THE ROLE OF REGULATORY T CELLS IN THE PATHOGENESIS AND PERSISTENCE OF *BRUCELLA ABORTUS* IN THE REPRODUCTIVE SYSTEM OF FEMALE MICE

A Dissertation

by

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ABSTRACT

Stealthy intracellular bacterial pathogens are known to establish persistent and sometimes life-long infections. Some of these pathogens also have a tropism for the reproductive system thereby increasing the risk of reproductive disease and infertility. To date, the pathogenic mechanism involved remains poorly understood. Here, we demonstrated that *Brucella abortus,* a notorious reproductive pathogen, has the ability to infect the non-pregnant uterus, sustain infection, and induce inflammatory changes during both acute and chronic stages of infection. In addition, we discovered that chronically infected mice had a significantly reduced number of pregnancies compared to naïve controls. To investigate the immunologic mechanism responsible for uterine tropism, we explored the role of regulatory T cells (Tregs) in the pathogenesis of *Brucella abortus* infection. We showed that highly suppressive CD4+FOXP3+TNFR2+ Tregs contribute to the persistence of *Brucella abortus* infection and that inactivation of Tregs with TNFR2 antagonistic antibody protected mice by significantly reducing bacterial burden both systemically and within reproductive tissues. These findings confirm a critical role for Tregs in the pathogenesis of persistence induced by *B. abortus*. Results from this study indicate that adverse reproductive outcomes can occur as sequelae of chronic *Brucella* infection in non-pregnant animals, and that fine-tuning Tregs activity may provide novel immunotherapeutic and prevention strategies against brucellosis and potentially other intracellular bacterial infections.

DEDICATION

To the One. The omnipotent and omniscient who has made this sojourn memorable. I also dedicate this work to my loving parents Mr. Aderemi Adetunji and Mrs. Nurat Adetunji, my ever-supportive husband Dr. Toriq Mustapha and beloved daughter Salmaa Mustapha.

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NOMENCLATURE

ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD25	Cluster of Differentiation 25
CFU	Colony Forming Unit
DPI	Days Post Infection
FOXP3	Forkhead Box P3
H&E	Hematoxylin and Eosin
Iba-1	Ionized Calcium Binding Adaptor Molecule 1
IHC	Immunohistochemistry
IL10	Interleukin 10
IL12	Interleukin 12
PBS	Phosphate Buffered Saline
S19	Strain 19
Teffs	T Effector Cells
TGFb	Transforming Growth Factor Beta
Th1	T Helper Type 1 Cells
Th2	T Helper Type 2 Cells
TNFa	Tumor Necrosis Factor Alpha

TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
Tregs	Regulatory T Cells
USA	United States of America
virB2	Virulent Determinant Gene B2

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CHAPTER I

INTRODUCTION

Epidemiology of Brucellosis

Brucellosis is a zoonosis of almost worldwide distribution that poses a significant threat to the health and welfare of animals and humans [1-3]. With over half a million new human cases being reported annually, brucellosis remains one of the top neglected zoonotic diseases as classified by the World Health Organization [2, 4]. The disease is endemic in high-risk regions, which include countries in Asia, South and Central America, Middle East, Mediterranean, and Africa, particularly countries with resource-limited settings and insufficient veterinary and public health programs [5-7]. In low-risk regions like North America and some European countries, the true incidence of brucellosis appears to be underestimated due to the non-specific clinical manifestations, under-diagnosis, and misdiagnosis of the disease [2, 7, 9]. Brucellosis is a bacterial zoonosis caused by intracellular, facultative, Gram-negative coccobacilli of the genus *Brucella*. Currently, there are 12 recognized species of *Brucella* with varying preferential hosts (Table 1). Three of these species are highly pathogenic to their target hosts, as well as humans, and they include *B. abortus* (cattle), *B. melitensis* (sheep and goats), and *B. suis* (pigs) [1, 8-11]. Even though different *Brucella* species have preference for specific hosts, certain species have the capability to infect multiple hosts [3], causing observable clinical disease.

Table 1. Preferential hosts and zoonotic potential of the recognized *Brucella* species [12, 13].

***Brucella* Species**	**Zoonotic potential**	**Host Preference**
B. abortus	High	Cattle, Bison, Elk
B. melitensis	High	Sheep, Goat
B. suis	Moderate	Pig
B. canis	Mild	Dog
B. neotomae	None	Desert wood rat (*Neotomae lepida*)
B. pinnipedialis	Mild	Sea Lions, Seals
B. ovis	None	Sheep
B. ceti	Mild	Whales, Dolphins
B. microti	None	Common voles (M*icrotus alvaris*)
B. inopinata	Not known	Human
B. vulpis	Not known	Fox
B. papionis	Not known	Baboon

Brucellosis is naturally transmitted in animals through oral routes (e.g. consumption of contaminated materials including aborted fetuses and fetal membranes), as in the case of *B. abortus* and *B. melitensis*, or via sexual transmission as noted in other *Brucella* species such as *B. ovis*, *B. suis*, and *B. canis* [14, 15]. In humans, transmission of the disease includes the consumption of unpasteurized dairy products (e.g. milk and

cheese), accidental exposure to *Brucella* vaccines (*B. abortus* RB51 and S19, *B. melitensis* Rev-1), and exposure of the mucous membranes and skin wounds to contaminated animal products and infectious genital secretions [2, 6, 16, 17]. These means of brucellosis transmission place certain individuals at increased risk of *Brucella* infection including veterinarians, laboratorians, ranchers, farmers, abattoir workers and meat processors, due to constant exposure of these individuals to potentially infected animals and products or to laboratory strains of the pathogen. The infectious dose of *Brucella* is reputed to be as low as 10 bacteria, making it highly contagious and one of the top laboratory-acquired infections in the world [18, 19]. Additionally, human to human transmission of brucellosis, although rare, can range from transplacental, bone marrow transplantation, and blood transfusion, to suspected sexual transmission cases [1, 20].

Due to the significant public health threat and the crippling socioeconomic effects posed by brucellosis, several control and eradication strategies have been established, particularly in developed countries. These strategies include mandatory vaccination programs for animals and a test and slaughter policy complemented with a reimbursement scheme for farmers to facilitate effective eradication of the disease, especially in livestock [21, 22]. However, in several developing countries, where these eradication strategies are difficult to implement, brucellosis continues to be a significant socioeconomic and public health concern [23]. Even in some developed countries where vaccination and eradication programs are strictly followed, reintroduction of brucellosis by wildlife and feral hogs into disease-free livestock is often reported, for example in the

United States [24-26]. Unfortunately, despite years of research, there are currently no available vaccines for the prevention of brucellosis in humans. However, three vaccines are available for use in animals, including *B. abortus* RB51, *B. abortus* S19, and *B. melitensis* Rev-1. Nevertheless, these vaccines have notable drawbacks including spontaneous abortion in pregnant animals, increased risk of infection in humans during accidental exposure, potential to cause disease in vaccinated animals, and interference with diagnostic tests [27, 28]. Therefore, several efforts are underway in the search of and the development of an improved, safe, and efficacious vaccine that will overcome the drawbacks of the currently available vaccines for the control and prevention of brucellosis in animals and humans.

Even though brucellosis results in high morbidity, mortality due to the disease is rare. Human brucellosis can be effectively treated with the use of a long course of antibiotics, such as six weeks of rifampin or doxycycline (Solera, 2000). Despite effective treatment of brucellosis with antibiotics, relapses of the infection can occur leading to varying chronic complications including osteoarticular, cardiac, and neurological disease [7, 29-31].

Clinical manifestations of brucellosis in humans include flu-like illness, characterized by undulating fever, night sweats, fatigue, arthralgia, and myalgia [1, 6]. The most common complications associated with brucellosis are reproductive, manifesting as increased risk of adverse obstetric outcome, such as spontaneous abortion and stillbirths in pregnant women [32, 33]. Other manifestations, usually associated with chronicity of the infection, include osteoarticular, neurologic, and cardiac diseases [7,

30]. In animals, clinical signs associated with brucellosis are mostly a result of reproductive complications and they include spontaneous abortion, stillbirth or birth of weak calves, retained placentas, reduced milk yield, increased reproductive cycle and infertility. In male animals, seminal vesiculitis, epididymitis, and orchitis have been reported [34-36].

Reproductive Complications Associated with *Brucella* Infection

Generally, brucellosis is considered a multi-organ disease by virtue of recovery of the pathogen from almost all tissues, particularly during experimental infections. The colonization of all tissues is due to *Brucella*'s special preference for the phagocytic cells of the reticuloendothelial system (RES), which are widely distributed in all tissues of the body [11, 37]. A putative preferential localization of *Brucella*, particularly the highly pathogenic species, is the reproductive system of both male and female animals. In the males, *Brucella* has been recovered from the testes, epididymis, and seminal vesicles, while in the females, the placenta and mammary glands are the major organs of *Brucella* recovery [3]. For example, several studies have shown the survival and replication of *Brucella* spp. in the pregnant uterus and placenta. As many as 10^{12} colony forming units of this pathogen have been recovered in the utero-placental unit of infected animal models (mice, cattle, and goats) that had classical pregnancy complications [3, 38]. Hence, tropism of *Brucella* to the reproductive tract can account for the severe complications associated with the disease in natural animal hosts including spontaneous abortion, stillbirth, infertility in females and orchitis and epididymitis in the males [35, 36, 38-42].

Statement of the Problem

It is important to note that until this date, investigation of the pathogenic mechanisms of *Brucella* colonization and survival in the female reproductive system have revolved around the use of pregnant animal models [39-41], with little or no information about the pathogenic effects of these organisms on the non-pregnant reproductive system. Hence, the capability of *Brucella* to colonize, infect, sustain infection, and induce persistent inflammation in the uterus of non-pregnant animals has not yet been investigated. Additionally, very little is known about the mechanisms involved in the preference of *Brucella* for the reproductive system. The findings of this current study have contributed to the understanding of the mechanisms of chronic brucellosis and *Brucella*-induced reproductive disease in animals and humans.

It is well documented that pregnant animals are more susceptible to *Brucella* infection. However, until this date, the mechanisms involved are incompletely understood [3, 42, 44, 45]. Unraveling these mechanisms requires an understanding of the factors not only needed for *Brucella* growth and survival, but also those commonly present in the reproductive system of both sex types. Previous studies have suggested the important role of nutrient metabolism in the persistence of *Brucella*, including substrates such as glycerol, lactate, glutamate, fructose, and most importantly erythritol which supports the growth and survival of *Brucella* in tissues [46].

Previously, it was assumed that erythritol, a four-carbon alcohol found in fetal fluids of ruminants stimulates the growth of *Brucella* spp. in the placenta of these animals, leading to abortion [47]. However, not all animals and humans have erythritol

in their placentas, yet they are still susceptible to *Brucella* infection and abortion [44]. In addition, genetic experimentation has disproved the relationship between erythritol metabolism and the virulence of *Brucella* species [48]. Another hypothetical mechanism of *Brucella* tropism and pathogenesis, particularly during pregnancy, is the involvement of placental trophoblasts. Placental trophoblasts are cells at the maternal-fetal interface that support the growth and development of the fetus [49]. Studies have suggested that these cells permit the unrestricted replication and survival of *Brucella* spp., which ultimately leads to cell death and the initiation of abortion [45,50, 51]. However, the multiplicity of infection (MOI) used to infect the placental trophoblasts in these studies was unrealistically high, leading to a higher number of infected cells, and ultimately, abortion. Additionally, other related studies have shown the specific mechanism by which placental trophoblasts perpetuate *Brucella*-induced placentitis and fetal loss. For example, a recent study from Byndloss et al., demonstrated that infection of placental trophoblasts with *B. abortus* resulted in cell death and fetal loss that was mediated by endoplasmic reticulum stress [51]. However, more recent identification of the contribution of maternal immune cells to pathogen persistence has weakened the erythritol and trophoblast theories. For example, previous studies have shown the protective roles of the maternal immune system during pregnancy, including the initiation of tolerance to the genetically foreign fetus (allogeneity) [52]. However, the suppression of maternal immunity that facilitates fetal tolerance during pregnancy may predispose to pathogen invasion, especially those pathogens with preference for reproductive tissues [53, 54]. Moreover, during normal human and mouse pregnancy,

there is an increase in the systemic population of Tregs, which peaks at midgestation [53, 54]. In a mouse model of listeriosis and salmonellosis, it was found that pregnant mice at midgestation were more susceptible to *Listeria monocytogenes* and *Salmonella typhimurium* infection [54].

Given the importance of Tregs in maintaining the balance between immunological tolerance of allogeneic fetal antigens and immune activation necessary for host defense against foreign pathogens, this current study hypothesized that Tregs play a critical role in the pathogenesis of *Brucella*-induced pregnancy complications.

Goals and Experimental Approach

It is well established that *Brucella* has a tropism for the pregnant reproductive system and that infection is a significant problem during pregnancy because of the adverse outcomes that include spontaneous abortion and stillbirth. Hence, to understand the mechanisms involved, numerous studies have focused on the experimental infection of pregnant animal models, with little or no information about the effects of *Brucella* on the non-pregnant reproductive system. However, *Brucella* has also been associated with other reproductive problems that do not necessarily involve pregnancy. For example, it has been reported that *Brucella* infection is associated with increased risk of infertility in humans as well as cycling problems in animals [33].

Therefore, this study aimed to determine if *Brucella* can colonize the non-pregnant uterus and sustain infection; induce persistent inflammation that results in decreased fertility during chronic stages of infection; and to identify the immunologic mechanism that facilitates survival and persistence of the pathogen.

Additionally, as mentioned earlier, the mechanism behind the adverse obstetric outcomes of *Brucella* infection during pregnancy is still poorly understood. Therefore, an investigation of the interaction between *Brucella* and the maternal immune cells that sustain fetal tolerance is paramount to further delineate the mechanisms of *Brucella*-induced pregnancy complications.

The mouse model was used in this study as an effective tool in understanding the pathological manifestations as well as immunologic responses to *Brucella* infection. Although these animals are not natural hosts of *Brucella* spp., their use is more economical and practical (e.g. availability of reagents and other immunological tools), and they have proven to be a valuable model to study the pathogenesis of brucellosis [43]. Furthermore, even though mice do not present with the classical abortion observed in *Brucella*-infected natural hosts, studies have shown significant similarities in the pathogenesis of placental infection in mice and ruminants with *B. abortus*. For example, in the cow and mouse, there is preferential growth of brucellae within the rough endoplasmic reticulum of the trophoblast cell, resulting in placental damage, fetal infection, and fetal loss [42].

CHAPTER II

MATERIALS AND METHODS

Ethics Statement

This study was conducted according to the recommendations in the *U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* [36]. Texas A&M University Institutional Animal Care and Use Committee (IACUC) approved this research under the Animal Use Protocol IACUC 2018-0046.

Animals

ICR female mice, aged 6-8 weeks, were purchased from the Texas Institute of Genomic Medicine (College Station, Texas, USA) and acclimatized in the Texas A&M University Laboratory Animal Resources and Research Biosafety Level 2 or TAMU Animal Biosafety Level 3 facilities (College Station, Texas, USA) for a week prior to experimental infection. For the breeding study, stud male mice with proven breeding performance were used.

Bacterial Strains

The strains used in this study were the virulent strain *B. abortus* S2308 (National Animal Disease Center, USDA ARS, USA), live-attenuated vaccine strain *B. abortus* S19 (National Veterinary Sciences Laboratories, Ames, Iowa, USA), and *B. abortus*

2308*ΔvirB2* [27]. To prepare inocula, these strains were grown in Tryptic Soy Agar (TSA: BD Biosciences, New Jersey, USA) at 37°C for 3-5 days. Inoculum was prepared according to published studies [37], and doses of inoculum were confirmed retrospectively by counts of distinct colonies of the inoculum residue.

Experimental Infection

To assess bacterial colonization and to characterize the gross and histopathological lesions induced by these strains of *B. ab*ortus in non-pregnant mice, four groups of animals were inoculated intraperitoneally with $1X10^6$ CFU of either S2308, S19, 2308*ΔvirB2* or PBS (A-A). Mice were monitored daily for signs of disease. At 3, 7, and 13 days post infection, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation, and a full necropsy was performed. At each time point, uterine and splenic samples were aseptically collected and processed for bacterial isolation, histopathology, and immunohistochemical analyses. For the chronic infection study (A-B), female ICR mice at 6-8 weeks of age were inoculated with 10^6 CFU of the virulent strain S2308 intraperitoneally. Eight weeks after infection, mice were housed in cages containing male mice bedding to synchronize estrus, and subsequently set up with stud males of similar strain. The presence of vaginal plugs confirmed mating. At gestation day 18, mice were euthanized, pregnancy outcome was assessed, and tissues were collected for bacterial recovery.

TNFR2/Tregs Antagonism

Non-pregnant ICR mice were inoculated intraperitoneally (IP) with $1X10_6$ CFU of S19, and treated with four doses of either 100µg of hamster anti-mouse TNFR2 antagonistic antibody [70], an isotype IgG control, or PBS via IP inoculation. At 14 days post infection, mice were euthanized, and tissues were collected for flow cytometry analysis, bacterial enumeration, and histopathology.

Bacterial Colonization

To determine the degree of colonization of *B. abortus* S2308, S19, and 2308*ΔvirB2* in the uterus compared to the spleen of non-pregnant mice, tissues harvested at 3, 7, and 13 days post infection were weighed, homogenized and serially diluted in sterile PBS. Dilutions were plated on Farrell's media and incubated for 3-5 days at 37°C. Plates were monitored daily for growth and *Brucella* was identified based on morphological characteristics. The recoverable bacteria were enumerated as $\log_{10}$ CFU/g of uterine and splenic tissues.

Histopathology

To evaluate the histological changes associated with *B. abortus* S2308, S19, and 2308*ΔvirB2* infection in the uterus of non-pregnant mice, sections of uterus from all mice were fixed in 10% neutral buffered formalin, routinely processed using xylene and graded concentrations of alcohol, and embedded in paraffin wax. Uterine sections of 5µm thickness were stained with hematoxylin and eosin (H&E) and examined via light

microscopy by a board certified veterinary anatomic pathologist (Martha E. Hensel at Texas A&M University) in a blinded fashion (Table 2). Uterine and splenic samples from the non-infected group were used as negative controls. The histopathological processing and tissue evaluation for all the samples were performed in the BSL2 (samples infected with the virulent strain were validated for the absence of select biological agent prior to processing in the BSL2).

Table 2. Histopathological description of the spleen of the animals used in the study [56].

Spleen	**Score**	**Description**
Histiocytic inflammation	0	None
	1	Minimal—One focus per 4x objective
	2	Mild—Two to four foci per 4x objective
	3	Moderate—Five to 10 foci per 4x objective
	4	Marked—>10 per 4x objective
Neutrophilic accumulation	0	None
	1	Minimal— few cells identified
	2	Mild—multiple small foci <10 cells
	3	Moderate—1-2 foci of >10 cells
	4	Marked—Multiple foci of >10 cells
Necrosis	0	None
	1	Minimal—one focus per 10x objective
	2	Mild—Two to four foci per 10x objective

Table 2 Continued

Spleen	Score	Description
Necrosis	3	Moderate—Five to ten foci per 10x objective
	4	Marked— >10 foci per 10x objective

Immunohistochemistry

The presence of *Brucella* antigen in the uterine tissues of infected mice was evaluated in formalin-fixed paraffin embedded tissues via immunohistochemistry. Briefly, uterine sections of 5µm thickness were deparaffinized and rehydrated. Antigen retrieval was performed using the 2100 Antigen Retriever System according to the manufacturer's instructions (Aptum Biologics Ltd, Southampton, United Kingdom), followed by blocking of endogenous peroxidase activity with Bloxall Blocking Solution (Vector Laboratories Inc., Burlingame, California, USA). Sections were subsequently incubated in 2% normal goat serum to reduce non-specific binding and background staining. Primary antibodies (rabbit anti-*Brucella* polyclonal IgG; 1:400 – Biocompare, South San Francisco, California, USA, or Microglia/Macrophage-specific protein Iba-1; 1:300 – Invitrogen, Carlsbad, California, USA) and rabbit IgG control (negative control) were incubated overnight at 4°C. Secondary antibody (biotinylated anti-rabbit IgG) was used for 30 minutes at room temperature. The avidin-biotin peroxidase system was used for detection (Vectastain Elite ABC HRP Kit; Vector Laboratories Inc., Burlingame, California, USA), and visualization was done by 3,3'-diaminobenzidine – DAB substrate (Betazoid DAB Chromogen Kit, Biocare Medical, Pacheco, California, USA). Uterine

sections were finally counterstained with hematoxylin, dehydrated, mounted, and examined using light microscopy.

Quantification of Inflammatory Response

The percentage stain area of macrophages in the uterus of naïve and infected non-pregnant mice was estimated using the image processing software Image J [37]. Briefly, multiple images (at least five) from each uterine section were obtained at 60X magnification and Iba-1 expression [111] was evaluated as percent positively stained macrophages.

Antibodies and Flow Cytometry

To determine the population and kinetics of systemic regulatory T cells in mice, the spleen of each animal was processed and analyzed by flow cytometry analysis using previously published protocols [57]. Briefly, the spleen was dissociated by mechanical disruption using a Miltenyi Tissue Dissociator (Miltenyi Biotec) to obtain a single cell suspension. Cell suspensions were then stained for flow cytometry analysis using fluorophore-conjugated mouse antibodies against surface and intracellular Tregs molecules (efluor450 anti-mouse CD4 clone GK1.5; phycoerythrin cyanine 5 PECy5 anti-mouse FOXP3 clone: FJK16s; and phycoerythrin PE hamster anti-mouse TNFR2 - CD120b clone TR75-54.7). Prior to intracellular staining of FOXP3, cells were fixed and permeabilized with the FOXP3 transcription factor buffer set by BD Biosciences according to the manufacturer's instruction (BD Biosciences, San Jose, California,

United States). BD Biosciences LSR Fortessa X-20 was used to collect and analyze data, and approximately $1X10_6$ cells were acquired. Flowjo software was used to analyze data. Cells infected with the virulent strain S2308 were inactivated using an in-house validated protocol (by inactivation with formaldehyde for 1 hour at 4°C) before analysis on the flow cytometer.

Statistical Analysis

The recoverable $\log_{10}$ bacterial CFU/g of uterine and splenic tissues, percentage of Tregs, splenic weight, and macrophage staining intensity on uterine tissues between mice of all treatment groups were analyzed using the Mann Whitney test, Two-way or One-way ANOVA and Tukey's multiple comparisons test depending on the data sets analyzed. GraphPad Prism software (La Jolla, California, USA) was used for the analyses.

Data Availability

Bacterial strains and other datasets presented in this study are available with no restrictions from the author.

Competing Interests

The author declares no competing interests.

CHAPTER III

TROPISM OF *BRUCELLA ABORTUS* FOR THE NON-PREGNANT UTERUS IS DRIVEN BY POTENT REGULATORY T CELLS AND TREATABLE WITH TNFR2 ANTIBODIES

Summary

Stealthy intracellular bacterial pathogens are known to establish persistent and sometimes life-long infections. Some of these pathogens also have a tropism for the reproductive system thereby increasing the risk of reproductive disease and infertility. To date, the pathogenic mechanism involved remains poorly understood. Here, we demonstrated that *Brucella abortus*, a notorious reproductive pathogen, has the ability to infect the non-pregnant uterus, sustain infection, and induce inflammatory changes during both acute and chronic stages of infection. In addition, we discovered that chronically infected mice had a significantly reduced number of pregnancies compared to naïve controls. To investigate the immunologic mechanism responsible for uterine tropism, we explored the role of regulatory T cells (Tregs) in the pathogenesis of *Brucella abortus* infection. We show that highly suppressive CD4+FOXP3+TNFR2+ Tregs contribute to the persistence of *Brucella abortus* infection and that inactivation of Tregs with TNFR2 antagonistic antibody protected mice by significantly reducing bacterial burden both systemically and within reproductive tissues. These findings confirm a critical role of Tregs in the pathogenesis of persistence induced by *B. abortus*. Results from this study indicate that adverse reproductive outcomes can occur as

sequelae of chronic infection in non-pregnant animals, and that fine-tuning Tregs activity may provide novel immunotherapeutic and prevention strategies against intracellular bacterial infections such as brucellosis.

Author Summary

Bacterial pathogens have developed several pathogenic mechanisms to establish persistent infections in their hosts. Several of these pathogens are also known to cause reproductive complications including stillbirth and infertility. We proposed that understanding the pathogenesis of these complications will help to devise novel therapeutic and prevention strategies to control the infection. Here, for the first time, we demonstrated that *Brucella* has the ability to colonize and induce long-lasting inflammatory changes in the uterus of non-pregnant mice. We also showed that there is a persistent chronic infection in these tissues with the wildtype strain *Brucella abortus* 2308, which resulted in a significantly reduced number of pregnancies when compared to non-infected controls. These findings suggest that the tropism of *Brucella* for the female reproductive system is independent of pregnancy, and that adverse reproductive outcomes can occur as sequelae of chronic infection in non-pregnant animals. In an effort to identify the immunologic mechanism responsible for uterine tropism, we demonstrated that highly suppressive regulatory T cells (Tregs) expressing TNFR2 contribute to the pathogenesis of *Brucella abortus* infection, and that inactivation of Tregs with antagonistic antibody protected mice by significantly reducing bacterial burden both systemically and within reproductive tissues. Therefore, this result confirms

a critical role for Tregs in *Brucella* persistence and also suggests that eliminating host Tregs activity may provide novel immunotherapeutic and prevention strategies against intracellular bacterial infections including brucellosis.

Introduction

Despite the presence of functional host defenses, some intracellular bacterial pathogens develop various survival mechanisms to cause persistent and occasionally life-long infections. A number of these pathogens are known to have tropism for the pregnant reproductive system thereby increasing the risk of complications including spontaneous abortion and stillbirth. To begin to understand the underlying mechanisms that culminate into the observed adverse reproductive complications, this study focused on *Brucella abortus*, an intracellular bacterial pathogen primarily known to cause reproductive diseases including infertility and severe pregnancy complications, particularly in livestock, and most recently described in humans [1-3]. *Brucella* pathogenesis has been extensively studied using mouse and other natural host animal models [4]. Several of these studies have shown the survival and replication of *Brucella* spp. in various organs of infected animals [11, 37]. Of particular importance is the tropism of *Brucella* for the pregnant uterus. For example, up to 10^{13} colony forming units of *Brucella* have been recovered in the utero-placental unit of infected animal models (mice, cattle, and goats) that had classical pregnancy complications such as spontaneous abortion [1, 9, 11]. However, until this study, investigation of the pathogenic mechanisms of *Brucella* colonization and survival in the female reproductive

system have revolved around pregnant animal models [38-42], with little or no information about the pathogenic effects of these organisms on the non-gravid reproductive system. Hence, the impact of *Brucella* infection on the reproductive tract of non-pregnant animals is unknown.

Therefore, the objectives of this current study were to determine if *Brucella* 1) can colonize the non-pregnant uterus and sustain a chronic infection; 2) induce persistent pathology composed of highly suppressive host regulatory T cells (Tregs) which express TNFR2 that hamper infection elimination and contribute to infertility; and 3) identify the possible therapeutic benefit of selective host Treg cells elimination through TNFR2 antagonistic antibody monitored by pathogen elimination. Specifically, we investigated the contribution of regulatory T cells (Tregs) to the pathogenesis of *Brucella* infection in non-pregnant mice based on the increasing evidence that Tregs-mediated immune suppression perpetuates chronic infections, including a possible role of the most suppressive TNFR2 Tregs [58-61]. The findings of this study contribute to the understanding of cellular mechanisms of *Brucella*-induced reproductive disease in animals and humans, as well as the sequelae of chronic infection.

Results

Brucella abortus Persists in the Uterus of Non-pregnant Mice during Acute Infection

To assess bacterial colonization and to characterize the gross and histopathological lesions induced by virulent *B. abortus* strain 2308 (S2308), live-attenuated vaccine strain S19 (S19), and attenuated mutant strain 2308*ΔvirB2* (*virB2*),

four groups of five mice each were inoculated intraperitoneally with $1X10_6$ CFU (A.1). Uterine colonization was evaluated at 3, 7, and 13 days post infection (dpi). By 13dpi, both S19 and S2308 persisted in the uterus, maintaining the infection, while *virB2* was completely cleared (Fig 1A). Interestingly, all the uteri were grossly unremarkable with no evidence of lesions, regardless of the treatment group or time post-inoculation. However, despite the unremarkable gross appearance in the uterus, both S19 and S2308 induced inflammation in which the severity was highly correlated with the virulence of the challenge strain; S2308 induced a more severe inflammation characterized by macrophage infiltration compared to S19 (Fig 1C and F).

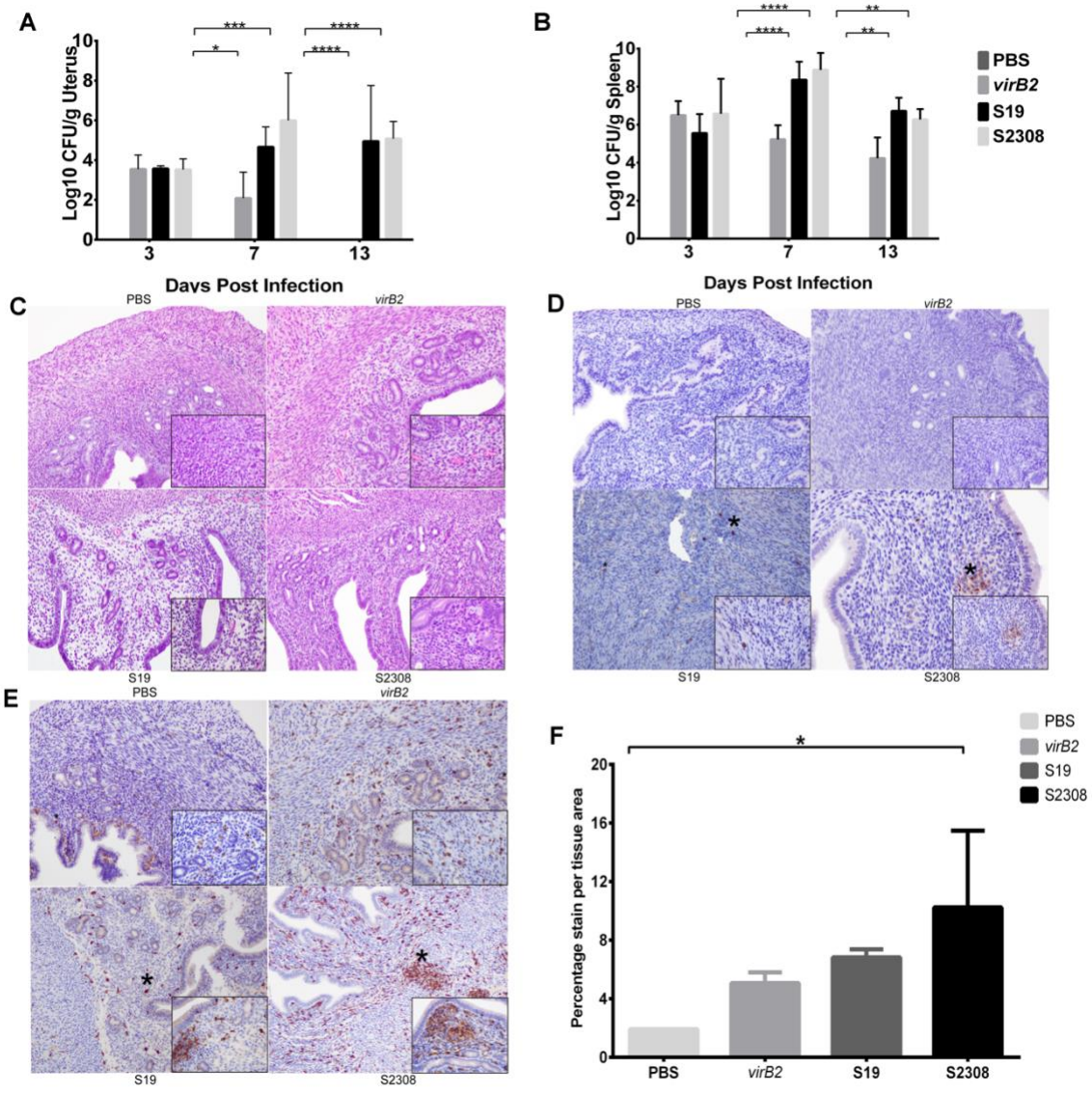
The presence of *Brucella* antigen in the uterine tissues of infected mice was also evaluated. At all time-points, macrophages in the endometrium and myometrium of mice infected with S19 and S2308 demonstrated strong intracytoplasmic immunolabeling for *Brucella*-specific antigen (Fig 1D). However, mice infected with *virB2* had less antigen at 3dpi and 7dpi, and by 13dpi, no immunolabeled signal was observed in the uterus of these mice (Fig 1D). Mice in the PBS group were negative for *Brucella* antigen at all time points. Strong positive Iba-1 immunolabelling (macrophage-specific protein) was also detected in the uterus of all groups (Fig 1E), but significantly higher in uterus samples of animals infected with the virulent strain, S2308 (Fig 1F).

To demonstrate consistent and typical infection by these *Brucella* strains, spleens were also collected from the corresponding animals. Remarkable levels of colonization were present in the spleen of all infected mice at all time points (Fig 1B). In accordance with previous studies [62-63] by two weeks post infection, all the strains persisted in the

spleen, but *virB2* was maintained at a significantly lower level than S19 and S2308 due to its attenuation.

Figure 1. ***Brucella abortus*** **colonized the uterus and induced endometritis in non-pregnant ICR mice.**
Mice were injected intraperitoneally with 1x10$_6$ CFU of either PBS, *B. abortus* mutant 2308*ΔvirB2* (*virB2*), live-attenuated vaccine strain S19, or wild-type *Brucella abortus* S2308. Uterine samples were collected at 3, 7, and 13 days post infection, and bacteria were enumerated via culture. **A-B.** Bar graphs represents average (n=5) with SD. Statistical differences between the groups were determined using Two-way ANOVA and Tukey's multiple comparisons test. ** P<0.01 ***P<0.001 **** P<0.0001.
C. Representative micrographs (Hematoxylin and eosin stain) of normal uterus from control and infected non-pregnant mice at 13 days post infection (dpi), respectively. Mild to moderate neutrophilic and histiocytic infiltration of the endometrium, and expansion of the endometrial tissue with clear space (edema) was evident. Asterisks represent edema and inflammatory cell infiltration. *Brucella* antigen distribution (**D**) and positive immunolabelling of macrophages (**E**) in the uterus of the respective mice at 13dpi. Asterisks indicate *Brucella* antigens or Iba-1 positive macrophages, respectively.
F. Percentage stain area in the uterus of non-pregnant mice. Estimation of stain area intensity was performed by Image J analysis. Significance was estimated using One-way ANOVA and Tukey's multiple comparisons post hoc test. *P < 0.05.

Figure 1 Continued



Virulent B. abortus 2308 Induced Chronic Endometritis in Non-pregnant Mice

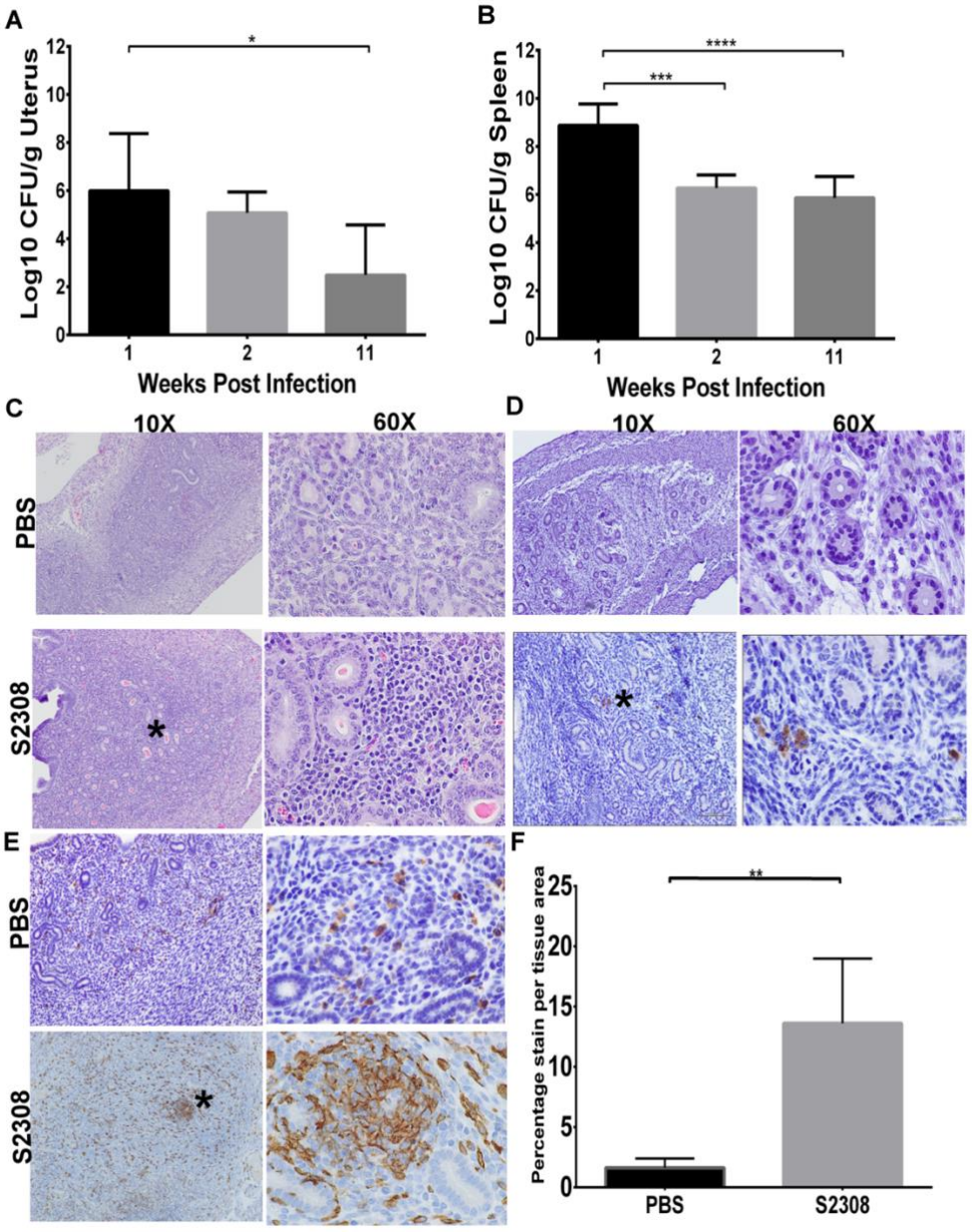
The pathogenesis of adverse reproductive outcomes during *Brucella* infection is unclear but may be due to a range of factors including sustained inflammation of the uterus of infected animals. Previous results from this study have demonstrated that *B. abortus* can colonize the uterus and induce acute histiocytic endometritis in non-pregnant mice (Fig 1). However, it is not known whether *Brucella* can persist in the uterus of non-pregnant mice for long periods and possibly induce complications that negatively impact future reproductive performance. Interestingly, infection with virulent *B. abortus* strain S2308 persisted in the uterus of all infected mice at 11 weeks post infection and induced a long-lasting inflammation (Fig 2A, C-F). Although no observable gross lesions were detected, histopathological evaluation of the uterine samples revealed multifocal areas of inflammation (Fig 2C). Positive immunostaining of *Brucella*-specific antigen (Fig 2D), as well as Iba-1 positive macrophages within the endometrium was also observed (Fig 2E-F). These results demonstrate that *B. abortus* not only persisted for long periods in the uterus of non-pregnant mice, but also induced a long-lasting inflammatory response.

Figure 2. Virulent B. abortus 2308 induced chronic endometritis in non-pregnant mice.

Bacterial (Log10 CFU/g) recovery in the uterus (A) and spleen (B) of non-pregnant mice (n= 11) infected with *B. abortus* S2308 and uninfected controls inoculated with PBS. *P<0.01. Bar graphs represents average (n=5-10) with SD. The black, light gray and dark gray colors indicates 1, 2, and 11 weeks post infection, respectively. Statistical differences between the groups were determine using One-way ANOVA and Tukey's multiple comparisons test. ***P<0.001 **** P<0.0001. C. Inflammatory cell infiltrates composed of macrophages and lymphocytes in the endometrium of infected non-pregnant mice. Positive immunolabelling of *Brucella* antigen (D) and Iba1-specific macrophages (E) in the endometrium of infected non-pregnant mice. F. Percentage stain per tissue area of macrophages in the endometrium of the respective mice. Estimation of stain area intensity was performed by Image J analysis. Significance was estimated using Mann Whitney test ** P<0.01.

*Asterisks: Inflammatory cell infiltrates (C), *Brucella* antigens (D) or Iba-1 positive macrophages (E).

Figure 2 Continued



Chronic Infection with Virulent B. abortus Reduced the Number of Successful Pregnancies in Mice

The adverse effect of chronic experimental *Brucella* infection on reproductive success in mice has not yet been established. In this current study, we sought to determine if chronic brucellosis, particularly with the virulent strain S2308, negatively impacts pregnancy success (A.2). Non-pregnant mice were either challenged with the virulent strain S2308 or inoculated with PBS as negative controls. After eight weeks of infection, all mice were exposed to male bedding for 3-5 days to synchronize estrous, and were subsequently mated with age-matched, stud males of the same strain. At gestation day 18, the number of chronically infected mice that had successful pregnancies was significantly lower than the uninfected control animals (Fig 3B). This finding suggests that S2308 persisted in the uterus of infected mice, resulting in chronic infection and inflammation that contributed to a significantly reduced rate of pregnancies. Interestingly, the chronically infected pregnant mice had a similar number of live pups as the uninfected controls, despite the fact that *Brucella* was recovered from the reproductive and gestational tissues (Fig 2A, C). The persistent infection and inflammatory lesions apparently decreased the probability of the pregnancy. Therefore, adverse reproductive outcomes resulted as sequelae of chronic infection and chronicity of infection negatively impacted reproductive performance in these animals.

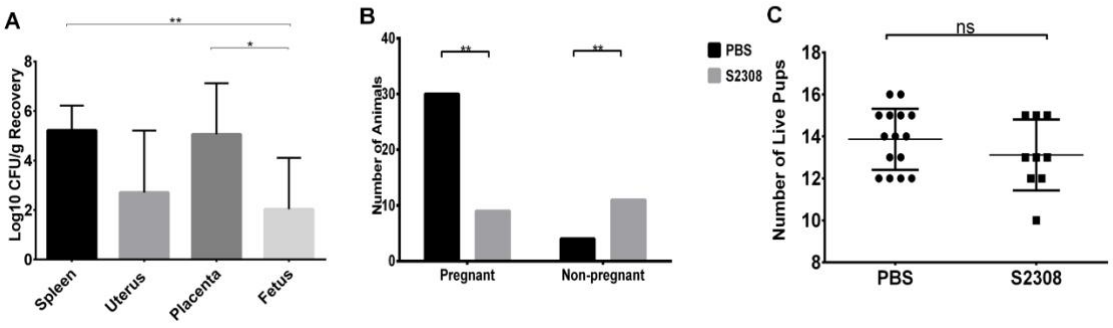


Figure 3. *Brucella abortus* S2308 persisted in the uterus of chronically infected non-pregnant mice reducing the number of successful pregnancies.
A. Bacterial recovery in the chronically infected mice (animals were infected for 8 weeks prior to breeding and tissues were evaluated on GD18) that had successful pregnancies. **B.** Number of animals that were either naïve or challenged with S2308 and that had successful pregnancies. Fisher's exact test. **C.** Number of live pups in those chronically infected mice that had successful pregnancies. Mann-Whitney test. Error bars indicate the standard deviation.

Regulatory T cells Expand and Promote the Persistence of Brucella abortus in Mice

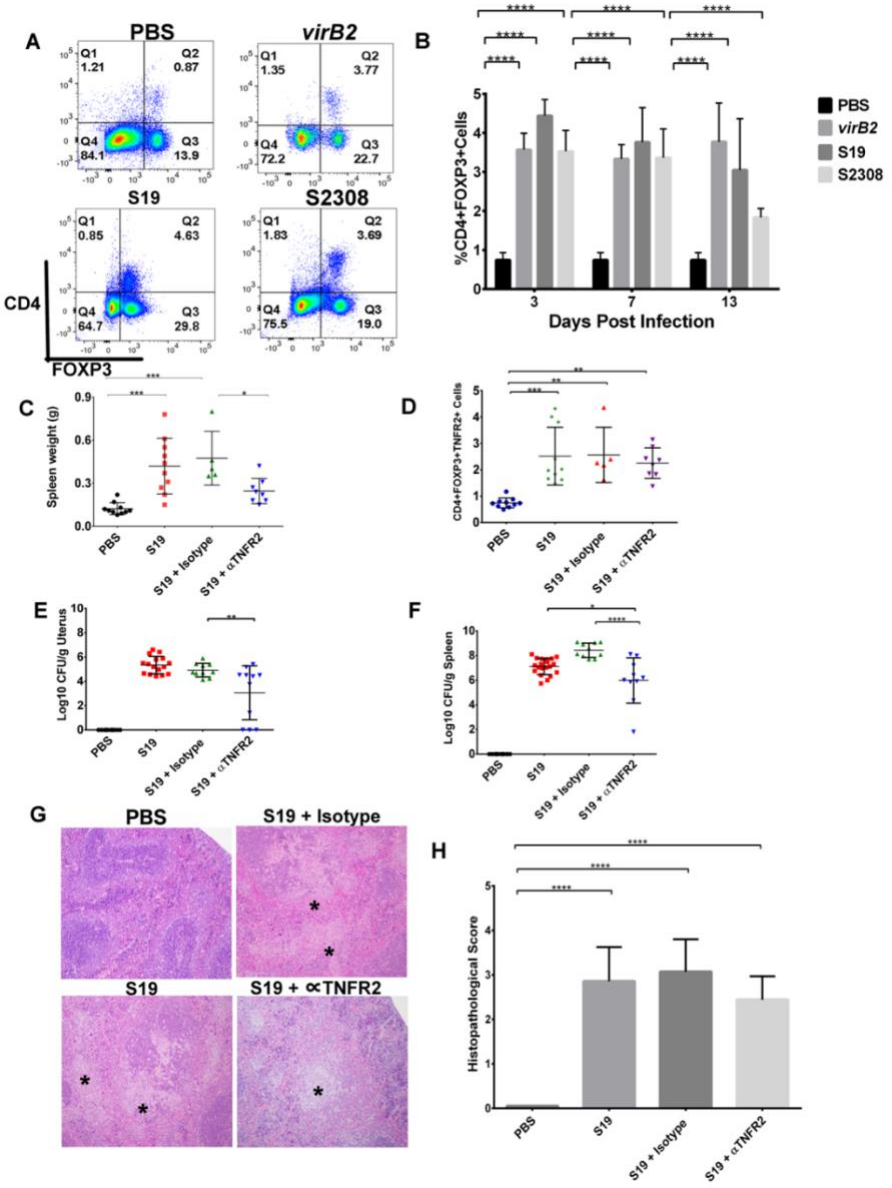
There is increasing evidence that Tregs enhance susceptibility to and persistence of various infectious diseases including brucellosis, tuberculosis, and listeriosis, amongst others [58-61, 64]. Therefore, to identify immunologic mechanisms that contribute to the tropism and persistence of *Brucella* in the uterus, we investigated the role of regulatory Tregs in the pathogenesis of brucellosis (A.3-4). Irrespective of the virulence of the infecting *Brucella* strain, CD4+FOXP3+ Tregs were expanded significantly in the spleen of all infected mice at all time-points compared to uninfected controls (Fig 4A-B). Given that an expansion of Tregs induces immunosuppression and increased susceptibility to infection, we examined bacterial burden in the uterus and spleen of infected mice. Overall, *Brucella* stimulated a significant, systemic expansion of Tregs which was

associated with increased bacterial burdens in the spleen and uterus of infected animals (Fig 1A-B).

Activated Tregs suppress the proliferation, activation, and effector functions of other immune cells [64-65]. In mice, activated Tregs express Tumor Necrosis Factor Receptor II (TNFR2), and this Tregs subset has been shown to inhibit the proliferation of effector T cells [20, 22-24]. Additionally, TNFR2 signaling either causes an expansion or contraction of Tregs, depending on the initiating signal [66-71]. We therefore hypothesized that counteracting TNFR2 expression on Tregs will inactivate these cells and their functions. However, mice treated with TNFR2 antagonistic antibody (which has been proven to functionally reduce the activation and proliferation of Tregs [66]) had a marginal reduction of TNFR2+ Tregs (Fig 4D), but a significant reduction in bacterial burden in the uterus and spleen compared with mice without treatment (Fig 4E-F). In addition, splenomegaly, one of the classical manifestations of *Brucella* infection [55, 62, 63] was significantly reduced in treated animals at 14pi (Fig 4C). Histopathological evaluation of the spleen also revealed a marginal reduction in inflammation in the spleen of animals treated with TNFR2 antagonistic antibody, which was evident by only mild obliteration of the splenic parenchyma (Fig 4G-H). In summary, the specific effects of the TNFR2 antagonistic antibody on Tregs inactivation was confirmed using an isotype antibody control, which showed similar results as observed in mice infected with S19 only. Therefore, our data highlight the crucial role of Tregs in the persistence of *Brucella* both systemically and in reproductive tissues.

Figure 4. A systemic expansion of CD4+FOXP3+ regulatory T cells is associated with increased bacterial burden in Brucella-infected mice.
A-B. Percentage of FOXP3+ T cells among CD4+ splenocytes in non-pregnant mice infected with either *Brucella abortus* Δ*virB2*, S19, and S2308. Bar graphs represent an average (n= 9) with standard deviation. Spleen weight **(C)**, percentage of CD4+FOXP3+TNFR2+ Tregs in the spleen **(D)**, and CFU/g recovery in the uterus **(E)** and spleen **(F)** of respective animals. TNFR2 antagonistic antibodies marginally diminishes splenic pathology in mice infected with *B. abortus* S19 **(G-H)**. Statistical differences between all groups were analyzed using One-way ANOVA followed by Tukey's multiple comparisons tests. Statistical software used was GraphPad Prism software (La Jolla, California, USA).

Figure 4 Continued.



Mechanism of Tregs-induced Persistence of Brucella abortus in Mice

The current study aimed to understand the role of immunosuppressive Tregs in the pathogenesis of brucellosis. The specific mechanism (for example, the inhibition of pro-inflammatory cytokines) of Tregs-induced immunosuppression that facilitates the colonization of *Brucella abortus* and the pathologic manifestations in nonpregnant mice was also explored.

Since the findings of the current study demonstrated a role of Tregs in the persistence of *Brucella abortus* in mice, we wanted to investigate the specific mechanism involved. Several mechanisms of Tregs-induced immunosuppression have been characterized including the modulation of dendritic cell maturation and function, cytolysis, metabolic disruption, or secretion of inhibitory cytokines such as TGFb, IL10, and IL35. We hypothesized that expanded Tregs favor the persistence of *B. abortus* through the induction of inhibitory cytokines (TGFb and IL10). Therefore, treatment with TNFR2 antagonistic antibody, which inactivates Tregs, will lead to a reduction of inhibitory cytokines and an elevation of pro-inflammatory cytokines needed to initiate pathogen clearance. To test this hypothesis, groups of mice were inoculated with *B. abortus* vaccine strain S19 and subsequently treated with TNFR2 antagonistic antibody, isotype control or PBS as previously described. At 14dpi, mice were euthanized and the spleens were processed to obtain single cell suspensions and cytokine expression was quantified using flow cytometry analyses.

In corroboration with previous studies, we found that CD4+ T cells were induced to a significantly higher percentage in the spleen of all infected animals at 14dpi (Fig 5).

However, contrary to expectations, our hypothesis that Tregs-induced persistence of *B. abortus* occurred through the induction of inhibitory cytokines was not supported because even though Tregs were eliminated through TNFR2 antagonistic antibody treatment, only a marginal reduction in the level of inhibitory cytokines was observed (Fig 5).

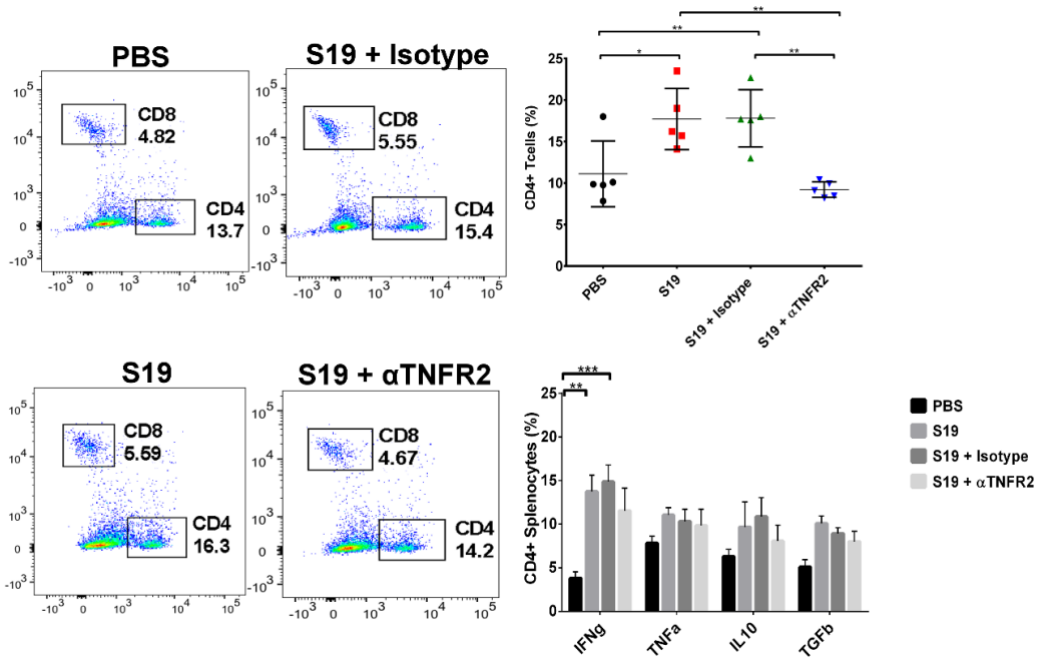


Figure 5. Effector CD4+ T cells are the predominant T cell response during B. abortus infection and a major producer of protective cytokines.
Percentage of CD4+ and CD8+ splenocytes and cytokines produced by each cell population in mice infected with *B. abortus* S19 with or without treatment with TNFR2 antagonistic antibodies.

Discussion

Historically, *Brucella* is known to have an exceptional tropism for the reproductive tissues of livestock, with consequential adverse pregnancy outcomes. Hence, brucellosis is considered a significant problem in pregnant animals. Several of the experimental studies delineating the implications of *Brucella* infection on the female reproductive system have investigated pregnant animal models (e.g. ruminants and rodents) [38-42, 72]. However, in reviewing the literature, little or no data was found on the pathogenic effects of *Brucella* on the non-pregnant reproductive system. Therefore, one of the objectives of this study was to assess whether *Brucella abortus* can infect, sustain infection, cause persistent inflammation of non-pregnant uterus, and subsequently impact successful pregnancy in infected animals. For the initial acute infection study, mice were infected with *B. abortus* strain 19 (S19: live-attenuated vaccine strain), *B. abortus* S2308 (S2308: virulent strain), and as a control, *B. abortus* 2308*ΔvirB2* (2308*ΔvirB2*: an attenuated strain with a defect in intracellular replication and persistence in tissues) [73, 74]. Both S19 and S2308 are smooth strains that are known to colonize reproductive tissues causing spontaneous abortion in their target species (cattle), with the rate of abortion being significantly higher with S2308 compared to S19 [3].

Results from this current study demonstrate that *Brucella abortus* has a tropism for the reproductive organs in mice that is not exclusively associated with pregnancy despite the historical assumption that brucellosis is mainly a significant problem during pregnancy. These findings provide new evidence that pregnancy is not a prerequisite for

significant reproductive complications associated with *Brucella* infection. Another important finding was that colonization and tropism of all the strains (2308*ΔvirB2*, S19, and S2308) to the uterus of infected non-pregnant animals were dependent on virulence (Fig 1A), because S19 and S2308 persisted and sustained the infection, which also indicated that even though S19 is an attenuated vaccine strain, it has the capability to invade and colonize the uterus of non-pregnant mice almost at the same level as the virulent strain, S2308 (Fig 1A). Previous studies have demonstrated that despite S19 being an attenuated strain, it can still invade the uterus and placenta of pregnant animals, resulting in spontaneous abortion, particularly in livestock [39, 75, 76]. The results from this current study and others not only confirm the undesired side effect of S19 as a vaccine, but also that the tropism of *B. abortus* S19 to the uterus of mice is independent of the reproductive status. In contrast, 2308*ΔvirB2* which is attenuated both *in vitro* and *in vivo* did not survive and was completely cleared from the uterus of non-pregnant mice by two weeks post infection. The result of this current study corroborates previous experimental studies using *virB* mutants of *Brucella*, which demonstrated the lack of survival and replication of these mutants in both *in vitro* and *in vivo* models of infection [73, 74].

Interestingly, in the absence of gross lesions, S19 and S2308 infected mice demonstrated histologic evidence of an increased inflammatory response evident at 7dpi (compared to those at 3dpi or uninfected controls) and increased by 13dpi. It is important to highlight that physiologic cellular infiltration of the mouse uterus occurs during the normal estrous cycle (which consists of the diestrus, estrus, metestrus, and proestrus

stages) [77, 78]. The quantity and composition of cells infiltrating the lamina propria of the uterus vary considerably with estrous stages [77, 78]. Unfortunately, the stage of estrous was not synchronized in this study, so it was a challenge to determine how much of the neutrophilic infiltration of the endometrium was related to *Brucella*-induced inflammatory change versus the stage of estrous. In contrast to studies that have investigated neutrophil fluctuations with estrous cycle, little is reported on the presence of or changes in macrophage numbers by estrous stage. This leads to dual hypotheses: *Brucella* induces macrophage infiltration into the uterus or resident macrophages phagocytose *Brucella*. Based on the relative increase in macrophage number in the uterus of mice infected with wildtype strain S2308, we favor the former hypothesis that *Brucella* induces an inflammatory reaction within the uterus. It can also be hypothesized that positive immunolabeling of *Brucella* antigens in the uterine macrophages (Fig 2D-F) of infected animals is a result of phagocytosis by those cells that are normally present during the various stages of the estrous cycle. However, the moderate numbers of macrophages in the uterine sections, as estimated via immunohistochemistry and quantitative image J analysis, signifies an inflammatory response to *Brucella* infection in these mice (Fig 1F, 2F). Besides, one of the first lines of innate defense against *Brucella* is the migration and accumulation of macrophages to phagocytose the bacteria. It is important to note that the effect of *Brucella* infection on the estrous cycle or how the estrous cycle impacts the severity of *Brucella* infection in non-pregnant mice is beyond the scope of this study. Hence, future studies exploring the influence of varying estrous stages on the susceptibility of mice to *Brucella* infection are required.

Immunohistochemical staining was used to detect *Brucella*-specific antigen in the uterus of infected animals. Strong intracytoplasmic immunolabelling of *Brucella* antigen was observed inside uterine macrophages of animals infected with S19 and S2308 (Fig 1C-D, 2D). The antigen distribution was consistently associated with the areas of inflammation present in these animals. The antigen was also present extracellularly within uterine tissues (which usually occurs when intracellular bacteria are released after cell death), similar to what is observed in other infected tissues. Interestingly, there was no significant difference in the distribution of *Brucella* antigens in the uterus of animals infected with either the live-attenuated vaccine strain (S19) or the virulent strain (S2308), indicating that S19 has similar capability as the virulent strain (S2308) to colonize and persist in the uterus of non-pregnant mice. As expected, *Brucella* antigen was observed only at early time points (3 and 7dpi), but not at 13dpi in 2308*ΔvirB2*-infected mice, indicating that the bacteria cannot sustain infection like the wild type, which is consistent with its attenuated phenotype (Fig 1). The findings of the current study may therefore support the hypothesis that S19 and S2308 enhance inflammation, particularly accumulation of macrophages, in the uterus of non-pregnant mice.

To establish the biological relevance of the survival and persistence of *B. abortus* in the uterus of non-pregnant mice, we conducted further experiments to demonstrate that *B. abortus* not only persisted for long periods, but also induced chronic inflammation. When chronically infected mice were exposed to stud males, an adverse reproductive outcome was observed, which was characterized by a significantly reduced

number of successful pregnancies when compared to uninfected control animals. The persistent macrophage infiltration of the endometrium may have contributed to the impaired reproductive performance in these animals. In addition, previous studies have shown that various factors associated with chronic endometritis, including persistent inflammatory processes, induction of immune mediators, and hormonal imbalance interfere with gamete or embryo physiology, reduced endometrial receptivity, and implantation failure [79-81]. Therefore, further studies are required to explore the cellular and molecular mechanisms of *Brucella*-induced endometritis and characterize the specific reproductive consequences.

Another objective of the current study was to identify the immunologic mechanism that drives susceptibility to and persistence of *Brucella* infection in non-pregnant mice. One of the many functions of regulatory T cells (Tregs) is to control the activity of the immune system by downregulating an excessive immune response to infection [54, 58-61, 64]. Hence, Tregs function as a double-edged sword having both protective and detrimental effects on host immune defense against invading pathogens. Activated Tregs suppress the proliferation, activation, and effector functions of other immune cells [65, 67]. In mice, activated Tregs express Tumor Necrosis Factor Receptor II (TNFR2), and this Tregs subset has been shown to inhibit the proliferation of effector T cells [66, 68-70]. Additionally, TNFR2 signaling causes either an expansion or contraction of Tregs, depending on the initiating signal [70, 71]. We investigated the critical involvement of Tregs in *Brucella* pathogenesis, based on the evidence that Tregs play a role in the regulation of immunity to infection [58-61, 64]. We hypothesized that

Brucella takes advantage of the Treg-cell mediated immunosuppression for unrestricted colonization and survival. Interestingly, our results showed that irrespective of the virulence of the infecting strain, *Brucella abortus* induced a significant systemic expansion of Tregs that was associated with increased bacterial burden in the uterus and spleen of infected animals. A possible mechanism by which *Brucella* drives the expansion of Tregs could be through the induction of tumor necrosis factor (TNF) on antigen presenting cells. TNF is a ligand of TNFR2 and the interaction between these two molecules results in the proliferation of Tregs [71]. Previous studies have shown that expanded Tregs can enhance the progression and chronicity of diseases primarily by limiting the effector functions of immune cells that are critical in the clearance of pathogens [54, 58-61]. This observation led us to conclude that a systemic expansion of activated Tregs by *Brucella* is one of the various mechanisms by which *Brucella* exploits the host immune system to enhance its colonization and survival. We confirmed that the immunosuppressive Tregs induced during *Brucella* infection were activated based on their expression of TNF receptor type II (TNFR2), a member of the TNF receptor superfamily (TNFRSF) [68, 69]. Previous studies have shown that TNFR2 is expressed by a unique subset of mouse splenic Tregs that have an activated phenotype with potent suppressive activity [66]. Hence, we hypothesized that blocking TNFR2 expression on Tregs will eliminate these cells and allow the proliferation of effector T cells [70], which will reduce *Brucella* colonization and survival in tissues. The TNFR2 antagonistic antibody used in this study was developed in-house (obtained from Dr. Denise L.

Faustman at Harvard Medical School) and has been shown to suppress Treg proliferation, while enabling the expansion of effector T cells [70].

We previously showed that the live-attenuated vaccine strain *B. abortus* S19 (S19) induced similar colonization, histopathologic changes, and expanded Tregs as the virulent strain S2308 (Fig 1-3). Therefore, S19 was used as a surrogate infection agent for the Tregs antagonism study. It is interesting to note that the significantly reduced bacterial burden and tissue inflammation in infected mice that were treated with TNFR2 antagonistic antibody was due to the inactivation of Tregs by this antibody, because mice treated with isotype control had comparable results to S19-infected mice (Fig 4E-H). This finding was in corroboration with previous studies in other disease models that showed that elimination of Tregs reversed susceptibility to infection [58-61]. Taken together, these findings demonstrate that Tregs have a crucial role in immune suppression during *Brucella* infection, as well as suggest the escape of *Brucella* from host immune surveillance.

The potential mechanism (inhibition of pro-inflammatory cytokines) of Tregs-induced immunosuppression that facilitates the colonization of *Brucella abortus* in mice was investigated. The pro-inflammatory cytokines (TNFa and IFNg) evaluated in this study were based on several studies indicating the crucial role of these cytokines in controlling *Brucella* infection [41], while the anti-inflammatory cytokines (IL10 and TGFb) were based on indirect immunosuppressive mechanisms of Tregs. Contrary to expectations, results from this study were unable to demonstrate that inactivation of Tregs with TNFR2 antagonistic antibody either reduced anti-inflammatory or increased

pro-inflammatory cytokines levels in the spleen. Some possible explanations for this finding might including the dose and timing of the TNFR2 antagonistic antibody treatment, or a redundancy of cytokine production by other immune cells. Although the potent Tregs mediating suppression were eliminated with TNFR2 antagonistic antibodies, the desired activation of effector cells for pathogen elimination might still be expected to maintain inflammation for effective function.

In conclusion, the current study has provided some insights into understanding the mechanisms involved in the persistence and pathogenesis of *Brucella* in non-pregnant mice. Particularly, the findings herein demonstrated for the first time that *Brucella* is not only a pathogen that is of reproductive significance during pregnancy, but that adverse reproductive outcomes can also occur as sequelae of chronic infection in non-pregnant animals. In addition, we showed a critical involvement of potent TNFR2+ expressing Tregs in the persistence of *Brucella* infection in non-pregnant mice. Future investigations are required to identify the mechanism by which *Brucella* specifically activates Tregs and the potential implications during pregnancy. Overall, the insights from the findings of this study will help to establish immunotherapeutic strategies and approaches to control intracellular bacterial infections with known predilection for the uterus including brucellosis.

CHAPTER IV

THE ROLE OF REGULATORY T CELLS IN *BRUCELLA*-INDUCED PLACENTITIS AND FETAL RESORPTION IN THE ALLOGENEIC PREGNANT MOUSE MODEL

Summary

Maternal regulatory T cells (Tregs) are known to sustain tolerance to the allogeneic fetus. However, the immune suppression associated with pregnancy has been implicated in the enhanced susceptibility to invading pathogens. Here we demonstrate that a systemic expansion of CD4+FOXP3+Tregs was associated with increased bacterial burden and pathology in the spleen and reproductive tissues of allogeneic pregnant mice during infection with *Brucella abortus*, an intracellular bacterial pathogen that causes spontaneous abortion in livestock. However, depletion of activated Tregs with an antagonistic antibody did not protect these pregnant mice from experimental *Brucella* infection as there was no significant difference in the bacterial burden both systemically and within reproductive tissues between treated and untreated groups of animals. Also, pregnant mice treated with only the antagonistic antibody without infection had fetal resorption rates comparable to those with *Brucella* infection, further reinforcing the importance of Tregs in fetal tolerance. These findings corroborate the pivotal role for activated Tregs during allogeneic pregnancy and how certain pathogens take advantage of pregnancy-induced immunosuppression for survival and persistence. Therefore, critical regulation of Tregs activity is needed to create a balanced system that

ensures pregnancy success as well as effectively controlling the invasion of prenatal pathogens.

Introduction

One of the most widely distributed bacterial zoonoses implicated in pregnancy complications is brucellosis [3], which is caused by a facultative intracellular bacterial pathogen of the genus *Brucella*. Notably, *Brucella* spp. are pathogens of the reproductive tract that cause spontaneous abortion, infertility, and stillbirth in animals, particularly livestock. Additionally, pregnant women infected with *Brucella* spp. have also been reported to have a significant risk of adverse obstetric effects [32-33]. Despite abortion being one of the major pathological manifestations of brucellosis in animals and humans, the pathogenic mechanisms of *Brucella*-induced abortion are poorly understood [3, 38, 42, 44, 45].

Although previous studies have proposed potential mechanisms by which *Brucella* causes adverse pregnancy outcome, an interaction between the pathogen and the maternal immune cells that sustain fetal tolerance has not yet been characterized [45, 50, 51]. It is important to note that during pregnancy, the developing fetus expresses foreign paternal antigens, making it prone to recognition and potential rejection by the maternal immune system. However, several cellular and molecular mechanisms at both the maternal-fetal interface and systemic level have been established to allow fetal tolerance and survival. A notable mechanism is the expansion of immunosuppressive regulatory T cells (Tregs), which occurs both systemically and locally at the feto-

maternal interface in humans and mice [52-54]. Tregs are a distinct subset of CD4+ T cells that are characterized by the expression of several surface markers, including CD4 and CD25 (interleukin (IL) 2 receptor α-chain), as well as the transcription factor Forkhead box P3 (FOXP3), which is constitutively expressed, and is responsible for Treg differentiation and function [67, 87]. These cells suppress the activation, proliferation, and effector functions of immune cells, making them important in the prevention of autoimmunity and particularly, the maintenance of fetal tolerance during pregnancy [67]. Several studies have demonstrated the importance of Tregs in maintaining pregnancy using pregnant animal models and varying experimental manipulation to investigate the specific relationship between Tregs and fetal survival [54, 85]. Additionally, other studies have illustrated diminished Tregs level in women with spontaneous miscarriage, pre-term birth, and pre-eclampsia [88-91].

Despite the obvious importance of Tregs in maintaining a successful pregnancy, it has been suggested that the suppression of maternal immunity that facilitates fetal tolerance during pregnancy predisposes to pathogen invasion, especially those pathogens with preference for reproductive tissues [54]. For example, in a mouse model of listeriosis and salmonellosis, it was found that pregnant mice were more susceptible to disseminated *Listeria monocytogenes* and *Salmonella typhimurium* infections [54]. The infection susceptibility in these mice was attributed to expanded Tregs which naturally occurred during pregnancy. This finding suggests the existence of a pregnancy-induced compromise in host defense against pathogens, particularly those pathogens that are well-known for proficient intracellular survival and placental cell

invasion [54, 92]. Given the importance of Tregs in maintaining the balance between immunological tolerance of foreign fetal antigens and immune activation necessary for host defense against invading pathogens, this current study aimed to establish the role of Tregs in the pathogenesis of *Brucella*-induced pregnancy complications in the allogeneic pregnant mouse model. The findings of this study have contributed to our understanding of how *Brucella* spp. exploit the immunosuppression induced by Tregs to enhance its colonization, particularly during pregnancy, ultimately leading to pathologic manifestations including placentitis and fetal resorption. Therefore, a critical regulation of Tregs activity is needed to create a balanced system that ensures pregnancy success as well as effectively controlling the invasion of prenatal pathogens.

Results

Brucella abortus Induced Fetal Resorption and Placentitis in Allogeneic Pregnant Mice

Brucella is generally known to cause reproductive disease in natural animal hosts including livestock. To evaluate the reproductive complications induced by *Brucella abortus* during allogeneic pregnancy (A.5), ICR female mice were bred with C57BL/6 mice, and subsequently infected at gestation day 5 (GD5) intraperitoneally with 10_6 CFU of virulent *B. abortus* strain 2308 (2308), live-attenuated vaccine strain S19 (S19), and attenuated mutant strain 2308*ΔvirB2* (*virB2*). Pregnancy outcome was assessed on GD8, 12, and 18. A reduction in the number of implantation sites (embryos), as well as embryo resorption was evident in mice infected with S19 and 2308 beginning at 7-days

post infection (dpi) or GD12 (Fig 6A). Since *Brucella* is known to have a tropism for the pregnant reproductive tissues, we found that the bacterial burden in the pregnant uterus was not only virulence-dependent but continued to increase significantly across the gestation period (Fig 6B-C). Histopathological assessments were also conducted in the gestational tissues of allogeneic pregnant mice infected with S19 and 2308. At 7dpi, embryos in these infected mice were completely resorbed with loss of tissue architecture and structural details, compared to uninfected controls. At 13dpi, there was necrotic placentitis, with much of the placental tissues completely obliterated by necrotic debris and inflammation (Fig 6D). Immunohistochemical analysis also revealed the presence and distribution of *Brucella* antigen (brown pigment) in placental trophoblasts (Fig 6D). Taken together, these findings demonstrated that *Brucella* colonized the systemic and gestational tissues of allogeneic pregnant mice in a virulence-dependent manner, causing adverse reproductive outcomes that manifested as placentitis and fetal resorption.

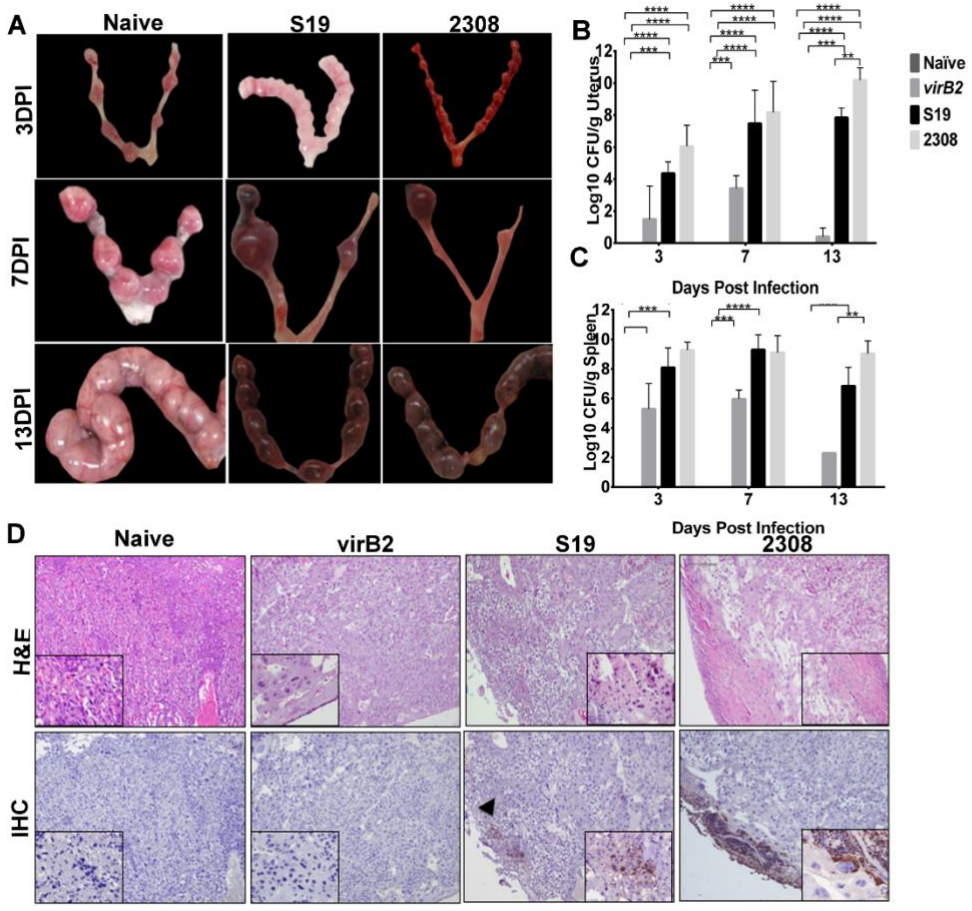


Figure 6. *Brucella abortus* induced fetal resorption and placentitis in allogeneic pregnant mice.

Allogeneic pregnant mice were infected intraperitoneally with 1x10$_6$ CFU of either PBS, *B. abortus* mutant 2308*ΔvirB2* (*virB2*), live-attenuated vaccine strain S19, or wild-type *Brucella abortus* 2308. The spleen and gestational tissues were evaluated at 3, 7, and 13 days post infection for bacteria enumeration, histopathology, and immunohistochemistry. **A.** Fetuses from mice of the respective groups at the stated days post infection. CFU/g recovery of *Brucella* in the spleen **(B)** and uterus **(C)** of the infected mice. Significance was estimated using One-way ANOVA and Tukey's multiple comparisons post hoc test. *P < 0.05. **D.** Representative micrographs (Hematoxylin and eosin - H&E stain and Immunohistochemistry-IHC) of placenta from control and infected allogeneic pregnant mice at 13 days post infection (dpi)/Gestation day 18. Inflammation of the placenta (H&E) and *Brucella*-specific antigen identified as brown pigments (IHC).

CD4+FOXP3+ Tregs contribute to susceptibility of infection by Brucella in allogeneic pregnant mice

Given that Tregs sustain fetal tolerance by suppressing overactive maternal immune cells, we hypothesized that naturally immunosuppressive Tregs produced during normal pregnancy indirectly contribute to susceptibility to *Brucella*-induced fetal resorption. To test this hypothesis, allogeneic pregnant mice were infected at gestation day 5 (GD5) intraperitoneally with 10_6 CFU of virulent *B. abortus* strain 2308 (2308), live-attenuated vaccine strain S19 (S19), and attenuated mutant strain 2308*ΔvirB2* (*virB2*) as previously described (A.6) [41, 93]. In contrast to previous studies that described a bell-curve expansion of splenic Tregs during mice gestation, we observed that the kinetics of Tregs expansion in our model of pregnant mice was constant across the gestation period because splenic Tregs were not significantly different on GD8, 12, or 18 [53, 54, 85, 94, 95]. Interestingly, S19 and 2308 infection further resulted in significant quantitative Treg expansion in the spleen of allogeneic pregnant mice compared to uninfected controls at 3-days post infection (dpi) (Fig 7A-B). However, at 7dpi, Tregs were expanded to similar levels in both infected and naïve allogeneic pregnant mice. At 13dpi, Tregs expansion appeared to be dependent on the virulence of the infecting strain, because Tregs expansion was significantly higher in animals infected with the more virulent 2308 (Fig 7B). This finding demonstrates that infection with *Brucella abortus* further expanded Tregs population in allogeneic pregnant mice beyond the level that naturally occurs in uninfected mice. Interestingly, the level of

systemic expansion of Tregs corresponded to bacterial burden in the spleen and uterus of infected allogeneic pregnant animals (Fig 6B-C).

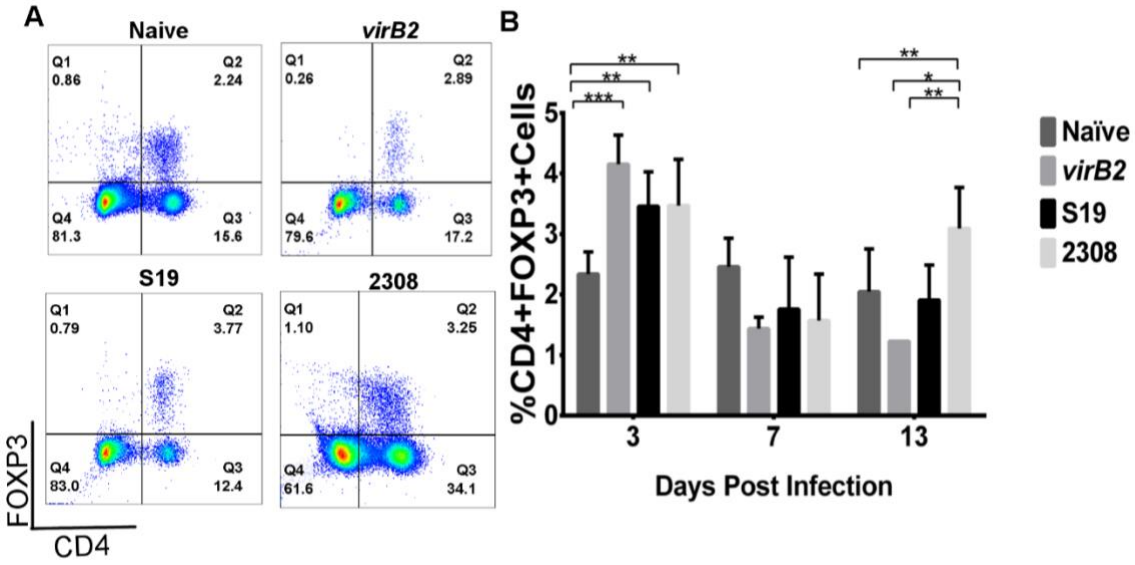


Figure 7. CD4+FOXP3+ Tregs are associated with *Brucella* infection susceptibility in allogeneic pregnant mice.
Allogeneic pregnant mice were inoculated intraperitoneally with $1x10_6$ CFU of either PBS, *B. abortus* mutant 2308*ΔvirB2* (*virB2*), live-attenuated vaccine strain S19, or wild-type *Brucella abortus* 2308. Spleens were evaluated at 3, 7, and 13 days post infection for Tregs characterization using flow cytometry **A.** Representative flow diagram of splenic FOXP3+ Tregs at 3dpi. **B.** Percentage of CD4+FOXP3+ T cells among splenocytes in infected and uninfected allogeneic pregnant mice. Significance was estimated using One-way ANOVA and Tukey's multiple comparisons post hoc test. *P < 0.05.

Inactivating Tregs with TNFR2 Antagonistic Antibody did not Protect Against the Complications of Brucella Infection in Allogeneic Pregnant Mice

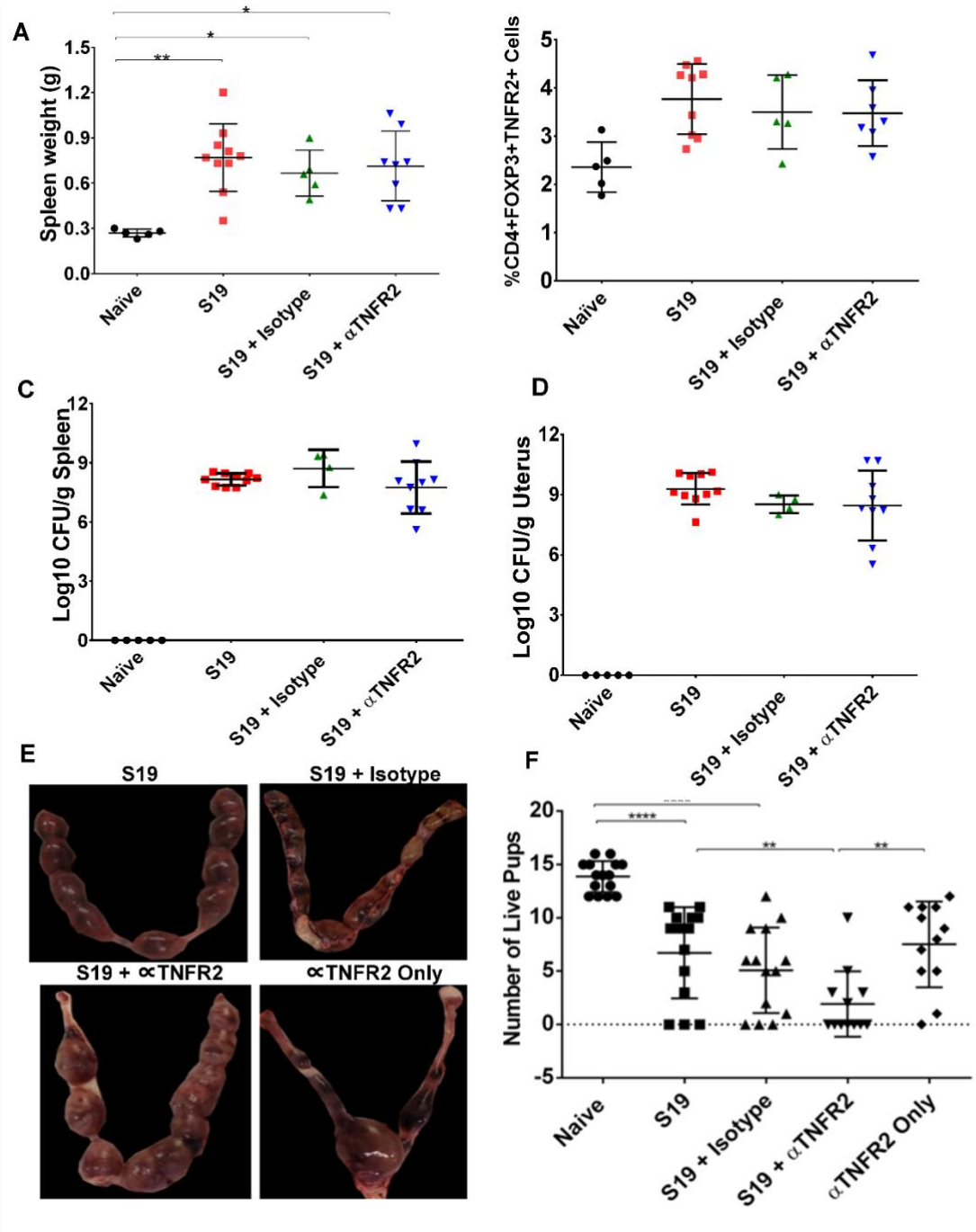
As previously demonstrated in the current study, Tregs may play a significant role in the pathogenesis of *Brucella* infection during allogeneic pregnancy (Fig 7). To confirm that the Tregs induced during *Brucella* infection were functional and suppressive, we demonstrated the expression of Tumor Necrosis Factor Receptor II (TNFR2) on CD4+FOXP3+ splenic T cells (Fig 8). In accordance with the current results, previous studies have shown that activated and maximally suppressive Tregs express TNFR2 [66, 68-70]. Also, TNFR2 signaling either causes an expansion or inactivation of Tregs, depending on the initiating signal [70, 71]. Therefore, we hypothesized that blocking TNFR2 expression would eliminate Tregs (A.7). To test this hypothesis, ICR females were bred with C57BL/6 males. At gestation day 5 (GD5), mice were infected with *B. abortus* vaccine strain S19 intraperitoneally and subsequently with four doses of 100ug TNFR2 antagonistic antibody at GD6, 9, 12, and 15. Mice in the control groups were inoculated with either TNFR2 antagonistic antibody, isotype control, or PBS. At GD18, mice were euthanized and evaluated for pregnancy outcome, splenic Tregs characterization, bacterial burden in the systemic and reproductive tissues, and histopathology.

As previously observed, infection of allogeneic pregnant mice with S19 induced fetal resorption, splenomegaly, systemic Tregs expansion, and increased bacterial burden (Fig 8A-D). Additionally, treatment of S19-infected allogeneic pregnant mice with

TNFR2 antagonistic antibody neither protected the animals from systemic and reproductive bacterial colonization nor the complications associated with S19 infection (fetal resorption and placentitis) (Fig 8E-F). In contrast to previous studies that suggested that embryo resorption does not immediately occur after Tregs ablation [54], we found in this current study that allogeneic pregnant animals inoculated with only TNFR2 antagonistic antibody had fetal resorption rates comparable to that of S19 infection only. This finding not only indicated that the TNFR2 antagonistic antibody used in this study specifically targeted activated Tregs, but also corroborated the crucial role of Tregs in maintaining allogeneic pregnancy.

Figure 8. Inactivating Tregs with TNFR2 antagonistic antibody did not protect against the complications of *Brucella* infection in allogeneic pregnant mice.
A-B. Gestational tissues from allogeneic pregnant mice infected with $1x10_6$ CFU of the live-attenuated vaccine strain S19 with or without ∝TNFR2 treatment. Fetal resorption was observed in all groups of animals regardless of the treatment. **C.** Percentage of CD4+FOXP3+ T cells among splenocytes in infected and uninfected allogeneic pregnant mice. **D.** Percentage of TNFR2+ cells among CD4+FOXP3+ splenocytes in the respective groups of animals. CFU/g recovery of *Brucella* in the spleen **(E)** and uterus **(F)** of the infected mice. Significance was estimated using One-way ANOVA and Tukey's multiple comparisons post hoc test. *$P < 0.05$.

Figure 8 Continued.



Discussion

Even though the developing fetus expresses foreign paternal antigens, special mechanisms have been put in place to prevent the rejection of the fetal tissue by the maternal immune system. One of the well-known mechanisms is an expanded population of regulatory T cells (Tregs) at both the systemic and the maternal-fetal interface that confer tolerance to the developing fetus [54, 97, 98]. Several reports have shown that the systemic expansion of Tregs in mice and humans follow a bell-curve representation across the gestation period that is characterized by a peak in expansion at mid-gestation and a steady decline in Tregs population towards the end of the gestation period [53, 95, 99]. However, the findings of this study are in contrast to the observation reported by previous studies, because the kinetics of Tregs during gestation in the mouse model used in the current study demonstrated a constant Tregs population across the gestation period. A possible explanation for the discordant results might be the use of a different mouse model (e.g. ICR females X C57BL6 males were used in the current study while C57BL6 females X BALB/C mice were used in other studies) and mating conditions. For example, a common mating combination used in previous studies involve C57BL6 females and Balb/C males, while ICR females and C57BL6 males were used in the current study.

Many studies have suggested that the immune suppression that is crucial for pregnancy success may also compromise host defense against invading pathogens [54, 108]. Therefore, the current study hypothesized that pregnancy-induced immunosuppression contributes to susceptibility to *Brucella* infection. Interestingly, we

found that a systemic expansion of Tregs was associated with increased bacterial burden and pathology in both systemic and reproductive tissues of allogeneic pregnant mice. This finding supports the evidence from previous studies that reported an increased susceptibility of pregnant mice to *Listeria* and *Salmonella* infections [54].

Another important finding in the current study was that *Brucella* infection triggered a further increase in the population of systemic Tregs compared to uninfected pregnant controls. However, this result was in contrast to a previous study that used *Listeria monocytogenes* as an infection model, and which showed that the percentage of systemic Tregs was similar in both infected and uninfected pregnant mice [54, 108]. It is well-established that Tregs function to suppress the activation and proliferation of effector T cells, and since effector T cells are critical in the clearance of intracellular pathogens such as *Brucella* [100, 101], we speculated that a systemic expansion of activated Tregs by *Brucella* represents one of the various mechanisms by which the pathogen exploits the host immune system to enhance its persistence. This explanation has been suggested by previous studies in other infectious disease models including tuberculosis, malaria, and leishamaniasis [58, 59, 64].

We also confirmed that the immunosuppressive Tregs induced during *Brucella* infection were activated and highly suppressive due to their expression of TNF receptor type II (TNFR2), a member of the TNF receptor superfamily (TNFRSF [104, 105]. As we have shown previously, the live-attenuated vaccine strain *B. abortus* S19 (S19) induced similar quantitative expansion of immunosuppressive Tregs as the virulent 2308. Therefore, S19 was used as a surrogate infection agent for the rest of the current

study. As previously observed, S19 induced a significant systemic expansion of CD4+FOXP3+TNFR2+ Tregs in infected mice compared to uninfected controls. Previous studies have shown that TNFR2 is expressed by a unique subset of mouse splenic Tregs that have an activated phenotype and potent suppressive activity [66], and that TNFR2 activates either the expansion or contraction of Tregs depending on the signaling trigger of agonism or antagonism [66, 71, 106, 107]. We therefore hypothesized that blocking TNFR2 expression on Tregs will allow the activation and proliferation of effector T cells and hence, a reduction in *Brucella* colonization. Several TNFR2 antagonistic antibodies have been identified, which suppressed Treg proliferation, and in turn enabled the expansion of effector T cells [70]. The important features of the TNFR2 antagonistic antibodies used in this study include no requirement for Fc binding, binding to the same region of the receptor, expressing dominance over TNF-mediated agonism, and hampering intracellular NF-kB activation and phosphorylation that is obligatory for TNFR2 signaling–mediated cell proliferation [70]. Contrary to expectations, the current study did not find a significant difference in bacterial burden in both the spleen and uterus of treated and untreated pregnant animals. Additionally, both treated and untreated groups of animals also exhibited placentitis and similar rates of fetal resorption (Fig 2). The significant resorption rates observed in uninfected pregnant animals that received the antagonistic TNFR2 antibody may be explained by the fact that the antagonistic antibody inactivated Tregs, which are also important in sustaining fetal tolerance. Hence, this finding corroborates previous reports of the crucial role of Tregs in pregnancy success. Taken together, it can be concluded

that targeting activated Tregs during S19 infection is detrimental during pregnancy. Therefore, further studies are required to critically regulate Tregs activity in order to create a balanced system that ensures pregnancy success as well as effectively controls the invasion of prenatal pathogens.

Additionally, this current study stimulates a very important question “How does *Brucella* stimulate such a massive Treg response?” “What ligand binds TNRF2 on Tregs to enhance proliferation during *Brucella* infection? (A-H). *Brucella* induces the expression of Tumor Necrosis Factor (TNF), which is a pleiotropic cytokine that modulates inflammation and immunity [109, 110]. TNF mediates its biological functions through its receptors: TNFR1 and TNFR2. Although TNF has been shown to have proinflammatory effects, there are several reports signifying its immunosuppressive effects through interaction with TNFR2. Therefore, we speculate that *Brucella* utilizes the immunosuppressive effects of TNF via its TNFR2 receptor to modulate host immune response for replication and survival.

CHAPTER V

DISCUSSION

Brucellosis is a widely distributed bacterial zoonosis that causes adverse obstetrical outcomes including spontaneous abortion, infertility, and stillbirth [3]. Consequently, investigation of the pathogenic mechanisms of *Brucella* colonization and survival in the female reproductive system has revolved around the use of pregnant animal models [39-41], with little or no information about the pathogenic effects of this pathogen on the non-pregnant reproductive system. In these studies, we demonstrated the capability of *Brucella* to colonize, sustain infection, and induce persistent inflammation that translated into infertility in non-pregnant animals. Additionally, we demonstrated the contribution of immunosuppressive TNFR2+Tregs to the pathogenesis of persistence of *Brucella* infection in non-pregnant and pregnant mice. [58-61].

Murine Models of *Brucella*-induced Reproductive Disease

Non-Pregnant Murine Model

To begin to understand the impact of *Brucella* on the female reproductive system, 6-10-week-old female ICR mice were utilized throughout the current studies. ICR (Institute for Cancer Research) mice are inbred strains derived from the outbred strain stock ICR. The reproductive advantages possessed by this mouse strain include high prolificity, pregnancy success, and good mothering capability, which are essential qualities required for the interpretation of the findings of our subsequent fertility studies. Additionally, the ICR mouse strain has been extensively used in the understanding of

bacterial colonization, pathogen clearance, host immune responses, as well as reproductive complications associated with *Brucella* spp. in females [40, 41]. In the current studies, the use of ICR strain to understand the pathogenesis of acute and chronic *Brucella* infection generated reproducible results in all infected animals which were characterized by high bacterial burden and pathological changes in splenic tissues. For the first time, the current study has also demonstrated the use of this mouse strain for understanding the pathogenesis of chronic infection, as well as sequela of *Brucella* infection in the reproductive system of non-pregnant mice.

Syngeneic and Allogeneic Pregnant Murine Model

Syngeneic pregnancy is generated through the mating of similar strains of mice while allogeneic pregnancy is produced by mating different strains of mice (e.g. ICR female x C57BL6 male). To demonstrate the adverse obstetric outcome of chronic brucellosis in mice, the syngeneic pregnancy was utilized. Generally, mating ICR females and stud males results into a high reproductive success as recorded by the Texas Institute for Genomic Medicine and other studies investigating reproductive performance in mice strains [40, 41]. Hence, this mating strategy was used to determine the likelihood of pregnancy occurrence in mice chronically infected with *Brucella abortus*. While most studies investigated pregnancy outcome during acute *Brucella* infection, we demonstrated for the first time that mice that had been chronically infected with the virulent strain 2308 are less likely to get pregnant, potentially due to the persistent uterine inflammation induced by this *Brucella* strain as shown in Figs 2-3. Overall, the current studies generated some interesting findings including the fact that the tropism of

Brucella abortus for the reproductive organs in mice is not exclusively associated with pregnancy despite the historical assumption that brucellosis is mainly a significant problem during pregnancy. Hence, the findings provide new evidence that pregnancy is not a prerequisite for significant reproductive complications associated with *Brucella* infection.

Additionally, to date, the pathogenic mechanisms of *Brucella*-induced pregnancy complications are still poorly understood. To begin to unravel the mechanisms involved, these current studies utilized an allogeneic pregnant mouse model. These type of pregnancy models are typically utilized in experimental studies that investigate how the divergence of maternal and fetal MHC molecules influence immune regulation during health and disease [54]. Since Tregs are known to maintain the balance between immunological tolerance of foreign fetal antigens and the immune activation necessary for the protection of the dam against pathogen invasion [54, 108], the current studies investigated the role of Tregs in the susceptibility of allogeneic pregnant mice to *Brucella*-induced systemic infection and pregnancy complications. Specifically, the findings thereof have contributed to our understanding of how *Brucella* spp. exploits the immunosuppression induced by Tregs to enhance its colonization in splenic and reproductive tissues during pregnancy, ultimately leading to pathologic manifestations including placentitis and fetal resorption (Figs 5-8). We hence speculated that Tregs mediated their suppressive activity through indirect mechanism of induction of inhibitory cytokines including IL10 and TGFb. However, based on the findings of the current study, there was no statistically significant difference between inhibitory

cytokine induction among all groups of mice regardless of the treatment status, meaning that the inactivation of Tregs in mice through TNFR2 antagonism did not result in reduced level of inhibitory cytokine as anticipated. Therefore, future investigations are required to establish the exact mechanisms by which immunosuppressive Tregs induce persistence of *Brucella* in tissues of animals. For example, future studies may explore the contribution of a direct mechanism (e.g. cell-to-cell contact) of Tregs immunosuppression through a co-culture system of Tregs and *Brucella*-specific effector T cells, and evaluating the impact of Tregs on effector T cell proliferation and functions. To perform this experiment, $FOXP3^{GFP+}$ transgenic mice which express green fluorescent protein (GFP) under the control of the FOXP3 promoter may be utilized. In these mice, FOXP3 expression can be detected in GFP+CD4+ lymphocytes. Spleens from S19 infected and uninfected groups (negative controls) can then be processed from these mice and wildtype controls to obtain single cell suspension. Splenocytes will be sorted for GFP+ cells (which will represent the Tregs population) and CD4+T cells only, which represent the effector T cell (Teffs) population. A co-culture system can then be established in which GFP+Tregs will be co-cultured with CD4+Teffs and heat-killed *B. abortus* 2308 will be used as the activating agent. Based on previous findings from the current study, it is expected that GFP+Tregs from S19 infected mice will suppress the proliferation and cytokine production of CD4+Teffs.

The Role of Tregs in the Pathogenesis of Persistence of *Brucella* Infection in Mice

Impact of TNFR2+ Tregs on Systemic and Local Uterine Infection

Previous studies have shown that Tregs-mediated immune suppression facilitate persistent infections including those caused by *Leishmania* spp. and *Mycobacterium tuberculosis* [58-61]. Until date, very little is known about the role of Tregs in *Brucella* pathogenesis in animals [81]. Hence, we aimed to investigate a possible role of the most suppressive TNFR2+ Tregs in the pathogenesis of persistence of *Brucella abortus*.

In non-pregnant mice, we have demonstrated for the first time that there is a systemic expansion of potent Tregs during infection with either the commercially available live-attenuated vaccine strain S19 or the virulent strain 2308. These expanded Tregs were shown to contribute to the persistence of *Brucella* in tissues such as the spleen and uterus because eliminating these potent Tregs resulted into decreased bacterial colonization in these organs (Fig 9). This finding is significant because as extensively discussed, *Brucella* is a pathogen that causes reproductive disease including infertility. Devising a therapeutic strategy to reduce bacterial burden and the associated complications of the reproductive system will limit the spread of the disease and enhance control and prevention. In summary (Fig 9), we designed a hypothetical model where we speculate that infection of mice with *B. abortus* S19 upregulates TNFa expression on antigen presenting cells like macrophages or dendritic cells [71, 96]. TNF is a pleiotropic cytokine that modulates inflammation and immunity. This cytokine typically mediates its biological functions through its receptors: TNFR1 and TNFR2. Although TNF has been shown to have proinflammatory effects, there are several reports signifying its

immunosuppressive effects, which may be in part, through the capacity of TNF in association with IL-2 to activate and expand mouse CD4+CD25+ Tregs through interaction with TNFR2. Therefore, we speculate that *Brucella* utilizes the immunosuppressive effects of TNF via its TNFR2 receptor to modulate the host immune system to its advantage for replication and survival (Fig 9). Based on the findings of the current study, the hypothetical model also speculates that an increase in Tregs during *Brucella* infection leads to colonization and pathology in tissues probably through a reduction in the effector functions and proliferation of *Brucella*-specific T effector cells. Future investigations are required to confirm or refute the hypothesis of contribution of Tregs and *Brucella*-specific T effector cells interaction in the pathogenesis of persistence of *B. abortus* infection. For example, evaluating the upregulation of inhibitory receptors of Tregs during *Brucella* infection, such as CTLA4 and PDL1, which directly downregulates effector T cells that are *Brucella*-specific could be evaluated. This experiment can be performed in conjunction with that stated above (page 62) to specifically analyze the expression of surface inhibitory receptors on Tregs and see if the upregulation of these inhibitory receptors corresponds with decreased proliferation and effector functions of *Brucella*-specific Teffs. If the hypothesis is true, it can be expected that there will be an increased expression of CTLA4 and PDL1 on Tregs when analyzed by flow cytometry and this increased expression will be directly correlated with reduced functions of *Brucella*-specific Teffs.

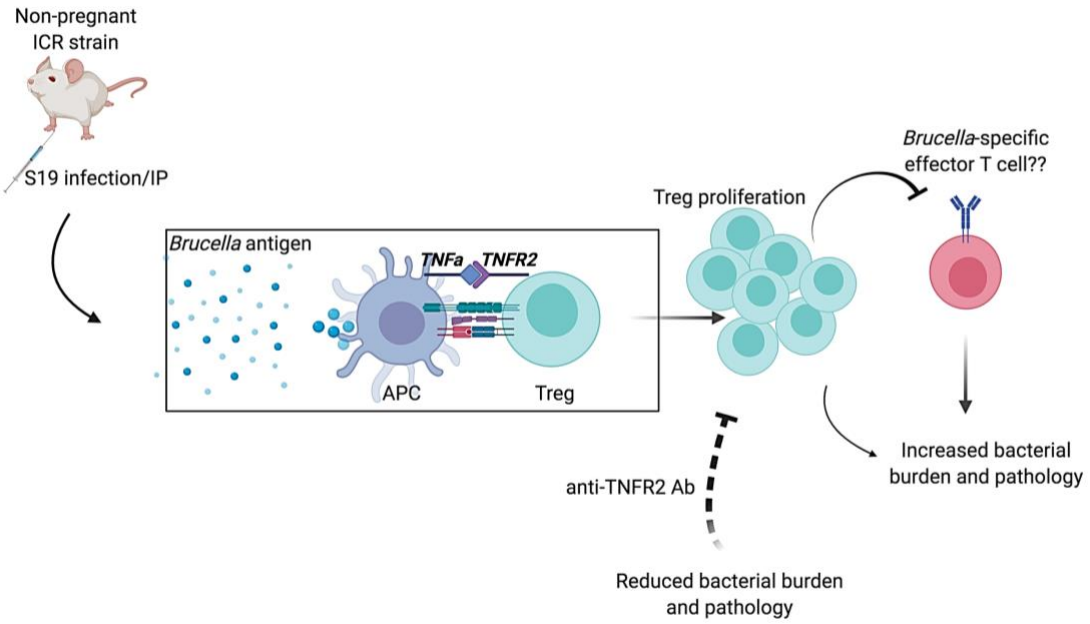


Figure 9. A model of how *Brucella* induces regulatory T cell expansion to evade host immune response and establish its niche in the tissues of non-pregnant mice. Upon infection with *Brucella abortus* S19, *Brucella* antigens are processed and presented to Tregs by antigen presenting cells (APCs), while TNF expression is upregulated on APCs as well. TNF ligand binds to its receptor (TNFR2) on activated Tregs which have been induced upon infection. TNF-TNFR2 ligand-receptor interaction further enhances Tregs activation and proliferation. An expanded Treg population causes increased *Brucella* burden and pathology in tissues including the spleen and uterus. Blocking TNFR2 expression on activated Tregs resulted into reduced bacterial burden and pathology in the spleen and uterus of treated mice.

Despite abortion being one of the major pathological manifestations of brucellosis in animals and humans, the pathogenic mechanisms of *Brucella*-induced abortion are poorly understood [3, 38, 42, 44, 45]. To begin to understand the mechanism behind the adverse obstetric effects associated with brucellosis, the current studies also investigated the role of Tregs because of the wealth of evidence that these cells confer tolerance to the developing fetus [54, 97, 98]. However, a number of studies have also suggested that the immune suppression that is crucial for pregnancy success may compromise host defense against invading pathogens [54, 108]. Therefore, the current study hypothesized that pregnancy-induced immunosuppression contributes to susceptibility to *Brucella* infection. Unlike what was observed in non-pregnant animals where eliminating Tregs resulted in reduced bacterial burden and pathology (Fig 9), immunosuppressive Tregs seemed to act differently during allogeneic pregnancy. For example, even though there was a further increase in systemic Tregs that was associated with disease burden and adverse pregnancy effects in infected pregnant animals, eliminating highly suppressive TNFR2+ Tregs resulted into fetal resorption that was comparable to S19 infection only. In other words, fetal resorption that occurred with only the anti-TNFR2 antagonistic antibody treatment was similar to that observed during S19 infection. A possible explanation for this is that pregnancy may induce the expression of TNFR2 on other cells that are also critical for sustaining fetal tolerance, and blocking this receptor may have contributed to the fetal resorption observed. Another possible explanation for this result is that inactivation of Tregs may have led to the enhanced proliferation of effector T cells and their functions thereby creating an

inflammatory storm that resulted in fetal death. Future studies are required to unravel the exact mechanism involved. For example, studies can prove that inactivation of Tregs by the antibody actually leads to the proliferation and effector functions of *Brucella*-specific effector T cells. Specifically, upon S19 infection of mice, and after intraperitoneal inoculation of the standard dose regimen of TNFR2 antagonistic antibodies (100ug/mouse: A.4), single cell suspension from the spleen can be analyzed to characterize, quantify, and compare the population of Tregs and Teffs. It can be expected that inactivation of Tregs through TNFR2 antagonism will allow Teffs to proliferate. Therefore, animals that received the TNFR2 antibody dose regimen will have a higher Teffs population than those that received the isotype antibody control or PBS. Nevertheless, findings from the current study further confirmed that immunosuppressive Tregs are required for sustenance of the allogeneic fetus and that elimination of Tregs in addition to S19 infection worsened pregnancy complications (Figs 8 &10). Therefore, it can be concluded that targeting activated Tregs during S19 infection is detrimental during pregnancy. Hence, further studies are required to critically regulate Tregs activity in order to create a balanced system that ensures pregnancy success as well as effectively controlling the invasion of *Brucella abortus*. For example, future studies may explore the use of different concentrations of anti-TNFR2 antibody and the number of inoculated doses at different gestation periods in pregnant mice. Another way of understanding the mechanism involved is to administer most of the doses of anti-TNFR2 antibody immediately prior to breeding and less doses of the antibody during the gestation and

infection period. This method may result in less fetal resorption because there will be more Tregs available during the gestation period to confer fetal tolerance.

Furthermore, like every great research endeavor, there are more questions than answers. The findings from the current study stimulate a very important question "How does *Brucella* stimulate such a massive Tregs response?" "What ligand binds TNRF2 on Tregs to enhance proliferation during *Brucella* infection? (Fig 10 and A.8). *Brucella* induces the expression of Tumor Necrosis Factor (TNF), a p cytokine known to regulate immune response to inflammation [109, 110]. TNF mediates its biological functions through its receptors: TNFR1 and TNFR2. Although TNF has been shown to have proinflammatory effects, there are several reports signifying its immunosuppressive effects through interaction with TNFR2. Therefore, we speculate that *Brucella* utilizes the immunosuppressive effects of TNF via its TNFR2 receptor to modulate host immune response for replication and survival (A.8). In future investigations, it will be interesting to conduct *in vitro* studies to characterize and quantify the expression of TNFa in a co-culture system of *Brucella*-activated macrophages/dendritic cells and *Brucella*-specific Tregs, which will help to answer the question of the specific mechanism by which TNFR2+Tregs are activated during *Brucella* infection. For example, to specifically address this question, mouse macrophage cell line (e.g. J774) can be infected with *B. abortus* S19 and the supernatant evaluated for the expression of TNFa using ELISA technique. When increased expression of TNFa is confirmed, then a co-culture system with Tregs can be established. Specifically, *Brucella*-specific TNFR2+Tregs can be sorted from the spleen of S19 infected mice and co-cultured with macrophages that have

been infected with heat-killed *B. abortus*. If our hypothesis is true, it is expected that there will be an enhanced proliferation of TNFR2+Tregs which may indicate the TNFa-TNFR2 interaction and involvement in *Brucella* pathogenesis. In conclusion, if an increased expression of TNFa on macrophages/dendritic cells corresponded to enhanced proliferation of TNFR2+Tregs in *Brucella*-infected mice in the co-culture system, then our hypothesis of the involvement of TNFa and TNFR2+Tregs interaction in the pathogenesis of *Brucella* infection may be true.

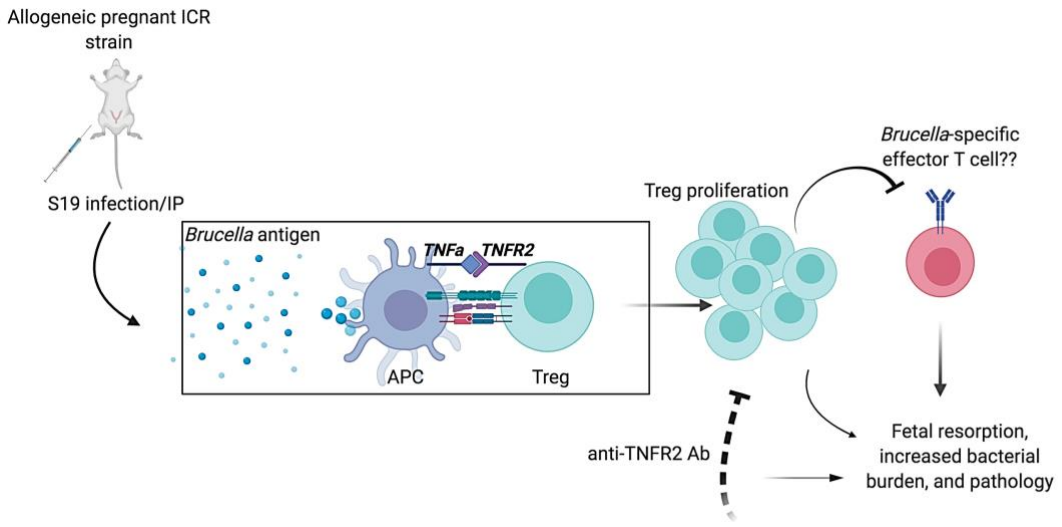


Figure 10. A model of how *Brucella* induces regulatory T cell expansion to evade host immune response and establish its niche in the tissues of allogeneic pregnant mice. Intraperitoneal infection of allogeneic pregnant mice with *Brucella abortus* S19, *Brucella* antigens are processed and presented by antigen presenting cells (APCs) to Tregs, and TNF expression is upregulated on APCs as well. TNF ligand binds to its receptor (TNFR2) on activated Tregs which have been induced upon infection. TNF-TNFR2 ligand-receptor interaction further enhances Tregs activation and proliferation. An expanded Treg population causes fetal resorption, increased *Brucella* burden and pathology in tissues including the spleen and uterus. However, blocking TNFR2 expression on activated Tregs resulted into more fetal resorption and pathology, and no change in bacterial burden between treated and control groups of allogeneic pregnant mice.

Mechanism of Tregs-induced Immunosuppression

Since the findings of the current studies indicate an important role of potent Tregs in the pathogenesis of brucellosis, further experiments were conducted to identify the specific mechanisms of immune suppression utilized by Tregs. As previously mentioned, Tregs mediate their suppressive activities through either direct (e.g. cell-to-cell contact) or indirect (induction of anti-inflammatory cytokines) mechanisms. The current study proposed that Tregs-induced immunosuppression that facilitates the colonization of *Brucella abortus* occurred through the effects of anti-inflammatory cytokines (A.8).

However, unexpectedly, we found that Tregs-induced persistence of *B. abortus* did not occur through the induction of anti-inflammatory cytokines because even though Tregs were inactivated through TNFR2 antagonistic antibody treatment, only a marginal reduction in the level of inhibitory cytokines was observed (Fig 5). We then speculated that this finding may have occurred because of the redundancy of these cytokines (for example, IL10 can also be produced by other immune cells including monocytes, mast cells, B cells, and other types of T cells). Therefore, in treated animals, there may have been a compensatory induction of IL10 and/or TGFb by the other immune cells. Future studies are required to characterize and quantify TNFR2+Tregs-specific IL10 and/or TGFb in treated vs untreated animals at different time points of infection in an *ex vivo* culture system. This can potentially be done by processing spleen samples from different groups of animals to obtain single cell suspensions. The obtained splenocytes can then be cultured in the presence of *Brucella* antigen and thereafter analyzed by flow

cytometry for the expression of TNFR2, IL10, and/or TGFb. Results will confirm or refute the hypothesis that Tregs-associated persistence of *Brucella* infection occurs through TNFR2 antagonism and is dependent on the indirect mechanism of immunosuppression of inhibitory cytokine induction (IL10 and/or TGFb).

CHAPTER VI

CONCLUSIONS

In conclusion, the current study has provided insights into understanding the mechanisms involved in the persistence and pathogenesis of *Brucella* in non-pregnant and pregnant mice. Particularly, the findings herein demonstrated for the first time that *Brucella* has a tropism for the female reproductive system that is not dependent on pregnancy, and that adverse reproductive outcomes can occur as sequelae of chronic infection in non-pregnant animals. In addition, we showed a critical involvement of Tregs in the persistence of *Brucella* infection in non-pregnant mice. Future investigations are required to identify the mechanism by which *Brucella* specifically activates Tregs and the potential implications during pregnancy. Overall, the insights from the findings of this study will help to establish immunotherapeutic strategies and approaches to control intracellular bacterial infections with known predilection for the uterus including brucellosis.

Another interesting finding in the current study was that potent Tregs also contributed to increased susceptibility of pregnant mice to *Brucella* infection. Unfortunately, an attempt to treat infected animals by inactivating Tregs did not produce the expected results because uninfected animals given the antagonistic antibody also aborted, indicating that activated Tregs are required for fetal tolerance as shown in previous studies. Therefore, further studies are required to critically regulate Tregs activity

in order to create a balanced system that ensures pregnancy success as well as effectively controls the invasion of prenatal pathogens.

Another significant finding of the current study was the undesired effect of S19 as a vaccine. Previous studies have demonstrated that despite S19 being an attenuated strain, it can still invade the uterus and placenta of pregnant animals, resulting in pathological manifestation of spontaneous abortion, particularly in livestock. Pregnancy complications were also observed in the current study with S19 infection at a comparable level to the virulent strain S2308. Collectively, the current research work has provided insights into the understanding of strain-specific pathogenic mechanism of S19 infection during pregnancy. For example, the results of the current study confirm that S19 infection induced immunosuppressive Tregs in both non-pregnant and pregnant animals. However, while the inactivation of Tregs significantly reduced bacterial burden in the spleen and uterus of non-pregnant mice, there was no statistically significant difference in bacterial colonization in the tissues of treated vs non-treated pregnant mice. This finding suggests that different mechanisms of Tregs immunosuppression are involved during *Brucella* infection in non-pregnant vs pregnant animals (Figs 8-9), and that the mechanism may be independent of TNFR2 antagonism during pregnancy.

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APPENDIX A

EXPERIMENTAL DESIGN OF THE STUDIES INVESTIGATING THE ROLE OF REGULAOTRY T CELLS IN THE PATHOGENESIS OF *BRUCELLA* INFECTION IN NON-PREGNANT AND PREGNANT MICE

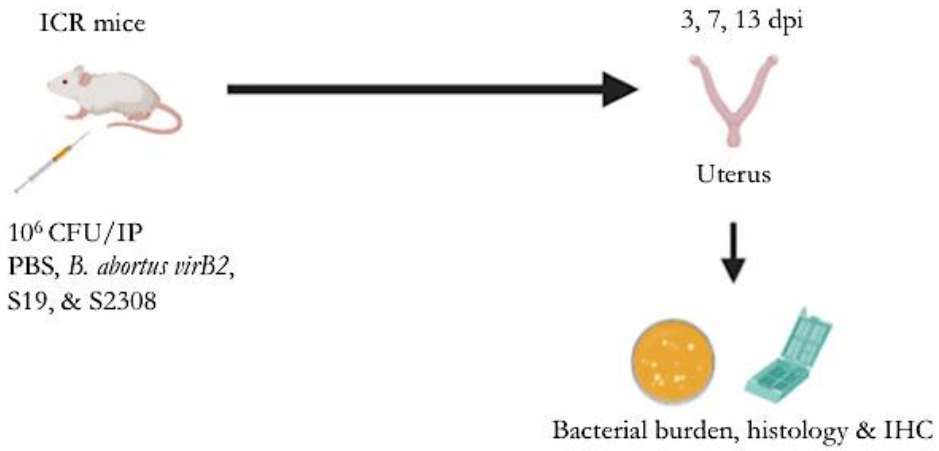

Figure A.1. Experimental design of acute infection of the uterus by *Brucella abortus*. 6-8-week old virgin ICR mice were inoculated intraperitoneally (IP) with either PBS,= attenuated mutant strain *B. abortus* virB2, live-attenuated vaccine strain S19, and the= virulent strain 2308. At 3, 7, and 13 days post infection, uterine samples were collected= to assess bacterial burden, histopathological changes, and *Brucella*-specific antigen= distribution.

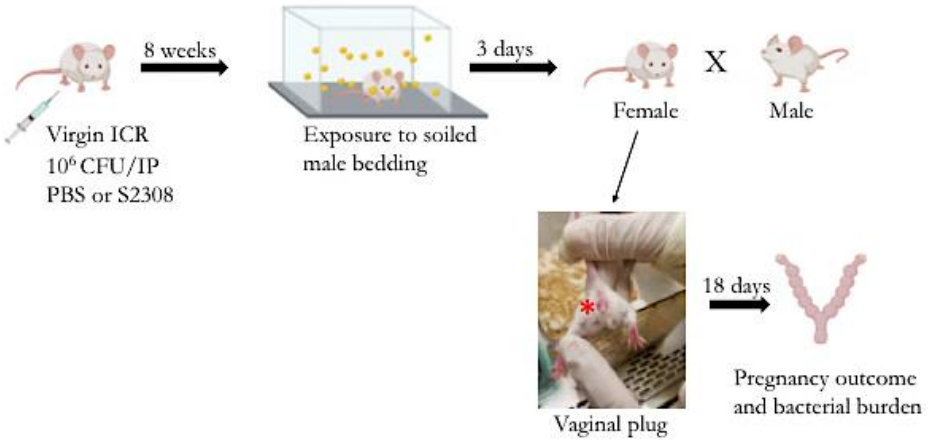

Figure A.2. Experimental design of adverse pregnancy outcome as a sequela of chronic=*Brucella* infection in non-pregnant mice.
To evaluate the biological relevance of chronic *Brucella* infection**,** 6-8-week old virgin= ICR mice were inoculated with either PBS or the virulent strain 2308 for 8 weeks to= establish chronic infection. The animals were then exposed to soiled male bedding for 3= days to synchronize estrus and thereafter were bred using age-matched studs. At 18 days= of gestation, pregnancy outcome and bacterial burden were assessed.

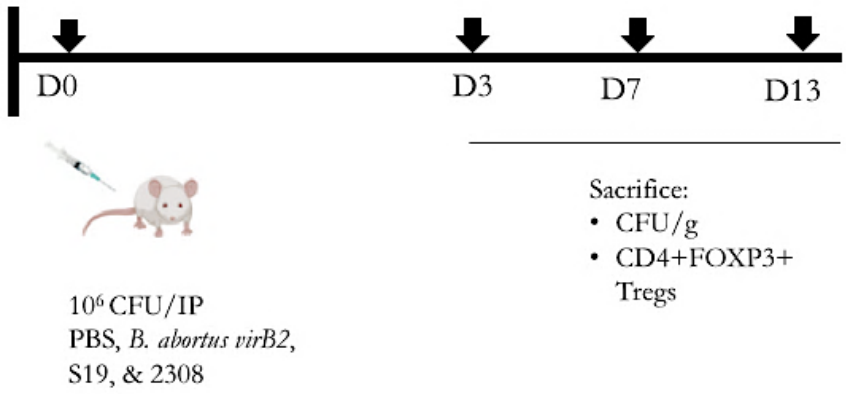

Figure A.3. Experimental design of the role of regulatory T cells (Tregs) in acute *Brucella* infection in non-pregnant mice.
6-8-week old virgin ICR mice were inoculated intraperitoneally (IP) with either PBS,= attenuated mutant strain *B. abortus virB2*, live-attenuated vaccine strain S19, and the= virulent strain 2308. At 3, 7, and 13 days post infection, spleen and uterine samples were= collected to assess bacterial burden and splenic Tregs characterization.

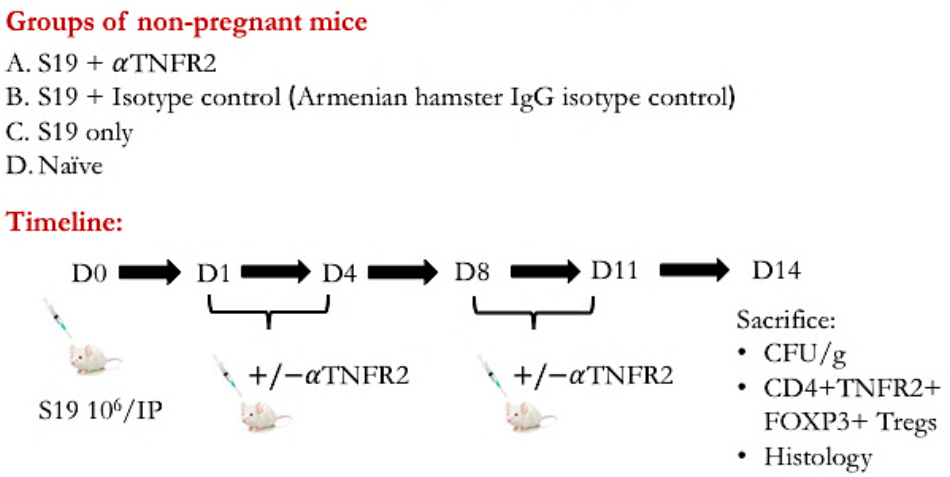

Figure A.4. Experimental design of the impact of regulatory T cells inactivation on the=persistence of *Brucella abortus* in the tissues of non-pregnant mice.
6-8-week old virgin ICR mice were inoculated intraperitoneally (IP) with either PBS or= the live-attenuated vaccine strain S19, the animals received biweekly doses of either= anti-TNFR2 antagonistic antibody or the isotype control. At 14 days post infection,= spleen and uterine samples were collected to assess bacterial burden, splenic Tregs= characterization and histopathologic changes.

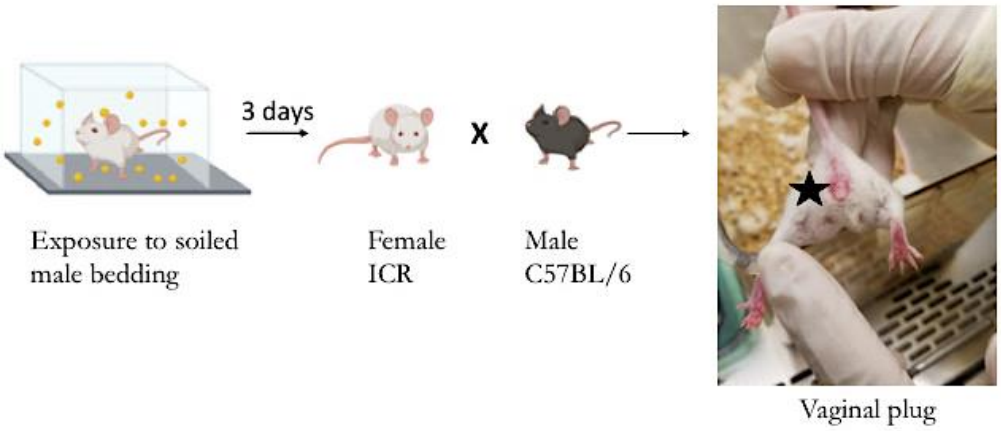

Figure A.5. Experimental design of the generation of allogeneic pregnancy.
To generate allogeneic pregnancy**,** 6-8-week old virgin ICR mice were exposed to soiled= male bedding for 3 days to synchronize estrus and thereafter bred using age-matched= C57BL/6 studs. Observation of vaginal plugs confirmed mating.

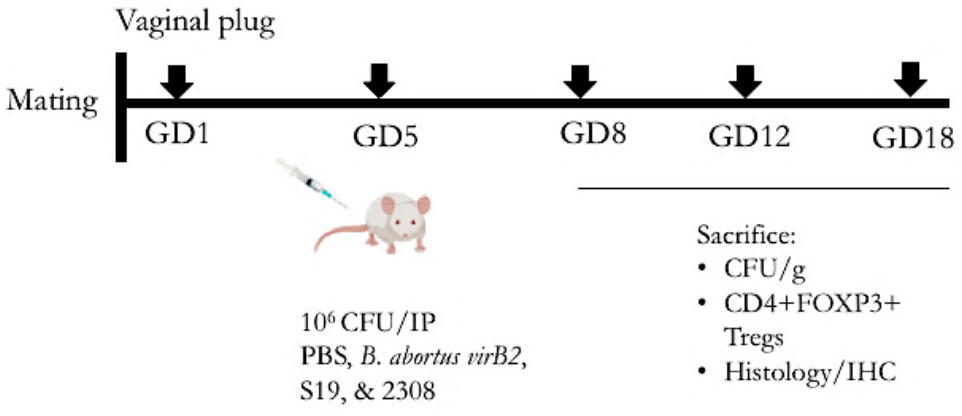

Figure A.6. Experimental design of the role of regulatory T cells during *Brucella* infection=in pregnant mice.
Allogeneic pregnancy was generated using the method described in A-D. Plugged mice= were inoculated intraperitoneally (IP) with either PBS, attenuated mutant strain *B. abortus virB2*, live-attenuated vaccine strain S19, and the virulent strain 2308. At 3, 7,= and 13 days post infection which corresponded to gestation day (GD) 8, 12, and 18,= spleen and uterine samples were collected to assess bacterial burden and splenic Tregs= characterization.

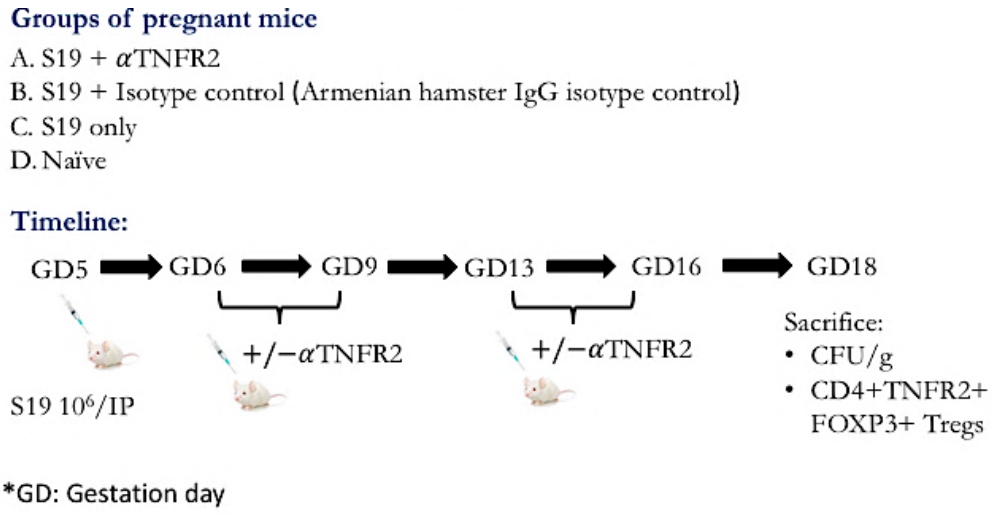

Figure A.7. Experimental design of the impact of regulatory T cells inactivation on the=persistence of *Brucella abortus* in the tissues of non-pregnant mice.
Allogeneic pregnancy was generated using the method described in A-D. Plugged mice= were inoculated intraperitoneally (IP) with either PBS or the live-attenuated vaccine= strain S19. At 14 days post infection which corresponded to gestation day (GD) 18,= spleen and uterine samples were collected to assess bacterial burden and splenic Tregs= characterization.

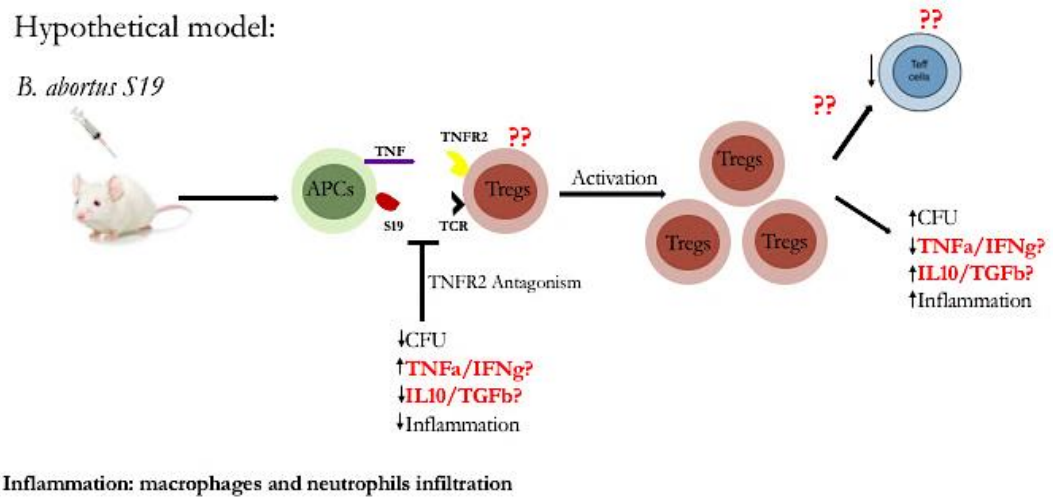

Figure A.8. Proposed model of how *Brucella* induces regulatory T cell expansion to evade=host immune response and establish its niche in tissues.
Upon infection with *Brucella abortus* S19 for example, *Brucella* antigens are processed and presented by antigen presenting cells (APCs) and TNF expression is upregulated on APCs as well. TNF ligand binds to its receptor (TNFR2) on activated Tregs which have been induced upon infection, and TNF-TNFR2 ligand-receptor interaction further enhances Tregs activation and proliferation. An expanded Treg population causes increased colonization and survival of *Brucella* by virtue of indirect immunosuppressive mechanisms of inhibitory cytokines (IL10 and TGFb) and possibly by directly suppressing *Brucella*-specific effector T cell response.