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Alternative Strategies for Vaccination to Brucellosis

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Abstract

Brucellosis remains burdensome for livestock and humans worldwide. Better vaccines for protection are needed to reduce disease incidence. Immunity to brucellosis and barriers to protection are discussed. The benefits and limitations of conventional and experimental brucellosis vaccines are outlined, and novel vaccination strategies needed to ultimately protect against brucellosis are introduced.

Keywords

vaccine; mucosal immunity; bacteria

1. Brucellosis Overview

The most common zoonotic disease worldwide, brucellosis, is ranked third of eight most neglected zoonotic diseases [1]. This global disease remains problematic for countries on the Mediterranean rim, Middle East, Central Asia, South America, and the states in the USA that border Mexico [2-4]. Estimates claim that for some countries, brucellosis exceeds 500 cases/1×10^6 inhabitants [2, 5]. Brucellosis is often not recognized or is misdiagnosed resulting in underreporting by as much 26-fold [5, 6]. This unmitigated persistence of brucellosis results from chronic exposure to *Brucella*-infected livestock [7], often following consumption of unpasteurized milk or other dairy products [4, 8].

Brucellosis is caused by the Gram-negative cocciobacilli from the highly homogenous genus, *Brucella* [9], which contains 10 - 12 species [10,11], of which *B. melitensis*, *B. abortus*, and *B. suis* are the ones causing human disease [12-14]. In 1886, the British physician, David Bruce, first identified small Gram-negative cocciobacilli obtained from patients hospitalized with “Malta Fever” [15], and called these bacteria, *Micrococcus melitensis* [16]. A unique attribute of *Brucella* is their 94% DNA homology among species [6, 14, 17] allowing immunity to cross-protect across heterologous *Brucella* species. Most *Brucella* species have two circular chromosomes of varying size [18]. Each species is

discriminated by its surface lipopolysaccharide imparting serological specificity [8]. Notably, *Brucella* LPS is weakly endotoxic with poor toll-like receptor 4 (TLR4) agonist activity due to the long fatty acid acylation of its lipid A. This feature adds to the stealth qualities of *Brucella* [8].

The primary route of infection for humans is by an oral exposure [4, 19], yet an airborne infection can occur resulting from exposure to *Brucella*-infected livestock [9]. Brucellosis can be lethal in livestock by inducing abortion [8, 19-21]; however, brucellosis is rarely fatal (<0.5% of cases) in humans, but instead results in a debilitating, systemic disease [3-5, 7], attributed to mostly to *B. melitensis* or *B. abortus* [22, 23]. Acute disease in humans often presents as a febrile disease with generic flu-like symptoms such as chills, malaise, headaches, hepatomegaly, and splenomegaly [4,5,7,9]. Despite oral exposures, intestinal disease rarely occurs [24, 25]. Chronic disease symptoms include a relapsing, undulant fever, chronic fatigue, malaise, and can produce positive *Brucella* blood cultures [7,9,24]. Brucellosis exacerbation can lead to more serious endocarditis and arthritis [26-29]. *Brucella* infections can be resolved with antibiotic treatment, but this requires a prolonged two-antibiotic regimen [9, 24]. Despite this rigorous treatment, it is not a guarantee that infection will be resolved as evidenced by sequelae occurring in ~16% of the infected patients [24], of which, 50% have a persistent bacteremia [24]. What remains enigmatic following oral exposure is the absence of *Brucella* replication or pathology in the gut [19, 25], questioning the significance of oral infections. Recent data combined with past studies implicate the oro-pharyngeal tissues as the site where infections initiate in humans [30-34].

2. Correlates of Protection to *Brucella* Infections

2.1. Th1-type immunity required for protection to brucellosis

Brucella exposure by any route of infection will cause a bacteremia and progress to a systemic infection [5,9,24]. *Brucella*'s primary target is macrophages [35], and once in these cells, brucellae become difficult to eliminate from the intracellular compartment. This pathogen has many stealth features enabling such infection including its low endotoxic LPS [8]; its ability to avoid fusion with lysosomes [35]; its ability to dampen dendritic cell maturation [36] and to interfere with TLR2 and TLR4 signaling via TcpB, an analog for mammalian Toll/interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP) [37,38]; and to interfere with MHC class I [39] and class II antigen presentation [40,41]. Thus, macrophages need to be activated by IFN-γ [42,43] and TNF-α [23, 44] to effectively eliminate intracellular brucellae. Hence, IFN-γ is required for resolving *Brucella* infections of macrophages [45, 46] in an IL-12- [47] and TNF-α-dependent manner [48].

Brucella's astute capacity to avoid innate immune detection also impairs subsequent development of a suitable adaptive immune response further accentuating the importance of cellular immunity for protection against brucellosis. Immunity to *Brucella* requires the stimulation of IFN-γ for protection as evidenced by disease exacerbation and impotency of vaccines in IFN-$\gamma^{-/-}$ mice [49-51]. The source of IFN- γ can be $CD4^+$ [51-59], $CD8^+$ T cells [50, 58-60], or both [61]. Both T cell subset responses have been studied after *Brucella* infections in various immunization and challenge paradigms, but their role in *Brucella* infections remains controversial. The relevance of $CD4^+$ or $CD8^+$ T cell subsets depends on

the vaccine and the route of administration. Some studies show that $CD4^+$ T cells as the major source of IFN-γ, and critical in thwarting brucellosis [52-56]. Thought to be less important than $CD4^+$ T cells, $CD8^+$ T cells' role was assumed to be solely cytotoxicity [57]. The notion of $CD8^+$ T cells being dispensable in protection was evident by the exquisite protection against virulent *B. melitensis* challenge retained in $CD8^{-/-}$ mice orally immunized with a purine auxotrophic *B. melitensis* mutant still retained [54]. The importance of $CD4^+$ T cells for protection is evident after low-dose exposure of MHC class $II^{-/-}$ mice to virulent *B. melitensis* and subsequent treatment with antibiotics. At 9 wks post-immunization, MHC class $II^{-/-}$ mice were subjected to virulent challenge. At 50 days post-challenge, the mice had considerably more wild-type brucellae colonization of their spleens with concomitant reduction in IFN- γ -producing cells than did similarly treated wild-type mice [54]. In contrast, MHC class $II^{-/-}$ and $TAP1^{-/-}$ mice nasally infected with virulent *B. melitensis*, and then treated with antibiotics. Both showed protection equivalent to that of similarly immunized and challenged wild-type mice [61] implicating that as long as a source of IFN-g-producing cells is present, either $CD4^+$ or $CD8^+$ T cells can mediate this protection. At the minimum, these findings reveal the significance of $CD4^+$ T cells in providing protection against wild-type *Brucella* and some mutant *Brucella* strains. In contrast, a protective role for $CD8^+$ T cells has been reported as early as 1989, when adoptively transferred $CD4^+$ or $CD8^+$ T cells from S19 *B. abortus*-immunized mice conferred significant protection against virulent *B. abortus* challenge [58]. Immunization with a Δ*norD ΔznuA B. abortus* mutant induced IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, further implicating the type of mutant used to influence T cell responses [59]. This was accentuated by the observation that mice vaccinated with *ΔznuA B. melitensis* mutant were protected in a $CD8^+$ T cell-dependent fashion since mice deficient in CD4, but not CD8, were resistant to virulent *B. melitensis* challenge [50]. Others have suggested that brucellae persistence is due to the lack of memory $CD8^+$ T cells [60], unlike *ΔznuA B. melitensis*-vaccinated mice, which were able to generate memory $CD8^+$ T cells and protected against virulent challenge [50].

2.2. Th17 immunity in brucellosis

Evidence from the limited work on IL-17's role in brucellosis indicates that this cytokine has minimal impact upon protection against systemic brucellosis in immunocompetent animals [49, 50,62]. As described below under **3. Mucosal Vaccinations**, the importance of IL-17 upon mucosal vaccination heightened when IL-17 compensated for the absence of IFN- γ [49,50]. Considered a primary source of IL-17, γδ T cells were found to be important for initial defense against *B. abortus* infections; however, resistance to infection was linked to γδ T cells' production of IFN-γ, not IL-17 [46], and IL-17 did not contribute to the development of osteoarthritis in mucosally or systemically *Brucella* challenged $IFN\text{-}\gamma^{-/-}$ mice [28]. Resistance to virulent nasal challenge with *B. melitensis* was also minimally affected in $IL\text{-}17RA^{-/-}$ and $IL\text{-}23p19^{-/-}$ mice [60].

Another source of Th17 cells resides with those T cells producing IL-22. Examination of IL-22 production revealed that mucosal immunization with *ΔznuA B. melitensis* mutant resulted in stimulation of IL-22 in immunocompetent and $IFN\text{-}\gamma^{-/-}$ mice [49,50]. Prominent IL-17 production was observed with antigen-restimulated $CD8^+$ T cells [50]. The stimulation of IL-22 is associated with protection of the mucosal epithelium by enhancing

the epithelial barrier and increasing defensins production [rev. in 63]. The stimulation of IL-22 may contribute to resistance to mucosal *Brucella* infections, and in particular, may account for the lack of oral infections as IL-22 production was elevated in orally vaccinated and nasally challenged mice [49]. Parenteral challenge of IL-22$^{-/-}$ mice with virulent *B. melitensis* 16M had minimal impact upon resistance to brucellosis [53]. Further studies are needed to determine IL-22's entire contribution to mucosal protection to brucellosis.

2.3. B cell immunity in brucellosis

Anti-*Brucella* polysaccharide antibodies are thought to be less important [53,64] than cell-mediated immunity in protection to brucellosis [8,10,13,65]. Although, passively transferred *anti-Brucellas* antiserum could reduce splenic *B. abortus* S19 colonization [58], as could passive transfer of an O-polysaccharide-specific monoclonal antibody in preventing infection with virulent *B. abortus* [66]. Further inquiry into B cells' role in immunity has found that B cell-deficient (μMT) mice were more resistant to *B. abortus* infection than were wild-type mice [64]. In fact, antibodies facilitated the uptake of GFP-expressing *B. abortus* into wild-type B cells but not in B cell-deficient (Jh$^{-/-}$) mice [67]. Hence, *B. abortus* can infect B cells, resulting in the production of B cell-derived TGF-β1, having anti-inflammatory properties that may exacerbate chronic *Brucella* infections [67].

3. Mucosal Vaccinations

Thus far, we described the importance of eliciting cell-mediated immunity, be it CD4$^+$ or CD8$^+$ T cell-dependent that produces IFN-γ to protect against virulent *Brucella* challenge. *Brucella* infections produce a systemic disease regardless of the route of infection [3-5,7,10]. By convention brucellosis vaccinations are administered parenterally to livestock [65]. As a result, consideration of mucosal administration is often overlooked despite the fact that the majority of livestock and human infections occur mucosally. An interesting caveat is that even with oral exposures with *Brucella* not producing a gut infection [25, 68], brucellae can enter via the cellular prion protein on Peyer's patch microfold cells [69]. While unable to sustain an intestinal infection, oral gavage with live brucellosis vaccines has been successfully used for brucellosis vaccination of laboratory animals [49,70-73], livestock [74-76], red deer [77], and nonhuman primates [73]. In contrast, the naso-oropharyngeal lymph nodes (LNs) that drain the oral cavity and upper respiratory tract are susceptible to *Brucella* infections [78] as a result of animals sniffing or licking *Brucella*-infected aborted fetuses and/or infected placental tissues [79,80]. In humans, past clinical cases of brucellosis involved occurrence of pharyngitis [30-34], which can result in brucellae sequestration in the cervical LNs (CLNs) [34]. Such evidence suggests that the oral cavity and associated LNs and tonsils serve as the first site of mucosal exposure. This is consistent with the notion of oral exposures from consumption of contaminated dairy products, and these findings are recapitulated in experimental brucellosis in sheep [81] and in rhesus macaques [82].

3.1. Oral vaccination strategies for brucellosis

Only a handful of studies has examined the possibility of using mucosal vaccination strategies for protection against brucellosis to understand immune mechanisms of brucellosis resistance. When orally are vaccinated with the *B. abortus* RB51 cattle vaccine, mice show

reduced splenic brucellae colonization by only ~ 2 logs following virulent oral *B. abortus* challenge, and a little protection against parenteral challenge [72]. *B. neotomae*, a pathogen of wood rats and humans [83], was used as a heterologous species to orally vaccinate against *B. abortus*, and was irradiated to prevent its replication, while still maintaining its immunogenicity. Immunized mice were subsequently challenged parenterally with virulent *B. abortus* reducing brucellae colonization of spleen, livers, and lungs [70] to levels similar as others have shown for live attenuated *Brucella* mutants tested in mice. When orally administered, the purine auxotrophic mutant, Δ*purEK B. melitensis* 16M (WR201) elicited potent protection in the lungs and reduced brucellae colonization of systemic tissues after nasal challenge with virulent *B. melitensis* 16M [71]. Such evidence demonstrates that if a suitable mutant is developed, it can be used to confer protection. In this same vein, outstanding protection against nasal challenge with virulent *B. melitensis* 16M was achieved in mice orally vaccinated with the Δ*znuA B. melitensis* mutant [49]. These orally vaccinated mice exhibited almost complete protection to brucellae colonization of systemic tissues. Interestingly, such protection was reduced, but not completely abrogated in IFN-$\gamma^{-/-}$ mice [49]. This remaining resistance in IFN-$\gamma^{-/-}$ mice orally vaccinated with Δ*znuA B. melitensis* was attributed to IL-17 since upon exogenous neutralization of IL-17, protection was abated [49]. IL-17 was less important in wild-type mice since in vivo IL-17 neutralization did not affect protection [49]. IL-17 had a much greater role in the IFN-$\gamma^{-/-}$ mice, given the absence of IFN-γ, and possibly, in compensating for this deficiency, elevations in IL-17-producing T cells were detected following in vitro antigen restimulation [49]. Such increases were greater than those detected for wild-type mice. One negative aspect for both mutants used in these studies was the high dose inoculum, ~10^{11} CFUs, required for protection [49,71], especially when considering vaccination of humans; however, this dose is a similar order of magnitude used for oral vaccination of cattle [75,76].

3.2. Nasal vaccination strategies for brucellosis

A mucosal alternative to oral vaccination is immunizing via the nasal route. To test its effectiveness, nasal vaccination strategies were applied using RB51 or RB51 overexpressing *Brucella*'s superoxide dismutase (RB51-SOD). Neither vaccine proved effective in protecting against nasal challenge with virulent *B. abortus* in contrast to mice nasally vaccinated with the smooth live cattle vaccine, strain 19 (S19) [84]. When combined with TLR agonists as adjuvants, nasal RB51 did improve efficacy [62]. Others found that nasal vaccination is effective against pulmonary challenge with virulent *B. melitensis* 16M [50]. A single, nasal dose of Δ*znuA B. melitensis* conferred potent protection against pulmonary *B. melitensis* challenge as evidenced by >50% of the mice having no detectable brucellae in their lungs or spleens [50]. This immune protection was IFN-g-dependent since IFN-$\gamma^{-/-}$ mice vaccinated with this mutant showed reduced efficacy, and what efficacy remained was abated subsequent in vivo IL-17 neutralization [50] similar to IFN-$\gamma^{-/-}$ mice orally immunized with Δ*znuA B. melitensis* [49]. This protective response was found to be $CD8^+$ T cell-dependent in the lungs since Δ*znuA B. melitensis* nasally vaccinated $CD8^{-/-}$ mice showed no efficacy against pulmonary challenge with virulent *B. melitensis* in contrast to similarly vaccinated wild-type and $CD4^{-/-}$ mice [50]. Protection in the spleen was only partially compromised in Δ*znuA B. melitensis*-vaccinated $CD8^{-/-}$ mice, suggesting $CD4^+$ T cells may compensate for the absence of $CD8^+$ T cells. Likewise, $CD8^{-/-}$ mice nasally

vaccinated with Rev. 1 lost protection in the lungs and spleen to pulmonary challenge [50]. Protection in the lungs was mediated by a pulmonary effector memory $CD8^+$ T cells producing IFN-γ, TNF-α, and/or granzyme B [50]. Hence, both the vaccination strategy, e.g., route, and the vaccine used contribute to the type of T cell response generated for protection. This latter study [50] showed that the type of T cell needed for protection may also vary with immune compartment or tissue as systemic tissues were protected by both $CD4^+$ and $CD8^+$ T cells, whereas $CD8^+$ T cells were required for the lungs. Additional work is needed to devise similar vaccination strategies for livestock.

4. Livestock and Experimental Vaccines

Brucellosis in livestock can result in fetal loss due to abortion, weak offspring, or reduced fertility [13]. This can impact livestock and milk production, with the latter often becoming contaminated with brucellae. The only commercial vaccines available for brucellosis are those for livestock [13,65]. No live or subunit vaccine has been approved for use in humans. Worldwide, three vaccines are currently used: the rough *B. abortus* mutant, RB51, used in cattle; the smooth vaccine, *B. abortus* S19, also used in cattle; and the smooth vaccine, *B. melitensis* Rev. 1, used in goats and sheep. These vaccines are normally given by the s.c. route. The oral *B. suis* strain 2 (S2) is administered via drinking water, and is used only in China [85]. *4.1 B. abortus S19 cattle vaccine*.

Brucellosis in cattle was especially problematic in the US and many other European and South American countries during the 20th century. In the 1930s, as part of the “New Deal” sought to improve livestock industry and improve human health in the US, the State-Federal Cooperative Brucellosis Eradication Program was established to facilitate the elimination of brucellosis from livestock herds [86]. Brucellosis caused disease in livestock handlers inrural communities, and also affected urban areas because of the consumption of contaminated milk [31,86]. This program began with test and slaughter to screen for infected animals, and subsequently incorporated vaccination of seronegative animals to aid reducing the economic losses associated with contaminated milk and decline in beef cattle production [86,87].

In 1923, the cattle S19 vaccine arose initially as a spontaneous attenuated mutant [88], which was later found to be the result of the loss of the erythritol catabolic genes [89]. S19 vaccination of cattle proved highly efficacious against abortions caused by *B. abortus* [90], while having minimal impact upon normal calf births [91]. Importantly, S19 vaccine could reduce infections in animals already infected with *B. abortus* [87]. Although S19 was instrumental in the elimination of brucellosis in the US [87], the vaccine is only ~70% efficacious in cattle [92]. S19 is normally given s.c. to heifer calves, but adults can be vaccinated using a reduced dose administered either s.c. or via the conjunctival route. Pregnant heifers orally vaccinated with S19 showed equivalent efficacy against *Brucella*-induced abortion as heifers vaccinated via the s.c. route [74, 75] demonstrating that S19 can be used as an oral vaccine. S19 has been used in humans in the former Soviet Union, where vaccination with S19 reduced *B. melitensis* infections in livestock workers [93], but no description of side-effects was included in this report. When similar vaccinations were performed in the US with S19 or with the live, attenuated small ruminant vaccine, *B.*

melitensis Rev. 1, both vaccines caused reactogenicity in humans [94,95], abrogating further testing in humans.

4.2 B. abortus RB51 cattle vaccine

The current vaccine used in the US for protection to brucellosis is the rough *B. abortus* RB51, developing by repeated passage of wild-type *B. abortus* 2308 selected on rifampin- and penicillin-containing media [96]. RB51 exhibits a mutation that results in the interruption of the wboA glycosyltransferase, an enzyme important for O-Ag biosynthesis [97]. Bearing a rough phenotype readily enables serological diagnosis of *B. abortus*-infected animals, since cattle vaccinated with RB51 and who are not infected with wild-type *B. abortus*, do not produce antibodies to *Brucella*'s O-Ag (LPS) [98]. This significant accomplishment permits serological herd surveillance. Furthermore, RB51 has a vaccine efficacy similar to that of S19 for preventing *Brucella*-induced abortion and fetal infection [87,99], not compromising the ability to vaccinate against brucellosis. However, variations in RB51's efficacy have been suggested to be age-related. Calves vaccinated at > 5-6 months of age are more resistant to *Brucella*-induced abortion during adult pregnancies than heifer calves vaccinated at 3 months of age [87,100]. In the US and in other countries, RB51 is used instead of S19 vaccine as part of a brucellosis eradication program, and is more effective where the prevalence of brucellosis is low [101]. Oral and s.c. RB51 vaccination also showed equivalent efficacy against *Brucella*-induced abortion and brucellae colonization in pregnant heifers [76].

4.3 B. melitensis Rev. 1 small ruminant vaccine

Brucellosis is also problematic for small ruminants, e.g., goats and sheep, mostly infected by *B. melitensis* [13,65,87]. *B. melitensis* Rev. 1 vaccine strain was originally derived from an avirulent streptomycin-resistant strain. This isolated revertant regained its sensitivity to streptomycin [102], and protects against *Brucella*-induced abortion in goats [103]. Rev. 1 is typically administered to sheep and goats either as a s.c. injection or applied via the conjunctiva [86], and is never given to pregnant animals so as to avoid vaccine-induced abortion [65, 87]. Rev. 1 is a smooth vaccine complicating serological surveillance in differentiating between vaccinated and naturally infected animals [87]. Incidentally, conjunctival administration can confer protection without producing a long-term antibody response [65].

4.4 B. suis strain 2 (S2) swine vaccine

Developed in China in the early 1950s, the *B. suis* strain 2 (S2) vaccine was attenuated as a result of serial passage of a virulent isolate from an aborted *B. suis*-infected fetal pig [85]. S2 vaccination is routinely done in China to curb *B. suis* and other *Brucella* infections in swine, and has significantly reduced brucellosis since its introduction in 1971 [85]. S2 is administered orally via the drinking water [85].

4.5 Subunit vaccines for brucellosis

The discussion thus far has focused on live attenuated vaccines for brucellosis, in large part because of their success in controlling disease and recapitulating aspects of immunity

required for protection [13,65,87]. Generally, subunit vaccines for brucellosis have not been able to achieve immunity to the level obtained with a live vaccine [65, 104]. Ordinarily, protein or DNA vaccines yield one- to two-log reduction in splenic colonization from a level of 5-6 log colonization by virulent *Brucella*. Subunit vaccines clearly have the advantage of avoiding side-effects associated with live vaccines, and readily facilitate serological surveillance in vaccinated livestock. Yet, another advantage is the ability to impact multiple *Brucella* species because their proteins are highly conserved. Unfortunately, no successful subunit vaccine for brucellosis has been developed despite the multiple efforts. Large scale evaluation of different protein candidates in livestock is cost-prohibitive, thus, initial screens have been performed in laboratory animals. In mice, the use of the immunodominant protein, S-adenosyl-L-homocysteine hydrolase, resulted in 2-log reduction in splenic colonization [105]. The combination of two proteins, Omp16 and Omp19 resulted in nearly 2-log protection [106]. Similar success was also achieved using DNA vaccine approaches with the combination of bp26 plus trigger factor [107], IalB [108], omp25 [108], and ribosomal protein L9 [109].

On the other hand, some success was achieved using a heterologous vaccine vector, influenza virus [110]. In this study, 50 pregnant cows (3-4 months of gestation) were subdivided into 5 groups: one group dosed s.c. with PBS in 20% Montanide Gel01 adjuvant (unvaccinated control); one group s.c. vaccinated with S19; one group s.c. vaccinated with RB51; and the last two groups primed-boosted either s.c. or vaccinated initially via the conjunctival route with H5N1-L7/L12 & omp16, and 28 days later boosted with a heterologous H1N1-L7/L12 & omp16. At 5-6 months gestation, pregnant heifers were challenged s.c. with virulent wild-type *B. abortus* 544. S19 and RB51 conferred 89% (8/9) and 70% (7/10) protection against abortion, respectively; and the s.c. and conjunctival flu virus vaccinated heifers showed 90% (9/10) and 80% (8/10) protection against abortion, respectively. Control heifers showed a 70% incidence of abortion. The vaccinates displayed a similar magnitude of protection against both infection and *Brucella*-induced abortion, and all of the control (from the adjuvant-dosed heifer) fetuses and calves were culture-positive for *Brucella* [110]. Hence, alternative vaccine vectors that mimic immunity required for protection to *Brucella* are needed to further subunit vaccine approaches.

5. Conclusion

Brucellosis is commonly viewed as a problem impacting livestock health and economic loss for livestock producers having an economic impact attributed to losses in milk and meat production, and because of its zoonosis, brucellosis also remains a threat to human health. Eradication programs seldom reimburse livestock producers for animal loss. Current vaccines for livestock are only about 70% efficacious. Hence, better vaccines are needed to abrogate brucellosis in livestock, the primary source of infection. Development of enhanced vaccines is dependent on a better understanding of *Brucella*'s pathogenesis, particularly in animals that are naturally susceptible to *Brucella* infections [10]. If a live vaccine is to be developed, it will require a DIVA component to allow the ability to distinguish vaccinated from infected animals during surveillance. Any subunit vaccine must achieve an efficacy better than conventional live vaccines. To date, most protein and DNA vaccines have not achieved the efficacy of conventional brucellosis vaccines [104], likely attributable to the

lack of identifying adjuvants suitable to produce the desired protective immune response. Improved efficacy may be accomplished via a novel vaccine delivery system used as suggested by recent studies that showed higher efficacy using an attenuated influenza-based vector [110]. Certainly, vaccine formulation and route of delivery can influence outcomes as suggested by our studies [49,50,59]. Consideration of alternative methods rather than reliance on parenteral methods for vaccination can lead to vaccination strategies that produce improved efficacy and long-term memory response. Such improvements in protection came about by considering brucellosis as a mucosal disease, rather one that solely produces a systemic disease. Empowering mucosal approaches could harness additional lymphocytes to protect against infection, particularly since most infections occur following a mucosal exposure. Vaccine formulations that license a strong response by both $CD4^+$ and $CD8^+$ T cells, rather than the reliance on a single T cell subset may hold the answer to immune protection.

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