**Vaccination with a $\Delta norD \Delta znuA$ *Brucella abortus* mutant confers potent
protection against virulent challenge**

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Abstract

There remains a need for an improved livestock vaccine for brucellosis since conventional vaccines are only ~70% efficacious, making some vaccinated animals susceptible to *Brucella* infections. To address this void, a vaccine capable of evoking protective immunity while still being sufficiently attenuated to produce minimal disease, is sought. In this pursuit, the $\Delta norD \Delta znuA$ *B. abortus-lacZ* (termed as znBAZ) was developed to be devoid of functional *norD* and *znuA* *B. abortus* genes, and to contain the *lacZ* as a marker gene. The results show that znBAZ is highly attenuated in mouse and human macrophages, and completely cleared from mouse spleens within eight weeks post-vaccination. Producing less splenic inflammation, znBAZ is significantly more protective than the conventional RB51 vaccine by more than four orders of magnitude. Vaccination with znBAZ elicits elevated numbers of IFN- γ^+ , TNF- α^+ , and polyfunctional IFN- γ^+ TNF- α^+ CD4 $^+$ and CD8 $^+$ T cells in contrast to RB51-vaccinated mice, which show reduced numbers of proinflammatory cytokine-producing T cells. These results demonstrate that znBAZ is a highly efficacious vaccine candidate capable of eliciting diverse T cell subsets that confer protection against parenteral challenge with virulent, wild-type *B. abortus*.

Keywords: *Brucella abortus*, attenuation, live vaccine, mouse, T cells

Running title: *Brucella* double mutant vaccine

1. Introduction

Brucellosis is a global zoonotic disease that poses a severe public health challenge as well as a major economic burden [1, 2]. *Brucella* is the causative agent of both animal and human brucellosis [3]. Aside from livestock, natural reservoirs for *Brucella* include various wildlife species perpetuating disease transmission between wildlife and livestock [4, 5]. As long as these reservoirs persist, brucellosis will remain a public health threat. To mitigate livestock disease and reduce human infections, brucellosis vaccination programs are essential. The combination of a livestock vaccination program with culling of infected animals has proven successful to eradicate brucellosis from the majority of states within the US [6, 7]. However, this approach cannot always be adopted by countries unable to provide restitution for the disposed livestock or limited by cultural beliefs [5].

Brucellosis is the most common worldwide zoonotic disease as described by the World Health Organization (WHO) [8], and WHO assists many countries in their efforts to manage this disease. These countries would certainly benefit the most if an efficacious vaccine, aimed in preventing *Brucella* infections in animals, can be developed. Although brucellosis vaccines are commercially available for livestock, a portion of the vaccinated animals still remains susceptible to *Brucella* infections. Hence, the development of an improved vaccine is warranted to protect livestock and minimize zoonotic transmission [2] since no vaccines currently exist for humans.

Brucellosis in humans begins as an acute disease that often goes chronic if left untreated [1, 9-13]. For its acute disease, patients typically present flu-like symptoms including an undulating fever, sweats, headache, back pain, and physical weakness [1, 2]. For some patients, brucellosis becomes chronic [9] presenting symptoms of recurrent fever, joint pain, and fatigue,

that can also become debilitating evidenced by complications such as sacroiliitis, arthritis, spondylitis, osteomyelitis, and bursitis [1, 2, 10]. Today, approximately half a million new human cases occur annually worldwide [1], but since this disease often goes underdiagnosed and underreported, it is believed that the disease incidence is as much as 26-fold higher than reported [11]. The primary mode of exposure in humans is by the oral route following consumption of unpasteurized dairy products. Occupational exposures are especially problematic for shepherds, abattoir workers, veterinarians, dairy-industry professionals, and microbiology laboratorians [12]. While regimens can last up to six weeks using two antibiotics, such treatment is not a guarantee that the *Brucella* infection will be resolved, and relapse rates of ~5-10% in antibiotic-treated patients are observed [13, 14]. This is further evidenced that an improved livestock vaccine is needed to aid in reducing human disease.

To address these needs, we sought to develop an efficacious live, brucellosis vaccine. The current animal vaccines, RB51, S19, and Rev-1 are all infectious to humans [5]. A such new, live vaccine candidate must be safe, highly attenuated, and immunogenic. We have previously shown that deletion of the high-affinity zinc uptake system (*znuA*) gene from *B. abortus* resulted in significant attenuation with similar protective efficacy as RB51 or S19 in preventing splenic brucellae colonization of mice [15]. Initial attempts to improve its effectiveness by introducing a second mutation resulted in the $\Delta znuA \Delta purE$ *B. abortus* mutant. Although this double mutant was rapidly cleared from mice, this strain was overattenuated and unable to confer potent protection [16]. Seeking a strain that would persist longer in the host, the virulence gene, *norD* [17] was deleted. Herein, we describe the $\Delta norD \Delta znuA$ *B. abortus-lacZ* strain exhibiting reduced virulence, but it is highly protective against virulent wt *B. abortus* challenge.

2. Materials and methods

2.1 *Bacteria, medium and plasmid*

Wild-type *B. abortus* strain 2308 and *B. abortus* vaccines, $\Delta znuA$ mutant, S19, and RB51, were inoculated with Difco Potato Infusion Agar (PIA) (DIFCO, Sparks, MD) and cultured with *Brucella* Broth (BB) for liquid growth as previously described [15, 16]. *E. coli* strain S17-1 λ pir was used for cloning and conjugation, as previously described [15].

The suicide plasmid used for *Brucella norD* mutant construction was pUCML, derived from plasmid pCVD442 [15], with the ampicillin marker replaced by kanamycin resistance marker. The plasmid was expanded in the *E. coli* strain S17-1 λ pir in Lysogeny broth (LB) medium containing kanamycin (50 μ g/ml).

2.2 *Construction of $\Delta norD \Delta znuA B. abortus$ -lacZ*

To generate the $\Delta norD \Delta znuA B. abortus$ -lacZ mutant, the $\Delta znuA B. abortus$ strain was further manipulated first by the deletion of *norD* followed by the insertion *lacZ*. The methods used to construct and select for these changes were similar to those used to delete *znuA* from *B. abortus* [15]. Briefly, two pairs of primers amplifying flanking *norD* DNA sequences were designed (**Appendix A1**), which was based on wt *B. abortus* strain 2308. Restriction SacI and XbaI enzyme sites were fused to the 5' ends of primer norD-dn-F and norD-dn-R, respectively, which then used to amplify the downstream DNA sequence (985 bp). Another pair of primers of norD-up-F and norD-up-R was used for amplifying *norD* upstream DNA sequence (1013 bp), with the XbaI and SalI sites integrated to their 5' ends.

The *norD* upstream and downstream DNA fragments were subjected to digestion with SacI/XbaI and SalI/XbaI, respectively, and then they were cloned to pUCML between SacI and

SallI sites. The new plasmid was named pUD_norD. Thus, the inner DNA sequence of *norD* (1176 bp) was removed in-frame. Plasmid pUD_norD contained in *E. coli* S17-1 was used to conjugate with $\Delta znuA$ *B. abortus* 2308. This *norD* mutation was confirmed by PCR using the norD-ckF and norD-ckR primers (**Appendix A1**), and showed a smaller DNA fragment (418 bp) corresponding to the mutant *norD* differing from wt *norD* (1594 bp). Conditions and procedures used for the *norD* interruption were similar to those previously described [15, 16]. Thus, a double mutant strain, $\Delta norD \Delta znuA$ *B. abortus* was obtained.

Since $\Delta norD \Delta znuA$ *B. abortus* strain retains its LPS, a DIVA (differentiating infected from vaccinated animals) gene was sought. The *lacZ* gene was selected since *Brucella* does not naturally possess the *lac* metabolic pathway. *lacZ* was inserted into the BAB1_0048 site since we have observed no negative impact upon *Brucella*'s virulence when reporter genes are inserted here (data not shown). The *lacZ* expression was placed under the control by the pBAD promoter as previously described [22]. This new strain was designated as $\Delta norD \Delta znuA$ *B. abortus-lacZ*, and abbreviated to znBAZ.

To determine whether *lacZ* was expressed, aliquots of znBAZ and wt *B. abortus* 2308 were spotted onto PIA plates containing X-gal in the absence or presence of arabinose (**Appendix A2**). The results showed the *lacZ* gene in znBAZ was constitutively active regardless of the presence of arabinose. Thus, *lacZ* expression readily enables identification of znBAZ from wt *Brucella* strains because wt *Brucella* lacks this activity (**Appendix A2**).

2.3 Characterization and evaluation of znBAZ attenuation in macrophages

RAW264.7 cells (American Type Culture Collection, Manassas, VA) and human macrophages were used to evaluate znBAZ survival in comparison to wt *B. abortus* strain 2308,

RB51 vaccine, and the single $\Delta znuA$ *B. abortus* mutant. Human macrophages were isolated as previously described [18], and approval for use of human peripheral blood was obtained from the University of Florida Institutional Review Board. Infection conditions were identical to those previously described [15, 16]. After overnight culture in complete medium (CM; RPMI 1640, 10% fetal bovine serum [Atlanta Biologicals, GA], 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate), cells were infected with a bacteria-to-macrophage ratio of 30:1 for 1 h at 37°C. Wells were washed twice with CM without antibiotics and then incubated with 50 µg/ml of gentamicin (Life Technologies) for 30 min at 37°C. After washing twice, fresh CM without antibiotics (1.0 ml/well) was added, and cells were incubated for an additional 4, 24, or 48 h.

2.4 Evaluation of znBAZ attenuation and protective efficacy in mouse model

All animal care and procedures were in accordance with institutional policies for animal health and well-being and approved by Montana State University and University of Florida Institutional Animal Care and Use Committee (IACUC). Animal experiments with live wt *Brucella* and mutant strains were conducted in an Animal Biosafety Level III (ABSL-3) facility. Female BALB/c mice (Frederick Cancer Research Facility, National Cancer Institute, MD), 7 to 9 weeks (wks) old, were used throughout the study. All animals were maintained in individually ventilated cages under HEPA-filtered barrier conditions of 12 h of light and 12 h of darkness in the ABSL-3 facility and provided with food and water ad libitum.

All *Brucella* strains were grown overnight in BB at 37°C. RB51 and znBAZ cells were pelleted, washed twice in sterile phosphate-buffered saline (PBS), and diluted to 1×10^8 cells/200 µl in sterile PBS. The actual viable inoculum was confirmed by serial dilution tests on PIA, and

200 µl of this suspension was administered to mice via intraperitoneal (i.p.) injection. In some experiments, a second dose of znBAZ was given 4 wks post-primary immunization. All mice were allowed at least 8 wks to clear the vaccine prior to challenge. The challenge strain, *B. abortus* 2308, was diluted in sterile PBS in which 200 µl of bacterial suspension contained 5×10^4 CFUs, and the immunized and PBS-dosed control mice were subsequently challenged i.p. with 200 µl brucellae/mouse. The challenge dose was also confirmed by plating bacterial dilutions on PIA.

To measure the extent of *in vivo* tissue colonization, 1×10^8 CFUs znBAZ in 200 µl sterile PBS were given i.p. to BALB/c mice, and a separate group of mice was infected i.p. 5×10^4 CFUs of wt *B. abortus* 2308 for comparison. Splenic weights and CFU counts were assessed at 2, 4, 8, and 12 wks post-infection. Individual spleens were removed and mechanically homogenized in 3 ml of sterile Milli-Q water. Diluted samples of splenic homogenates were incubated for 3-5 days at 37°C in 5% CO₂ on PIA, bacterial colonies were enumerated, and CFUs per spleen were calculated [15].

2.5 Flow cytometry and cytokine analysis

Intracellular IFN-γ and TNF-α expression levels by immune lymphocytes were measured by flow cytometry similar to that previously described [19]. Groups of mice i.p. vaccinated with znBAZ, RB51, or PBS were evaluated 21 days after vaccination. For intracellular IFN-γ detection, splenic lymphocytes from individual mice were stimulated *in vitro* with 10^9 CFUs of heat-killed RB51 (HKRB51) overnight, followed by 5 ng/ml PMA and 500 ng/ml ionomycin and simultaneously treated with 10 µg/ml brefeldin A (BioVision, Milpitas, CA) during the last 5 h of culture. Cells were then stained with PE-Cy-5 anti-CD4 (BDBiosciences) and Pacific Orange

anti-CD8 T cell (Life Technologies) mAbs, washed, and then fixed with IC Fixation Buffer (eBioscience). Subsequently, cells were permeabilized with Permeabilization Buffer (eBioscience), and stained with FITC-labeled anti-IFN- γ (eBioscience) and Brilliant Violet 421™-labeled TNF- α (Biolegend). Fluorescence was acquired on LSRFortessa flow cytometer with BD FACSDiva software (BD Biosciences, San Jose, CA, USA). All samples were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.6 Serum IgG anti- β -galactosidase (β gal) antibody levels.

To generate anti- β gal Abs, five mice were i.m. vaccinated with 100 μ g pBudCE4.1 vector (Invitrogen Corp.) bearing the molecular adjuvant, lymphotactin (LTN) on days 0, 7, and 14 [20]. The plasmid DNA was injected into the tibialis anterior muscles of the two hind legs, as previously described [20]. Two wks after the last boost, mice were bled and collected individual serum was analyzed for induced anti- β gal Abs by standard ELISA methods [21].

2.7 Statistical analysis

The statistical differences were calculated by Tukey-Kramer Multiple Comparisons Test to evaluate differences among variations of *in vitro* infection and *in vivo* splenic weight, as well as colonization by znBAZ, Δ znuA 2308, S19, RB51, or wt 2308 at the 95% confidence interval.

3. Results

3.1. *znBAZ* is attenuated in both mouse RAW264.7 and primary human macrophages

To evaluate the extent of *znBAZ*'s attenuation, a virulence assay was performed using murine RAW264.7 macrophages. RAW264.7 cells were infected at a 30:1 ratio of bacteria to macrophages with *znBAZ*, $\Delta znuA$ *B. abortus*, RB51, or wt *B. abortus* 2308 to measure their ability to replicate (**Fig. 1A**). Infected macrophages were harvested at different time points (4, 24, and 48 h) post-infection by water lysis to measure the extent of brucellae colonization on PIA. The results showed that both *znBAZ* and $\Delta znuA$ *B. abortus* did not replicate in RAW264.7 macrophages in sharp contrast to the rapid increase of wt *B. abortus* 2308 and RB51 vaccine. Of note, *znBAZ* was significantly more attenuated than $\Delta znuA$ *B. abortus* (**Fig. 1A**), suggesting that introduction of the second mutation further debilitates the brucellae. Thus, these results show that *znBAZ* is highly attenuated, and similar to its parental $\Delta znuA$ *B. abortus* strain, it is unable to replicate within RAW264.7 macrophages.

A similar analysis was performed using primary human macrophages to measure *znBAZ*'s ability to replicate. As with RAW264.7 murine macrophages, primary human macrophages were infected at 30:1 *znBAZ* to macrophage ratio, and the extent of brucellae replication was measured at 24 and 48 h post-infection compared to RB51 vaccine and wt *B. abortus* 2308. Strain *znBAZ* was significantly attenuated relative to RB51 and wt *B. abortus* 2308 (**Fig. 1B**).

3.2. *znBAZ* is avirulent in the mouse

To measure *znBAZ*'s virulence *in vivo*, BALB/c mice were infected i.p. with 1×10^8 CFUs of *znBAZ* or RB51. Since *znBAZ* is a smooth strain, we also included comparisons to

mice dosed i.p. with 1×10^7 CFUs of *B. abortus* S19 vaccine. The mice were sampled for extent of brucellae colonization at 1, 2, 4, 6, and 8 wks post-infection (**Fig. 2**). Strain znBAZ exhibited a clearance rate similar to mice infected with RB51, and no brucellae were detectable in the spleen by 8 wks post-infection. In contrast, mice infected with S19 showed a modest decline 4 wks post-infection, but remained infected for at least 8 wks with still more than 1×10^5 CFUs/spleen. **These results show that znBAZ is attenuated in mice, similar to RB51.**

3.3. znBAZ protects against wt B. abortus challenge and induces anti- β gal antibodies

Since it was unclear the degree to which znBAZ's virulence was reduced, one group of mice was vaccinated i.p. twice with with 10^8 CFUs znBAZ, once on day 0 and second 4 wks later. Likewise, one group of mice was dosed twice with sterile PBS. Simultaneous with the znBAZ group receiving its boost, separate groups of mice were vaccinated i.p once with znBAZ or RB51. Eight wks post-primary or post-secondary immunization, all mice were challenged i.p. with 5×10^4 CFUs of virulent *B. abortus* 2308. Four wks post-challenge, individual spleens were assessed for bacterial burden (**Fig. 3A**). Although both znBAZ and RB51 showed significantly reduced splenic colonization ($P < 0.005$), a single dose of znBAZ was more effective in reducing brucellae colonization by 132-fold, while RB51 only reduced colonization by 6.8-fold. When two doses were administered, znBAZ was even more effective conferring 15,600-fold reduction in splenic colonization (**Fig. 3A**).

Another measure of protection is determining the extent of splenic inflammation following challenge. Both RB51- and znBAZ-vaccinated mice showed significantly reduced splenic weights when compared to PBS-dosed mice ($P < 0.001$; **Fig. 3B**). Furthermore, znBAZ was more effective than RB51 since znBAZ-vaccinated mice showed significantly reduced

splenic weights when compared to RB51-vaccinated mice ($P = 0.003$), regardless of the number of znBAZ doses used (**Fig. 3B**). These collective findings show that znBAZ is a potent brucellosis vaccine candidate.

Since the znBAZ encodes β gal as a DIVA, mice twice vaccinated with znBAZ were individually bled and assessed for serum IgG anti- β gal endpoint titers. As a positive control for serum IgG anti- β gal responses, a separate group of mice was vaccinated by the i.m. route using a eukaryotic DNA expression plasmid as previously described [20]. znBAZ induced serum IgG anti- β gal antibodies at levels similar to those induced with the *lacZ*-encoded DNA vaccine, and both were significantly greater than levels detected in the RB51-immunized mice (**Fig. 3C**). Thus, the β gal expressed by znBAZ is immunogenic and can potentially aid in distinguishing znBAZ-vaccinated animals from animals naturally infected with *B. abortus*.

3.4 Strain znBAZ significantly enhances the number of IFN- γ - and TNF- α -producing CD4⁺ and CD8⁺ T cells

To ascertain the T cells and cytokines induced following znBAZ vaccination, groups of mice were i.p. dosed with PBS or vaccinated once with RB51 or znBAZ, and splenic lymphocytes were assessed for IFN- γ and TNF- α production by flow cytometry. The znBAZ-vaccinated mice showed significantly elevated numbers of IFN- γ ⁺, TNF- α ⁺, and polyfunctional IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells when compared to naive or RB51-vaccinated mice (**Fig. 4A-D**). Likewise, znBAZ-vaccinated mice showed significantly elevated numbers of IFN- γ ⁺, TNF- α ⁺, and polyfunctional IFN- γ ⁺ TNF- α ⁺ CD8⁺ T cells when compared to naive or RB51-vaccinated mice (**Fig. 5A-D**). Hence, these results show that znBAZ vaccination is more apt to stimulate

both CD4⁺ and CD8⁺ T cells than RB51-vaccinated mice. In fact, the potency of this vaccine becomes evident by the 4.4-, 5.1-, and 6.8-fold increase in the numbers of IFN- γ ⁺, TNF- α ⁺, and IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells, respectively, when compared to RB51-vaccinated mice (**Fig. 4B-D**). The same was true regarding vaccine potency evident by the 5.8-, 3.9-, and 10.8-fold increase in the numbers of IFN- γ ⁺, TNF- α ⁺, and IFN- γ ⁺ TNF- α ⁺ CD8⁺ T cells, respectively, by znBAZ-vaccinated mice compared to RB51-vaccinated mice (**Fig. 5B-D**).

4. Discussion

Livestock brucellosis still remains problematic for many countries [rev. in 23]. Despite their availability, conventional brucellosis vaccines are only approximately 70% efficacious [24, 25]. Although the smooth vaccine, *B. abortus* S19, has been replaced with the rough RB51 vaccine in the US [26], S19 is still used in many countries including India [27] and Argentina [28]. S19 tends to be more pathogenic and can cause abortion in 3.2% of pregnant cows following subcutaneous vaccination [29] or 100% following i.v. injection [30]. In contrast, RB51 showed reduced abortion when given i.v. [31]. In addition to its efficacy, a significant advantage of RB51 is that it does not seroconvert vaccinated animals as does S19 [23]. Hence, any new, smooth vaccine would require having a DIVA to distinguish vaccinated from naturally infected animals.

To develop effective and safe vaccines, significant efforts have focused on targeting the deletion of various *Brucella* virulence genes [rev. in 5]. Although these mutants can show retarded growth in macrophages, many vary in their ability to confer protection [5]. While single mutants can be highly effective, concerns of reversion or recombination with wt *Brucella* often dampen enthusiasm for their application. Thus, the introduction of a second mutation is done to negate these concerns as done here for znBAZ. This approach has been shown to be highly effective in reducing the virulence of the other pathogens, particularly *Salmonella* [32].

The introduction of two mutations into *B. abortus* was previously tested with the double mutant strain, $\Delta znuA \Delta purE$ *B. abortus* [16]. The *znuA* gene encodes a high affinity-binding protein that is a key cofactor at acquiring Zn^{2+} from phagosomes [33], which is required for the activity of Cu, Zn superoxide dismutase enabling *Brucella* to resist oxidative stress [34]. Zinc is also a co-factor for other enzymes. Hence, zinc homeostasis plays an essential role in *Brucella*

pathogenesis [35] as evidenced by the reduced virulence observed for *znuA* *Brucella* mutants [15, 36]. The *purE* encodes an enzyme required for purine biosynthesis pathway, and this auxotrophic mutant is significantly attenuated requiring purine supplementation for growth [37, 38]. Together, these two mutations in *B. abortus* failed to confer improved efficacy against wt challenge [16], although the $\Delta znuA \Delta purE$ *B. abortus* was cleared from the host with similar kinetics to the *znuA* single mutant [15]. It became evident that the introduction of *purE* mutation overattenuated the $\Delta znuA$ *B. abortus* reducing this strain's efficacy.

To circumvent this issue of overattenuation, in this study, we tested a second candidate virulence gene, *norD*, in combination with the *znuA* mutation. As part of the *norEFCBQD* operon, *norD* encodes for a nitric oxide reductase, and when mutated, *Brucella* cannot survive under anaerobic denitrifying conditions [17]. Since this gene only partially reduces wt *B. abortus* virulence, we hypothesized that *norD* mutant may be suitable when paired with the *znuA* mutation leading to the creation of the $\Delta norD \Delta znuA$ *B. abortus* double mutant. Since this new double mutant strain retains its LPS and cannot be distinguished from animals previously exposed to wt *Brucella*, the DIVA gene, *lacZ*, was introduced to facilitate monitoring of induced anti- β gal antibody responses.

Upon examination of its vaccine properties, the znBAZ mutant showed reduced virulence *in vitro* when compared to its parental $\Delta znuA$ *B. abortus* strain, RB51 vaccine, and wt *B. abortus* 2308. The *in vivo* studies revealed that znBAZ's virulence was similar to RB51, but significantly less than S19. Importantly, znBAZ was cleared from the spleen within 8 wks. These traits pointed to znBAZ being a safe vaccine. Evaluation of its protective efficacy showed that znBAZ performed much better than vaccine RB51. A single dose of znBAZ strain produced

132-fold reduction in wt brucellae colonization, nearly 20-fold more protective than RB51. The $\Delta znuA \Delta purE$ *B. abortus* strain required two doses, and even that provided minimal protection (0.79 log units) unlike the single $\Delta znuA$ *B. abortus* provided 1.6 - 1.8 log units of protection [16]. znBAZ's efficacy was notably augmented when animals were vaccinated twice providing more than 100-fold improvement in vaccine efficacy or nearly 16,000-fold reduction in brucellae colonization, and certainly more effective than the $\Delta znuA$ *B. abortus* strain [15, 16]. Collectively, the data demonstrate that the paired *znuA* and *norD* deletions act synergistically to produce a strain that proves to be effective against virulent, wt *B. abortus* challenge.

To investigate how this protection was mediated, additional analysis was performed to measure the types of proinflammatory cytokine responses induced. Vaccination with znBAZ stimulated both TNF- α and IFN- γ noted by the elevations in cytokine-producing CD4⁺ and CD8⁺ T cells. Past studies have shown the importance of both TNF- α and IFN- γ for protection to *B. abortus* [39-41]. In addition to potently inducing TNF- α and IFN- γ , the znBAZ induced greater numbers of IFN- γ ⁺, TNF- α ⁺, and polyfunctional IFN- γ ⁺ / TNF- α ⁺ CD4⁺ and CD8⁺ T cells than those stimulated by RB51 vaccination. Although the numbers of cytokine-producing CD4⁺ T cells exceeded CD8⁺ T cells in znBAZ-vaccinated mice, about one-third of the total IFN- γ -producing TCR β ⁺ cells were CD8⁺ T cells. It has been presumed that CD4⁺ T cells are the major source for IFN- γ and TNF- α [42,43], and only a handful of studies has implicated the importance of CD8⁺ T cells [40,44]. The route of vaccination may be a contributing factor in CD8 T cell elevations [19, 45, 46]. Alternatively, attributes of the mutant strain used for vaccination may influence the T cell bias as evidenced here by the enhanced vaccine potency, and the contributions of both CD4⁺ and CD8⁺ T cells in znBAZ-vaccinated mice compared to those

induced T cell responses by RB51-vaccinated mice. Interestingly, it was also observed that polyfunctional CD4⁺ and CD8⁺ T cells producing IFN- γ and TNF- α were induced by znBAZ to a much greater extent than RB51. Similar polyfunctional T cells were found to be induced by various infectious agents, and these are believed to be more responsive to infections [47-50]. Hence, znBAZ induces a more potent and diverse T cell response than RB51 which may account for the improved vaccine efficacy by znBAZ in conferring protection against virulent, wt *B. abortus* challenge. This level of efficacy coupled with its low residual virulence, makes this vaccine candidate worthy of assessment in brucellosis sensitive livestock.

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Figure legends

Figure 1. $\Delta norD \Delta znuA$ *B. abortus-lacZ* cannot replicate in mouse and human macrophages.

(A) Wild-type *B. abortus* strain 2308, RB51 vaccine, $\Delta znuA$ *B. abortus*, and $\Delta norD \Delta znuA$ *B. abortus-lacZ* were used to infect murine RAW264.7 macrophages at a bacteria-to-macrophage ratio of 30:1. After 1 h of incubation followed by 30 min of treatment with gentamicin, infected macrophages were incubated in fresh medium for 0, 4, 24, or 48 h. Infected macrophages were water lysed, and supernatants were diluted for CFU enumeration. Statistical differences between mutant or vaccine strains and wt *B. abortus* strain were calculated using Student's *t*-test, and the significant differences are indicated: $*P < 0.05$ and $**P < 0.01$, for $\Delta norD \Delta znuA$ *B. abortus-lacZ*, $\Delta znuA$ *B. abortus*, or RB51 versus wt *B. abortus* 2308; and $^{\dagger}P < 0.05$, for $\Delta norD \Delta znuA$ *B. abortus-lacZ* and $\Delta znuA$ *B. abortus* versus RB51. In a similar fashion, (B) wild-type *B. abortus* 2308, RB51 vaccine, and $\Delta norD \Delta znuA$ *B. abortus-lacZ* were used to infect human macrophages at a bacteria-to-macrophage ratio of 30:1, and the bacterial CFUs were assessed as describe in (A). $\Delta norD \Delta znuA$ *B. abortus-lacZ* was unable to achieve the level of colonization achieved by the wt *B. abortus* 2308 or RB51 vaccine. Values are the means of two independent experiments $\pm$ standard errors of the means. Differences in brucellae replication by $\Delta norD \Delta znuA$ *B. abortus-lacZ* versus wt *B. abortus* 2308 ($*P < 0.05$) or RB51 ($^{\dagger}P < 0.05$) are indicated.

Figure 2. Double mutant $\Delta norD \Delta znuA$ *B. abortus-lacZ* is completely cleared by 8 weeks after infection. BALB/c mice (5 mice/group/time point) were i.p. dosed with 1×10^8 CFUs of $\Delta norD \Delta znuA$ *B. abortus-lacZ* or RB51, or 1×10^7 CFUs of S19. At weeks 1, 2, 4, 6, and 8 post-

administration, individual spleens were assessed for colonization. Depicted is the mean of each group per time point $\pm$ SEM. Differences in colonization of $\Delta norD \Delta znuA B. abortus-lacZ$ ($***P \leq 0.001$) or RB51 ($^{###}P < 0.001$) versus wt *B. abortus* 2308 were determined.

Fig. 3. $\Delta norD \Delta znuA B. abortus-lacZ$ is highly protective against parenteral challenge and vaccinated mice produce anti- β gal Abs. BALB/c mice were i.p. immunized with 1×10^8 CFUs $\Delta norD \Delta znuA B. abortus-lacZ$ (10/group), RB51 (5/group), or sterile PBS (10/group). One group (n=5) of $\Delta norD \Delta znuA B. abortus-lacZ$ mice was given a second i.p. dose (1×10^8 CFUs) of vaccine and one group of PBS-treated mice (n=5), a second PBS dose 4 wks post-primary administration. Each group was rested 8 wks after primary or booster vaccination, and then challenged i.p. with 5×10^4 CFUs wt *B. abortus* 2308. Four wks post-challenge, individual spleens were assessed for **(A)** extent of colonization (CFUs) and **(B)** splenic weights. Two doses of $\Delta norD \Delta znuA B. abortus-lacZ$ vaccine conferred the greatest protection against wt *B. abortus* challenge. Values are the means of individual mice $\pm$ SEM; for colonization, $*P < 0.001$, $**P = 0.005$ vs PBS-dosed mice, and $^{\dagger\dagger}P \leq 0.008$ vs RB51-vaccinated mice; and for splenic weights, $*P < 0.001$ vs PBS-dosed mice, and $^{\dagger}P = 0.003$ vs RB51-vaccinated mice. **(C)** Sera were collected from individual mice three weeks after the second dose of $\Delta norD \Delta znuA B. abortus-lacZ$ or 7 wks after RB51 vaccination, and evaluated for IgG anti- β gal endpoint titers (Log_2) by standard ELISA methods. As a positive control, mice were i.m. immunized three times with a DNA vaccine encoding *lacZ* gene and lymphotactin, and bled two weeks after the third dose; $*P = 0.011$ versus RB51-vaccinated mice.

Fig. 4. Intraperitoneal vaccination with $\Delta norD \Delta znuA B. abortus-lacZ$ stimulates the production of IFN- γ - and TNF- α -producing CD4⁺ T cells. BALB/c mice (6/group) were i.p. immunized with 1×10^8 CFUs $\Delta norD \Delta znuA B. abortus-lacZ$, RB51, or sterile PBS, and 21 days later, (A) CD4⁺ T cells were evaluated for intracellular IFN- γ and TNF- α production by flow cytometry. $\Delta norD \Delta znuA B. abortus-lacZ$ vaccination augmented the total number of (B) IFN- γ ⁺, (C) TNF- α , and (D) polyfunctional IFN- γ ⁺ TNF- α ⁺ CD4⁺ T cells; * $P = 0.002$, ** $P \leq 0.009$ versus $\Delta norD \Delta znuA B. abortus-lacZ$ -vaccinated mice.

Fig. 5. Intraperitoneal vaccination with $\Delta norD \Delta znuA B. abortus-lacZ$ stimulates the production of IFN- γ - and TNF- α -producing CD8⁺ T cells. Lymphocytes from BALB/c mice i.p. immunized as described in Fig. 4 were also evaluated for (A) CD8⁺ T cell IFN- γ and TNF- α production by flow cytometry. $\Delta norD \Delta znuA B. abortus-lacZ$ vaccination augmented the total number of (B) IFN- γ ⁺, (C) TNF- α , and (D) polyfunctional IFN- γ ⁺ TNF- α ⁺ CD8⁺ T cells; * $P \leq 0.003$, ** $P = 0.009$, *** $P = 0.017$ versus $\Delta norD \Delta znuA B. abortus-lacZ$ -vaccinated mice.

REFERENCES

- [1] Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. *Lancet Infect Dis* 2006; 6:91–99.
- [2] Roth F, Zinsstag J, Orkhon D, Chimed-Ochir G, Hutton G, Cosivi O, Carrin G, Otte J. Human health benefits from livestock vaccination for brucellosis: case study. *Bull World Health Organ* 2003; 81: 867–876.
- [3] Yang X. Brucellosis. *Conn's Current Therapy 2013* Edited by Edward T Bope, Rick D Kellerman, and Robert E Rakel. 2013:Page 77-81.
- [4] Olsen SC. Brucellosis in the United States: role and significance of wildlife reservoirs. *Vaccine*. 2010;28 Suppl 5:F73-6.
- [5] Yang X, Skyberg JA, Cao L, Clapp B, Thornburg T, Pascual DW. Progress in *Brucella* vaccine development. *Front Biol*. 2013;8:60-77.
- [6] Ebel ED, Williams MS, Tomlinson SM. Estimating herd prevalence of bovine brucellosis in 46 USA states using slaughter surveillance. *Prev Vet Med*. 2008;85:295-316.
- [7] Treanor JJ, Johnson JS, Wallen RL, Cilles S, Crowley PH, Cox JJ, et al. Vaccination strategies for managing brucellosis in Yellowstone bison. *Vaccine*. 2010;28 Suppl 5:F64-72.
- [8] WHO. Zoonoses and veterinary public health. Neglected zoonotic diseases - brucellosis. 2012; <http://www.who.int/zoonoses/diseases/brucellosis/en/index.html>.
- [9] Castano MJ, Solera J. Chronic brucellosis and persistence of *Brucella melitensis* DNA. *J Clin Microbiol*. 2009;47:2084-9.
- [10] Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. *Lancet Infect Dis*. 2007;7:775-86.
- [11] Dean AS, Crump L, Greter H, Schelling E, Zinsstag J. Global burden of human brucellosis: a systematic review of disease frequency. *PLoS Negl Trop Dis* 2012; 6:e1865.
- [12] Atluri VL, Xavier MN, de Jong MF, den Hartigh AB, Tsolis RE. Interactions of the human pathogenic *Brucella* species with their hosts. *Annu Rev Microbiol*. 2011;65:523-41.
- [13] Hall WH. Modern chemotherapy for brucellosis in humans. *Rev Infect Dis*. 1990;12:1060-99.

- [14] Memish Z, Mah MW, Al Mahmoud S, Al Shaalan M, Khan MY. *Brucella* bacteraemia: clinical and laboratory observations in 160 patients. J Infect. 2000;40:59-63.
- [15] Yang X, Becker T, Walters N, Pascual DW. Deletion of *znuA* virulence factor attenuates *Brucella abortus* and confers protection against wild-type challenge. Infect Immun. 2006;74:3874-9.
- [16] Yang X, Thornburg T, Walters N, Pascual DW. $\Delta znuA\Delta purE$ *Brucella abortus* 2308 mutant as a live vaccine candidate. Vaccine. 2010;28:1069-74.
- [17] Loisel-Meyer S, Jimenez de Bagues MP, Basseres E, Dornand J, Kohler S, Liautard JP, et al. Requirement of *norD* for *Brucella suis* virulence in a murine model of *in vitro* and *in vivo* infection. Infect Immun. 2006;74:1973-6.
- [18] Skyberg, JA, Rollins MCF, Holderness JS, Marlenee NL, Schepetkin IA, Goodyear A, Dow SW, Jutila MA, Pascual DW. Nasal Acai polysaccharides potentiate innate immunity to protect against pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* infections. PLoS-Pathogens 2012; 8:e1002587.
- [19] Clapp B, Yang X, Thornburg T, Walters N, Pascual DW. Nasal vaccination stimulates CD8⁺ T cells for potent protection against mucosal *Brucella melitensis* challenge. Immunol Cell Biol. 2016; 94:496-508.
- [20] Yamanaka H, Hoyt T, Yang X, Bowen R, Golden S, Crist K, Becker T, Maddaloni M, Pascual DW. A parenteral DNA vaccine protects against pneumonic plague. Vaccine 2010; 28:3219-3230.
- [21] van Ginkel F W, Liu C-G, Simecka J, Dong J-Y, Greenway T, Frizzell RA, Kiyono H, McGhee JR, Pascual DW. Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and β -galactosidase. Hum Gene Therapy 1995; 6:895-903.
- [22] Curtiss R 3rd, Wanda SY, Gunn BM, Zhang X, Tinge SA, Ananthnarayan V, Mo H, Wang S, Kong W. *Salmonella enterica* serovar Typhimurium strains with regulated delayed attenuation in vivo. Infect Immun. 2009;77:1071-1082.

- [23] Goodwin ZI, Pascual DW. Brucellosis vaccines for livestock. *Vet Immunol Immunopath.* 2016; (In press).
- [24] Lubroth J, Rweyemamu MM, Viljoen G, Diallo A, Dungu B, Amanfu W. Veterinary vaccines and their use in developing countries. *Rev Sci Tech Off Int Epiz.* 2007; 26:179–201.
- [25] Olsen SC. Immune responses and efficacy after administration of a commercial *Brucella abortus* strain RB51 vaccine to cattle. *Vet Ther.* 2000; 1:183-191.
- [26] Lord VR, Schurig GG, Cherwonogrodzky JW, Marcano MJ, Melendez GE. Field study of vaccination of cattle with *Brucella abortus* strains RB51 and 19 under high and low disease prevalence. *Am J Vet Res.* 1998;59:1016-20.
- [27] Chand P, Chhabra R, Nagra J. Vaccination of adult animals with a reduced dose of *Brucella abortus* S19 vaccine to control brucellosis on dairy farms in endemic areas of India. *Trop Anim Health Prod* 2015; 47:29-35.
- [28] Aznar MN, Samartino LE, Humblet MF, Saegerman C. Bovine brucellosis in Argentina and bordering countries: update. *Transbound Emerg Dis* 2014; 61:121-33.
- [29] Beckett FW, MacDiarmid SC. The effect of reduced-dose *Brucella abortus* strain 19 vaccination in accredited dairy herds. *Br Vet J* 1985;141:507-14.
- [30] Taylor AW, McDiarmid A. The stability of the avirulent characters of *Brucella abortus*, strain 19 and strain 45/20 in lactating and pregnant cows. *Vet Rec.* 1949; 61:317–318.
- [31] Palmer MV, Cheville NF, Jensen AE.. Experimental infection of pregnant cattle with the vaccine candidate *Brucella abortus* strain RB51: pathologic, bacteriologic, and serologic findings. *Vet Pathol.* 1996; 33:682–691.
- [32] Laniewski P, Mitra A, Karaca K, Khan A, Prasad R, Curtiss R, 3rd, et al. Evaluation of protective efficacy of live attenuated *Salmonella enterica* serovar Gallinarum vaccine strains against fowl typhoid in chickens. *Clin Vaccine Immunol.* 2014;21:1267-76.
- [33] Patzer SI, Hantke K. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol Microbiol.* 1998;28:1199-210.
- [34] Gee JM, Valderas MW, Kovach ME, Grippe VK, Robertson GT, Ng WL, et al. The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative

killing by murine macrophages and wild-type virulence in experimentally infected mice. *Infect Immun.* 2005;73:2873-80.

[35] Sheehan LM, Budnick JA, Roop RM, 2nd, Caswell CC. Coordinated zinc homeostasis is essential for the wild-type virulence of *Brucella abortus*. *J Bacteriol.* 2015;197:1582-91.

[36] Kim S, Watanabe K, Shirahata T, Watarai M. Zinc uptake system (*znuA* locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. *J Vet Med Sci.* 2004;66:1059-63.

[37] Drazek ES, Houngh HS, Crawford RM, Hadfield TL, Hoover DL, Warren RL. Deletion of *purE* attenuates *Brucella melitensis* 16M for growth in human monocyte-derived macrophages. *Infect Immun.* 1995;63:3297-301.

[38] Alcantara RB, Read RD, Valderas MW, Brown TD, Roop RM, 2nd. Intact purine biosynthesis pathways are required for wild-type virulence of *Brucella abortus* 2308 in the BALB/c mouse model. *Infect Immun.* 2004;72:4911-7.

[39] Zhan Y, Cheers C. Control of IL-12 and IFN- γ in response to live or dead bacteria by TNF and other factors. *J Immunol.* 1998; 161:1447–1453.

[40] Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon- γ is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. *Immunology* 2001; 103:511-518.

[41] Macedo GC, Magnani DM, Carvalho NB, Bruna-Romero O, Gazzinelli RT, Oliveira SC. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. *J Immunol.* 2008; 180:1080-1087.

[42] Vitry MA, De Trez C, Goriely S, Dumoutier L, Akira S, Ryffel B, Carlier Y, Letesson JJ, Muraille E. Crucial role of gamma interferon-producing CD4⁺ Th1 cells but dispensable function of CD8⁺ T cell, B cell, Th2, and Th17 responses in the control of *Brucella melitensis* infection in mice. *Infect Immun.* 2012; 80:4271-4280.

[43] Yingst SL, Izadjoo M, Hoover DL. CD8 knockout mice are protected from challenge by vaccination with WR201, a live attenuated mutant of *Brucella melitensis*. *Clin Dev Immunol.* 2013; 2013:686919.

- [44] Durward-Diioia M, Harms J, Khan M, Hall C, Smith JA, Splitter GA. CD8⁺ T cell exhaustion, suppressed gamma interferon production, and delayed memory response induced by chronic *Brucella melitensis* infection. *Infect Immun*. 2015; 83:4759-4771.
- [45] Clapp B, Skyberg JA, Yang XH, Thornburg T, Walters N, Pascual DW. Protective live oral brucellosis vaccines stimulate Th1 and Th17 cell responses. *Infect Immun*. 2011;79:4165-74.
- [46] Dabral N, Martha-Moreno-Lafont, Sriranganathan N, Vemulapalli R. Oral immunization of mice with gamma-irradiated *Brucella neotomae* induces protection against intraperitoneal and intranasal challenge with virulent *B. abortus* 2308. *PLoS One* 2014; 9:e107180.
- [47] Masopust D, Ha SJ, Vezys V, Ahmed R. Stimulation history dictates memory CD8 T cell phenotype: implications for prime-boost vaccination. *J Immunol*. 2006; 177:831-839.
- [48] Gómez CE, Perdiguero B, Cepeda MV, Mingorance L, García-Arriaza J, Vandermeeren A, Sorzano CÓ, Esteban M. High, broad, polyfunctional, and durable T cell immune responses induced in mice by a novel hepatitis C virus (HCV) vaccine candidate (MVA-HCV) based on modified vaccinia virus Ankara expressing the nearly full-length HCV genome. *J Virol*. 2013; 87:7282-7300.
- [49] Dietrich J, Roy S, Rosenkrands I, Lindenstrøm T, Filskov J, Rasmussen EM, Cassidy J, Andersen P. Differential influence of nutrient-starved *Mycobacterium tuberculosis* on adaptive immunity results in progressive tuberculosis disease and pathology. *Infect Immun*. 2015; 83:4731-4739.
- [50] Mou Z, Li J, Boussoffara T, Kishi H, Hamana H, Ezzati P, Hu C, Yi W, Liu D, Khadem F, Okwor I, Jia P, Shitaoka K, Wang S, Ndao M, Petersen C, Chen J, Rafati S, Louzir H, Muraguchi A, Wilkins JA, Uzonna JE. Identification of broadly conserved cross-species protective *Leishmania* antigen and its responding CD4⁺ T cells. *Sci Transl Med*. 2015; 7:310ra167.

Fig. 1

CFUs/ 10^6 RAW264.7 Macrophages

CFUs/ 10^5 Human Macrophages

Fig. 2

Fig. 3

Fig. 4

Fig. 5