-*Brucella* mouse serum at an MOI of 100. The image was cut from the microscope field, contrasted, and saturated using hue tool to obtain suitable color separation (magnification, ×200). Similar results were obtained with C57BL/6 and BALB/c mice.

Surface *N*-formyl-perosamine homopolysaccharides are responsible for *Brucella* camouflage. Since innate mouse opsonins failed to promote the phagocytosis of smooth *B. abortus* by murine PMNs, we then explored the presence of blocking components on the bacterial surface. First, we tested the ability of murine PMNs to phagocytize the rough *B. abortus* 2308 Δ*per* mutant lacking surface *N*-formyl-perosamine sugars (*Br*-LPS O chain and native hapten [NH] polysaccharide) and the smooth *B. abortus* 2308 Δ*wadC* mutant displaying a defect in the core oligosaccharide (Fig. 4). While the core Δ*wadC* mutant was not recognized by murine PMNs and behaved as the parental strain did, the rough Δ*per* mutant was readily phagocytized in the presence of normal but not inactivated mouse serum (Fig. 5A). High numbers of intracellular rough *B. abortus* Δ*per* bacteria did not cause obvious alterations in mouse PMNs at early times (<1 h) of infection (Fig. 5A, inset), paralleling the results obtained with antibody-opsonized smooth brucellae (Fig. 3C).

To confirm the role of *N*-formyl-perosamine sugars in the *Brucella* camouflage, we then tested the phagocytosis of *Yersinia enterocolitica* O:9. The O chain and NH surface molecules of this bacterium are identical to those of *B. abortus* (16, 28). *Y. enterocolitica* O:9 was not phagocytized by mouse PMNs in the presence of normal mouse serum (Fig. 5B). However, this bacterium was readily internalized in the presence of anti-*Brucella* antibodies. These results demonstrate that the surface *N*-formyl-perosamine polysaccharides were the moieties responsible for the *B. abortus* camouflage for opsonization.

***B. abortus* is resistant to the bactericidal action of mouse immune serum and PMNs.** Since it has been shown that *B. abortus* Δ*wadC* and Δ*per* mutants are more sensitive to the bactericidal action of bovine serum than the parental strain (14), we then asked whether murine serum components and PMNs were capable of killing intracellular *Brucella*. The Δ*wadC* mutant was resistant to the action of normal mouse serum, as was the virulent *B. abortus* 2308 strain (Fig. 5C). Moreover, the presence of anti-*Brucella* antibodies did not have a significant effect on the viability of

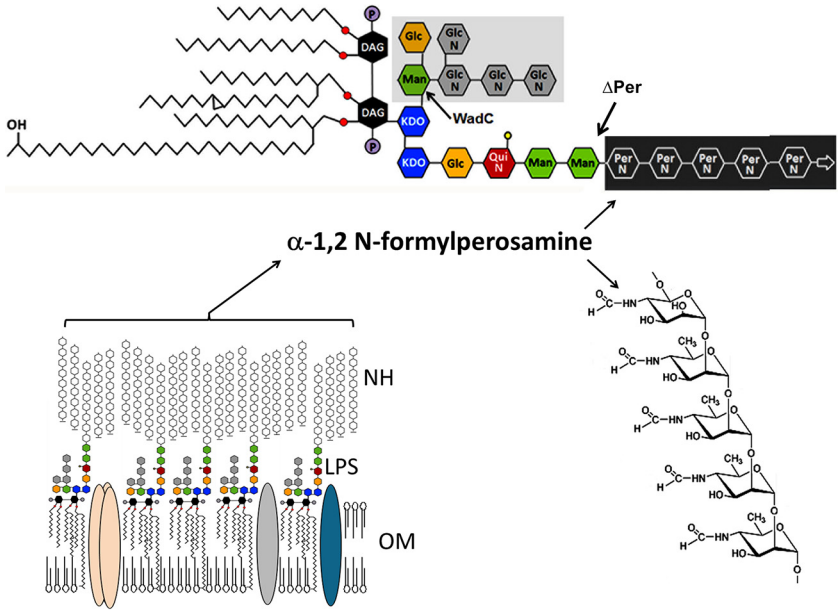

FIG 4 Schematic structure of *B. abortus* outer membrane showing the LPS and NH. The O polysaccharide and native hapten (NH) are unbranched linear homopolymers of α-1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl units (*N*-formyl-perosamine) with an average chain length of 96 to 100 glycosyl subunits (28). While the NH is not directly bound to the lipid membrane, the O polysaccharide is linked to a core bifurcating oligosaccharide composed of βGlcN-6-βGlcN-4-βGlcN(-6-βGlcN)-3-αMan(-6-αGlc)-5-KDO_1(−1-KDO_1)-lipid A immersed in the outer membrane. Branching from KDO_2 is αPerNFo-(-2PerNFo)$_n$-2PerNFo-2-αMan-3-αMan-3-βQuiNAc-4-βGlc-4-KDO_2-4-KDO_2 (59). The KDO_1 is linked to the lipid A composed of a backbone of diaminoglucose (DAG) disaccharide, substituted with phosphates (P) and amide and ester-linked long-chain saturated ($C_{16:0}$ to $C_{18:0}$) and hydroxylated (3-OH-$C_{12:0}$ to 29-OH-$C_{30:0}$) fatty acids (17, 60). The lipid A is bound to the outer membrane. KDO, ketodeoxyoctulosonic acid; Man, mannose; QuiN, acetyl-quinovosamine; Glc, glucose (Glc); PerNFo, *N*-formyl-perosamine. The Δ*wadC* mutation precludes the incorporation of the βGlcN-6-βGlcN-4-βGlcN(-6-βGlcN)-3-αMan(-6-αGlc)-oligosaccharide to the KDO_1 (marked by a gray area) while the Δ*per* mutation precludes the incorporation of αPerNFo-(-2PerNFo)$_n$-2PerNFo-homopolymer (61).

the bacteria (Fig. 5C). Although serum components opsonized the rough Δ*per* mutant, no bactericidal activity was recorded by mouse serum in the absence or presence of PMNs (Fig. 5D).

***N*-Formyl-perosamine homopolysaccharides hamper the binding of murine heat-labile serum components.** Since bacterial opsonization commonly occurs via activation and binding of various heat-labile serum factors such as complement and fibronectin (29), we then asked whether *B. abortus* was capable of interacting with these components. As presented in Fig. 6A, *B. abortus* was capable of adsorbing a significant number of human serum proteins, including complement components. Under the same experimental conditions mouse serum constituents were barely adsorbed by smooth or rough *B. abortus* strains (Fig. 6A). The small amounts of mouse serum proteins adsorbed by *B. abortus* strains, revealed as faint bands, corresponded to serum albumin, platelet factor 4, and mannose binding protein. With the exception of the last protein, no other opsonins or complement-related proteins were detected by this proteomic approach.

In spite of the overall lack of affinity of the *Brucella* cell envelope for mouse serum factors, it was revealed by Western blotting that the Δ*per* mutant adsorbed heat-labile complement and fibronectin in larger amounts than the smooth counterpart strain 2308 (Fig. 6B). This was consistent with the phagocytosis of *B. abortus* Δ*per* by murine PMNs in the presence of normal but not inactivated serum (Fig. 5A). These results demonstrate that phagocytosis of the Δ*per* strain by murine PMNs occurs through opsonization of small amounts of heat-labile serum components such as complement and fibronectin and that the O chain and NH polysaccharides hamper the access of these components to surface bacterial molecules.

Intracellular *B. abortus* induces the cell death of mouse PMNs. We have demonstrated that *B. abortus* induces premature cell death of human PMNs (6). Therefore, we explored the effect of both the antibody-opsonized smooth *B. abortus* 2308 and the rough mutant *B. abortus* Δ*per* on mouse PMNs. As shown in Fig. 7, both internalized bacteria readily induce the cell death of murine PMNs, paralleling the phenomenon observed with human PMNs.

DISCUSSION

It has been shown that naive human, guinea pig, bovine, rat, caprine, and canine PMNs readily phagocytize both smooth and rough *Brucella* species (3–5, 24–26, 30). Moreover, PMNs from some of these animals (humans, guinea pigs, rats, and cows) are capable of ingesting *Brucella* in the presence of inactivated normal serum or even in the absence of serum (3, 4, 6). In general, the overall behavior of human PMNs is rather similar to that observed with bovine PMNs (4), a phenomenon that is commensurate with the coevolution of *B. abortus* and its host.

In this work we have shown that mouse normal serum components do not opsonize smooth *B. abortus* and that mouse PMNs, in the absence of specific antibodies, do not phagocytize this bacterium. This phenomenon is specific, and the molecules responsible for the *Brucella* camouflage are *N*-formyl-perosamine surface homopolysaccharides (Fig. 4). This is relevant since in addition to the hindrance function, these perosamine polysaccha-

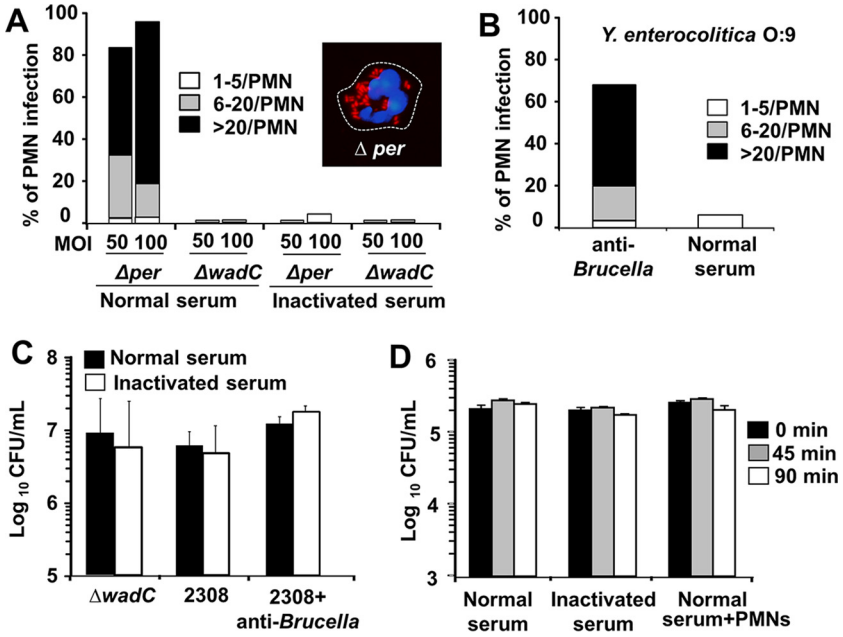

FIG 5 Surface *N*-formyl-perosamine polysaccharides are responsible for *Brucella* camouflage. (A) Smooth *B. abortus* Δ*wadC* and rough Δ*per* mutants were incubated with BALB/c bone marrow PMNs at the indicated MOIs in the presence of normal or inactivated mouse serum, and phagocytosis was recorded as described in the legend of Fig. 2. The inset corresponds to a PMN with internalized *B. abortus* Δ*per* (MOI of 50); the infected cell was fixed and mounted with ProLong Gold Antifade reagent with DAPI and observed under the fluorescence microscope at a magnification of ×200. The image was cut from the microscope field, contrasted, and saturated using the hue tool to obtain suitable color separation. (B) *Y. enterocolitica* O:9 (MOI of 100) was incubated with BALB/c bone marrow PMNs in the presence of normal serum or serum with anti-*Brucella* antibody (2%), and the phagocytosis was recorded. (C) *B. abortus* 2308 and the Δ*wadC* mutant (10^6 CFU) were incubated with normal or inactivated mouse serum or normal mouse serum containing antibodies against *Brucella*, and bacterial viability was recorded after 45 min of incubation. (D) *B. abortus* Δ*per* was incubated in the presence of normal or inactivated mouse serum or with BALB/c bone marrow PMNs in normal serum, and the bacterial viability was estimated at the indicated times.

rides display other biological properties related to virulence, such as dimerization of major histocompatibility complex (MHC) class II, blocking of MHC class II antigen presentation, and protection against a collection of bactericidal substances (16, 31–33).

The absence of NH and O-chain polysaccharides uncovers potential targets in the *Brucella* cell envelope, such as outer membrane proteins and phospholipids, as well as ketodeoxyoctulosonic acid (KDO) and lipid A phosphate groups, present in the innermost sections of the core moiety (14, 34). Still, mouse PMNs fail to ingest the rough *B. abortus* Δ*per* mutant in the absence of classical heat-sensitive serum opsonins, such as complement and fibronectin. This is relevant since mouse PMNs are capable of ingesting latex beads and other bacteria, such as *S. aureus* and *E. coli*, in the presence or absence of complement, albeit the phagocytosis is more efficient in the former case (22, 23). In the absence of opsonization by specific antibodies or complement, PMNs may

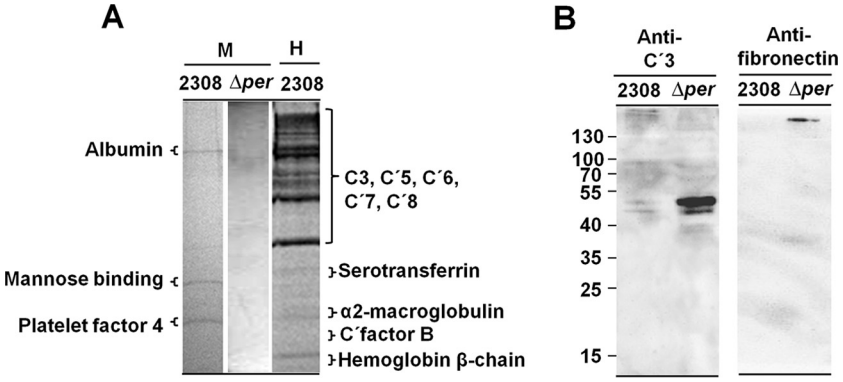

FIG 6 Normal mouse serum proteins do not opsonize smooth *B. abortus*. (A) Coomassie blue-stained 10% SDS-PAGE gel of mouse (M) and human (H) proteins (10 μg/well) eluted from the surface of *B. abortus* 2308 or the Δ*per* mutant after incubation of the respective serum with viable bacterial cells. Individual lines, corresponding to the different eluted fractions, were cut out and separated from the main gel as indicated in the figure; then each gel line was horizontally sliced (about 2-mm slices, from top to bottom), and the identities of proteins in each slice were determined by proteomic analysis. (B) Western blotting of mouse proteins (10 μg/well) eluted from the surface of *B. abortus* 2308 or the Δ*per* mutant after incubation of the respective serum with viable bacterial cells. Western blots were developed with monoclonal antibodies against mouse C′3 or monospecific rabbit IgG anti-fibronectin.

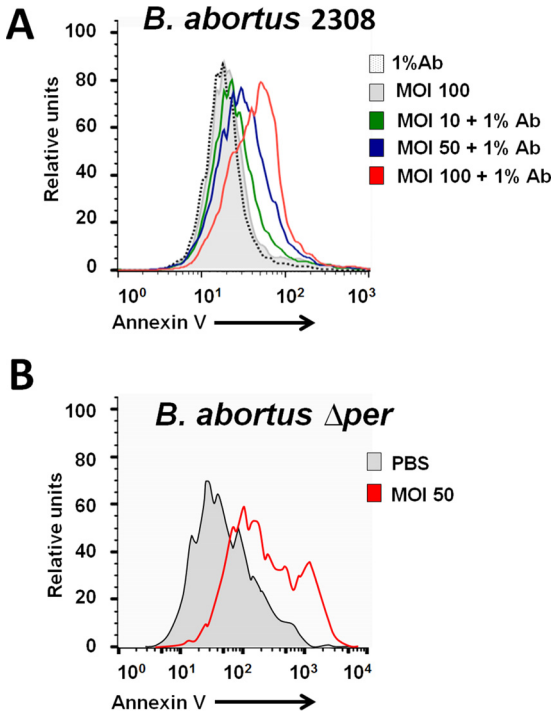

FIG 7 Induction of mouse PMN cell death after phagocytosis of antibody-opsonized smooth *B. abortus* 2308 or the rough Δ*per* mutant. (A) Heparinized blood of C57BL/6 mice was incubated with *B. abortus* 2308-GFP or with *B. abortus* 2308-GFP opsonized with antibody (Ab) against *Br*-LPS at the indicated MOIs for 2 h, and then the PMN population was gated and analyzed by cytometry using annexin V as a cell death marker. Negative controls were treated with either PBS or antibody alone. Similar results were observed with bone marrow-derived PMNs. (B) Bone marrow PMNs of BALB/c mice were incubated with the rough mutant *B. abortus* Δ*per* at an MOI of 50 or with PBS for 4 h, and the PMN population was gated and analyzed by cytometry using annexin V as a cell death marker. The figure represents one experiment of at least four repetitions.

also ingest microorganisms through the pathways of other PMN pattern recognition receptor (PRRs), such as β2 integrins, C-type lectins, or scavenger or pentraxin receptors (22, 35, 36). This explains the failure of mouse PMN PRRs to recognize putative *B. abortus* pathogen-associated molecular patterns (PAMPs), such as outer membrane lipoproteins, adhesion-like proteins, ornithine-containing lipids, flagellar structures, and phospholipids, among the most conspicuous elements on the surface of brucellae, which in other bacteria are constitutive PAMPs and targets for recognition (7, 8, 14, 27, 37).

Although *Brucella* organisms are more resistant than other Gram-negative bacteria to the killing action of human serum and PMNs, these components are still able to kill about 20 to 30% of virulent smooth *B. abortus* bacteria after 90 min (3–5, 38). This bactericidal activity is even more conspicuous and efficient in the case of rough *B. abortus* bacteria (3). In contrast, both smooth and rough *B. abortus* strains were totally resistant to the killing actions of mouse PMNs and complement even in the presence of antibodies.

Therefore, it seems that the lack of *B. abortus* recognition and the failure to kill this bacterium work on at least three different levels of the innate immune system: (i) the absence of binding of natural opsonins to the surface of smooth *Brucella* bacteria, (ii) the lack of recognition of putative PAMPs by murine PMN PRRs, and (iii) the virtually absolute resistance of *Brucella* to the killing action of mouse complement—by either the alternative or classical pathway—and PMNs.

Regarding the interaction of *Brucella* with human complement, it has been demonstrated that the bacteria are considerably more resistant to the killing action of human serum than other Gram-negative bacteria (5, 39). In spite of this, the binding of human complement components to the surface of *B. abortus* was conspicuous and commensurate with the opsonization of this bacterium by normal serum. This is in clear contrast to the absence of opsonization and brucellicidal activity observed by mouse serum. This is striking since it has been proposed that bacterial opsonization by mouse complement is similar to that of human complement and that the mouse has significant quantities of complement activity and high serum levels of C3 as well as other complement proteins (40). However, genetic and structural differences have been demonstrated between human and mouse complement C3 and C4 components (41). For instance, while human C3 is readily inhibited by compstatin, mouse C3 is resistant to this compound due to structural differences in amino acid residues 329 to 534 (42). Likewise, mouse C4 does not have classical C5 convertase activity due to differences with the human beta-chain segments of C4 and other regions of the molecule contributing to C5 binding (43).

Although the differences between murine and human surface PMN PRRs have not been explored in detail, there are some discrete features that may be relevant. While in humans the complement receptor proteins CR1 and CR2 are two different molecules, in mouse PMNs, they constitute a single chimeric CR1/CR2 molecule with different affinities for various complement components (44). In addition, the affinity of the G protein-coupled receptor for the chemoattractant and activator formyl-Met-Leu-Phe (fMLP) is lower in mouse than in human PMNs (45). Likewise, while the human PMN receptor CXCR1 uses as the substrate CXCL8, the mouse counterpart uses CXCL6 (46). Finally, some receptors present in mouse PMNs (e.g., the sialic acid receptor CD33, FcγRIIIA, FcγRIII, and FcγRIV) are absent in humans, and vice versa (e.g., Toll-like receptor 10 [TLR-10], L-selectin binding to E-selectin, and FcγRIIA) (47–52).

Finally, the almost absolute resistance to the killing action of mouse complement and PMNs is linked to the absence of activation of these elements by the putative *Brucella* PAMPs (5, 7, 8, 14, 27, 37), as well as to the bacterial resistance to the microbicidal substances of PMNs (33, 34). Regarding this, decreased lytic activity of mouse complement in comparison to that of the human counterpart has been described (53). With respect to PMNs, there are several differences in microbicidal components between human and mouse. For instance, mouse PMNs lack defensins (54), and the functions of several proteases and ROS activation differ between PMNs of mice and humans (55–57). Whether some of these differences are related to the absence of *Brucella* recognition and killing by murine serum components and PMNs remains to be investigated.

We have previously shown that *Brucella* induces premature death of human PMNs (6). Commensurate with this, *B. abortus* also induces the death of these leukocytes once the bacteria have been internalized via Fc receptors or, in the case of the Δ*per* mutant strain, via other serum opsonins. As proposed (6), this phenomenon may favor the nonphlogistic removal of infected dying

PMNs by Mϕ and DCs, favoring the dispersion of *Brucella* in the organism following a Trojan horse effect. In this sense, the premature death of mouse PMNs parallels that of human PMNs, and therefore it is a useful model to explore this hypothesis.

The mouse model has provided a substantial body of information concerning the pathobiology and immunology of brucellosis (12, 13). Still, our approach has revealed significant differences between mice and other hosts in front-line elements of innate immunity that may have a profound influence on downstream mechanisms of the immune response. This is not trivial since the outcome of brucellosis in a given animal species may be determined during the initial stages of immune recognition. Therefore, the differences between the immune system of mice and that of other mammals (58) should prevent us from making direct extrapolations and encourage us to dissect the mechanisms behind them.

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HOST RESPONSE AND INFLAMMATION

Neutrophils Dampen Adaptive Immunity in Brucellosis

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ABSTRACT *Brucella* organisms are intracellular stealth pathogens of animals and humans. The bacteria overcome the assault of innate immunity at early stages of an infection. Removal of polymorphonuclear neutrophils (PMNs) at the onset of adaptive immunity against *Brucella abortus* favored bacterial elimination in mice. This was associated with higher levels of interferon gamma (IFN-γ) and a higher proportion of cells expressing interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS), compatible with M1 macrophages, in PMN-depleted *B. abortus*-infected (PMNd-*Br*) mice. At later times in the acute infection phase, the amounts of IFN-γ fell while IL-6, IL-10, and IL-12 became the predominant cytokines in PMNd-*Br* mice. IL-4, IL-1β, and tumor necrosis factor alpha (TNF-α) remained at background levels at all times of the infection. Depletion of PMNs at the acute stages of infection promoted the premature resolution of spleen inflammation. The efficient removal of bacteria in the PMNd-*Br* mice was not due to an increase of antibodies, since the immunoglobulin isotype responses to *Brucella* antigens were dampened. Anti-*Brucella* antibodies abrogated the production of IL-6, IL-10, and IL-12 but did not affect the levels of IFN-γ at later stages of infection in PMNd-*Br* mice. These results demonstrate that PMNs have an active role in modulating the course of *B. abortus* infection after the adaptive immune response has already developed.

KEYWORDS *Brucella*, *Brucella abortus*, interferon gamma, adaptive immunity, brucellosis, native immunity, neutralizing antibodies, neutrophils

Polymorphonuclear neutrophils (PMNs) are essential elements of innate immunity and the first line of defense against microbial invaders. These cells phagocytize and destroy bacteria, release cytokines, and activate elements of the innate immune response (1). However, PMNs also modulate components of adaptive immunity, a phenomenon that has gained considerable attention in recent years (2, 3).

Neutropenic murine models have been used to dissect the roles of PMNs during innate and adaptive immune responses against microbial infections. The selective depletion of PMNs by means of antibodies is the most common and widespread model (4–8). A second model includes a mutant mouse strain named Genista, which is devoid of mature PMNs (4, 9–11). Both models have advantages and drawbacks, though they generally display good correlation and render similar results (4, 11). Neutropenia in the anti-PMN depletion model is transient and cannot be maintained beyond 1 week. Still, the advantage of this model is that the neutropenic condition can be induced at any stage of an infection (12–14).

We have used both the Genista and anti-PMN models to explore the role of PMNs and innate immune response during the onset of *Brucella abortus* infection (4, 15). *Brucella* organisms are intracellular stealth pathogens of animals and humans that avoid the activation of innate immunity, remaining in several tissues for protracted periods (15–17). *B. abortus* readily invades PMNs, resisting the killing action of these

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leukocytes (15, 18–22). This correlates with the resistance and modification of the bacterial cell envelope components, which barely promote the generation of reactive oxygen species and proinflammatory cytokines in the infected PMNs (15, 19). In addition, through its lipopolysaccharide (*Br*-LPS), *B. abortus* mediates in a nonphlogistic manner the premature cell death of PMNs and induces the expression of "eat me" signals on these cells (19, 21). The absence of PMNs at the onset of *B. abortus* infection stimulates the recruitment of monocytes/dendritic cells, favors the activation of B and T lymphocytes, and promotes the production of Th1 cytokines (4).

The course of human brucellosis parallels that observed in mice (16, 23). In the mouse model, brucellosis is divided into four phases according to the bacterial colonization of the target organs, the pathological signs, and the profile of the immune response (17, 23). The first phase corresponds to the onset of infection (also known as the incubation stage), which typically lasts 2 to 3 days. During this phase, the production of proinflammatory cytokines and the activation of innate immunity are negligible (4). The acute phase follows, lasting 2 to 3 weeks. Active bacterial replication and high levels of Th1 cytokines characterize this phase (23, 24). Then, the chronic steady phase, lasting from 8 to 11 weeks, corresponds to the plateau of the infection. Finally, the chronic declining phase is characterized by the gradual elimination of bacteria. This phase may last months or even years (23, 24). During the acute and chronic phases, large amounts of anti-*Br*-LPS antibodies are produced (25). At these stages, the bone marrow (BM) is colonized by *Brucella* organisms, maintaining for protracted periods high bacterial loads within BM PMNs and, to a minor extent, in monocytes and stem cells (17). This is significant, since PMNs in other target organs, such as the spleen, do not harbor *Brucella* (26).

Here, we describe how PMNs modulate adaptive immunity in the initial stages of acute murine brucellosis. The results presented here reinforce our previous hypothesis (4) and give new insights into the role that PMNs have in shaping the immune response during brucellosis.

RESULTS

The absence of PMNs enhances the removal of *B. abortus* in mice. We have shown that the absence of PMNs at the onset of *B. abortus* infection enhances bacterial removal after several days (4). Following this, we explored whether the absence of PMNs has any influence at the onset of adaptive immunity, once Th1 cytokines and specific antibodies have developed (21). For this, the protocols described in Fig. S1A and B in the supplemental material were followed.

After the sixth day of infection (1 day after PMN depletion), we observed an initial increase of bacterial loads in the spleens of PMNd-*Br* mice (Fig. 1A). This outcome agreed with our previous results (4). After 14 days of *B. abortus* infection (9 days post-PMN depletion), the numbers of CFU in the spleens of PMNd-*Br* mice reached values similar to those of the non-PMN-depleted controls (Fig. 1A); however, PMNd-*Br* mice showed more efficient bacterial removal (Fig. 1B). This phenomenon was more conspicuous after 30 days of infection (15 days of PMN depletion) (Fig. 2).

RB6-8C5 antibody partially depletes a subpopulation of monocytes (27) (see Table S1 in the supplemental material). Therefore, we repeated the experiment using the anti-PMN antibody from the 1A8 clone, which is supposed to be highly specific for murine PMNs (27). Similar results using this antibody were observed (see Fig. S2 in the supplemental material). However, the elimination of bacteria was more evident in BM, regardless of the antibody used to deplete PMNs (Fig. 3). This was striking, since during the chronic stages, the presence of *Brucella* organisms in the BM is marked (17), and in contrast to other tissues, BM retains a proportion of PMNs after depletion of these cells (see Table S1).

It is worth mentioning that the 1A8 antibody has several drawbacks in comparison to the RB6-8C5 antibody. To achieve significant PMN depletion, very high doses of 1A8 antibody (500 μg/mouse) were required. In spite of this, depletion seldom reached more than 95% of blood PMNs (see Table S1), and neutropenia was not as steadily

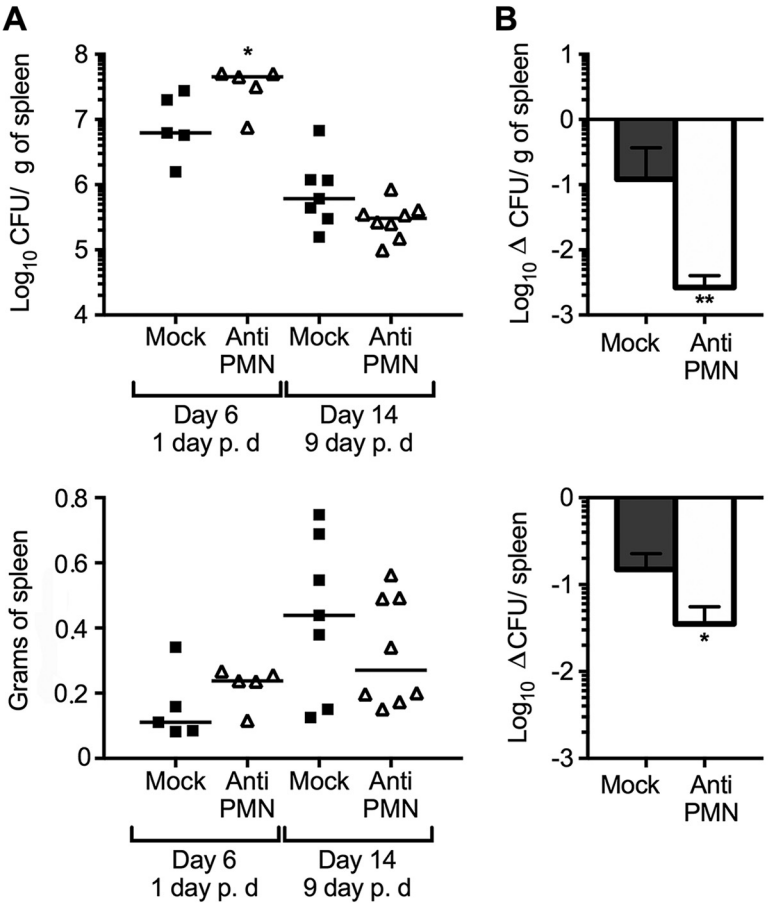

FIG 1 PMN depletion at the onset of adaptive immunity promotes *Brucella* removal. C57BL/6 mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W. After 5 days of infection, one group of mice was depleted of PMNs by means of i.p. injection of RB6-8C5 anti-PMN. (A) At the indicated times, the numbers of CFU per spleen and spleen weights were determined. Each symbol represents one animal, and the lines represent the medians for each group. p. d, postdepletion. (B) Rates of change in CFU per spleen (Δ CFU/spleen) and CFU per spleen weight (Δ CFU/g of spleen) were calculated over time using the following equations: Δ CFU/spleen = mean CFU 14 days/CFU 6 day ± standard deviation (SD) and Δ CFU/g of spleen = mean CFU/g of spleen 14 days/6 days ± SD. The error bars represent standard deviations. *, $P < 0.05$, and **, $P < 0.01$, in relation to the mock-treated controls.

maintained as with the RB6-8C5 antibody. Similar results have been reported by other authors (27). Regardless of this, the overall elimination of bacteria was more efficient in the PMNd-*Br* mice than in the infected controls.

***B. abortus* infection enhances cytokine production in neutropenic mice.** At the onset of *B. abortus* infection, the levels of proinflammatory cytokines are negligible. This agrees with the stealth strategy of *Brucella* (15). However, once an infection has been established (after 5 days), there is an increase in production of interferon gamma (IFN-γ), the most relevant cytokine for mounting an efficient immune response against *Brucella* sp. infections (28, 29). As expected, after 6 days of infection, the levels of IFN-γ were already high in the mock-treated control mice (Fig. 4). Still, the amounts of IFN-γ doubled in PMNd-*Br* mice (1 day of PMN depletion), with negligible or low production of other cytokines (Fig. 4). After 14 days of infection (9 days of PMN depletion), the levels of IFN-γ decreased, but the regulatory interleukin 10 (IL-10) and other cytokines, such as IL-12 and IL-6, significantly increased (Fig. 4). Similar results for the levels of IFN-γ were observed using the 1A8 antibody for PMN depletion (see Fig. S3 in the supplemental material).

Unexpectedly, the day after PMN depletion (6 days of *B. abortus* infection), the PMNd-*Br* mice showed clinical symptoms, such as lethargy, piloerection, anorexia, and

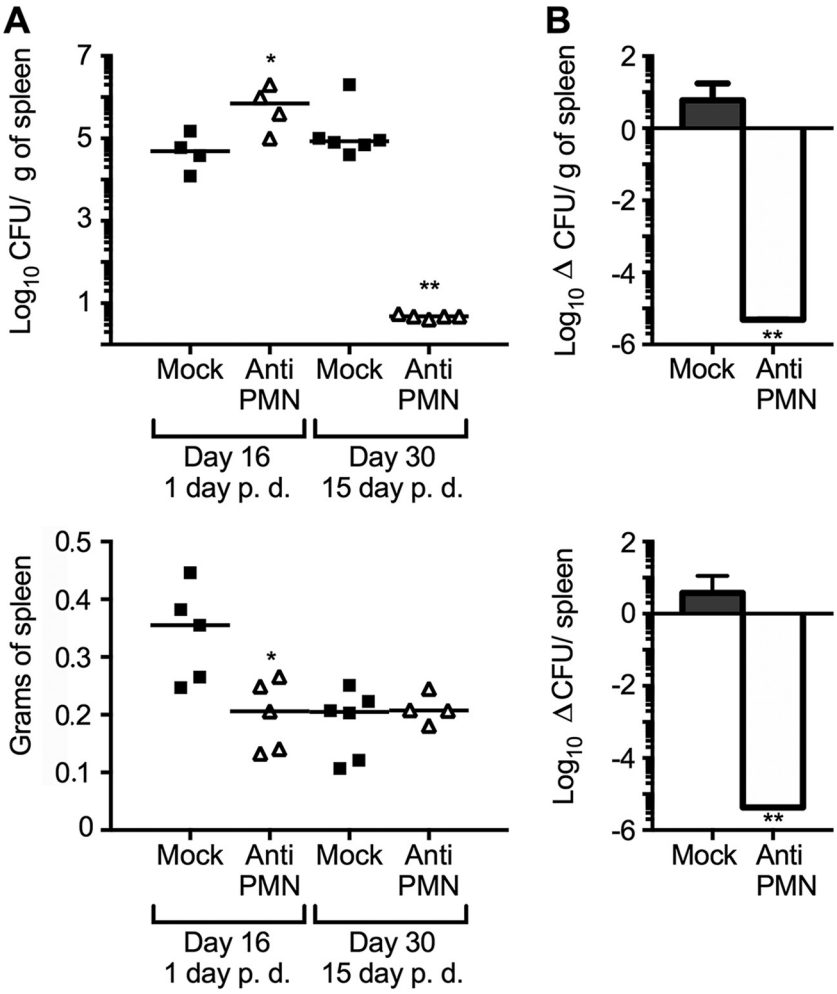

FIG 2 PMN depletion during the acute infection period promotes *Brucella* removal. C57BL/6 mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W. After 15 days of infection, one group of mice was depleted of PMNs by means of i.p. injection of RB6-8C5 anti-PMN. (A) At the indicated times, the numbers of CFU per spleen and spleen weights were determined. Each symbol represents one animal, and the lines represent the medians for each group. (B) Rates of change in CFU per spleen (Δ CFU/spleen) and CFU per spleen weight (Δ CFU/g of spleen) were calculated over time using the following equations: Δ CFU/spleen = mean CFU 30 days/CFU 6 day ± SD and Δ CFU/g of spleen = mean CFU/g of spleen 30 days/16 days ± SD. The error bars represent standard deviations. **, $P < 0.01$ in relation to the mock-treated controls.

general malaise. Weight loss of the PMNd-*Br* mice was evident after 14 days of infection (9 days of PMN depletion) (Fig. 5). However, 30 days after infection (25 days of PMN depletion), the weight of the PMNd-*Br* mice increased in comparison to the mock-treated controls, suggesting health improvement due to better bacterial removal (Fig. 5). Similar results were obtained with the 1A8 antibody.

The absence of PMNs promotes the premature resolution of spleen inflammation in infected mice. The removal of PMNs at the onset of *B. abortus* infection induces premature granulomatous inflammation and follicular hyperplasia of the spleen characterized by augmented infiltration of epithelioid histiocytes (4). In contrast, the removal of PMNs after the immune response has been established induces a different pathological effect in infected mice (Fig. 6). As expected, after 6 days of infection (1 day of PMN depletion), PMNd-*Br* mice showed no significant differences in spleen inflammation (Fig. 6A and B). However, the absence of PMNs at the acute stages of adaptive immunity favored the fast resolution of spleen inflammation (Fig. 6). Indeed, after 14 days of infection (9 days of PMN depletion), PMNd-*Br* mice showed lower numbers

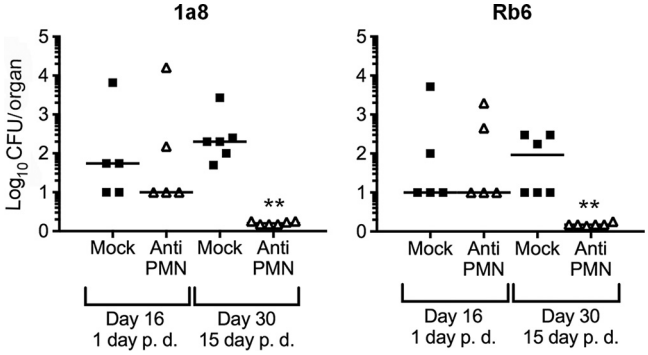

FIG 3 Late PMN depletion diminishes bacterial loads in BM. C57BL/6 mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W, and at 15 days postinfection, one group of mice was depleted of PMNs by means of i.p. injection of 1A8 or RB6-8C5 anti-PMN. At the indicated times, the number of CFU per BM was determined. Each symbol represents one animal. **, $P < 0.01$ in relation to the mock-treated controls.

of granulomas, reduced vasodilation, lower follicular hyperplasia, and less hyperemia (Fig. 6C and D) than the spleens of the mock-treated controls (Fig. 6E). As shown previously (4), the depletion of PMNs alone did not induce pathological alterations in the target organs of noninfected mice.

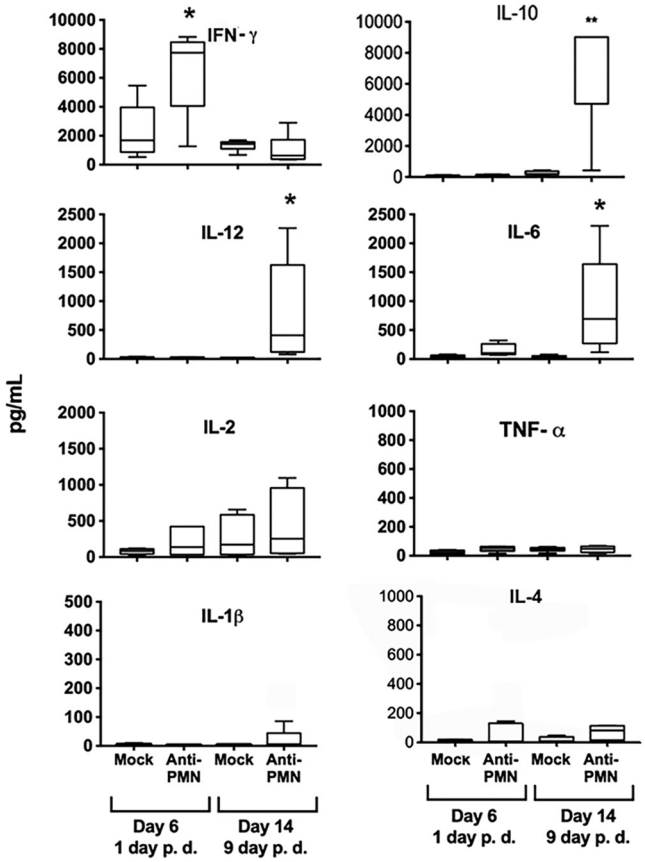

FIG 4 PMN depletion after *B. abortus* infection increases the levels of cytokines. C57BL/6 mice were infected by the i.p. route with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W. After 5 days of infection, one group of mice was depleted of PMNs by means of i.p. injection of RB6-8C5 anti-PMN. The levels of various cytokines were determined by ELISA in the sera of all the mice at 6 and 14 days postinfection (1 and 9 days postdepletion, respectively). Bars represent the value distribution, while the median values are indicated by the horizontal lines within bars. Whiskers above and below the bars represent the error values. *, $P < 0.05$, and **, $P < 0.01$, in relation to the mock-treated controls.

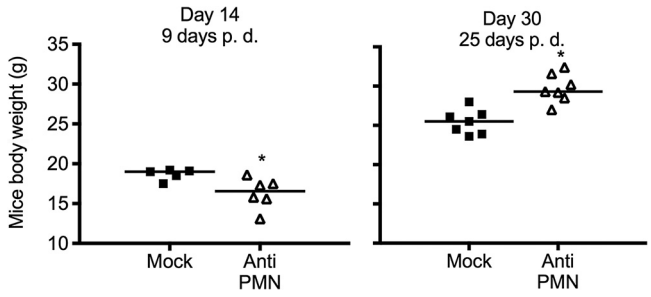

FIG 5 PMN depletion after *B. abortus* infection induces reduction in body weight. C57BL/6 mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W and depleted of PMNs with RB6-8C5 antibodies 5 days postinfection. Mouse body weights at day 14 postinfection (9 days postdepletion) and day 30 postinfection (25 days postdepletion) are shown. Each symbol represents one animal. *, $P < 0.05$ in relation to the mock-treated controls.

Neutropenic mice show lower antibody responses against *B. abortus* antigens. It has been demonstrated that IFN-γ influences the immunoglobulin isotypes against *Brucella* antigens (30). Therefore, we investigated if the reduced bacterial loads in the neutropenic mice could be due to higher antibody titers or to an increase of specific antibody isotypes against *Br*-LPS, the most relevant antigen in brucellosis (16). In comparison to the mock-treated controls, the PMNd-*Br* mice displayed lower antibody agglutination titers after 21 and 30 days of infection (16 and 25 days of PMN depletion, respectively) (Fig. 7A). These titers correlated with the generally smaller amounts of the immunoglobulin isotypes against *Br*-LPS at both times and was more evident after 30 days of infection (25 days of PMN depletion) (Fig. 7B). A similar trend was recorded after 30 days of infection (25 days of PMN depletion) when the 1A8 antibody was used for PMN depletion. However, it was less conspicuous than that observed with the

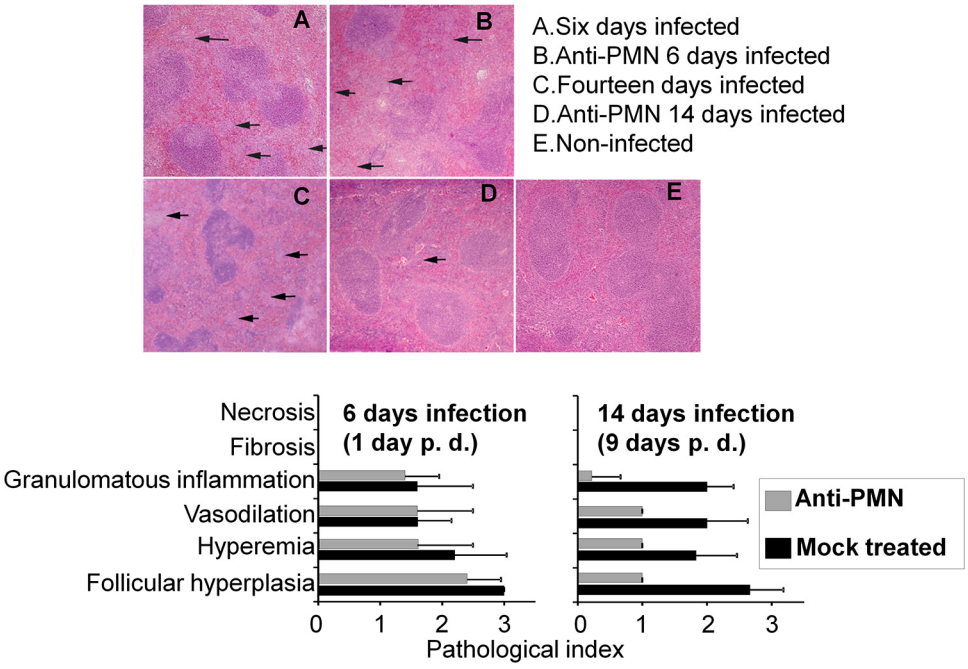

FIG 6 PMN depletion during the immune response favors the premature resolution of inflammation. (Top) Spleens from *B. abortus* 2308W-infected C57BL/6 mice were processed for histological examination and stained with hematoxylin and eosin, and the pathological parameters were observed under a microscope (magnification, ×10). The arrows indicate the presence of granulomas. (Bottom) Semiquantitative estimation of spleen inflammation by evaluating the pathological index. All values in the lower right panel at 14 days of infection (9 days after PMN depletion) were significant ($P < 0.01$ in relation to the mock-treated controls). The error bars represent standard deviations.

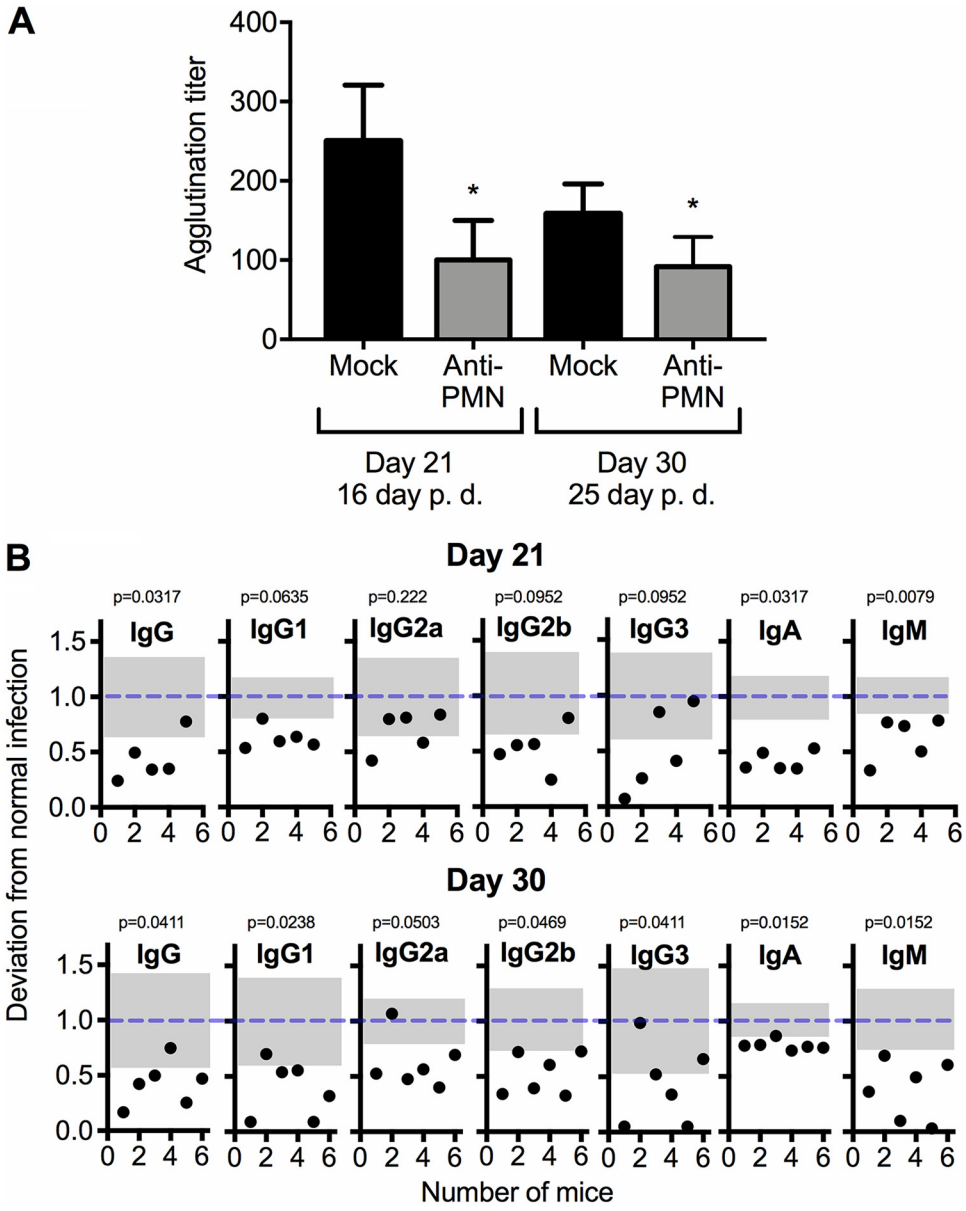

FIG 7 The specific antibody response is depressed in PMN-depleted mice. C57BL/6 mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of *B. abortus* 2308W, and at 5 days of infection, one group of mice was depleted of PMNs by means of RB6-8C5 anti-PMN i.p. injection. (A) Agglutination titers against *Brucella* cells. (B) Isotype antibody responses against *Br*-LPS. Each black dot represents one animal. The dashed lines show the average normalized value of the mock-treated controls, and the gray areas represent the standard deviations of the mock-treated controls. The cutoff and range values of ELISA optical densities are provided in Materials and Methods. *, $P < 0.05$ in relation to the mock-treated controls.

RB6-8C5 antibody, and a significant increase of IgG3 production was observed (see Fig. S4 in the supplemental material).

The absence of PMNs promotes M1 macrophage polarization. *Brucella* organisms manipulate the peroxisome proliferator-activated receptor gamma (PPARγ) pathway to avoid M1 macrophage polarization and benefit from a nutrient-rich environment of alternatively activated M2 macrophages (31). Since polarization towards M1 macrophages is promoted by IFN-γ, we explored the proportion of M1 cells in the PMNd-*Br* mice. As shown in Fig. 8A and B, the relative amounts of lymph node $Ly6C^+/Ly6C^{Hi}$ cells were enhanced in the PMNd-*Br* mice after 9 days of infection. When $Ly6C^+/Ly6C^{Hi}$ cells were analyzed for intracellular IL-6 and inducible nitric oxide

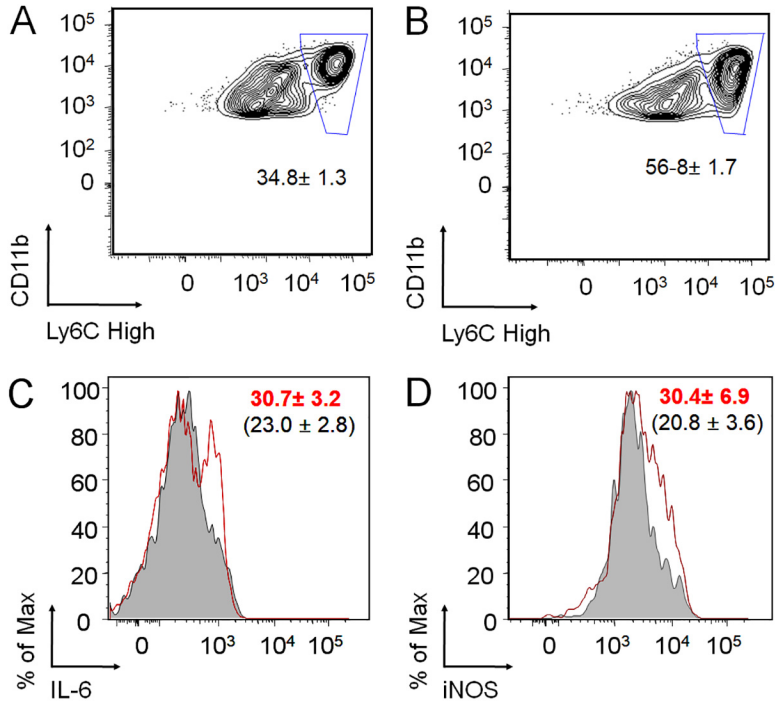

FIG 8 Absence of PMNs promotes M1 macrophage polarization. Lymph node leukocytes of C57BL/6 mice were analyzed by flow cytometry for CD11b, Ly6C, IL-6, and iNOS markers. (A) Lymph node leukocytes of infected mice sorted by $CD11b^{+}/Ly6^{Hi}$. (B) Lymph node leukocytes from PMN-depleted infected mice sorted by $CD11b^{+}/Ly6^{Hi}$. (C) $Ly6C^{+}$ and $Ly6C^{Hi}$ cells from lymph nodes were analyzed in the presence of intracellular IL-6 by flow cytometry. (D) $Ly6C^{+}$ and $Ly6C^{Hi}$ cells from lymph nodes were analyzed for the presence of intracellular iNOS by flow cytometry. The gray areas and the numbers within parentheses correspond to *B. abortus*-infected mice. The red lines marking areas and the numbers in red correspond to PMN-depleted infected mice.

synthase (iNOS) (markers for M1 macrophages), the proportion of macrophages displaying these markers was higher in the PMNd-*Br* mice (Fig. 8C and D).

Anti-*Brucella* antibodies abrogate IFN-γ production at the onset, but not at later times, of infection. Since the large amounts of IFN-γ were inversely correlated with the antibody titers in the PMNd-*Br* mice, we injected nonsterilizing amounts of anti-*Brucella* antibodies at different infection times. Anti-*Brucella* serum given 1 day before infection completely abrogated the IFN-γ response in mice and lowered the bacterial loads (Fig. 9), regardless of the presence or absence of PMNs. However, if the same antibody regime was given after 6 days of infection, the levels of IFN-γ remained unchanged (Fig. 9B). Moreover, after treatment with the corresponding antibodies at 6, 9, and 12 days after infection (see Fig. S1), the levels of IFN-γ were not significantly different at 14 days of infection (Fig. 9C), although the bacterial loads were still lower than in the mock-treated controls (Fig. 9E).

Anti-*Brucella* antibodies abrogate IL-6, IL-10, and IL-12 cytokines in neutropenic mice. Since IL-6, IL-10, and IL-12 cytokines were considerably elevated in PMNd-*Br* mice at later stages of acute infection (day 14 of infection; day 9 postdepletion) (Fig. 4), we explored the effects of anti-*Brucella* antibodies at these times in PMNd-*Br* mice. In contrast to IFN-γ, anti-*Brucella* antibodies dampened the levels of IL-6, IL-10, and IL-12 in the PMNd-*Br* mice (Fig. 10; compare with Fig. 4). Other cytokines, such as IL-4, tumor necrosis factor alpha (TNF-α), and IL-1β, remained close to background levels at all times and in all experiments (Fig. 4 and 10).

DISCUSSION

We have shown that PMN removal before the development of adaptive immunity promotes the elimination of *B. abortus* from target organs at the onset of infection (4).

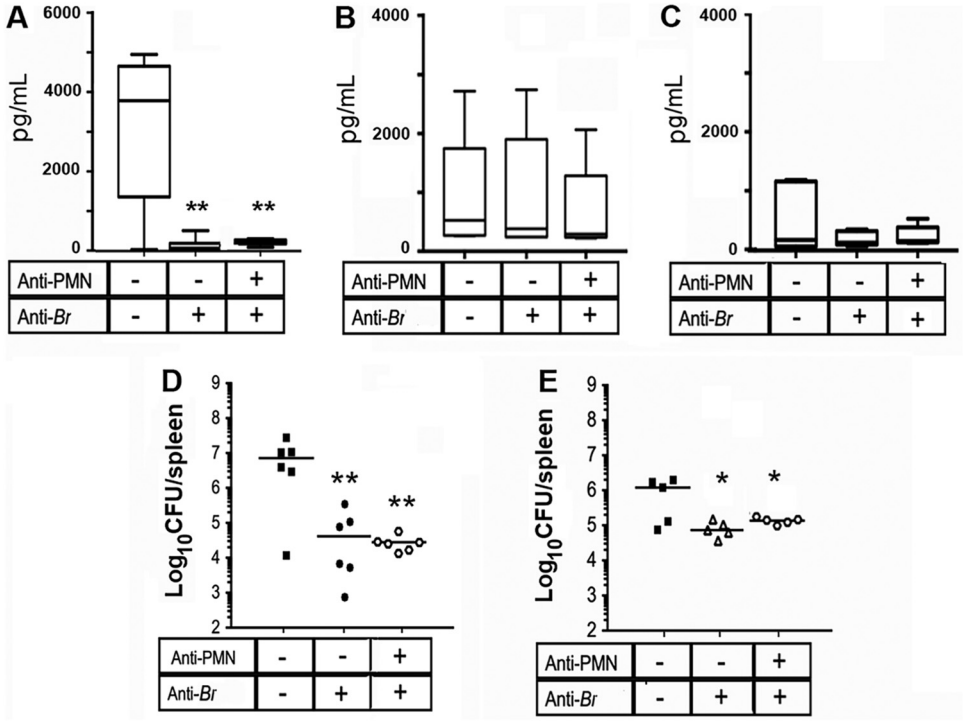

FIG 9 Anti-*Brucella* antibodies dampen IFN-γ production at the onset, but not at later times, of infection. (A) C57BL/6 mice were i.p. injected (+) or not (−) either with immune mouse sera against *Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN (RB6-8C5) 1 day before infection and 2 days after infection, and IFN-γ was measured in the sera of the mice by ELISA after 5 days of infection (see Fig. S1C). (B) Mice were i.p. injected (+) or not (−) either with immune mouse sera against *Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN 5 days after infection, and IFN-γ was measured after 6 days of infection (see Fig. S1D). (C) Mice were i.p. injected (+) or not (−) either with immune mouse sera against-*Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN 6, 9, and 12 days after infection, and IFN-γ was measured after 14 days of infection (see Fig. S1E). (D) Bacterial counts corresponding to the experiment shown in panel A. (E) Bacterial counts corresponding to the experiment shown in panel C. (A to C) Median values are indicated by the horizontal lines within bars. (D and E) Each symbol represents one animal, and the lines represent the medians for each group. *, $P < 0.05$, and **, $P < 0.01$, in relation to the mock-treated controls.

This phenomenon is linked to the efficient recruitment of macrophages and dendritic cells, stronger activation of $CD4^+$ and $CD8^+$ T lymphocytes, and the concomitant increase of IFN-γ (4). Here, we have complemented these findings and demonstrated that the absence of PMNs, after adaptive immunity has fully developed, also favors the efficient elimination of *B. abortus* in mice.

These results seem counterintuitive, mainly when they are compared to the positive role that PMNs play in controlling other bacterial infections, such as those by *Salmonella*, *Yersinia, Legionella*, and *Listeria* organisms (7, 8, 11). In the case of *Brucella* sp. infections, the primary microbicidal function of PMNs is not achieved. Rather, *Brucella* organisms induce the premature death of PMNs in a nonphlogistic manner (19) and dampen the regulatory influence that PMNs have on adaptive immunity at different stages of an infection.

It is known that M1 macrophages are the first line of defense against intracellular pathogens, including *Brucella* organisms (32, 33). The higher production of IFN-γ is correlated with the activation of these cells, the resolution of inflammation, and the efficient elimination of bacteria in PMNd-*Br* mice. Under the influence of IFN-γ, M1 macrophages differentiate, increase their microbicidal activity, and amplify Th1 polarization of $CD4^+$ lymphocytes by IL-12 production (33, 34).

Higher secretion of IFN-γ was a common feature in both the PMNd-*Br* mice at the onset of infection (4) and PMNd-*Br* mice after adaptive immunity had emerged. In spite of this, some significant differences were observed. For instance, the levels of IFN-γ produced at the onset of infection were lower (4) than those recorded once adaptive

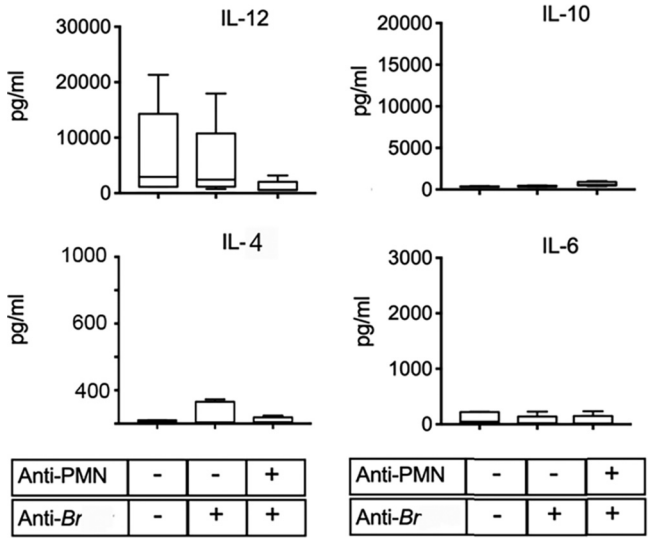

FIG 10 Anti-*Brucella* antibodies dampen proinflammatory cytokines in neutropenic mice at acute stages of infection. C57BL/6 mice were i.p. injected (+) or not (−) either with immune mouse sera against *Brucella* or with a mixture of immune mouse sera against *Brucella* and anti-PMN (RB6-8C5) at 6, 9, and 12 days after infection, and the various proinflammatory cytokines were measured in the sera of the mice by ELISA after 14 days of infection. Median values are indicated by the horizontal lines within the bars. In comparison with cytokine levels in neutropenic infected mice shown in Fig. 4 (far right column in each graph), the levels of IL-12, IL-10, and IL-6 were significantly lower ($P < 0.01$) in neutropenic mice treated with anti-*Brucella* antibodies.

immunity had developed. While in the former case the levels of IFN-γ were not associated with sickness, in the latter case weight loss and cachexia were observed in the neutropenic infected mice. This was an unexpected clinical feature. Indeed, *Brucella*-infected mice seldom show sickness during the early days of infection (23). It seemed, therefore, that the very high levels of IFN-γ (close to 7,500 pg/ml) and the subsequent activation of the immune system were not without a price (35).

It has been shown that IL-12 is an essential cytokine to retain a Th1 response in brucellosis (36). The higher levels of IL-12 at later times of acute infection in the PMNd-*Br* mice revealed no shift toward a Th2 response. Moreover, cytokine IL-4 always remained close to background levels. The negligible amount of IL-4 in the sera and spleen cells of infected mice during brucellosis is a well-known feature and delineates the Th1 predominant immune response (15, 23). It is also known that removal of IL-4 depresses anti-*Brucella* antibody response, indirectly favoring the Th1 response (37).

The rise of IL-10 and IL-6 in the PMN-depleted infected mice at later times of infection correlated with the decreasing levels of IFN-γ. The lack of IL-10 has been related to lower *B. abortus* survival at early stages of infection and linked to the regulation of IFN-γ at later stages (31, 37). Likewise, IL-6 limits the recruitment of innate immune cells and therefore represents a critical element in the regulation of inflammation (38). A rise in IL-6 has also been observed at the onset of infection in PMNd-*Br* mice, including the Genista strain (4).

It is worth noting that anti-*Brucella* antibodies dampened IFN-γ only when given before infection. This seems to be related to the fast removal of bacteria, which hampers the development of adaptive immunity, a trend observed previously with other bacteria (39, 40). However, after the initiation of adaptive immunity, anti-*Brucella* antibodies did not influence the levels of IFN-γ, regardless of the presence or absence of PMNs or the number of bacteria. This agrees with previous data showing that the levels of IFN-γ generated are independent of the bacterial load in *B. abortus*-infected mice (4). In contrast, anti-*Brucella* antibodies dampen IL-6, IL-10, and IL-12 in neutropenic mice at later stages of acute infection (compare Fig. 4 with Fig. 10), a fact that correlates with the lower numbers of bacteria in the treated mice.

It was clear that the efficient elimination of bacteria in the neutropenic mice was not linked to the rise of antibodies or to increased levels of specific immunoglobulin isotypes against *Brucella* antigens. On the contrary, lower antibody titers were observed after 3 and 4 weeks of infection. This may be the result of a stronger cellular immunity, promoted by the high levels of IFN-γ during the acute phase of infection. The fact that IFN-γ exerts a regulatory influence on the production of immunoglobulin isotypes against *Brucella* antigens supports this (30). In addition, mice devoid of B cells (and thus deprived of antibodies) eliminate *B. abortus* more efficiently, which is linked to higher levels of IFN-γ and to a stronger cellular Th1 response (41). Although the lower bacterial loads could have had some influence on the antibody titers, this seems unlikely. It has been shown that once antibodies are produced, they remain at the same high levels, regardless of the number of *Brucella* organisms present in the target organs (42). Likewise, an increase in bacterial loads was recorded in the PMNd-*Br* mice on the first day after PMN removal (Fig. 1 and 2).

The ability of *Brucella* organisms to produce chronic infection is linked to their long persistence in the BM (17, 43). In humans and mice, colonization of the BM by *Brucella* organisms causes neutropenia, thrombocytopenia, anemia, pancytopenia, and other pathological signs (17, 43, 44). The bacterium resides within BM monocytes, PMNs, and, to a lesser extent, granulocyte-monocyte progenitors (17). Therefore, the abrogation of the *B. abortus* infection in the BM of PMNd-*Br* mice was intriguing, considering the significant number of PMNs remaining in the BM after repeated injections of anti-PMN (see Table S1). Whether anti-PMN antibodies remove mostly mature and functional PMNs from the BM remains to be studied.

The precise routes by which PMNs regulate other cells of the immune system remain elusive, and those proposed for other pathogens do not match our observations (45, 46). For instance, in a murine model of *Legionella pneumophila* infection, PMN depletion led to more Th2 skewing and more disease (8). This is striking, since in both bacterial diseases IFN-γ plays a central role (28, 29, 47), and the pathogenic mechanisms and intracellular life cycles of the two bacteria display some resemblances (48). Other regulatory mechanisms, such as direct contact between PMNs and lymphocytes, macrophages/monocytes, and dendritic cells, have been discussed (4). Regulation through PMN cytokines seems unlikely, since the amounts of proinflammatory cytokines released by *B. abortus*-infected PMNs are negligible (19).

One alternative mechanism that explains the phenomenon observed here is related to the "Trojan horse" hypothesis (19, 24). This mechanism proposes that prematurely dying *Brucella*-infected PMNs displaying "eat me" signals are readily phagocytized by cells of the mononuclear phagocytic system in a nonphlogistic manner (19). This opens a window for the intracellular trafficking of brucellae to the endoplasmic reticulum and eventual replication in these phagocytic cells. This delays the activation of the adaptive immune system, allowing the stealthy organism to establish a long-lasting infection (11, 15, 24). In the absence of PMNs, this mechanism is shattered, allowing mononuclear phagocytic cells to interact directly with the bacterium in a proinflammatory manner. This allows strong activation of the immune system, reflected by increased release of IFN-γ by $CD4^+$ and $CD8^+$ cells and polarization of macrophages toward M1, which is central to combating intracellular parasites. This proposal fits the Occam's razor principle of parsimony, previous experimental data, and the results presented here.

MATERIALS AND METHODS

Ethics. Experimentation with mice was conducted following the guidelines of the Comité Institucional para el Cuido y Uso de los Animales of the Universidad de Costa Rica (CICUA-019-16) and in agreement with the corresponding law (Ley de Bienestar de los Animales de Costa Rica; law 9458 on animal welfare). Mice were housed in the animal facility of the Veterinary Medicine School of the National University of Costa Rica. The mice were kept in cages with food and water *ad libitum* under biosafety conditions.

Generation of neutropenic mice. Inbred C57BL/6 mice (18 to 21 g) were used in the experiments. Neutropenic mice were generated as previously described (4, 15). Briefly, mice were depleted of PMNs by means of intraperitoneal (i.p.) injection of 100 μg of rat anti-mouse Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5; Bio X Cell) or 500 μg of anti-mouse Ly6G (clone 1A8; BD Biosciences) in 0.1 ml phosphate-buffered saline

(PBS). PMN depletion was confirmed by the absence of $CD11b^+$ $Ly6G^+$ cells by flow cytometry and microscopic examination of blood, spleen, lymph nodes, and BM (4) (see Table S1). A single i.p. injection of anti-PMN antibody resulted in the depletion of PMNs from blood, spleen, and lymph nodes for at least 3 days (see Table S1). PMN depletion in the BM was achieved only to 25 to 30% (see Table S1). Differences in depletion were observed between RB6-8C5 and 1A8 antibodies. In order to maintain the neutropenic stage, mice were injected with the indicated antibody every 3 days according to the following protocols: (i) depletion at the onset of innate immunity, (ii) depletion at the onset of adaptive immunity, and (iii) depletion at the acute phase of infection (see Fig. S1). In all experiments, nonimmune rat IgG was used and administered at the same concentrations and by the same route as the anti-PMN antibodies (mock-treated controls). After 8 days from the first anti-PMN injection, the mice developed antibodies against the anti-PMN antibody (4). A detailed time course protocol and kinetics for the RB6-8C5-PMN depletion after *B. abortus* infection has been reported previously (4).

***Brucella abortus* infection.** Mock-treated and PMN-depleted mice were i.p. infected with 0.1 ml of PBS containing 10^6 CFU of virulent *B. abortus* 2308W (49) as described previously (4). Bacterial colonization was determined in spleens and BM of mice collected at the indicated times, following previously published protocols (4, 17, 50). Serial dilutions of infected macerated tissues were plated on Trypticase soy agar and incubated at 37°C for 72 h in the presence 5% CO_2, and bacterial CFU were determined (50). Spleens from the mice were processed for histopathological studies as described previously (51). Blinded evaluation of histopathology slides was performed. The inflammatory stage was evaluated using a semiquantitative scoring system (31).

Antibody and cytokine determination. Murine hyperimmune serum production against *Brucella* antigens and IgG purification were carried out following previously published protocols (21). PMN-depleted mice and the mock-treated control mice were bled at different times, serum was separated from cells, and antibody titration was carried out in 96-well round-bottom plastic plates as described previously (21). After titration, immune sera were stored at −20°C in aliquots. Western blotting revealed that most antibody recognition was directed against *Br*-LPS.

For isotype antibody determination against *Br*-LPS, enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well plates (Nunc) as previously described (52). Briefly, the 96-well plates were coated with 0.1 ml of 10-μg/ml *Br*-LPS. Mouse serum was diluted 1:200 in blocking buffer (PBS with 0.4% bovine serum albumin [BSA] and 0.05% Tween 20) and then incubated on plates for 1 h at 37°C, followed by extensive washing (PBS with 0.05% Tween 20). Secondary horseradish peroxidase (HRP) antibody conjugates against mouse IgG, IgM, IgG1, IgG2a, IgG2b, and IgA (all from Sigma-Aldrich) at the adjusted dilution in the blocking buffer were used for immunoglobulin isotyping. After washing the plates, the reaction was developed with HRP substrate (Sigma-Aldrich), and the optical density was measured at 450 nm. Serial dilutions of the murine hyperimmune serum (positive-control serum) and the respective conjugates were performed in order to establish the optimal cutoff value for each conjugate in comparison to sera from uninfected mice. The negative-control serum optical density for each conjugate was adjusted to 0.110 ± 0.025 nm, while the positive-control serum optical density was adjusted to 1.200 ± 0.150 nm. The cutoff value was estimated at 0.200 nm.

The levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, and TNF-α cytokines were measured in sera by ELISA (eBioscience), according to the manufacturer's specifications.

Flow cytometry. Flow cytometry was carried out as previously described (4). Phycoerythrin (PE) anti-CD11b (M1/70), Alexa Fluor 488 anti-Ly6C (AL-21), and PE cyanine 5.5 anti-Ly6G (1A8) antibodies were purchased from BD Biosciences, and 1A8 and RB6-8C5 neutralizing antibodies were from Bio X Cell. Blood, spleen, and bone marrow cells were prepared as described previously (4, 17). Popliteal, inguinal, and mesenteric lymph nodes were prepared as described previously (53) and processed for flow cytometry as described previously (4). Intracellular staining was performed with allophycocyanin (APC) anti-iNOS (clone CXNFT) and PE anti-IL-6 (clone MP5-20F3) with the respective isotype controls, all from Invitrogen. Before staining with different antibody mixtures, cells were preincubated on ice for at least 10 min with the anti-mouse CD16/CD32 (clone 2.4G2) monoclonal antibody to block Fc receptors (BD Biosciences). Multiparameter fluorescence-activated cell sorter (FACS) analysis was performed, using a Guava easyCyte flow cytometer (Millipore). The FACS data were analyzed using Flow Jo software, version 10.4. For each experiment, control mice were included to define the proper gates. Blood was stained directly with the antibodies and lysed with BD FACS lysing buffer (BD Biosciences). If the mice had been previously treated with PMN-depleting antibodies, the blood samples were washed thoroughly (four times) with PBS to remove anti-Ly6G from the sera before the staining process. All the samples were washed and resuspended in PBS prior to acquisition.

Statistics. The data were processed in Microsoft Office Excel. To determine statistical significance, comparison of two samples was performed by a Mann-Whitney test, and multiple comparisons were established by a Kruskal-Wallis test using the GraphPad software package (version 7.0; GraphPad, La Jolla, CA, USA). For antibody isotype comparison, values were normalized by adjusting the measurements of the different scales to a notionally common scale. For all tests, P values of <0.05 and <0.01 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00118-19.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 3, PDF file, 1.5 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 5, PDF file, 0.01 MB.
SUPPLEMENTAL FILE 6, PDF file, 1.1 MB.

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We have no conflicts of interest to disclose.

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Annex 3

Annex. 3.1.

Barquero-Calvo, E., Mora-Cartín, R., Arce-Gorvel, V., de Diego, J. L., Chacón-Díaz, C., Chaves-Olarte, E., … Moreno, E. (2015). *Brucella abortus* Induces the Premature Death of Human Neutrophils through the Action of Its Lipopolysaccharide. *PLoS Pathogens*, *11*(5), e1004853. https://doi.org/10.1371/journal.ppat.1004853

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RESEARCH ARTICLE

Brucella abortus Induces the Premature Death of Human Neutrophils through the Action of Its Lipopolysaccharide

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Abstract

Most bacterial infections induce the activation of polymorphonuclear neutrophils (PMNs), enhance their microbicidal function, and promote the survival of these leukocytes for protracted periods of time. *Brucella abortus* is a stealthy pathogen that evades innate immunity, barely activates PMNs, and resists the killing mechanisms of these phagocytes. Intriguing clinical signs observed during brucellosis are the low numbers of *Brucella* infected PMNs in the target organs and neutropenia in a proportion of the patients; features that deserve further attention. Here we demonstrate that *B*. *abortus* prematurely kills human PMNs in a dose-dependent and cell-specific manner. Death of PMNs is concomitant with the intracellular *Brucella* lipopolysaccharide (*Br*-LPS) release within vacuoles. This molecule and its lipid A reproduce the premature cell death of PMNs, a phenomenon associated to the low production of proinflammatory cytokines. Blocking of CD14 but not TLR4 prevents the *Br*-LPS-induced cell death. The PMNs cell death departs from necrosis, NETosis and classical apoptosis. The mechanism of PMN cell death is linked to the activation of NADPH-oxidase and a modest but steadily increase of ROS mediators. These effectors generate DNA damage, recruitments of check point kinase 1, caspases 5 and to minor extent of caspase 4, RIP1 and Ca^{++} release. The production of IL-1β by PMNs was barely stimulated by *B*. *abortus* infection or *Br*-LPS treatment. Likewise, inhibition of caspase 1 did not hamper the *Br*-LPS induced PMN cell death, suggesting that the inflammasome pathway was not involved. Although activation of caspases 8 and 9 was observed, they did not seem to participate in the initial triggering mechanisms, since inhibition of these caspases scarcely blocked PMN cell death. These findings suggest a mechanism for neutropenia in chronic brucellosis and reveal a novel *Brucella*-host cross-talk through which *B*. *abortus* is able to hinder the innate function of PMN.

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Author Summary

The absence of obvious clinical symptoms during the early stages of brucellosis is linked to the *Brucella* stealthy strategy and its non-canonical PAMPs, which are low PRRs agonists. Still, there are clinical profiles that require explanation. For instance –despite the fact that neutrophils readily ingest *Brucella* during the onset of infection, brucellosis courses without neutrophilia, and just a low number of infected neutrophils are present in target organs. In the chronic phases, a significant proportion of the patients display absolute neutropenia and bone marrow pancytopenia linked to the myeloid cell linage. Examination of the *Brucella* infected bone marrow reveals granulomas and phagocytosis of myeloid cells. Based on these observations we explored the fate of native neutrophils during their interaction with *Brucella*. We found that the bacterium induces the premature cell death of neutrophils without inducing proinflammatory phenotypic changes. This event was reproduced by the lipid A of the *Brucella* LPS and depends on NADPH-oxidase activation and low ROS formation. We believe that this phenomenon explains –at least in part– the hematological and histological profiles observed during brucellosis. In addition, it may be that dying *Brucella*-infected neutrophils serve as "Trojan horse" vehicles for infecting phagocytic cells without promoting activation.

Introduction

Polymorphonuclear leukocytes (PMNs) represent a key cellular component of the host's antibacterial arsenal. Once in the circulation, the average lifespan of PMNs is close to 5.4 days, period after which they undergo spontaneous apoptosis [1]. This is in frank contrast to the previously reported short lifespans of a few hours for these cells [2]. Then, these dead PMNs are removed by phagocytic cells laying in the reticuloendothelial system, such as monocytes (Mo), macrophages (Mϕ) and dendritic cells (DCs) [3]. This physiological phenomenon does not induce proinflammatory signals and is regarded as a constitutive mechanism to maintain leukocyte homeostasis [4].

Upon bacterial infection, PMNs are activated and migrate into tissues, where they may survive three to five days to perform their phagocytic, microbicidal and proinflammatory functions [1,5]. These events are part of the innate immune response commonly triggered by pathogen-associated molecular patterns (PAMPs) [6] or by danger signals that guide the PMNs response [7].

A variety of microbes have evolved strategies to influence the timing and mode of PMN cell death [8–10]. For instance, *Shigella flexneri* kills PMNs by necrosis, a process characterized by the release of tissue-injurious granular proteins. This contributes to disruption of the intestinal epithelial barrier, leading to the dysentery observed in shigellosis and allowing the bacterium to enter its colonic host cells [11]. Similarly, *Pseudomonas aeruginosa* infections may cause lysis or oncosis of PMNs, leading to persistent infections by depleting these cells and contributing to the pulmonary pathophysiology by facilitating bacterial extracellular replication [12,13]. Others, such as the obligate intracellular *Anaplasma phagocytophilum* and *Chlamydia pneumoniae* are able to inhibit PMN cell death to achieve intracellular replication within these leukocytes [14,15].

Brucella microorganisms are stealthy alpha-protobacterial intracellular pathogens of mammals, including humans [16,17]. In the early stages of infection, *Brucella* minimizes the host proinflammatory response, opening an immunological window that allows this bacterium to invade and reach sheltered intracellular niches before adaptive immunity becomes effective [16,18,19]. Once established, *Brucella* organisms survive and extensively replicate within the intracellular milieu of Mo, Mϕ, DCs and placental trophoblasts [20,21]. As part of its parasitic strategy, *Brucella* inhibits apoptosis and prolongs the life of these infected mononuclear phagocytic cells [16,22]. Although *Brucella* is readily internalized by PMNs [23,24], the bacterium survives inside the phagosomes of these cells resisting their killing action including oxidative components and isolated lysosomal extracts [16,25,26].

During the course of human and animal brucellosis, there are several clinical and pathological features related to PMNs which biological mechanisms remain unclear. Among the most striking signs are the neutropenia observed during chronic brucellosis, the absence of recruitment of PMNs at the site of infection and the low numbers of *Brucella* infected PMNs in the target organs [16,27–30]. Moreover, PMNs have an unexpected influence in dampening the immune response against intracellular *Brucella* infection and strengthen the notion that PMNs actively participate in regulatory circuits shaping both innate and adaptive immunity [19].

In an attempt to improve our understanding of the mechanisms underlying the fate of PMNs during brucellosis, we have explored the outcome of these leukocytes upon interaction with *Brucella abortus*. Our findings reveal a novel microbial-host cross-talk through which *B. abortus* is able to hinder and evade host innate PMN response and suggest a mechanism by which *Brucella* may hamper the presence of infected PMNs in the target organs and promote neutropenia during chronic brucellosis.

Results

B. abortus resists the killing action of PMNs

Confirming previous reports [16,18,31], *Brucella* is more resistant than other bacteria to the killing action of PMNs (Fig 1A). This resistance is not related to reduced bacterial internalization, since at multiplicity of infection (MOI) of 5, both *B. abortus* and *Salmonella enterica*, were phagocytized at similar rates. Due to the toxic effects mediated by *Salmonella* on PMNs, higher MOIs of this bacterium were precluded. Compared to latex beads, fluorescent *B. abortus*-GFP was internalized more efficiently by PMNs at different MOIs, suggesting an active PAMP receptor-mediated phagocytosis (Fig 1B). Early phagocytosis of *B. abortus*-GFP (MOI < 50) was not accompanied by obvious PMN shape changes such as nuclear rounding, chromatin condensation, cell fragmentation, degranulation (Fig 1C) or myeloperoxidase activity [16]. This observation is in agreement with previous reports [16,25,31,32]. Only when high loads of *B. abortus*-GFP were tested (MOI > 50) changes in nuclear morphology was detected in a proportion of PMNs containing more than 50 bacteria/cell (Fig 1C).

B. abortus infection induces PMN cell death in a dose-dependent manner

After infection with *Brucella*-GFP, PMN cell death was assessed by flow cytometry using Annexin V and AquaDead as markers. After two hours of incubation (MOI = 10), *Brucella* infected PMNs (whole blood or purified PMNs, see below) became positive for both markers, following a bacterial dose dependence (Fig 2). This phenomenon did not require live bacteria, since similar effects were observed in PMNs exposed to equivalent doses of live or heat killed *B. abortus* (HKBA) (Fig 3).

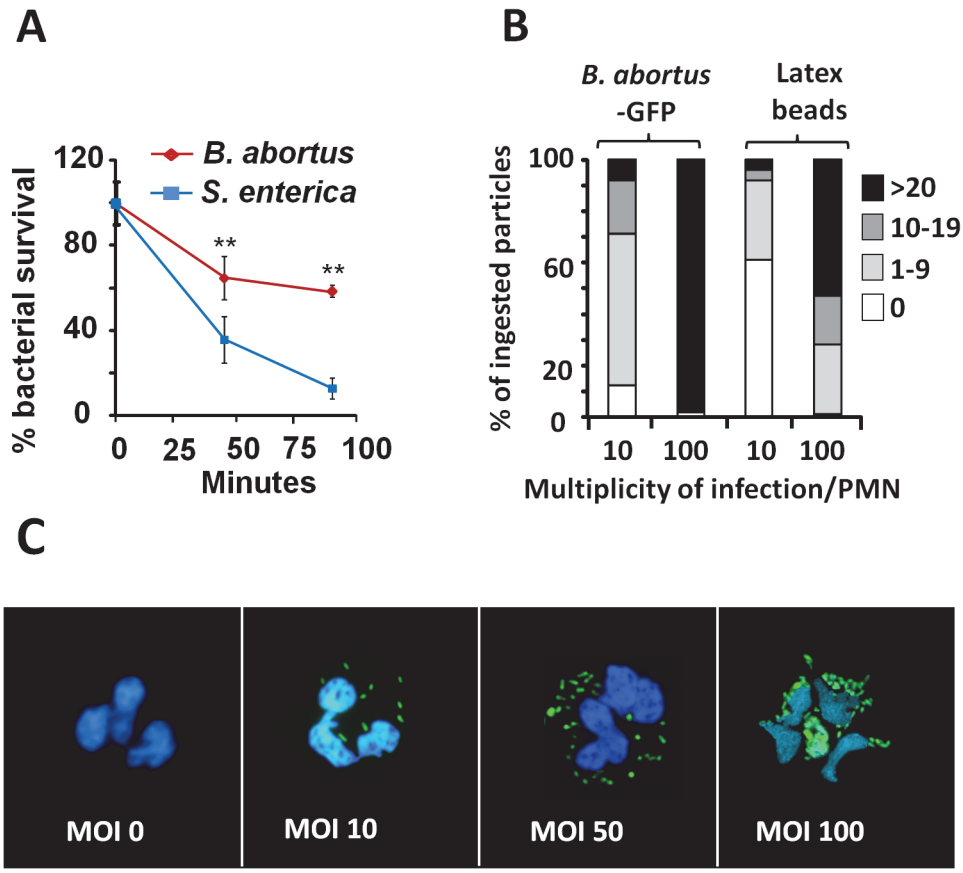

Fig 1. *B. abortus* is partially resistant to the killing action of PMNs. (A) PMNs were isolated from blood and incubated with *B. abortus* or *S. enterica* (MOI 5) and CFUs determined at different time points. (B) Heparinized blood was incubated with *B. abortus*-GFP or fluorescent latex beads for two hours (MOI 10 or 100). Blood smears were then fixed and mounted with ProLong Gold Antifade Reagent with DAPI. At least 100 PMNs were counted per sample and the number of intracellular bacterial or latex particles determined in each PMN and the proportion expressed as % of phagocytized particles. (C) Human PMNs infected with different MOI of *B. abortus*-GFP and stained as in "B". Microscope images are at 400 × magnification. Representative PMNs with DAPI-stained nuclei and intracellular green fluorescent *B. abortus* were photographed under the microscope 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 then merged using Adobe Photoshop 8 software. Experiments were repeated at least three times. Values of $p<0.01$ (**) are indicated.

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B. abortus releases *Br*-LPS inside vacuoles of PMNs

We have demonstrated that *B. abortus* sheds non-toxic *Br*-LPS inside cells and that this molecule traffics in vacuoles and influences antigen presentation to T cells [33–35]. Following this, we explored the shedding of *Br*-LPS inside PMNs by live *B. abortus*. For this purpose, we used a double labeling fluorescence method [36]. First PMNs were infected with *B. abortus*-RFP at a MOI of 5. After 1 h of incubation, PMNs were permeabilized and treated with anti-*Br*-LPS FITC-antibody and counterstained with DAPI. This approach revealed that significant amounts of *Br*-LPS molecules (green fluorescence) were released intracellularly by live (red fluorescent) *Brucella* in the proximity of bacteria-containing PMN phagosomes (Fig 4). Almost all *B. abortus*-RFP infected PMNs exhibited this pattern after 1 h infection, most strikingly evident by immunofluorescence in cells containing between 2–3 bacteria/PMN.

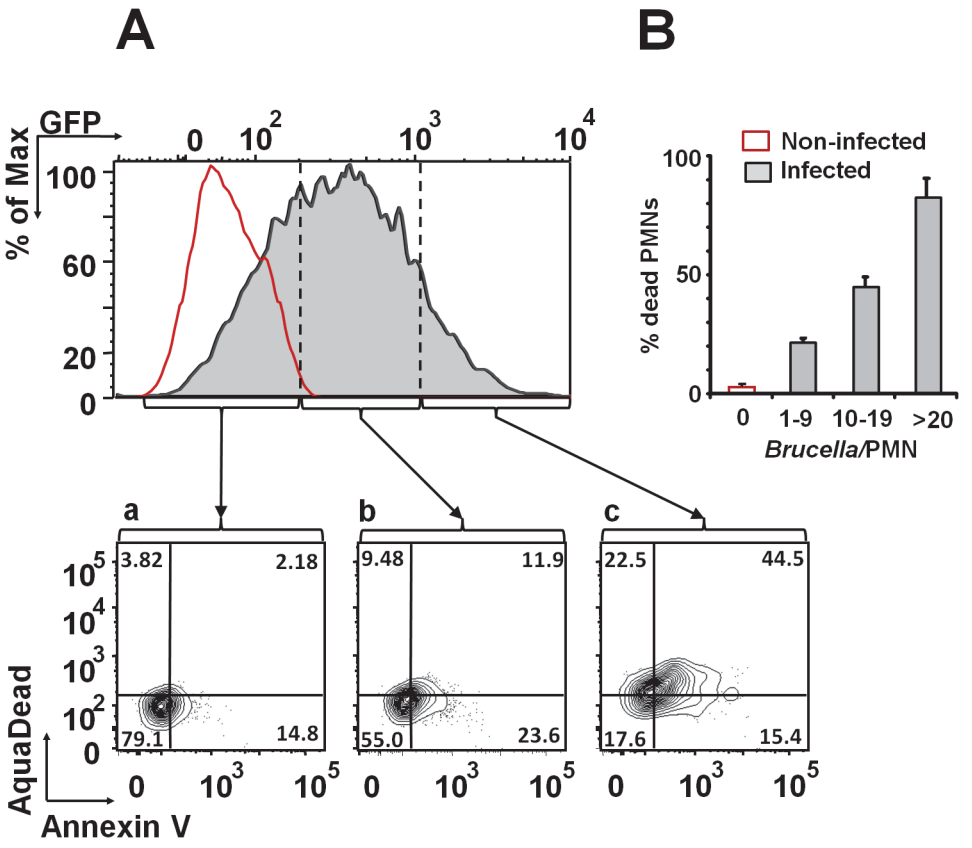

Fig 2. *B. abortus* infection induces PMN cell death in a dose dependent manner. (A) Heparinized blood was incubated with *B. abortus*-GFP (MOI 10) for two hours and PMNs population analyzed for cell death using AquaDead and Annexin V markers. GFP fluorescence intensity was used to differentiate among three categories: (a) low, (b) intermediate and (c) high infection. (B) Percentages of PMNs positive for any marker in relation to the number of internalized bacteria are shown. Experiments were repeated at least three times.

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In order to determine if *Br*-LPS was released inside vacuoles or translocated to the cytosol, *B. abortus* infected PMNs were subjected to immunodetection of *Br*-LPS by electron microscopy. Regular osmium tetroxide staining of infected PMNs (1 hour) demonstrated that all phagocytized *B. abortus* reside inside phagosomes, and just a few of them within phagolysosomes, confirming previous results [37]. As expected, sensitive staining for detection of immunogold particles revealed the presence of *Br*-LPS in the bacterial cells. However, vesicles in the proximity of the ingested *Brucella* also contained gold particles, indicating the presence of *Br*-LPS within vacuoles (Fig 5). In some cells, immunogold stained *Br*-LPS molecules were detected within a phagosome containing partially digested bacteria or in the proximity of cell membrane ruffle-like structures (Fig 5E). Gold particles were practically absent in the cytosol and not detected in the extracellular milieu (Fig 5A).

Br-LPS specifically induces the cell death of PMNs

Intracellular *Br*-LPS influences the antigen presentation of Mϕ without affecting the survival of these cells [34,35]. Therefore, we assessed the effects of *Br*-LPS on PMNs cell survival. As demonstrated in Fig 6A, *Br*-LPS induced PMN cell death in a dose-dependent manner in blood or in purified (see the results presented in the next sections) PMNs. This effect was specific for PMNs since other cells, such as lymphocytes, treated and gated under the same conditions, did

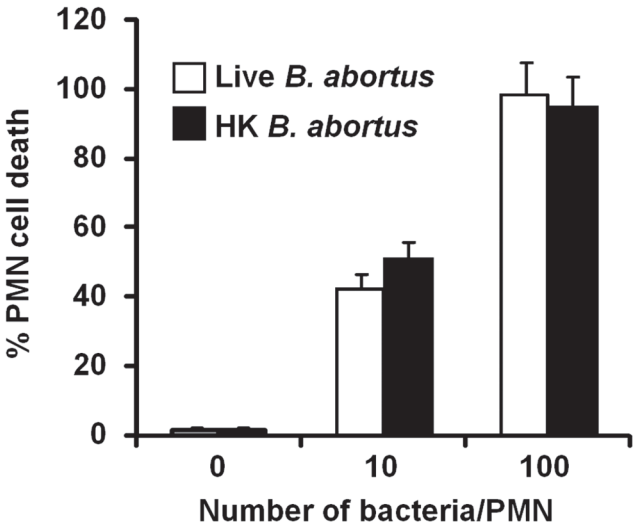

Fig 3. Live and heat-killed *B. abortus* induce PMN cell death. Heparinized blood was incubated with live or heat-killed (HK) *B. abortus* for two hours (10 and 100 bacteria/PMN). PMN population was analyzed by flow cytometry for cell death using AquaDead and Annexin V markers, as described in Fig 2. Percentages of PMNs positive for any marker were determined. Experiments were repeated at least three times. No significant differences were detected between live and HK *B. abortus*.

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not display death cell markers (Fig 6B). Consistent with previous observations [22,38], *Br*-LPS did not induce cell death of Mϕ, Mo and DCs. In contrast, *Escherichia coli* LPS (*Ec*-LPS) did

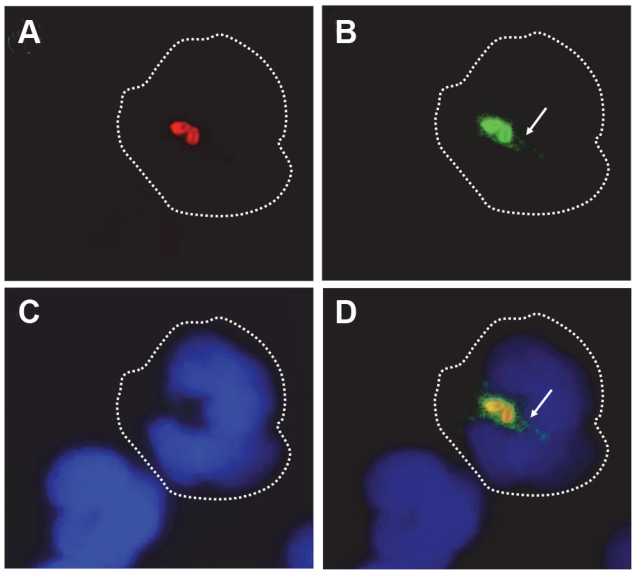

Fig 4. *Br*-LPS is released inside PMNs. Heparinized blood was incubated with *B. abortus*-RFP for one hour (MOI 2). Blood smears were fixed, stained with anti-*Brucella* LPS FITC (green) and mounted with ProLong Gold Antifade Reagent with DAPI. (a) *B. abortus*-RFP, (b) IgG-FITC anti-*Brucella* LPS staining, (c) PMN DAPI staining and (d) merged images. Shed *Brucella* LPS (white arrow) is pointed. Representative PMNs with DAPI-stained nuclei and intracellular *B. abortus* were photographed under the microscope 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 then merged using Adobe Photoshop 8 program. Microscope images are at 1000 × magnification.

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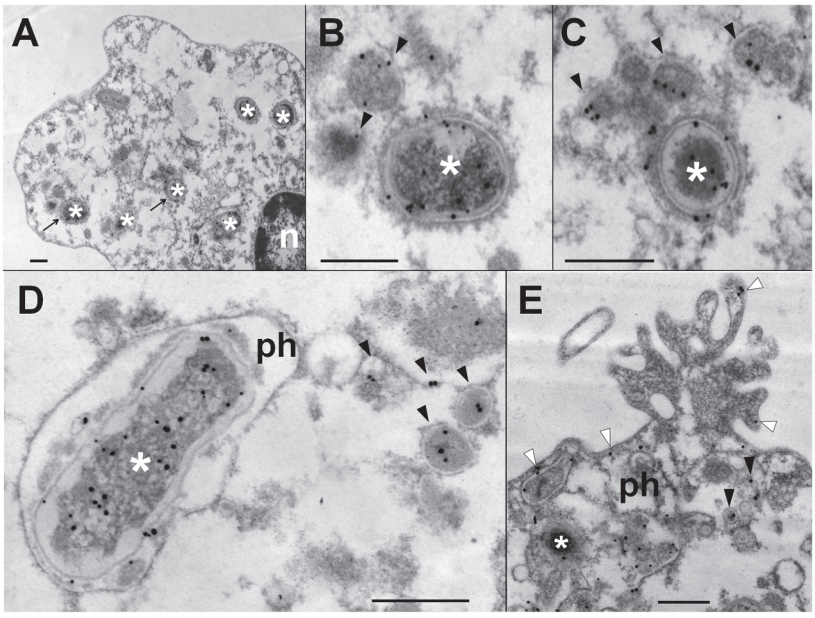

Fig 5. *Br*-LPS released inside cells is mostly found within vacuolar compartments of PMNs. Purified human PMNs 5 ×10^6 were infected with *B*. *abortus* 2308 at MOI 20. After one hour incubation, infected cells were fixed and processed for immunogold staining and electron microscopy. Detection of *Br*-LPS was performed using mouse IgG anti Br-LPS in combination with protein-A/protein-G colloidal gold 15 nm. (A) PMN (n, nucleus of cell) with intracellular *B*. *abortus* (white asterisk) and immunogold detection of *Br*-LPS. (B) and (C) correspond to amplified sections pointed with arrows from "A" panel; *B*. *abortus* (white asterisk) and immunogold detection of *Br*-LPS inside vacuoles (pointed by black arrow heads). (D) *B*. *abortus* (white asterisk) within a phagosome (ph) and vacuoles containing immunogold labeled *Br*-LPS (black arrow heads). (E) PMN membrane ruffle showing immunogold detection of *Br*-LPS associated to the membrane (white arrow heads) and *B*. *abortus* (white asterisk) debris inside a phagosome (ph) and immunogold detection of *Br*-LPS inside vacuoles (black arrow heads). No colloidal gold particles were observed when IgG purified from normal mouse serum was used for controlling the specificity of the reaction. Bar represents 500 nm.

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not induce cell death in blood (Fig 6A) or in purified PMNs under the same experimental conditions.

In view of the relatively high amounts of *Br*-LPS added to induce PMN cell death, a quantitative determination of the *Br*-LPS interacting with these cells was performed. For this purpose, purified PMNs were incubated with *Br*-LPS and the associated amounts determined by Western blotting (Fig 7). In order to have a saturating positive control, the assay was also performed in the presence of human antibodies against *Br*-LPS. The estimated quantities of associated *Br*-LPS in the absence of antibodies ranged between 5–25 ng/10^6 PMNs. Likewise, the amounts of associated *Br*-LPS in the presence of antibodies were between 10–50 ng/10^6 PMNs. This result indicates that the actual quantities of *Br*-LPS interacting with PMNs under these experimental conditions corresponded just 0.05–0.25% of the total *Br*-LPS added. As expected, antibodies increased close to 10 times the quantities of *Br*-LPS associated to PMNs through the concourse of Fc receptors. It should be noticed that the molecules associated to PMNs corresponded to the lower molecular weight (~30–40 MW) fraction of *Br*-LPS. This indicates that among all the *Br*-LPS molecules available, just specific classes are selected by PMNs.

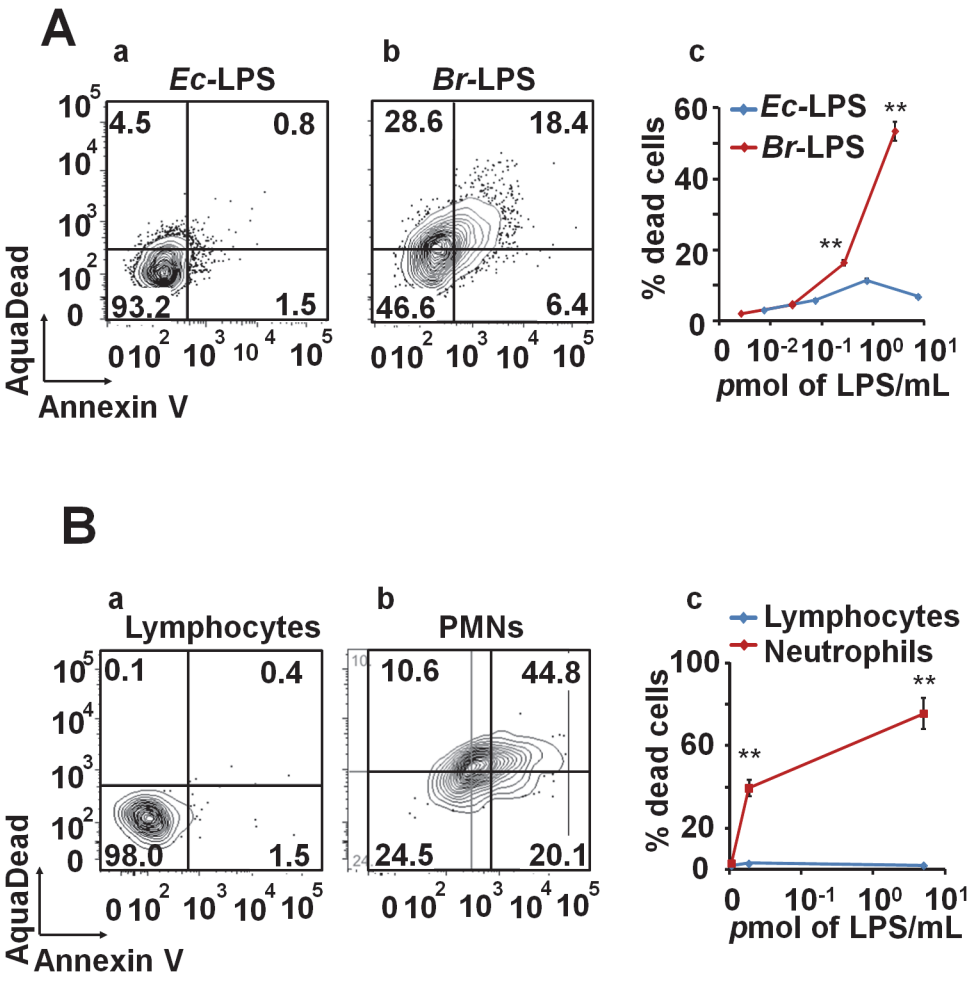

Fig 6. *Br*-LPS induces cell death of PMN in a dose dependent manner. (A) Heparinized blood was incubated with 100μg/mL of (a) *Ec*-LPS (corresponding to 7.5 pmol/mL) or (b) *Br*-LPS (corresponding to 3 pmol/mL) for two hours and the PMN population was analyzed by AquaDead and Annexin V markers as in Fig 2. (c) Percentages of PMNs positive for any marker treated and with various concentrations of LPS are shown. (B) Human blood was incubated with 3 pmol/mL of *Br*-LPS for two hours. (a) Lymphocyte and (b) PMN populations were analyzed by AquaDead and Annexin V markers. (c) Percentages of lymphocytes and PMNs positive for any marker treated and with various concentrations of *Br*-LPS for are shown. Experiments were repeated at least three times. Values of $p<0.01$ (**) are indicated.

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The lipid A moiety of the *Br*-LPS is responsible for the induction of PMN cell death

The non-toxic *Br*-LPS is built of an O-chain constructed of N-formyl perosamine sugar homopolymer, a positively charged core oligosaccharide and a lipid A containing a diaminoglucose disaccharide backbone substituted with long chain hydroxylated, cyclic and non-hydroxylated fatty acids (S1 Fig). In an attempt to identify the moiety responsible for inducing the PMN cell death, we first tested the biological action of different LPSs that shared at least some of the *Br*-LPS structural features [18,39–45]: i) *Yersinia enterocolitica* O:9 LPS displays the same O-chain homopolymer as *Br*-LPS but has different lipid A and core oligosaccharide; ii) *Ochrobactrum anthropi* LPS shares the lipid A structural features with *Br*-LPS but possesses different O chain and core oligosaccharide; iii) *B. abortus* ΔWadC LPS displays the same lipid A and O chain as the *Br*-LPS, but has a different core oligosaccharide; finally, iv) the overall structure of *Ec*-LPS

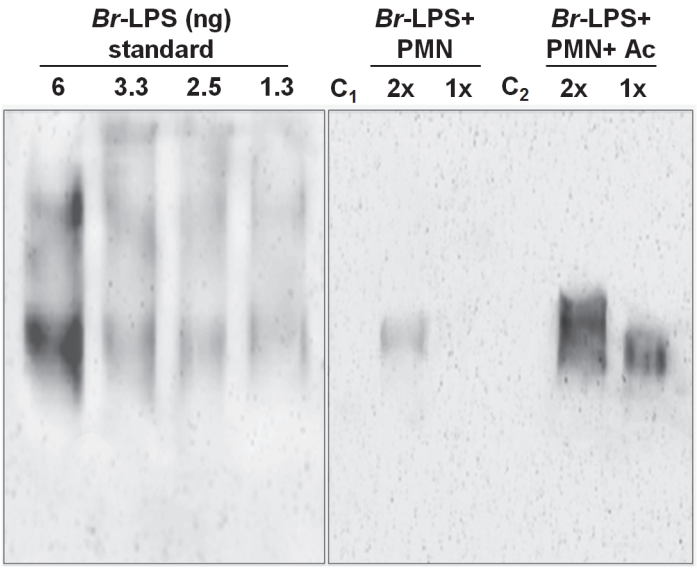

Fig 7. Quantities of *Br*-LPS interacting with PMNs. The quantities of *Br*-LPS associated to PMNs were determined by Western blotting using a monoclonal antibody against *Br*-LPS conjugated with peroxidase enzyme. All wells were loaded with 15 μL of the respective preparation. The amounts of purified *Br*-LPS in the left panel were used to estimate the quantities based on a standard curve ranging from 0.06 ng to 12 ng (only wells from 1.3–6 ng are shown). The right panel corresponds to the assay: purified PMNs were incubated with *Br*-LPS and the associated amounts determined by Western blot (Br-LPS+PMNs). In order to have a saturating positive control, the assay was also performed in the presence of human antibodies against *Br*-LPS (*Br*-LPS+PMNs+Ac). Controls included the assay performed with *Br*-LPS in the absence (C_1) or presence of human antibodies (C_2) but in the absence of PMNs. PMNs alone did not show any signal. Notice that the *Br*-LPS molecules associated to PMNs corresponded to the lower molecular weight fraction (~30–40 MW). The amounts of *Br*-LPS were estimated to be in the range of 5–25 ng/10^6 PMNs, corresponding to less than 0.25% of the original *Br*-LPS added. The amounts of associated *Br*-LPS in the presence of antibodies were between 10–50 ng/10^6 PMNs. These estimated quantities were from four different experiments. The read-out of the corresponding bands was performed by densitometry.

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differs from that of *Br*-LPS, but it shares the lipid A and core features with *Y*. *enterocolitica* O:9 LPS. As shown in Fig 8A, LPSs from *B*. *abortus* ΔWad*C* and *O*. *anthropi* sharing the same lipid A structure as the *Br*-LPS were able to induce cell death more readily than other LPSs. A similar pattern of PMN cell death was observed when these cells were treated with increasing quantities of purified *B*. *abortus* 2308 lipid A (Fig 8B). Altogether, these results demonstrate that the lipid A of *Br*-LPS is the moiety responsible for inducing the premature cell death of human PMNs.

Blocking of CD14 molecule prevents the *Br*-LPS-induced PMN cell death

It is well known that the coordinated interaction of CD14, MD-2/TLR4 molecules mediate LPS recognition in mammalian cells [46] and that binding of these membrane molecules may promote cell survival or cell death depending on the context [47,48]. Therefore, we explored the roles of TLR4 and CD14 *Br*-LPS-induced the cell death in PMNs.

When TLR4 or CD14 receptors were blocked with specific antibodies prior to the exposure of blood with *Ec*-LPS, the secretion of TNF-α was significantly abrogated (Fig 9A), indicating that the amounts of antibodies used were suitable. Despite of the lower amounts of TNF-α induced by *Br*-LPS as compared to those stimulated by *Ec*-LPS, the blocking of TLR4 does not have any effect on the action of the former bacterial molecule on blood cells. This phenomenon

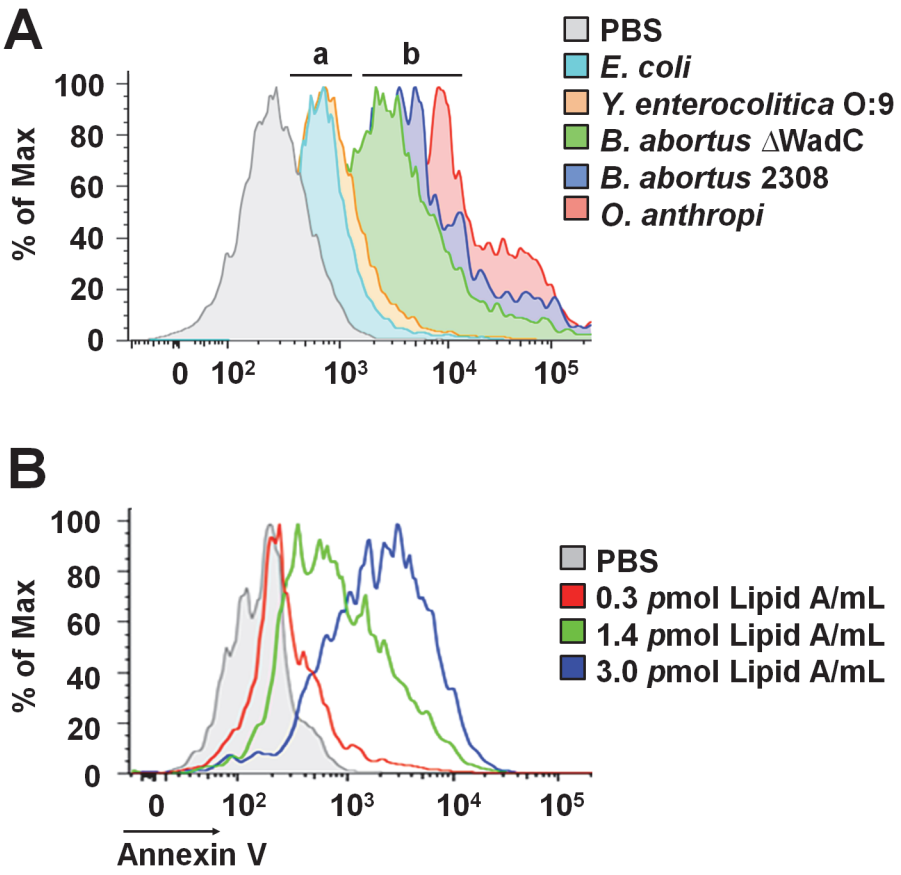

Fig 8. *Brucella* lipid A induces PMNs cell death in a dose dependent manner. (A) Heparinized blood was incubated for two hours with LPSs of *Y. enterocolitica* O:9 (3 pmol/mL), of *E. coli* (7.5 pmol/mL), of *B. abortus* 2308 (3 pmol/mL), of *B. abortus* ΔWadC (3 pmol/mL) and of *O. anthropi* (2 pmol/mL), all corresponding to 100μg/mL of LPS. The LPSs differed in at least one of the moieties (O-chain, core and lipid A) with *B. abortus* 2308 LPS: (a) LPSs possessing lipid As that differ from *B. abortus* 2308 LPS, (b) LPSs possessing lipid As structures similar to *B. abortus* 2308 LPS. (B) Heparinized blood treated with different concentrations of *B. abortus* 2308 lipid A for two hours. In all assays, PMN population was gated and analyzed by Annexin V marker and the geometric means of histograms displayed as relative units. Experiments were repeated at least three times.

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is consistent with previous findings demonstrating that *Br*-LPS is a poor agonist of the MD-2/TLR4 pathway [16,43]. Likewise, when TLR4 was blocked, PMN cell death mediated by *Br*-LPS was not inhibited (S2 Fig). In contrast, anti-CD14 antibodies significantly inhibited the secretion of TNF-α (Fig 9A) in blood as well as PMN cell death induced by *Br*-LPS (Fig 9B). Since anti-CD14 treatment of blood could modulate other leukocytes and influence the dead of PMNs, we then performed the experiment using purified PMNs (Fig 9C) to confirm the blocking effect of anti-CD14. In preparations of purified human PMNs, blocking of CD14 totally abolished the *Br*-LPS induction of cell death after a short incubation (Fig 9C). Anti-CD14 alone or low amounts of this antibody (≤1μg) did not have any observable effect in PMN cell death.

Br-LPS-induced PMN cell death correlates with low ROS formation

The low and slow kinetics of ROS formation induced by *Br*-LPS correlates with the kinetics of the PMN cell death observed (Fig 10A). Although it is dose dependent, this profile is in clear

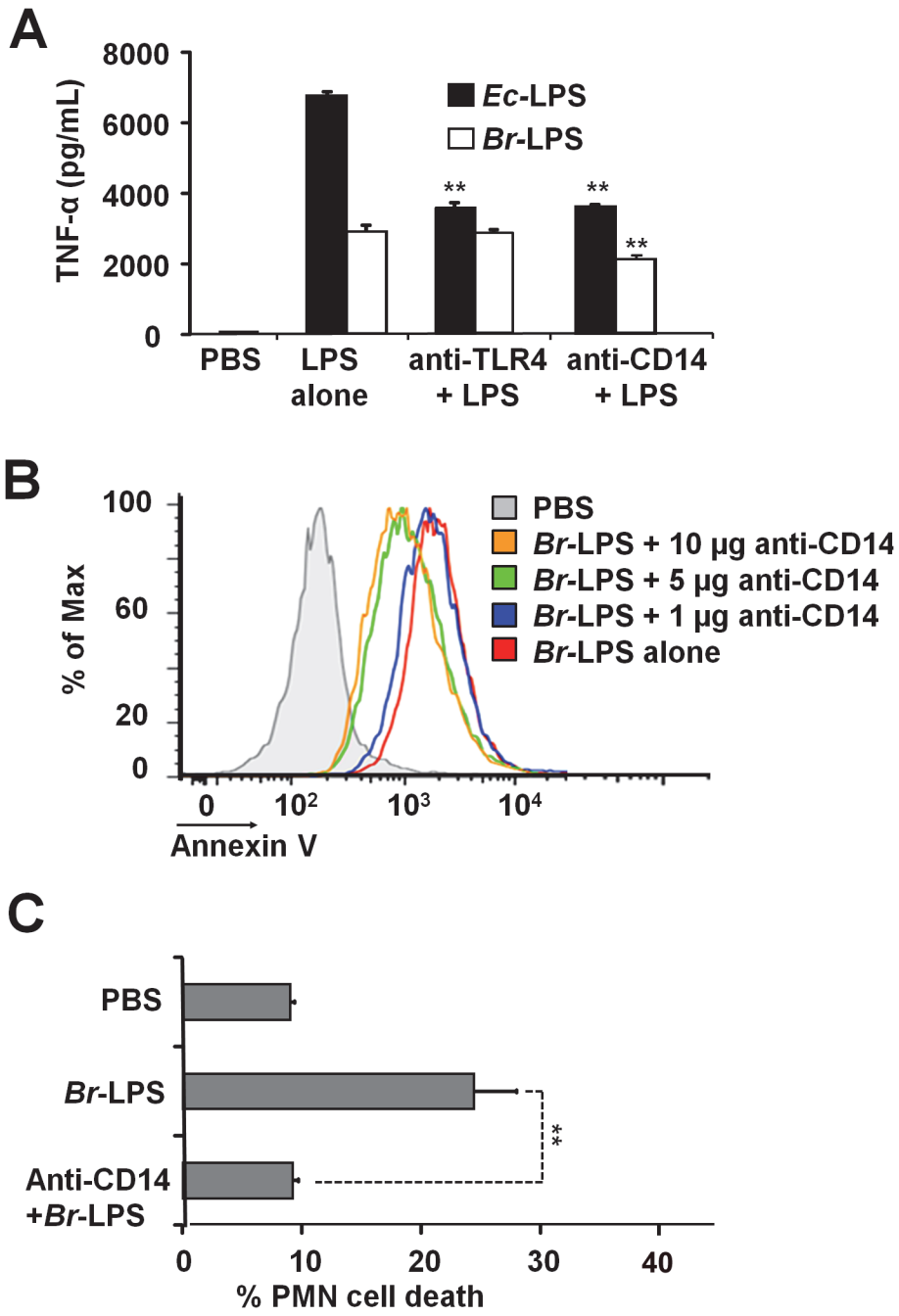

Fig 9. Neutralization of CD14 protects against *Br*-LPS-induced PMN cell death. (A) Heparinized blood was incubated for two hours with 0.4 pmol/mL of *Ec*-LPS or 3 pmol/mL of *Br*-LPS. Prior to LPS stimulation, some samples were previously treated with anti-TLR4 (1 µg/mL) or anti-CD14 (5µg/mL) antibodies and TNF-α secretion quantified in plasma by ELISA. Values of $p<0.01$ (**) are indicated in relation to their respective LPS control. (B) Heparinized blood was incubated with *Br*-LPS (3 pmol/mL) alone or previously neutralized with different quantities of anti-CD14. PMN population was gated and analyzed by Annexin V marker. Geometric means of histograms displayed as relative units. Experiments were repeated at least three times. (C) Purified PMNs were incubated for two hours with *Br*-LPS (1.5 pmol/mL). Prior to LPS stimulation, some samples were previously treated with anti-CD14 (5µg/mL) antibodies and PMN population gated and analyzed by AquaDead marker. Anti-CD14 alone does not have any significant effect in PMN cell death. Value of $p<0.01$ (**) is indicated in relation to the *Br*-LPS control.

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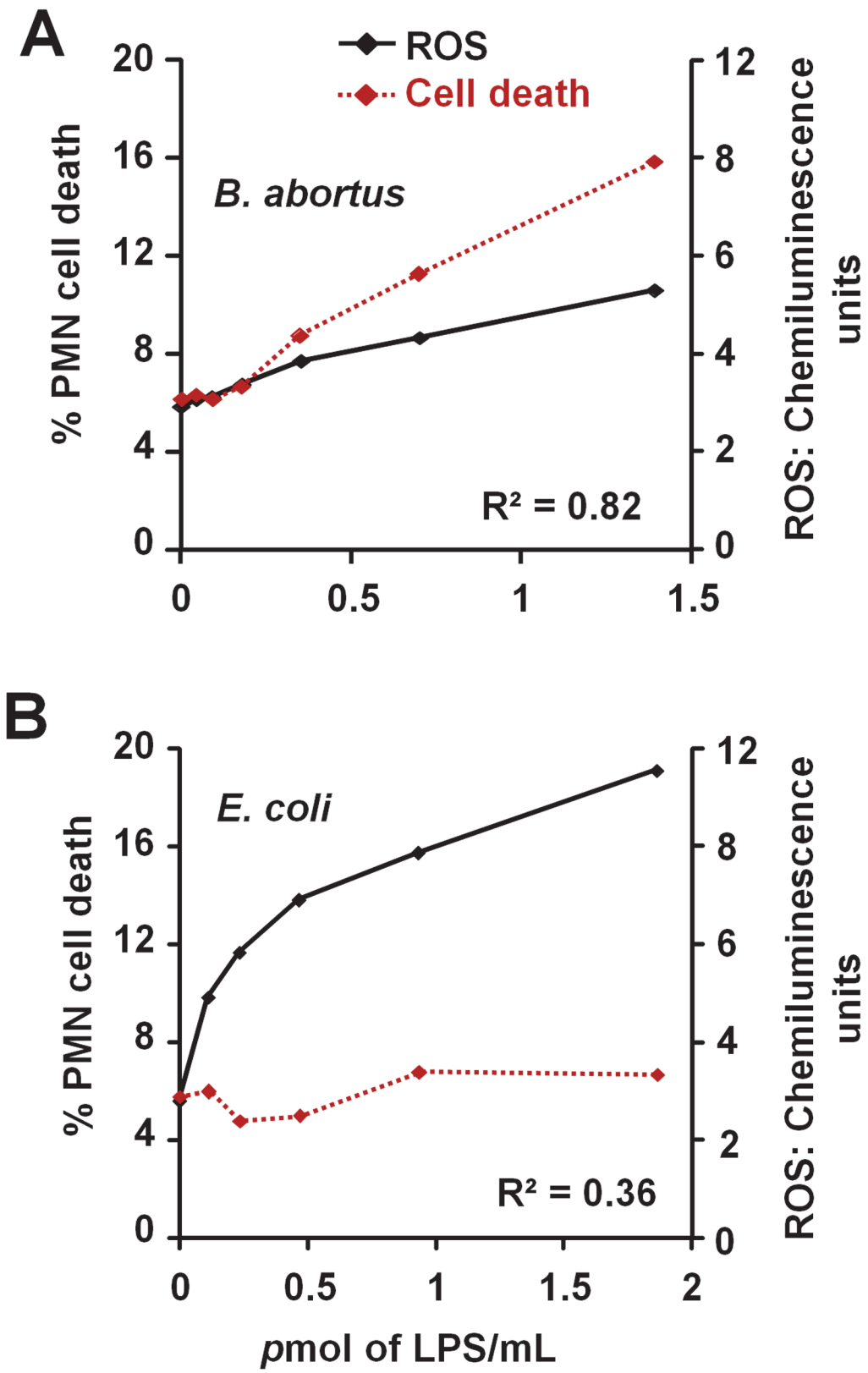

Fig 10. *Br*-LPS-induced PMN cell death correlates with low ROS formation. Purified human PMNs were seeded on serum-uncoated plates and treated with various concentrations of *Br*-LPS or *Ec*-LPS for 6.5 h. (A) ROS kinetics production was monitored for 90 minutes by luminol-amplified

chemiluminescence and the maximum obtained value for each LPS concentration plotted (black line). (B) Cell death of purified PMNs was monitored by evaluation of Sytox green fluorescence (shown as percentage of cell death relative to PMA-induced cell death) (red dotted line). Figure represents the outcome of a single experiment. Similar results were obtained in repeated experiments. Correlation R^2 was obtained by using the Excel tool facility.

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contrast and opposite to the kinetics of ROS formation and cell death measured in *Ec*-LPS treated PMNs (Fig 10B). *Brucella*-infected PMNs did not undergo NETosis or display typical signs of apoptosis or necrosis (Fig 1C). Likewise, the doses of *Br*-LPS that promoted PMN cell death failed to induce NETosis (S4 Fig) or degranulation, as demonstrated before [49]. Therefore, this phenomenon seems to be specifically mediated by *Br*-LPS and its lipid A. This also agrees with previous data demonstrating that these bacterial molecules barely induce degranulation or activation of PMNs [49].

Br-LPS triggers PMN cell death through the action of NADPH-oxidase and ROS mediators

Many of the cell death features displayed by PMNs are unique for these leukocytes [50–52]. Since microscopically the *Brucella*-induced PMN cell death does not fit with any of the classical cell death types described for these phagocytes, then we investigated the action of several chemical inhibitors (Fig 11). Among the most conspicuous were the NADPH-oxidase inhibitor, acetovanillone (apocynin) [53] and the superoxide and hydrogen peroxide scavengers, tiron and catalase, respectively [54,55]. These chemicals almost completely abrogated the *Br*-LPS-induced PMN cell death.

Since inhibition of the check point kinase 1 (Chk1) significantly prevented the cell death of *Br*-LPS treated PMNs, then we explored the induction of DNA damage. One hour after *B. abortus* infection, the fragmentation of PMN DNA was already evident (S5A Fig). The DNA damage induced by *B. abortus* infection or by *Br*-LPS treatment was reversed by pan-caspase inhibition (S5B Fig), suggesting the participation of caspase-activated DNase (CAD) [56].

Blocking of caspase 5 and to minor extent of caspase 4, prevented cell death; however, specific inhibition of caspases 1 had very little effect. Although related to caspase 1, caspases 5 and 4 have different substrates than caspase 1, and the activation of the former caspases induce cell death independently from the later [57]; therefore not necessarily linked in function. This result, suggests the absence of inflammosome recruitment in the *Brucella* induced PMNs cell death. The modest action of BAPTA/AM and Necrostatin-5 indicates partial involvement of Ca++ and the RIP1 kinase/FADD cell death routes [58].

Caspase-8 and caspase-9 are important mediators of cell death through the extrinsic and intrinsic pathways [59,60]. As shown in Fig 12, both caspases became activated after treatment of PMNs with *Br*-LPS. In spite of this, specific inhibitors for these caspases had little effect in preventing the death of PMNs (Fig 11). This effect was specific for PMNs since caspase triggering was not observed in other blood cells, such as lymphocytes (S3 Fig). This suggests the downstream recruitment of caspases 8 and 9, after the initial cell death triggering mechanisms. Other inhibitors, such as those used for preventing necrosis, apoptosome formation or the activity of Ca^{++} dependent-ATPase or MAP-, tyrosine- or PI3-kinases did not have any effect in blocking the action of *Br*-LPS (Fig 11).

Brucella and *Br*-LPS induce low levels of proinflammatory cytokines in PMNs

Pro-inflammatory TNF-α, IL-1β and IL-6 cytokines and IL-8 chemokine, may influence the life of PMNs, either prolonging or inducing the death of these phagocytic leukocytes [61–64].

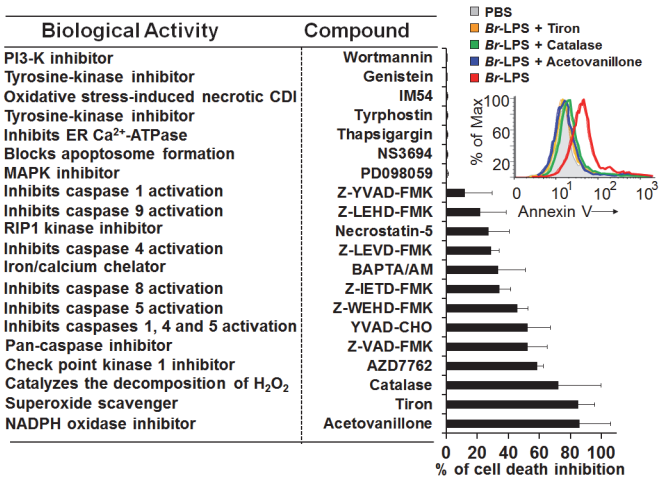

Fig 11. Inhibitory action of various compounds on the *Br*-LPS-induced PMN cell death. Prior to *Br*-LPS stimulation, samples were treated with wortmanin (50 nM), genistein (100 μM), IM-54 (10 μM), tyrphostin (250 μM), thapsigargin (50 nM), NS3694 (10 μM), PD098059 (50 μM), Z-YVAD-FMK (10 μM), Z-LEHD-FMK (10 μM), necrostatin-5 (10 μM), Z-LEVD-FMK (10 μM), BAPTA/AM (10 μM), Z-IETD-FMK (10 μM), Z-WEHD-FMK (10 μM), YVAD-CHO (50 μM), Z-VAD-FMK (10 μg/mL), AZD7762 (30 μM), catalase (2800 U/mL), tiron (2 mg/mL), acetovanillone (100 μg/mL) or PBS. After treatment with the inhibitory compounds, samples were incubated with *Br*-LPS (1.5 pmol/mL) for 2 hours. Samples were further processed and analyzed by cytometry for cell death with Annexin V as described above. Geometric means of histograms displayed as relative units. In the upright corner, the read out procedure of the inhibitory action of tiron, catalase and acetovanillone is presented. Values were estimated as relative units of the geometric means of histograms. Each experiment was repeated at least three times.

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Therefore, we assessed the release of these cytokines and chemokines in whole blood cell preparations or purified PMNs after *Brucella* or *Br*-LPS treatment. *S. enterica* was included as a

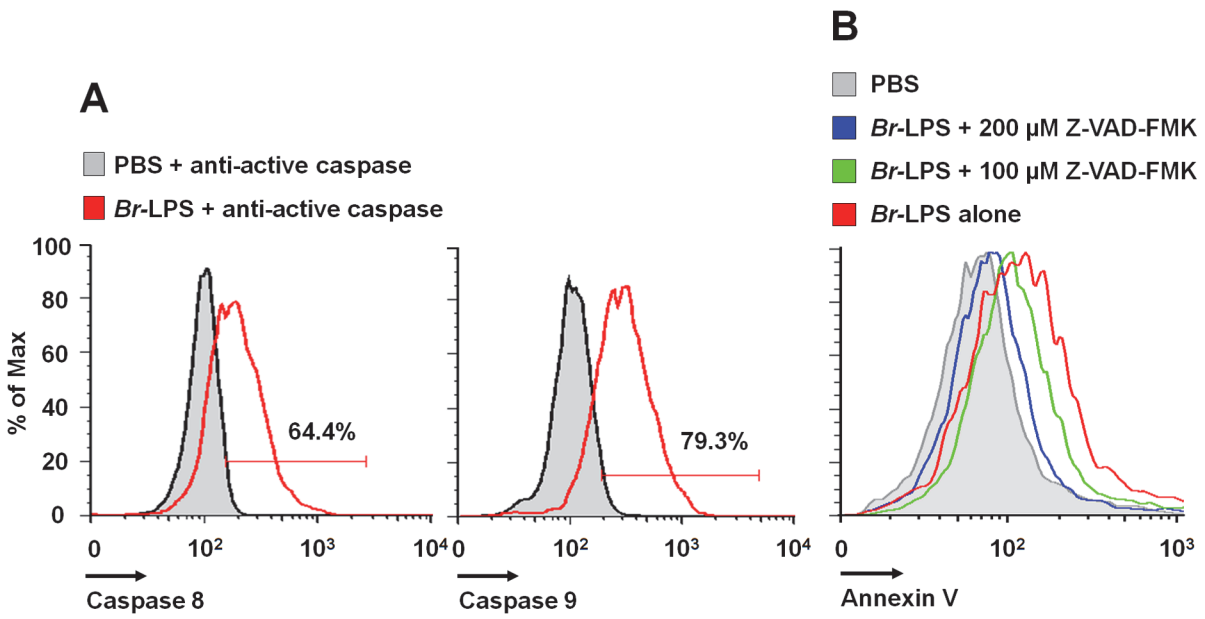

Fig 12. *Br*-LPS induces activation of caspase 8 and 9 in PMNs. (A) Heparinized blood was incubated with 0.3 pmol/mL of *Br*-LPS or PBS for 30 minutes and stained with anti-active caspase 8 or anti-active caspase 9. PMNs population was analyzed by each caspase marker (B) Heparinized blood samples were treated with Z-VAD-FMK or PBS for 1 hour and then incubated with *Br*-LPS (1.5 pmol/mL) for 2 hours. PMNs population was analyzed by Annexin V. Geometric means of histograms are displayed as relative units. Experiments were repeated at least three times.

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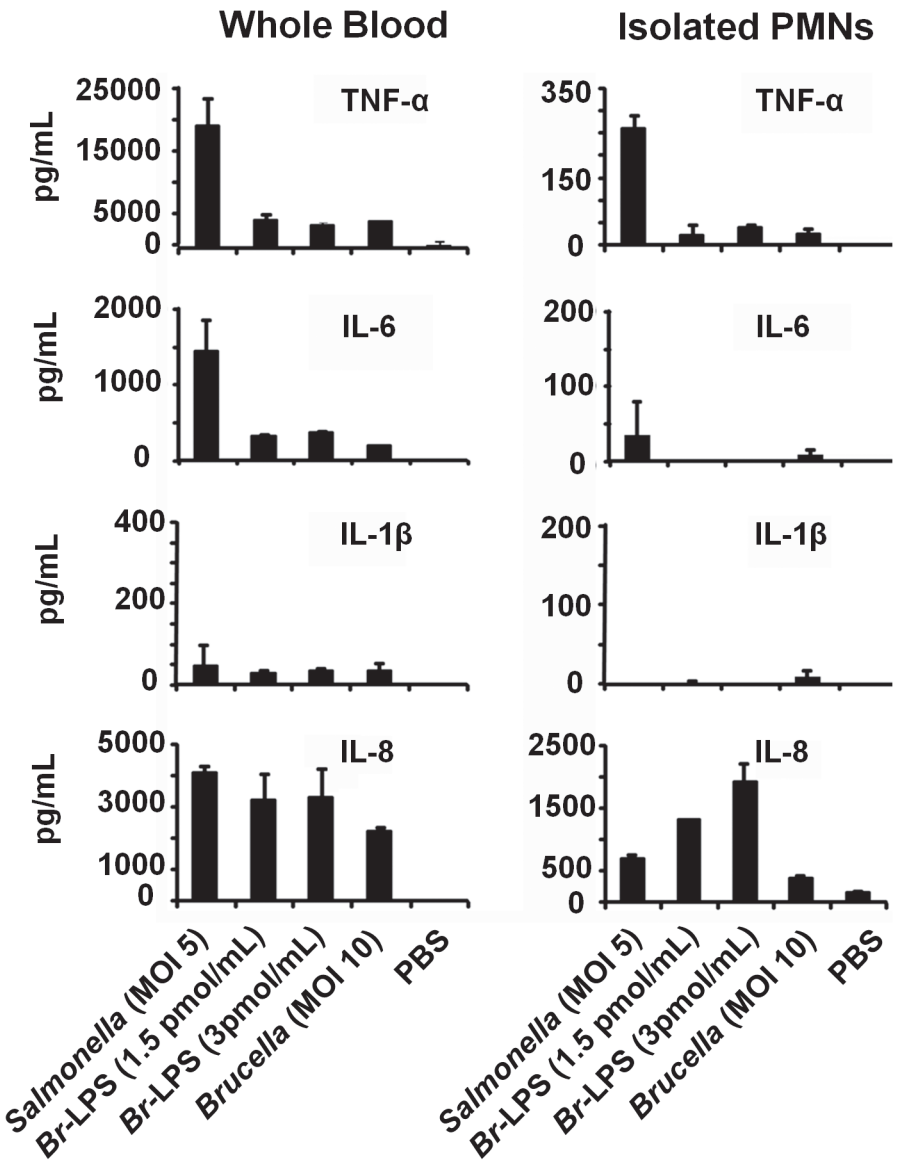

Fig 13. Cytokine differences between blood and purified PMNs infected with *B. abortus* or stimulated with *Br*-LPS. The level of the indicated cytokines was determined by ELISA in the plasma of heparinized blood or in the culture supernatants of purified PMNs after treatment with *S. enterica*, *B. abortus* or *Br*-LPS at various concentrations for two hours. Experiments were repeated at least three times.

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control. As shown in Fig 13, there were significant quantitative differences in cytokine production between blood and purified PMNs. *Salmonella* stimulates the release of cytokines by PMNs (Fig 13), induces degranulation and does not prematurely promote the cell death of these cells [65] Regardless whether blood or purified PMNs were tested, the levels of TNF-α, IL-1β and IL-6 were comparatively low after *Brucella* infection or *Br*-LPS treatment. This result is consistent with the low cytokine production by *Brucella* infected or *Br*-LPS treated macrophages [66–69], or by *Brucella* infected mice at early time points of infection [16].

The concentrations of IL-8 induced by *Brucella* or *Br*-LPS in purified PMNs were significantly higher than the levels of the other cytokines (Fig 13). This is striking since it has been established that the chemoattractant IL-8, rather than inducing cell death, promotes PMNs

survival [61,70]. Transcription of IL-8 is constitutive in PMNs, making the synthesis of this chemokine readily available after simulation [71]. Still, the cell death remains evident in both PMN preparations, being more conspicuous in blood that in purified PMNs.

Discussion

The consensus in Gram negative bacterial infections is that the endotoxic LPS molecule and other PAMPs, engage PMNs into activation and prolongation of their life span [63]. This phenomenon is linked to the activation of other cells such as Mϕ, Mo and DCs. In purified PMNs, stimulation of TLR2 and TLR4 with agonists modestly inhibits apoptosis, while in the presence of Mϕ, Mo and DCs, the inhibition of PMN cell death is very potent [47,72]. PMNs are able to use this time delay to recruit other cells and to promote proinflammatory events to eliminate the invading bacteria [73] through actions that involve the respiratory burst [5]. It has been shown that high levels of ROS inhibit caspases activities, suggesting that reactive oxygen species may prevent these proteases from functioning optimally in PMNs [74]. During these processes, some PMNs degranulate, others undergo NETosis, while others may die by necrosis or oncosis, triggering proinflammatory signals [51,52].

In contrast, upon invasion *Brucella* resists the killing action of PMNs and prematurely induces the cell death of these phagocytes. The *Brucella*-induced PMN cell death occurs without bacterial replication [23,24] and without promoting those classical phenotypic changes associated with NETosis, degranulation, necrosis, oncosis or classical apoptosis. The cell death of *Brucella*-infected PMNs seems to be triggered after active phagocytosis of the microorganism followed by the intracellular release of the *Br*-LPS inside cell vacuoles, either by alive or death bacteria. Although the process by which *B. abortus* sheds *Br*-LPS inside the cells has not been elucidated, it is likely that it occurs through blebbing of outer membrane fragments enriched in *Br*-LPS, a phenomenon that is well known in *Brucella* [75]. This is significant, since *Br*-LPS is capable to circulate in the body and reside inside phagocytes for months without being destroyed [33], and consequently, capable to exert its biological action on PMNs *in vivo*.

There are reports claiming that *B. abortus* and *Brucella* lipoproteins activate PMNs [76]. However, in those experiments the *ex vivo* PMNs viability was less than expected and the assays were performed with heat killed bacteria and lipoproteins acylated in the *E. coli* background; thus preventing comparison with our results, as explained before [17]. The interaction between the lipid A of *Br*-LPS and PMNs mostly precludes TLR4, as well as other TLRs, as demonstrated before for Mϕ [18,43,67,77,78]. However, it does not exclude the CD14 molecule, since antibodies against the later co-receptor abrogates the PMN cell death and to less extent the release of TNF-α, suggesting some signaling through this co-receptor. The interaction of *Br*-LPS with intracellular CD14 molecules is feasible, since this lipoprotein is also found inside PMN vesicles [79] and in concordance with the transport of *Br*-LPS to CD14 containing lipid rafts in Mϕ membranes [80]. Moreover, in agreement with our results, it has been shown that in Mϕ, *Brucella* signals through CD14 for the production of low amounts of TNF-α [81]. Finally, the involvement of CD14 in the induction of cell death is not without precedent and it has been demonstrated that direct binding of LPS to CD14 –without the concourse of TLR4– prompts apoptosis in DCs [48].

We have demonstrated in murine Mϕ that *Br*-LPS follows the classical endocytic pathway used by protein antigens but with a slower kinetics [80]. Then, *Br*-LPS is transported to cellular compartments enriched in MHC-II and recycled to the cell surface, where it forms dense macrodomains. Once in the cell membrane, the *B*r-LPS macrodomains segregate several lipid-raft components and interfere with the MHC-II presentation of peptides to specific CD4+ T cells [80]. The initial release of *Br*-LPS inside PMN phagosomes and its subsequently transit

within vacuoles seems to occur by a similar mechanism proposed for Mϕ [82]. Likewise, in some infected PMNs, *Br*-LPS was also observed in cell membrane ruffles-like structures. However, the biogenesis and life span between infected human PMNs and murine Mϕ is rather different: while in the former leukocytes *Brucella* induces premature cell death, in the later it prologues the life span and protects against apoptosis [16,22]. Moreover, the amounts of *Br*-LPS internalized by Mϕ are comparatively much higher than those ingested by PMNs [33]. This difference may be linked to the numbers of CD14 surface molecules present in Mo and Mϕ, which are from 30–40 times more abundant than in PMNs [83]. However, the amounts of intracellular *Br*-LPS available in PMNs at early times of cell infection may be considerably larger than in Mϕ; since the former leukocytes ingest larger number of *Brucella* organisms than the latter, which internalize just a few bacteria [84]. These and other differences make quite difficult to perform a detail experimentation of the intracellular trafficking of *Br*-LPS inside PMNs, and alternative methodological approaches would be required. For the moment, this is beyond of our possibilities.

The dose-dependent *Br*-LPS-induced PMN cell death correlates with a modest but steadily increase of ROS mediated by NADPH oxidase. This seems to be the main triggering mechanisms by which the lipid A of *Br*-LPS induces the premature cell death of human PMNs. It is worth noting that several of the molecular pathways causing PMN cell death are dependent on ROS generation. While large amounts of ROS may inhibit caspases, promote necrosis or cause NETosis [74,85,86], low amounts may induce PMN cell death [52].

DNA damage by oxygen radicals is a well-known phenomenon in a variety of cells, including PMNs [52]; and even small amounts of ROS may induce DNA alterations. The recorded DNA fragmentation of *B. abortus* and *Br*-LPS treated PMNs recruited Chk1, a protein that coordinates the DNA damage response at the initiation of cell cycle [87]. Although in other cells inhibition of Chk1 induces apoptosis [87], it is likely that in non-dividing cells –such as PMNs – this protein has a terminal role and its function is not to arrest the cell cycle, but to promote cell death. The PMN DNA fragmentation was partially reversed by pan-caspase inhibitors; event that suggests the participation of CAD [56].

At first glance, the profile of inhibitory substances suggests that caspase-8 could be extrinsically activated through the RIP1 kinase/FADD route [88]. In addition, the mobilization of Ca^{++} may activate several death signals, including the calcium-activated cysteine protease calpain that cleaves and thereby activates a number of molecules that have important functions in the apoptosis processes [89].

As already recorded in human monocytes [68] the amounts of IL-1β induced by *B. abortus* and its *Br*-LPS in PMNs are rather low. In addition, inhibition of caspase 1 did not block the *Br*-LPS mediated PMN cell death. These two observations, together with the low cytokine induction by *Brucella* and its *Br*-LPS in PMNs, seem to preclude the role of the inflammasome pathway in the premature death of these leukocytes. Though this seems relevant, it has been reported that caspase-1 induced pyroptotic cell death does not function in PMNs [90]. Moreover, upon inflammasome activation the amounts of IL-1β produced by purified PMNs are rather low [91]. This may be linked to the fact that human neutrophils express key components of the inflammasome machinery at non-canonical intracellular sites [91]. A general proposal of the mechanisms for the induction of the premature PMN cell death generated during *B. abortus* infection is presented in S6 Fig.

It is well known that under certain circumstances, proinflammatory cytokines produced by leukocytes during Gram negative endotoxemia are capable of inducing programmed cell death [92,93]. Among these, the TNF-α is the most conspicuous cytokine generating apoptosis through binding to its cognate TNFR1 [94,95]. However, it is unlikely that TNF-α is the signal that promotes the *Br*-LPS-induced PMN cell death. First, the amounts of proinflammatory

cytokines –including TNF-α– produced upon exposure of PMNs to *Brucella* or *Br*-LPS were very low (Fig 13). Second, it is well known that *Brucella* and *Br*-LPS are low agonist of pattern recognition receptors and low activators of NF-κB [16,43,67]. Third, under similar experimental conditions the *Ec*-LPS –which induces the production of much higher quantities of TNF-α – does not promote premature PMN cell death (Figs 6A and 9). Finally, under the same experimental conditions *Br*-LPS did not induce the death of lymphocytes (Fig 6B) which are also susceptible to the pro-apoptotic effect of TNF-α [95].

It is worth noting that the amounts of IL-8 induced by *B. abortus* in purified PMNs were higher than other cytokines. It has been shown that this chemokine, rather that promoting cell death, delays spontaneous and TNF-α-induced apoptosis of human PMNs in a dose dependent manner [70]. The delay in apoptosis is mainly mediated through the interaction of IL-8 with its cognate RII receptor, while the RI receptor may provide an added effect. Still, PMNs died after *Brucella* infection or *Br*-LPS treatment, precluding the influence of IL-8 in a delimited population of PMNs. The different levels of cytokines detected in whole blood versus purified PMNs exposed to *Brucella* or *Br*-LPS may reflect the participation of Mo, DCs and serum components (e.g. complement) present in blood, which may have served as an additional stimuli and sources of cytokines, including IL-8.

For many years it has been recognized that a proportion of patients with chronic brucellosis display absolute neutropenia [27,28]. It has been also shown that the invasion of *Brucella* organisms induces significant hematological chages in the bone marrow, involving pancytopenia and phagocytosis of blood elements (including PMNs) by resident Mϕ [96–98]. In addition, during the accute phase of brucellosis there is a conspicuous absence of infected PMNs in the target organs, a phenomenon that is in clear contrast to the presence of *Brucella* inside Mϕ and DCs [29]. The fact that *Brucella* and its *Br*-LPS specifically induce the premature cell death of PMNs may explain, at least in part, these clinical signs.

Dying PMNs display "eat-me" signals. Therefore, they are readily removed by phagocytic cells. Then, it is likely that *Brucella* infected PMNs may serve as "Trojan horse" vehicles for dispersing the bacterium to other organs; hence, contributing to the long lasting infections observed in brucellosis [99]. The *Brucella*-induced cell death –without significant activation of PMNs and their non-phlogistic removal by Mϕ and DCs– would help to hamper the promotion of proinflammatory signals (S7 Fig). This mechanism may represent a seminal component of the stealthy strategy used by *Brucella* organisms [16] to spread in its host while avoiding innate immunity.

Materials and Methods

Ethics

Human fresh blood was obtained in the blood bank of the Charité Hospital, Berlin, following a protocol approved by the Charité Hospital, Berlin Ethical Committee. Fresh blood was also obtained from normal healthy volunteer donors through the "Etablissement Français du Sang" following their approval and in agreement with the "French Ethics Committee on Human Experimentation F11", within a convention EFS-08-21-2012 with Institut National de la Santé et de la Recherche Médicale, signed. All blood donors involved were informed about the study and provided written consents.

Bacterial strains, LPSs and lipid A preparations

Virulent *B. abortus* (2308), *B. abortus*-GFP (2308) [100], transgenic *B. abortus*-RFP (2308) with an integrated chromosomal gene coding for the red fluorescent protein from *Discosoma* coral (provided by Dr. Jean-Jacques Letesson; Unité de Recherche en Biologie Moléculaire,

Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium) and *Salmonella enterica* sv. Typhimurium (SL1344) were grown in tryptic Soy or Luria Bertani broths as previously described [16]. Bacterial cells were washed three times by centrifugation in Hanks or PBS solution before the assays. Purified LPSs were prepared from *B. abortus* (2308), *B. abortus wadC* (2308), *E. coli* (0127), *Y. enterocolitica* O:9 (MY79), *O. anthropi* (LMG 3331T) as reported before [43,77]. *B. abortus* lipid A was prepared by mild acid hydrolysis from *Br*-LPS and solubilized as described elsewhere [101]. All the *Brucella Br*-LPS and lipid A preparations were above 98% pure and devoid of contaminant proteins, free lipids and cyclic glucans.

Neutrophil purification

PMNs were purified by Histopaque and Percoll gradients from blood of healthy donors as previously described [16,50]. Cell preparations were composed from 95–98% of granulocytes. Cell viability was >90%. PMN preparations were maintained at 4°C in PBS or autologous plasma, and used within the first hour after extraction. Under our conditions, PMN spontaneous apoptosis was just evident after 5–7 hours after purification.

Bactericidal activity

Bactericidal activity was measured as previously described [16]. Briefly, *B. abortus* or S. *enterica* were mixed with 500 μL of purified human PMNs ($1x10^6$ PMNs/mL) at a MOI of 5 bacteria/PMN and incubated under mild agitation for 90 minutes. Control bacteria were incubated in the absence of PMNs to quantify bacterial replication during the experiment. Viable CFU were determined at 0, 45 and 90 minutes of incubation by lysing cells with 0.1% triton and plating samples in tripticase soy agar. The percentage of bacterial survival was calculated.

Phagocytosis assay

Human heparinized blood or purified PMNs were incubated with *B. abortus*-GFP or fluorescent latex beads for two hours at 37°C a multiplicity of infection (MOI) of 10–100 bacteria or beads/cell, under mild agitation. Smears were fixed with methanol and mounted with ProLong Gold Antifade Reagent with DAPI. One hundred PMNs were counted per sample and the number of particles determined to calculate the percentage of phagocytosis.

PMN cell death assays

PMN cell death was analyzed by treating human whole blood or isolated PMNs with different bacterial strains and LPSs. Human heparinized blood (100 or 500 μL) collected with lithium heparin was incubated for 2 hours at 37°C in agitation (200–300 rpm) with each treatment. Bacteria were tested at MOIs of 1, 10 or 100 bacteria/PMN. In the case of LPS or lipid A, blood samples were treated at concentrations from $3x10^{-3}$ to $3x10^{1}$ pmol/mL. After incubation, blood samples were lysed for 5–10 min in 900 μL of red blood cell lysis buffer (NH_4Cl 8.02 gm, $NaHCO_3$ 0.84gm and EDTA 0.37gm/L, pH 7.2). Cells were washed with ice cold PBS and re-suspended in 100 μL of Annexin V Binding Buffer (BD). 5 μL of Annexin V (BD) and 2 μL of AquaDead (Invitrogen) (diluted 1/20 in PBS) were added and incubated for 30 min on ice in the dark. Cells were washed once with ice cold PBS, re-suspended in 200 μL of paraformaldehyde 3% and incubated for 30 min at room temperature. Samples were then diluted 1:2 with PBS and acquired for analysis within 1 hour.

Intracellular detection of *Br*-LPS

For intracellular detection of *Br*-LPS a double labeling fluorescence approach was performed [36]. Human heparinized blood was incubated with *B. abortus*-RFP (red) for one hour (MOI 2) under mild agitation. Blood smears were fixed and permeabilized with methanol, stained with anti-*Brucella* LPS FITC (green) and mounted with ProLong Gold Antifade Reagent with DAPI (blue). Samples were observed by fluorescent microscopy (Olympus BH-2) under 1000 × magnification. *Br*-LPS shed by *Brucella* is shown in green staining around red bacteria.

Intracellular detection of *Br*-LPS was also performed in *B. abortus* infected PMNs by immunogold detection under the electron microscope. Briefly, purified human PMNs 5 $\times 10^6$ were infected with *B. abortus* 2308 at MOI 20. After 1 hour incubation at 37°C under mild agitation, cells were washed and the pellet fixed with 200 μl of 2.5% glutaraldehyde in phosphate buffer 0.05M pH 7.4 (PB) at 4°C for 1 hour. Cells were pelleted at 3000 rpm for 10 min, washed in PB and suspended in 50 μL of PB. Then fixed cells were incubated at 40°C for 5 minutes, and 100 μL of 3% low melting agarose at 40°C added. The temperature was lowered, and 5 volumes of 2.5% glutaraldehyde in PB were added to the solid agarose block and incubated overnight at 4°C. Agarose blocks containing the fixed infected PMNs were processed for inclusion in Spurr resin for immunogold staining and for electron microscopy as described elsewhere [102]. For detection of *Br*-LPS, human IgG or mouse IgG with specificity against the O chain polysaccharide [103] were used in combination with protein-A/protein-G colloidal gold 15 nm (EY Laboratories, Inc.). Purified mouse and human IgGs from normal serum were used for controlling the specificity of the reaction. Finally, PMNs sections were stained following the led citrate procedure described by Reynolds [104] [101] and observed under a Hitachi H 7100 electron microscope.

Quantitation of *Br*-LPS interacting with PMNs

In order to determine the amount of *Br*-LPS interacting with human PMNs, 10 μg (0.3 pmol) of *Br*-LPS were incubated with $1x10^6$ PMNs in 500 μL of HBSS at 37°C for 1 h under mild rotation in the presence or absence of human IgG anti-*Br*-LPS. PMNs were washed three times with HBSS to remove the excess of *Br*-LPS, and then the cell pellet lysed with deionized water containing 50 μg/mL of DNAase and 125 μg/mL of proteinase K (Fisher Scientific) at 37°C for 1 h under mild rotation. Cell lysate was incubated with SDS-PAGE sample buffer and subjected to Western blotting. *Br*-LPSs bands were revealed with a monoclonal antibody against the O chain polysaccharide of the *Br*-LPS conjugated with peroxidase [105]. Controls included the assay performed with *Br*-LPS in the absence or presence of human antibodies but in the absence of PMNs, and PMNs alone. Quantitation of *Br*-LPS bound to PMNs was estimated in relation to a standard curve of purified *Br*-LPS ranging from 0.1 ng to 12 ng. The read-out of the bands was performed by densitometry with the support of ImageJ software (http://imagej.net).

ROS detection

Isolated PMNs ($1x10^5$) were re-suspended in 50 μL of Hanks Balanced Salt Solution (HBSS +1% FBS) per well of a 96-uncoated serum well plate. Cell suspension was supplemented with Reactive Oxygen Species (ROS) Detection Reagents (Invitrogen) and stimulated with phorbol myristate acetate (40 nM), *Br*-LPS (0.03–3 pmol/mL), *Ec*-LPS (0.09–7.5 pmol/mL) in 50 μl HBSS+1% FBS or left untreated. The kinetics of ROS production was monitored with a Victor Perkin Elmer luminometer at 37C for 90 min.

NET formation and cell cytotoxicity assay

Isolated PMNs ($1x10^5$) were re-suspended in 500 μL of RPMI medium (10 mM HEPES +1% FBS without glutamine) and let sit on 24-well plates for 30 min at 37°C. Cells were stimulated with phorbol myristate acetate (40 nM) (Sigma), *Br*-LPS (0.7–100 μg/ml), *Ec*-LPS (0.7–100 μg/ml) or left untreated in RPMI medium. After 6h 30 minutes, cell cytotoxicity was measured by Sytox (0.3 μM) (Invitrogen) staining with a fluorometer. Some cells were fixed in paraformaldehyde 8% and observed with a Leica inverted fluorescence microscope to evaluate the nuclear morphologies and NET spreading.

Cytokine quantitation

The levels of TNF-α, IL-8, IL-1β and IL-6 were measured by ELISA (eBioscience) in heparinized human blood (plasma) or supernatant of isolated PMNs treated with different stimuli according to manufacturer's specifications.

Determination of caspase 8 and 9 activation

Heparinized human blood (500 μL) was incubated with *B. abortus* LPS (10 μg/mL) or PBS for 30 minutes under mild agitation and stained directly and incubated with anti-active caspase 8 or anti-active caspase 9 using Guava Caspase 8 FAM & Caspase 9 SR Kit (Millipore) according to manufacturer's specifications and quantitated by flow cytometry. PMNs population was gated by forward light scatter and side light scatter parameters and analyzed by each caspase marker.

DNA fragmentation assays

PMNs were isolated as previously described [106] and incubated for one hour with *B. abortus* (MOI 100) or 10 μg/mL (0.3 pmol) of *Br*-LPS in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK). Cycloheximide (Sigma-Aldrich) was used as a positive control for DNA fragmentation. After incubation, PMN cell death was measured by using a DNA fragmentation ELISA (Roche) according to manufacturer's specifications. For microscopic analysis, heparinized blood was incubated with *B. abortus*-RFP for 2 hours (MOI 100). Red blood cells were lysed and total leucocytes prepared, fixed and stained with APO-BrdU TUNEL Assay Kit (Invitrogen) according to manufacturer's specifications. Cells were centrifuged on a microscope slide by using a Cytospin 2 (Shandon) and mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen).

TLR4 and CD14 neutralization

TLR4 and CD14 cell receptors were neutralized (before *Br*-LPS or *Ec*-LPS treatments) by incubating isolated PMNs or heparinized human blood with 1 μg of anti-hTLR4-IgG (clone W7C11) or 5 μg of anti-CD14-IgA (clone D3B8) antibodies (InvivoGen), for 20 minutes and one hour respectively. No inhibitory or stimulatory signals were observed with mouse monoclonal IgG1 (anti-bovine IgG, Sigma-Aldrich), or enriched mouse and human immunoglobulin preparations. Receptor blockage was verified in side controls by measuring TNF-α secretion after treating blood with 5 μg/mL *Ec*-LPS (Fig 9A).

PMN cell death inhibition assays

Heparinized human blood samples (350 μL) were pre-incubated with one of the following compounds for 1 hour: IM-54 (Enzo Life Sciences), Wortmanin, Genistein, Tyrphostin, PD098059, Necrostatin-5, Z-VAD-FMK, AZD7762, Catalase, Tiron, Acetovanillone (Sigma),

BAPTA/AM, YVAD-CHO, NS3694, Thapsigargin (Calbiochem), Z-IETD-FMK, Z-WEHD-FMK, Z-LEVD-FMK, Z-LEHD-FMK (BioVision), Z-YVAD-FMK (Santa Cruz). Concentrations were utilized according to previous reports and standardized to our conditions for optimal inhibitory performance. After treatment with the inhibitory compounds, samples were incubated with *Br*-LPS (1.3 pmol/mL) for 2 hours. Samples were further processed and analyzed by cytometry for cell death with Annexin V as described above.

Flow cytometry and FACS analysis

PMN or lymphocyte populations were gated as indicated (S8 Fig) and analyzed for cell death of caspase activation by flow cytometry. FACS analysis was performed using a FACSCanto system (BD Biosciences) or Guava easyCyte (Millipore). FACS data were analyzed using FlowJo software (Tree Star, Inc.). For each experiment, control samples were included to define the proper gates.

Statistical analysis

Values were expressed as means ± standard error, and compared using Student's t test for determining the statistical significance in the different assays. Values of $p< 0.05$ were considered statistically significant.

Supporting Information

S1 Fig. Schematic structure of smooth *B. abortus Br*-LPS. The O-polysaccharide is an unbranched linear homopolymer of α-1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl units (*N*-formylperosamine) with an average chain length of 96 to 100 glycosyl subunits [105]. The O-polysaccharide is linked to a core bifurcating oligosaccharide composed of βGlcN-6-βGlcN-4-βGlcN(-6-βGlcN)-3-αMan(-6-αGlc)-5-KDO1(-2-KDO2)-Lipid A; branching from KDO1 is αPerNFo-[-2PerNFo]$_n$-2PerNF-2-αMan-3-αMan-3-βQuiNAc-4-βGlc-4-KDO2-4-KDO1 [44]. The KDO1 is linked to the lipid A composed of a backbone of diaminoglucose (DAG) disaccharide, substituted with phosphates (P) and amide and ester-linked long chain saturated ($C_{16:0}$ to $C_{18:0}$) and hydroxylated (3-OH-$C_{12:0}$ to 29-OH-$C_{30:0}$) fatty acids [42,107]. Ketodeoxyoctulosonic acid (KDO), mannose (Man), Acetyl-quinovosamine (QuiN), glucose (Glc).
(TIF)

S2 Fig. Neutralization of TLR4 does not protect against *Br*-LPS-induced PMN cell death. Heparinized blood was incubated with *Br*-LPS (3 pmol/mL) alone or previously neutralized with anti-TLR4 and PMN population gated and analyzed by Annexin V marker. Geometric means of histograms displayed as relative units. Experiments were repeated at least three times.
(TIF)

S3 Fig. *Br*-LPS induces little activation of caspase 8 and 9 in lymphocytes. Heparinized blood was incubated with 0.3 pmol/mL of *Br*-LPS or PBS for 30 minutes and stained with anti-active caspase 8 or anti-active caspase 9. Lymphocyte population was gated by forward light scatter and side light scatter parameters and analyzed for each caspase marker. Geometric means of histograms are displayed as relative units. Experiments were repeated at least three times.
(TIF)

S4 Fig. Cell death promoted by *Br*-LPS failed to induce NETosis. Isolated PMNs were stimulated with PMA (40nM) or *Br*-LPS (3 pmol/mL). (A) NET formation induced by PMA, or (B)

cell cytotoxicity induced by *Br*-LPS was analyzed under the fluorescent microscope. (C) Cell morphology of PMA treated cells, or (D) *Br*-LPS treated cells were observed using phase contrast. NET formation is clearly seen in "A", while in "B" cell death without NET formation is observed. Microscope images are at 400 × magnification. Figure represents the outcome of a single experiment. Similar results were obtained in repeated experiments by looking NET spreading
(TIF)

S5 Fig. *Brucella* and *Br*-LPS induces PMNs DNA fragmentation. (A) Heparinized blood was incubated with *B. abortus*-RFP for 2 hours (MOI 100). Red blood cells were lysed and total leucocytes prepared, fixed and stained with APO-BrdU TUNEL Assay Kit according to manufacturer's specifications. Cells were centrifuged and mounted with ProLong Gold Antifade Reagent with DAPI. (a) *B. abortus*-RFP, (b) PMN DAPI staining (c) TUNEL positive nucleus and (d) merged images. Images were cut from microscope field, contrasted and saturated using Hue tool to obtain suitable color separation. Images were then merged using Adobe Photoshop 8 software. Microscope images are at 1000 × magnification. (B) Purified blood PMNs were incubated with *B. abortus* (MOI 100) or *Br*-LPS (0.3 pmol/mL) in the presence or absence of a pan-caspase inhibitor (Z-VAD-FMK) for one hour. Cycloheximide was used as a positive control. PMN DNA fragmentation was measured by Cellular DNA Fragmentation ELISA (Roche). Values of $p<0.01$ (**) are indicated.
(TIF)

S6 Fig. Proposed model for the premature cell death of Brucella infected PMNs. After *Brucella* invasion, the bacterium is readily phagocytized by resident PMNs [24] resisting the killing mechanisms mediated by these leukocytes [31]. Once inside phagosomes, the bacterium releases non-toxic *Br*-LPS, probably in the form of outer membrane fragments [75]. Then, the *Br*-LPS fuses with the cell membrane of PMNs, binds to CD14 lipoprotein and is transported inside the cytoplasm of PMNs within endocytic vacuoles. During this process, the *Br*-LPS does not interact with TLR-4; then, avoiding activation of PMNs. In the course of this action, NADPH oxidase is progressively recruited promoting the slow generation of controlled amounts of ROS mediators. These effectors induce oxidative damage of nuclear DNA inducing molecular fragmentation and the recruitment of Chek1 protein, which is the main responsible for coordinating the DNA damage response at the initiation of the cell cycle. In PMNs –which are non-dividing effector cells– Chek1, rather than arresting the cell cycle, may recruit cell death executioner caspases which in course promote the activation of caspase-activated Dnases (CAD), contributing to the damage of DNA. At the same time, some of the ROS effectors may act as second messengers and induce the activation of caspases 5 and to minor extend caspase 4, but not caspase 1, excluding the participation of the inflammasome pathway. ROS may also induce the recruitment of the RIP1 kinase/FADD cell death routes, caspase 8 and promote the release of Ca^{++} to the cytosol. These mediators, will also recruit cell death executioner caspases and together with ROS mediators trigger additional death effector mechanisms (e.g. activation of calpains and cathepsins). Finally, the activation of the initiator caspase 9 of the intrinsic cell death pathway will be activated downstream by caspase 8 contributing to the premature PMN cell death mechanism. During this process, the infected PMNs expose "eat-me" signals (e.g. phosphatidylserine) on the surface that promote their phagocytosis by Mϕ or DCs.
(TIF)

S7 Fig. PMN cell death modulation. After danger signal or PAMP recognition, PMNs become activated, cell death delayed and inflammatory response promoted. Under noninfectious conditions, PMNs die spontaneously and are phagocytized by DCs and MØ under non-

inflammatory conditions. Following PMNs ingestion of *Brucella*, PMNs are quickly primed for cell death and phagocytized by DCs and MØ where *Brucella* replicates intensively under a non-inflammatory environment.
(TIF)

S8 Fig. PMNs and lymphocytes gating strategy. (A) PMN or lymphocyte cell populations were gated by forward light scatter and side light scatter parameters from total blood leucocyte population. (B) GFP negative or GFP positive population (infected with *B. abortus*-GFP) were selected and (C) analyzed for cell death by AquaDead and Annexin V markers.
(TIF)

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Author Contributions

Conceived and designed the experiments: EBC AGB JPG EM. Performed the experiments: EBC RMC VAG JLdD CCD ECO. Analyzed the data: EBC RMC CGV JLdD AGB CCD JPG EM. Contributed reagents/materials/analysis tools: EBC JLdD ECO CGV AGB JPG EM. Wrote the paper: EBC JPG EM.

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Annex 4

Annex. 4.1.

Gutiérrez-Jiménez, C., Hysenaj, L., Alfaro-Alarcón, A., Mora-Cartín, R., Arce-Gorvel, V., Moreno, E., … Barquero-Calvo, E. (2018). Persistence of *Brucella abortus* in the Bone Marrow of Infected Mice. *Journal of Immunology Research*, *2018*, 5370414. https://doi.org/10.1155/2018/5370414

Annex. 4.2.

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Research Article

Persistence of *Brucella abortus* in the Bone Marrow of Infected Mice

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Brucellosis is a zoonotic bacterial infection that may persist for long periods causing relapses in antibiotic-treated patients. The ability of *Brucella* to develop chronic infections is linked to their capacity to invade and replicate within the mononuclear phagocyte system, including the bone marrow (BM). Persistence of *Brucella* in the BM has been associated with hematological complications such as neutropenia, thrombocytopenia, anemia, and pancytopenia in human patients. In the mouse model, we observed that the number of *Brucella abortus* in the BM remained constant for up to 168 days of postinfection. This persistence was associated with histopathological changes, accompanied by augmented numbers of BM myeloid GMP progenitors, PMNs, and $CD4^+$ lymphocytes during the acute phase (eight days) of the infection in the BM. Monocytes, PMNs, and GMP cells were identified as the cells harboring *Brucella* in the BM. We propose that the BM is an essential niche for the bacterium to establish long-lasting infections and that infected PMNs may serve as vehicles for dispersion of *Brucella* organisms, following the Trojan horse hypothesis. Monocytes are solid candidates for *Brucella* reservoirs in the BM.

1. Introduction

Brucellosis is a zoonotic bacterial infection caused by members of the genus *Brucella* [1]. In humans, the disease is long-lasting, displaying a variety of clinical and pathological manifestations that may persist for months or years [2–5]. If the infection is not properly treated, it may cause death.

The ability of *Brucella* organisms to develop chronic infections is linked to their competence to invade the mononuclear phagocyte system, where they replicate within the endoplasmic reticulum [6]. In addition, the poor proinflammatory responses induced at the onset of the infection [7], together with the capacity of *Brucella* organisms to extend the life of infected cells, are factors that contribute to the pathogenicity of this microorganism [7, 8].

The persistence of *Brucella* organisms in humans occurs in the lymph nodes, spleen, liver, bone marrow (BM), reproductive organs, and joints [9, 10]. The bacterium is isolated from the BM in about half of the human patients with brucellosis [4]. However, in all brucellosis cases, the BM displays histopathological alterations, whether or not the bacterium is isolated from this tissue. Common hematological signs are neutropenia, thrombocytopenia, and anemia, and in severe cases, pancytopenia has also been

reported [4, 5, 11]. In most patients, the BM cellular alterations ameliorate or disappear after antibiotic treatment [4]. Moreover, brucellosis transmission by BM transplantation from seemingly healthy donors has been reported [12]. These data indicate that even in those cases in which the bacterium is not isolated from the BM, it still may be present, hidden within cells.

Following experimentation in mice, it has been proposed the BM may be the most relevant tissue for *Brucella* persistence [13]. In addition, *Brucella canis* has been shown to persevere in the BM at chronic stages of mouse infection [14]. Here, we describe the persistence of *Brucella abortus* in cells of the mice BM and propose that this tissue is essential for establishing long-lasting chronic infections.

2. Materials and Methods

2.1. Infection Protocols. B. abortus 2308W expressing red fluorescent protein from *Discosoma* coral (*B. abortus*-RFP), provided by Jean-Jacques Letesson (University Notre-Dame de la Paix, Namur, Belgium) was used in all experiments. BALB/c mice were supplied by the Escuela de Medicina Veterinaria, Universidad Nacional, Costa Rica, and Laboratorio de Ensayos Biológicos, Universidad de Costa Rica. C57BL/6 mice were purchased from Charles River Laboratories (Les Oncins, France), housed under specific pathogen-free conditions, and handled in accordance with French and European guidelines.

Mice were infected by the intraperitoneal route (i.p.) with 10^6 bacterial colony forming units (CFU) of *B. abortus*-RFP. At different phases of the infection, the spleen, liver, lymph nodes, and bone marrow (BM) were collected. Then, the organs subjected to bacterial counts, histopathological examination, and cells analyzed by flow cytometry, as described elsewhere [15, 16]. Experimentation in mice was conducted following the guidelines and consent of 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 Animal Welfare Law of Costa Rica (Law 9458). All animals were kept in cages with food and water *ad libitum* under biosafety containment conditions.

BM cells were also isolated and infected ex vivo in the presence of anti-*Brucella* antibodies, following previous protocols [16]. Briefly, BM cells were isolated from the tibia and femur of *B. abortus*-RFP-infected mice at 8 and 30 days of postinfection by flushing bones with HBSS (no calcium, no magnesium) or RPMI medium. BM cells were then incubated with *B. abortus*-RFP at MOI of 50 bacteria/cell at 37°C for 2 hours, washed with PBS, suspended in HBSS, and subjected to examination. The number of CFUs infecting enriched BM-derived PMNs was estimated by lysing the cells and counting bacteria in agar plates [17].

2.2. Immunofluorescence. BM cells (50 to 100 μl resuspended in DMEM at a concentration of 10^6 cells/ml) were loaded on alcian blue-coated coverslips (Sigma) and incubated for 20 min at 37°C to allow cell attachment. Twenty minutes Antigenfix (Diapath) was used for fixation. Once fixed onto coverslips, cells were washed with PBS and slides were mounted using ProLong Gold Antifade reagent containing DAPI (Thermo Fisher Scientific). Slides were observed with confocal microscope (Leica TCS SP8) as described before [18]. Image analyses were performed using the ZEN 2011 software.

2.3. Histopathology. For histopathological studies, the spleen, lymph nodes, and BM from infected and PBS-treated mice were fixed in 10% neutral buffered formalin, processed and stained with hematoxylin and eosin or Giemsa stain [19]. The histopathological score (from 0 (negative) to 4 (severe)) was determined by semiquantitative analysis as previously described [20–22].

2.4. Flow Cytometry. For flow cytometric analyses, cell surface markers were stained using the following antibodies: BV421 anti-CD11b (M170), BV711 anti-Ly6G (1A8), BV785 anti-F4/80 (BM8), and BV570 anti-CD4 (RM4–5) antibodies were purchased from BioLegend; AF647 anti-CD34 (RAM34), BV711 anti-CD8α (53-6.7), and BV650 anti-CD3 (245-2CII) from BD Biosciences; and Alexa Fluor 488 and APC both anti-CD115 (AFS98), PE anti- Ly6G (1A8), Ef450 anti-CD45R/B220 (RA3-6B2), PE Cy7 anti-CD19 (1D3), and AF700 anti-CD44 (1M7) antibodies from eBiosciences; and APC Cy7 anti-CD16/32 (2.4G2), BV510 anti-Sca-1 (D7), and BV605 CD117/c-kit (2B8) from BD Biosciences. An antibody staining scheme is provided in Table S1. Cells were identified according to the staining scheme and the percentage of each cell type determined in relation to all living cells of bone marrow at 8 and 30 days of postinfection. Cell viability was evaluated using Fixable Viability Dye UV (eBiosciences). Cells were fixed with Antigenfix for 20 min before the acquisition. Multiparameter flow cytometry was performed using a FACS LSRII UV (BD Biosciences) or Guava easyCyte (Millipore). Flow cytometry data were analyzed using the FlowJo software, version 10.0.7 (Tree Star Inc.).

2.5. Statistics. One-way analysis of variance (ANOVA) followed by Dunnett's test or multivariate analysis of variance (MANOVA) was used to determine statistical significance in the different assays. The JMP (https://www.jmp.com) and GraphPad Prism software (https://www.graphpad.com) were used for statistical analysis. Data were processed in Microsoft Office Excel 2015.

3. Results

According to bacterial loads, histopathological alterations, and immune response, murine brucellosis has been divided into four stages: onset of infection, acute phase, chronic phase, and chronic declining phase [23] (Figure 1(a)). After infection, *B. abortus* CFU counting was performed from the spleen, lymph nodes, and BM during the lapse of 168 days of postinfection (Figure 1(a)). Bacterial loads and kinetic profiles of the spleen and lymph nodes were similar. A significant bacterial increase was observed in the lymph nodes and spleen at the chronic steady phase III (28 days of postinfection), followed by a decrease in the bacterial numbers at the chronic declining phase IV and until the end of the experimentation. In the BM, *B. abortus* infection steadily

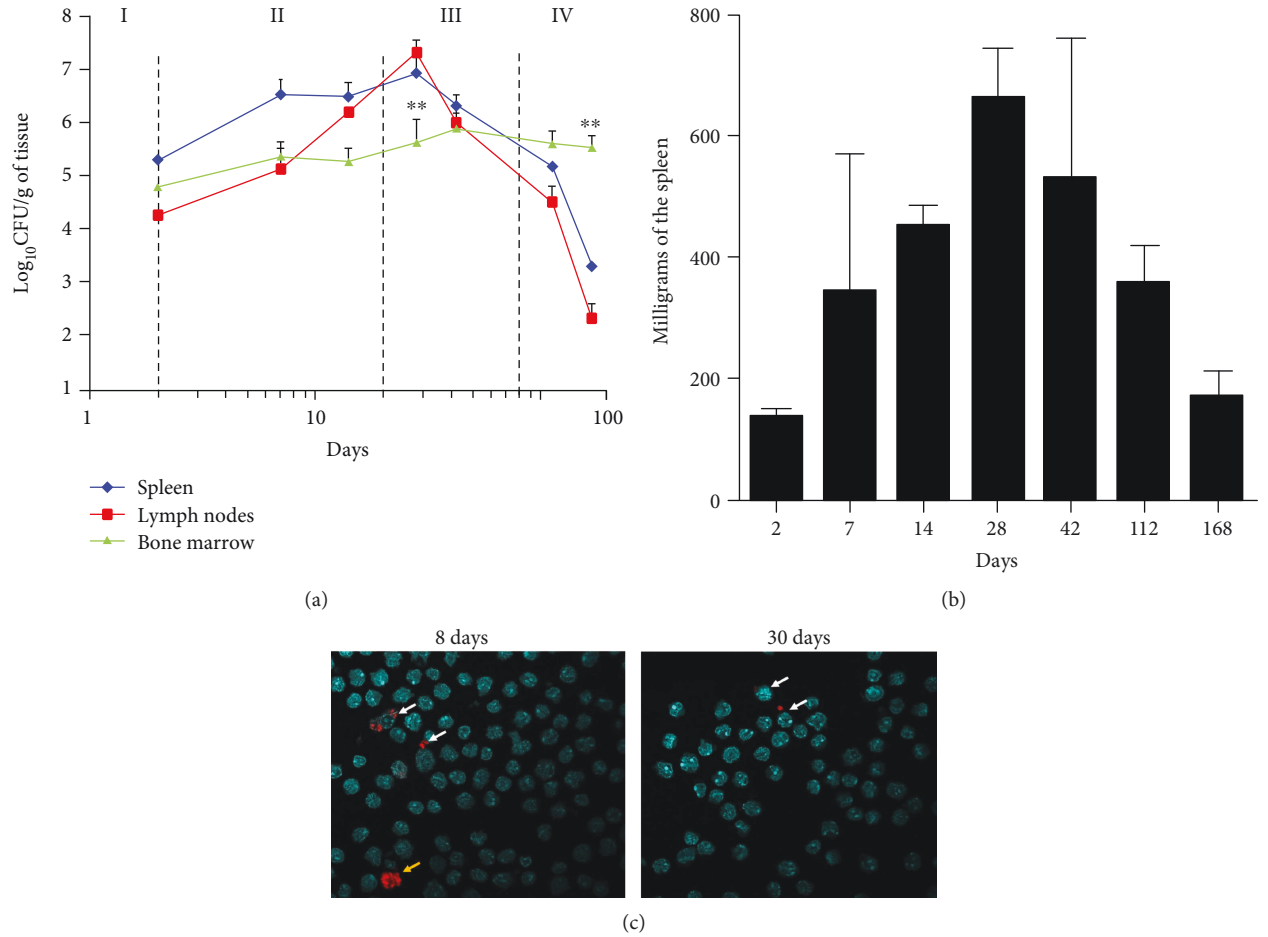

Figure 1: *B. abortus* persists in bone marrow during the course of infection. Mice were infected with *B. abortus*-RFP. (a) Spleen, lymph nodes, and BM were collected, and CFUs determined at different phases of infection [23]: the onset of infection (I), the acute phase (II), the chronic steady phase (III), and the chronic declining phase (IV). Each bar is the mean (±1 SD) of an experiment. Values of $^{**}p < 0.01$ are indicated in relation to spleen and lymph node bacterial loads. (b) Before CFU determination, the spleens were weighted at each time of examination. (c) BM cells were isolated from the tibia and femur of *B. abortus*-RFP- (red intracellular bacteria) infected mice at 8 and 30 days of postinfection mounted using ProLong Gold containing DAPI (blue nuclei). Microscope images are captured at 60x magnification confocal microscope.

persisted throughout all four phases, until day 168, when the CFU/g loads were significantly higher than those of lymph nodes and spleen. Similar results were obtained with C57BL/6 mice (not shown). The weight of the spleens increased until day 28 and then decreased until the end of the experiment, following a pattern similar to that of the kinetics of the CFU count (Figure 1(b)). Even though the number of CFU/g of BM was relatively high, the absolute numbers of *B. abortus* BM-infected cells were low at 8 (acute phase) and 30 (chronic phase) days of postinfection, suggesting that few infected cells harbored many bacteria (Figure 1(c)). However, a high number of bacteria was observed in some cells, a phenomenon that may account for the discrepancy between the CFU/g and the number of infected cells.

It has been demonstrated that most *Brucella*-infected human patients display histopathological alterations, whether or not the bacterium is isolated from the BM [4]. As shown in Figure 2(a), granulomatous inflammation was more severe and diffuse at acute stages than the multifocal chronic phase in the BM, spleen, and liver. At the acute phase, the inflammatory process was characterized by coalescing to diffuse inflammation with larger and cell-rich granulomas, while in the chronic phase, granulomatous inflammation was multifocal with smaller and fewer cellular lymphohistiocytic aggregates. Epithelioid macrophages predominate during the inflammatory process at early stages, reducing in number with chronicity. Classical granuloma formation was observed more clearly in the spleen and liver, while bone marrow developed an epithelioid macrophage-rich aggregate with scattered lymphocytes, which reduced its size and cellularity over time. Compared to the spleen, bone marrow granulomatous inflammation was more severe in the first two weeks of infection. After four weeks of infection, the spleen and bone marrow presented similar inflammation scores, though the granulomatous inflammation decreased in both tissues afterward (Figure 2(b)).

The cellular changes in the BM of infected mice were estimated by flow cytometry. At 8 days of postinfection, we observed changes in the hematopoietic cell population. At day 8 of postinfection, the percentage megakaryocyte-

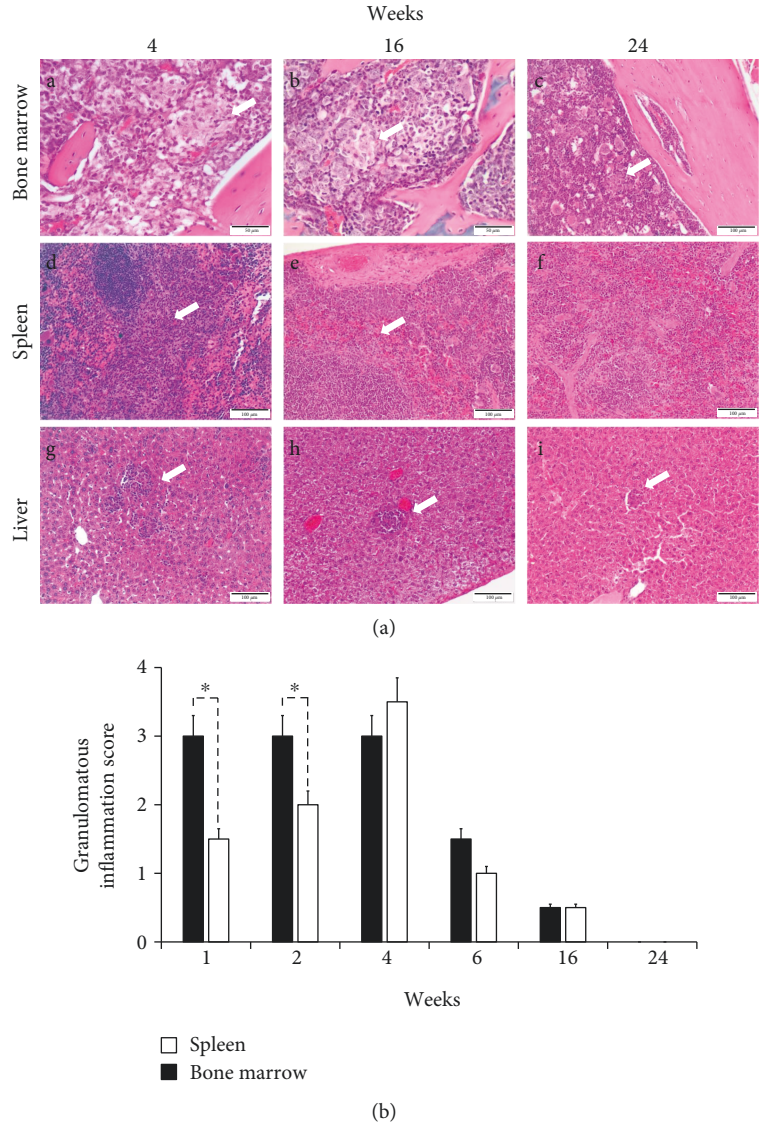

Figure 2: *Brucella abortus* induces a granulomatous inflammation in bone marrow. (a) Mice were infected with *B. abortus*-RFP. The spleen, liver, and BM were collected at different phases of infection and subjected to histopathological examination. (b) Granulomatous inflammation was scored from 0 (negative) to 4 (severe) [19] in BM over time. Each bar is the mean (±1 SD) of an experiment. Value of $^{*}p < 0.05$ is indicated in relation to BM and spleen granulomatous inflammation.

erythrocyte progenitor (MEP) decreased compared to the BM of noninfected mice (data not shown). Contrarily, the percentage of granulocyte-monocyte progenitors (GMP) significantly increased. Likewise, neutrophils (PMN) and $CD4^{+}$ lymphocyte populations significantly increased at 8 days of postinfection (Figure 3(a)). The increase of $CD8^{+}$ cells was evident, but not significant ($p < 0.05$).

In order to estimate the proficiency of BM cells to internalize *B. abortus*, we performed an ex vivo infection. For this, BM cells were infected with *B. abortus*-RFP in the presence of anti-*Brucella* antibodies. As shown in Figure 3(b), close to 32% of the BM cells were infected; of these, over 90% were identified as PMNs [16].

Flow cytometry analysis of BM from infected mice rendered three main cell types containing *B. abortus*: monocytes, PMNs, and GMPs (Figure 3(c)). At 8 days of postinfection, the proportion of PMN-containing bacteria was greater than other cells. Strikingly, the number of infected PMNs dramatically decreased after 30 days. The proportion of infected monocytes remained similar at 8 and 30 days of postinfection. Although at early stages of infection close to 3% of the GMP-contained bacteria, the number of infected cells practically disappeared at later times (Figure 3(c)).

4. Discussion

At initial stages of infection, *Brucella* invades target organs, before a strong activation of the innate immune system and stimulation of antimicrobial mechanisms [7, 24]. This immunological gap allows the bacterium to colonize, replicate, and hide within cells of the mononuclear phagocyte system. Linked to this is the observation that *B. abortus*

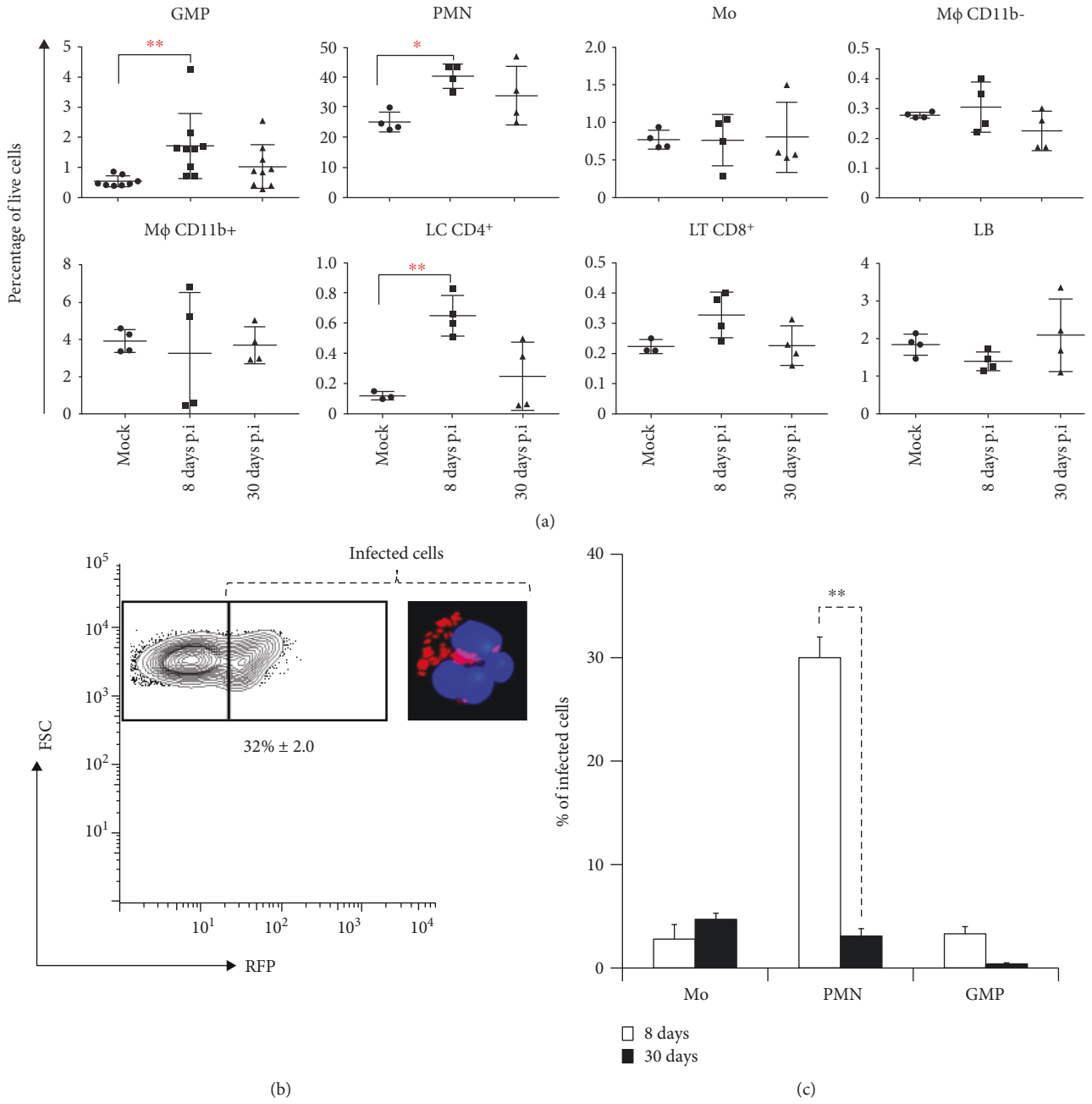

Figure 3: Bone marrow leukocyte variation at different stages of infection. (a) BM cells from *B. abortus*-infected mice were collected and subjected to multiparameter flow cytometry analysis. Cells were identified according to the staining scheme (Table S1) and the percentage of each cell type determined in relation to all living cells of bone marrow at 8 and 30 days of postinfection. Values of $^{*}p < 0.05$ or $^{**}p < 0.01$ are indicated in relation to control noninfected mice at 8 and 30 days of postinfection. (b) Whole BM cells were collected and infected ex vivo with *B. abortus*-RFP. Infected cells were gated based on the RFP (red) positivity, and the total percentages of infected cells were quantified. (c) BM cells from *B. abortus*-RFP-infected mice were collected and subjected to multiparameter flow cytometry analysis. Infected cells were gated based on the RFP (red) positivity, identified and quantified according to the staining scheme (Table S1) at 8 a 30 days of postinfection. Each bar is the mean (±1 SD) of an experiment. Values of $^{**}p < 0.01$ are indicated.

infection remains sequestered within BM cells for a protracted period, without significant changes in the bacterial loads. These phenomena propose a mechanism for *Brucella* persistence.

Granuloma formation, commonly observed in long-lasting infections, is an attempt to eliminate the microorganisms [25, 26]. In tuberculosis, it has been proposed that granulomas provide a bacterial safety shelter from the host immune response [27]. The higher number of granulomas in the BM and the permanence of these structures indicate the struggle of immune cells for eliminating *B. abortus*. This is also depicted by the significantly higher number of $CD4^{+}$ lymphocytes in the BM at early stages of infection, which in brucellosis correlates with Th1 polarization [28].

The most abundant infected BM cells at the acute phase of murine infection (once antibodies against *Brucella* have developed) were PMNs. This result is reminiscent of the ex vivo infection of BM cells. Indeed, we have demonstrated that a large proportion of *ex vivo B. abortus*-infected BM cells are PMNs and that these leukocytes are unable to kill the ingested bacteria [16]. Despite this, it is unlikely that PMNs are the main reservoirs for *Brucella* in the BM. Indeed, *B. abortus* does not replicate in these cells and these infected leukocytes died prematurely [16]. Rather, PMNs may serve as vehicles for dispersing the bacterium, functioning as Trojan horses, as previously proposed [16, 18].

A small proportion of GMP cells in the BM were also infected at the acute phase of infection. This is unexpected since uncommitted progenitors such us GMP cells are not yet considered phagocytic cells [29]. However, at later time points, the proportion of infected cells was negligible. Moreover, the total number of GMP cells increased at early times of infection, diminishing afterward. These cellular variations correlate with the pathological changes of the BM. A similar phenomenon has been observed in human brucellosis cases [4, 30].

To our knowledge, this is the first time that myeloid oligopotent progenitor stem cells, lacking a developed phagocytic machinery, have been shown to become infected with *Brucella* organisms. Even though it is common to observe extramedullary hematopoiesis in the spleen of *Brucella*-infected mice [23], here we demonstrate for the first time *Brucella*-infected hematopoietic oligopotent stem cells residing in the BM. During emergency myelopoiesis, self-renewing GMPs in patches (pGMPs) build GMP clusters and differentiate into clustering GMPs (cGMPs). These GMP clusters can differentiate into mature cells until complete disappearance of the GMP clusters [31, 32]. Moreover, it has been shown that the increasing number of myeloid progenitors can promote microbial persistence in the organism [33]. All these findings make us speculate that *B. abortus* infects myeloid oligopotent progenitor stem cells and may interfere to induce GMP differentiation into infected-differentiated cells. Whether the reduced number of infected nonphagocytic erythrocytes and B cells [34, 35] originates from BM-infected progenitor cells remains unknown.

Despite the histopathological changes of the *B. abortus*-infected BM, and the low numbers of infected monocytes, the proportion of these leukocytes remained constant and persistent. It is well known that during granuloma formation, monocytes differentiate into macrophages, epithelioid cells, and dendritic Langerhans-type giant cells [36]. Moreover, *Brucella* is able to survive in monocytes and inhibits their programmed cell death [8]. Join-Lambert et al. [37] showed that *Listeria monocytogene*-infected myeloid cells in the bone marrow play a crucial role in the pathophysiology of meningoencephalitis by releasing infected cells into the circulation. Therefore, BM monocytes are firm candidates for *Brucella* reservoirs in the BM. These cells may be the source of the frequent relapses observed in antibiotic-treated individuals, even several years after the primo infection [38, 39].

5. Conclusions

Bacterial persistence, chronicity, and relapses are major problems in brucellosis. Within this context, we concluded (i) that loads of *B. abortus* in the BM remain constant and are long lasting; (ii) that *B. abortus*-infected BM displays histopathological modifications associated with augmented numbers of multipotent progenitor and active hematopoietic stem cells, PMNs, and $CD4^+$ lymphocytes during the acute phase of the infection; and (iii) that the three types of infected cells in the BM are monocytes, PMNs, and GMP cells. In addition, we hypothesize that (iv) BM PMNs may serve as vehicles for dispersion of *Brucella*, following the Trojan horse hypothesis; (v) that *B. abortus*-infected myeloid oligopotent progenitor cells may differentiate into mature infected cells; and (vi) that monocytes are the most likely *Brucella* reservoirs in the BM and that these cells may be the source of the frequent relapses observed in antibiotic-treated individuals.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Supplementary Materials

Table S1: antibody staining scheme used for identifying each cell population. *(Supplementary Materials)*

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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

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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 leucocytes (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 (Mϕs) 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 Mϕs 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 *Brucell*a-infected PMNs are readily phagocyted by murine Mϕs in a non-phlogistic manner, and that bacteria delivered through PMNs, extensively replicate inside Mϕs. The experiments shown here, are a proof of concept for the "Trojan horse" proposal, which states that *Brucella*-infected PMNs serve as vehicles for Mϕ 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 Mϕs were harvested and cultured as previously described (19). *B. abortus*-RFP infection (MOI 50) of 2×10^5 RAW 264.7 or peritoneal Mϕ monolayers was performed by using the gentamicin protection assay to avoid extracellular bacteria (20). Additionally, RAW 264.7 or peritoneal Mϕs 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 Mϕ 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 μg/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 Mϕs monolayers were calculated retroactively by lysing the PMNs and counting bacteria in agar plates. Controls of co-cultivated non-infected PMN with Mϕ 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 Mϕs 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). Mϕ 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 Mϕs 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 Flour 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 Log_{10} 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.

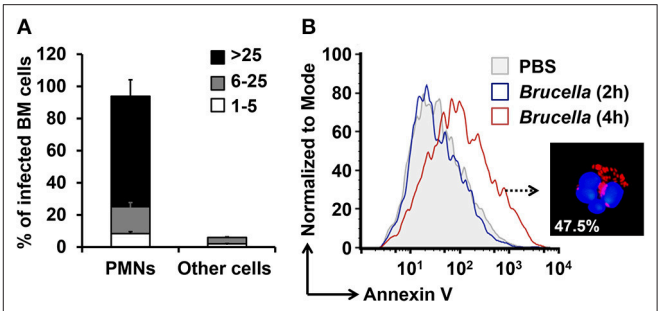

FIGURE 1 | *B. abortus* infect PMNs and promote the exposure of phosphatidylserine. BM cells were incubated with *B. abortus*-RFP (MOI 50) for 4 h. **(A)** Cells were mounted using Prolong Gold containing DAPI (blue nuclei). At least 200 PMNs were counted per sample. The percentage of infection and the number of intracellular bacteria (1–5, 6–25, or >25) per cell was determined by fluorescence microscopy. Cell infections were confirmed by flow cytometry. **(B)** The PMN population analyzed by flow cytometry was gated using anti-Ly6G as PMNs cell marker and analyzed by Annexin V as a cell death marker. These experiments were repeated at least three times.

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

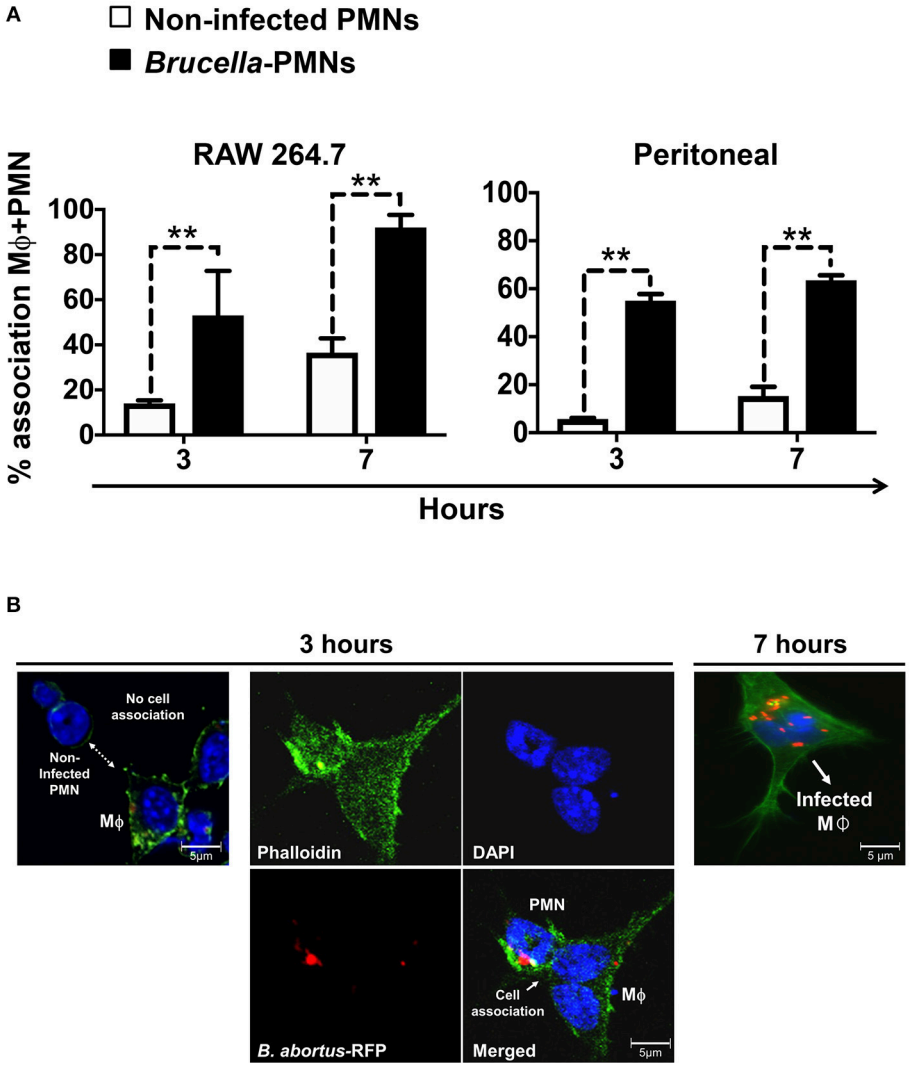

FIGURE 2 | *B. abortus*-infection increase the association of PMN with Mϕs. **(A)** Non-infected or *B. abortus*-RFP-infected PMN were incubated with RAW or peritoneal Mϕs (1:1) at different time points and PMN-Mϕ interactions were quantified. Cells were stained (DAPI, for nuclei; phalloidin-FITC for actin filaments), fixed and mounted with MOWIOL. At least 200 PMNs were counted and the percentage of PMN-Mϕ cell association determined. Values of $p < 0.01$ (**) are indicated in relation to Mϕs incubated with non-infected PMNs. **(B)** RAW Mϕ 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 μg), 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 Mϕs 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 Mϕs, 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 Mϕs in the replication kinetics. In any case, in both experiments, *Brucella*-PMN-infected Mϕs 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 Mϕs. 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 Mϕs significantly diminished (**Figure 6A**). Moreover, bacterial replication was reduced in RAW Mϕs at all-time points (**Figure 6B**), displaying the profile

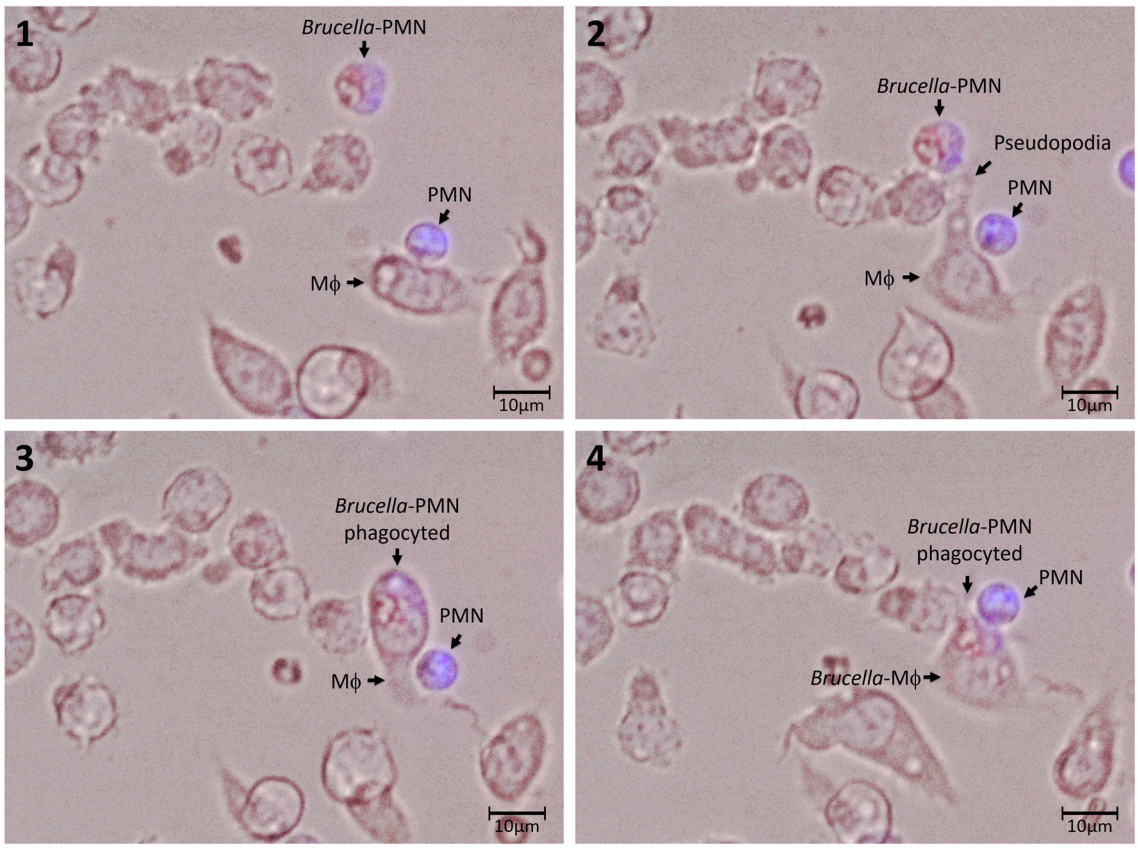

FIGURE 3 | Association and uptake of *B. abortus*-infected PMN by Mϕs. PMNs were incubated with *B. abortus*-RFP (red) (MOI of 50) for 1.5 h; then, cells were pelleted and washed with PBS to remove extracellular bacteria. *Brucella*-infected-PMNs were suspended in DMEM without gentamicin and added to 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,

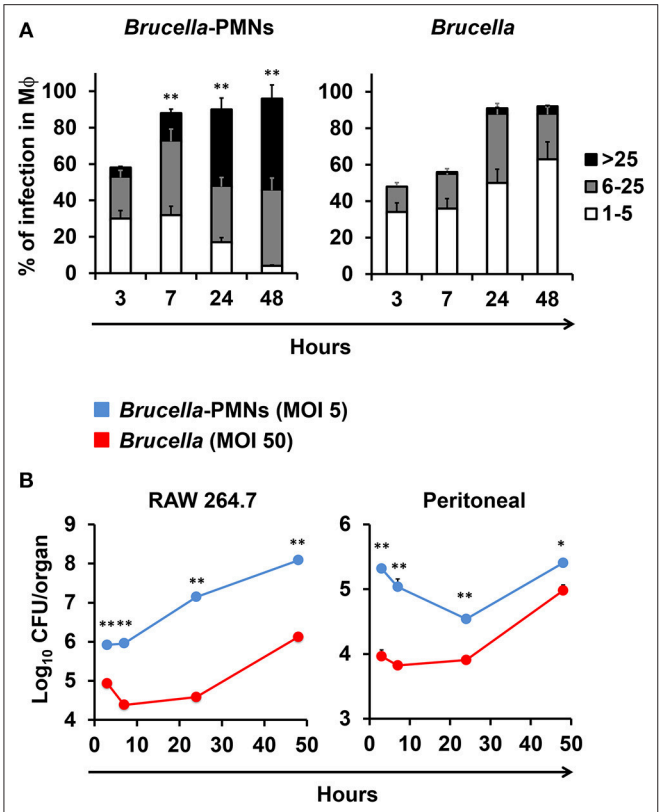

FIGURE 4 | *B. abortus*-infected PMN promote *Brucella* infection of Mϕs. **(A)** RAW Mϕs were incubated with *B. abortus*-RFP alone or with *B. abortus*-infected PMN, then cells were stained (DAPI, for nuclei; phalloidin-FITC for actin filaments), fixed and mounted with MOWIOL, and the number intracellular bacteria (1–5, 6–25, or >25) per Mϕ was determined. The proportion of infection and the number of intracellular bacteria was determined by fluorescence microscopy. At least 200 cells were counted per sample. Values of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated in relation to Mϕs incubated with non-infected PMNs. **(B)** RAW and peritoneal Mϕs were infected with *B. abortus*-RFP alone (MOI 50) or with *B. abortus*-RFP infected PMNs (MOI 5) and CFU determined at different time points. These experiments were repeated at least three times.

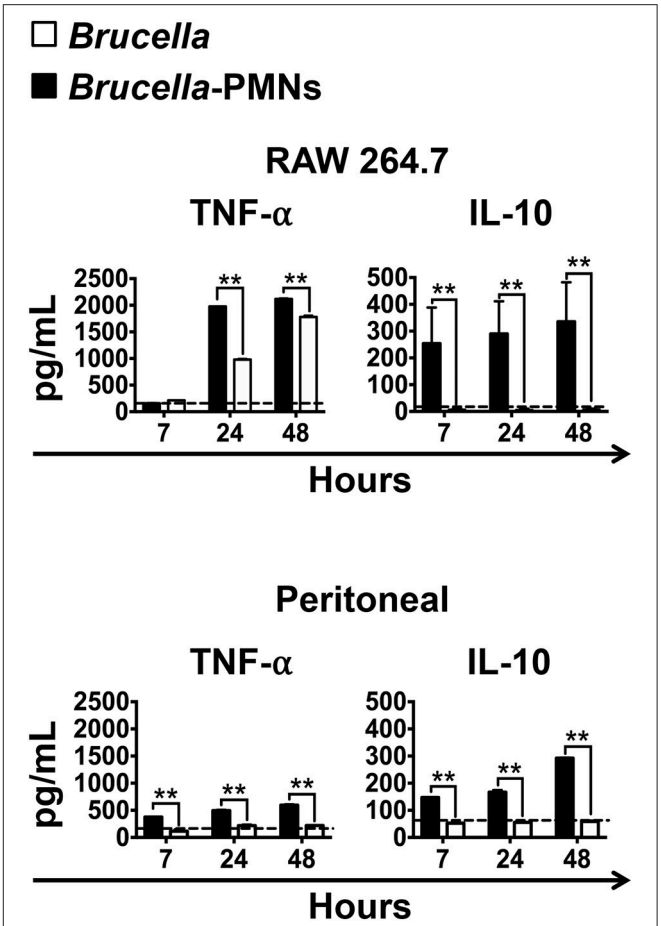

FIGURE 5 | High production of IL-10 and low production of TNF-α in the supernatant of infected Mϕs at initial stages of infection. RAW and peritoneal Mϕs were infected with *B. abortus*-RFP (MOI 50) alone or with *B. abortus*-infected PMNs (MOI 5), and the supernatants collected at different time points. The level of the cytokines was determined by ELISA in the culture supernatants of infected Mϕs. Values of $p < 0.01$ (**) are indicated in relation to Mϕs infected with *Brucella* alone. The backgrounds of the cytokine production by Mϕs co-cultivated with non-infected PMNs are indicated by dashed lines. These experiments were repeated at least three times.

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 a to 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

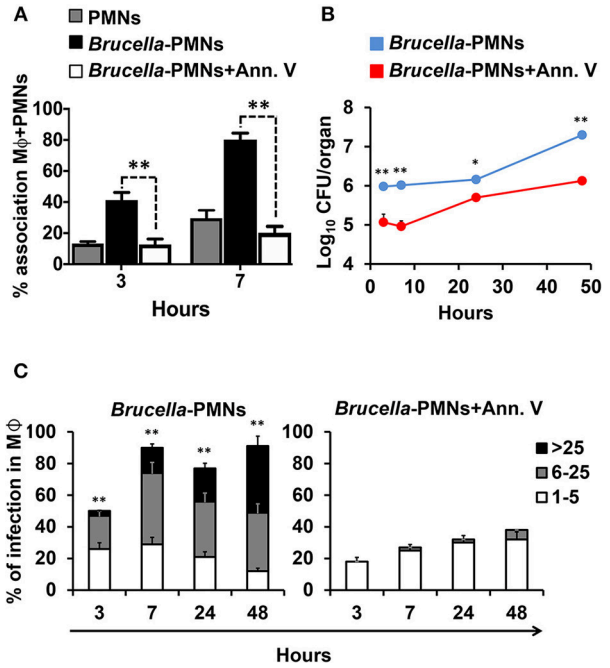

FIGURE 6 | Blockage of PS on the surface of *Brucella*-infected PMNs hampers Mϕ *B. abortus* infection. **(A)** *B. abortus* infected PMNs were pre-treated or not with Annexin V before co-incubation with RAW Mϕs, and cell association estimated. The red dashed line corresponds to the mean value of non-infected PMNs **(B)** *B. abortus* infected PMNs (MOI 5) were pre-treated or not with Annexin V before co-incubation with RAW Mϕs and CFU determined at different time points. **(C)** *B. abortus* infected PMNs were pre-treated or not with Annexin V before co-incubation with RAW Mϕs and the number of infected Mϕs and intracellular bacteria within these cells estimated at different time points. Cells were stained (DAPI for nuclei; phalloidin-FITC for actin filaments), fixed and mounted with MOWIOL. At least 200 cells were counted in each experiment. The percentage of infection and the number of intracellular bacteria (1–5, 6–25, or >25) per Mϕ was determined by fluorescence microscopy. Values of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated in relation to Mϕs incubated with PMNs treated with Annexin V. These experiments were repeated at least three times.

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 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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