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Vaccination evokes gender-dependent protection against tularemia infection in C57BL/6Tac mice

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ABSTRACT

Francisella tularensis (*Ft*) is a Category A biothreat agent for which there currently is no FDA-approved vaccine. Thus, there is a substantial effort underway to develop an effective tularemia vaccine. While it is well established that gender can significantly impact susceptibility to primary infection, the impact of gender on vaccine efficacy is not well established. Thus, development of a successful vaccine against tularemia will require an understanding of the impact gender has on vaccine-induced protection against this organism. In this study, a role for gender in vaccine-induced protection following *Ft* challenge is identified for the first time. In the present study, mucosal vaccination with inactivated *Ft* (i*Ft*) LVS elicited gender-based protection in C57BL/6Tac mice against respiratory challenge with *Ft* LVS. Specifically, vaccinated male mice were more susceptible to subsequent *Ft* LVS challenge. This increased susceptibility in male mice correlated with increased bacterial burden, increased tissue inflammation, and increased proinflammatory cytokine production late in post-challenge infection. In contrast, improved survival of i*Ft*-vaccinated female mice correlated with reduced bacterial burden and enhanced levels of *Ft*-specific Abs in serum and broncho-alveolar lavage (BAL) fluid post-challenge. Furthermore, vaccination with a live attenuated vaccine consisting of an *Ft* LVS superoxide dismutase (SodB) mutant, which has proven efficacious against the highly virulent *Ft* SchuS4 strain, demonstrated similar gender bias in protection post-*Ft* SchuS4 challenge. Of particular significance is the fact that these are the first studies to demonstrate that gender differences impact disease outcome in the case of lethal respiratory tularemia following mucosal vaccination. In addition, these studies further emphasize the fact that gender differences must be a serious consideration in any future tularemia vaccine development studies.

1. Introduction

It is well documented that gender can play an important role in determining the outcome of primary infection in that the gender of a host can significantly affect susceptibility to infection [1]. Epidemiological studies have shown that males and females handle infections differently [2,3]. Most notably, males can be at increased risk of susceptibility to major bacterial and viral infections versus females [4,5]. In the case of primary infection, consistent correlations between sex, immunity, and protection have been observed. For example, females have greater humoral and cell-mediated immune responses to antigenic stimulation by infectious agents as compared to males [6–8]. In contrast, males have higher levels of expression of pattern-recognition receptors for bacterial lipopolysaccharide, which has been linked to heightened production of proinflammatory cytokines and a greater incidence of lethal systemic inflammation observed in males [9]. The existence of gender bias in the immune response to infectious diseases is further supported by numerous in vivo studies focused on *Mycobacterium marinum* [10], *Streptococcus pneumoniae* [11], *Streptococcus pyogenus* [12], *Plasmodium chabaudi* [13], and *Mycoplasma pulmonis* [14]. A similar tendency has been seen in humans against numerous pathogens including: *Mycobacterium tuberculosis* [15], Influenza virus [16] and community-acquired pneumonia [17] in which men are more susceptible than women.

In contrast to the above, the impact of sex on vaccine-induced protection has received substantially less attention and is thus less clear. This lack of clarity is also exacerbated by inconsistencies between the limited numbers of investigations completed. For example, in several studies, women appeared to exhibit better responses to vaccination than men. This was the case for influenza,

Abbreviations: *Ft*, *F. tularensis*; i*Ft*, inactivated *Ft*; SodB, superoxide dismutase B; Ab, antibody; mAb, monoclonal Ab; Ag, antigen; i.n., intranasal; i.d., intradermal; BAL, bronchial alveolar lavage fluid; MTD, median time to death.

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hepatitis A and B, and herpes simplex (HSV)-2 vaccines [18,19] [20]. In addition, when using the 23-valent pneumococcal polysaccharide vaccine (PPV23), vaccine efficacy was higher in females versus males [21]. In contrast to the above studies, men demonstrated superior Ab responses to diphtheria, measles, and smallpox vaccines when compared to women [19,22]. Men were also better protected against diphtheria and tetanus than their female counterparts [23,24].

Thus, while a great deal is known regarding the impact of gender on primary infection, the impact of gender on protection following vaccination is substantially less clear. Furthermore, based on the limited number of studies that have been done in this regard, results suggest the infectious agent itself may also influence the role gender plays in vaccine-induced immunity and protection [5].

Francisella tularensis (*Ft*), the causative agent of tularemia, is a gram negative, intracellular pathogen. *Ft* has also been used as bio warfare agent due primarily to its high virulence and ability to be aerosolized [25–27]. Most notably however, clinical incidence due to primary infection and progression of tularemia in endemic areas is significantly higher in males than in females. While this may reflect differences in pathogen exposure through hunting and outdoors professional activities (CDC – http://www.cdc.gov/tularemia/statistics/agesex.html), [28], gender differences could also be a contributing factor. In addition, there is no approved tularemia vaccine and thus substantial efforts are underway to develop one. Therefore, we sought to fill a critical knowledge gap in tularemia vaccine development and investigate the impact of gender on tularemia vaccine efficacy. We demonstrate for the first time that while we observe no difference in the susceptibility of naïve male versus female mice to *Ft* challenge, female mice, which are first vaccinated with either inactivated or attenuated *Ft* vaccine are more resistant to infection as compared to their male counterparts. Results of experiments examining humoral and cellular immune responses following vaccination of male versus female mice are also support this conclusion.

2. Materials and methods

2.1. Mice

Pathogen-free, 6-to-8-week-old male and female C57BL/6Tac mice were purchased from Taconic Farms. Mice were housed in sterile microisolator cages in the animal biosafety level 2 (ABSL-2) and ABSL-3 facilities at the Albany Medical Center (AMC). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Albany Medical College.

2.2. Ft *organisms*

Ft LVS and *Ft* SchuS4 were cultured aerobically at 37 °C in modified Mueller-Hinton broth (MHB) or agar (Becton Dickinson, Sparks, MD) supplemented with ferric pyrophosphate and Iso-Vitalex (Becton Dickinson, Sparks, MD). *Ft* LVS SodB mutant was grown in Brain-Heart Infusion (BHI) medium and the active mid-log phase bacteria were harvested and used for immunization.

2.3. *Generation of* i*Ft immunogen*

Ft LVS grown in MHB was inactivated using paraformaldehyde as previously described [29,30]. Inactivation was verified by plating a 100 μl sample (1×10^9 i*Ft* organisms) on chocolate agar plates (Becton, Sparks, MD) for 7 days. The protein concentration of i*Ft* was estimated by Lowry's method, the i*Ft* preparations were stored at −20 °C in PBS.

2.4. *Immunization and challenge studies*

Prior to immunization, each mouse was anesthetized by intraperitoneal (i.p.) injection of 20% ketamine plus 5% xylazine. In the case of i*Ft* vaccination, mice were subsequently administered intranasally (i.n.) either 20 μl of PBS (control) or i*Ft* (1500 ng) in 20 μl of PBS. Unless, otherwise indicated, mice were immunized on day 0 and boosted on day 21. Immunized mice were then challenged on day 35 i.n. using $1–10 \times LD_{50}$ of *Ft* LVS. In this case, $1 \times LD_{50}$ is equivalent to 800 CFU of *Ft* LVS administered i.n. In the case of immunizations using live attenuated vaccine, an attenuated *Ft* LVS SodB mutant organism was utilized as the vaccine. Specifically, 1×10^3 CFU of *Ft* LVS SodB in 50 μl of PBS were administered intradermally (i.d.) followed by an i.n. boost with 1×10^3 CFU in 20 μl of PBS on day 21 post-primary immunization. Mice were then challenged i.n. with 20–30 CFU of *Ft* SchuS4 in 20 μl of PBS on day 42 post-primary immunization. The challenged mice were subsequently monitored for survival for a minimum of 25 days using death as an endpoint.

2.5. *Quantification of bacterial burden*

Following immunization and challenge, mice were euthanized at various time intervals as indicated in the individual figures and bacterial burden in the lungs, liver and spleen of infected mice was monitored as previously described [29].

2.6. *Serum lactate dehydrogenase (LDH) assay*

Serum concentrations of LDH were measured using a lactate dehydrogenase activity assay kit (Sigma–Aldrich, St. Louis, MO). Standards and serum samples were diluted according to manufacturers protocol and 50 μl/well were added to plates along with NAD substrate. Plates were incubated at 37 °C and read at 450 nm using a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA). LDH activity was determined using following equation: LDH activity = $B \times$ sample dilution factor/(reaction time) $\times V$, where B = amount of NADH generated (nmole) and V = sample volume (mL) added to the well.

2.7. *Histopathology*

Lung, liver and spleen from i*Ft*-vaccinated and *Ft* LVS-challenged mice were excised and processed for histology as previously described [29]. Disease severity in the tissues was then assessed based upon cellular infiltration, thickening of alveolar septa, and airway passage congestion.

2.8. *Cytokine quantification in tissues and broncho-alveolar lavage (BAL) fluid*

Tissue homogenates were obtained as indicated above when measuring bacterial burdens. Supernatants were then collected and stored at −20 °C for cytokine analysis. Luminex assay was performed to determine in vivo cytokine levels of interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17 (IL-17), and monocyte chemoattractant protein-1 (MCP-1) to assess inflammation.

2.9. *Assessment of humoral immune responses*

Anti-*Ft* Ab production in response to immunization and/or *Ft* infection in i*Ft*-immunized mice was measured by enzyme

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