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

Raju Sunagar, **Sudeep Kumar**, **Brian J. Franz**, and **Edmund J. Gosselin***
Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY 12208, United States

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.

Keywords

Tularemia; Gender differences; Host susceptibility; Intranasal vaccine; Inactivated vaccine; Humoral immunity; Proinflammatory cytokines

* Corresponding author. Tel.: +1 5182625562; fax: +1 5182626161. gossele@mail.amc.edu (E.J. Gosselin).

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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, 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

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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.

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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 linked immunosorbent assay (ELISA) as previously described [29,30].

2.10. Statistical analysis

Statistical data for bacterial clearance and cytokine production was generated using analysis of variances by two-way ANOVA or Mann–Whitney two-tailed test on post-challenge day 7, which is the peak of infection. In the case of survival, significance was determined using a log-rank (Montel-Cox) test. The data are expressed as mean ± standard deviation (SD). Differences between the experimental groups were considered statistically significance at a $P < 0.05$. The data were analyzed using GraphPad prism (v6.0) software (GraphPad Software, San Diego, CA).

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3. Results

3.1. Naive male and female mice are equally susceptible to primary infection with Ft

Initially, we sought to determine if there was a difference in susceptibility of naïve male versus female mice to challenge with *Ft* LVS. In this case, infection of naïve mice with $1 \times LD_{50}$ (Fig. 1A), $2 \times LD_{50}$ (Fig. 1B) or $4 \times LD_{50}$ (Fig. 1C) of *Ft* LVS resulted in no significant differences in survival between male and female mice.

3.2. Increased survival of female versus male mice is observed following challenge of vaccinated mice with Ft *LVS*

Given the relatively short time to death of 7–10 days following *Ft* LVS challenge (Fig. 1), it was possible any impact of gender on the adaptive immune response, and thus survival, may not be evident when challenging naïve mice with *Ft*. Thus, to address this possibility, male and female C57BL/6Tac mice were vaccinated i.n. with i*Ft* and subsequently challenged i.n. with *Ft* LVS. Similar to naïve mice, both male and female PBS-immunized mice were equally susceptible to *Ft* LVS infection, succumbing to infection by day 10. However, a marked difference in susceptibility to infection was observed among male and female mice following vaccination and subsequent challenge with $4 \times LD_{50}$. Male mice were significantly more susceptible to *Ft* LVS challenge than their female counterparts, as evidenced by their decreased survival and increased weight loss (Fig. 2A and B). This difference was also evident in terms of extended survival, but not weight loss, when challenging with $10 \times LD_{50}$, in that vaccinated female mice had an extended median time to death (MTD) (12 days), which was significantly higher than that of their male counterparts (9.5 days) (Fig. 2C and D).

3.3. Decreased bacterial burden is observed in vaccinated female versus male mice

The above differences in body weight loss and survival influenced by gender prompted us to also examine bacterial burden as a reflection of bacterial control. Specifically, we assessed bacterial burden in i*Ft*-vaccinated male versus female mice challenged with a sublethal dose ($1 \times LD_{50}$) of *Ft* LVS. Consistent with survival studies, mice of both sexes receiving PBS i.n. exhibited significantly higher bacterial numbers in the lungs, liver, and spleen compared to vaccinated mice (Fig. 3). However, bacterial numbers recovered from the lungs, livers, and spleens of vaccinated female mice were substantially lower than those observed in the same tissues of vaccinated male mice particularly on day 7 post-challenge. Collectively, vaccinated female mice not only exhibited a reduced bacterial burden as compared to their male counterparts, but also exhibited more rapid clearance of bacteria from the above tissues compared to the vaccinated males (Fig. 3).

3.4. Vaccinated female mice exhibit a reduced inflammatory response than male mice following Ft *LVS challenge*

To provide further support for the above observed gender differences in response to *Ft* LVS challenge following vaccination, tissue inflammation was assessed in i*Ft*-vaccinated male and female mice by measuring the serum concentration of LDH, which provides a means of quantitation for tissue destruction and inflammation. Serum samples for LDH measurements

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were taken on day 5 and 7 post-challenge with sublethal dose of *Ft* LVS. While no significant differences in the serum LDH concentration between the vaccinated and unvaccinated groups were observed on day 5, on day 7 male and female mice in the PBS-immunized groups, along with i*Ft*-vaccinated males, exhibited significantly higher levels of LDH in serum versus vaccinated female mice, indicating more severe inflammation and tissue destruction in the PBS and vaccinated male mice as compared to the i*Ft*-vaccinated female mice (Fig. 4). Furthermore, histological examination of lung tissues from both PBS and i*Ft*-vaccinated male and female mice with a sublethal dose ($1 \times LD_{50}$) of *Ft* LVS exhibited more severe inflammation in PBS-immunized male and female mice as well as i*Ft*-vaccinated male mice as compared to i*Ft*-vaccinated female mice on day 7 post-challenge (Supplemental Fig. 1B–E). Specifically, tissue damage and necrotic lesions were observed in the lung tissue of i*Ft*-vaccinated males. In contrast, in i*Ft*-vaccinated female mice, mild inflamma-tory foci in the lungs were observed, with histopathology being less severe overall versus i*Ft*-vaccinated male mice.

Supplemental Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.04.054.

3.5. Proinflammatory cytokine levels are reduced in female versus male mice, following iFt-*vaccination and* Ft *LVS challenge*

Several studies have demonstrated that male mice produce significantly higher levels of proinflammatory cytokines as compared to female mice following in vivo LPS exposure [31,32]. We have previously observed that regulated IFN-γ production strongly correlates with increased protection during *Ft* infection [29]. Thus we also evaluated the proinflammatory cytokine responses including that of IFN-γ in lung homogenates of PBS and i*Ft*-vaccinated male versus female mice prior to and following *Ft* LVS challenge. For the most part, there were no significant differences in cytokine profiles between vaccinated male and female mice pre-challenge (Supplemental Fig. 2). However, while early in the infection post-challenge both i*Ft*-vaccinated male and female mice showed similar levels of cytokine production, at 7 days post-challenge, the levels of IFN-γ, IL-6, and MCP-1 were significantly elevated in i*Ft*-vaccinated male mice. In contrast, i*Ft*-vaccinated female mice displayed regulated production of proinflammatory cytokines in tissues and BAL fluid (Fig. 5A and D). Furthermore, proinflammatory cytokines measured in spleen and liver homogenates exhibited similar differences to that of the lungs (Fig. 5B and C).

Supplemental Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.04.054.

*3.6. i*Ft-*Vaccinated female mice exhibit elevated anti-*Ft Ab titers as compared to their male counterparts following Ft *LVS challenge*

Given that i*Ft*-vaccinated female mice exhibit better protection, lower bacterial burden, and reduced histopathology, as compared to their male counterparts, we hypothesized that the humoral immune response, which, in some cases, can mediate protection against *Ft* infection [29,33], may also be superior to that of male mice. Accordingly, we observed that i*Ft* vaccination generated elevated levels of *Ft*-specific Abs in female mice as compared to their

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male counterparts both prior to (Supplemental Fig. 3A) and post-*Ft* LVS challenge. Specifically, vaccinated female mice produced higher titers of *Ft*-specific IgA and IgG2c in serum as well as in BALF by day 7 post-challenge, as compared to the vaccinated male mice (Supplemental Fig. 3B and C).

Supplemental Fig. 3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.04.054.

3.7. Gender-based protection is also observed in mice vaccinated with a live attenuated Ft vaccine (SodB mutant) and subsequently challenged with the highly virulent Ft *SchuS4 organism*

Having observed differences in survival in male versus female mice following i*Ft* vaccination and subsequent *Ft* LVS challenge, we sought to address the question as to whether a gender-based difference would also be observed when immunizing and challenging with *Ft* SchuS4. Unlike *Ft* LVS, *Ft* SchuS4 is highly virulent for humans and therefore is a model organism against which most *Ft* vaccines are being tested. Importantly, the i*Ft* immunogen used to immunize mice in *Ft* LVS challenge experiments does not induce protection against *Ft* SchuS4 challenge. Thus, to address the above question, it was necessary to choose a vaccination model, which had been previously shown to generate protection against infection with *Ft* SchuS4. Thus, in this case, mice were immunized with a live attenuated vaccine (SodB mutant derived from *Ft* LVS), which has been shown to provide clearly measurable, although incomplete, protection against *Ft* SchuS4 infection in C57BL/6J mice [34]. To first verify that the *Ft* SodB organism can function similar to i*Ft* generated from *Ft* LVS, *Ft* SodB was similarly inactivated and used as immunogen in an *Ft* LVS challenge experiment. In fact, the use of i*Ft* generated from *Ft* SodB not only generated improved survival in female versus male mice, but also the level of protection was superior to that of i*Ft* generated from wildtype *Ft* LVS (Fig. 6A and B). Most importantly however, in the case of *Ft* SchuS4 challenge, female mice vaccinated with live attenuated *Ft* SodB mutant showed increased survival versus their male counterparts, exhibiting a higher MTD of 20 days as compared to the male mice, with an MTD of 14 days (Fig. 6C and D).

4. Discussion

Multiple studies by a number of research groups have reported gender-based susceptibility to numerous pathogens and infectious diseases. However, studies on sex bias in tularemia infection have not been published. In general, males of many species are more susceptible than females to primary bacterial, viral, and fungal infections [11,35,36]. In contrast, in this study we show for the first time that both naïve male and female C57BL/6Tac mice are equally susceptible to *Ft* LVS tularemia (Fig. 1). However, prior mucosal immunizations with inactivated or live *Ft* vaccine results in a gender-based protection in the case of both *Ft* LVS and *Ft* SchuS4 challenge. Specifically, vaccinated male mice develop severe clinical disease and a significantly higher mortality rate, which correlates with increased weight loss as compared to immunized female mice (Fig. 2). Importantly, this implies tularemia vaccine efficacy will vary based on gender, which has been observed in clinical trials involving other infectious agents. Specifically, the efficacy of HSV-2 vaccination was 73% in women and

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only 11% in men [20]. Similarly, another study using PPV23 to prevent hospitalization due to *S. pneumoniae* demonstrated vaccine efficacy to be 68% in females and 34% in males [21]. However, in contrast to the above, vaccinated men are better protected against diphtheria and tetanus, which correlates with higher levels of toxin-specific Ab, which is required for protection in both cases [23,24]. The above differences in protection based on gender may reflect, in part, the type of immune response required for protection, suggesting that not just the individual's gender, but also the type of infectious agent involved are important factors in determining which gender is best protected against a given infection following vaccination. For example, when Ab alone is required for protection, males seem to be better protected than females. While this paradigm does not hold true in the case of HSV-2 and *S. pneumoniae*, where Ab is also known to play an important role in protection, the mechanism of protection is more complex, in that both Ab and cellular immune components (phagocytic cells) are required to resolve infection [37,38]. Thus, in the case of vaccination, further investigation will be required to more clearly define correlations between the specific gender protected and the immune component(s) required for protection against specific infections.

In regard to *Ft* infection, it is known that both cellular and humoral immunity can play important roles in resolving this infection [29,30,39], similar to the cases with HSV-2 and *S. pneumoniae*. Specifically, it has been established that cell-mediated immunity (CMI) plays a critical role in protection against tularemia [39]. For example, both CD4 and CD8T cells can proliferate and produce IFN-γ in response to a number of *Ft* proteins [40]. Additionally, depletion of CD4 T-cells, CD8 T-cells, or IFN-γ abolishes vaccine-induced immunity against type A *Ft* (SchuS4) infection [41]. Thus the production of IFN-γ by Th1 cells is thought to be key to developing protective immunity against *Ft*. In fact, in the case of IFN-γ production, we do show a difference between males and females (Fig. 5), which may help explain our observed differences in protection between genders. In contrast to cellular immunity, the role of humoral immunity in the resolution of *Ft* infection and protection remains controversial, although a number of investigations have demonstrated that humoral immunity can play a role in protection against tularemia, in particular *Ft* LVS (type B) infection. The latter is consistent with the observation that *Ft* has been shown to have an extracellular phase [33,42]. In addition, this controversy may be partially explained by studies from our laboratory, which demonstrated that while both Ab and IFN-γ can be critical for vaccine-induced protection [29], the need for Ab can be overcome, when IFN-γ levels are sufficiently high [30]. In regard to the generation of humoral immunity to *Ft* in these studies, we did observe elevated levels of *Ft*-specific IgA IgG and IgG2c in i*Ft*-vaccinated female mice in response to i*Ft* vaccination (Supplemental Fig. 3A), as well as *Ft* LVS infection on day 7 post-*Ft* LVS challenge (Supplemental Fig. 3B and C), which, as discussed above, may or may not be a significant contributor to the enhanced protection that we observed in female mice. In addition to the above, vaccinated female mice showed significantly less bacterial burden in the lung, liver, and spleen versus their vaccinated male counterparts (Fig. 3). Improved control of bacterial replication and early clearance of *Ft* LVS in vaccinated female mice was also reflected in the extent of tissue inflammation, as measured by serum LDH, and tissue pathology. In regard to the latter, more extensive cellular infiltration and associated cell death was apparent in the lungs of vaccinated male mice versus vaccinated

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female mice (Fig. 4 and Supplemental Fig. 1). More specifically, the increased bacterial load at day 7 observed in male mice likely contributes to the increased cytokine levels and inflammation observed. The latter also being reflected in LDH values obtained. Importantly, our laboratory as well as other laboratories, have demonstrated that non-protected mice (in this case male mice, Fig. 5) generally exhibit elevated levels of proinflammatory cytokines later in infection (5–7 days post-challenge), which we also observed in these studies [29,30,34,43]. Furthermore, the improved survival of vaccinated female mice following challenge with the highly virulent *Ft* SchuS4 strain (Fig. 6) indicates that the gender differences in disease susceptibility are not unique to *Ft* LVS, suggesting that the increased susceptibility of male mice to tularemia following vaccination is not *Ft* strain specific.

In regard to the broader mechanism(s) by which gender bias in protection against infection is mediated, it is firmly established that disparities in immune responses between males and females to infectious disease challenge are, in part, the result of the actions of reproductive hormones. Both in vitro and in vivo studies have revealed that testosterone, a male hormone, can be immunosuppressive and down-regulate immunoglobulin and cytokine production [44]. In contrast, estrogen, a female hormone, restores cellular immunity in injured male mice via suppression of interleukin-6 production [45]. Estrogen also suppresses MCP1 expression in murine macrophages, thus exerting anti-inflammatory effects potentially via prevention of macrophage accumulation [46]. Numerous studies suggest that mice succumb to tularemia infection due to excessive inflammation [29,30,47]. If estrogen limits and testosterone exacerbates such inflammation, this may explain why immunized female mice are less susceptible than immunized male mice to tularemia challenge in our studies.

In summary, we demonstrate for the first time that gender differences impact disease outcome in the case of lethal respiratory tularemia following mucosal vaccination. Specifically, female mice generate a more robust humoral immune response following vaccination. In turn, female mice are also better protected than their male counterparts against *Ft* challenge, where a key mechanism for protection requires both humoral and cellular immunity can play key roles in protection [30,39]. Taken together, the results presented in these studies demonstrate for the first time significant differences between males and females in susceptibility to Ft, reinforcing the importance of gender consideration in vaccine development against *Ft* and most likely other pathogens as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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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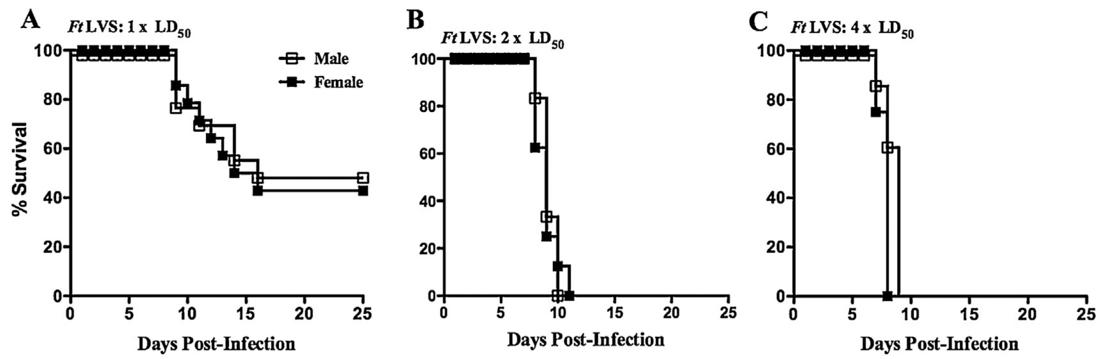

Fig. 1.

Naive male and female mice are equally susceptible to *Ft* infection. Naive C57BL/6Tac male and female mice were challenged i.n. with $1 \times LD_{50}$ (A), $2 \times LD_{50}$ (B), or $4 \times LD_{50}$ (C) of *Ft* LVS and subsequently monitored for 25 days for survival. The survival data represent combined data from two separate experiments (a combined total of 8–14 mice/group).

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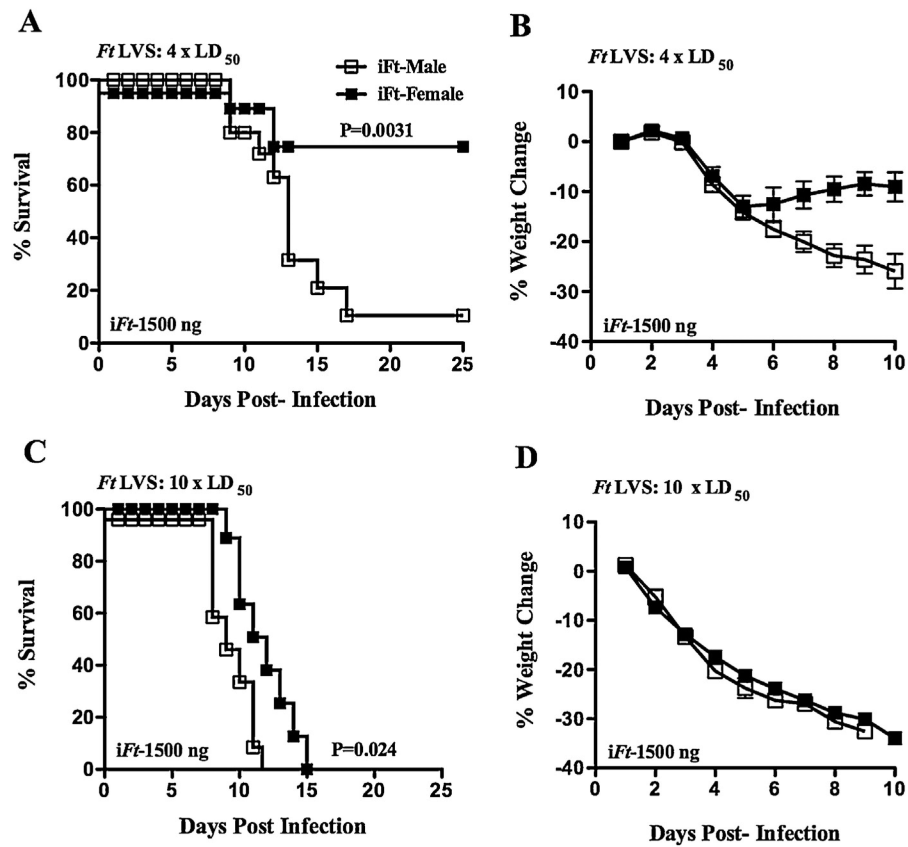

Fig. 2.

Improved survival of female versus male mice challenged with *Ft* LVS following i*Ft* vaccination. C57BL/6Tac male and female mice were immunized i.n. with either 20 µl of vehicle (PBS), 20 µl of PBS containing 1500 ng of i*Ft* on day 0 and were then boosted with the same i*Ft* preparations on day 21. Mice were then challenged i.n. on day 35 with 4 × LD_{50} (A and B) or 10 × LD_{50} (C and D) *Ft* LVS, and subsequently monitored for 25 days for survival. The mice were also weighed at the indicated times post-infection to monitor the progression of infection (B and D). The survival data represent combined data from two independent experiments (a combined total of 12–16 mice/group).

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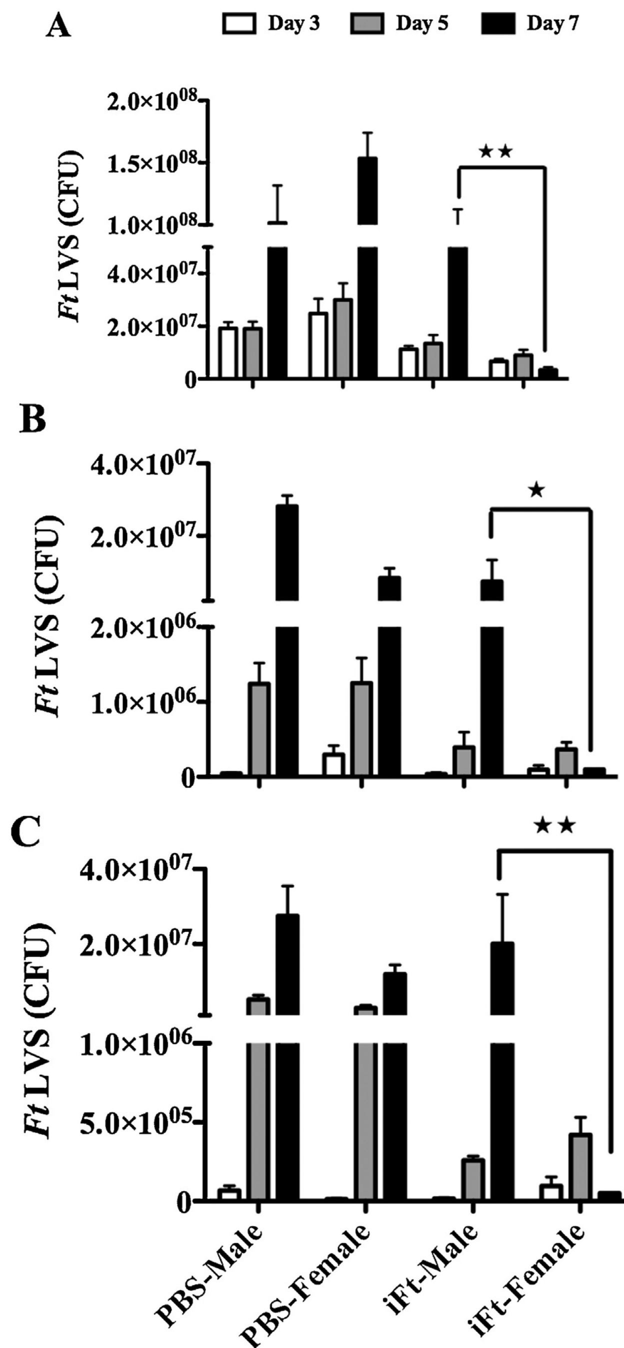

Fig. 3.

Lower bacterial burden is observed in vaccinated female versus male mice. C57BL/6Tac male and female mice were immunized i.n. with 20 μL of PBS (vehicle) or 20 μL of PBS containing 1500 ng i*Ft* on day 0 and subsequently boosted with the same on day 21. On day 35 mice were given a sublethal challenge i.n. with *Ft* LVS [850 CFU (~1 × LD_{50})]. Bacterial burdens from lung (A), liver (B), and spleen (C) were determined on days 3, 5, and 7 post-challenge. The data presented represent the average bacterial count of 3 mice sacrificed at

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each time point ± SD and are from a single experiment. Similar results were obtained in two independent experiments. *$P < 0.05$, **$P < 0.01$.

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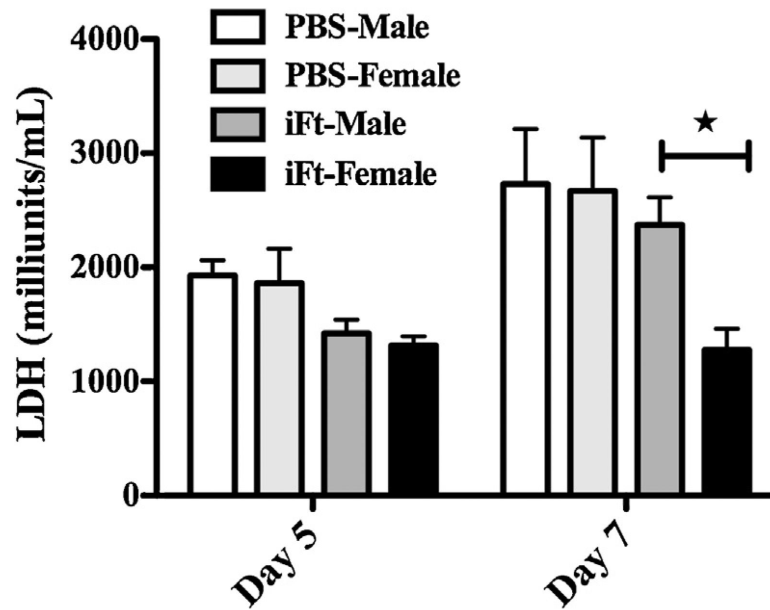

Fig. 4.

Vaccinated female mice exhibit less inflammation than male mice as indicated by reduced lactate dehydrogenase release in serum following *Ft* LVS infection. C57BL/6Tac male and female mice were immunized and challenged as described in Fig. 3. Serum concentrations of LDH were determined at the indicated time points post-infection. The data are depicted as mean ± SD (4 mice per group) and are representative of two independent experiments. *$P < 0.05$.

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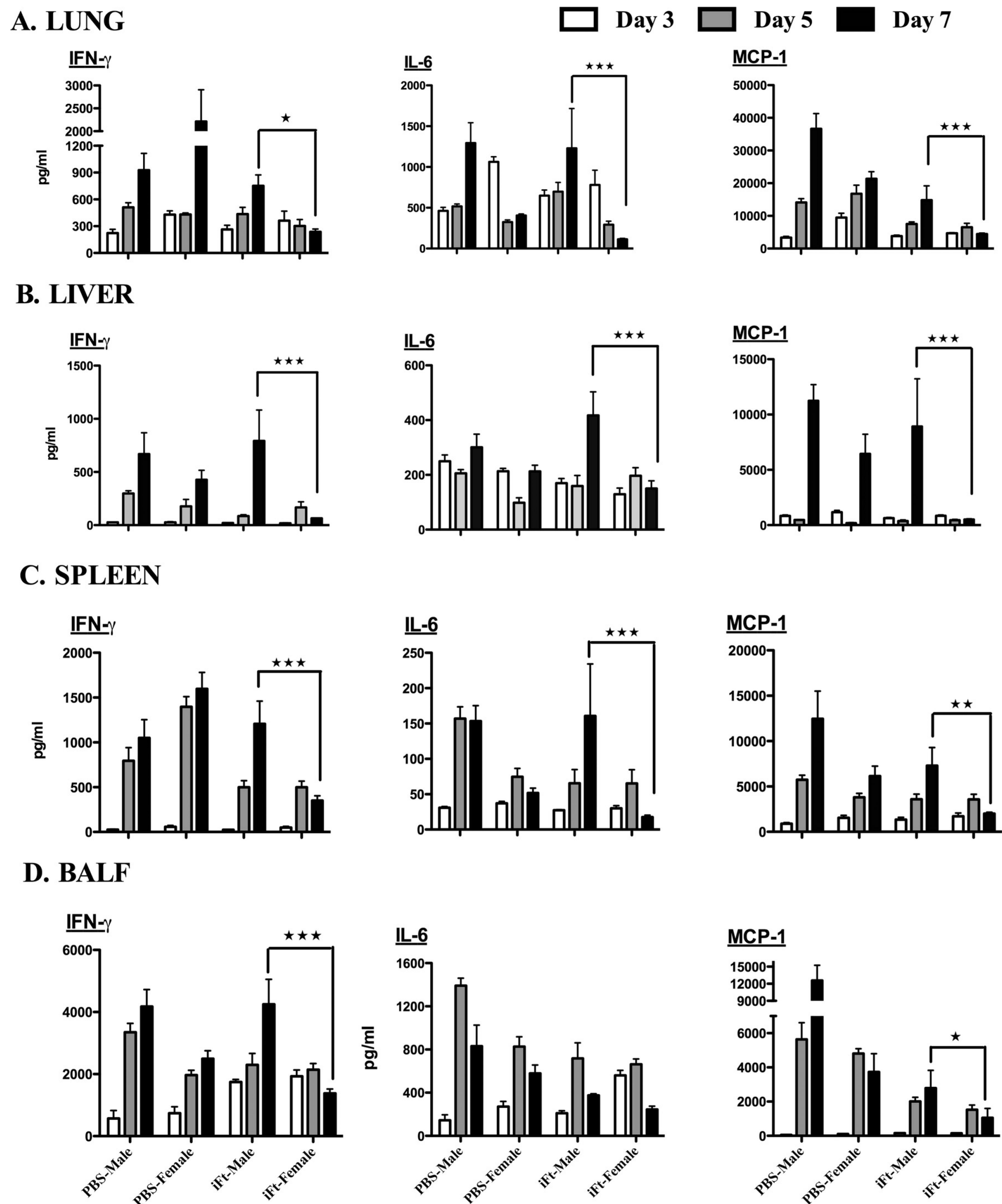

Fig. 5.

Proinflammatory cytokine levels are reduced in female versus male mice, following i*Ft*-vaccination and *Ft* LVS challenge. C57BL/6Tac male and female mice were immunized and challenged as described in Fig. 3. The levels of proinflammatory cytokines from tissue homogenates and BAL fluid were determined on days 3, 5, and 7 post-challenge using Luminex assay. All the cytokines measured are listed in Section 2 and were all detected in these studies. In this figure however, only cytokines levels for which differences between male and female mice were observed are presented and include: IL-6, IFN-γ, and MCP-1.

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Cytokine levels in the lungs (A), liver (B), spleen (C), and BAL fluid (D) are presented. The data are presented as mean ± SD (4 mice per group) and are representative of two independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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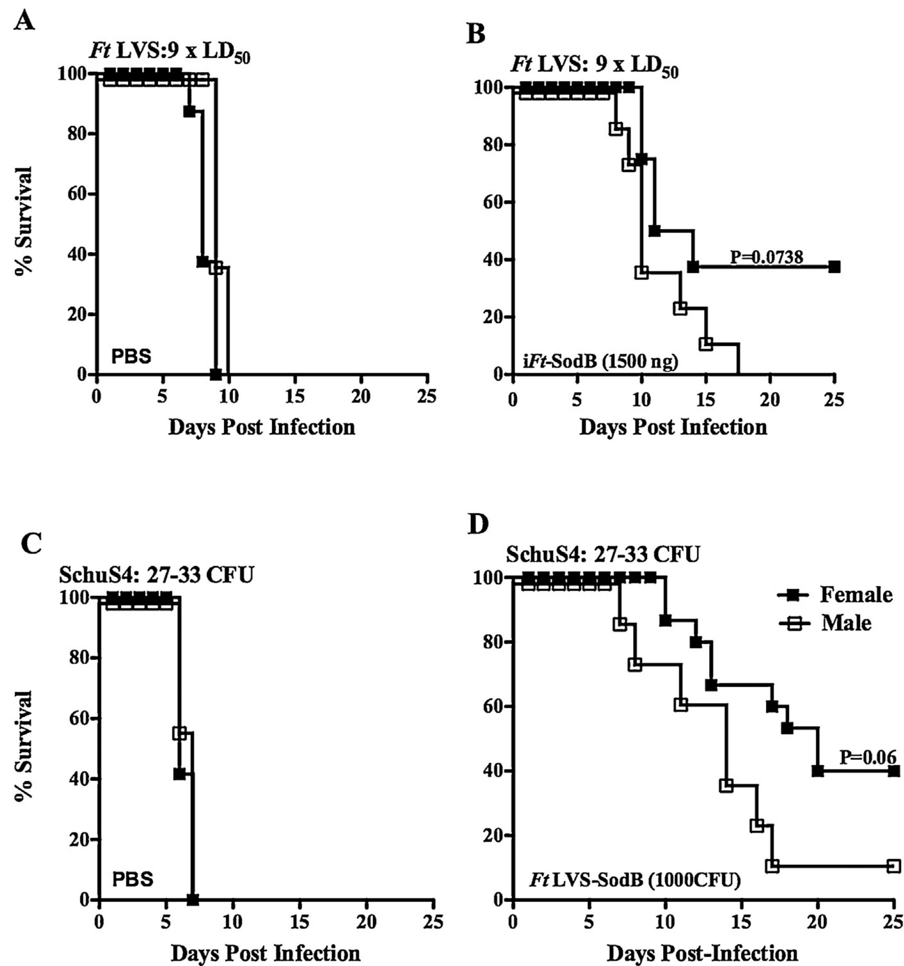

Fig. 6.

Gender-based protection is also observed in mice vaccinated with a live attenuated *Ft* vaccine (SodB mutant) and subsequently challenged with the highly virulent *Ft* SchuS4 organism. Upper panel – C57BL/6Tac male and female mice were immunized i.n. with either 20 μl of vehicle (PBS) or 20 μl of 1500 ng of i*Ft*-SodB on day 0, and boosted on day 21. Mice were then challenged i.n. on day 35 with 9 × LD_{50} CFU *Ft* LVS (A and B) and subsequently monitored for 25 days for survival. Lower panel – C57BL/6Tac male and female mice were immunized i.d. with either 50 μl of vehicle (PBS) or 50 μl of 1 × 10^3 CFU of attenuated *Ft* LVS SodB mutant (C and D) on day 0 and boosted i.n. on day 21 with either 20 μl of vehicle (PBS) or 20 μl of 1 × 10^3 CFU of attenuated *Ft* LVS SodB mutant. Mice were then challenged i.n. on day 42 i.n. with 27–33 (~15 to 25 × LD_{50}) CFU of *Ft* SchuS4 and subsequently monitored for 25 days for survival. Panels A and B represent one experiment containing 8 mice/group, while panels C and D are combined results from two independent experiments with a total of 16 mice/group.