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Review Article

Characteristics and Intracellular Life of Brucella Organism: A Review

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ABSTRACT

Brucella is a Gram-negative, facultative intracellular bacteria that causes zoonotic brucellosis in humans and various animals. These pathogens are affecting domestic animals (cattle, goat, sheep, pig, dogs and camel), human and wild animals. Humans are accidental hosts of brucellosis acquiring the infection commonly from contact with infected animals, aborted materials and consumption raw milk. *Brucella* do not produce classical virulence factors, and their capacity to successfully replicate within a variety of host cells, to persist for prolonged periods within host cells and to evade the host immune response at the same time underlies their pathogenicity. The virulence factors of *Brucella* are involved in intracellular survival and replication within mononuclear phagocytic cells, preferentially macrophages in the host and hampers the intracellular trafficking and ability to prevent recognition by the host defense system. All these comprehensions of *Brucella* can inhabit inside the phagocytes of infected host to promote their survival, persistence and multiplication.

Keywords: Brucella; Immunity; Intracellular survival; Pathogenicity; Virulence factors

INTRODUCTION

Brucellosis, is known by many names including Melitococcosis, undulant fever, Malta fever, Mediterranean fever (in man); contagious abortion, infectious abortion, epizootic abortion (in animals); Bang's disease (in cattle) [1]. Brucellosis is one of the most common zoonotic diseases worldwide, caused by Gram-negative and intracellular bacteria called *Brucella*, that cause a chronic disease of domestic, wild animals and humans [2]. The ability of these bacteria to invade, survive for long periods of time and multiply within host cells is critical for disease causation [3]. Traditionally, the genus *Brucella* consisted of six recognized species, grouped according to their primary host preferences, that is, *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (wood desert rats). Recent isolates from human (*B. inopinata*), aquatic mammals (*B. pinnipedialis* and *B. ceti*), and a common vole (*B. microti*) are recognized as new species, bringing the current number to 10 species in the genus [4].

Brucellosis in animals results in abortion and other disease manifestations. Human infection usually results from direct contact with tissues or blood from infected animals or by consumption of contaminated animal products, including unpasteurized milk and cheeses [5]. In fact, half of the million new human cases are estimated to occur annually [6]. Brucellosis in humans typically presents with fever, sweat fatigue and headache. However, chronic brucellosis may affect many host organs, leading to arthritis, orchitis, hepatitis, encephalomyelitis, and endocarditis [7]. In a study of over 75 diseases affecting livestock, brucellosis was determined to be one of the 10 most important in terms of impact on impoverished people [8].

Many developing countries lack the resources to establish control programs, and brucellosis remains endemic in much of the developing world including the Middle East, Asia, Africa, and South America, and in the United States where foci of disease remain because of persistent infection in wildlife species [4].

An important aspect of *Brucella* infection is its ability to persist and replicate within phagocytic cells of the reticuloendothelial system as well as in non-phagocytic cells such as trophoblasts. This ability involves a temporary fusion of the *Brucella*-containing vacuole with the lysosome, and subsequent exclusion of the lysosomal proteins, following this process, the *Brucella*-containing vacuole becomes associated with the endoplasmic reticulum. These endoplasmic reticulum-associated compartments are the niche for intracellular replication of *Brucella* in macrophages, epithelial cell lines and placental trophoblasts, once inside this compartment, the bacteria can establish chronic infection [9].

Prevention of Brucellosis in the human population relies on control of disease in the animal reservoir. A prerequisite to the development of improved vaccines and treatment strategies to

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control the disease in animal reservoir is the study of the immune response of different host species to *Brucella* infection [10]. Intracellular nature of the organisms makes it difficult in control of the disease to be completely eliminated by the host immune responses or by antimicrobial drugs, this may be due incomplete understanding of mechanistic events involved in the process of intracellular survivability [11]. Therefore, this review outlines the pathogenesis, virulence factors, mechanisms that permit intracellular survival and evasion from host immune responses as the basis to prevent and treat brucellosis.

General characteristics of *Brucella* organism

Brucella is very small (0.5–0.7 μm × 0.6–1.5 μm), faintly stained Gram-negative coccoid rods, with a microscopic appearance of 'fine sand', that lack endospores, capsules, or native plasmids [2]. Primary culture of *Brucella* reveals punctate, non-pigmented, and non-haemolytic colonies. Colonies of smooth (S) *Brucella* strains are raised, convex, circular and translucent. After sub-cultivation or prolonged culture (>4 days), the colony morphology of *Brucella* may become less convex and opaquer with a dull, dry, yellowish-white granular appearance. These changes are caused by the dissociation of *Brucella* from smooth to rough (R) forms. *Brucella* species stain weakly with safranin. Although *Brucella* is a strict aerobe, some strains require carbon dioxide, especially on primary isolation. *Brucella* is non-motile and generally oxidase-positive and urease-positive [12].

Brucella is an intracellular pathogen, during an infection it survives and multiplies in macrophages; the bacteria adapt to the acidic pH, low levels of oxygen, and low levels of nutrients [13]. Bacterial cells are able to survive for a prolonged time in water, aborted fetus, soil, dairy products, meat, dung, and dust [14].

For isolation of *Brucella* species, the enrichment and selective media such as Tayer-Martin's medium or Farrell's medium are commonly used. The colonies mature after four to six days of incubation at temperature of 37°C. They can also grow at 28°C, but poorly and slowly. Moreover, these bacteria can grow in both aerobic atmosphere and in 10% CO_2; while, their growth is enhanced without additional CO_2 on a serum dextrose agar. For culture of blood or body fluid a biphase medium called Castaneda should be used. Castaneda consists of two phases: liquid and solid closed in bottle. Liquid medium contains 1-2% of sodium citrate. Sample (5-10 ml) is added to the medium and incubated in 37°C in perpendiculary standing bottle in 10% carbon dioxide atmosphere [15]. Culture of bone marrow may have a higher yield than routine cultures of blood [7].

Pathogenicity and clinical presentation

B. melitensis is considered the most virulent *Brucella* for humans with a few organisms (10 to 100) being sufficient to cause a debilitating chronic infection and is considered one of the most important zoonotic diseases worldwide, Although *B. abortus, B. suis and B. canis* are potential agents of this disease [9]. *B. abortus* and *B. suis* infections usually affect occupational groups, while *B. melitensis* infections occur more frequently than the other types in the general population. The greatest prevalence in man is found in those countries with a high incidence of *B. melitensis* infection among goats, sheep, or both species [1]. In most cases, human infections occur through ingestion of contaminated milk and unpasteurized dairy products. However, occupational exposure of mucosa or skin abrasions to fluids and tissues from aborted materials of infected animals is also an important source of infection [9].

In human Brucellosis, fever, joint pain, night sweats and constitutional symptoms including malaise, anorexia, weakness and weight loss are the most common symptoms. Osteoarticular complications and spondylitis are the most difficult to treat and can be associated with neurological complications. The reproductive system is the second most common site of focal complication with epididymoorchitis and spontaneous abortion reported. Endocarditis is the primary cause of mortality from *Brucella* infection, although cardiac complications are observed [3].

Animals Brucellosis is a widespread reproductive disease, commonly causing abortion, death of young ones, stillbirth, retained placenta or birth of weak calves, delayed calving, male infertility, and marked reduction in milk yield. It infects almost all domestic species except cats, which are naturally resistant to *Brucella* infection. In bulls, the disease is characterized by fever, vesiculitis, orchitis, and epididymitis. In severe cases, it can also be the reason for testicular abscesses, metritis or that can lead to lifetime infertility. In horse *B. abortus* and *B. suis* have been isolated. The disease usually manifests itself in the form of fistulous bursitis, "poll evil" and "fistulous withers." Abortions are rare but they do occur [16]. Horses acquire the infection from cattle or swine, but transmission from horses to cattle has also been proven. Man can contract the infection from horses that have open lesions. In general, horses are more resistant to the infection [1].

Pathogenesis

Fifty-five years ago, it was known that *Brucella* penetrate the mucosal epithelium and are transported as free bacteria, or within phagocytic cells, to regional lymph nodes. Localization within regional lymph nodes results in lymph node hypertrophy, lymphatic and reticuloendothelial hyperplasia, and inflammation. If bacteria are not localized and killed within regional lymph nodes draining the site of infection, they replicate and spread via blood or lymph to other lymphoreticular tissues and organs such as the spleen, reproductive tract, and/or mammary gland [17]. Bacteria can spread in a host through the lymph nodes and then translocate to the preferred tissues in reproductive tract [18-20]. There, *Brucella* induces acute or chronic infection of reproductive tract that leads to abortion or severe reproductive tract diseases [2].

Intracellular trafficking and survival of *Brucella*: In order to reach its target cells, *Brucella* needs to cross mucosal barriers of the respiratory, genitourinary or digestive tract resulting in dissemination of the organism to lymphoid and reproductive organs. Putative receptors include extracellular matrix proteins, the cellular prion protein, syndecans, and integrins. Interestingly, an integrin binding sialoprotein found to be up regulated in the Peyer's patch during *Brucella* invasion, is also highly expressed in bone matrix and trophoblastic, potentially explaining *Brucella's* preference for these host sites [18].

Regardless of the specific mechanism, *Brucella* are able to rapidly cross the mucosal barrier with minimal activation of the host immune system. Tall like Receptors (TLR) signaling pathways are subverted indicating that *Brucella* reduce or PAMPs that typically trigger immune system activation. Furthermore, the down regulation of cytokine expression within the intestine shortly after infection suggests *Brucella* are able to actively manipulate the host's immune response for its benefit [18].

Brucella survives within non-phagocytic cells up to 72 hours after infection, overcomes the epithelial barrier and then penetrates the

phagocytic cells [6,17]. In macrophages the pathogen avoids the host immune response; therefore, it can multiply and spread to other tissues using cellular tropism [19]. *Brucella* is internalized by phagocytosis that requires a moderate recruitment of actin filaments upon activation during interaction of *Brucella* and receptors on the surface of the macrophage cell membrane. Opsonized *Brucella* is internalized via complement receptors whereas non-opsonized bacteria interact with lectin and fibronectin receptors [21]. Non-opsonized *Brucella* can survive and replicate inside cells; in contrast, opsonization of bacteria or IFN-activation of macrophages enhance intracellular killing of bacteria inside the host cell [22]. Lipids rafts, which are cholesterol-rich microdomains in the cell membrane of macrophages, also participate in bacterial internalization, and additionally these rafts contribute to directing intracellular trafficking of bacteria [20,23].

Soon after internalization, the *Brucella*-containing phagosome interacts with early and late endosomes. The majority of phagocytosed *Brucella* is destroyed by bactericidal action of free radicals of oxygen, nitric oxide and enzymes inside phagolysosomes. However, a certain number of bacteria resists these bactericidal mechanisms, and after transient fusion with the lysosome can actively exclude lysosomal proteins and redirect the *Brucella*-containing phagosome (BCV) to the endoplasmic reticulum, where the organism is capable of replicating [24]. Importantly, acidification of the BCV does not injure the bacteria, but it triggers expression of bacterial genes that are essential for intracellular survival during the early stages of infection [25].

BCV seizure of Endoplasmic reticulum (ER) membranes and components is accompanied by structural characteristics and functional restructuring of the ER. BCVs later accumulate autophagic features and exhibit lysosome-associated membrane protein-1 positivity, constituting a distinctive aspect of the intracellular *Brucella* life style [9]. Lysosomal-induced acidification of the BCV is a necessary signal for induction of *Brucella's* primary virulence relatedness factors, the T4SS. Regulation of vacuolar trafficking and subsequent survival and replication of *Brucella* within macrophages is dependent on a functional T4SS [26]. Laboratory strains of *Brucella melitensis, Brucella abortus or Brucella suis* with mutations in the T4SS are unable to ultimately exclude lysosomal markers and survive within the macrophage [3,26].

Bacteria are able to survive and replicate in DCs similarly to macrophages, although intracellular growth tends to be more prominent in DCs. Furthermore, bacteria can inhibit maturation of DCs compromising DCs antigen presentation and cytokine secretion. As a result, DCs have two important aspects which transform them in excellent carriers for *Brucella*: High tolerance for bacteria development, and migratory properties which could support pathogen spreading [27].

The behavior of *Brucella* in non-phagocytic cells has been better investigated in epithelial cell lines, although *Brucella* is capable of invading epithelial [28] and trophoblatic cells [29], it is much less invasive than other facultative intracellular bacterial pathogens such as *Salmonella enterica* [30]. *Brucella* invades epithelial cells *via* recruitment of actin filaments and rearrangement of the host cell membrane. Intracellular trafficking of *Brucella* in epithelial cells is similar to that observed in phagocytes [26]. Trophoblastic cells are key target cell of *Brucella* infection during late phase of gestation in ruminants, the capacity to replicate rapidly and extensively in trophoblasts can compromise the integrity of the placenta and infection of fetus, resulting in abortion or birth of weak offspring [31]. Growth of *Brucella* inside trophoblasts is apparently enhanced in the presence of high concentrations of steroid hormones and erythritol during the final third of gestation, in addition, hormonal changes in infected placentas may affect the occurrence of abortion since an increase in prostaglandin, estrogen and cortisol, and decrease in progesterone levels mimic what happens during parturition [9].

Virulence factors and Host Immune Response to *Brucella* infection

Establishment of persistent infection is dependent on the initial interactions between *Brucella* and the host. *Brucella* do not to cause overt toxicity, but instead stealthy invade. As a result, *Brucella* lack classical bacterial virulence factors including exotoxins, endotoxic LPS, cytolysins, a capsule, functional flagella, fimbriae, plasmids, and inducers of apoptosis, instead *Brucella* virulence factors function in three ways: to hide *Brucella* from immune detection, to protect from any brucellacidal mechanisms employed by the host, and to disrupt the host immune response [3]. That is why *Brucella* is frequently called as "nasty bugs" based on their unusual virulence characters [32].

Lipopolisaccharide (LPS): LPS consists of lipid A, oligosaccharide core and O-antigen and it serves all three necessary functions for *Brucella:* Hiding the organism, protecting from bactericidal mechanisms, and disrupting the immune response [33]. Lipopolisaccharide is different and non-classical in *Brucella* as compared to other Gram-negative bacteria, Lipopolisaccharide from *Brucella* strains is less toxic and less active than the classical LPS isolated from *E. coli* and *Salmonella* allowing evasion of innate immunity, Classical LPS causes a high pyrogenicity, while non-classical LPS shows low pyrogenicity, being a weak inducer of tumor necrosis factor [34].

In lipid A of *Brucella abortus* diaminoglucose is fundamental component instead of glucosamine, lipid A is connected to the core by amide bonds, instead ester and amide bounds [35]. Similar to other related α-Proteobacteria, *Brucella* possess LPS with a non-canonical lipid A characterized by very long chain fatty acids [36]. In strains with smooth colonies, the smooth LPS, (S-LPS) contains: lipid A, that consists of two types of aminoglycose, and fatty acid besides -hydroxymiristic acid, core comprises mannose, glucose, quinovosamine, and O-chains are composed of 4-formamido-4,6-dideoxymannose, The O-side-chain of *Brucella* LPS protects smooth bacteria from both extracellular and intracellular bactericidal components, *Brucella* are resistant to complement, cationic peptides, and neutrophil extracts, induction of IL-10 production, and interference with MHC class II antigen presentation [3].

LPS may also play a role in inhibition of apoptosis by the interaction of the O-chain with TNF-α (tumor necrosis factor), thus, dead cells do not release specific factors, therefore they do not activate the immune system and *Brucella* are able to avoid host immune surveillance [37]. Furthermore, smooth LPS provides resistance to complement and antimicrobial peptides such as alpha-defensins and lactoferrins. Smooth LPS also confers resistance to nitric oxide, free radicals and lysozyme, important antibacterial mechanisms of macrophages and neutrophils. Therefore, *Brucella* smooth LPS may be considered a virulence factor required for resistance against both extra and intracellular antimicrobial mechanisms of the host [9]. The O-chain connects with lipid rafs on the macrophage surface and the bacteria enter the cell. *Brucella* strains with R-LPS, for example *B. ovis* or *B. canis* do not connect with lipid rafs and rapidly connect with lysosomes [23].

Urease: Urease is a multi-subunit enzyme that decomposes urea to carbonic acid and to ammonium form, through nitrogen metabolism, which results in increase of pH due to ammonia production as a result of urea hydrolysis [38]. It is responsible for urease activity and its inactivation cause attenuation of strains in mice when the organism is inoculated via the digestive tract [9]. This feature of the *Brucella* enables its survival in acid environment. That urease may protect *Brucella* during passage through the digestive tract (stomach), when the bacteria access their host through the oral route. Urease is produced by all bacteria belonging to the genus *Brucella* but *B. ovis* [6]. *B. ovis* does not express urease, potentially explaining why the primary route of infection for this species is venereal rather than oral [38].

Type IV secretion system (virB T4SS): Perhaps T4SS is the best and main *Brucella* virulent factor which encoded by the virB operon and contains totally 12 genes (VirB1-12) placed on chromosome II [39]. *Brucella* probably use both stationary and exponential stages for intracellular survival, with stationary-phase physiology providing *Brucella* with benefits for adapting to the harsh conditions encountered in the phagosome and exponential stages associated with replication under favorable conditions. Molecular mechanisms may regulate adaptation. For example, VirB has been found to be maximally expressed during exponential growth, but expression is repressed upon entry into the stationary phase [40].

Pathogen-associated molecular patterns (PAMPs): PAMPs have been defined as virulence factors and that are feeble stimulators of toll-like receptors (TLRs), which are contributing to *Brucella* in a stealthy nature at the initial stage of infection. *Brucella* species are hidden to early detection by innate immunity, the absence of PAMPs expression in the cell envelop *Brucella* outer membrane lipopolysaccharide, ornithine-containing lipids, lipoproteins and flagellin, which minimally activate the innate immunity, The TLRs have an extracellular leucine-rich repeat (LRR) domain which recognizes and binds to specific antigen ligands, *Brucella* toll-interleukin receptor (TIR) domain is established in both the cytoplasmic regions of TLRs and adaptor proteins. TIR domain consist of BtpA and BtpB proteins, which are considered as virulence factors and are accountable for mediating the signaling cascades of innate immune recognition [41].

Superoxide dismutase and catalase: Since oxidative killing is the primary mechanism employed by host phagocytes to control replication of intracellular pathogens, it has been found that *Brucella* have multiple mechanisms to detoxify free radicals. The production of enzymes is the main line of defense and counteracting reactive oxygen intermediates. These enzymes include superoxide dismutase (SOD) and catalase [42]. Macrophages with *Brucella* produce reactive oxygen intermediates (ROIs), this is a primary mechanism of destruction of the bacteria ingested, and it also prevents their intracellular replication. The following ROIs: O^{2-} (superoxide), H_2O_2 (hydrogen peroxide), OH– (hydroxyl radical) are very detrimental for cell structure. SOD (metalloenzyme) is encoded by sod sequence. An enzyme contains iron, magnesium, or zinc and copper at its active site 43(Benov and Fridovich, 1994). SOD is responsible for dismutation of O^{2-} (superoxide) to H_2O_2 (hydrogen peroxide) and O_2 (oxygen) – transfer from one molecule to another ($2O^{2-}+2H^+ \rightarrow H_2O_2 + O_2$) and suggesting that it plays a crucial role in *Brucella* intracellular Survival [43, 44]. Catalase decomposes hydrogen peroxide into oxygen and water. Catalase activity is limited to the periplasmic space, where together with Cu-Zn SOD leave external sources of ROI unchanged [20]. Catalase is not necessary virulence factor, the other enzymes can compensate lack of this enzyme in catalase mutants, e.g. alkyl hydroperoxide reductase or enzymes involved in DNA repair mechanisms [45].

Cyclic β-1-2-glucans (CβG): CβG is an osmoregulated periplasmic polysaccharide of the plant pathogens. *Brucella* species classified within the 2 subdivision of Proteobacteria, which includes Agrobacterium and Rhizobium, also contain cyclic β (1–2) glucan. In *Brucella*, this sugar is produced by cyclic β (1–2) glucan synthetase encoded by cgs but is not osmotically regulated [10]. Glucans are constituents of the bacterial periplasm with osmoregulatory and cholesterol sequestering activity and are required for survival of *Brucella* in non-phagocytic cells and in vivo in mice. Cyclic β (1-2) glucans of *Brucella* prevent phagosome maturation by interfering with lipid rafts, thus altering protein expression in the vacuolar membrane and excluding lysosomal proteins from the BCV 9(Xavier *et al.,* 2010) and it is independent from VirB and type IV secretion systems [17]. *B. abortus* CβG impacts intracellular trafficking by acting on lipid rafs on macrophage surface. These glucans participate in control of the phagosome-lysosome fusion. Mutants are destroyed in phagolysosome and they are not able to multiply [46].

Cytochrome oxidase and Nitric oxide reductase (NorD): Cytochrome oxidase is an enzyme facilitating *Brucella's* survival inside the macrophages, where oxygen availability is limited. There are two operons in genome encoding two types of high oxygen-affinity oxidases: cytochrome cbb3-type and cytochrome bd (ubiquinol oxidases) oxidases. Cytochrome cbb3 oxidase is expressed in vitro and allows for colonization of anoxic tissues (maximal action in micro aerobiosis). Cytochrome bd oxidase is expressed during intracellular multiplication and enables adjustment to the replicative niche by restraining the creation of oxidative free radicals and detoxification of compartment inside the cell [6]. NorD is used in the reduction of nitrate to dinitrogen gas which is an essential process for bacteria in case of oxygen deficiency inside the cell; this process allows for respiration of nitrate [47].

Alkyl hydroperoxide reductase: Alkyl hydroperoxide reductase (Ahp) attempt protection against oxygen radical and reactive nitrogen. AhpC and AhpD are organized in an operon under one promoter control. AhpC mutants are more sensitive to peroxide killing and are vulnerable to spontaneous mutagenesis [45], and may also protect against oxidative killing; in other bacteria, it protects against low levels of hydrogen peroxide and can detoxify the oxidizing compound peroxynitrite [17].

***Brucella* virulence factor A**- *Brucella* virulence factor A is induced in macrophages, through phagosome acidification. Presumably this protein is involved in forming the replication intracellular niche [48].

***Brucella* virulence-related regulatory (BvrR) and sensory (BvrS) system**- This regulator system is required for recruitment of GTPases and actin filaments, and for maintaining the integrity of the bacterial outer membrane. Mutant strains lacking BvrR/BvrS are unable to invade phagocytic and non-phagocytic cells because they do not recruit GTPases (Cdc42), so they persist extracellularly and in consequence they do not infect the cell [28]. This regulator system is also important for intracellular survival, since mutant strains are unable to inhibit phagosome-lysosome fusion and intracellular trafficking [49]. These proteins affect the transcription of the membrane proteins: Omp3b (Omp22) or Omp3a (Omp25a)

and have the influence on other non-protein membrane molecules and hence on functional and structural membrane homeostasis, BvrR/BvrS mutants show structural changes in LPS, but O-chains seem to be undisturbed [50].

The host immune response against intracellular pathogens such as *Brucella* is divided into innate and adaptive immunity [51]. The innate immune system includes anatomical barriers, complement system and cellular populations, such as phagocytes, and innate lymphocyte subsets (natural killer and γδ T cells). The adaptive immunity, also known as the acquired immunity is composed of T lymphocytes, and B lymphocytes [52,53].

Innate Immunity

In case of brucellosis as well as in other diseases, the innate immune system will act as the first line of host defense, responsible for preventing replication, reducing the initial number and killing of the microorganism, besides creating conditions for the generation of an effective adaptive immune response. This first line of defense includes phagocytosis of pathogens by cells such as neutrophils, macrophages and DC, death by natural killer (NK)-cells, secretion of cytokines and chemokines, recognition of molecules typical of a microbe PAMPs by PRRs and activation of the complement system [3,54].

Neutrophils: Neutrophils are the most numerous and important short-lived phagocytes in innate immune response. Murine, human, and bovine neutrophils efficiently take up *Brucella,* internalizing far more bacteria than macrophages. However, *Brucella* infection of neutrophils stimulates only minimal degranulation and respiratory burst activity compared to *Salmonella* infection. As a result, bacterial survival rates for *Brucella* in human neutrophils are approximately 70%, compared to less than 25% for *Salmonella*. Murine studies have shown that neutrophils play a negligible role in *Brucella* infection since depletion of neutrophils by treatment with a monoclonal antibody does not influence bacterial load in the spleen [36]. *B. abortus* is killed more efficiently in the absence of neutrophils than in their presence. It was suggested that neutrophils limit and regulate the activation of adaptive immune response against intracellular *B. abortus* infection, mainly throughout decreasing T lymphocytes activation [36].

In humans, the survival of *Brucella* in neutrophils during early infection has been observed, suggesting that the transportation of *Brucella* to lymphoid tissues can be mediated by neutrophils and also, human neutrophils have been implicated in potential mechanisms of tissue damage during liver brucellosis, since hepatic cell apoptosis was significantly enhanced by stimulation with supernatants from *Brucella*-infected neutrophils [55].

Neutrophils typically play a vital role in the innate immune response to bacterial pathogens; *Brucella* stimulate only limited neutrophil activation and have the ability to resist their killing mechanisms. Recent evidence also indicates that *B. abortus* actively induces cell death of human neutrophils, further limiting the capacity of neutrophils to kill invading *Brucella* and stimulate an appropriate immune response. This is consistent with the neutropenia observed in some human patients [36]. Therefore, activation of neutrophils seems not to be associated with protective immunity against *B. abortus,* but rather, it appears to be related to tissue damage and down regulation of adaptive immune response. By inducing neutrophil death, *Brucella* may be better able to reach their preferred replicative niche. Neutrophil fragments containing *Brucella* are removed by macrophages and dendritic cells.

Macrophages: Macrophages employ a number of strategies to directly kill pathogens including phagocytosis and autophagy with subsequent degradation by hydrolytic lysosomal enzymes, oxidative burst with subsequent killing by reactive oxygen and nitrogen species, and antimicrobial peptides with various mechanisms of action. Macrophages also function to stimulate and shape the adaptive immune response *via* cytokine production and antigen presentation. The efficacy of these direct and indirect mechanisms against *Brucella* depends on the host species, the *Brucella* species, and a multitude of factors specific to the individual infected. These factors ultimately determine the outcome of infection [56]. *Brucella* actually utilize the autophagy pathway to their benefit in order to spread from cell to cell [26]. Autophagy is defined as the process of cellular degradation, capture and removal of intracellular microbes through delivery of pathogens to lysosomes for destruction, which can lead to host intracellular innate immunity [57].

The bactericidal activity of macrophages is enhanced following activation of LPS, TNF-α, and IFN all function to activate macrophages. Interestingly, a study of *B. abortus* infection in murine macrophages found that pre-activation of macrophages prior to infection resulted in the death of 80% more *Brucella* compared to untreated macrophages. However, if macrophages were activated after *Brucella* had already established a replicative niche, the *Brucella* were resistant to the enhanced bactericidal mechanisms. This has important implications since *Brucella* infection of natural host's results in limited TLR stimulation *via* LPS and limited TNF-α production. If macrophage activation is dependent on IFN- production, this occurs later during infection likely after *Brucella* have established a replicative niche. A last-ditch effort employed by macrophages unable to control intracellular bacterial replication is induction of apoptosis. *Brucella* inhibits this bactericidal mechanism as well, facilitating the establishment of chronic infection. In murine macrophages, *B. abortus* prevents apoptosis by a mechanism independent of TLR signaling [36].

Dendritic cells (DCs): DCs are the primary cell type involved in presentation of antigen to naive T lymphocytes. As such DCs are a bridge between innate and adaptive immunity and are essential in shaping a protective Th1 response. A study of human DCs subjected to in vitro infection with *B. abortus, B. suis,* and *B. melitensis* found that regardless of the species, *Brucella* are actually better able to invade and persist in DCs than in macrophages, the ability to persist in DCs may represent a specific virulence strategy of *Brucella,* allowing for dissemination throughout the host within these highly-migratory cells [27]. While persistence of *Brucella* in murine DCs has also been demonstrated, evidence for survival in bovine DCs is lacking [58]. Once established within murine or human DCs, *Brucella* interferes with cell maturation, production of TNF-α and IL-12, and presentation of antigen to T lymphocytes [27,36,58]. The inhibition of DCs maturation appears to be at least partially due to *Brucella*'s ability to prevent TNF-α production in human DCs [27].

TLR signaling serves to activate bactericidal mechanisms of phagocytes, stimulate cytokine release, and enhance antigen-presenting properties of DCs so that the adaptive immune system can be primed. *Brucella* LPS is only a weak stimulator of TLR4 due to modification of the lipid A in the LPS molecule and active interference with TLR signaling *via* the BtpA/TcpB and BtpB proteins [59]. These proteins also interfere with TLR2 signaling but do not inhibit signaling by TLR9. Several studies have used TLR-deficient mice to elucidate the importance of TLR signaling to the

outcome of *Brucella* infection. These experiments have suggested that TLR9 plays a central role in determining host resistance [58,60]. In macrophages from Zebu cattle, which are better able to control *B. abortus* replication in vitro than macrophages from European cattle, TNF-α and IL-12 expression is stimulated by infection. In macrophages from the more susceptible breed, *B. abortus* appears to inhibit TNF-α and IL-12 expression below basal levels [58].

Additionally, IL-1, IL-6, and IL-8 are common components of the general cytokine response to gram negative bacteria. *Brucella abortus*-infected mice produce minimal IL-1, IL-6, and TNF- in comparison to *Salmonella*-infected mice, this effect does not appear to be due to active inhibitory mechanisms, but instead is likely related to the poor induction of TLR signaling by *Brucella*. While levels of pro-inflammatory cytokines induced by *Brucella* infection in mice are more than 10-fold lower than that induced by *Salmonella*, IL-1, IL-6, and TNF-α are all still present in murine brucellosis models [36].

Natural killer (NK): NK cells are activated by *Brucella* or their antigenic fractions and are thought to be important in the activation of B-cells and consequently to antibody production. However, even though NK-cells may be activated following infection, they seem to be not crucial in controlling brucellosis in mice, since its depletion in vivo does not affect the course of infection [61].

In patients with acute brucellosis, normal numbers of NK cells are present; however, they show a functional deficit in cytotoxicity [62]. There is evidence that the deficit in cytotoxicity is due to impairment of NK cell maturation. This may be a direct inhibitory effect or may be due to *Brucella's* inhibition of TNF-α and IL-12 production by macrophages and DCs, as both cytokines serve to activate NK cells. The pathogenic implications for this deficit in NK cell function are unknown but may contribute to intracellular survival of *brucella* in human hosts [3].

Adaptive Immunity

The innate immune system serves to limit *Brucella* replication and stimulate an adaptive immune response, which is more effective at clearing infection. Adaptive immunity is defined by antigen-specific recognition of pathogens by T and B lymphocytes and secreted antibody. The adaptive immune response attempts to control *Brucella* infection by three primary mechanisms. First, IFN- produced by CD4+ and CD8+ T cells serves to activate macrophages, enhancing their bactericidal capacity. Second, cytotoxic CD8+ T cells directly kill infected macrophages. Third, B lymphocytes secrete antibody, which has limited effect on the outcome of infection but is useful for diagnosis of disease [52].

Th1 cells are a subset of CD4+ T lymphocytes that produce the cytokines IFN- and IL-2 and are primarily involved in activating macrophages and CD8+ T cells. The major competing immune response is the Th2 response which is characterized by CD4+ T lymphocytes that produce the cytokines IL-4, IL-5, IL-10, and IL-13 and function to stimulate B cells to produce antibody. The importance of the Th1 immune response in *Brucella* infection was first realized in the mouse model and has since been observed in humans and natural hosts. IFN- is the principal cytokine secreted from in vitro cultures of splenocytes, T cells, and peripheral blood mononuclear cells of mice, humans, and cattle stimulated with *Brucella* antigen [63].

Neutralization of IFN: In mice infected with *B. abortus* results in increased bacterial colonization of the spleen. Deficits in IFN- production and the Th1 response in whole are also observed in some ruminant species following vaccination with *B. abortus* strain RB51, and this is believed to explain the poor efficacy of the vaccine in elk and water buffalo [17]. Neutralization of IL-10 results in higher levels of IFN- , TNF-α, and IL-6 production and a reduction in *Brucella* replication; however, increased disease pathology is observed as a result of the high levels of pro-inflammatory cytokines. A murine study indicates that *B. abortus* may induce regulatory T cells to produce IL-10 early in infection. In the absence of this early IL-10 expression, *Brucella* is unable to escape the phagolysosome and establish a replicative niche within the macrophage [64].

CD8+ T cells function to inhibit *Brucella* replication within macrophages via both IFN- production and Perforin-mediated cytotoxicity. CD8+ T cell numbers seem to increase during certain stages of infection, likely to compensate for lapses in CD4+ T cell responses. This has been observed in mice as well as in human patients with chronic brucellosis. Although their numbers increase, CD8+ T may not be able to control infection [65].

B. melitensis actively inhibits the CD8+ T cell response via secretion of the virulence factor TcpB. TcpB is released by *B. melitensis* within infected macrophages and functions to exclude phosphatidylinositols from the immune synapse formed during macrophage- CD8+ T cell binding. This prevents CD8+ T cell mediated killing, but also has effects beyond the immediate survival of *Brucella* within the contacted macrophage. The dampening of the resulting immune response appears to prevent the differentiation of CD8+ effector T cells into a long-lived memory pool. In a mouse model, few CD8+ T cells retained a memory cell phenotype during chronic *Brucella* infection. The few persisting memory cells displayed an exhausted phenotype characterized by deficiencies in IFN-, TNF-α, and IL-2 production [3].

CONCLUSION

Brucellosis is one of the most important zoonotic disease and causes major economic loss in animal husbandry sector. Most of the disease causative pathogens are efficiently removed by host immune cells, but *Brucella* has capability to establish a chronic or persistent infection. Virulence factors of *Brucella* are involved mainly for its intracellular survivability and replication within mononuclear phagocyte cells, which hampers the intracellular trafficking and ability to prevent recognition by both innate and adaptive immune response in their host but mechanistic events involved in this process are not fully understood and require further study in order to design suitable prevention and control measures to counter this important zoonotic disease.

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