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Differences In The M1 And M2 Macrophage Subtypes Between The Sexes Determine Susceptibility To Francisella Tularensis Infection

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DIFFERENCES IN THE M1 AND M2 MACROPHAGE SUBTYPES

BETWEEN THE SEXES DETERMINE SUSCEPTIBILITY

TO *FRANCISELLA TULARENSIS* INFECTION

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Dedication

To my lovely chicas

DIFFERENCES IN THE M1 AND M2 MACROPHAGE SUBTYPES BETWEEN THE SEXES

DETERMINE SUSCEPTIBILITY TO FRANCISELLA TULARENSIS INFECTION

by

MICHELLE A SANCHEZ, B.Sc.

THESIS

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Abstract

Francisella tularensis is the causative agent of the human disease tularemia. It is highly infectious with as few as 10 microorganisms via inhalation causing a lethal infection. *F. tularensis* infects a variety of cell types, including macrophages and neutrophils, since it needs to enter, survive and proliferate in order to cause pathogenicity. Disease is the result of over activating the host's own inflammatory response initiated by the macrophage's response to infection.

Known differences exist in the intensity of the inflammatory response between the sexes which leads to differences in sensitivity to autoimmune and infectious disease. Males tend to be more susceptible to infectious diseases whereas females tend to be more susceptible to autoimmune diseases. In contrast, our preliminary data demonstrated that female mice were more susceptible than male mice to the infectious disease *F. tularensis*.

We hypothesized that female macrophages respond to *F. tularensis* infection by generating a more intense inflammatory response which makes females more susceptible to *F. tularensis* disease. We determined the relative ratio of M1/M2 subtypes *in vivo* and their subsequent response to *F. tularensis* infection *in vivo* and *ex vivo*. Splenic macrophages were isolated and differentiated *in vitro* into M1 and M2 subtypes in order to directly compare differences in the intracellular response between males and females in response to *F. tularensis* infection. Our results demonstrated that females possess a higher abundance of M1 macrophages and that these M1 macrophages are more inflammatory in response to *F. tularensis* infection.

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Chapter 1: Introduction

1.1 *Francisella tularensis* **and Tularemia disease**

Francisella tularensis, the causative agent of tularemia, is highly contagious and can cause human infections with as few as 10 microorganisms via the inhalation route [1-4]. *F. tularensis* is a gram-negative intracellular coccobacillus and is categorized as a tier 1 select agent by the Centers for Disease and Control and Prevention (CDC) and category A pathogen by the National Institutes of Health (NIH) [1]. Select agents have the potential to be a major public health problem causing severe disease and lethality [5]. Select agents also include agents of viral hemorrhagic fevers such as filoviruses (Ebola, Marburg), *Bacillus anthracis* (anthrax), *Yersinia pestis* (plague) and more highly dangerous pathogens [6].

There are four strains of *F. tularensis* of significant public health import, subspecies (subsp.) *tularensis, holartica, novicida* and *mediasiatica.* F. *tularensis* subsp. *tularensis* (type A) is the most commonly acquired in North America and the most highly virulent for humans. It persistently infects many small mammals, including domestic animals like cats and rabbits, from which it can be transferred to humans. *F. tularensis* subsp. *tularensis* is estimated to cause 70% of tularemia clinical cases in humans in North America [7, 8]. The disease has been reported in all states in the United States except for Hawaii [9, 10]. Infections in Arkansas, Oklahoma and Missouri account for approximately 50% of the cases of tularemia reported in the US each year (Figure 1) [9, 10].

Type B strains, primarily found in Europe and Asia, e.g., *F. tularensis* subsp. *holarctica,* are less virulent, due to its slower dissemination and cause a milder form of the disease than subsp. *tularensis* [5]. Type B is associated with semiaquatic rodents such as muskrats and beaver in North America and, therefore, is rarely transmitted to humans with the decline of

trapping of these animals. A live attenuated vaccine was developed in 1942 called *F. tularensis* live vaccine strain (LVS) [5]. LVS was derived from *F. tularensis* subsp. *holarctica* and has shown some efficacy in humans and in animal models. However, LVS is not licensed for general human use and is restricted to military personnel and first responders [11].
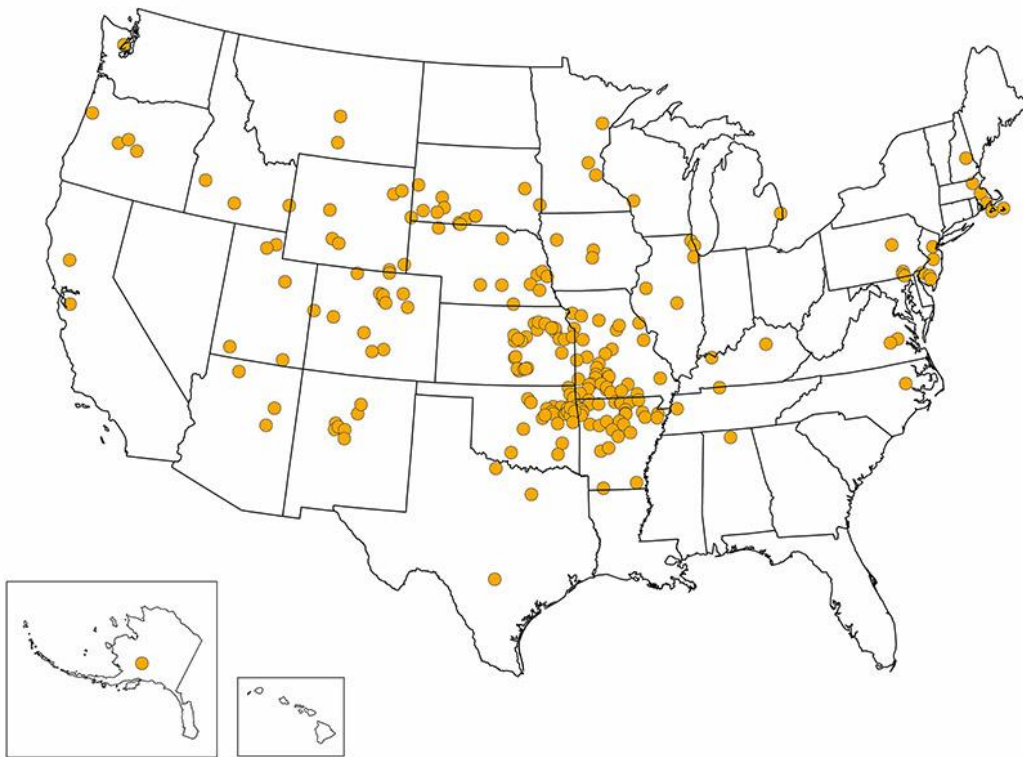
1 dot placed randomly within county of residence for each reported case
Figure 1. reported case of tularemia in the United States [12].

F. tularensis infection is transmitted through multiple routes (Figure 2): 1) handling the carcass of an infected animal; 2) the bite of an infected arthropod vector such as a tick, deer fly or flea; 3) ingesting meat from a contaminated animal; 4) drinking contaminated water; and, 5) inhalation of aerosol droplets, which causes the most severe disease. Since close contact with infected animals is required for the majority of transmissions, tularemia

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infection commonly occurs during summer in the south-central US due to the high exposure to infected ticks. In the northeastern US, infection peaks during winter due to the hunting season. The incident of disease is dramatically increased when a warm winter is followed by a wet summer causing the tick population to proliferate [9, 13, 14].

Ticks are the most common vector for arthropod-mediated tularemia transmission in the United States while mosquito-mediated transmission is most common in Europe. Tick transmission is more frequently associated with type A strains than type B strains. *Ixodes, Dermancentor,* and *Ambylomma* tick species transmit tularemia to humans and are present throughout the states, including Texas [9, 15]. Type B strains have been isolated from streams, ponds, lakes and rivers, from which the disease can be spread by drinking contaminated water. *F. tularensis* can survive in water and animal carcasses for long periods [5, 9]. Aerosol transmission has been documented as a result of mowing lawns or working on farms due to the prolonged survival of *F. tularensis* in the soil after death of an infected animal [9, 14, 15].
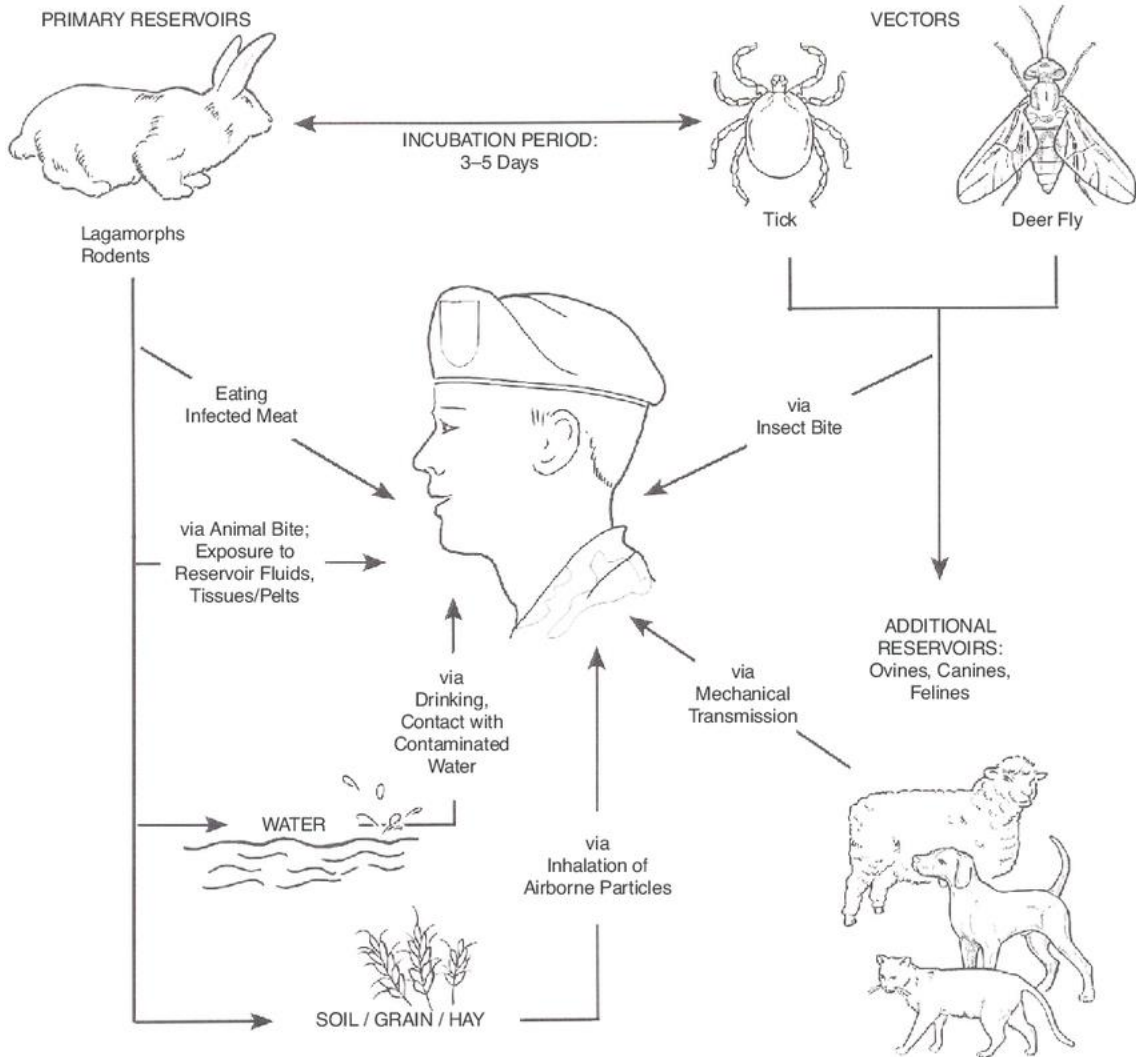
Figure 2. Transmission of *Francisella tularensis* [16].

There are six major clinical manifestations related to primary site of the infection (Figure 3). Ulceroglandular, which is the most common, starts as a painful papule at the site of the infection, such as a tick bite or direct inoculation of the bacteria into the skin, which later becomes an ulcer. Glandular tularemia is similar to ulceroglandular, but without an ulcer, and can also develop from the bite of a tick, deer fly or from handling sick or deceased animals [9, 17]. Oropharyngeal tularemia is characterized by swelling of lymph glands in the neck and is caused from eating contaminated food or drinking contaminated water. Oculoglandular

tularemia is acquired when a person touches the eyes with infected fingers or contaminated water gets in the eyes and develops as painful conjunctivitis and swelling of lymph glands in front of the ear. Typhoidal tularemia results from ingestion or inhalation of the bacteria causing very serious side effects, e.g., septic shock, with hepatomegaly and splenomegaly usually apparent. Pneumonic tularemia results from inhalation of aerosolized bacteria and is the most severe manifestation of tularemia due to the high mortality rate (>30% if untreated).

Figure 3. Clinical symptoms of tularemia, top left: ulceroglandular [18], bottom left: oculoglandular [19] and right: glandular tularemia panel [19].

Tularemia is primarily treated with the antibiotics gentamicin, streptomycin, doxycycline or ciprofloxacin. However, they must be given immediately following exposure and for prolonged time; the treatment with antibiotics usually lasts 10 to 21 days [9, 17]. Despite the necessary rapid response, pneumonic tularemia is often misdiagnosed thereby delaying treatment. Furthermore, we do not fully understood factors that influence susceptibility to tularemia.

1.2 Mechanism of *Francisella tularensis* **pathogenesis**

F. tularensis is a cytosol-dwelling pathogen that needs to enter, survive and proliferate in order to cause pathogenicity [20]. It infects a variety of host cells, including macrophages, dendritic cells (DC), polymorphonuclear neutrophils, hepatocytes, endothelial, and type II alveolar lung epithelial cells [21].

F. tularensis binds to various immune receptors, such as the mannose receptor cluster of differentiation 206 (CD206), complement receptor 3 (CR3) consisting of (CD11b), the scavenger receptor-A (SR-A), and Fcγ receptors (CD16/32), triggering phagocytosis [22, 23]. Among those receptors, Fcγ receptors are the main phagocytic receptors involved during uptake of *F. tularensis* [22]. Following uptake, the bacterium will reside within the phagosome, called the *Francisella*-containing phagosome (FCP) [20]. The FCP sequentially acquires markers of early and late endosomes such as early endosome antigen 1 (EEA-1), CD63, lysosomal associated membrane protein 1 (LAMP-1), LAMP-2 and Rab7 [24]. The acquisition of these markers suggest that the FCP are subject to maturation into a bactericidal phagolysosome. However, the FCP does not fuse with lysosomes; instead, the bacterium physically disrupts the phagosomal membrane and escapes into the host cell cytosol [25]. This phagosomal escape is dependent on *MglA*, a master transcription factor, and is critical for intracellular survival and proliferation [24]. Indeed, *F. tularensis* mutants deficient in the *MglA* gene product do not survive inside the cell and do not cause pathogenesis. *F. tularensis* achieves phagosomal escape by expressing a type VI-like secretion system that helps in disrupting the phagosomal membrane [26]. Other factors also help in the phagosomal exit such as acid phosphatases and pyrimidine biosynthetic genes [27, 28].

The presence of *F. tularensis* in the cytosol of macrophages is recognized by innate

pattern-recognition receptors (PRRs) such as the Nod-like receptors (NLRs), NLRP3 and interferon-inducible protein 2 (AIM2) [20]. PRRs recognize pathogen-associated molecular patterns (PAMP) or danger-associated molecular patterns (DAMP) to initiate the innate immune response [29]. Recognition of cytosolic *F. tularensis* by the PRRs, NLRP3 or AIM2 results in formation of the multiprotein complex, the inflammasome, which initiates inflammation. Once activated, the inflammasome matures the precursor of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 [30]. Following secretion, these in turn initiate a cascade of inflammatory cytokine production from neighboring cells.

The continued production of these inflammatory cytokines results in an imbalance in the immune response, called a cytokine storm. This overproduction of inflammatory plus dysregulated anti-inflammatory cytokines causes severe side effects such as edema, hypovolemia, pneumonia and death. As such, *F. tularensis* is part of a small but deadly class of pathogens that cause disease by triggering an overactive pro-inflammatory immune response rather than excessive replication of the *F. tularensis* bacteria or production of bacterial toxins [31].

This cytokine storm includes the overproduction of a number of cytokines, including Interferon (IFN)-γ , IL-6, and tumor necrosis factor-α (TNF-α). IFN-γ is a potent macrophage activating factor inducing anti-LVS activity in resident peritoneal macrophages infected with *F. tularensis* [32]. Activated macrophages have many potential antimicrobial effector activities not present in resting macrophages, including downregulation of transferrin receptors for decreasing intracellular ion, degradation of tryptophan, and production of effector molecules such as reactive oxygen and nitrogen intermediates [33].

Recombinant IFN-γ stimulates both resident and inflammatory macrophages to inhibit

bacterial growth in dose-dependent manner [32]. Interestingly, resident macrophages required higher concentrations of IFN-γ for bacterial growth inhibition than do inflammatory macrophages [32]. In addition, nitric oxide (NO) production by activated macrophages correlates directly with killing a variety of infectious targets in murine systems, including *F. tularensis*, and is a quantitative index of macrophage activation and killing capacity [32].

IFN-γ-activated macrophages kill a number of intracellular and extracellular targets; however, this effector function can be altered by the nature of the macrophage population [34]. Classically-activated M1 type macrophages are induced by IFN-γ and lipopolysaccharide (LPS) and are readily identified by the presence of the surface marker CD80 [35, 36]. M1 type macrophages secrete pro-inflammatory cytokines, including TNF-α and IL-1β, and serve immunostimulatory functions including elimination of intracellular microbes and induction of the T helper 1 (Th1) response [35]. Alternatively-activated M2 type macrophages are activated by IL-4, IL-10, or immune complexes to secrete anti-inflammatory cytokines, such as transforming growth factor β (TGF-β), and display heterogeneous surface markers including CD163, CD200R, and CD206 [37] . M2 type macrophage functions are anti-inflammatory or pro-resolving in nature and include debris clearance, matrix remodeling and tissue repair [38].

1.3 Sex differences in immune response

The production of inflammatory cytokines contributes to infection resistance by making the host environment less suitable causing undesirable symptoms such as fever, swelling, vomiting [39]. However, in the case of tularemia, the overproduction of these same inflammatory cytokines adversely affects the host itself, ultimately causing disease and death. Therefore, the intensity of inflammation is directly tied to survival from *F. tularensis* infection.

Host immunity plays a key role in defining resistance and susceptibility to infection [40]. Differences in the intensity of the inflammatory response have been observed between male and female immune responses. These differences in intensity of the immune response between the sexes leads to differences in sensitivity to autoimmune and infectious diseases [40-42]. Males tend to be more susceptible to infectious disease whereas females tend to be more susceptible to autoimmune disease.

Differences between the male and female immune response to bacterial infections have been noted at various levels. In addition to a marked difference between males and females in B cell activity and antibody production in response to bacterial infection, there are also differences between frequency, severity, and outcome of severe sepsis and septic shock [43]. Sex based differences have been observed in animal studies in susceptibility to *Mycobacterium marinum* infection in mice where males showed higher disease severity, bacterial burden and mortality than female mice [43, 44]. Similarly, rats administered *Vibrio vulnificus*-derived lipopolysaccharide (LPS) led to endotoxic shock in male rats, causing a mortality rate of 82%, while females exhibited only a 21% mortality rate [43, 45]. Interestingly, female sepsis patients exhibit higher levels of the anti-inflammatory cytokine

IL-10 than do age and disease severity matched male patients [43, 46]. In another study, *Escherichia coli*-derived LPS administration in mice elicited higher circulating levels of interleukin-6 (IL-6) in male than in female mice. Taken together, prevailing data suggests that females are less susceptible to the development of bacterial infection and subsequent bacteremia and/or sepsis while males exhibit greater incidence and severity of bacterial infections and are far more likely to develop lethal sequelae [43]. In the case of *F. tularensis*, while cases are more frequent in males than females, the frequency of exposure is also likely higher in males due to non-biological gender differences between males and females, such as increased risk behavior for infection [47].

Females produce stronger cellular and humoral immune reactions which may result in a higher incidence of autoimmune diseases, such as multiple sclerosis (MS), atherosclerosis, arthritis, systemic lupus erythematosus and insulin-dependent diabetes mellitus as compared to males [40, 48-50]. These differences in susceptibility may be caused by differences in immune responses between the sexes and/or their reproductive phases which are accompanied by variations in sex hormones, as is the case where estrogens increases antibody production [50]. In contrast, males are more susceptible to infections because of behaviors that make them more suitable to acquisition of the infection and their sex steroid hormones that can affect resistance genes [40].

There are also differences between cell-mediated immune responses between the sexes. Reliance on subsets of helper T cells (Th1 or Th2) varies between sexes where females exhibit higher Th2 responses, such as higher interleukin (IL) production of IL-4, IL-5, IL-6 and IL-10 than males. Consequently, females of many species might produce elevated immune responses even against self-tissues and develop autoimmune diseases as compared

to males [40].

Hormones, such as estrogens, generally enhance both cell-mediated and humoral immune responses and can modulate immune function in females that might contribute to resistance against infection. Sex steroids circulating in the body can alter the immune system by influencing the survival or death of cells and lymphocytes as well as differentiation of activated immune cells. Sex steroid hormones modify major histocompatibility complex (MHC) class II expression on antigen presenting cells (APCs). APCs from female mice are more efficient at presenting peptides than are APCs from males suggesting that MHC expression may be different between the sexes. In addition, castration of males increases antigen presentation by immune cells and testosterone-treatment of females decreases antigen presentation [40].

In addition, sex differences in the production of pro-inflammatory cytokines and chemokines have been documented following *in vitro* LPS challenge of isolated rodent peripheral monocytes and macrophages [43]. *In vitro* studies using peritoneal macrophages show that cells derived from young male mice produce higher levels of IL-1β and IL-6 following LPS challenge than do similarly treated cells derived from females [43]. However, these results are contradicted by another study in which peripheral monocytes from female mice produced more IL-6 than monocytes from males, even though these same cells from females produced less TNF-α than males cells did [43]. Another study demonstrated that female-derived splenic macrophages secreted higher levels of the anti-inflammatory cytokine IL-10 than did cells derived from males [51]. Therefore, conflicting results from previous reports cannot solely be used to prove/disprove our hypothesis.

Chapter 2: Preliminary Data

2.1 Evaluation of survival in male and female mice

Male and female C57BL/6 mice were inoculated intradermally with 8e5 colony forming units (CFU) of live vaccine strain (LVS) bacteria. The LVS infection was monitored over the course of two weeks. Female mice were more sensitive to *F. tularensis* infection than male mice (Figure 4). This difference could be explained by one of 3 possibilities: 1) differences in bacterial burdens between males and females, 2) differential susceptibility due to production of different sex hormones, 3) differences in the immune response to the bacterium. i.e., the cytokine storm.
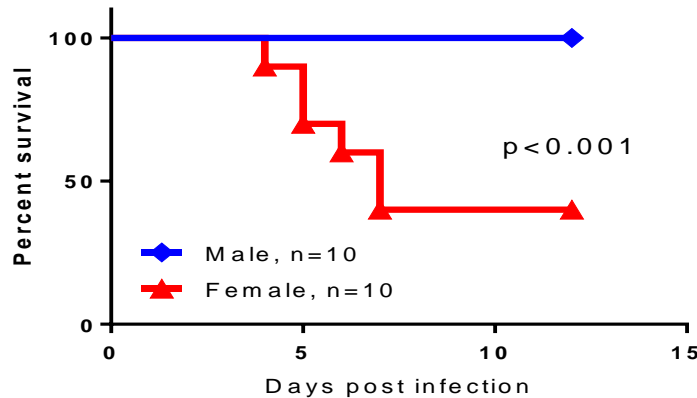
Figure 4. Male and female C57BL/6 mice were infected intradermally with 8e5 cfu of *F. tularensis* LVS bacteria and infection was monitored by weighting the animals daily over the course of 2 weeks. (N=10). Significant difference (p<0.001, Mantel-Cox test) between male and female.

2.2 Bacterial burden in target organs

The first possibility is that the bacterial burden may be higher in females causing more severe symptoms leading to death. Spleen, liver and lung organs were harvested from infected mice and bacterial burden was determined during the course of the infection in order to compare bacterial growth in each sex. No difference in bacterial growth was observed (Figure 5).
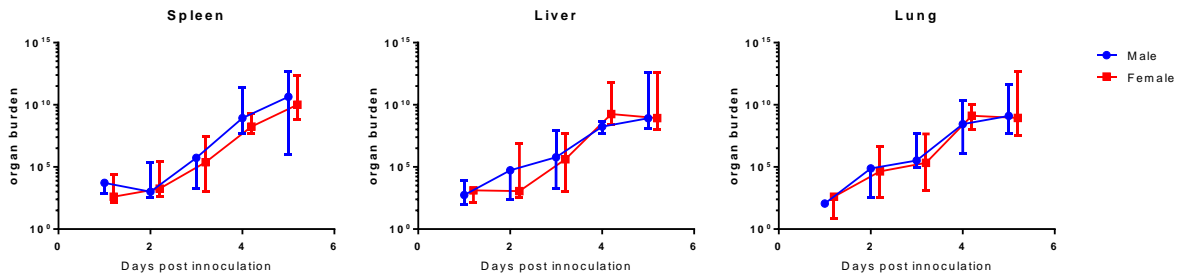
Figure 5. Male and female C57BL/6 mice were infected with 1e6 cfu of *F. tularensis* LVS intradermally and organs were harvested 3 days post-inoculation. Bacterial load was determined during the course of infection with LVS. No significant differences were detectable by ANOVA (n=4 mice per time point).

2.3 Differential susceptibility due to sex hormones

As described in 1.3, the immune system responds to sex hormones. Therefore, to determine their role in this sex difference, the gonads were removed in young mice in order to deplete the sex hormones. Both males and females without gonads were more susceptible to *F. tularensis* infection compared with intact animals (Figure 6). However, removal of the sex hormones did not alter the dichotomy observed in the intact animals.
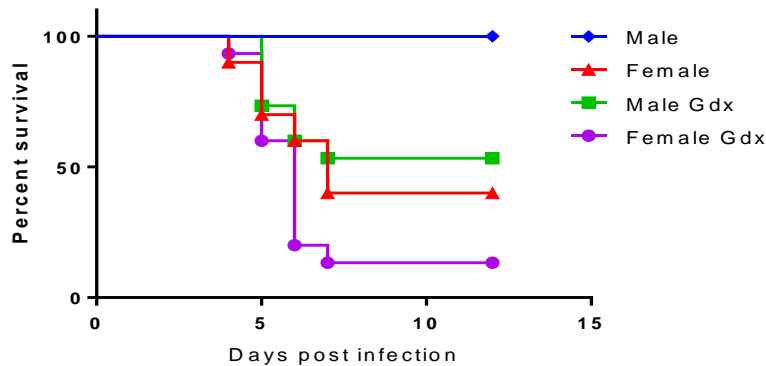

Figure 6. In male and female C57BL/6 young mice, gonads were removed. Then male and female mice were infected with 8e5 cfu of *F. tularensis* LVS and percent survival was observed over the course of two weeks from intact and gonadectomized mice. Significant differences (p<0.0006, Mantel-Cox test) between wild type and gonads mice and between the sexes within each group.

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2.4 Cytokine storm analyzed by multiplex ELISA assay

In order to determine whether differences in the immune response may explain the sex difference observed in outcome, we quantified the serum levels of 19 cytokines in each mouse over time on an individual basis. Out of the 19 cytokines analyzed, five pro-inflammatory cytokines and the anti-inflammatory cytokine IL-10 showed a temporal response to the infection. Overall female mice had higher serum levels than male mice of all these cytokines, indicating a more intense cytokine storm (Figure 7). Therefore, we hypothesized that immune cells responding to infection, may be responsible for this difference in susceptibility between the sexes.

Figure 7. Male and female C57BL/6 mice were infected with 1e$_6$ cfu of F. *tularensis* LVS intradermally and blood was collected at days 0, 3 and 5 post-infection and at the time of termination (T). Serum was analyzed by Milliplex quantifying 19 different cytokines in each mouse over time on an individual basis. Significant differences were observed (p<0.005, two-way ANOVA, n=4-10) between the main effect (sex) of the following analytes: IL-6,IL-1β, IL-10 and TNF-α.

2.5 Myeloid growth and differentiation factors

In addition, during the analysis of serum cytokines, growth, differentiation and chemotactic factors were also measured after challenge with *F. tularensis* before inoculation, at days 3 and 5 after inoculation and at termination. Female mice had also higher serum levels specifically of myeloid growth and differentiation factors compared with male mice (Figure 8), suggesting a role specifically for myeloid cells in this alteration in the immune response.
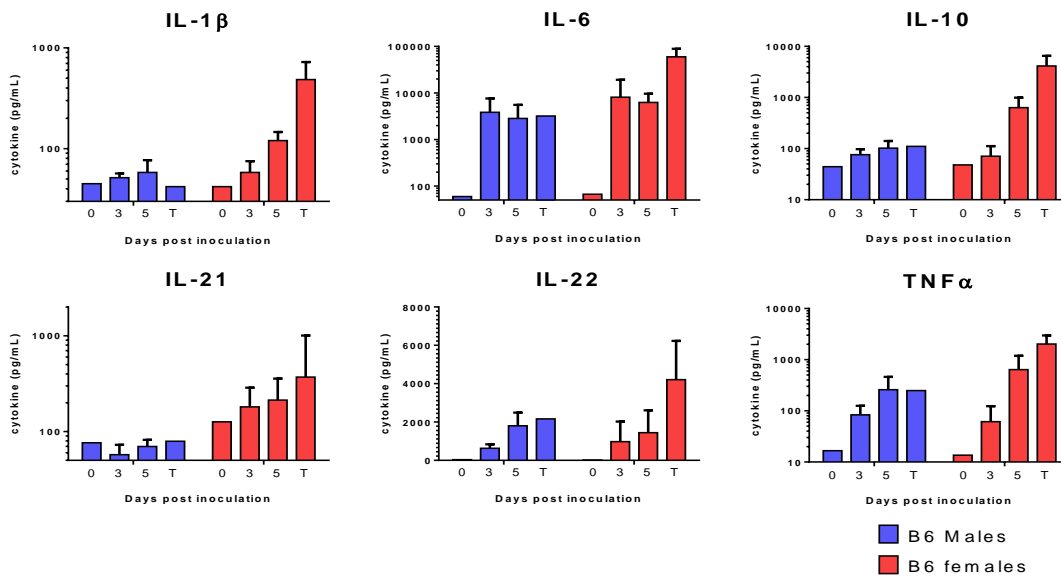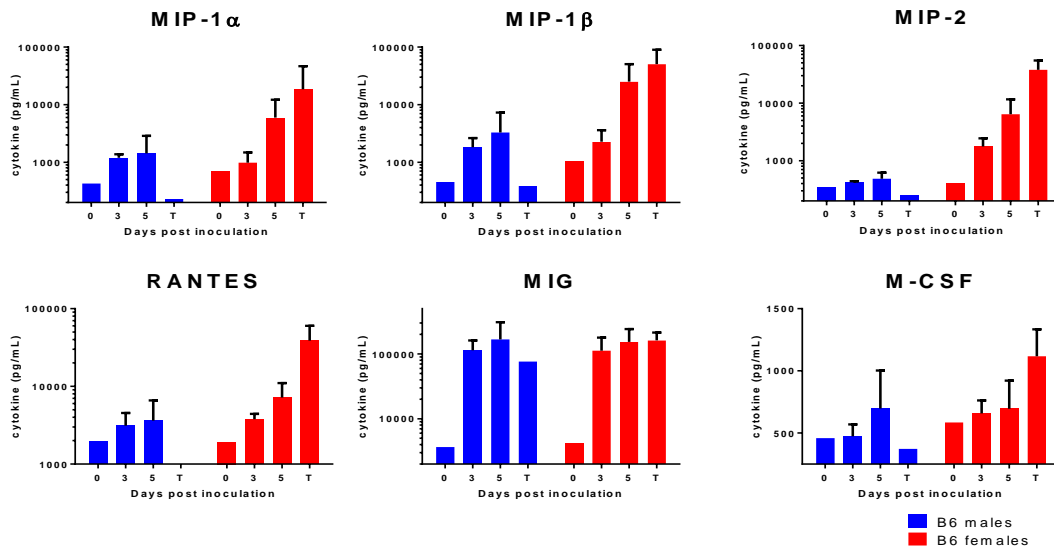

Figure 8. Male and female C57BL/6 mice were infected with 1e6 cfu of *F. tularensis* LVS intradermally. Blood was collected at day 0, 3 and 5 post-infection and at the time of termination (T). Serum was analyzed by Milliplex quantifying myeloid growth and differentiation factors in each mouse over time on an individual basis. Significant differences were observed (P<0.05, two-way ANOVA, n=4-10) between the main effect (sex) of the following analytes: MIP-1α, MIP-1β and MIP-2.

2.6 Hypothesis

Despite *F. tularensis* being an infectious disease, it causes disease much as an autoimmune disorder with an imbalance in inflammation in females. Therefore, we hypothesized that female macrophages respond to *F. tularensis* infection by generating a more intense inflammatory response which makes females more susceptible to *F. tularensis* disease.

2.7 Specific Aims and Approach

Based on the preliminary data in Chapter 2, we proposed to test our hypothesis that the female inflammatory response makes them more susceptible to *F. tularensis* disease through:

Aim 1: Profiling male and female immune cells after infection with *F. tularensis.*

1a) Analyze immune cells responding to infection by flow cytometry.

1b) Determine differences in the presence of inflammatory and regulatory macrophages between males and females.

1c) Analyze the temporal activation of macrophages in males and females.

1d) Analyze cytokine and chemokine responses of males and females responding to infection.

Aim 2: Identify the mechanisms by which macrophages control susceptibility to *F. tularensis.*

2a) Determine the relative percentages of M1 vs M2 macrophages in males and females.

2b) Compare the cellular response of M1 and M2 macrophages between males and females.

2c) Analyzing the different signaling pathways activated in males and females.

Chapter 3: Materials and methods

3.1 Reagents

The antibodies used for flow cytometry assay are; FITC-labeled anti-CD80 (Tonbo biosciences), PE-Cy7-labeled anti-CD23 (BD biosciences), APC-labeled anti-CD11b (Tonbo biosciences), PACB-labeled anti-CD11b (Tonbo biosciences),PE-CF594-labeled anti-CD45R/B220 (BD biosciences), CD16/CD32 (Fc shield) (Tonbo biosciences), APC-labeled anti-LY6G (BD biosciences), Alexa flour 700-labeled anti-LY6C(Bio legend) and PE-labeled anti-IL-6 (BD biosciences). Pac Blue-labeled anti-CD11b (Biolegend), PERCP-labeled anti-siglec F (BD biosciences), PECY7-labeled anti-CD23 (BD biosciences), APC-labeled anti-CD3e (TONBO), APC-CY7-labeled anti-LY6G (BD biosciences), Alexa flour 700-labeled anti-LY6C(BD biosciences), FITC-labeled anti-iNOS (eBioscience), PE-labeled anti-Arginase (eBioscience), Cytofix/Cytoperm (BD biosciences),Ciprofloxacin 500 μg /ml (Sigma) and gentamicin 50 mg/ml (Fisher) will be used to grow macrophages and bacteria, respectively. Mueller-Hinton agar plates supplemented with 1% IsoVitaleX enrichment (BD biosciences), 5% defibrinated sheep blood (Quad five) and 10% glucose (Sigma) will be used to grow bacteria. Red Blood cells (RBC) lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, 0.04% EDTA, pH 7.4), FACS buffer (4% inactivated FBS (Hyclone) and 0.4% 2 mM EDTA in 1X PBS (Fisher)). Cytofix/Cytoperm plus kit (BD Biosciences) for internal staining. Invitrogen IL-6 mouse kit (Thermo Fisher), Invitrogen IL-10 mouse kit (Thermo Fisher) will measure responses to bacterial infection. Macrophages will be grown in Dulbecco's modified Eagles' medium (DMEM) (Corning) supplemented with 10% inactivated FBS, 1% glutamine (Hyclone) and 1% Amphotericin B (Hyclone).1% penicillin/streptomycin (Lonza), RNeasy plus mini kit (Qiagen). LPS (100ng/mL, Sigma-Aldrich L2880), IFN-γ (20ng/mL, eBioscience), IL-4 (20ng/mL,

eBioscience). CYBRFast 1-step RT-qPCR HI-ROX Kit (Tonbo). iNOS, GAPDH and CD206 primers (Sigma). Diphtheria toxin from *Corynebacterium* (1 mg, Sigma Aldrich). Vacuum manifold for 96 well filter plates (abcam), BCA protein Assay kit (Thermo), Protease inhibitor cocktail (Sigma), 9-plex Multi-Pathway Total Magnetic Bead Kit (Millipore), 9-Plex Multi-Pathway Signaling Mag Bead Kit-Phosphoprotein (Millipore), Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore), heparinized capillary tubes (Fisher).

3.2 Bacteria culture

F. tularensis holarctica LVS was obtained from Stanford University and grown on Mueller-Hinton plates supplemented with 1% of IsoVitaleX, plus 5% defibrinated sheep blood, at 37 °C for 2 days. After the second day of incubation, bacteria were scrapped into 15% glycerol in PBS, aliquoted and stored at -80°C until further use.

3.3 Mouse infections

Female and male C57BL/6 mice and CD11b-hDTR mice (6 to 8 weeks old) were purchased from Jackson Laboratories, breed and maintained at UTEP's animal facility following OLAW guidance and animal protocol (A-201809-1) approved by UTEP's Institutional Animal Care and Use Committee (IACUC). For injection, bacterial stocks were diluted to the indicated concentration in sterile PBS. Mice anesthetized with 3-5% isoflurane inhalation were injected intradermally in the flank above the hind quarters with 50 ul of 8e5 – 1e6 cfu bacteria.CD11b-hDTR mice injection diphtheria toxin was diluted to the indicated concentration in sterile PBS. Mice anesthetized with 3-5% isoflurane inhalation were injected peritoneally into the peritoneal cavity with 15ng/g/day of diphtheria toxin. Animals were monitored every 12 hours post-infection for the first 48 hours, and every 8 hours thereafter. All animals were weighed before inoculation and every morning thereafter. An animal was considered terminal and humanely

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euthanized per AVMA standards when it had lost 20% of its baseline weight. In addition, animals were checked for clinical symptoms of disease and considered terminal when lethargic and immobile with prodding.

3.4 Mouse retro-orbital blood collection

During infection course, blood was collected every other day through retro-orbital route alternating the eyes. Approximately 40 µL of blood was collected into heparinized capillary tubes and transferred to a 1.5 mL microcentrifuge tube. Blood samples were centrifuged at 1000x g for 10 minutes at 4°C. Plasma was transferred to a new microcentrifuge tube and stored at -80°C for future analysis for cytokine storm.

3.5 Milliplex Assay for cytokine storm

Collected plasma samples were analyzed using Mouse Cytokine/Chemokine Magnetic Bead Panel kit following the manufacture's protocol. Plates were read on MAGPIX with xPONENT software.

3.6 Macrophage Polarization

Mice were euthanized in a CO_2 chamber followed by cervical dislocation. Spleens were harvested from male and female mice and homogenized by passage through a 70 µm cell strainer, then centrifuged for 10 min. Red Blood Cells (RBC) were lysed with RBC lysis buffer and lymphocytes distributed into Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% inactivated FBS, 1% glutamine,1% Amphotericin B, 1% penicillin and 10% of L929 growth factor and placed into T-75 flasks (Falcon). The next day, media was replaced with media containing either LPS+ IFN-γ (M1) or IL-4 (M2) but without L929 growth factor for polarization purposes; media was left overnight. The next day splenocytes were passaged and 5e5 macrophages seeded per well into 96-well plate with Dulbecco's modified Eagles' medium

(DMEM) supplemented with 10% inactivated FBS, 1% glutamine and 1% Amphotericin B and left overnight.

3.7 Macrophage infection

Splenocytes were infected with 1e10 colony-forming unit (cfu) of *F. tularensis holarctica* LVS (a multiplicity of infection (MOI) of 30) in DMEM supplemented with 10% inactivated FBS, 1% glutamine and 1% Amphotericin B for 2 hours. Once the 2 hours period of incubation were done, the cell growth media was removed and 3 washes with 1X PBS were performed and replaced with gentamicin (0.1µg/mL) was administered for 1 hour and 30 minutes. Media was removed and replaced after time point with media containing gentamicin at a lower dose (0.01µg/mL) and plate was incubated at 37°C, in 5% CO_2 atmosphere for 24 hours. After 24 hours of incubation, 100 ul of supernatant was collected and placed at -20°C until further analysis. Supernatant was replaced with 100 ul of DMEM supplemented with 10% inactivated FBS, 1% glutamine and 1% Amphotericin B and the plate was incubated for another 24 hours. After 48 hours of incubation, 100ul of supernatant was collected and placed at -20°C.

3.8 Enzyme Linked ImmunoSorbent Assay

Collected supernatants were analyzed using the Invitrogen IL-6 kit and IL-10 kit following the manufacture's protocol.

3.9 RNA extraction

After supernatant was collected after 48 hrs, cells attached to 96 well plate were detached with trypsin 0.05% incubation at 37°C, in 5% CO_2 atmosphere for 2 minutes or until all the cells detached from the bottom. Once the cells were detached trypsin was neutralized with DMEM supplemented with 10% inactivated FBS, 1% glutamine and 1% Amphotericin B, and transferred to a microcentrifuge of 1.5 mL and centrifuged for 10 min at 1500 rpm. Supernatant was

removed and the cell pellet was processed for RNA extraction following manufacture's protocol for RNeasy plus mini kit extraction. Once the RNA was obtained for each sample, it was quantified by absorbance using the NanodropᵀᴹSpectrophotometer. Samples were stored at the -80°C until further analysis.

3.10 Real-time RT-PCR

Master mix was made, containing RTase 1 µL, CYBRFast 10 µL (Tonbo Bio), 1 µM each of forward and reverse of target primer for each reaction. Another master mix was made, containing RNA sample (10 ng) and RNA-free water for each sample. Both master mixes were added to an Optical quality 96-well PCR plate (Applied Biosystems). Negative controls contained master mix and just RNA-free water. PCR plate was run following lab's protocol on StepOne Plus (Applied Biosystems).

3.11 Flow cytometry

Mice were euthanized in a CO_2 chamber followed by cervical dislocation. Spleens were harvested from male and female mice and homogenized by passage through a 70 µm cell strainer, then centrifuged for 10 min. Red Blood Cells (RBC) were lysed with RBC lysis buffer and lymphocytes distributed into Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% inactivated FBS, 1% glutamine, 1% Amphotericin B, 1% penicillin and then 200µL of each cell suspension was added to a 96 wells plate. Fc receptors were neutralized with FC shield (Tonbo Bio) for 15 min at 4°C. After incubation, buffer was removed and fluorochrome conjugated antibodies were added at 0.5 µg/1e6 cells: PE-CF594-labeled anti-CD45R, Pac Blue-labeled anti-CD11b, PERCP-labeled anti-siglec F, PECY7-labeled anti-CD23, APC-labeled anti-CD3e, APC-CY7-labeled anti-LY6G, Alexa flour 700-labeled anti-LY6C and incubated for 20 min at 4°C. For the control standard, 1 ul of a single antibody was added to the cells. After

incubation, cell suspensions were centrifuged, and the buffer was removed. Cells were perm

wash and fixed using Cytofix/Cytoperm kit to stain intracellular with FITC-labeled anti-iNOS

PE-labeled anti-Arginase. The cell suspension was resuspended in Dulbecco's modified Eagle's

medium without phenol red (DMEM) and enriched with 5% FBS and 1% Penicillin/streptomycin

(Lonza) and passed through a 70 µm cell strainer and collected in 5 ml conical tubes to analyze

with Beckman Coulter Gallios Flow Cytometer.

3.12 BCA Protein assay

Macrophages were polarized and infected in step 3.4 and 3.5, following infection media was

removed and 9-plex Multi-Pathway Total Magnetic Bead Kit and 9-Plex Multi-Pathway

Signaling Mag Bead Kit-Phosphoprotein was used following the manufacture's

recommendations. Shortly lysis buffer was added to the controls and infected wells, protein

concentration was measure using BCA protein assay following manufacture's protocol.

3.13 Milliplex for Multi-pathways Assay

Collected cell lysates were analyzed using 9-plex Multi-Pathway Total Magnetic Bead Kit and 9-

Plex Multi-Pathway Signaling Mag Bead Kit-Phosphoprotein kit following the manufacture's

protocol. Plates were read on MAGPIX with xPONENT software.

Chapter 4: Results

4.1 Profiling male and female immune cells after infection with *F. tularensis*.

4.1a Analyzing immune cells responding to infection by flow cytometry

The proportion and/or activation of different immune cell populations were quantified (Figure 9). Little difference was observed between males and females in the immune cell populations analyzed, except for the macrophage population. A significant difference between male and female cell population was found in the macrophages population. This difference in the macrophage population led us to hypothesize that inflammatory M1 type macrophages might cause the intense inflammation previously seen in female mice (Figure 4).
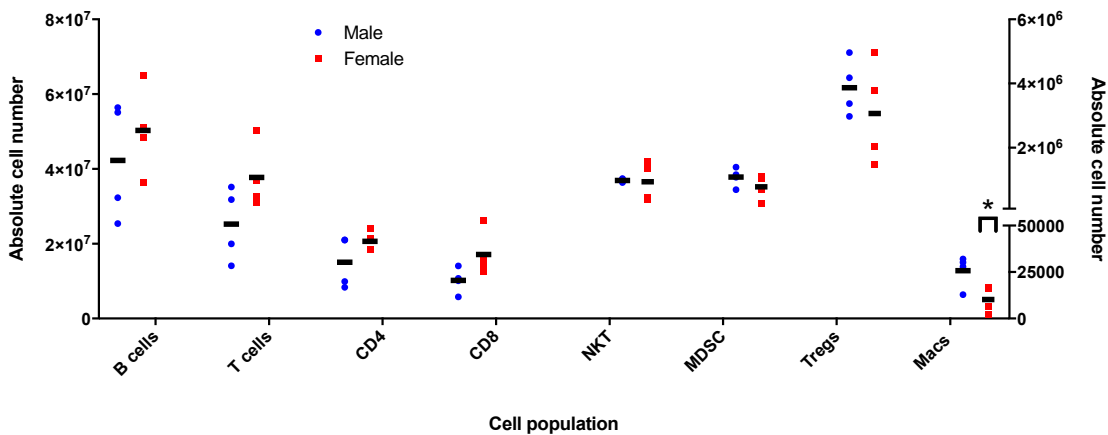

Figure 9. Male and female C57BL/6 mice were infected with 1e$_6$ cfu of *F. tularensis* LVS intradermally. Spleens were harvested 3 days post-inoculation. Splenocytes were stained with antibodies to identify various immune cell population and analyzed with the Beckman Coulter Gallios flow cytometer. Significant differences ($p<0.0001$, multiple t test) between cell population are indicated by a (*).

4.1b Determining differences in the presence of inflammatory and regulatory macrophages between males and females

F. tularensis causes an excessive inflammatory response and elevated serum levels of myeloid growth and differentiation factors (Figure 8) in male and female infected mice. This significant differences between macrophages (Figure 9) between male and female led us to

determine the ability of macrophages to produce either the cytokine IL-6 (inflammatory) or IL-4 (regulatory or anti-inflammatory) macrophages. Macrophages producing these cytokines were determined by flow cytometric intracellular cytokine staining during the peak response (72 hours) to infection. A significantly greater number of IL-6 producing macrophages was observed in female in comparison with male mice (p=0.01, n=4; Figure 10), while no difference was observed in IL-4 producing macrophages.
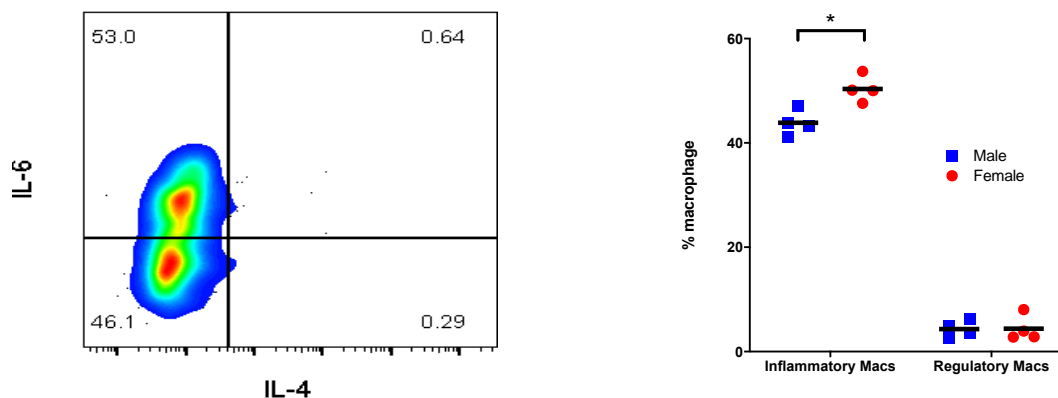

Figure 10. **A**. Male and female C57BL/6 mice were infected with 1e$_6$ cfu of *F. tularensis* LVS. Spleens were harvested 3 days post-inoculation or the peak response (72 hours) to LVS challenge. Macrophages were determined by flow cytometric staining of CD11b+ B220- cells and intracellular cytokine staining and analyzed with the Beckman Coulter Gallios flow cytometer **B.** Significant differences (p=0.01, Mann Whitney test) between male and female are represented by a (*). (N=4).

4.1c Analyzing the inflammatory immune cells differently active in males and females

Since our data suggest that there is a significant different in the IL-6 producing macrophages from female as compared to male, we wanted to compare the percentage of macrophages producing IL-6 between male and female mice over the peak response (72 hours). Significant differences between the sexes were observed at 36, 48, and 60 hours post-infection (Figure 11). At the 36 and 48 hours post-infection time points, a greater percentage of macrophages from female mice produced IL-6 as compared to those from male mice (p < 0.05). However, at 60 hours post-infection, a greater percentage of macrophages from male mice produced IL-6 as

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compared to those from female mice, indeed IL-6 production peaked at this time. To the contrary, macrophages from female mice ceased to produce IL-6 at this time point. Therefore, female mice had an earlier, faster and stronger inflammatory response as compared to male mice.
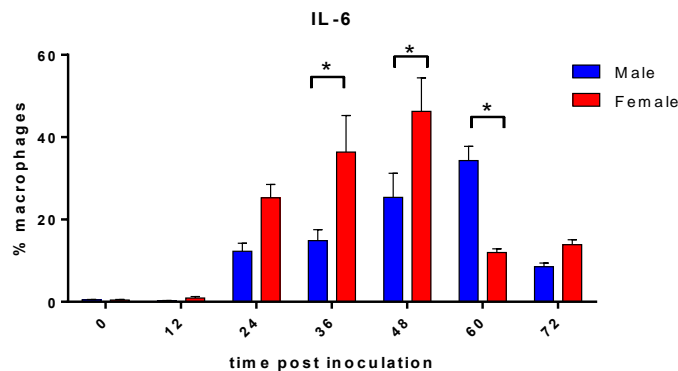

Figure 11. Male and female C57BL/6 mice were infected with 1e$_6$ cfu of *F. tularensis* LVS. Spleens were harvested at the indicated times post-infection. Macrophages were identified as CD11b+ B220- cells and were stained for intracellular flow cytometry and analyzed on the Beckman Coulter Gallios flow analyzer. Significant differences (p< 0.05, multiple t test) between male and female are indicated by a (*).

4.**1d Analyzing Cytokine and chemokine analysis of males and females responding to infection**

Our data suggests that inflammatory macrophages are responding differently between males and females possibly resulting in the difference between their sensitivity to *F. tularensis*-mediated disease. Therefore, depletion of these macrophages might eliminate the observed sexual dimorphism. In order to deplete macrophages, we used a transgenic mouse line routinely used as a macrophage deficient mouse strain (CD11b-hDTR). The CD11b-hDTR mouse expresses the human diphtheria toxin receptor (DTR) under control of CD11b promoter. CD11b is constitutively expressed in macrophages and, therefore, the human DTR is expressed in macrophages. Upon injection of diphtheria toxin (DT) into the mice, the toxin will bind to the

DTR and be internalized where it will induce apoptosis in those cells making the mice deficient in macrophages.

Diphtheria toxin (15ng/g/day) was injected intraperitonially in CD11b-hDTR female and male mice 2 days prior to infection with LVS. Diphtheria toxin administration was repeated every other day after first dose to prevent rederivation of new macrophages from the bone marrow. LVS was injected intradermally after the first treatment with diphtheria toxin. Female and male mice were weighed daily, and their external body temperature was measured every day as well for the duration of the time course. Initial experiments revealed that these mice were much more sensitive to *F. tularensis* infection than wild type mice; therefore, the dose of *F. tularensis* administered had to be reduced to 5e5 cfu LVS instead. Compared to wild type mice (Figure 4), CD11b-DTR mice infected with *F. tularensis* showed no difference in survival between males and female (Figure 12).

Survival after *F.tularensis* challenge
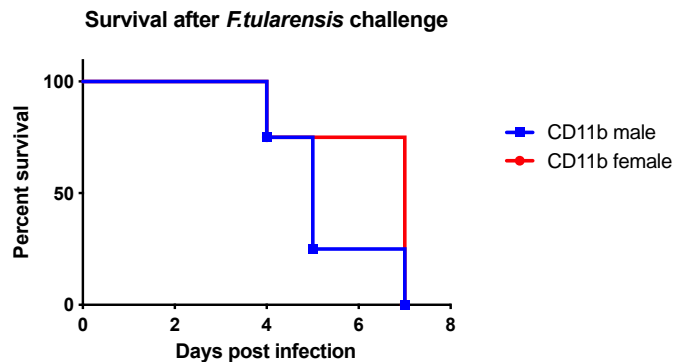
Figure 12. Diphtheria toxin was injected intraperitonially in male and female CD11b-hDTR mice 2 days prior to infection with 5e5 cfu of *F. tularensis* LVS. Diphtheria toxin administration was repeated every other day to prevent rederivation of new macrophages from the bone marrow. LVS was injected intradermally after the first treatment with diphtheria toxin and female and male mice were weighted daily and external temperature was measured every day as well for the duration of time course. (n=5). No statistical difference was found by Mantel-Cox test.

Plasma samples collected from infected mice were analyzed for systemic production of cytokines and chemokines using Milliplex assay. As opposed to the increased production of

cytokines in wild type female mice (Figure 7), similar levels of the cytokines IFN-γ, IL-6 and

TNFα were observed from female and male CD11b-DTR mice (Figure 13). Therefore, removal

of macrophages normalizes the sensitivity of males and females and their chances of survival.
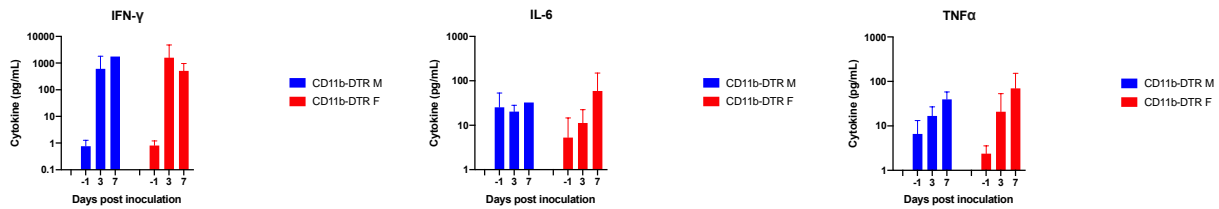

Figure 13. Male and female CD11b-hDTR mice were infected with 5e5 cfu of *F. tularensis* LVS. Blood was collected retro orbital at days -1 and 3 and at termination (T) time using heparinized capillary tubes to collect approximately 40 µL of blood from each animal. Blood was processed and plasma was collected. Plasma samples were analyzed for systemic production of cytokines and chemokines using Milliplex assay. (N=5) No statistical difference was found by ANOVA.

4.2 Identify the mechanisms by which macrophages control susceptibility to *F. tularensis*.

4.2a. Determine the relative percentages of M1 vs M2 cells in males and females

Having determined that differential macrophage responses lead to different susceptibility

to disease, we next sought to determine the cellular mechanism controlling this discrepancy.

Therefore, we first analyzed differences in the presence of M1 and M2 macrophage subsets

between males and females. The percentage of M1 type macrophages was elevated in female

mice compared to that in male mice, suggesting that this difference could result in the stronger

immune response Figure 14, *p<0.005. No statistically significant difference was found in the

percentage of M2 type macrophages between the sexes.
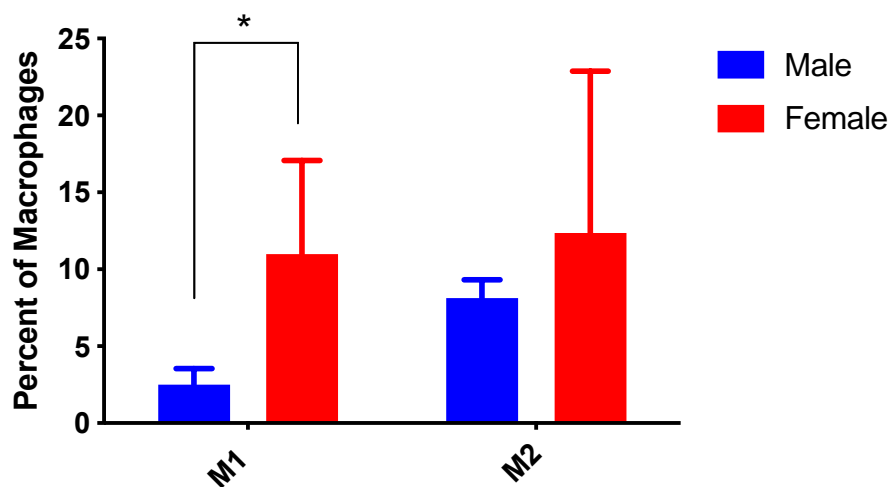
Male vs Female Mouse M1/M2 Macrophages

Figure 14. Male and female C57BL/6 mice were infected with 1e$_6$ cfu of LVS intradermally and spleens were harvested 3 days post-inoculation. Macrophages were identified as CD11b+ B220- cells and were stained with antibodies in order to identify the macrophages subtypes (CD80 for M1 and CD23 for M2) and analyzed on the Beckman Coulter Gallios flow cytometer. Data represents the mean (SD). Significant differences ($p < 0.005$, Mann Whitney test) between male and female in M1 macrophages are indicated by a (*).

4.2b. Compare cellular response of M1 and M2 macrophages from males and females

We next sought to determine whether the inflammatory cytokine response was different between M1 and M2 macrophages from males and females. IL-6 produced by M1 type macrophages in infected female's splenocytes is higher than in infected male's splenocytes ($p < 0.0005$) whereas IL-10 produced by M2 type macrophages in female cells is significantly elevated than in male cells ($p < 0.001$) (Figure 15). This shows a stronger immune response against *F. tularensis* in females as compared to males due to higher levels of IL-6 and IL-10.
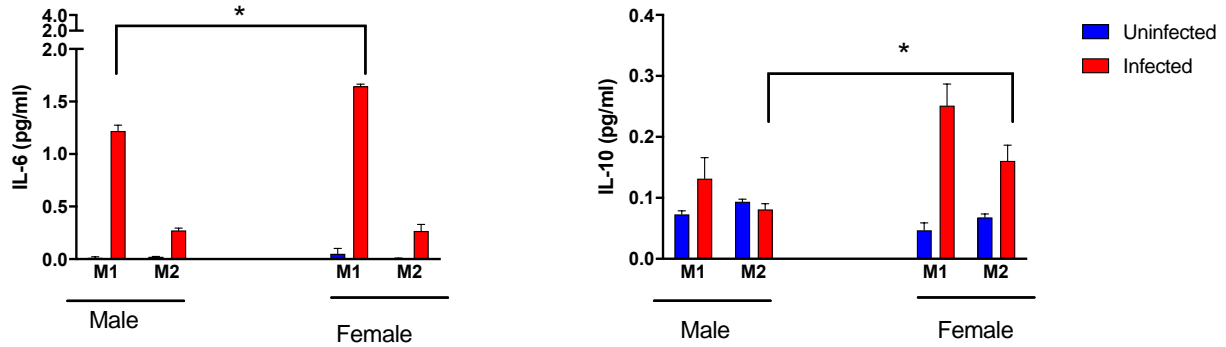
Figure 15. Spleens from naïve male and female mice polarized using M1 or M2 cytokine cocktails prior to infection with 1e10 cfu of *F. tularensis holartica* LVS. Supernatant was collected at 48 hours post-infection and analyzed for IL-6 and IL-10 production by ELISA. Significant differences (p<0.0005,p<0.001, multiple t-test) between male and female in IL-6 and IL-10 respectively are indicated by a (*).

Polarization was verified by analysis of the expression of iNOS (M1) using the β-Actin

housekeeping gene as a control. Figure 16 shows the fold change ($2^{\wedge}$- $\Delta\Delta Ct$) in iNOS in M1

and M2 type macrophages before *LVS* challenge. After polarization, iNOS expression in M1

polarized macrophage was elevated compared with M2 polarized macrophages in both sexes,

suggesting that the *in vitro* polarization was successful.
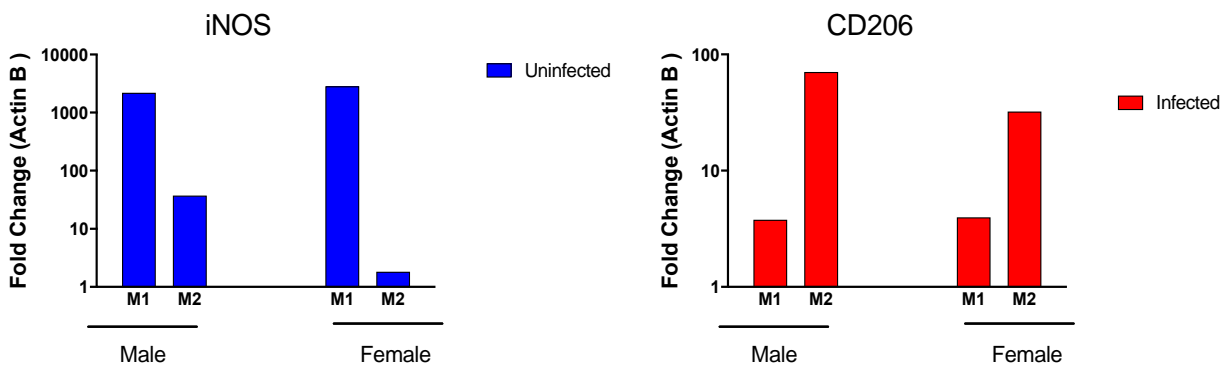
Figure 16. **A.** RNA harvested from polarized macrophages was analyzed by real time RT-PCR using iNOS primer for M1 macrophages (A) or CD206 primer for M2 macrophages (B) and Actin β as housekeeping gene. Data is represented as fold change ($2^{\wedge}\Delta\Delta CT$).

Since LPS is used to polarize M1 macrophages and *F. tularensis* contains LPS, it was

possible that infected M2 macrophages could have repolarized to M1 macrophages. Therefore,

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we analyzed the expression of iNOS and CD206 48 hours post infection by real time RT-PCR. Fold change in CD206 in infected cells for male M2 macrophages is much higher than M1 macrophages in infected cells from males. There is also an increase of CD206 in M2 from males than in females as shown in figure 16. Showing higher levels of M2 polarized macrophages in males suggesting that males might be protected from the cytokine storm caused by *Francisella tularensis* infection.

4.2c Analyzing the different signaling pathways activated in males and females

We sought to investigate what signaling pathway was being differentially activated by M1 and M2 male and female macrophages infected with *F. tularensis* LVS. These kits measure the total and phosphorylated proteins of 9 different transcription factor allowing us to determine the activation of different cellular pathways. Based on our results, CREB and ERK 1/2 activation index was statistically different between males and females, particularly for ERK 1/2. M1 type macrophage from females have a higher activation of ERK 1/2 compared with M1 type macrophages from males (figure 18). ERK 1/2 activation has been associated with polarization of toward the M1 type macrophages. We concluded that the higher ERK 1/2 activation in females results in more proinflammatory macrophages compared with males; therefore, secreting more IL-6 and eliciting greater inflammation (Figure 15).

Figure 18. *In vitro* polarized macrophages infected with LVS were lysed in buffer containing protease inhibitor cocktail and phosphatase inhibitor. The lysates were analyzed using a 9-plex Multi-Pathway Total Magnetic Bead Kit and 9-Plex Multi-Pathway Signaling Mag Bead Kit-Phosphoprotein multiplex ELISA kit. Significant differences (*$p < 0.05$ and **$p < 0.005$, two-way ANOVA) were observed between male and female macrophages.

In contrast, ERK 1/2 activation in M2 type macrophages was significantly higher in males than in females (Figure 19). It has been suggested that activation of ERK 1/2 in macrophages triggers the secretion of more IL-10 whereas, if ERK 1/2 is inhibited, there is more production of pro-inflammatory cytokines such as IL-12 [52]. The high activation of ERK 1/2 seen in male M2 type macrophages could be support this finding where the higher activation of ERK 1/2 produces more IL-10. Therefore, males are better protected from detrimental consequences of LVS.
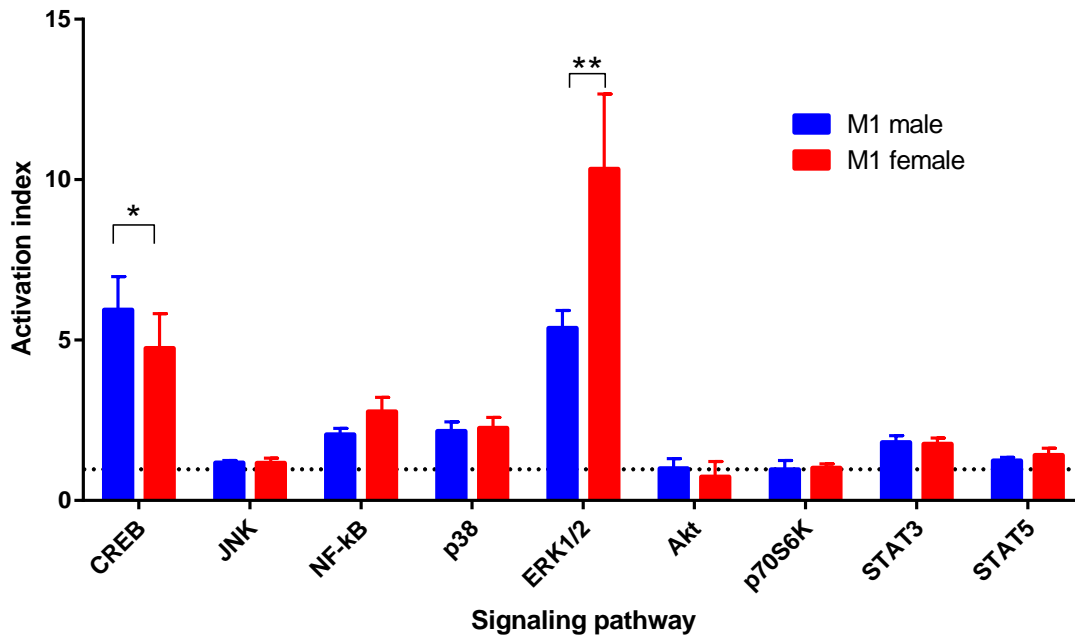
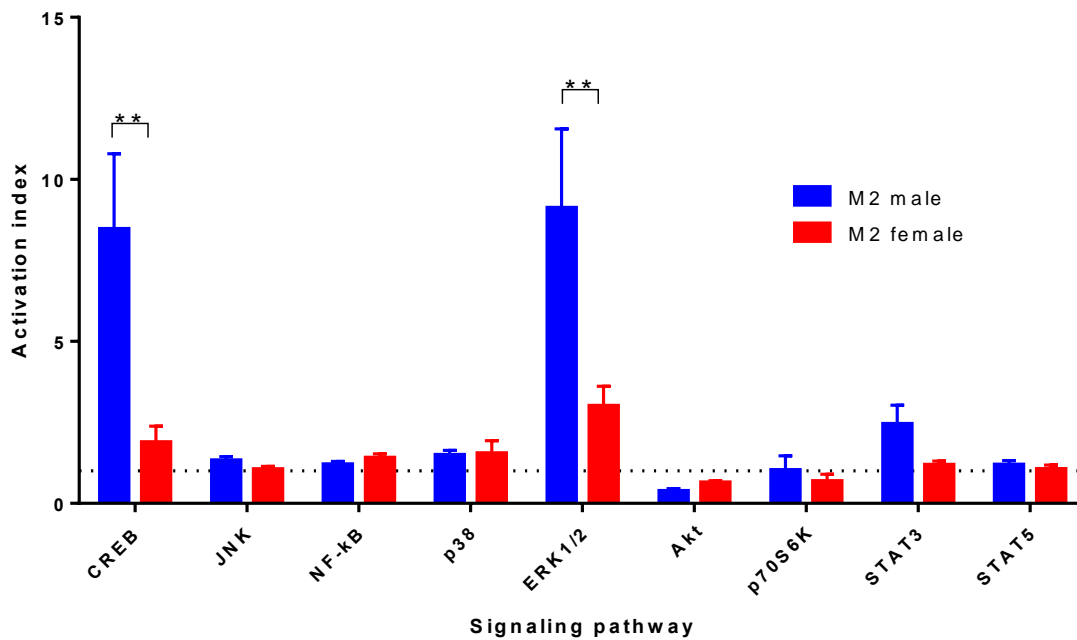Figure 19. *In vitro* polarized macrophages infected with LVS were lysed in buffer containing protease inhibitor cocktail and phosphatase inhibitor. The lysates were analyzed using a 9-plex Multi-Pathway Total Magnetic Bead Kit and 9-Plex Multi-Pathway Signaling Mag Bead Kit-Phosphoprotein multiplex ELISA kit. Significant differences (**$p<0.005$, two-way ANOVA) were observed between male and female macrophages.

Chapter 5: Discussion and future work

Males and females differ in susceptibility to various infectious diseases, with males being more susceptible to infectious diseases [40].This is attributed to males generally exhibiting lower immune responses than females [40] [53].

Our data suggests that females are more susceptible to *F. tularensis* infection in comparison to males due to their stronger and more rapid immune response. Although it has been demonstrated that females are less susceptible to the development of bacterial infections following bacteremia or sepsis, males show greater incidence and severity of bacterial infections and they are far more likely to develop lethal consequences [54]. In several mycobacterial infections, including *M. lepraemurium, M. marinum and M. intracellulare*, male mice were more susceptible to infection [42]. In *M. intracellulare* infection in mice, males had more severe gross lesions in the visceral organs as wells as increased numbers of microbes in the lungs, liver and spleen compared with females. Examination of peritoneal macrophages from male mice infected with *M. intracellulare* showed more rapid growth of phagocytosed organisms suggesting that female peritoneal macrophages possess more potent antibacterial activity [42].

In our study, we showed that C57BL/6 female mice infected with LVS do poorly to infection and consequently infection is detrimental as compared to males. Importantly and distinct from other infectious diseases, an excessive inflammatory response resulting in a sepsis like response is a key feature of death from *F. tularensis* [55]. Differences in the production of pro-inflammatory cytokines between sexes might therefore have a lethal consequence [54]. Female mice infected with *F. tularensis* showed an increase in serum levels of pro-inflammatory cytokines such as IL-6, IL-1β, and TNFα. It has been demonstrated that development of sepsis is mainly driven by the overproduction of these cytokines [53]. Septic shock is mediated mostly by the

activation of macrophages and the subsequent overproduction of pro-inflammatory mediators [54]. Our study showed a difference between the involvement of two phenotypes of macrophage, where females exhibited higher levels of inflammatory macrophage subtype (M1) as compared to males.

Since females tend to mount higher innate, cell mediated and humoral immune responses than males, elevated immunity in female represents a balance between immune responses conferring protection and causing pathology [56]. Several studies showed that excessive pro-inflammatory responses known as a cytokine storm contribute drastically to morbidity and mortality [56]. Our study shows that females mount a higher cytokine storm mainly elicited by pro-inflammatory subtype macrophage (M1). We found that there is a higher percentage of M1 type macrophages activated females responding to *F. tularensis* infection than in males, likely leading to the elevated cytokine storm.

It has been suggested that macrophage depletion might enhance survival by removing the bacterial (*F. tularensis*) replication location [55]. Our data showed that when macrophages are depleted the level of pro-inflammatory cytokines IFN-γ, IL-6 and TNFα were normalized between the sexes suggesting that infected macrophages where the source of the differential cytokine response. This data suggested that removal of macrophages would improve the chances of survival in female mice. Indeed, while mice depleted of macrophages were more sensitive to infection, the sex differences where ablated.

In vitro studies where peritoneal macrophages were used, it was shown that cells from male mice produce higher levels of IL-6 following LPS challenge than similarly treated cells from females [57]. However, in our study *in vitro* studies demonstrated that macrophages from females produce higher levels of IL-6 following LVS challenge than cells from males. In addition, we have

demonstrated that females macrophages secrete higher levels of anti-inflammatory cytokine IL-10 than do macrophages from males as it has been shown previously in another studies [58]. Studies reveal that higher pro-inflammatory responses are correlated with mortality during infection, as seen in our study [56]. These discrepancies are likely due to differential signaling and activation of macrophages responding to varying stimuli. Specifically, macrophages responding to *F. tularensis* infection primarily activated the CREB and ERK 1/2 signaling pathways.

Overall, elevated immunity among females creates a double edge sword which might be beneficial against infectious disease but also might be detrimental in terms of risk of developing an autoimmune diseases [49]. We demonstrated that a stronger and faster immune response elicited by M1 type macrophage is detrimental against *F. tularensis* in female mice whereas in male mice there is no lethal consequence.

Studies have shown that IFN-γ is essential for survival from respiratory *F. tularensis* infections and alveolar macrophages are required for protection against infectious doses with the microorganism [54]. Based on this, future work might include testing androgen functions as this sex steroid hormones differentially impact the outcome of influenza A virus infection in mice. Influenza A dysregulates cytokine and chemokine production in females, causing significantly more susceptibility to weight loss, hypothermia and death as compared to males [56]. In that same study, administration of high dose of estradiol or an estrogen receptor α (ERα) antagonist suppresses the production of cytokines and chemokines and increases survival following viral infection [56].

In addition, neutrophils have shown to be essential for survival of intravenous or intradermal *F. tularensis* infection. However, it has been suggested that neutrophils are detrimental to the host following pulmonary *F. tularensis* infection due to induction of overwhelming

inflammation [55]. Indeed, this secondary tropism may explain the lethality seen in the absence of macrophages. Future studies may investigate the role of neutrophils by depleting them and see the cellular response, production of pro-inflammatory cytokines and survival within sexes.

As stated, LPS and LVS stimulation of macrophages resulted in opposite responses of cytokine production. Since LVS stimulates TLR-2 (while LPS stimulates LPS), it would be ideal to look at that pathway in better detail such as downstream targets of ERK 1/2 pathway. Previous studies with *M. tuberculosis,* which activates TLR-2 signaling pathway, demonstrated that activation of ERK promotes secretion of IL-10 and decreases secretion of IL-12. On the contrary, inhibition of ERK decreases secretion of IL-10 while increases secretion of IL-12. However, there are conflicting reports on the polarizing activity of ERK which promotes polarization from M2 to M1 macrophages enhancing IL-12 and IFN-γ production [52, 59]. Specifically identifying the signaling pathway(s) involved in this differential and opposite response thereby remains a challenge to be addressed.

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Appendix

<u>List of publications and manuscripts</u>

1. Ramos Muniz, M. G., Palfreeman, M., Setzu, N., **Sanchez, M. A**., Saenz Portillo, P., Garza, K. M., Gosselink, K. L., … Spencer, C. T. (2018). Obesity Exacerbates the Cytokine Storm Elicited by *Francisella tularensis* Infection of Females and Is Associated with Increased Mortality. *BioMed research international*, *2018*, 3412732. doi:10.1155/2018/3412732.

2. Activation of M1 subtype macrophages in female mice infected with F.tularensis might be detrimental to the host due to elevated cytokine storm. Tentatively submission date April 2020. Manuscript in preparation.

Vita

Michelle Arlene Sanchez Guillen was born on September 11, 1994 in Denver Colorado but lived in Ciudad Juarez Chihuahua for almost 15 years. She acquired her Bachelor of Science degree in Microbiology in Spring 2017 from the University of Texas at El Paso (UTEP). In August 2017, she was accepted to the Master of Science program at UTEP under the mentorship of Dr. Charles T Spencer. For the last three years, her research has focus on finding the differences in immunological responses between male and female elicited by *Francisella tularensis.*

Furthermore, she has one co-author manuscripts and one more in preparation, she has presented her research at multiple local and national meetings. After graduation, she hopes to continue working with infectious disease and immunology research related.

This thesis was typed by Michelle Arlene Sanchez Guillen.