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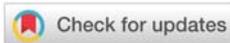
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## Research Article

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

## 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 ease of genetic modification, makes it a bioterror concern. The overall goal is to generate a safe and effective vaccine for humans. 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 (CD4 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 Toll Like Receptors (TLRs) 2, 4 and 9. The addition of TLR 2 or TLR 4, and a trend with TLR9 agonists, when combined with IN strain 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 enhance protection and clearance in the lung against strain 2308 challenge (IN), by activating the DC1 and CD4 Th1 and CD8 immune response. This study showed that protection was not enhanced by combining all TLR agonists. The group with non-significantly greater clearance 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 one of the top 5 zoonotic diseases worldwide [1]. It is easily transmitted to humans from infected animals, through contact with aborted fetal material, consumption of unpasteurized dairy products, and/or ingestion and inhalation of infected materials. The ability of *Brucella* to be easily aerosolized, combined with its virulence, and potential to be genetically modified, make this pathogen a candidate for use as a biological weapon [1]. At this time, there is no efficacious vaccine for humans.

As a model for disease in humans and cattle, mouse models initially focused on intraperitoneal (IP), intramuscular (IM), or intravenous (IV) routes of vaccination and challenge [2-4]. None of these 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 irrespective of 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 but not aerosol challenge [2]. In an attempt to establish mucosal

protection, Surendran et al. [5] vaccinated mice IN using strain RB51, but they were not protected against IN challenge with strain 2308 [5]. Furthermore, systemic IP vaccination and IN boost did not enhance clearance in the lung or mediastinal lymph node when challenged with strain 2308, nor did priming with intramuscular (IM) or intradermal (ID) vaccination followed by IN boost [5]. However, IP primary vaccination followed by secondary IN boost using *B. abortus* strain RB51SOD, a strain over expressing superoxide dismutase, showed significant clearance in the spleen, but not in the lung [5]. By contrast, using a *B. melitensis* vaccine strain administered IP, Kahl-McDongah et al., demonstrated protection in the lung against aerosolized challenge with either *B. melitensis* or *B. abortus* [6]. These studies support that the ability to protect the host against *Brucella* is dependent upon which strain, route, and dosage of vaccine and challenge strain is 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 protective CD4 Th1

CD8 adaptive response. *Brucella* interferes with DC function, and therefore does not sufficiently activate T-cells, compared to uninfected cells [7-9]. Previous studies have demonstrated a role for TLR 2, 4, 6, and 9 *in vitro* and *in vivo* for *Brucella* mediated DC function [10-14]. As a first step, we demonstrated protection in the lung could be improved by using TLR 2 and 4, and a trend for TLR9, agonists individually, when combined with strain RB51 [11]. Therefore, 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 vaccination would upregulate the protective immune response against IN challenge with *B. abortus* strain 2308. Protection was assessed based on clearance 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 our approved protocol (10-009-CVM) by 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) were anesthetized with 0.4 mL of xylazine-ketamine (0.2 mg/mL and 0.9 mg/mL) mixture; serum was collected by retro-orbital bleeding. Mice were vaccinated with 20  $\mu$ L phosphate buffered saline (PBS control) or *B. abortus* strain RB51 ( $4 \times 10^8$  CFU/mouse) alone, or in combination with TLR agonists 2 and 4, or TLR agonists 2, 4, and 9 (all TLR agonists: Invivogen San Diego, CA). Concentrations were TLR2 agonist PAMCSKK4 (5  $\mu$ g/mouse), TLR4 agonist ultrapure *E. coli* 0111:B4 LPS (10  $\mu$ g/mouse), and TLR9 agonist CpG (30  $\mu$ g/mouse) These doses were chosen based on previous studies which demonstrate protection at this dose for TLR2 and TLR4 with a trend for TLR9 in the lung (8, 9). The four groups are: PBS control, experimental RB51: RB51; strain RB51 with TLR 2 4 agonists: RB51TLR24; and RB51 with the TLR 2 4 9 agonists: RB51TLR249 groups. Six weeks post vaccination, mice were anesthetized and challenged IN with strain 2308 ( $2 \times 10^4$  CFUs/mouse). Mice were euthanized two weeks post challenge using 1.4 mL of xylazine-ketamine mixture. 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<sub>2</sub> (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 [3,5,10].

**Antibody titers.** 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  $\mu$ g/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  $\mu$ L 2% BSA (Sigma, St. Louis, MO) in TBS at 37°C for 1 hour, and then plates were washed. The bronchoalveolar lavage (BAL) samples were not diluted. Serum samples were diluted 1:100 for identification of IgA and IgG1, and a dilution of 1:100 and 1:200 were used for IgG2a in 2% BSA (Fisher Scientific). Fifty  $\mu$ L per well was added to each well, and duplicate samples were incubated for 4 hours at room temperature (RT). 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 incubated, and then plates were washed. TMB was added at 100  $\mu$ L per well (Microwell peroxidase substrate, Kirkgaard & Perry Laboratories, Gaithersburg, MD). The reaction was stopped using 100  $\mu$ L per well of 0.185 M sulfuric acid (Fisher Scientific) to stop the reaction. 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. Briefly, red blood cells were lysed with 2 mL ACK lysis buffer (Lonza, Walkersville, MD) (150 mM NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, 0.1 mM Na<sub>2</sub>EDTA) for 2 minutes [15]. Samples were washed and resuspended in RPMI 1640 (Cellgro, Manassas, VA) complete media at  $5 \times 10^6$  cells/ml. Splenocytes ( $5 \times 10^5$  cells/well) were cultured in 96 well plates and stimulated for 5 days with media, ConA (0.5 $\mu$ g/ 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  $\mu$ M 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 [15].

**Processing of Bronchoalveolar Lavages (BALs).** BALs were spun at 250 x g for 5 minutes to isolate cells; BALs from mice were combined from either 2 mice (mice #4-5 per group) or 3 mice (mice #1-3 per group) to ensure enough cells for flow cytometry [11]. BAL supernatants were frozen at -80°C for cytokine and immunoglobulin analysis [11].

**Flow cytometry.** BAL and splenocyte samples were aliquoted for flow cytometry and proliferation assays [16]. As performed previously, because cell numbers were limited on some samples,  $5 \times 10^5$  cells/sample were stained for flow cytometry [5,7,10,16]. Stain combinations were: DC1: APC conjugated anti-CD11c (BioLegend, San Diego, CA), PE conjugated anti -I-A/I-E (BD Pharmingen, 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-CD8 $\alpha$  (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-CD8 $\alpha$  (BioLegend), PE conjugated anti-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-CD8 $\alpha$  (BioLegend); T3: PE conjugated anti-B220 (BD Pharmingen), FITC conjugated anti-CD69 (BioLegend) [5,8,10].

Cytokines as determined by ELISAs. BALs, splenocyte supernatants, organ homogenates (lung, liver, spleen, MLN) were stored at -80°C. TNF- $\alpha$ , IL-12p70, IFN- $\gamma$ , IL-10, IL-4, IL-17 were measured using indirect sandwich ELISAs (BD Pharmingen) [3,8,9]. 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, and then plates were washed 4 times with 0.5% Tween PBS wash buffer. Samples and recombinant cytokines for a standard curve were added at 50  $\mu$ L per well. All samples and standards were run in duplicate. Plates were incubated for 3 hours at RT. Samples were then removed, and plates were washed 4 times with wash buffer. The primary antibody was added at the specified concentration, and incubated for 45 minutes at RT. The plates were washed again (4 times), and the horse radish peroxidase conjugated secondary antibody was applied for 30 minutes at room temperature. The plates were then washed again (8 times) before the TMB substrate solution (Dako, Carpinteria, CA) was added. The reaction was 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  $p < 0.05$ ; 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):** There were no significant differences between treatment groups based on CFUs of any of the organs (Figure 1).

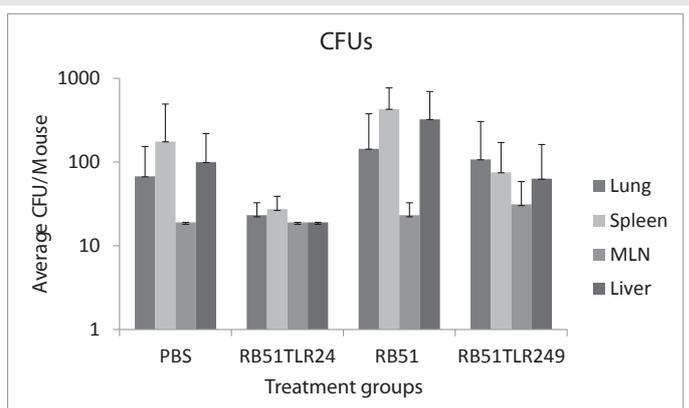
**Increased total cell numbers in BAL and spleen by non-protected groups:** In the BAL, the RB51 group ( $1.97 \times 10^5$  cells  $\pm 4.35 \times 10^4$ ) had significantly higher total cells versus the PBS ( $1.94 \times 10^4$  cells  $\pm 1.38 \times 10^4$ ) and RB51TLR24 ( $3.77 \times 10^4$  cells  $\pm 3.24 \times 10^3$ ) groups. The RB51TLR249 group ( $9.52 \times 10^4$  cells  $\pm 3.18 \times 10^4$ ) had significantly more cells vs. the PBS treatment. Comparatively, the RB51 ( $1.52 \times 10^7$  cells  $\pm 4.35 \times 10^4$ ) and RB51TLR249 ( $1.42 \times 10^7$  cells  $\pm 3.18 \times 10^4$ ) had significantly more splenocytes than the PBS group ( $8.64 \times 10^6$  cells  $\pm 1.38 \times 10^4$ ).

**Vaccination induces higher antibody titers in BAL and serum:** In both the BAL and serum, post 2308 challenge

samples had a mixed IgG2a and IgG1 response, with lower amounts of IgA, suggesting a mixed Th1 and Th2 response against strain RB51 (Figure 2A-E). Antibody production against strain 2308 was lower than against strain RB51. It followed the same pattern, but had lower OD values vs. strain RB51 induced response (data not shown).

**DC recruitment:** RB51TLR24 and RB51 had higher levels of CD11b DC whereas RB51TLR249 had increased CD103 DC. In the BAL and spleen, DC subtype (CD103 DC, CD11b, and CD8 $\alpha$  resident DC), as well as up regulation 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 identified based on CD11c+ staining. In the BAL, (Figure 3) RB51 and RB51TLR249 had significantly increased CD11c high than PBS and RB51TLR2. RB51 had significantly increased CD11c high CD11b high in BAL compared to RB51 and RB51TLR249. Similarly, in the spleen, RB51 and RB51 TLR249 had increased CD11c high CD11c medium compared to PBS and RB51TLR24. Additionally, RB51TLR24 had increased CD11c high CD11b high vs. RB51. In the spleen, only RB51TLR24 upregulated CD11c high CD11b cells vs. RB51 and RB51TLR249 group (Figure 3C-D).

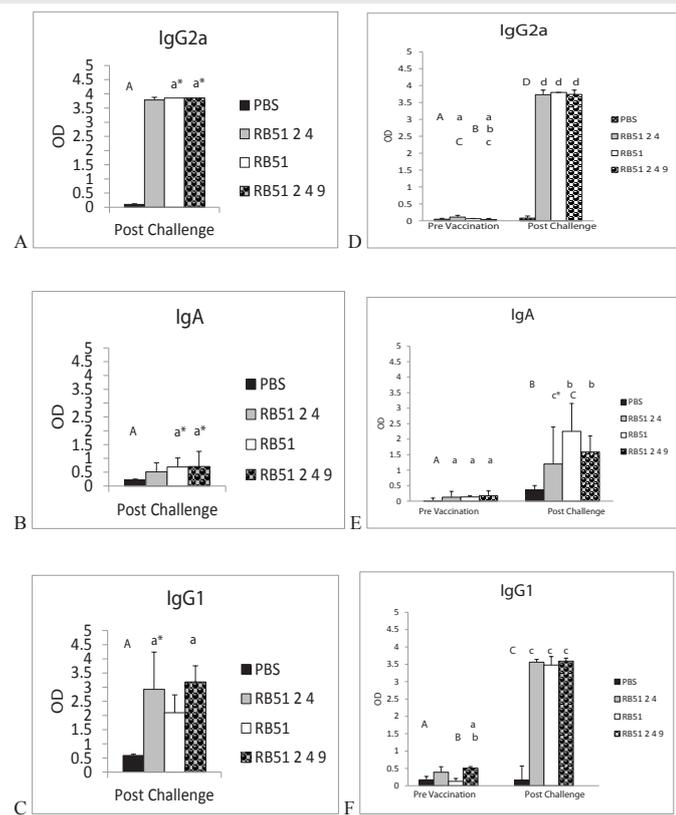
In assessing the remaining DC subsets (CD103, CD8  $\alpha$ ), the RB51TLR249 group had overall significantly greater CD11c low MHC class II high CD103 DC than most other treatment groups in the BAL (Figure 4A-B). By comparison, in the spleen, the RB51TLR24 and RB51TLR249 groups had greater CD11c high CD8 $\alpha$  high than the RB51 group (data not shown). The RB51 and RB51TLR249 groups had increased CD11c low MHC class II high CD8 $\alpha$  cells compared to PBS and RBTLR24 groups (Figure 4C), suggesting differences in TLR mediated activation and protection and response to infection.



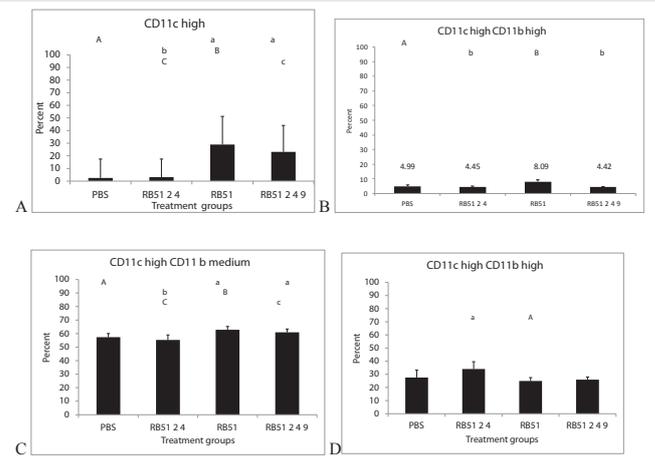
**Figure 1:** Average colony forming units isolated per organ and clearance of *B. abortus* strain 2308 following intranasal vaccination and challenge. Mice were vaccinated IN with PBS, strain RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), or strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ( $2 \times 10^4$  CFU/ mouse). Fourteen days post challenge, mice were euthanized. Lungs, liver, spleen, and MLN were collected and homogenized and colony forming units (CFUs) were determined. 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  $\pm$  SD.

RB51TLR2 upregulated CD11c high CD86 in BAL (Figure 5A-C) vs. RB51 and RB51TLR249 had increased CD11c low CD40 and CD11c low CD86 CD40. RB51TLR249 and RB51 had increased CD11c high MHC class II low CD40 and CD11c low MHC class II high vs. the PBS and RB51TLR2 groups (Figure 5D-F, data not shown). Additionally, the RB51 group in the spleen had higher CD11c low CD86+ CD40- cells than the RB51 TLR249 group (data not shown). RB51 and RB51TLR249 did not upregulate CD11c compared to RB51TLR24. Additionally, the RB51TLR249 and RB51 groups induced more activation of cells via CD40 expression, while the RB51TLR24 group more consistently upregulated through CD86 expression, potentially due to differences in TLR mediated activation and/or levels of infection.

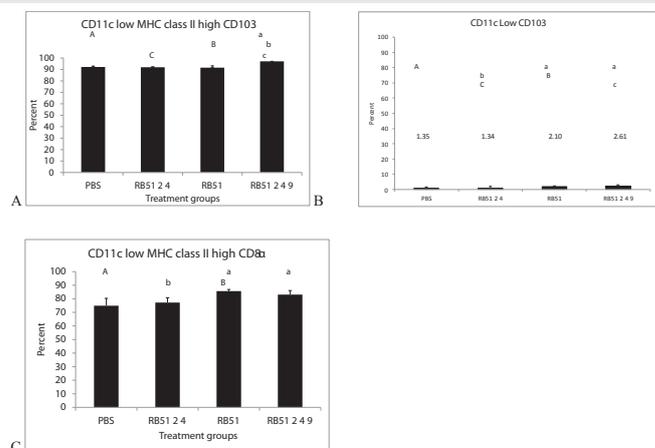
**Infection associated with increased activated T cells:** To assess the immune response associated with clearance/chronic infection, identify possible correlates of protection, and determine how the vaccine groups differentially stimulated immunity, lymphocytes were assessed for subsets and activation by flow cytometry. Lymphocyte subsets (CD4, CD8, B cells (B220), and activation/phenotype (CD69 early activation,



**Figure 2:** Immunoglobulin isotypes against strain RB51 were measured in the bronchoalveolar lavage (BAL) and serum by ELISA. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> CFU/mouse). Fourteen days post challenge, mice were euthanized. Immunoglobulin levels were determined in the BAL for A) IgG2a, B) IgA, C) IgG1 and serum for D) IgG2a, E) IgA, F) 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:** CD11c high cells in the bronchoalveolar lavage and spleen. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> CFU/mouse). Fourteen days post challenge, mice were euthanized, and A) CD11c high and CD11c low DC were determined by flow cytometry analysis. CD11c high and CD11b DC expression were determined by from the BAL or spleen B) CD11c high CD11b high as a percent of total CD11c high cells in the BAL, C) Percent of CD11b cells which were CD11b medium in the spleen, D) 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).



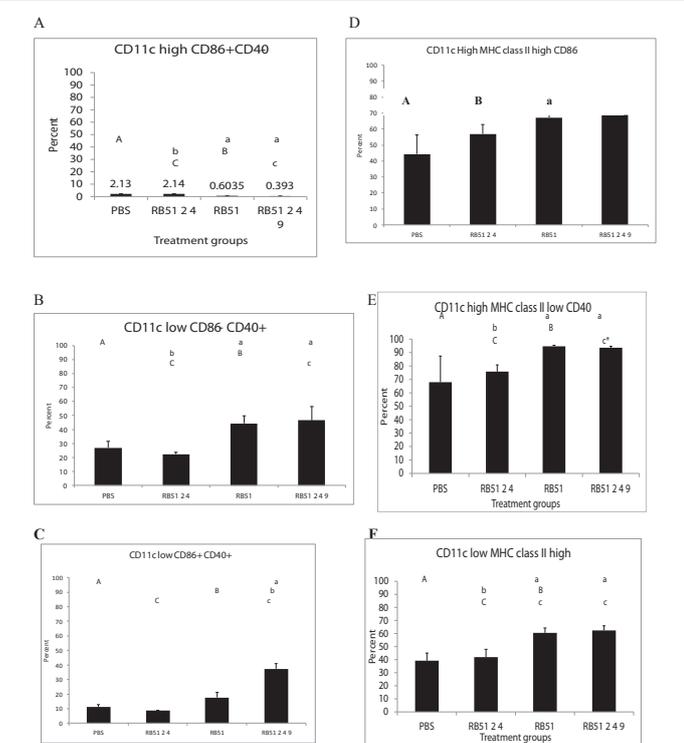
**Figure 4:** CD103 CD11c low DC in the bronchoalveolar lavage (BAL) and spleen. Mice were vaccinated IN with PBS, strain RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), or strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 (2x10<sup>4</sup> 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, C) CD11c low MHC class II high CD8a 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).

CD45RB effector, CD62L naïve, CD44 memory, CD25 T-reg or activation) were determined for BAL and spleen samples (Figure 6A-E).

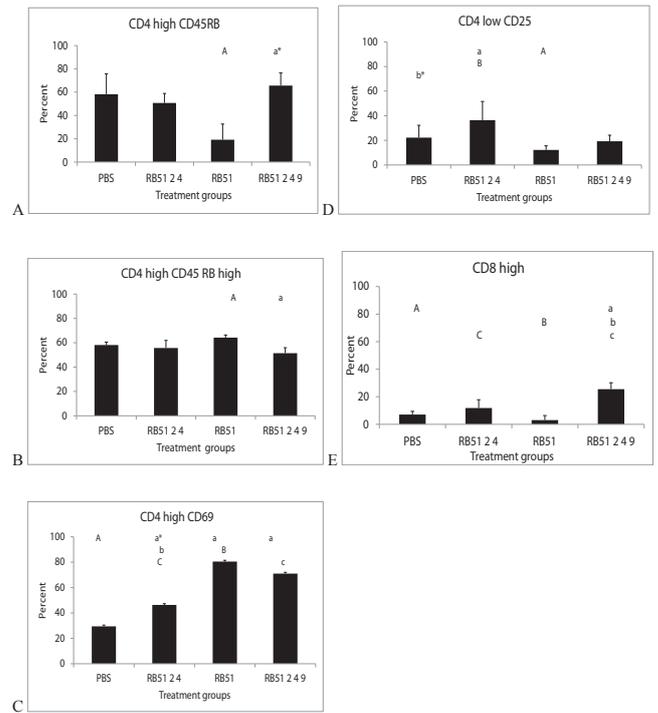
Overall, there was a pattern that the RB51TLR249 had increased CD4 effector and CD8 high activation in the BAL. Correspondingly, there was a decrease in CD4 effector cells in the spleen of RB1TLR249 (Fig 6B-E). Neither PBS nor RB51 groups upregulated CD8 high CD45RB high (data not shown). RB51TLR2 had increased CD4 low CD25 (Figure 6D) and increased CD4 low (data not shown) compared to RB1TLR249, suggesting differences in TLR induced immune responses as well as levels of infection. Overall, RB51TLR249 groups tended to induce increased CD4 and CD8 CD69 and CD45RB expression, while the RB51TLR24 group induced higher CD25 expression but also CD69 expression in CD4 low and CD8 low T cells. The PBS and RB51 groups did not upregulate CD8 high.

**Changes in naïve and memory responses in the spleen:**

Naïve and memory T-cell status was based on CD62L (naïve) and CD44 (memory) expression in the spleen. For this study, we used CD44 to identify memory cells and CD62L to stain naïve cells as the cell populations were assessed in the spleen. We recognize the central memory cells are CD62L, and are



**Figure 5:** DC activation based on CD86 and CD40 expression in the bronchoalveolar lavage (BAL) and spleen. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> CFU/ mouse). Fourteen days post challenge, mice were euthanized, and CD11c high or CD11c low and CD40 and CD86 or MHC class II high or MHC class II low expression were determined by flow cytometry analysis from the BAL (A-C): A) CD11c high CD86+ CD40-, B) CD11c low CD86- CD40+ C) CD11c low CD86+ CD40+; and in the spleen (D-F): D) CD11c high MHC class II high CD86 E) CD11c high MHC class II low CD40 F) CD11c low MHC class II high 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).



**Figure 6:** Lymphocyte activation based on CD4 high CD45RB, CD69, CD4 low CD25 expression in the spleen and CD8 high lymphocytes in the BAL. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> CFU/ mouse). Fourteen days post challenge, mice were euthanized, and CD4 high and CD4 low, CD45RB and CD69, CD8 high expression were determined by flow cytometry analysis from the BAL (A, E) and spleen (B-D): A) CD4 high CD45RB in the BAL, B) CD4 high CD45RB, C) CD4 high CD69 high, D) CD4 low CD25 in the spleen and E) CD8 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).

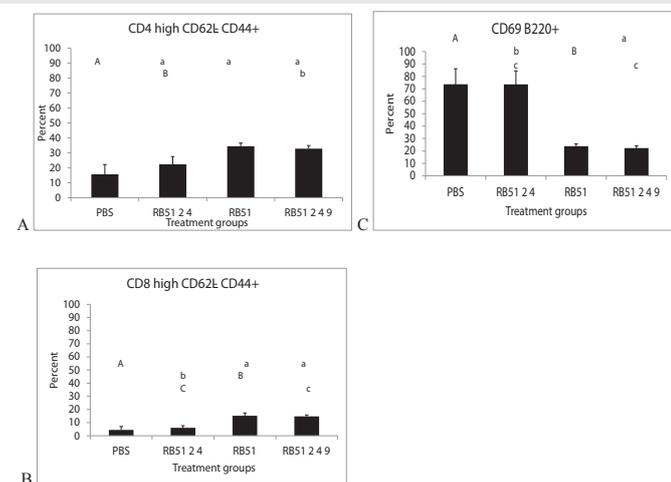
located predominantly in the lymph nodes, not in the spleen. Therefore, for the purpose of this study, we refer to CD62L+ cells as naïve. We recognize that there is the unlikely potential that the CD62 cells in the spleen could be central memory cells. Overall, the PBS and RB51TLR24 groups had higher naïve cells than the RB51 and RB51TLR249 groups in comparison, potentially indicating a naïve response, reflective of lack of induction and declining infection, respectively (Fig 7A-B, data not shown). Besides significant changes noted, the RB51 group 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 to have increased CD62 naïve cells (data not shown), and the RB51 and RB51TLR249 groups had increased CD44 memory cells (Figure 7). The functional significance of the CD4 and CD8 high and low subsets needs to be determined.

**T and B-cell activation:** PBS and RB51TLR24 groups had significantly higher percentages of both B cells (data not shown) and activated (CD69) B cells compared to the RB51 and RB51TLR249 groups (Figure 7C). These data were not associated with differences in antibody production in the serum or IL-4 levels in any organ.

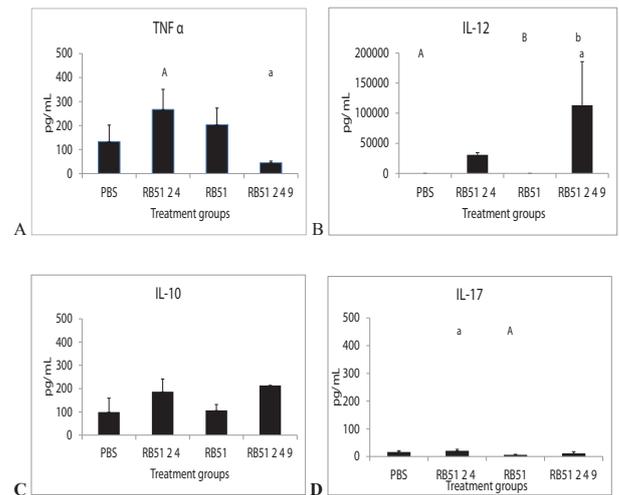
**RB51TLR2 induces DC1 CD4 Th1 CD8 cytokine profile in BAL and lung:** The specific cytokine profile reflects the nature of the immune response. TNF- $\alpha$  and IL-12, are consistent with a Th1 response, and have been shown to provide protection against *Brucella* infection [8,17]. Production of IFN- $\gamma$ , a Th1 cytokine by immune cells, has been shown to be essential in control and clearance of *Brucella* [18]. Production of IL-4 supports 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; it 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 [19,20].

In the BAL, RB51TL2 induces TNF- $\alpha$  and IL-17 in the BAL, and IL-12 consistent with a DC1 CD4 Th1 CD8 and IL-17 response. RB51TLR9 has increased IL-12 in the BAL (Figure 8-11). In the BAL and lung, IFN- $\gamma$  and IL-4 were not detected (Figure 9A-D, data not shown). In the spleen, no IL-4 production was detected in any groups (Figure 10A-E, data not shown). In the MLN, significant changes are demonstrated. PBS had significantly increased TNF-alpha and IL-17 in te MLN compared to RB51TLR249 (Figure 11A- F).

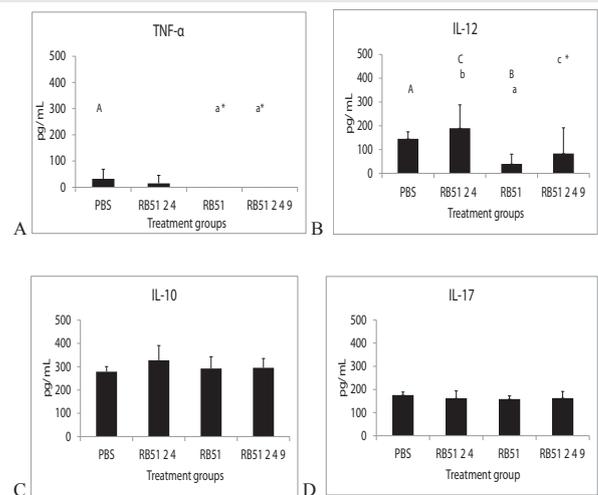
**Cytokine production from stimulated splenocytes:** Insufficient samples were present for TNF- $\alpha$  and IL-17 determination. For IL-12 production, there was a pattern that for media, ConA, and IR2308 stimulated samples, that the RB51 and RB51TLR24 samples were greater than the PBS stimulated splenocytes (Figure 12A). There was a pattern that RB51TLR249 stimulated increased IL-10 in RB51TLR249 vs. RB51 and RB51TLR24 suggesting possible T-regulatory involvement.



**Figure 7:** Memory and naïve lymphocytes in the spleen. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> 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+, C) CD69 as a percentage of B220. 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:** Cytokine production in BAL as determined by ELISAs. Mice were vaccinated IN with PBS, strain 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 with *B. abortus* strain 2308 (2x10<sup>4</sup> CFU/ mouse). Fourteen days post challenge, mice were euthanized. BAL was collected. Cytokines were measured by ELISA A) TNF- $\alpha$ , 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 9:** Cytokine production in lungs as determined by ELISAs. Mice were vaccinated IN with PBS, strain 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<sup>4</sup> CFU/ mouse). Fourteen days post challenge, mice were euthanized. Lungs were collected and homogenized. Cytokines were measured by ELISA A) TNF- $\alpha$ , 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).

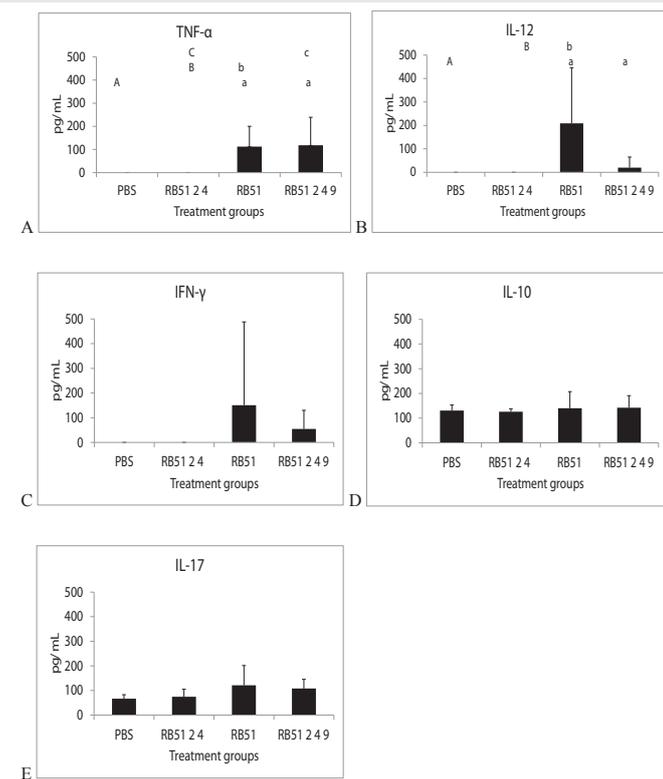
For IFN- $\gamma$  production, there was a pattern for the RB51TLR24, RB51 and RB51TLR249 stimulated splenocytes induced higher IFN- $\gamma$  than PBS stimulated splenocytes (Fig 12B). Overall, the RB51TLR24 group, and in many cases the RB51

group, induced higher production of Th1 type cytokines (IL-12 and IFN- $\gamma$ ), similar to the pattern of the organ homogenates. The addition of TLR 9 (TLR 2 4 9) did not induce as strong of a Th1 response.

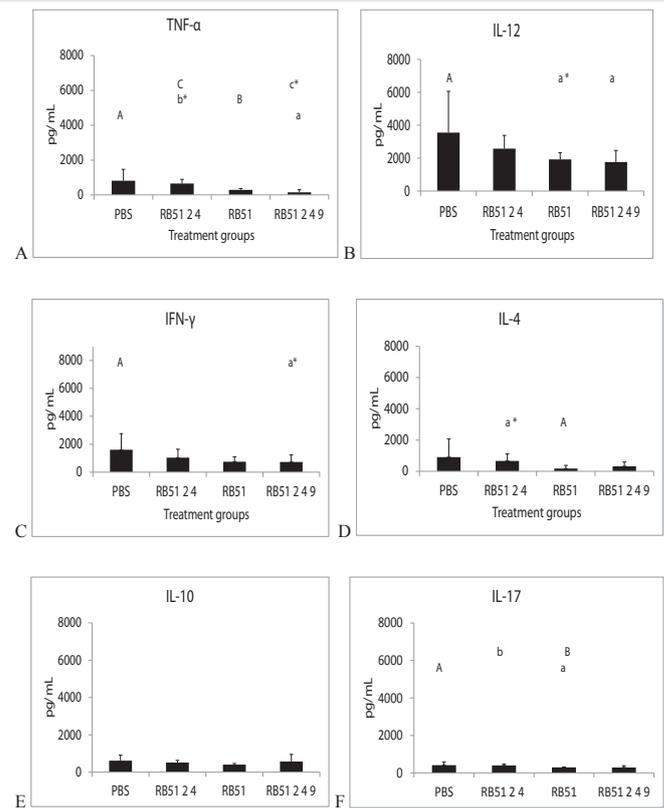
Splenocyte stimulated IL-4 production was present but low (data not shown). There was a pattern with Con A, that the RB51 group was significantly greater than the RB51TLR249 group. With IR2308 stimulation, the PBS and RB51 groups were significantly greater than the RB51TLR249 group. With IRRB51 stimulation, there was a pattern for the PBS group to be greater than the RB51 groups to be significantly greater than the RB51TLR249 groups (data not shown). This suggests a partial Th2 response by the PBS and RB51 groups following infection/challenge.

In assessing the IL-10 response, there was a pattern that RB51TLR24 induced more IL-10 than PBS or RB51TLR249 groups, suggesting possible differences in induction of immunity associated with TLR9 and/or response to infection (Figure 12C).

The total amount of splenocyte supernatants was limited;



**Figure 10:** Cytokine production in spleens as determined by ELISAs. Mice were vaccinated IN with PBS, strain 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 ( $2 \times 10^4$  CFU/ mouse). Fourteen days post challenge, mice were euthanized. Spleens were collected and homogenized. Cytokines were measured by ELISA A) TNF- $\alpha$ , B) IL-12, C) IFN- $\gamma$ , 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  $\pm$  SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ( $0.05 < p < 0.1$ ).

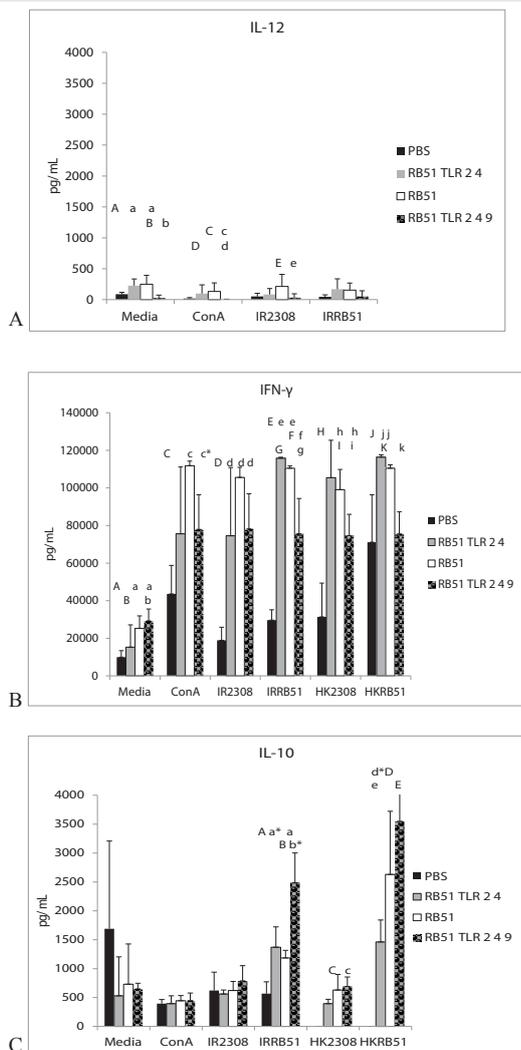


**Figure 11:** Cytokine production in MLN as determined by ELISAs. Mice were vaccinated IN with PBS, strain RB51 with TLR 2 and 4 agonists (RB51TLR24), strain RB51 (RB51), or strain RB51 with TLR 2, 4 and 9 agonists (RB51TLR249). Mice were then challenged 6 weeks post vaccination IN with *B. abortus* strain 2308 ( $2 \times 10^4$  CFU/ mouse). Fourteen days post challenge, mice were euthanized. MLN were collected and homogenized. Cytokines were measured by ELISA A) TNF- $\alpha$ , B) IL-12, C) IFN- $\gamma$ , 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  $\pm$  SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ( $0.05 < p < 0.1$ ).

therefore, some samples (i.e., PBS) could not be tested for IL-17 production. When stimulated with IR2308 and IRRB51, the amount of IL-17 increased dramatically for both the RB51 and RB51TLR249 groups. However, these were the only two groups with samples to test. The production of IL-17 was not significantly different between groups (data not shown). The RB51TLR249 group also induced high IL-17 production when stimulated by HK2308 and HKRB51 (data not shown), however this was the only group tested. The RB51 and RB51TLR249 groups induced IL-17, suggesting a potential role in the immune response associated with vaccination and/or infection.

## Discussion

In analyzing our results, and the lack of efficacy of the RB51TLR agonists against strain 2308 challenge, it is apparent that the level of infection in the control mice is lower than our previously published studies [11]. We did back calculate our challenge dose, and it was accurate. Possible explanations for the decreased titer in control, and potentially other mice, would be unknowingly administering a lower dose to all mice through a pipetting error. After examining our records,



**Figure 12:** Cytokine production by stimulated splenocytes as determined by ELISAs. Mice were vaccinated IN with PBS, strain 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 ( $2 \times 10^4$  CFU/ mouse). Fourteen days post challenge, mice were euthanized. Splenocytes were collected, and stimulated with either media, ConA, irradiated strain 2308 (IR2308), irradiated strain RB51 (IRRB51), heat killed strain 2308 (HK2308) or heat killed strain RB51 (HKRB51) ( $10^6$  CFU/106 CFU/well). Cytokines were measured by ELISA, A) IL-12 B) TNF- $\alpha$ , 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  $\pm$  SD. Capital letters denote significant differences to the same lower case letter, and the addition of asterisk indicates a trend ( $0.05 < p < 0.1$ ).

we did not identify an error in dosing, administration, etc., and despite that the dose in the BALB/c mice is lower than expected, the reported results are statistically significant. We may have seen more differences both in protection and correlates of protection/chronic infection, if the mice had maintained a higher dose of strain 2308. In addition, while the numbers of mice for the study were not extensive, this is the same or similar to other similar publications, and our results are statistically significant [11,21,22].

While not statistically significant, based on clearance, strain RB51 with TLR2 and 4 agonists (RB51TLR24 group) appeared to be the most promising. There were significant differences in

the immune responses in both treatment groups (RB51TLR24, RB51TLR249), as well as strain RB51 alone, supporting important differences in the pathways, mechanisms of the TLR vaccine induced pathways, and the immune response associated with subsequent infection/challenge.

In assessing the mechanisms of how the different vaccines with TLR agonists induced immune responses, we propose that there appears to be a pattern for both DC subsets and activation, lymphocyte responses as well as cytokine production. Based on our previous study by Surendran et al. [10], we proposed that the addition of the TLR2 or TLR4 agonist with strain RB51 induced immunity against respiratory challenge with strain 2308 was associated with a CD103 DC mediated response. We proposed that the CD103 DC was present in the initial response, and stimulated a protective immune response based on cytokine production in the lung/organs. As infection declined, the CD103 DC and cytokine production decreased, whereas persistently infected mice still had increased CD103 DC and cytokine levels. We proposed that the CD103 DC were most critical for protection in the initial response. In this study, at 2 weeks post-challenge, there appeared to be a role for CD103 as well as possibly CD11b. In this study, we propose but cannot prove that the RB51TLR24 vaccine group had already induced a CD103 response and transitioned to a CD11b response, whereas the RB51TLR249 group still had an increased CD103 response, but had not transitioned. By contrast, the RB51 group, which appeared to overall poorly stimulate a protective response, induces a CD11b response. The exact mechanism for the RB51 response is not clear, but our data suggest that the RB51 induced response may be different than those induced by RB51 with various TLR agonists.

In our model, RB51 and RB51TLR249, had significantly increased CD11c low, CDlow, MHC class II low, CD11c lo MHC class II high, CD11c low CD40 vs. the PBS and RB51TLR24 groups. By comparison, RB51TLR2 had increased CD11c high, (CD11c I cd86 high, CD49) in the BAL and increased CD11c CD86+ cells. We proposed that because of differences in TLR mediated activation and infection, that RB51TLR249 and RB51 activate cells in response to infection differently than RB51TLR24. In other models, a role has been shown for the CD11c high cells to be more functional with respect to activating T-cells [23-27]. Additional studies are needed to identify the differences in these DC subsets in protection.

Some of the differences in DC activation and cytokine production within different organs may reflect the differential pathways by which the vaccines  $\pm$  TLR agonists induced immune responses and response to challenge/infection. For instance, in the BAL, the RB51TLR24 group induced higher levels of TNF- $\alpha$  and IL-17, but non-significantly lower levels of IL-12 vs. the RB51TLR249 group at this time point. Additionally, splenocytes from the RB51TLR24 vaccinated mice consistently had increased IFN- $\gamma$  production vs. the RB51TLR249 stimulated splenocytes. This supports the overall higher CD4 Th1 CD8 Tc1 response by the RB51TLR24 vs. other groups. The question still remains as to why overall clearance by the RB51TLR24 group was not higher. Possible explanations include that the RB51TLR24 group induced response may not

be great enough (i.e. it is a DC1 CD4 Th1 CD8 Tc1 response, but it is not high enough, or that some aspect of combining the TLR2 and 4 agonists stimulated different types of responses, which limited the overall response). It is possible that our combined dose of TLR agonists was not optimal despite our and other previous studies (REF).

In examining more closely the differences between the RB51, RB51TLR24, RB51TR249 groups for mechanisms associated with the differential TLR activation, Chandran et al. [28], demonstrated that TLR2 agonists combined with influenza HA peptide stimulated IL-2 and IL-17 dependent activation, whereas the TLR4 agonist with HA peptide stimulated decreased numbers of CD4 cells, but higher percentages of IFN- $\gamma$  producing cells. Other studies have demonstrated a role for TLR2 in Th1, Th2, Th17 or T-reg responses [10,12,20,28,29]. Additionally, Mori et al., have also demonstrated a role for TLR9 and IL-17 [30].

Our previous studies in TLR KO mice demonstrated that the TNF- $\alpha$  production was TLR2 and TLR9 dependent, whereas IL-12 was TLR2 and TLR4 dependent [10,11]. Based on our data in this study, in the BAL, we see that the RB51TLR24 pathway induces significantly increased TNF- $\alpha$  production vs. the RB51TLR249 pathway, and also the former has increased IL-17 vs. RB51 without TLR agonists. These data support the ability of the RB51TLR24 group to promote a stronger DC1 response vs. the RB51TLR249 group.

In regards to the subsequent T-cell response, strain RB51 has previously demonstrated to show a role for CD25 cells in response to infection. It is not clear if the CD25 response is a primary response associated with infection, which could potentially limit the protective immune response vs. a mechanism for resolving the immune response. Several investigators (Pasquali et al. [31], Xavier et al., [32], Corsetti et al [33], have demonstrated a role for some combination of CD4, CD25 and IL-10 in limiting the protective immune response. Collectively, these studies support that the RB51TLR24 group with the increased CD4 CD25 population in our model, could be limiting the protective immune response.

If Xavier/Tsolis and Corsetti et al's theories are accurate for our models, we would expect differences in IL-10 responses and clearance. Our time point was 2 wks post challenge, and ideal time points would be earlier. Thus, an alternative explanation, based on Martirosya et al.'s study [34], in which they determined that there is a CD4 CD25 CD62 CD44 CD43 IFN- $\gamma$  producing cytotoxic cells in infection. The functionality of the cells must be assessed in order to define their role in regulating (promoting/inhibiting) the immune response to *Brucella* [35,36].

Based on these data, we propose that the differences in DC function are also associated with increased DC activation and ultimately T-cell activation and function and protection. Although additional studies are needed to further define the mechanisms of the TLR mediated immune responses, potential protection and also lack of protection, these data do support that the RB51TLR24 responses can upregulate immunity.

Enhancement of immunity is needed to upregulate protection. Additional studies are warranted on the mechanisms by which the TLRs activate immunity and protection, as well as the roles of IL-10, CD4 CD25 and Th17 cells in immunity.

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