Vaccine 31 (2013) 4103–4110

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Protection to respiratory challenge of *Brucella abortus* strain 2308 in the lung

Naveen Surendran[a,1], Nammalwar Sriranganathan[b], Stephen M. Boyle[b], Elizabeth M. Hiltbold[c], Nancy Tenpenny[a], Michelle Walker[a], Kurt Zimmerman[b], Stephen Werre[b], Sharon G. Witonsky[a,*]

[a] *Department of Large Animal Clinical Sciences, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342, USA*
[b] *Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA 24061-0342, USA*
[c] *Department of Microbiology and Immunology, Wake Forest University, Winston-Salem, NC 27157, USA*

ARTICLE INFO

Article history:
Received 5 April 2013
Received in revised form 21 June 2013
Accepted 25 June 2013
Available online 8 July 2013

Keywords:
Brucella
Dendritic cell
Respiratory

ABSTRACT

Brucella is amongst the top 5 causes of zoonotic disease worldwide. Infection is through ingestion, inhalation or contact exposure. *Brucella* is characterized as a class B pathogen by Centers of Disease Control and Prevention (CDC). Currently, there are no efficacious vaccines available in people. Currently available USDA approved vaccines for animals include *B. abortus* strain RB51 and *B. melitensis* Rev1. Protection is mediated by a strong innate and CD4 Th1, CD8 Tc1 immune response. If protective vaccines can be developed, disease in people and animals can be controlled. While strain RB51 protects in cattle, and against intraperitoneal challenge in mice, it does not protect against respiratory challenge. Therefore, we assessed the efficacy of strain RB51 combined with different TLR agonists, and O-side chain from LPS, to enhance protection against respiratory challenge with strain 2308. We hypothesized that TLR agonists and O-side chain would enhance protection. Strains RB51 with TLR2 agonist, RB51 with TLR4 agonist and strain 19 provided significant protection in the lung. Protection using strain RB51 with TLR agonists was associated with increased IgG2a and IgG1 in the (bronchoalveolar lavage) BAL and serum, and increased IgA (serum). Splenocytes from strain RB51 with TLR2 vaccinated mice up-regulated antigen specific interferon-gamma and TNF-alpha production. Vaccination and challenge resulted in significant increases in activated dendritic cells (DCs), and increased CD4 and CD8 cells in the BAL. Overall, this study demonstrates the ability of TLR agonists 2 and 4 to up-regulate strain RB51 mediated protection in the lung to respiratory challenge against strain 2308.

1. Introduction

Brucella is a Gram negative intracellular bacterium, of the alpha-2-proteobacteria class [1–3]. It is one of the top 5 zoonotic diseases worldwide, causing disease in people and animals through ingestion, inhalation or contact exposure; animals are the reservoir of infection [2]. It is a bioterror threat because of its ability to be mutated readily, its pathogenicity, and that it can be readily aerosolized [4]. Thus, Centers of Disease Control (CDC) and Prevention characterizes it as a Category B pathogen. Currently there are no efficacious human vaccines available [5]. Vaccine strains for animals include United States Department of Agriculture (USDA) approved strain RB51 and *B. melitensis* Rev1 for small ruminants [6]. Protection is mediated by a strong cell mediated immune response, including a CD4 Th1 and CD8 with associated IFN-gamma production [7,8]. A vaccine that protects against respiratory challenge to *Brucella* would be ideal in both humans and animals [9,10].

Previous studies demonstrated that mice vaccinated intraperitoneally (IP) with *B. abortus* strain RB51 were protected against IP challenge with strain 2308 [10,11]. We predicted that intranasal vaccination would also protect against intranasal challenge. However, strain RB51 did not protect [5], and neither altering the route or dose, nor changing priming/boosting of vaccination, enhanced protection in the lung. Based on our studies in TLR KO mice [12], we demonstrated a role for TLR2, TLR4 and TLR9 in DC activation and function, and we also demonstrated a TLR4 dependent effect in clearance of strain RB51 from the lung at day 14 post-infection, and

* Corresponding author at: Department of Large Animal Clinical Sciences, Phase II, Duck Pond Dr., Blacksburg, VA 24061-0442, USA. Tel.: +1 540 231 4621; fax: +1 540 231 1676.
E-mail addresses: switonsk@vt.edu, switonsky@yahoo.com (S.G. Witonsky).
[1] Current address: Rochester General Hospital Research Institute, Rochester, NY 14621, USA.

0264-410X/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.vaccine.2013.06.078

a TLR2 dependent effect in clearance of strain 2308 from the spleen at day 42 post-infection. Thus, we hypothesized that TLR agonists would up-regulate strain RB51 mediated immunity in the lung. For this study, mice were vaccinated with strain RB51 with or without TLR agonists 2, 4 or 9 administered concurrently, and mice were challenged intranasally with strain 2308. We determined protection/clearance as well as identified potential immune correlates of protection/persistence. A preliminary role for IL-17 was also identified.

2. Materials and methods

2.1. Mice

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

2.2. Bacterial strains

Live attenuated smooth strain 19, rough *B. abortus* strains RB51, RB51WboA and virulent smooth strain 2308 were from our stock culture collection. All experiments with *Brucella* were performed in our CDC approved (C2003 1120-0016) Biosafety Level (BSL)-3 Infectious Disease Unit (IDU) facility [6,11].

2.3. Experimental design for challenge study

Serum was collected, and BALB/c mice ($n = 5$/group) were vaccinated IN, under xylazine-ketamine anesthesia, with either *B. abortus* rough strain RB51 (4×10^8 CFUs/mouse) alone, or simultaneously in combination with one of the TLR agonists from Invivogen (San Diego, CA) (TLR2 agonist 5 μg/mouse PAMCSKK4, TLR4 agonist 10 μg/mouse ultrapure *E. coli* 0111:B4 LPS or TLR9 agonist 30 μg/mouse CpG), strain RB51WboA, which is strain RB51 with a complemented *wboA* gene (4×10^8 CFUs/mouse), or strain 19 (2×10^5 CFUs/mouse) in 20 μl phosphate buffered saline (PBS) [13]. One group was given strain RB51 (4×10^8 CFUs/mouse) intratracheally (IT) to determine if protection was enhanced with IT vaccination which would potentially deposit the bacteria further in the lung. Six weeks post-vaccination, serum was collected, and all mice were challenged IN with smooth strain 2308 (2×10^4 CFUs/mouse). Mice were euthanized at day 14 post-challenge. Serum, bronchoalveolar lavage (BAL), lung, mediastinal lymph node (MLN) and spleen were collected. Minimum level of detection for CFUs was 20. Any sample with 0 colonies present was recorded as 19 CFU (Fig. 1) [5,11]. Lung homogenates were frozen for cytokine analysis [11].

2.4. Serology

Strain RB51 isotype (IgG1, IgG2a, and IgA) specific antibodies were determined on serum from pre-vaccinated, post-vaccinated and post-challenged mice, as well as BALs from post-challenged mice. Strain 2308 isotype (IgG1, IgG2a and IgA) specific antibodies were determined on BAL from post-challenged mice [11,14].

2.5. Staining and flow cytometry

Cells from BAL and splenocytes were counted, and aliquots were stained for FACS analysis [15,16]. Stain combinations were (1) APC conjugated anti-CD11c (Biolegend, Pacific Blue conjugated anti-CD86 (Biolegend), biotin conjugated anti-CD40, Streptavidin APC-Cy7 (Biolegend), FITC CD11b (Invitrogen), and PE conjugated anti I-A/I-E(BD Pharmingen; (2) biotinylated conjugated anti-CD103 (Pharmingen/Invitrogen), Streptavidin APC-Cy7; Pacific Blue conjugated anti-CD8 (Biolegend), APC conjugated anti-CD11c, and PE conjugated anti-CD4 (Pharmingen; (3) Texas Red CD4 (Caltag/Invitrogen), Pacific Blue conjugated anti-CD8 alpha, PE anti-CD44 (Pharmingen/Invitrogen), FITC conjugated anti-CD62 (Pharmingen/Invitrogen); (4) Texas Red conjugated anti-CD4, Pacific Blue conjugated anti-CD8 alpha, PE conjugated anti-CD45RB (Pharmingen); (5) Texas Red conjugated anti-CD4, Pac Blue conjugated anti-CD8 alpha, FITC conjugated anti-CD69 (Pharmingen/Invitrogen) (6) PE conjugated anti-B220 (Pharmingen/Invitrogen), FITC conjugated anti-CD69. The company for each antibody cited is listed the first time only. Cells were washed and analyzed by BD FACSAria™ flow cytometer [15,16].

2.6. Cytokine quantitation of splenocytes

Splenocytes (5×10^5/well) were stimulated as previously described [11] with media, ConA (0.5 μg/well), irradiated (IR) or heat-killed (HK) strains (10^6 CFU/well) of strain RB51 or strain 2308. Supernatants were harvested at 5 days, and stored at −70 °C until cytokine analysis by ELISAs was performed [11].

2.7. ELISA cytokine analysis

Samples (BAL, splenocytes supernatants, lung homogenates) were assayed for TNF-α, IL-12_{p70} (bioactive form of IL-12), IFN-γ, IL-10, IL-4 and IL-17 (lung only) cytokine levels using indirect sandwich ELISAs (BD Pharmingen) [7,16,17].

2.8. Statistical analysis

To assess significant differences in clearance, treatments were compared using Kruskal–Wallis test followed by Dunn's procedure for multiple comparisons. All statistical analysis was done using SAS system (NC, USA). Significance was determined at $p < 0.05$. To assess differences in serology, flow cytometry, and cytokines in the lung, a one way ANOVA was performed using the GLIMMIX procedure for SAS. The Benjamini and Hochberg procedure was used to control for false discovery rate. For splenocyte and BAL cytokine data, the nonparametric ANOVA using the Exact Kruskal–Wallis test was used to assess significance.

3. Results

3.1. Strain 19 and TLR agonists enhance protection in lung

Mice vaccinated with strain RB51 + TLR2 agonist (referred to as RB51TLR2), RB51 + TLR4 agonist (referred to as RB51TLR4) or strain 19 all demonstrated significant clearance in the lung at day 14 ($p < 0.05$) compared to PBS control (Fig. 1). Strain RB51 with TLR9 agonist (referred to as RB51TLR9) demonstrated a trend ($p = 0.075$) toward clearance in the lung. No significant differences in clearance were noted with lung, spleen or MLN with any other vaccines.

Clearance in each organ was also assessed (Fig. 1). Minimum level of detection was 20 CFU/organ. RB51TLR2 vaccine yielded maximum clearance of strain 2308 from lung and MLN (4/5 mice), followed by RB51TLR4 and strain 19, each with 2/5 from lung and 3/5 from MLN (Fig. 1).

3.2. TLR agonists and strain RB51WboA induce higher antibody titers against strain RB51 in BAL

Most significantly, strains RB51WboA, RB51TLR2, RB51TLR4 and RB51TLR9 all induced significantly higher IgG1 post strain 2308