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REVIEW

The Role of Neutrophils in Brucellosis

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SUMMARY Brucellosis is a bacterial disease of domestic animals and humans. The pathogenic ability of *Brucella* organisms relies on their stealthy strategy and their capacity to replicate within host cells and to induce long-lasting infections. *Brucella* organisms barely induce neutrophil activation and survive within these leukocytes by resisting microbicidal mechanisms. Very few *Brucella*-infected neutrophils are found in the target organs, except for the bone marrow, early in infection. Still, *Brucella* induces a mild reactive oxygen species formation and, through its lipopolysaccharide, promotes the premature death of neutrophils, which release chemokines and express "eat me" signals. This effect drives the phagocytosis of infected neutrophils by mononuclear cells that become thoroughly susceptible to *Brucella* replication and vehicles for bacterial dispersion. The premature death of the infected neutrophils proceeds without NETosis, necrosis/oncosis, or classical apoptosis morphology. In the absence of neutrophils, the Th1 response exacerbates and promotes bacterial removal, indicating that *Brucella*-infected neutrophils dampen adaptive immunity. This modulatory effect opens a window for bacterial dispersion in host tissues before adaptive immunity becomes fully activated. However, the hyperactivation of immunity is not without a price, since neutropenic *Brucella*-infected animals develop cachexia in the early phases of the disease. The delay in the immunological response seems a *sine qua non* requirement for the development of long-lasting brucellosis. This property may be shared with other pathogenic alphaproteobacteria closely related to *Brucella*. We propose a model in which *Brucella*-infected polymorphonuclear neutrophils (PMNs) function as "Trojan horse" vehicles for bacterial dispersal and as modulators of the Th1 adaptive immunity in infection.

KEYWORDS *Brucella*, brucellosis, neutrophils, neutropenia, native immunity, adaptive immunity, Trojan horse

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INTRODUCTION

In 1902, the British bacteriologist and surgeon Sir Percy William Bassett-Smith (1861–1927) of the Royal Naval Hospital studied the plasma-opsonizing properties in Mediterranean fever patients infected with "*Micrococcus melitensis*" (*Brucella melitensis*) (1). This bacterium was the etiological agent of Mediterranean fever, or Malta fever, later known as brucellosis, discovered in 1887 by a team of investigators under the leadership of Surgeon Captain David Bruce on the island of Malta. According to Bassett-Smith's experience, one of the most prominent and constant features of human brucellosis was the "relative reduction of the number, as well as the phagocytic activity, of the polymorphonuclear white cells." He related this phenomenon to the "causation of the prolonged course and recurrent nature of the disease that can last for decades" (2), as suffered by Alice C. Evans (1881–1975), the brilliant bacteriologist who championed the procedure of milk pasteurization and found that *M. melitensis* and the bacillus of Bang's disease (*Brucella abortus*) were related. She acquired the infection in 1922 and experienced this long-lasting and recurrent disease for 22 years (3). Surgeon Bassett-Smith, who knew the methods described by the Nobel Prize laureate Élie Metchnikoff (1845–1916), noted that polymorphonuclear neutrophils (PMNs) were capable of phagocytizing a large number of *M. melitensis* organisms without lysis of these leukocytes (4). Moreover, the process could proceed in the absence of specific opsonins (antibodies), as then proposed by Metchnikoff (5). Later, the American bacteriologist Irvin Forest Huddleson (1893–1965) extended and illustrated these observations in 1933 (6).

The first comprehensive hematological profiles, carried out in 300 human brucellosis patients in 1939, demonstrated that 40 to 50% of patients with active brucellosis displayed absolute and relative neutropenia (7). The correlation between bacteremia and neutropenia was established a few years later (8). Based on clinical and experimental data, some investigators suggested in 1941 that through a bacterium-extractable substance, *Brucella* organisms were "toxic" to PMNs (9). While some supported this proposal, others denied it, raising a significant controversy among leading brucellologists of the time (8, 10). Finally, in 1943, investigators observed that in contrast to other Gram-negative bacteria, *Brucella* organisms did not prime for the so-called Shwartzman reaction, a response that recruits large numbers of PMNs in the skin after the second injection of endotoxin (11).

Amid the 20th century, brucellosis became a global and relevant zoonotic problem. Due to its high prevalence in humans, a significant number of clinicians studied the disease. One of the best known American medical brucellologists of the epoch was Wesley W. Spink (1904–1988), from the University of Minnesota. He had ample experience with hundreds of human brucellosis cases and with experimental models (8). In 1951, one of his students, Abraham I. Braude (1917–1984), who later became a renowned scientist in his own right, observed that soon after invasion, *Brucella*-infected PMNs bound Kupffer cells gathering around them in the liver sinusoids. After a few hours, the Kupffer cells were remarkably full of bacteria, while the liver sinusoids were devoid of *Brucella*-infected PMNs (12). These events suggested that the mononuclear phagocytic cells in the affected organs phagocytized the circulating, infected PMNs. Spink claimed that PMNs played a significant role in the pathogenesis of the disease and reported that "phagocytosis of brucellae by these leukocytes may be detrimental to the host since the bacteria may not be killed but protected by this intracellular localization" (8). Other studies revealed that *Brucella* organisms caused mononuclear granulomas, transient pancytopenia, and hemophagocytosis after the invasion of the bone marrow (BM) (8, 10). These results suggested that infected cells in the hematopoietic tissues (including PMNs) were removed, mainly after long-lasting infections. In his classic book *The Nature of Brucellosis*, Wesley W. Spink proposed that "systemic dissemination of the infection may be dependent, in part, upon the circulation within the bloodstream of these phagocytes [PMNs], containing viable brucellae" (8).

In the last 3 decades of the 20th century, various groups unequivocally demon-

strated that *Brucella* organisms were naturally resistant to the killing action of PMNs (13–15) and that together with resident macrophages (Mϕs) and dendritic cells (DCs), PMNs were the first cells to encounter and internalize *Brucella* organisms after mucosal invasion (16, 17). All of these phenomena agreed with the low proinflammatory response at early times in the infection, the lack of endotoxic symptoms, and the absence of coagulopathies in brucellosis patients and animals (18, 19).

Despite these remarkable clinical and experimental observations and the significant role that PMNs play against other bacterial diseases, the function of these leukocytes in brucellosis remained unexplored for several decades. On the one hand, cellular microbiologists focused on unveiling the intracellular life cycle of *Brucella* organisms in Mϕs, DCs, and epithelial cells but not in PMNs (20, 21). As expected, the absence of *Brucella* replication in PMNs precluded these cells from studies in cellular microbiology. On the other hand, most studies in brucellosis concentrated on cells of the acquired immune system rather than on PMNs participating in the early proinflammatory responses (22, 23). In recent years, however, renewed interest in the role that PMNs have in *Brucella* infections has increased. Here, we critically review most of the work done in clinical and experimental brucellosis concerning PMNs and establish a model for the function of these leukocytes in the disease.

THE DISEASE NAMED BRUCELLOSIS

Members of the genus *Brucella*, mainly *Brucella melitensis*, *Brucella suis*, *Brucella abortus*, and *Brucella canis*, are pathogens responsible for a worldwide disease known as brucellosis, which affects domestic animals and humans (24). The high prevalence of the infection in low- and middle-income nations has a detrimental impact on public health and causes significant economic losses (25). In high-income countries, where the disease has been eradicated from livestock, brucellosis exists in wildlife and dogs (26) and is an "exotic" human infection, often overlooked or confused with other chronic illnesses (27). This matter is not trivial, since a large proportion of immigrants and refugees in high-income countries arrive from areas where brucellosis is endemic. Other *Brucella* organisms, such as *Brucella ovis*, a pathogen of rams, *Brucella ceti* and *Brucella pinnipedialis,* infecting marine mammals, and *Brucella neotomae* and *Brucella microti,* parasites of *Cricetidae* rodents, are of less zoonotic relevance and therefore not considered dangerous human pathogens (24).

As suggested by their names, the zoonotic *Brucella* species have different host preferences: *B. melitensis* for goats and sheep, *B. abortus* for cattle, *B. suis* for pigs, and *B. canis* for dogs. The various *Brucella* organisms are capable of cross-infecting different animal species, but such infections are, with some exceptions, mostly sporadic and not epizootic. The zoonotic *Brucella* species display some differences in virulence. The most dangerous is *B. melitensis*, and the least dangerous is *B. canis*, with *B. suis* and *B. abortus* standing somewhere in the middle (24, 28, 29). Still, the genetic relatedness of the species is very close, with a DNA similarity near 98 to 99%. Consequently, they show similar phenotypic characteristics and possess the same virulence factors required for infection, intracellular life, and dispersion (24). Regardless of the bacterial species causing brucellosis, the clinical symptoms observed in infected humans are the same, and the same antibiotic regimen is used to treat the disease, namely, a combination of doxycycline and streptomycin or a combination of doxycycline and rifampin for at least 7 weeks (8, 10, 19).

Brucella organisms generally invade through the mucosal membranes by direct contact with infected animals or their secretions. Although several studies have shown that *Brucella* organisms are capable of trespassing the respiratory and oral tract, the conjunctiva, lachrymal ducts, and vaginal and preputial mucosa, the exact mechanisms of epithelial invasion in these tissues remain unknown (17). It has been shown that epithelial cells readily phagocytize *Brucella* organisms (21). In the ligated ileal loop model, lymphoepithelial M-like cells phagocytize and transfer bacteria to the submucosal plexus when infected with large numbers of brucellae (16). However, this effect may be the consequence of the model, and it is doubtful that invasion occurs through

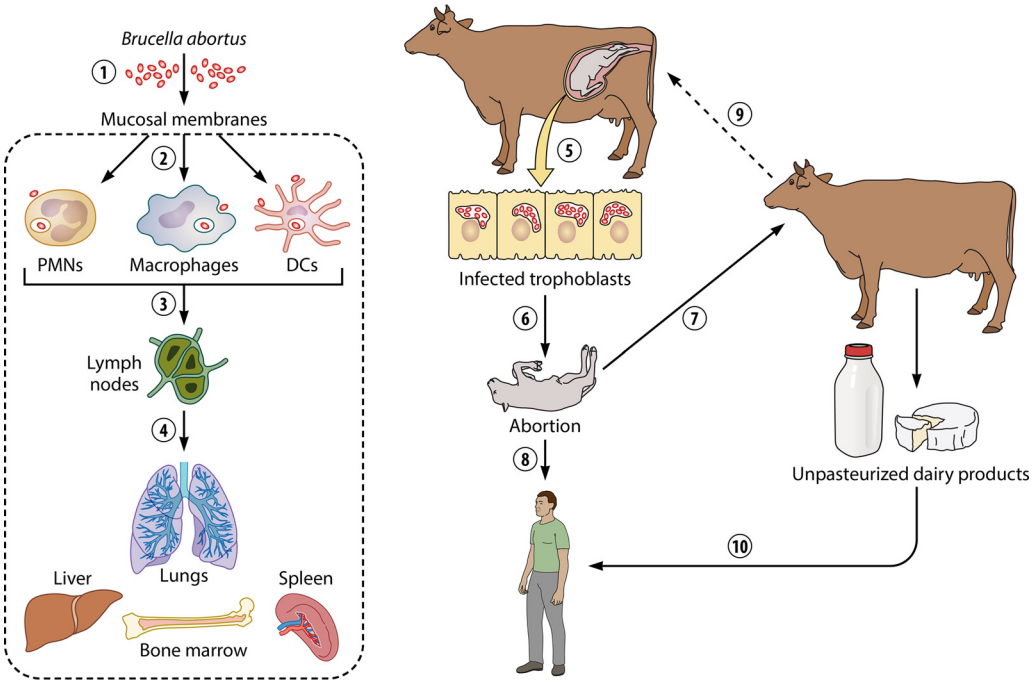

FIG 1 Cycle of *Brucella abortus* in the bovine host and humans. *Brucella* organisms infect through the mucosal membranes (1); once inside, local professional phagocytes such as Mϕs, DCs, and PMNs internalize the invading bacterium (2). From here, the bacterium moves to regional lymph nodes (3), and then it spreads to different organs of the reticuloendothelial system, such as the lungs, spleen, liver, and BM (4). In the pregnant animal, *Brucella* invades the placental trophoblasts, replicating extensively within these cells, causing placentitis (5) and abortion in the last trimester of pregnancy (6). The fetus becomes a source of infection for other animals (7) and humans (8). If the newly infected animal is pregnant, the abortive bacterial cycle continues (9). Humans may become infected by direct contact with secretions of animals with brucellosis or through ingestion of unpasteurized, contaminated dairy products (10).

the intestinal route, since it is rather inefficient compared with other routes (30). Moreover, *Brucella* is markedly sensitive to gastric juices, and no association between achlorhydria and brucellosis has been observed (8).

Brucellosis is a disease that progresses with a long incubation period that may last weeks, months, or even years (8, 10). Experimental eye infection in bovines induces a delay of 6 to 8 days, before the appearance of a local mild inflammatory response. The inflamed submucosa contains mainly mononuclear phagocytes, plasma cells, metachromatic mastocytes, eosinophilic leukocytes, and just a few PMNs (17). After initial replication at this site, the bacteria drain into the regional lymph nodes. During this period, the infected animals do not show signs of disease (31), and in pregnant animals, the bacteria replicate extensively in placental trophoblasts, causing placentitis and abortion in the last trimester. In males, the bacteria invade the testes and cause orchiepididymitis. The aborted fetus is the primary source of infection for other animals (31). Humans commonly acquire the bacterium from infected animals or through the ingestion of unpasteurized, contaminated dairy products (Fig. 1) (32).

In contrast to infection in the preferred natural host, brucellosis in humans is a grave debilitating disease (8, 10). The Mexican brucellologist Maximiliano Ruiz-Castañeda (1892–1992) described 34 different symptoms and signs (10), and Spink described about the same number (8). The disease progresses without the characteristic endotoxic symptoms of other bacterial infections and is seldom associated with distinctive clinical or laboratory markers. In its mild form, the disease displays a nonpathognomonic collection of symptoms, such as fever, joint pain, myalgia, headache, and neurasthenia, among others, and, therefore, is difficult to diagnose. If not properly treated, the bacterium may invade and replicate in vital organs, such as the bone marrow (BM), the heart, and the brain, causing a severe syndrome, even death (8, 10, 33).

The natural replicative niche of *Brucella* organisms corresponds to the intracellular environment of animal cells. Alternatively, they are better defined as facultatively extracellular intracellular pathogens rather than facultative intracellular parasites (32). It is therefore not surprising that *Brucella* species are closely related and share functional and structural properties with other animal and plant cell-associated alphaproteobacterial parasites, such as *Bartonella*, *Rickettsia*, *Anaplasma*, *Afipia*, *Wolbachia*, *Ochrobactrum*, *Sinorhizobium*, and *Agrobacterium* (24, 34). The primary host cells in which *Brucella* organisms replicate are monocytes (Mo), Mϕs, DCs, and placental trophoblasts (21). Infected at much lower rates are B lymphocytes, fibroblasts, osteoblasts, granulocyte-progenitor cells, hepatocytes, and erythrocytes (35–40). In the course of brucellosis, mononuclear phagocytic leukocytes are recruited at the site of infection and are the main effectors engaged by adaptive immunity (41–43). As already mentioned, PMNs also ingest *Brucella* organisms in high numbers, but their function in brucellosis departs from that of mononuclear phagocytic cells.

As with other chronic bacterial infections, the resistance to pathogenic *Brucella* organisms relies on strong cell-mediated immunity. This response involves the activation of the bactericidal mechanisms and antigen-presenting functions of Mϕs and DCs and the concomitant activation and expansion of $CD4^+$ and $CD8^+$ T cells, which are the principal immune effectors in brucellosis (22). An adequate Th1 immunity, with significant production of gamma interferon (IFN-γ), interleukin-12 (IL-12), IL-6, and other cytokines, is critical for the clearance of *Brucella* infections. Studies in animals and humans have demonstrated that IFN-γ is the central cytokine in the adaptive immune response against brucellosis. Animals deficient in this cytokine or its receptor are highly susceptible to infection (44). Although antibodies play some role against brucellosis, clinical observations and experimentation in animals have shown that they may be dispensable, together with B cells (45). Like other cell-associated alphaproteobacterial pathogens, in order to establish long-lasting infections, *Brucella* has evolved different strategies to evade innate and adaptive immune responses (23, 46). Vaccination with *B. melitensis* Rev1 and *B. abortus* S19 live vaccines effectively protect against ovine/caprine brucellosis and bovine brucellosis, respectively (47). *Brucella* extracts or attenuated strains have been indiscriminately and dangerously used to vaccinate humans. Despite these efforts, no suitable vaccines are available for humans (47).

THE ROLE OF PMNs IN BACTERIAL INFECTIONS

In this section, we summarize some of the most relevant information on the function of PMNs within the context of bacterial diseases. For further details on the role of PMNs, we recommend comprehensive reviews published elsewhere (48–52).

PMNs are essential components of the innate immune system devoted to the control of microbial infections and are the first leukocytes to be recruited at the invasion sites (53). PMNs are also the primary cell effectors in the innate immune response and play a role in regulating adaptive immunity (48, 54). PMN homeostasis maintains a delicate balance between granulopoiesis in the bone marrow (BM), storage, release, intravascular margination, clearance, and destruction (48). In the BM, the production of PMNs corresponds to 5×10^{10} to 10×10^{10} cells/day, following a maturation process that includes invagination of the nucleus and lysosomal granule formation (55). Mature PMNs migrate from the BM to peripheral blood and the mononuclear phagocytic system and beneath the mucous membranes (56). The half-life span of PMNs is open to dispute, ranging from close to 6 h to 6 days and subject to various circumstances (51, 57), such as their location in tissues, which include the BM, reticuloendothelial system, lymphatic organs, and blood. Chronic diseases may prolong or shorten the life span of PMNs, a phenomenon that depends on the course of the illness and the type of pathogen.

After the bacterial invasion of tissues, PMNs become rapidly activated by cytokines and chemotactic signals, migrating through endothelial membranes of the small blood vessels to exert their microbicidal properties (53). Recruitment of PMNs at a site of infection is a crucial phenomenon of the innate immune response. Chemoattractants,

such as chemokines, cytokines, complement-derived fragments, leukotrienes, and microbial components such as N-formylated peptides, influence leukocyte migration through the activation of G protein-coupled receptors on the PMN membrane (50).

PMNs are powerful professional phagocytes capable of ingesting and killing microbes intracellularly by activating hydrolytic enzymes, cationic microbicidal peptides, and reactive oxygen species (ROS). On their surface, PMNs express receptors for complement proteins, immunoglobulins (Fc), integrins, selectins, cell adhesion molecules (CAMs), and mannose, as well as scavenger receptors, which function as adherence molecules or for phagocytosis. Activated PMNs may degranulate and discharge ectosomes, microbicidal substances in the surroundings, or discharge PMN-extracellular traps (NETs). These last elements are composed of sticky DNA, histones, and other proteins that entrap and kill microorganisms through their cationic or enzymatic action (58). PMNs also produce chemokines, cytokines, prostaglandins, lipoxins, and other lipid mediators to attract, activate, and regulate cells of the immune system required for defense (49).

In many bacterial diseases, the primed, activated PMNs extend their life span and enhance their microbicidal activity (53, 58, 59). Cytokines such as IFN-γ can activate and prolong the life of these leukocytes in infections (60). After the bacterial invaders are destroyed, and the inflammatory response slowly resolves, a careful elimination of the recruited PMNs occurs. This process, which avoids further harm and favors tissue repair, initiates when the recruited PMNs undergo apoptosis at the resolution site. The dying PMNs express "eat me" signals such as phosphatidylserine on the cell surface, engaging Mϕs and DCs to phagocytize these apoptotic leukocytes in manners that do not promote proinflammatory responses, generally known as nonphlogistic processes (61). Indeed, phagocytic cells with internalized apoptotic PMNs are not fully activated and hamper the release of proinflammatory agonistic signals but promote the production of regulatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) (56, 62). In other cases, PMNs become fully proinflammatory: they enhance their microbicidal activities, release cytokines and chemokines, degranulate, undergo NETosis, or die by necrosis or oncosis (52).

Several bacteria have evolved strategies to influence the function of PMNs (63, 64). The canine and zoonotic pathogen *Anaplasma phagocytophilum*, a close phylogenetic relative of *Brucella*, evades the host immune response by furtive strategies (65, 66) and actively invades PMNs by a process in which the bacterium takes control of the lipid raft domain-containing glycosylphosphatidylinositol-anchored protein. Once inside PMNs, *Anaplasma* modifies the vesicular biogenesis to create a unique intracellular membrane-bound compartment that allows their replication in seclusion from lysosomal destruction (65). To survive within PMNs, *Anaplasma* hampers the activation of NADPH oxidase and the subsequent degranulation of these cells. Furthermore, *A. phagocytophilum* lacks genes for the synthesis of lipopolysaccharide (LPS), cyclic β-glucans (CβG), peptidoglycan, flagella, and fimbria. Therefore, this bacterium lacks some of the essential pathogen-associated molecular patterns (PAMPs) required to trigger the innate immune response through the action of pattern recognition receptors, known as PRRs (65).

Bartonella henselae and *Afipia felis*, pathogens that are close relatives of *Brucella* organisms, inhibit human PMN oxidative function and survive within these leukocytes (67). In cats (the preferred mammal host), bacteremia progresses without neutrophilia or endotoxicity (68). Likewise, a significant characteristic of trench fever bacteremia caused by *Bartonella quintana* is the absence of septic shock symptoms such as disseminated intravascular coagulation, neutrophilia, or organ failure (69). This phenomenon is related to the absence of agonistic effects of its nonendotoxic LPS on Toll-like receptor 4 (TLR4). It is noteworthy that *Bartonella* LPS shares many structural features with *Brucella* LPS (*Br*-LPS) (70–73), which departs from the classical endotoxic LPSs (74). It seems, therefore, that these closely related intracellular alphaproteobacteria share several features associated with a stealthy strategy to invade host cells.

Gram-negative bacteria from other class subdivisions follow different strategies. For

instance, *Shigella flexneri* kills PMNs by necrosis, a process characterized by the release of tissue-injurious granular proteins (75). This event allows the bacterium to disrupt the epithelial barrier and enter its colonic host cells and cause dysentery. Likewise, *Pseudomonas aeruginosa* strains are toxic and cause lysis and oncosis of PMNs. The depletion of PMNs by *P. aeruginosa* contributes to the pathophysiology of the disease by facilitating bacterial extracellular replication and persistent infections (76, 77).

Although PMNs efficiently internalize chlamydiae, a significant proportion of the ingested bacteria survive inside these cells (78) and use these leukocytes as "Trojan horse" vehicles for bacterial dispersion into different organs (79). *Chlamydia trachomatis* prevents the activation of PMNs by releasing a protease-like activating factor that targets and releases a formyl peptide receptor-2 on the surface of PMNs. The cleavage of this protein dampens the G protein-coupled receptor signal and prevents the downstream activation of PMNs. The protease-like activating factor suppresses the oxidative burst, interferes with chemical-mediated activation of PMNs and NET formation, and enables the pathogen to survive inside PMNs for extended periods (80).

Even Gram-positive bacteria such as *Neisseria gonorrhoeae*, which promotes the intense recruitment of PMNs at the infection site, display some evasion strategies. The fact that a proportion of *N. gonorrhoeae* organisms can be cultured from PMN-rich exudates is an indication that at least some bacteria resist the microbicidal mechanisms of PMNs. It seems, therefore, that some of the ingested gonococci are able to express significant amounts of virulence factors, such as pili, fimbria, Opa proteins, lipooligosaccharide (LOS), and other unknown components, which protect them from oxidative and nonoxidative antimicrobial activities and modulate PMN phagocytosis as well as the release of antimicrobial components (81).

Besides the primary function of PMNs in innate immunity, these cells also regulate inflammation and adaptive immunity in various diseases. These effects may be stimulatory or inhibitory, and they may promote or dampen the adaptive immune response (48, 82–86). As expected, the stimulatory or inhibitory effects exerted on T and B lymphocytes, antigen-presenting cells (87–94), or NK cells (95, 96) are reliant on the disease and the infection model. Moreover, there are significant variations in the immune profiles between acute and chronic processes (97–99). The regulatory mechanisms exerted by PMNs on the immune response are discussed below in the context of brucellosis.

BRUCELLA VIRULENCE STRATEGIES

The overall virulence of *Brucella* is expressed by three different but related properties (Fig. 2): (i) the bacterial ability to control its intracellular life to replicate in host cells (20, 21), (ii) the bacterial capacity to behave as a stealthy pathogen at the onset of infection to avoid early immune activation (46, 100, 101), and (iii) the resistance of *Brucella* organisms to bactericidal substances (102, 103). These properties probably evolved from ancestral characteristics of free-living alphaproteobacteria.

Control of Intracellular Life by *Brucella* Organisms

The ability of *Brucella* organisms to replicate inside Mϕs, Mo, DCs, and epithelial cells is directly related to the pathogenicity of these bacteria (Fig. 2A). Once inside permissive host cells, the *Brucella*-containing vacuole interacts with components of the endocytic and secretory pathways, such as early endosomes and vacuoles that intersect with the endoplasmic reticulum, where the bacterium replicates. In this process, the bacterium avoids fusion with lysosomes in a nontoxic encounter, extending the life of Mϕs and Mo and promoting the maturation of DCs (100, 104–106). *Brucella* ends its intracellular cycle within vacuoles of the autophagocytic apparatus, followed by the release of competent bacteria ready to infect PMNs and other cells (107). Once in PMNs, brucellae survive and persist within the phagosomes, resisting the bactericidal activities of these leukocytes for a protracted period (13–15).

The bacterial factors involved in the entry of *Brucella* organisms into cells are just partially known. PMNs and other professional phagocytes readily ingest opsonized

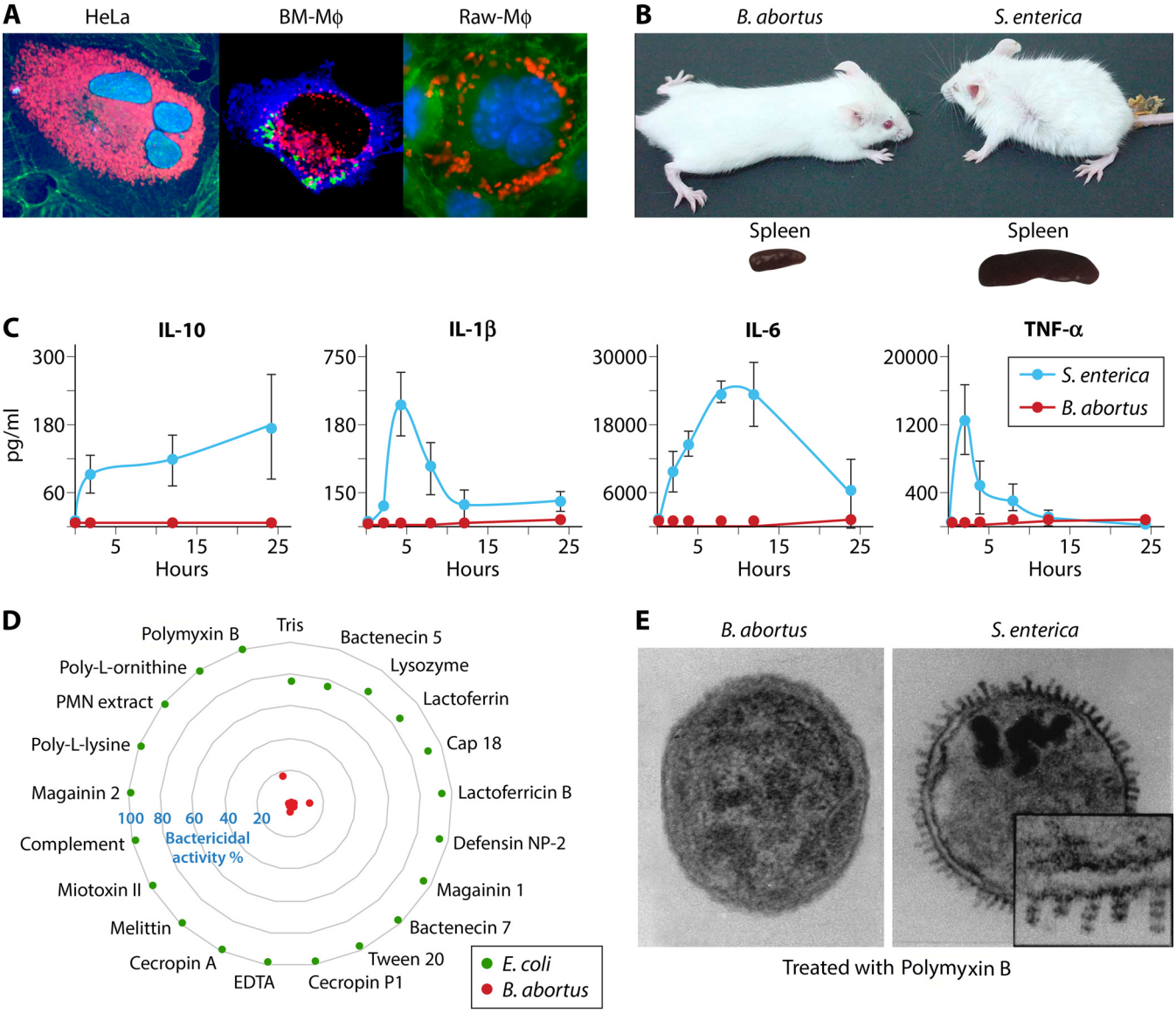

FIG 2 Three main virulent properties expressed by *Brucella* organisms. (A) *B. abortus* replicates extensively in dividing epithelial HeLa cells (left panel, bacteria in red, nuclei in blue, actin cytoskeleton in green) and in BM Mϕ (middle panel, bacteria in green, calnexin-positive compartments in blue, Cop-II compartments in red) or in dividing Raw Mϕ (right panel, bacteria in red, nuclei in blue, cytoskeleton in green). (Photos courtesy of Esteban Chaves [left], Jean Pierre Gorvel [middle], and Esteban Chaves and Pamela Altamirano-Silva [right], reproduced with permission.) (B and C) The capacity of *B. abortus* to behave as a stealthy pathogen is revealed by the absence of endotoxic symptoms and low spleen inflammation at the onset of infection (24 to 48 h) in *B. abortus*-infected mice in comparison with *Salmonella enterica* serovar Typhimurium-infected mice (B) and the negligible amounts of proinflammatory cytokines produced in comparison to *S. enterica* Typhimurium infection in mice at the onset of infection (C). (Panel C adapted from reference 100, published under the terms of the Creative Commons Attribution license [http://creativecommons.org/licenses/by/2.0].) (D) *B. abortus* resists the attack of an extensive collection of bactericidal substances, several of them expressed by cells. (E) While the outer membrane of *B. abortus* remains intact upon treatment with cationic peptides (polymyxin B), *S. enterica* Typhimurium demonstrates "blebbing," chromatin condensation, and death (adapted from reference 102). Note in panel A that *Brucella* replicates extensively without inducing noticeable toxic effects in cells. Note in panel B that while the *Salmonella*-infected mouse (multiplicity of infection [MOI], 10^3) shows the characteristic endotoxic cachexia symptoms, the *Brucella*-infected mouse (MOI, 10^6) looks unaffected.

Brucella with antibodies and complement through Fc and complement receptors (21). *Brucella* lacks classical surface virulence factors such as pili, fimbria, capsules, endotoxic moieties, toxins, or motile flagella (32). Still, several mutations in genes coding for various proteins (e.g., BvrS/BvrR, Omp25/Omp31 family, Omp2b, CGH, Efp, CcmC, BmaC, BtaE) and *Br*-LPS determinants (O chain, core, and lipid A) affect the binding of and penetration by *Brucella* to host cells (108–115). For most of these proteins, however, there is no demonstration of specific attachment to a cellular receptor. Furthermore, several of these mutations induce pleiotropic defects in the cell envelope, complicating matters even more.

Three exceptions are the following: (i) the O-chain of the *Br*-LPS that binds scavenger lectin-like receptors in Mϕs, (ii) the BmaC membrane protein that binds to fibronectin and the cognate receptor in epithelial cells, and (iii) BtaE, a polar protein that recognizes hyaluronic acid (41, 113, 115, 116). *Br*-LPS binds to C-type lectins on the surface of Mϕs, DCs, and PMNs (41, 117), an anticipated event, as mannose residues and sugars with mannose configuration are present in the *Br*-LPS core oligosaccharide and the perosamine O-chain homopolymer (Fig. 3A). Both BmaC and BtaE localize at the new pole of the bacterial surface, suggesting that this region may be functionally involved in adhesion, consistent with the inherent polarization of *Brucella* organisms (113, 115). The entrance of *Brucella* into cells involves the recruitment of several molecular determinants. Mϕs commonly ingest *Brucella* organisms through a zipper-like phagocytosis mechanism, with moderate recruitment of actin filaments and activation of the cyclic AMP/kinase pathway, followed by phosphorylation of the transcription factor CREB. In epithelial cells, *Brucella* organisms penetrate by phagocytosis via moderate recruitment of actin filaments, activation of small GTPases of the Rho subfamily, such as Cdc42, Rac, and Rho, and signals mediated by second messengers that include tyrosine kinase (Tyr-K), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3-K) (21).

Brucella organisms have adapted their physiology for intracellular life (118). Therefore, it is understandable that the vast majority of the so-called virulence factors described are related to cell cycle and metabolism (119, 120). However, some molecules are strictly required for transit from extracellular to intracellular environments, trafficking, and replication within cells. *Br*-LPS is one of the main factors involved in virulence. *Brucella* rough mutants display disruptive intracellular biogenesis, do not evade lysosomal fusion in Mϕs and DCs, are readily destroyed by PMNs, and are attenuated in animal models (13, 21, 121). The mode by which *Br*-LPS modulates the biogenesis of phagosomes in Mϕs and epithelial is barely known. In Mϕs, *Br*-LPS is released within phagocytic compartments. Once inside, *Br*-LPS binds to the vacuolar membrane and hampers several functions, including antigen presentation and activation (122).

A significant *Brucella* constituent involved in virulence corresponds to the two-component regulatory BvrR/BvrS system, which works as a master regulator of other systems (114, 123–126). The *bvrR* and *bvrS* gene mutants are attenuated and readily destroyed by PMNs and other cells (Fig. 4A). Coordinated functions between the BvrR and BvrS proteins are required to modulate the bacterial physiology and the shift from extracellular to intracellular life. This system controls the homeostasis of components of the cell envelope, such as outer membrane proteins and *Br*-LPS. Since BvrR/BvrS mutants display cell envelope defects, they are also sensitive to the microbicidal action of PMNs. The phosphorylated regulatory protein BvrR interacts with the genes coding for quorum-sensing VjbR and the type IV secretion system VirB, essential for intracellular life. The BvrS/BvrR system also controls pathways related to carbon and nitrogen metabolism.

Like other cell-associated alphaproteobacteria (127), *Brucella* requires the VirB type IV secretion system for intracellular survival. Attenuated *Brucella* VirB mutants are readily killed by Mϕs, DCs, and epithelial cells, since they do not avoid the fusion of *Brucella*-containing vacuoles with lysosomes (106, 128). The function of the VirB system is connected with the BvrS/BvrR and the VjbR complexes (123, 125), all essential for virulence (129). There are several putative effector molecules tentatively associated with the VirB apparatus. The mechanisms of transfection and the function of most of these molecules are still unclear (130), and very probably, they do not play a role in PMN parasitism, since VirB mutants survive for a protected period in these leukocytes (Fig. 4A).

Although the flagellum-like apparatus is not conspicuous on the bacterial cell, it is required at later stages of *Brucella* infection (131). Finally, *Brucella* periplasmic cyclic β-glucan (CβG) prevents fusion of the *Brucella*-containing vacuole with lysosomes by modifying cholesterol-rich lipid rafts present on the vacuolar membrane and is in control of the endosomal maturation process (132).

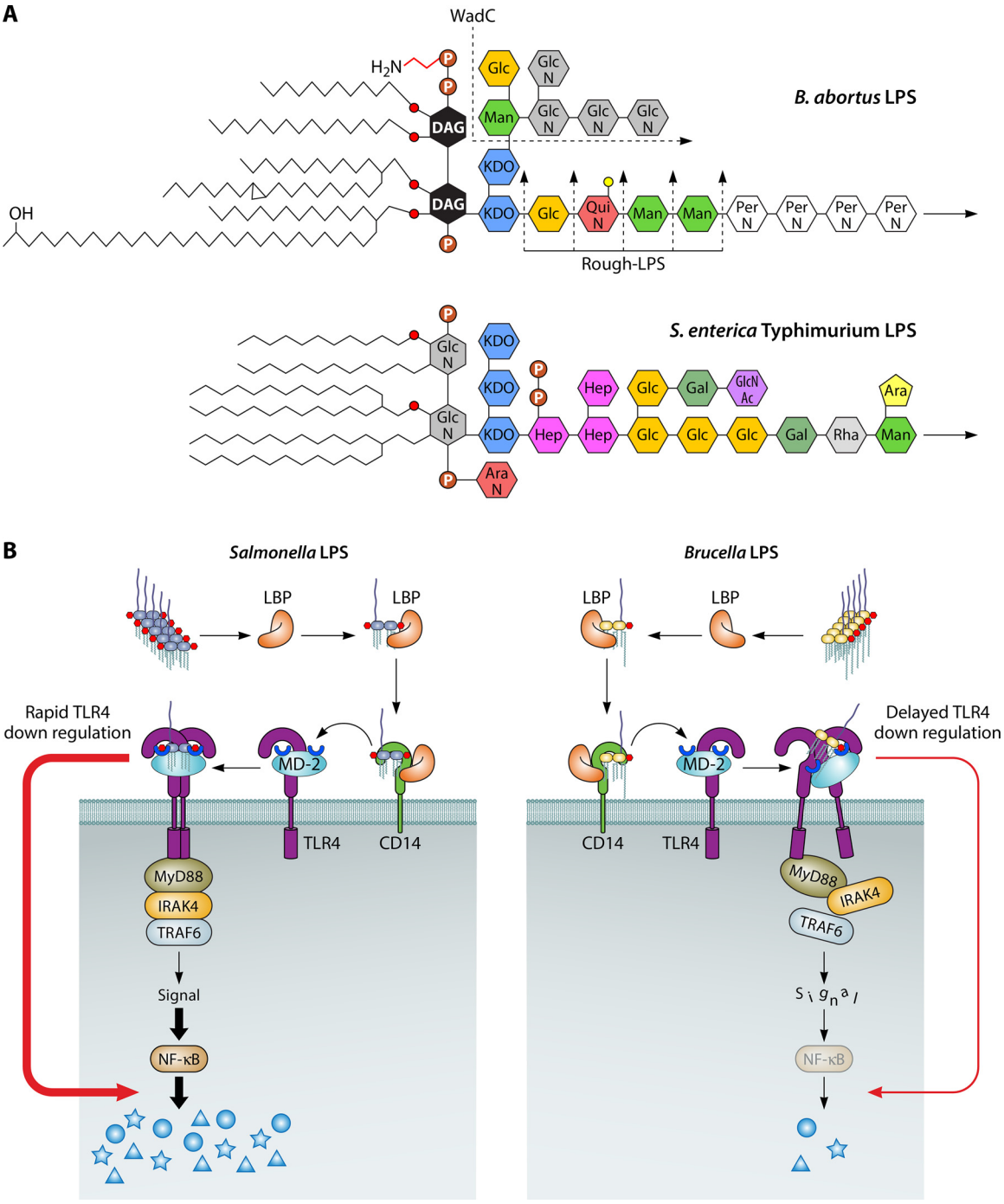

FIG 3 Noncanonical *Br*-LPS determines several virulence properties of *Brucella* species. (A) Comparison of the schematic structures of the LPSs from *B. abortus* and *S. enterica* Typhimurium. *Br*-LPS displays a nonendotoxic lipid A composed of a diaminoglucose (DAG) backbone replaced by long-chain fatty acids up to 30 C long and a core oligosaccharide with low-density negative charges and an O-chain polysaccharide composed of a homopolymer of N-formyl-perosamine sugars (70, 71, 150, 151). In contrast, *S. enterica* LPS has an endotoxic lipid A composed of a glucosamine (GlcN) backbone replaced by shorter-chain fatty acids with a core oligosaccharide with high-density negative charges and an O chain with ramifications (74). The *B. abortus* WadC mutant lacks positively charged GlcN sugars, exposing the negative charges of 2-keto-3-deoxyoctulosonic acid (KDO) and phosphates, while the *B. abortus* rough-LPS mutants, which may occur at different levels, lack the protective O-chain polysaccharide. Man, mannose; Glc, glucose; QuiN, quinovosamine; PerN, *N*-formylperosamine; AraN, arabinosamine; Hep, heptose; Gal, galactose; GlcNAc, acetyl-glucosamine; Rha, rhamnose; Ara, arabinose; P, phosphate; P-H_2N, ethanolamine phosphate. (B) The classical endotoxic *Salmonella* LPS readily binds to the lipid-binding protein (LBP) and CD14 receptor that transfer the LPS to the MD-2 coreceptor required for activation of TLR4 and downstream events that result in the induction of NF-κB and production of proinflammatory cytokines. In contrast, *Br*-LPS barely promotes the synthesis of proinflammatory cytokines due to its defective MD-2 binding, lack of TLR4 activation, and subsequent triggering of downstream events. Similar nonagonistic effects have been found for other *Brucella* molecules, such as flagellin, lipoproteins, and ornithine-containing lipids (135, 143, 144).

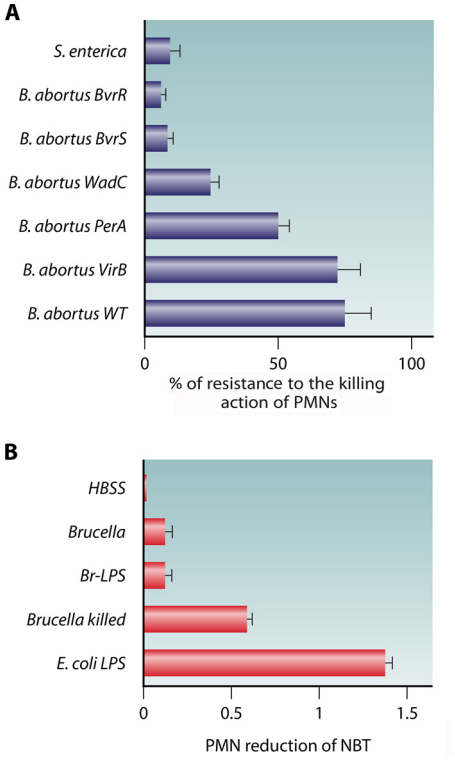

FIG 4 *Brucella* is resistant to the killing action of PMNs and barely induces activation of these leukocytes. (A) Sensitivity to the bactericidal action of human PMNs (after 1.5 h) against *S. enterica* and *B. abortus* mutants. The *B. abortus* BvrR and *B. abortus* BvrS mutants have membrane and lipid A-fatty acid defects; the *B. abortus* WadC mutant has a core defect; the *B. abortus* PerA mutant lacks O-chain polysaccharide. The *B. abortus* VirB mutant is a smooth bacterium defective in the type IV secretion apparatus. Note that resistance to the bactericidal action of human PMNs is similar in the virulent *B. abortus* wild type (WT) and the attenuated *B. abortus* VirB mutant. (B) In contrast to *E. coli* LPS and killed *Brucella*, live *B. melitensis* and *Br*-LPS promote a meager reduction of nitroblue tetrazolium (NBT) of goat's PMNs (Hanks' balanced salt solution [HBSS]). The NBT reduction indirectly measures ROS activity as well as the activity of oxidoreductases such as NADPH. (Based on data from reference 204.)

Brucella Organisms Behave as Stealth Pathogens

Brucella organisms barely induce proinflammatory responses at the onset of infection (Fig. 2B and C) (100), a characteristic related to the structure of their putative PAMPs. Various types of PRRs, such as TLRs and NODs, barely recognize *Brucella* PAMPs, since these bacterial molecules display molecular modifications (46, 100, 133–135). Despite some initial controversies on the role of TLR4 in murine brucellosis (136), evidence for low agonistic activity of *Br*-LPS is robust and extensive (100, 106, 135, 137, 138). As in other alphaproteobacterial pathogens (71, 73), this phenomenon is linked to the nonconventional core-lipid A moiety of *Br*-LPS (Fig. 3A). *Br*-LPS does not fit and barely binds to the MD-2 coreceptor of TLR4. This event hampers the recycling and activation of TLR4 and the subsequent induction of NF-κB that promotes the synthesis of a collection of proinflammatory cytokines (Fig. 3B) (137). Moreover, *Br*-LPS induces just low quantities of ROS mediators (Fig. 4B), and the blocking of TLR4 does not hamper the interaction of *Br*-LPS with PMNs (139).

A similar controversy has been observed for TLR2 and TLR9 (100, 137, 140–142). While some authors do not find any effect on bacterial replication when one of these TLRs is absent, others find a slight difference in the bacterial loads. It seems evident that the different Myd88-dependent TLRs cooperate in the control of brucellosis at later stages. It has been shown that TLR2 and TLR4 cooperate in Mϕ survival at later stages of *B. abortus* infections (100) and in the survival of *B. microti*-infected mice. This event is significant, since in contrast to other *Brucella* species, *B. microti* causes bacteremia and displays strong pathogenicity for mice (142).

Likewise, the *Brucella* nonmotile flagellum-like apparatus (131) is not agonistic for TLR5, a property shared with various alphaproteobacterial pathogens (143, 144). *Brucella*-derived outer membrane fragments containing large amounts of lipoproteins induce low proinflammatory responses compared to conventional PAMPs (100). Ornithine-containing lipids that are potent PAMPs in other bacteria (145) do not have a detectable agonistic function during *Brucella* infections (135). Other molecules, such as β-cyclic glucans (CβG), have both pro- and anti-inflammatory properties (146).

The *Brucella* outer membrane works as a shield for PAMPs such as CpG, peptidoglycan, and CβG (138, 147). This property is related to the high hydrophobicity of the *Brucella* outer membrane, which results from the long hydrocarbon aliphatic chains (up to 30 C atoms) replacing phospholipids, ornithine lipids, lipoproteins, and the lipid A moiety (135, 148). Some of these long-chain fatty acids may span the *Brucella* outer membrane, making the bacterium strongly resistant to disruption (102).

In any case, the overall mild agonistic effect that *Brucella* organisms exert over PPRs is reflected in the low activation of infected Mϕs, DCs, and PMNs and the deficient production of proinflammatory cytokines at the onset of infection (Fig. 2C) (46, 100). These experimental observations correlate with the often-prolonged incubation time, the nonendotoxic clinical manifestations, and the absence of distinctive proinflammatory markers in brucellosis patients (8, 10, 149). Consequently, live *Brucella* organisms and their putative PAMPs barely activate PMNs and other immune cells (Fig. 4B), giving these furtive pathogens time to invade, replicate, and spread through different tissues before the activation of adaptive immunity.

Brucella Organisms Are Resistant to Bactericidal Substances

The resistance of *Brucella* organisms to bactericidal molecules of physiological fluids and cells is due, in part, to the structure of its cell envelope. The positively charged *Br*-LPS core oligosaccharide, composed of amino sugars, and the O-chain and NH homopolymers, built of nonreducing *N*-formyl-perosamine residues (Fig. 3A) (150, 151), form a shield, masking the available negative charges on the smooth brucella surface (152). This property makes the invading bacterium "invisible" to various PRRs and resistant to the microbicidal action of cellular cationic microbicidal molecules of PMNs and other cells (102, 103, 148, 153, 154). Indeed, the outer membrane of virulent brucellae is resistant to complement, oxidative substances, defensins, bactenecins, cathelicidins, lysozyme, phospholipases, lactoferrin, and PMN extracts, among others (Fig. 2D and E). The sensitivity of cell envelope *Brucella* mutants to these substances is linked, directly or indirectly, to modifications of *Br*-LPS (114, 133, 138).

One attractive characteristic not directly linked to the structure of the cell envelope is the resistance of *Brucella* organisms to DNA alkylating stress mediated by phagocytic cells. Genes involved in repair and base excision repair pathways are required by the bacterium to confront alkylation stress within phagosomes (155). This event is commensurate with the absence of bacterial replication within PMNs, a characteristic that favors resistance to the killing action.

Evolution of *Brucella* Virulence Mechanisms

The close phylogenetic relatedness of *Brucella* organisms with other cell-associated alphaproteobacteria has allowed comparison of the structure and function of several systems (138). *Ochrobactrum* spp. are the closest phylogenetic relatives of *Brucella* spp. These bacteria share with *Brucella* pathogens structural and functional features, such as lipid A, ornithine-containing lipids, flagellin, lipoproteins, CβG, and orthologous systems such as VirB, BvrS/BvrS, and VjbR, among others (34, 138, 143, 156). Still, the PAMPs of this opportunistic pathogen keep some structural properties that induce proinflammatory responses (138). Upon infection, *Ochrobactrum* kills neutropenic mice, promotes the release of much higher cytokine secretion, and induces neutrophilia and recruitment of PMNs. *Ochrobactrum* LPS has more negative charges in the core oligosaccharide than *Br*-LPS, a property that makes the outer membrane of this opportunistic bacterium more susceptible to the recognition of microbicidal cationic sub-

stances. In comparison to *Br*-LPS, which barely binds MD-2/TLR4 molecules, *Ochrobactrum anthropi* LPS binds with a high affinity to the MD-2 coreceptor of TLR4. Consequently, *O. anthropi* LPS triggers NF-κB (138) and induces a more robust cytokine response and activation of Mϕs and PMNs than *Br*-LPS. This behavior reveals that in *Brucella*, the putative PAMPs have been eliminated, modified, or hidden compared to free-living alphaproteobacteria.

The *B. abortus* WadC core mutant directly proves the critical role that *Br*-LPS has in virulence (133). The core oligosaccharide of this attenuated mutant lacks the positively charged glucosamine branch linked to the second 2-keto-3-deoxyoctulosonic acid (KDO) while keeping the O chain bound to the first KDO (Fig. 3A); hence, the negatively charged molecules become exposed. Consequently, bactericidal molecules of PMNs and other cells can attack and kill the WadC mutant more efficiently (Fig. 4A). The WadC mutant also induces higher production of proinflammatory cytokines in infected phagocytic cells, a phenomenon that is reproduced by its *Br*-LPS. Like the *Ochrobactrum* LPS, the WadC LPS binds to the MD-2 coreceptor of TLR4 with higher affinity, triggering the activation of NF-κB. As expected, the WadC mutants are also killed more efficiently by PMNs than virulent wild-type (WT) *Brucella* organisms (Fig. 4A).

These examples suggest that the free-living *Brucella* alphaproteobacterial ancestor evolved and adapted to infect animal cells by fine-tuning its virulence mechanisms. Some of these adaptations involved structural and functional modifications of the surface molecules and PAMPs and other virulent systems such as VirB, BvrS/BvrR, and VjbR. Thanks to these molecular adaptations, *Brucella* organisms were capable of circumventing the activation of PMNs, became stealthy pathogens, and avoid the early recognition of the innate immune system. This strategy opened a "window" for the dispersion and expansion of *Brucella* organisms before activation.

PMN RECRUITMENT IN THE COURSE OF BRUCELLOSIS

Mucosal epithelial cells, Mϕ, DCs, and PMNs are the first cells to encounter *Brucella* organisms in the mucous membranes (16, 157). Despite this, brucellosis seldom progresses with the characteristic neutrophilia of many other bacterial infections (Fig. 5) (19, 31, 100, 101, 158–161). At the onset of systemic infection (the first 48 h) of experimental murine brucellosis, there is no recruitment of PMNs at the infection site, and the number of PMNs in blood or target organs, such as the spleen and liver, remains low. Five days after infection, at the beginning of the acute brucellosis phase (43), there are still negligible numbers of infected PMNs in the target organs but significant recruitment of infected mononuclear phagocytes (42). After 2 weeks of bacterial replication in cells of the reticuloendothelial system, granulomas composed of lymphocytes and infected Mϕs and DCs become evident, while PMNs are at low numbers and are not infected (42, 162).

The recruitment of PMNs depends on the route of infection. Natural *Brucella* infection through the skin is rare, since these organisms cannot cross this epithelial barrier (8, 10). Despite this, experimental subcutaneous inoculation of *Brucella* organisms into the skin of humans induces the recruitment of PMNs and histiocytes to the site of infection, within a few hours (33). Likewise, subcutaneous *Brucella* injection in mice promotes transient recruitment of PMNs at the site of infection. However, this effect is 10 times lower than the recruitment of PMNs induced by subcutaneous injection of *Salmonella* (100). Infection with high doses of bacteria (10^7 CFU) by the intradermal route in the footpad of mice recruits some PMNs and fibroblasts in the skin (35). However, intradermal administration of *Brucella* crude extracts in mice after 21 days of bacterial infection promotes only the migration of Mϕs at the injection site after 24 and 48 h, with no early or late migration of PMNs (163). A similar inhibitory effect occurs in PMN mobility in the presence of *Brucella* extracts in human patients (164). Moreover, intradermal infections with *Brucella* organisms or extracts containing *Br*-LPS and other components do not promote a Shwartzman reaction or the characteristic immediate local inflammatory response and recruitment of PMNs observed after the injection of Gram-negative bacteria or enterobacterial endotoxin (6, 8, 10, 165).

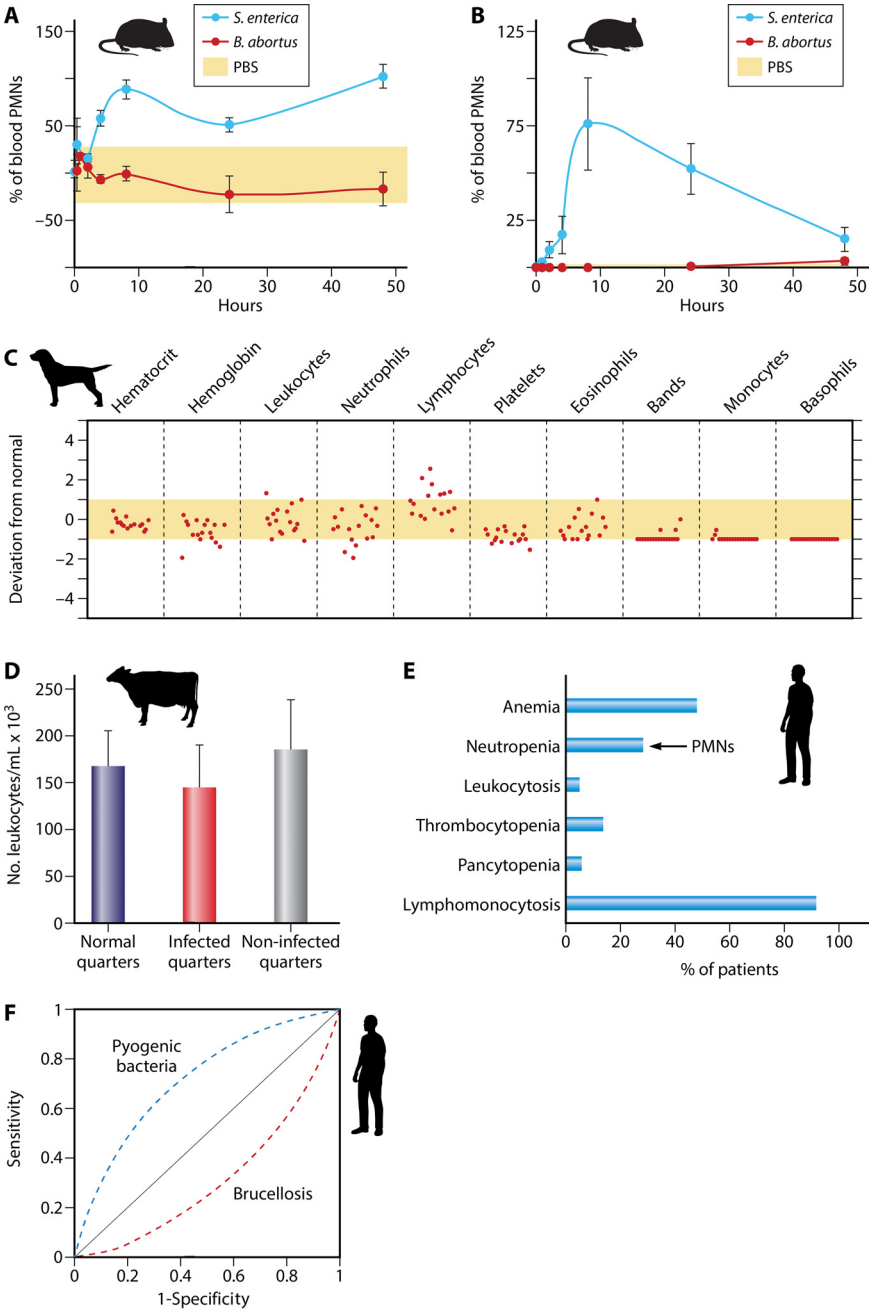

FIG 5 Leukocyte counts in brucellosis. (A) Blood PMN counts in *S. enterica* Typhimurium- and *B. abortus*-infected mice. The yellow area demarcates average blood PMN normal values. PBS, phosphate-buffered saline. (Adapted from reference 100, published under the terms of the Creative Commons Attribution license [http://creativecommons.org/licenses/by/2.0].) (B) PMN counts in the peritoneal fluids of five mice infected with *S. enterica* Typhimurium or *B. abortus*. The yellow area (close to the *x* axis) depicts the normal maximum upper and lower limits. (Adapted from reference 100, published under the terms of the Creative Commons Attribution license [http://creativecommons.org/licenses/by/2.0].) (C) Hematological profiles of 17 *B. canis*-infected dogs. The average maximum upper and lower limits correspond to the yellow area for each cell type. Each circle represents one dog, numbered from 1 to 17, with number 1 being the farthest to the left in each panel. (Adapted from reference 101.) (D) Leukocyte counts in infected quarters (with isolation of *Brucella* spp.) and noninfected quarters of udders of 18 cows with brucellosis and comparison with quarters from udders from noninfected cows. (Based on data from reference 158.) (E) Blood cell profile of 530 human patients with brucellosis. Neutropenia, due to the absolute low PMN number (<2,500/mm^3), is observed in about one-third of the patients, while relative lymphomonocytosis is observed in close to 90% of the patients. (Based on data from reference 159.) (F) Comparison of receiver operating characteristic (ROC) curves estimated by regression analysis of lymphocyte/PMN ratios in human patients with brucellosis and infected with pyogenic bacteria. Note the frequent neutropenia as a predictive indicator for brucellosis diagnoses in contrast to pyogenic bacterial infections. (Based on data from references 160 and 161.)

As stated before, the natural route of *Brucella* infection is through mucosal membranes. In humans and the preferred host, the mucosal infection seldom promotes dense PMN recruitment within the next 6 to 8 days (157). *Brucella* infection of mice by the intravenous, intraperitoneal, intranasal, intragastric, or oral route barely promotes the recruitment of PMNs in the blood (Fig. 4A), peritoneum (Fig. 5B), lungs (35), regional lymph nodes (35), spleen (35, 42, 162), or gastric mucosa (166). Intratracheal inoculation with a high number of *Brucella* organisms (10^8 CFU) induces microgranulomas and necrosis in the lungs and granulomas in the liver in the presence of PMNs. However, this does not occur with a lower number of infecting bacteria ($<10^6$ CFU) (167).

We ignore why the subcutaneous inoculation of *Brucella* organisms promotes transient recruitment of PMNs at the site of infection while systemic infections by other routes commonly do not. Although mast cells do not phagocytize *Brucella* organisms, these bacteria are capable of inducing degranulation of these leukocytes by an unknown mechanism (168). Resident mast cells, beneath the skin, recruit PMNs to release cytokines and chemokines and coordinate the movement of these phagocytic leukocytes to the site of infection (169). Therefore, it is feasible that the subcutaneous inoculation of large numbers of *Brucella* organisms promotes the degranulation of mast cells and the concomitant recruitment of PMNs in the skin. It seems that live *Brucella* organisms are required to recruit PMNs in the skin, since the injection of bacterial extracts or *Br*-LPS at this site does not induce the same effect.

Epididymitis, orchitis, placentitis, osteoarthritis, BM pancytopenia, meningoencephalomyelitis, and other severe tissue pathologies are mainly observed in long-lasting brucellosis cases (8, 10). While the recruitment of mononuclear phagocytes at the infection site is a constant feature, the arrival of PMNs is related to the disease's duration and is reliant on the local cytokine and chemokine response and tissue injury (35, 37, 170–174). Mϕs, DCs, and fibroblasts produce chemotactic signals, while intensely infected tissues generate damage-associated molecular patterns known as DAMPs. These two substances are potent mediators for recruiting PMNs and other inflammatory cells (174).

Abortion is the consequence of placental destruction and cytokine release in the last trimester of pregnancy. At this stage, there is an intense *Brucella* parasitism of trophoblasts, characterized by necrosis and the presence of a high number of extracellular bacteria, accompanied by a conspicuous inflammatory exudate and cellular debris (Fig. 5) (170, 172, 175–177). After an abortion, the infected placenta shows vasculitis and necrotizing placentitis, with infiltration of Mϕs and PMNs with a large number of intracellular *Brucella* organisms (Fig. 6A and B). The destruction of the trophoblast layer promotes the release of DAMPs and cytokines and the subsequent recruitment of PMNs (175, 178, 179). The release of proinflammatory mediators by *B. abortus*-infected bovine trophoblasts *in vivo* and *in vitro* is dampened at an earlier but not later stage of the infection (171).

In contrast to the infected placenta, the lungs and other tissues of aborted fetuses from bovines with brucellosis display a predominant inflammatory infiltrate of Mϕs with just a few PMNs (176). Likewise, *B. canis*-infected dogs do not show neutrophilia or significant hematological alterations (Fig. 5C), and the placentae of bitches after abortion display a low inflammatory reaction, with very few or no PMNs (180). *Brucella*-infected mice also develop moderate multifocal necrotic placentitis (181). The infiltrate is not particularly purulent; still, the infected mouse placenta shows *Brucella* within trophoblasts and signs of inflammation, with infiltration of few PMNs and an abundance of other immune cells (181). Purulent placentae associated with endometritis and abortion are rare outcomes in murine brucellosis (182), and many pups come to term and are born alive (28, 182, 183).

At the initial stages of testicular *Brucella* infection in men, dogs, bulls, and rams, the inflammatory exudates of the epididymis and cortical testicular tissue seldom show a PMN predominance (31, 184). Later, severe epididymitis and orchitis, with infiltration of PMNs and mononuclear cells with intracellular bacteria, become evident (185). As in

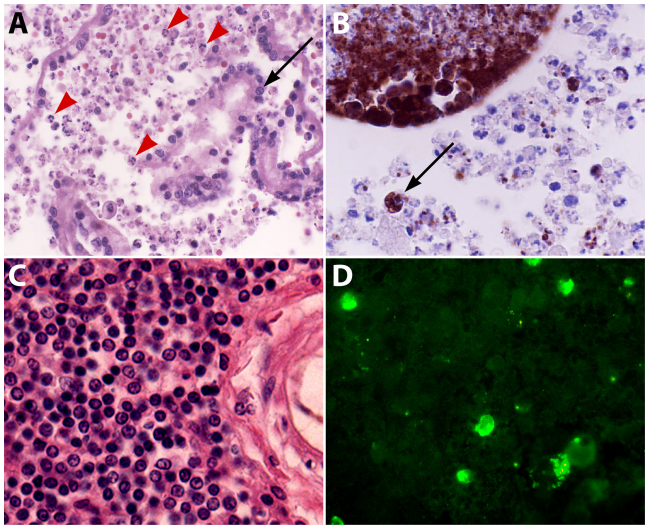

FIG 6 Histopathological findings in the placenta and brain of a *Brucella ceti*-infected striped dolphin. (A) Placental villi with mononuclear and PMN inflammatory infiltrate (red arrowheads) and some detached placental epithelial cells (arrow). Hematoxylin and eosin (HE) stain. Magnification, ×40. (B) Immunoperoxidase labeling of intracellular *Brucella* antigens inside inflammatory PMNs and mononuclear cells (dark brown) invading the placental villi (example indicated by an arrow). Immunohistochemistry. Magnification, ×40. (C) Mononuclear infiltrate in infected brain meninges with the absence of PMNs. HE stain. Magnification, ×40. (D) Immunofluorescence of infected mononuclear cells (in green) in the cerebrospinal fluid of infected meninges. HE stain. Magnification, ×40. The expected outcomes of stranded striped dolphins with brucellosis are abortion, meningoencephalomyelitis, and death caused by the invading bacteria (172). (All photos courtesy of Rocio Gonzalez-Barrientos and Gabriela Hernández-Mora, reproduced with permission.)

other tissues, the recruitment of PMNs may be due to the combination of cytokines and DAMPs released by the injured testicular tissue.

B. abortus and *B. melitensis* also have a strong tropism for the sinuses of the mammary gland in ungulates (175, 186). Despite this, there are no classical signs of mastitis, and macroscopically the udders do not demonstrate significant pathological signs. Although PMNs and other cells in the udder of *Brucella*-infected animals may harbor intracellular bacteria, PMNs are not particularly abundant in this tissue (170, 186). The infected quarters of cows do not shed more leukocytes than normal or noninfected quarters (Fig. 5D). Resident udder PMNs display significantly lesser bactericidal activity and lower ROS formation against *Brucella* than those induced by other pathogens such as *Escherichia coli*, *Salmonella enterica*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Listeria monocytogenes,* and *Mycobacterium bovis* (187, 188). This picture contrasts with human breast brucellosis. In rare cases of long-lasting disease, the bacterium may invade patients' breasts, with the generation of granulomas containing giant cells and PMNs (189).

Neurobrucellosis in humans and animals such as dolphins seldom progresses with neutrophilia and inflammatory exudates in the cerebrospinal fluid, and the meninges have mononuclear cells of lymphocytic and macrophagic origin, some of them containing *Brucella* and bacterial debris but seldom PMNs (Fig. 6C and D) (172, 190). This observation is relevant, since parenchymal nerve and glial cells do not become infected with *Brucella* organisms and inflammation of the brain is limited to the blood vessels and the meninges. *Brucella* invasion of the brain is a severe disease that may cause death (172).

In contrast to what happens in bacterially induced arthritis (191), *Brucella* infections in humans are not a frequent cause of bacterial septic or reactive arthritides, and most brucellosis patients with arthritis do not show significant hematological changes. *Brucella* organisms are seldom isolated from the mononuclear cell-enriched synovial liquids that generally are devoid of PMNs (192, 193). In long-lasting brucellosis arthritis

and bursitis, PMNs may infiltrate the synovial fluid. Inflammation of the joints such as arthritis, bursitis, and hygromas may also be present in old bovines with a long history of brucellosis, mainly from low-income countries (194, 195). The most frequent histopathological lesions in bursitis with the isolation of *Brucella* from the synovial fluid correspond with multifocal necrosis, hemorrhage, and signs of a granulomatous process with a lymphohistiocytic inflammatory infiltrate containing Mϕs and some PMNs (194). As expected, joint infections also involve the local immune response and release of DAMPs due to tissue destruction (174).

B and T lymphocytes are dispensable in brucellar arthritis, while CXCR2 (receptor for IL-8/CXCL8 chemokine) is necessary for focal PMN recruitment and inflammation in the joints of mice (196). Although murine PMNs may participate in arthritis, they do not seem important in *Brucella* elimination, and CXCR2 in cells is not necessary for the clearance of these microorganisms. *Brucella*-infected human fibroblast-like synoviocytes produce proinflammatory cytokines and chemokine CXCL8, and supernatants from these infected cells recruit PMNs and Mo (197). Likewise, human osteoblasts upregulate their cytokine and CXCL8 production after coculture with supernatant from *Brucella*-infected Mo. In turn, *Brucella*-infected osteoblasts also induce proinflammatory cytokines and CXCL8 by Mo (36).

It seems that the ability of *Brucella* organisms to develop long-lasting infections is linked to their persistence in the BM (198, 199). In humans, the BM shows histopathological alterations such as granulomas and an augmented number of inflammatory cells. Later, neutropenia, thrombocytopenia, anemia, and, in severe cases, pancytopenia, myelodysplasia, and hemophagocytosis are common BM alterations (198, 200, 201). *Brucella* organisms can persist in the BM of infected mice for protracted periods and induce significant changes in this tissue. Granulomas and augmented numbers of BM myeloid granulocyte-monocyte progenitors, PMNs, and $CD4^+$ lymphocytes are present during the acute phase (199). The most abundant infected BM cells at the early phases of murine infection (once antibodies against *Brucella* have developed) are PMNs, followed by granulocyte-monocyte progenitors and Mo. At later times, the number of infected PMNs in the BM decreases considerably, and the numbers become similar to those of infected Mo. The neutropenia observed in about one-third of patients with long-lasting infections (Fig. 5E and F) may be due to the sustained death of *Brucella*-infected PMNs and granulocyte-monocyte progenitor cells in the BM (39). Monocytes and granulocyte-monocyte progenitors are likely to serve as host cells for *Brucella* replication, remaining in this hematopoietic tissue for protracted periods. It is unlikely that PMNs function as bacterial reservoirs in the BM, since the number of these infected cells significantly decreases after the initial infection periods, along with the fact that *Brucella* organisms do not replicate in these leukocytes. Instead, according to the Trojan horse model, PMNs may function as vehicles for dispersion (39).

Repeated injections of antibodies against PMNs eliminate these leukocytes from blood, lymphatics, and other organs of mice but only partially remove them from the BM in *Brucella*-infected mice (93). Nevertheless, those PMNs that remain in the BM barely become infected (93). This result suggests that anti-PMN antibodies select functional and fully phagocytic BM PMNs, leaving a population of resting, not fully phagocytic PMNs in the BM. This observation is relevant since BM Mϕs phagocytize bacterial antigen-laden PMNs and subsequently present antigen to $CD8^+$ T lymphocytes to generate memory T cells (202).

The clinical and experimental observations demonstrate that the recruitment of PMNs in brucellosis depends on the route of infection, the course of the disease, infected organs, and tissue destruction. At the onset of infection, and during the long incubation period, the recruitment of PMNs at the infection site and target organs is low. At these sites, mononuclear phagocytes and lymphocytes are the primary inflammatory cells. Throughout this period, *Brucella* organisms avoid the recruitment and full activation of PMNs, behaving as silent pathogens. Once the disease becomes evident and clinical signs appear, the infection progresses without neutrophilia hematological alterations or coagulopathies (19, 31, 100, 101). Like the brain or udder, some organs

seldom present septic PMN infiltration, despite the large numbers of bacteria present in these tissues. Later, PMNs may arrive at the damaged, infected organs, such as the testes, placenta, joints, or BM. However, PMNs do not contribute to the resolution of the *Brucella* infection, and these leukocytes are dispensable for bacterial clearance and the cellular immune response (87, 93).

RESISTANCE OF *BRUCELLA* TO THE BACTERICIDAL ACTION OF PMNs

After infection, *Brucella* organisms are opsonized and quickly phagocytized by naive human, bovine, caprine, rat, and canine PMNs (6, 14, 15, 100, 203–207). Human and bovine PMNs internalize opsonized *Brucella* cells with complement or immunoglobulins or with integrins, C-type lectins, and fibronectin-binding receptors (6, 41, 117, 208, 209). In contrast, murine PMNs require specific antibodies to phagocytize smooth (but not rough) *Brucella* organisms, since *N*-formyl-perosamine oligosaccharides of the LPSs and related native haptens constitute a shelter for complement and fibronectin opsonization in these animals (205).

Brucella organisms can live in large numbers inside PMNs and resist the bactericidal action of these leukocytes (6, 13, 16, 100, 203, 209, 210). Still, *Brucella* resistance to PMNs is high but not absolute (Fig. 4A), and it depends upon various factors. *Brucella* is slightly more susceptible to IFN-γ-activated PMNs and PMN-granule extracts supplemented with H_2O_2 and halide (13, 117, 211). The bactericidal response of activated PMNs correlates with larger amounts of superoxide anion and H_2O_2 secretion. The production in *Brucella* organisms is regulated, and catalase mutant bacterial strains have high sensitivity to H_2O_2 (212). Extensive contacts with O^{2-}, myeloperoxidase-H_2O_2-halide, and other components of the ROS system are lethal for *Brucella* organisms (14). Therefore, it seems that activated PMNs may circumvent, in part, the furtive behavior of *Brucella* once adaptive immunity has initiated and killed a proportion of the bacteria.

The bactericidal activities of PMNs also depend on the animal species and *Brucella* strain. For instance, bovine PMNs are more bactericidal than those of humans and caprine, and all these cells are more bactericidal than those of guinea pigs and mice (13–15, 204, 205). Likewise, bovine PMNs are more bactericidal after ingesting antibody-opsonized *Brucella* in the presence or absence of complement (208). In contrast, murine and guinea pig PMNs show low bactericidal activity against *Brucella*, regardless of antibody opsonization (13, 205). *B. melitensis*, *B. abortus*, *B. canis*, and *B. suis* display similar resistance against PMNs (117); however, the rough counterparts and some other attenuated mutants show increased sensitivity to these leukocytes.

The *Brucella* VirB attenuated mutants are resistant to PMNs for at least 2 h (Fig. 3C). In contrast, some mutants (e.g., HtrA) susceptible to PMNs keep their virulence in mice (210). This difference demonstrates decoupling between the capacity of *Brucella* to cause systemic infections and its resistance to PMNs and suggests that PMNs are dispensable for immune defense in brucellosis. It also indicates that the ability to control intracellular trafficking is independent of bacterial resistance to PMNs. The VirB mutants inside PMNs persist longer in the skin (35). This resistance suggests that *Brucella* organisms do not require the type IV secretion machinery for survival inside PMNs but rather depend on the other two virulent strategies. The relatively neutral conditions of PMN phagosomes in comparison to those of Mϕs may preclude the turning on of the *Brucella* type IV secretion system in PMNs (213). Indeed, the expression of the VirB system requires the acidic conditions of the Mϕ phagosomes (123). Therefore, the vacuolar milieu of PMNs constitutes a shelter for the bacterium rather than a replication niche.

The unique structure of the *Brucella* cell envelope protects these bacteria from the bactericidal action of PMNs; defects in this layer favor the destruction of these pathogens (133, 153, 203, 205). Rough *Brucella* mutants lack the outer membrane protective O-polysaccharide layer and display pleiotropic membrane alterations, exposing otherwise hidden molecular determinants (118, 152, 214, 215). As a consequence, rough mutants are more susceptible to bactericidal substances and PMNs (13, 15). As ex-

plained before, WadC exposes negative charges on its outer membrane, a property that makes the WadC mutant susceptible to the cationic bactericidal molecules of PMNs (133). Likewise, the smooth *Brucella* BvrR and BvrS mutants that display *Br*-LPS alterations and cell envelope defects show high susceptibility to bactericidal substances and PMNs (114, 124, 153). After the phagocytosis of these cell envelope-defective *Brucella* mutants, PMNs degranulate and generate a more robust ROS response than when infected with the fully virulent counterparts (13). Some investigators proposed that a nucleotide-like material derived from *Brucella* extracts was responsible for blocking PMN degranulation, with preferential inhibition of primary granule release (216). However, this proposal does not explain why the attenuated mutant brucellae that also produce this material, or even killed organisms, do not inhibit degranulation (13) or ROS activation (Fig. 4B).

As mentioned before, the opportunistic *Ochrobactrum* is more sensitive than *Brucella* organisms to bactericidal molecules and PMNs (138, 217), and ochrobacteriosis progresses with moderate to high neutrophilia in humans and mice (138, 218–220). In contrast to *Brucella*, *Ochrobactrum* replicates in neutropenic mice and promotes the secretion of cytokines but still less than *Salmonella* (138). These differences are due in part by the broader diversity of PAMPs in *Ochrobactrum*, which binds PRRs, promoting the activation of NF-κB (138, 221).

PMN DEACTIVATION BY *BRUCELLA* ORGANISMS

Vigorous proinflammatory activities characterize the biological properties of PMNs during bacterial infections. Among the most conspicuous are the robust production of cytokines and chemokines, oxidative burst with potent ROS generation, degranulation, the release of ectosomes, and NET formation (222). Even heavily infected *Brucella* PMNs, with up to 25 to 50 bacteria/cell, barely become activated or display significant phenotypic alterations (6, 16, 139, 205). *Brucella*-infected PMNs do not undergo NETosis, degranulation, necrosis, oncosis, or classical apoptosis morphology, and large quantities of *Br*-LPS do not promote noticeable phenotypic changes associated with activation (100, 139, 223). Antibiotics used to treat brucellosis, such as doxycycline, streptomycin, and rifampin, which influence the bactericidal functions of PMNs (224), do not promote the activation or degranulation of these *Brucella*-infected cells (225). These phenomena correlate with low ROS formation (Fig. 4B), negligible activation of the monophosphate pathway, and low myeloperoxidase activity of *Brucella*-infected PMNs (100, 188, 203, 204, 225, 226). These processes may be partially overcome, just by very high numbers of *Brucella* organisms (>100/PMN) (227). Moreover, PMNs from human patients with an early evolution of brucellosis do not show the robust activation observed in other bacterial diseases (228–230). In long-lasting *Brucella* infections, the PMNs of some patients show augmented migration against zymosan but diminished chemokinesis against specific antigens. Likewise, the phagocytosis mediated by PMNs diminishes, and the oxidative burst of unstimulated leukocytes is not augmented before treatment (164).

PMNs infected with *Brucella* organisms or treated with *Br*-LPS practically do not release proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6 (139) or regulatory IL-10 (231). This phenomenon is consistent with the low cytokine production of *Brucella*-infected mice at early time points of the infection (100, 137, 232). The small amounts of cytokines, particularly IL-1β, and the absence of caspase-1 involvement preclude the generation of the inflammasome pathway in *Brucella*-infected PMNs (139). One exception is the release of low but consistent amounts of IL-8 (CXCL8) (139). This chemokine, constitutively produced by PMNs, is readily available after *Brucella* infection (233). The CXCL8 may function as a "find me" signal for *Brucella*-infected PMNs by Mϕs (234).

The mechanisms behind the overall low activation of PMNs relate to the furtive nature of *Brucella* organisms. On the one hand, the putative *Brucella* PAMPs are not agonistic for PMN PRRs, hampering the activation of these leukocytes. On the other hand, *Br*-LPS promotes the premature cell death of infected PMNs (139). These two

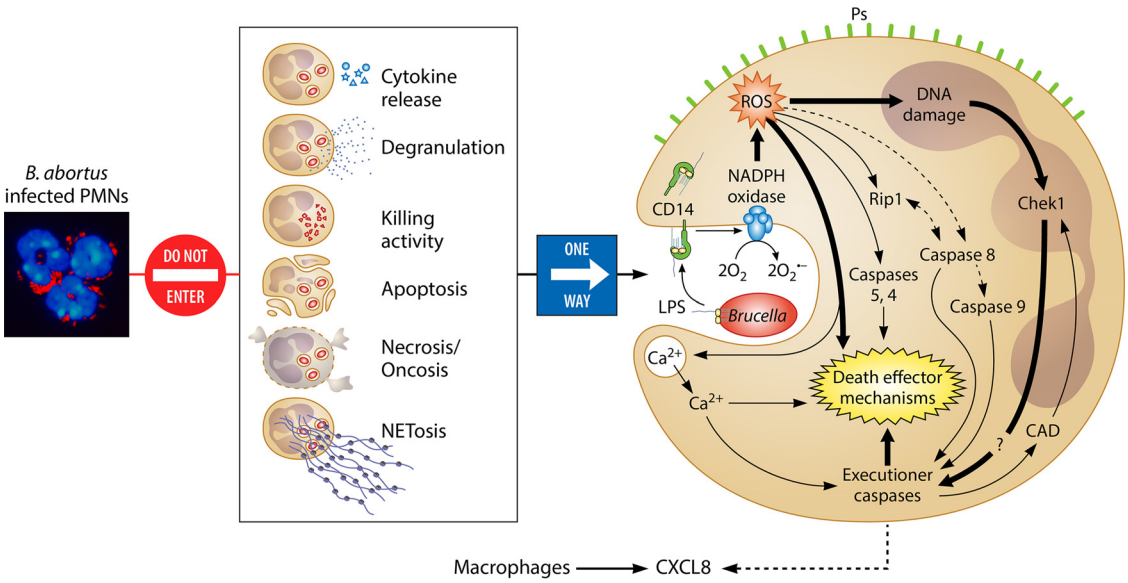

FIG 7 *Brucella* organisms do not activate PMNs but induce the premature cell death of these leukocytes. PMNs (nuclei in blue) heavily infected with *B. abortus* (in red) do not display significant phenotypic changes characteristic of degranulation, apoptosis, necrosis/oncosis, or NETosis and do not display significant bactericidal activities or release proinflammatory cytokines. However, through the release of nonendotoxic *Br*-LPS inside infected PMNs, *B. abortus* organisms promote the premature cell death of these leukocytes. After fusion with cell membranes, *Br*-LPS interacts with CD14 lipoprotein, progressively recruiting the action of NADPH oxidase and promoting the slow generation of controlled amounts of ROS mediators, which induce oxidative fragmentation of nuclear DNA and the recruitment of Chek1 protein, which is mainly responsible for coordinating the DNA damage after the activation of caspase-activated DNases (CAD). At the same time, some of the ROS effectors may induce recruitment of the RIP1 kinase/FADD cell death routes and caspase 8 and promote the release of Ca^{2+} to the cytosol. These mediators 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 initiator caspase 9 of the intrinsic cell death pathway is activated downstream by caspase 8, contributing to the premature PMN cell death mechanism. In this process, the infected PMNs release chemokine CXCL8 to attract mononuclear phagocytic cells and expose "eat me" signals (e.g., phosphatidylserine [Ps]) on the surface to promote their phagocytosis. Since *Br*-LPS does not interact with TLR4 on PMNs, then these cells do not become activated through this pathway. (Adapted in part from supplemental material for reference 139, published under the terms of the Creative Commons Attribution license [http://creativecommons.org/licenses/by/2.0]. The photo [far left] is reproduced from reference 205.)

effects work together, triggering a short-term, nonphlogistic process that allows *Brucella* dispersion and infection of other cells.

BRUCELLA-INFECTED NEUTROPHILS AS VEHICLES FOR MACROPHAGE INFECTION

Intracellular *Brucella* organisms are nontoxic for host cells (Fig. 2A); instead, they inhibit apoptosis and prolong the life of Mo and Mϕs, induce the maturation of DCs, and do not hamper mitosis of the infected cells (100, 104–106, 235). Despite this, *Brucella* provokes the premature cell death of human and mouse PMNs in a dose-dependent manner, an event that is reproduced by *Br*-LPS and its lipid A (Fig. 6). The premature cell death of *Brucella*-infected PMNs initiates after phagocytosis of the bacterium and the subsequent intracellular release of *Br*-LPS. Once inside PMNs, *Br*-LPS fails to interact with TLR4; instead, it moves to CD14 lipoprotein domains in the plasma membrane or intracellular vesicles, as observed in Mϕs (122). Then, the nonendotoxic lipid A moiety of *Br*-LPS recruits NADPH oxidase, which promotes the controlled generation of small amounts of ROS, triggering the cell death mechanisms (Fig. 7). This phenomenon occurs without the conspicuous phenotypic and functional changes characteristic of PMN apoptosis morphology, NETosis, or oncosis (39, 139, 223, 226). Alternatively, the *Brucella*-infected PMNs proceed to nonphlogistic premature cell death that resembles, in some features, apoptosis, but without condensation of the nucleus or cell fragmentation (139). First, the *Brucella*-infected PMNs release CXCL8 chemokine as a "find me" signal, and second, they translocate phosphatidylserine to the membrane external surface as an "eat me" signal (Fig. 7) (39). As demonstrated in other systems, dying PMNs still perform oxidative metabolism, release CXCL8, and

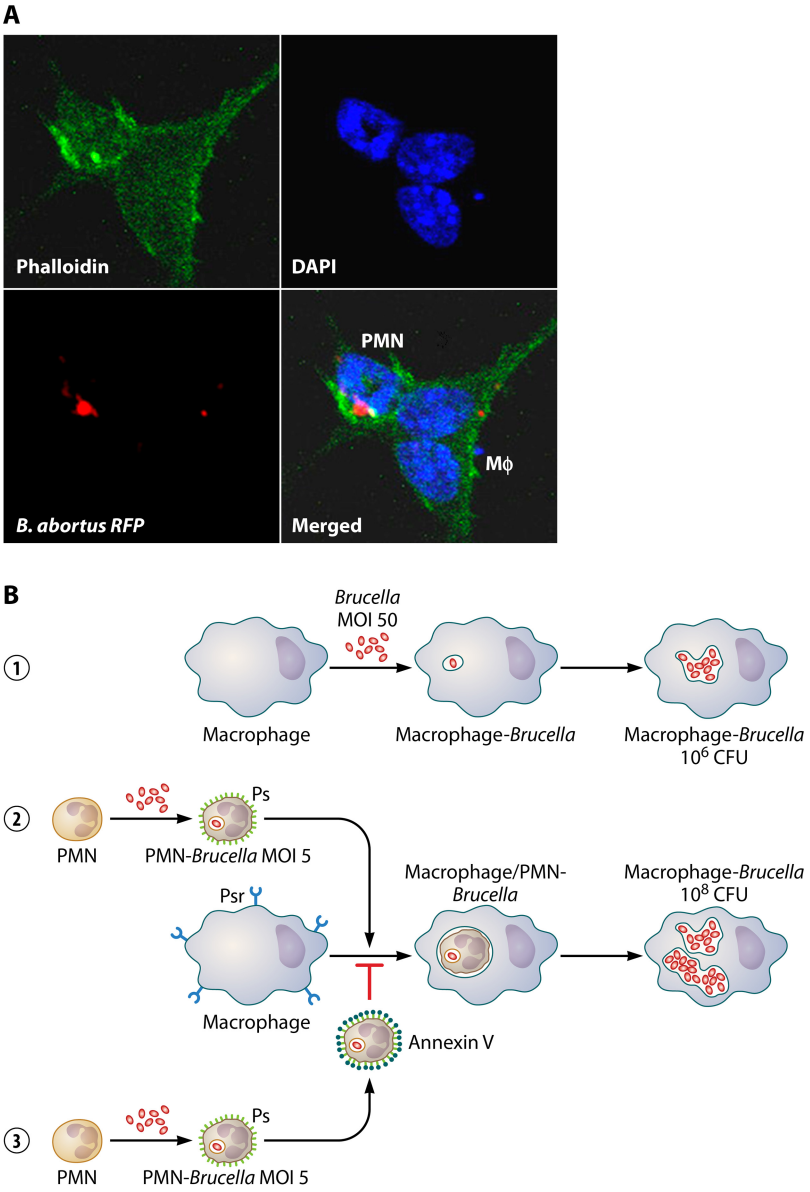

FIG 8 PMNs are efficient vehicles for dispersing *Brucella* organisms. (A) The Mϕ (cell with two nuclei and the actin cytoskeleton in green) phagocytizes a *Brucella*-infected PMN (cell with a donut-shaped nucleus, actin cytoskeleton in green, and with intracellular bacteria in red) with high efficiency. RFP, red fluorescent protein. (Adapted from reference 39, published under the terms of the Creative Commons Attribution license [http://creativecommons.org/licenses/by/2.0].) (B) *B. abortus* propagates at higher rates in Mϕs colonized through infected PMNs (1). *Brucella* infects Mϕs and replicates intracellularly (2). Mϕs recognize *Brucella*-infected PMNs, exposing phosphatidylserine (Ps), through their Ps receptors (Psr). Once the infected PMNs are phagocytized, bacteria move to the Mϕ phagosomes and replicate intracellularly at rates 100- to 1,000-fold higher than in Mϕs infected with bacteria alone (3). The blockage of Ps with annexin V on the PMN surface hampers the recognition of Mϕ-Psr and the subsequent bacterial colonization.

express phosphatidylserine on the external surface in a time-dependent manner (61, 236). After this process, Mϕs find infected PMNs and specifically detect phosphatidylserine on these cells (Fig. 8A). Then, Mϕs internalize the *Brucella*-infected PMNs in a nonphlogistic manner by a homeostatic-like procedure for PMN recycling (see Movie S1 in the supplemental material) (39, 51). Finally, at early times of infection, Mϕs release low quantities of proinflammatory cytokines but larger amounts of regulatory IL-10 (39).

The invasion of Mϕs by *Brucella* organisms via infected PMNs is 100 to 1,000 times

more efficient than when these mononuclear phagocytic cells become infected with bacteria alone (Fig. 8B). After several hours, once bacteria are inside their replicative niche in Mϕs, a simultaneous release of TNF-α and regulatory IL-10 cytokines is produced. Bacteria inside endoplasmic reticulum-derived compartments are protected from destruction, even if Mϕs become activated afterward (100, 106). As expected, blockage of phosphatidylserine on the surface of the *Brucella*-infected PMNs hinders their association with Mϕs and hampers phagocytosis (Fig. 9B). It seems, therefore, that the infected PMNs may serve as efficient Trojan horse vehicles for bacterial dispersal, as suggested by Wesley W. Spink 70 years ago on the basis of clinical observations (8).

BRUCELLA-INFECTED NEUTROPHILS DAMPEN ADAPTIVE IMMUNITY

As in other diseases caused by intracellular bacteria, the activation of cell-mediated immunity followed by the release of agonistic cytokines and recruitment of mononuclear phagocytic cells is essential for eliminating *Brucella* organisms (22, 23). However, before this process, PMNs and other elements of innate immunity that are essential for controlling the first stages of infection recognize invading bacteria (48). Besides the central role exerted by PMNs in the innate immune response, these leukocytes also modulate adaptive immunity (48, 80–83). In most bacterial infections, the absence of PMNs favors bacterial replication and increases lethality (99, 100, 237–239). In murine brucellosis, however, the absence of PMNs (either in neutropenic mutant Genista mice or in mice depleted of PMNs with anti-Ly6G antibodies) does not favor *Brucella* replication but instead promotes the elimination of these pathogens from the target organs and fast resolution of the disease (87, 93). This phenomenon correlates with the transient and greater liability for infection observed in some *Brucella*-susceptible mouse strains. In these animals, the recruitment of PMNs in the spleen results from a higher bacterial burden, without efficient bacterial elimination from this organ (240).

After *Brucella* infection of neutropenic mice, there is a mild transient increase in bacterial numbers in the spleen at the onset of infection, a phenomenon that is rapidly reversed after 2 to 3 days (87). At this time, the absence of PMNs provokes the exacerbation of cell immunity, with augmented spleen swelling and higher infiltration of Mϕs and DCs. The numbers of $CD8^+$ $CD4^+$ B cells, monocytes, and Th1 cytokines significantly increase in both the *Brucella*-infected, neutropenic Genista mice and the PMN-depleted mice. Removal of PMNs in infected mice, once the adaptive immune response has initiated, promotes an even faster resolution of the spleen inflammation. These phenomena are associated with the potent activation of the cellular immune response, with increasing levels of IFN-γ, IL-12, and IL-6 and efficient recruitment of Mo, Mϕs, and DCs, as well as enhanced Mϕ differentiation toward M1-type cells (87, 93). At later times of the *Brucella* infection, once the number of bacteria has diminished in the neutropenic mice, the amounts of IFN-γ decrease, while IL-12, IL-10, and IL-6 increase and IL-4, IL-1β, and TNF-α remain at background levels. In murine brucellosis, augmented IL-10 is related to the $CD25^+$ $CD4^+$ T cell function that balances proinflammatory and anti-inflammatory cytokines (231). Likewise, the increase of IL-10 and IL-6 cytokines at later times of infection in neutropenic mice is linked to the decrease of IFN-γ. Low levels of IL-10 and large amounts of IFN-γ correlate with reduced *Brucella* survival, while the opposite connects to the resolution of the infection at later times (231, 241). The rise of IL-6 at later stages of infection limits recruitment of innate immune cells, and it is a critical regulatory event in inflammation (242).

Neutropenic infected mice dampen the overall antibody titers and all the immunoglobulin isotype responses against *Brucella* antigens, a phenomenon that is commensurate with the recorded robust cell-mediated immunity. Therefore, the enhanced removal of *Brucella* in these mice is not due to increased levels of antibodies but to a more efficient cellular response. The low levels of antibodies correlate with the absence of IL-4 and the rise of the Th1 response (241). Furthermore, the presence of anti-*Brucella* antibodies in neutropenic infected mice did not affect the levels of IFN-γ, although they dampen the IL-6, IL-12, and IL-10 response. Taken together, this indicates that PMNs play a significant role in regulating adaptive immunity during the infection process (87, 93).

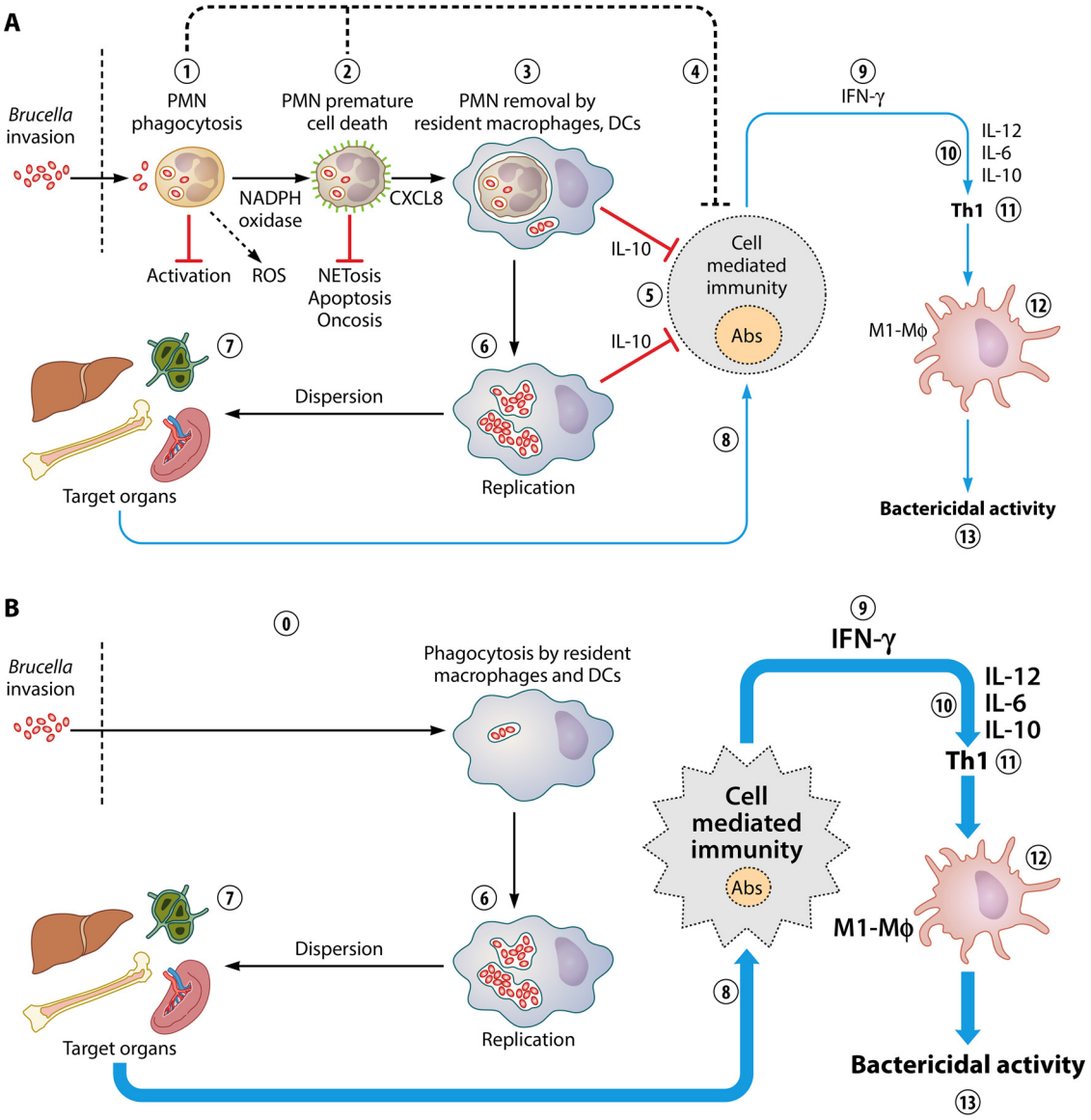

FIG 9 The Trojan horse hypothesis and modulation of the Th1 response. (A) After the invasion of *Brucella* organisms, resident PMNs phagocytize the bacteria without inducing significant activation or degranulation (1) nor signs of necrosis, oncosis, or NETosis. Infected PMNs, however, release chemokine CXCL8 and expose phosphatidylserine on the cell surface as an "eat me" signal (in green) (2). Then, in the primarily affected organs, such as the liver, spleen, and BM, Mϕs and DCs phagocytize the *Brucella*-infected PMNs in a nonphlogistic manner (3). Infected PMNs may have a direct regulatory influence over cells of the adaptive immune response by an unknown mechanism that may involve lipoxins and other mediators (3). Those Mϕs colonized by *Brucella*-infected PMNs control adaptive immunity by releasing the regulatory cytokine IL-10 (5). This event allows bacterial replication inside mononuclear phagocytic cells such as Mϕs, Mo, and DCs (6), opening a window for bacterial dispersion in the target organs (7). Then, the adaptive immune response begins, with activation of the cell-mediated immunity and release of anti-*Brucella* antibodies (8), significant quantities of IFN-γ (9), and the subsequent release of IL-12, IL-6, and the regulatory IL-10 (10), promoting a regulated Th1 response (11). The Th1 immune response induces polarization toward M1-type Mϕs (12), which exert an effective bactericidal action, controlling the infection (13). (B) In the absence of PMNs, there is no such regulatory function over the immune system, allowing the direct interaction of Mϕs with the bacterium (0). The fast activation of Mϕs and other mononuclear cells narrows the window for the immune system to respond, favoring an exacerbation of Th1 cell-mediated immunity with very large amounts of IFN-γ, IL-12, and IL-6 cytokines, smaller amounts of antibodies, and increased polarization of bactericidal M1-type Mϕs. The large amounts of IL-10 control the cytokine storm and promote the fast resolution of inflammation. As a consequence of this enhanced activation, the number of bacteria in the target organs decreases, favoring fast resolution of the infection.

Under the effect of IFN-γ and IL-12 cytokines, CD4$^+$ lymphocytes become activated, promoting Th1 polarization and the differentiation of Mϕs to M1-type cells, with increased brucellicidal function (243–245). Since in murine brucellosis, all these factors increase in the absence of PMNs, then the obvious conclusion is that these leukocytes

exert modulatory functions on the adaptive immune response (Fig. 8). However, the hyperactivation of adaptive immunity, with IFN-γ levels that exceed 8,000 pg/ml, is not without a price, since the neutropenic *Brucella*-infected mice develop cachexia in the early phases of the disease (93). Therefore, the physiological removal of infected PMNs in brucellosis may keep the immune response's equilibrium, avoiding the cytokine storm, which may be lethal (see Movie S2 in the supplemental material).

Given the role that PMNs have in controlling many bacterial infections, the inhibitory effect that these leukocytes have on the immune response against *Brucella* organisms is counterintuitive (87, 99, 237–239). In legionellosis, the absence of PMNs, rather than promoting a Th1 response, led to Th2 skewing and more disease (99). This shift is a remarkable phenomenon, since IFN-γ also plays a central role in the immune response in legionellosis (246) and the intracellular life cycle of *Legionella pneumophila* resembles that of *Brucella* organisms (247). Likewise, the absence of PMN apoptosis is linked to the slow activation of naive $CD4^+$ T cells in *M. tuberculosis* infections (88), and the role of PMNs as antigen-presenting cells in other bacterial infections (92) does not fit with the inhibitory functions observed in brucellosis. Mechanisms like regulation through PMN cytokines or the release of ectosomes or exosomes (248) seem unlikely due to the absence of degranulation and the negligible amounts of regulatory and proinflammatory cytokines released by *Brucella*-infected PMNs (100, 139, 231).

Despite this, there are inhibitory actions throughout the immune system mediated by PMNs that offer alternative mechanisms. Activated or dying PMNs hamper the proliferation of IFN-γ-producing T cells by the release of arginase, which, over time, depletes extracellular L-arginine in a NO-dependent manner (82). Similarly, subsets of mature and activated PMNs inhibit T cell proliferation through the release of ROS in the immunological synapse between the PMNs and T cells (94). In cancer patients, activated PMNs suppress cytokines and inhibit T cell functions through ROS generation (90). PMNs instructed by *Mycobacterium*-infected DCs produce IL-10 and shut down Th17 cells through their IL-10 receptor (249). Primed PMNs may compete for the availability of antigen with DCs and Mϕs and then hamper antigen presentation in lymph nodes (85). PMN-mediated T cell regulation in adjuvant immunization may be dependent on prostanoids. Indeed, adjuvant-primed PMNs control the spread of T cell responses to distal lymph nodes. This effect depends on prostanoids, since, in neutropenic mice, there is mobilization of, and an increase in, $CD4^+$ T cell response-dependent IL-2 or IFN-γ production (86). Although these mechanisms somewhat fit with inhibitory regulatory functions, they require fully activated PMNs, which is not the case in brucellosis.

One alternative that deserves attention is the release by PMNs of anti-inflammatory lipid mediators, such as lipoxins, particularly LXA4 (250). 5-Lipoxygenase mediates the synthesis and production of lipoxins through a mechanism that involves the interaction of PMNs with the endothelium and platelets in the vascular lumen or resident tissue mucosal cells (250). It has been demonstrated that *Brucella*-infected, 5-lipoxygenase knockout mice deficient in the synthesis of lipid mediators, such as lipoxin LXA4, release larger amounts of IFN-γ and IL-12 cytokines than infected wild-type mice, promoting the fastest resolution of inflammation and more efficient removal of bacteria in the target organs (251). This phenomenon, which is reminiscent of the Th1 modulatory function exerted by PMNs in murine brucellosis, may be due to the inhibitory action that lipoxins exert on Mϕs, Mos, and DCs and on the differentiation of T effector cell responses as well as T helper activities (250, 252). Therefore, in the absence of PMNs and the concomitant lower levels of lipoxins, the exacerbation of the Th1 response may be the anticipated outcome in neutropenic mice.

THE TROJAN HORSE HYPOTHESIS AND MODULATION OF THE Th1 RESPONSE

A comprehensive model of the role of PMNs in the *Brucella* infection process is illustrated in Fig. 9A. After invasion through mucous membranes, resident Mϕs/DCs and PMNs phagocytize *Brucella* organisms. While the former are suitable host cells for *Brucella* replication (253, 254), the latter leukocytes do not kill the bacterium and avoid the proinflammatory functions at the onset of the infection. The removal of infected

PMNs by phagocytic cells occurs mainly in the affected organs, such as the liver, spleen, and BM. Then, *Brucella* organisms are delivered inside mononuclear phagocytic cells through the infected PMNs. Once inside, the bacteria escape to their replicating niche without significant activation of the host Mϕs, Mo, or DCs (Fig. 9A). The internalization of *Brucella*-infected dying PMNs promotes the release of IL-10 and hampers the activation of the phagocytic mononuclear cells early in the infection process (255–257). The regulatory IL-10 cytokine dampens Mϕ effector functions and limits the production of IFN-γ (241). This nonphlogistic process at the onset of infection opens a window for bacterial dispersion before the adaptive immune system becomes fully activated. Later in the infection, the Th1 adaptive immunity fully develops with a predominant production of IFN-γ and other cytokines that promote Mϕ polarization toward M1-type cells, which are the most brucellicidal cells (243–245). This model does not rule out the direct influence of *Brucella*-infected PMNs on immune cells as a complementary mechanism (e.g., through lipoxins or other mediators).

In the absence of PMNs, mononuclear phagocytic cells interact directly with free *Brucella* organisms that display lower invasion efficiency (Fig. 8B). The direct recognition and phagocytosis of the bacterial cells promote a faster and more powerful Th1 immune response, with the release of larger amounts of IFN-γ and other cytokines and stronger polarization toward M1-type Mϕs. The exacerbated immune response readily eliminates brucellae from the target organs. After that, larger IL-10 and IL-6 amounts are released by the neutropenic host, modulating the enhanced immune response and resolving the infection in a shorter period.

A short time after infection, PMN-containing brucellae aggregate around Kupffer Mϕs in the liver sinusoids, which then become fully infected with bacteria (12). Likely, this phenomenon also occurs in other organs of the mononuclear phagocytic system (42). The replication of *Brucella* organisms in the primarily affected organs induces granulomas composed mainly of Mϕs and DCs (epithelioid cells), many of them heavily infected, with just a few PMNs present (43). Most PMNs in these granulomatous inflammations do not harbor bacteria (42), suggesting that phagocytic cells of the mononuclear phagocytic system remove the infected PMNs.

FINAL REMARKS

Experimental designs related to infectious diseases must fit the realm and reality of the episodes to unveil the hidden mechanisms behind the disease; otherwise, the results become a curiosity and are irrelevant in the context and scope of the events (258). In this direction, the use of neutropenic experimental murine models has contributed significantly to defining the role of PMNs at the onset of *Brucella* infection or in the early phases of the disease, explaining some of the outcomes observed in human and animal brucellosis. While PMNs are dispensable for combating *Brucella*, these infected PMNs dampen the adaptive immune response against brucellosis. Nevertheless, PMNs are needed to modulate the cytokine storm that risks the lives of infected animals, as demonstrated by weight loss in neutropenic infected mice (93). Although PMNs are not critical for bacterial clearance, they still modulate the immune response to avoid an exacerbated reaction.

Our comprehensive model is compatible with a large number of clinical observations, which include a lack of overt clinical signs at the onset of infection and a long incubation period, which in humans and domestic ruminants may last from weeks to months (31, 259). The absence of neutrophilia in the initial phases and the concomitant neutropenia in a significant number of long-lasting brucellosis cases suggest the effective removal of *Brucella*-infected PMNs by mononuclear phagocytic cells of the reticuloendothelial system. It is noteworthy that *Br*-LPS, the molecule that triggers the premature cell death of PMNs, circulates for several months in mice without being destroyed (122), a condition that may be associated with the neutropenia observed in some brucellosis patients.

This scenario is not without precedent. For instance, other microbial pathogens also use PMNs as Trojan horse vehicles for dispersion (79, 260), and the presence of CD14

on PMNs and DCs has been related to LPS-induced apoptosis (261, 262). Some of the strategies followed by *Brucella* organisms are present in other pathogenic alphaproteobacteria related to *Brucella*, such as *Anaplasma* and *Bartonella*. These bacteria also display furtive strategies used to avoid activation of the innate immune system and the microbicidal action of PMNs (65, 66, 69, 263). Finally, some investigations have shown the negative role of PMNs in controlling chronic bacterial infections (97).

Once installed in humans, brucellosis is a long-lasting bacterial infection that is seldom eliminated by the sole action of the immune system and requires an aggressive dual antibiotic treatment for several weeks (8, 10, 149). If not treated, the bacteria may remain in tissues for extended periods and cause relapses, some of them many years apart (264, 265). Immunized animals with the live attenuated-*Brucella* vaccines can mount an efficient cellular adaptive immune response against brucellosis (266), and activated phagocytic cells can eliminate the pathogenic organisms (254, 267).

The stealthy strategy followed by *Brucella* organisms opens a window into the innate immune response and gives the bacteria time to establish and hide within host tissues before adaptive immunity becomes fully activated (46). This event is a *sine qua non* requirement for the development of long-lasting brucellosis. Finally, our model describing *Brucella*-infected PMNs as Trojan horse vehicles for bacterial dispersal and as modulators of the Th1 adaptive immunity in the infection fits with the "Occam's razor" principle of parsimony, in which the most straightforward explanation that agrees with the experimental data is also the most probable one.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, MP4 file, 1.5 MB.

SUPPLEMENTAL FILE 2, MP4 file, 1.9 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.3 MB.

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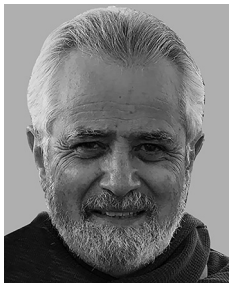

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