Unraveling the host innate immune response to a respiratory model of *Brucella abortus*

Naveen Surendran

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirement of the degree of

Doctor of Philosophy
in
Biomedical & Veterinary Sciences

Sharon G. Witonsky
Nammalwar Sriranganathan
Stephen M. Boyle
Kurt L. Zimmerman
Elizabeth (Hiltbold) Schwartz

May 24, 2010

Blacksburg, Virginia

Keywords: *Brucella abortus*, innate immunity, dendritic cells, vaccine strains RB51 and RB51SOD, toll-like receptors, intranasal vaccination

Unraveling the host innate immune response to a respiratory model of *Brucella abortus*

Naveen Surendran

Abstract

Brucella are Gram-negative intracellular bacteria that cause abortion and infertility in livestock and chronic disease in humans. The Centers for Disease Control and Prevention (CDC) categorizes them as class B pathogens due to their zoonotic potential. Currently, there are no efficacious *Brucella* vaccines for humans available. Very few studies have focused on identifying protective vaccines against respiratory exposure. Protection by *B. abortus* rough vaccine strains RB51 and RB51SOD is through strong $CD4^+$ Th_1 and $CD8^+$ Tc_1 adaptive immunity. However, limited information is available on how they stimulate innate immunity. This knowledge is critical for improving these vaccines for their potential use in humans.

Dendritic cells (DCs) play a crucial role bridging innate and adaptive immunity. Therefore, enhancing the ability of rough vaccine strains to induce DC maturation and function could be critical for upregulating protective T-cell responses. Herein, we demonstrated that live vaccine strain RB51 induced significantly better ($p \leq 0.05$) DC maturation and function *in vitro* and upon intranasal inoculation *in vivo* compared to strain RB51SOD or strain 2308. Due to safety concerns of live vaccines, irradiated and heat killed vaccines were also tested; only live strain RB51 infected DCs induced significant ($p \leq 0.05$) DC function based on TNF -α and IL-12 secretion.

DC activation occurs through Toll-like receptors (TLRs) 2, 4 and 9. Our study reported that strain RB51 induced significant ($p \leq 0.05$) DC activation compared to strain 2308 , which was not dependent on a specific TLR. However, strain RB51 induced TNF – α production was TLR2

and TLR9 dependent and IL-12 production was TLR2 and TLR4 dependent. TLR4 KO mice had significantly ($p \leq 0.05$) higher number of strain RB51 colonies present at day 14 post infection.

By unraveling the innate immune responses to *Brucella*, the ultimate goal of these studies is to develop a protective vaccine for animals and people against respiratory challenge. As such, we tested several vaccination strategies. Despite enhanced DC activation and function achieved by vaccine strains, they failed to protect mice against intranasal challenge with strain 2308. Future experiments will address host-pathogen interaction at the lung microenvironment and elucidate immune mechanisms that will enhance protection against aerosol exposure.

Dedication

To

My Dear Father

Mr. Surendran Ramakrishnan

(1948-2010)

Acknowledgements

My sincere gratitude and heart-felt thank you goes first of all to my advisor Dr. Sharon G. Witonsky for being a wonderful mentor for my dissertation research. Her exceptional mentoring skills, knowledge, patience, perceptiveness, tireless perfectionism, ethics, kindness, care and support inspired and guided me through the PhD program and shaped my research career.

Among my committee members, I am indebted to Dr. Nammalwar Sriranganathan for his scientific and personal support throughout the program as well as for encouraging me to develop a research philosophy. I would like to thank Dr. Stephen M. Boyle for his valuable questions which always helped me to address research problems from different perspectives. Dr. Kurt L. Zimmerman, with his knowledge and expertise in pathology, was of immense help to me in trying to understand the histopathological lesions while going through innumerable slides. I would like to thank Dr. Elizabeth (Hiltbold) Schwartz for giving me the new direction in research by introducing us to the world of dendritic cells. I would also like to thank the deceased member of my committee, Dr. Robert B. Duncan Jr. who supported me in the initial stages of my research. I thank Dr. Ramesh Vemulapalli for sharing his valuable time to be my external reviewer.

Center for Molecular Medicine and Infectious Diseases (CMMID) was an excellent place to work with cheerful and kind neighbors; together they fostered a good research environment. I extend my thanks to Heather Lawler who taught me the basic bench top skills in the laboratory at the beginning of my program. I thank Dr. Rochelle Lewis who was my colleague in the lab for her sense of humor and company. I would like to thank Ms. Betty Mitchell for the invaluable help with organ preparation and CFU calculations. I was fortunate to get help from Bettina Heid, a highly resourceful person with tremendous knowledge in lab techniques. She taught me how to

trouble shoot efficiently, where to find information and how important it is to be detail-oriented. I extend my sincere thanks to her for the help. My Bio-Safety level -3 (BSL-3) lab experiences would not have been complete without Ms. Kay Carlson. As the BSL-3 lab supervisor, she inducted me into the laboratory and safe guarded BSL-3 principles while taking pain to explain and teach us all of those safety practices. I would also like to thank Ms. Nancy Tenpenny, glass ware staff Ms. Doris Tickle and Ms. Debbie Saville and the animal care staff for their helping hand at times of need.

I cannot forget and am thankful to the informative sessions I had with Dr. Stephen Werre who helped with the statistical analysis of most of my data. I would like to thank Ms. Melissa Makris for her great effort to help me with flow cytometry and analysis of results. I also acknowledge the help from Joan Kalnitsky, the previous flow laboratory technician.

There is no better word to acknowledge the exceptional support provided by the Research and Graduate Studies for the administrative, financial and emotional needs of my graduate education. I sincerely extend my thanks to Dr. Roger Avery and to Ms. Becky Jones, Ms. Cindy Booth and Ms. Tara Vipperman Craig. I belong to the Department of Large Animal Clinical Sciences and I acknowledge the invaluable help from Ms. Ruth Meade in arranging and organizing all my paper work and helping me to meet the conference deadlines. As the department head, Dr. David Hodgson was of great help in my professional development.

I cherish my friendship with all the different people I interacted with or received help from during my five year PhD program in Virginia Tech. I would like to thank Dr. Ranga Appuhamy, Dr. Vahida and family, Dr. Ashish Ranjan, Drs. Pradeep, Bisi and family, Dr. Sunish Mohanan, Dr. Joby and family, Karthik and family, Dr. Binu Velayudhan and family, Dr. Gopakumar and family, Dr. Subbiah and family, Dr. Ramanathan Kasimanickyam, Dr. Parthiban

Rajasekaran, Dr. Mohammed Naguieb Seleem, Dr. Sheela Ramamoorthy, Clifton Cassidy, Dr. Andrew Herbert, Sumanth Kumar, Abdul Gafoor, Neeta Jain, Deena Khan, Cheryl Ryder and Alba Hall.

I thank my father for giving me the motivation, courage and confidence and my mother for her unconditional support, love and sacrifice. I thank my wife Saritha for her love, encouragement, care and patience; my sister and family for their great support; and to Saritha's parents and brother for being supportive and caring.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Acronym	Expansion
BMDC	Bone marrow derived dendritic cells
IN	Intranasal
MLN	Mediastinal lymph node
IP	Intraperitoneal
SC	Subcutaneous
ID	Intradermal
IM	Intramuscular
PI	Post infection
TLR	Toll-like receptors
MyD88	Myeloid differentiation factor 88
TRIF	TIR-domain-containing adapter-inducing interferon-β
IFN – γ	Interferon – gamma
TNF – α	Tumor necrosis factor – alpha
IL – 12, 4, 10	Interleukin – 12, 4, 10
HK	Heat killed
IR	Irradiated
MOI	Multiplicities of infection
SOD	Superoxide dismutase
DC	Dendritic cell

CHAPTER 1

Literature Review

Historical overview: Brucellosis is a world-wide zoonotic disease affecting both domesticated and wild animals including marine mammals, which is caused by bacterial organisms of the genus *Brucella* (1). The first description of clinical conditions characteristic of brucellosis was written as early as 450 B.C. by Hippocrates (99). Recently, Capasso et al. found vertebral lesions suggestive of brucellosis in skeletal remains of Roman residents buried alive at Herculaneum by the tremendous volcanic eruption of Mount Vesuvius in August 79 A.D (20). In 1751, Cleghorn, a British army surgeon stationed in Mediterranean island of Minorca, described chronic cases of relapsing febrile illness and related it to Hippocrates's description of a similar disease (99). However, the cause of the disease was unknown until 1887, when Sir David Bruce first isolated *Micrococcus melitensis* from the spleen of a British soldier who died from maltese fever in Malta (81, 99, 111). Ten years later, M. L. Hughes published a monograph detailing the clinical and pathological conditions in 844 human patients and coined the term "undulant fever" to describe the relapsing nature of the fever (99). In that same year, a Danish investigator Bang identified "Bacillus of abortion" (i.e., *B. abortus*) from placentas and fetuses of cattle affected with contagious abortion (81). The first recognized human case of brucellosis in the United States of America (USA) was reported in 1898 in an army officer based in Puerto Rico (81). It was only by 1905 that *Brucella* was recognized as a zoonotic agent by Zammit after isolating *B. melitensis* from goat's milk (81, 111). In 1918, Alice Evans showed that Bang's organism was identical to that described by Bruce in 1887 and renamed the genus to *Brucella* in honor of Sir David Bruce (81). *B. suis* was isolated in 1914 by Traum from an aborted pig fetus in United States (US) (81). During 1953-66, three more species of *Brucella* were identified from sheep (*B. ovis*), desert

wood rat (*B. neotomae*) and dogs (*B. canis*) (81). The concept of land based distribution of brucellosis was changed by 1994 when a bacterial isolate from the aborted fetus of a bottle nose dolphin was characterized as nontypical *Brucella spp*. (45) Since then new *Brucella* species have been isolated from different marine mammals (*B. cetaceae* and *B. pinnipediae*)(45). By April 2003, zoonotic nature of marine *Brucellae* was documented by showing its ability to cause abortions in cattle and neurologic disease in humans (45). As of 2009, eight different *Brucella* species have been recognized (81).

General characteristics of *Brucella*: *Brucella spp.* are small (0.5 to 0.7 μm by 0.5 to 1.5 μm), nonmotile, nonsporulating, nonfermenting, microaerophilic Gram-negative coccobacilli (99, 110). Although classically considered as facultative intracellular organisms, they can survive in open environment and bacteriological media to some extent. *Brucellae* grow best on trypticase soybased or other enriched media with a typical doubling time of 2 hours (99). Growth occurs aerobically and is enhanced by 5-10% CO_2. *Brucellae* produce urease, oxidize nitrite to nitrate, and are oxidase and catalase positive (99). The genus *Brucella* belongs to the order Rhizobiales within the class alpha-proteobacteria along with *Ochrobactrum*, *Rhizobium*, *Rhodobacter*, *Agrobacterium*, *Bartonella* and *Rickettsia* (81, 110). Although DNA hybridization studies carried out within the genus revealed high degree of homology (>90%) between the different species and it was proposed that *Brucella* should be grouped as biovars of a single species, the current classification based on host specificity and pathogenicity is preferred (81). At present, eight *Brucella* species are recognized; six of them affect terrestrial animals: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* and two affect marine mammals: *B. cetaceae* and *B. pinnipediae* (81, 110). Different Brucella species are further subdivided into biovars (*B. abortus*, *B. melitensis* and *B. suis* into seven, three and five biovars respectively) based on serotyping,

phage typing, dye sensitivity to basic fuchsin and thionin, CO_2 requirement, H_2S production and metabolic properties (81, 99).

The genome sequencing efforts of various *Brucella* species/strains are underway and complete genome sequences of 4 species are already published (81). Each species within the genus has a similar genome size of approximately 3.28Mb. The genome consists of two circular chromosomes. The G+C content of Chromosome I for all genomes is 57.2% and for chromosome II is 57.3% (81).

Transmission, pathogenesis and diagnosis in domestic animals: The main pathogenic *Brucella* species for domestic animals are *B. abortus* (cattle), *B. melitensis* (goats) and *B. suis* (swine) (111). These three *Brucella* species cause abortion in pregnant animals and infertility in males due to orchitis and epididymitis (99). *B. ovis* and *B. canis* are responsible for ram epididymitis and canine brucellosis respectively (99). Different *Brucella* strains have also been isolated from a wide variety of wildlife species such as bison, elk, wild boar, fox, hare, reindeer, caribou, ibex, and wildlife are considered as an important reservoir for zoonotic brucellosis (45). Besides affecting productivity, the presence of brucellosis in a herd, region or country causes restrictions in animal movement and trade, thus resulting in huge economic losses (45).

Brucella infection in a susceptible animal depends on dose, exposure route, virulence of the strain, age and gestational stage of the animal. Typically, *B. abortus* is transmitted through oral route by ingesting infected placenta, fetal tissues or fluids containing high concentrations of organisms (99). *Brucellae* can also enter the host through skin abrasions or cuts, conjunctiva, mucosa and respiratory tract. Congenital and sexual transmissions have also been documented in domestic cattle (103). Sexually mature cattle are susceptible to infection and among those pregnant animals are the most susceptible (34). The increased susceptibility of pregnant cattle is

thought to be related to the concentration of sugar erythritol in the gravid bovine uterus (34, 105).

Upon ingestion, the organisms reach the gastro intestinal tract, and they are phagocytosed by lymphoepithelial cells of gut-associated lymphoid tissue and gain access to submucosa (99). In the submucosa, organisms are rapidly ingested by neutrophils and phagocytosed by macrophages (99). Inside macrophages, *Brucella* escape death by inhibiting phago-lysosomal fusion, survive and reach the reticuloendothelial system of local lymph nodes, this leads to local lymphadenopathy eventually resulting in bacteremia (25, 99). *Brucella* then spread through the circulation to the spleen, liver, mammary gland, joints, kidneys, bone marrow and reproductive tract establishing a systemic infection (99).

In ruminants, *Brucella* target embryonic and trophoblastic tissue with high concentrations of erythritol in such tissues as pregnant uterus, fetal tissues and male genital tract (105). *B. abortus* infection in cattle may cause late term abortions, still births, retained placentas, sterility, lymphoplasmacytic mastitis and tissue granulomas (34). The infected animal will shed virulent *Brucella* through milk, aborted secretions and afterbirth (34).

Brucellae can be cultured from bones, joints, eyes and brain in adult cattle and from the stomach, lung and spleen of the bovine fetus (33). Culture of *Brucella* from aborted material, milk or tissues collected at autopsy provides a definitive diagnosis (34). Serology is usually the most practicable of diagnosis methods. In cattle, World Health Organisation (WHO) recommends the Rose Bengal plate Test (RBT) for screening and ELISA or complement fixation for confirmation of infected individual animals. Screening of milk samples by milk ring test or ELISA is useful for surveillance (34). No single serological test is reliable for confirmation of infection in individual animals in sheep, goats and pigs (34). Serological tests should be used on

a herd or flock basis. A “rough-specific” antigen must be used for *B. canis* serology owing to the rough nature of the pathogen (34).

Prevention, control and eradication of animal brucellosis: According to WHO, animal brucellosis is best prevented by careful herd management and hygiene (34). Vaccination is useful for prevention and control of infection. *B. abortus* strains 19 and RB51 are recommended for prevention of bovine brucellosis (34). *B. melitensis* Rev 1 is recommended for prevention of *B. melitensis* infection in sheep and goats (34). Vaccine efficacy may be limited in the face of heavy exposure. Eradication can only be achieved by test-and slaughter combined with effective prevention measures and control of animal movements (34, 85).

Use of mouse model in *Brucella* research to study host immune response: Murine brucellosis is widely accepted as an established model to study the host immune response to experimental *Brucella* infection (98, 114). Mice are inexpensive, easy to house, handle and have a short generation interval which makes them the preferred model to study brucellosis compared to the high costs and long time span of experiments in natural hosts. Moreover, the murine immune system has been extensively characterized and the availability of a variety of inbred mouse strains minimizes individual animal variations. This allows all researchers to work on genetically identical mouse strains worldwide. Therefore, mice have been used as a preliminary step in the analyses of vaccines against brucellosis.

Intracellular adaptation of *Brucella*: *Brucella* is an intracellular pathogen that infects professional phagocytic cells such as macrophages and dendritic cells as well as non professional phagocytes such as trophoblasts (105). *Brucellae* are described as pathogenic bacteria without classical virulence factors such as exotoxins, cytolysins, capsules, fimbria, plasmids, endotoxic LPS or inducers of apoptosis (47, 110). The pathogenicity of *Brucella* is due to its ability to

adapt to the environmental conditions encountered in its intracellular replicative niche including low levels of nutrients and oxygen, acidic pH and reactive oxygen intermediates (63). This ability is believed to be responsible for establishment of chronic infection. *Brucella* had a long standing co-evolution with its replication niche which makes the pathogen well adapted to the intracellular environment. For instance, *Brucella* has the ability to control its own intracellular trafficking to avoid lysosomal degradation, replicate extensively within the host cell and not induce apoptosis (25, 48, 50). *Brucella* expresses a non-canonical LPS with very low endotoxicity which plays an essential role in the entry of the organism into the phagocytic cell through interactions with particular receptors within the lipid rafts located on the host cell plasma membrane (21, 65). Although immediately after entry *Brucella* containing vacuoles (BCV) within the host cell interact with the early compartments of endocytic pathway, *Brucella* with an intact O-side chain on its LPS avoids fusion of the BCV with lysosome (25). An additional mechanism by which *Brucella* avoids lysosomal fusion is by secreting cyclic β-1,2-glucan which extracts cholesterol from lipid rafts of vacuole membrane preventing phagosomal maturation (6). After surviving the early destruction within the macrophages, BCVs continues to interact with endocytic pathway until vacuolar acidification occurs which is required for intracellular expression of type IV secretion system (25). At this stage BCVs segregate themselves from the endocytic pathway and start to physically interact with the endoplasmic reticulum (ER) to become mature replication proficient vacuoles (25, 105). *Brucellae virB* mutants fail to acquire ER markers and become ER – derived organelles that ultimately fuse with lysosomes (25, 105). Therefore, *Brucella* possesses VirB type IV secretion machinery as well to reach its replication permissive niche for intracellular survival (25). Moreover, recently it has been shown that

Brucella uses its LPS and lipidated outer membrane proteins (L-OMP19) to inhibit MHC class II antigen expression of host cells to prevent antigen presentation to T-lymphocytes (9, 65).

***Brucella* virulence factors:** *Brucella* uses a number of virulence factors/mechanisms for avoiding or suppressing bactericidal responses and for invading and surviving within the host cell.

Lipopolysaccharides (LPS): LPS is vital to the structural and functional integrity of the Gram-negative bacterial outer membrane (21). The LPS is composed of Lipid A, a core oligosaccharide, and an O-side chain polysaccharide (65). LPS of rough *Brucella* strains do not have O-side chain (108). In contrast to enterobacteria, such as *Escherichia coli* (*E. coli*), *Brucella spp.* possesses a nonclassical LPS (21). *B. abortus* lipid A has a diaminoglucose backbone (rather than glucosamine) and acyl groups are longer (C18–C19 or C28 rather than C12 and C14) and are linked to the core by simple amide bonds (rather than ester and amide bonds) (21, 65). Highly purified *B. abortus* LPS is several hundred times less active and toxic than the classical *E. coli* LPS and is a poor inducer of respiratory burst, bactericidal nitrogen intermediates and lysozyme secretion (102). *Brucella* O-side chain blocks deposition of complement factor C1Q to the outer membrane protein targets and impairs anti-microbial host responses (65). *Brucella spp.* are resistant to a large variety of anti-bacterial proteins, including defensin NP-2, lactoferrin, cecropines, lysozyme, bactenecin-derived peptides and the defensin-like antibiotic polymyxin B, as well as to crude lysosomal extracts from polymorphonuclear leukocytes (75, 104). *Brucella* LPS forms LPS macrodomains enriched with MHC II molecules which inhibit efficient antigen presentation and downregulate T-cell activation (65). Moreover, pathogenic *Brucella* smooth strains enter cells using their LPS to interact with cell surface lipid rafts to avoid fusion with

lysosomes (6, 25). Rough *Brucella* strains which lack the O-side chain of LPS do not enter the cell through lipid rafts. Instead, they fuse rapidly with lysosomes and get lysed (25).

The two-component BvrR/BvrS system: The two-component BvrR/BvrS system is crucial for the control of virulence, cell invasion and intracellular replication (70). This system turns on essential genes for invasion and intracellular survival once the bacteria switch from extracellular to intracellular mode of life inside the host cell (70). The *bvr*R and *bvr*S genes encode proteins which regulate the composition of the outer membrane (49). Mutation of *bvr*R/*bvr*S system results in lack of expression of Omp 25 and Omp 22 from outer membrane (71). Both *bvr*R and *bvr*S mutants are less invasive than the wild-type strain (70, 71). Both mutants fail to replicate within phagocytic or nonphagocytic cells and are degraded by lysosomal fusion. Studies using macrophages and dendritic cells show that Omp25 inhibits TNF-α release from human dendritic cells (12). Dysfunction of BvrR and BvrS also diminishes the characteristic resistance of *B. abortus* to bactericidal cationic peptides and increases its permeability to surfactants (70).

Cyclic β-1,2 glucan: The *B. abortus* genome encodes a high molecular weight (316.2 kDa) inner-membrane protein encoded by the cyclic β-1,2 glucan synthetase gene (*cgs*) (71). The CβG interferes with cellular trafficking by acting on lipid rafts of host cell membrane and controls vacuole maturation by avoiding fusion with lysosomes, and thus allowing intracellular *Brucella spp*. to survive and reach its replication niche (6). *Brucella spp. cgs* mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells (71).

Type IV secretion system: The type IV secretion system, encoded by the *vir*B region, is a key virulence factor for *Brucella spp.* (106) The *vir*B region is composed of 12 genes that form an operon specifically induced by phagosome acidification in cells after phagocytosis (36, 37, 53). Although no effectors have yet been identified, similarities with plant pathogen *Agrobacterium*

tumefaciens suggest that *Brucella spp.* use their type IV secretion system to secrete effector molecules into the host cytosol (22, 29, 30, 106). Upon entering the macrophage through lipid rafts, the *Brucella* containing vacuole (BCV) avoid fusion with lysosomes and start interacting with endoplasmic reticulum (ER) to reach their replicative niche in the ER (26, 27). The acquisition of ER membranes requires a functional *vir*B apparatus for sustained interactions and fusion events between the BCV and ER elements (26). *Brucella vir*B mutants have shown to loose their ability to multiply in HeLa cells (27, 86). However, Billard et al. documented that type IV secretion system is not involved in the inhibition of DC maturation (14).

Protective antigens of *Brucella* - O-side chain: The oligosaccharide chain (O-side chain) is the most exposed, major antigenic determinant of *Brucella spp.(46)* This N-formylperosamine O-polysaccharide of LPS stimulates the major proportion of antibody response in animals and humans infected with pathogenic *Brucella* species (83). Although protection against brucellosis is mainly cell mediated, Arraya et al. demonstrated that passive transfer of immune serum with O-sidechain antibodies conferred protection in mice against virulent *Brucella* challenge (5, 78). Moreover, studies in mice by Vemulapalli et al. using *B. abortus* vaccine strain RB51 expressing *wboA* gene, which expresses low quantities of O-side chain, demonstrated enhanced protection against challenge with virulent strain 2308 compared to mice vaccinated with strain RB51 not expressing O-side chain (122, 125). However, it seems antibody mediated protection is dependent on the host animal as O-side chain antibodies in bovines do not additionally enhance protection (83).

Outer membrane proteins (OMPs): OMPs were identified as early as 1980s by using monoclonal antibodies (MAb) and immunogold techniques (31, 127). Two major *B. abortus* OMPs were identified and designated as group 2 and 3 proteins based on molecular mass

representing 36-38 and 25-27 kDa OMPs respectively (127). Additionally, several low molecular weight proteins including Omp10, Omp16 and Omp19 have been identified as minor OMPs and as lipoproteins (31). Group 2 and 3 proteins have shown to be strongly associated with peptidoglycan (31). Group 2 proteins were also identified as porin proteins (31). Omp31 from *B. suis*, *B. melitensis* and *B. ovis* was shown to be a hemin binding protein (HBP), which is expressed under reduced iron conditions and helps obtain iron from the host (35). However, group 2 and 3 OMPs from rough *B. abortus* and *B. melitensis* did not protect against smooth *B. abortus* and *B. melitensis* challenge in mouse models (31, 78). Additionally, major OMPs only induced low antibody levels and served as poor immunogens in *B. abortus* infected cattle. Gonzalez et al. demonstrated that the outer membrane proteins are more exposed on rough strains than on smooth *Brucella* due to the absence of O-polysaccharide of the LPS (46). Therefore, the lack of steric hindrance caused by O-side chain to MAbs against major OMPs might explain the protection afforded by major OMPs in mice against rough *B. ovis* challenge infection (17, 24). In contrast, Zwerdling et al. and Pasquevich et al. both have shown that minor outer membrane proteins such as Omp16 and Omp19 were immunostimulatory (97, 130). Pasquevich et al. demonstrated that both Omp16 and Omp19 in its unlipidated version stimulated antigen specific $CD4^+$ and $CD8^+$ T-cells to provide systemic and oral protection to *B. abortus* infection in mice (97). In summary, major OMPs appear to be less relevant as protective antigens against smooth *Brucella* infection. Although recently, major OMP25 have shown to play a potential role as virulence factors by limiting the host response while inhibiting TNF-alpha secretion from DCs upon infection with smooth *B. suis* (12).

Superoxide dismutase (SOD): *Brucella* Cu/Zn SOD is a protective periplasmic antigen (123). Reactive oxygen intermediates (ROI) are harmful to *Brucella* and production of ROIs is one

mechanism adopted by the host to limit intracellular replication of *Brucella* (43, 59). SODs are a family of metallo-enzymes that catalyze the dismutation of superoxide into hydrogen peroxide and molecular oxygen, thus preventing damage to *Brucella* by ROIs (76). *Brucella* Cu/Zn SOD with copper and zinc at their active sites is encoded by *sodC* gene and is highly conserved among *Brucella* biovars (112). However, the inability to produce Cu/Zn SOD by *B. abortus* does not significantly impair its virulence in mice and mutants were able to establish chronic infection in mice (66, 115). Therefore, SOD cannot be considered a virulence factor of *Brucella* (110). Antigenic properties of *Brucella* Cu-Zn SOD have been demonstrated under several experimental conditions. Recombinant *E. coli* expressing *Brucella* Cu/Zn SOD and strain RB51 overexpressing SOD have been shown to protect mice against challenge with pathogenic *Brucella* (93, 123). SOD specific IFN-gamma levels have been detected in vaccinated mice (123).

L7/L12 ribosomal proteins: $CD4^+$ T cells play an important role in protecting against *Brucella* infection. Ribosomal preparations have been used as vaccines against several pathogens, including *B. abortus*, conferring some degree of protection. Oliveira et al. demonstrated that in mice recombinant *B. abortus* L7/L12 protein stimulated CD4 Th_1 - cell response with IFN-γ secretion (90, 91). Antibody and delayed type hypersensitivity (DTH) responses to this protein have also been demonstrated in cattle and mice (64, 91). However, it is not clear whether such subunit vaccinations will provide long term protection in the host.

Antibodies to *Brucella*: The significance of humoral immunity in murine brucellosis has been demonstrated by many passive-transfer experiments (5, 78). *Brucella* LPS and O-antigen of the *Brucella* LPS are the two immunodominant structures against which antibodies are shown to be produced (5, 78, 94). Passive transfer of sera containing LPS antibodies to mice protected against

challenge with virulent *B. abortus* (5, 32, 78). Antibodies to *Brucella* O-antigen reduced bacterial infection in mice or conferred partial protection against virulent *Brucella* infection in murine models (32). IgG2a and IgG3 are the dominant antibody isotypes detected in mice suggesting a Th_1 immune response against brucellosis (39, 113).

B. abortus infection induces production of IgM, IgG1, IgG2a and IgA antibody isotypes in both milk and sera of cattle (84). Although humoral immunity plays a role in resistance to brucellosis, the data suggest that cell mediated immunity is most critical. *B. abortus* vaccine strain RB51 lacking the O-side chain of LPS, which therefore does not induce any O-side chain antibodies, still provides good protection (52, 108, 109). Therefore, while passive transfer studies in mice support a role for humoral immunity, based on these other studies, CMI provides adequate immunity.

Cell Mediated Immunity to *Brucella*: Similar to most intracellular bacterial infections, T-cell mediated immunity plays a significant role in protecting against virulent *Brucella* infection (52). This is best demonstrated by results from *B. abortus* vaccine strain RB51 studies. Protection conferred by strain RB51 can only be transferred by immune T cells and not by antibodies (52, 109). Protective functions of adaptive immune response in brucellosis can be classified in to 2 mechanisms (62). The first mechanism is IFN-γ production by $CD4^+$, $CD8^+$ and γδ T-cells which stimulates macrophage antimicrobial activity and hampers intracellular survival of *Brucella*. The second mechanism of T cell mediated immunity is the lysis of infected cells by specific $CD8^+$ and γδ T cells.

Some of the studies which demonstrated the critical role of CMI were adoptive transfer experiments. In these studies, $CD4^+$, $CD8^+$ and whole T-cell populations from immunized BALB/c mice which were transferred into infected mice enhanced protection indicating that both

$CD4^+$ and $CD8^+$ T-cell subsets are involved in protection (4). Additionally, the critical role of IFN-γ in resistance to *Brucella* infection has been demonstrated by *in vivo* antibody neutralization experiments (79, 89). Although IFN-gamma can be produced by CD4, CD8, NK and gamma delta cells, CD4 cells are the major T-cell population based on number and they secrete most of the IFN- γ; all of this suggests a critical role for CD4 T-cells and associated IFN—γ production (62). However, experiments with $\alpha\beta^{-/-}$ and $\beta 2\text{-}m^{-/-}$ mice infected with *B. abortus* strain 19 suggest that CD8 T-cell deficient mice have decreased clearance compared to CD4 T-cell deficient mice and wild type mice implying a more critical role of CD8 vs. CD4 T-cells (89). Mouse models of brucellosis have revealed that *Brucella* resistant C57BL/6 mice require IFN-γ throughout the course of infection and mice died in its absence (79). In comparison, *Brucella* susceptible BALB/c mice which failed to produce IFN-γ after first weeks of infection relied on CD8 T-cells and TNF-α to control infection (79). In cattle younger than 1 year, the major T-cell population is γδ T cells suggesting a significant role of γδ T cells in *Brucella* infected calves although their role has not been characterized *in vivo* (116).

B. abortus induces a CD4 Th_1 and CD8 T_{C1} immune response and inhibits Th_2 type immune responses. The mechanisms associated with regulation of CD4 Th1 and CD8 Tc1 responses are less clear. DC mediated cytokines such as IFN-γ, IL-12 and TNF- α often direct the T-cell response towards a CD4 Th1 CD8 Tc1 response (62). *In vivo* depletion of endogenous IL-12, which is produced by DCs and macrophages, exacerbated *Brucella* infection and reduced IFN-γ production (128). Additionally, decreased TNF-α, via TNF-α-receptor knockout mice ($TNF\text{-}r^{-/-}$), were also severely deficient in IL-12 production; these mice had aggravated *Brucella* infection (129).

***Brucella* vaccines – live vaccines:** Prevention is better than cure. Historically the most successful vaccines against brucellosis were live attenuated vaccines compared to killed vaccines (42). Live attenuated vaccines provide long lasting cell mediated immunity and *Brucella* can replicate within the host leading to a longer half-life and better immune response, and thus making it more efficacious and less expensive (42). Compared to subunit or DNA vaccines, live attenuated vaccines contain intact bacteria with all the immunogenic components that can be involved in protection making it more efficient (62). However, some live attenuated vaccines may cause abortion in pregnant animals and safety concerns limit their use in humans.

Live vaccines - *B. abortus* strain 19: This vaccine has been used extensively in brucellosis eradication program in the United States, prior to the introduction of strain RB51 in 1996. Strain 19 is a live attenuated smooth strain (85). The molecular basis of attenuation is not known. This was first described in 1930 (18). Anecdotal references indicated that strain 19 was originally isolated from the milk of a Jersey cow as a virulent strain in 1923. But after being kept in the laboratory for over a year at room temperature it developed a deletion in the erythritol gene (41); this attenuated the strain (3). While strain 19 conferred protection against virulent *B. abortus* in cattle, abortions can develop in pregnant animals (10). Additionally, it has the disadvantage of inducing O-side chain antibodies that can interfere with diagnostic tests to differentiate infected and vaccinated animals (109).

***B. melitensis* Rev. 1:** Rev. 1 vaccine is a live attenuated spontaneous mutant derived from virulent *B. melitensis* (109). The strain is resistant to streptomycin (38). It stimulates protection against *B. melitensis* infection in sheep and goats and also protects rams against *B. ovis* infection (109). The use of Rev. 1 in cattle indicates that it provides better protection than strain 19 (109).

Depending upon the dose administered, abortion occurs with variable frequency (109). Rev. 1 is a smooth strain and it interferes with diagnostic tests.

***B. suis* strain 2:** This is a live attenuated smooth strain derived from biovar 1 of *B. suis.* It is used as an oral vaccine in China to protect cattle, goats, sheep and pigs (15, 80). Although it induces O-side chain antibodies, they disappear by one year post vaccination (80).

***B. abortus* strain 45/20:** *B. abortus* smooth strain 45/0 was isolated from a cow in 1922 (109). After 20 passages in guinea pigs, rough strain 45/20 bearing at least one unknown mutation was obtained. This strain protects guinea pigs and cattle from *Brucella* infection (109). However, when used as a live vaccine, strain 45/20 was not stable and reverted back to smooth virulent form (108). The reversion to smooth strain resulted in vaccine induced antibodies which interfered with diagnostic tests. This defeated the purpose of using rough strains.

***B. abortus* strain RB51:** Vaccine strain RB51 is a stable, rifampin-resistant, rough mutant of *B. abortus* strain 2308 (108). It was derived by serial passage of parental strain 2308 on Trypticase soy agar supplemented with varying concentrations of rifampin and pencillin (108). “R” stands for rough and “B” stands for *Brucella;* 51 refer to an internal laboratory nomenclature used at the time it was derived and not the passage number (108). Strain RB51 is devoid of O-side chain and is stable after multiple passages *in vitro* and *in vivo* through various species of animals (108). Colonies of strain RB51 are rough in morphology as indicated by their ability to absorb crystal violet as well as auto-agglutinate while in suspension (108). Biochemically, strain RB51 has the ability to use erythritol unlike strain 19 (108). In February 1996, the USDA Animal Plant Health Inspection service (APHIS) approved the use of *B. abortus* strain RB51 as the official calf hood vaccine for protection against brucellosis. The recommended dose for calves between the ages of

4-12 months vaccinated subcutaneously (SC) is 1-3.4 X 10^{10} organisms (117). It induces protection in cattle against virulent *B. abortus* at a level similar to that conferred by strain 19.

There are a number of advantages for strain RB51 over other vaccines. It does not produce clinical signs post vaccination; there are no local reaction at the site of injection (28). It is rapidly cleared from blood stream as early as 2 weeks post inoculation and it is not shed in the nasal secretions, saliva or urine. Thus it is unable to spread from vaccinated to non-vaccinated animals through these routes (28, 108). Pregnant cattle can be safely vaccinated SC with 10^9 organisms of strain RB51 without inducing abortion or placentitis. Mouse studies revealed that protective immunity induced by strain RB51 is solely mediated by T-cells with a polarized type 1 cytokine profile which is the desired type of protection against intracellular pathogens (52). In a murine model, strain RB51 protected against challenge with *B. abortus*, *B. melitensis*, *B. suis*, and *B. ovis* (108). Moreover, the lack of O-side chain with strain RB51 prevents O-antigen specific antibody formation and interference with diagnostic tests (108).

Although strain RB51 is extremely stable, the exact nature for its avirulence is not known (108). It is thought that the strain possesses at least two mutations in its LPS biosynthetic pathway. One being the presence of an IS711 element in the *wbo*A gene responsible for synthesis of O-side chain (124). Complementation experiments using *wbo*A gene showed that the strain produces O-side chain while maintaining a rough phenotype, but the O-side chain remains in the cytoplasm indicating the possibility for at least one more mutation (121). This second mutation is thought to be in the *wzt* gene that codes for an ABC type transporter which is involved in the translocation of the O-side chain across the inner membrane of *Brucella* (44).

Recombinant strain RB51 vaccine overexpressing homologous protective antigens such as *B. abortus* Cu/Zn SOD (superoxide dismutase; approximately 10 times the normal level),

designated RB51SOD, induced significantly increased protection against challenge with virulent strain 2308 in BALB/c mice (123). Complementation of strain RB51 with a functional copy of *wbo*A gene, RB51wboA, produced intracytoplasmic O-side chain and completely protected mice against virulent strain 2308 infection (122). Additionally, recombinant strain RB51SODWboA, which overexpressed SOD with simultaneous expression of O-side chain in the cytoplasm, induced better protection than strain RB51 or RB51SOD against strain 2308 challenge (119). The ability of strain RB51 to induce CD4 Th_1 and CD8 T_{C1} polarized response with high levels of IFN-γ made it an attractive candidate for heterologous expression of protective antigens belonging to other intracellular pathogens. Development of strain RB51 as a vector for expression of heterologous antigens has met with success when *E. coli*, Mycobacterium and *Neospora caninum* antigens were successfully expressed in strain RB51 (96, 100, 101, 121, 124).

Vemulapalli et al. had demonstrated that strain RB51 exposed to an appropriate minimum dose of gamma radiation were unable to replicate but retained their ability to stimulate Th1 immune responses and protected mice against virulent challenge with strain 2308 (107). Additionally, Magnani et al. showed that irradiated *B. melitensis* protected against virulent *B. melitensis* challenge (74). By contrast, Lee et al., found that irradiated (higher dose of irradiation) strain RB51 with or without IL-12 as an adjuvant, did not protect against strain 2308 challenge (68, 69).

Killed vaccines: Killed vaccines can be safer alternatives to live attenuated vaccine strains and a variety of killed vaccines have been developed for protection against brucellosis. However, killed vaccines without adjuvants had only limited success and protection compared to live attenuated strains. *B. melitensis* H38 was smooth formalin killed vaccine in mineral oil adjuvant used for vaccination in goats and sheep (109). It protected against abortions but induced positive

serology to vaccine and caused unacceptable local reactions at the inoculation site (109). *B. abortus* strain 45/20 when used as a bacterin incorporated in adjuvants gave varying results regarding protective efficacy and positive serology (109). Most investigators considered that two vaccinations were necessary for protection. Strain 45/20 did not induce abortions when used as bacterin (109). However, batch to batch variations in properties of the vaccine, variability of reported protection, severe local reactions and unpredictable serology prompted the discontinuation of this killed vaccine (109).

Recombinant vector vaccines: *Brucella* protective antigens such as Cu/Zn SOD were expressed using vaccinia virus and insect baculovirus vectors although these vaccines were not successful in eliciting effective protection against virulent *Brucella* challenge (7, 120). Similarly, Gram-negative soil bacterium *Ochrobactrum anthropi*, closest genetic relative of *Brucella*, had been used to express Cu/Zn SOD antigen (51). Vaccination of mice with recombinant *O. anthropi* induced mixed Th_1-Th_2 immunity with high IFN-γ and IL-4 levels. It was non-protective unless co-administered with CpG adjuvant which polarized the cytokine response to Th_1 profile (51).

Subunit vaccines: The concept of subunit vaccines in brucellosis is based on generation of memory Th_1 cells by immunization with T-cell antigen (62). The strategy is to identify those *Brucella* antigens that are responsible for T-cell mediated response. Until now periplasmic binding protein P39, bacterioferritin and L7/L12 proteins, Omp 31 have been purified and tested as subunit vaccines with adjuvants (2, 90). Mice vaccinated with these proteins showed only a partial protection when challenged. The enzyme lumazine synthase from *Brucella spp*. (BLS) is highly immunogenic and stable. Goldbaum et al. (2007), showed that a recombinant chimera of 10 copies of protective antigen OMP31 on a scaffold of BLS (rBLSOmp31) provided good protection level against *Brucella ovis* (23). The rBLSOmp31 vaccine induced greater protection

than vaccination with co-delivery of both recombinant proteins (rBLS + rOmp31) (23). Additionally, the former protected similarly compared to control vaccine *Brucella melitensis* strain Rev.1 (23). The chimera induced humoral as well as BLS and peptide specific T-cell responses (23, 40, 118). Recently, Pasquevich et al. (2009) demonstrated that immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific $CD4^+$ and $CD8^+$ T-cells as well as systemic and oral protection against *Brucella abortus* challenge (97).

DNA vaccines: DNA vaccines involve the injection of plasmid DNA encoding protective antigens in to the host. No other cellular or subcellular components are included in the vaccine (109). The type of immune response elicited depends upon the antigen's characteristics, route of administration and the presence of immunostimulatory DNA sequences. So far, DNA vaccines expressing various *Brucella* antigens such as Cu/Zn SOD, BLS, L7/L12, P39, heat shock protein GroEL and OMPs have been tested by different research groups in mice with variable levels of protection upon challenge infection (2, 64, 67, 118). However the question remains as to whether DNA vaccines encoding *Brucella* antigens would induce an effective long term protection.

Zoonosis: Zoonosis is any infectious disease that can be transmitted (in some instances, by a vector) from non-human animals, both wild and domestic, to humans or vice versa (1). Brucellosis is an established zoonosis (34). All *Brucella* species with the exception of *B. ovis* and *B. neotomae* can infect humans (34). *B. melitensis* is the most important zoonotic agent among *Brucella* species although most human cases of brucellosis are caused by *B. abortus*. *B. melitensis*, *B. suis* and *B. abortus* are the most infectious of the genus in their order of pathogenicity (34). The incidence of human disease is closely related to the prevalence of infection in livestock and to the practices that allow potential exposure of humans to infected

animals or their products (45). The risk group is Abattoir workers, meat inspectors, animal handlers, veterinarians, and lab workers (61).

Consumption of unpasteurized cow, small ruminant or camelid milk and milk products is considered to be the main route of infection (45). Clinically symptoms are, in the acute form (<8 weeks from illness onset), nonspecific and "flu-like" symptoms including fever, sweats, malaise, anorexia, headache, myalgia, and back pain (34, 45). In the undulant form (<1 year from illness onset), symptoms include undulant fevers, arthritis, and epididymo-orchitis in males (34). Neurologic symptoms may occur acutely in up to 5% of cases. In the chronic form (>1 year from onset), symptoms may include chronic fatigue syndrome, depression, and arthritis. Human-to-human transmissions by tissue transplantation or sexual contact have occasionally been reported but are very rare (34). Therefore, control and eradication of the disease from the natural animal reservoirs have important public health implications (1, 34, 45). A definitive diagnosis in acute human brucellosis includes, isolation of *Brucella* from blood or other tissues is definitive (34). However, culture is often negative, especially in long-standing disease. Serology is the most generally useful diagnostic procedure. The Rose Bengal test (RBT), tube agglutination and ELISA procedures are recommended (34). Methods which differentiate IgM and IgG can distinguish active vs. past infection. The critical element in the treatment of all forms of human brucellosis is the administration of effective antibiotics for an adequate length of time (34). Treatment of uncomplicated cases in adults and children eight years of age and older are by using a combination of antibiotics; doxycycline 100 mg twice a day for six weeks + streptomycin 1 g daily for two to three weeks or doxycycline 100 mg twice a day for six weeks + rifampicin 600–900 mg daily for six weeks (34). However, for these acute cases the relapse rate is 10-20 % and in chronic phase, eradication is difficult since *Brucella spp*. are localized intracellularly and

most antibiotics do not actively pass through cell membranes (34). There are no safe and effective commercially available vaccines to protect against human brucellosis.

Human brucellosis vaccines: Safe and protective vaccines against human brucellosis are not commercially available (34). However, numerous vaccines have been tested in people in the past with limited success. *B. abortus* strain 19-BA was used in the former USSR (109). This strain 19 derived vaccine (1×10^9 CFUs) given by skin scarification (epicutaneous route) induced protection for a shorter duration (5-6 months, maximum up to 1 year) but with a high frequency of hypersensitivity reactions occurring in 76% of those vaccinated (34, 109). Attenuated strains of *B. abortus* 84-C and 104-M were also given epicutaneously or as aerosols in USSR and China respectively (109). Although considered effective, these vaccines induced serious adverse reactions and are no longer in use. Emphasis for safer non living vaccines led to the development of subunit vaccines for use in humans. The French developed a vaccine utilizing phenol-insoluble peptidoglycan fraction of *B. melitensis* M15 which was administered subcutaneously and supposedly offered protection for 2 years (34, 109). However, conclusive evidence of protective efficacy is not available and the vaccine is not at present in production. Another sub-cellular fraction namely "*Brucella* chemical vaccine" (BCV) was developed from an acetic acid extracted polysaccharide-protein fraction in Russia (34). This vaccine given intramuscularly does not elicit severe hypersensitivity reactions but evidence of protective efficacy from controlled clinical trials is not available.

Renewed interest in *Brucella* as a potential bio-terror weapon illustrates the need for developing an effective vaccine against human brucellosis.

Bioterrorism: *Brucella* has been traditionally considered as a biological weapon (95). *B. melitensis* and *B. suis* have been developed experimentally as biological weapons by many state

sponsored programs during World War II (95). Brucellosis remains the most common anthropozoonosis worldwide and its significance as a potential bioterrorism agent makes it in to the category B biodefense research list of Center for Disease Control and Prevention (CDC) (95). Although *Brucella* can enter the human host through skin abrasions or cuts, the conjunctiva or by consuming unpasteurized dairy products, the most important means of transmission in a bioterrorism event is airborne transmission (61). They are relatively stable in aerosol form and a small inoculum (10-100 bacteria) will induce human disease (61). The organism is easily obtained worldwide in contrast to other agents and easy to develop antibiotic resistant strains (95). The disease is severely debilitating, infectious to both humans and livestock, has vague clinical characteristics delaying rapid diagnosis and requires combined antibiotic regimen for a prolonged period to treat the disease (34, 45). Additionally, there are no human vaccines available. According to Godfroid et al., in a theoretical model of a bioterrorist attack and in the absence of an intervention program for 100,000 persons exposed, a *B. melitensis* cloud would result in 82,500 cases of brucellosis requiring extended therapy, with 413 deaths. The economic impact of such a brucellosis bioterrorist attack would cost $ 477.7 million per 100,000 persons exposed (45). Therefore, the development of a vaccine for brucellosis suitable for humans would be an ideal solution to prepare for a bioterror threat. One of the theoretical vaccine targets for the future that could be considered for humans, whose efficacy has been proven in animals, is *B. abortus* strain RB51.

Introduction and rationale

In spite of the documented evidence that an infectious aerosol dose of 10-100 *Brucella* can cause human disease (16), its potential use as a bioterror agent and the absence of an efficacious

vaccine for use in humans, very few studies have focused on vaccine efficacy associated with respiratory challenge. Most *Brucella* studies have predominantly focused on non-respiratory routes of vaccination and challenge, such as vaccinating animals intraperitoneal (IP), subcutaneous (SC), or intravenous (IV) followed by IP or (IV) challenge infection (60, 108). By contrast, Mense et al. demonstrated that intranasal (IN) inoculation of virulent *B. melitensis* 16M can cause chronic infection in BALB/c mice (77). In a different study, Ficht et al. also demonstrated that aerosol infection with *B. abortus* caused chronic infection at lower intranasal doses compared to IN *B. melitensis* infection (4×10^2 vs. 1×10^4 CFUs/mouse) respectively (61). These studies demonstrated that *Brucella* species can cause chronic infection either via or subsequent to respiratory infection. However, contrary to the fact that IP vaccination protected against brucellosis in mouse models, IP vaccination did not protect against aerosol challenge. Both Ficht et al. and Olsen et al. failed to show clearance of *B. melitensis* and/or *B. abortus* from lung upon aerosol challenge following IP vaccination with protective vaccine strains including *B. abortus* strain RB51 (61, 92).

B. abortus strain RB51 is a USDA approved live attenuated rough vaccine used in the United States and many other countries against cattle brucellosis. Another live attenuated *B. abortus* strain RB51SOD which overexpresses Cu-Zn superoxide dismutase had been shown to elicit better protection than strain RB51 when vaccinated IP against IP challenge with virulent *B. abortus* in mice (123). Protection against brucellosis induced by both these vaccine strains is mediated through a strong $CD4^+$ Th_1 and $CD8^+$ Tc_1 adaptive immune response (52). Nevertheless, vaccine strain RB51 failed to protect mice against IN challenge with virulent *B. abortus* when vaccinated IP (92).

Part of the reason for this lack of protection may be due to the route of vaccination. Based on continuously developing knowledge, it is expected that mucosal vaccination would enhance mucosal protective immune response against aerosol challenge (82). Therefore, an IN vaccination with either vaccine strains RB51 or RB51SOD was expected to protect mice against IN challenge with virulent *B. abortus*. However, preliminary data from our laboratory demonstrated that intranasal vaccination alone with strains RB51 or RB51SOD would not elicit protection against intranasal challenge with virulent *B. abortus* strain 2308 in BALB/c mice. This finding warranted further exploration into the events which led to lack of protective immunity elicited by these vaccine strains. Although it has been proven that both vaccine strains induce protection through T-cell mediated immunity, limited information is available on how they stimulate innate immune response which results in protective CMI. This knowledge is critical to improving these protective animal vaccines for their ultimate use in humans against aerosol brucellosis infection.

A robust innate immune response is necessary to initiate a strong adaptive immune response. Dendritic cells (DCs) and macrophages are the two antigen presenting cells of the innate immune system (8). DCs are the better antigen presenting cells (APC) and are more susceptible to *Brucella* infection. DCs recognize and capture antigen, and subsequently migrate to secondary lymphoid organs (8). There the DCs present the antigens to naïve T-lymphocytes, thus resulting in the initiation of specific adaptive immune responses (8). Based on DC activation status and the cytokines they produce, DCs prime T cell phenotype and function (Th1/Th2 or regulatory T cells or Th17 cells) (8). Inadequate DC activation characterized by high expression of MHC class II and costimulatory markers and limited cytokine production might lead to T-cell tolerance (72). Thus, DCs play a crucial role in bridging the innate and adaptive immune

response by acting as the key mediator. Therefore, the enhanced ability of rough vaccine strains for inducing DC maturation and function could be critical for a protective T-cell response.

There are no published data on how rough vaccine strains RB51 or RB51SOD affect DC maturation, activation and function. However, there are contradictory data on the effects of strain 2308 on DC maturation (13, 73, 130). Previous studies have established the use of murine bone marrow derived dendritic cells (BMDCs) as a model system for studying the effects of bacterial infection on DC phenotype in vitro (73). In **chapter 2** of this dissertation, we discuss the effect of vaccine strains RB51, RB51SOD and pathogenic *B. abortus* strain 2308 on DC phenotype and cytokine production using murine BMDCs.

Although it is important to improve the protective ability of live rough vaccine strain RB51 by delineating its innate immune activation ability, safety concerns limit their ultimate use in humans. Therefore, ideally, heat killed (HK) or irradiated (IR) strain RB51 vaccine which still induces efficacious protective immune responses has the potential as a safer human vaccine. Previous studies have shown that both HK and IR *B. abortus* strains induce Th1 immunity (54, 56, 107). However, the differential ability of live, HK and IR rough and smooth strains of *B. abortus* to stimulate BMDC activation and function at the same doses has not been reported in literature. **Chapter 3** of my dissertation presents the data from our *in vitro* study designed to determine whether HK and IR strain RB51 stimulated comparable innate responses to live vaccine strain RB51 for exploring their use as vaccine in people and animals.

The above mentioned *in vitro* studies helped us delineate rough vs. smooth *B. abortus* strain mediated DC activation and function. However, in an accidental or deliberate aerosol *Brucella* exposure, the organism is directly delivered to the pulmonary airways and airway epithelium. Although pulmonary DCs comprise only a small fraction of innate immune cells in

lung compared to alveolar macrophages, they have the unique ability to migrate to the draining lymph node with the captured antigen to activate naïve T-cells (57). However, it is expected that pulmonary DCs behave differently in some significant respects to BMDCs. Given the inability of vaccine strains RB51 and RB51SOD to protect against IN challenge with pathogenic strain 2308 upon IN vaccination, it is crucial to understand the differential ability of these vaccine strains given IN to stimulate innate immunity *in vivo*. Additionally these studies will also provide information as to whether *B. abortus* pathogenic strain 2308 limits the proinflammatory response in the lungs. The ability to minimize the innate immune response may allow both *Brucella* species to subvert the immune response and allow for systemic spread. To our knowledge, no studies have been published which characterize the in vivo innate immune response including the associated histopathological changes to IN inoculation of either *B. abortus* pathogenic strain 2308 or rough vaccine strains RB51 or RB51SOD. In **chapter 4**, we evaluated the differential ability of *B. abortus* rough vaccine strains RB51, RB51SOD and smooth pathogenic strain 2308 to elicit pulmonary DC activation and function *in vivo*. We also assessed the vaccine and virulent strain induced histopathological changes in lung at day 3, 5, 7 and 14 post infection (PI).

While considering DCs as the major mediator of host innate response, in order for the activation to occur, DCs must first recognize rough and/or smooth strains of *B. abortus.* DCs recognize microbes via host cell membrane receptors called Toll-Like Receptors (TLRs) (58). Upon recognition of microbial products, TLRs transduce signals via common adaptor molecules to activate their host cells (58, 87). Published literature suggest that *B. abortus* signals through multiple TLRs such as TLR2 (outer membrane proteins), TLR4 (lipopolysaccharide) and TLR9 (CpG DNA) (11, 19, 55, 88, 126, 130). However, there are contradictory data on the most crucial TLRs in recognition of *B. abortus* by DCs. Weiss et al. demonstrate that *Brucella* signals through

TLR2, TLR4, and MyD88; the latter is most critical for clearance (126). Although TLR2 and TLR4 both signal through MyD88, their studies suggest an additional role for TLR9 molecule. Subsequently, Oliveira et al. also suggested a prominent role for TLR9 in DC, IL-12 production and *Brucella* clearance (88). Zwerdling et al. provided data suggests that *Brucella* signals through TLR2 and TLR4 (130). Despite how individual TLRs activate a cell, there is a dearth in information whether a difference in TLR preference exists between rough and smooth strains of *Brucella* in mediating DC activation. Identifying a differential TLR activation, if it exists, between *B. abortus* rough vaccine strain RB51 and smooth strain 2308 will help us to improve innate immune stimulating ability of strain RB51 by using TLR agonist adjuvants. In order to address this question, we infected TLR2, TLR4, TLR9 KO BALB/c BMDCs and wild type control BMDCs with rough strain RB51 and smooth strain 2308 to analyze the difference in DC activation and function. Additionally, no published studies have addressed the role of TLRs in the clearance of rough or smooth *B. abortus* strains from intranasally infected mice. It is critical to know if the pulmonary clearance of smooth *B. abortus* is directly related to recognition of bacteria through a particular TLR to identify the best strategy to induce enhanced clearance. In our study, we infected BALB/c control mice as well as TLR2, TLR4, TLR9 KO mice with either vaccination dose of strain RB51 or challenge dose of strain 2308 to assess TLR dependent clearance of *Brucella* strains. **Chapter 5** of my dissertation discuss the role of TLR2, TLR4 and TLR9 in the differential activation of DCs upon infection with *B. abortus* rough and smooth strains *in vitro* and in the clearance of a *Brucella* challenge *in vivo*.

Concurrent with unraveling the innate immune response to *Brucella* vaccine and pathogenic strains, the ultimate goal of the experiments which constitute this dissertation is to develop a protective vaccine for animals and people against respiratory challenge with *Brucella.*

Although our initial efforts with IN vaccination of strain RB51 or RB51SOD failed to protect against IN challenge with strain 2308, we performed subsequent experiments to identify alternate vaccination strategies which might yield protection. Those strategies included testing different vaccination routes, doses, booster vaccination, various prime-boost strategies (involving systemic and IN vaccination routes) using *B. abortus* vaccine strains RB51 and RB51SOD to protect against intranasal exposure to pathogenic *B. abortus* 2308. **Chapter 6** describes the results and conclusions from those experiments.

In summary, experiments from this dissertation research determined the extent to which *B. abortus* vaccine and pathogenic strain mediated DC activation and function *in vitro* and *in vivo* as well as the roles played by various TLRs in inducing DC response *in vitro* and pulmonary clearance *in vivo*. Experiments were also designed to test different vaccination strategies to protect against an aerosol challenge with virulent strain *B. abortus.*

References

1. **Acha, P., Szyfres, B. (ed.).** 2001. Zoonoses and communicable diseases common to man and animal. , vol. I, Bacterioses and Mycoses. Pan American Health Organization, WHO.
2. **Al-Mariri, A., A. Tibor, P. Mertens, X. De Bolle, P. Michel, J. Godefroid, K. Walravens, and J. J. Letesson.** 2001. Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. Infect Immun **69:**4816-4822.
3. **Alton, G. G.** 1978. Recent developments in vaccination against bovine brucellosis. Aust Vet J **54:**551-557.

4. **Araya, L. N., P. H. Elzer, G. E. Rowe, F. M. Enright, and A. J. Winter.** 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J Immunol **143:**3330-3337.

5. **Araya, L. N., and A. J. Winter.** 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect Immun **58:**254-256.

6. **Arellano-Reynoso, B., N. Lapaque, S. Salcedo, G. Briones, A. E. Ciocchini, R. Ugalde, E. Moreno, I. Moriyon, and J. P. Gorvel.** 2005. Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. Nat Immunol **6:**618-625.

7. **Bae, J. E., G. G. Schurig, and T. E. Toth.** 2002. Mice immune responses to *Brucella abortus* heat shock proteins. Use of baculovirus recombinant-expressing whole insect cells, purified *Brucella abortus* recombinant proteins, and a vaccinia virus recombinant as immunogens. Vet Microbiol **88:**189-202.

8. **Banchereau, J., and R. M. Steinman.** 1998. Dendritic cells and the control of immunity. Nature **392:**245-252.

9. **Barrionuevo, P., J. Cassataro, M. V. Delpino, A. Zwerdling, K. A. Pasquevich, C. Garcia Samartino, J. C. Wallach, C. A. Fossati, and G. H. Giambartolomei.** 2008. *Brucella abortus* inhibits major histocompatibility complex class II expression and antigen processing through interleukin-6 secretion via Toll-like receptor 2. Infect Immun **76:**250-262.

10. **Beckett, F. W., and S. C. MacDiarmid.** 1985. The effect of reduced-dose *Brucella abortus* strain 19 vaccination in accredited dairy herds. Br Vet J **141:**507-514.

11. **Berguer, P. M., J. Mundinano, I. Piazzon, and F. A. Goldbaum.** 2006. A polymeric bacterial protein activates dendritic cells via TLR4. J Immunol **176:**2366-2372.

12. **Billard, E., J. Dornand, and A. Gross.** 2007. *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. Infect Immun **75:**4980-4989.

13. **Billard, E., J. Dornand, and A. Gross.** 2007. Interaction of *Brucella suis* and *Brucella abortus* rough strains with human dendritic cells. Infect Immun **75:**5916-5923.

14. **Billard, E., J. Dornand, and A. Gross.** 2008. VirB type IV secretory system does not contribute to *Brucella suis*' avoidance of human dendritic cell maturation. FEMS Immunol Med Microbiol **53:**404-412.

15. **Blasco, J. M., C. Marin, M. P. Jimenez de Bagues, and M. Barberan.** 1993. Efficacy of *Brucella suis* strain 2 vaccine against *Brucella ovis* in rams. Vaccine **11:**1291-1294.

16. **Bossi, P., A. Tegnell, A. Baka, F. Van Loock, J. Hendriks, A. Werner, H. Maidhof, and G. Gouvras.** 2004. Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. Euro Surveill **9:**E15-16.

17. **Bowden, R. A., A. Cloeckaert, M. S. Zygmunt, and G. Dubray.** 1995. Outer-membrane protein- and rough lipopolysaccharide-specific monoclonal antibodies protect mice against *Brucella ovis*. J Med Microbiol **43:**344-347.

18. **Buck, J. M.** 1930. Studies of vaccination during calfhood to prevent bovine infectious abortion. J. Agric. Res. **41:**667.

19. **Campos, M. A., G. M. Rosinha, I. C. Almeida, X. S. Salgueiro, B. W. Jarvis, G. A. Splitter, N. Qureshi, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira.** 2004.

Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. Infect Immun **72:**176-186.

20. **Capasso, L.** 1999. Brucellosis at Herculaneum (79 AD). Int. J. Osteoarchaeol. **9:**277-288.

21. **Cardoso, P. G., G. C. Macedo, V. Azevedo, and S. C. Oliveira.** 2006. *Brucella spp* noncanonical LPS: structure, biosynthesis, and interaction with host immune system. Microb Cell Fact **5:**13.

22. **Cascales, E., and P. J. Christie.** 2003. The versatile bacterial type IV secretion systems. Nat Rev Microbiol **1:**137-149.

23. **Cassataro, J., K. A. Pasquevich, S. M. Estein, D. A. Laplagne, C. A. Velikovsky, S. de la Barrera, R. Bowden, C. A. Fossati, G. H. Giambartolomei, and F. A. Goldbaum.** 2007. A recombinant subunit vaccine based on the insertion of 27 amino acids from Omp31 to the N-terminus of BLS induced a similar degree of protection against *B. ovis* than Rev.1 vaccination. Vaccine **25:**4437-4446.

24. **Cassataro, J., C. A. Velikovsky, S. de la Barrera, S. M. Estein, L. Bruno, R. Bowden, K. A. Pasquevich, C. A. Fossati, and G. H. Giambartolomei.** 2005. A DNA vaccine coding for the *Brucella* outer membrane protein 31 confers protection against *B. melitensis* and *B. ovis* infection by eliciting a specific cytotoxic response. Infect Immun **73:**6537-6546.

25. **Celli, J.** 2006. Surviving inside a macrophage: the many ways of *Brucella*. Res Microbiol **157:**93-98.

26. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med **198:**545-556.

27. **Celli, J., and J. P. Gorvel.** 2004. Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr Opin Microbiol **7:**93-97.

28. **Cheville, N. F., M. G. Stevens, A. E. Jensen, F. M. Tatum, and S. M. Halling.** 1993. Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. Am J Vet Res **54:**1591-1597.

29. **Christie, P. J.** 2004. Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems. Biochim Biophys Acta **1694:**219-234.

30. **Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales.** 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol **59:**451-485.

31. **Cloeckaert, A., P. de Wergifosse, G. Dubray, and J. N. Limet.** 1990. Identification of seven surface-exposed *Brucella* outer membrane proteins by use of monoclonal antibodies: immunogold labeling for electron microscopy and enzyme-linked immunosorbent assay. Infect Immun **58:**3980-3987.

32. **Cloeckaert, A., I. Jacques, N. Bosseray, J. N. Limet, R. Bowden, G. Dubray, and M. Plommet.** 1991. Protection conferred on mice by monoclonal antibodies directed against outer-membrane-protein antigens of *Brucella*. J Med Microbiol **34:**175-180.

33. **Colby, L. A.** 1997. The humoral immune response of Elk (*Cervus elaphus nelsoni*) and mice to vaccination with *Brucella abortus* strain RB51. Virginia Polytechnic Institute and State University.

34. **Corbel, M. J. (ed.).** 2006. Brucellosis in humans and animals. World Health Organization.

35. **Delpino, M. V., J. Cassataro, C. A. Fossati, F. A. Goldbaum, and P. C. Baldi.** 2006. *Brucella* outer membrane protein Omp31 is a haemin-binding protein. Microbes Infect **8:**1203-1208.

36. **Delrue, R. M., C. Deschamps, S. Leonard, C. Nijskens, I. Danese, J. M. Schaus, S. Bonnot, J. Ferooz, A. Tibor, X. De Bolle, and J. J. Letesson.** 2005. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. Cell Microbiol **7:**1151-1161.

37. **Delrue, R. M., M. Martinez-Lorenzo, P. Lestrate, I. Danese, V. Bielarz, P. Mertens, X. De Bolle, A. Tibor, J. P. Gorvel, and J. J. Letesson.** 2001. Identification of *Brucella* spp. genes involved in intracellular trafficking. Cell Microbiol **3:**487-497.

38. **Elberg, S. S., and K. Faunce, Jr.** 1957. Immunization against *Brucella* infection. VI. Immunity conferred on goats by a nondependent mutant from a streptomycin-dependent mutant strain of *Brucella melitensis*. J Bacteriol **73:**211-217.

39. **Elzer, P. H., R. H. Jacobson, K. H. Nielsen, J. T. Douglas, and A. J. Winter.** 1994. BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. Immunol Lett **42:**145-150.

40. **Estein, S. M., M. A. Fiorentino, F. A. Paolicchi, M. Clausse, J. Manazza, J. Cassataro, G. H. Giambartolomei, L. M. Coria, V. Zylberman, C. A. Fossati, R. Kjeken, and F. A. Goldbaum.** 2009. The polymeric antigen BLSOmp31 confers protection against *Brucella ovis* infection in rams. Vaccine **27:**6704-6711.

41. **Ewalt, D. R., P. F. Ross, and J. B. Payeur.** 1990. Identification of *Brucella abortus* strain 19 by decreased ability to utilize erythritol as determined by gas liquid chromatography. J Vet Diagn Invest **2:**120-122.

42. **Ficht, T. A., M. M. Kahl-McDonagh, A. M. Arenas-Gamboa, and A. C. Rice-Ficht.** 2009. Brucellosis: the case for live, attenuated vaccines. Vaccine **27 Suppl 4:**D40-43.

43. **Gee, J. M., M. W. Valderas, M. E. Kovach, V. K. Grippe, G. T. Robertson, W. L. Ng, J. M. Richardson, M. E. Winkler, and R. M. Roop, 2nd.** 2005. 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 **73:**2873-2880.

44. **Godfroid, F., A. Cloeckaert, B. Taminiau, I. Danese, A. Tibor, X. de Bolle, P. Mertens, and J. J. Letesson.** 2000. Genetic organisation of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (wbk). Res Microbiol **151:**655-668.

45. **Godfroid, J., A. Cloeckaert, J. P. Liautard, S. Kohler, D. Fretin, K. Walravens, B. Garin-Bastuji, and J. J. Letesson.** 2005. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet Res **36:**313-326.

46. **Gonzalez, D., M. J. Grillo, M. J. De Miguel, T. Ali, V. Arce-Gorvel, R. M. Delrue, R. Conde-Alvarez, P. Munoz, I. Lopez-Goni, M. Iriarte, C. M. Marin, A. Weintraub, G. Widmalm, M. Zygmunt, J. J. Letesson, J. P. Gorvel, J. M. Blasco, and I. Moriyon.** 2008. Brucellosis vaccines: assessment of Brucella melitensis

lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS ONE **3:**e2760.

47. **Gorvel, J. P., and E. Moreno.** 2002. *Brucella* intracellular life: from invasion to intracellular replication. Vet Microbiol **90:**281-297.

48. **Gross, A., A. Terraza, S. Ouahrani-Bettache, J. P. Liautard, and J. Dornand.** 2000. In vitro *Brucella suis* infection prevents the programmed cell death of human monocytic cells. Infect Immun **68:**342-351.

49. **Guzman-Verri, C., L. Manterola, A. Sola-Landa, A. Parra, A. Cloeckaert, J. Garin, J. P. Gorvel, I. Moriyon, E. Moreno, and I. Lopez-Goni.** 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proc Natl Acad Sci U S A **99:**12375-12380.

50. **He, Y., S. Reichow, S. Ramamoorthy, X. Ding, R. Lathigra, J. C. Craig, B. W. Sobral, G. G. Schurig, N. Sriranganathan, and S. M. Boyle.** 2006. *Brucella melitensis* triggers time-dependent modulation of apoptosis and down-regulation of mitochondrion-associated gene expression in mouse macrophages. Infect Immun **74:**5035-5046.

51. **He, Y., R. Vemulapalli, and G. G. Schurig.** 2002. Recombinant Ochrobactrum anthropi expressing *Brucella abortus* Cu,Zn superoxide dismutase protects mice against *B. abortus* infection only after switching of immune responses to Th1 type. Infect Immun **70:**2535-2543.

52. **He, Y., R. Vemulapalli, A. Zeytun, and G. G. Schurig.** 2001. Induction of specific cytotoxic lymphocytes in mice vaccinated with *Brucella abortus* RB51. Infect Immun **69:**5502-5508.

53. **Hong, P. C., R. M. Tsolis, and T. A. Ficht.** 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect Immun **68:**4102-4107.

54. **Huang, L., A. M. Krieg, N. Eller, and D. E. Scott.** 1999. Induction and regulation of Th1-inducing cytokines by bacterial DNA, lipopolysaccharide, and heat-inactivated bacteria. Infect Immun **67:**6257-6263.

55. **Huang, L. Y., J. Aliberti, C. A. Leifer, D. M. Segal, A. Sher, D. T. Golenbock, and B. Golding.** 2003. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptor 2 dependent. J Immunol **171:**1441-1446.

56. **Huang, L. Y., K. J. Ishii, S. Akira, J. Aliberti, and B. Golding.** 2005. Th1-like cytokine induction by heat-killed *Brucella abortus* is dependent on triggering of TLR9. J Immunol **175:**3964-3970.

57. **Jakubzick, C., F. Tacke, J. Llodra, N. van Rooijen, and G. J. Randolph.** 2006. Modulation of dendritic cell trafficking to and from the airways. J Immunol **176:**3578-3584.

58. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. Annu Rev Immunol **20:**197-216.

59. **Jiang, X., B. Leonard, R. Benson, and C. L. Baldwin.** 1993. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. Cell Immunol **151:**309-319.

60. **Jimenez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant

RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect Immun **62:**4990-4996.

61. **Kahl-McDonagh, M. M., A. M. Arenas-Gamboa, and T. A. Ficht.** 2007. Aerosol infection of BALB/c mice with *Brucella melitensis* and *Brucella abortus* and protective efficacy against aerosol challenge. Infect Immun **75:**4923-4932.

62. **Ko, J., and G. A. Splitter.** 2003. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev **16:**65-78.

63. **Kohler, S., V. Foulongne, S. Ouahrani-Bettache, G. Bourg, J. Teyssier, M. Ramuz, and J. P. Liautard.** 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. Proc Natl Acad Sci U S A **99:**15711-15716.

64. **Kurar, E., and G. A. Splitter.** 1997. Nucleic acid vaccination of *Brucella abortus* ribosomal L7/L12 gene elicits immune response. Vaccine **15:**1851-1857.

65. **Lapaque, N., I. Moriyon, E. Moreno, and J. P. Gorvel.** 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Curr Opin Microbiol **8:**60-66.

66. **Latimer, E., J. Simmers, N. Sriranganathan, R. M. Roop, 2nd, G. G. Schurig, and S. M. Boyle.** 1992. *Brucella abortus* deficient in copper/zinc superoxide dismutase is virulent in BALB/c mice. Microb Pathog **12:**105-113.

67. **Leclerq, S., J. S. Harms, G. M. Rosinha, V. Azevedo, and S. C. Oliveira.** 2002. Induction of a th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus* GroEL heat-shock gene. J Med Microbiol **51:**20-26.

68. **Lee, I. K., S. C. Olsen, and C. A. Bolin.** 2001. Effects of exogenous recombinant interleukin-12 on immune responses and protection against *Brucella abortus* in a murine model. Can J Vet Res **65:**223-228.

69. **Lee, I. K., S. C. Olsen, M. Kehrli, and C. A. Bolin.** 1999. The adjuvant effect of a single dose of interleukin-12 on murine immune responses to live or killed *Brucella abortus* strain RB51. Can J Vet Res **63:**284-287.

70. **Lopez-Goni, I., C. Guzman-Verri, L. Manterola, A. Sola-Landa, I. Moriyon, and E. Moreno.** 2002. Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. Vet Microbiol **90:**329-339.

71. **Lopez-Goni, I., Moriyon, I. (ed.).** 2004. *Brucella*: Molecular and Cellular Biology, 1 ed. Horizon Bioscience, England.

72. **Lutz, M. B., and G. Schuler.** 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol **23:**445-449.

73. **Macedo, G. C., D. M. Magnani, N. B. Carvalho, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira.** 2008. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. J Immunol **180:**1080-1087.

74. **Magnani, D. M., J. S. Harms, M. A. Durward, and G. A. Splitter.** 2009. Nondividing but metabolically active gamma-irradiated *Brucella melitensis* is protective against virulent *B. melitensis* challenge in mice. Infect Immun **77:**5181-5189.

75. **Martinez de Tejada, G., J. Pizarro-Cerda, E. Moreno, and I. Moriyon.** 1995. The outer membranes of *Brucella spp.* are resistant to bactericidal cationic peptides. Infect Immun **63:**3054-3061.

76. **McCord, J. M., B. B. Keele, Jr., and I. Fridovich.** 1971. An enzyme-based theory of obligate anaerobiosis: the physiological function of superoxide dismutase. Proc Natl Acad Sci U S A **68:**1024-1027.

77. **Mense, M. G., L. L. Van De Verg, A. K. Bhattacharjee, J. L. Garrett, J. A. Hart, L. E. Lindler, T. L. Hadfield, and D. L. Hoover.** 2001. Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. Am J Vet Res **62:**398-405.

78. **Montaraz, J. A., A. J. Winter, D. M. Hunter, B. A. Sowa, A. M. Wu, and L. G. Adams.** 1986. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun **51:**961-963.

79. **Murphy, E. A., J. Sathiyaseelan, M. A. Parent, B. Zou, and C. L. Baldwin.** 2001. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology **103:**511-518.

80. **Mustafa, A. A., and M. Abusowa.** 1993. Field-oriented trial of the Chinese *Brucella suis* strain 2 vaccine on sheep and goats in Libya. Vet Res **24:**422-429.

81. **Nene, V., Kole, Chittaranjan. (ed.).** 2009. Genome Mapping and Genomics in Animal-Associated Microbes, 1 ed. Springer-Verlag, Berlin Heidelberg.

82. **Neutra, M. R., and P. A. Kozlowski.** 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol **6:**148-158.

83. **Nielsen, K.** 2002. Diagnosis of brucellosis by serology. Vet Microbiol **90:**447-459.

84. **Nielsen, K., and J. R. Duncan.** 1988. Antibody isotype response in adult cattle vaccinated with *Brucella abortus* S19. Vet Immunol Immunopathol **19:**205-214.

85. **Nielsen, K., Duncan, J. R. (ed.).** 1990. Animal Brucellosis. CRC press, Inc., Florida.

86. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol Microbiol **33:**1210-1220.

87. **O'Neill, L. A.** 2006. How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol **18:**3-9.

88. **Oliveira, S. C., F. S. de Oliveira, G. C. Macedo, L. A. de Almeida, and N. B. Carvalho.** 2008. The role of innate immune receptors in the control of *Brucella abortus* infection: toll-like receptors and beyond. Microbes Infect **10:**1005-1009.

89. **Oliveira, S. C., and G. A. Splitter.** 1995. CD8+ type 1 CD44hi CD45 RBlo T lymphocytes control intracellular *Brucella abortus* infection as demonstrated in major histocompatibility complex class I- and class II-deficient mice. Eur J Immunol **25:**2551-2557.

90. **Oliveira, S. C., and G. A. Splitter.** 1996. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. Vaccine **14:**959-962.

91. **Oliveira, S. C., Y. Zhu, and G. A. Splitter.** 1994. Recombinant L7/L12 ribosomal protein and gamma-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4+ T cells. Immunology **83:**659-664.

92. **Olsen, S. C., W. R. Waters, and W. S. Stoffregen.** 2007. An aerosolized *Brucella spp.* challenge model for laboratory animals. Zoonoses Public Health **54:**281-285.

93. **Onate, A. A., R. Vemulapalli, E. Andrews, G. G. Schurig, S. Boyle, and H. Folch.** 1999. Vaccination with live Escherichia coli expressing *Brucella abortus* Cu/Zn

superoxide dismutase protects mice against virulent *B. abortus*. Infect Immun **67:**986-988.

94. **Palmer, D. A., and J. T. Douglas.** 1989. Analysis of *Brucella* lipopolysaccharide with specific and cross-reacting monoclonal antibodies. J Clin Microbiol **27:**2331-2337.

95. **Pappas, G., P. Panagopoulou, L. Christou, and N. Akritidis.** 2006. *Brucella* as a biological weapon. Cell Mol Life Sci **63:**2229-2236.

96. **Pasnik, D. J., R. Vemulapalli, S. A. Smith, and G. G. Schurig.** 2003. A recombinant vaccine expressing a mammalian *Mycobacterium sp.* antigen is immunostimulatory but not protective in striped bass. Vet Immunol Immunopathol **95:**43-52.

97. **Pasquevich, K. A., S. M. Estein, C. G. Samartino, A. Zwerdling, L. M. Coria, P. Barrionuevo, C. A. Fossati, G. H. Giambartolomei, and J. Cassataro.** 2009. Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infect Immun **77:**436-445.

98. **Plommet, M.** 1987. Brucellosis and immunity: humoral and cellular components in mice. Ann Inst Pasteur Microbiol **138:**105-110.

99. **Purcell, B. K., Hoover, D. L., Friedlander, A.M. (ed.).** 2007. Medical aspects of biological warfare. Department of Defense, Office of The Surgeon General, US Army, Borden Institute., Washington D.C.

100. **Ramamoorthy, S., N. Sanakkayala, R. Vemulapalli, R. B. Duncan, D. S. Lindsay, G. S. Schurig, S. M. Boyle, R. Kasimanickam, and N. Sriranganathan.** 2007. Prevention of lethal experimental infection of C57BL/6 mice by vaccination with *Brucella abortus* strain RB51 expressing *Neospora caninum* antigens. Int J Parasitol **37:**1521-1529.

101. **Ramamoorthy, S., N. Sanakkayala, R. Vemulapalli, N. Jain, D. S. Lindsay, G. S. Schurig, S. M. Boyle, and N. Sriranganathan.** 2007. Prevention of vertical transmission of *Neospora caninum* in C57BL/6 mice vaccinated with Brucella abortus strain RB51 expressing *N. caninum* protective antigens. Int J Parasitol **37:**1531-1538.

102. **Rasool, O., E. Freer, E. Moreno, and C. Jarstrand.** 1992. Effect of *Brucella abortus* lipopolysaccharide on oxidative metabolism and lysozyme release by human neutrophils. Infect Immun **60:**1699-1702.

103. **Ray, W. C., R. R. Brown, D. A. Stringfellow, P. R. Schnurrenberger, C. M. Scanlan, and A. I. Swann.** 1988. Bovine brucellosis: an investigation of latency in progeny of culture-positive cows. J Am Vet Med Assoc **192:**182-186.

104. **Riley, L. K., and D. C. Robertson.** 1984. Ingestion and intracellular survival of *Brucella abortus* in human and bovine polymorphonuclear leukocytes. Infect Immun **46:**224-230.

105. **Roop, R. M., 2nd, B. H. Bellaire, M. W. Valderas, and J. A. Cardelli.** 2004. Adaptation of the *Brucellae* to their intracellular niche. Mol Microbiol **52:**621-630.

106. **Roux, C. M., H. G. Rolan, R. L. Santos, P. D. Beremand, T. L. Thomas, L. G. Adams, and R. M. Tsolis.** 2007. *Brucella* requires a functional Type IV secretion system to elicit innate immune responses in mice. Cell Microbiol **9:**1851-1869.

107. **Sanakkayala, N., A. Sokolovska, J. Gulani, H. Hogenesch, N. Sriranganathan, S. M. Boyle, G. G. Schurig, and R. Vemulapalli.** 2005. Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diagn Lab Immunol **12:**1429-1436.

108. **Schurig, G. G., R. M. Roop, 2nd, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol **28:**171-188.

109. **Schurig, G. G., N. Sriranganathan, and M. J. Corbel.** 2002. Brucellosis vaccines: past, present and future. Vet Microbiol **90:**479-496.

110. **Seleem, M. N., S. M. Boyle, and N. Sriranganathan.** 2008. *Brucella*: a pathogen without classic virulence genes. Vet Microbiol **129:**1-14.

111. **Seleem, M. N., S. M. Boyle, and N. Sriranganathan.** Brucellosis: a re-emerging zoonosis. Vet Microbiol **140:**392-398.

112. **Stabel, T. J., Z. Sha, and J. E. Mayfield.** 1994. Periplasmic location of *Brucella abortus* Cu/Zn superoxide dismutase. Vet Microbiol **38:**307-314.

113. **Stevens, T. L., A. Bossie, V. M. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta.** 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature **334:**255-258.

114. **Sulitzeanu, D.** 1965. Mechanism of immunity against *brucella*. Nature **205:**1086-1088.

115. **Tatum, F. M., P. G. Detilleux, J. M. Sacks, and S. M. Halling.** 1992. Construction of Cu-Zn superoxide dismutase deletion mutants of *Brucella abortus*: analysis of survival in vitro in epithelial and phagocytic cells and in vivo in mice. Infect Immun **60:**2863-2869.

116. **Tizard, I. R. (ed.).** 2008. Veterinary Immunology: An Introduction, 8 ed. Elsevier.

117. **USDA-APHIS.** 2003. Brucellosis eradication: Uniform methods and Rules, p. 121. *In* USDA-APHIS (ed.). USDA-APHIS.

118. **Velikovsky, C. A., F. A. Goldbaum, J. Cassataro, S. Estein, R. A. Bowden, L. Bruno, C. A. Fossati, and G. H. Giambartolomei.** 2003. *Brucella* lumazine synthase elicits a

mixed Th1-Th2 immune response and reduces infection in mice challenged with *Brucella abortus* 544 independently of the adjuvant formulation used. Infect Immun **71:**5750-5755.

119. **Vemulapalli, R., A. Contreras, N. Sanakkayala, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2004. Enhanced efficacy of recombinant *Brucella abortus* RB51 vaccines against *B. melitensis* infection in mice. Vet Microbiol **102:**237-245.

120. **Vemulapalli, R., S. Cravero, C. L. Calvert, T. E. Toth, N. Sriranganathan, S. M. Boyle, O. L. Rossetti, and G. G. Schurig.** 2000. Characterization of specific immune responses of mice inoculated with recombinant vaccinia virus expressing an 18-kilodalton outer membrane protein of *Brucella abortus*. Clin Diagn Lab Immunol **7:**114-118.

121. **Vemulapalli, R., Y. He, S. M. Boyle, N. Sriranganathan, and G. G. Schurig.** 2000. *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infect Immun **68:**3290-3296.

122. **Vemulapalli, R., Y. He, L. S. Buccolo, S. M. Boyle, N. Sriranganathan, and G. G. Schurig.** 2000. Complementation of *Brucella abortus* RB51 with a functional wboA gene results in O-antigen synthesis and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect Immun **68:**3927-3932.

123. **Vemulapalli, R., Y. He, S. Cravero, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun **68:**3286-3289.

124. **Vemulapalli, R., Y. He, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2002. *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet Microbiol **90:**521-532.

125. **Vemulapalli, R., J. R. McQuiston, G. G. Schurig, N. Sriranganathan, S. M. Halling, and S. M. Boyle.** 1999. Identification of an IS711 element interrupting the wboA gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immunol **6:**760-764.

126. **Weiss, D. S., K. Takeda, S. Akira, A. Zychlinsky, and E. Moreno.** 2005. MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. Infect Immun **73:**5137-5143.

127. **Winter, A. J.** 1987. Outer membrane proteins of *Brucella*. Ann Inst Pasteur Microbiol **138:**87-89.

128. **Zhan, Y., and C. Cheers.** 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infect Immun **63:**1387-1390.

129. **Zhan, Y., Z. Liu, and C. Cheers.** 1996. Tumor necrosis factor alpha and interleukin-12 contribute to resistance to the intracellular bacterium *Brucella abortus* by different mechanisms. Infect Immun **64:**2782-2786.

130. **Zwerdling, A., M. V. Delpino, P. Barrionuevo, J. Cassataro, K. A. Pasquevich, C. Garcia Samartino, C. A. Fossati, and G. H. Giambartolomei.** 2008. *Brucella* lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. Microbes Infect **10:**1346-1354.

Chapter 2

Live *Brucella abortus* rough vaccine strain RB51 stimulates enhanced innate immune response *in vitro* compared to rough vaccine strain RB51SOD and virulent smooth strain 2308 in murine bone-marrow derived dendritic cells.

Naveen Surendran[a], Elizabeth M. Hiltbold[c], Bettina Heid[a], Nammalwar Sriranganathan[b], Stephen M. Boyle[b], Kurt L. Zimmerman[b], Melissa R. Makris[b], Sharon. G. Witonsky[a*].

(Revised version accepted to Veterinary Microbiology)

Abstract

Brucella spp. are Gram-negative, coccobacillary, facultative intracellular pathogens. *B. abortus* strain 2308 is a pathogenic strain affecting cattle and humans. Rough *B. abortus* strain RB51, which lacks the O-side chain of lipopolysaccharide (LPS), is the live attenuated USDA approved vaccine for cattle in the United States. Strain RB51SOD, which over-expresses Cu-Zn superoxide dismutase (SOD), has been shown to confer better protection than strain RB51 in a murine model. Protection against brucellosis is mediated by a strong CD4+ Th_1 and CD8+ Tc_1 adaptive immune response. In order to stimulate a robust adaptive response, a solid innate immune response, including that mediated by dendritic cells, is essential. As dendritic cells (DCs) are highly susceptible to *Brucella* infection, it is possible that pathogenic strains could limit the innate and thereby adaptive immune response. By contrast, vaccine strains could limit or bolster the innate and subsequent adaptive immune response. Identifying how *Brucella* vaccines stimulate innate and adaptive immunity is critical to enhance vaccine efficacy. The ability of rough vaccine strains RB51 and RB51SOD to stimulate DC function has not been

characterized. We report that live rough vaccine strain RB51 induced significantly better ($p \leq 0.05$) DC maturation and function compared to either strain RB51SOD or smooth virulent strain 2308, based on costimulatory marker expression and cytokine production.

Keywords: BRUCELLA ABORTUS; DENDRITIC CELLS; INNATE IMMUNITY

Introduction

Brucella spp. are small coccobacillary, Gram-negative, facultative intracellular α2-proteobacteria (Acha, 2001). Brucellosis is a world-wide zoonotic disease affecting a broad range of hosts including humans, cattle, goats, sheep, pigs, dogs and marine mammals (Corbel, 2006). In addition to its potential use as a bioterror agent, it causes infertility, abortion and reduced productivity in livestock, all resulting in massive economic losses to industry. Brucellosis is one of the most common anthropozoonoses in the world with more than half a million new human cases reported annually (Pappas et al., 2006). In humans, it can cause acute infection characterized by undulant fever and general malaise; this can evolve into chronic and debilitating disease if untreated quickly. Three of the most infectious species in humans are *B. melitensis*, *B. abortus* and *B. suis*. Of these, smooth *B. abortus* strain 2308 is one of the species affecting cattle.

In contrast to these smooth strains, mutant strains have been studied for their potential use as vaccines. *B. abortus* strain RB51 is a spontaneous naturally occurring mutant of *B. abortus* 2308 and lacks the N-formylperosamine O-polysaccharide of LPS (Schurig et al., 1991). *B. abortus* strain RB51SOD was generated by overexpressing the gene encoding Cu-Zn SOD on a broad host range plasmid (Vemulapalli et al., 2000). Also derived from *B. abortus* is a

rough strain 45/20, whose mutation is unknown and reversible (Schurig et al., 1991), therefore, it is not a good candidate for human vaccine (Schurig et al., 1991).

By contrast, both strains RB51 and RB51SOD have stable mutations and thus are potential vaccine candidates for people. Both strain RB51 and RB51SOD carry an IS711-disrupted *wbo*A gene (putative glycosyl transferase gene, resulting in the lack of the O-side chain), as well as resistance to rifampicin (Vemulapalli et al., 1999).

Challenge studies in mice demonstrated that strain RB51SOD protects better than strain RB51 (Vemulapalli et al., 2000). Enhanced protection by strain RB51SOD is thought to be at least partially mediated by increased CD8 cytotoxic T- lymphocyte activity (He, unpublished, Va Tech). However, there are no published studies assessing the mechanism of enhanced protection between strain RB51 and strain RB51SOD. It has not been demonstrated whether strain RB51SOD upregulates DC mediated immunity versus strain RB51. It is possible that even if strain RB51SOD does not upregulate and/or limits DC mediated function, the adaptive immune response still enhances CD8 function such that strain RB51SOD provides greater protection than strain RB51 in murine models. It is also possible that overexpressed SOD is functional, acting to decrease inflammation that could result in decreased innate, including DC function. Unpublished data (manuscript in preparation) demonstrates that strain RB51SOD does not stimulate as significant inflammatory pulmonary infiltrate when administered intranasally compared to strain RB51. These data support, despite the fact that strain RB51SOD provides better immunity in mouse IP vaccination and IP challenge studies, the increased protection is not associated with enhanced DC function compared to strain RB51. Additional information regarding strain RB51SOD's ability to protect *in vivo* arises from a report by Olsen et al., (22) in which strain RB51SOD did not show better protection than RB51 in bison against challenge with *B. abortus.*

Thus, although murine IP challenge studies demonstrated that strain RB51SOD has enhanced protection vs. strain RB51, there are other data that question the mechanism and extent of protection provided by strain RB51SOD.

In the studies described here we used both rough strains RB51 and RB51SOD. Smooth pathogenic and rough vaccine strains can infect DCs and macrophages. Upon infection, *Brucella* infect mononuclear phagocytes and prevent phago-lysosome fusion. Thus, *Brucella* find a replicative niche within phagocytes which provide a means for infected phagocytes to disseminate bacteria throughout the body (Celli, 2006). In comparing the roles of macrophages and DCs, although murine macrophages serve as classic *in vitro* models for *Brucella* infection studies, their role is limited as to the innate immune response. By contrast, as dendritic cells (DCs) bridge the innate and adaptive immune response and DCs are highly susceptible to *Brucella* infection, DCs are a better model for assessing *Brucella*-mediated innate immune responses (Billard et al., 2005). DCs are critical in recognizing, capturing and presenting antigen to naïve and memory T-cells to stimulate the adaptive immune response. Upon pathogen recognition, DCs mature, upregulating both co-stimulatory molecules and cytokine production. The nature of the DC response (i.e., cytokines) dictates the direction of the T-cell response (i.e., T-helper-1 (Th1); Th2, Th17 or regulatory T-cells (T-regs). During this process, DCs often migrate from the site of antigen exposure to secondary lymphoid organs.

As DCs have been demonstrated to be an important cellular target for *Brucella* infection (Billard et al., 2005), DC infection studies are warranted to define the mechanisms of activation and/or inhibition involving both smooth and rough *Brucella* strains.

For the majority of intracellular bacteria such as *Salmonella*, *Listeria*, and *Francisella* (Bosio and Dow, 2005; Brzoza et al., 2004; Svensson et al., 2000), infection will induce DC

maturation. By contrast, pathogenic smooth *B. abortus* strain 2308, possessing the smooth O-chain of LPS, only weakly stimulates DC maturation compared to rough *B. abortus* strain 45/20 (Billard et al., 2007b).

In order to have a reliable *in vitro* model, we used the already established murine BMDC model, including controls, to characterize the effects of *B. abortus* infection on BMDC phenotype and cytokine production. Our goal for this study was to determine the differential ability of live *B. abortus* rough and smooth strains to induce BMDC activation and function. We hypothesized that live rough vaccine strains, as opposed to smooth virulent strain 2308, would stimulate increased BMDC activation and function based on costimulatory molecule cell surface expression and cytokine production.

Materials and Methods

Mice: Female 6-8 weeks old BALB/c mice were obtained from Charles River Laboratories Inc., Wilmington, MA. Mice were used under animal care protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Dendritic cell preparation: Bone marrow-derived DCs (BMDCs) were generated, as previously described (Inaba et al., 1992). Briefly, tibias and fibulas of 7-8 weeks old BALB/c mice were incised and bone marrow (BM) cells removed. Following red blood cell lysis and filtration, the cells were resuspended and plated in RPMI 1640 complete media with 10% non heat-inactivated fetal bovine serum and 20ng/ml rGM-CSF (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C in 5% $C0_2$. Fresh media containing rGM-CSF was added at days 2, 4 and 5 and harvested on day 6. The cells harvested on day 6 were typically 70% $CD11c^+$ and displayed low

levels of MHC class II, CD40 and CD86, consistent with immature DCs. Flow cytometry was performed to confirm immature DC status (Inaba et al., 1992).

***Brucella* strains:** Live attenuated rough *B. abortus* strains RB51, RB51SOD and virulent smooth strain 2308 were used from our stock culture collection (Schurig et al., 1991; Vemulapalli et al., 2000). All experiments with *Brucella* were performed in our CDC approved Biosafety Level (BSL)-3 facility.

Infection experiments: On day 6, DCs were harvested and plated at 5 X 10^5 cells/well in 24 well plates and infected with strain RB51, RB51SOD or strain 2308 at each of the three multiplicities of infection (MOI) 1:1 (DC:*Brucella*), 1:10 and 1:100. Infection was enhanced by a short spin at 1300 rpm (400 x g) for 5 minutes at room temperature. The infected cells were incubated for 4 hours at 37°C in 5% $C0_2$. The infection was terminated by washing the cells with gentamicin (Sigma-Aldrich, St. Louis, MO) at 30µg/ml. The cells were then incubated for an additional 20 hours in complete media with 10ng/ml rGM-CSF and 30µg/ml gentamicin. Control samples were maintained by incubating cells with media (negative control) or *Escherichia coli* LPS *0111:B4* (Sigma) (positive control) (100ng/ml) following the exact same procedure for infection. In replicate experiments, at each time point for each treatment, an aliquot of cells was collected to determine bacteria cell numbers. Cells were washed to remove non-intracellular bacteria, and total BMDCs were counted. BMDCs were then lysed by treating with 1 ml/well of 0.1% Triton x-100 in sterile distilled water for 10 minutes, mixed well and 10-fold serial dilutions were plated onto TSA plates. Intracellular bacteria were counted and number of bacteria per cell was determined.

Viability and infection controls: To quantitate and assess viability, at each time point and with each treatment, Trypan blue was used to differentiate viable and dead cells. Total live and dead BMDC numbers were determined.

Staining and flow cytometry: The cells were harvested 24 hours following infection, and they were stained with the following monoclonal antibodies at 0.1 - 0.2 μg per million cells for FACS analysis: PE-Texas red conjugated anti-CD11c, Biotin-conjugated anti-CD40, Streptavidin Tri-color conjugate, PE-conjugated anti-CD86 were all acquired from Caltag (Invitrogen), and PE-conjugated anti I-A/I-E, acquired from BD Pharmingen, San Jose, CA. Cells were washed and analyzed by BD FACSAria™ flow cytometer.

Cytokine analysis: For cytokine measurement, culture supernatants from *Brucella* infected BMDCs were collected after 24 hours of incubation and stored at -80°C. TNF-α, IL-12_{p70} (bioactive form of IL-12) and IL-4 cytokine levels were subsequently measured using indirect sandwich ELISAs (BD Pharmingen).

Statistical analysis: A normal probability plot was generated to assess if each of the outcomes followed an approximate Gaussian distribution. As the data had a Gaussian distribution, the effect of treatments on expression of various DC maturation and activation markers were tested using a mixed model ANOVA with treatment as a fixed effect and day as a blocking factor (Tukey procedure for multiple comparisons). After a logarithmic (to base e) transformation, TNF-α data was also analyzed using the above mentioned procedure. For IL-12_{p70}, the treatments were compared using the exact Kruskal-Wallis test. The main p-value for this test which applies to the overall dataset for the effect of variable treatments (including samples from all different MOIs per treatment) was > 0.05 (0.0889). By this method, all different MOIs were analyzed together; therefore, there was no consideration if only certain MOIs have a significant effect. As

the pattern of IL-12_{p70} secretion between different treatments was similar to TNF-α and we used Dunn's procedure for two-way comparisons as a post hoc test on IL-12_{p70} data. Significance was set at $p \leq 0.05$. All analyses were performed using the SAS system (Cary, NC, USA).

Results

Harvested bone marrow cells at day 6 were found to be predominantly (70%) $CD11c^+$ immature dendritic cells: $CD11c^+$ expression on the harvested cells was determined to calculate the yield and percentage of BMDCs following 6 days of culture and after each treatment. Fig. 1A depicts the level of $CD11c^+$ expression on bone marrow cells prior to and following each treatment as a percentage of total cells collected (harvested). Bone marrow cells were gated based on size and granularity and almost 70% of the total gated cells expressed $CD11c^+$ on day 6. Following overnight infection with all treatments except LPS, the percentage of $CD11c^+$ cells increased to 84-90% (Fig. 1A) of total gated cells compared to day 6 media only ($p<0.05$). By contrast, with *E. coli* LPS treatment, only 71.65% of cells were $CD11c^+$. In addition, >99% of all $CD11c^+$ cells from all treatments were positive for expression of CD11b (data not shown). On day 6, non-stimulated (day 6) $CD11c^+$ BMDCs expressed an immature phenotype based on surface expression of characteristic maturation markers MHC class II, CD40 and CD86 (Fig.1B). LPS significantly upregulated DC maturation markers MHC class II, CD40 and CD86 compared to unstimulated BMDCs (24 hr media and day 6) ($p<0.05$). Media only (24 hr media) samples had similar expression patterns of surface markers as that of day 6 unstimulated BMDCs.

Rough vaccine strain RB51 significantly up-regulated MHC class II expression on BMDCs compared to strain 2308: LPS stimulated DC maturation characterized by significant upregulation of MHC class II high expression compared to media only (Fig. 1B) ($p<0.05$). At

MOI 1:1, none of the *Brucella* strains induced significant upregulation of MHC class II expression on BMDCs compared to media. A dose related increase in upregulation of MHC class II was observed with rough and smooth strain-infected BMDCs at both MOIs of 1:10 and 1:100 with only some doses and treatments being significantly different than 24 hr media only (Fig.2A). Rough vaccine strain RB51 induced significantly ($p<0.05$) higher upregulation of BMDC MHC class II high expression at both 1:10 and 1:100 MOIs compared to media only. By contrast, strain RB51SOD and strain 2308 induced significant ($p<0.05$) increases in MHC class II high only at MOI 1:100 (Fig.2A). Furthermore, strain RB51 infected BMDCs at MOI 1:100 induced significantly higher expression of MHC class II high compared to strain 2308 infected BMDCs at MOI 1:100 ($p = 0.0079$) (Fig.2A). In addition, to the increased percentage of CD11c that upregulated MHC high class II expression, strain RB51 induced a significant ($p<0.05$) increase in total number of MHC class II high expressing DCs at 1:100 MOI compared to LPS treated BMDCs (Fig. 2B). Both LPS and strain RB51 induced significantly greater MHC class II expressing cells than media only. Fig. 2C demonstrates the enhanced MHC class II expression by strain RB51 infected BMDCs compared to strain 2308 ($p <0.05$). Although strain RB51SOD induced higher average expression of MHC II on DCs than strain 2308 at MOIs 1:10 and 1:100, it was not statistically significant.

Rough vaccine strain RB51 infected immature BMDCs induced higher expression of costimulatory molecules CD40 and CD86 compared to strain RB51SOD and strain 2308: Strain RB51 consistently had higher expression levels of each costimulatory molecule CD40 and CD86 at MOIs 1:10 (data not shown) and 1:100 compared to strain RB51SOD or virulent strain 2308 (Fig. 3A and 3B). Both strain RB51 and RB51SOD induced significantly greater CD40 expression ($p <0.05$) on infected DCs compared to media only (Fig.3A). Interestingly, strain

2308 along with rough strain RB51 at MOIs 1:10 and 1:100 promoted significant ($p<0.05$) upregulation of CD86 on infected DCs compared to media only. In addition at MOI 1:10, strain RB51 infected DC-CD86 expression was significantly ($p<0.05$) greater than strain RB51SOD infected DCs (data not shown). At 1:100, strain RB51 induced significantly greater ($p<0.05$) CD86 expression levels on infected DCs ($p<0.05$) compared to LPS induced levels (Fig.3B). At MOIs 1:10 and 1:100, strain RB51 induced significant ($p<0.05$) upregulation of $CD40^{+}/CD86^{+}$ coexpression on infected DCs compared to media. At MOI 1:100, strain RB51 induced CD40/CD86 coexpression was even significantly higher than LPS positive control, strain RB51SOD and strain 2308 infected BMDCs (p values: 0.0036, 0.049 and 0.021 respectively) (Fig.3C).

DC functional analysis: Strain RB51 induced higher IL-12 and TNF-α secretion than other treatments: Rough strain RB51 at MOI 1:100 induced significantly greater ($p\leq0.05$) production of TNF-α compared to strain RB51SOD and strain 2308 infected DCs at all MOIs (Fig. 4A). IL-12 production was also significantly higher ($p\leq0.05$) with strain RB51 at MOI 1:100 compared to strain 2308 at all MOIs and strain RB51SOD at MOI 1:1 and 1:10 (Fig. 4B).

Discussion

In our study, we determined the dose dependent BMDC phenotypic maturation upon *Brucella* infection. At a MOI 1:1 (DC:*Brucella*), strain 2308 infected DCs had non-significantly increased MHC class II high expression above the media control. At 1:10, strain 2308 infected BMDCs induced significantly higher CD86 expression compared to media. At a MOI 1:100, strain 2308 induced significant levels of all maturation markers (CD40, CD86, MHC class II) on infected BMDCs compared to media control. At MOIs of 1:10 and 1:100 strain RB51 stimulated

significantly higher expression of MHC class II than strain 2308. At 1:100, strain RB51 infected BMDCs had greater CD40/CD86 expression than strain 2308 (Figures 2A-C, 3A-3C). Additionally, strain RB51 stimulated enhanced BMDC function more than strain 2308 as well based on TNF-alpha and IL-12p70 cytokine production. These data illustrated that strain RB51 enhanced BMDC maturation and function greater than strain 2308.

In comparing our results with others, recent publications show conflicting reports on the effects of strain 2308 on DC maturation. These differences can at least partially be explained based on differing cell and *Brucella* concentration. Billard et al. (Billard et al., 2007a, b) and Salcedo et al. (Salcedo et al., 2008) reported that smooth strain 2308 inhibited DC maturation whereas Zwerdling et al. (Zwerdling et al., 2008) and Macedo et al. (Macedo et al., 2008), who both used higher concentrations of DCs, reported that smooth strain 2308 induced DC maturation. In our studies, we also found that there was a dose dependent response. Having shown the differential ability of *Brucella* rough and smooth strains in DC maturation, the questions remain as to what are the minimal requirements of DC activation/function needed to promote CD4 Th_1 immunity. Recently it has been shown that DCs can bias the T-cell response towards a CD4 Th_1, Th_2 T-reg or Th_{17} phenotype/population. The requirements by DCs and T-cells for each specific DC mediated T-cell responses are not fully understood.

With regard to tolerance/anergy, certain types of immature and mature DCs can induce tolerance (Steinman et al., 2003). Some immature DCs in peripheral tissues expressing low levels of MHC class II and co-stimulatory molecules can induce T-cell anergy or regulatory T-cells (Jonuleit et al., 2000). In addition, other murine BMDC studies show that stimulated DCs can induce tolerance (Akbari et al., 2001). Despite the high expression of MHC class II and costimulatory markers, these tolerogenic DCs do not produce proinflammatory cytokines,

particularly IL-12_{p70}; they are referred to as "semimature DCs" (Lutz and Schuler, 2002). Other studies have shown that Gram-negative bacterial pathogens such as *Bordetella pertussis* and antigen such as ovalbumin can stimulate DC maturation with associated IL-10 production; these DCs induce pathogen specific T-regs (Akbari et al., 2001; McGuirk et al., 2002).

Although we did not determine IL-10 levels, it is possible that strain 2308 infected DCs promoted tolerance by inducing T-regs. Strain 2308 infected DCs induced a high level of expression of MHC class II and costimulatory markers compared to media control at MOIs of 1:10 and 1:100, but did not induce significant IL-12_{p70} or TNF-α. Thus it is possible that strain 2308 induced a T-reg response. Baldwin et al. (Fernandes and Baldwin, 1995) demonstrated that *in vivo* neutralization of IL-10 using anti IL-10 monoclonal antibodies in BALB/c mice improved resistance to *B. abortus* infection. This suggested that *Brucella* strain 2308 normally induced a DC mediated IL-10 directed T-reg response, which allowed for chronic infection. Thus, these data suggested that smooth *Brucella* strains could induce tolerogenic DCs producing IL-10, which could stimulate a non-protective T-cell response, resulting in chronic infection.

We assessed differences in strain mediated DC function based on TNF-alpha, IL-12 and IL-4 production. TNF-alpha is a proinflammatory cytokine primarily involved in host defense and DC maturation. DCs producing IL-12 direct the T-cell response to a CD4 Th_1 mediated response. IL-4 dictate a CD4 Th_2 mediated response. Therefore we used these cytokines to assess the differential ability of *Brucella* strains to stimulate DC function. The data presented here established that smooth strain 2308 and rough strain RB51SOD did not induce DC maturation compared to strain RB51 at the same MOI. Both strains RB51SOD and 2308 infected DCs failed to secrete significant amounts of TNF-α or IL-12 at all MOIs compared to strain RB51 infected

BMDCs at MOI 1:100 (Figure 4A and 4B). The lack of TNF-α and IL-12 secretion by strains RB51SOD and 2308 infected DCs support weaker DC function.

By comparison, studies with strain 2308 infected DCs by Billard et al. (Billard et al., 2007a) and Salcedo et al. (Salcedo et al., 2008) also corroborated the impaired DC - TNF-α and IL-12 secretion. Billard et al. (Billard et al., 2007a) additionally determined that *Brucella* outer membrane protein Omp25 blocked TNF-α secretion by smooth strain infected DCs. Omp-25 is expressed on both rough and smooth strains of *B. abortus*, yet RB51 infected DCs at MOI 1:100 stimulated higher TNF-α secretion. This suggested that there were multiple mechanisms regulating TNF-alpha production.

Analyzing the IL-12 (IL-12_{p70}) secretion, only strain RB51 at MOI 1:100 induced DCs to produce substantial levels of IL-12_{p70} (Figure 4B). For a protective CD4 Th_1 and CD8 Tc_1 T-cell response, DC derived IL-12_{p70} is required. Thus, these data suggested that if cell-mediated immunity (CMI) was dictated only by the DC response that only strain RB51 would trigger a protective CD4 Th_1 response compared to strain RB51SOD or strain 2308. This further explains the better innate immune stimulation by vaccine strain RB51 compared to strain RB51SOD.

As strain RB51SOD and strain 2308 infected BMDCs did not induce significant IL-12 or TNF-alpha to direct a CD4 Th_1 response, IL-4 production was assessed. However, none of the strains induced IL-4 secretion. These data suggested that smooth vs. rough strains do not induce an increased DC mediated IL-4 response which would bias towards a CD4 Th_2 polarization.

Clearly, these data do support that strain RB51 vs. strains RB51SOD and 2308 have an increased vs. decreased bias towards a DC mediated CD4 Th_1 CD8 Tc_1 response. As these differences for strains RB51SOD and 2308 are not mediated by IL-4 and a CD4 Th_2 response,

the differences could still be mediated by either IL-10 biasing towards a T-reg or IL-17 biasing towards a Th_{17} response.

With this study, for the first time, we evaluated the *in vitro* innate immune response of immature murine BMDCs to *B. abortus* rough vaccine strains RB51 and RB51SOD infection compared to smooth strain 2308. Understanding the rough vaccine vs. smooth pathogenic strain induced changes in DC phenotype will help identify the mechanism(s) by which rough *Brucella* strains modulate the immune response towards a CD4 Th_1 profile. It will also allow us to begin to define differences in DC function between rough strains (RB51 vs. RB51SOD). This study demonstrated that rough vaccine strain RB51 induced strong DC maturation and function compared to strain RB51SOD or pathogenic strain 2308 at corresponding MOIs. Additionally, there is a dose dependent positive correlation between infection dose and DC phenotypic maturation following rough or smooth *Brucella* infection.

There are several possible explanations for these differences between smooth and rough strains. Some likely explanations include differences in viability/infectivity. Additional differences in *Brucella* specific components/factors (i.e. LPS, outermembrane proteins, virulence factors) could alter DC mediated activation and function.

In order to determine that the differences in DC mediated function were not due to differences in viability of *Brucella* and/or BMDCs, DC viability and *Brucella* numbers were analyzed. DC viability was analyzed in all experiments at 24 hrs. These data showed that at 4hrs there were relatively similar levels of *Brucella*: BMDCs. Data are from 1 of 3 replicates and the counts denote number of intracellular *Brucella* per 100 cells. For just the 1:100 MOI: at 1 hr, *Brucella*: BMDCs for RB51 were 35,254; RB51SOD 16,000; 2308 4,535. For 4hrs, RB51 6,330; RB51SOD 8,760; 2308 19,420; At 24 hrs, strain RB51 124; RB51SOD 378; strain 2308 2,125.

Therefore, while initial 1 hr time point and other data (Pei and Ficht, 2004) supported that rough strains were internalized more rapidly than smooth strains, by 4 hrs, which was when BMDCs were washed with gentamicin, there were much fewer rough vs. smooth strains of bacteria. At 24 hrs, rough strains were still less than smooth strains. This suggested that although rough strains may be internalized and could possibly have more bacteria within activated DCs at 1 hour, by 4 hrs and 24 hrs smooth strains remained higher than rough strains in sufficient numbers to stimulate BMDCs. These data would suggest that even though the number of rough bacteria was greater earlier in the response, as the smooth bacteria had higher numbers at 24 hrs, should have been sufficient to stimulate the BMDCs, if the stimulation differences between strains were regulated only by numbers of bacteria. Thus, the differences are attributable to other characteristics/components of the rough vs. smooth bacteria. It is possible that rough strains could stimulate the BMDCs with large numbers of cells early and that smooth strains are inhibitory and at greater numbers of bacteria at the later time point exert an even more significant inhibitory response. However, there are still characteristically different properties between smooth and rough strains which are associated with the differences in BMDC function.

Some of the additional potential differences to be explored include LPS, VirB, outermembrane protein expression, and *Brucella* Toll Interleukin 1 receptor family containing protein (Btp1). Barquero-Calvo et al. (Barquero-Calvo et al., 2009) recently demonstrated that LPS from smooth *Brucella* does not bind to TLR signaling molecule MD2 as well as *Salmonella* LPS. This supports that LPS has a role in lack of stimulation by smooth strains. Both rough *B. abortus* vaccine strains lack the O-side chain of the LPS making them rough. However, by treating human immature DCs either with rough LPS (100 ng) from *B. abortus* rough strain 45/20 or with smooth LPS (200 ng) from strain 2308, Billard et al. (Billard et al., 2007b)

established that *Brucella* LPS does not stimulate DC maturation and there are no differences in DC maturation between rough or smooth *Brucella* LPS, thus ruling out the possibility for implicating these strains of rough LPS in enhanced DC activation.

Besides LPS, Type IV secretion system encoded by VirB is a virulence factor involved in the control of host defense. VirB controls maturation of the *Brucella*-containing vacuole into a replication permissive organelle. Unfortunately, recent studies have shown that VirB mutants similar to wild type *Brucella* do not control DC maturation (Billard et al., 2008). Thus, this supports the notion that VirB does not have a significant effect in DC mediated function. Another component that has been demonstrated to down-modulate DC maturation through TLR2 is Btp1 (Salcedo et al., 2008). Btp1 decreased CD40 and CD80 activation of DCs as well as decreasing DC mediated IL-6, IFN-beta, TNF alpha, IL-12 production (13). However, it is unlikely that rough vaccine vs. smooth pathogenic strains differ with respect to Btp1 expression. Thus Btp1 is unlikely to have a significant contributing role in the differences between rough and smooth strains.

There is potential that variations in composition or expression of outer membrane proteins on rough vs. smooth strains which could explain differences in DC function. Zwerdling et al. (Zwerdling et al., 2008) and Pasquevich et al. (Pasquevich et al., 2009) both have shown that outer membrane proteins Omp16 and Omp19 are immunostimulatory. Pasquevich et al. (Pasquevich et al., 2009) demonstrated that both Omp16 and Omp19 in its unlipidated version stimulates specific CD4+ and CD8+ T-cells to provide systemic and oral protection to *B. abortus* infection. Additionally, Gonzalez et al. (Gonzalez et al., 2008) demonstrated that the outer membrane proteins are more exposed on rough strains than on smooth *Brucella* due to the absence of O-polysaccharide of the LPS. These recent findings suggested that the relatively more

exposed Omps on the surface of rough vaccine strains RB51 and RB51SOD could stimulate stronger DC maturation and function compared to smooth strain 2308. Additionally, Zwerdling et al. (Zwerdling et al., 2008) described lipidated Omp19 upregulating DC maturation.

The above mentioned discussion provides a few plausible reasons to explain the differences between rough and smooth strains. With regard to differences between strains RB51 and RB51SOD, at this point, we can only speculate as to why strain RB51SOD does not stimulate as well as strain RB51. We do not expect differences in LPS or omps which could be present between rough and smooth strains to be present between strains RB51 and RB51SOD. Therefore, this suggests that there are other possible explanations for differences between strain RB51 and RB51SOD. Strain RB51SOD overexpresses SOD and this was demonstrated by western blot analysis. In future studies, the next step would be to determine whether SOD is functional. As SOD downregulates oxidative damage, it is expected that superoxide (SO) would be decreased. Theoretically, if SOD decreases SO and oxidative inflammation, the inflammatory response associated with infection would be decreased. Decreased DC activation and function could be a consequence. Consistent with this explanation is our preliminary data (not shown, manuscript in preparation) which demonstrates that mice infected IN with strain RB51SOD vs. RB51 show markedly decreased inflammatory infiltrate in the lungs. The next step, in future studies, would be to determine in these experiments whether mice infected with strain RB51SOD vs. RB51 have decreased SO and cytokine mediators in the lungs. This would also support our theory. These findings substantiate our data which demonstrates that strain RB51SOD induces substantially less DC activation and function. Our proposed theory, which will be investigated in future studies, is that strain RB51SOD overproduces functional SOD which downregulates the inflammatory response, and subsequently also including DC mediated activation and function.

While these data are not consistent with the studies demonstrating that strain RB51SOD protects better than strain RB51 in mice vaccinated IP and challenged IP with *B. abortus* strain 2308 (Vemulapalli et al., 2000), Olsen demonstrated that strain RB51SOD when administered subcutaneously did not protect bison against *B. abortus* strain 2308 (Olsen et al., 2009). Thus, although strain RB51SOD provided better protection in mice, it did not provide better protection in bison. This provides support that strain RB51SOD is not a superior vaccine in all species/trials.

Additionally, it is still possible that while strain RB51SOD does not upregulate DC activation and function in a DC mediated CD4 Th_1 response, it is still possible that strain RB51SOD could provide enhanced protection. There are a few possible explanations for these results. It is possible that SOD is an immunodominant antigen, and despite the limited DC response, because SOD is immunodominant, there is still a marked CD4 and CD8 *Brucella* specific response, which results in enhanced protection vs. strain RB51. Another possibility is that strain RB51SOD may direct the DC mediated T-cell response towards a Th_{17} response, which could still result in enhanced bacterial clearance. These possible explanations for strain RB51SOD enhanced protection will be investigated in future studies. In this paper, we have focused on demonstrating that these differences between strain RB51 and RB51SOD do exist. These data provide information on why in some challenge models, strain RB51SOD may not be a more protective vaccine. In addition, if one's goal were to identify a more efficacious vaccine, then generating a recombinant RB51SOD vaccine that upregulated DC function would be ideal based on previous mouse model data (Vemulapalli et al., 2000). However, based on the data presented here, choosing another vaccine or vector other than strain RB51SOD may allow for

enhanced protection. Thus these data provide valuable information on the introductory mechanisms of strain RB51SOD mediated protection.

In conclusion, our study demonstrated that live *B. abortus* rough vaccine strain RB51 stimulated enhanced murine BMDC maturation responses *in vitro* compared to rough strain RB51SOD and virulent smooth strain 2308. The BMDC maturation response upon infection with rough or smooth *B. abortus* strains was dose dependent with maximum DC maturation response to rough strain RB51 at MOIs 1:10 and 1:100. Further experiments are needed to understand the mechanism of enhanced DC function associated with strain RB51; the causes for reduced DC maturation by strain RB51SOD, and the limited ability of strain 2308 to induce DC maturation and function.

Acknowledgements: The authors acknowledge Dr. Stephen Werre for help with the statistical analysis of the data.

References

Acha, P., Szyfres, B. 2001. Zoonoses and communicable diseases common to man and animal. (Pan American Health Organization, WHO).

Akbari, O., DeKruyff, R.H., Umetsu, D.T., 2001, Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat Immunol 2, 725-731.

Barquero-Calvo, E., Conde-Alvarez, R., Chacon-Diaz, C., Quesada-Lobo, L., Martirosyan, A., Guzman-Verri, C., Iriarte, M., Mancek-Keber, M., Jerala, R., Gorvel, J.P., Moriyon, I., Moreno, E., Chaves-Olarte, E., 2009, The differential interaction of *Brucella* and

ochrobactrum with innate immunity reveals traits related to the evolution of stealthy pathogens. PLoS ONE 4, e5893.

Billard, E., Cazevieille, C., Dornand, J., Gross, A., 2005, High susceptibility of human dendritic cells to invasion by the intracellular pathogens *Brucella suis*, *B. abortus*, and *B. melitensis*. Infect Immun 73, 8418-8424.

Billard, E., Dornand, J., Gross, A., 2007a, *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. Infect Immun 75, 4980-4989.

Billard, E., Dornand, J., Gross, A., 2007b, Interaction of *Brucella suis* and *Brucella abortus* rough strains with human dendritic cells. Infect Immun 75, 5916-5923.

Billard, E., Dornand, J., Gross, A., 2008, VirB type IV secretory system does not contribute to *Brucella suis'* avoidance of human dendritic cell maturation. FEMS Immunol Med Microbiol 53, 404-412.

Bosio, C.M., Dow, S.W., 2005, *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. J Immunol 175, 6792-6801.

Brzoza, K.L., Rockel, A.B., Hiltbold, E.M., 2004, Cytoplasmic entry of *Listeria monocytogenes* enhances dendritic cell maturation and T cell differentiation and function. J Immunol 173, 2641-2651.

Celli, J., 2006, Surviving inside a macrophage: the many ways of *Brucella*. Res Microbiol 157, 93-98.

Corbel, M.J. 2006. Brucellosis in humans and animals (World Health Organization).

Fernandes, D.M., Baldwin, C.L., 1995, Interleukin-10 downregulates protective immunity to *Brucella abortus*. Infect Immun 63, 1130-1133.

Gonzalez, D., Grillo, M.J., De Miguel, M.J., Ali, T., Arce-Gorvel, V., Delrue, R.M., Conde-Alvarez, R., Munoz, P., Lopez-Goni, I., Iriarte, M., Marin, C.M., Weintraub, A., Widmalm, G., Zygmunt, M., Letesson, J.J., Gorvel, J.P., Blasco, J.M., Moriyon, I., 2008, Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and O-polysaccharide synthesis and export. PLoS ONE 3, e2760.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., Steinman, R.M., 1992, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176, 1693-1702.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., Enk, A.H., 2000, Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med 192, 1213-1222.

Lutz, M.B., Schuler, G., 2002, Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 23, 445-449.

Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T., Oliveira, S.C., 2008, Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. J Immunol 180, 1080-1087.

McGuirk, P., McCann, C., Mills, K.H., 2002, Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J Exp Med 195, 221-231.

Olsen, S.C., Boyle, S.M., Schurig, G.G., Sriranganathan, N.N., 2009, Immune responses and protection against experimental challenge after vaccination of bison with *Brucella abortus* strain RB51 or RB51 overexpressing superoxide dismutase and glycosyltransferase genes. Clin Vaccine Immunol 16, 535-540.

Pappas, G., Panagopoulou, P., Christou, L., Akritidis, N., 2006, *Brucella* as a biological weapon. Cell Mol Life Sci 63, 2229-2236.

Pasquevich, K.A., Estein, S.M., Samartino, C.G., Zwerdling, A., Coria, L.M., Barrionuevo, P., Fossati, C.A., Giambartolomei, G.H., Cassataro, J., 2009, Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infect Immun 77, 436-445.

Pei, J., Ficht, T.A., 2004, *Brucella abortus* rough mutants are cytopathic for macrophages in culture. Infect Immun 72, 440-450.

Salcedo, S.P., Marchesini, M.I., Lelouard, H., Fugier, E., Jolly, G., Balor, S., Muller, A., Lapaque, N., Demaria, O., Alexopoulou, L., Comerci, D.J., Ugalde, R.A., Pierre, P., Gorvel, J.P., 2008, *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS Pathog 4, e21.

Schurig, G.G., Roop, R.M., 2nd, Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991, Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

Steinman, R.M., Hawiger, D., Nussenzweig, M.C., 2003, Tolerogenic dendritic cells. Annu Rev Immunol 21, 685-711.

Svensson, M., Johansson, C., Wick, M.J., 2000, *Salmonella enterica* serovar typhimurium-induced maturation of bone marrow-derived dendritic cells. Infect Immun 68, 6311-6320.

Vemulapalli, R., He, Y., Cravero, S., Sriranganathan, N., Boyle, S.M., Schurig, G.G., 2000, Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun 68, 3286-3289.

Vemulapalli, R., McQuiston, J.R., Schurig, G.G., Sriranganathan, N., Halling, S.M., Boyle, S.M., 1999, Identification of an IS711 element interrupting the wboA gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin Diagn Lab Immunol 6, 760-764.

Zwerdling, A., Delpino, M.V., Barrionuevo, P., Cassataro, J., Pasquevich, K.A., Garcia Samartino, C., Fossati, C.A., Giambartolomei, G.H., 2008, *Brucella* lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. Microbes Infect 10, 1346-1354.

Figure legends

Figure 1: Bone marrow cells after 6 days of culture are predominantly CD11c$^+$ immature dendritic cells. Bone marrow cells isolated from BALB/c mice were cultured for 6 days in 10% RPMI medium with rGM-CSF (20ng/ml). The cells were harvested on day 6 and analyzed by flow cytometry for CD11c$^+$ marker expression using fluorescent labeled CD11c$^+$ antibody. Gated CD11c$^+$ cells were further analyzed for their maturation status using anti MHC class II, CD40 and CD86 antibodies. A: Comparison between percentages of CD11c$^+$ expressed by bone marrow cells harvested on day 6 to those treated with *B. abortus* rough and smooth strains for 24 hours at different MOIs (DC:*Brucella*; 1:1, 1:10, 1:100). Day 6 DCs treated with *E.coli* LPS (100ng/ml) or media alone for 24 hours served as positive and negative controls respectively. B: As an internal control to assess conditions: Comparison of the percentages of CD11c$^+$ DCs

expressing the surface maturation markers MHC class II, CD40 and CD86 harvested on day 6 to those treated for 24 hours with negative control media alone or positive control *E. coli* LPS (100 ng/ml). Two asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to data represented by an asterisk (*). For A and B, data represents means ± standard deviations of 3 independent experiments.

Figure 2: *B. abortus* Rough vaccine strain RB51 significantly upregulates MHC class II expression on immature BMDCs. Immature BMDCs were infected with either *B. abortus* rough vaccine strains (RB51 or RB51SOD) or with smooth strain 2308 at MOIs (DC:*Brucella*) 1:1, 1:10 and 1:100. At 24 hours post infection (p.i.), BMDCs were analyzed for MHC class II expression. A: Comparison of the percentage of $CD11c^+$ cells (BMDCs) expressing MHC class II high on its surface across different treatment groups. Media and *E. coli* LPS were the negative and positive controls respectively. Double characters **, ## represent statistically significant difference at $p \leq 0.05$ with the corresponding single character representation (*, #). Data represents means ± standard deviations of 3 independent experiments. B: Histogram of a single representative experiment (of 3) showing total MHC class II expression by RB51 infected BMDCs (MOI 1:100) in comparison to media and *E.coli* LPS controls. Filled grey histogram: media control; continuous thick line: *E.coli* LPS; broken thick line: strain RB51. C: Histogram of the same single representative experiment (of total 3) showing total MHC II expression by strains RB51 (broken thick line), RB51SOD (dotted line) or 2308 (continuous thin line) infected BMDCs at MOI 1:100. Double characters (**, ##) denotes statistically significant data at $p \leq 0.05$ compared to data represented by single character (*, #).

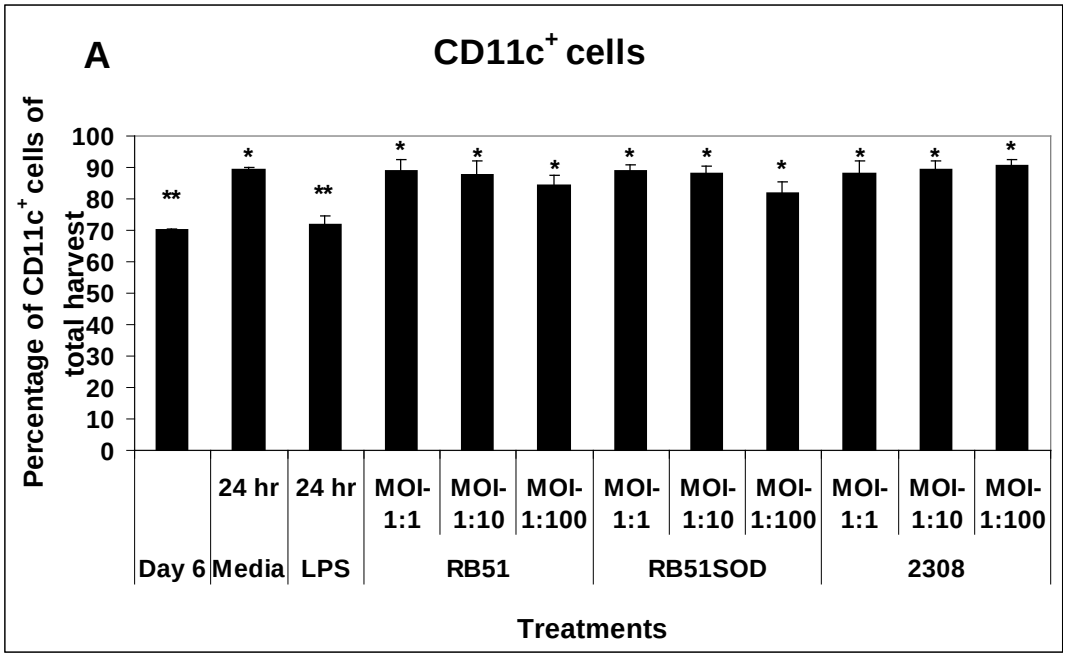
Figure 3: ***B. abortus* rough vaccine strain RB51 significantly upregulates costimulatory marker expression on immature BMDCs.** Immature BMDCs were infected with either *B.*

abortus rough vaccine strains RB51 or RB51SOD or smooth strain 2308 at MOIs (DC:*Brucella*) 1:1, 1:10 and 1:100 at 24 hours post-infection and analyzed for costimulatory marker CD40 and CD86 expression. A: Histogram of a single representative experiment (of 3) showing the CD40 expression on $CD11c^+$ BMDCs infected by strains RB51, RB51SOD and 2308 at MOI 1:100 in comparison to media and LPS controls. . For A, filled grey histogram: media control; continuous thick line: *E.coli* LPS; broken thick line: strain RB51; dotted line: strain RB51SOD; and continuous thin line: strain 2308. Two asterisks (**) denotes statistically significant difference at $p \leq 0.05$ compared to data represented by an asterisk (*) B: Histogram of same single representative experiment (of 3) showing the CD86 expression on $CD11c^+$ BMDCs infected by strains RB51, RB51SOD or 2308 at MOI 1:100 in comparison to media and LPS controls. For B, filled grey histogram: media control; continuous thick line: *E.coli* LPS; broken thick line: strain RB51; dotted line: strain RB51SOD; and continuous thin line: strain 2308. Two characters (**, ##) denotes statistically significant data at $p \leq 0.05$ compared to data represented by single character (*, #) C: Comparison of the percentage of $CD40^+/CD86^+$ coexpression on $CD11c^+$ cells (BMDCs) across different treatment groups. Media and *E. coli* LPS are the negative and positive controls respectively. Double characters ** and ## represent statistically significant change at $p \leq 0.05$ with the corresponding single character representation (*, #). Data represents means ± standard deviations of 3 independent experiments.

Figure 4: *B. abortus* rough vaccine strain RB51 induces higher TNF-α and IL-12 secretion. To assess DC function, TNF-α (A) and IL-12_{p70} (B) levels in 24 hour culture supernatants of *B. abortus* strain RB51, RB51SOD or 2308 infected BMDCs at various MOIs were analyzed using indirect sandwich ELISA. *E. coli* LPS and media treated cell supernatants served as the positive and negative controls respectively. The limits of detection for both the cytokines were 15pg/ml.

The TNF-α results represent means ± SEM of 3 independent experiments. The IL-12_{p70} results represent medians and ranges of 3 independent experiments. Two asterisks (**) denotes statistically significant difference at $p \leq 0.05$ compared to data represented by an asterisk (*).

Figure 1


A
CD11c+ cells
Percentage of CD11c+ cells of total harvest
100
90
80
70
60
50
40
30
20
10
0
Day 6
24 hr Media
24 hr LPS
MOI-1:1
MOI-1:10
MOI-1:100
MOI-1:1
MOI-1:10
MOI-1:100
MOI-1:1
MOI-1:10
MOI-1:100
RB51
RB51SOD
2308
Treatments

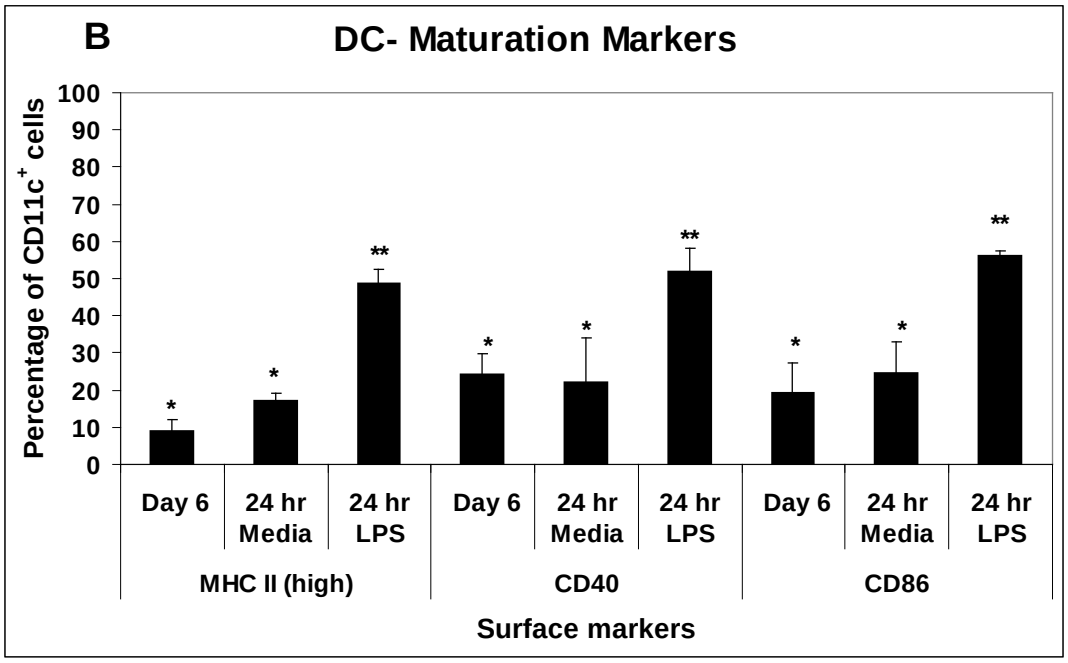
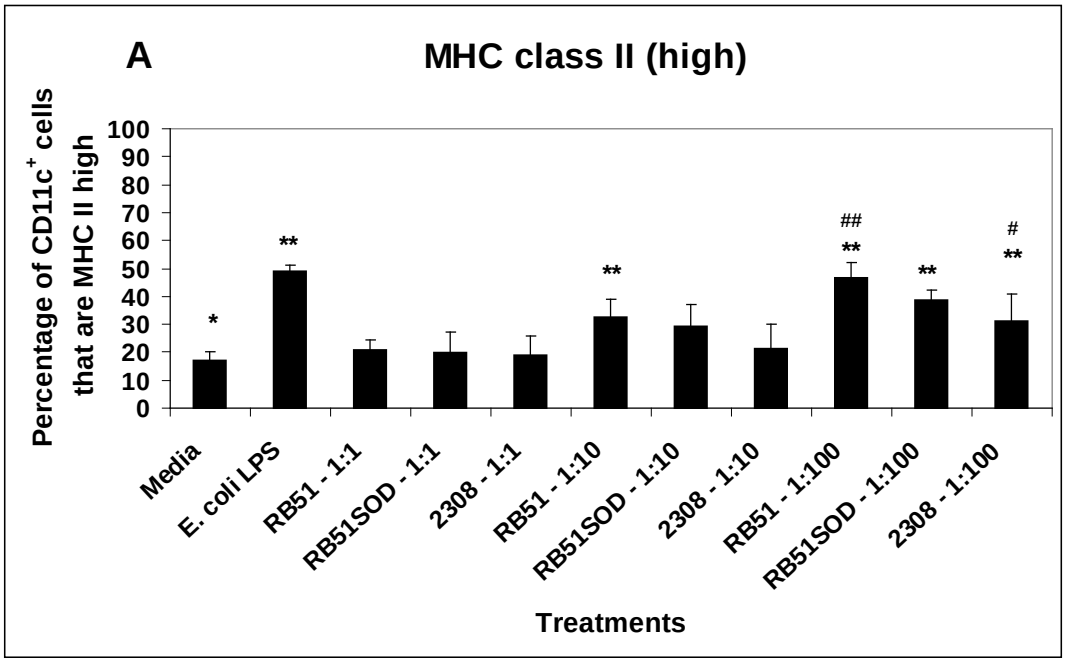
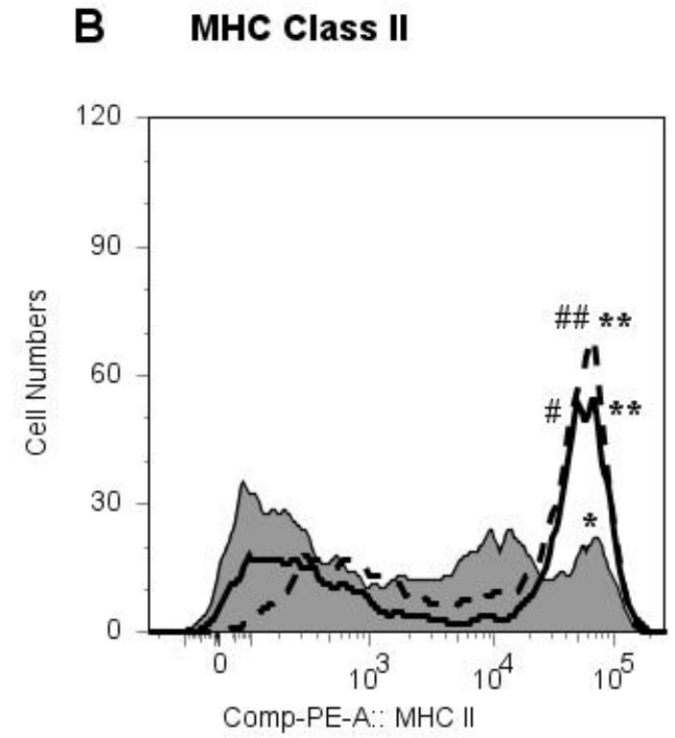

B
DC- Maturation Markers
Percentage of CD11c+ cells
100
90
80
70
60
50
40
30
20
10
0
Day 6
24 hr Media
24 hr LPS
Day 6
24 hr Media
24 hr LPS
Day 6
24 hr Media
24 hr LPS
MHC II (high)
CD40
CD86
Surface markers

Figure 2


A
MHC class II (high)
Percentage of CD11c+ cells that are MHC II high
100
90
80
70
60
50
40
30
20
10
0
*
**
**
##
**
**
#
**
Media
E. coli LPS
RB51 - 1:1
RB51SOD - 1:1
2308 - 1:1
RB51 - 1:10
RB51SOD - 1:10
2308 - 1:10
RB51 - 1:100
RB51SOD - 1:100
2308 - 1:100
Treatments


B
MHC Class II
120
90
60
30
0
Cell Numbers
**
**
*
0
10^3
10^4
10^5
Comp-PE-A:: MHC II

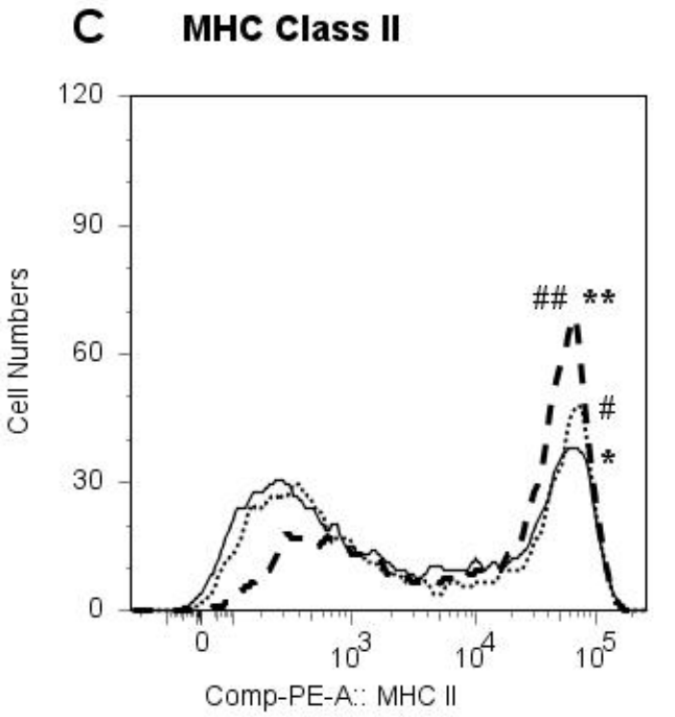
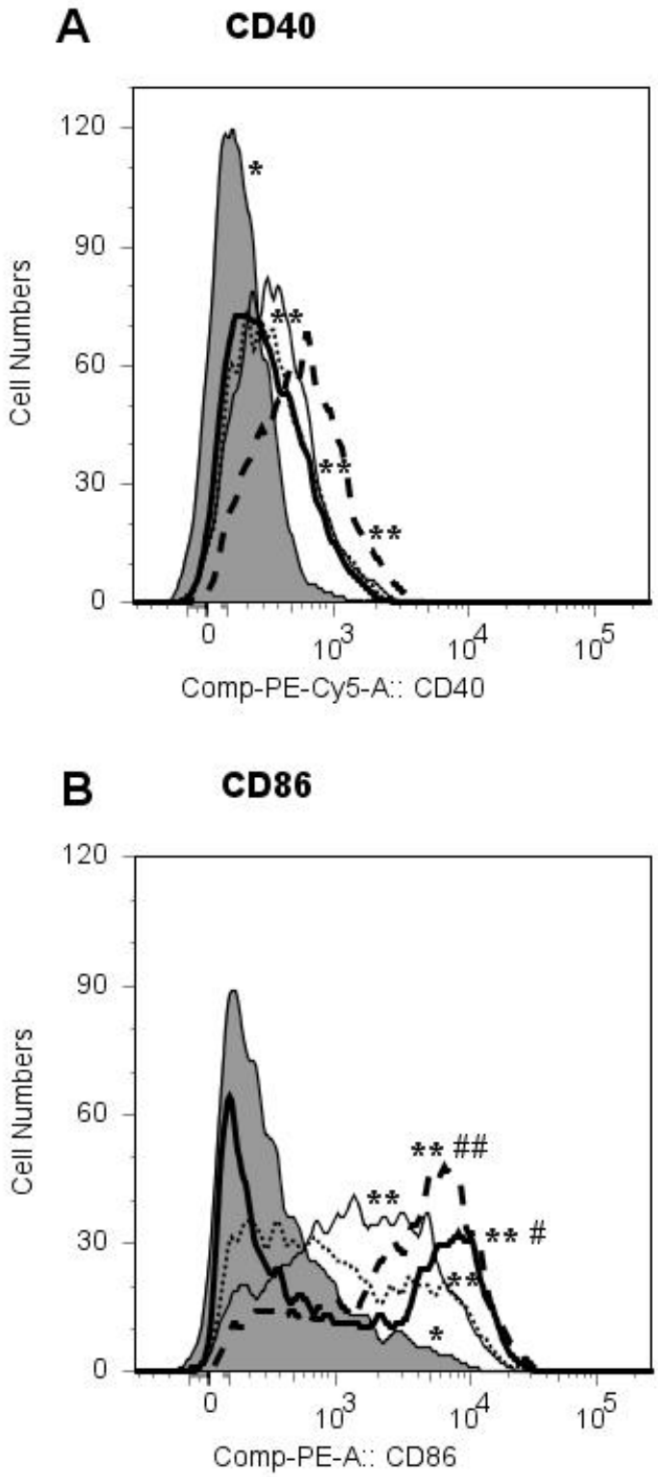

C
MHC Class II
120
90
60
30
0
Cell Numbers
**
#
*
0
10^3
10^4
10^5
Comp-PE-A:: MHC II

Figure 3


A
CD40
120
90
60
30
0
Cell Numbers
*
**
**
**
0
10^3
10^4
10^5
Comp-PE-Cy5-A:: CD40
B
CD86
120
90
60
30
0
Cell Numbers
** ##
**
** #
**
*
0
10^3
10^4
10^5
Comp-PE-A:: CD86

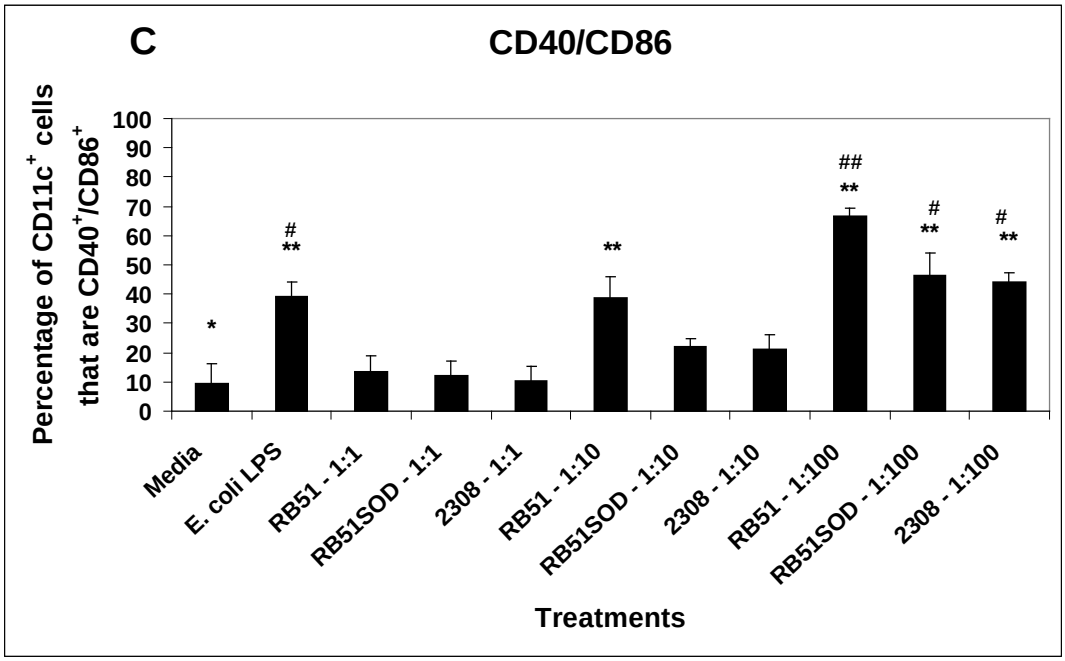
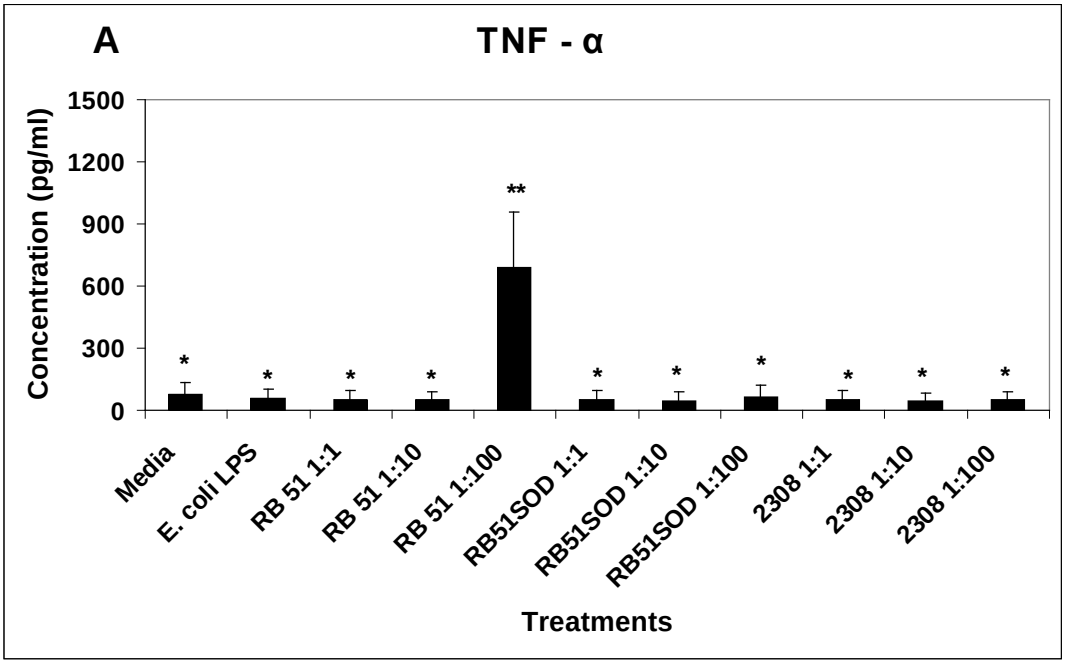
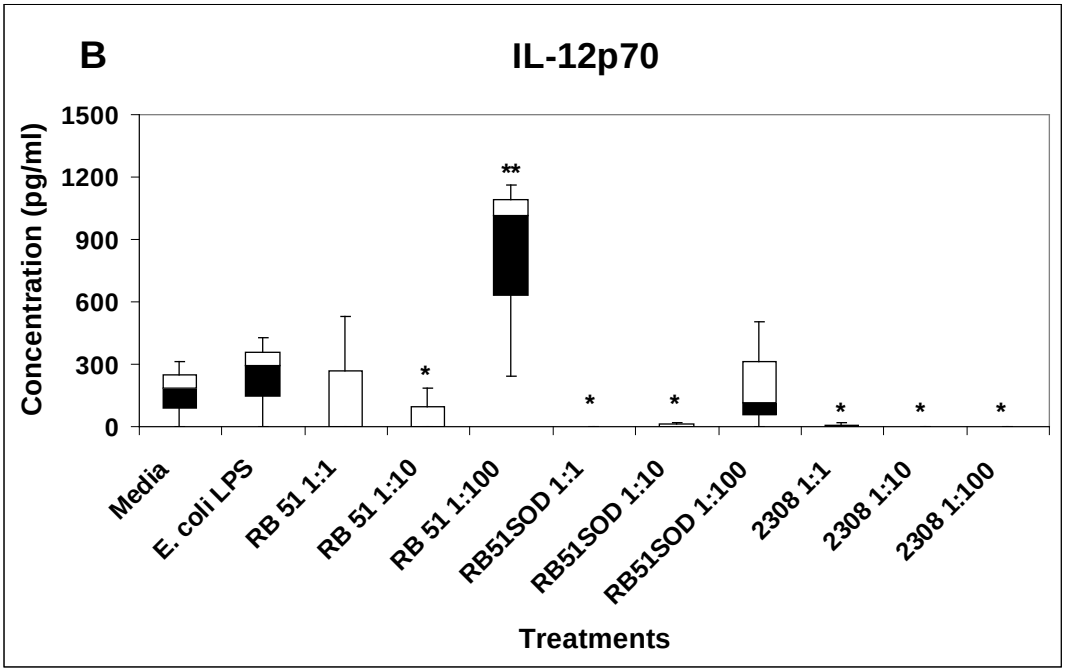

C
CD40/CD86
Percentage of CD11c+ cells that are CD40+/CD86+
100
90
80
70
60
50
40
30
20
10
0
*
#
**
**
##
**
#
**
#
**
Media
E. coli LPS
RB51 - 1:1
RB51SOD - 1:1
2308 - 1:1
RB51 - 1:10
RB51SOD - 1:10
2308 - 1:10
RB51 - 1:100
RB51SOD - 1:100
2308 - 1:100
Treatments

Figure 4


A
TNF - α
Concentration (pg/ml)
1500
1200
900
600
300
0
Media
E. coli LPS
RB 51 1:1
RB 51 1:10
RB 51 1:100
RB51SOD 1:1
RB51SOD 1:10
RB51SOD 1:100
2308 1:1
2308 1:10
2308 1:100
Treatments


B
IL-12p70
Concentration (pg/ml)
1500
1200
900
600
300
0
Media
E. coli LPS
RB 51 1:1
RB 51 1:10
RB 51 1:100
RB51SOD 1:1
RB51SOD 1:10
RB51SOD 1:100
2308 1:1
2308 1:10
2308 1:100
Treatments

Chapter 3

Heat killed and gamma-irradiated *Brucella* strain RB51 stimulate enhanced dendritic cell activation but not functioncompared to virulent smooth strain 2308

Running title: Heat killed and irradiated strain RB51stimulate BMDCs.

Naveen Surendran[a], Elizabeth M. Hiltbold[c], Bettina Heid[a], Nammalwar Sriranganathan[b], Stephen M. Boyle[b], Kurt L. Zimmerman[b], Sharon G. Witonsky[a*].

(Submitted to FEMS immunology and Medical Microbiology)

Keywords: BRUCELLA ABORTUS; DENDRITIC CELL; INNATE IMMUNITY

Abstract:

Brucella spp. are Gram-negative, facultative intracellular bacterial pathogens that cause abortion in livestock and undulant fever in humans worldwide. *B. abortus* strain 2308 is a pathogenic strain that affects cattle and humans. Currently, there are no efficacious human vaccines available. However, *B. abortus* strain RB51 is a live attenuated rough vaccine against bovine brucellosis which is approved by the USDA. Live strain RB51 induces protection via $CD4^+$ and $CD8^+$ T- cell mediated immunity. To generate an optimal T-cell response, strong innate immune responses by dendritic cells (DCs) are crucial. Because of safety concerns, using live vaccine strain RB51 in humans is limited. Therefore in this study, we analyzed the differential ability of same doses of live, heat-killed (HK) and gamma-irradiated (IR) strain RB51 in inducing DC activation and function. Smooth strain 2308, live strain RB51 and LPS were used as controls. Studies using mouse bone marrow derived DCs revealed that, irrespective of viability, strain RB51 induced higher DC activation than smooth strain 2308. Live strain

RB51 induced significantly ($p \leq 0.05$) higher DC maturation than HK and IR strains, and only live strain RB51 infected DCs (at MOI 1:100) induced significant ($p \leq 0.05$) TNF -α and IL-12 secretion.

1. Introduction

B. abortus is a Gram-negative, facultative intracellular bacterium that causes abortion in cattle and undulant fever in humans (Corbel, 2006). Brucellosis, the disease caused by *Brucella spp*., is one of the five most prevalent human bacterial zoonoses in the world with more than half a million human cases reported annually (Pappas et al., 2006). *Brucella* species are easy to aerosolize and can be genetically modified to create antibiotic resistant strains. Therefore, they are ideal agents for development as bioterror weapons (Pappas et al., 2006). Consequently, the Center for Disease Control and Prevention (CDC) categorizes them as Class B pathogens. There are no human vaccines available to date. If untreated this disease is devastating in humans and animals.

B. abortus strain 2308 is a phenotypically smooth strain possessing a surface exposed O-side chain of lipopolysaccharide (LPS); this is an immunodominant antigen referred to as O-antigen (Schurig et al., 1991). As with most intracellular bacterial infections, protection against *Brucella* involves both a $CD4^+$ T-helper-1 (Th_1) and $CD8^+$ cytotoxic T-cell-1 (Tc_1) response (He et al., 2001). *B. abortus* strain RB51 is a live attenuated stable rough phenotypic mutant derived from virulent strain 2308. Strain RB51 lacks the O-side chain in its LPS (Schurig et al., 1991). Live vaccine strain RB51 protects animals by inducing a cell mediated CD4 Th_1 and $CD8^+$ Tc_1 gamma interferon (IFN-γ) response (He et al., 2001). Despite the knowledge that strain RB51 stimulates protective cell mediated immunity (CMI), there is limited information regarding how

B. abortus strains induce innate immune responses which result in protective CMI. To develop a human vaccine, additional knowledge is needed on how strain RB51 stimulates the innate response.

Dendritic cells (DCs) are the sentinel cells of the innate immune system and their interaction with naïve T-cells following antigen capture determines the specificity and polarization of T-cell mediated immunity (Banchereau and Steinman, 1998). In addition, DCs are highly susceptible to *Brucella* infection making them a valuable model for assessing *Brucella* mediated immune responses (Billard et al., 2005). In our previous study (Surendran et al., under review), we demonstrated that rough strain RB51 induced significantly higher DC maturation and function compared to smooth virulent strain 2308. This enhanced DC activation and function caused by live vaccine strain RB51 could be the critical initial defining point for a successful T-cell mediated adaptive immune response. Because safety concerns of live vaccines limit their use in people, the efficacy of safer heat killed (HK) or irradiated (IR) vaccines should be considered (Plotkin, 2005).

HK *B. abortus* is an established CD4 Th_1 promoting stimulus. It stimulates cytotoxic CD8 T-lymphocytes even in the absence of CD4 T-cell help (Finkelman et al., 1988; Street et al., 1990). By comparison, IR strain RB51 induced CD4 Th_1 type responses and if used at one log higher dose than live strain RB51, it protected against virulent *B. abortus* challenge in a mouse model (Sanakkayala et al., 2005). With this study, we wanted to determine whether HK and IR strain RB51 stimulated comparable innate responses to live vaccine strain RB51 for exploring their use as a vaccine in people and animals. To assess innate immunity we examined the ability of HK and IR *B. abortus* rough strain RB51 and smooth strain 2308 to stimulate murine bone marrow derived DC (BMDC) activation and function based on cell surface expression of

costimulatory molecule and cytokine production. This study assessed simultaneously, for the first time, the differential ability of live, HK and IR rough and smooth strains of *B. abortus* at same doses to stimulate DC activation and function.

2. Materials and Methods

2. 1. Mice: Female 6-8 weeks old BALB/c mice were obtained from Charles River Laboratories Inc., Wilmington, MA. Mice were used under animal care protocols approved by Institutional Animal Care and Use Committee at Virginia Tech.

2. 2. Dendritic cell preparation: Bone marrow-derived DCs (BMDCs) were generated, as previously described (Inaba et al., 1992). Briefly, tibias and fibulas of 7-8 week old BALB/c mice were incised and bone marrow (BM) cells removed. Following red blood cell lysis and filtration, the cells were resuspended and plated in RPMI 1640 complete media with 10% non heat-inactivated fetal bovine serum and 20ng/ml rGM-CSF (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C in 5% CO_2. Fresh media containing rGM-CSF was added at days 2, 4 and 5 and harvested on day 6. The cells harvested on day 6 were typically 70% $CD11c^+$ and displayed low levels of MHC class II, CD40 and CD86 expression, consistent with immature DCs. Flow cytometry was performed to confirm DC activation status (Inaba et al., 1992).

2. 3. *Brucella* strains: Stock cultures of live attenuated rough *Brucella abortus* vaccine strain RB51 and virulent smooth strain 2308 from our culture collection (Schurig et al., 1991; Vemulapalli et al., 2000) were stored at -80°C. An aliquot each of strain RB51 and strain 2308 were subjected to gamma irradiation using a ^{60}Co source irradiator with a radiation output of 2,200 Rads/minute (Model 109-68R by J.L. Shepherd and associates, San Fernando, CA) for 3 hours (396 kilorads of gamma radiation). Another aliquot of strain RB51 and strain 2308 were

subjected to heat killing by incubating in an 80°C water bath for 60 minutes. Irradiated and heat killed bacterial preparations were confirmed to be nonviable by plating aliquots on TSA plates and confirming lack of growth following 4 days of incubation. All experiments with *Brucella* were performed in our CDC approved (C2003 1120-0016) Biosafety Level (BSL)-3 facility.

2. 4. Infection experiments: On day 6, DCs were harvested and plated at 5 X 10^5 cells/well in 24 well plates and stimulated with live, IR or HK strain RB51 or strain 2308 at 1:10 (DC:*Brucella*) or 1:100 CFUs/well (i.e. 5 X 10^6 or 5 X 10^7 CFU equivalents/well of irradiated or heat killed *B. abortus*). Stimulation was enhanced by a short spin at 1300 rpm (400 x g) for 5 minutes at room temperature. The stimulated cells were incubated for 4 hours at 37°C in 5% CO_2. Then cells were washed with media containing gentamicin (Sigma, St. Louis, MO) 30μg/ml. The stimulated cells were incubated for an additional 20 hours in complete media with 10ng/ml rGM-CSF and 30μg/ml gentamicin. Control samples were maintained by incubating cells with media (negative control) or *Escherichia coli* LPS *0111:B4* (Sigma) (positive control) (100ng/ml) following the same protocol (Sanakkayala et al., 2005).

Viability and infection controls: To quantitate and assess viability, at each time point and with each treatment, Trypan blue was used to differentiate viable and dead cells. Total live and dead BMDC numbers were determined.

2. 5. Staining and flow cytometry: The cells were harvested 24 hours following infection, and they were stained with the following monoclonal antibodies at 0.1 - 0.2 μg per million cells for FACS analysis: PE-Texas red conjugated anti-CD11c, Biotin-conjugated anti-CD40, Streptavidin Tri-color conjugate, PE-conjugated anti-CD86 were all acquired from Caltag (Invitrogen), and PE-conjugated anti I-A/I-E, acquired from BD Pharmingen, San Jose, CA. Cells were washed and analyzed by BD FACSAria™ flow cytometer (Sanakkayala et al., 2005).

2. 6. Cytokine analysis: For cytokine measurement, culture supernatants from *Brucella* infected BMDCs were collected after 24 hours of incubation and stored at -80°C. TNF-α, IL-12_{p70} and IL-4 cytokine levels were subsequently measured using indirect sandwich ELISAs (BD Pharmingen) (Sanakkayala et al., 2005).

2. 7. Statistical analysis: As the data had a Gaussian distribution, the effect of treatment on expression of various DC maturation and activation markers was tested using a mixed model ANOVA with treatment as a fixed effect and day as a blocking factor (Tukey procedure for multiple comparisons). After a logarithmic (to base e) transformation, TNF-α data was also analyzed using the above mentioned procedure. For IL-12_{p70}, the treatments were compared using the exact Kruskal-Wallis test. The main p-value for this test which applies to the overall dataset for the effect of variable treatments (including samples from all different MOIs per treatment) was > 0.05 (0.0889). By this method, as different MOIs are analyzed together, there is no consideration if only certain MOIs potentially have significant effect. As the pattern of IL-12_{p70} secretion between different treatments was similar to TNF-α we used the Dunn's procedure for two-way comparisons as a post hoc test. Significance was set at $p \leq 0.05$. All analyses were performed using the SAS system (Cary, NC).

3. Results

3. 1. Day 6 harvested BMDCs show an immature phenotype: $CD11c^+$ expression on the harvested cells was determined to calculate the yield and percentage of BMDCs following 6 days of culture. BM cells were gated based on size and granularity and almost 70% of the total gated cells expressed $CD11c^+$ on day 6. $CD11c^+$ BMDCs expressed an immature phenotype based on surface expression of characteristic maturation markers MHC class II, CD40 and CD86 (Fig.

1A). Following 24 hour incubation with different treatments, the percentage of $CD11c^+$ cells within the DC gate increased to 81-90% of total gated cells ($p<0.05$) except for LPS treatment (71.65±2.74%). In addition, >99% of all such $CD11c^+$ cells were positive for expression of CD11b characteristic of myeloid origin of DCs (data not shown). Following LPS overnight treatment, BMDCs treated had a mature BMDC phenotype based on MHC class II high, CD40 and CD86 expression ($p<0.05$).

3. 2. HK or IR *B. abortus* rough vaccine strain RB51 induces greater DC maturation than smooth strain 2308: To evaluate how HK or IR *Brucella* affected DC maturation, immature BMDCs were stimulated with either HK or IR rough vaccine strain RB51 or smooth pathogenic strain 2308 at 1:10 (DC: *Brucella*) or 1:100 CFU equivalents. Additional controls included media only and LPS treated BMDCs as well as live strain RB51 and 2308-infected (at MOI 1:10 or 1:100) BMDCs. Immature BMDCs treated overnight with media alone retained their immature phenotype with reduced surface expression of MHC class II and CD40, CD86 costimulatory markers compared to LPS (Fig. 1A).

3. 3. BMDC – MHC class II high expression: Immature BMDCs stimulated with HK strain RB51 (HKRB51) at both 1:10 ($p=0.0542$) (not shown) and 1:100 ($p=0.0018$) CFU equivalents showed significant up-regulation of MHC class II high expression compared to media control (Fig. 1B). In addition, at corresponding doses of 1:10 and 1:100 HKRB51 had higher mean (not statistically significant) MHC class II high expression than HK strain 2308 (HK2308) stimulated BMDCs (Fig. 1B). Furthermore, both HKRB51 and HK2308 stimulated DCs showed a non-significant dose related increase in MHC class II high expression at 1:100 compared to 1:10. However, live strain RB51 infected BMDCs had greater MHC class II high expression than HKRB51 (not significant) and HK2308 ($p\leq0.05$) at corresponding doses (Fig. 1B).

Irradiated (IR) strain RB51 (IRRB51) induced a relatively higher but not significant MHC class II high expression than IR strain 2308 (IR2308) stimulated BMDCs at corresponding doses. At 1:100, IRRB51 induced significantly ($p \leq 0.05$) higher MHC class II high expression than media (Fig. 1B). Moreover, IRRB51 induced mean DC-MHC class II high expression level was lower (not significant) than that induced by HKRB51 at respective doses (Fig. 1B). At both MOIs, live strain RB51 induced higher MHC class II high expression on BMDCs compared to IRRB51 with significant differences ($p \leq 0.05$) at MOI 1:100 (Fig. 1B). Live strain RB51 at 1:100 also induced significantly higher ($p<0.05$) MHC class II high expression than live strain 2308 at same dose (Fig 1B).

3. 4. BMDC – Costimulatory marker expression: The expression levels of costimulatory molecules CD40 and CD86 (independent and co-expression) were also analyzed to assess the effect of live vs. HK or IR *Brucella* on DC maturation. Fig. 1C shows CD40 expression on live, HK and IR *Brucella* infected BMDCs. Only live, but not HK or IR, strain RB51 infected BMDCs at MOI 1:100 induced significantly higher CD40 expression than media control ($p \leq 0.05$). By comparison, HKRB51 infected BMDCs had a dose related higher mean CD40 expression compared to HK2308 infected BMDCs at corresponding doses but it was not statistically significant. At 1:100, HKRB51 induced DC - CD40 expression reached closer to significance ($p \leq 0.06$) compared to media control.

In comparing CD40 and CD86 expression, results were similar. At 1:100, HKRB51 and IRRB51induced greater CD86 expression ($p \leq 0.05$) vs. media only (Fig. 1D). HKRB51 induced non significantly higher DC - CD86 expression than HK2308 at both doses respectively. By contrast, at both 1:10 and 1:100 both live *Brucella* strains (RB51 and 2308) induced significantly ($p \leq 0.05$) higher CD86 expression on infected DCs compared to media. In addition, live strain

RB51 induced CD86 expression was significantly higher ($p \leq 0.05$) than both HK and IR rough and smooth strain induced CD86 levels at respective MOIs (Fig. 1D). At MOI 1:10, live strain 2308 induced CD86 level was significantly higher ($p \leq 0.05$) than HK2308 induced levels at MOI 1:10 equivalent and at MOI 1:100, live strain 2308 induced CD86 level was significantly higher ($p \leq 0.05$) than both HK and IR rough and smooth strains induced CD86 levels with MOI 1:100 equivalent. Fig. 1E illustrates the CD40/ CD86 co-expression analyses on immature BMDCs treated with HK and live *Brucella* strains which were similar to CD86 expression. HKRB51 induced higher non significant mean CD40/CD86 co-expression than HK2308 at both 1:10 and 1:100. At 1:100, HKRB51 induced significantly higher levels of CD40/CD86 ($p \leq 0.05$) compared to media.

By comparison, strain IRRB51 induced greater DC - CD86 and CD40/CD86 expression than media at a dose 1:100 ($p \leq 0.05$). However, strain IRRB51 compared to strain HKRB51 stimulated BMDCs were not significantly different than each other at either doses. Strain IRRB51 had lower mean values, but not statistically significant, of each costimulatory molecule expression and followed the same pattern of CD40, CD86 and CD40/86 expression as that of HKRB51 stimulated DCs (Fig. 1C-E).

3. 5. DC functional analysis: HK or IR *B. abortus* rough vaccine strain RB51 do not induce significant TNF-α and IL-12 secretion: TNF-α is an inflammatory cytokine that plays an important role in the defense against intracellular pathogens and is essential for DC maturation. IL-12 production by DCs is critical for a protective CD4 Th_1 type immune response and clearance of intracellular bacteria (Huang et al., 2001). To determine DC function based on cytokine secretion, TNF-α, $IL\text{-}12_{p70}$ and IL-4 secretion from the antigen treated BMDC culture supernatants were analyzed using indirect ELISA. Neither HK nor IR rough strain RB51

produced significant amounts of TNF-α or IL-12 at both doses compared to media control (Fig. 2A & 2B). Only live strain RB51 at a MOI 1:100 induced BMDCs to secrete a significantly higher amount of both TNF-α and IL-12 ($p \leq 0.05$). Irrespective of viability or dose, strain 2308 did not induce significant levels of TNF-α or IL-12 from infected BMDCs. (Fig. 2A & 2B). None of the strains induced detectable levels of IL-4 cytokine (data not shown).

4. Discussion

We have recently submitted another manuscript (Surendran et al., Veterinary Microbiology, 2010; accepted) in which we determined that vaccine strain RB51 upregulated DC activation and function using our *in vitro* BMDC model. In that study, we determined that the differences in DC mediated function were not due to differences in viability of *Brucella* and/or BMDCs.DC viability and *Brucella* numbers were analyzed at 1, 4 and 24 hrs. These data showed that at 4 hrs there were relatively similar levels of *Brucella*: BMDCs. Data were from 1 of 3 replicates and the counts denoted number of intracellular *Brucella* per 100 cells. For the 1:100 MOI: at 1 hr, *Brucella*: BMDCs for strain RB51 were 35,254 and strain 2308, 4,535. For 4 hrs, *Brucella*: BMDCs for strain RB51 was 6,330 and strain 2308, 19,420; At 24 hrs, *Brucella*: BMDCs for strain RB51 was 124; strain 2308, 2,125. These data substantiated that our model permitted both rough and smooth *Brucella* strains to infect and stimulate BMDCs. Thus, increased activation associated with increased numbers of rough strains appeared unlikely. The results reflected effects of strain differences on BMDC function.

Collectively, both data from the other submitted manuscript and these data presented here showed that regardless of viability, rough vaccine strain RB51 induced enhanced DC maturation compared to smooth virulent strain 2308. Additionally, live strain RB51 induced DC maturation

and function greater than its respective HK or IR strain. Furthermore, at MOI 1:100, live strain 2308 induced almost equal or greater expression of DC maturation markers as that of HK or IRRB51 at the same dose. However, none of the smooth strains, regardless of viability or dose, induced DC function based on cytokine production.

Based on these data, live strain RB51 provided optimal DC activation and function based on up-regulation of MHC class II, CD40, CD86 and TNF-alpha and IL-12 production compared to media control (Fig. 1, 2). At MOI 1:100, the IR and HK strains significantly up-regulated MHC class II and CD86 greater than media; however neither CD40 expression or cytokine production was greater than media. Additionally at MOI 1:100, IR strain RB51 induced significantly less MHC class II and CD86 expression than live strain RB51. These data all supported that live strain RB51 up-regulated DC function significantly better than HK or IR strains of RB51. However, the question remains as to whether non-live *Brucella* strains can protect against challenge and thus be used as alternative "safe" strains for people and animals. Additionally, as *Brucella* has been used as an adjuvant (Golding et al., 1995), the effect of viability on DC function, T-cell function and overall protection is a concern.

HK *Brucella* is an established adjuvant and carrier that promotes a Th_1 protective immune response (Finkelman et al., 1988; Street et al., 1990). IR strain RB51 has been shown to stimulate antigen-specific Th_1 immune responses (Oliveira et al., 1994; Sanakkayala et al., 2005). In order to generate a strong Th_1 response, enhanced DC activation with associated IL-12 secretion is critical (Golding et al., 2001). As DCs are a major source of IL-12 and an important cellular target for *Brucella* infection (Billard et al., 2005; Huang et al., 2001), our aim in this study was to differentially analyze DC immune activation potential of inactivated (HK or IR) vs.

live vaccine and pathogenic strains of *B. abortus* using the *in vitro* murine BMDC model. This would provide additional information on the potential of IR or HK vaccines for human use.

Based on our data, which demonstrated that while HK and IR strain RB51 induced upregulation of costimulatory molecules but not TNF-alpha or IL-12 production, the question remains as to whether live vs. HK or IR strains can also upregulate T-cell function and ultimately protect against challenge. In comparing *Brucella*, with other live strains of intracellular organisms such as *Listeria monocytogenes* (Muraille et al., 2005) and *Chlamydia trachomatis* (Rey-Ladino et al., 2005), live strains induced higher levels of DC maturation compared to their HK or UV-IR forms respectively. Muraille et al. (Muraille et al., 2005) and Takemori et al. (Tsunetsugu-Yokota et al., 2002) showed that the T-lymphocytes primed by HK *Listeria* or *Mycobacterium* pulsed DCs did not fully differentiate and that only infection with live organisms induced long term $CD8^+$ T-cell mediated immunity. Additionally, only live *Listeria* and Bacillus Calmette-Guierin (BCG) strain of *Mycobacterium* protected against challenge. (Muraille et al., 2005)

In comparing our data with results from other laboratories, we found that our data was in contrast with data presented by Zwerdling et al. (Zwerdling et al., 2008) and Macedo et al. (Macedo et al., 2008). Their results showed that DC - cytokine secretion was not dependent upon bacterial viability and HK *B. abortus* 2308 (at 10^8 or 10^9 bacteria/ml) induced DC maturation and TNF-α and IL-12 secretion in a dose dependent fashion. The probable reasons for this discrepancy could be the lower DC (5×10^5 cells/ml), HK and IR cell concentrations used in our study. Our studies with live bacteria do support that live bacteria induce a dose dependent upregulation of DC costimulatory molecule expression and cytokine production (Surendran et al., in press; Veterinary Microbiology). In this study, there was a dose dependent response

between 1:10 and 1:100 for HK and IR, but while higher doses stimulated more co-stimulatory molecule expression, neither the HK or IR strains induced DC cytokine production at doses tested here in. With live strains, there appears to be a threshold of DC activation needed for cytokine production (Surendran et al., accepted; Veterinary Microbiology). In this study, for an appropriate comparison between strains, we used the same doses of live, heat killed and irradiated strains RB51 and 2308 for infecting the DCs. Besides the differences in DC activation and function reported by Zwederling and Macedo (Macedo et al., 2008; Zwerdling et al., 2008), our results were also different than those reported by Vemulapalli (Sanakkayala et al., 2005) and Datta (Datta et al., 2006).

Vemulapalli et al., found that both HK and IR strain RB51 induced similar DC activation and IR vs. HK strain RB51 induced increased IL-12 secretion which correlated with protection against strain 2308. Using a *Listeria* model, Datta (Datta et al., 2006) confirmed similar findings. By contrast, Lee et al., (Lee et al., 1999) found that IR strain RB51 with or without IL-12 as an adjuvant, did not protect against strain 2308 challenge. These conflicting results could possibly be explained based on the fact that other groups stimulated for 24 hrs while we stimulated for 4 hrs. Mechanistically, some of these differences between HK vs. IR vs. live strains in induced DC and T-cell function and protection could be due to the amount and nature of antigen being processed and presented as well as the extent to which DCs are stimulated.

In a different model, findings by Kalupahana et al., (Kalupahana et al., 2005) using HK and live *S. typhimurium* supported the above premise by showing that prolonged contact with HK bacteria was necessary to obtain similar DC activation and function achieved by live strains in a shorter period. Additionally, in contrast to the 65°C, 30 minutes heat inactivation by Vemulapalli et al. (Sanakkayala et al., 2005), we used a higher temperature of 80°C for 1 hour.

Theoretically, although not likely, additional heating may have disrupted the *Brucella* cell envelopes (Barquero-Calvo et al., 2007) and exposed large amounts of *Brucella* LPS, lipoproteins, peptidoglycan, DNA and other molecules recognized by innate immunity. Additional differences between IR and HK could be due that IR may stimulate a better DC mediated CD8 response than HK (Datta et al., 2006).

Besides differences in the ability of IR vs. HK to stimulate more CD8 vs. CD4 mediated immune responses, and the role of IR vs. HK in protection, regulators which influence DC function and protection are TNF-alpha, IL-12 and IL-10. As previously stated, TNF-alpha production is critical for maximal IL-12 production and CD4 Th1 response. If either is decreased, DC mediated T-cell responses and potentially protection could be decreased. Another mechanism by which protection would be decreased would be through an IL-10 mediated T-regulatory response which down-regulated IL-12 production by DCs (Huang et al., 2001). (McGuirk et al., 2002). Correspondingly, HK and/or IR strains may suboptimally stimulate BMDCs at a given dose which might induce them to become tolerogenic DCs (semimature DCs) with the inability to produce proinflammatory cytokines (Lutz and Schuler, 2002). As others have shown that both HK and IR strains of *B. abortus* induced similar levels of IL-10 (Sanakkayala et al., 2005), we did not determine the ability of HK or IR strains to induce IL-10 secretion from BMDCs. However, it is possible that live vs. HK or IR strains may induce different levels of IL-10 that could influence DC and T-cell function and protection. Thus, our findings along with already published studies suggest multiple mechanisms for differences between live vs. IR vs. HK strains induced DC function, T-cell function and protection. Additional studies are warranted to further investigate these mechanisms as well as their impact on protection.

In conclusion, these studies demonstrated that with the goal of comparing equal doses and duration of treatment: 1) irrespective of viability, *B. abortus* attenuated vaccine strain RB51 induced enhanced DC maturation compared to corresponding pathogenic strain 2308; 2) live strains stimulated greater DC activation and function compared to inactivated strains at same dose and 3) neither HK or IR strain RB51 stimulated strong DC functional response based on cytokine production at tested doses. Potentially higher doses of or prolonged stimulation with HK or IR strain RB51 could cause BMDCs to produce significant amounts of TNF-α and IL-12 cytokines *in vitro* and confer protection against challenge with pathogenic strain 2308 *in vivo*. Hence, both HK and IR strains could be considered as alternatives to live attenuated strain RB51. In addition, or as an alternative approach, another method of enhancing the innate response could be to use appropriate TLR agonists to upregulate DC mediated responses. These studies are warranted as ideally HK or IR vaccine strains with optimal DC and subsequent T-cell function and protection would be optimal for human use (Huang et al., 2003; Huang et al., 2005; Macedo et al., 2008).

Acknowledgements: The authors acknowledge Dr. Stephen Werre for help with the statistical analysis of the data and Melissa Makris for help with the flow cytometry.

References:

Banchereau, J., Steinman, R.M., 1998, Dendritic cells and the control of immunity. Nature 392, 245-252.

Barquero-Calvo, E., Chaves-Olarte, E., Weiss, D.S., Guzman-Verri, C., Chacon-Diaz, C., Rucavado, A., Moriyon, I., Moreno, E., 2007, *Brucella abortus* uses a stealthy strategy to

avoid activation of the innate immune system during the onset of infection. PLoS ONE 2, e631.

Billard, E., Cazevieille, C., Dornand, J., Gross, A., 2005, High susceptibility of human dendritic cells to invasion by the intracellular pathogens *Brucella suis*, *B. abortus*, and *B. melitensis*. Infect Immun 73, 8418-8424.

Billard, E., Dornand, J., Gross, A., 2007, *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. Infect Immun 75, 4980-4989.

Corbel, M.J. 2006. Brucellosis in humans and animals (World Health Organization).

Datta, S.K., Okamoto, S., Hayashi, T., Shin, S.S., Mihajlov, I., Fermin, A., Guiney, D.G., Fierer, J., Raz, E., 2006, Vaccination with irradiated *Listeria* induces protective T cell immunity. Immunity 25, 143-152.

Finkelman, F.D., Katona, I.M., Mosmann, T.R., Coffman, R.L., 1988, IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. J Immunol 140, 1022-1027.

Golding, B., Inman, J., Highet, P., Blackburn, R., Manischewitz, J., Blyveis, N., Angus, R.D., Golding, H., 1995, *Brucella abortus* conjugated with a gp120 or V3 loop peptide derived from human immunodeficiency virus (HIV) type 1 induces neutralizing anti-HIV antibodies, and the V3-*B. abortus* conjugate is effective even after CD4+ T-cell depletion. J Virol 69, 3299-3307.

Golding, B., Scott, D.E., Scharf, O., Huang, L.Y., Zaitseva, M., Lapham, C., Eller, N., Golding, H., 2001, Immunity and protection against *Brucella abortus*. Microbes Infect 3, 43-48.

He, Y., Vemulapalli, R., Zeytun, A., Schurig, G.G., 2001, Induction of specific cytotoxic lymphocytes in mice vaccinated with *Brucella abortus* RB51. Infect Immun 69, 5502-5508.

Huang, L.Y., Aliberti, J., Leifer, C.A., Segal, D.M., Sher, A., Golenbock, D.T., Golding, B., 2003, Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptor 2 dependent. J Immunol 171, 1441-1446.

Huang, L.Y., Ishii, K.J., Akira, S., Aliberti, J., Golding, B., 2005, Th1-like cytokine induction by heat-killed *Brucella abortus* is dependent on triggering of TLR9. J Immunol 175, 3964-3970.

Huang, L.Y., Reis e Sousa, C., Itoh, Y., Inman, J., Scott, D.E., 2001, IL-12 induction by a TH1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10. J Immunol 167, 1423-1430.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., Steinman, R.M., 1992, Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176, 1693-1702.

Kalupahana, R.S., Mastroeni, P., Maskell, D., Blacklaws, B.A., 2005, Activation of murine dendritic cells and macrophages induced by *Salmonella enterica* serovar Typhimurium. Immunology 115, 462-472.

Lee, I.K., Olsen, S.C., Kehrli, M., Bolin, C.A., 1999, The adjuvant effect of a single dose of interleukin-12 on murine immune responses to live or killed *Brucella abortus* strain RB51. Can J Vet Res 63, 284-287.

Lutz, M.B., Schuler, G., 2002, Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 23, 445-449.

Macedo, G.C., Magnani, D.M., Carvalho, N.B., Bruna-Romero, O., Gazzinelli, R.T., Oliveira, S.C., 2008, Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. J Immunol 180, 1080-1087.

McGuirk, P., McCann, C., Mills, K.H., 2002, Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J Exp Med 195, 221-231.

Muraille, E., Giannino, R., Guirnalda, P., Leiner, I., Jung, S., Pamer, E.G., Lauvau, G., 2005, Distinct in vivo dendritic cell activation by live versus killed *Listeria monocytogenes*. Eur J Immunol 35, 1463-1471.

Oliveira, S.C., Zhu, Y., Splitter, G.A., 1994, Recombinant L7/L12 ribosomal protein and gamma-irradiated *Brucella abortus* induce a T-helper 1 subset response from murine CD4+ T cells. Immunology 83, 659-664.

Pappas, G., Panagopoulou, P., Christou, L., Akritidis, N., 2006, *Brucella* as a biological weapon. Cell Mol Life Sci 63, 2229-2236.

Plotkin, S.A., 2005, Vaccines: past, present and future. Nat Med 11, S5-11.

Rey-Ladino, J., Koochesfahani, K.M., Zaharik, M.L., Shen, C., Brunham, R.C., 2005, A live and inactivated *Chlamydia trachomatis* mouse pneumonitis strain induces the maturation of dendritic cells that are phenotypically and immunologically distinct. Infect Immun 73, 1568-1577.

Sanakkayala, N., Sokolovska, A., Gulani, J., Hogenesch, H., Sriranganathan, N., Boyle, S.M., Schurig, G.G., Vemulapalli, R., 2005, Induction of antigen-specific Th1-type immune responses by gamma-irradiated recombinant *Brucella abortus* RB51. Clin Diagn Lab Immunol 12, 1429-1436.

Schurig, G.G., Roop, R.M., 2nd, Bagchi, T., Boyle, S., Buhrman, D., Sriranganathan, N., 1991, Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol 28, 171-188.

Street, N.E., Schumacher, J.H., Fong, T.A., Bass, H., Fiorentino, D.F., Leverah, J.A., Mosmann, T.R., 1990, Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. J Immunol 144, 1629-1639.

Tsunetsugu-Yokota, Y., Tamura, H., Tachibana, M., Ogata, K., Honda, M., Takemori, T., 2002, Selective expansion of perforin-positive CD8+ T cells by immature dendritic cells infected with live Bacillus Calmette-Guerin mycobacteria. J Leukoc Biol 72, 115-124.

Vemulapalli, R., He, Y., Cravero, S., Sriranganathan, N., Boyle, S.M., Schurig, G.G., 2000, Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun 68, 3286-3289.

Zhan, Y., Cheers, C., 1998, Control of IL-12 and IFN-gamma production in response to live or dead bacteria by TNF and other factors. J Immunol 161, 1447-1453.

Zwerdling, A., Delpino, M.V., Barrionuevo, P., Cassataro, J., Pasquevich, K.A., Garcia Samartino, C., Fossati, C.A., Giambartolomei, G.H., 2008, *Brucella* lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. Microbes Infect 10, 1346-1354.

Figure legends:

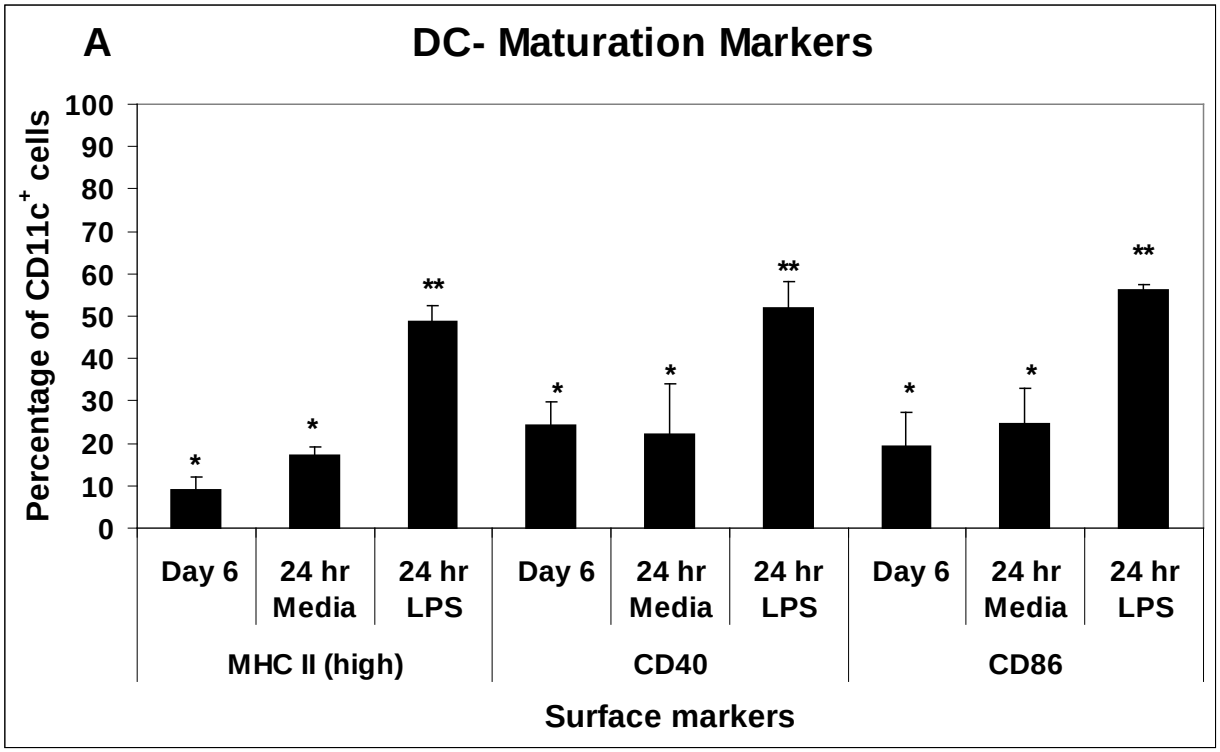
Fig. 1A: Day 6 harvested BMDCs show an immature phenotype. Bone marrow cells isolated from BALB/c mice were cultured for 6 days in 10% RPMI medium with rGM-CSF (20ng/ml). Cells were harvested on day 6 and analyzed by flow cytometry for $CD11c^+$ marker expression using fluorescent labeled $CD11c^+$ antibody. The figure compares the percentages of $CD11c^+$ DCs expressing the surface maturation markers MHC class II, CD40 and CD86 harvested on day 6 to that treated overnight with (negative control) media alone or (positive control) *E. coli* LPS (100 ng/ml). Two asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to data represented by an asterisk (*). Data represents means ± standard deviations of 3 independent experiments.

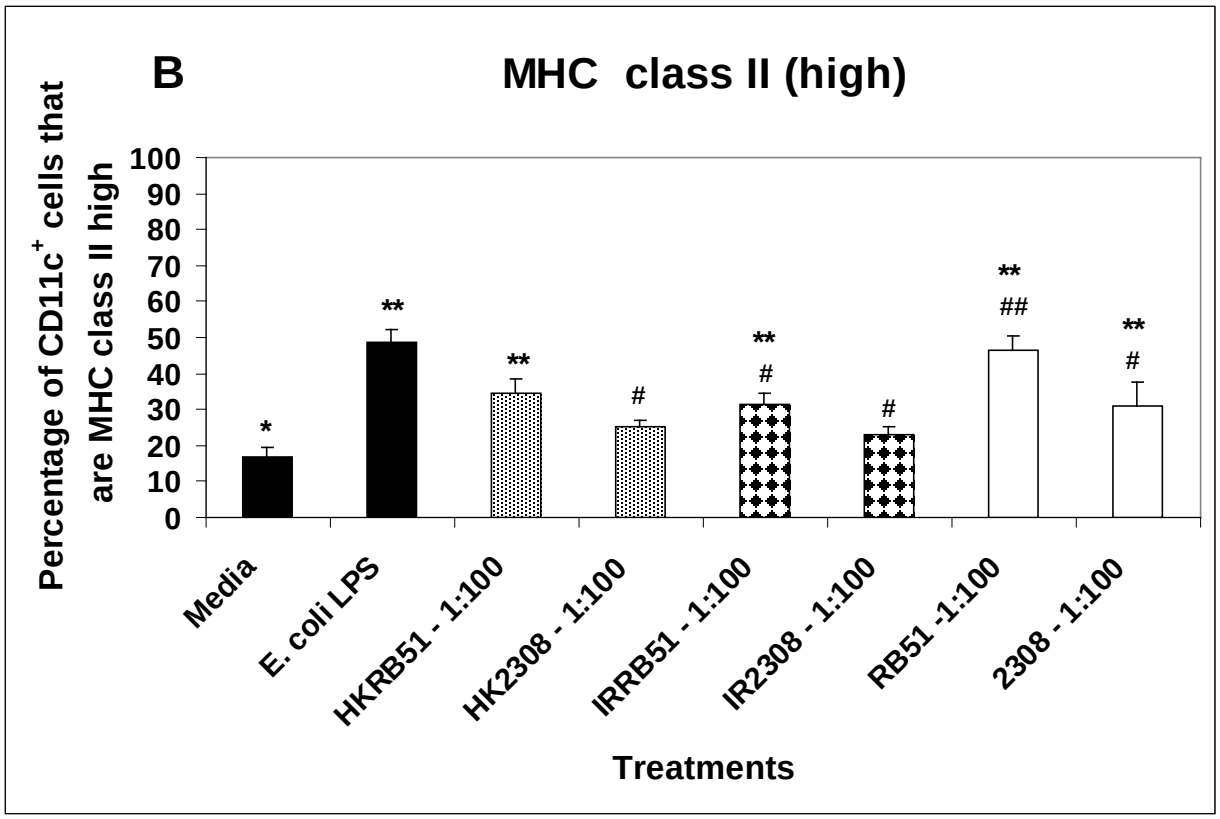
Fig. 1B-1E: Heat killed or irradiated *B. abortus* rough vaccine strain RB51 induced greater DC maturation than corresponding smooth strain 2308. Immature BMDCs were infected with live, HK or IR *B. abortus* rough vaccine strain RB51 or corresponding smooth strain 2308 at the given doses (DC:*Brucella*) 1:10 (not shown) or 1:100. At 24 hours post-incubation, the cells were analyzed for the DC surface expression of MHC class II, CD40 and CD86. Figures 1B-1E represent comparisons of the percentage of $CD11c^+$ cells (BMDCs) expressing MHC class II high, CD40, CD86 and coexpression of $CD40^+/CD86^+$ respectively across different treatment groups. Media and *E. coli* LPS are the negative and positive controls respectively. Double characters **, ##, aa represent statistically significant change at $p \leq 0.05$ with the corresponding single characters *, # and a respectively. Data represents means ± standard deviations of 3 independent experiments.

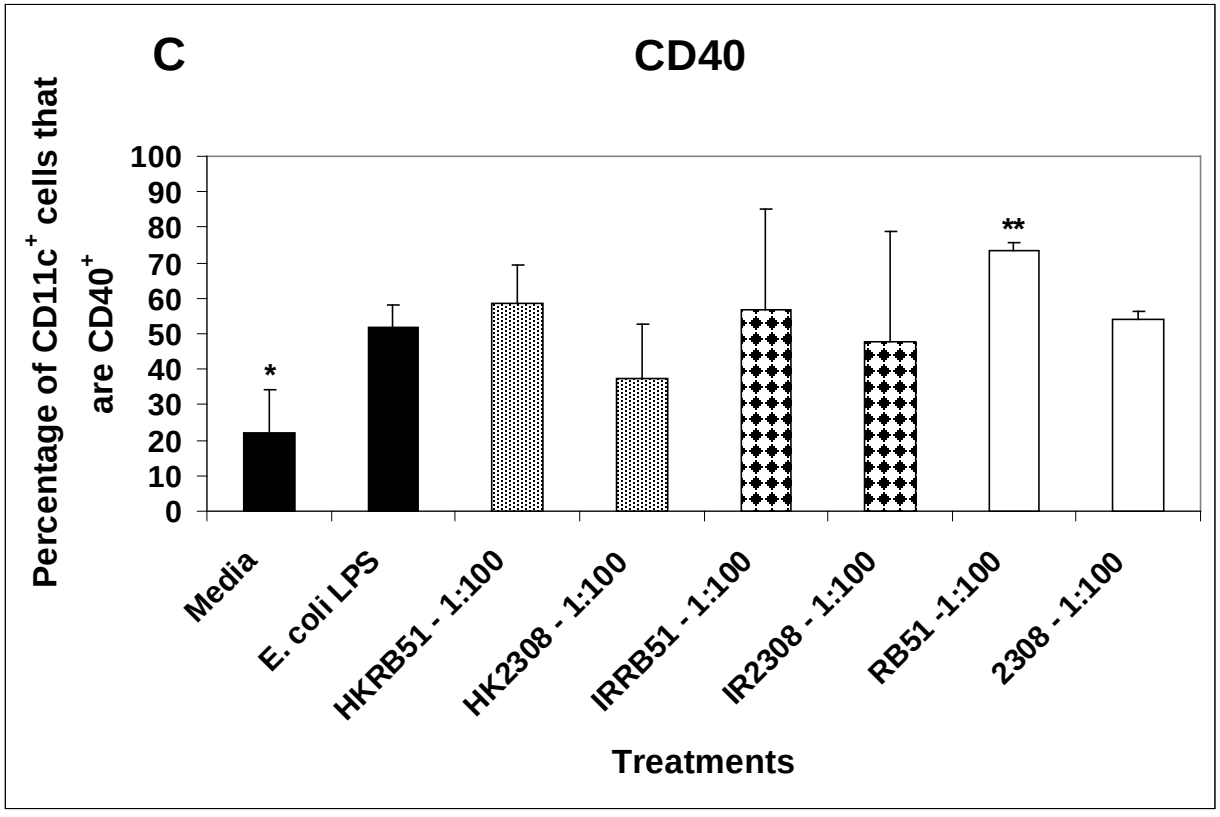
Fig. 2: Heat killed or irradiated *B. abortus* rough vaccine strain RB51 do not induce significant TNF – α and IL-12 secretion. To assess DC function, TNF-α (A) and IL-12_{p70} (B) levels from the culture supernatants of live, HK or IR *B. abortus* strain RB51 or 2308 infected

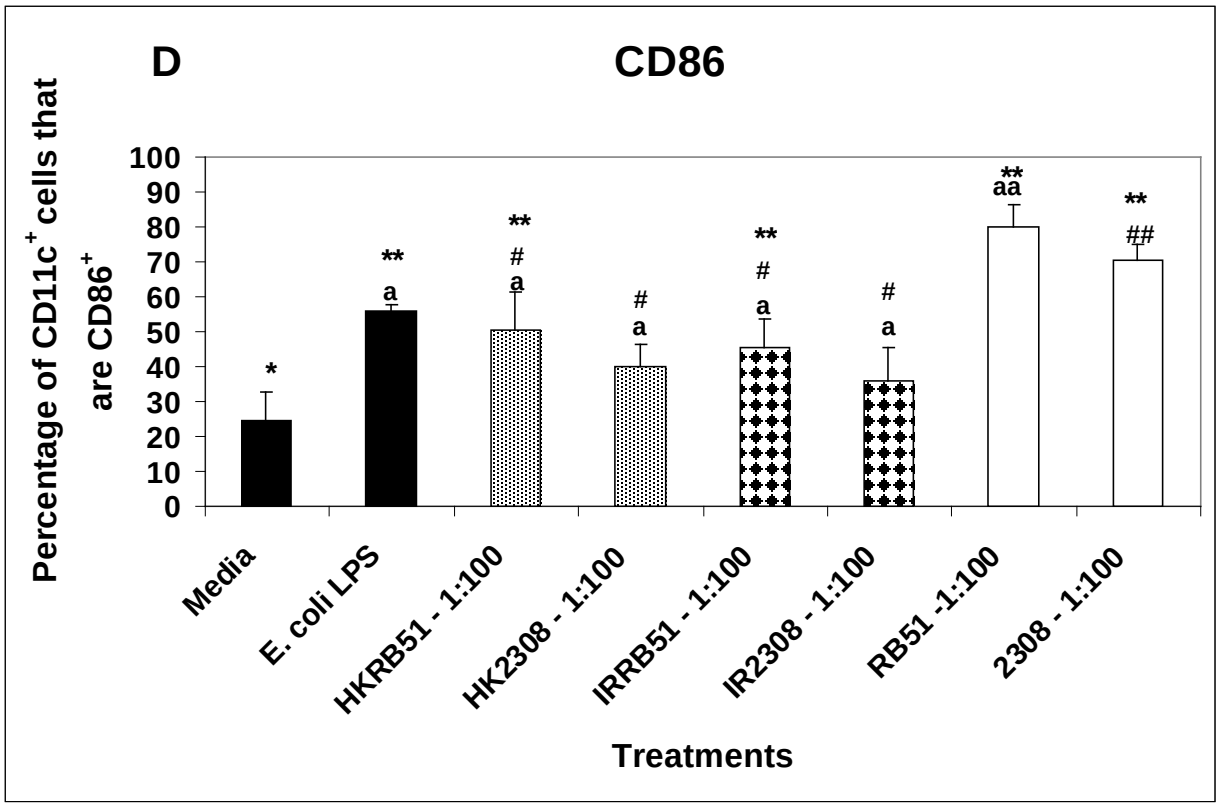
BMDCs at the given doses were analyzed using indirect sandwich ELISA. *E. coli* LPS and media treated cell supernatants served as the positive and negative controls respectively. The limits of detection for both the cytokines were 15pg/ml. The TNF-α result represents means ± SEM of 3 independent experiments. The IL-12_{p70} result represents medians and range of 3 independent experiments. Two asterisks (**) denotes statistically significant data at $\leq p 0.05$ compared to data represented by an asterisk (*).

Figure 1


A
DC- Maturation Markers
Percentage of CD11c+ cells
100
90
80
70
60
50
40
30
20
10
0
*
*
**
*
*
**
*
*
**
Day 6
24 hr Media
24 hr LPS
Day 6
24 hr Media
24 hr LPS
Day 6
24 hr Media
24 hr LPS
MHC II (high)
CD40
CD86
Surface markers


B
MHC class II (high)
Percentage of CD11c+ cells that are MHC class II high
100
90
80
70
60
50
40
30
20
10
0
*
**
**
#
**
#
#
**
##
**
#
Media
E. coli LPS
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 -1:100
2308 - 1:100
Treatments


C
CD40
Percentage of CD11c+ cells that are CD40+
100
90
80
70
60
50
40
30
20
10
0
*
**
Media
E. coli LPS
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 -1:100
2308 - 1:100
Treatments


D
CD86
Percentage of CD11c+ cells that are CD86+
100
90
80
70
60
50
40
30
20
10
0
Media
E. coli LPS
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 -1:100
2308 - 1:100
Treatments

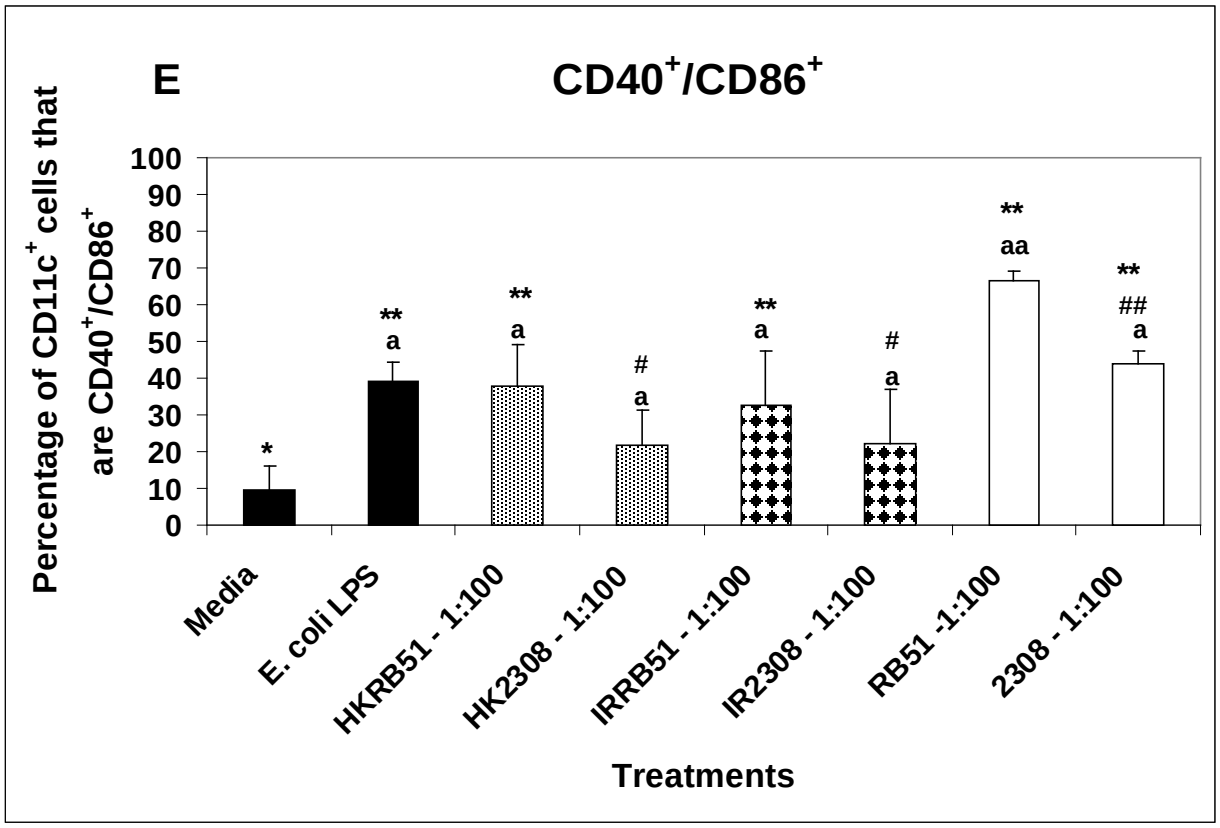
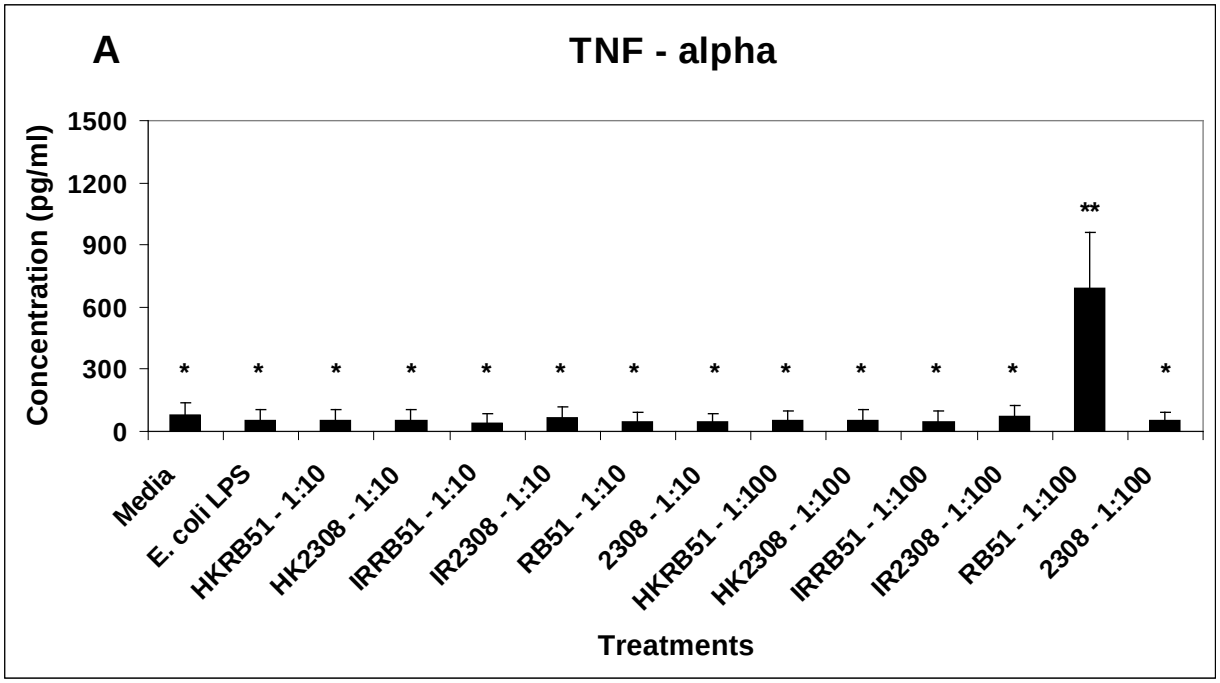
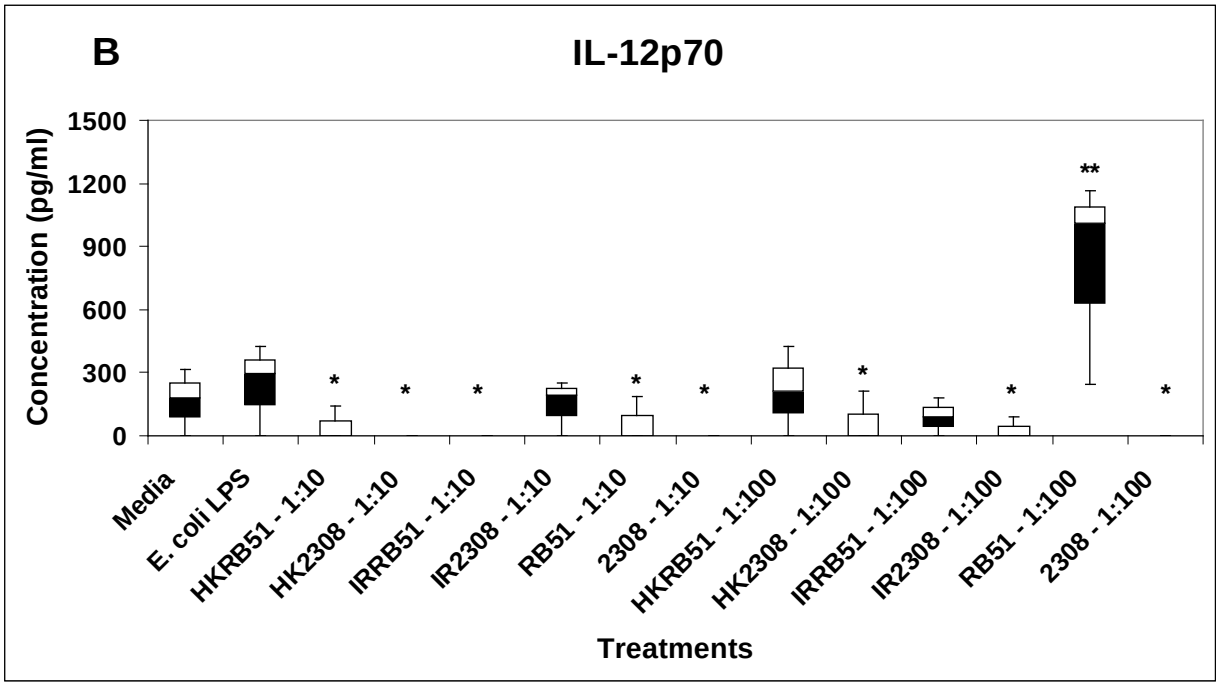

E
CD40+/CD86+
Percentage of CD11c+ cells that are CD40+/CD86+
100
90
80
70
60
50
40
30
20
10
0
Media
E. coli LPS
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 -1:100
2308 - 1:100
Treatments

Figure 2


A
TNF - alpha
Concentration (pg/ml)
1500
1200
900
600
300
0
Media
E. coli LPS
HKRB51 - 1:10
HK2308 - 1:10
IRRB51 - 1:10
IR2308 - 1:10
RB51 - 1:10
2308 - 1:10
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 - 1:100
2308 - 1:100
Treatments


B
IL-12p70
Concentration (pg/ml)
1500
1200
900
600
300
0
Media
E. coli LPS
HKRB51 - 1:10
HK2308 - 1:10
IRRB51 - 1:10
IR2308 - 1:10
RB51 - 1:10
2308 - 1:10
HKRB51 - 1:100
HK2308 - 1:100
IRRB51 - 1:100
IR2308 - 1:100
RB51 - 1:100
2308 - 1:100
Treatments

Chapter 4

The ability of *Brucella abortus* rough vaccine and smooth pathogenic strains to elicit innate immunity in a murine respiratory model.

Running title: *B. abortus* RB51 induces strong innate immunity in lung

Naveen Surendran[1], Kurt Zimmerman[2], Mohamed N. Seleem[2], Nammalwar Sriranganathan[2,] Stephen M. Boyle[2,] Elizabeth M. Hiltbold[3], Heather Lawler[1], Bettina Heid[1], Sharon G. Witonsky[1*].

(To be submitted to Vaccine)

Abstract

B. abortus strain RB51 is a live attenuated rough strain approved as the official vaccine against bovine brucellosis in the United States and many other countries. Another *B. abortus* rough vaccine candidate RB51SOD, which overexpresses its own Cu-Zn superoxide dismutase, has shown to provide better protection upon IP vaccination against pathogenic *B. abortus* strain 2308 in murine model of brucellosis. However, there are no approved vaccines against human brucellosis and inhalation of aerosolized *Brucella* organisms is one route of human infection. Currently, limited information is available on how *Brucella* stimulates pulmonary immunity upon aerosol infection. In this study, we assessed the ability of intranasally delivered rough vaccine strains RB51, RB51SOD and smooth pathogenic strain 2308 to induce innate response characterized by pulmonary dendritic cell (DC) activation and function *in vivo*. We also evaluated the histopathological changes associated with intranasal inoculation of vaccine as well as pathogenic strains. Our results show that rough strain RB51 has superior ability ($p \leq 0.05$) in

stimulating DC activation and function based on surface expression of MHC class II, CD40 and IFN-γ production compared to rough strain RB51SOD and smooth pathogenic strain 2308. In addition, rough strain RB51 induced more proinflammatory histopathological changes in lung compared to the other two *Brucella* strains.

Introduction

Brucellosis is a worldwide zoonotic disease (4) caused by *Brucella spp*., Gram-negative facultative intracellular bacteria. Brucellosis has a huge economic impact on the animal industry since infection results in abortions, still-births and infertility in livestock. Brucellosis can be transmitted from animals to humans as animal tissues are the source of the pathogen. Around the globe, more than 500,000 human infections are reported annually (20). Clinical syndromes in humans include chronic fatigue, undulant fever, reproductive disorders and general malaise (4). *Brucella spp*. are a major potential bio-terror threat, as they are zoonotic, highly infectious, readily aerosolized, and *Brucella* can be genetically manipulated to create antibiotic resistant strains. The Centers for Disease Control and Prevention (CDC) classify *Brucella spp.* as category B agents. Inhalation of infected aerosols is implicated as a route for human exposure both through natural as well as intentional exposure (10, 14). Currently, there are no approved human vaccines available.

Murine brucellosis is widely accepted as an established model for studying the host immune response to experimental *Brucella* infection (i.e., vaccination and challenge) (15, 23, 29). In spite of the importance of aerosol exposure as a route of infection and *Brucella spp.* use as a potential bioterror threat, very few studies have focused on a respiratory route of infection for vaccine efficacy studies (9, 19). Ficht *et al.*, (9) and Olsen *et al.*, (19) failed to show clearance

from lung following intraperitoneal (IP) vaccination and aerosol challenge with *B. melitensis* and/or *B. abortus* respectively. Part of the reason for lack of protection may be due to the IP vaccination route. Based on past experience, it is expected that mucosal vaccination would enhance a protective immune response against aerosol challenge (17, 21). In order to further understand the protective immune responses against *Brucella*, more basic background information is needed. Most *Brucella* studies have predominantly focused on vaccinating via IP, subcutaneous (SC), intravenous (IV) or intramuscular (IM) routes with challenge occurring primarily by IP exposure (8, 23). However, few studies have discerned novel information on innate or adaptive protective immune responses particularly against respiratory challenge. While models have established chronic infection, they were not successful in showing respiratory protection (1, 9, 12, 13, 28). Therefore, to develop an efficacious vaccination regime, assessment of the innate immune response following vaccination is critical. As *B. abortus* strain RB51 has been successfully developed for use in animals (24) and is a vector for the expression of multiple homologous and heterologous antigens (30), we investigated the innate response in mice to vaccine and pathogenic strains of *B. abortus*.

The three *B. abortus* strains used for this study are pathogenic strain 2308, and vaccine strains RB51 and RB51SOD. *B. abortus* strain 2308, with the O-side chain of lipopolysaccharide (LPS), is one of the smooth, pathogenic, zoonotic *Brucella* species affecting cattle and humans (15). *B. abortus* strain RB51, which lacks the O-side chain of LPS, is the rough, live attenuated vaccine strain approved for use in cattle by the United States Department of Agriculture (USDA) for cattle (23). The other *B. abortus* rough strain RB51SOD, which overexpresses its own copper-zinc superoxide dismutase (SOD), has been shown to be more efficacious than strain RB51 against strain 2308 challenge in murine IP vaccination and challenge models (29). To our

knowledge, no studies have been published which characterize the *in vivo* innate immune response including the associated histopathological changes to IN inoculation of either *B. abortus* pathogenic strain 2308 or rough vaccine strains RB51 or RB51SOD. This information will enhance our knowledge on differences between the immune responses associated with IN inoculation of vaccine vs. pathogenic strains. With this knowledge, we may have the ability to both develop an efficacious human brucellosis vaccine against aerosol infection as well as provide information on how *B. abortus* pathogenic strain 2308 limits the immune response in the lungs.

In these studies, we assessed *in vivo* the ability of IN delivered rough vaccine strains RB51 and RB51SOD vs. pathogenic strain 2308 to induce an innate response. In our previous *in vitro* study, we demonstrated that rough vaccine strain RB51 elicited enhanced BMDC maturation and function compared to strain RB51SOD and pathogenic strain 2308 (Surendran et al., under review). Here we evaluated the differential ability of *B. abortus* rough vaccine strains RB51, RB51SOD and smooth pathogenic strain 2308 to elicit pulmonary DC activation and function *in vivo*. We also assessed the vaccine and virulent strain induced histopathological changes in lung, liver and spleen at day 3, 5, 7 and 14 PI. We hypothesized that live rough vaccine strains, as seen *in vitro*, would stimulate increased DC activation and function based on upregulation of costimulatory marker expression and cytokine production. Additionally we expected rough strains would induce more proinflammatory histopathological changes compared to smooth pathogenic strain 2308.

Materials and Methods

Mice: Female 6-8 weeks old BALB/c mice were obtained from Charles River Laboratories Inc., Wilmington, MA. Mice were used under animal care protocols approved by Institutional Animal Care and Use Committee at Virginia Tech.

Bacterial strains, plasmids and oligonucleotides: Live attenuated rough *B. abortus* strains RB51, RB51SOD and virulent smooth strain 2308 used for clearance study were from our stock culture collection (23, 29). *Brucella* strains were routinely grown at 37°C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco). Chloramphenicol (Cm) was used at a final concentration of 22µg/ml for growing strain RB51SOD. Primers used in this study are listed in Table 1.

Recombinant DNA methods: Recombinant DNA methods (DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis) were performed according to standard techniques (22). The polymerase chain reaction (PCR) was performed using Platinum PCR SuperMix High Fidelity (Invitrogen) and a Gradient Mastercycler® (Eppendorf). Oligonucleotides were purchased from Sigma-Genosys (Sigma-Aldrich). Restriction and modification enzymes were purchased from Promega. QIAprep Spin Miniprep Kit from QIAGEN was used for plasmid extractions and QIAGEN PCR cleanup kit was used for restriction enzymes removal and DNA gel extraction.

Vectors construction: *Brucella* strains expressing GFP and HA peptides were generated to study innate immune response as well as CD4 and CD8 antigen specific response to IN inoculated bacterial strains. Briefly, *Trc* promoter (25) with downstream short peptides (IYSTVASSL-PKY) MHC class I and (VKQNTLKLAT) MHC class II (5, 11) was constructed

and amplified in two steps using primers (T-F and T-R1) in the first step. The PCR amplicon was gel purified and used as a template for the second PCR cycle using primers (T-F and T-R2).

The amplified promoter fusion (Trc::MHC) was purified and cloned into *Bam*HI and *Sal*I restriction sites of promoterless pNS vector (27) to form the pNSMHC construct. A promoterless Green Fluorescence Protein gene (GFP) was excised from pGFPuv vector (BD Biosciences Clontech) and cloned in frame downstream of the promoter in the multiple cloning site area of pNSMHC to form (pNSMHC/GFP). The *Brucella* sodC gene with its own promoter was amplified from *B. suis* using primers Sod-F and Sod-R (Table 1). sodC gene was cloned into pNSMHC/GFP vector to form pNSMHC/GFP+SOD. The plasmids were sequenced to confirm the correct sequence. Transformation of *B. abortus* strains 2308 and RB51 was done by electroporation with a Gene Pulser (BTX) set at 2.4 KV, 25 µF and 200 Ω as described previously (26). Recombinant RB51, RB51SOD and 2308 strains expressing GFP were detected under UV light. SOD expression was confirmed by SDS-PAGE and Western blot. The strain that harbors pNSMHC/GFP was named RB51pNSMHCGFP, while the strain that harbors plasmid pNSMHC/GFP+SOD was named RB51pNSMHCGFPSOD. Strain 2308 that harbors pNSMHCGFP was named as 2308pNSMHCGFP (Table 2). These strains will be designated as strains RB51, RB51SOD and 2308 respectively in this manuscript.

All experiments with *Brucella* were performed in our CDC approved (C2003 1120-0016) Biosafety Level (BSL) -3 Infectious Disease Unit (IDU) facility.

Table 1. Primers used for amplification

Name	Size in bp	Source of DNA	Primers Name	Primer sequence
TrcMHC	450	*pNSTrc*	T-F1	5`-CCCGTCGACATTCTGAAATGAGCTGTTGACAAT-3`
			T-R1	5`-GTACTTCGGGAGCGACGAAGCAACGGTCGAGTAGA TGCCATGATGATGATGATGATGAGCCAT-3`
			T-R2	5`-CCCGGATCCGGTAGCGAGCTTGAGGGTGTTCTGCTT AACGTACTTCGGGAGCGACGAAGCAAGGGT-3`
sodC	750	*B. suis*	Sod-F	5`- GGGAAGCTTCCCTCTAGAATAATTTCGGGGTGG AGACATAGTT-3`
			Sod-R	5`- GGGACTAGTTTATTCGATCACGCCGCAGGC -3`

Mice infection and clearance study: BALB/c mice (n=4 per treatment group per time point) were infected IN, under light xylazine-ketamine anesthesia IP, with either of the *B. abortus* rough strains RB51, RB51SOD (4 X 10^7 CFUs/mouse) or with smooth strain 2308 (2 X 10^3 CFUs/mouse) in 35 µl phosphate buffered saline (PBS). Mice were euthanized on day 7, 14 (rough strains) or 16 (smooth strain), and day 42 post infection with a lethal dose of xylazine-ketamine IP. Lung, MLN and spleen were collected. Single cell suspensions of organs collected were serially diluted and plated on to Tryptic soy agar (TSA) plates and incubated for 5 days at 37° C and 5% CO_2. Bacterial colony forming units (CFUs) were counted and CFUs/mouse organ were calculated.

Innate immune response experimental design: BALB/c mice (n=8 total mice per treatment group per time point) were infected IN with the dose (CFUs) described in Table 2. Non-infected (PBS 35µl IN) age matched BALB/c mice (n=2 mice per time point) served as control. The experiment was performed in blocks for each day of the experiment. Mice (n=2 mice per treatment group) were euthanized by IP injection of xylazine hydrochloride and ketamine hydrochloride at days 3, 5, 7 and 14 post infection (PI).

Table 2. Dosage and route of administration of *Brucella* strains for innate experiment.

	Brucella strains	Route of inoculation	Dose (CFUs/Mouse) in 35 μl PBS
Experimental	RB51pNSMHCGFP	IN	4×10^9
	RB51pNSMHCGFPSOD	IN	4×10^9
	2308pNSMHCGFP	IN	2×10^5
Control	PBS	IN	35 μl PBS

Collection and preparation of samples: Broncho-alveolar lavage (BAL), mediastinal lymph node (MLN), spleens, lung and liver were collected at the time of euthanasia. BAL was spun at 250 x *g* for 5 minutes to isolate the cells and the supernatant was frozen for cytokine analysis. The BAL samples from same treatment group were pooled at each time point. The spleens were excised and divided equally for flow cytometry, and histology. The entire MLN was used for flow cytometric analysis. Briefly, spleen and MLN were dissociated with sterile metallic screens and a 3 ml syringe plunger. Cells were washed and enumerated using a hemocytometer. Cells were resuspended at 5×10^6 cells/ml in saline for flow cytometry.

Tissue processing and staining: Portions of the lung, spleen, and liver were collected for histological examination. Sections were fixed in formalin and embedded in paraffin (31). A blinded histopathologic review of lung from all IN infected mice was performed by a board certified anatomic and clinical pathologist (KZ). SW reviewed all spleen and liver samples followed by joint review (SW and KZ) of these tissues.

Tissue reaction scores: Based on characterization of pulmonary changes, lungs were characterized based on pneumonia, vascular change, septa thickening, as well as changes to the pleura, large airway and alveoli. For all parameters, severity was graded as 0-5 (0-1 no change, 1-2 minimal, 2-3 mild, 3-4 moderate, 4-5 severe, >5 marked or extreme). In addition to severity for all parameters, pneumonia was characterized based on severity, pattern, distribution, percent

involvement and type of inflammation. For vascular change, the presence and type of vessel wall changes were noted. For septa thickening, the infiltrative cell type was noted. Alveolar changes were characterized based on debris/protein, type 2 hyperplasia, grade of inflammation (0-5) and type of inflammation. Large airway changes were assessed based on hyperplasia, necrosis, and severity (0-5) of inflammation, peri-lymphoid hyperplasia, peri-edema, and type of inflammatory cells present. Pleura were graded on inflammation (0-5), fibrosis (0-5), and inflammatory cell type. Any additional comments were noted. Averages of percentage of interstitial pneumonia based on KZ assessment were determined, and are reported. Averages of severity, percentage involvement of pneumonia and other changes are reported (Table 3).

Staining and flow cytometry: The cells were stained with approximately 0.5μg of the appropriate stain per 0.5 x 10^6 cells using the following monoclonal antibodies for FACS analysis: FITC-conjugated anti-CD11c, PE-conjugated anti I-A/I-E (BD Pharmingen), TR-conjugated anti-CD11c, PE-conjugated anti-CD40 and Biotin-conjugated anti-CD80 (Caltag). The samples were washed and stained with biotinylated antibody if required. Cells were washed and analyzed by EPICS XL Flow cytometer (Coulter, Hileah, FL).

Cytokine analysis: For cytokine measurement, BAL supernatants from *Brucella* infected mice stored at -80°C were used. IFN- γ, TNF-α, IL-12_{p70} (bioactive form of IL-12) and IL-4 cytokine levels were measured using indirect sandwich ELISAs (BD Pharmingen).

Statistical analysis: Kruskal-Wallis non-parametric test was used to analyze the significance of the DC activation marker expression. Statistical significance of cytokine production in BAL was analyzed by mixed model ANOVA followed by Dunnett's procedure for multiple comparisons. Statistical significance of histopathological lung changes was tested using a general linear model of ANOVA accessing effects of treatment, day and interaction of treatment-day on severity and

percentage of involvement data. Tukey 95% confidence interval pairwise comparisons were used to test for differences in severity and involvement among all levels of treatment. All statistical analysis was done using SAS system (NC, USA) and Minitab software (PA, USA).

Results

***B. abortus* rough vaccine strains cleared more quickly than wild type strain 2308 from IN infected BALB/c mice:** IN doses were chosen relative to already established vaccine strain RB51, RB51SOD (4 X 10^8 CFUs/mouse, IP) and challenge strain 2308 (2 X 10^4 CFUs/mouse, IP) doses. To assess clearance and/or development of chronic infection, BALB/c mice were infected IN with 4 X 10^7 CFUs of either of the rough strains (RB51 or RB51SOD) or with 2 X 10^3 CFUs of pathogenic strain 2308. Clearance was assessed at day 7, 14 and 42 PI from lungs, MLN and spleen for rough vaccine strains (Fig. 1 A). *Brucella* strains RB51 and RB51SOD had marked clearance in the lung with approximately 10^4 decreases in titers by day 14 PI. In the MLN and spleen, titers were approximately 10^3 and 10^2 respectively by day 7. Titers persisted at day 14 PI with spleen and MLN at 10^3 before clearance at day 42. Clearance was complete from all organs by day 42 PI. By comparison, the bacterial load progressively increased for smooth strain 2308 reaching a plateau by day 16 PI (Fig. 1B). Mice remained chronically infected with increased CFUs in spleen and MLN at day 116 PI, whereas clearance was complete in lung by day 116 PI. For strain 2308, live bacteria were not cultured from the MLN until day 16 time point although the titers in spleen were > 80 times the MLN titers. Splenic colonization for strain 2308 was almost 6 times greater than lung titers by day 16. By day 42, splenic colonization for strain 2308 remained higher compared to lung and MLN colonization, which were similar. As of day 42, mice infected with smooth strain 2308 were chronically infected in lung, spleen

and MLN. By comparison, by day 7, mice infected IN with higher doses of rough strains had live bacteria that were cultured from MLN with greater numbers than those recovered from spleen. With rough strains, titers were similar in all organs by day 14 and completely cleared by day 42 (Fig. 1A). These data suggest differences in bacterial kinetics for systemic spread for rough and smooth strains *in vivo*.

Rough strain RB51 significantly upregulated CD11c$^+$/MHC class II high and CD40 expression: The ability of rough GFP expressing strains RB51, RB51SOD and smooth GFP expressing strain 2308 vs. saline control to induce DC activation was evaluated by assessing MHC class II, CD40 and CD80 expression on CD11c$^+$ cells (DCs) from BAL, MLN and spleen at day 3, 5, 7 and 14 PI. In BAL (Fig. 2A), of the total gated granulocytes, strain RB51 infected mice induced significantly ($p \leq 0.05$) higher percentage of CD11c$^+$ cells expressing MHC class II high than PBS inoculated mice at all post infection time periods (day 3, 5, 7 & 14) tested. The CD11c$^+$/MHC class II high expression induced by strain RB51 was also significantly ($p \leq 0.05$) higher than smooth strain 2308 induced expression at day 5, 7 and day 14 PI. RB51 also induced significantly ($p \leq 0.05$) higher MHC class II high expression greate r than strain RB51SOD at day 5 and day 7. At day 14 PI, strain RB51SOD induced population of CD11c$^+$/MHC class II high cells were significantly higher ($p \leq 0.05$) than induced by strain 2308 infected mice. On average 90-98% of the gated CD11c$^+$ cells in BAL, from all the different treatment groups at all post infection time points were positive for CD11b expression. This suggested that they were DCs of myeloid origin (data not shown).

Similarly, strain RB51 infected mice had significantly higher ($p \leq 0.05$) CD11c$^+$/MHC class II high expressing cells compared to PBS mice in MLN at day 3 and 7 ($p \leq 0.05$) and 14 ($p=0.052$) (Fig. 2B). Strain RB51SOD induced CD11c$^+$/MHC class II high expression was only

significantly ($p \leq 0.05$) higher than PBS control at day 7 PI. In the splee n, there were no significant changes in $CD11c^+$/MHC class II high cells between any treatments at any time points (data not shown). Smooth strain 2308 did not induce significant percentages in $CD11c^+$/MHC class II high expression in BAL or MLN compared to PBS control. Assessing DC activation in BAL, MLN and spleen, we determined that at day 7 PI strain RB51 stimulated statistically significant ($p \leq 0.05$) increased expression of $CD11c^+/CD40^+$ cells in BAL compared to PBS and strain 2308 treated mice (data not shown). Total cellularity was not assessed. No other significant findings were detected.

Strain RB51 infected mice induced increased IFN-γ secretion in BAL: To assess potential innate function, IFN-γ and IL-4 cytokine levels in the BAL supernatants were measured from infected and control mice at day 3, 5, 7 and 14 PI. RB51 infected mice IN (Fig. 3) had higher IFN-γ production in BAL at both day 5 (non significant) and 7 PI (significant, $p \leq 0.05$) compared to PBS control. Neither rough strain RB51SOD nor smooth strain 2308 infected mice induced significantly increased IFN-γ production compared to PBS control at any time points (Fig. 3). Both RB51 and RB51SOD had significantly decreased IFN-γ production compared to PBS control at day 3. None of the *Brucella* strains or PBS control mice induced detectable levels of IL-4 in BAL supernatants from IN infected mice at any of the time points tested (data not shown).

Altogether, these data demonstrated that rough vaccine strain RB51 had superior ability *in vivo* to induce DC activation and function compared to rough strain RB51SOD and smooth pathogenic strain 2308.

Strain RB51 infected mice developed interstitial pneumonia with vasculitis: The most significant histopathologic changes were noted in the lungs with much less significant changes in

the spleen and liver. In the lung only strain RB51 induced significant (p=0.0003) histopathological changes in terms of severity of pneumonia compared to PBS control (Figure 4). Strain RB51 effect on severity was not influenced by day of treatment or by interaction of day and treatment type. Strain RB51 induced severity of pneumonia was significantly higher than both strain RB51SOD (p=0.027) and strain 2308 (p=0.015) induced pneumonia (Figure 4). However, no other treatment differences were seen for PBS, strain RB51SOD, and strain 2308 for severity or for percentage of lung involvement between any of the groups. Table 3 illustrates average pulmonary changes based on severity and percentage involvement of pneumonia as well as severity of vascular change, septa thickening and debri/protein. Overall, strain RB51 induced significantly higher severity of pneumonia than PBS, strain RB51SOD and strain 2308 at all time points. At all days, strain RB51 induced significantly higher average severity but non-significant increases in percentage of pneumonia, severity of vascular change with lymphocytic perivascular changes, septal thickening and debris/protein compared to both strains RB51SOD and strain 2308, with the following exceptions at day 14. At day 14, strain RB51SOD induced minimally greater percentage involvement of pneumonia and debri/protein than strain RB51. In addition, only strain RB51 infected mice, except for 1 strain RB51SOD infected mouse had lymphocytic perivascular infiltrate. This was most noticeable at day 3 and 5, but also present at days 7 and 14 PI.

Histopathologic review of livers and spleens was performed by SW. In the liver at day 3 PI changes were very minimal in all *Brucella* infected mice compared to saline controls. At day 5 PI, there was a mild increase in small multi-focal lymphocytic infiltrates within the hepatic parenchyma in both strain RB51 and strain RB51SOD infected mice. By day 7 PI, infiltrates were still present, but contained increased numbers of macrophages. Limited changes were

present in strain 2308 infected mice at day 5 PI, but by day 7 PI, some mice had multi-focal infiltrates containing neutrophils and lymphocytes within the liver parenchyma.

A limited number of samples from the spleens were available for analysis. At day 3 post-infection (PI), there was a mild increase in follicular activity in all treatment groups (PBS, strain RB51, strain RB51SOD, strain 2308). At day 5 PI, follicular activity of all *Brucella* infected mice was mildly increased compared to saline control group. In addition, there was a mild increase in extramedullary hematopoiesis (EMH) in the strain RB51 and 2308 inoculated mice. Some of the mice from strain RB51SOD and strain 2308 infected mice had increased numbers of macrophages present. These changes persisted at day 7 PI.

Discussion

Inhalation of aerosolized *Brucella* organisms is one of the routes of human infection in intentional exposure/bioterrorism leading to brucellosis (4, 20). In this study, for the first time, we analyzed the innate immune response and associated histopathological changes due to IN infection with *B. abortus* rough vaccine strains RB51, RB51SOD and smooth virulent strain 2308 expressing GFP in a BALB/c mouse model. Our results were consistent with in vitro studies (2, 3) which showed an enhanced DC activation and function induced by rough vs. smooth strains of different *Brucella* species. However, in our studies, strain RB51 displayed significantly better ability to induce DC activation and IFN-γ secretion *in vivo* compared to strain RB51SOD. Histopathological analysis also revealed enhanced inflammatory response with strain RB51 infected mice versus strain RB51SOD and strain 2308 inoculated mice. (13). Rough strains were also cleared completely while smooth strains caused chronic infection.

Overall these data support the potential use of strain RB51 as a vaccine strain to protect against respiratory challenge. These data also raise questions on the subdued immune response induced by strain RB51SOD and the mechanisms of immune subversion by strain 2308. In this study, systemic clearance of rough and smooth strains following intranasal infection was determined. We used these data to select doses and time points, and to identify differences between vaccine strains, which were cleared and pathogenic strains which resulted in chronic infection. Further challenge experiments would be necessary to confirm vaccine induced protection. In comparing dissemination of rough vs. smooth strains, for rough strains, clearance from the lung (by DCs and other cells as well as killing of strain RB51) was associated with dissemination to spleen and lymph node by day 14 PI. Clearance was complete from all organs by day 42 PI. With smooth strains, replication likely occurred in lungs as titers in the lungs increased between day 7 and 16 PI. Comparing dissemination between smooth and rough strains, dissemination systemically appeared to be delayed in smooth strains in that titers in spleen and MLN were less than lung at day 7 PI. One possible explanation for these differences is based on possible epithelial cell replication (1, 9, 19, 28). Ferrero et al. (6), showed that smooth strains exhibited marked intracellular replication ability in human lung epithelial cell lines. Our data supports this possible explanation for allowing smooth *Brucella* strains to replicate in the lungs, possibly both within epithelial cells as well as DCs. This replication could allow smooth strain 2308 to have delayed clearance from the lung compared to rough strains. Additionally if the immune response to strain 2308 is delayed compared to rough strains, it is possible that strain 2308 resided in the lung and replicated in DCs and other cells without rapid systemic activation and spread to other organs, as occurs with rough strains.

In comparing dissemination of smooth and rough strains in spleen and MLN, rough strains followed an expected/predicted pattern of clearance whereby MLN and spleen titers were increased at day 7, plateaued at day 14 and were cleared by day 42 PI. As Day 7 titers are higher in the MLN than spleen the data suggest that rough strains may spread to MLN prior to spleen. Comparatively, with smooth strain 2308, by day 7 PI, *Brucella* had disseminated to the spleen but not to the MLN. From that point, titers increased by day 16 PI. As titers persisted at day 42, this was considered to be a chronic infection.

Our initial clearance studies demonstrated the ability to clear rough vs. smooth *Brucella* strains as well as established parameters for respiratory model of vaccine and challenge experiments. To identify differences in innate immune responses between vaccine and pathogenic strains, we focused on using DCs in this study as they are a critical cell population in initiating the innate and regulating the adaptive immune response. Jakubzick et al. (7) had demonstrated that following aerosol exposure, pulmonary DCs vs. alveolar macrophages efficiently traffic to the lung draining lymph node (MLN) with captured antigen to initiate the adaptive immune response. Pulmonary DCs were $CD11c^+$, $CD11b^+$ and expressed moderate to high levels of MHC class II based on maturation/activation status. By contrast, macrophages were $CD11c^+$, $CD11b^-$ and low levels of MHC class II (7). In the MLN, the percentage of $CD11c^+$ DCs that expressed CD11b marker was lower than in the BAL. This could have been due to downregulation of CD11b upon maturation and migration to the lymph node.

In our study, interestingly, we observed that IN infection with strain RB51 induced significant ($p \leq 0.05$) upregulation of $CD11c^+$/MHC class II high expressing cells in BAL compared to that induced by PBS at all days PI; to strain RB51SOD on day 5 & 7 PI, and to strain 2308 on day 5, 7 & 14 PI. These data supported that strain RB51 had a better ability to

activate DCs *in vivo* (Fig. 2A). In addition, strain RB51 induced significantly ($p \leq 0.05$) higher costimulatory marker expression in terms of $CD11c^+/CD40^+$ expressing cells in BAL (data not shown) at day 7 PI compared to PBS and strain 2308 inoculated mice. In contrast, strain RB51SOD had significantly higher MHC class II high expression at day 14 PI. Smooth strain 2308 did not induce any higher than baseline levels of $CD11c^+$/MHC class II high expressing cells in BAL similar to PBS inoculated mice. This suggested that smooth strain 2308 did not activate DCs or the innate immune response as well.

In MLN, strain RB51 [day 3 and 7 ($p \leq 0.05$) and 14 PI ($p=0.052$)] and RB51SOD (day 7 PI) induced significantly greater $CD11c^+$ MHC class II high expression than PBS treated mice. These data suggested that many of the antigen captured immature DCs matured while trafficking from lung to MLN (Fig. 2B). In considering all the strains, strain RB51 induced persistent DC activation in BAL and MLN on all days PI tested (except for day 5 PI in MLN) compared to control mice. Thus, strain RB51 induced the best overall DC activation compared to all the other strains. In contrast, smooth pathogenic strain 2308 did not stimulate significant DC maturation at any time points in either BAL or MLN. Strain RB51SOD stimulated significant DC maturation only at day 7 (MLN) and 14 (BAL) PI vs. PBS control. These data supported that strain RB51 induced more significant DC activation than strain RB51SOD; therefore strain RB51 should be considered for use as a vaccine vs. strain RB51SOD.

In addition to delineating DC activation, DC function was also assessed based on cytokine function in the BAL. The nature of the DC response (i.e., DC activation and cytokine profiles) modulates the outcome of the resultant adaptive immune response. For *Brucella* infection, IFN-γ plays a pivotal role in mediating an effective protective response (16). The significantly higher IFN-γ level detected from BAL of strain RB51 infected mice on day 7 PI

gave additional proof for the enhanced innate immune stimulation achieved by strain RB51 (Fig. 3) compared to smooth strain 2308 and rough strain RB51SOD. The source of IFN-γ may be NK cells, DCs or T-cells activated and recruited to the lung upon DC mediated antigen presentation. As significant differences in DC activation in BAL were detected from strain RB51 infected mice at day 5 PI, but IFN-γ levels were increased but not significant until day 7 PI, this still suggested any of the above cell populations as a source of IFN-γ. In future studies, intracellular cytokine staining or RT-PCR of isolated populations would be needed to detect the contribution by specific cell populations. These data supported that there was a bias toward a Th_1 response in lungs. Additionally, there was no detectable IL-4 secretion in BAL from both *B. abortus* rough or smooth strain infected mice ruling out a Th_2 mediated immune response. The BAL - IFN-γ level reached baseline levels by day 14 PI, in agreement with the documented cessation of IFN-γ production that begins after the first week of *B. abortus* infection in BALB/c mice (15). There was a significant decrease in IFN-γ production by strain RB51 and RB51SOD inoculated mice in BAL compared to saline control. Averages of strain 2308 induced IFN-γ levels were also lower although not significantly different. This supported that either a) saline induced some level of reaction given IN that still caused a mild immune and inflammatory response associated with IFN-γ and/or b) infection caused consumption of baseline levels of IFN-γ prior to the host's ability to make IFN-γ. Both are possibilities. However, as the levels of PBS induced IFN-γ were relatively constant in the BAL over time, it suggests that explanation b is more likely. Additional studies are needed to more closely track acute changes in specific cell populations over time.

Besides assessing innate response based on DC activation and cytokine production in BAL, the innate response was also assessed based on histopathology (Table 3). Our data supported that strain RB51 infected mice overall had more significant changes regardless of time

as strain RB51 infected mice induced higher severity of pneumonia compared to all the other treatment groups. Strain RB51 infected mice overall had significantly greater scores in severity of pneumonia, but nonsignificant increase in percentage of multifocal lymphocytic histiocytic pneumonia with increased severity of vascular change including fibrinoid necrosis and perivascular infiltrate. Only strain RB51 infected mice, except for 1 strain RB51SOD infected mouse, had lymphocytic perivascular infiltrate compared to the other treatment groups. Additionally, overall strain RB51 infected mice had greater, but non significant, severity of septal thickening due to macrophages and lymphocytic infiltrate along with increased severity of debri and protein in the airways. By comparison, strain RB51SOD infected mice had less severe changes than strain RB51. Finally, strain 2308 infected mice had the least severe average changes in pneumonia and percentage involvement overall as well as other parameters compared to the other strains.

Minimal changes were present in the liver and spleen from the infected mice. Interestingly, whereas only strain RB51 infected mice had lymphocytic perivascular infiltrate in the lung, none of the strain 2308 infected mice had perivascular infiltrate. Based on the parameters assessed, strain RB51 had more severe changes for all parameters vs. strain RB51SOD and strain 2308. The difference in the perivascular infiltrate of strain RB51 was striking. The mechanism for it is not known. Possible explanations include differences in endothelial cell activation with or without direct infection of cells, and/or differences in DC activation, cytokine production by DCs, macrophages, NK cells; any of these changes could cause differences in cell recruitment. Thus, the mechanism of infiltrate and its corresponding role in the innate immune response and associated protection warrants further investigation.

Overall, these data supported that strain RB51 enhanced DC activation, cytokine production and inflammatory responses *in vivo* compared to strains RB51SOD and 2308. While both rough strains were cleared following IN infection and strain 2308 was not, these data also supported that some rough strains may enhance innate immune function and protect better than pathogenic smooth strains that cause persistent infection. There are likely different factors affecting clearance vs. ability to stimulate protective immunity. In this study as well as others (Surendran et al., accepted; Veterinary Microbiology; Sriranganathan and Boyle, personal communication), strain RB51SOD did not stimulate innate response as well as strain RB51. *In vitro* studies (Surendran et al., accepted; Veterinary Microbiology) showed that strain RB51 had greater DC activation and function in BMDC mouse model compared to strain RB51SOD. Possible explanations for the decreased response to RB51SOD, which are being investigated, include: whether over-expressed SOD was functional; if it could decrease the inflammation and subsequent immune response and/or whether SOD biases the DC mediated T-cell response to a reduced Th1 response compared to strain RB51. Preliminary studies by others (Sriranaganathan and Boyle, personal communication) support the latter. Although mouse studies supported that strain RB51SOD was more protective (29), Olsen et al. (18) demonstrated in bison that strain RB51 showed greater protection compared to strain RB51SOD. There are no other published studies assessing innate response to strain RB51 vs. RB51SOD. These studies are the first to assess innate response to strain RB51 and RB51SOD both *in vitro* and *in vivo*. As part of these differences in function, the mechanism for the perivascular infiltrate and its role in the innate response of strain RB51 warrants additional investigation. The results have a significant impact on what vaccines may be used in the future both for *Brucella* as well as using *Brucella* as a platform for multivalent vaccines.

Acknowledgements: The authors acknowledge Dr. Ramanathan Kasimanickyam and Dr. Stephen Werre for help with the statistical analysis of the data and Ms. Joan Kalnitsky for help with flow cytometry.

References

1. **Bhattacharjee, A. K., L. Van de Verg, M. J. Izadjoo, L. Yuan, T. L. Hadfield, W. D. Zollinger, and D. L. Hoover.** 2002. Protection of mice against brucellosis by intranasal immunization with *Brucella melitensis* lipopolysaccharide as a noncovalent complex with *Neisseria meningitidis* group B outer membrane protein. Infect Immun **70:**3324-3329.
2. **Billard, E., J. Dornand, and A. Gross.** 2007. *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. Infect Immun **75:**4980-4989.
3. **Billard, E., J. Dornand, and A. Gross.** 2007. Interaction of *Brucella suis* and *Brucella abortus* rough strains with human dendritic cells. Infect Immun **75:**5916-5923.
4. **Corbel, M. J. (ed.).** 2006. Brucellosis in humans and animals. World Health Organization.
5. **Cunliffe, S. L., J. R. Wyer, J. K. Sutton, M. Lucas, G. Harcourt, P. Klenerman, A. J. McMichael, and A. D. Kelleher.** 2002. Optimization of peptide linker length in production of MHC class II/peptide tetrameric complexes increases yield and stability, and allows identification of antigen-specific CD4+T cells in peripheral blood mononuclear cells. Eur J Immunol **32:**3366-3375.

6. **Ferrero, M. C., C. A. Fossati, and P. C. Baldi.** 2009. Smooth *Brucella* strains invade and replicate in human lung epithelial cells without inducing cell death. Microbes Infect **11:**476-483.

7. **Jakubzick, C., F. Tacke, J. Llodra, N. van Rooijen, and G. J. Randolph.** 2006. Modulation of dendritic cell trafficking to and from the airways. J Immunol **176:**3578-3584.

8. **Jimenez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect Immun **62:**4990-4996.

9. **Kahl-McDonagh, M. M., A. M. Arenas-Gamboa, and T. A. Ficht.** 2007. Aerosol infection of BALB/c mice with *Brucella melitensis* and *Brucella abortus* and protective efficacy against aerosol challenge. Infect Immun **75:**4923-4932.

10. **Kaufmann, A. F., M. D. Fox, J. M. Boyce, D. C. Anderson, M. E. Potter, W. J. Martone, and C. M. Patton.** 1980. Airborne spread of brucellosis. Ann N Y Acad Sci **353:**105-114.

11. **Kreuwel, H. T., J. A. Biggs, I. M. Pilip, E. G. Pamer, D. Lo, and L. A. Sherman.** 2001. Defective CD8+ T cell peripheral tolerance in nonobese diabetic mice. J Immunol **167:**1112-1117.

12. **Mense, M. G., R. H. Borschel, C. L. Wilhelmsen, M. L. Pitt, and D. L. Hoover.** 2004. Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques (*Macaca mulatta*). Am J Vet Res **65:**644-652.

13. **Mense, M. G., L. L. Van De Verg, A. K. Bhattacharjee, J. L. Garrett, J. A. Hart, L. E. Lindler, T. L. Hadfield, and D. L. Hoover.** 2001. Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. Am J Vet Res **62:**398-405.

14. **Miller, C. D., J. R. Songer, and J. F. Sullivan.** 1987. A twenty-five year review of laboratory-acquired human infections at the National Animal Disease Center. Am Ind Hyg Assoc J **48:**271-275.

15. **Murphy, E. A., M. Parent, J. Sathiyaseelan, X. Jiang, and C. L. Baldwin.** 2001. Immune control of *Brucella abortus* 2308 infections in BALB/c mice. FEMS Immunol Med Microbiol **32:**85-88.

16. **Murphy, E. A., J. Sathiyaseelan, M. A. Parent, B. Zou, and C. L. Baldwin.** 2001. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology **103:**511-518.

17. **Neutra, M. R., and P. A. Kozlowski.** 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol **6:**148-158.

18. **Olsen, S. C., S. M. Boyle, G. G. Schurig, and N. N. Sriranganathan.** 2009. Immune responses and protection against experimental challenge after vaccination of bison with *Brucella abortus* strain RB51 or RB51 overexpressing superoxide dismutase and glycosyltransferase genes. Clin Vaccine Immunol **16:**535-540.

19. **Olsen, S. C., W. R. Waters, and W. S. Stoffregen.** 2007. An aerosolized *Brucella spp.* challenge model for laboratory animals. Zoonoses Public Health **54:**281-285.

20. **Pappas, G., P. Panagopoulou, L. Christou, and N. Akritidis.** 2006. *Brucella* as a biological weapon. Cell Mol Life Sci **63:**2229-2236.

21. **Pilette, C., Y. Ouadrhiri, V. Godding, J. P. Vaerman, and Y. Sibille.** 2001. Lung mucosal immunity: immunoglobulin-A revisited. Eur Respir J **18:**571-588.

22. **Sambrook J, F. E. a. M. T.** 1989 Molecular cloning. A Laboratory Manual., vol. 2nd ed. Cold Spring Harbor Laboratory Press, New York.

23. **Schurig, G. G., R. M. Roop, 2nd, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol **28:**171-188.

24. **Schurig, G. G., N. Sriranganathan, and M. J. Corbel.** 2002. Brucellosis vaccines: past, present and future. Vet Microbiol **90:**479-496.

25. **Seleem, M., M. Ali, M. W. Abd Al-Azeem, S. M. Boyle, and N. Sriranganathan.** 2007. High-level heterologous gene expression in *Ochrobactrum anthropi* using an A-rich UP element. Appl Microbiol Biotechnol **73:**1123-1127.

26. **Seleem, M., Rajasekaran P, Ali M, Boyle SM & Sriranganathan N** 2008. Simple method for transformation of *Ochrobactrum anthropi*. World Journal of Microbiology and Biotechnology **24:**2111-2114.

27. **Seleem, M. N., R. Vemulapalli, S. M. Boyle, G. G. Schurig, and N. Sriranganathan.** 2004. Improved expression vector for *Brucella* species. Biotechniques **37:**740, 742, 744.

28. **Smither, S. J., S. D. Perkins, C. Davies, A. J. Stagg, M. Nelson, and H. S. Atkins.** 2009. Development and characterization of mouse models of infection with aerosolized *Brucella melitensis* and *Brucella suis*. Clin Vaccine Immunol **16:**779-783.

29. **Vemulapalli, R., Y. He, S. Cravero, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun **68:**3286-3289.

30. **Vemulapalli, R., Y. He, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2002. *Brucella abortus* RB51: enhancing vaccine efficacy and developing multivalent vaccines. Vet Microbiol **90:**521-532.

31. **Witonsky, S. G., R. M. Gogal, Jr., R. B. Duncan, and D. S. Lindsay.** 2003. Immunopathologic effects associated with Sarcocystis neurona-infected interferon-gamma knockout mice. J Parasitol **89:**932-940.

Figure legends

Figure 1. Bacterial clearance from BALB/c mice following IN infection with *B. abortus* rough and smooth strains. BALB/c mice (n=4) were infected IN with strains RB51, RB51SOD (4 X 10^7 CFUs/mouse) and 2308 (2 X 10^3 CFUs/mouse). Mice were euthanized and the number of bacteria recovered from lung, mediastinal lymph node (MLN) and spleen were counted on day 7, 14 and 42 post infection for rough strains (A) and on day 7, 16 and 42 post infection for strain 2308 (B). Results represent mean values ± standard deviation.

Figure 2. *In vivo* dendritic cell (DC) maturation in response to IN infection with *B. abortus* rough and smooth strains. On day 3, 5, 7 and 14 following IN infection with *B. abortus* rough (RB51, RB51SOD; 4 X 10^9 CFUs/mouse) and smooth strains (2308; 2 X 10^5 CFUs/mouse) expressing GFP, broncho-alveolar lavage (BAL) and mediastinal lymph node (MLN) were collected and stained with anti-mouse CD11c and MHC class II (I-A/I-E) antibodies. PBS inoculated mice served as control. (A) Percentage of gated cells in BAL expressing $CD11c^+$/MHC class II high markers from different treatment groups at day 3, 5, 7 and 14 post infection. Data represent results pooled from BAL samples of 2 mice per time point per treatment group from 4 independent experiments. The graph shows median ± 75^{th} percentile. (B)

Percentage of gated cells in MLN expressing $CD11c^+$/MHC class II high markers from different treatment groups at day 3, 5, 7 and 14 post infection. Data represent results from MLN samples of 2 mice per time point per treatment group from 4 independent experiments. The graph shows median ± 75^{th} percentile. Double characters ** and ## represent statistically significant change at $p \leq 0.05$ with the corresponding single character representation (*, #). For MLN – $CD11c^+$/MHC class II high expression at day 14, p values for RB51, RB51SOD and 2308 in comparison to PBS control mice were 0.052, 0.061 and 0.093 respectively.

Figure 3. IFN-γ secretion in BAL following IN infection with *B. abortus* rough and smooth strains. On day 3, 5, 7 and 14 following IN infection with *B. abortus* rough (RB51, RB51SOD; 4 X 10^9 CFUs/mouse) and smooth strains (2308; 2 X 10^5 CFUs/mouse) expressing GFP, broncho-alveolar lavage (BAL) was collected and IFN-γ level assessed by indirect sandwich ELISA. PBS inoculated mice served as control. Data represent results from pooled BAL samples of 2 mice per time point per treatment group from 4 independent experiments. The graph shows mean values ± standard deviation. Two asterisks (**) denote statistically significant data at $p \leq 0.05$ compared to data represented by an asterisk (*)

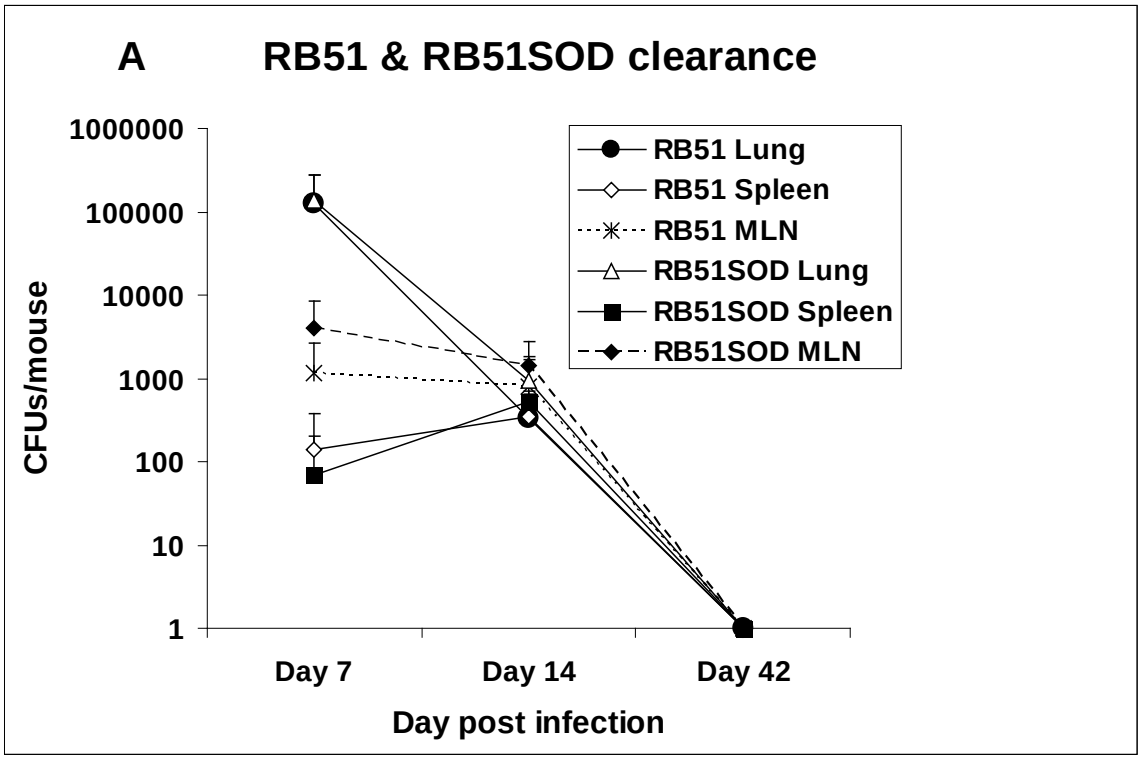
Figure 4. Variation in pneumonia severity score following IN infection with *B. abortus* rough and smooth strains. Strain RB51 induced significantly more severe pneumonia compared to PBS, RB51SOD and 2308 treatments. Line represents mean severity score bracketed by 95% confidence interval. Two asterisks (**) denote statistically significant data at $p \leq 0.05$ compared to data represented by an asterisk (*)

Table 3. Histopathological changes in lung tissue after intranasal inoculation with saline or rough and smooth strains of *B. abortus*. Based on histopathological pulmonary changes at day 3, 5, 7 or 14 post infection with saline, rough or smooth strains of *B. abortus*; lungs were

characterized based on pneumonia, vascular change, septa thickening, as well as changes to the pleura, large airway and alveoli. For all parameters, average (± standard deviation) severity was graded as 0-5 (0-1 no change, 1-2 minimal, 2-3 mild, 3-4 moderate, 4-5 severe, >5 marked or extreme).

Figure 5. Histopathology of lungs from BALB/c mice following intranasal infection with *B. abortus* strains compared to saline control. BALB/c mice were infected with saline or respective rough RB51, RB51SOD and smooth strain 2308. At days 3, 5, 7 and 14 post-infection, samples were collected for clearance, flow cytometry and histopathology. Samples from day 5 lung at 400 x magnification are shown here. (A) PBS control with minimal changes. (B) Strain RB51 infected mouse depicting mild lymphocytic perivascular infiltrate with fibrinoid necrosis of cell wall along with multifocal lymphocytic and histiocytic interstitial pneumonia. (C) Strain RB51SOD infected mouse with mild endothelial hypertrophy/activation and mild but decreased perivascular infiltrate and multifocal lymphocytic and histiocytic interstitial pneumonia. (D) Strain 2308 infected mouse with mild perivascular infiltrate and multifocal lymphocytic and histiocytic interstitial pneumonia.

Figure 1


A
RB51 & RB51SOD clearance
1000000
100000
10000
1000
100
10
1
CFUs/mouse
RB51 Lung
RB51 Spleen
RB51 MLN
RB51SOD Lung
RB51SOD Spleen
RB51SOD MLN
Day 7
Day 14
Day 42
Day post infection

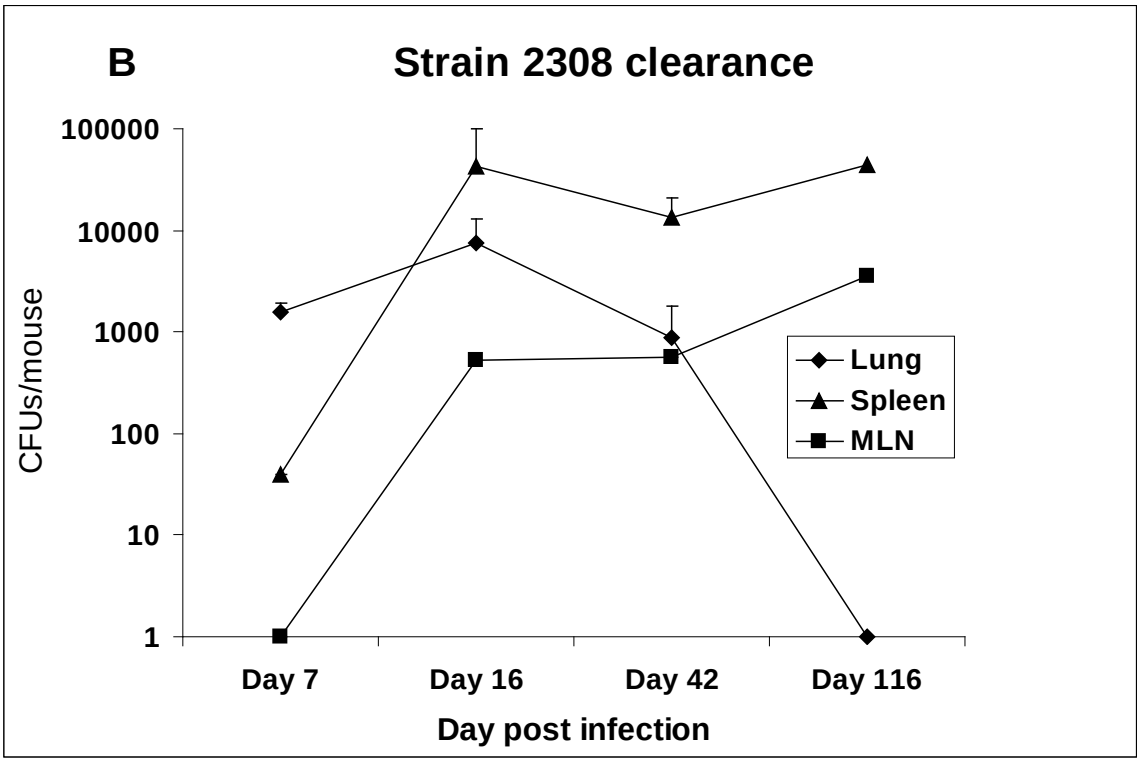
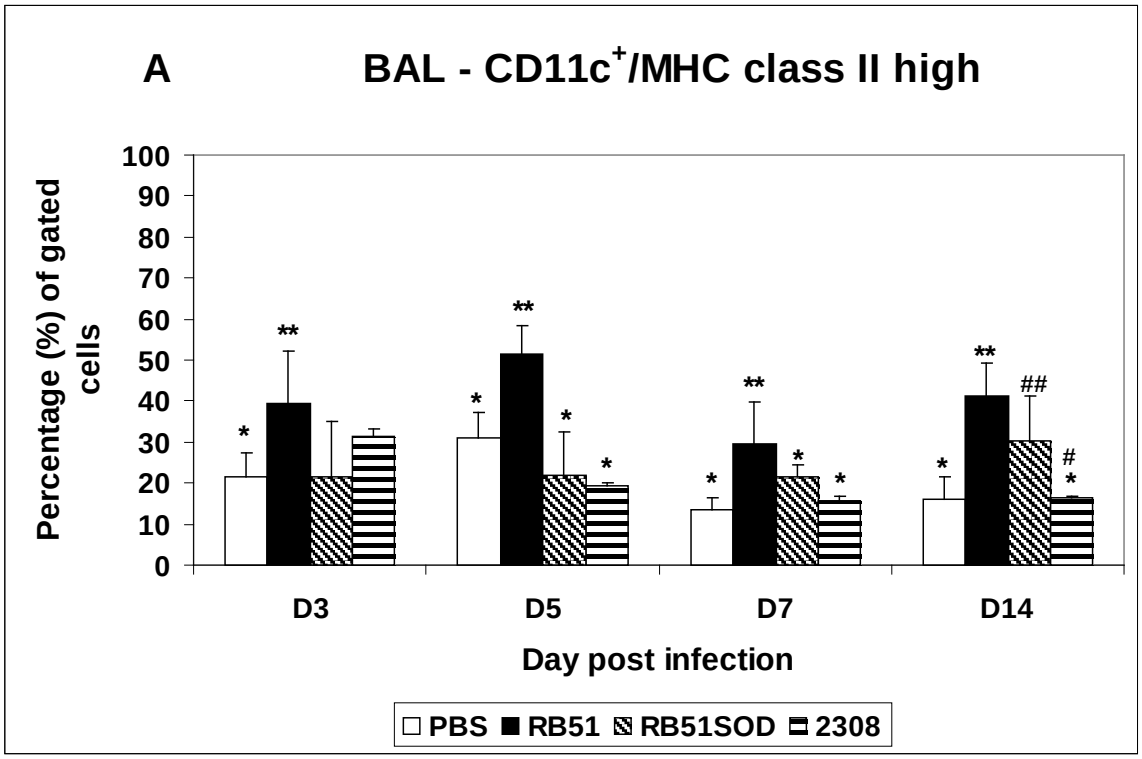

B
Strain 2308 clearance
100000
10000
1000
100
10
1
CFUs/mouse
Lung
Spleen
MLN
Day 7
Day 16
Day 42
Day 116
Day post infection

Figure 2


A
BAL - CD11c+/MHC class II high
Percentage (%) of gated cells
100
90
80
70
60
50
40
30
20
10
0
D3
D5
D7
D14
Day post infection
PBS
RB51
RB51SOD
2308

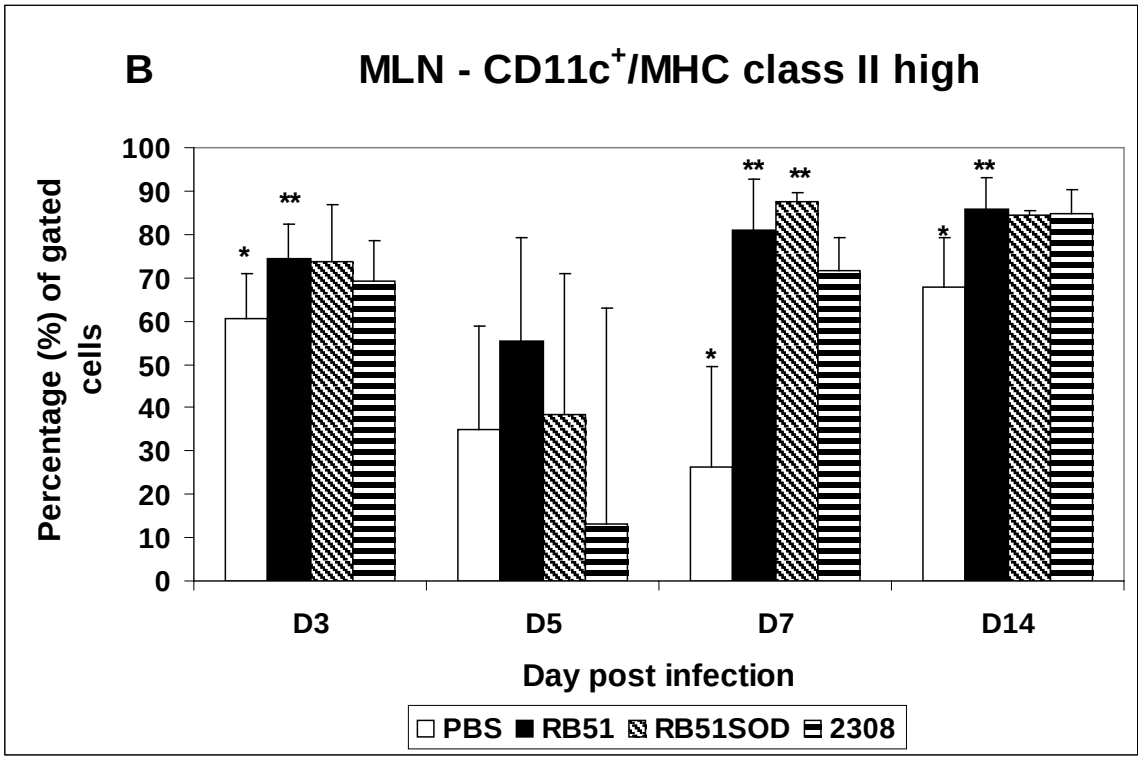

B
MLN - CD11c+/MHC class II high
Percentage (%) of gated cells
100
90
80
70
60
50
40
30
20
10
0
D3
D5
D7
D14
Day post infection
PBS
RB51
RB51SOD
2308

Figure 3

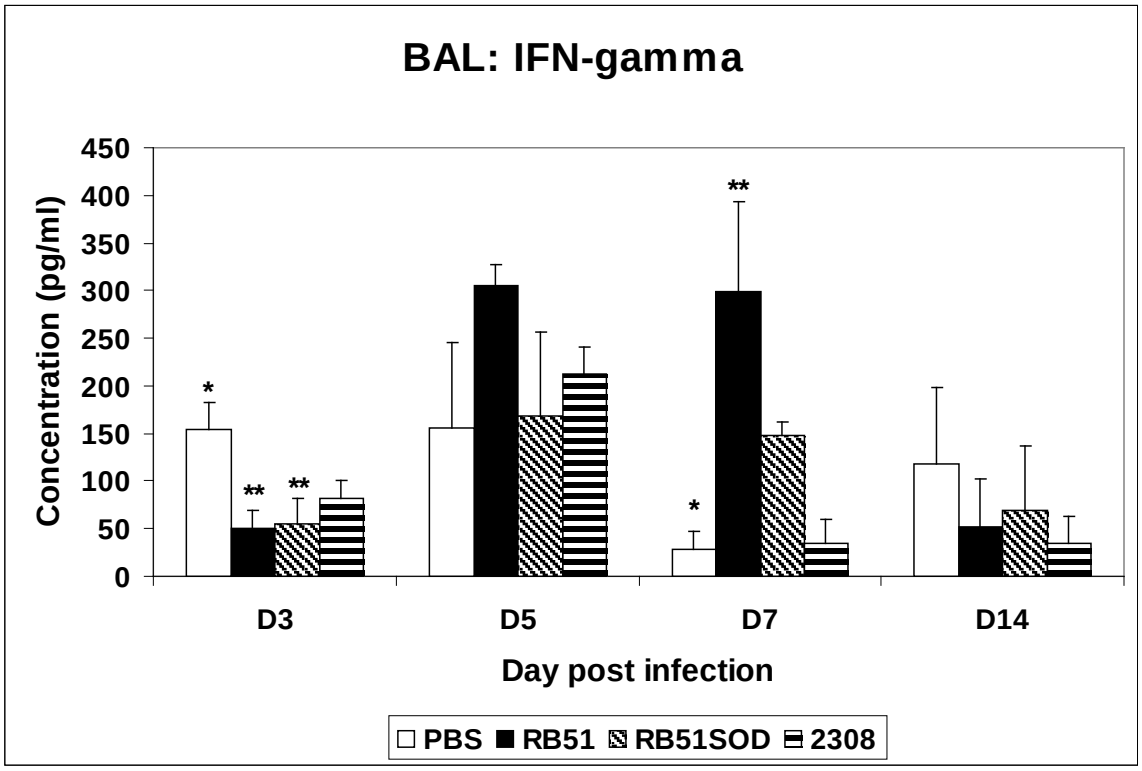

BAL: IFN-gamma
Concentration (pg/ml)
450
400
350
300
250
200
150
100
50
0
*
**
**
*
**
D3
D5
D7
D14
Day post infection
PBS
RB51
RB51SOD
2308

Figure 4

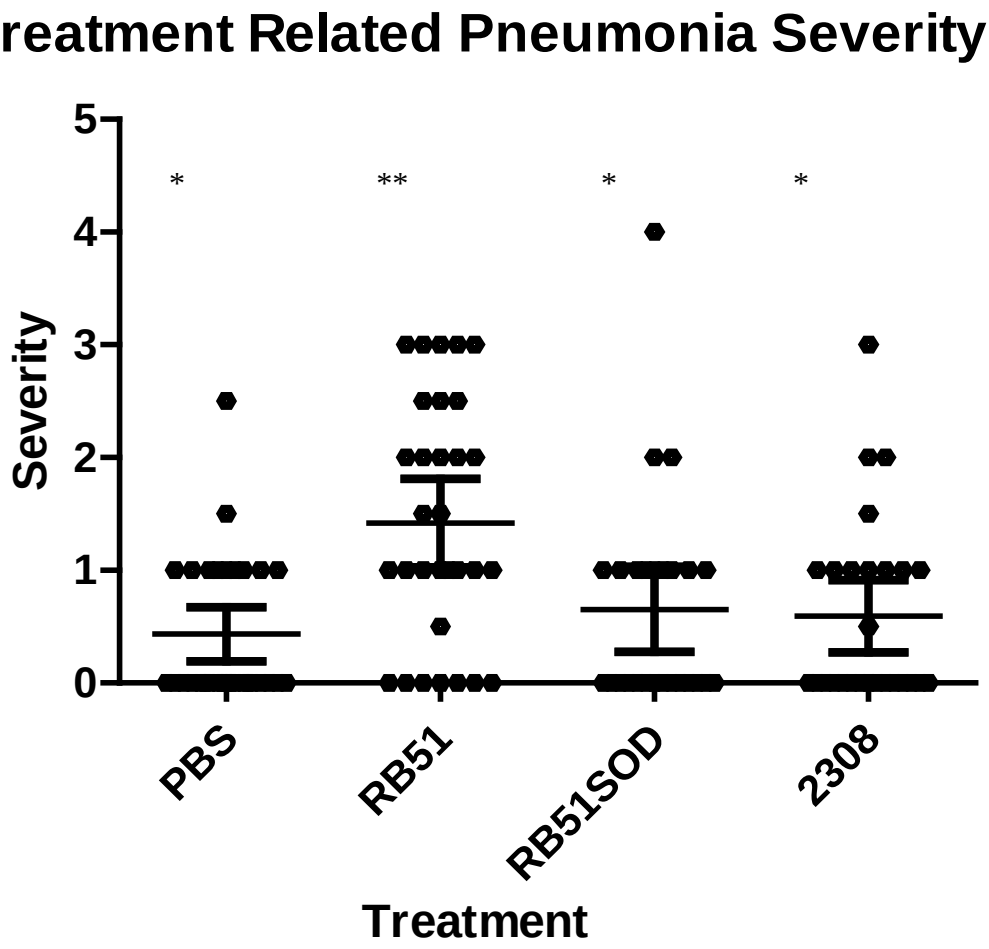
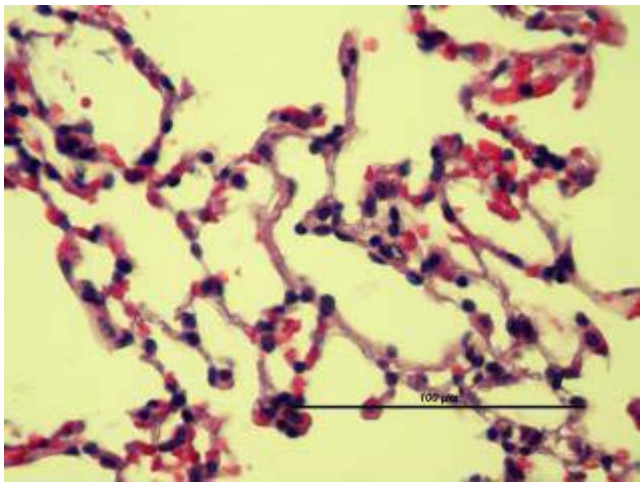

Treatment Related Pneumonia Severity
Severity
5
4
3
2
1
0
*
**
*
*
PBS
RB51
RB51SOD
2308
Treatment

Table 3

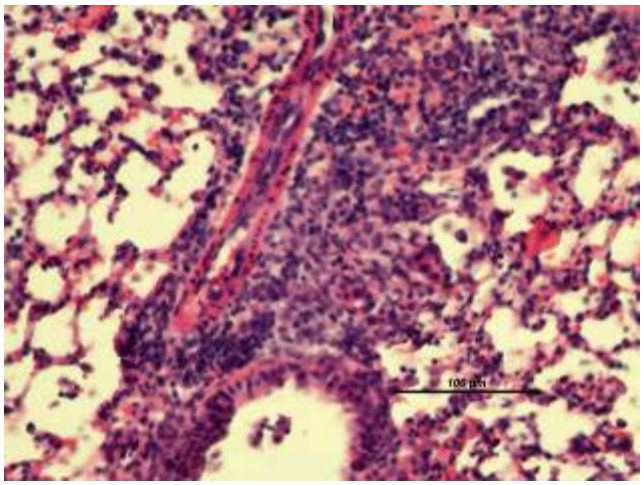
Day post infection	Treatment	Pulmonary Changes				
		Pneumonia		Vascular change	Septa thickening	Debris/protein
		Severity	Percentage (%) involvement			
3	PBS	**0.55** (0.72)	**5** (6.6)	**0.33** (0.70)	**0.22** (0.44)	**0.33** (0.5)
	RB51	**1.5** (1.19)	**17.25** (20.54)	**0.57** (1.0)	**1.0** (1.41)	**0.875** (1.24)
	RB51SOD	**0.875** (1.45)	**12.5** (27.64)	**0.375** (0.74)	**0.22** (0.35)	**0.57** (0.75)
	2308	**0.56** (0.72)	**7.87** (11.41)	**0.142** (0.37)	**0.428** (0.78)	**0.285** (0.75)
5	PBS	**0.375** (0.51)	**8.1** (12.51)	**0.125** (0.35)	**0.25** (0.46)	**0.5** (0.53)
	RB51	**1.68** (1.27)	**24.3** (24.99)	**0.5** (0.75)	**1.5** (1.41)	**1.125** (0.83)
	RB51SOD	**0.57** (0.53)	**12.14** (17.76)	**0** (0)	**0.57** (0.53)	**0.285** (0.48)
	2308	**0.625** (1.0)	**20** (29.76)	**0** (0)	**0.5** (0.75)	**0.375** (0.74)
7	PBS	**0.285** (0.48)	**2.857** (4.8)	**0** (0)	**0.285** (0.48)	**0.142** (0.37)
	RB51	**1.07** (0.93)	**15.71** (16.93)	**0.142** (0.37)	**0.857** (0.89)	**0.714** (0.75)
	RB51SOD	**0.375** (0.51)	**9.3** (20.77)	**0** (0)	**0.375** (0.51)	**0.375** (0.51)
	2308	**0.44** (0.52)	**8.33** (13.22)	**0** (0)	**0.375** (0.51)	**0.125** (0.35)
14	PBS	**0.857** (0.94)	**16.42** (23.22)	**0.285** (0.75)	**0.57** (1.13)	**0.285** (0.75)
	RB51	**1.375** (0.91)	**19.75** (14.70)	**0.57** (0.97)	**1.28** (0.75)	**0.57** (0.53)
	RB51SOD	**0.75** (0.95)	**20** (33.66)	**0** (0)	**0.75** (0.95)	**0.57** (0.5)
	2308	**0.9** (0.89)	**16** (25.34)	**0** (0)	**1** (1.0)	**0.33** (0.57)

Figure 5

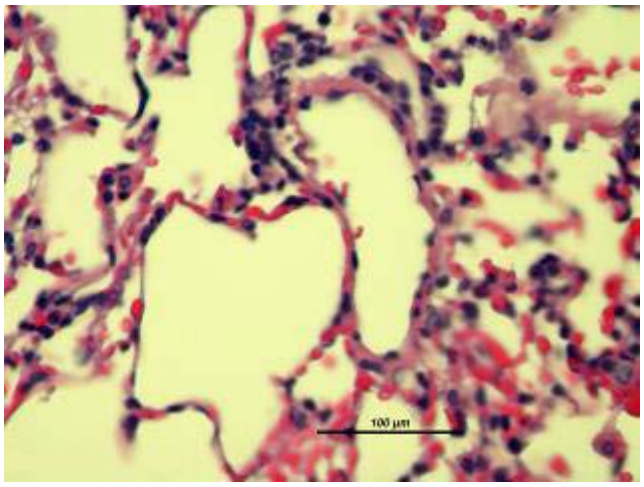
A. PBS



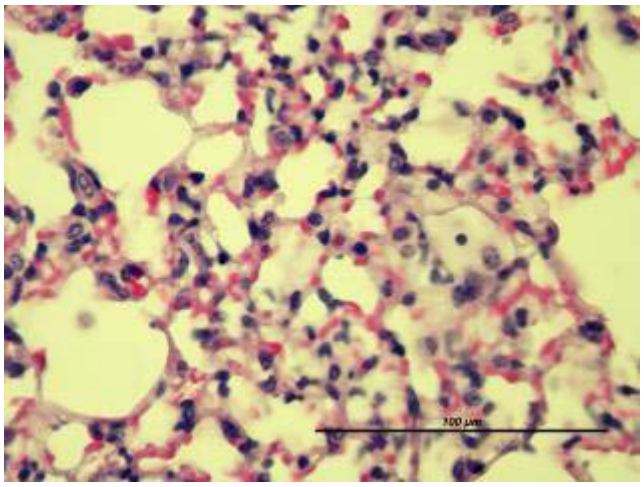
B. RB51



C. RB51SOD



D. Strain 2308



Chapter 5

Role of TLRs in *Brucella abortus* mediated murine dendritic cell activation *in vitro* and clearance of pulmonary infection *in vivo*.

Running title: Interaction of *B. abortus* with TLR KO BMDC *in vitro* and TLR KO mice *in vivo*

Naveen Surendran[*], Elizabeth M. Hiltbold[†], Bettina Heid[*], Shizuo Akira[‡], Theodore J. Standiford[§], Nammalwar Sriranganathan[¶], Stephen M. Boyle[¶], Kurt L. Zimmerman[¶], Melissa R. Makris[¶], Sharon G. Witonsky[*1]. (To be submitted to Journal of Immunology)

Abstract

Brucellosis is worldwide zoonoses affecting 500,000 people annually with no approved human vaccines available. Live attenuated *B. abortus* vaccine strain RB51 protects cattle through CD4 and CD8 T-cell mediated responses. However, limited information is known regarding how *Brucella* stimulates innate immunity. While *Brucella* is known to bind TLR2, TLR4 and TLR9, studies report conflicting results as to level of importance of each TLR. Furthermore, the most critical toll like receptor(s) (TLRs) for vaccine strain RB51 mediated DC activation is not known. Additionally, the most critical TLR for clearance is not known in response to aerosol/respiratory infection of *Brucella*. Therefore, in this study, we assessed the differential ability of strain RB51 and strain 2308 to stimulate murine bone marrow derived dendritic cell (BMDCs) activation and function from TLR2, TLR4 or TLR9 knockout and control BALB/c mice. Complementary *in vivo* experiments were also conducted to differentially assess the role of TLRs on clearance following intranasal infection of pathogenic strain 2308. We determined that strain RB51 induced significant ($p \leq 0.05$) DC activation compared to strain 2308 that was

independent of a specific TLR. However, strain RB51 induced TNF – α production was TLR2 and TLR9 dependent and IL-12 production was TLR2 and TLR4 dependent. TLR4 KO mice had significantly ($p \leq 0.05$) delayed pulmonary clearance of strain RB51 *in vivo* at day 14 post infection. Nevertheless, clearance of strain 2308 was not significantly different among organs between control and KO mice.

Introduction

Brucellosis is worldwide zoonoses affecting 500,000 people annually (1, 2). The causative organism is a Gram-negative, facultative intracellular bacterium from the genus *Brucella* classified under α-2 Proteobacteria (3). *B. melitensis*, *B. suis* and *B. abortus* are the most virulent species in order of pathogenicity (4). Of these, *B. abortus* causes infertility and abortion in cattle and undulant fever in humans (4). *Brucella* can be easily aerosolized, genetically modified to make antibiotic resistant strains and has the potential to be used as a bio-terror weapon (2). The Center for Disease Control and Prevention (CDC) categorizes it as a class B pathogen. There are no approved human vaccines available.

B. abortus strain RB51 is an efficacious live attenuated rough vaccine approved by United States Department of Agriculture (USDA) against bovine brucellosis (5). Strain RB51, which lacks the O-side chain lipopolysaccharide (LPS) is a mutant of pathogenic *B. abortus* strain 2308 (5). Protection afforded by strain RB51 is mediated through IFN-γ secreting CD4 T-helper$_1$ (Th_1) and cytotoxic CD8 T - cells (Tc_1) (6). In order to stimulate a robust adaptive response, a solid innate response is needed. However, the means by which *B. abortus* induces this response have not been fully elucidated.

The initial host defense to any bacterial infection involves innate immune cells, including neutrophils, natural killer (NK) cells, macrophages and dendritic cells (7). Barquero-Calvo et al. demonstrated that neutrophils do not have a significant role in control of *B. abortus* (8). Although a good source of IFN-gamma, NK cells do not contribute to antigen presentation whereas both macrophages and dendritic cells (DCs) are efficient antigen presenting cells (APCs) (7). As DCs compared to macrophages are 100 times more efficient in antigen capture; have the ability to migrate from peripheral tissue to draining lymph node with the antigen to present the processed antigen to naïve T-cells; produce higher IL-12; have higher rates of *Brucella* infection; and are a critical link between innate and adaptive immunity, DCs are an ideal model for studying *Brucella* mediated innate immune response (7, 9, 10).

DC activation is initiated by Toll-like receptors (TLRs) recognizing pathogen associated molecular patterns (PAMPs) (11). Subsequently, TLRs transduce signals via common adaptor molecules to activate DCs to produce proinflammatory cytokines (12). There are contradictory data on the most crucial TLRs in recognition of *B. abortus* by DCs. Weiss et al. (13) demonstrated that *Brucella* signals through TLR2, TLR4, and MyD88. As the latter is most critical for clearance, this suggests a role for TLR9, which also signals through MyD88. Subsequently, Oliveira et al.(14) and Huang et al. (15) suggested a prominent role for TLR9 in DC mediated IL-12 production and *Brucella* clearance. Zwerdling et al. (16) also suggests that *Brucella* signals through TLR2 and TLR4. Altogether, published literature suggests that *B. abortus* signals through multiple TLRs, including TLR2 (outer membrane proteins), TLR4 (lipopolysaccharide), and TLR9 (CpG DNA). Downstream MyD88 is implicated as the most critical adapter protein involved in signaling, which leads to NF-κB activation, DC function and ultimately *Brucella* clearance *in vivo* (17, 18). Despite these published TLR studies, there is a

dearth of information on whether a differential ability of TLRs for activating DCs exists between rough and smooth *Brucella* strains. If present, innate activation of strain RB51 could be enhanced by using TLR agonists as adjuvants. To answer this question, we infected TLR2, TLR4, TLR9 KO BALB/c mice derived bone marrow DCs (BMDCs) along with wild type control BMDCs to analyze differences in activation and function between *B. abortus* rough strain RB51 and smooth strain 2308 infected BMDCs.

Additionally, assessing *Brucella* clearance from TLR KO mice following respiratory exposure will also provide critical information both on whether vaccine strains can be upregulated through TLR agonists as well as whether respiratory clearance of *B. abortus* strain 2308 is altered compared to other routes of challenge. With either natural or intentional (bioterror) aerosol exposure, *Brucella* is directly delivered to the pulmonary airway and airway epithelium. Currently, there are no published studies which have addressed the role of TLRs in the clearance of rough or smooth *B. abortus* strains from intranasally infected mice (19). Therefore, to address the role of TLRs in *B. abortus* clearance in a pulmonary infection, we infected BALB/c as well as TLR2, TLR4, TLR9 KO mice with strain RB51 and 2308 to assess TLR dependent clearance. Our overall aim for this study was to determine the role of TLR2, TLR4 and TLR9 in the differential activation of DCs upon infection with *B. abortus* rough and smooth strains *in vitro* and in the clearance of *Brucella* challenge *in vivo*.

Materials and Methods

Mice: Female 4-6 weeks old BALB/c mice were obtained from Charles River Laboratories Inc., Wilmington, MA. TLR4 and TLR9 knockout (KO) mice breeding pairs in BALB/c background were obtained from S. Akira (Department of Host Defense, Research Institute for Microbial

Diseases, Osaka University, Japan). TLR2 KO mice breeding pairs in BALB/c background were obtained through Dr. Akira from Dr. Standiford (University of Michigan, Ann Arbor, MI). Female, 6-8 week old control and KO mice were used for all the experiments under animal care protocols approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Generation of Bone marrow derived DCs: Bone marrow-derived DCs (BMDCs) were generated, as previously described (20). Briefly, tibias and fibulas of 7-8 weeks old BALB/c control and KO mice were incised and bone marrow (BM) cells removed. Following red blood cell lysis and filtration, the cells were resuspended and plated in RPMI 1640 complete media with 10% non heat-inactivated fetal bovine serum and 20ng/ml rGM-CSF (Invitrogen, Carlsbad, CA). The cells were incubated at 37°C in 5% $C0_2$. Fresh media containing rGM-CSF was added at days 2, 4 and 5 and harvested on day 6. The cells harvested on day 6 were typically 70% $CD11c^+$ and displayed low levels of MHC class II, CD40 and CD86, consistent with immature DCs. Flow cytometry was performed to confirm immature DC status.

***Brucella* strains:** Live attenuated rough *B. abortus* strain RB51 and virulent smooth strain 2308 were used from our stock culture collection (5). All experiments with *Brucella* were performed in our CDC approved (C2003 1120-0016) Biosafety Level (BSL)-3 facility.

DC Infection experiments: On day 6, control and TLR2, TLR4 and TLR9 KO DCs were harvested and plated at 5 X 10^5 cells/well in separate 24 well plates and infected with strain RB51 or strain 2308 at each of the three multiplicities of infection (MOI) 1:1 (DC:*Brucella*), 1:10 and 1:100. Infection was enhanced by a short spin at 1300 rpm (400 x g) for 5 minutes at room temperature. The infected cells were incubated for 4 hours at 37°C in 5% $C0_2$. The infection was terminated by washing the cells with gentamicin (Sigma, St. Louis, MO) at 30μg/ml. The cells were then incubated for an additional 20 hours in complete media with

10ng/ml rGM-CSF and 30μg/ml gentamicin. Control samples were maintained by incubating cells with media (negative control) or *Escherichia coli* LPS *0111:B4* (Sigma) (positive control) (100ng/ml) following the exact same procedure for infection. In replicate experiments, at each time point for each treatment, an aliquot of cells was collected to determine bacteria cell numbers. Cells were washed to remove non-intracellular bacteria, and total BMDCs were counted. BMDCs were then lysed by treating with 1 ml/well of 0.1% Triton x-100 in sterile distilled water for 10 minutes, mixed well and 10-fold serial dilutions were plated onto TSA plates. Intracellular bacteria were counted and number of bacteria per cell was determined.

Viability and infection controls: To quantitate and assess viability of control and KO BMDCs at 24 hour post infection with each treatment, Trypan blue was used to differentiate viable and dead cells. Total live and dead BMDC cell numbers were determined.

Staining and flow cytometry: The cells were harvested 24 hours following infection, and they were stained with the following monoclonal antibodies at 0.1 - 0.2 μg per million cells for FACS analysis: APC conjugated anti-CD11c, pacific blue-conjugated anti-CD86 (Biolegend, San Diego, CA), Biotin-conjugated anti-CD40, Streptavidin Tri-color conjugate, were all acquired from Caltag (Invitrogen), and PE-conjugated anti I-A/I-E, acquired from BD Pharmingen, San Jose, CA. Cells were washed and analyzed by BD FACSAria™ flow cytometer.

Cytokine analysis: For cytokine measurement, culture supernatants from *Brucella* infected BMDCs were collected after 24 hours of incubation and stored at -80°C. TNF-α, IL-12_{p70} (bioactive form of IL-12) IFN-γ and IL-4 cytokine levels were subsequently measured using indirect sandwich ELISAs (BD Pharmingen).

Mice infection and clearance study: Age matched female control and KO BALB/c mice were infected intranasally (IN), under light xylazine-ketamine anesthesia IP, with either *B. abortus*

rough strains RB51 (4 X 10^8 CFUs/mouse) or with smooth strain 2308 (2 X 10^4 CFUs/mouse) in 25 µl phosphate buffered saline (PBS). Mice were euthanized on day 14 and day 42 post infection (PI) with a lethal dose of xylazine-ketamine IP. Day 42 post infection time point was not performed for TLR2 KO mice. At the time of euthanasia, lung, MLN and spleen were collected. Single cell suspensions of organs collected were serially diluted and plated on to Tryptic soy agar (TSA) plates and incubated for 5 days at 37° C and 5% CO_2 (21). Bacterial colony forming units (CFUs) were counted and CFUs/mouse were calculated.

Statistical analysis: The effect of TLRs and treatments on expression of various DC maturation and activation markers was tested using mixed model ANOVA followed by Tukey's procedure for multiple comparisons with date of experiment as a blocking factor. Statistical significance of cytokine production in BMDC supernatant and effect of TLRs on *in vivo* clearance of *Brucella* strains was assessed by exact Kruskal-Wallis test followed by Dunn's procedure for multiple comparisons. Significance was set at $p \leq 0.05$. All analyses were performed using the SAS system (Cary, NC, USA).

Results

***E. coli* LPS downregulates CD11c surface expression on bone marrow derived DCs through TLR4.** To determine that the differences in DC mediated activation and function were not due to differences in viability of *Brucella* and/or BMDCs, DC viability and *Brucella* numbers were analyzed. Our data showed that at 4hrs there were relatively similar levels of *Brucella*: BMDCs (data not shown). After overnight incubation, the percentage yield of $CD11c^+$ cells from uninfected media control and strain 2308 infected cells ranged from 81-92 % and 79-91 % (all 3 MOIs of 2308 from control and KO mice) respectively. The percentage yield of

$CD11c^+$ cells was significantly ($p \leq 0.05$) lower for *E. coli* LPS treated control as well as KO mice derived cells (57–81%) compared to uninfected media treated cells possibly due to activation induced downregulation and a marginal nonsignificant increase in cell death (Figure 1). LPS induced CD11c expression was also significantly lower ($p \leq 0.05$) than strain 2308 (all MOIs) in wild type mice and strain RB51 (at MOI 1:1, 1:10 in wild type mice and also for TLR2 KO at 1:100) induced CD11c expression except for TLR4 KO mice. However, *E. coli* LPS induced CD11c expression was significantly ($p \leq 0.05$) higher for TLR4 KO (81%) mice compared to control, TLR2 and TLR9 KO mice suggesting that the LPS induced CD11c downregulation is predominantly mediated through TLR4 (Table I). At MOI 1:1 and 1:10, strain RB51 induced CD11c expression (80-88%) of control and KO cells were similar to media (data not shown) whereas at MOI 1:100, strain RB51 induced CD11c expression was significantly ($p \leq 0.05$) lower than media treated cells except for TLR9 KO cells, showing a dose response (Figure 1). For BALB/c control, the level of RB51 (MOI 1:100) induced CD11c expression was even significantly lower than strain 2308 (MOI 1:100) induced expression (Figure 1). Nevertheless, at MOI 1:100, strain RB51 induced CD11c expression was not significantly different between TLR4 KO and other mouse cells.

Rough strain RB51 up-regulated MHC class II expression in control and TLR KO BMDCs. Immature BMDCs from control and KO mice were treated with all 3 MOIs of strain RB51 or strain 2308 to analyze the role of TLRs and the effect of infection on DC maturation. As expected, control and KO BMDCs except TLR4 KO cells upregulated significant ($p \leq 0.05$) MHC class II expression upon stimulation with *E. coli* LPS compared to respective media treated BMDCs (Figure 2A). LPS mediated MHC class II expression was significantly ($p \leq 0.05$) higher with control, TLR2 KO and TLR9 KO BMDCs compared to TLR4 KO BMDCs (Table I). At

MOI 1:100, strain RB51 induced significantly higher MHC class II expression of TLR2 ($p=0.0556$), TLR4 ($p\leq0.05$) and TLR9 ($p\leq0.05$) KO BMDCs compared to respective BMDCs treated with media (Figure 2A) (Figure 3). Only TLR4 KO BMDCs expressed significantly greater ($p\leq0.05$) MHC class II up -regulation with strain RB51 at 1:10 (data not shown) compared to media and strain RB51 1:100 compared to LPS (Figure 2A). Strain 2308 did not induce significant up-regulation of MHC class II compared to media at any of the doses tested with the exception of TLR4 KO BMDCs at MOI 1:100 (Figure 2A) (Figure 3).

In comparing control and KO mice BMDCs for their maturation response based on MHC class II expression with respect to treatments with strain RB51 or strain 2308, strain RB51 at MOI 1:100 induced significantly ($p\leq0.05$) higher MHC class II expression in TLR 9 KO BMDCs compared to TLR2 KO BMDCs and at MOI 1:1 and 1:10 compared to TLR4 KO BMDCs (strain RB51 induced MHC class II is more TLR2 and 4 dependent than TLR9 dependent). Additionally, strain 2308 induced MHC class II expression was significantly ($p\leq0.05$) higher with TLR9 KO BMDCs compared to TLR4 KO BMDCs at MOI 1:10 (Table I). (Strain 2308 induced MHC class II expression is more TLR4 than TLR9 dependent).

Rough strain RB51 induced significantly higher costimulatory marker CD40 and CD86 expression compared to smooth strain 2308 in control and TLR KO BMDCs. Control as well as TLR KO BMDCs infected with strain RB51 induced significantly higher ($p\leq0.05$) CD40, CD86 expression and $CD40^+/CD86^+$ co-expression at both 1:10 and 1:100 MOIs compared to media (Figure 2B – 2D). At MOI 1:100, strain RB51 induced CD40, $CD40^+/CD86^+$ (also at MOI 1:10 for TLR4 KO), and CD86 expression levels were significantly ($p\leq0.05$) higher than LPS induced upregulation of the same markers from respective BMDCs. Additionally, BMDCs from each type of mice induced statistically significant ($p\leq0.05$) up-regulation of all co-stimulatory

markers (except TLR4 KO CD40) when infected with strain RB51 at MOI 1:100 compared to strain 2308 infected (MOI 1:100) BMDCs from the same type of mice (Figure 2B – 2D). $CD40^+/CD86^+$ co-expression was significantly higher for strain RB51 infected BMDCs even at MOI 1:10 compared to strain 2308 infected mice at MOI 1:10 (except for TLR2 KO BMDCs).

By comparison, strain 2308 induced significantly higher CD40 and CD86 (also at MOI 1:10 for CD86 expression with TLR4 KO) expression in all BMDCs compared to media treated cells, only at MOI 1:100 (Figure 2B-C) (Figure 3). However, $CD40^+/CD86^+$ co-expression was significantly higher for strain 2308 infected BMDCs (except TLR2 KO at MOI 1:10) at both MOIs 1:10 and 1:100 compared to media (Figure 3). Additionally at MOI 1:100, TLR4 KO BMDCs infected with strain 2308 induced significantly higher ($p \leq 0.05$) CD40, CD86 and $CD40^+/CD86^+$ expression than *E. coli* LPS treated TLR4 KO BMDCs (Figure 2B – 2D) (Figure 3).

While comparing the level of expression of co-stimulatory markers on BMDCs from control and TLR KO mice treated with different treatments, we found that CD40, CD86 and $CD40^+/CD86^+$ co-expression on TLR4 KO BMDCs were significantly ($p \leq 0.05$) decreased compared to control and TLR2, TLR9 KO BMDCs (Table I) (Figure 3). Additional significant differences with LPS are also listed in Table 1. Interestingly at MOI 1:100 both strains RB51 and 2308 induced CD86 and $CD40^+/CD86^+$ co-expression was significantly ($p \leq 0.05$) decreased for TLR2 KO mice compared to BALB/c control, TLR9 and TLR4 KO BMDCs (except for strain 2308 stimulated TLR4 KO BMDC - CD86 expression) (Table I) (Figure 3).

Rough strain RB51 induced DC – TNF-α secretion is TLR2 and partly TLR9 dependent. TNF-α is an essential cytokine released by DCs undergoing maturation. Results from our previous work (Surendran et al., under review) have established that while smooth strain 2308

inhibited TNF-α secretion by interfering with DC maturation and function, rough strain RB51 at MOI 1:100 induced significantly higher ($p \leq 0.05$) TNF -α secretion compared to media control. In this study, we further investigated the role of TLR2, TLR4 and TLR9 in *Brucella* induced DC – TNF-α secretion. Figure 4A shows that both BALB/c control and TLR4 KO BMDCs infected with strain RB51 at MOI 1:100 secreted similar levels of TNF-α which was significantly ($p \leq 0.05$) higher than respective media, *E. coli* LPS controls as well as TLR2 KO BMDCs. Strain RB51 1:100 infected TLR9KO BMDCs secreted significantly greater ($p \leq 0.05$) TNF-α compared to respective media and LPS controls; however, the amount secreted was only one-fifth of the BALB/c control and TLR4 KO cells. Furthermore, TNF-α induced by TLR9 KO BMDCs was not significantly higher than that induced by TLR2 KO BMDCs.

Rough strain RB51 induced DC - IL-12$_{p70}$ secretion is TLR2 and TLR4 dependent. IL-12 is a key cytokine produced mainly by DCs upon pathogen recognition and subsequent maturation. IL-12 helps to establish a Th_1 response. We previously demonstrated (Surendran et al., under review) that rough strain RB51 induced significantly ($p \leq 0.05$) higher IL -12 secretion from BMDCs compared to smooth strain 2308. Figure 4B illustrates that both BALB/c control and TLR9 KO BMDCs secreted similar levels of IL-12$_{p70}$ in response to strain RB51 (MOI 1:100) stimulation which was significantly ($p \leq 0.05$) higher than respective media and *E. coli* LPS controls as well as TLR2 and TLR4 KO BMDCs. Irrespective of the type of mice BMDCs, strain 2308 did not induce significant production of IL-12$_{p70}$ at any of the MOIs tested (Figure 4B).

Pulmonary clearance of rough strain RB51 is TLR4 dependent in vivo. To determine the role of TLRs in the clearance of rough and smooth *Brucella* strains from IN infected animals, TLR2, TLR4 and TLR9 KO mice were infected IN either with rough strain RB51 (4×10^8 CFUs/ mouse) or with smooth strain 2308 (2×10^4 CFUs/mouse). Bacterial clearance from lung,

MLN and spleen were compared to BALB/c control mice day 14 PI. As demonstrated in Figure 5A, TLR4 KO mice showed significantly ($p \leq 0.05$) reduced clearance of strain RB51 from lungs compared to BALB/c control and TLR9 KO mice. TLR2 KO mice also showed non-significantly reduced strain RB51 clearance in lung compared to BALB/c control or TLR9 KO mice. However, neither control nor KO mice showed significant difference in clearance of strain RB51 from MLN or spleen.

Figure 5B illustrated the difference in day 14 PI clearance of strain 2308 from IN infected control and KO mice. Although not statistically significant, TLR2 KO mice showed reduced clearance of strain 2308 from lung compared to control BALB/c and TLR4, TLR9 KO mice. Splenic and MLN clearance of strain 2308 were also not significantly different among control and KO mice owing to the variation in bacterial CFUs recovered from respective organs.

Discussion

Published literature suggests that over the last few years several laboratories addressed the question of how the pathogenic *Brucella spp*. interact with the murine innate immune system (10, 13-18, 22-27). The majority of those studies focused on identifying the different TLRs involved in the host recognition of *Brucella*. There were additional efforts for identifying the specific *Brucella* associated molecular patterns which bound to the associated TLR to determine whether that interaction stimulated or inhibited the immune response. In contrast only few laboratories studied the role of different TLRs in the control of *Brucella* infection *in vivo* (13, 17, 18, 27). It has been established that pathogenic *B. abortus* signals through multiple TLRs such as TLR2 (outer membrane proteins), TLR4 (lipopolysaccharide) and TLR9 (CpG DNA) (13, 16, 18, 24). However, to our knowledge, none of the labs have studied whether there is a difference

in TLR preference between live attenuated rough vaccine strains and pathogenic smooth strain in stimulating or subverting the DC mediated innate immune activation and function. Additionally, despite the fact that brucellosis can be transmitted by accidental/intentional aerosol exposure and that vaccination strategies did not protect in the lung (28, 29), no published studies focused on the role by specific TLRs in the pulmonary clearance of either vaccine or pathogenic strains. Answering such questions is critical for improving the efficacy of existing vaccines and for developing a successful vaccine strategy against aerosol/intranasal *Brucella* infection.

Previous work (Surendran et al., under review) showed that live vaccine strain RB51 (MOI 1:100) significantly enhanced BMDC activation and function compared to smooth pathogenic strain 2308 based on costimulatory marker expression and cytokine production. While reaffirming the previous results, these data showed that strain RB51 induced DC activation (based on MHC class II, CD40 and CD86 expression) was not mediated only through one specific TLR or that signaling occur through all the different TLRs (TLR2, 4 and 9) to initiate DC activation, possibly because they all signal through MyD88. However, based on the exhibited TLR dependence in cytokine production, lack of specific TLR dependency for DC activation may be explained as DC activation requires only minimal stimulation through the TLRs potentially because they all commonly signal through MyD88. In just assessing DC activation, as has been demonstrated by others, this supports the involvement of the common downstream adapter molecule, MyD88 (13, 17, 18). Although not significantly higher than strain 2308 mediated DC activation, strain RB51 induced significant CD40 and CD86 expression on BMDCs even at MOI 1:10, irrespective of TLR KO status, compared to media control. However, only at the highest MOI of 1:100, was pathogenic strain 2308 able to induce significant upregulation of costimulatory molecules CD40 and CD86 on BMDCs compared to media

control, irrespective of TLRKO status. This suggested that, not withstanding the higher dose requirement, wild type *Brucella* also mediated DC activation through a combination of TLRs or there exists a compensatory mechanism in each specific KO mice through other TLR pathways, leading to downstream activation of an adaptor molecule or there are other TLR 2, 4, and 9 independent pathways for DC activation. The possible reasons for a significantly low activation of DCs with smooth virulent strain could be attributed to various immune evasive survival mechanisms that *Brucella* utilize such as lipid raft entry, inhibition of phagosome-lysosome fusion and its ability to impair antigen presentation.

Comparing strain 2308 and strain RB51 mediated signaling, interestingly, even at MOI 1:100 strain 2308 failed to stimulate significant MHC II expression compared to media except with TLR4 KO BMDCs. Results from Forestier et al.(30) were similar in murine macrophage cell lines. As LPS signals through TLR4 (12), our data consistent with other studies support that strain 2308 impairs the MHC class II expression on murine BMDCs through TLR4 mediated pathway. Additionally, by comparing lipid A profiles of strain 2308 and strain RB51 using mass spectrometry, Campos et al. (27) showed that strain 2308 lipid A signals more through TLR4. Therefore, TLR4 plays potentially an inhibitory role in strain 2308 induced MHC class II expression on BMDCs.

As mentioned above, DC function was TLR dependent. In agreement with previous studies (22, 23), smooth strain 2308 did not induce significant production of TNF-α from wild type or any of the TLR KO mice. However, the rough strain RB51 (MOI 1:100) induced TNF-α secretion was TLR2 and to some extend TLR9 dependent. Using heat killed *B. abortus* 2308 to stimulate DCs, Huang et al. (24) and Macedo et al. (18) showed that DC – TNF-α secretion is TLR2 dependent and the latter also showed that DC - TNF-α secretion is TLR9 independent. The

viability, and rough nature of strain RB51 leading to increased initial uptake by DCs might have engaged TLR9 to mediate significant production of TNF-α upon activation. The role of MyD88 cannot also be ruled out.

Following the same pattern, only rough strain RB51 (MOI 1:100) induced significant IL-12 $_{p70}$ production from stimulated BMDCs which was TLR2 and TLR4 dependent. This contradicted results from groups using heat killed *B. abortus,* who showed that DC mediated IL-12 secretion was predominantly TLR9 dependent (15, 18). Possible differences in these results could be due differences in strain and viability. While enhanced initial uptake of live strain RB51 and engagement of TLR9 leading to DC activation is possible, results from our study showed that DC induced IL-12 secretion required both TLR2 and TLR4. Recent studies by Krummen et al. (31), demonstrated that combinations of TRIF- and MyD88-dependent TLR ligands acted in synergy for inducing DCs to release IL-12 and DC activation remained unchanged regardless of TLR synergy. TLR4 is the only receptor which signals through both TRIF and MyD88 upon stimulation with *Brucella* (17, 32). In addition, Macedo et al. (18) had shown that only IL-12 could partially rescue host susceptibility to *Brucella* infection in MyD88 deficient mice and Huang et al. (10) demonstrated that DCs are the major producers of IL-12. Taken together, IL-12 production by DCs plays a crucial role in host innate immune resistance against brucellosis and TLR4 act as one of the important receptor in DC mediated IL-12 secretion. The fact that we found both TLR2 and TLR4 to be involved in DC: IL-12 production suggests a role for MyD88 in IL-12 production. Altogether, strain RB51 induced significant DC function, which was TLR dependent and suggested a predominant role for MyD88 and possibly TRIF. It is noteworthy that TLR2 was indispensable for TNF-α and IL-12_{p70} production by DCs.

Concurrently, for the first time, we analyzed the role of TLRs with respect to virulent *Brucella* clearance *in vivo* from an intranasally (IN) infected mice. Results demonstrated that there was no significant delay in clearance of strain 2308 at day 14 post intranasal (IN) inoculation from wild type or TLR KO mice. Although not statistically significant, it is intriguing to note that TLR2 KO mice had a reduced clearance of strain 2308 from lung at day 14 post infection while such a difference was not observable in spleen or MLN. Similarly, pulmonary clearances for other intracellular Gram-negative bacteria such as *Legionella* (33) and *Francisella* (34) have shown to be significantly decreased in TLR2 KO mice. In contrast, recent publications assessing *Brucella* clearance from spleen show conflicting reports. Weiss et al. (13), using IP infection with B. *abortus* strain 19 showed that TLR2, TLR4 and TLR2/TLR4$^{-/-}$ KO but not MyD88 KO mice clear infection as efficiently as wild type mice. By comparison, Campos et al. (27), who used strain 2308 (IP infection) at one log higher dose (10^5 CFUs) than our IN dose, reported reduced splenic clearance bacteria from TLR4 KO mice. While outlining the importance of MyD88 in strain 2308 (10^6 CFUs) clearance from spleen, Macedo et al. (18), also showed that TLR9 KO mice had significantly reduced clearance. Although using *B. melitensis* 16M, Copin et al. (17) also demonstrated a moderate reduction in clearance of infection from spleen in TLR4, TLR9 KO mice and a significantly reduced clearance from MyD88 mice. Part of the reason for these discrepancies could be the use of different mouse strain, route of inoculation and the dosage. However, given the importance an IN route of infection and lack of effective vaccines against aerosol challenge, reduced strain 2308 pulmonary clearance from TLR2 KO mice (non significant) is a valuable observation for vaccine development which require further studies.

Conversely, pulmonary clearance of rough strain RB51 was significantly delayed in TLR4 KO mice compared to wild type, TLR2 and TLR9 KO mice. No statistically significant difference was observed in spleen and MLN. Previous studies from our lab (Surendran et al., under review) analyzing the *in vivo* pulmonary innate immune responses to IN inoculated strain RB51 has revealed that the vaccine strain induce enhanced DC response compared to virulent strain 2308. Although we can only speculate at this time, considering these data on the role of TLR 4 in strain RB51 infected DC IL-12 secretion, it is probable that in the TLR4 KO mice, the impaired IL-12 secretion and subsequent subdued immune response could be the explanation for the observed reduction in pulmonary clearance of strain RB51. Given the information, in future studies TLR agonists can be assessed along with vaccine strain RB51 for their ability to enhance protection against intranasal *Brucella* challenge.

In conclusion, strain RB51 is capable of inducing significant innate immune response characterized by BMDC maturation and function compared to pathogenic strain 2308. Strain RB51 induced DC maturation is not dependent on an individual TLR, yet DC function characterized by TNF-α secretion is TLR2 and in part TLR9 dependent and IL-12 secretion is TLR2 as well as TLR4 dependent. Virulent strain 2308 stimulated significant DC activation only at the highest MOI irrespective of TLR KO status but without inducing pro inflammatory or Th_1 cytokine secretion. The study also gives novel insights into the TLR dependent clearance of *B. abortus* pathogenic strain 2308 as well as vaccine strain RB51 clearance from IN infected mice. Results from this study will give new direction to develop vaccine strategies against IN *Brucella* infection. Future studies should address the potential role of downstream adaptor molecule MyD88 with respect to vaccine strain induced DC activation and pulmonary clearance of rough and smooth bacteria.

Acknowledgements: The authors acknowledge Dr. Stephen Werre for help with the statistical analysis of the data.

References

1. Acha, P., Szyfres, B. , ed. 2001. *Zoonoses and communicable diseases common to man and animal.* . Pan American Health Organization, WHO.
2. Pappas, G., P. Panagopoulou, L. Christou, and N. Akritidis. 2006. *Brucella* as a biological weapon. *Cell Mol Life Sci* 63:2229-2236.
3. Nene, V., Kole, Chittaranjan., ed. 2009. *Genome Mapping and Genomics in Animal-Associated Microbes*. Springer-Verlag, Berlin Heidelberg.
4. Corbel, M. J., ed. 2006. *Brucellosis in humans and animals*. World Health Organization.
5. Schurig, G. G., R. M. Roop, 2nd, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan. 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. *Vet Microbiol* 28:171-188.
6. He, Y., R. Vemulapalli, A. Zeytun, and G. G. Schurig. 2001. Induction of specific cytotoxic lymphocytes in mice vaccinated with *Brucella abortus* RB51. *Infect Immun* 69:5502-5508.
7. Tizard, I. R., ed. 2008. *Veterinary Immunology: An Introduction*. Elsevier.
8. Barquero-Calvo, E., E. Chaves-Olarte, D. S. Weiss, C. Guzman-Verri, C. Chacon-Diaz, A. Rucavado, I. Moriyon, and E. Moreno. 2007. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. *PLoS ONE* 2:e631.

9. Billard, E., C. Cazevieille, J. Dornand, and A. Gross. 2005. High susceptibility of human dendritic cells to invasion by the intracellular pathogens *Brucella suis*, *B. abortus*, and *B. melitensis*. *Infect Immun* 73:8418-8424.

10. Huang, L. Y., C. Reis e Sousa, Y. Itoh, J. Inman, and D. E. Scott. 2001. IL-12 induction by a TH1-inducing adjuvant in vivo: dendritic cell subsets and regulation by IL-10. *J Immunol* 167:1423-1430.

11. Janeway, C. A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annu Rev Immunol* 20:197-216.

12. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.

13. Weiss, D. S., K. Takeda, S. Akira, A. Zychlinsky, and E. Moreno. 2005. MyD88, but not toll-like receptors 4 and 2, is required for efficient clearance of *Brucella abortus*. *Infect Immun* 73:5137-5143.

14. Oliveira, S. C., F. S. de Oliveira, G. C. Macedo, L. A. de Almeida, and N. B. Carvalho. 2008. The role of innate immune receptors in the control of *Brucella abortus* infection: toll-like receptors and beyond. *Microbes Infect* 10:1005-1009.

15. Huang, L. Y., K. J. Ishii, S. Akira, J. Aliberti, and B. Golding. 2005. Th1-like cytokine induction by heat-killed *Brucella abortus* is dependent on triggering of TLR9. *J Immunol* 175:3964-3970.

16. Zwerdling, A., M. V. Delpino, P. Barrionuevo, J. Cassataro, K. A. Pasquevich, C. Garcia Samartino, C. A. Fossati, and G. H. Giambartolomei. 2008. *Brucella* lipoproteins mimic dendritic cell maturation induced by *Brucella abortus*. *Microbes Infect* 10:1346-1354.

17. Copin, R., P. De Baetselier, Y. Carlier, J. J. Letesson, and E. Muraille. 2007. MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during *Brucella melitensis* infection. *J Immunol* 178:5182-5191.

18. Macedo, G. C., D. M. Magnani, N. B. Carvalho, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira. 2008. Central role of MyD88-dependent dendritic cell maturation and proinflammatory cytokine production to control *Brucella abortus* infection. *J Immunol* 180:1080-1087.

19. Neutra, M. R., and P. A. Kozlowski. 2006. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 6:148-158.

20. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693-1702.

21. Vemulapalli, R., Y. He, S. Cravero, N. Sriranganathan, S. M. Boyle, and G. G. Schurig. 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. *Infect Immun* 68:3286-3289.

22. Billard, E., J. Dornand, and A. Gross. 2007. *Brucella suis* prevents human dendritic cell maturation and antigen presentation through regulation of tumor necrosis factor alpha secretion. *Infect Immun* 75:4980-4989.

23. Billard, E., J. Dornand, and A. Gross. 2007. Interaction of *Brucella suis* and *Brucella abortus* rough strains with human dendritic cells. *Infect Immun* 75:5916-5923.

24. Huang, L. Y., J. Aliberti, C. A. Leifer, D. M. Segal, A. Sher, D. T. Golenbock, and B. Golding. 2003. Heat-killed *Brucella abortus* induces TNF and IL-12p40 by distinct

MyD88-dependent pathways: TNF, unlike IL-12p40 secretion, is Toll-like receptor 2 dependent. *J Immunol* 171:1441-1446.

25. Pei, J., J. E. Turse, and T. A. Ficht. 2008. Evidence of *Brucella abortus* OPS dictating uptake and restricting NF-kappaB activation in murine macrophages. *Microbes Infect* 10:582-590.

26. Salcedo, S. P., M. I. Marchesini, H. Lelouard, E. Fugier, G. Jolly, S. Balor, A. Muller, N. Lapaque, O. Demaria, L. Alexopoulou, D. J. Comerci, R. A. Ugalde, P. Pierre, and J. P. Gorvel. 2008. *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. *PLoS Pathog* 4:e21.

27. Campos, M. A., G. M. Rosinha, I. C. Almeida, X. S. Salgueiro, B. W. Jarvis, G. A. Splitter, N. Qureshi, O. Bruna-Romero, R. T. Gazzinelli, and S. C. Oliveira. 2004. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. *Infect Immun* 72:176-186.

28. Kahl-McDonagh, M. M., A. M. Arenas-Gamboa, and T. A. Ficht. 2007. Aerosol infection of BALB/c mice with *Brucella melitensis* and *Brucella abortus* and protective efficacy against aerosol challenge. *Infect Immun* 75:4923-4932.

29. Olsen, S. C., W. R. Waters, and W. S. Stoffregen. 2007. An aerosolized *Brucella spp.* challenge model for laboratory animals. *Zoonoses Public Health* 54:281-285.

30. Forestier, C., F. Deleuil, N. Lapaque, E. Moreno, and J. P. Gorvel. 2000. *Brucella abortus* lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. *J Immunol* 165:5202-5210.

31. Krummen, M., S. Balkow, L. Shen, S. Heinz, C. Loquai, H. C. Probst, and S. Grabbe. Release of IL-12 by dendritic cells activated by TLR ligation is dependent on MyD88 signaling, whereas TRIF signaling is indispensable for TLR synergy. *J Leukoc Biol*.

32. O'Neill, L. A. 2006. How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 18:3-9.

33. Archer, K. A., and C. R. Roy. 2006. MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of *Legionella pneumophila* in a mouse model of Legionnaires' disease. *Infect Immun* 74:3325-3333.

34. Malik, M., C. S. Bakshi, B. Sahay, A. Shah, S. A. Lotz, and T. J. Sellati. 2006. Toll-like receptor 2 is required for control of pulmonary infection with *Francisella tularensis*. *Infect Immun* 74:3657-3662.

Figure legends

Figure 1: *E. coli* LPS downregulated CD11c expression on bone marrow derived cells. Bone marrow cells isolated from BALB/c control and TLR KO mice were cultured for 6 days in 10% RPMI medium with rGM-CSF (20ng/ml). The cells were harvested on day 6 and treated for 4 hours with either *E. coli* LPS (100 ng/ml) or *B.abortus* rough or smooth strains (DC:*Brucella*; 1:1, 1:10, 1:100). Cells were incubated for an additional 20 hours in gentamicin containing media and analyzed for $CD11c^+$ marker expression using fluorescent labeled $CD11c^+$ antibody. Cells treated with media served as the control. Asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to corresponding media control (*). (##) denotes statistically significant data at $p \leq 0.05$ compared to corresponding *E. coli* LPS (#). Alphabets ‘aa’ represents

statistically significant data at $p \leq 0.05$ compared to corresponding strain 2308 stimulated DCs at MOI 1:100 'a'. Data represents means ± standard deviations of 3 independent experiments.

Figure 2: Rough strain RB51 up-regulated MHC class II and co-stimulatory marker expression in control and TLR KO BMDCs. Immature BMDCs from control and KO mice were infected with either *B. abortus* rough vaccine strain RB51 or with smooth strain 2308 at MOIs (DC:*Brucella*) 1:1, 1:10 and 1:100. At 24 hours post infection, BMDCs were analyzed for MHC class II, CD40, CD86 expression and CD40+/CD86+ co-expression. Media and *E. coli* LPS were the negative and positive controls respectively. A: Comparison of the percentage of $CD11c^+$ cells (BMDCs) expressing MHC class II high on its surface across different treatment groups. B: Comparison of the percentage of CD40 expression on $CD11c^+$ cells (BMDCs) across different treatment groups. C: Comparison of the percentage of CD86 expression on $CD11c^+$ cells (BMDCs) across different treatment groups. D: Comparison of the percentage of $CD40^+/CD86^+$ coexpression on $CD11c^+$ cells (BMDCs) across different treatment groups. Asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to corresponding media control (*). (##) denotes statistically significant data at $p \leq 0.05$ compared to corresponding *E.coli* LPS(#). Alphabets 'aa' represents statistically significant data at $p \leq 0.05$ compared to corresponding strain 2308 stimulated DCs at MOI 1:100 'a'. Alphabets 'bb' represents statistically significant data at $p \leq 0.05$ compared to corresponding strain 2308 stimulated DCs at MOI 1:10 'b'. Data represents means ± standard deviations of 3 independent experiments.

Figure 3: Rough strain RB51 activated BMDCs irrespective of TLR KO status. Immature BMDCs from control and KO mice were infected with either *B. abortus* rough vaccine strain RB51 or with smooth strain 2308 at MOI (DC:*Brucella*) 1:100. Histograms of single representative experiment (of 3) from each type of BMDCs for total MHC class II expression,

CD40 expression and CD86 expression. Filled grey histogram: BALB/c media control; continuous thick line: BALB/c; continuous thin line: TLR2 KO; broken thick line: TLR4 KO; dotted line: TLR9 KO.

Figure 4: Rough strain RB51 induced DC – TNF-α and IL-12 secretion was TLR dependent. To assess DC function, TNF-α (A) and IL-12_{p70} (B) levels in 24 hour culture supernatants of *B. abortus* strain RB51 or 2308 infected BMDCs at various MOIs were analyzed using indirect sandwich ELISA. *E. coli* LPS and media treated cell supernatants served as the positive and negative controls respectively. The limits of detection for both the cytokines were 15pg/ml. The TNF-α and IL-12_{p70} results represent medians and 75^{th} percentile of 3 independent experiments. Asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to corresponding media control (*). (##) denotes statistically significant data at $p \leq 0.05$ compared to corresponding *E.coli* LPS(#). Alphabets 'cc' represents statistically significant data at $p \leq 0.05$ compared to alphabet 'c'.

Figure 5: Pulmonary clearance of rough strain RB51 is TLR4 dependent. Bacterial clearance from TLR 2, TLR4, TLR9 KO and BALB/c control mice following IN infection with A: *B. abortus* rough strain RB51 and B: smooth strain 2308. Mice were infected IN either with strain RB51 (4×10^8 CFUs/mouse) or with strain 2308 (2×10^4 CFUs/mouse) and the number of bacteria recovered from lung, mediastinal lymph node (MLN) and spleen were counted on day 14 post infection (PI). Asterisks (**) denotes statistically significant data at $p \leq 0.05$ compared to corresponding media control (*). Results represent median ± 75^{th} percentile.

Figure 1

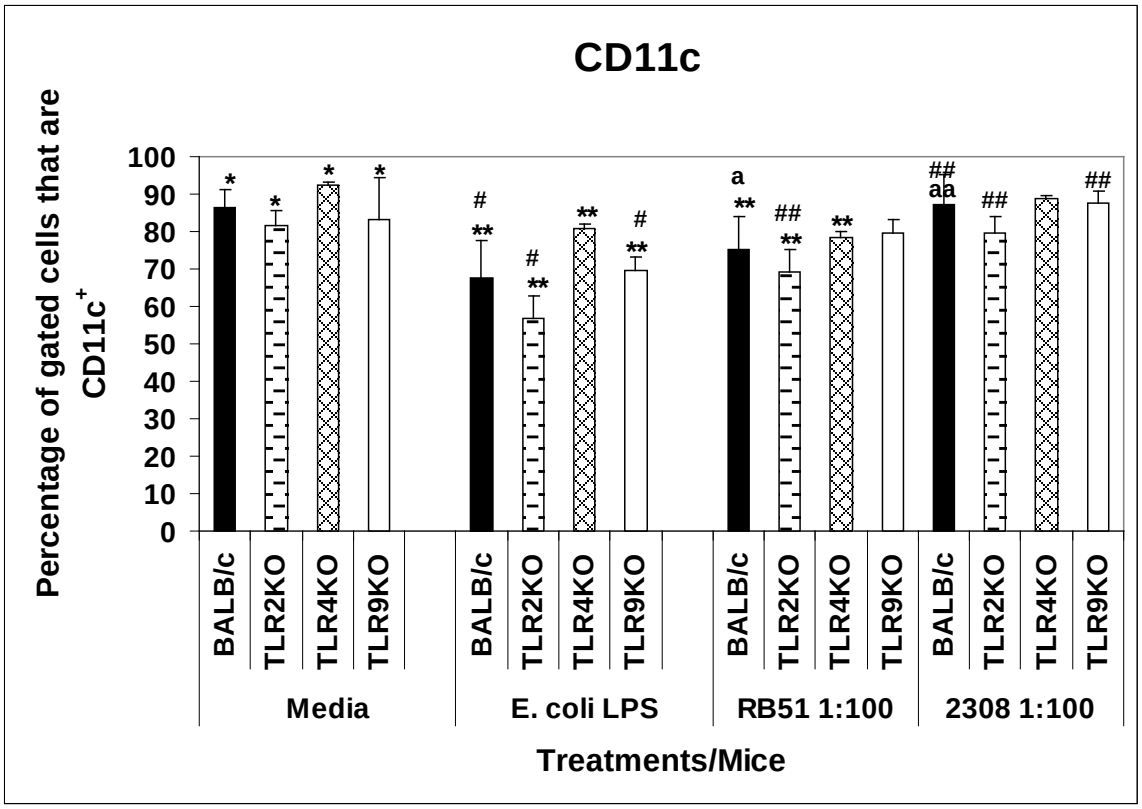
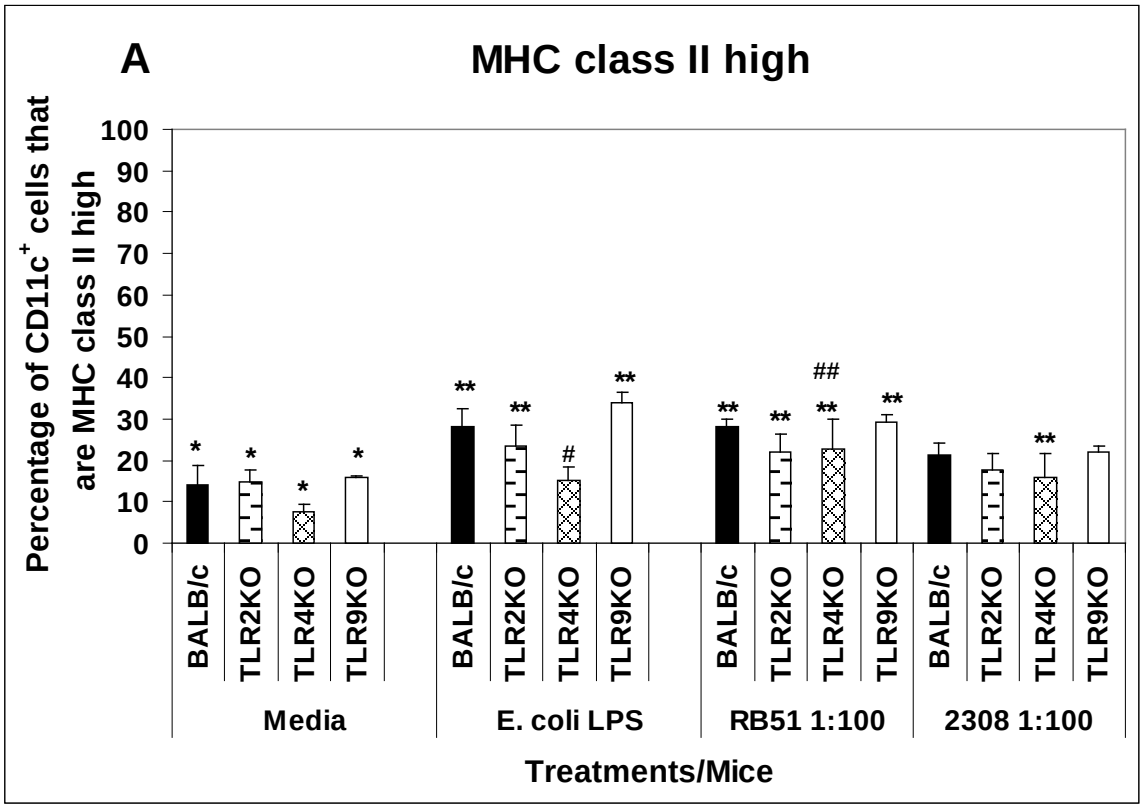
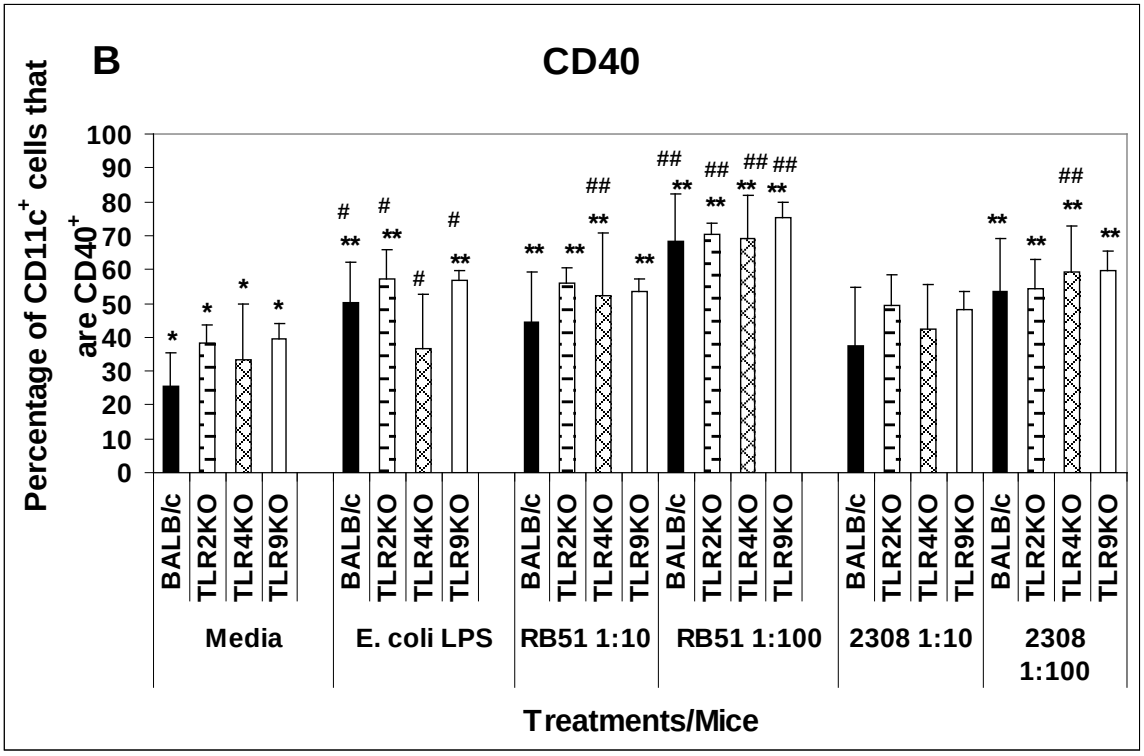

CD11c
Percentage of gated cells that are CD11c+
100
90
80
70
60
50
40
30
20
10
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
E. coli LPS
RB51 1:100
2308 1:100
Treatments/Mice

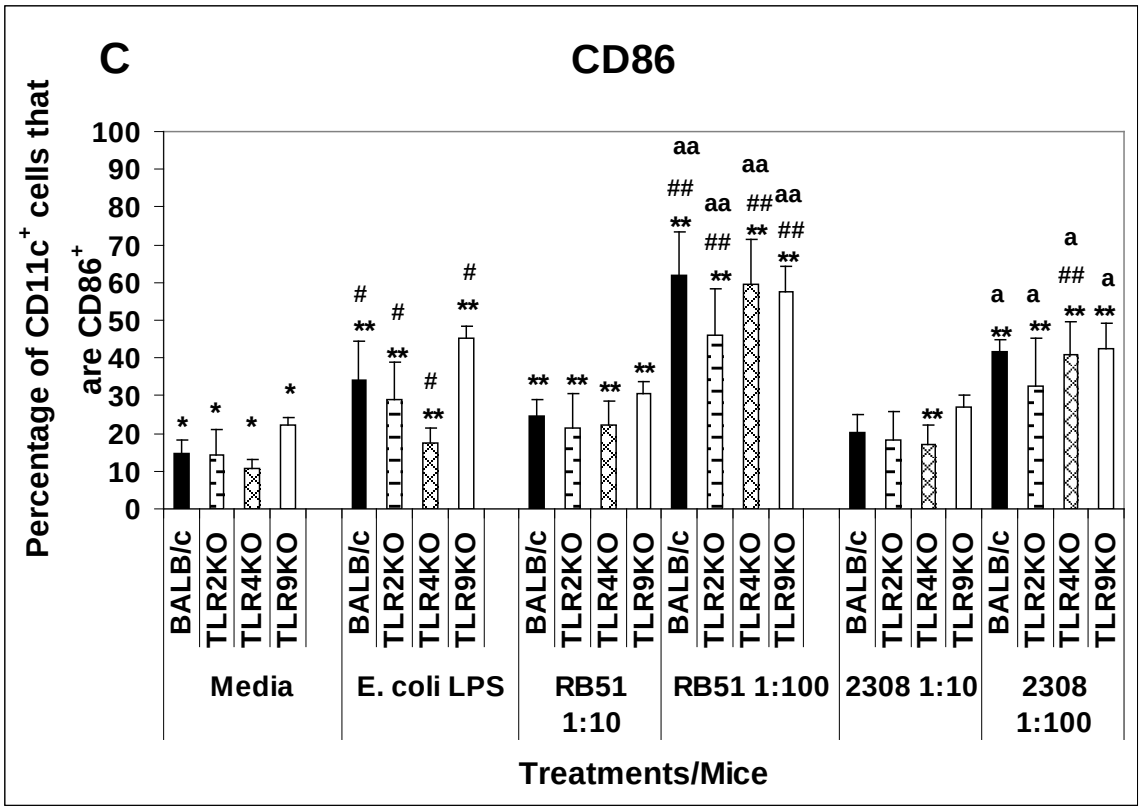
Table I: Comparison of significant changes among BALB/c control and TLR KO mice for BMDC cell surface marker expression.

Treatment	Mice comparison	BMDC cell surface expression				
		CD 11c	MHC II	CD40	CD86	$CD40^+/CD86^+$
Media	TLR9 KO vs. BALB/c	--	--	--	0.0087	0.039
	TLR9 KO vs. TLR2 KO	--	--	--	0.0044	--
	TLR9 KO vs. TLR4 KO	0.0467	0.0456	--	0.039	0.034
E. coli LPS	BALB/c vs. TLR4 KO	0.0323	0.0006	0.0109	0.0014	<0.0001
	TLR2 KO vs. TLR4 KO	<0.0001	0.0384	0.0129	0.0461	<0.0001
	TLR9 KO vs. TLR4 KO	0.0105	<0.0001	0.0052	<0.0001	<0.0001
	TLR9 KO vs. TLR2 KO	0.0414	0.0078	--	0.0017	<0.0001
	TLR9 KO vs. BALB/c	--	0.0416	--	<0.0001	0.0103
	BALB/c vs. TLR2 KO	--	--	--	0.0217	0.0191
RB51 1:10	TLR9 KO vs. TLR2 KO	--	--	--	--	0.0043
	TLR9 KO vs. TLR4 KO	--	--	--	--	0.0353
RB51 1:100	BALB/c vs. TLR2 KO	--	--	--	0.0008	0.0036
	TLR4 KO vs. TLR2 KO	--	--	--	0.0047	0.0029
	TLR9 KO vs. TLR2 KO	--	0.0191	--	0.0169	0.0073
2308 1:10	TLR9 KO vs. BALB/c	--	--	--	0.00234	--
	TLR9 KO vs. TLR2 KO	--	--	--	0.0029	0.0149
	TLR9 KO vs. TLR4 KO	--	0.0463	--	--	0.0176
2308 1:100	BALB/c vs. TLR2 KO	--	--	--	0.05	0.0194
	TLR4 KO vs. TLR2 KO	--	--	--	--	0.0036
	TLR9 KO vs. TLR2 KO	--	--	--	0.03	0.0378

Figure 2


A
MHC class II high
Percentage of CD11c+ cells that are MHC class II high
100
90
80
70
60
50
40
30
20
10
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
E. coli LPS
RB51 1:100
2308 1:100
Treatments/Mice


B
CD40
Percentage of CD11c+ cells that are CD40+
100
90
80
70
60
50
40
30
20
10
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
E. coli LPS
RB51 1:10
RB51 1:100
2308 1:10
2308 1:100
Treatments/Mice


C
CD86
Percentage of CD11c+ cells that are CD86+
100
90
80
70
60
50
40
30
20
10
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
E. coli LPS
RB51 1:10
RB51 1:100
2308 1:10
2308 1:100
Treatments/Mice

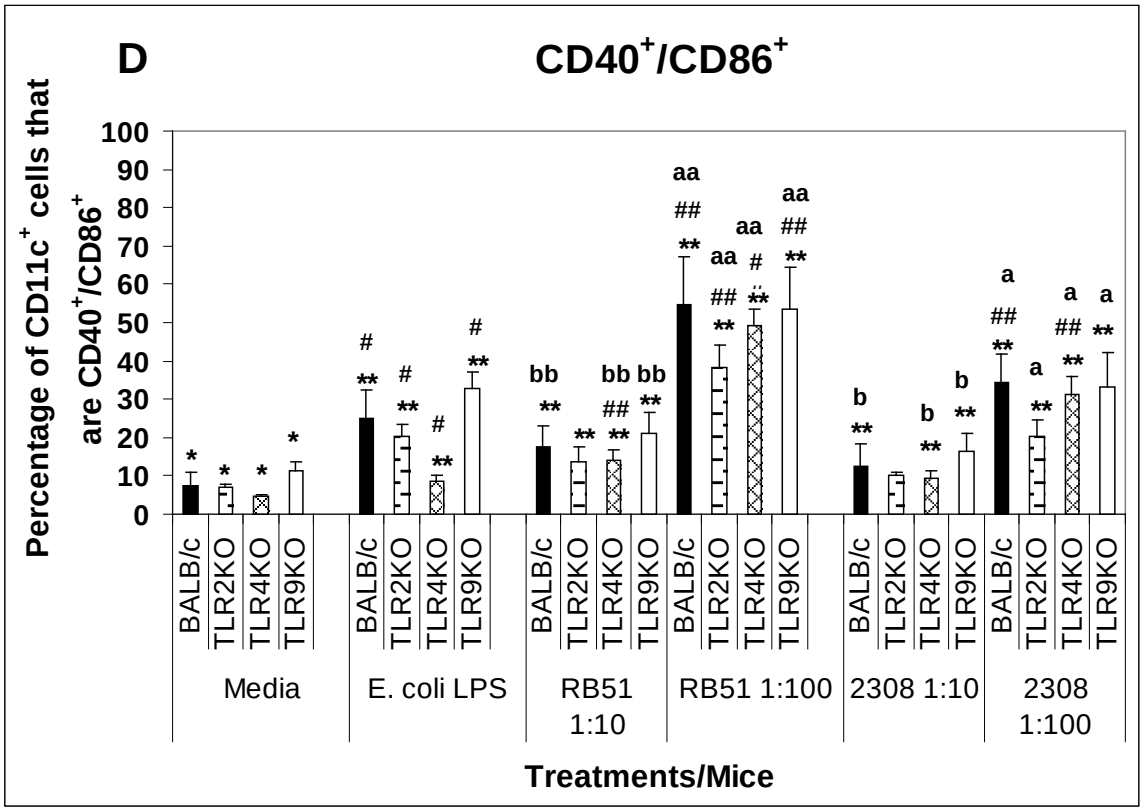

D
CD40+/CD86+
Percentage of CD11c+ cells that are CD40+/CD86+
100
90
80
70
60
50
40
30
20
10
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
E. coli LPS
RB51 1:10
RB51 1:100
2308 1:10
2308 1:100
Treatments/Mice

Figure 3

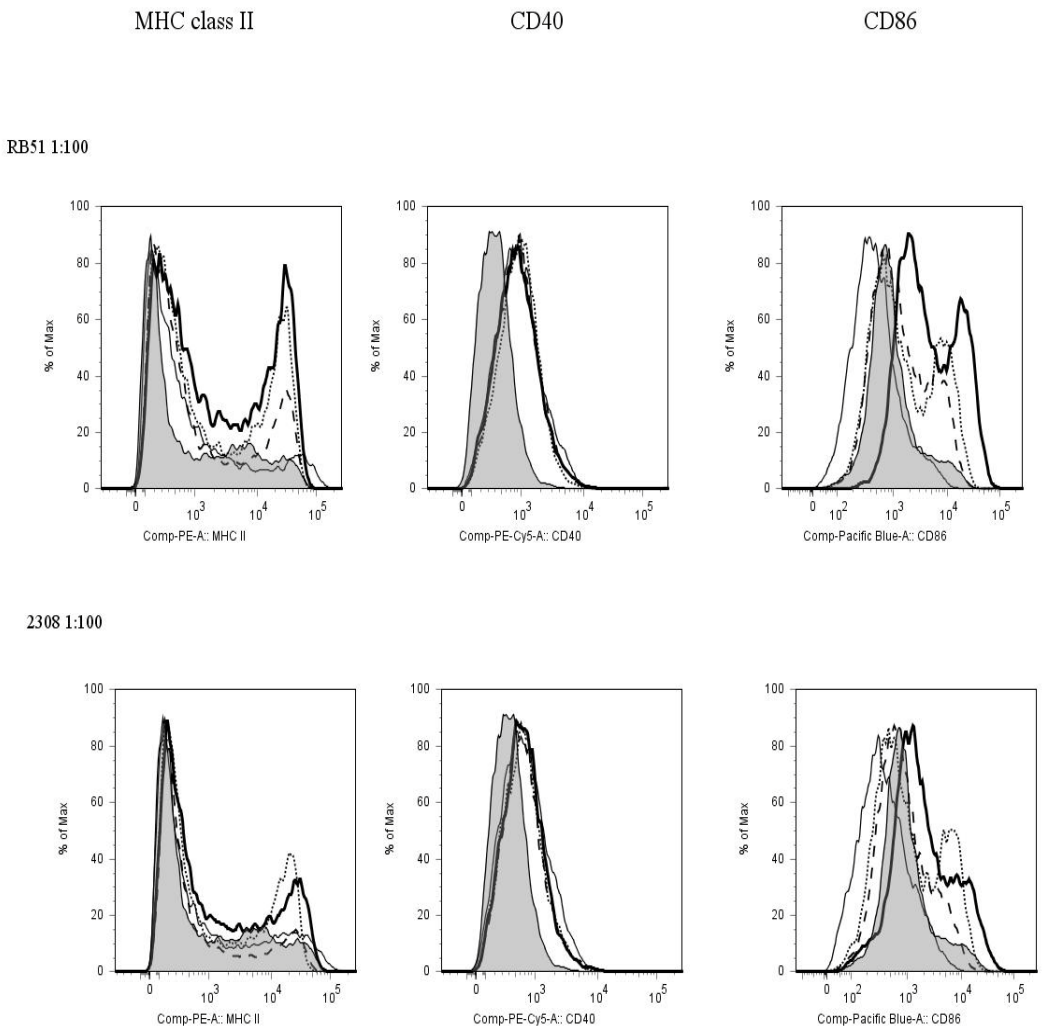

MHC class II
CD40
CD86
RB51 1:100
2308 1:100
% of Max
Comp-PE-A:: MHC II
Comp-PE-Cy5-A:: CD40
Comp-Pacific Blue-A:: CD86

Figure 4

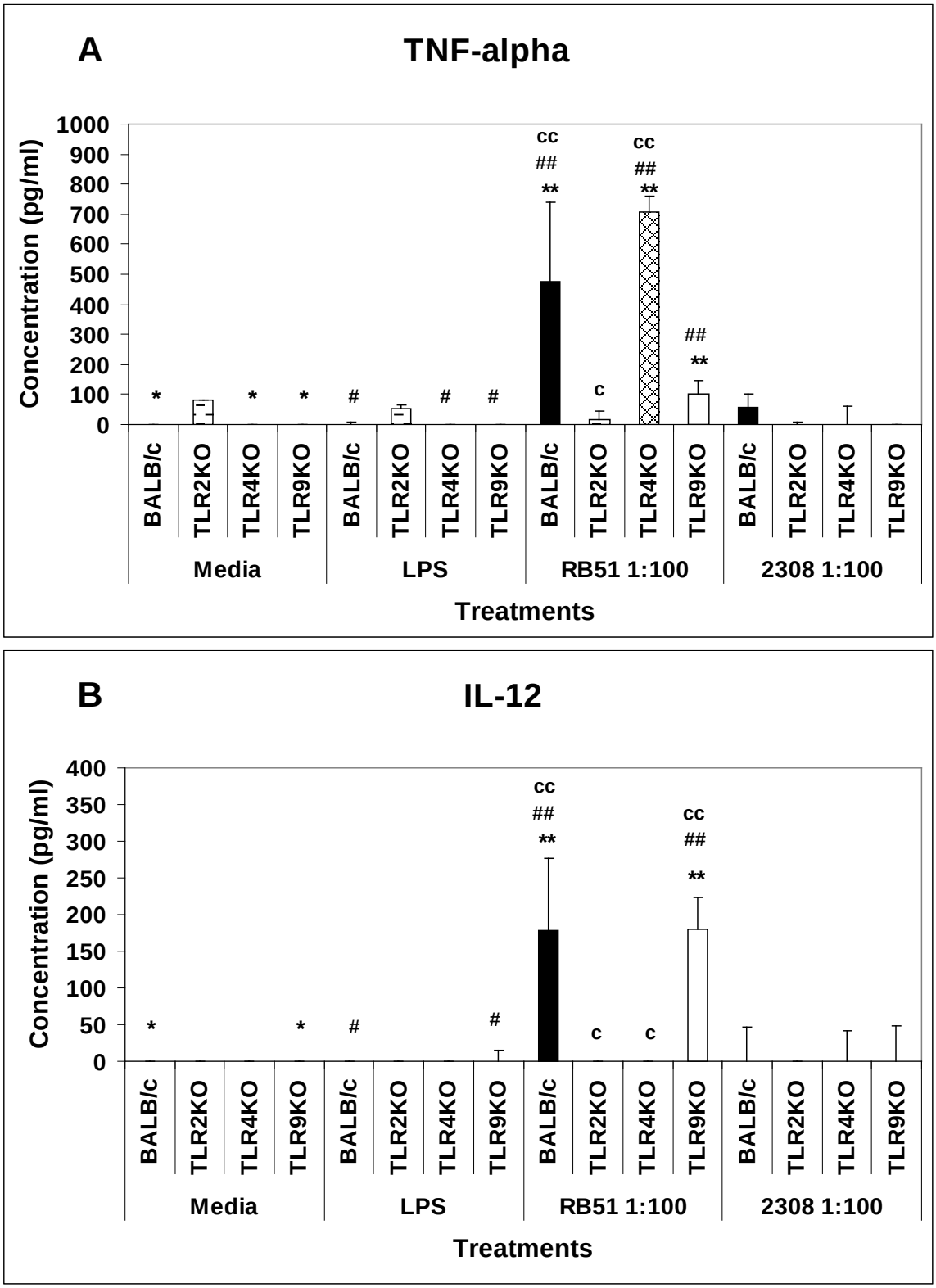
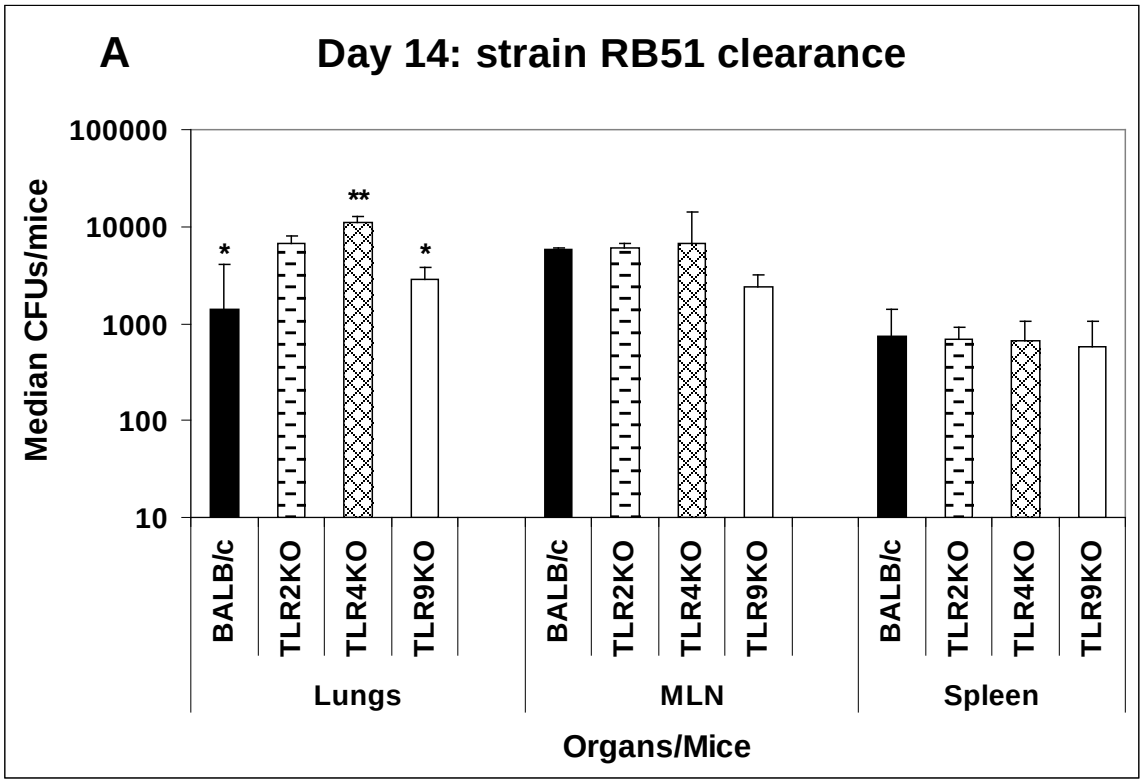
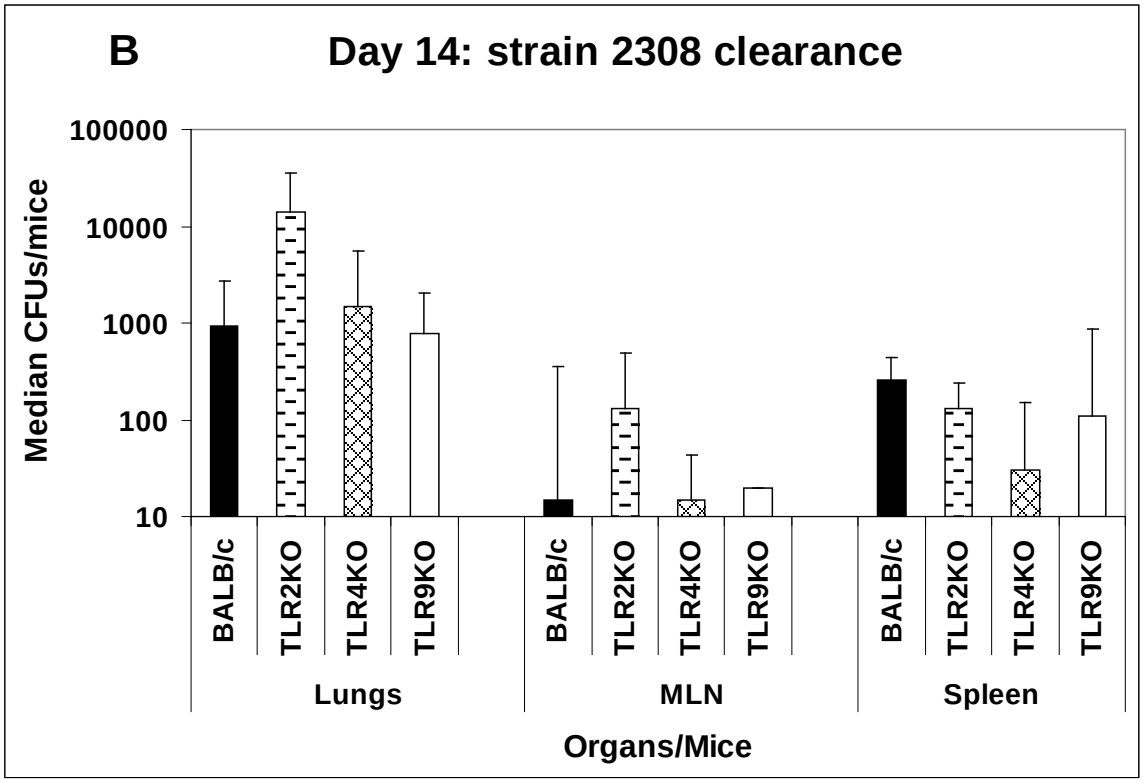

A
TNF-alpha
Concentration (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
BALB/c
TLR2KO
TLR4KO
TLR9KO
Media
LPS
RB51 1:100
2308 1:100
Treatments
B
IL-12
Concentration (pg/ml)
400
350
300
250
200
150
100
50
0
Treatments

Figure 5


A
Day 14: strain RB51 clearance
Median CFUs/mice
100000
10000
1000
100
10
*
**
*
BALB/c
TLR2KO
TLR4KO
TLR9KO
Lungs
MLN
Spleen
Organs/Mice


B
Day 14: strain 2308 clearance
Median CFUs/mice
100000
10000
1000
100
10
BALB/c
TLR2KO
TLR4KO
TLR9KO
Lungs
MLN
Spleen
Organs/Mice

Chapter 6

Efficacy of vaccination strategies against intranasal challenge with *Brucella abortus* in BALB/c model.

Running title: Intranasal vaccine against *B. abortus* respiratory infection.

Naveen Surendran[1], Nammalwar Sriranganathan[2], Heather Lawler[1], Stephen M. Boyle[2], Elizabeth M. Hiltbold[3], Bettina Heid[1], Kurt Zimmerman[2], Sharon G. Witonsky[1*]. (To be submitted to Infection and Immunity)

Abstract

Brucellosis is a zoonotic disease with affecting 500,000 people worldwide annually. Inhalation of infected aerosol is one of the major routes of disease transmission in humans. Currently there are no licensed human vaccines available. *B. abortus* strain RB51 is a USDA approved live attenuated vaccine against cattle brucellosis. *B. abortus* strain RB51SOD has been shown to be more efficacious compared to strain RB51 against *B. abortus* pathogenic strain 2308 in mouse models. However, there is lack of information on the ability of these vaccine strains to protect against intranasal challenge. In this study, with the long term goal to develop protective vaccine for animals and people against respiratory challenge of *Brucella* spp., we tested a number of different vaccination strategies in mice against intranasal infection with strain 2308. Our strategies included strains RB51 or RB51SOD, evaluating the efficacy of different vaccination routes, doses, boosting, and various prime-boost strategies against strain 2308 challenge. Despite using multiple protocols to enhance mucosal and systemic protection, our results revealed that neither vaccine strains provided respiratory protection against intranasal

pathogenic *Brucella* infection. Further studies will address host-pathogen interaction at the lung microenvironment and elucidate immune mechanisms that will enhance protection to aerosol infection.

Introduction

Brucella is the most common zoonotic agent worldwide (1). Although *B. melitensis* is the most important zoonotic agent among *Brucella* species, most human cases are caused by *B. abortus* (4). *B. melitensis*, *B. suis* and *B. abortus* are the most infectious of the genus in the order of their pathogenicity (4). Incidence of human disease correlates closely both with prevalence of infection of livestock animals and risk factors for human exposure to infected animals and/or related products (8). The highest risk group is abattoir workers, meat inspectors, animal handlers, veterinarians, and lab workers (16). Clinical syndromes in humans include chronic fatigue, undulant fever, reproductive disorders and general malaise (4). Around the globe, more than 500,000 human infections are infected annually (13).

Brucella is considered a biological weapon due to its propensity for air borne transmission and highly infectious nature (13). *Brucella* is relatively stable in aerosol form and a small inoculum (10-100 microorganisms) will induce disease (3). The organism is easily obtained and antibiotic resistant strains can be easily developed (13). Therefore, Center for Disease Control and Prevention (CDC) included *Brucella* species as a category B biodefense agent. At present, safe and protective vaccines licensed for use against human brucellosis are not commercially available.

To date, vaccination and experimental challenge studies with live *Brucella* strains have predominantly focused on intraperitoneal (IP) delivery in mouse models (7). In spite of the

importance of aerosol exposure, very few studies have focused on assessing protection against respiratory challenge (2, 8, 16). Previously, Mense et al. (10) demonstrated that aerosol infection of BALB/c mice with *Brucella spp* establishes chronic respiratory and systemic infection of spleen and liver. In separate studies, Kahl-McDonagh et al. (8) and Olsen et al. (12) described aerosol infection with *B. melitensis* 16M and/or *B. abortus* 2308 respectively. However, IP vaccination with live attenuated vaccine strains was not protective (8, 12). Part of the lack of efficacy may be due to the immune response associated with vaccination route. Published literature shows that mice vaccinated IP protected against IP challenge with *Brucella* (7), and mice vaccinated orally with *B. abortus* strain RB51 protected against oral *B. abortus* strain 2308 challenge (14). Therefore, protection might be optimum when vaccination and challenge routes are identical such that associated local and systemic immune responses may be optimal. Hence, we hypothesized that IN vaccination with live attenuated vaccine would enhance local mucosal and therefore overall protective immune response against IN *Brucella* challenge. Additionally, it is expected that mucosal vaccination would enhance mucosal protective immune response against aerosol challenge.

No studies have been published assessing the efficacy of IN vaccination with *B. abortus* vaccine strains against IN challenge with *B. abortus* in a mouse model. In this study, we assessed protection due to IN vaccination with *B. abortus* live attenuated rough vaccine strains RB51 (15) and RB51SOD (over expressing Cu-Zn superoxide dismutase) against IN challenge with *B. abortus* smooth pathogenic strain 2308 (17). We also assessed the effect of route and prime-boost vaccination strategies against IN strain 2308 challenge.

Materials and Methods

Mice: Female 6-8 weeks old BALB/c mice were obtained from Charles River Laboratories Inc., Wilmington, MA. Mice were used under animal care protocols approved by Institutional Animal Care and Use Committee at Virginia Tech.

Bacterial strains: Live attenuated rough *B. abortus* strains RB51, RB51SOD and virulent smooth strain 2308 used were from our stock culture collection. All experiments with *Brucella* were performed in our CDC approved (C2003 1120-0016) Biosafety Level (BSL) -3 Infectious Disease Unit (IDU) facility.

Experimental design: The experimental designs employed to assess different routes and prime boost protocols is outlined in Table 1.

Table 1. Experimental design of vaccination and challenge studies

Groups	Vaccine strain	Route of vaccination	Vaccine dose (CFUs/mouse)	Booster dose?	Route of booster dose	IN - strain 2308 infection dose (CFUs/mouse)
1	RB51	IN	4×10^9	No	--	2×10^5
	RB51SOD	IN	4×10^9	No	--	2×10^5
2	RB51	IN	4×10^9	Yes	IN	2×10^5
	RB51SOD	IN	4×10^9	Yes	IN	2×10^5
3	RB51SOD	IN	4×10^9	Yes	IN	2×10^4
4	RB51SOD	IN	1×10^7	No	--	2×10^4
	RB51SOD	IN	1×10^8	No	--	2×10^4
	RB51SOD	IN	1×10^9	No	--	2×10^4
5	RB51SOD	IP	4×10^8	No	--	2×10^4
	RB51SOD	IM	4×10^8	No	--	2×10^4
	RB51SOD	ID	4×10^8	No	--	2×10^4
	RB51SOD	SC	4×10^8	No	--	2×10^4
6	RB51SOD	IP	4×10^8	Yes	IP	2×10^4
	RB51SOD	IP	4×10^8	Yes	IN	2×10^4
	RB51SOD	IM	4×10^8	Yes	IN	2×10^4
	RB51SOD	ID	4×10^8	Yes	IN	2×10^4
	RB51SOD	IN	4×10^8	Yes	IP	2×10^4
	RB51SOD	IN	4×10^8	Yes	IM	2×10^4

Group 1: To assess vaccine efficacy with single intranasal vaccination and the effect of day, BALB/c mice (n=5) were immunized intranasally (IN), under xylazine-ketamine anesthesia, with either *B. abortus* rough strains RB51 or strain RB51SOD (4 X 10^9 CFUs/mouse) in 35 µl phosphate buffered saline (PBS). PBS inoculated mice served as control with all experiments unless otherwise mentioned. Clearance of vaccine strains was determined prior to challenge. Six weeks post vaccination, control as well as vaccinated mice were infected IN with smooth strain 2308 (2 X 10^5 CFUs/mouse). Mice were euthanized at day 14 and day 41 post infection (to determine if there was a delay in clearance) with a lethal dose of xylazine-ketamine IP. Lung, mediastinal lymph node (MLN) and spleen were collected. Single cell suspensions of organs collected were serially diluted and plated on to Tryptic soy agar (TSA) plates and incubated for 5 days at 37° C and 5% CO_2. Bacterial colony forming units (CFUs) were counted and CFUs/mouse were calculated (8, 17).

Group 2: To assess the effect of intranasal boosting and day, mice (n=5 per day per treatment group) were primed (4 X 10^9 CFUs/mouse) and boosted (4 X 10^9 CFUs/mouse) IN 4 weeks apart. Clearance of vaccine strains was determined prior to boosting and challenge. Mice (n=5) were challenged 4 weeks post boost with virulent strain 2308 (2 X 10^5 CFUs/mouse). CFUs/mouse were calculated at day 14 and day 41 (to determine if there was a delay in clearance) as before.

Group 3: To determine whether challenge dose was limiting assessment of protection, the challenge dose was decreased to 2 X 10^4 CFUs/mouse, that used for IP challenge studies. Strain RB51SOD was used as it has been demonstrating to be more efficacious than strain RB51 in mouse models. Mice (n=8 per group) were vaccinated and boosted IN (4 X 10^9 CFUs/mouse)

with strain RB51SOD as in Group 2, challenged IN with strain 2308 (2 X 10^4 CFUs/mouse) and day 14 PI CFU/mouse were determined.

Group 4: To identify the optimal vaccine dose, five mice (n=5) per group were given 1 of the 3 doses IN (1 X 10^7, 1 X 10^8, or 1 X 10^9 CFUs/mouse) of strain RB51SOD). Six weeks post vaccination mice were challenged IN with strain 2308 (2 X 10^4 CFUs/mouse). Day 14 post infection clearance was assessed as above.

Group 5: To identify the optimal systemic vaccination route, mice (n=5 per group) were vaccinated subcutaneously (SC), intraperitoneally (IP), intradermally (ID) or intramuscularly (IM) with strain RB51SOD (4 X 10^8 CFUs/mouse) in sterile PBS. Six weeks post vaccination, mice were challenged IN with strain 2308 (2 X 10^4 CFUs/mouse) and clearance (CFUs/mouse) was determined at day 14 post challenge as above.

Group 6: To optimize the prime-boost protocol, mice were vaccinated with strain RB51SOD (4 X 10^8 CFUs/mouse) by different prime boost protocols. The study was completed in 2 blocks/experiments. In the 1st block/experiment, mice (n=8 per group) were primed and boosted (IP/IP and IP/IN) 4 wks apart. In the 2nd block/experiment, mice (n=6 per group) were primed and boosted (IM/IN, ID/IN, IN/IP, IN/IM) at same time interval. Mice were challenged IN with strain 2308 (2 X 10^4 CFUs/mouse) 4 weeks following post boost vaccination. Day 14 clearance was assessed based on CFUs/mouse to determine protection.

Statistical analysis: Statistical significance of the effect of single IN vaccine, IN vaccine - IN boost, different vaccine doses and route of systemic vaccination on were done by comparing the treatments using ANOVA followed by Tukey's procedure for multiple comparisons. IP vaccine – IP boost and IP vaccine – IN boost study organs were analyzed using T-test for comparison of the effect of treatment. For systemic prime – IN boost as well as IN prime – systemic boost

studies, treatments were compared using Kruskal-Wallis test followed by Dunn's procedure for multiple comparisons. All statistical analysis was done using SAS system (NC, USA).

Results

In Group 1 to assess vaccine efficacy with single intranasal vaccination and the effect of day, single intranasal vaccination with vaccine strains RB51 and RB51SOD did not protect against IN challenge with pathogenic strain 2308 at day 14 (Figure 1A) or day 41 (Figure 1B) in lung, spleen or MLN. Both strain RB51 and strain RB51SOD induced almost a log clearance of strain 2308 at day 14 PI from spleen but that was not significantly different from PBS control mice (Figure 1A) (Although the overall p-value from ANOVA for the effect of treatments vs. organ was 0.08, PBS vs. RB51 comparison in spleen demonstrated a trend toward clearance with a p value of 0.08 and in lung, PBS vs. RB51SOD had a p value of 0.03).

As vaccinated mice showed almost 1 log clearance in the spleen, in **Group 2**, the effect of boosting the mice IN was determined. Figures 2A and 2B illustrate the clearance of *B. abortus* pathogenic strain from spleen, lungs and MLN at day 14 and 41 PI respectively. Mice boosted intranasally did not show significant increase in clearance from any organs tested at both day 14 and day 41 PI compared to control (Figure 2A and 2B) (The overall p value for ANOVA was 0.0643 for the effect boost vs. treatment and among the individual comparisons, PBS vs. RB51 in spleen yielded a p value of 0.013). As there was some non significantly enhanced clearance from the spleen, we assessed whether the challenge dose was too high to demonstrate vaccine efficacy **(Group 3)**; the challenge dose was decreased 1 log to 2 X 10^4 CFUs/mouse. Nevertheless, intranasal vaccination of strain RB51SOD did not show significant protection in any organs.

Group 4: As the initial vaccine dose, which had been chosen based on that used for IP vaccination and protection studies did not protect, we sought to identify a more efficacious vaccine dose. Thus, the effect of vaccine dose on clearance was determined in **Group 4** experiments. Figure 3A demonstrated that there was no significant difference in clearance of strain 2308 between IN doses (10^7, $10^{8,}$ 10^9 CFUs/mouse) in any of the organs tested.

As IN vaccination had not proven efficacious, the effect of systemic vaccination route on challenge was determined **(Group 5).** In Figure 3B, none of the systemic vaccination routes induced significant clearance of strain 2308 from the organs tested when compared to PBS control. Furthermore, clearance of strain 2308 from spleen, lungs or MLN was not significantly different between any of the different vaccination routes tested.

To determine if prime-boost vaccination strategies would enhance clearance, mice were vaccinated and challenge as described **(Group 6).** Similar to homologous IN prime-boost strategy (Figure 2A-B), homologous IP prime and IP boost strategy using strain RB51SOD (4 X 10^8 CFUs/mouse) failed to provide significant clearance of strain 2308 (2 X 10^4 CFUs/mouse) from mice at day 14 PI compared to control mice (Figure 4A). As it is predicted that the most effective vaccine strategy which will provide optimal protection against IN infection might require heterologous prime-boost combinations that involve both systemic and mucosal delivery, we tested IP vaccine – IN boost strategy with strain RB51SOD to determine protection. No significant improvement in bacterial clearance from lung or MLN was obtained with this vaccination strategy although the vaccine RB51SOD induced significant clearance of strain 2308 from spleen ($p=0.0146$) (Figure 4B). Based on the lack of protection, the experiment was repeated to include other systemic vaccination routes. As shown in Figure 4C, IM and ID systemic priming followed by IN boost did not protect mice against IN pathogenic 2308

infection in any of organs. However, there was a trend towards significant clearance in spleen ($p=0.086$) from IM vaccinated and IN boosted mice. As systemic priming might not prime the immune system for subsequent mucosal vaccination and as mucosal immunization could potentially prime the immune system for both systemic and mucosal responses, in a subsequent study we reversed the heterologous prime-boost strategy for an IN prime followed by systemic booster vaccination. Different groups of mice primed IN with strain RB51SOD were given a booster dose of strain RB51SOD either IP or IM before challenging IN with virulent strain 2308. Nonetheless, the vaccination strategy was not protective against IN strain 2308 challenge as the bacterial CFUs/mouse recovered from any organs at day 14 PI were not significantly lower than PBS control group (Figure 4D).

Discussion

Published studies have established that aerosol or IN exposure of mice (or macaques) with even low (10^2 - 10^3) doses of pathogenic *B. abortus* strain 2308 or *B. melitensis* 16M caused chronic pulmonary or systemic infection (8-10). To date, most vaccine efficacy studies against IN *Brucella* challenge infection focused on IP vaccination. As this approach was not efficacious, another strategy is needed (8, 12). Based on other models, mucosal delivery of vaccines should enhance protection (11). Therefore, we determined whether IN vaccination of *B. abortus* rough vaccine strains RB51 and/or RB51SOD was efficacious against IN challenge with smooth pathogenic strain 2308.

In preliminary studies, we assessed clearance of both vaccine and pathogenic strains to establish our model. Vaccine doses of IN administered vaccine strains RB51 and RB51SOD ranged from 10^7 to 10^9 CFUs/mouse. As previously shown for vaccines delivered via IP, vaccine

strains given IN were completely cleared from lungs, MLN and spleen by 4-6 weeks post vaccination (data not shown). In contrast, pathogenic strain 2308 administered IN did not clear until day 116 from lungs; MLN and spleen remained infected even at the lowest inoculum dose of 2 X 10^3 CFUs/mouse (data not shown). Based on some models (10, 12) using 1 log higher vaccine and challenge doses for IN administered agents, we followed that protocol for the initial challenge studies. However, IN vaccination followed by IN challenge did not elicit significant protection either at day 14 or 41 PI in any of the organs tested, compared to the traditional IP vaccine – IP challenge model (7). The reasons for this lack of protection in this and the subsequent studies described here, despite IN and/or systemic boosting with the same vaccine strains could be manifold. Possible explanations for the lack of clearance include insufficient dose to generate protection, pathogen persistence, and poor ability of vaccine to stimulate CD4 Th1 response in mucosal environment with a CD4 Th2 bias.

As stated, some of the differences could be dose and species dependent clearance. Whereas decreasing the *B. abortus* challenge dose did not improve clearance, Smither et al. (16) demonstrated that decreasing the *B. melitensis* 16M challenge dose to 4 X 10^3 CFUs/mouse did enhance protection with IN vaccine *B. melitensis* Rev. 1. In addition, Kahl McDonagh et al. (8) demonstrated that aerosol *B. melitensis* infection in mice diminished more rapidly compared to *B. abortus*. Thus, the lower challenge dose and species dependent difference in clearance might have led to improved protection by *B. melitensis* Rev. 1 against IN *B. melitensis* challenge. As smooth vaccine strains such as *B. melitensis* Rev. 1 enhanced protection against *B. melitensis* 16M, identifying the mechanisms of induced protection to see if anything may be applied to the *B. abortus* model is warranted.

Another possible explanation is the inability of the vaccines to stimulate a sufficient CD4 Th1 or Th17 response in a mucosal CD4 Th2 biased environment (5). Interestingly, the vaccine doses tested did not enhance protection possibly due to the inability to surmount the bias towards a Th2 response (5). As an additional IN booster dose also failed to enhance clearance, it is possible that the vaccine does not override a Th2, or T-reg status in the lung.

It was possible that regardless of dose, intranasal vaccination alone did not stimulate sufficient CD4 Th1 or Th17 muscoal immunity, but possibly with systemic vaccination, a strong Th1 memory or Th17 response could be elicited. However, in agreement with the IP vaccine – IN challenge experiment results by Kahl McDonagh et al. (8) and Olsen et al., (12) none of the systemic vaccination routes (SC, IM, ID or IP) tested using live attenuated *B. abortus* strain RB51SOD provided significant protection against *B. abortus* IN challenge infection. Even IP prime and boost vaccination did not protect mice against IN challenge. Broadly, possible explanations for the lack of protection include the vaccine's inability to stimulate appropriate innate and/or adaptive immune response in the mucosal or systemic environment. More specifically, rough vaccine strains may be unable to stimulate appropriate TLR induced responses to stimulate sufficient DC response, which may or may not be affected by the mucosal environment. This could affect the ability to elicit a sufficient CD4 Th1 and CD8 Tc1 response as well as possibly Th17 induced responses.

Ideally, vaccination at a single site would provide protection not only at the relevant mucosal surface, but also throughout the body (11). However, optimal protection is likely to require both mucosal and systemic immune effectors, and the most effective mucosal vaccine strategies might be prime–boost combinations that involve both mucosal and systemic delivery (11). Our rationale for the IN prime – systemic boost was that the CD4 Th1 response induced by

systemic boost could potentially overcome a CD4 Th2 response elicited by initial intranasal vaccination. We tested both systemic prime – IN boost and IN prime – systemic boost vaccination strategies and failed to show protection against IN challenge. Additional studies will focus on the underlying mechanism for the lack of protection.

Besides the inability of the vaccines to induce an apparent protective immune response, the ability of intracellular pathogens to remain sequestered from the immune response is another possible reason for lack of protection. In fact, strain 2308 has recently been shown to infect and replicate within respiratory epithelial cells without inducing cell death or cytotoxicity (6). Thus this is another possible mechanism by which strain 2308 could persist in the respiratory epithelium without detection or clearance.

In summary, all of the vaccination strategies tested failed to induce significant protection in mice against *B. abortus* strain 2308 IN challenge from spleen (except IP/IN), lungs and MLN. We tested both the *B. abortus* rough strains RB51 and RB51SOD as IN vaccines against an IN strain 2308 challenge infection. We found that strain RB51SOD did not induce significant improvement in pathogen clearance compared to strain RB51 upon IN vaccination as thought based on IP vaccine-challenge studies. Results from our studies and currently available literature suggest that development of a protective vaccine against IN/aerosol challenge requires additional research focused on the ability of vaccines to elicit specific immunity based on route of administration, as well as elucidating pulmonary immune responses against IN delivered pathogens.

Acknowledgement: The authors would like to acknowledge Betty Mitchell for her invaluable help in organ preparation and CFU calculations. The authors would also like to thank Dr. Ramanathan Kasimanickyam and Dr. Stephen Werre for their help with statistical analysis.

References

1. **Acha, P., Szyfres, B. (ed.).** 2001. Zoonoses and communicable diseases common to man and animal. , vol. I, Bacterioses and Mycoses. Pan American Health Organization, WHO.
2. **Bhattacharjee, A. K., M. J. Izadjoo, W. D. Zollinger, M. P. Nikolich, and D. L. Hoover.** 2006. Comparison of protective efficacy of subcutaneous versus intranasal immunization of mice with a *Brucella melitensis* lipopolysaccharide subunit vaccine. Infect Immun **74:**5820-5825.
3. **Bossi, P., A. Tegnell, A. Baka, F. Van Loock, J. Hendriks, A. Werner, H. Maidhof, and G. Gouvras.** 2004. Bichat guidelines for the clinical management of brucellosis and bioterrorism-related brucellosis. Euro Surveill **9:**E15-16.
4. **Corbel, M. J. (ed.).** 2006. Brucellosis in humans and animals. World Health Organization.
5. **Eisenbarth, S. C., D. A. Piggott, J. W. Huleatt, I. Visintin, C. A. Herrick, and K. Bottomly.** 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med **196:**1645-1651.
6. **Ferrero, M. C., C. A. Fossati, and P. C. Baldi.** 2009. Smooth *Brucella* strains invade and replicate in human lung epithelial cells without inducing cell death. Microbes Infect **11:**476-483.

7. **Jimenez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect Immun **62:**4990-4996.

8. **Kahl-McDonagh, M. M., A. M. Arenas-Gamboa, and T. A. Ficht.** 2007. Aerosol infection of BALB/c mice with *Brucella melitensis* and *Brucella abortus* and protective efficacy against aerosol challenge. Infect Immun **75:**4923-4932.

9. **Mense, M. G., R. H. Borschel, C. L. Wilhelmsen, M. L. Pitt, and D. L. Hoover.** 2004. Pathologic changes associated with brucellosis experimentally induced by aerosol exposure in rhesus macaques (*Macaca mulatta*). Am J Vet Res **65:**644-652.

10. **Mense, M. G., L. L. Van De Verg, A. K. Bhattacharjee, J. L. Garrett, J. A. Hart, L. E. Lindler, T. L. Hadfield, and D. L. Hoover.** 2001. Bacteriologic and histologic features in mice after intranasal inoculation of *Brucella melitensis*. Am J Vet Res **62:**398-405.

11. **Neutra, M. R., and P. A. Kozlowski.** 2006. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol **6:**148-158.

12. **Olsen, S. C., W. R. Waters, and W. S. Stoffregen.** 2007. An aerosolized *Brucella spp.* challenge model for laboratory animals. Zoonoses Public Health **54:**281-285.

13. **Pappas, G., P. Panagopoulou, L. Christou, and N. Akritidis.** 2006. *Brucella* as a biological weapon. Cell Mol Life Sci **63:**2229-2236.

14. **Pasquali, P., A. Rosanna, C. Pistoia, P. Petrucci, and F. Ciuchini.** 2003. *Brucella abortus* RB51 induces protection in mice orally infected with the virulent strain *B. abortus 2308*. Infect Immun **71:**2326-2330.

15. **Schurig, G. G., R. M. Roop, 2nd, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet Microbiol **28:**171-188.

16. **Smither, S. J., S. D. Perkins, C. Davies, A. J. Stagg, M. Nelson, and H. S. Atkins.** 2009. Development and characterization of mouse models of infection with aerosolized *Brucella melitensis* and *Brucella suis*. Clin Vaccine Immunol **16:**779-783.

17. **Vemulapalli, R., Y. He, S. Cravero, N. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 2000. Overexpression of protective antigen as a novel approach to enhance vaccine efficacy of *Brucella abortus* strain RB51. Infect Immun **68:**3286-3289.

Figure legends

Figure 1. IN vaccination – IN challenge study. Mice were vaccinated IN with either *B. abortus* strain RB51 (solid black) or strain RB51SOD (checkered) and at 6 weeks post vaccination challenged IN with *B. abortus* strain 2308. Day 14 **(A)** and day 41 **(B)** post infection (PI) bacterial clearance was assessed from spleen, lungs and MLN as CFUs/mice. PBS vaccinated (white) mice served as control. Each column on the graph represents mean CFUs ± standard deviation.

Figure 2. IN vaccination – IN boost – IN challenge study. Mice were vaccinated IN with either *B. abortus* strain RB51 (solid black) or strain RB51SOD (checkered) and at 4 weeks post vaccination, mice were boosted IN with the same dose of respective vaccine strains. Four weeks after booster vaccination, mice were challenged IN with *B. abortus* strain 2308. Day 14 **(A)** and day 41 **(B)** post infection (PI) bacterial clearance was assessed from spleen, lungs and MLN as

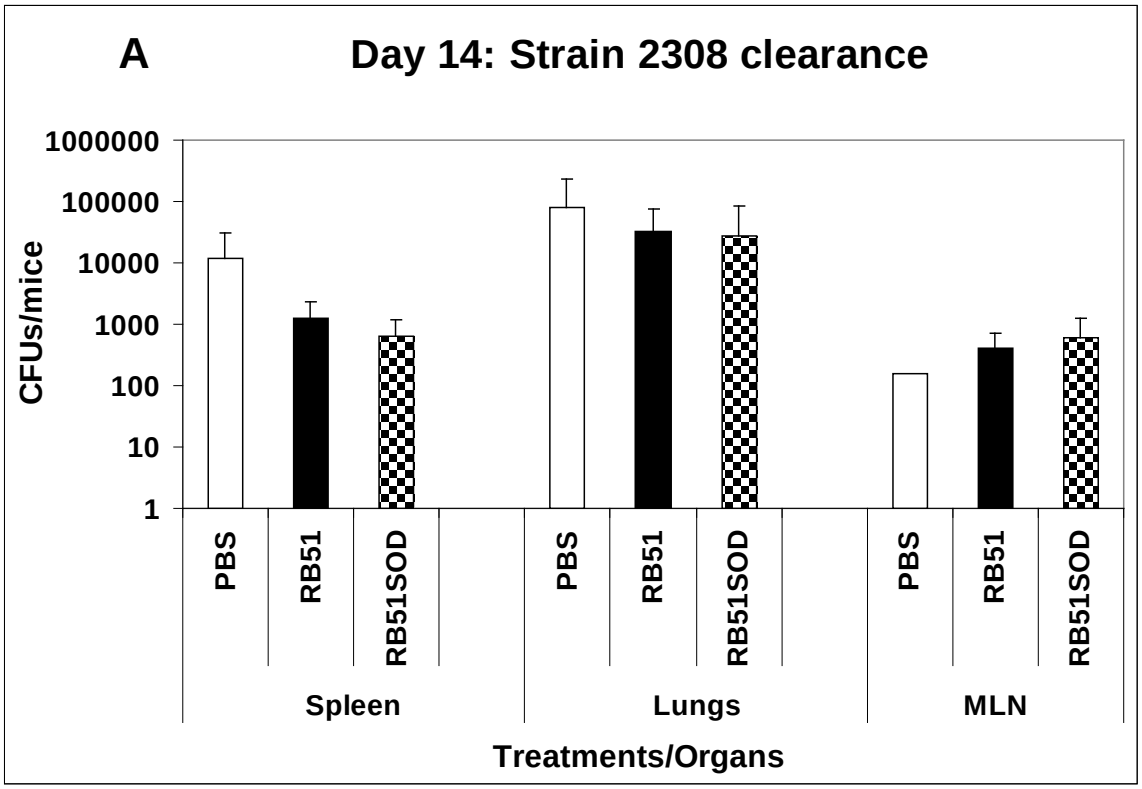
CFUs/mice. PBS vaccinated (white) mice served as control. Each column on the graph represents mean CFUs ± standard deviation.

Figure 3. Optimal dose and route of vaccination. **A.** Mice were vaccinated IN with any of the 3 different doses of *B. abortus* strain RB51SOD; 1 X 10^9 (dashed horizontal), 1 X 10^8 (light vertical) and 1 X 10^7 (large grid) CFUs/mouse respectively. After 6 weeks post vaccination, mice were challenged IN with *B. abortus* strain 2308 and day 14 post infection bacterial clearance was assessed from spleen, lungs and MLN. **B.** Mice were vaccinated with strain RB51SOD through any of the four different routes of vaccination; subcutaneous (SC – dotted), intradermal (ID – crossed lines), intraperitoneal (IP – upward diagonal lines), intramuscular (IM – horizontal brick). After 6 weeks post vaccination, mice were IN challenged with strain 2308 and day 14 post infection CFUs/mice from spleen, lungs and MLN were assessed. PBS vaccinated (white) mice served as control. Each column on the graph represents mean CFUs ± standard deviation.

Figure 4. Prime-boost vaccination strategy. Mice were vaccinated with strain RB51SOD by different prime boost protocols. IP prime-IP boost **(A)** as well as IP prime-IN boost **(B)** vaccination strategies were tested against IN challenge infection with strain 2308. Prime and boost vaccinations were given 4 weeks apart to mice, and virulent strain 2308 challenge was given IN, 4 weeks post booster vaccination. Day 14 post infection bacterial clearance was assessed from spleen, lungs and MLN as CFUs/mice. The heterologous prime-boost study was repeated with additional systemic routes for strain RB51SOD priming other than IP such as ID and IM followed by IN boosting **(C)** at 4 weeks post vaccination and IN challenge with strain 2308 at 4 weeks post booster dose. Day 14 post infection bacterial clearance was assessed from spleen, lungs and MLN; intradermal (ID – crossed lines), intraperitoneal (IP – upward diagonal lines), intramuscular (IM – horizontal brick), PBS control (white). Similarly additional sets of

mice were primed IN and boosted **(D)** either intraperitoneal (IP – upward diagonal lines) or intramuscular (IM – horizontal brick) and challenged IN with virulent strain 2308. Day 14 post infection bacterial clearance was assessed from spleen, lungs and MLN. Each column on the graphs represents mean CFUs ± standard deviation. ** depicts statistically significant data compared * at p value < 0.05.

Figure 1


A
Day 14: Strain 2308 clearance
CFUs/mice
1000000
100000
10000
1000
100
10
1
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
Spleen
Lungs
MLN
Treatments/Organs

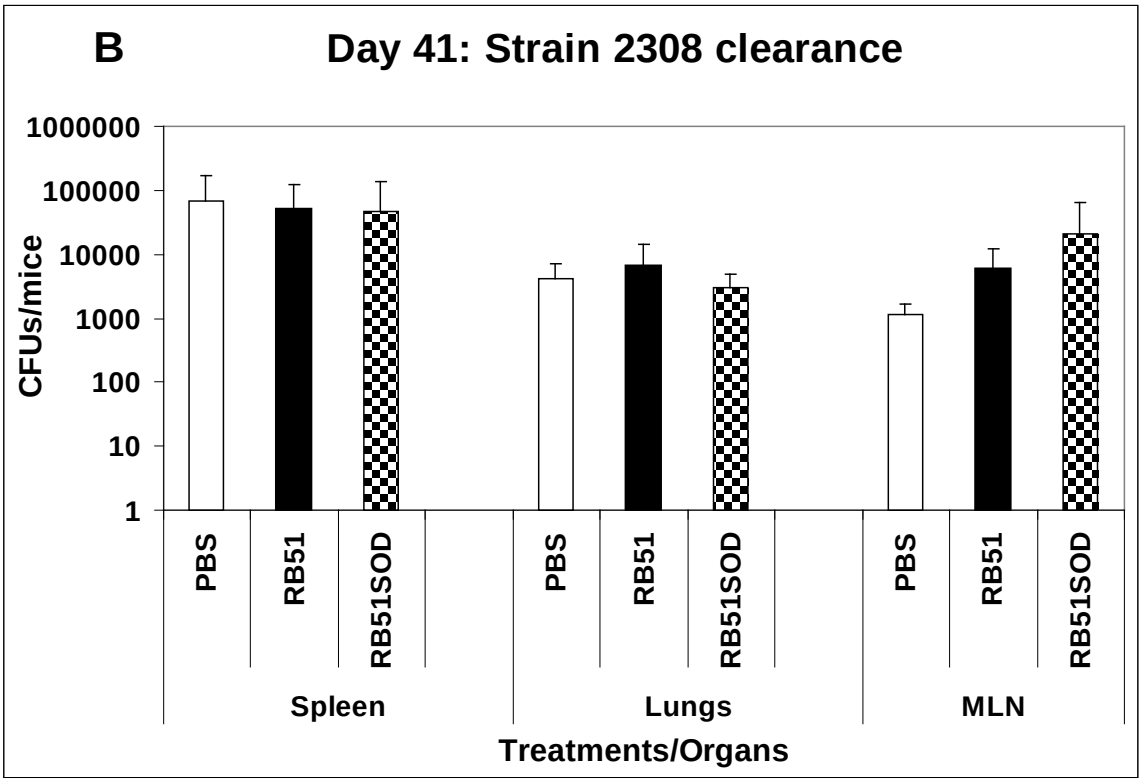
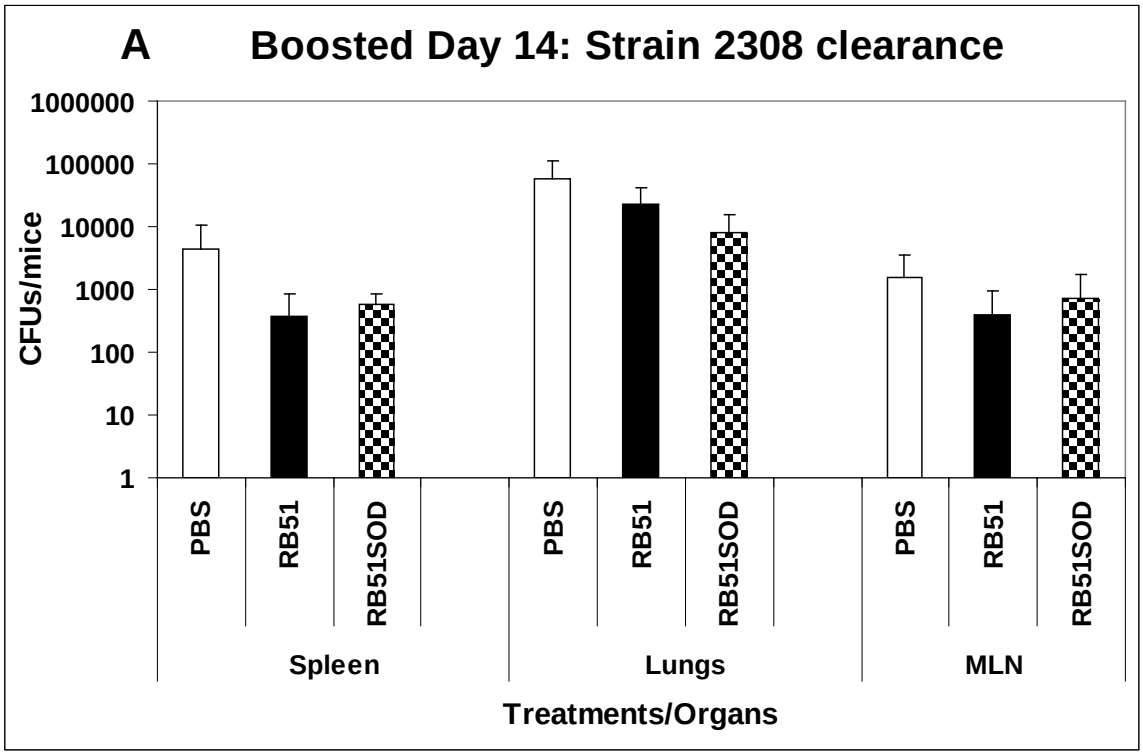

B
Day 41: Strain 2308 clearance
CFUs/mice
1000000
100000
10000
1000
100
10
1
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
Spleen
Lungs
MLN
Treatments/Organs

Figure 2


A
Boosted Day 14: Strain 2308 clearance
1000000
100000
10000
1000
100
10
1
CFUs/mice
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
Spleen
Lungs
MLN
Treatments/Organs

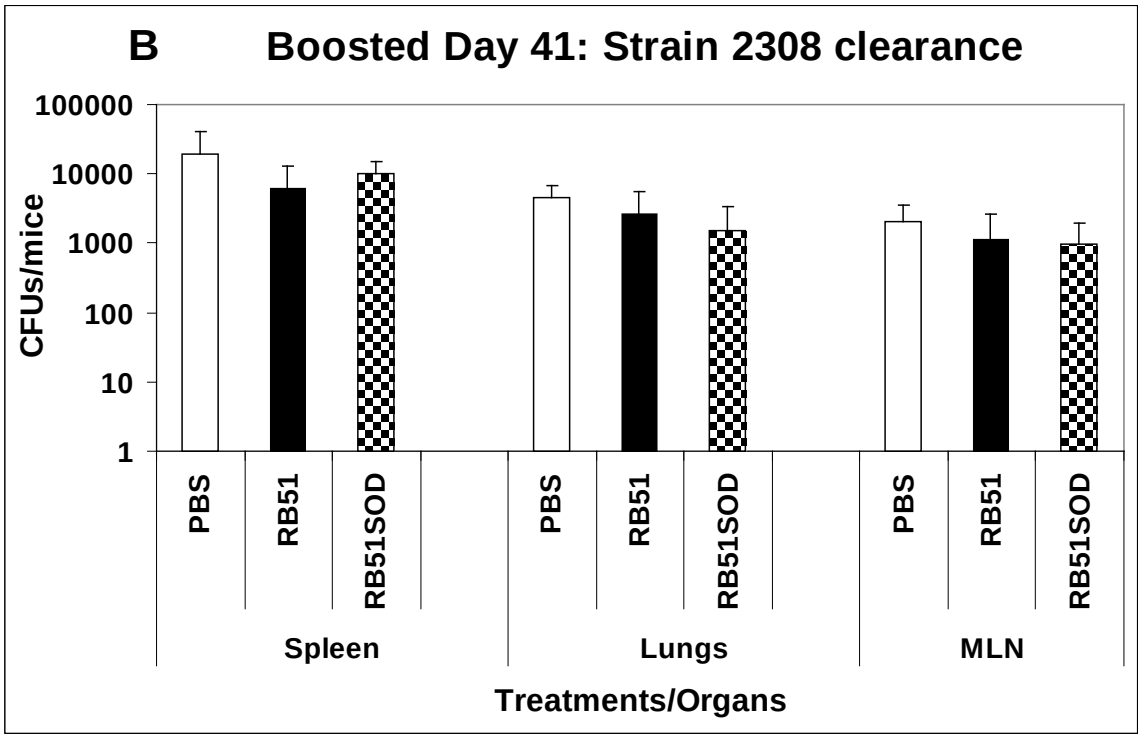
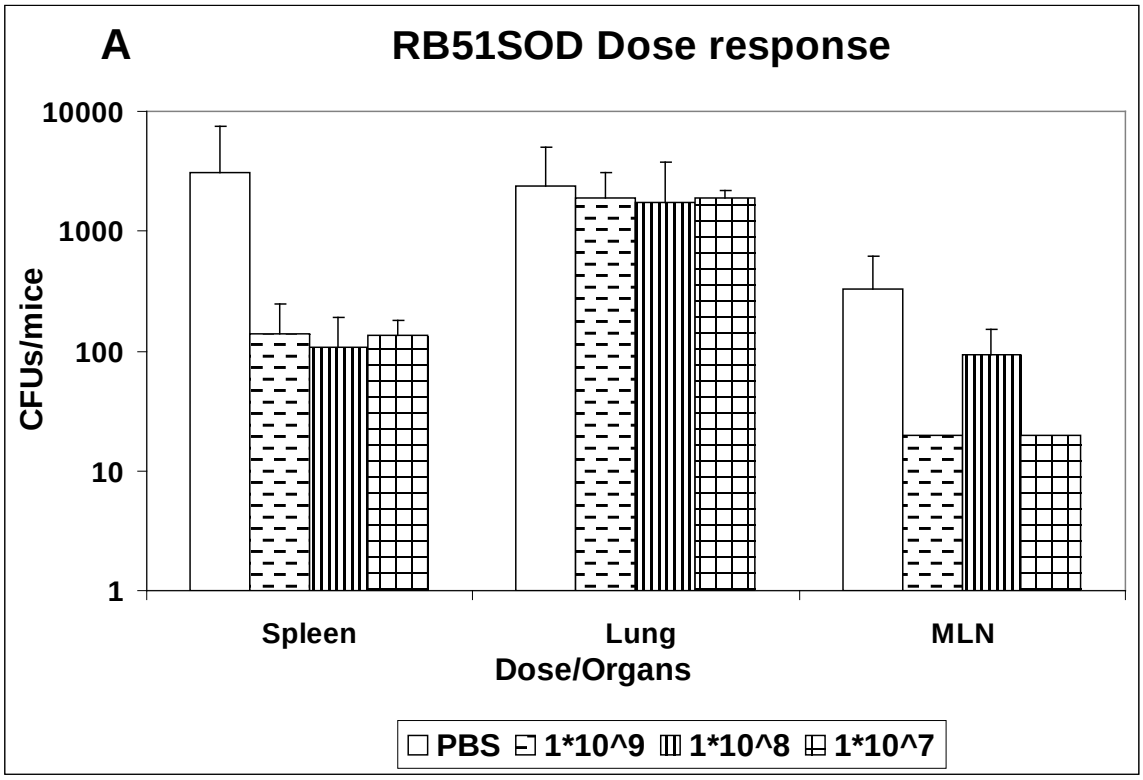

B
Boosted Day 41: Strain 2308 clearance
100000
10000
1000
100
10
1
CFUs/mice
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
PBS
RB51
RB51SOD
Spleen
Lungs
MLN
Treatments/Organs

Figure 3


A
RB51SOD Dose response
10000
1000
100
10
1
CFUs/mice
Spleen
Lung
MLN
Dose/Organs
PBS
1*10^9
1*10^8
1*10^7

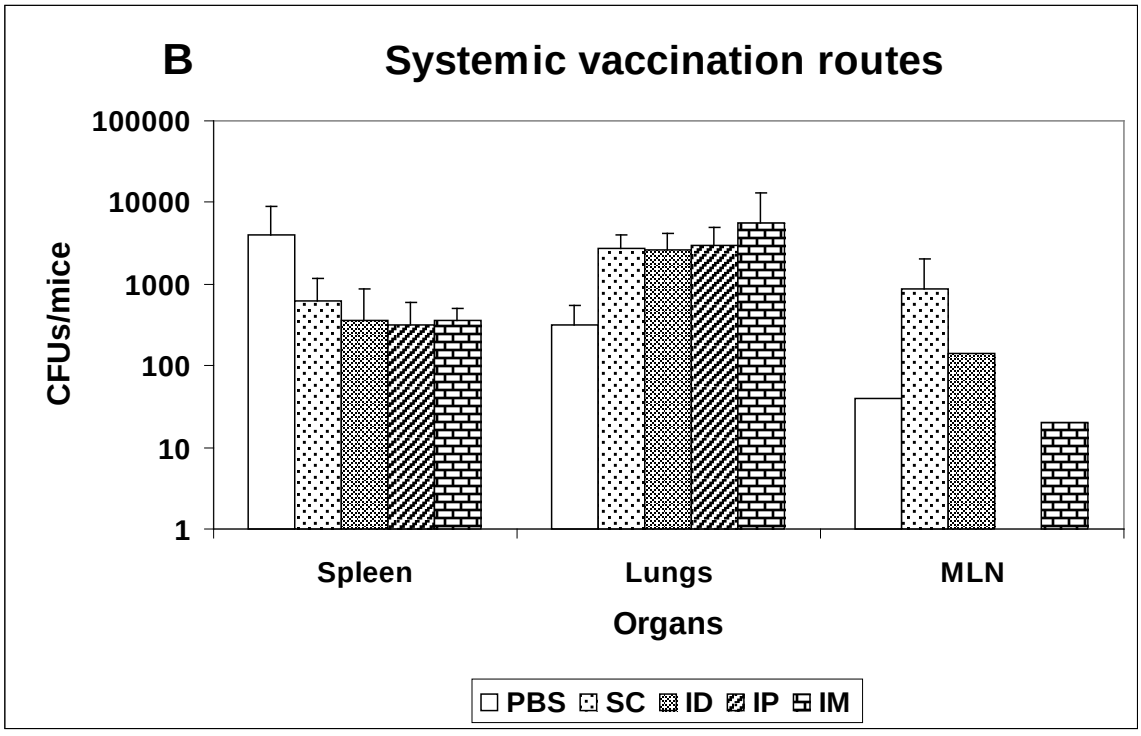
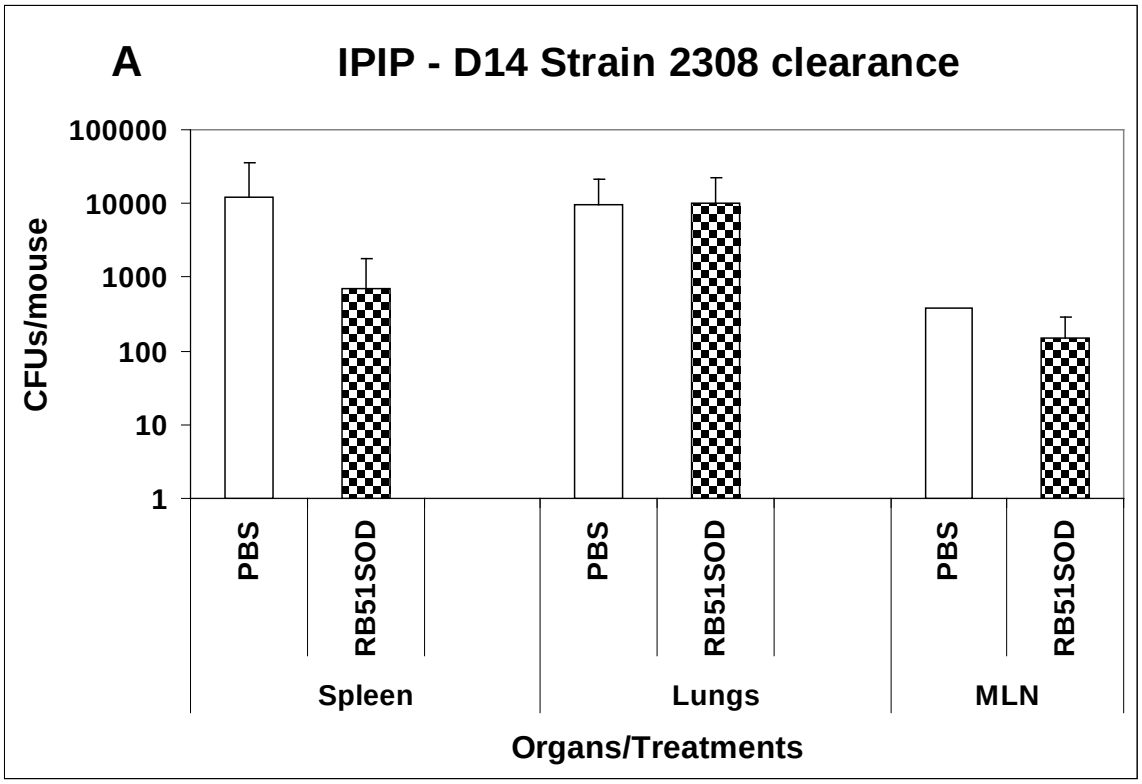
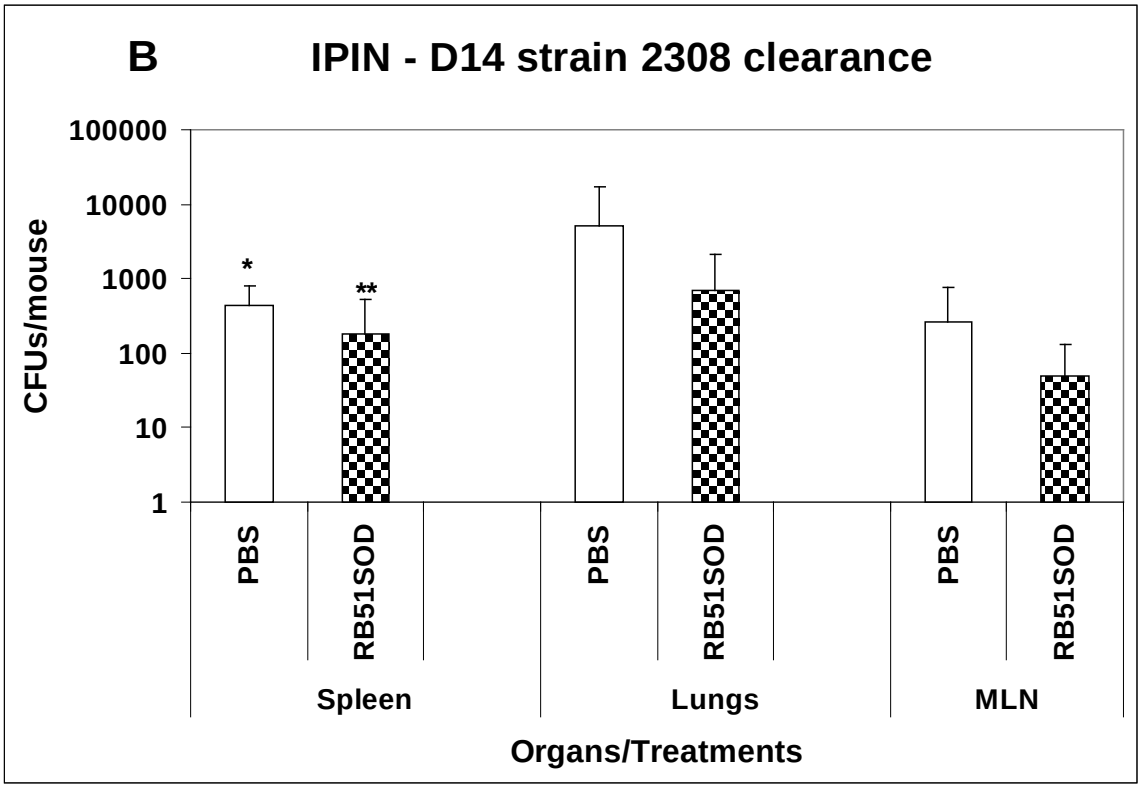
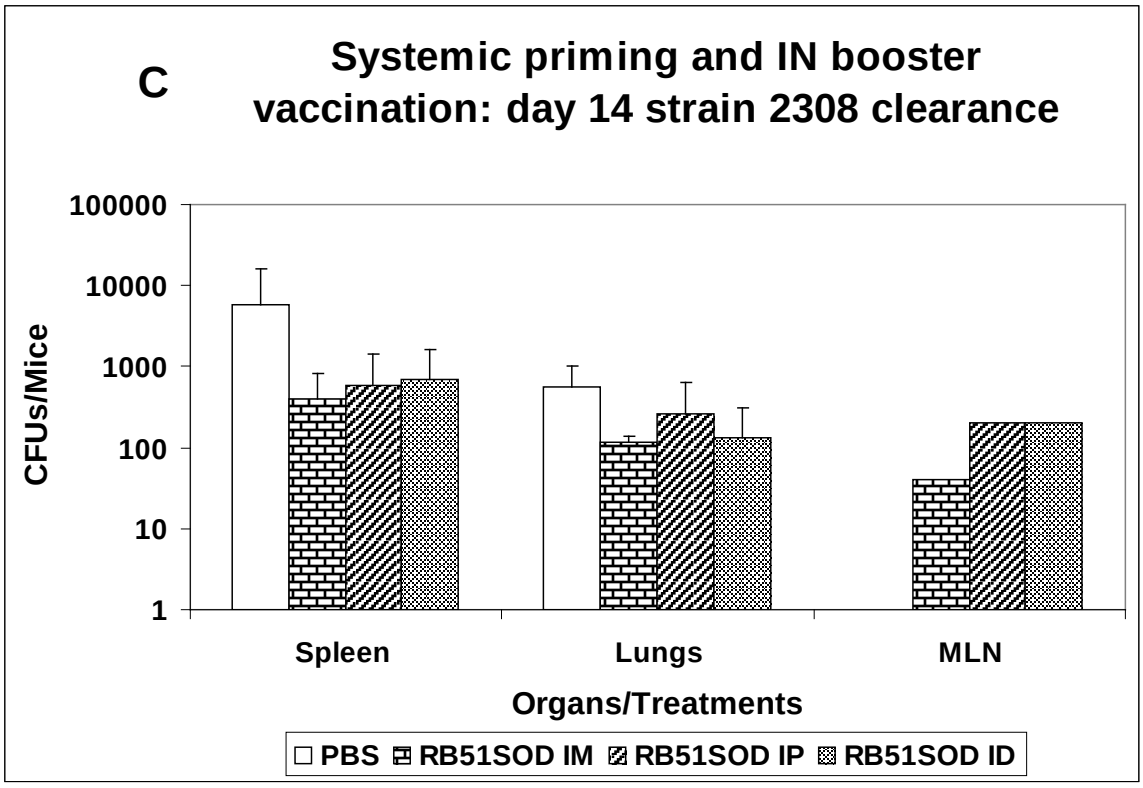
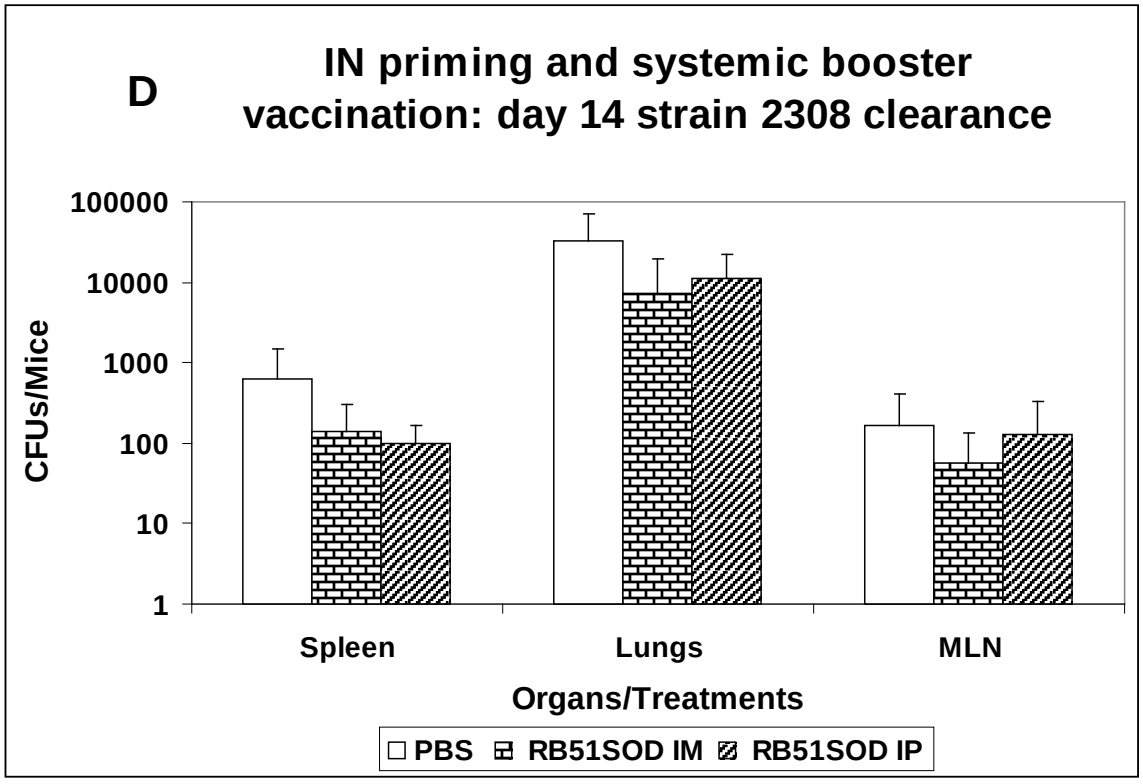

B
Systemic vaccination routes
100000
10000
1000
100
10
1
CFUs/mice
Spleen
Lungs
MLN
Organs
PBS
SC
ID
IP
IM

Figure 4


A
IPIP - D14 Strain 2308 clearance
CFUs/mouse
100000
10000
1000
100
10
1
PBS
RB51SOD
PBS
RB51SOD
PBS
RB51SOD
Spleen
Lungs
MLN
Organs/Treatments


B
IPIN - D14 strain 2308 clearance
CFUs/mouse
100000
10000
1000
100
10
1
*
**
PBS
RB51SOD
PBS
RB51SOD
PBS
RB51SOD
Spleen
Lungs
MLN
Organs/Treatments


C
Systemic priming and IN booster vaccination: day 14 strain 2308 clearance
100000
10000
1000
100
10
1
CFUs/Mice
Spleen
Lungs
MLN
Organs/Treatments
PBS
RB51SOD IM
RB51SOD IP
RB51SOD ID


D
IN priming and systemic booster vaccination: day 14 strain 2308 clearance
100000
10000
1000
100
10
1
CFUs/Mice
Spleen
Lungs
MLN
Organs/Treatments
PBS
RB51SOD IM
RB51SOD IP

Overall Summary and Discussion

"Prevention is better than cure" – As the idiom goes, vaccines have proved to be the most effective tool in the containment and eradication of many infectious diseases. In 1996, USDA approved *B. abortus* vaccine strain RB51 for use against brucellosis in cattle. Later, another rough vaccine strain RB51SOD was shown to induce better protection against brucellosis in mouse models. In this dissertation, we compared both the vaccine strains for their ability to induce innate immune response in a mouse model. Despite the documented ability of strain RB51SOD to induce better protection than strain RB51, our results showed that vaccine strain RB51 induced better DC activation and function *in vitro* based on murine bone marrow derived dendritic cell activation and function compared to strain RB51SOD and virulent strain 2308. Our *in vivo* study results, using a respiratory model of infection, corroborated the enhanced ability of strain RB51 to induce increased pulmonary DC activation and innate immune function compared to strain RB51SOD and strain 2308. Although our study did not elucidate the mechanism for this subdued DC response to strain RB51SOD, the reasons could be manifold. Histopathology results from our *in vivo* study clearly showed that strain RB51SOD when inoculated intranasally did not elicit substantial proinflammatory response compared to strain RB51. Theoretically, over expression of superoxide dismutase (SOD) could be limiting the inflammatory response and thus the activation of innate immune cells *in vitro* or *in vivo*. Although not the primary objective of this study, the scope of our *in vivo* study was limited in that we did not analyze the role played by other innate immune cells such as neutrophils, alveolar macrophages and natural killer cells in response to intranasally administered vaccines. Moreover, we did not analyze the type of DC subsets involved in the strain RB51 induced enhanced DC activation. Also, our *in vivo* results did not provide conclusive evidence on which cells were

predominantly involved in the strain RB51 induced IFN-γ secretion (Th_1 response). While identifying that intranasal inoculation with neither vaccine strains nor smooth strain 2308 induced Th_2 mediated IL-4 secretion, we did not analyze the vaccine or pathogen mediated IL-10, IL-17, IL-6 and/or TGF-β secretion as potential indicators of a T-regulatory or Th_{17} responses. Furthermore, assessing the level of T-cell activation achieved by strain RB51 induced activated DCs in the lung environment is critical to improve efficacy of strain RB51 for use as an IN vaccine. Therefore, future studies will focus on systematically elucidating the pulmonary immune mechanisms against IN administered vaccine or pathogenic strains.

In spite of the observed differences in stimulating innate immunity, a single dose of either of the vaccine strains RB51 and RB51SOD given IP, protects mice against IP challenge with pathogenic strain 2308. Considering the importance of aerosol transmission of *Brucella* and the lack of protective vaccines, we tested a variety of vaccination strategies including intranasal vaccination using either strain RB51 or RB51SOD against intranasal challenge with virulent strain 2308 in a mouse model.

In contrast to the intraperitoneal (IP) vaccination and challenge models, intranasal (IN) vaccination as well as the different systemic, homologous and heterologous vaccine strategies failed to confer protection against IN infection. The probable reasons for this lack of protection could primarily include the bias towards Th_2 response in lung environment, the inability of vaccine strains to induce strong Th_1 response in lung and/or the ability of virulent *Brucella* to establish persistent yet undetected infection of respiratory epithelium. Our results suggested that the vaccination route, dose of IN vaccination or dose of pathogen challenge did not have an effect on pulmonary pathogen clearance. However, our *in vivo* TLR KO studies provided additional information on TLR dependence in DC function and pulmonary clearance.

Our *in vitro* TLR KO studies revealed that the vaccine strain RB51 has specific TLR requirements in inducing DC activation based on upregulation of costimulatory molecules (TLR2 dependent) and DC function based on TNF-α (TLR2 and TLR9 dependent) and IL-12 (TLR2 and TLR4 dependent) production. Although TLR2 KO mice induced significantly higher DC activation compared to media control, the activation levels were significantly lower than control and TLR9 KO mice. In our in vivo KO clearance studies, the pathogen load was comparatively higher in TLR2KO mice infected IN with strain 2308 at day 14 post infection compared to control, TLR4 and TLR9 KO mice. Moreover, pulmonary clearance of strain RB51 was found to be TLR4 dependent. Taken together, TLR2 had a major role in strain RB51 induced DC activation and TNF-α and IL-12 secretion as well as pulmonary clearance of strain 2308. Additionally TLR4 was involved in strain RB51 clearance as well as DC:IL-12 secretion. Partial TLR9 dependence on strain RB51 induced DC:TNF-α secretion was also noted. While these data provided some information, questions remained as to whether an individual TLR agonist can improve protection and/or whether compensatory mechanisms exist in single TLR KO mice to stimulate innate immune response. Nevertheless, the enhanced DC activation capability of strain RB51 and TLR dependent pulmonary clearance pattern of IN infected mice provide additional tools to enhance vaccine efficacy. In future studies, TLR agonists could be tested to improve the protective ability of strain RB51 against intranasal *Brucella* challenge.

As safety concerns might limit the use of live vaccine strain RB51 in humans, the use of heat killed or irradiated strain RB51 should be considered. In our study comparing similar doses of heat killed or irradiated to live strains (positive control), heat killed and irradiated vaccine strain RB51 induced less DC activation than live strain 2308. Our data showed that DC activation achieved by the former treated strains was higher than corresponding smooth strain

2308. As similar doses were used, higher doses and an increased infection time might improve the DC activation and function capacity of inactivated strains. If an optimal dose and TLR agonist could be identified in subsequent studies, a combination of inactivated vaccine strain RB51 with the identified TLR agonist could be tested to protect against intranasal *Brucella* infection. Altogether, my dissertation research provided important information on vaccine and pathogen induced innate immune activation which produced valuable insights into the different ways to improve currently approved animal brucellosis vaccine strain RB51.