Ability of TLR Agonists to Upregulate *Brucella abortus* Strain RB51 Mediated Protection in a Murine Respiratory Model

Michelle Walker

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Sharon Witonsky, Chair

Isis Kanevsky

Kurt Zimmerman

Nammalwar Sriranganathan

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Abstract

Brucella abortus is amongst the top 5 zoonotic diseases worldwide. The overall goal of this research is to generate a safe and effective vaccine for humans. *Brucella abortus* strain RB51, approved for use in cattle, provides protection by initiating a strong T-helper 1 (Th1) type response is a candidate vaccine. Based on a model for aerosol exposure mice were vaccinated intranasally (IN) with strain RB51 and challenged IN with *B. abortus* strain 2308, strain RB51 did not protect. Protection against *Brucella* is mediated through TLRs 2, 4 and 9. The addition of TLR 2 or TLR 4 and a trend with TLR9 agonists with intranasal RB51 vaccination significantly increased bacterial clearance in the lung after strain 2308 challenge. Therefore, we hypothesized that combining TLR agonists 2, 4, and 9 with strain RB51 IN would upregulate protection and clearance in the lung against strain 2308 challenge (IN), by upregulating the DC1 and CD4 Th1 and CD8 immune response. This study showed that protection is not upregulated by combining all TLR agonists. Overall the addition of TLR 2 and 4 vs. TLR 2, 4 and 9 agonists affects the immune response and impacts the level of clearance. Our data support the development of a DC1 Th1 CD8 response, based on serology, and both DC and T-cell activation and function by the group which received the TLR 2 and 4 agonists and to a lesser degree the group receiving TLR 2, 4, and 9 agonists. Additional studies are warranted to further define the differential mechanisms and endpoints of protection.

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Table of Contents

List of Figures

Chapter 1

Review of Literature:

Brucella abortus is a zoonotic disease, which infects cattle, elk, bison, and humans. It is a Gram-negative facultative intracellular bacterium. It is endemic in many parts of the world in both people and animals, in part because of the difficulty in diagnosing and treating cases. This contributes to a population of chronically infected animals, which then serve as a reservoir for infection. The economic losses in cattle are devastating to farmers. Fortunately, extensive programs have been used to eliminate *Brucella* from domestic herds in the United States, and decrease infection in herds world-wide. However, it is still present in wild life reservoirs nationally and internationally. Additionally, due to potential aerosol exposure, its low infectious dose, and our ability to mutate it readily, creates concern for *Brucella* to be used as a bioterror agent. Centers for Disease Control and Prevention (CDC) classify it as a class B pathogen. While there is a United States Department of Agriculture (USDA) approved vaccine for cattle, there is currently no efficacious commercially available vaccine to protect humans against natural or intentional exposure.

Brucella species

There are multiple species of *Brucella,* which infect an array of hosts. The three top zoonotic species of *Brucella,* include, *B. melitensis,* which infects sheep, goats, swine, and cattle, and is the most pathogenic to humans, *B. suis,* which infects pigs and humans, and *B. abortus,* which infects cattle, swine, horses and humans. There are other *Brucella* strains which do not readily cause infection in humans, such as *B. canis* which infects dogs, *B. ovis* which infects sheep, *B. neotomae* which infects the *Neotoma lepida* (desert wood rat), and *B. pinnipedialis* and

B. certi, which infect cetaceans and pinnipeds (seals) respectively (1). *Brucella* species have host preferences, but can infect other animal species. For instance, *B. abortus*, primarily infects cattle, but can infect humans, feral swine and horses (2). *Brucella* spp. fall under the family Brucellaceae, order Rhizobiales, class Alphaproteobacteria and phylum Proteobacteria (3). It shares the same class as other disease causing bacteria such as *Bartonella*, *Rickettsia*, and *Ehrlichia* (3). *Brucella* has two chromosomes, one of which is smaller; however, both contain essential genes (3). Comparison of sequenced *Brucella* genomes show they are similar in size and in nucleotide composition (3). The different strains, especially the zoonotic strains, are very similar. The similarity in the genome most likely allows for cross species infection (3).

Brucella **epidemiology**

Brucella is endemic in many areas, including the Mediterranean, Middle East, South and Central America, Africa, and Asia, making it a concern for human health, as there is no vaccine for humans (4). Consumption of contaminated food, which can be obtained via international importation or acquired during international travel, typically cause *Brucella* cases in non-endemic areas (5). Many human cases of Brucellosis in the United States were reported after visiting or consuming unpasteurized dairy products from Mexico (6). Often importation of infected animals has allowed for the spread of disease to areas which were previously considered *Brucella* free, as was the case in the Balkans (1). In another instance, infected goats exported to Vietnam were found to be from the United Arab Emirates as part of a breeding program (1). Developing countries have limited resources for detection of infected animals which contributes to further disease. To eliminate disease, countries must implement vaccination and culling practices in herd animals.

Bioterrorism

Brucella is classified as a class B pathogen by the CDC because it is a potential bioterror threat. It has the ability to infect humans when aerosolized with a low infectious dose, and ease of genetic modification (7). The spread of *Brucella* can be devastating in people and animals, both because of the low infectious dose, and the difficulty in diagnosing cases based on non-specific influenza like symptoms (7). The mortality rate of *Brucella* may not be high, but it can cause extensive chronic febrile disease, and would place a great strain on a region's resources for health care (8). Aerosol exposure would be a very effective/efficient method for delivery of select agents such as *Brucella* due to the high infectivity. Intentional contamination of food could also pose a threat, as *Brucella* naturally survives in dairy products (8). Characteristics such as low infectious dose and aerosol delivery make it an agent of interest for use in biological programs.

Historically *Brucella* strains have been developed and tested for use as biological weapons. It was one of the first agents Japan experimented with in the Manchuria Unit around World War II, and in 1952, the United States weaponized *B. suis*, with the intent to harm others as an act of war. This developed agent was used in field testing in 1952 (5). In the former Soviet Union, *Brucella* was also developed as part of their offensive arsenal (5). It was estimated that if 50 kg of *B. suis* were released upwind from a city with 500,000 people, 125,000 people could be effected and cause 500 deaths (8). The cost on a population for such an attack was estimated to be $477.7 million per 100,000 exposed (8). Due to *Brucella* being endemic in many areas, isolating this agent from infected animals would not be difficult (5). Intentional release of *Brucella* as a biological agent could impact the food supply as exposure of herd animals to this bacterium could cause major losses due to abortions, and culling of infected animals in the meat

and dairy industry. If this agent were misused on a large scale, it would have a marked impact on both humans and animals. In addition to its ability to cause new infection in animals and people, *Brucella* also has the ability to cause chronic disease due to its persistence within the host. Many patients have spondylitis, while others have symptoms of chronic fatigue syndrome, musculoskeletal pain and undulant fever (9).

Cellular entry and intracellular trafficking

Brucella most commonly enters the host through mucosal surfaces of the digestive, reproductive, and respiratory tracts. *Brucella* enters dendritic cells (DC) and macrophages by phagocytosis and lipid rafts, and epithelial cells through lipid rafts (10-12). Internalization of the bacteria is rapid and dependent upon a functional VirB system (11). The BvrR/BvrS two component system is necessary for virulence and invasion of HeLa cells *in vivo* (13). Entry into monocytes and epithelial cells is dependent on protein 41, a surface protein which interacts with sialic acid residues (14). Many of the bacteria that enter phagocytic cells undergo phagolysosomal fusion and are killed. The surviving bacteria will form the *Brucella* (10) containing vacuole (BCV) and deviate from normal host intracellular trafficking. Under normal host trafficking the vacuole will undergo phagolysomal fusion which normally kills any bacteria inside the vacuole (12). BCVs interact with early compartments of the endocytic pathway (13). Early acidification of the lysosome activates the VirB operon which controls virulence genes and the type 4 secretion system (T4SS) (15). Acquisition of late endosomal proteins allows trafficking and fusion to the endoplasmic reticulum (ER) to form a replicative vacuole (16). *Brucella* can cross the epithelial barriers of mucosal surfaces to disseminate through the host (17). It can also be transported to the draining lymph nodes by infected DCs and macrophages

(17). Upon cell death, the bacteria are released into the extracellular environment, and can infect new cells.

Adaptations to survive in the host

The VirB operon is important in *Brucella* virulence and trafficking as it codes for T4SS. VirB mutants can enter HeLa cells in an *in vitro* model, but are unable to change the cellular trafficking, to form the BCV and undergo phagolysomal fusion, compared to pathogenic *B. abortus* strain 2308 (18). These mutants are attenuated in this experimental model, and the mutants are unable to replicate. Thus far 3 proteins have been identified VceA, VcC, and RicA which are secreted by *Brucella* into macrophage cell lines. VceA and VcC, were secreted into the cytoplasm of J774 macrophage like cells, and found to be regulated with the T4SS, but their function has not yet been characterized (19). RicA, was found to interact with a Rab2, a GTPase; however, its effects are still unknown (20). The VirB operon is important for the survival and replication of the bacteria in the host.

Brucella is an organism which has evolved to evade/limit the immune system of the host via multiple mechanisms. One of the major pathways by which the immune system is stimulated, and by which Brucella limits the immune response is lipopolysaccharide LPS. The LPS of *Brucella* does not stimulate the innate immune response as effectively as LPS from other bacteria, such as *E. coli* or *Salmonella.* The LPS is anchored to the membrane by Lipid A, and has an inner and outer sugar based core, and a chain consisting of sugar molecules (21). The *Brucella* oligosaccharide composition differs from *E. coli* or *Salmonella*, and contributes to its low toxicity (22). *Brucella* LPS is less toxic than that of *E.coli.* Using the rabbit pyrogen test, the dose of purified *B. abortus* LPS needed to induce 50% of the maximal increase in temperature was about 10,000 fold greater than using purified LPS from *E.coli* (23). Additional experiments

in mice showed that *E.coli* LPS was about 268 times more lethal in mice compared to *B. abortus* purified LPS (23). Lastly, when cytokines IL-1β and TNF-α, cytokines implicated in endotoxic shock, were examined *in vitro* using collected human monocytes, the *E. coli* LPS was about 1000 fold more potent at inducing IL-1β and 400 fold at inducing TNF-α (23). *B. abortus* LPS interferes with normal MHC class II presentation of antigens in murine macrophages *in vitro* and *in vivo* (22). Mutant strains missing the O-side chain have a rough colony phenotype, and are attenuated in the host *in vivo*, and in cells *in vitro* (24, 25). Many of these rough mutant strains do undergo phagolysomal fusion, and do not traffic normally inside the cell. The unique structure and composition of the LPS allows it to limit the host immune response.

Part of the mechanism by which *Brucella* limits the immune response is by interfering with signaling from the Toll Like Receptors (TLR) by mimicking the Toll/ interleukin-1 receptor (TIR) domain. This protein is a TIR containing protein designated as TcpB, and was found to interfere with the TLR 2 and TLR 4 pathways. *In vitro*, TcpB was shown to interact with, and increase the ubiquination of, phosphorylated signaling adaptor molecule MyD88-adapor-like (MAL) using human embryonic kidney cells (HEK) 293 (26). TcpB reduces the activation of NF-κB in a dose dependent manner (26). This is one specific mechanism by which *Brucella* is able to survive undetected in the host.

Down regulation of the immune response

Brucella is well adapted to survive in the host, as there are multiple ways in which it is able to circumvent the host's defenses. *B. suis* prevents human dendritic cells from maturing *in vivo*. After infection, human DC do not upregulate activation/maturation cell surface markers as well as *E.coli* infected DC (27). Human DC cells infected with wild type *B. suis* induced comparable levels of proliferation compared immature DCs when exposed to naïve T cells (27).

The function of the infected DC was impaired, they had reduced TNF-α production (27). Additionally in another study, *B. suis* can prevent spontaneous cell death in human monocytes by IFN-γ and Fas-induced apoptosis (28). Also, *B. abortus* strain 2308 LPS is unique in its lipid A structure, and does not induce DC maturation or function of murine bone marrow dendritic cells, compared to the LPS from a smooth LPS mutant BaΔ*wadC* , which has a core defect (29). This core defect caused a difference in migration pattern when anti-O-PS monoclonal antibodies were run as a western blot and lack of reactivity to anti-core antibodies (29). Murine DCs exposed to LPS of strain 2308, did not upregulate CD40, CD86 and MHC class II as well as DCs exposed to the mutant strain BaΔ*wadC* LPS or *E. coli* LPS (29). More recently, *in vitro* human CD4+ T cells undergo increased apoptosis when infected with strain 2308, thus potentially reducing the immune response, due to reduced CD4+ T cell numbers in patients infected with *Brucella* (30). The bacteria lipoproteins were potentially responsible for initiating apoptosis in these cells, as treatment with recombinant lipidated omp19 increased apoptosis while treatment with *B. abortus* LPS did not (30). *Brucella* is able to down-regulate the host immune system, as well as controlling cell for its own replicative purposes.

B. abortus **in cattle**

B. abortus targets the reproductive organs of cattle. *Brucella* readily metabolizes erythritol which is found in the placenta, which is thought to be one reason why *Brucella* infects cells within the placenta (31). In bulls, it causes orchitis and epidymitis, and can result in sterility (32). In most animal species, *Brucella* replicates in the mammary glands and reproductive organs of the host, but it can be isolated from other tissues in the host. There have been a few instances where bulls have transmitted *B. abortus* during mating (32). Non-pregnant cows rarely show any symptoms of disease, which visually makes it difficult to identify infected

animals (33). Abortions in infected cows occur during the fifth and eight month of pregnancy (32). Cows with placentitis, usually have necrotic cotyledons, which cause yellow-brown flocculent discharge containing large numbers of bacteria (32). Cattle typically become infected via licking or inhaling *Brucella* from the aborted fetus and placental material. Diagnosis in cattle is based on serological tests such as the Rose Bengal test and milk ring tests which measure antibodies in the serum and milk respectively (33). Control of *Brucella* in cattle limits exposure and disease in humans.

B. abortus **in humans**

Humans typically contract *B. abortus* through consumption of contaminated dairy products, contact with pregnant cows and aborted fetuses, and ingestion, inhalation, or contact exposure from working in slaughter houses. In humans, *Brucella* causes a fever and flu-like symptoms, which is easily misdiagnosed by doctors and difficult to treat. Clinical signs can persist for weeks or months (34). *Brucella* can effect cardiovascular, nervous, and musculoskeletal systems (17, 34). It causes both acute and chronic disease, and relapses are common (34). Diagnosis is based on patient history, blood culture, and serologic tests (34). Serologic testing uses agglutination tests to confirm the presence of *Brucella* antibodies (34). ELISA can be used to detect antibodies to LPS, as well as the use of PCR to identify the presence of *Brucella* (34). Treatment consists of doxycycline for 6 weeks and streptomycin for 2 to 3 weeks, or doxycycline and rifampicin for 6 weeks (35, 36). Patient compliance is often difficult due to the length of treatment and cost of drugs. In this case, prevention of disease in at risk populations would be ideal compared to treatment.

Current vaccines

Currently, there are no efficacious commercially available vaccines for humans; however, two strains of *B. abortus* have been developed and approved for vaccination in cattle. *B. abortus* strain 19 was the first attenuated strain developed as a vaccine for cattle to control Brucellosis, and it is still used in many countries today. This strain expresses the O-side chain on its LPS, giving the colonies a smooth appearance. The production of antibodies to the O-side chain may enhance protection, but it causes vaccinated animals to test positive, using serologic tests for the O-side chain. Side effects of this vaccine include abortion in pregnant cows, and disease in humans. This limits its use in the US in cattle and humans (37). In mice, strain 19 typically takes 12 weeks to clear in BALB/c mice when given at 5 x 10^6 CFU intraperitoneally (IP); this increased persistence causes immune stimulation and resistance to pathogenic challenge (38). In addition to strain 19, *B. abortus* strain RB51 is a newer developed vaccine strain used in the United States, and approved by the USDA to prevent Brucellosis in cattle (39). This strain lacks the O-side chain, and does not induce antibody production to LPS, which theoretically eliminates false positives, due to the lack of the O-side chain antibodies for detection in serologic tests. Strain RB51 was proven to be stable, and it does not revert back to virulence based on multiple passages both *in vitro* and *in vivo* (37). This strain induced protection when administered IP at $3x10^8$ CFUs 5 wks post vaccination in BALB/cByJ mice against virulent strain 2308 at $5x10^4$ CFU administered intravenously (IV) (40). Another vaccine, rough *B. abortus* strain RB51SOD, which over-expresses its Copper-Zinc superoxide dismutase (SOD) also, provides protection in mice. Mice were vaccinated with $4x10^8$ CFU of strain RB51SOD or RB51 IP, and challenged with strain 2308 $2x10^4$ CFU IP at 7 weeks post inoculation. Mice inoculated with strain RB51SOD vs. strain RB51, had significantly lower CFUs of *B. abortus* strain 2308 recovered

from the spleen (41). These three strains, strain RB51, RB51SOD, and strain 19, have stimulated a strong cell mediated immune (CMI) response to protect against infection (42-44).

Immune response

Cells of the innate immune system become activated by pathogen associated molecular patterns (PAMPS) on *Brucella sp.* which are recognized by Toll-like receptors (TLRs) on antigen presenting cells. Each TLR recognizes a specific PAMP, TLR 2 recognizes lipoproteins, TLR 4 recognizes LPS, and TLR 9 recognizes CpG DNA. These TLRs and signaling molecule MyD88 play a role in control and clearance of *Brucella sp*. (25, 45, 46). Campos et al. demonstrated TLR 4 knockout (KO) mice (C3H/HeJ) challenged IP with strain 2308, had significantly higher CFUs in the spleens compared to wild type mice (C3H/HePas) (25). In this same experiment, IFN-γ production was reduced in splenocytes collected from KO mice, compared to wild type mice when stimulated with *B. abortus* LPS (25). Huang et al., showed production of TNF-α in the serum which was significantly reduced using TLR2 or MyD88 KO mice, compared to wild type mice when stimulated IP with heat killed *B. abortus* strain 2308 (45). A similar effect was observed when cultured splenocytes from WT, TLR2 and MyD88 KOs were stimulated with heat killed (HK) *B. abortus* strain 2308*;* there was reduced TNF-α found in the media (45). Secretion of IL-12p40 by splenic DCs *in vivo* was decreased in MyD88 KO mice compared to TLR2 and TLR4 KO mice, suggesting other TLRs which signal through MyD88 are involved in signaling for IL-12 production (45). Macedo et al. showed the MyD88 KO mice also produced significantly less IFN-γ, IL-12 and TNF-α when stimulated with HK *B. abortus* strain 2308, compared to wild type mice (47). In experiments done by Copin et al., there was significantly less clearance of *B. melitensis* in MyD88 or TLR4 KO mice on BALB/c background and TLR9 KO mice on a C57BL/6 background, compared to wild type mice (46).

These experiments demonstrated the importance of TLR 2, 4, and 9 for control of *Brucella* infection though induction of Th1 cytokines needed for clearance. With strong stimulation of the TLRs, APCs such as DCs become activated, and will migrate to the draining lymph nodes, and present antigen to activate naïve T helper cells.

While there are a plethora of DC subsets, based on our interest in a respiratory model, this introduction will emphasize the response to respiratory exposure. There are both resident and recruited DC populations in the lung, the latter of which can be recruited during inflammation from an infection or other inhaled molecule. Some DC bias towards a specific type of adaptive immune response, whereas others are more influenced by antigen, DC maturation status, and cytokines present. DC are identified by the expression of CD11c on their surface as well as other markers which designate a specific subtype. CD103 DC are present in the lung and can migrate after capturing antigen to the mesenteric lymph nodes (48). DC expressing CD8-alpha are resident DCs found primarily in the spleen and lymph nodes (48). DC which express CD11b are recruited and derived from monocytes under inflammatory conditions; once they become activated they migrate to the lymph nodes (49). Activation and maturation of DC can be measured by upregulation of MHC class II molecules, CD40, CD80 and CD86 on the surface of CD11c+ DC. Cytokine production reflects DC function and subset/phenotype; TNF-α, IL-12, IFN-γ produced by DC direct CD4 Th1 and CD8 T cells needed for protection and clearance of *Brucella*. DC which take on a DC2 phenotype produce IL-4 to polarize naïve CD4 T cells to Th2 type of response, and DC which secrete TGF-β cause polarization to T-regulatory cells (T-regs). Polarization of Th17 cells is dependent upon IL-6, TGF-β, and IL-23 production (50). *Brucella sp.* poorly induces DC activation, which causes the DC to insufficiently activate CD4 Th1 and

CD8 T cells. Activation of the innate immune system is necessary to stimulate an appropriate adaptive immune response.

Protection and clearance of *B. abortus* is dependent upon a strong CMI response, including CD4 Th1, CD8 T_C cytotoxic T cells, IL-12 and IFN-γ production. Strain RB51 has been shown *in vitro* to stimulate DC upregulation of co-stimulatory molecules and produce TNF-α and IL-12 (51). In turn, DCs activate naïve T-helper cells to a Th1 phenotype which produce IFN-γ. Secretion of IFN-γ by T cells protects the host against intracellular pathogens (52). CD8+ T cells are needed to lyse infected cells, and both CD8 and CD4 T cells generate memory cells (52). Although T cells still provide the most protection, antibodies generated during infection against *B. abortus,* help control infection (53). Cloeckaert et al, demonstrated that mice given monoclonal antibodies against outer membrane protein had decreased bacterial loads in the spleen at 7 days, vs. mice not given antibodies (54). Passive transfer experiments demonstrated sera from mice infected with strain 19 given to naïve mice, which were then infected with strain 19 or strain 2308, had protection in the spleen, compared to mice receiving naïve sera (53). Adone et al. recently determined that antibodies from *B. melitensis* infected strain B115 in mice, protected mice against strain 2308 and *B. melitensis* strain 16M, based on the significant reduction of bacterial numbers in the spleen (55). Antibodies help by opsonizing extracellular bacteria, but as it was shown that opsonized strain 2308 could still survive and replicate in murine peritoneal macrophages (56). Thus while antibodies are beneficial, a protective CMI response is most critical for clearance.

Challenges and immune response of the lung mucosa

The lung mucosa provides a unique immunological environment. Activation of a strong CD4 Th1 response in the lung is potentially difficult due to the Th2 nature of the mucosal

environment (57). Some mucosal DC seem to differ from those found systemically as they tend to induce more of a Th2 or T-reg response (57). In a study performed in rats, DC harvested from the lung have higher levels of IL-10 mRNA *in vivo* which can down regulate the immune response, or encourage either a Th2 or T-reg response. However, if the DCs are treated with GM-CSF, which increases IL-12p35 and decreases IL-10 mRNA production, this directs a Th1 response (58). Additionally, when respiratory DC were pulsed with ova albumin (OVA), or OVA and GM-CSF *in vitro*, and then adoptively transferred into rats, OVA challenged rats receiving OVA pulsed DC only vs. DC pulsed with OVA and GM-SCF showed more Th2 specific antibodies (58). Without additional signals such as cytokines, and antigen lung DC seem to naturally induce a Th2 or T-reg type response.

Both CD103 and CD11b DC take up antigen and migrate to the lymph nodes and present a high level of activation markers to activate CD4 and CD8 T cells (59). CD11b DC, which are monocytes derived, tend to increase in number later in infection after activated CD103 DC migrated to the MLN to present antigen (59). CD8α DCs tend to induce CD8 cytotoxic T cell response in the MLN, and CD103 DCs were also shown to be necessary to induce CD8 T cell immunity (59). CD103 DCs are highly versatile, as they can present to both CD4 and CD8 T cells as well as to resident CD8α DCs; they can be critical for optimal protection against pathogens such as influenza (59). In a study done looking at CD103 DCs, when stimulated with OVA peptide antigen, CD103 DCs tended to prime naïve CD4 T cells to a Th1 type of response and induce higher levels of IL-2, TNF-α, IFN-γ, and IL-17A, thus making them important in intracellular infection (60). When CD11b high DCs were used to prime CD4 T cells, the T cells induced a Th2 type response secreting IL-4, IL-10, and IL-6 (60, 61). CD8α DCs could also induce a Th1 response when stimulated with GM-CSF, while CD11c+ CD8α- DCs induced T-

reg cells (62). DCs which are CD8α have shown to cross present viral antigen, and were the primary DCs to prime CD8 T cells to induce a Th1 type of response (49).

Besides DCs, other innate cell types present in the lung include macrophages, natural killer cells, and neutrophils, all of which are part of the first line of defense against infection. Macrophages, like DCs, can become infected by *Brucella* and are also APCs (63). Macrophages can uptake antigens and become stimulated into either a M1 pro-inflammatory macrophage or M2 anti-inflammatory (64). M1 macrophages tend to be microbicidal; they phagocytize bacteria and secrete inflammatory cytokines such as TNF-α, IL-6, and IL-12 (65). M2 macrophages modulate the immune response by secreted cytokines such as IL-10 and IL-4, they do phagocytize, but they have compromised ability to kill intracellular pathogens compared to M1 macrophages (65, 66). Neutrophils also phagocytize and degranulate to kill bacteria; however, infiltration to the site of infection and degranulation can increase inflammation by damaging the surrounding cells and tight junctions (67). In response to the environment, epithelial cells can produce cytokines and chemokines such as IL-12, IL-23, GM-CSF, MIP-2, and CXCL1, to attract and activate innate immune cells (68). Th17 may also play a role in control of *Brucella* infection in the lung mucosa. As previous studies have shown a role for Th17 cells in the control of *Francisella tularensis* and *Chlamydia muridarum*, the production of IL-17 can regulate the production of IL-12p70 from DC and increase the immune response to intracellular pathogens (69). IL-17 can also induce epithelial cells to produce chemokines such as IL-8 and antimicrobial protein human β-defensin2 (69). Besides IL-17 influencing responses, T-regulatory cells (T-regs) do play a role in preventing excessive inflammation in the lung, and are able to downregulate the activity of specific cells through secretion of IL-10 and by cell to cell contact (70). The secretion of IL-10 and TGF-β can induce T cells to become T-reg cells and can

suppress antigen specific immune response (70). T-reg cells can prevent excess damage in some conditions, but, because they potentially can down regulate the immune response, this could hinder bacterial clearance (70). These data demonstrate that the T cell response is strongly influenced by the innate response, particularly by DCs. Collectively, the DC and T cell response reflect the overall immune response both in the lung and the rest of the body.

Past respiratory vaccine challenges

The ability to protect against an intranasal or aerosol route of challenge varies based on which vaccine and challenge strains of *Brucella* are used, routes and doses used, and endpoints assessed. Protection has previously been demonstrated using intraperitoneal (IP) vaccination with *B. abortus* strain RB51, and IP challenge with *B. abortus* strain 2308 (41). However, IP vaccination did not necessarily provide protection against an aerosolized challenge (71). Intranasal vaccination of strain RB51 alone did not provide protection to an IN challenge using strain 2308 (72). Systemic vaccination and IN boost did not enhance clearance in the lung and MLN; however, IP and IN boost using strain RB51SOD showed significant clearance in the spleen (72). Due to the inability of rough *Brucella* vaccine strains to stimulate a strong protective immune response, and the bias towards a Th2 response in the lung, upregulation of DCs via TLR agonists 2 4 and 9, may enhance the immune response to vaccination, and thus provide protection against respiratory challenge with strain 2308. Intranasal vaccination with strain RB51 with the addition of TLR2 or TLR4 agonists did significantly, with a trend for TLR9 reduced CFU counts in the lung (72). If protection cannot be provided through TLR agonists, an alternate approach is to use other vaccines/strains to enhance protection. Kahl-McDongah et al, used a *B. melitensis* vaccine strain administered IP, and was able to demonstrate protection

against aerosolized challenges of both *B. melitensis* and *B. abortus* (73). These data suggest that both route, as well as strains used, influence immunity and protection.

Mouse model

Mouse models have been developed to test the efficacy of *B. abortus* vaccines for cattle. When BALB/c mice, or C57BL/6 were infected, they develop lesions in the spleen and liver similar to humans (74, 75). For many reasons, they are now an established model to measure the immune response against *Brucella* infection (74). The ease of handling and housing makes mice a good laboratory model. In addition, the costs are markedly reduced for mice vs. cattle. Furthermore, there are many murine reagents available, thus minimizing restrictions in experimental design/planning. Mice have been used as a model for many years in the laboratory, and have been highly characterized, based on genetics, strain, and immune responses. Using inbred strains decreases genetic variation, and decreases variability in outcome responses, due to differences in genetics. Thus, the mouse model is a valuable tool in studying the immune response and vaccine development. Therefore, we have employed a BALB/c mouse model to assess the ability of TLR agonists to upregulate strain RB51 mediated protection against respiratory challenge of strain 2308 (76).

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Chapter 2

Ability of TLR agonists to upregulate *Brucella abortus* strain RB51 mediated protection in a murine respiratory model

M. Walker[1], N. Sriranganathan[2], I. Kanevsky [4], K. Zimmerman[2], N. Tenpenny[3], M. Makris[3], S. Werre[3], S. Witonsky[1].

[1]Department of Large Animal Clinical Sciences, [2]Department of Biomedical Sciences and Pathobiology, [3]Research and Graduate Studies, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA. [4]Department of Dairy Science, College of Agricultural and Life Sciences, Virginia Tech, Blacksburg, VA, USA.

Abstract

Brucella abortus is a Gram-negative intracellular zoonotic bacterium that causes infertility and abortion in cattle and undulant fever in humans. Its low dose of infectivity, ability to be aerosolized and easily genetically modified causes it to be a bioterror concern. The overall goal of this research is to generate a safe and effective vaccine for humans as none currently exists. One candidate vaccine is *Brucella abortus* strain RB51, which was approved for use in cattle and provides protection by initiating a strong T-helper 1 (Th1) response. Based on a model for aerosol exposure, mice were vaccinated intranasally (IN) with strain RB51 and challenged IN with *B. abortus* strain 2308. However, strain RB51 did not protect. Protection against *Brucella* is mediated through TLRs 2, 4 and 9. The addition of TLR 2 or TLR 4 and a trend with TLR9 agonists with intranasal RB51 vaccination significantly increased bacterial clearance in the lung after strain 2308 challenge. Therefore, for this study, we hypothesized that combining TLR agonists 2, 4, and 9 with strain RB51 IN would upregulate protection and clearance in the lung

against strain 2308 challenge (IN), by upregulating the DC1 and CD4 Th1 and CD8 immune response. This study showed that protection is not upregulated by combining all TLR agonists. The group with the most non-significant improved clearance, based on decreased colony forming units (CFUs) in the organs, was strain RB51 and TLR 2 and 4 agonists. Additional studies are warranted to further define the differential mechanisms and endpoints of protection and chronic infection.

Introduction

Brucella is an important zoonotic disease, as it is endemic across the globe, and it is a risk to humans (1). It is easily transmitted to humans from infected herd animals, through contact with aborted fetal material, consumption of unpasteurized dairy products, and inhalation, ingestion, or contact working as an abattoir. Because *Brucella* can easily be aerosolized, combined with its virulence, and potential to be genetically modified, it is also a candidate for use as a biological weapon. There is no commercially available efficacious vaccine for human use.

As a model for humans and cattle, mouse models initially focused on intraperitoneal (IP), intramuscular (IM), or intravenous (IV) routes of vaccination and challenge. None of these first models were used to assess protection against respiratory infection. When laboratories began using these established mouse models to investigate respiratory infection and protection, there were differences in the ability of vaccination to provide protection against respiratory challenge of different vaccine and challenge strains. Initially, Olsen et al. had shown that vaccination of mice IP with *B. abortus* strain RB51 provided protection against *B. abortus* strain 2308 IP challenge (2). However, when mice were vaccinated IP or IN with strain RB51 and IN challenge against strain 2308, these mice were not protected in the lung (2-4). Additionally, mice vaccinated IN using strain RB51 did not protect against IN challenge with strain 2308 (3-5). Furthermore, systemic IP vaccination and IN boost did not enhance clearance in the lung or mediastinal lymph node against strain 2308, nor did priming with intramuscular (IM) or intradermal (ID) vaccination followed by IN boost (5). However, IP prime and IN boost using *B. abortus* strain RB51SOD, a strain over expressing superoxide dismutase, showed significant clearance in the spleen, but not lung (5). By contrast, using a *B. melitensis* developed vaccine

strain administered IP, Kahl-McDongah et al., demonstrated protection in the lung against aerosolized challenge of *B. melitensis* and *B. abortus* (6). These studies support that the ability to protect the host against *Brucella* is dependent upon both which vaccine and challenge strains are used, what routes of vaccination and challenge, and what dosage used. A vaccine that can stimulate the innate and adaptive immune response including creating long term memory is needed to provide protection against infection.

To develop an efficacious vaccine, knowledge is needed regarding the protective immune response against *Brucella*. Protection is mediated by TLRs 2, 4, 6, and 9 which can initiate a strong DC1 response and subsequently stimulate a protective CD4 Th1, CD8 adaptive response. *Brucella* interferes with DC function and therefore does not sufficiently activate T-cells compared to uninfected cells (7). As previous studies have demonstrated a role for TLR 2, 4, 6, and 9 *in vitro* and *in vivo Brucella* mediated DC function and protection in the lung improved by using TLR 2 and 4 individually combined with strain RB51, the objective of this study was to determine if the addition of TLR agonists 2, 4, and 9 to intranasal (IN) strain RB51 vaccination, will enhance protection and clearance against strain 2308 IN challenge. We hypothesized that the addition of TLR agonists 2, 4, and 9 with IN *B. abortus* strain RB51 would upregulate the protective immune response to IN challenge against *B. abortus* strain 2308. Protection was assessed based on determining clearance in the organs and development of a DC1 CD4 Th1 CD8 response.

Materials and Methods

Mice. Female BALB/c mice, 5-8 weeks old, from Charles River Laboratories Inc., Wilmington, MA were used in accordance to the approved Institutional Animal Care and Use Committee at Virginia Tech.

Bacterial strains. Live *B. abortus* strain 2308 and strain RB51 were obtained from our stock culture collection. Experiments were performed in our Biosafety level (BSL)-3, Centers for Disease Control approved (C2003 1120-016) facility (3, 5).

Experimental design. BALB/c mice (n=5/group) anesthetized with 0.4 mL of xylazine-ketamine 0.2 mg/mL and 0.9 mg/mL was collected by retro orbital bleeding. Mice were vaccinated with 20 µL phosphate buffered saline (PBS) or *B. abortus* strain RB51 ($4x10^8$ CFU/mouse) alone, or in combination with TLR agonists 2 and 4, or TLR agonists 2, 4, and 9 (Invivogen San Diego, CA): Concentrations were TLR2 agonist PAMCSKK4 (5ug/mouse), TLR4 agonist ultrapure *E. coli* 0111:B4 LPS (10ug/mouse), or TLR9 agonist CpG (30ug/mouse) (8, 9). The group receiving PBS only will be referred to as PBS, the group receiving strain RB51 only, will be referred to RB51, the group receiving strain RB51 and TLR 2 4 for agonists, will be referred to as RB51TLR24, and lastly the group receiving strain RB51 and TLR 2 4 9 agonists, will be referred to as RB51TLR249. Six weeks post vaccination, mice were anesthetized and challenged IN with strain 2308 ($2x10^4$ CFUs/mouse). Mice were euthanized two weeks post challenge using 1.4 mL of xylazine-ketamine. Bronchoalveolar lavage (BAL), serum, lung, spleen, mediastinal lymph node (MLN) were collected. Organs were homogenized using a tissue homogenizer, and then serially diluted and plated on Tryptic soy agar (TSA) plates. Plates were incubated for 5 days at 37°C and 5% CO_2(8). Minimum level of detection of CFU's was 20.

Samples with no colonies present were recorded as 19 CFUs. Organ homogenates were frozen for cytokine analysis (3, 5, 8).

Antibody ELISA. BAL and serum were stored at -80°C until analyzed. Plates were coated with either heat killed strain 2308 or heat killed strain RB51 at 1 µg/well. Plates were washed with Tris buffered saline TBS (Fisher Scientific, Fairlawn, NJ) and 0.05% Tween 20 (Fisher Scientific). Plates were blocked with 200 µL 2% BSA (Sigma St. Louis, MO) in TBS at 37°C for 1 hour then washed. The BAL was not diluted. Serum was diluted to 1:100 for identification of IgA, IgG1, and a dilution of 1:100 and 1:200 were used for IgG2a in 2% BSA (Fisher Scientific). 50 µL per well was added in each well and duplicate samples were incubated for 4 hours at room temperature. Plates were washed, and isotype specific anti-mouse horseradish peroxidase conjugates antibodies (IgG1, IgG2a, IgA) (Southern biotechnology associates Inc., Birmingham, Alabama) were added at 1:4000 dilution. Plates were washed, and TMB was added at 100 µL per well (Microwell peroxidase substrate, Kirkgaard & Perry Laboratories, Gaithersburg, MD) was used to for color development with peroxidase conjugates, and then stopped using 100 µL per well of 0.185 M sulfuric acid (Fisher Scientific). Antibody levels in serum of pre-vaccinated, and serum and BAL of post-challenged mice were measured by ELISA using OD values (3).

Splenocyte isolation. Splenocytes were isolated as previously described, and red blood cells were lysed with 2 mL ACK lysis buffer (Lonza, Walkersville, MD) (150 mM NH_4Cl, 1 mM $KHCO_3$ 0.1 mM Na_2EDTA) for 2 minutes (10). Samples were washed and resuspended in RPMI 1640 (Cellgro, Manassas, VA) complete media at 5 x 10^6 cells/ml. Splenocytes (5 x 10^5 cells/well) were cultured in 96 well plates and stimulated for 5 days with media, ConA (0.5µg/ well) (Sigma), irradiated (IR) or heat killed (HK) strain 2308 or RB51 at 10^6 CFU/ well. RPMI

1640 media (Cellgro) was supplemented with 2mM L-glutamine (Cellgro), 10% heat-inactivated fetal calf serum (Atlanta biological, Lawrenceville, GA), penicillin/streptomycin (Cellgro), and 50 µM 2-mercaptoethanol (Sigma). After 5 days, supernatants were harvested as previously described by Vemulapalli et al., and stored at -80°C until cytokine ELISAs could be performed (10).

Processing of BALs. BALs were spun at 250 x g for 5 minutes to isolate cells; BALs from mice were combined in either 2 mice (mice #4-5 per group) or 3 mice (mice #1-3 per group) to ensure enough cells for flow cytometry (9). BAL supernatants were frozen at -80°C for cytokine and immunoglobulin analysis (9).

Flow cytometry. BAL and splenocyte samples were aliquoted for flow cytometry and proliferation assays (11). As performed previously, because cell numbers were limited on some samples, 5 x 10^5 cells/sample were stained for flow cytometry (5, 7, 8, 11). Stain combinations were: DC1: APC conjugated anti-CD11c (BioLegend, San Diego, CA), PE conjugated anti -I-A/I-E (BioLegend, Franklin Lakes, NJ), biotin conjugated anti-CD40 (BioLegend), Pacific Blue conjugated anti-CD86 (BioLegend), PE-Cy7 conjugated anti-CD11b (BioLegend), PerCP conjugated Streptavidin (BioLegend); DC2: APC conjugated anti-CD11c (BioLegend), PE conjugated anti-I-A/I-E (BioLegend), Pacific Blue conjugated anti-CD8α (BioLegend), Biotin conjugated anti-CD103 (BD Pharmingen), PE-Cy7 conjugated anti-CD11b (BioLegend), PerCP conjugated Streptavidin (BioLegend); T1: APC conjugated anti-CD25 (BioLegend), PE-Cy7 conjugated anti-CD69 (BD Pharmingen) or FITC (BioLegend) conjugated anti-CD69, Texas Red conjugated anti-CD4 (Caltag Laboratories Invitrogen Camarillo, CA), Pacific Blue conjugated anti-CD8α (BioLegend), PE conjugated ant--CD45RB (Caltag Laboratories); T2: PE conjugated anti-CD44 (BD Pharmingen), Texas Red conjugated anti-CD4 (Caltag Laboratories Invitrogen),

FITC conjugated anti-CD62L (BD Pharmingen), Pacific Blue conjugated anti-CD8α (BioLegend); T3: PE conjugated anti-B220 (BD BPharmingen), FITC conjugated anti-CD69 (BioLegend) (5, 8, 12).

ELISAs to measure cytokines. BAL, splenocyte supernatants, organ homogenates (lung, liver, spleen, MLN) were stored at -80°C. TNF-α, IL-12p70, INF-γ, IL-10, IL-4, IL-17 were measured using indirect sandwich ELISAs (BD Pharmingen)(3, 12, 13). Plates (Nunc, Roskilde, Denmark) were coated overnight with the respective capture antibodies for each cytokine at specified concentrations in their respective coating buffers. Plates were then blocked with 2% BSA in PBS for 30 minutes at 37°C then washed 4 times with 0.5% Tween PBS wash buffer. Samples and recombinant cytokines for a standard curve were added at 50 µL per well and run in duplicate. Plates were incubated for 3 hours at room temperature. Samples were then removed, and plates were washed 4 times with wash buffer. The primary antibody against the cytokine was added at specified concentration and incubated for 45 minutes at room temperature. The plates were washed again (4 times) and the secondary antibody which was horse radish peroxidase conjugated was applied and incubated for 30 minutes at room temperature. The plates were then washed again (8 times) before the TMB substrate solution (Dako, Carpinteria, CA) was applied and incubated to develop blue color, then stopped with 0.185 M sulfuric acid (Fisher). The plates were then read at 450 nm. Averages from duplicate wells were calculated.

Statistical analysis. Organ CFUs were analyzed using Wilcoxon/Kruskal-Wallis Tests followed by Dunn procedure for multiple comparisons with JMP software. Significant differences were determined at $p<0.05$. Trends were marked for $p>0.05-<0.1$, and standard deviations were reported. For flow cytometry results from BAL and splenocytes, samples were

analyzed using one way ANOVA followed by Tukey-Kramer procedure performed using GLIMMIX procedure for SAS (NC, USA).

Results

Protection based on colony forming units (CFU). Organs were homogenized and plated; bacterial colonies were counted after 5 days. There were no significant differences between treatment groups in clearance based on CFUs of any of the organs. The RB51TLR24 vaccinated group had the fewest CFUs in the lung, spleen, and liver compared to the other treatments; however, it was not significantly lower compared to the other groups (Fig. 1). The strain RB51 group had non-significantly higher CFUs in lung, spleen, and liver than the other vaccine groups (Fig. 1). The RB51TLR249 group showed a non-significant increase in CFUs compared to the RB51TLR24 group, indicating the addition of TLR 9 non-significantly negatively affected clearance (Fig. 1). While not significant, based on clearance, the RB51TLR24 group appeared the most promising vaccine.

Total cell numbers in BAL and spleen. In the BAL, the RB51 group (1.97×10^5 cells ± 4.35×10^4) had significantly more cells versus the PBS (1.94×10^4 cells ± 1.38×10^4) and RB51TLR24 (3.77×10^4 cells ± 3.24×10^3) groups. The RB51TLR249 group (9.52×10^4 cells ± 3.18×10^4) had significantly more vs. the PBS treatment. Comparatively, in the spleen, there were significantly less cells in the PBS group (8.64×10^6 cells ± 1.38×10^4) compared to the RB51 (1.52×10^7 cells ± 4.35×10^4) and RB51TLR249 (1.42×10^7 cells ± 3.18×10^4) groups. For the RB51 group, there was a trend towards higher cells compared to the RB51TLR24 group (8.51×10^6 cells ± 3.2×10^3). Overall, the RB51 group had significantly higher total cells in the BAL and spleen, suggesting an increased in response, based on cell number only.

Vaccination induces higher antibody titers in BAL. In the BAL, the mice in vaccine groups RB51 and RB51TLR249 induced a trend for higher IgG2a antibodies, consistent with a Th1 response against strain RB51, post strain 2308 challenge (Fig. 2A). Antibodies against strain

2308 had a similar pattern, wherein the RB51TLR24 group was significantly higher, and the RB51TLR249 was a trend higher than the PBS group (data not shown). The RB51 and RB51TLR249 groups induced a trend for higher IgA production vs. PBS against strain RB51, consistent with a Th2 response, compared to the PBS group (Fig. 2B). The RB51 group was the only treatment to non-significantly induce higher IgA production against strain 2308 compared to the PBS group, supportive of a Th2 response (data not shown). The RB51TLR249 group significantly, and RB51TLR24 group non-significantly induced higher IgG1 vs. the PBS group against strain RB51, consistent with a Th2 response (Fig. 2C). There was a similar pattern with IgG1 antibodies against strain 2308. The RB51TLR249 group induced non-significantly higher IgG1 production against strain 2308 compared to the PBS group (data not shown). Additionally, the RB51TLR24, RB51 and RB51TLR249 groups produced more antibodies (higher OD values) against strain RB51 than strain 2308. All groups had non-significantly enhanced IgG1 and IgG2a antibody production compared to the PBS group. The immune response to strains 2308 and RB51 based on antibody production suggests a possible mixed Th1 and Th2 response from the post-vaccination post challenge samples.

Vaccination induces higher antibody titers in the serum. Antibody production in the serum measured post challenge showed increased antibody titers in the vaccinated vs. PBS group. Following strain 2308 challenge, all groups induced significantly more IgG2a and IgG1 production against both strain 2308 and strain RB51 antigens compared to PBS (Figures 3A & 3B, and data not shown). Again, similar to the BAL, the OD value for antibodies against strain RB51 were higher than 2308, suggesting a greater antibody response to strain RB51. The RB51 group induced significantly higher IgA production against strain RB51 compared to the PBS group, and a trend higher than the RB51TLR24 group (Fig. 3C), and showed a similar pattern

against strain 2308. The RB51 group tended to induce more IgA production in the serum and BAL, and had an increased cell number in both the BAL and spleen, but did not induce protection, suggesting either a delayed response or reduced immune function. Production of IgA, IgG2a, and IgG1 by the RB51 group suggests a mixed immune response based on antibody production alone. Comparatively, the RB51TLR24 group induced a stronger Th1 response based on antibody production.

Phenotypic analysis of DC subsets. In the BAL and spleen, DC subtype (CD103 DC, CD11b, and CD8α resident DC), as well as upregulation of activation markers (MHC class II, CD40 and CD86) were assessed to determine if there were immune correlates of protection and/or differences in vaccine induced responses. DC were determined based on CD11c+ staining. In the BAL and similarly in spleen, the RB51 and RB51TLR249 groups were significantly higher than the PBS and RB51TLR24 groups in CD11c high DC, potentially associated with response to infection (Fig. 4). Assessing differences in DC subtypes, the CD11c high CD11b high DC in the BAL as a percent of total CD11c high cells in the BAL, the RB51 group was a significantly higher than the RB51TLR24 and RB51TLR249 groups, and a trend higher than the PBS group suggesting that the RB51 group induced CD11b cells (Fig. 5A). Comparatively, the RB51 group induced significantly higher CD11c low CD11b high cells compared to all other groups (data not shown). In the spleen, the RB51 group was significantly higher than the RB51TLR24 and PBS groups in CD11c high CD11b medium cells in the spleen. The RB51TLR249 group was significantly greater than the PBS and RB51TLR24 groups. The RB51 group did not upregulate CD11b expression as well (Fig. 5B). When assessing for CD11c high CD11b high in the spleen, the RB51 group was significantly lower than the RB51TLR24 group (Fig. 5C), indicating lack of induction of CD11c high by the RB51 group. The CD11b DC

expression was different among the groups, suggesting possible differences in vaccine induced immunity.

Assessing the CD103 response in the BAL, the RB51TLR249 group had significantly greater CD11c low MHC class II high CD103 DC than the other treatment groups (Fig. 6A). The RB51 group was significantly higher in CD11c low MHC class II low CD103 cells than PBS and RB51TLR24 groups, while the RB51TLR249 group was significantly higher than the RB51TLR24 group, but only a trend lower than the PBS group (data not shown) in the BAL. For CD11c low CD103 cells in the spleen, the RB51TLR249 group was significantly higher than the PBS and RB51 TLR24 groups, and the RB51 group was a trend higher than PBS and RB51TLR24 groups (Fig. 6B), suggesting differences in how TLRs induced DC mediated immunity.

In the spleen, CD11c high and low DC were examined for subtypes to determine if there was a particular subtype associated with protection or persistent infection. It was found that the RB51 group had a significantly lower percentage of CD11c high CD8α high DC compared to the RB51TLR24 and RB51TLR249 groups (data not shown), suggesting that the TLR agonists may induce CD11c high CD8α high DC. In the spleen, the RB51 group was significantly higher than the PBS and RB51TLR24 groups, and the RB51TLR249 group was also significantly higher than the PBS group in CD11c low MHC class II high CD8α (Fig. 7).

DC activation. DC activation in the BAL was assessed based on both CD11c high and CD11c low populations. In the CD11c high population, the RB51 and RB51TLR249 groups were significantly higher than the PBS and RB51TLR24 groups, potentially due to differences in TLR activation and infection status (Fig. 4). A similar pattern of CD11c low expression was

observed as the CD11c high DC in both the BAL and spleen. Similar to in the spleen, the RB51 and RB51TLR249 groups were significantly higher than the PBS and RB51TLR24 groups in CD11c high in the BAL (data not shown). The PBS and RB51TLR24 groups had significantly higher CD11c high CD86+ CD40- DC than the RB51 and RB51TLR249 groups in the BAL (Fig. 8a). The RB51 group in the spleen was significantly higher than the RB51 TLR249 group in CD11c low CD86+ CD40- (data not shown). The RB51 group did not upregulate CD40 to have double positive CD40 CD86 expressing DC compared to the RB51 TLR249 group. The RB51TLR249 group was significantly higher than the PBS and RB51TLR24 groups and a trend higher than RB51 group, and the RB51 group was a trend higher than the PBS group in CD11c low CD86- CD40+ in the BAL (Fig. 8b) and a similar pattern of expression was seen in the spleen. The RB51TLR249 group was significantly higher than the PBS, RB51TLR24, and RB51 groups in CD11c low CD86+ CD40+ DC, and the RB51 group was a trend higher than the RB51TLR24 and PBS groups in the BAL. This was similar to the pattern in the spleen for CD11 c high DC in the BAL (Fig. 8c). Upregulation of CD86 and CD40 were potentially due to differences in how the pathways are stimulated and levels of infection. The less protected groups tend to induce expression of CD86 and CD40. Additionally, the RB51TLR24 group tended to induce CD86 upregulation vs. RB51TLR249 which tended to induce CD40 expression. The differences in DC subsets and activation are the result of how the different TLRs stimulated immune responses.

In the BAL and spleen, activation was also measured by MHC class II high expression. While there were no differences inCD11c high groups, there were significant different in MHC class II high (data not shown), All groups expressed a high percentage (90% or more) of CD11c high MHC class II high CD40 or CD86, supporting activation in the BAL, potentially due to

infection (data not shown). A similar expression pattern was seen in the spleen with CD11c low DC. Similar to CD11c high DC, the RB51 and RB51TLR249 groups were significantly higher than the PBS and RB51TLR24 groups in CD11c low MHC class II high indicating DC activation from bacterial stimulation (data not shown). Similarly to the BAL, the RB51 and RB51TLR249 groups were significantly higher than the PBS group in CD11c high MHC II high CD86 in the spleen (Fig. 9a). The RB51TLR24 group was a trend higher compared to the PBS group, and a trend lower compared to the RB51TLR249 group (Fig. 9a). In the spleen, the RB51 and RB51TLR249 groups were significantly higher than the PBS group in CD11c high MHC class II low CD40, and the RB51 group was significantly higher than the RB51TLR24 group. The RB51TLR249 group was also a trend higher than the RB51TLR24 group (Fig. 9b). This pattern was similar to the BAL. The RB51 and RB51TLR249 groups had significantly more DC expressing MHC class II and CD40 vs. the PBS group, the RB51 group was significantly higher, while RB51TLR249 was a trend higher than RB51TLR24 (Fig. 9b). This was possibly due to active infection in the BAL and spleen. The RB51 TLR249 and RB51 groups induced more activation of cells via CD40 expression while the RB51TLR24 group more consistently upregulated through CD86 expression.

Similar to CD11c high DC, the RB51 and RB51TLR249 groups were significantly higher than the PBS and RB51TLR24 groups in CD11c low MHC class II high indicating DC activation from bacterial stimulation in the spleen (Fig. 10). Unlike in CD11c high DC, the RB51TLR24 group was significantly higher than the RB51 and RB51TLR249 groups, and the PBS group was a trend higher than the RB51TLR249 group in CD11c low MHC class II high CD86 (data not shown). The RB51TLR24 group induced more CD86 expression compared to the RB51 and RB51TLR24 groups. The latter two groups were better able to upregulate CD40.The less

protected groups, RB51 and RB51TLR249, tended to induced higher levels of MHC class II activation, over the more protected RB51TLR24 group. This was possibly due to persistent infection.

Clearance associated with increased activated T cells. To assess the immune response associated with clearance/chronic infection, identify possible correlates of protection, and determine how the vaccine groups differentially stimulated immunity, lymphocytes were assessed for subsets and activation by flow cytometry. Lymphocyte subsets (CD4, CD8, B cells (B220), and activation/phenotype (CD69 early activation, CD45RB, CD62L naïve, CD44 memory, CD25 T-reg or activation) were determined for BAL and spleen samples.

The groups were examined for CD45RB effector cells in the CD4 and CD8 high and low populations of lymphocytes. In the BAL, the RB51TLR249 group was significantly increased in CD8 high cells vs. all the other groups. Additionally, the PBS group was significantly lower than the RB51 group and a trend lower than the RB51TLR249 group in CD8 low CD45RB low (data not shown). The RB51 group expressed levels of CD8 high CD45RB low, but did not upregulate CD8 high CD45RB high (data not shown). The RB51TLR249 group showed a trend for increased effector cells. The RB51TLR249 group induced a trend for higher CD4 high CD45RB high cells in the BAL (Fig. 11a). The RB51TLR249 group had a trend towards significantly lower CD4 high CD45RB low compared to RB51 the group (data not shown). The RB51 group did not upregulate CD45RB compared to the RB51TLR249 group. By comparison, in the spleen, the RB51 group was significantly higher than the RB51TLR249 group in CD4 high CD45RB high (Fig. 11b), and was significantly lower than the RB51TLR24 and RB51TLR249 groups in CD4 high CD45RB low (data not shown). Similar to CD4 high CD45RB high in the spleen, the RB51 group was significantly higher in CD4 low CD45RB high cells compared to the

RB51TLR24 and RB51TLR249 groups, and a trend higher than the PBS group (data not shown). The least protected groups demonstrated higher CD45RB effector T cells.

Besides CD45RB to detect effector cells, CD69 can be used to detect early activation. The RB51TLR24 group had a trend, and the RB51 and RB51TLR249 groups were significantly higher than the PBS group in CD4 high CD69 in the spleen (Fig. 12). The RB51TLR24 group was significantly lower than the RB51 and RB51TLR249 groups (Fig. 12). Comparatively, the RB51TLR24 group was significantly higher than all other groups in CD4 low CD69 (data not shown). These data suggest that chronically infected groups have increased CD4 high activation and CD45RB populations present. There may be differences in high and low populations associated with infection. Consistent with the increased CD8 CD25 status and CD4 low CD69, the RB51TLR24 was also significantly higher than the RB51TLR249 group in CD8 low CD69 cells; indicating increased early activated T cells (data not shown). The RB51TLR24 group was able to induce more CD4 low and CD8 low activated T cells, possibly due to persistent infection, and/or a difference in how TLRs activate the immune response and/or associated with infection status.

The CD4 and CD8 lymphocytes were also examined for CD25 expression as a measure of activation and/or T-reg cells. In the spleen, there were differences in CD4 low cells, including CD4 CD25 cells, which could suggest activation or a regulatory state. The RB51TLR24 group was significantly higher than the PBS and RB51TLR249 groups in CD4 low T cells, and the RB51 group was a trend higher than the RB51TLR249 group (data not shown). The RB51TLR24 group was significantly higher than the RB51 group and a trend higher than the RB51TLR249 group in CD4 low CD25 expression (Fig. 13), and showed a similar pattern to CD8 low CD25 expression. The addition of TLR 2 4 agonists induced more CD4 low and CD4

low CD25 T cells in the spleen, potentially associated with a higher level of T-regulatory or activated T cells. Cytokine production and/or gene expression of the CD25 cells is needed to know the cell phenotype.

In addition to the CD25 response by the CD8 cells, effector and activation status were also determined. The PBS and RB51TLR24 groups were significantly higher in CD8 high cells compared to the RB51 and RB51TLR249 groups, suggesting more recruitment of CD8 T cells to the BAL (Fig. 14). Assessing early activation response, the RB51 group significantly, and had trends for the RB51TLR24 and RB51TLR249 groups to be more activated based on CD8 high CD69 than PBS, suggesting increased activation of these groups (data not shown). CD8 low cells were also assessed. The RB51TLR24 group was significantly higher than the RB51 group, and a trend higher than the RB51TLR249 groups in CD8 low T cells (data not shown). Overall, the less protected groups tended to induce increased CD4 and CD8 CD69 and CD45RB expression, while the more protected RB51TLR24 group induced higher CD25 expression but also CD69 expression in CD4 low and CD8 low T cells.

Clearance and lack of infection associated with decreased CD44+ increased CD62+ expression. Naïve and memory T-cell status was based on CD62L (naïve) and CD44 (memory) expression in the spleen. For this study, we have used CD44+ to represent memory cells and CD62L+ to represent naïve cells as the cell populations we assessed were in the spleen. We recognize the central memory cells are CD62L+, and are located predominantly in the lymph nodes, not in the spleen. Therefore, for the purpose of this study, we refer to CD62L+ cells as naïve. We recognize that there is the unlikely potential that the CD62+ cells in the spleen could be central memory cells. There was a pattern that the PBS group had a significantly higher percentage of CD4 high CD62L high cells vs. the other treatments, especially the RB51 and

RB51TLR249. The pattern was present by the CD4 and CD8 high cells as well, suspect of a higher percentage of naïve cells (data not shown). Overall, the PBS and RB51TLR24 groups had higher naïve cells in comparison, potentially reflective of lack of induction and declining infection, respectively. In the CD4 high quadrant gate, which assessed CD44 and CD62 expression, the RB51 and RB51TLR249 groups were significantly higher in CD62L-and CD44+ cells compared to the PBS and RB51TLR24 groups (Fig. 15a), suggestive of a memory T cells response by the latter groups. There was a similar pattern of expression of CD4 low CD62L-CD44+ cells, by the PBS and RB51TLR24 groups (data not shown) to the CD4 high gate. For the PBS group, the percentage of CD4 low cells were significantly higher compared to all other groups in the spleen, suggesting its ability to recruit CD4 low cells to the spleen (data not shown).

In assessing the CD8 high and CD8 low response, there were similar patterns to the CD4 response; the PBS and RB51TLR24 groups tended to be higher in CD62 naïve cells, whereas the RB51 and RB51TLR249 groups tended to have higher CD44 memory expression. For the CD8 high CD62L-CD44+ (Fig. 15b) which was gated on CD8 low cells and then CD44 or CD62L expression the RB51TLR249 group was significantly higher than PBS and RB51TLR24 groups for CD8 low CD62L- CD44+ expression, likely due to persistent infection (data not shown). In the second quadrant gate, the PBS and RB51TLR24 groups were significantly higher than the RB51 and RB51TLR249 groups in CD8 low CD62L+ and CD44+, suggesting a mixed population of memory and naïve cells (data not shown). This pattern CD62L+ and CD44+ expression was a similar pattern to CD4 high, CD4 low, and CD8 high. The RB51TLR24 group was significantly higher than the PBS in CD62L+ and CD44- indicating the ability of TLR 2 4 to have increased active naïve cells (data not shown) at this time point. By comparison the

RB51TLR249 group was significantly higher than the PBS and RB51TLR24 groups in CD8 low CD44 high cells. The RB51 group was a trend higher than the PBS group, but was significantly higher than the RB51TLR24 group in CD8 low CD44 high (data not shown). Collectively, there was a tendency for the CD4 and CD8 cells in the PBS and RB51TLR24 groups for increased CD62+ naïve cells, and the RB51 and RB51TLR249 groups had increased CD44 memory cells (data not shown). We propose that the increased naïve cells in the PBS group were due to lack of induction of an immune response, and the RB51TLR24 group has a declining infection at this time point. By comparison, we proposed that the increased memory (CD44) response in the RB51 and RB51TLR249 groups was due to persistent infection. The functional significance of the CD4 and CD8 high and low subsets still needs to be determined.

Clearance and lack of protection associated with increased CD69+ B cells. When spleens were examined for B cells using B220 as a marker, it was found that the PBS and RB51TLR24 groups had significantly higher percentages of B cells (data not shown), including activated B cells (CD69) compared to the RB51 and RB51TLR249 groups (Fig. 16). Based on flow cytometry alone the PBS and RB51TLR24 groups induced higher levels of activated B cells. However, these were not consistent with antibody production in the serum or IL-4 levels.

Cytokine production in organs. The specific cytokine profile reflects the nature of the immune response. TNF-α and IL-12, are consistent with a Th1 response, and have been shown to provide protection against *Brucella* infection (12, 14). Production of IFN-γ, a Th1 cytokine by immune cells, has been shown to be essential in control and clearance of *Brucella* (15). Production of IL-4 indicates a Th2 response, and IL-10 can be consistent with a Th2 or T-regulatory (T-reg) response. IL-17 is associated with a Th17 response, and has shown to have a

role in protection or exacerbation of different infectious diseases, as well as having a role in some non-infectious chronic diseases (16, 17).

In the lung, there were no significant differences in TNF-α production. The PBS group was a trend higher compared to the RB51 and RB51TLR249 group (Fig. 17A). For IL-12 production, the PBS group produced significantly more IL-12 than RB51 group (Fig. 17B). The RB51TLR24 group produced significantly higher IL-12 compared to the RB51 group and a trend higher than the RB51TLR249 group indicating a stronger Th1 type of response (Fig. 17B). There was no IFN-γ or IL-4 production (data not shown), and no significant differences in IL-10 or IL-17 production (Fig. 17C & 17D) between groups.

Next in the spleen, the PBS and RB51TLR24 groups produced significantly less TNF-α compared to the RB51 and RB51TLR249 groups (Fig. 18A). The RB51 group was significantly higher than the PBS and RB51TLR24 groups in IL-12 production indicating the RB51 group induced a Th1 type of response. There were no significant differences in IFN-γ production (Fig. 18C); groups produced either very low or non-detectable amounts. There was also no IL-4 production detected in any groups (data not shown). There were no significant differences in IL-17 and IL-10 production between the different groups (Fig. 18D & 18E).

In the MLN, the PBS group was significantly higher in TNF-α than the RB51TLR249. The RB51TLR24 group was a trend higher in TNF-α compared to the RB51 and RB51TLR249 (Fig. 19A). The PBS group was also significantly higher in IL-12 than the RB51TLR249 group, and a trend higher than the RB51TLR249 group in IFN-γ (Fig. 19B &19C). The RB51TLR24 group was a trend higher than the RB51 group in IL-4 production (Fig. 19D). There were no significant differences between groups in IL-10 production (Fig. 19E). The PBS and

RB51TLR24 groups were significantly higher than the RB51 group for IL-17 production (Fig. 19F).

Last in the BAL, the RB51TLR24 group was significantly higher in TNF-α than the RB51TLR249 (Fig. 20A). The RB51TLR249 group was significantly higher than the PBS and RB51 groups in IL-12 (Fig. 20B). IFN-γ and IL-4 were not detected (data not shown). IL-10 was produced, but there were no significant differences between any groups (Fig. 20C). The RB51TLR24 group produced significantly more IL-17 compared to the RB51 group.

Cytokine production from stimulated splenocytes. Splenocytes were stimulated and the supernatants collected; however there were not enough samples to determine all cytokine levels. Insufficient samples were present for TNF-α and IL-17 determination. There were significant differences for IL-12 production there was a pattern that for media, ConA, and IR2308 stimulated samples, the RB51 and RB51TLR24 were greater than PBS stimulated splenocytes (21A).The RB51 group tended to be greater than RB51TLR249 as well.

For IFN-γ production, there was a pattern of the RB51TLR24, RB51 and RB51TLR249 stimulated splenocytes to induce higher IFN-γ than PBS stimulated splenocytes. In the same cases RB51TLR249 was less than RB51 and RB51TLR24 significant differences are marked (Fig 21B). Overall, the RB51TLR24 group induced higher production of Th1 type cytokines (IL-12 and IFN-γ), similar to pattern of the organ homogenates. The addition of TLR 9 (TLR 2 4 9) did not induce as strong of a Th1 response.

Stimulation of the splenocytes with ConA increased IL-4 production in the RB51 group, as it was significantly higher than the RB51TLR249 group, and a trend higher than the RB51TLR24 and PBS groups (data not shown). Splenocytes stimulated with IR2308 showed

significantly higher IL-4 production in the PBS and RB51 groups compared to RB51TLR249 group (data not shown). There was a trend for the PBS group to be higher than the RB51 group, and the RB51 group to be higher than the RB51TLR249 groups when cells were stimulated with IRRB51 (data not shown). The vaccinated groups with TLR 2 4 and TLR 2 4 9 agonists induced less IL-4 production.

In assessing the IL-10 response, splenocytes stimulated with IRRB51 induced significantly higher IL-10 production in the RB51TLR249 groups compared to the PBS groups, and there was a trend for the RB51TLR249 groups to be higher compared to the RB51 group (Fig. 21C). The RB51TLR249 group showed addition of the agonists increased IL-10 production, supporting a T-reg response or Th2 response. The RB51TLR24 group induced a trend towards higher IL-10 production compared to the PBS group (Fig. 21D). For splenocytes stimulated with HKRB51, the RB51TLR249 group produced significantly more IL-10 than the RB51TLR24 group, and the RB51 group produced a trend higher compared to the RB51TLR24 group (Fig. 21D). Comparatively, RB51TLR24 had lower IL-10 production.

The total amount of splenocyte supernatants was limited; therefore, some samples could not be tested for IL-17 production. There were no samples left from the PBS group. From the data we collected, there were no significant differences in media stimulated cells. The RB51 group induced non-significantly higher IL-17 than the RB51TLR24 group when stimulated with ConA (data not shown). When stimulated with IR2308 and IRRB51, the amount of IL-17 increased dramatically for both RB51 and RB51TLR249 groups; these were the only two groups with sample to test, and the production of IL-17 was not significantly different (data not shown). The RB51TLR249 group also induced high IL-17 production when stimulated by HK2308 and

HKRB51 (data not sown), however this was the only group tested. The RB51 and RB51TLR249 groups induced IL-17, suggesting a potential role in immunity.

Discussion

Protection was assessed based predominantly on clearance. Possible immune correlates of protection were also determined, as well as the differential abilities of vaccines to stimulate protective responses. The addition of TLR 2 and 4 agonists to strain RB51 vaccination non-significantly provided the most clearance/protection in comparison to the other groups. Surendran et al. previously showed individually added TLR agonists 2 or 4, with a trend for TLR 9, combined with strain RB51 vaccination, offered significant protection in the lung against strain 2308 by decreasing the bacterial load by more than a log compared to the PBS group. In our study, combining TLR agonists 2 and 4 appeared most beneficial; however, levels of protection were not significant compared to any of the other groups as compared to previous studies by Surendran et al. using TLR 2 and 4 agonists individually with strain RB51 (8, 9). The use of all the agonists together (TLR 2, 4, and 9) did not improve clearance based on CFU counts, and comparatively altered the immune response. These differences, as well as possible mechanisms for lack of protection, will be presented and discussed.

In assessing the cellular response based on total cell numbers, the increased cell numbers in the RB51 and RB51TLR249 groups (data not shown) could be from increased bacterial presence causing the recruitment of cells in the spleen and BAL. By contrast, for the PBS group, based on the lack of clearance and decreased cell numbers, it is likely that this group did not mount a sufficient immune response. The most promising group, RB51TLR24, had comparatively lower cell numbers possibly due to a resolving/declining immune response as there was increased bacterial clearance compared to the other groups.

Generally all vaccinated post challenged mice generated significant titers vs. PBS for IgG2a, IgG1, and IgA. Generally the RB51 and RB51TLR249 groups generated more IgA than PBS and in some cases RB51TLR24. {Surendran, 2013 #156}Additionally, all vaccinated groups had a pattern for higher antibody production based on OD against strain RB51 vs. challenge strain 2308 (Fig. 2A-3C). The limited ability to produce antibodies against strain 2308 could be because mice were vaccinated with strain RB51 or it could be associated with the limited ability of the host to respond to challenge. Vaccination with strain RB51 alone will generate antibodies against surface membrane proteins, while antibodies against strain 2308 could also stimulate antibodies against the O-side chain (18). It is possible that antibodies against strain RB51 may not bind as well to strain 2308, due to hindrance from the O-side chain (19-21). These data for the mixed but higher IgG2a vs. IgG1 responses are similar to previous studies (Fig. 2A-3C). The RB51TLR24 was the only group that showed significantly higher IgG2a production in comparison to PBS against strain 2308 in the BAL, supporting a Th1 type of response which could have helped with protection (Fig. 3A). Collectively, our data suggests that the RB51TLR24 group was better able to induce IgG2a and a Th1 response vs. strain RB51 which induced more IgA. Additional investigation would be necessary to further define the Th1 vs. Th2 responses and if protective antibodies could be generated.

DC subtype was examined to identify the most protective subtypes and the associated of immune response generated (i.e. Th1 vs. Th2). In the BAL and spleen, the RB51 and RB51TLR249 groups had increased CD11c high cells compared to the rest of the groups (Fig. 4) like due to persistent infection and reflective of increased cell numbers. The addition of TLR 9

agonist, via the TLR agonist 2 4 9 group may induce a less protective immune response thus leading to persistent infection, CD11c high cells were similar to those of the RB51 group.

The predominate DC subtype present for the PBS, RB51TLR24, and RB51 groups, was CD11b DC at this particular time point. There were also differences between the groups in CD11b expression. CD11b DC tended to be recruited monocytes which can transition into DC due to inflammation (22). These DC can migrate to draining lymph nodes and present antigens to activate naïve T cells. The RB51 and RB51TLR249 groups were high in CD11c high CD11b medium (data not shown) which could reflect an inability to upregulate CD11b, while the more protected group RB51TLR24 had a higher percentage of CD11c high CD11b high cells (data not shown) in the spleen which could prove to be correlated with increased protection.. The RB51 group tended to be higher in CD11b high in the BAL (Fig. 5A) possibly due to higher level of infection, and the RB51TLR24 group was high in the spleen (Fig. 5C), while the RB51TLR249 group tended to induce CD11b low in both the BAL and spleen. The addition of the TLR9 agonist via the RB51TLR249 group may have induced a limited immune response in which mice did not upregulate CD11b DC in the BAL, even with persistent infection.

The PBS and RB51TLR24 groups had increased CD11c low MHC class II high CD11b cells in the BAL and spleen which could indicate activation of these DC (data not shown); this gate did not differentiate CD11b cells into low, medium and high, and the functionality of CD11b low vs. high was not specifically determined. Overall, the RB51TLR24 groups had a CD11b population which appeared to be more activated, based on MHC class II expression and cytokine production. The RB51TLR24 group in the BAL, had the most TNF-α production compared to the other groups, and showed upregulated MHC class II expression and the increased CD11b DC may provide better protection. The PBS group also had higher CD11c low

MHC class II low CD11b cells (data not shown), demonstrating the lack of protection by the control group in the spleen. Others have demonstrated functional differences in CD11c low and CD11c high, where in the CD11c high cells tend to provide protection and CD11c low cells, may not be as protective (23). However, the functionality of the subsets would need to be determined by cytokine production or other assays to know the overall contribution to the response.

The next subtype assessed was CD103 DC. When evaluating CD103 activation, in contrast, the RB51TLR249 group had the highest percentage of CD11c low MHC class II high CD103 cells (Fig. 6A). The MHC class II high expression, the increased CD4 high CD45RB high effector cells, and increased IL-12 production in the BAL, suggested these cells were activated. The CD103 DC displayed characteristics of providing protection; however clearance was delayed at this time point for the RB51TLR249 group. The addition of all three TLR agonists did not enhance DC activation and function as we did not see a reduction in clearance. This could be because there was a delay in recruiting the CD103 DC and migration, or they were still increased due to infection, or they were not as protective. In a previous study assessing the effects of individual TLRs on protection, the most protected with the agonists added groups had fewer CD103 DC than less protected groups (9). It was proposed that the CD103 DC were protective, and that there was a pattern between the CD103 DC levels, cytokine levels in the lung, and protection. More protected mice had decreased CD103 and CD8α DC and increased Th1 cytokine production vs. less protected mice had higher CD103 and CD8α DC and reduced cytokine production. The previous results are potentially consistent with these results as the more protected RB51TLR24 mice have lower CD103 DC and higher CD11b DC, and the less protected RB51TLR249 mice had higher CD103 DC and CD8α DC. Alternatively, the CD103

DC might not be protective, and the CD11b cells were. Additional time points and studies are needed to assess the progression of the immune response.

Previous models demonstrate CD103 and CD11b DC tend to take up antigen, upregulate activation markers, migrate to the lymph nodes and present antigen to activate CD4 and CD8 T cells (24). During infection, depending on the type of pathogen, CD11b DC increased in the lung and, activated CD103 DC migrated from the lung to the MLN to present antigens (24). In one study, CD103 DC were present earlier and CD11b DC were increased later (24). For maximal CD8 T-cell activation, both CD103 and CD8α DC were needed (24). In another model assessing functional differences between the CD103 and CD11b DC, Furuhashi et al. found that CD103 DC stimulated with OVA peptide antigen, tended to prime naïve CD4 T cells to a Th1 type of response and induce higher levels of IL-2, TNF-α, IFN-γ, and IL-17A (25). Whereas CD11b DC primed CD4 T cells to a Th2 type response secreting IL-4, IL-6, and IL-10 (25-27). These studies suspect that there could be could be functional differences between the CD11b and CD103 DC subsets which could influence protection and activation of a protective immune response based on previous findings.

By comparison, in trying to determine the functional significance of the CD11c low CD11b high and CD11c low CD11b high populations, others have identified in their models, that CD1lc high cells tended to activate T cells, while CD11c low cells were less activated, and did not drive T cell responses (23). This population of DC which was also seen during these experiments could have contributed to the lack of clearance due to their inability to upregulate CD4. However, in an OVA model, both CD11c high CD11b low and CD11c low CD11b hi b DC induce naive CD4 T cell proliferation but only with OVA. CD11c high CD11b low DC induced higher levels of IL-12 and IFN-γ comparatively to CD11c low CD11b high (28). This

study also mentioned that CD8α DC could have a regulatory function in their OVA model if exposed to IL-10 (28). CD8α DC could also induce a Th1 response when stimulated with GM-CSF, while CD11c+ CD8α- DC induced T-reg cells (29). It is not clear whether the CD11c high CD11b low and CD11c low CD11b high populations are similarly dysfunctional in our model. Additional studies are needed to define the functionality of specific subsets.

Overall, DC from the RB51 and RB51TLR249 groups typically had increased CD11c high and CD11c low cells in the spleen and BAL (Fig. 4), which was consistent with the increased cell numbers. With some exceptions, these two groups tended to display activated CD11c low and high DC, and were usually upregulated for MHC class II CD40 or CD86.There was a tendency for the RB51TLR249 to upregulate through CD40 in the BAL. There were a few exceptions to the following patterns which were observed. By comparison the PBS and RB51TLR24 groups were able to upregulate CD86 in both CD11c high CD86+ CD40- cells (Fig. 8A), and RB51 in the CD11c low CD86+ CD40- cells (data not shown). In the spleen the less protected CD11c high groups upregulated both MHC class II low CD40 and CD11c MHC class II high CD86. It is not clear if the CD11c MHC class II low CD40 cells have different function. The upregulation of activation markers in the RB51 and RB51TLR249 groups could also be from persistent infection due to delayed clearance, as they had non-significantly higher CFUs, and also tended to produce less Th1 cytokines than more protected groups.

For the RB51 and RB51TLR249 groups' CD11c low cells tended to express CD11c high and CD11c low MHC class II high CD40 (BAL) and CD40 and CD86 (BAL and spleen) DC. However, these groups were not the strongest producers of Th1 response, based on IFN-γ by the splenocytes, IFN- γ, TNF-α and IL-12 in the lung homogenates. By comparing an *L. donovani* model Owens et al demonstrated CD11c MHC class II high induced IL-10 and IFN-γ and limited

protection. The functionality of this subset in our *Brucella* model warrants further investigation. The addition of the TLR agonists induced different immune responses which were identified with each endpoint assessed. The addition of TLR 2 and 4 agonists seemed to enhance clearance, potentially through upregulating a Th1 response, based on cytokines (IL-12 and TNF-α,) and IgG2a production, which likely led to non-significantly improved protection. The RB51TLR24 group induced increased IL-17 in the MLN and BAL, which possibly played a role in protection. In previous studies, production of IL-17 and IL-17 dependent cells provided protection during pulmonary infection with *K. pneumonia*, and IL-17 could similarly provide protection in our model (17). The addition of the TLR 9 agonist to this combination seemed to reduce the ability to protect by inducing a mixed response based on cytokine production of both Th1 cytokines and IL-10 a regulatory cytokine. The use of TLR 2 as an agonist was previously shown to induce IgG1, and both DC and B cell maturation demonstrated a mixed Th1/Th2 response (30). A TLR 4 agonist typically induces a Th1 response in mice by generating IgG2a antibodies and strong IL-12 production (30). The TLR 9 agonist has also been shown to induce a Th1 response based cytokines (30, 31). These TLRs all signal through MyD88 pathway while TLR 4 can also signal through the TRIF pathway (31). The use of these agonists should support a protective Th1 response based on previous studies, but this was not necessarily seen with this model.

Individually the TLR agonists exert different effects on the immune system, when used separately or together they can also drive activation of the immune system by upregulation of surface markers on immune cells and inducing cytokine production. In a study, when CpG (TLR9) and LPS (TLR 4) were added separately to cultures, both were shown to upregulate CD40, CD80, CD86, and MHC class II in bone marrow derived DC (32). Use of TLR 9 with CD40 (mCD40L) agonists induced strong B cell proliferation, as well as upregulation of CD69

and CD86 *in vitro* (33). The TLR 9 agonist induced increased IgM and IgA with mCD40L (33). The addition of TLR 9 to vaccination in our study could have induced higher antibody production, but this was not protective at this time point. *In-vivo* C57BL/6 mice were injected with CpG TLR 9 agonist or LPS TLR 4 agonists, and both agonists together were able to upregulate CD80 and CD86 in DC (32). Individually, TLR 2 agonists showed the ability to activate both Th1 CD4 T cells and activation of CD8 T cells or Th2 CD4 T cells to activate B cells immune response, as well as mucosal immunity by inducing IgA (31). TLR2 agonists can also induce DC maturation and upregulation of co-stimulatory and antigen presentation molecules and production of pro-inflammatory cytokines such as IL-12, TNF-α and IFN-γ (30). A TLR 4 agonist was also shown to activate both Th1 and CTL responses, as well as antibody production (31). This response could also vary with the type of LPS used. Individually the TLR agonists used in combination with vaccination as adjuvants should drive an appropriate protective Th1 CMI immune response. TLR agonists can also work in synergy. For example, the use of TLR 4, and TLR 9 on murine DC, showed increased expression of IL-12 together, vs. agonists used individually (34). Additionally it was found there was an optimal time for delivery of the agonists to achieve the highest level of synergy (34). By comparison, in another study measuring the effects of different TLR agonists on proliferation of mouse B cell showed the opposite effect, and activation by B cells showed TLR 2 and TLR alone seemed to induce more CD86 than the TLR 9 agonist (33). In this study TLR 9 did not always induce upregulation of CD86 in DC and B cells.

Overall when examining the DC for subtype and activation we found the most protected group RB51TLR24 had higher CD11b DC in comparison to RB51 and RB51TLR249. While the RB51TLR249 group, which was slower to clear at this time point induced significantly higher

DC expressing CD103 compared to PBS, RB51TLR24, and RB51 groups. The RB51TLR24 group did not upregulate traditional activation markers such as MHC class II, CD40 and CD86 as readily has the RB51 and RB51TLR249 groups; however, at this time point the immune response could have been resolving, while in the RB51 and RB51TLR249 groups it was not. There did seem to be a correlation between the addition of the TLR9 agonist and upregulation of CD40 in our study and in DC examined by Sheng et al (32).

In assessing the ability of DC to induce CD4 and CD8 activation and function, in the BAL, there was a trend for the RB51TLR249 group to induce higher CD4 high and CD8 high CD45RB effector cells, (Fig. 11A). This was likely due to the addition of TLR9 in the RB51TLR249 group altering the cellular response, potentially associated with the lack of protection causing increased bacterial presence. In the BAL, the RB51 group was unable to upregulate CD45RB, based on the low CD45RB high, and increased CD4 high CD45RB low cells present (data not shown). By contrast, in the spleen, the RB51 group was able to upregulate CD4 high and CD4 low for CD45RB high (Fig. 11b). The RB51TLR24 and RB51TLR249 groups were significantly higher in CD4 low CD45RB low (data not shown). By comparison our results showed similarities to Surendran et al. challenge study (9). Consistent with increased effector cells, the less protected groups had increased early activated cells based on CD4 and CD69 expression. This was most likely due to persistent infection (Fig. 12).

Our study reported finding higher CD4 low CD25 and CD8 low CD25 cells in the RB51TLR24 group. The functionally and role of these cells needs to be further assessed based on expression of T reg makers such as FoxP3 as well as cytokine production of this subset.

In the spleen, the RB51TLR249 group had higher CD8 high cells, potentially due to chronic infection attracted CD8 cytotoxic cells (Fig. 14).). All groups other than the PBS group were able to induce CD8 high CD69 cells indicating early activation of CD8 T cells, but the TLR agonists did not increase cellular activation over the RB51 group only. The RB51TLR24 group had increased CD8 low CD69 activated T cells (data not shown). The addition of TLR 2 and 4 agonists seemed to induce early activation in CD8 low T cells compared to all 3 agonists, TLR 2, 4, and 9 which could have increased clearance and provided protection. Additional studies would be needed to assess the function of these cells including cell specific cytokine production and Cytotoxic T Lymphocyte (CTL) assays.

There were similar trends were between the groups for naïve and memory cells for both CD4 and CD8 high and low. Overall, the PBS and RB51TLR24 groups were higher in naïve CD62L high and low and lower in CD44 (memory) expression, for both CD4 and CD8 T cells. These data were consistent with the lack of a protective response induced by the PBS group which was also seen previously (8, 9). In our study, the RB51 and RB51TLR249 groups tended to express less CD62L but more CD44+ for both CD4 and CD8 T cells (Figure15A & 15B), suggesting a memory cell response, which we propose is associated with persistent infection. Upregulation of CD4 in previous studies was linked with antigen specific T cells after exposure to antigens. In one study, mice inoculated with heat killed *Mycobacterium tuberculosis*, the CD4 high T cells tended to express higher levels of CD69 and CD45RB high indicating activated and memory type CD4 T cells, while the CD4 low T cells did not upregulate these markers (35). The CD4 high T cells collected from the LN had increased proliferation *in vitro* compared to CD4 low cells (35). CD4 high T cells represent T cells which were stimulated and became activated. The CD4 high T cells may provide more protection as they displayed increased activation. It is

possible in our study the most protected group RB51TLR24 was past this T cell activation and resolving infection while the RB51 and RB51TLR249 which displayed active T cells had not yet As for CD8 high vs. low cells, there did not seem to be any recent studies addressing differences in function in mice. In humans a CD8 low population was identified in peripheral blood. These CD8 low T cells displayed markers for activated effector cells such as CD25 and CD45RA (36). The CD8 low T cells were more proliferative compared. The functionality of the CD4 and CD8 high and low subset still needs to be determined in a *Mycobacterium tuberculosis* model that CD4 high t cells express CD69 and CD45RB are more functional than CD4 low cells based on activation and proliferation. Functional assessment of CD8 high and low cells are more limited. The functionality of these cells in our model still needs to be investigated Trautmann et al demonstrated a role for CD8 low T cells inducing higher IFN-γ, IL-4, and IL-10 in comparison to CD8 high T cells, and that these cells may have a role in chronic infection of Estein-Barr virus (EBV) (36).

When assessing B cell populations and activation status, the PBS and RB51TLR24 groups had a significantly higher percentage of both B cells, including activated B cells, in comparison to the RB51 and RB51TLR2 9 groups (Fig. 16 The PBS group did not produce high levels of antibodies after vaccination and challenge But, the RB51TLR24, RB51, and RB51TLR249 groups induced similar levels of IgG2a and IgG1 (Fig. 4A, 4B, 4D, 4E). There was not a noted IL-4 production supportive of Th2 response. These data contradict a previous similar study for the RB51TLR2 group where in the most protected mice had decreased activated B cells (1). However, in the Surendran et al. study protected mice showed significant clearance in the lung whereas in this study, there was not significant clearance. Thus infection may be a stimulus to activate B cells. Additional studies are needed to define the contribution of the

humoral response, and plaque forming cell (PFC) assays can be used to identify the number of antibody producing cells or B cell proliferative assays (37, 38) in addition to cytokine and antibody production. Functionality of T-regs can also be similarly assessed. These percentages of activated B cells did not correlate with antibody or cytokine production. Alternatively, as the PBS and RB51TLR24 mice were harvested on one day and the other groups on the second day, it is possible this was an effect of experiment days and difference in handling and staining of the cells.

In addition to serology suggesting that protection was associated with Th1 response based on IgG2a antibody production, organ cytokine levels and splenocyte supernatants also supported a Th1 response, and a role for Il-17. Th1 cytokines assessed included TNF-α, IL-12, IFN-γ. In reviewing the role for cytokines overall, and knowing that TNF-α was shown to be important for clearance of *Brucella* and activation of immune cells (12, 39), overall, with some exceptions, the RB51TLR24 group induced more TNF- α in the organs than the RB51 and RB51TLR249 groups, demonstrating activation of a Th1 type of response (Fig. 17A -20A). The RB51TLR24 group, which had the most non-significant protection/clearance, showed a trend for higher TNF-α production in the MLN, and significantly more TNF- α in the BAL, than the RB51TLR249 group (Fig. 19A & 20A). The increased production of TNF- α in the BAL by RB51TLR24 demonstrates a difference in how the different TLRs can induce cytokines, since the RB51TLR249 group was unable to increase production. The addition of the TLR 9 agonist seemed to alter the immune response, and decreased the ability of the mice to produce Th1 type cytokines. An exception was the spleen, where both the RB51 and RB51TLR249 groups had increased TNF- α production, which was significantly higher than the PBS and RB51TLR24 groups (Fig. 18A); however, this not associated with increased protection/clearance. Increased

TNF-α production by both the PBS and RB51TLR24 groups in the MLN showed that innate immune cells have migrated, and were activating other innate and adaptive immune cells, which could have a role in clearance. Overall, the PBS and RB51TLR24 groups induced more TNF-α production associated with a Th1 type of response. The production of TNF-α seemed to be more protective, and the addition of TLR9 reduced the ability of the cells to produce TNF-α.

IL-12 was also involved in a protective Th1 immune response against *Brucella* (39, 40). Similarly to TNF-α, the PBS and RB51TLR24 groups induced more IL-12 with some exceptions. In the lung, the RB51TLR24 group seemed to induce a Th1 response, based on increased IL-12 and TNF- α production (Fig. 17A & 17B). The RB51TLR24 group non-significantly had more IL-12 than either RB51 or RB51TLR249. The spleen was an exception to RB51TLR24 inducing more IL-12. The RB51 group induced higher IL-12 than the PBS group, and in the BAL, the RB51TLR249 group induced higher IL-12 than the PBS and RB51 groups, indicating a Th1 response (Fig. 20B). Increased IL-12 in the BAL by the RB51TLR249 group compared to the RB51TLR24 group suggests a difference in how TLRs could influence cell activation and cytokine production. However, because clearance was not significantly enhanced, it is possible that the immune response was delayed and/or non-protective for some other reason. It is also possible in the RB51 and RB51TLR249 groups, which did not enhance *Brucella* clearance, the continued antigen stimulation stimulated increased IL-12 production in the different organs. Overall, in most organs, the RB51TLR24 group seemed to induce a stronger Th1 response, whereas the addition of TLR 9 was associated with decreased production of Th1 cytokines.

It was previously demonstrated that IFN-γ production is necessary for clearance and control of *Brucella* (3-5). In this study, there was no IFN- γ production at this time point in lung

and BAL, by the methods used. In a previous study by Surendran et al., levels of IFN- γ were detected using similar methods (9). The lack of production may have prevented significant clearance between groups. BALB/c mice have been known to produce less IFN- γ compared to C57BL10 mice, and therefore have a more difficult time naturally clearing *Brucella* (15). In the MLN, the PBS group produced a trend more IFN- γ compared to the RB51TLR249 group (Fig. 19C), and the RB51TLR24 group also non-significantly produced IFN- γ as well, consistent with the Th1 type of response for TNF-α and IL-12, for both the PBS and RB51TLR24 groups. The production of TNF-α, IL-12 and IFN-γ by the RB51TLR124 group may have contributed to the increase in clearance. By using the organ homogenates, multiple cell types were present and cytokine production in the organ as a whole was assessed, encompassing both innate and adaptive immune response. The cytokines produced reflect the type of response the cells are generating. To understand the inability of the vaccines to protect, as well as decipher the differential ability of vaccines to stimulate immunity, cytokines for not only Th1 (IL-12, TNF-α, IFN-γ), but also Th2 (IL-4), T-reg (IL-10) and Th17 (IL-17) were measured.

In assessing the Th2 response, there was no significant difference in IL-4 production and there was overall no or limited production in the organs assessed. This along with Th1 cytokine production suggested that there was not a strong Th2 type of response. In the RB51TLR24 group it was likely the Th1 response was dominant, due to the greater production of Th1 vs. Th2 cytokines (TNF-α, IL-12 and IFN-γ vs. IL-4). Additionally, there were not higher levels of IgG1 compared to IgG2a to indicate a biased response. These results suggested a strong Th2 response was not generated.

In assessing the T-reg response, IL-10 was produced (Fig. 17C, 18D, 19E, 20C). However, the lack of differences between treatment groups suggested that while there may be a

role in influencing protection, there was not a critical role in the differential immune response between treatment groups. BALB/c mice infected with *Brucella* produce IL-10, and it can negatively affect clearance (41). The role of IL-10 should be further assessed as this could have inhibited clearance; one possible way may be the use of IL-10 knockout mice or depletion of IL-10 by antibodies to view dampening effects on the immune system.

In comparison, there may be a differential role for IL-17 in clearance/protection. IL-17 was produced, but there were no significant differences in IL-17 production in the lung and spleen (Fig. 17D & 18E). In the BAL and MLN, there was significantly more IL-17 produced by the RB51TLR24 group, which also had the highest non-significant level of clearance, thus supporting that IL-17 could enhance clearance and contribute to protection (Fig. 20D &19F). Previously, IL-17 was shown to provide protection against infection when IFN-γ production was impaired (16). IL-17 has shown to play a role in controlling infection at the mucosa of mice against *K. pheumoniae* in the lung and *C. rodentium* in the gut (17). Its role against *Brucella* infection should be further investigated.

Data from stimulated splenocytes also supports a role for a Th1, and possibly T-reg and Th17 response. For IL-12, the RB51 and RB51TLR24 groups had significantly higher ConA induced IL-12 than the RB51TLR249 group (Fig. 21A). This could be suggestive of differences in how the TLR9 pathway stimulates an immune response/protection. Additionally, based on the increased IL-12 production by the RB51 group, when stimulated with IR2308 (Fig. 21A), this could support a greater immune response in the spleen and/or could be due to ongoing infection. The RB51 group also tended to have higher DC activation in the spleen vs. the PBS group.

Although, IFN-γ was not detected in organ homogenates, when stimulated with antigen specific IR2308, all groups had increased IFN-γ production vs. the PBS group (Fig. 21B), the vaccinated groups were better able to produce IFN-γ which could lead to clearance. The RB51 and RB51TLR24 groups had increased IFN- γ production when stimulated with antigen specific IRRB51, HK2308 and HKRB51 compared to the RB51TLR249 and PBS groups supporting the antigen specific Th1 immune response. The RB51TLR249 group was greater than the PBS group, indicating an antigen specific Th1 response (Fig. 21B). The antigen specific IFN- γ production may have contributed to the enhanced non-statistically significant clearance/protection of the RB51TLR24 group, while clearance was not increased in the RB51TLR249 group, the latter group had lower levels of IFN-γ to some antigen (IRRB51, HKRB51, HK2308).

In assessing IL-4 production and the Th2 response based on splenocyte stimulated supernatants. There was a pattern that the RB51 group splenocytes had higher IL-4 when stimulated with media and ConA, while the PBS group splenocytes had higher levels when stimulated with any of the antigen specific mitogens. However, based on the relatively low levels of IL-4 produced, Based on the limited and/or lack of IL-4 production by organ homogenates, these data also indicate that a Th2 response was not the predominant response by the groups.

By comparison, in assessing a T-reg response, IL-10 was detected in the splenocyte supernatants and by all organ homogenates, but there were no significant differences in the organs or in the BAL. Overall there was a pattern that RB51TLR249 and RB51 stimulated more IL-10 production vs. PBS and sometimes RB51TLR24 with antigen specific stimulation (Fig. 21C). Both the RB51 and RB51TLR249 induced higher IL-10 compared to the RB51TLR24 group which led to decreased clearance in comparison. It is possible that the RB51 and

RB51TLR249 groups had a bias towards IL-10 production and T-regs with antigen specific stimulation that could have affected/contributed to persistent infection. Additional studies are needed to assess the role of T-regs and IL-10 in infection/protection. The literature supports production of IL-10 downregulates the immune response and reduces protection to infection (41). Our data supports that IL-10 produced by the RB51 and RB51TLR249 groups which could limit the immune response to reduce clearance of the bacteria.

In addition to measuring Th1, Th2 or T-reg responses, a superficial assessment of the Th17 responses was performed, with a limited number of samples. The RB51 and RB51TLR249 groups' stimulated splenocytes produced greater IL-17 when stimulated with antigen specific mitogens (IR2308 vs. ConA). This supports that *Brucella* can induce IL-17 production. Organ homogenates did contain levels of IL-17. In the BAL, the RB51TLR24 group has significantly higher IL-17 production versus to RB51, and has higher levels of clearance, may indicate a protective role for IL-17. These data are consistent with other published studies which demonstrated that vaccination with other strains could induce IL-17 which was protective against *Brucella* infection (16). IL-17 at the mucosal level was important in providing protection against other pathogens such as *K. pneumonia*, and needed to induce a Th1 response in *M. tuberculosis* and *Listeria* (17). Collectively, these data support that further studies are needed to define the role for IL-17 produced by the splenoctyes.

Overall, most significantly from this study, we saw that the most protected group RB51TLR24 seemed to induce higher levels of CD11b medium and high DC compared to the RB51 and RB51TLR249 groups. The RB51TLR24 group had lower levels of activated T cells; however, it did have higher CD25 expression, which based on cytokine profiles could be either T-reg cells as there was some IL-10 production or activated T cells as there was a reduction in

the CFUs. At this time point, it also had fewer activated DC likely due to resolving infection. The RB51TLR249 group at this time point induced the highest levels of CD103 DC which were previously implicated in providing protection. Additionally, this group tended to have more activated T cells likely due to persistent infection. The RB51TLR249 group did not produce high levels of protective Th1 cytokines such as IFN-γ, TNF-α, and IL-12 in many of the organs and after splenocyte stimulation. Instead, it showed higher production of IL-10 in the splenocyte supernatants. The addition of TLR9 did not enhance protection and may have altered signaling and weakened the Th1 response. Our data supports altered diminished Th1 response vs. increased Th2 or T-reg response. In past studies, RB51 has shown to induce a Th1/ CMI, response and was the main immune response responsible for protection (18). In this study the RB51 group did upregulate activation markers for DC and T cells, and did upregulate IL-12 and IFN-γ in the splenocyte supernatants supporting a shift to a Th1 or CMI response which was similar to past models and studies using mice but did not enhance clearance.

Overall the addition of TLR 2, 4, and 9 agonists as a single treatment with strain RB51 did not generate a stronger Th1 immune response or protection than the TLR 2 or 4 agonists used individually. There were changes to the immune response suggesting further evaluation on how TLRs when stimulated simultaneously can activate the immune response. Our most promising vaccine candidate used TLR 2 and 4 agonists, stimulated the most clearance and a stronger Th1 immune response in comparison to the agonists altogether. Neither the PBS control group nor the strain RB51 group mounted a sufficient immune response or enhanced clearance. Further exploration of the use of TLR 2 and 4 as a potential vaccine adjuvant with strain RB51 vaccination should be conducted. Additional studied focused on the mechanisms associated with the lack of protection of the other treatment groups are also warranted.

Figures

Figure 1

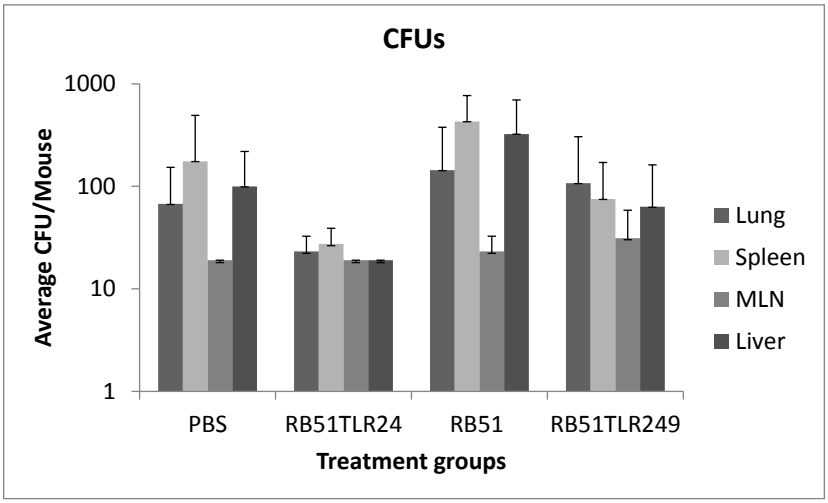


Figure 1: Average colony forming units isolated per organ and clearance of *B. abortus* strain 2308 following intranasal vaccination and challenge. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51) or, strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge, mice were euthanized. Lungs, liver, spleen, and MLN were collected and homogenized to measure the CFU. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as CFU +/- SD.

Figure 2

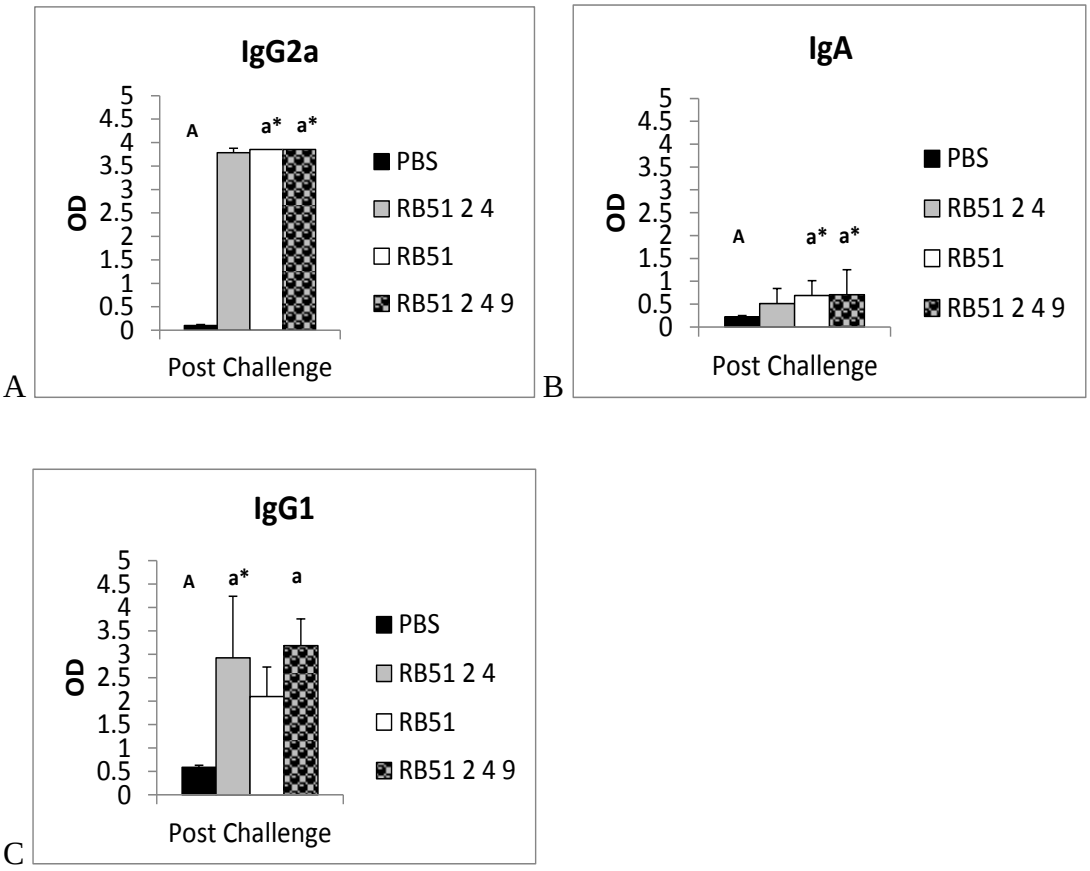


Figure 2: Immunoglobulin isotypes against strain RB51 were measured in the bronchoalveolar lavage by ELISA. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized. Immunoglobulin levels were determined the BAL for A) IgG2a, B) IgA, C) IgG1. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, significance $p<0.05$. All statistical analysis was done using SAS system (NC,

USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 3

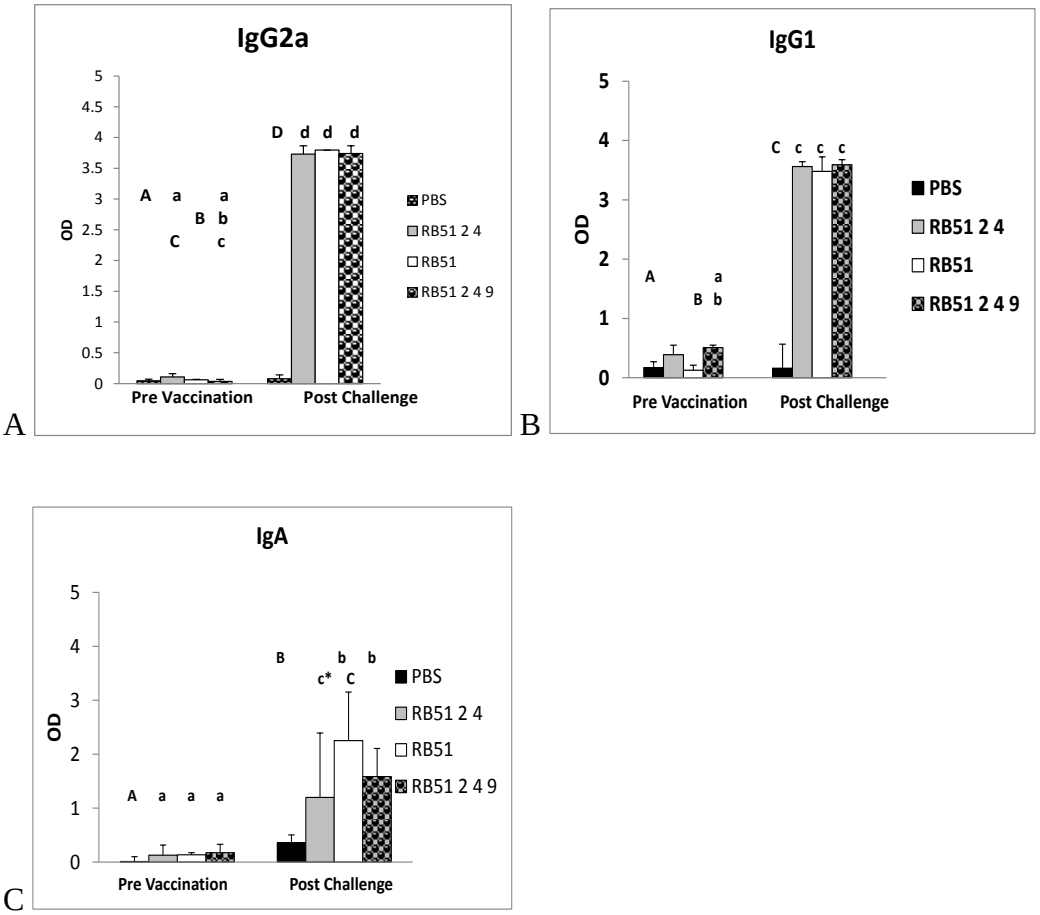


Figure 3: Immunoglobulin isotypes against strain RB51 were measured in the serum by ELISA. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized. Immunoglobulin levels on pre vaccinated and post challenged serum samples were determined for A) IgG2a, B) IgA, C) IgG1. Statistical

analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, significance $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 4

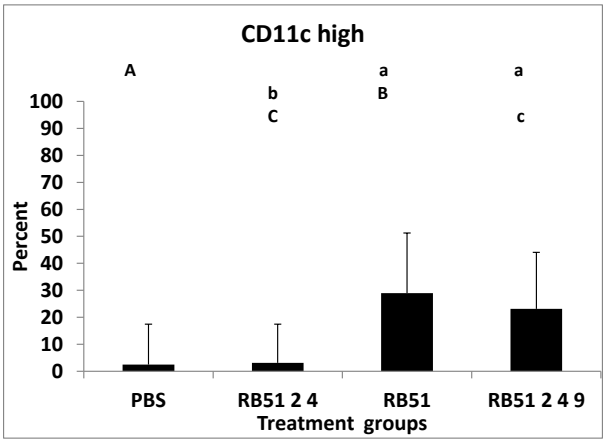


Figure 4: CD11c high cells in the bronchoalveolar lavage. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD11c low DC were determined by flow cytometry analysis. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 5

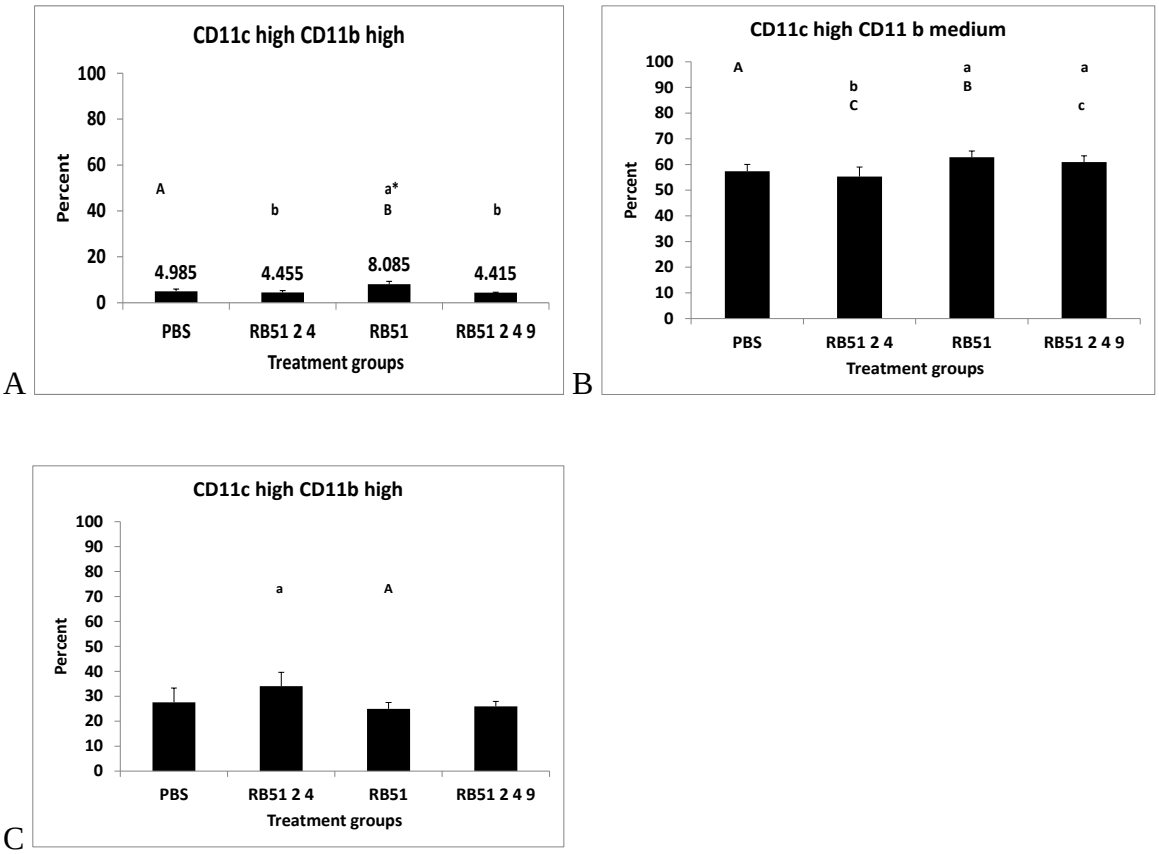


Figure 5: CD11c high cells were CD11b positive in the BAL. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD11b DC expression were determined by flow cytometry analysis from the BAL A) CD11c high CD11b high as a percent of total CD11c high cells in the BAL, B) Percent of CD11b cells which were CD11b medium in the spleen, C) percent of CD11b cells which were CD11b high in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA).

Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend (0.05<p<0.1).

Figure 6

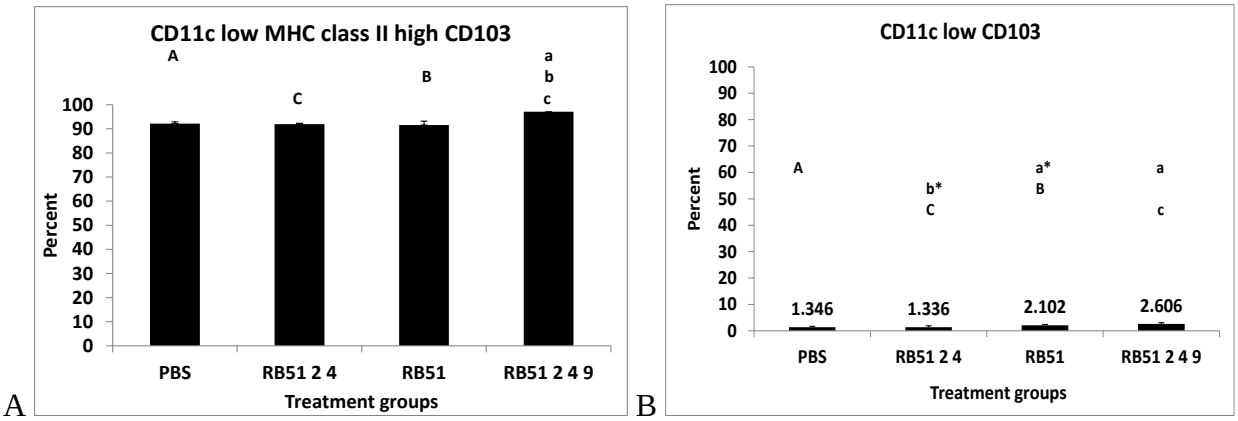


Figure 6: CD103 CD11c low DC in the bronchoalveolar lavage and spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD103 DC expression were determined by flow cytometry analysis from the BAL and spleen A) CD11c low MHC class II high CD103 in the BAL, B) CD11c low CD103 in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, p<0.05. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend (0.05<p<0.1).

Figure 7

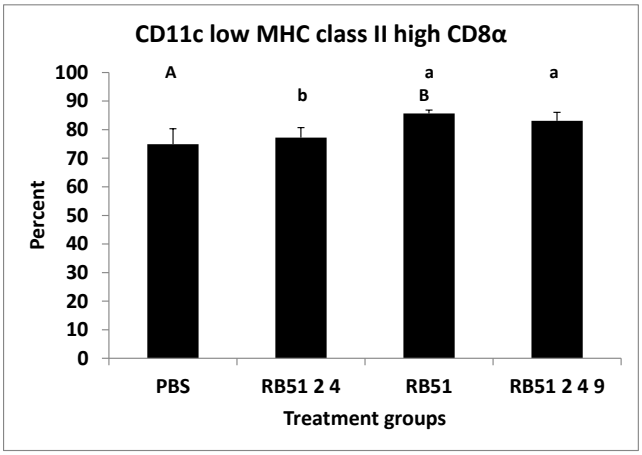


Figure 7: CD11c low MHC class II high CD8α expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD8α DC expression as a percentage of CD11c low DC were determined by flow cytometry analysis in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 8

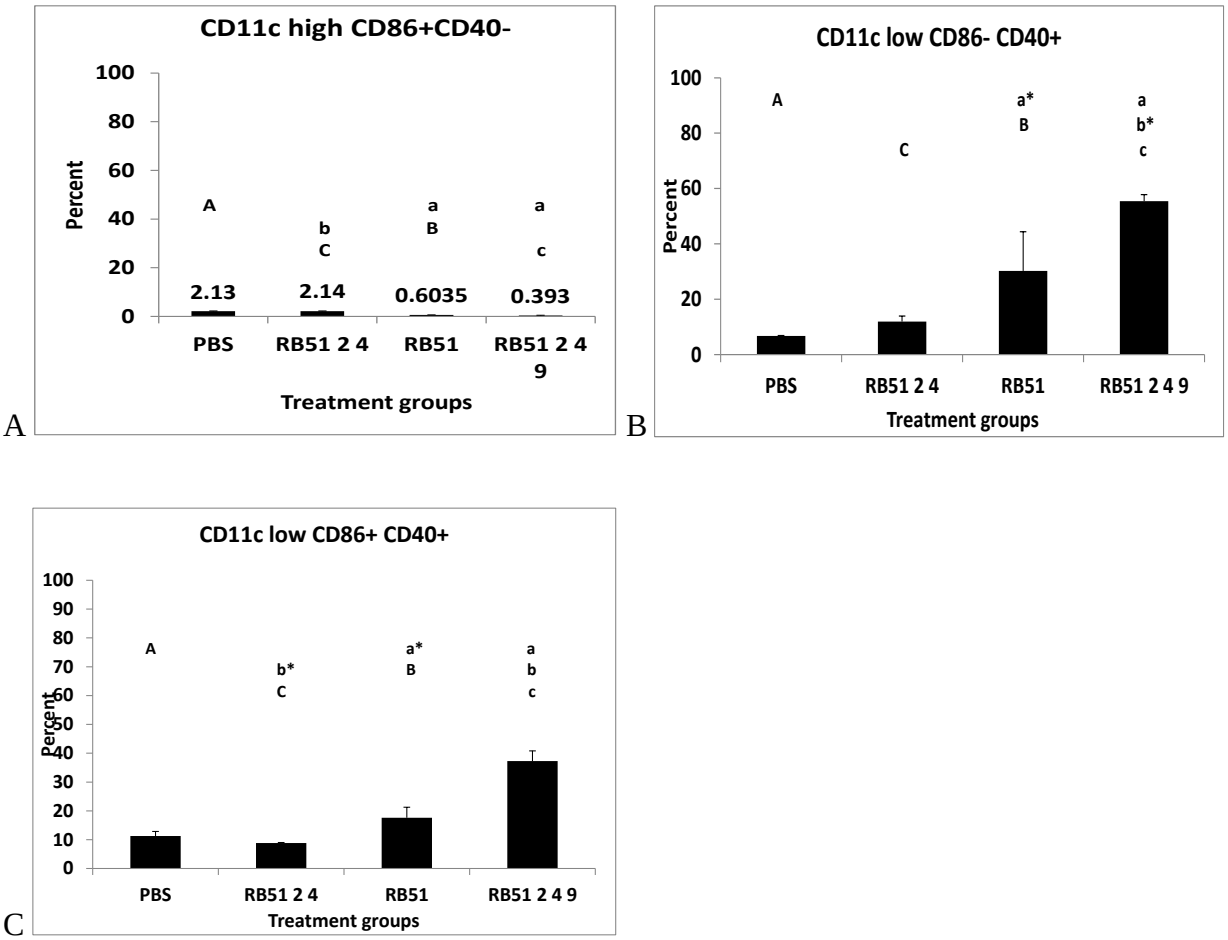
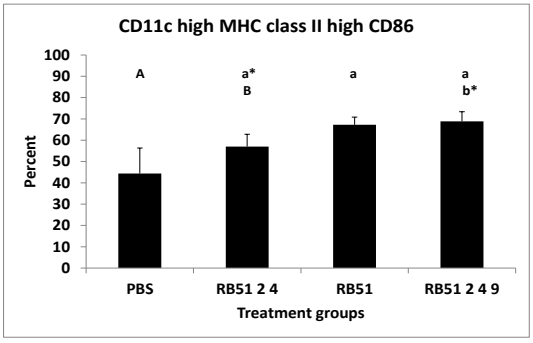


Figure 8: DC activation based on CD86 and CD40 expression in the bronchoalveolar lavage. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high or CD11c low and CD40 and CD86 expression were determined by flow cytometry analysis from the BAL A) CD11c high CD86+ CD40-, B) CD11c low CD86- CD40+, C) CD11c low CD86+ CD40+. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis

was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 9

A



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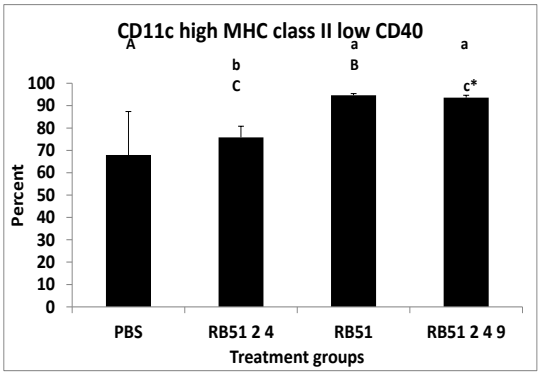


Figure 9: DC activation based on MHC class II, CD86 and CD40 expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high MHC class II and CD86 or CD40 DC expression were determined by flow cytometry analysis from the spleen A) CD11c high class II high CD86 B) CD11c high MHC class II low CD40. Statistical analysis was

performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 10

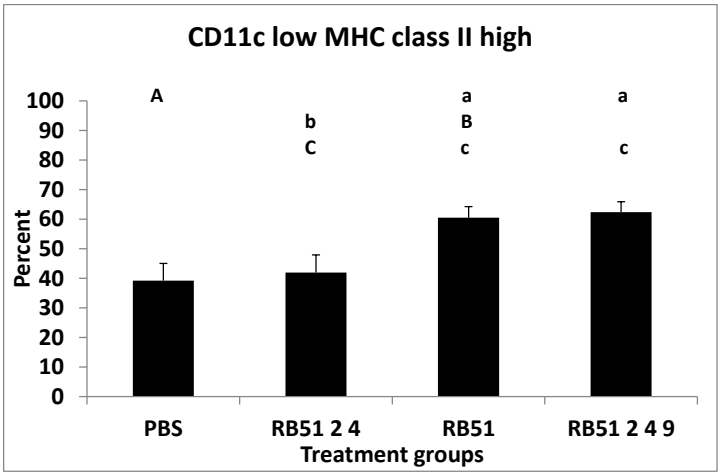
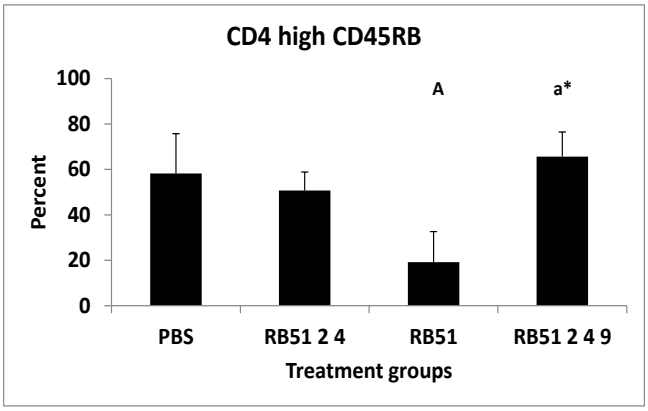


Figure 10: DC activation based on MHC class II high expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high MHC class II expression were determined by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 11

A



B

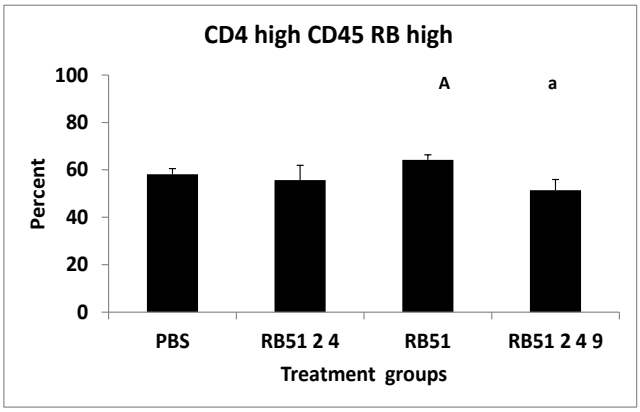


Figure 11: Lymphocyte activation based on CD4 high CD45RB. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). 14 days post challenge mice were euthanized, and CD4 high and CD45RB expression were determined by flow cytometry analysis from the BAL and spleen, A) CD4 high CD45RB in the BAL, B) CD4 high CD45RB high in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/-

SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend (0.05<p<0.1).

Figure 12

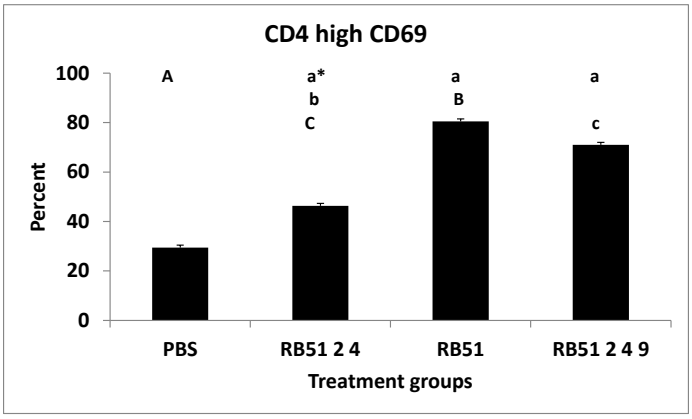


Figure 12: Lymphocyte activation based on CD4 high CD69 in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD4 high CD69 expression were determined by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend (0.05<p<0.1).

Figure 13

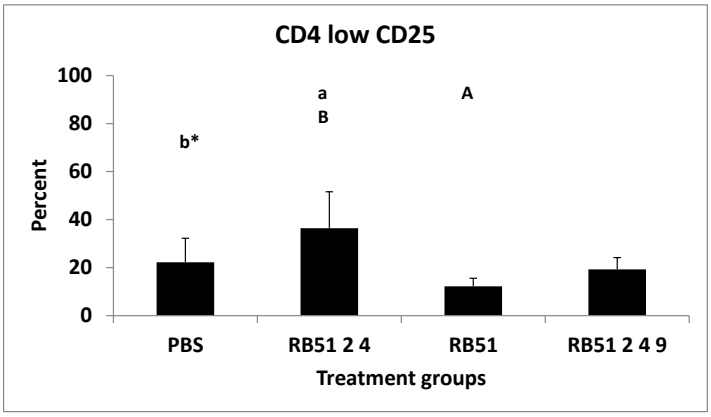


Figure 13: Lymphocyte activation based on CD4 low CD25 expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD4 low CD25 expression were determined by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 14

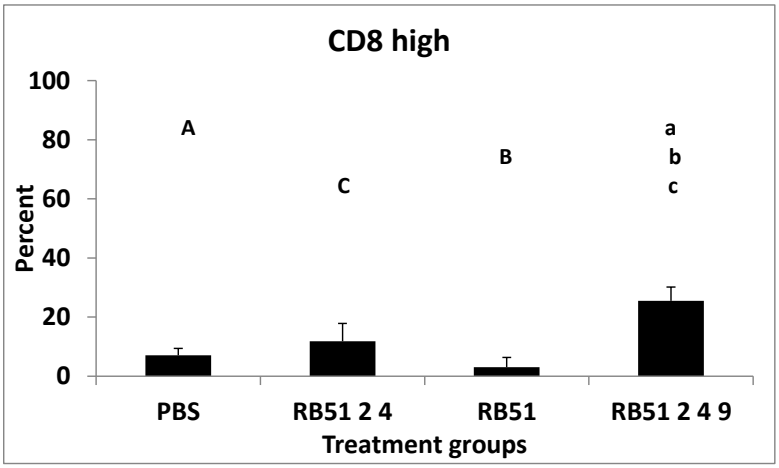


Figure 14: CD8 high lymphocytes in the BAL. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD8 high expression were determined by flow cytometry analysis from the BAL. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 15

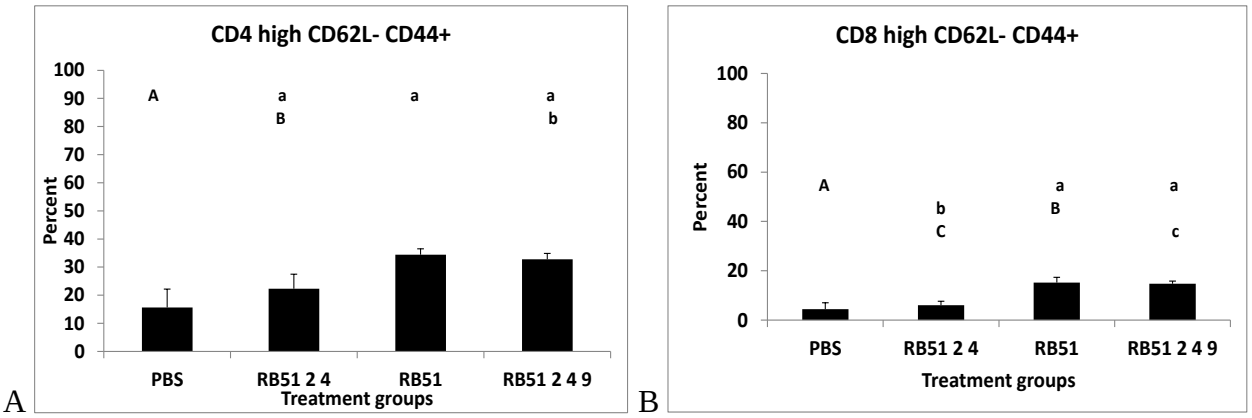


Figure 15: Memory and naïve lymphocytes in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD44 and CD62L expression in CD4 and CD8 lymphocytes were determined by flow cytometry analysis in the spleen A) CD4 high CD62L- CD44+, B) CD8 high CD62L- CD44+. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 16

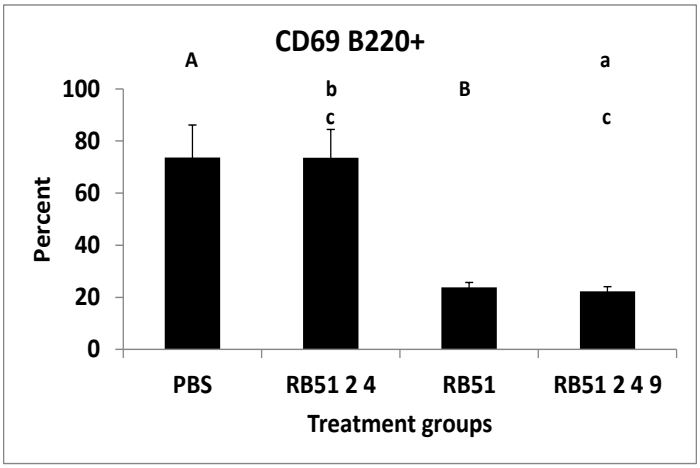


Figure 16: B lymphocyte activation in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and B220 was used to identify B lymphocytes and CD69 was used as a marker for activation by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 17

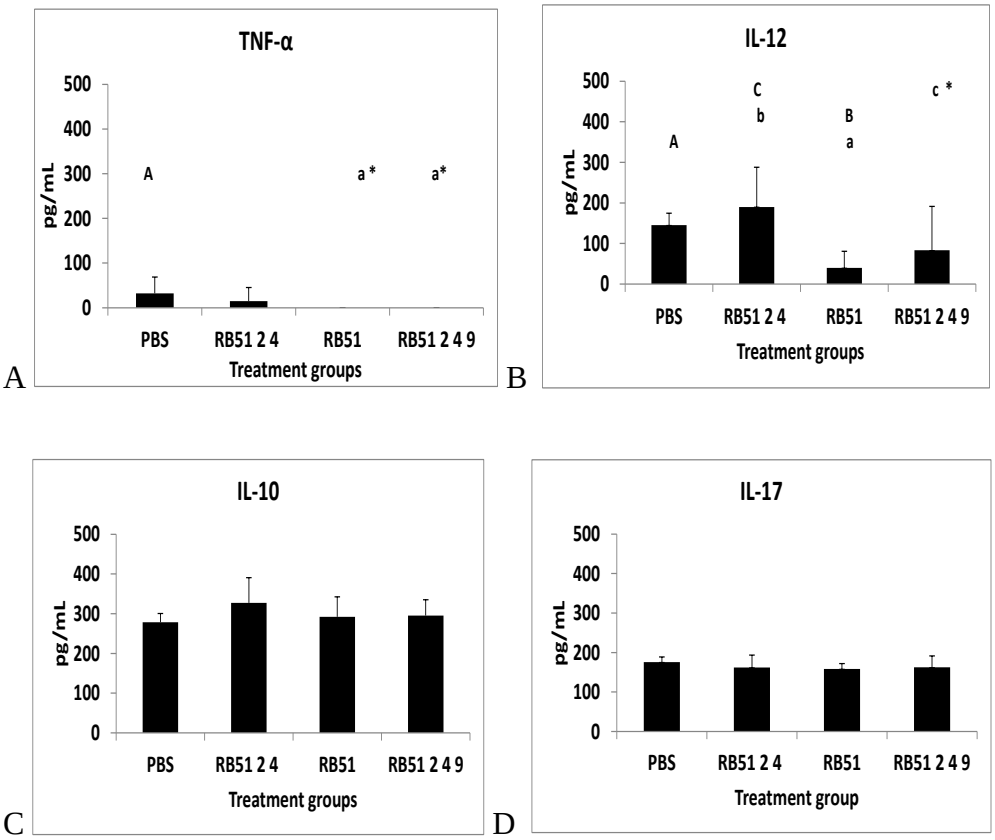


Figure 17: Cytokine expression by lung homogenates examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, lungs were collected and homogenized. Cytokines were measured by ELISA: A) TNF-α, B) IL-12, C) IL-10, D) IL-17. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant

differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 18

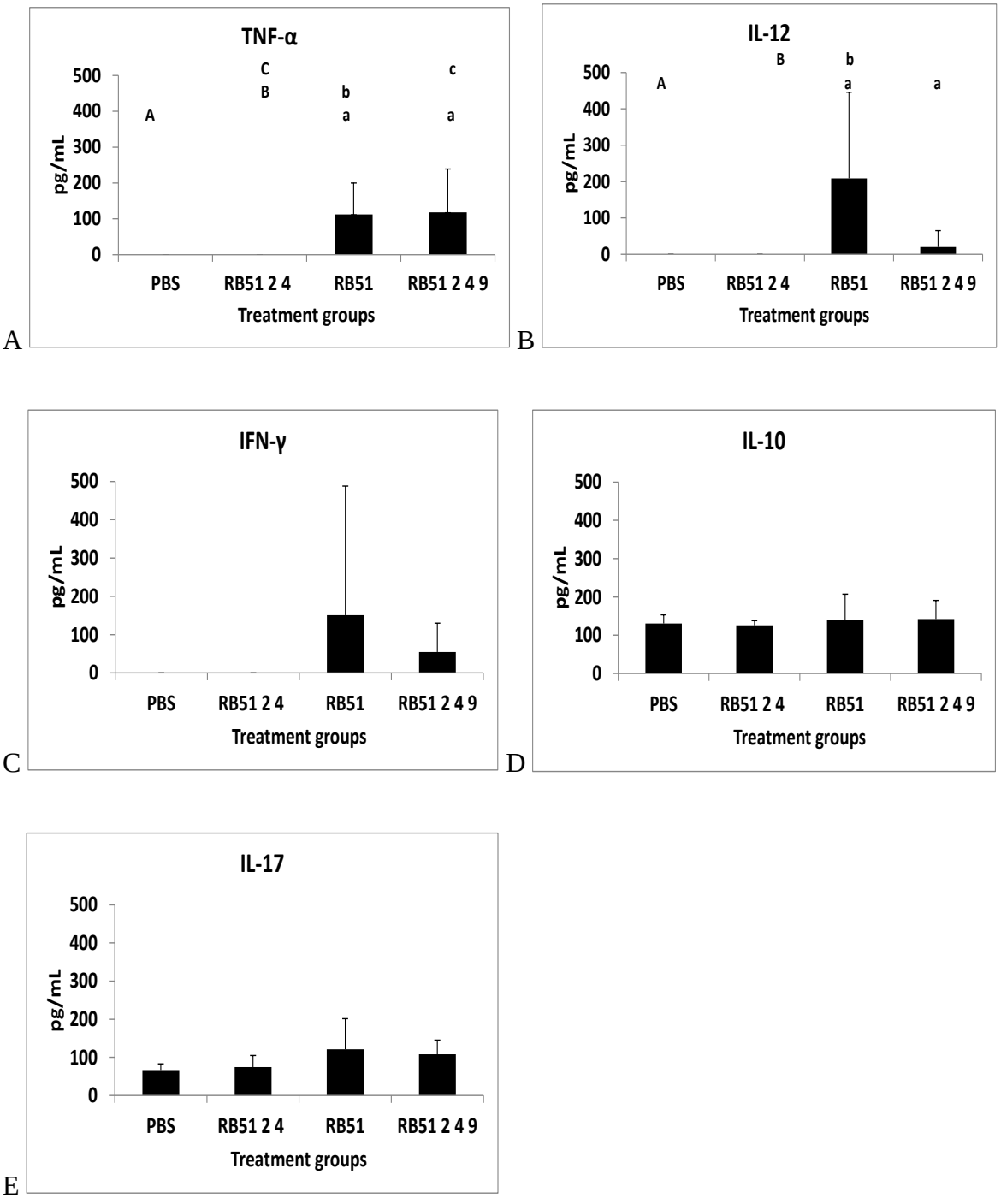
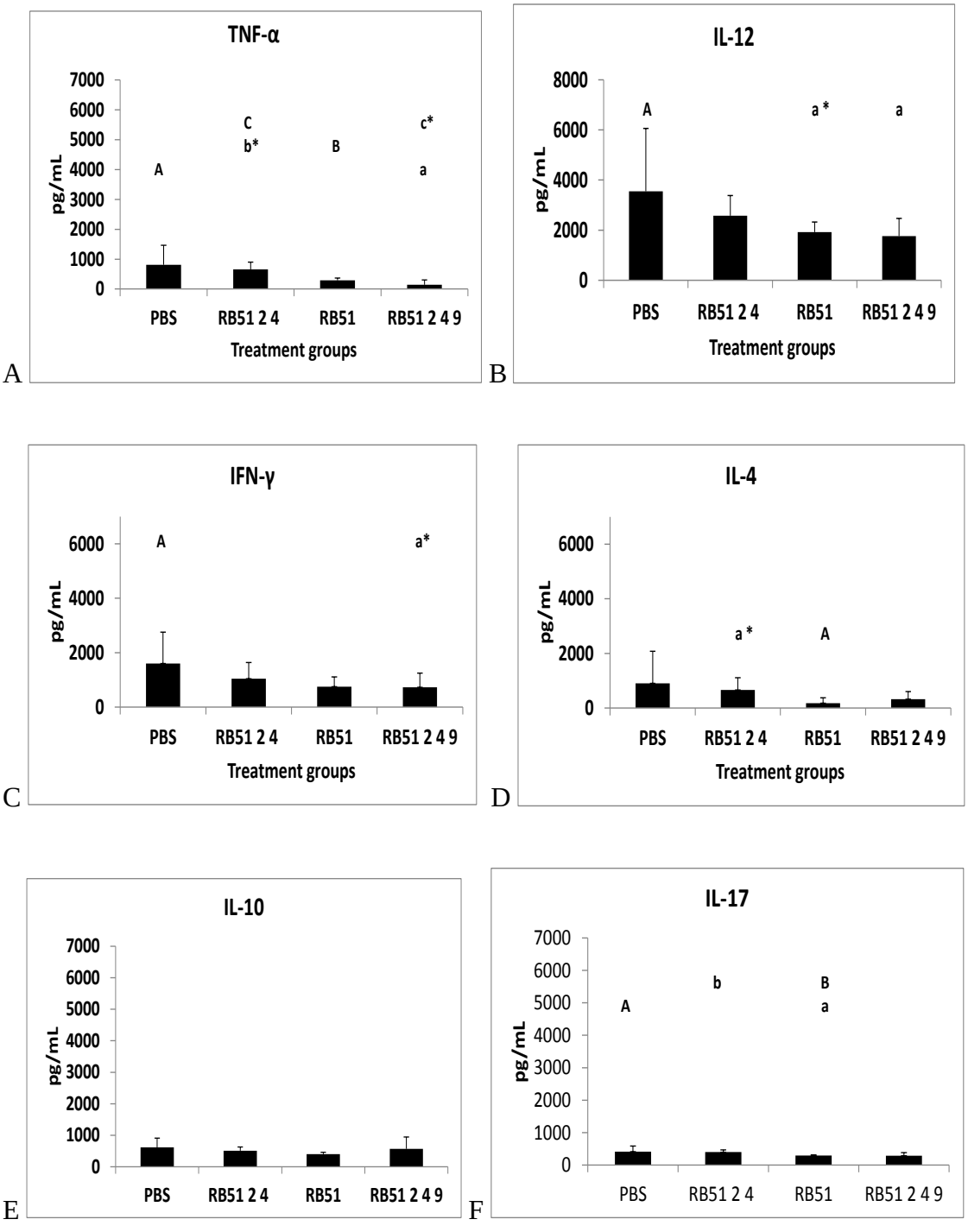


Figure 18: Cytokine expression by spleen homogenates examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, spleens were collected and homogenized, cytokines were measured by ELISA, A) TNF-α, B) IL-12, C) IFN-γ, D) IL-10, E) IL-17. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 19


A
TNF-α
pg/mL
7000
6000
5000
4000
3000
2000
1000
0
A
C
b*
B
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a
PBS
RB51 2 4
RB51
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Treatment groups
B
IL-12
pg/mL
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6000
4000
2000
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a*
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RB51
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Treatment groups
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IFN-γ
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Treatment groups
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IL-10
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PBS
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RB51
RB51 2 4 9
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IL-17
pg/mL
7000
6000
5000
4000
3000
2000
1000
0
A
b
B
a
PBS
RB51 2 4
RB51
RB51 2 4 9

Figure 19: Cytokine expression by MLN homogenates examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, MLN were collected and homogenized, cytokines were measured by ELISA, A) TNF-α, B) IL-12, C) IFN-γ, D) IL-4, E) IL-10, F) IL-17. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 20

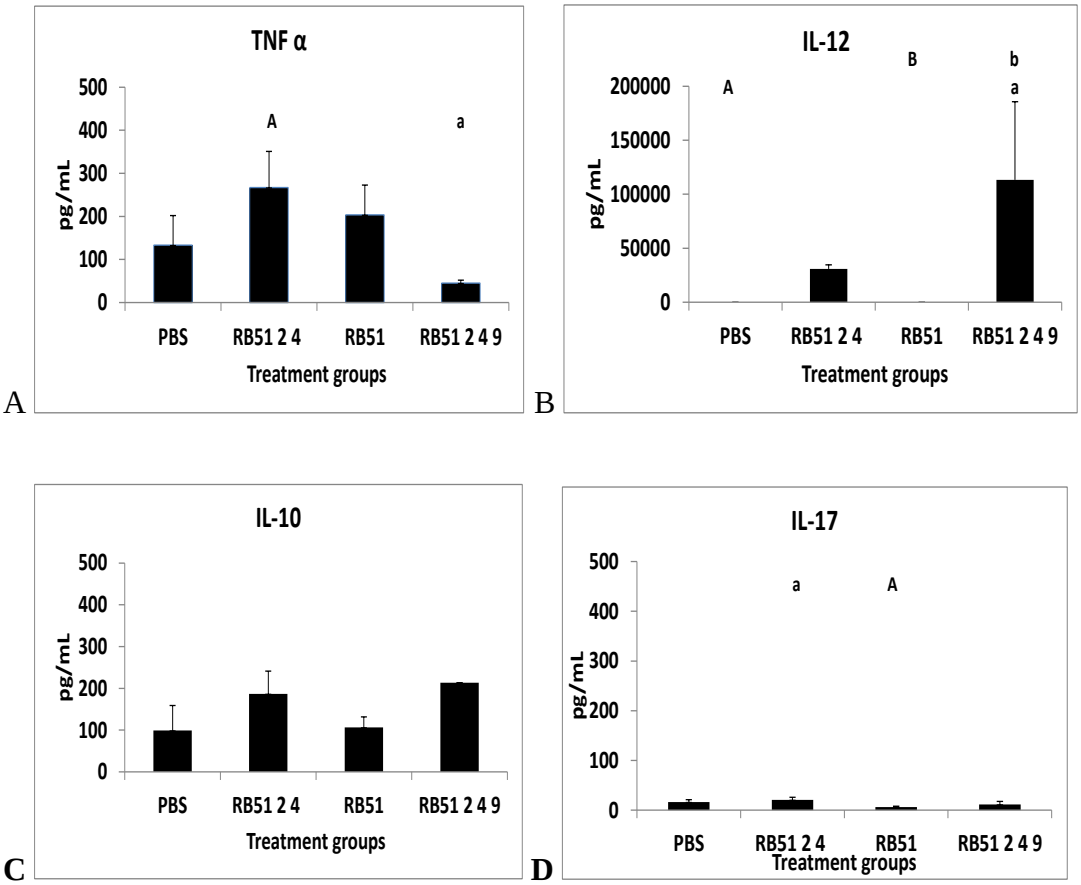
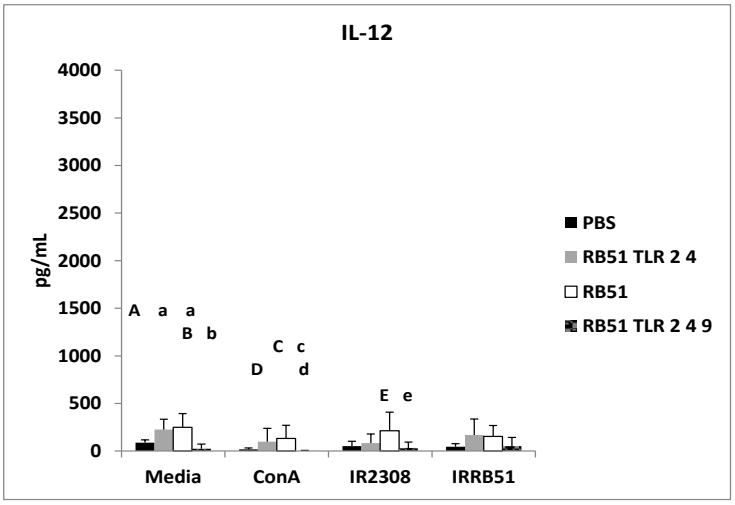
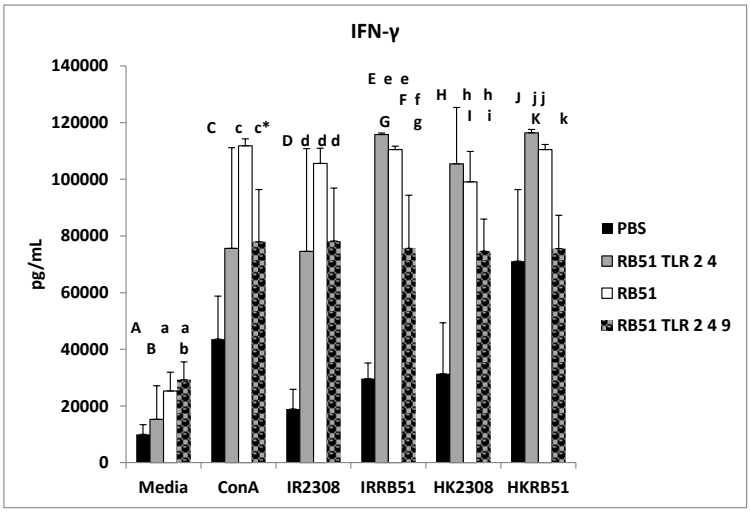


Figure 20: Cytokine expression by BAL homogenates examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, BAL were collected and cytokines were measured by ELISA, A) TNF-α, B) IL-12, C) IL-10, D) IL-17. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/-

SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 21

A 

B 

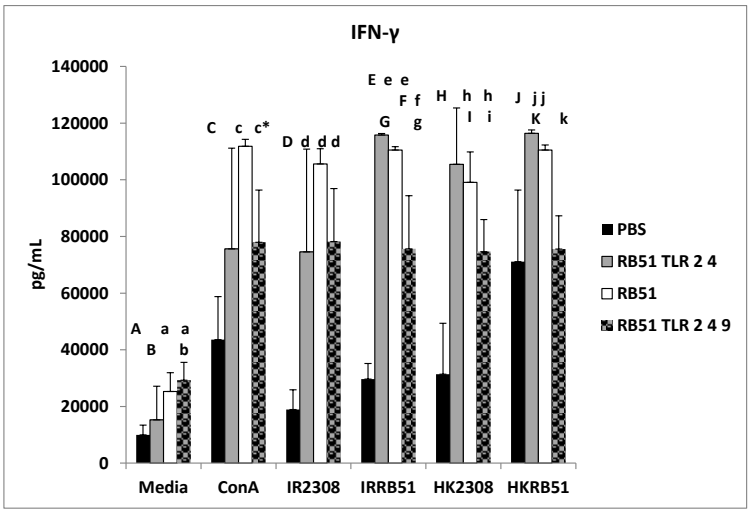
C 

Figure 21: Cytokine expression by stimulated splenocytes examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, splenocytes were collected and stimulated with either media, ConA, irradiated strain 2308, irradiated strain RB51, heat killed strain 2308 or heat killed strain RB51 (10^6 CFU106 CFU/well). Cytokines were measured by ELISA, A) IL-12 B) TNF-α, C) IL-10. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

References

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Chapter 3

Future Directions

The use of TLR agonists as vaccine adjuvants should be studied further. Current knowledge is limited on the ability of different agonists to perform as adjuvants when combined with strain RB51. In this study, the combination of TLR 2, 4, and 9 agonists did not enhance protection but did show a differences in the immune responses generated. Additional studies are warranted to discern the optimal agonists in combination with the vaccine and its effects to upregulate the immune response.

The use of multiple TLR agonists has not been extensively studied. Previous studies have shown that TLR agonists can work collectively to increase the immune response (1). The dose of the TLR agonists needs to be examined further to determine if there is a more optimal dose and/or time course for when the agonists are administered in combination with vaccination. The doses in this study were based on the individual doses which enhanced protection (2, 3). A study measuring varying doses and frequency of TLR agonists with vaccination when mixed together may show the optimal concentrations/timing to induce higher protection.

In addition, experiments over time would be ideal to further define the immune endpoints associated with the different treatment groups. There were differences in cell activation based on the different TLR agonists given to each group. With just one time point, the complete immune response over time cannot be observed. Some differences in activation could have been due to the delay in clearance, and the presence of increased bacterial loads causing stimulation to activate the immune cells. With multiple time points we could see if the less protected groups eventually show similar CFU, cell activation, and cytokine production at a later time point. A

study using more time points would provide a broader picture of the overall immune response with the addition of the TLR agonists to vaccination.

Mechanisms by which the TLRs can be regulated should also be further explored as TLRs influence cellular activation and function. Some of the differences in signaling could be associated with signaling molecules such as Triad3a, or cytokines such as TGF-β. Triad3a, which functions as an E3 ubiquitin-protein ligase (4, 5), can reduce TLR 4 and 9 expression. Additionally, TGF-β can reduce TLR4 activation of NF-κB (4). Intracellular signaling can be modulated in multiples ways such as protein degradation, phosphorylation, and inhibitory adaptor molecules (4). TLR 9 was the only agonist used which is located in the endocytic compartment instead of on the surface of the cell. There are many points during intracellular signaling which can be altered to change the response of the cells. Experiments examining how the addition of individual agonists added to this particular infection model should be conducted to measure the differences in immune cell activation and monitor differences in intracellular signaling. This would allow us to better understand how each individual TLR agonists alters the immune response. Once the mechanism by which the individual TLR agonists effect the cells are identified, then the cellular response of the agonists added together should be measured. For these experiments, measuring the upregulation activation markers and intracellular signaling would give us a better understanding of how the agonists together influence the immune cells such as DC. Measuring intracellular signaling could possibly be done using flow cytometry and upregulation of activation markers could be determined. A better understanding of how the TLR agonists activate the signaling cascades and any control mechanism would potentially allow for a more effective immune response and perhaps identification of the ideal agonists to use as a method of upregulating the immune response to *Brucella* infection.

There were significant differences in the cytokine profiles of the different groups. The increase in IL-17 after vaccination using TLR 2 and 4 agonists, as well as using TLR 2, 4, and 9 agonists collectively demonstrated the possibility of a role for Th17 cells in clearance and protection. Experiments examining the effects of IL-17 should be conducted. Using an *in vitro* model, infected cells such as DCs could be exposed to IL-17 and examined for the upregulation of activation markers, and their ability to activate T cells. Infecting IL-17 KO mice or using cells from the IL-17KO mice, and then infecting them would also provide information on if the removal of IL-17 would help or hinder clearance. These KO mice or cells could then be supplemented with IL-17 to see the effects on the immune response could be returned to normal levels.

IL-10 was also produced and stimulated splenocytes from the RB51TLR249 group induced higher levels of IL-10, a regulatory cytokine, which could have limited clearance. The addition of TLR 9 may have stimulated a T-reg response. T-reg cells can also be identified with other markers such as FoxP3 (forkhead box P3) in addition to IL-10 production to confirm T-reg cells. The effect of IL-10 production from the addition of the agonists should be further evaluated and could be examined similarly to IL-17.

Looking further into the different DC subtypes as well as expression markers should also be considered. In different models CD103 or CD11b DC can induce different immune responses such as CD4 vs. CD8 T cell activation, and suggests variation in offering protection against pathogens (6, 7). In addition, studies demonstrated possible differences in DC function with CD11c high vs CD11c low DC, suggest that CD11c high DC may be more functional, and thus provide better protection (8). Furthermore, CD11c low CD11b high could better activate CD4 T cells and CD11c high CD11b low subset did not (9). Our current knowledge of these subsets and

their function and role in immunity against *Brucella* is limited. These data and our results suggest assessing the functionality of these specific subsets is warranted.

In this study the focus was primarily on activation and function of the DCs and lymphocytes. While DC play a large role in the innate immune response, there are other immune cells which play a role as well. We did not examine how the TLR agonists affected the macrophages, neutrophils, or natural killer cells which all play roles in innate immune response and can influence the adaptive immune response. Examining the response of these other innate cells would also provide additional information on the effects of the TLR agonists with strain RB51 vaccination and how all of the innate immune cells come together to influence over all immunity to *Brucella*.

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Appendix

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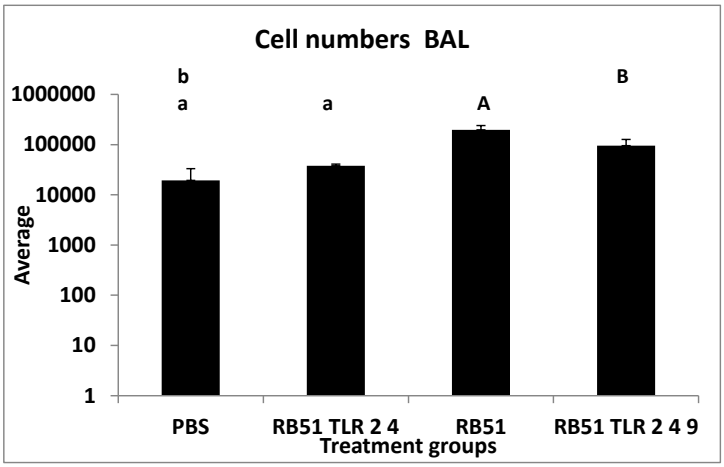

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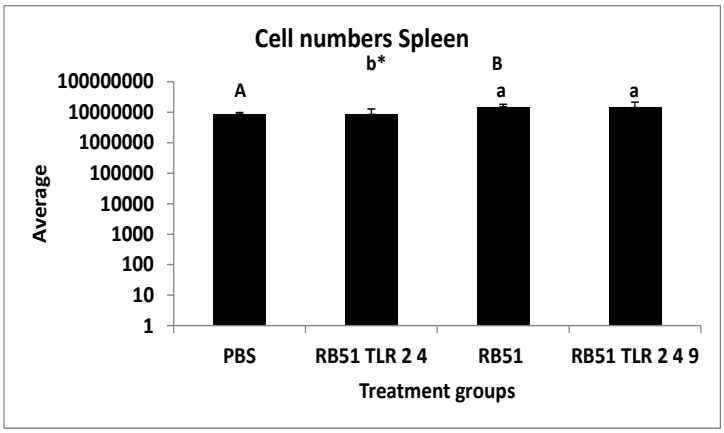


Figure 1: Average of cells recovered from the bronchoalveolar lavage and spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51) or, strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge, mice were euthanized. Cells were counted from the BAL and spleen. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as CFU +/- SD.

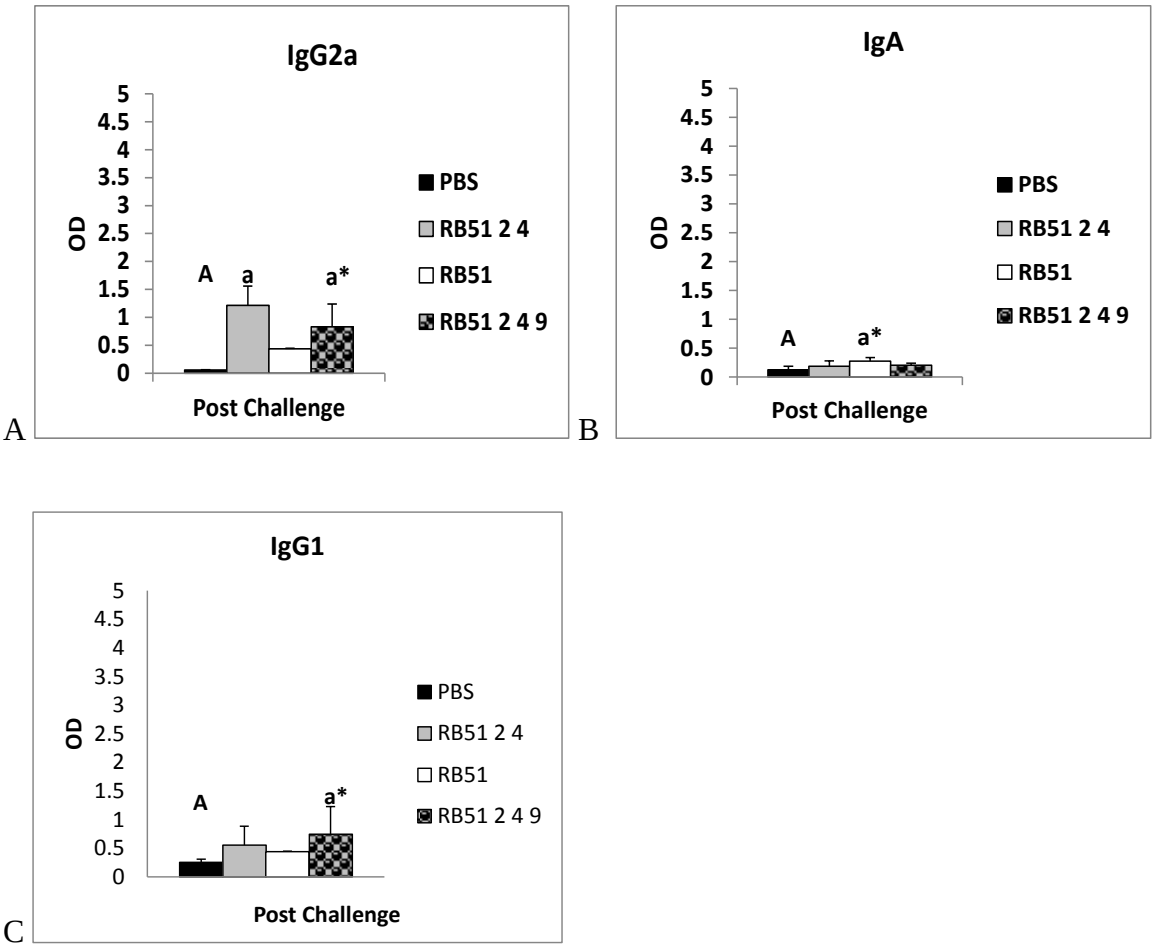
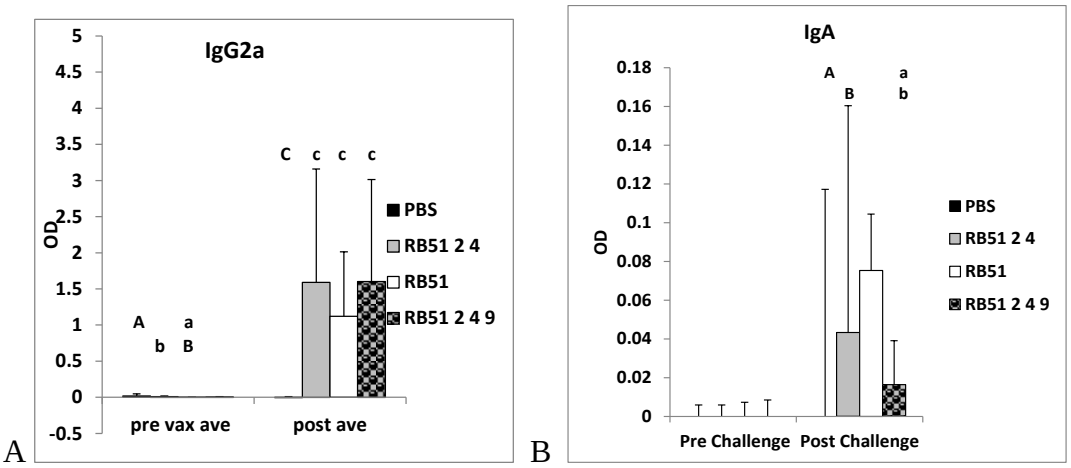


Figure 2: Immunoglobulin isotypes against strain 2308 were measured in the bronchoalveolar lavage measured by ELISA. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized. Immunoglobulin levels were determined the BAL for A) IgG2a, B) IgA, C) IgG1. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, significance $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).



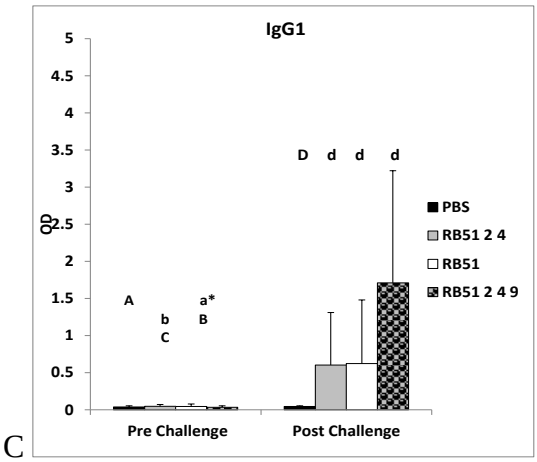


Figure 3: Immunoglobulin isotypes against strain 2308 were measured in the serum measured by ELISA. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized. Immunoglobulin levels on pre vaccinated and post challenged serum samples were determined for A) IgG2a, B) IgA, C) IgG1. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, significance $p<0.05$. All statistical analysis was done using SAS

system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

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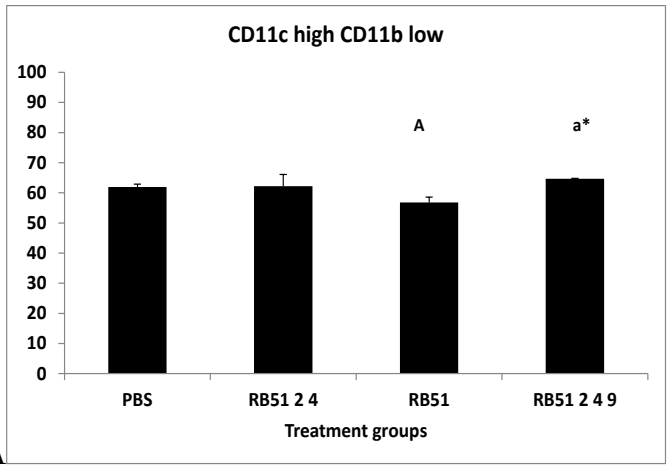
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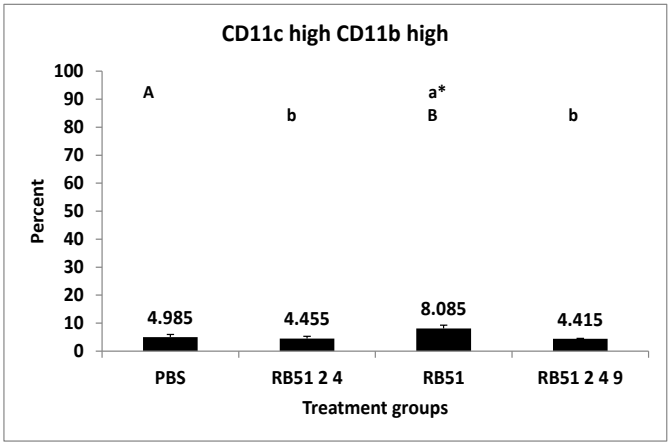
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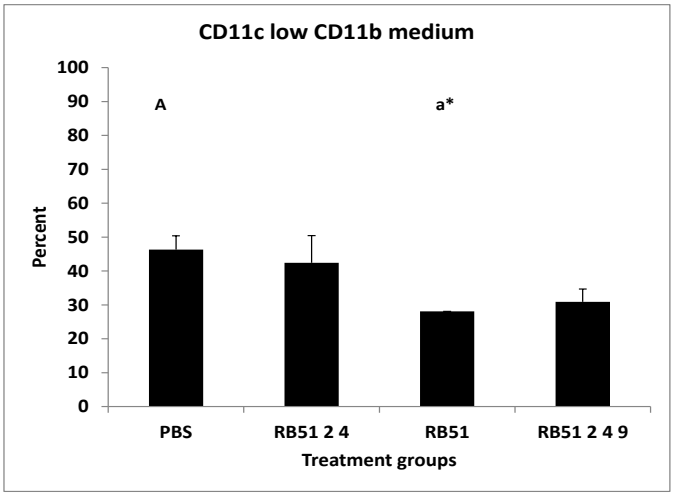
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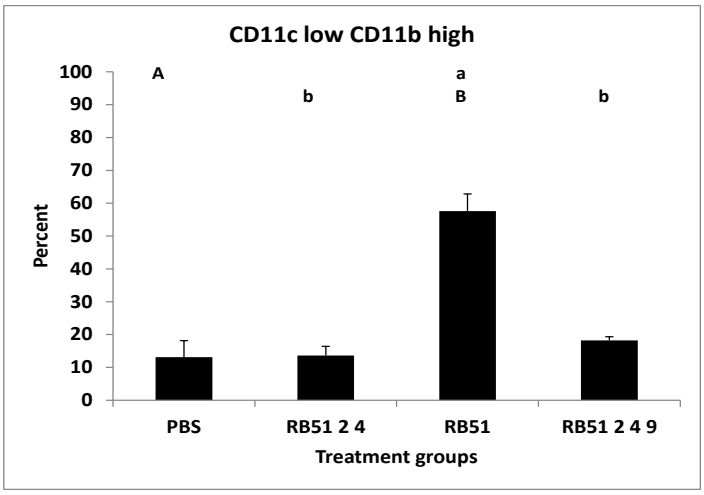
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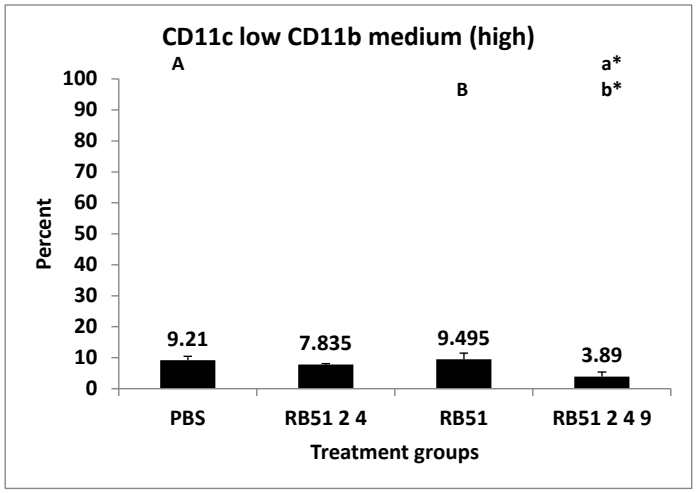
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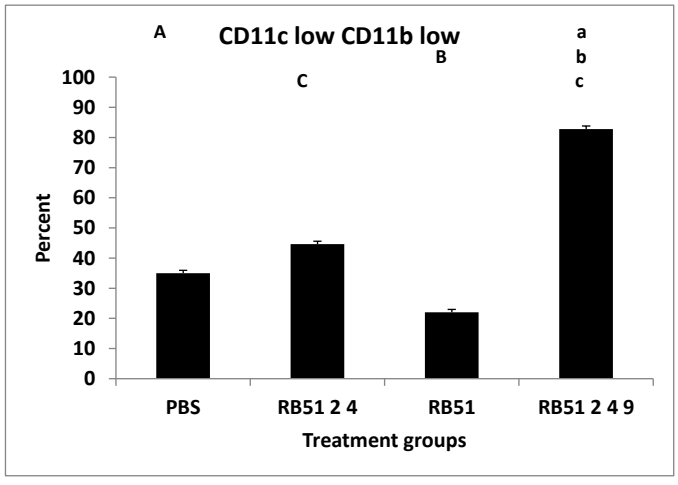
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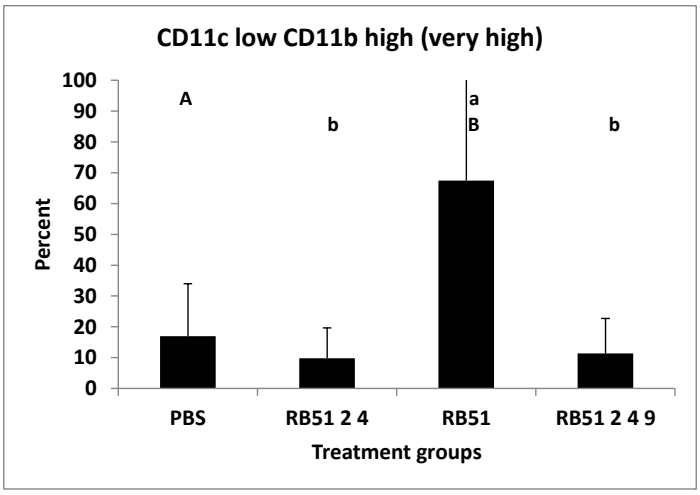
G 

Figure 4: CD11c DC expression CD11b high or low cells from the BAL. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD11b DC expression were determined by flow cytometry analysis from the BAL A) CD11c high CD11b low as a percent of total CD11c high cells in the BAL, B) percent of CD11c high cells which were CD11b high in the BAL, C) percent of CD11c low cells which were CD11b medium in the BAL, D) percent of CD11c low cells which were CD11b high in the BAL, E) percent of CD11c low cells which were CD11b medium in the BAL, F) percent of CD11c low cells which were CD11b low in the BAL, G) percent of CD11c low cells which were CD11b high in the BAL. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

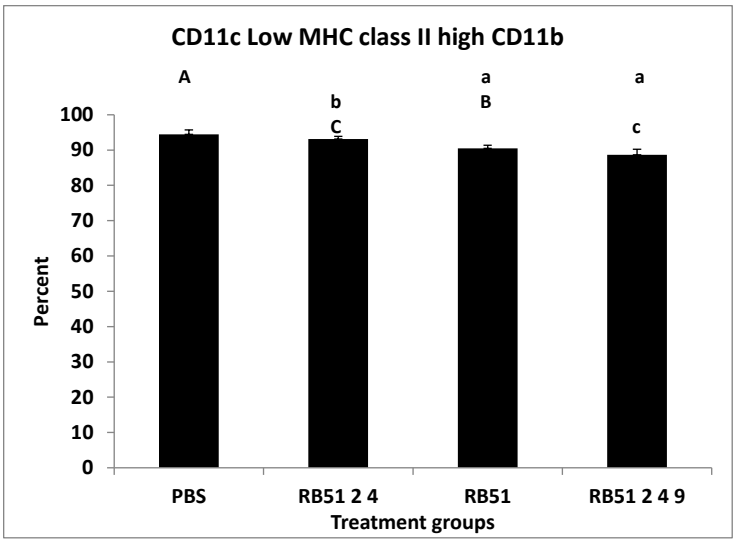


Figure 5: CD11c MHC class II CD11b expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD11b DC expression were determined by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

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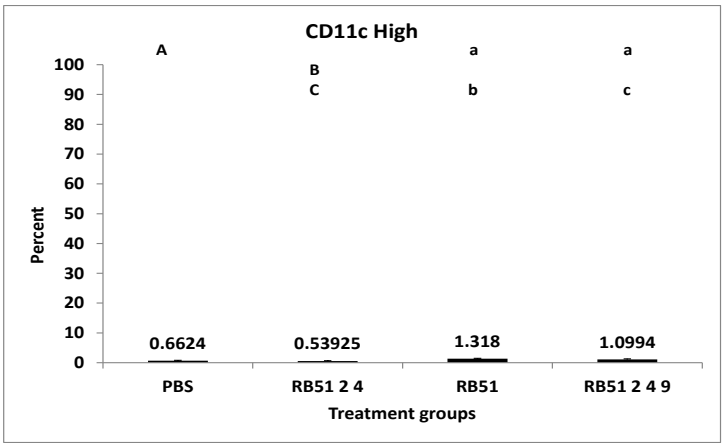

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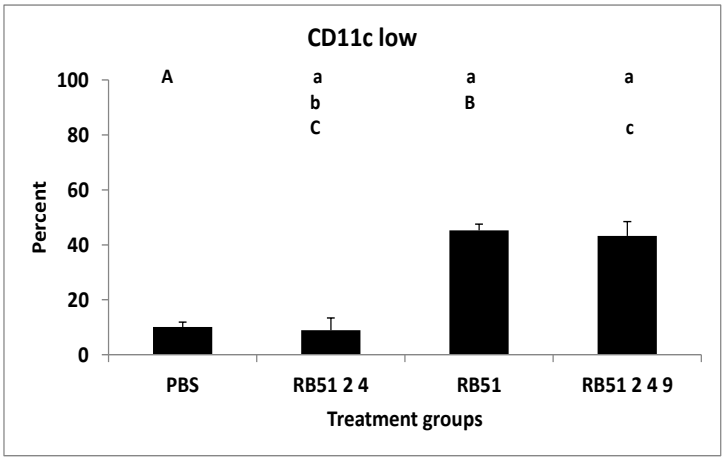

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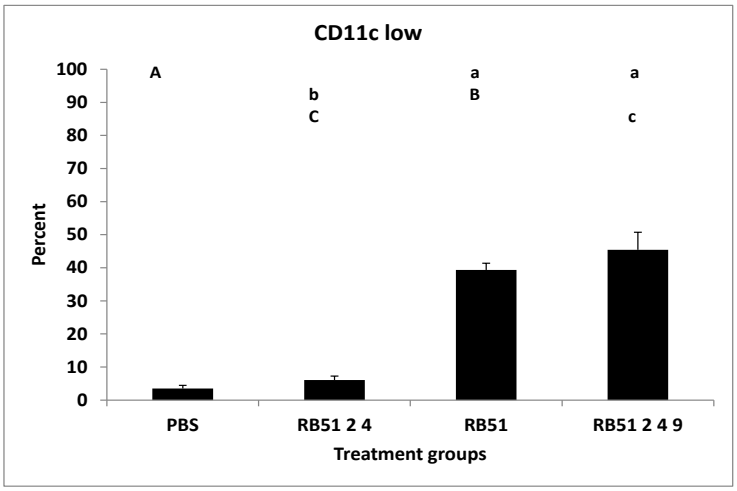


Figure 6: CD11c high and low expression from the spleen and BAL. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51

with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high and CD11c low DC were determined by flow cytometry analysis. A) CD11c high DC in the spleen, B) CD11c low DC in the spleen, C) CD11c low DC in the BAL. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

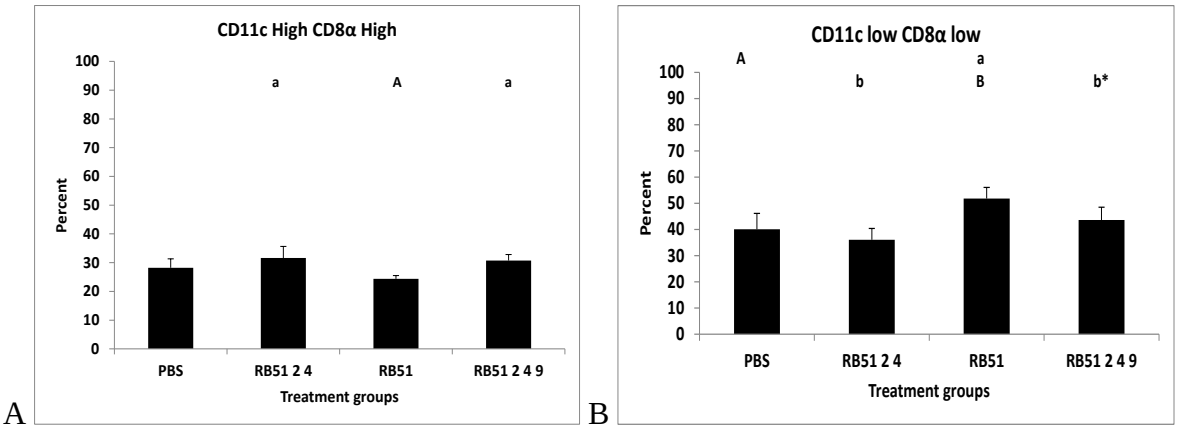
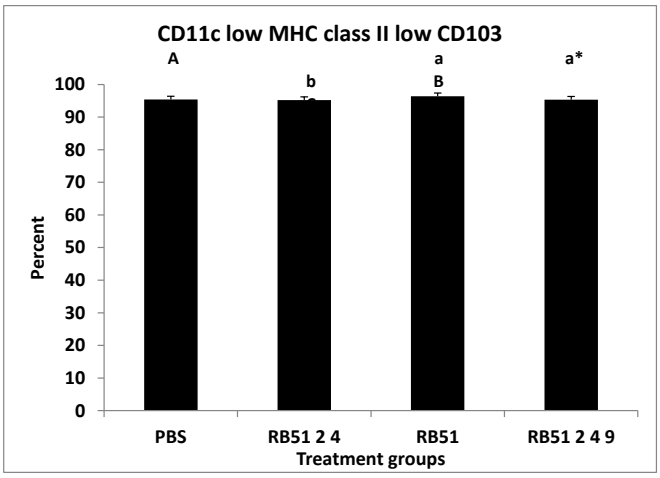
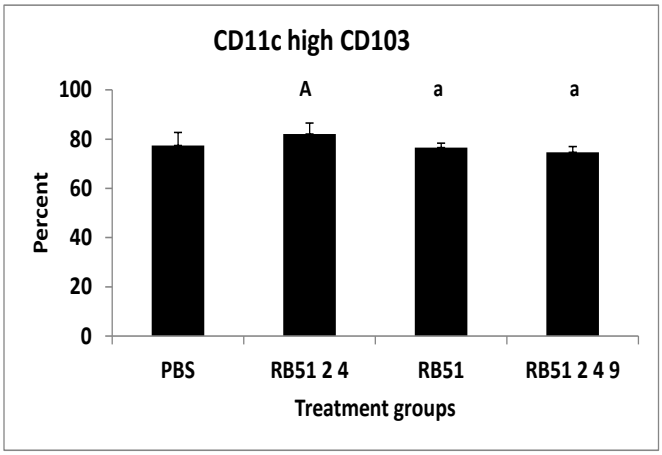


Figure 7: CD11c CD8α high and low DC expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD8α DC expression as a percentage of CD11c were determined by flow cytometry analysis in the spleen. A) CD11c low high CD8α high, B) CD11c low CD8α low DC. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$)

A



B



C

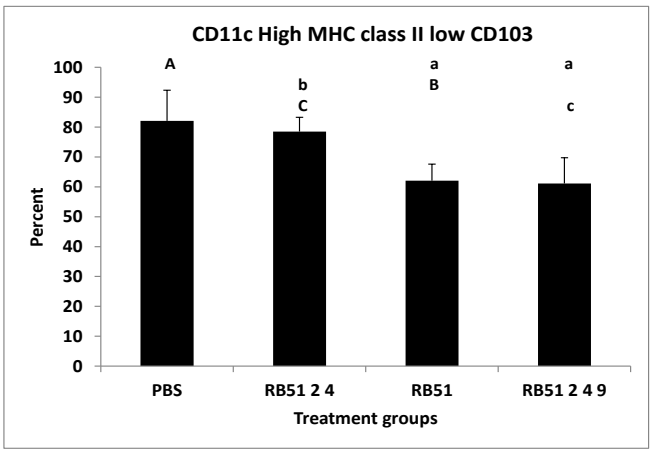
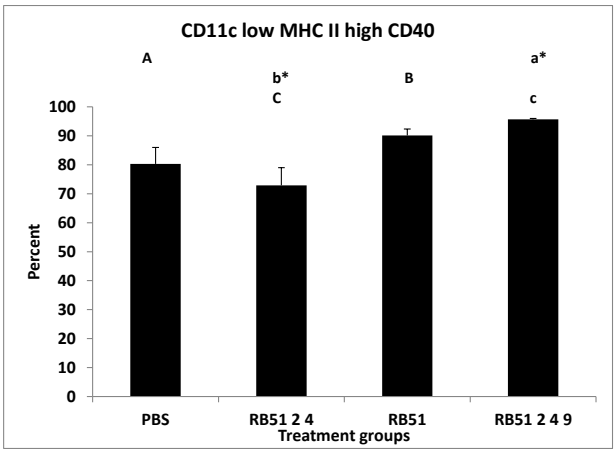


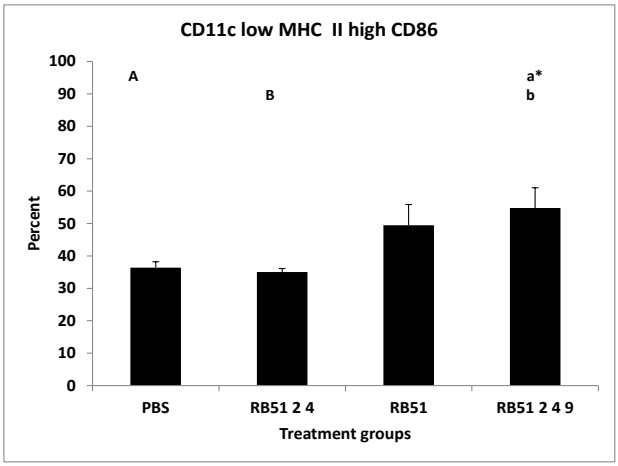
Figure 8: CD103 DC based on CD11c high or low in the bronchoalveoloar lavage and spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24),

strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high or low and CD103 DC expression were determined by flow cytometry analysis from the BAL and spleen A) CD11c low MHC class II low CD103 in the BAL, B) CD11c high CD103 in the spleen, C)CD11c high MHC class II low CD103 in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

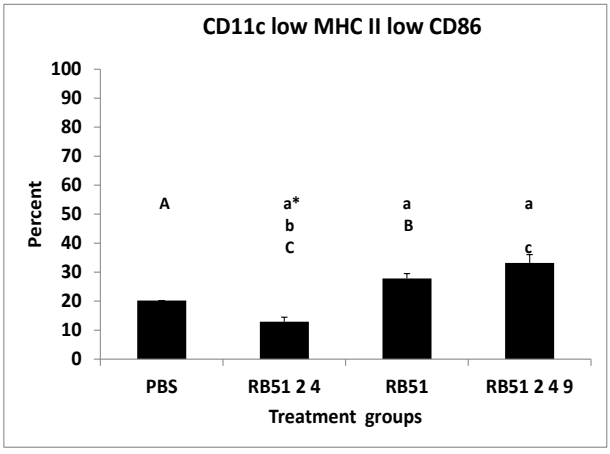
A


CD11c low MHC II high CD40
A
b*
C
B
a*
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

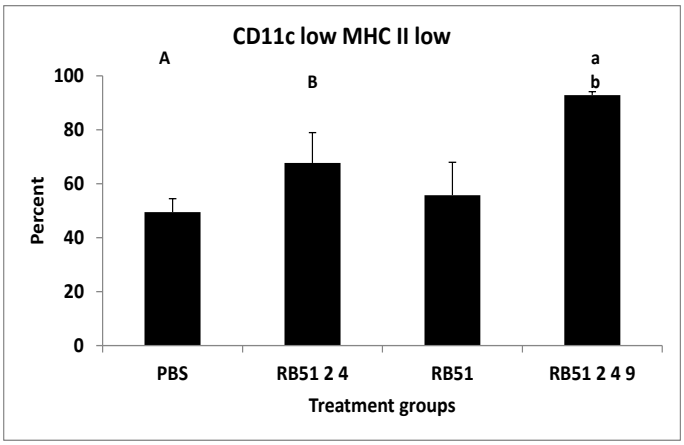
B


CD11c low MHC II high CD86
A
B
a*
b
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

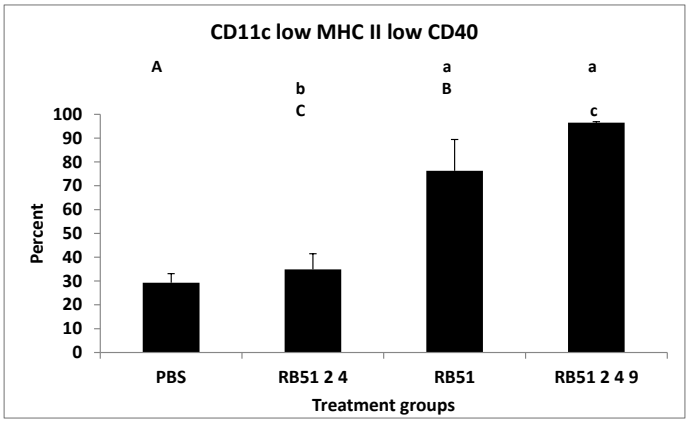
C


CD11c low MHC II low CD86
A
a*
b
C
a
B
a
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

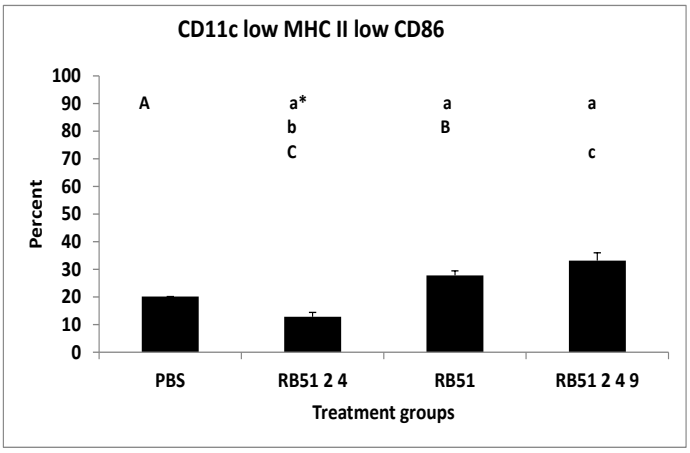
D


CD11c low MHC II low
A
B
a
b
100
80
60
40
20
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

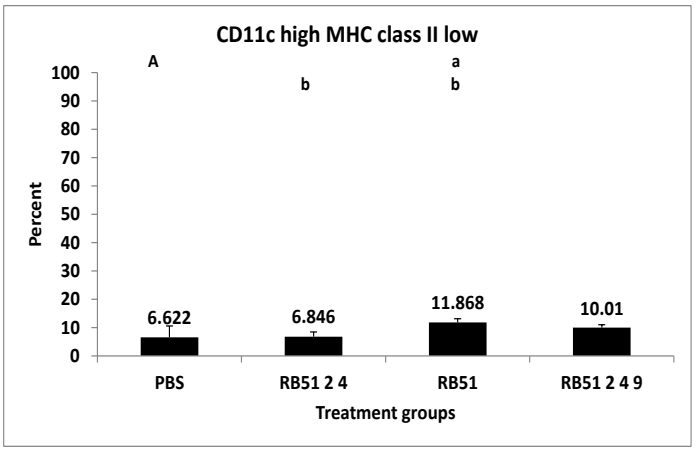
E


CD11c low MHC II low CD40
A
b
C
a
B
a
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

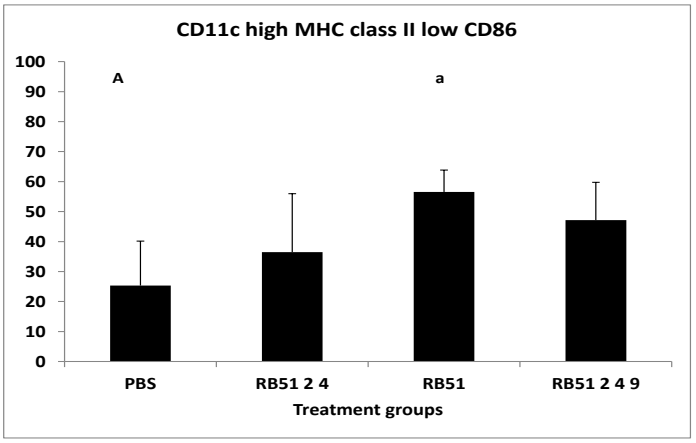
F


CD11c low MHC II low CD86
A
a*
b
C
a
B
a
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

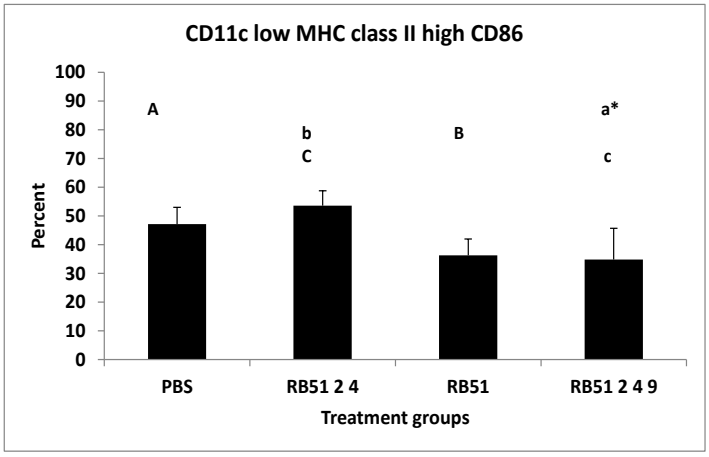
G

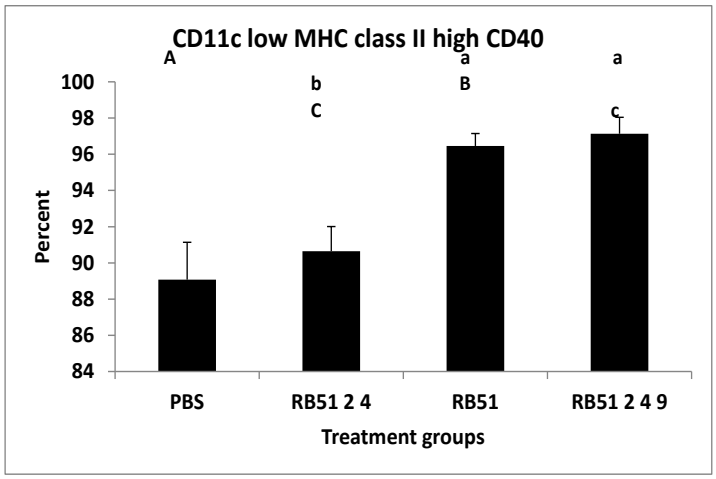

CD11c high MHC class II low
A
b
a
b
100
90
80
70
60
50
40
30
20
10
0
Percent
6.622
6.846
11.868
10.01
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

H

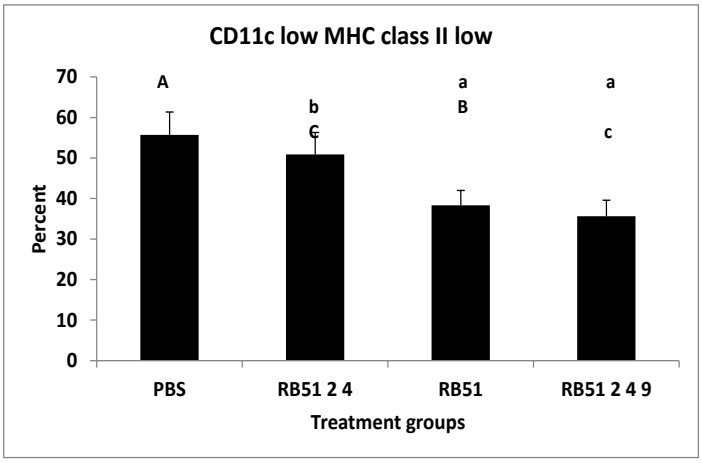

CD11c high MHC class II low CD86
A
a
100
90
80
70
60
50
40
30
20
10
0
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

I

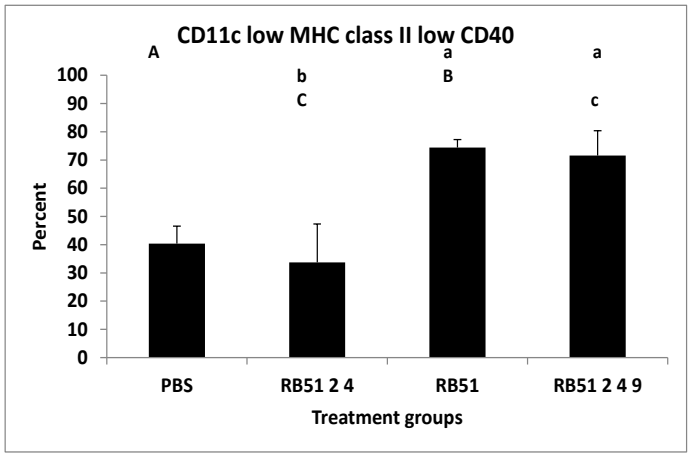

CD11c low MHC class II high CD86
A
b
C
B
a*
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups


CD11c low MHC class II high CD40
A
b
C
a
B
a
c
100
98
96
94
92
90
88
86
84
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

J


CD11c low MHC class II low
A
b
C
a
B
a
c
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

K


CD11c low MHC class II low CD40
A
b
C
a
B
a
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

L

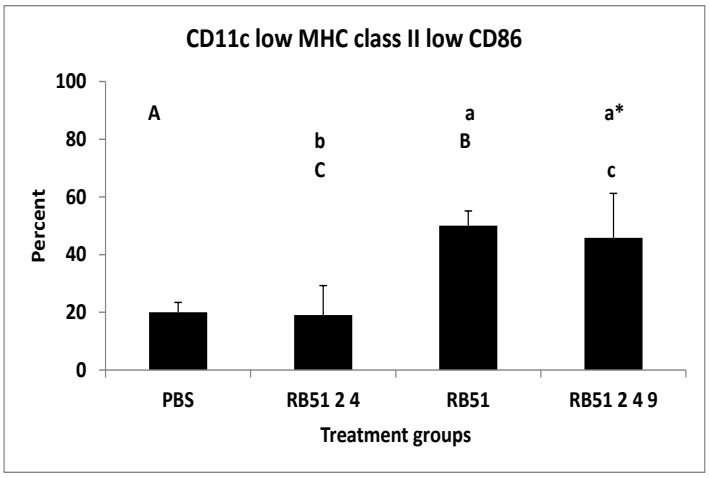
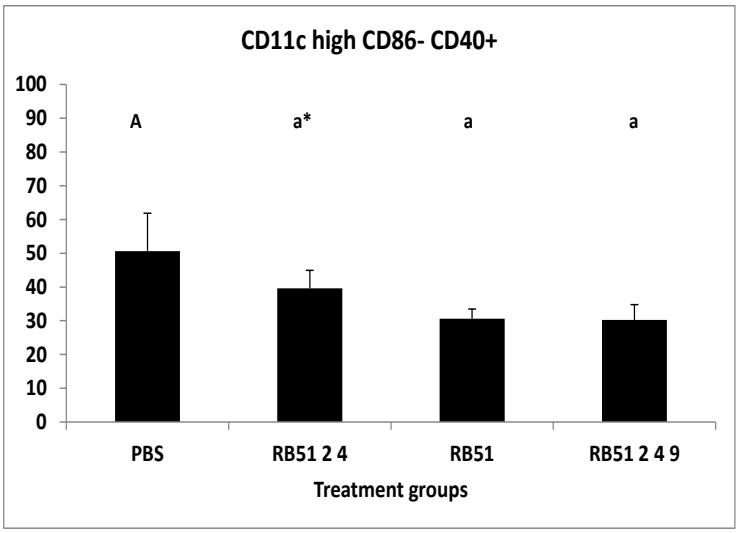
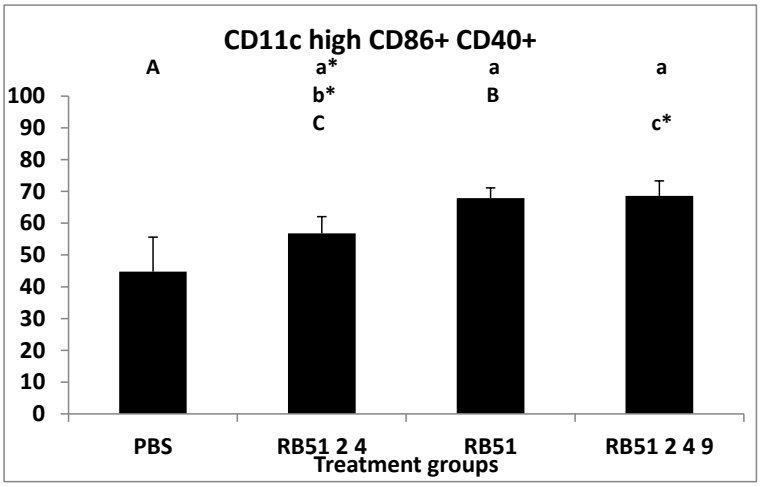
M 

Figure 9: Activation measured by CD11c high and low MHC class II expression in DC in the BAL and spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high MHC class II and CD86 or CD40 DC expression were determined by flow cytometry analysis from the spleen A) CD11c low class II high CD40 in the BAL B) CD11c low MHC class II high CD86 in the BAL, C) CD11c low MHC class II low CD86 in the BAL, D) CD11c low MHC class II low in the BAL, E) CD11c low MHC class II low CD40 in the BAL, F) CD11c low MHC class II low CD86 in the BAL, G) CD11c high MHC class II low in the spleen, H) CD11c high MHC class low CD86 in the spleen, I) CD11c low MHC class II high CD86 in the spleen, J) CD11c low MHC class II high CD40 in the spleen, K) CD11c low MHC class II low in the spleen, L) CD11c low MHC class II low CD40 in the spleen, M) CD11c low MHC class II low CD86 in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital

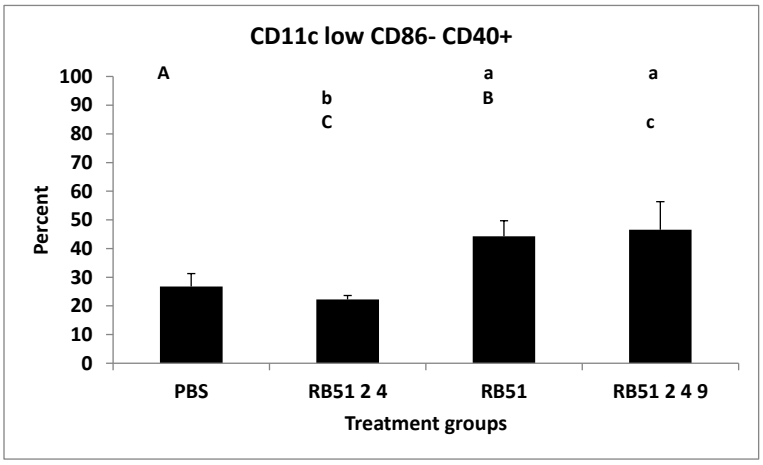
letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).


CD11c high CD86- CD40+
100
90
80
70
60
50
40
30
20
10
0
A
a*
a
a
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

A


CD11c high CD86+ CD40+
A
a*
b*
C
a
B
a
c*
100
90
80
70
60
50
40
30
20
10
0
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

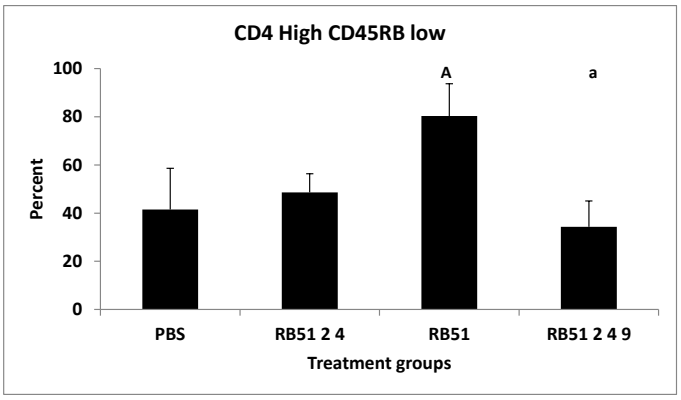
B


CD11c low CD86- CD40+
A
b
C
a
B
a
c
100
90
80
70
60
50
40
30
20
10
0
Percent
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

C

Figure 10: DC activation based on CD11c and CD86 and CD40 expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD11c high or CD11c low and CD40 and CD86 expression were determined by flow cytometry analysis from the spleen A) CD11c high CD86- CD40+, B) CD11c high CD86+ CD40+, C) CD11c low CD86- CD40+. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

A



B

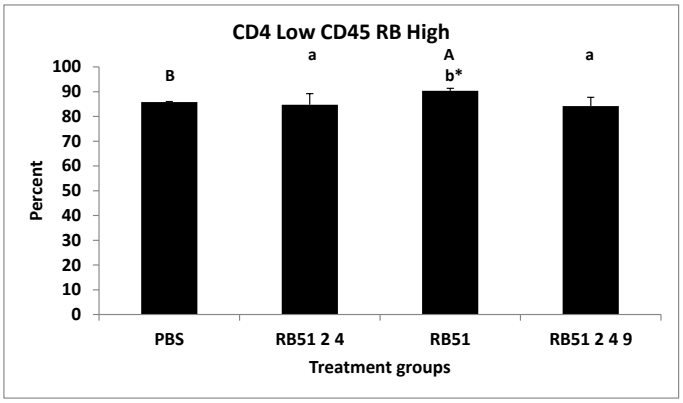
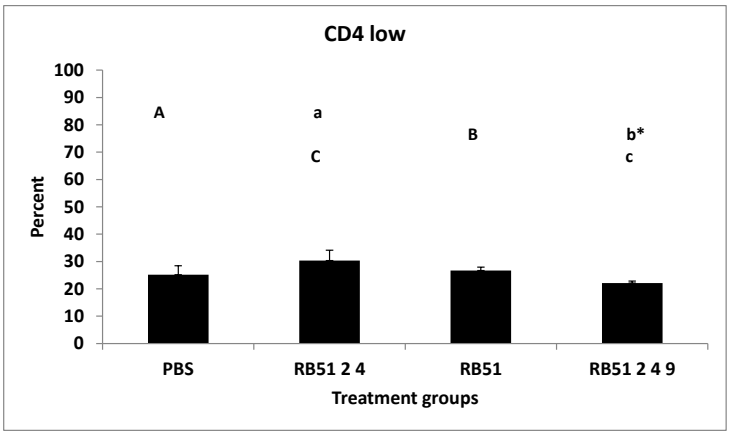
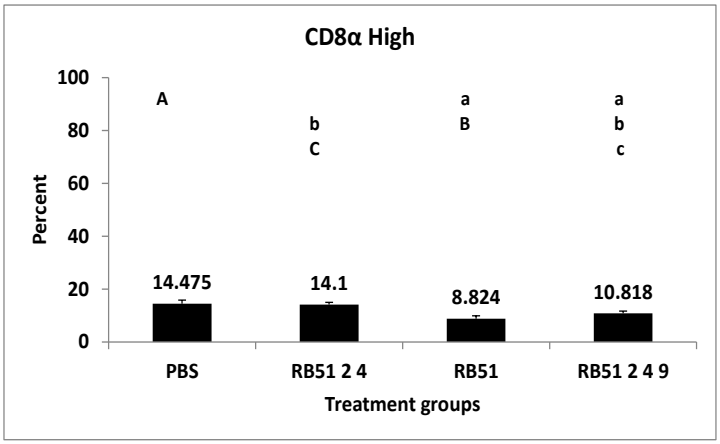


Figure 11: Lymphocyte activation based on CD4 high or low CD45RB expression from the BAL and spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). 14 days post challenge mice were euthanized, and CD4 high and CD45RB expression were determined by flow cytometry analysis from the BAL and spleen, A) CD4 high CD45RB low in the BAL, B) CD4 low CD45RB high in the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

A


CD4 low
Percent
100
90
80
70
60
50
40
30
20
10
0
A
a
C
B
b*
c
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

B


CD8α High
Percent
100
80
60
40
20
0
A
b
C
a
B
a
b
c
14.475
14.1
8.824
10.818
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

C

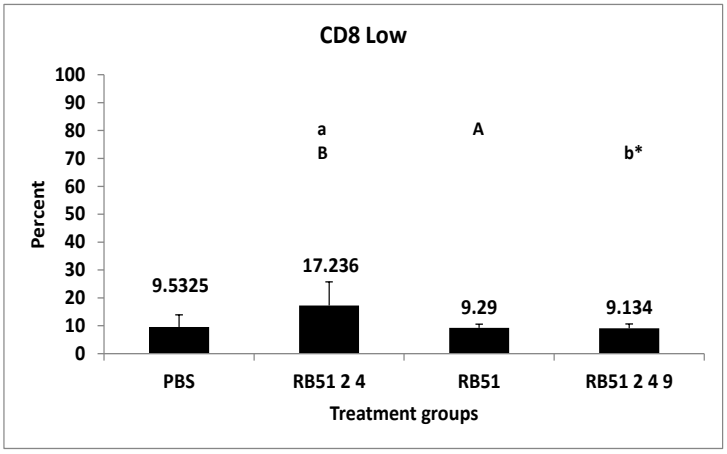
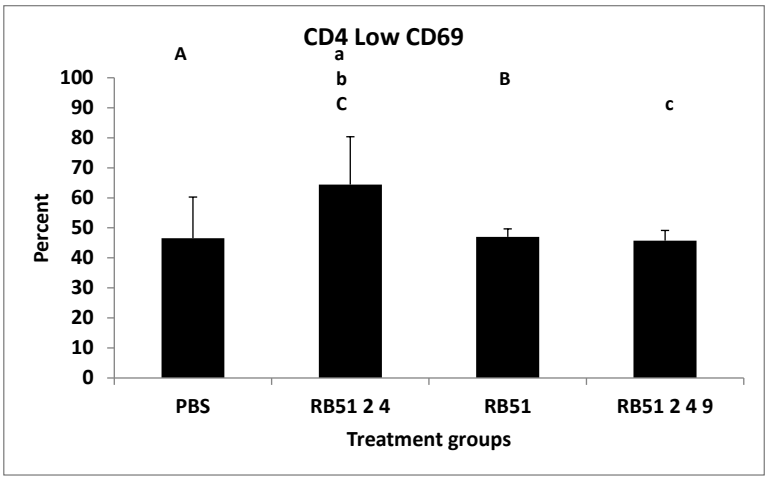
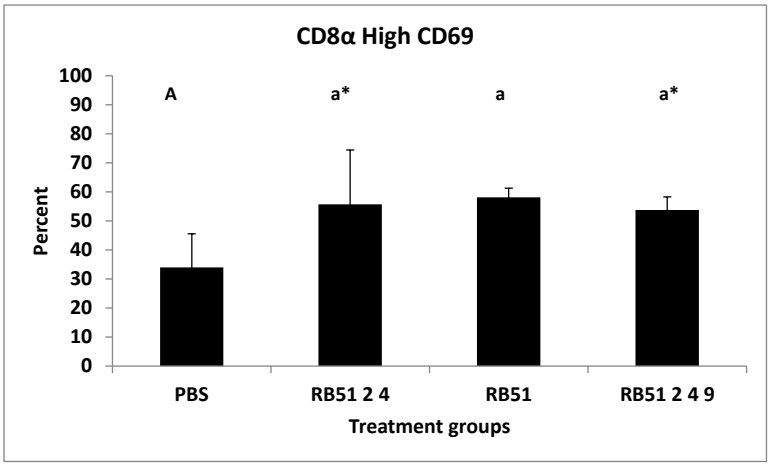

CD8 Low
Percent
100
90
80
70
60
50
40
30
20
10
0
a
B
A
b*
9.5325
17.236
9.29
9.134
PBS
RB51 2 4
RB51
RB51 2 4 9
Treatment groups

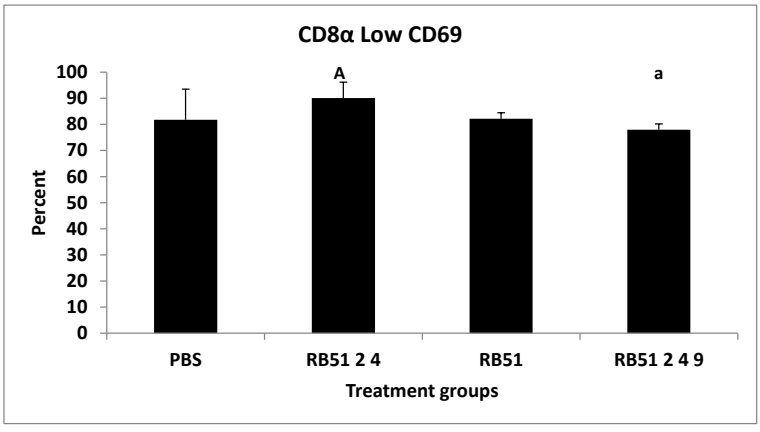
Figure 12: Expression of CD4 and CD8 lymphocytes in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD8 and CD4 high or low expression were determined by flow cytometry analysis from the Spleen, A) CD4 low expression, B) CD8α high expression, C) CD8α low expression. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).



A



B



C

Figure 13: CD69 expression by CD4 and CD 8 lymphocytes in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51),

strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD4 and CD8 CD69 expression were determined by flow cytometry analysis from the spleen, A) CD4 low CD69, B) CD8α CD69, C) CD8α low CD69. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

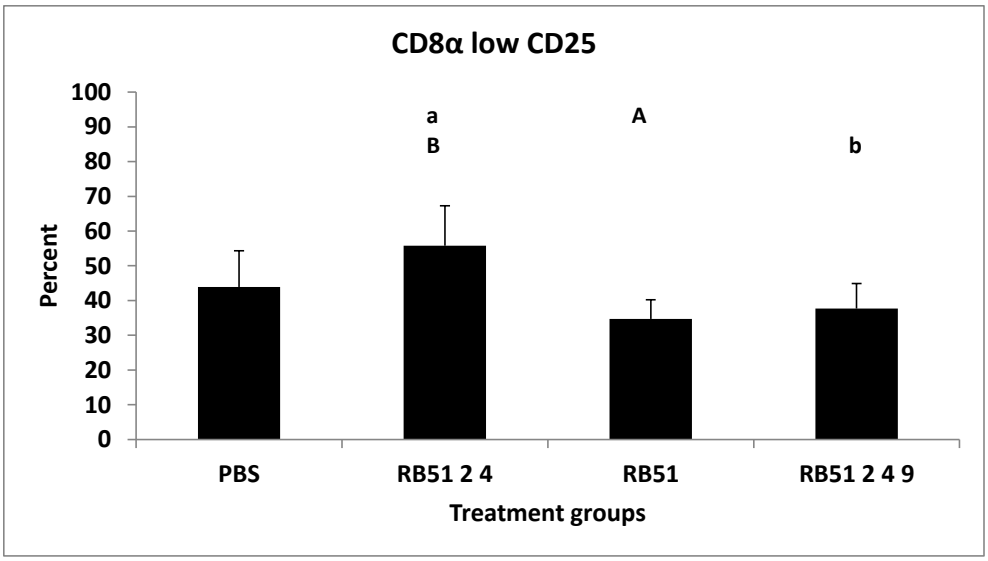
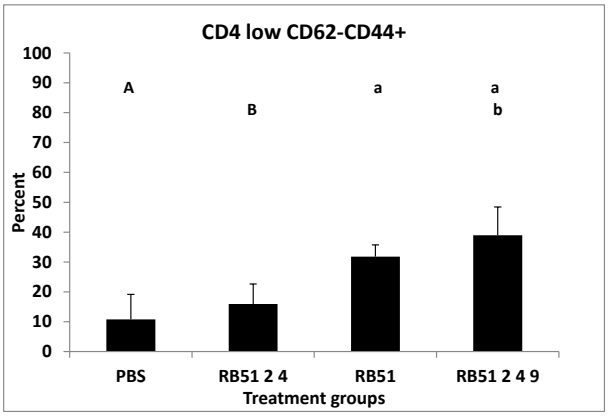


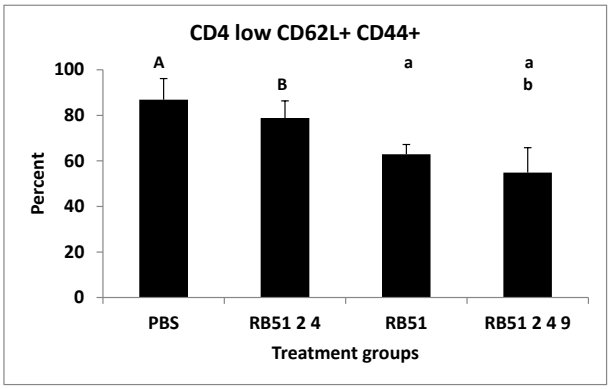
Figure 14: Lymphocyte activation based on CD8α low CD25 expression in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD8α low CD25 expression were determined by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

Figure 15:

A



B



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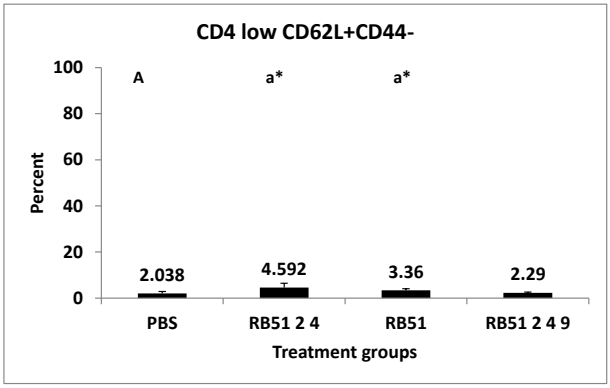

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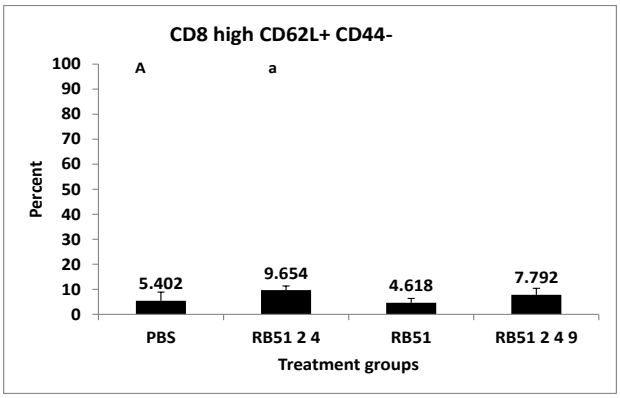


E

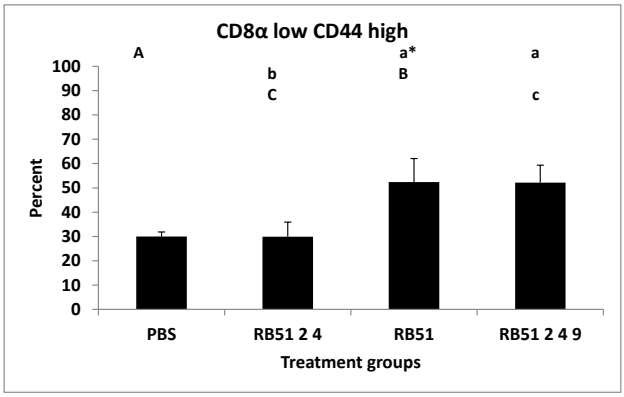


Figure 15: Expression of naïve and memory CD4 and CD8 α T cells in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and CD44 and CD62L expression in CD4 and CD8 lymphocytes were determined by flow cytometry analysis in the spleen A) CD4 low CD62L- CD44+, B) CD4 low CD62L+ CD44+, C) CD4 low CD62L+ CD44-, D) CD8α high CD62L+ CD44-, E) CD8α low CD44 high. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown

as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).

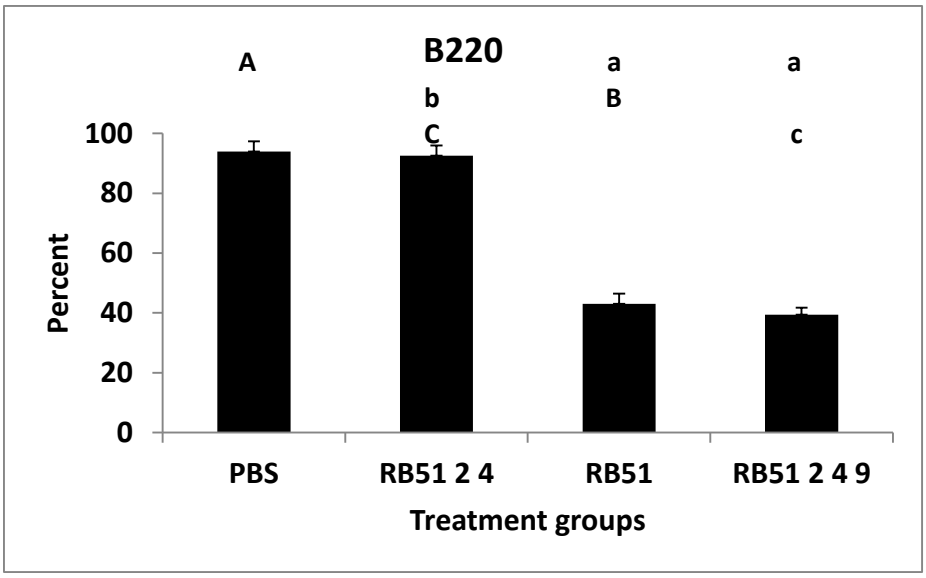
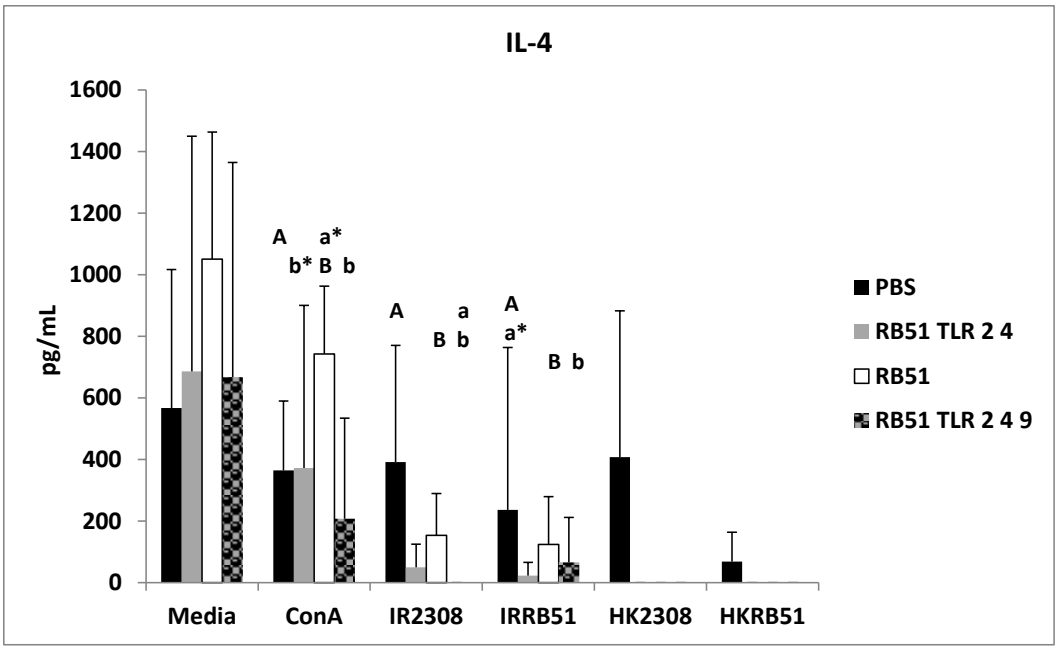


Figure 16: Cell expressing B220 as a marker for B cells in the spleen. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post challenge mice were euthanized, and B220 was used to identify B lymphocytes and CD69 was used as a marker for activation by flow cytometry analysis from the spleen. Statistical analysis was performed using one way ANOVA followed by Tukey-Kramer procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).



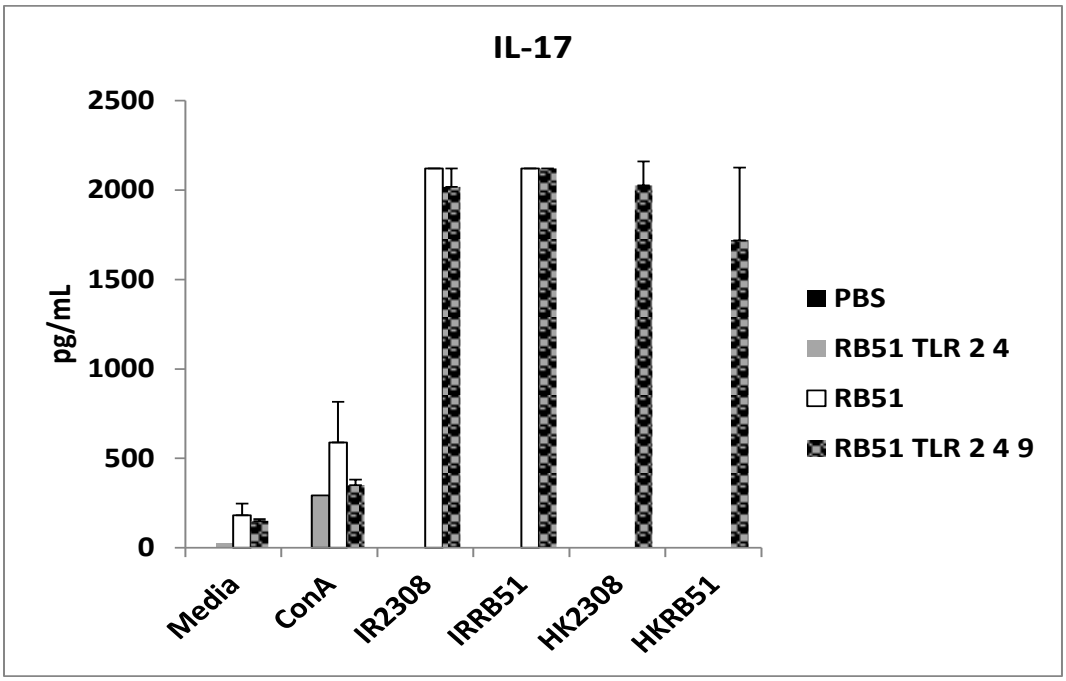


Figure 17: Cytokine expression stimulated splenocytes examined by ELISAs. Mice were vaccinated IN with PBS, RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ($2x10^4$ CFU/ mouse). Fourteen days post

challenge mice were euthanized, splenocytes were collected and stimulated with either media, ConA, irradiated strain 2308, irradiated strain RB51, heat killed strain 2308 or heat killed strain RB51 (10^6 CFU106 CFU/well). Cytokines were measured by ELISA, A) IL-4 B) IL-17. Statistical analysis was performed using the Kruskal-Wallis Test followed by Dunn's procedure for multiple comparisons, no significant differences were present, $p<0.05$. All statistical analysis was done using SAS system (NC, USA). Data are shown as +/- SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ($0.05<p<0.1$).