MECHANISMS OF IMMUNITY AND INFLAMMATION IN *BRUCELLA*-INDUCED ARTHRITIS

A Dissertation
Presented to
The Faculty of the Graduate School
at the
UNIVERSITY OF MISSOURI

In Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy
in Microbiology

By

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May 2018

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DEDICATION

To Mom and Dad and Grandma and Grandpa Lacey, who fostered my curiosity and creativity as a child so I could become a creative and curious adult.

ACKNOWLEDGEMENTS

I am grateful to all of those with whom I have had the pleasure to work with during these and other related projects. Assistance provided by the excellent staff at the University of Missouri, Laboratory for Infectious Disease Research was greatly appreciated. My special thanks are extended to Travis McCarthy for his constant support when I worked many long days in the BSL-3, and for always being willing to help when I had to radio out of the lab when I forgot something.

I would also like to express my gratitude to Dr. William Mitchell who scored the histology slides seen in the works here, and without whose work would not have made this dissertation possible. Thanks for teaching me about joint histology and answering all of my many questions.

I would like to acknowledge each of the members of my Dissertation Committee, Dr. Deborah Anderson, Charles Brown, Sheila Grant and Craig Franklin, as each of them has guided me to succeed in these scientific endeavors, and has provided me with a great deal of advice and encouragement. I would like to give a special thanks to Dr. Charles Brown for being there in moments of crisis and always being full of career and life advice. Thanks for encouraging me to “run with the big dogs.” You certainly kept my ambitious spirit alive through this process.

I would also like to thank my fellow graduate students in Connaway Hall. Without everyone’s commitment to scientific learning we would not have developed such a strong group. Thanks for sharing ideas, reagents and most of all friendship. A special thanks to Carrie Lasky (Williams) for helping me get

adapted to graduate school and encouraging me to do a lab rotation in Connaway Hall. Additionally, I would like to thank Alexis Dadelahi for her edits and comments during my comprehensive exam process and throughout this dissertation.

Finally, I would like to express my very great appreciation to my advisor and mentor Dr. Jerod Skyberg for taking a chance on me as a graduate student. I look up to you more than you know. This journey has challenged me further than I ever thought imaginable, and I am glad I had you as an advisor guiding me through the process. Thank you for your time in teaching me how to use the BSL-3 lab, editing several of my manuscripts, exposing me to the grant writing process and the review process, and helping me to prepare several formal presentations. I feel more than prepared for my next scientific journey because of you.

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Footnotes:

- A. Chapter II. This work was published as in *the Journal of Infectious Diseases* in 2016 with Carolyn A. Lacey as the first author, followed in authorship order by Lauren L. Keleher, William J. Mitchell, Charles R. Brown, and Jerod A. Skyberg. CAL conceived the project, designed, performed, analyzed experiments, and wrote the paper. LLK assisted with experiments. WJM scored histopathology. CRB analyzed experiments, and edited the paper. JAS conceived the project, designed, performed, and assisted with experiments, and edited the paper.
- B. Chapter III. This work was published as in *Infection and Immunity* in 2017 with Carolyn A. Lacey as the first author, followed in authorship order by William J. Mitchell, Charles R. Brown, and Jerod A. Skyberg. CAL conceived the project, designed, performed, analyzed experiments, and wrote the paper. WJM scored histopathology. CRB analyzed experiments, and edited the paper. JAS conceived the project, designed, performed, and assisted with experiments, and edited the paper.
- C. Chapter IV. This work was submitted for publication in 2018 with Carolyn A. Lacey as the first author, followed in authorship order by William J. Mitchell, Alexis S. Dadelahi, and Jerod A. Skyberg. CAL conceived the project, designed, performed, analyzed experiments, and wrote the paper. WJM scored histopathology. ASD provided reagents. JAS conceived the project, designed, performed, and assisted with experiments, and edited the paper.

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ABBREVIATIONS

	2-DG	2-Deoxy-glucose
A	AAMs	alternatively activated macrophages
	AGHS	aminoguanidine hemisulfate salt
	AIM2	absent in melanoma 2
	ASC	apoptosis-associated speck like protein
B	<i>B.a.</i>	<i>Brucella abortus</i>
	<i>B.m.</i>	<i>Brucella melatinsis</i>
	<i>B.s.</i>	<i>Brucella suis</i>
	Ba	<i>Brucella</i> agar
	BCA	bicinchoninic acid assay
	BCV	<i>Brucella</i> containing vacuole
	BMDMs	bone marrow derived Macrophages
	BSL-3	biosafety Level-3
C	CAMs	classically activated macrophages
	Casp1	caspase-1
	Casp11	caspase-11
	CDC	Centers for Disease Control and Prevention
	cDNA	complementary deoxyribonucleic acid
	CFUs	colony-forming units
	CM	Complete Media
	CPG DNA	cytidine-phosphateguanosine deoxyribonucleic acid
D	DAMPs	damage associated molecular patterns
	DNA	deoxyribonucleic acid
E	ELISA	enzyme-linked immunosorbent assay
	ER	endoplasmic reticulum
F	f.p.	footpad
	FACs	fluorescence-activated cells sorting
	FBS	fetal bovine serum
	FLS	fibroblast-like synoviocytes

G	GAPDH	glyceraldehyde 3-phosphate dehydrogenase
	GBP	guanylate binding proteins
H	H&E	Haemotoxylin and Eosin
	HMGB1	high mobility group box 1
I	i.p.	intraperitoneal
	IFN	interferon
	IFNAR	interferon alpha/beta receptor
	IHC	immunohistochemistry
	IL-18R	interleukin-18 receptor
	IL-18RA	interleukin-18 receptor antagonist
	IL-1R	interleukin-1 receptor
	ILCs	innate lymphoid cells
	iNOS	inducible nitric oxide synthase
K	KanR	kanamycin resistance
L	LAMP1	lysosome-associated membrane protein
	LDH	lactate dehydrogenase
	<i>lIdD</i>	lactate dehydrogenase (gene)
	LPS	lipopolysaccharide
M	M-CSF	macrophage colony-stimulating factor
	MMPs	matrix metalloproteinase
	mRNA	messenger ribonucleic acid
	MWCO	molecular weight cut-off
	MyD88	myeloid differentiation factor 88
N	NETs	neutrophil extracellular traps
	NK cells	natural killer cells
	NLRP3	NACHT, LLR and PYD domains-containing protein 3
	NLRs	nucleotide-binding oligomerization domain-like receptors
	NO	nitric oxide
	NOS2	nitric oxide synthase 2 (inducible)
O	OD	optical density
P	PAMPs	pathogen associated molecular patterns

	PBS	phosphate buffered saline
	PCR	polymerase chain reaction
	Perf	perforin
	PMN	polymorphonuclear
	PRRs	pathogen recognition receptors
R	Rag1	recombination activating gene 1
	RNA	ribonucleic acid
	ROS	reactive oxygen species
	RPMI medium	Roswell Park Memorial Institute medium
S	SD	standard deviation
	SEM	standard error of the mean
	SNAP	S-nitroso-N-acetyl-penicillamine
	sPBS	sterile phosphate buffered saline
	spp.	species
	SYK	spleen tyrosine kinase
T	T4SS	type IV secretion system
	TAK1	transforming growth factor- β -activated kinase-1
	TcpB	<i>Brucella</i> TIR-domain-containing protein
	TLR	Toll-like receptor
	TNFR	tumor necrosis factor receptor
	TRIF	TIR domain-containing adaptor-inducing interferon- β
	TUNEL	terminal deoxynucleotidyl transferase nick-end labeling
V	VLCFAs	very long chain fatty acid
W	WT	wildtype
T	TCR α	T-cell receptor alpha chain

CHAPTER I

Introduction

Human Brucellosis

The genus *Brucella* is a group of Gram-negative facultative intracellular coccobacilli that cause the zoonotic disease brucellosis. Human brucellosis is a systemic disease often referred to as undulant fever due to the characteristic interspersed fevers often displayed in infected patients (2). *Brucella* infects phagocytic cells of the reticuloendothelial system, particularly macrophages, as well as placental trophoblasts in pregnant animals (3). Clinical presentation in humans varies but generally consists of flu like symptoms such as malaise, chills and headaches, and, in acute forms, hepatomegaly and splenomegaly (4, 5). Disease manifestations typically present between 5 and 60 days post infection but brucellosis can also be asymptomatic in humans (6, 7). *Brucella* can infect almost any tissue and cause disease with relapse and lifelong complications, including arthritis, liver abscesses, and possible neurological disorders. While generally not life-threatening *Brucella* infection can cause endocarditis, which is a significant contributor to the 5% fatality rate associated with disease (1, 4, 8, 9). The most prevalent species infectious to humans are *B. melitensis*, *B. abortus* and *B. suis* which have preferred domestic animal reservoirs, but are not limited to, ruminants, bovine and swine, respectively (10, 11). *B. melitensis* is the most virulent species in humans and is most frequently reported as the cause of human disease (12). The genome of *Brucella spp.* are very similar to one another, exhibiting greater

than 99% amino acid identity between orthologs (13). *Brucella*'s genetic conservation and diverse range of hosts indicates these organisms are highly evolved for survival within mammals, although it remains unclear why there is such great diversity in host preference (14, 15).

Transmission to humans typically occurs through the consumption of unpasteurized milk or aerosol following contact with contaminated meat products, but may also spread through skin abrasions (16, 17). *Brucella spp.* infect a variety of wild and domestic livestock providing occupational hazards for veterinarians, meat processors and other individuals repeatedly exposed to animals (7, 17, 18). Brucellosis seldom spreads from person to person, and over 50% of human-to-human transfer cases are in patients less than one year old, indicating dissemination due to breastfeeding or vertical transmission. Rare cases of disease passage between humans through blood transfusions, bone marrow transplants and sexual transmission have also been reported. (12, 19, 20). Brucellosis is also highly contagious with an infectious dose as low as 10 bacteria, making brucellosis the top laboratory acquired infection in the world (21).

Due to *Brucella*'s ability to easily infect humans through aerosol and the potential to exhaust healthcare and agricultural resources, *Brucella* poses a major threat to public health and safety. Weaponizing *Brucella* may be of potential interest to terrorist organizations, thus *Brucella* has been designated a potential biological warfare threat. In fact, *Brucella* was the first biological agent to be weaponized by the United States, and the former Soviet Union tested *Brucella* in their offensive biological weapons program (22, 23). Thus, *Brucella spp.* have

been named non-Tier 1 select agents by the Centers for Disease Control (CDC) (24).

There are currently no human vaccines licensed to prevent human brucellosis and diagnosis can be difficult. The gold standard for *Brucella* diagnostics is through culture, however blood samples are often false negatives and growth is slow. Thus, serology is often used in conjunction with culture for rapid and accurate diagnosis (25). Uncomplicated cases of brucellosis are generally treated with a rigorous course of antibiotics, typically 6 weeks of doxycycline in combination with 2-3 weeks of an aminoglycoside (such as streptomycin or gentamycin), or 6 weeks of rifampin (26, 27). Despite antibiotic therapy resistant abscesses may still persist, and relapse of infection is common leading to chronic arthritis and endocarditis (5, 28, 29). Additionally, *Brucella* DNA can be detected in the patient's blood long after clinical manifestations have subsided, and some patients never clear infection which can lead to lifelong complications (28).

Animal Brucellosis

Brucella spp. are highly adapted to surviving in a variety of hosts. *B. canis* which preferentially infects dogs can cause brucellosis in humans; however, transmission is only mild when compared with other *Brucella* spp. Additionally, there are other species within the genus *Brucella* that have modest to no reported human transmission cases, including *B. ovis*, *B. neotomae* and *B. microti* (30). In fact, some species even infect marine mammals. *B. ceti* has been isolated in cetaceans such as dolphins, whales and porpoises and *B. pinnipediae* infects

pinnipeds which include sea lions and seals. Although rare, there is evidence that these marine species can also infect humans (Table 1) (31, 32). Despite *Brucella*'s diverse host tropism, agricultural animals are the main source of human infections. Brucellosis incidence in many countries has been reduced due to the use of live-attenuated vaccines in livestock; these consist of *B. abortus* vaccines, S19 and RB51 and *B. melitensis* vaccine Rev1. Although vaccination of animals reduces human disease incidence (33), these vaccines often present risks for veterinarians and animal caretakers as they cause disease in humans upon incidental exposure (34-36).

There is also risk for reintroduction of brucellosis to livestock as free-range wildlife can carry the disease. Populations of bison and elk often harbor *B. abortus* in the greater Yellowstone area, some feral swine in the southern United States test positive for *B. suis* and Alaskan caribou and reindeer also exhibit brucellosis positive titers (37, 38). Although brucellosis in animals is not associated with a unique set of symptoms, disease can present as mastitis, lameness and lymphadenitis (39). Additionally, there are rare cases of *B. melitensis* infected goats and sheep developing arthritis, infected horses can display local abscesses in bursae and dogs with *B. canis* can develop discospondylitis (40, 41). Nonetheless, the best characterized manifestations associated with animal brucellosis are placentitis followed by spontaneous abortion in pregnant females, generally in the 3rd trimester, and epididymitis and/or orchitis, which can lead to sterility in males (25, 40). Due to the abundance of brucellae in aborted fetal material, contact with birthing discharge or an infected fetus and/or placenta

Species	Zoonotic Potential	Host Preference
<i>Brucella melitensis</i>	High	Sheep, Goat
<i>Brucella abortus</i>	Moderate	Cattle, Bison, Elk
<i>Brucella suis</i>	Moderate	Pig
<i>Brucella canis</i>	Mild	Dog
<i>Brucella ovis</i>	Absent	Sheep
<i>Brucella neotomae</i>	Absent	Desert wood rat (<i>Neotomae lepida</i>)
<i>Brucella ceti</i>	Mild	Whales, Dolphins, Porpoises
<i>Brucella pinnipedialis</i>	Mild	Sealions, Seals
<i>Brucella microti</i>	Absent	Common voles (<i>microtus arvalis</i>)

Table 1. Host Preference for *Brucella* Species.

Brucella species and their potential to infect/cause human disease (zoonotic potential), and the preferential host reservoir associated with each species. Adapted from Xavier et al, 2010.(1)

remains the major source of natural host transmission, but animal to animal spread of disease can also occur through sex and consumption of infected milk or food (1).

Epidemiology of Brucellosis

With over 500,000 new cases annually, brucellosis is one of the most common zoonotic diseases worldwide, and has recently been designated as a neglected zoonotic disease by the World Health Organization (2, 42). Brucellosis remains an important global health issue, as it is endemic in the Mediterranean rim, Middle East, Central Asia, South America, and Africa and in some regions disease incidence exceeds 1,000 cases per year per 1 million inhabitants (8, 10, 43, 44). Frequency of disease has been drastically reduced in the United States and other countries with strong public and veterinary health programs (45). These policies often exercise vaccination laws, test-and-slaughter methods and surveillance programs to identify infected animals (33, 46). Elevated prevalence of brucellosis is generally associated with lower income countries which lack access to proper veterinary care and surveillance as well as compensatory policies for dealing with infected livestock. Additionally, endemic brucellosis represents a major economic destabilizer as the financial burden of infected animals affects the health, nutrition, and financial livelihood of many farmers in these regions (47).

The nonspecific presentation of brucellosis results in widespread misdiagnosis and underreporting of the disease, particularly in malaria-endemic areas where similarity in disease presentation results in infections often being ascribed to malaria (10). Delays in health-seeking behavior, variable performance

of serological tests and the lack of sufficient knowledge about the disease amongst health care staff in endemic areas also confounds diagnosis (10). Brucellosis in the United States is considered an imported disease with most cases arising from recent trips to Mexico (2, 8, 43, 48) ; however, several wild animal reservoirs remain in the United States and many regions around the world (38). Furthermore, brucellosis has been described as a re-emerging zoonosis and there are current social misconceptions about the nutritive quality of pasteurized milk (49-52). This has led to an increase in unpasteurized dairy consumption, potentially putting more individuals at risk for contracting zoonotic diseases (50-52).

***Brucella*-Induced Arthritis**

Joint inflammation in humans is observed upon infection with all the *Brucella* species that typically infect humans (53-55). Osteoarticular and musculoskeletal inflammation are the most common focal complications of human brucellosis and occur in 40-80% of *Brucella*-infected patients (56, 57). Arthritis is thought to arise from the hematogenous spread of *Brucella* to the joints, as viable brucellae can be found within the synovial fluid of infected patients (58, 59). Arthritis and osteoarticular complications can manifest as peripheral arthritis, sacroiliitis (inflammation of the lower spine), and spondylitis (inflammation of the vertebrae), which are most common in children, young adults and older adults, respectively (56, 57, 60, 61). Inflammation of the joint synovium is prevalent in infected individuals, with polymorphonuclear and mononuclear leukocytes found in and around synovial tissue (62). *Brucella*-induced arthritis is commonly treated with antibiotics; however, resolution of arthritis is often protracted and the disease can

relapse (57, 63). Untreated articular brucellosis can result in synovial rupture, bone destruction, cartilage degradation osteopenia and/or bursitis with the potential to cause permanent disability (61, 64-66). Additionally, articular brucellosis cases refractive to antibiotic treatment may require arthroscopic debridement and/or drainage of the joint (67). Bone erosion is often rapid and radiographic changes can be difficult to identify, thus individuals are typically diagnosed via radiological examination, MRI or bone scan, but may need surgery for proper diagnosis (68, 69).

Models of Brucellosis and *Brucella* Induced Arthritis

Mechanisms underlying arthritic responses induced by *Brucella* have been hindered by the lack of relevant mouse models, as wild-type mice infected with conventional doses (10^4 - 10^6 colony forming units [CFUs]) by systemic routes do not develop joint inflammation (58, 70). Although mice are the best studied model of brucellosis, they are naturally resistant to *Brucella* infection and develop disease differently than humans or ruminants. One study suggests that upon systemic infection, *B. melitensis* is the most virulent *Brucella* spp. in mice followed by *B. abortus* with *B. suis* remaining the least virulent (71). In contrast to human brucellosis, mice tend to develop a transient acute infection that peaks around 2-3 weeks. Mice will naturally resolve infection, but it may persist for over 6 months. (72, 73). Additionally, much of the sequelae observed in chronic human brucellosis has not been reported in WT murine infection models (73). Rats, guinea pigs and monkeys have also been used as laboratory research models of *Brucella* infection,

but to our knowledge do not develop arthritis (11). Furthermore, young cattle can develop arthritic complications upon administration of vaccine strain S19 directly into their joints (74). However, it is difficult to measure immune responses in cattle due to the limited availability of genetically similar animals, and logistical and financial complications inherent to working with cattle under BSL-3 conditions. Others have observed that WT BALB/c mice infected intraperitoneally (i.p.) with 1×10^7 CFUs of *B. melitensis* can develop articular brucellosis at 26 weeks post-infection. Studying immune responses in this model is challenging as arthritis development is sporadic, and it is difficult to experimentally modulate immune responses past 4 weeks post-infection (75). Additionally, detectable *Brucellae* were found in the bones and limbs of this model but obvious *Brucella* joint burdens were not evident, thus making it difficult to determine the effects of immune responses on *Brucella* clearance (75). Moreover, i.p. *Brucella* infection of IFN- γ -deficient mice results in arthritis of ankle joints as early as 15 days post-infection. It is suspected that IFN- γ deficiency allows for the hematogenous spread of *Brucella*, resulting in infection and subsequent inflammation of the joints (58).

Inflammation and the Inflammatory Response to *Brucella*

Inflammation is a natural process induced by the body's immune system in response to cellular damage or foreign antigen, and is a vital part of tissue homeostasis as it eliminates microbial pathogens, clears cellular debris and initiates tissue repair. During an inflammatory stimulus, such as *Brucella* infection, immune cells like neutrophils and monocytes are recruited to the site of the irritant

and secrete cytotoxic molecules. This is toxic to bacterial pathogens but also leads to tissue damage and release of damage associated molecular patterns (DAMPS) which can initiate increased recruitment of inflammatory infiltrates. Thus, inflammation has to be tightly regulated in order to prevent this vicious cycle of tissue debilitating immune cell recruitment (76). In the case of arthritis, inflammation is reoccurring leading to pathology of the joint.

The common attributes of inflammation are described as redness, warmth, pain, recruitment/activation of leukocytes, and localized leakage of plasma-protein-rich fluid into inflamed tissues (swelling) (77). Upon contact with an inflammatory stimulus, cytokines such as IL-1 and TNF- α are released by tissue resident cells surrounding the antigen. IL-1 and TNF- α signal through endothelial IL-1R and TNFR to induce rearrangement of actin and tubulin, allowing endothelial cells to open up gaps between tight junctions. This allows leakage of plasma proteins into the affected tissues. Furthermore, IL-1 and TNF- α production activate endothelial tissues to vasodilate and upregulate E-selectin and P-selectin (77). Upregulation of these molecules causes weak tethering of circulating leukocytes to the endothelium inducing a slow rolling motion and finally adhesion to endothelial integrins (78). Along with tissue activation, increased blood flow due to vasodilation culminates in leukocyte extravasation (78, 79). When the cell is stationary, it can migrate into the tissue when the proper signals are presented. This process not only aids in cell entry into the tissues but also helps activate inflammatory cells such as neutrophils (80).

As neutrophils adhere to the epithelial wall, migration into the tissue, or

diapedesis, is initiated through contact with neutrophil CXCR2 receptor ligands such as CXCL1 and CXCL2 (78, 79). Although circulating neutrophils are continuously present in the blood, the majority of neutrophils and neutrophil precursors are stored in the bone marrow. Thus, during an infection robust neutrophil egress from the bone marrow needs to occur. In addition to CXCR2 ligand release at or near the epithelium, these chemokines are also released into the blood and signal to the bone marrow to promote neutrophil maturation and release. Following passage through the epithelium, inflammatory cells such as neutrophils migrate to sites of infection or tissue damage guided by a chemokine gradient (78). Inflammatory cells can also follow other chemotactic gradients like those established by lipid mediators such as eicosanoids (81, 82).

Once in the tissue, bacteria can be eliminated or restricted by neutrophil phagocytosis, and or by trapping them in extruded DNA termed neutrophil extracellular traps (NETs) (83). Neutrophils can also elicit bactericidal activity by releasing reactive oxygen species (ROS), matrix metalloproteinase (MMPs) and granules such as myeloperoxidase, elastase, defensins, NO and β -glucuronidase. These proteins are typically released during neutrophil degranulation; however, lytic cell death, such as netosis, pyroptosis or necrosis results in robust granule and inflammatory protein release along with DAMPS. While in the tissue, neutrophils can also secrete additional chemokines and cytokines to amplify inflammatory cell recruitment (84, 85). Thus, it is critical for neutrophils to undergo apoptosis in order to turn off the inflammatory response and prevent pathology.

Monocyte migration is similar to neutrophils as chemotactic signals,

selectins and integrins promote their transmigration into infected, inflamed and/or damaged tissues. CCL2 is the most common chemokine involved in monocyte recruitment, consequently causing monocyte release from the bone marrow, diapedesis into affected tissues, and differentiation into macrophages (86). While in affected tissues macrophages, especially those primed with IFN- γ , phagocytose bacteria and make bactericidal molecules such as reactive nitrogen intermediates, ROS, and phagolysosomal enzymes (86-88). Macrophages are also strong producers of TNF- α , IL-6 and type I interferons and are thus inflammatory. Alternatively, macrophages also play an anti-inflammatory role as they are responsible for clearing dead and dying cells including apoptotic neutrophils (89). Macrophage efferocytosis of dead/dying cells not only reduces inflammatory DAMPs but leads to production of anti-inflammatory lipoxins, resolvins and protectins (90). Hence, inflammation is necessary for pathogen clearance but must be tightly regulated, as an endless cascade of inflammatory pathology can lead to disease such as rheumatoid arthritis (91).

While most pathogens are quickly identified and cleared by the innate immune system, *Brucella* evades extracellular immune recognition and lacks obvious pathogen associated molecular patterns (PAMPs). Therefore, an effective innate inflammatory response able to clear the pathogen is never mounted. Additionally, *Brucella* does not express proteins that directly harm host cells such as exotoxins, exoproteases or cytolytins; thus, pathology in most tissues is probably driven by a prolonged low-grade inflammatory response initiated by weak recognition of *Brucella* PAMPs (92). *Brucella* can however, induce robust

inflammatory responses in some tissues such as the placenta and the joints (93). Despite systemic studies and some investigations that involve the placenta, examination of tissue specific inflammatory responses to *Brucella* are rare. Considering different tissues respond more robustly to *Brucella* antigen, it is vital to understand how *Brucella spp.* evade and are recognized by the immune system and whether inflammation induced by these stimuli are helpful for the clearance of *Brucella* in various tissues. Understanding the immune response to *Brucella* could lead to new therapies or treatments for *Brucella*-induced arthritis and inflammatory disease in general.

Brucella's Evasive Intracellular Life Cycle

Brucella is a stealthy pathogen equipped with many mechanisms to evade recognition and clearance by the host's immune system. One of *Brucella's* most effective strategies of evasion begins by establishing itself in an intracellular niche. Host macrophages and monocytes typically phagocytose *Brucella* via zipper-like phagocytosis and through lipid rafts (94, 95). Over 90% of the brucellae are quickly killed by these macrophages, but some bacteria are able to resist killing and work to establish an intracellular niche capable of replication (96). *Brucella* can also invade non-phagocytic cells, such as B-cells and trophoblasts, by utilization of Rho GTPases (97) While in the cell's phagosome, *Brucella* expresses a VirB type IV secretion system (T4SS) that secretes important effector proteins that modulate the endocytic pathway. Expression of the T4SS is induced upon acidification of the phagosome; thus, rather than phagosomal fusion with the lysosome and

subsequent degradation of endosomal inhabitants, *Brucella* proteins reduce the lysosomal trafficking protein LAMP1 preventing phagolysosomal fusion (98, 99). After divergence from the phagolysosome fate, the phagosome containing *Brucella* traffics to the endoplasmic reticulum (ER), where it exchanges membrane components. This interaction makes the phagosomal membrane indistinguishable from the ER and other host cell organelles, allowing the development of a specialized compartment termed the *Brucella* containing vacuole capable of evading cytosolic detection. This vacuole then breaks free from the ER allowing safeguarded intracellular replication of the bacteria (99). Finally, *Brucella* sequesters glucose, lactate and other nutrients from the host cell in order to maintain its survival and replication (100, 101).

During chronic infection, *Brucella* preferentially replicates in alternatively activated macrophages (AAMs) which are cells typically involved in tissue repair. AAMs preferentially oxidize fatty acids as their energy source, allowing *Brucella* to exploit unutilized host cell glucose (100). *Brucella* may also replicate in classically activated macrophages (CAMs), or inflammatory macrophages which prefer glycolytic metabolism (100, 102, 103). However, during acute infection it is unknown if *Brucella* prefers cells undergoing glycolysis or oxidative phosphorylation. Inflammatory macrophages, or macrophages activated by IFN- γ , are required for *Brucella* clearance, but considering the bacterium may exploit these glycolytic cells to establish a niche of replication, macrophages and *Brucella* have a paradoxical relationship (104). Hence, it is vital that intracellular host

receptors recognize and mount an effective immune response in order to clear *Brucella*.

Immune Recognition of *Brucella*

Although *Brucella* evades the host immune system through intracellular invasion of phagocytes, pathogen recognition receptors (PRRs) are important initial responders to infection. PRRs recognize similar moieties on pathogens and therefore quickly signal and activate transcription factors to induce secretion of inflammatory cytokines, such as IL-1 and TNF- α . Toll-like receptors (TLRs) are some of the best studied PRRs, but there is discrepancy in the current literature as to what TLRs control *Brucella* infection (105). This may be due in part to *Brucella*'s ability to evade TLR activation by secreting proteins that block TLR signaling (106, 107). However, it is generally agreed that TLR2, TLR4, TLR6 and TLR9 can play an inflammatory role (108-112). TLR2 and TLR6 recognize *Brucella* lipoprotein (110), TLR4 can identify brucellar LPS (111) and TLR9 can recognize *Brucella* CpG DNA (109). These TLRs typically signal through the adaptor protein MyD88 in order to induce an inflammatory cytokine response (113). This robust inflammatory activity can be detrimental to the host as MyD88 signaling can result in arthritis and osteoclastogenesis in murine models of bacterium induced arthritis (114-116). However, it remains unknown if MyD88 or TLR signaling are involved in the induction of *Brucella*-induced arthritis.

Another important pathogen recognition system, especially for identifying intracellular pathogens, are inflammasomes. Inflammasomes use intracellular

PRRs such as AIM2 (absent in melanoma 2) and NLRP3 (NACHT, LRR and PYD domains-containing protein 3) to detect cytosolic threats. Upon recognition of pathogen associated molecular patterns (PAMP), or some DAMPS, the ASC (apoptosis-associated speck-like protein containing a CARD) adaptor protein is recruited and forms a complex known as the inflammasome. Inflammasomes proteolytically cleave the central protein caspase-1 into its active form. Caspase-1 then activates pro-inflammatory cytokines IL-1 β and IL-18 into their functional secreted structures, and can also induce a programmed form of lytic cell death called pyroptosis. Secretion of IL-1 β and IL-18 are known to contribute to systemic *Brucella* restriction, however the role of pyroptosis during brucellosis remains unknown (117). Inflammasomes can recognize intracellular *Brucella* and other vacuolar pathogens by utilizing host guanylate binding proteins (GBPs) (118). GBPs perturb the membrane of the pathogen containing vacuole therefore exposing antigen to the cytosol which may then be recognized by inflammasomes (119). Caspase-11, otherwise known as the non-canonical inflammasome, directly recognizes bacterial LPS in the host cytosol (120). Like caspase-1, caspase-11 can induce pyroptosis and subsequent DAMPS which can indirectly activate the canonical inflammasome (120, 121). Similar to *Brucella*'s weak stimulation of TLR4, *Brucella* LPS is not thought to strongly activate caspase-11 (122).

Considering MyD88 and caspase-11 can both act through Gram-negative LPS recognition, bacterial LPS is generally a strong activator of innate immune responses. However, due to *Brucella*'s unconventional LPS structure, it is considered a virulence factor that allows *Brucella* to evade immune recognition

(123, 124). Unlike enterobacteria which present a hexa-acylated lipid A and are therefore recognized by TLR4/MyD88 and caspase-11, *Brucella* expresses a hepta-acylated lipid A and contains unusual very long chain fatty acids (VLCFAs). Additionally, *Brucella* LPS has a core oligosaccharide which acts as a shield to block LPS lipid A identification (125). *Brucella* LPS also contains an O polysaccharide which results in smooth colony formation and reduced host cell death, allowing survival of the infected host cell reservoir (126).

Inflammatory Cytokines

Brucella induced arthritis is characterized by heavy recruitment of inflammatory cells to the joint and the musculoskeletal structures surrounding the articular space (62). Similarly, neutrophils are the most abundant cells in the synovial fluid in murine models of rheumatoid arthritis (127, 128). Upon *Brucella* infection, macrophages and resident cells such as osteoblasts can produce vigorous chemoattractants such as IL-6 which recruits T-cells, and CCL2 which recruits monocytes. Additionally, these cells can also manufacture CXCR2 ligands, such as CXCL1 and CXCL2 which stimulate migration of PMNs (66, 129-131). It is not known, however, if these cytokine/chemokines lead to increased pathogenesis in the joints of brucellosis patients. Unlike rheumatoid arthritis where autoantibodies and autoreactive T-cells mediate pro-inflammatory cell recruitment (132), our group previously demonstrated joint *Brucella* infection in IFN- γ deficient mice does not induce collagen auto reactivity. Rather, it is the host's immune response to *Brucella* that mediates focal inflammation (58). Although auto

reactivity to collagen was not observed, it is unknown if adaptive immune cells such as B and T cells play a role in *Brucella*-induced arthritis or if they are needed for mediating resolution.

Inflammasome activated IL-1 β and IL-18 can also induce cell mediated inflammation (120). IL-1 β is known to recruit and activate inflammatory macrophages and other inflammatory cells (133), while IL-18 stimulates cytotoxicity in NK cells and can prime T-cell IFN- γ production (134). IL-1 β and IL-18 are protective against systemic *Brucella* burdens but they may also elicit overwhelming joint inflammation, (117, 135) as they contribute to the development of rheumatoid arthritis (136-138). IL-1 β is elevated in the joints of brucellosis patients and can also induce inflammation in septic arthritis (66, 139). Additionally, IL-1 is partially responsible for joint inflammation in IFN- γ -deficient mice intraperitoneally infected with *Brucella*, but is also required for systemic *Brucella* clearance (58). It is currently unknown if IL-1 β and IL-18 induce *Brucella* clearance in the joints or if they mediate arthritis in WT infected mice.

IFN- γ is one of the best documented cytokines known to aid in the clearance of brucellosis (58, 140-142), as IFN- γ promotes macrophages production of ROS and nitric oxide (NO) both which work to eradicate intracellular *Brucella* (143). T-cells are thought to be the primary producers of IFN- γ during *Brucella* infection and are thus vital for the restriction of *Brucella* replication. Polymorphisms in IFN- γ are associated with an increased risk of contracting brucellosis and developing *Brucella*-induced arthritis (144-147), and T-cell exhaustion, seen during chronic brucellosis correlates with diminished IFN- γ production (148, 149). Similarly,

IFN- γ R^{-/-} and T-cell-deficient mice are individually unable to control systemic *Brucella* burdens (142). T-cell and/or NK cell induction of IFN- γ is often induced by the synergistic signaling of IL-12 and IL-18 through their respective receptors (150, 151); however, it is not known if this is the protective mechanism of IL-18 seen during systemic infection. Additionally, not much is known about the role of IFN- γ in the joint. Intraperitoneally infected IFN- γ -deficient mice develop arthritis but it is unclear if ablation of IFN- γ allows *Brucella* to access the joint, as it is protective in systemic infection, or if there is a local role for IFN- γ within joint tissues (58).

Apoptosis is typically the preferred mode of clearance of host cells infected with intracellular pathogens, as it can kill the infected host cell and the bacteria harbored inside while resulting in minimal damage to host tissues (152). Apoptosis can be a result of *Brucella* infected osteoblasts (152, 153); however, there are additional reports that *Brucella* infection enhances macrophage survival in order to maintain its intracellular niche (154). *In vitro* *Brucella* infection can also promote prolonged cell survival of synoviocytes, (155) a phenomenon also seen during rheumatoid arthritis (156). Although *Brucella* may inhibit host cell death to evade immune recognition, *Brucella* may need to induce host death in order to disseminate. Studies suggest some natural dissociation of *Brucella* LPS from smooth to rough occurs, promoting macrophage egress and allowing dissemination of smooth *Brucella* which did not dissociate (126, 157). Conflicting reports on *Brucella*'s induction of host cell survival indicates *Brucella* may only induce cell death in a tissue or cell specific manner and the nature of cell death induced by *Brucella* remains largely unexplored.

Purpose and Experimental Approach

Treatment of unresolved sterile arthritis such as rheumatoid arthritis or osteoarthritis involves eliminating or dampening host inflammatory responses. While inflammation may inflict severe articular pathology to the host during infectious arthritis, inflammation may also be vital for pathogen restriction. This creates a paradox when identifying appropriate treatments for brucellar arthritis as administration of anti-inflammatories may increase bacterial replication in the host. Thus, it is vital to characterize what inflammatory responses are helpful and harmful to the host. Thus, the focus of this research was to understand the inflammatory mediators inducing inflammation and arthritis, and to determine what inflammatory responses are needed in order to clear *Brucella* during brucellosis. We describe experimental *Brucella*-induced arthritis as an effective tool in understanding both adaptive and innate inflammatory responses to *Brucella* as swelling correlates with inflammation and can be monitored in longitudinal studies.

CHAPTER II

Materials and Methods

Bacterial strains and growth conditions

All experiments with live *Brucella* were performed in biosafety level 3 (BSL-3) facilities. *B. melitensis* 16M and *B. abortus* 2308, obtained from Montana State University (Bozeman, Montana), and *B. suis* 1330, obtained from BEI Resources (Manassas, VA), were grown for 3 days at 37°C/5%CO₂ on *Brucella* agar (Ba) (Becton Dickinson). Colonies were picked from Ba plates, and strains were cultured in *Brucella* broth (Becton Dickinson) overnight at 37°C with shaking. The overnight *Brucella* concentration was estimated by measuring the OD at 600 nm, and the inoculum was diluted to the appropriate concentration in sterile phosphate-buffered saline (sPBS). Actual viable titer was confirmed by serial dilution of the *Brucella* inoculum onto Ba plates.

Deletion and complementation of the *B. melitensis* L-lactate dehydrogenase gene (*lldD*)

The *lldD* gene in *B. melitensis* 16M (BMEI10377) was mutated using a nonpolar, unmarked gene excision strategy. Briefly, a 1250 bp gBlock fragment was synthesized by IDTDNA containing from 5'-3': ~600 bp upstream of *lldD*, then the first and last three codons of *lldD*, followed by ~600 bp downstream of *lldD* (Table 2). In addition, the 5' end of the gBlock fragment contained 30 bp

homologous to 30 bp upstream of the BamHI site in pNTPS139 and the 3' end of the gBlock contained 30 bp homologous to the 30 bp downstream of the Sall site of pNTPS139. The suicide plasmid pNTPS139 (158) was digested with BamHI and Sall. The gBlock fragment and digested pNTPS139 were then ligated using the NEB Hi-Fi DNA Assembly Kit according to manufacturer's instructions (New England Biolabs). This plasmid was then introduced into *B. melitensis* 16M, and merodiploid transformants were obtained by selection on *Brucella* agar plus 25 µg/ml kanamycin. A single kanamycin-resistant clone was grown overnight in *Brucella* broth and then plated onto *Brucella* agar containing 6% sucrose. Genomic DNA from sucrose-resistant, kanamycin-sensitive colonies was isolated and screened by PCR using primers (*lldD* screen forward, *lldD* screen reverse) in Table 3 for loss of the *lldD* gene. Genetic complementation of the *lldD* mutation was performed by expressing wild-type *lldD* from its native promoter in pBBR1MCS-2 (159). The *lldD* gene, along with the native promoter, was amplified from *B. melitensis* 16M genomic DNA by PCR using primers (*lldD* clone forward and *lldD* clone reverse) in Table 3. These primers also contained overlapping sequences to the SacI and XhoI sites of pBBR1MCS-2. The resulting PCR fragment was then ligated with SacI/XhoI digested pBBR1MCS-2 via the NEB Hi-Fi Assembly kit. This construct was sequenced and then introduced into *B. melitensis* $\Delta lldD$ by conjugation.

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GCGCCAGAAAGCTTCCTGCAGGATATCGTGCTTCATATTTGTCCGTTGCCGTGTTGTAGCGCATGGG
ATGGGTAAAGCGCCCCGTATTTTCCAGATCGTAATCCGTCCATTCCCGAAGCTTGGAAACCGTGTGT
TCGCGAAAAAAGGCAGGCGTAGTGCGCCGGTCCGTTGCCTCCAGGCAACGGCCTTGACGCCGTT
TTCACAAAATTCAAAGACGAGCCATGTTCCGGATCACCCCATGCACAACCGGGACAATCGAACCCA
TCCGGCTGATTGGCTTTCAAAGCGCACGGGCGCCAGCCAGCGGTGCACCGCTGGCCAGCAATTG
CTGGCCACAGCTTTTCAGCGCACCCCATCCGCCTGCCGCGCTGGAACGTTTGCCGATAAAACCGGT
TTTTTACCCTTGCTCATCTCTTCATCCTGTAAGCACAAATGCCTGGCCGCCATGACCTGCCAGCAAAT
TGTGCCGGGGCGGGAGCCAATAGCATAAGAAGAAAAGTGCACAGCTTCAATTAACCGCGAGCCATT
TGTTATCGAATTGCTTGTTGGGACTTGATTTCAAGCGCGCGATCCGTCATTGATTGGTAAAGAAAAC
TGACCACAAAGGGTGGGATGATGTCCGATTTCTGATCTATCCACCAACAGATATAAGGCCCCGATAAT
AGTCGGGCTTATTCCAGCTTTTAAATAAATATCTTGGTTTATTATGCCTGCGACTGAGTTAACACCA
GAAGCCGCGCAGCGATTAAGCTTTTTCAGGGCATAATACGCTAGCATCTTGGCAACTCTAACATAAT
GAAATTTTAAGTTAACACTTATATAAAATAACCAATATTTAAGCATATCAGTTTACATCTCCGACCGAG
CGGTATGAAAACCGTATATTTTTTGGGCTGGAGTGAAACACATGAAACGCGGTTGCGCCATTGCGG
TCATGATATGCGGTTTAATCACAAGCGTGAGCGCGGCATCAGCCGCTGACCTCATAGTTCAGGAACC
CGTTTTTGAGCCTTTACCCAGCCAGCCCTTGCGGGCTGGTATCTCCGGGGCGATATCGGCTACAA
TTTTAAATCGAAAACCGGCGGCAAGTGGGATTTCTGGAACCAAGTTGAAGAACCCTATCGCGGGGTT
GACGACACCTTCAACTATGATGATTTCTCGCTGAAAGGCGGAGCCTCCTATGGGGTCGGCGTCGGC
TATCGCTTCAACGACATCCCACTAGTGAGTCGTATTACGTAGCTTGG

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Table 2. gBlock construct for deletion of *lldD*.

Underlined regions denote homology to pNPTS139. Underlined/bold region denotes first 3 codons of *lldD*. Bold/italics region denotes last 3 codons of *lldD*.

Primer name	5'-3' sequence	Purpose
GAPDH-F	GTG GAC CTC ATG GCC TAC AT	qRT-PCR
GAPDH-R	GGG TGC AGC GAA CTT TAT TG	qRT-PCR
iNOS-F	TCC TGG ACA TTA CGA CCC CT	qRT-PCR
iNOS-R	CTC TGA GGG CTG ACA CAA GG	qRT-PCR
<i>lldD</i> screen-F	ATT TGT CCG TTG CCG TGT TG	Confirming deletion of <i>lldD</i>
<i>lldD</i> screen-R	TCC GCC TTT CAG CGA GAA AT	Confirming deletion of <i>lldD</i>
<i>lldD</i> clone-F*	<u>GGG CGA ATT GGA GCT</u> CAA ATT GTG CCG GGG CGG GAG CC	Complementation of <i>lldD</i>
<i>lldD</i> clone-R*	GTA CCG GGC CCC CCC <u>GAT</u> CAG AAA TCG ATC GAA TGG	Complementation of <i>lldD</i>

Table 3. Primers used in this study.

*Underlined regions denote homology to pBBR1MCS-2

Mouse strains

Experiments were conducted using 6- to 12-week-old age- and sex-matched mice on a C57BL/6 or BALB/c background. Breeding pairs of MyD88^{-/-}, caspase-1/11^{-/-}, caspase-11^{-/-}, IL-1R^{-/-}, IL-18^{-/-}, NLRP3^{-/-} and AIM2^{-/-} mice (on C57BL/6 backgrounds) and CXCR2^{-/-} mice (on a BALB/c background) were obtained from the Jackson Laboratory (Bar Harbor, ME). Breeding pairs of IFN-γ^{-/-} and heterozygous CXCR2^{+/-} mice (on C57BL/6 backgrounds) were obtained from Jackson Laboratory. Heterozygous CXCR2^{+/-} mice were intercrossed with IFN-γ^{-/-} mice to obtain IFN-γ^{-/-}/CXCR2^{-/-} mice. Breeding pairs of IL-1R/IL-18^{-/-} mice were gifted by Dr. Edward Miao (University of North Carolina, Chapel Hill, NC). Rag1^{-/-}, μMT^{-/-}, CCR2^{-/-}, Perf^{-/-} and Nos2^{-/-} mice on a C57BL/6 background were also purchased from Jackson Laboratory.

Mouse infections

In some experiments mice were infected i.p. with 200 μl of ~1x10⁵ CFUs of *B. melitensis* 16M in sPBS. In other studies mice were infected in both rear footpads with a volume of 50 μl containing 1x10³, 1x10⁴, 1x10⁵ or 1x10⁶ CFU of *B. melitensis*, *B. abortus* or *B. suis* (160). After infection, all mice were maintained in individually ventilated cages under high-efficiency particulate air-filtered barrier conditions of 12 h of light and 12 h of darkness in animal BSL3 facilities and were provided with sterile food and water. All studies were conducted in accordance with University of Missouri Animal Care and Use Committee guidelines.

Antibody neutralizations/ treatments

In order to neutralize IFN- γ , in i.p. infected mice, mice were treated with anti-IFN- γ antibody (clone XMG1.2, BioXCell) via intraperitoneal administration of 0.25 mg 1 day before infection and 3 times per week thereafter. Mice neutralized of IFN- γ during footpad infections were treated with anti-IFN- γ antibody (clone XMG1.2, BioXCell) via intraperitoneal administration of 0.5 mg 1 day prior and 3 days after *Brucella* infection. To neutralize IL-18 mice were administered 2.5 mg of anti-IL-18 antibody (clone YIGIF74-IG7, BioXCell) into the peritoneum one day prior to infection. NK cells and $\gamma\delta$ T-cells were neutralized via i.p. administration of 0.5 mg of anti-NK-cell antibody (clone PK136, BioXCell) or anti- $\gamma\delta$ T-cell antibody (clone UC7-13D5, BioXCell) respectively, 1 day prior and 3 days after *Brucella* infection. Control mice for antibody depletion experiments received Rat IgG (Southern Biotech) as an isotype control. For eicosanoid experiments mice received intraperitoneal delivery of 2 mg/kg indomethacin (Sigma) on day -1 and daily thereafter. To inhibit glycolysis *in vivo* mice were administered 100 mg/kg of 2-deoxyglucose i.p. (2-dG) on days 1-6 post-infection. For *in vivo* nitric oxide synthase inhibition, mice were given drinking water containing 2.5% aminoguanidine hemisulfate salt (AGHS, Sigma) (161) 14 days prior to infection and throughout the experimental time course.

Bacterial burdens

At various times after infection, mice were euthanized, and spleens and mouse paws (following skin removal) were harvested. Spleens and paws were

homogenized mechanically in sPBS (160). A series of 10-fold serial dilutions were performed in triplicate in sPBS and plated onto Ba. Plates were incubated 3-4 days at 37°C, colonies were enumerated, and the number of CFUs/tissue was calculated.

Macrophage generation and infections

Bone marrow derived macrophages (BMDMs) were derived by flushing the bone marrow from femurs and tibias of C57BL/6 mice with sPBS. Isolated bone marrow was then cultured in complete media (CM; RPMI 1640, 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 10 mM non-essential amino acids, 10 mM sodium pyruvate) containing 30 ng/ml recombinant murine M-CSF (Shenandoah). After 3-4 days of culture fresh CM containing 30 ng/ml M-CSF was added to culture flasks. Adherent cells were then collected at 6-7 days post-bone marrow harvest by adding 0.05% Trypsin (Life Technologies). Cells were plated at 1×10^6 cell/ml in fresh CM and left to adhere overnight. Cells were infected with a multiplicity of infection (MOI) of 100 *B. melitensis* 16M. Cells were infected for 6 hours, washed with sPBS, CM containing 50 µg/ml gentamicin was added, and cells were incubated for 30 mins. Cells were then washed with sPBS and left to incubate in CM containing 2.5 µg/ml of gentamicin for the remainder of the experiment. In some experiments 2-dG or SNAP was added as indicated. 10% FBS was used for all studies unless cell death was assayed or western blots were performed (Fig 23A-D, Fig 30G, and Fig 24C), in which case 2% FBS was used. Supernatants were harvested and intracellular *Brucella* in lysates were enumerated at 24 or 48

hours post-infection. Supernatants were used for L-Lactate quantification as described below. Measurements of IL-1 β were obtained using a Mouse IL-1 β ELISA Ready Set Go kit according to the manufacturer's instructions (Invitrogen) and LDH release was measured utilizing the Cytotoxicity Detection Kit plus (LDH) adhering to the vendor's protocols (Roche). Additionally, cell lysates and supernatants were used for western blots. To determine bacterial burdens BMDMs were washed with sPBS, lysed in ddH₂O and CFUs enumerated by serial dilutions onto Ba. For competitive index experiments, both Δ *lldD* and the *lldD* complemented strain (containing kanamycin resistance (KanR)) were combined in a 1:1 ratio and titered at the time of infection by plating inoculum on regular Ba and Ba containing 25 μ g/ml kanamycin. Phagocytosis was determined by plating cell lysate collected at 0 hr post-infection on regular Ba and Ba with kanamycin. Cell lysates were also collected at 48 hours post-infection, and plated onto regular Ba and Ba with kanamycin then CFUs were enumerated. The final competitive index was calculated by comparing the relative ratio of the two strains at 48 hrs post-infection relative to their phagocytosis.

Lactic acid quantification

In order to remove cell debris BMDM supernatants were centrifuged at 13,000 X g for 10 min. Supernatants were then deproteinated using a Vivaspin 500 10K MWCO filter (Fisher) according to the manufacturer's instructions. L-Lactate was measured using the L-Lactate kit (Eton Bio) according to the manufacturer's instructions.

LPS purification and transfection

LPS was purified from the *B. abortus* S19 vaccine strain (obtained from the University of Wyoming) using hot-phenol extraction (162). Briefly, S19 was suspended in deionized water at 66°C and then combined with 90% phenol (Sigma), preheated to 66°C. The suspension was stirred for 20 minutes, followed by cooling in an ice bath before centrifugation. The phenol layer was aspirated using a 16 gauge cannula and cellular debris removed via filtration using a Whatman #1 filter. The LPS was then precipitated via addition of methanol (Sigma) containing 1 % methanol saturated with sodium acetate (Sigma) and allowed to incubate for 2 hr at 4°C before centrifugation. The precipitate was stirred with deionized water for 18 hours at 4°C. Following incubation, the LPS was centrifuged, the supernatant reserved and maintained at 4°C, while the precipitate was resuspended in deionized water and stirred for 2 hours at 4°C. The supernatant solution was recovered as above, and pooled with the reserved supernatant sample. Trichloroacetic acid (sigma) was added to the supernatant containing the crude LPS, stirred for 10 minutes, and the precipitate removed via centrifugation. The supernatant solutions were dialyzed against deionized water and then freeze dried.

Peritoneal macrophages were isolated by injecting mice with 1 ml of aged thioglycolate (163). 4 days post-injection peritoneal macrophages were isolated by washing the peritoneal cavity with 10 mls of sPBS. Macrophages were resuspended in CM and cultured at 1×10^6 cells/ml and primed overnight with 1 µg/ml Poly I:C (Invivogen). Cells were washed and then cultured in Opti-MEM

reduced serum media (Thermo-Fisher). Purified *Brucella* LPS was packaged in Eugene 6 (Promega) at room temperature for 15 minutes according to manufacturer's instructions. Wells receiving 10 µg/ml LPS received a final concentration of 0.25% (v/v) Eugene 6, while wells receiving 100 µg/ml LPS received a final concentration of 2.5% Eugene. Cells were incubated for 9 hours at which time LDH release was measured as described above.

Immunoblots

Supernatants and BMDMs were evaluated at 24 hours post-infection. BMDMs were lysed in RIPA buffer (Thermo), harvested, and total protein normalized using BCA Protein Assay Kit according to the manufacturer's instructions (Thermo). Caspase-1 westerns were run on 15% acrylamide gels. Blots were probed with anti-Caspase-1 (p20) (casper-1) (Adipogen) diluted 1:500 and then with Peroxidase-conjugate Goat Anti-Mouse IgG (Jackson Immuno Research) diluted 1:5000. Detection was performed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo).

Extraction of RNA and RT-PCR analysis of joint homogenates

Joints were homogenized in TRI reagent (Sigma). 100 µl of chloroform (Sigma) was added to 500 µl of TRI reagent, incubated for 10 mins, and centrifuged at 15000 RPM for 15 mins at 4°C (163). The top RNA containing layer was harvested and mixed with 700 µl of isopropanol (Acros organics). RNA was further purified with an RNeasy Mini Kit (Qiagen). cDNA was generated using the

superscript III First Strand Synthesis System (Invitrogen) using oligodT primers. Primers for iNOS and GAPDH were designed using NCBI Primer Blast. Primer sequences are included in Table 3. Relative specific mRNA was quantified by measuring SYBR green incorporation during real-time quantitative reverse transcription-PCR (qRT-PCR) (performed in duplicate for each sample) using the comparative threshold method (164).

Assessment of pathology

Ankle swelling was evaluated in relation to basal joint measurements made prior to infection as have described (160). Ankle swelling was measured at various time points by collective measurements of both tibiotarsal joints. The difference of the recorded measurement from basal was reported as mean joint swelling. Histology was performed on both mouse ankles, in which skin was removed prior, and on mouse tails. Tissues were fixed in 10% buffered zinc formalin, decalcified in 15% formic acid, rinsed in tap water, dehydrated with an alcohol gradient and clearing agent then embedded in paraffin. Tissue sections (5 μ m) were mounted on glass slides, and serial sections were stained with hematoxylin and eosin (H&E) and covered with a coverslip with aqueous mounting medium. Tissues were evaluated for inflammation (neutrophils, macrophages, lymphocytes) which involved multiple tissues (synovial membrane, bone, tendons, skeletal muscle). Two sections at different levels of each ankle joint and associated structures were evaluated for each mouse by histopathology and scored for lesion severity (inflammation). The following scale was used: 0 = none (no inflammation); 1 =

minimal with inflammation involving < 5% of tissue; 2= moderate with focally extensive areas of inflammation (5% to 25% of tissue and involving 1 or more tissues); 3 = moderate to severe with focally extensive areas of inflammation (>25% to 50% of tissue and involving multiple tissues and mild distention of tissues associated with joint); 4 = severe with large confluent areas of inflammation (> 50% of tissue and involving all tissues and severe distention of tissues associated with joint) (160). For enzyme immunohistochemical (IHC) staining, mouse tail tissue sections (5µm) were mounted on Plus Charged glass slides and sections were deparaffinized using appropriate buffer and rinsing between each immunostaining step. Samples were rehydrated in xylene, absolute alcohol, 95% alcohol, and rinsed with distilled water. Endogenous peroxidase was blocked with 3% H₂O₂, 5% BSA and slides were incubated with 4.7 µg/ml of rat anti-Ly-6G antibody (clone 1A8, BioXCell) for 60 minutes. Slides were then incubated with secondary biotinylated donkey-anti-rat antibody, followed with incubation in streptavidin-HRP (Dako). Slides were developed with diaminobenzidine (DAB+, Dako) and lightly counterstained with hematoxylin 1 and coverslipped with aqueous mounting media. Three sections of tail at different tissue levels (bone and associated structures) were evaluated and scored for each mouse by a histopathologist. For neutrophil infiltration (IHC), the following scoring system was used (labeling of neutrophils in normal bone marrow was excluded from evaluation): 0 = none (no labeled cells); 1 = minimal numbers of labeled cells; 2 = moderate numbers of labeled cells; 3 = large numbers of labeled cells and skeletal muscle along with periarticular inflammation.

Flow cytometry

Rear legs were de-gloved, dislocated at the hip, muscle trimmed, and put into a sterile 4-mL PBS solution containing 125 U/mL collagenase (Sigma), 2 μ L/mL of DNase (1 U/mL; Thermo Scientific), and 4% fetal bovine serum (FBS; Sigma) and incubated for 1 hour at 37°C with shaking. Joint slurry was put into sterile dishes, stripped of surrounding tissue, strained through an 80- μ m mesh, and washed with complete medium. Cells were re-suspended in RPMI 1640 medium with 0.1 mM HEPES, and red blood cells were lysed with lysis buffer (final concentration, 0.15 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM ethylenediaminetetraacetic acid) (70). Cells were then washed and stained in fluorescence-activated cell-sorting (FACS) buffer (PBS and 2% FBS). Immunofluorescence staining was performed for 30 minutes at 4°C, using a combination of the following fluorochrome-labeled monoclonal antibodies from eBioscience: F4/80 (BM8), Ly-6G (1A8), CD11b (M1/70) and CD45.2 (104), CD4 (GK1.5), CD8a (2.43), and B220 (RA3-6B2). Stained cells were then washed with FACS buffer and fixed overnight in 4% paraformaldehyde at 4°C. After fixation TUNEL staining was conducted on some samples using the Click-iT plus TUNEL assay kit (Life technologies) according to the manufacturer's instructions then reconstituted in FACS buffer. Fluorescence was acquired on a CyAn ADP Analyzer (Beckman Coulter), and FlowJo (Tree Star) software was used for analysis. The following gates were used to detect the following cell types: Macrophages $\text{CD45.2}^+/\text{CD11b}^+/\text{F4/80}^+$, neutrophils $\text{CD45.2}^+/\text{CD11b}^+/\text{F4/80}^+$, CD4

T-cells CD45.2⁺/CD8⁻/CD4⁺, CD8 T-cells CD45.2⁺/CD4⁻/CD8⁺, B-cells CD45.2⁺/B220⁺.

Spleens were processed for intracellular flow cytometry by homogenizing splenic tissue in 900 µl of sterile PBS. Tissue homogenates were strained through an 80 µm mesh and washed with complete medium. Cells were resuspended in RPMI medium with 0.1 mM HEPES, and red blood cells were lysed. Spleen cells were then washed and resuspended in CM and cultured for 4 hours at 37°C/5%CO₂ with a cell stimulation cocktail containing phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and monensin (Ebioscience). Cells were then stained for surface markers (anti-CD4, clone GK1.5 anti-CD8, clone 2.43) and fixed in paraformaldehyde. Cells were permeabilized with 0.2% saponin prior to intracellular staining for IFN-γ (clone XMG1.2). Fluorescence was acquired on a CyAn ADP Analyzer (Beckman Coulter), and FlowJo (Tree Star) software was used for analysis

Ankle processing for cytokine measurements

Following skin removal, ankles from each mouse were excised, combined, and then homogenized *in toto* in 3 to 4 ml of sPBS treated with Halt protease inhibitor cocktail (Thermo Scientific) (160). Homogenized tissues were then centrifuged at 2,000 g for 5 min, supernatants were filter sterilized (0.2 µm pore) and stored at -70°C until analysis using a Luminex MagPix instrument with Milliplex magnetic reagents according to the manufacturer's instructions (Millipore). Luminex data were analyzed with Milliplex Analyst software (Millipore) and

normalized to total protein levels determined by a bicinchoninic acid (BCA) protein assay (Thermo Scientific).

Statistical analysis

Focal inflammation incidence was analyzed by log-rank analysis on incidence curves. Statistical analysis of the difference between 2 mean values was conducted using a two-tailed Student t test with significance set at $P \leq .05$, while comparisons of ≥ 3 mean values was done using ANOVA, followed by Tukey's test (when all means were compared to each other) or Dunnett's test (when samples means were only compared with a control group) as described in the Figure Legends with significance set at $P \leq .05$. The statistical significance of differences in histology scores was determined by a Mann–Whitney U test for 2 groups, or an ANOVA on ranks for ≥ 3 groups.

CHAPTER III

CXCR2 Mediates *Brucella*-Induced Arthritis in IFN- γ - Deficient Mice

ABSTRACT

Brucella spp. are facultative intracellular gram negative bacteria that cause brucellosis, a common global zoonosis. Infection of the joints is the most common focal complication of brucellosis in humans. The purpose of this study was to identify mediators of focal inflammation during brucellosis. Wild-type (WT) mice are naturally resistant to *Brucella* infection; therefore, we infected anti-IFN- γ -treated, or IFN- $\gamma^{-/-}$ mice with *Brucella* to induce osteoarticular and musculoskeletal inflammation as has been previously described. Mice were infected intraperitoneally with *B. melitensis* and the clinical course of disease, histopathologic changes, and cytokine levels were compared among groups. Rag1 $^{-/-}$ mice (B and T cell deficient) and μ MT $^{-/-}$ mice (B cell deficient) developed paw inflammation at a similar rate and severity as wild-type mice following infection with *B. melitensis* and treatment with anti-IFN- γ . Joints from *B. melitensis*-infected IFN- $\gamma^{-/-}$ mice had markedly increased levels of CCR2 and CXCR2 ligands. While anti-IFN- γ -treated CCR2 $^{-/-}$ and WT mice behaved similarly, anti-IFN- γ -treated CXCR2 $^{-/-}$ or IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice had strikingly reduced focal swelling relative to anti-IFN- γ -treated WT or IFN- $\gamma^{-/-}$ mice, respectively. Additionally, neutrophil recruitment was dependent on CXCR2. Collectively these data demonstrate adaptive immune cells and CCR2 are dispensable, while CXCR2 is necessary for *Brucella*-induced focal neutrophil recruitment and inflammation.

INTRODUCTION

Investigation of articular brucellosis has been hindered by the lack of relevant models, as wild-type mice infected with conventional doses of *Brucella* do not develop disease in the same way as humans or ruminants (165, 166). Previous work, has shown that genetic or antibody-mediated ablation of IFN- γ in mice results in joint colonization and development of arthritis within two to four weeks after intranasal or i.p. infection with $\sim 2 \times 10^4$ CFUs *B. melitensis* or *B. abortus* (165). While humans have an intact IFN- γ gene, a single nucleotide polymorphism (+874A/A) that potentially subverts optimal NF- κ B binding to the IFN- γ gene and correlates with diminished IFN- γ expression was found to be a brucellosis risk factor (141, 145-147). In addition, another IFN- γ polymorphism (5644A) associated with low IFN- γ production was found to be overrepresented in patients with focal complications of brucellosis (144). Human patients with chronic brucellosis also have an impaired IFN- γ response to *Brucella* antigen relative to patients at the onset of disease (148). Thus, suboptimal IFN- γ responses may predispose both humans and mice to focal brucellosis.

It has been reported that neutrophils are the most abundant cells in the synovial fluid of mice with experimental rheumatoid arthritis (127, 128). Examination of synovial fluid or tissue from brucellosis patients reveals a mixture of polymorphonuclear (PMN) and mononuclear leukocyte infiltrates (29, 57, 67, 167). Neutrophil activation and recruitment to the joints is stimulated by pro-inflammatory cytokine/chemokine secretion into the synovium, consequentially

leading to neutrophil release of granule contents, reactive oxygen species and other pro-inflammatory products that may drive inflammation (127).

Little information exists on the composition of cytokines/chemokines in the synovial fluid of human brucellosis patients. Previously, our group found that IL-1R^{-/-} mice have reduced musculoskeletal inflammation when infected with *Brucella* and depleted of IFN- γ (165). In addition, elevated levels of IL-1 β , CCL2 and CXCR2 ligands have been found in the synovial bursa of a human brucellosis patient with osteoarticular complications(66). In order to characterize the induction of *Brucella*-induced focal inflammation, we investigated the role of adaptive immunity and chemokine production. Here, we show that adaptive immune cells are dispensable, but CXCR2 is critical for *Brucella* induced-arthritis.

RESULTS

Inflamed *B. melitensis*-infected joints have increased levels of neutrophils

To characterize infiltrating cells, flow cytometry was performed on joints from naïve and *B. melitensis*-infected mice. A marked increase in hematopoietic cells (CD45.2⁺), and in particular neutrophils, (CD45.2⁺/Ly-6G^{hi}) was observed in *Brucella*-infected IFN- γ ^{-/-} mice (Fig 1A-B) relative to infected wild-type mice. To a lesser extent there was a slight, but statistically significant, increase in CD4⁺ T cells in *Brucella*-infected WT and IFN- γ ^{-/-} mice relative to naïve controls (Fig 1B). A significant increase in the proportion of CD45.2⁺/CD11b⁺/Ly6-G⁻/F4/80⁻ cells was also seen in infected IFN- γ ^{-/-} mice, relative to infected wild-type animals, which may indicate an influx of monocytes (data not shown).

Figure 1. T and B cells are not required for *Brucella*-induced focal inflammation.

Flow cytometry was performed on joints from naïve C57BL/6 and IFN- $\gamma^{-/-}$ mice or from C57BL/6 and IFN- $\gamma^{-/-}$ mice 28 days after i.p. infection with $\sim 1 \times 10^5$ CFUs of *Brucella melitensis* 16M (A-B) (n=4-5 mice/group). Within a cell type, means with the same letter are not significantly different from each other P<0.05. Error bars depict SEM. (C-D) Wild-type, μ MT $^{-/-}$ and Rag1 $^{-/-}$ mice (all on a C57BL/6 background) were infected intraperitoneally (i.p.) with $\sim 1 \times 10^5$ CFUs of *Brucella melitensis* 16M and treated with anti-IFN- γ (n=5-10 mice/group). Incidence of paw swelling (C) and average clinical scores (D) were recorded over time. Error bars depict SEM.

T and B cells are not required for Brucella-induced focal inflammation

To investigate a role for adaptive immune cells, wild-type, $\mu\text{MT}^{-/-}$ (B cell deficient), and $\text{Rag1}^{-/-}$ mice (B and T cell deficient) were infected i.p. with $\sim 1 \times 10^5$ CFUs of *B. melitensis* and treated with anti-IFN- γ antibody as described in Methods. To assess inflammation, mice were monitored for swelling and redness at the tibiotarsal junction. Anti-IFN- γ -treated wild-type, $\mu\text{MT}^{-/-}$, and $\text{Rag1}^{-/-}$ mice displayed similar incidence, degree of paw swelling and clinical scores (Fig 1C&D). *Brucella* joint tissue loads and neutrophil infiltration, as measured by flow cytometry, were also similar between WT and $\mu\text{MT}^{-/-}$ mice at day 28 post-infection (data not shown). Tissue bacterial burdens and joint neutrophil infiltration was not measured in $\text{Rag1}^{-/-}$ mice as 80% of animals succumbed to infection by day 28 (data not shown).

CCR2 and CXCR2 ligands are up-regulated in inflamed, B. melitensis-infected joints

To determine cytokines/chemokines that were induced during infection, paw homogenates from *B. melitensis* infected wild-type and IFN- $\gamma^{-/-}$ mice were assessed for T-cell-associated cytokines, inflammatory cytokines and chemokines by Luminex at day 23 and 30 post-infection (Fig 2A-C). In infected wild-type mice, IL-13 and IL-2 were the only cytokines enhanced relative to infected IFN- $\gamma^{-/-}$ animals (Fig 2A). Marked increases in the inflammatory cytokines, IL-1 β , IL-6 and TNF- α were seen in infected IFN- $\gamma^{-/-}$ mice at days 23 and 30 post-infection (Fig 2B). Additionally, the chemokines CXCL2, CCL2, and CCL3 were also significantly

Figure 2. CCR2 and CXCR2 ligands are up-regulated in inflamed, *B. melitensis*-infected joints.

Joint homogenates (n=5 mice/group) from wild-type and IFN- $\gamma^{-/-}$ mice infected i.p. with $\sim 1 \times 10^5$ CFUs *B. melitensis* 16M were assayed for T-cell-associated cytokines (A) inflammatory cytokines (B) and chemokines (C) by Luminex at 23 and 30 days post-infection and normalized by BCA. *P<0.05 as compared to WT mice. Error bars depicted SEM.

increased on day 23 and 30 in IFN- γ ^{-/-} versus WT mice, while CXCL1 was up-regulated at day 30 post-infection (Fig 2C).

CCR2 is not a critical mediator of *Brucella*-induced focal inflammation

CCR2 is the primary receptor for CCL2 signaling and recruitment of inflammatory monocytes (168). CCL2 was elevated in joint homogenates from infected IFN- γ ^{-/-} mice; therefore, we examined the role of CCR2 in *Brucella*-induced inflammation. Wild-type and CCR2^{-/-} mice (C57BL/6 background) were treated with anti-IFN- γ , infected i.p. with *B. melitensis* and monitored over time. Incidence, clinical scores, and swelling were similar in wild-type and CCR2^{-/-} mice Fig 3A-C). Additionally, tissue bacterial loads were comparable in CCR2^{-/-} and wild-type tissues (Fig 3D).

CXCR2^{-/-} mice display reduced clinical signs of *Brucella*-induced inflammation

CXCL1 and CXCL2 were elevated in *Brucella*-infected joints and are both CXCR2 ligands that can recruit neutrophils to the site of infection (169). To elucidate the role of CXCR2 in *Brucella*-induced inflammation, wild-type BALB/c and CXCR2^{-/-} mice (BALB/c background) were treated with anti-IFN- γ and infected i.p. with $\sim 1 \times 10^5$ CFUs of *B. melitensis*. CXCR2^{-/-} mice displayed delayed incidence, reduced clinical scores and swelling compared to wild-type mice (Fig 4A-C). CXCR2^{-/-} mice did lose more weight than wild-type animals (data not shown), which may explain some of the decreased paw thickness in these mice (Fig 4C).

Figure 3. CCR2 is not a critical mediator of *Brucella*-induced focal inflammation.

Wild-type and CCR2^{-/-} mice were infected i.p. with $\sim 1 \times 10^5$ CFUs of *B. melitensis* 16M and treated with anti-IFN- γ (n=5-10 animals/group). Incidence of paw swelling (A), clinical scores (B), and mean swelling (C) were recorded over time. On day 28 post-infection mice were sacrificed and *Brucella* CFUs were enumerated (D). Error bars depict SEM.

Figure 4. Reduced focal inflammation in *Brucella*-infected, anti-IFN- γ -treated, CXCR2^{-/-} mice.

Wild-type and CXCR2^{-/-} mice (both on a BALB/c background) were infected i.p. with $\sim 1 \times 10^5$ CFUs of *B. melitensis* 16M and treated with anti-IFN- γ . Incidence of paw swelling (A), clinical scores (B) and mean swelling (C) were recorded over time. On days 9, 21 and 28 post-infection *Brucella* colonization was determined (D). *P<0.05 as compared to WT mice. Error bars depict SEM. Data in A-C is from 5-10 mice/group and is representative of three independent experiments. CFU data in D) was obtained from 3-9 mice/group. Error bars depict SEM.

Bacterial loads in the spleen and joints of wild-type and CXCR2^{-/-} mice were similar at days 9 and 21 post-infection. However, at day 28, CXCR2^{-/-} mice had significantly lower CFUs compared to wild-type mice in both the joints and spleen (Fig 4D). We also found that joints from CXCR2^{-/-} mice had reduced levels of CCL2, IL-6, TNF- α and IL-1 β compared to wild-type mice at 28 days post-infection (data not shown).

To confirm our results obtained by anti-IFN- γ treatment, we generated IFN- γ ^{-/-}/CXCR2^{-/-} mice. Following infection with *B. melitensis*, IFN- γ ^{-/-}/CXCR2^{-/-} mice had delayed arthritis onset with only 16.6% developing arthritis by day 19 compared to 100% of IFN- γ ^{-/-} mice (Fig 5A). Additionally, IFN- γ ^{-/-}/CXCR2^{-/-} mice had reduced clinical scores with an average score of 1 versus 8.87 for IFN- γ ^{-/-} mice (Fig 5B). Swelling of the paws was also strikingly reduced in the IFN- γ ^{-/-}/CXCR2^{-/-} mice (Fig 5C). IFN- γ ^{-/-}/CXCR2^{-/-} mice did lose more weight compared to IFN- γ ^{-/-} mice; however, no significant change in bacterial loads in the spleen or joints between groups was noticed at days 18 and 25 post-infection (Fig 5D).

CXCR2 mediates neutrophil infiltration in musculoskeletal tissue

Gross swelling of mouse paws and tails was markedly reduced in IFN- γ ^{-/-}/CXCR2^{-/-} mice relative to IFN- γ ^{-/-} animals infected with *B. melitensis* (Fig 6A). Inflammation of mouse paws develops asymmetrically in *Brucella*-infected IFN- γ ^{-/-} mice, while tails typically develop multiple inflammatory foci (Fig 6A). Therefore to assess inflammation in the same tissue from all mice, histology was performed on mouse tails and scores were assigned as described in Methods. H&E staining of

Figure 5. CXCR2 mediates *Brucella*-induced inflammation in IFN- $\gamma^{-/-}$ mice. IFN- $\gamma^{-/-}$ and IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice (on C57BL/6 background, n=4-7/group) were infected i.p. with $\sim 1 \times 10^5$ CFUs *B. melitensis*. Incidence of paw swelling (A) clinical scores (B) and mean swelling (C) were recorded over time. Colonization of the joints and spleen was determined on day 18 and 25 post-infection (D). *P<0.05 as compared to IFN- $\gamma^{-/-}$ mice. Data in (A-C) is representative of three independent experiments. Error bars depict SEM.

Figure 6. CXCR2 mediates musculoskeletal neutrophil recruitment in *Brucella*-infected mice.

IFN- $\gamma^{-/-}$ and IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice were infected i.p. with $\sim 1 \times 10^5$ CFUs of *Brucella melitensis* 16M and sacrificed at day 25 post-infection. Representative tail images comparing IFN- $\gamma^{-/-}$ and IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice (**A**) Representative images of H&E stained, *B. melitensis*-infected IFN- $\gamma^{-/-}$ and IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ tail vertebral sections using a 4X (left) and 40X (right) objective lens (**B**) Representative images of immunohistochemistry sections using anti-Ly6G, (clone 1A8) indicating neutrophil infiltration using a 40X objective (**C**) Scoring of histopathology H&E (total inflammation) and immunohistochemistry (neutrophil infiltration) tail vertebra sections (6-7 mice/group) (**D**) (arrow) intervertebral disk, (B) bone, (MC) medullary cavity, (N) neutrophils *P< 0.05 as compared to sections from IFN- $\gamma^{-/-}$ mice.

mouse-tail sections from *B. melitensis*-infected IFN- $\gamma^{-/-}$ mice revealed severe areas of inflammation that involved bone, medullary cavity of bone, tendons, skeletal muscle and periarticular inflammation (Fig 6B). Osteomyelitis was observed in the medullary cavity of *B. melitensis*-infected IFN- $\gamma^{-/-}$ mice, which generally occurred near the growth plate, and in some instances may have led to erosion of the bone (Fig 6B). Osteomyelitis was also observed in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice infected with *B. melitensis*, but was much milder than that observed in IFN- $\gamma^{-/-}$ mice. Inflammation was sometimes localized to medullary cavity in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ in contrast to IFN- $\gamma^{-/-}$ mice that displayed extensive inflammation in bone, tendon, and skeletal muscle (Fig 6B&D). Immunohistochemistry was also performed using the neutrophil specific Ly-6G antibody (clone 1A8). Neutrophil infiltration was seen in both groups, however IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ had significantly reduced neutrophil recruitment in inflamed tissues relative to IFN- $\gamma^{-/-}$ mice (Fig. 6C& D). Flow cytometry also revealed a ~40% reduction of neutrophil infiltration in joints from IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$, relative to IFN- $\gamma^{-/-}$ mice (data not shown).

Reduced TNF- α and CCL3 levels in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice

Cytokines from *Brucella*-infected IFN- $\gamma^{-/-}$ and IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ joints were evaluated by Luminex to determine signaling pathways that might be regulated by CXCR2. At day 25 post-infection, both TNF- α and CCL3 were significantly reduced in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ compared to IFN- $\gamma^{-/-}$ joints (Fig 7A&B). IL-1 β levels were also lower, but not significantly in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice. Additionally, the anti-

Figure 7. Reduced levels of TNF- α and CCL3 in $IFN-\gamma^{-/-}$ mice lacking CXCR2.

$IFN-\gamma^{-/-}$ and $IFN-\gamma^{-/-}/CXCR2^{-/-}$ mice were infected i.p. with $\sim 1 \times 10^5$ CFUs of *B. melitensis*. Joint homogenates (n=6-7/group) were assayed for inflammatory cytokines (**A**) and chemokines (**B**) at 25 days post-infection by Luminex and normalized by BCA. * $P < 0.05$ as compared to $IFN-\gamma^{-/-}$ mice. Error bars depict SEM

inflammatory cytokine IL-10 was slightly up-regulated in IFN- $\gamma^{-/-}$ /CXCR2 $^{-/-}$ mice on day 18 post-infection relative to IFN- $\gamma^{-/-}$ mice (data not shown).

DISCUSSION

Brucellosis is one of the most common zoonotic diseases worldwide (8), and osteoarticular complications are the most frequent localized manifestations (56, 57). Here, we investigated mechanisms of inflammation during *Brucella* infection using our previously published mouse model (165). We found that T- and B-cell-deficient mice developed focal inflammation similarly to wild-type animals. We also did not observe a robust recruitment of T and B cells to infected joints. In contrast to rheumatoid arthritis, where auto-reactive T cells and B cells are the primary cause of pathogenesis (170, 171), T cell and antibody responses do not appear to be required for *Brucella*-induced inflammation, similar to what has been reported in other murine models of infectious arthritis, such as Lyme disease (172). While T and B cells were not required for the development of arthritis in our model, others have shown that Rag1 $^{-/-}$ mice are more susceptible to *B. melitensis* infection (173). B cell deficient mice are more resistant to *Brucella* infection (174); however, several other groups have shown that T cells mediate clearance of *Brucella* and are a major producer of IFN- γ (175, 176). Thus, in future studies we will determine if transfer of wild-type T cells to IFN- $\gamma^{-/-}$ mice can mediate resolution of *Brucella*-induced arthritis.

While adaptive immune cells are not required for *Brucella*-induced focal inflammation, we did find elevated levels of neutrophils in infected joints. CCR2,

and its ligand CCL2 are typically involved in the recruitment of neutrophils and inflammatory monocytes (128, 177). In a model of antigen-induced arthritis, CCR2-deficient mice developed significantly reduced arthritis and neutrophil infiltration within their joints, while administration of WT neutrophils to CCR2^{-/-} mice diminished protection (128). However, similar to what others have reported for *Borrelia burgdorferi*-induced arthritis (178), we did not find a prominent role for CCR2 in musculoskeletal brucellosis.

CXCR2 is important in neutrophil recruitment and subsequent antimicrobial functions including oxidative burst, degranulation, netosis, phagocytosis and is activated through the production of chemokines such as CXCL1 and CXCL2 (179, 180). Levels of CXCL1 and CXCL2 in *Brucella*-infected IFN- γ ^{-/-} joints correlated with the onset of inflammation. CXCR2 deficiency did not enhance susceptibility to infection as *B. melitensis* loads in the spleens and joints of CXCR2^{-/-} mice and control animals were similar. We also found that CXCR2^{-/-} mice controlled *Brucella* infection as well as wild-type mice in the absence of anti-IFN- γ treatment (data not shown). Therefore, CXCR2, under these conditions, was not required for the clearance of *Brucella*. Others have demonstrated that during chronic infection, neutrophils impede clearance of *B. abortus*, perhaps by reducing Th1 responses (181). Thus, reduced neutrophil recruitment in CXCR2-deficient animals is not likely to result in enhanced susceptibility to *Brucella* infection.

Mice lacking CXCR2, in both our anti-IFN- γ -treated and IFN- γ ^{-/-} models, displayed markedly reduced symptoms of focal inflammation relative to control animals. These findings may be clinically relevant as elevated levels of CXCL8,

a human chemokine that signals through CXCR2, were found in the synovial fluid of a human articular brucellosis patient (182). CXCR2 was required for neutrophil recruitment to *Brucella*-infected joints. Studies using collagen-induced, K/BXN, and LPS-induced models of arthritis suggest neutrophil depletion using anti-Gr1 antibodies is protective against disease (183, 184). However in experimental Lyme arthritis, neutrophil depletion, with RB6-8C5 antibody, results in early induction of arthritis and infiltration of PMN/leukocyte-like cells that do not express Gr-1 (185). Here we found that, CXCR2-deficient animals had reduced levels of CCL3 and TNF- α , compared to control animals. Some evidence suggests neutrophil recruitment can be dependent on CXCL2 inducing the release of CCL3 with subsequent induction of TNF- α (186). Future studies will determine the role of neutrophils and the signaling pathways that recruit them in *Brucella*-induced focal inflammation.

The signaling pathways and cells that produce the CXCR2 ligands, CXCL1 and CXCL2, have yet to be determined in our murine model, however it has been demonstrated that TLR2 is required for alveolar macrophages to produce CXCL1 in response to *B. abortus* infection (108). In addition to professional phagocytes, cells native to the joint may also be producing CXCR2 ligands. One study showed that when human fibroblast-like synoviocytes are infected with *B. abortus* they produce IL-6, CCL2, and CXCL8, in a dose-dependent manner. Additionally, these supernatants recruited human PMNs and monocytes *in vitro* (187). Furthermore, human osteoblasts up-regulate their production of CCL2, IL-6 and CXCL8 upon co-culture with supernatant from *B. abortus*-infected monocytes. In turn, media

conditioned by *B. abortus*-infected osteoblasts induced monocyte production of CXCL8, TNF- α , IL-6 and IL-1 β (129).

Collectively, our study suggests blocking or inhibition of CXCR2 may be a potential therapy for treating *Brucella*-induced inflammation. Genetic ablation of CXCR2 resulted in reduced arthritis in a mouse model of Lyme disease (178, 188). Similarly, blocking CXCR2 through various techniques has proven to be therapeutic in treating models of rheumatoid arthritis (189-191). In addition, treating mice with allosteric inhibitors of CXCR1 and CXCR2 reduced the number of neutrophils in the synovium in a mouse model of antigen-induced arthritis (192). Therefore, the targeting of CXCR2, or CXCR2 ligands, may have potential as a complementary therapy, in combination with antibiotics, for treating osteoarticular complications of brucellosis.

Figure 8. Model of *Brucella* Induced Arthritis in i.p. infected IFN- γ deficient mice.

Brucella infects the joint space and local tissues produce CXCL1 and/or CXCL2. The neutrophil CXCR2 receptor is activated by ligands CXCL1 and CXCL2. Thus, neutrophils become activated and are recruited to the joint.

CHAPTER IV

Temporal Role for MyD88 in a Model of *Brucella*-Induced Arthritis and Musculoskeletal Inflammation

ABSTRACT

Brucella spp. are facultative intracellular Gram-negative bacteria that cause the zoonotic disease brucellosis, one of the most common global zoonoses. Osteomyelitis, arthritis and musculoskeletal inflammation are common focal complications of brucellosis in humans; however, wild-type (WT) mice infected systemically with conventional doses of *Brucella* do not develop these complications. Here we report WT C57BL/6 mice infected via the footpad with 10^3 - 10^6 CFUs of *Brucella spp.* display neutrophil and monocyte infiltration to the joint space and surrounding musculoskeletal tissue. Joint inflammation is detectable as early as one day post-infection and peaks one to two weeks later after which WT mice are able to slowly resolve inflammation. B and T cells were dispensable for the onset of swelling but required for resolution of joint inflammation and infection. At early time points, MyD88^{-/-} mice display decreased joint inflammation, swelling, and pro-inflammatory cytokine levels relative to WT mice. Subsequently, swelling of MyD88^{-/-} joints surpassed WT joint swelling, and resolution of joint inflammation was prolonged. Joint bacterial loads in MyD88^{-/-} mice were significantly greater than WT by day 3 post-infection and at all time points thereafter. In addition, MyD88^{-/-} joint inflammatory cytokine levels on day 3 and beyond were similar to WT. Collectively this data demonstrates MyD88 signaling mediates early inflammatory responses in the joint, but also contributes to subsequent clearance

of *Brucella* and resolution of inflammation. This work also establishes a mouse model for studying *Brucella*-induced arthritis, musculoskeletal complications, and systemic responses, which will lead to a better understanding of focal complications of brucellosis.

INTRODUCTION

Mechanisms underlying arthritic responses induced by *Brucella* have been hindered by the lack of relevant mouse models, as wild-type mice infected with conventional doses (10^4 - 10^6 colony forming units [CFUs]) by systemic routes do not develop joint inflammation (58, 70). One study showed that BALB/c mice infected intraperitoneally (i.p.) with 1×10^7 CFUs of *B. melitensis* developed arthritis at 26 weeks post-infection (75). Additionally, others have previously reported that i.p. *Brucella* infection of IFN- γ -deficient mice resulted in arthritis of ankle joints as early as 15 days post-infection. Arthritis in this model was dependent on CXCR2 and IL-1R, but adaptive immune cells were not required for the induction of inflammation (58, 70). We suspected that IFN- γ deficiency allowed for the hematogenous spread of *Brucella*, resulting in infection and inflammation of the joint (58). Other mouse models of infectious arthritis have utilized footpad inoculation to localize pathogens, such as *Borrelia burgdorferi*, Chikungunya virus, and *Candida albicans*, to the joint and surrounding tissue to induce inflammation (172, 193, 194) Here we describe a model of *Brucella*-induced arthritis that is applicable in WT mice. Infection of the articular space and surrounding tissue is achieved by injecting *Brucella* adjacent to ankle joints, via footpad inoculation.

Myeloid differentiation factor 88 (MyD88) is an adaptor protein that relays Toll-like receptor (TLR), IL-1R and IL-18R signaling (195, 196). Once activated, most TLRs signal through MyD88 to induce cytokine production (113). This signaling can result in arthritis and osteoclastogenesis in murine models of bacterial-induced focal complications (114-116). Host cells reportedly recognize *Brucella* through TLR2, TLR4, TLR6 and TLR9 (108-112). To determine if MyD88 signaling is important for *Brucella*-induced arthritis, we investigated joint inflammatory responses in MyD88^{-/-} mice following footpad infection with *Brucella*. We show MyD88 regulates early arthritis development, but is also required to control bacterial burden and resolve inflammation at later stages of infection.

RESULTS

***Brucella* infection of the footpad results in arthritis and musculoskeletal inflammation in wild-type mice.**

The systemic spread of *Brucella* to the joints is thought to induce arthritis and musculoskeletal inflammation (58). Thus, we hypothesized that delivering *Brucella* adjacent to the joint would allow for bacterial colonization and subsequent arthritis. Therefore, we infected wild-type C57BL/6 mice (WT) in the rear footpads with 1×10^6 CFUs of *B. melitensis* 16M. H&E staining was conducted on mock-infected mice at day 3, and *Brucella*-infected mice at days 1, 3, 7, 14 and 28 post-infection (Fig. 9A-F). All footpad-infected mice exhibited signs of arthritis and musculoskeletal inflammation in the joint and surrounding muscle and soft tissues

Figure 9. WT mice develop arthritis and musculoskeletal inflammation following footpad infection.

WT, C57BL/6, mice were either mock-infected in each rear footpad with sterile PBS (sPBS) or infected with 1×10^6 *B. melitensis* 16M. Mock-infected mice were euthanized at day 3 (A), and *B. melitensis*-infected mice at days 1 (B), 3 (C), 7 (D), 14 (E) and 28 (F) post-infection. H&E staining was conducted on mouse joints. Representative images (100x) from mock treated, and infected mice are depicted. Amplified boxed regions (400x) showing inflammation within the joint space of *B. melitensis*-infected mice are displayed beneath the 100x image at each timepoint. Images are representative of joints from 4 to 5 mice per timepoint from one kinetic experiment. Similar pathology was observed at the day 3 and day 28 timepoints in 2-3 additional experiments.

relative to mock-infected mice (Fig. 9B-F). *Brucella*-infected C57BL/6 mice demonstrated moderate to focally extensive areas of inflammation and large numbers of neutrophils, including neutrophils in the joint space, one day after infection (Fig. 9B). Joint arthritis and musculoskeletal inflammation peaked from days 3-14, presenting with severe large confluent areas of inflammation (neutrophils, macrophages and fibrin in the joint space) (Fig. 9C-E). Inflammation began to resolve by day 28, with moderate to severe pathology with focally extensive areas of inflammation including macrophages, fibrin and few neutrophils in the joint space (Fig. 9F).

Footpad inoculation of mice with *B. melitensis*, *B. suis* or *B. abortus* results in dose dependent joint swelling, arthritis and musculoskeletal inflammation.

To determine if footpad inoculation with a lower dose or other *Brucella* species pathogenic in humans could also induce arthritis, we injected 1×10^5 *B. melitensis* 16M, *B. suis* 1330, or *B. abortus* 2308 into the footpads of wild-type C57BL/6 (WT) mice. Joint swelling was also measured as it is a characteristic manifestation of *Brucella*-induced arthritis and musculoskeletal inflammation (58, 70). Relative to *B. melitensis*-infected mice, swelling of joints was slightly, but significantly, greater in *B. abortus*-infected mice on day 2, and in *B. suis*-infected mice on day 2 and 11. However, all *Brucella* species displayed similar swelling kinetics and induced robust swelling of mouse ankles by day 2 post-infection (Fig. 10A). Additionally, we investigated if *B. melitensis* 16M at a dose of 1×10^3 or 1×10^4

Figure 10. WT mice infected via the footpad with *B. melitensis*, *B. abortus* or *B. suis* develop arthritis and musculoskeletal inflammation. WT, C57BL/6, mice were infected in each rear footpad with 1×10^5 *Brucella* spp., 1×10^4 *B. melitensis* 16M (**A,B,F-I**), 1×10^3 *B. melitensis* 16M or mock infected with sterile PBS (sPBS) (**C&D**) and evaluated for ankle swelling over time (**A&C**). Mice were euthanized on day 14 post-infection and joint and spleen bacterial loads were enumerated (**B&D**). $P < 0.05$ as compared to WT mice infected with 1×10^5 *B. melitensis* 16M (**A**). $P < 0.05$ as compared to mock infected WT mice (**C**) and error bars depict standard errors of the mean. Representative plots of H&E stained joint sections from mock-infected mice (**E**) or mice infected in the footpad with 1×10^5 *B. melitensis* 16M (**F**) 1×10^3 *B. suis* 1330 (**G**) 1×10^5 *B. abortus* 2308 (**H**), 1×10^4 *B. melitensis* 16M (**I**) or 1×10^3 *B. melitensis* 16M (**J**) are shown. Images (100x) are depicted with amplified boxed regions (400x) displayed beneath each image. Data from mice infected with 1×10^5 *B. melitensis* 16M is representative of 2-3 independent experiments while data from other *Brucella* strains is representative of one experiment.

could induce inflammation via footpad infection (Fig 10A&C). Although ankle swelling was evident, it was slightly delayed in mice infected with a dose of 1×10^4 CFUs when compared to mice infected with 1×10^5 *B. melitensis* (Fig. 10A). Similarly, mice infected with 1×10^3 *B. melitensis* also developed joint swelling, while no apparent swelling was in mice mock-infected with sterile PBS (Fig. 10C). Additionally, bacterial loads in joint homogenates were similar between mice infected with 1×10^5 CFUs of *B. abortus*, *B. suis*, or *B. melitensis* 14 days after infection (Fig. 10B). To determine if *Brucella* was able to colonize systemic tissues in this model, splenic bacterial loads were measured and *B. melitensis*, *B. abortus*, and *B. suis* were all found to similarly colonize the spleen following footpad inoculation with 1×10^5 CFUs. (Fig. 10B). Bacterial colonization was also detected in the joints and spleens of mice infected with 1×10^3 or 1×10^4 *B. melitensis* (Fig. 10B&D). On day 14 post-infection, histology was conducted on joint sections from mock-infected mice, along with mice infected with 1×10^3 - 1×10^5 *B. melitensis* or with 1×10^5 *B. suis* or *B. abortus* (Fig. 10E-J). All *Brucella*-infected mice (Fig. 10F-J) developed arthritis and musculoskeletal inflammation while no pathology was evident in sterile PBS-inoculated animals (Fig. 10E). All mice infected with 1×10^5 *Brucella spp.* had a similar degree of neutrophil and macrophage infiltration in and around the joint space (Fig. 10F-H). Mice infected with lower doses of *B. melitensis* (Fig. 10I&J) also developed arthritis and musculoskeletal inflammation. While doses of 1×10^3 - 1×10^6 CFUs all induced inflammation in this model, a dose of 1×10^6 was chosen for future studies as it was found to reliably generate detectable levels of joint cytokines in pilot studies (data not shown).

Adaptive immune responses mediate resolution of inflammation, but are not required for development of joint swelling.

To investigate the role of adaptive immune responses during focal inflammation, wild-type C57BL/6 (WT) and Rag1^{-/-} (B and T cell deficient) mice were infected in each hind footpad with 1x10⁶ CFUs of *B. melitensis* 16M, and ankle swelling was followed for 30 days post-infection. At days 1 and 3 post-infection both WT and Rag1^{-/-} mice had developed a similar degree of ankle swelling; however, at days 6 and 8 Rag1^{-/-} mice displayed a slight, but statistically significant, decrease in swelling compared to WT mice. After day 8 WT mice began to resolve joint swelling, but in Rag1^{-/-} mice swelling remained constant and was significantly greater than that present in WT mice by day 27 post-infection (Fig. 11A). Joint *Brucella* loads in Rag1^{-/-} mice were more than 1,000 fold greater than those in WT mice by day 30 post-infection (Fig. 11B). Joint inflammation was higher at day 30 in Rag1^{-/-}, relative to WT mice and joint levels of the pro-inflammatory cytokines CXCL1, IL-1 β , CCL2, and IL-6 were also significantly greater in Rag1^{-/-} mice compared to WT mice (Fig. 11C-E). In addition, joints from WT mice had reduced amounts of fibrin relative to joints from Rag1^{-/-} mice (Fig. 11E). These data demonstrate that adaptive immunity contributes to, but is not absolutely required for, the initiation of joint swelling, and subsequently mediates bacterial clearance and resolution of joint inflammation.

Figure 11. Adaptive immune responses are needed for inflammation resolution but not required for inflammation onset.

WT, C57BL/6, and Rag1^{-/-} mice were infected in each rear footpad with 1×10^6 *B. melitensis* 16M. Joint swelling was recorded over time (5-10 mice/group) (A). Mice were euthanized at day 30 post-infection and ankle joint bacterial loads (n=5-10 group) (B) and cytokine levels (n=4-5 mice/group) (D) were enumerated. H&E staining was conducted on joints at day 30 post-infection (n=5-6/group) and scored for inflammation severity (C&E). Representative images are depicted (100x) and amplified boxed regions are displayed beneath each image (400x) (E). $P < 0.05$ as compared to WT mice. Error bars depict standard errors of the mean. Data is representative of one experiment.

MyD88 initiates early joint swelling, but is also required for clearance of *Brucella* from the joint and resolution of joint swelling

To determine if MyD88 was involved in the initiation of joint inflammation, swelling of wild-type C57BL/6 (WT) and MyD88^{-/-} mouse ankles was measured over 28 days post *Brucella*-infection. MyD88^{-/-} mice infected with 1×10^6 *B. melitensis* 16M displayed reduced ankle swelling at days 1 and 3 post-infection (Fig. 12A). In contrast to the phenotype seen at day 1 and 3, MyD88^{-/-} joints had increased swelling compared to WT joints by day 7 and at all subsequent time-points post-infection (Fig. 12A). A similar effect of MyD88 on joint swelling was observed (albeit with delayed kinetics), when an infectious dose of 1×10^5 CFUs was used (Fig. 12B).

To explain the increase in swelling of MyD88^{-/-} joints after day 3 post-infection, we hypothesized that MyD88 is important for mediating bacterial clearance in the joint. Thus, bacterial loads were examined in ankle homogenates on days 1, 3, 7, 14 and 28 post-infection with 1×10^6 *B. melitensis* (Fig. 12C). While joint CFUs were similar between groups at day 1, by day 3 there were significantly greater bacterial loads in MyD88^{-/-} joints when compared to WT mice. This trend continued throughout the experimental time course (Fig. 12C).

MyD88 initiates early recruitment of inflammatory infiltrates following *B. melitensis* footpad infection.

To assess the role of MyD88 in joint inflammation, H&E staining was

Figure 12. MyD88^{-/-} mice have delayed joint swelling but increased joint *Brucella* burden.

WT, C57BL/6, and MyD88^{-/-} mice were infected in each rear footpad with 1×10^6 (A) or with 1×10^5 *B. melitensis* 16M (4-5 mice/group) (B) and swelling was recorded over time. At days 1, 3, 7, 14 and 28, mice infected with 1×10^6 *B. melitensis* were euthanized and bacterial loads were enumerated in the ankle joint (3-4 mice/group/timepoint) (C). $P < 0.05$ as compared to WT mice. Error bars depict standard errors of the mean. CFU data in (C) is from one kinetic experiment. Swelling curves are representative of two independent experiments.

conducted on wild-type C57BL/6 (WT) and MyD88^{-/-} joints 3 days after infection with 1x10⁶ CFUs of *B. melitensis* 16M (Fig. 13A). Consistent with our swelling data, MyD88^{-/-} mice displayed visibly reduced inflammatory infiltrates, including macrophages and neutrophils (Fig. 13A). Inflammation severity scores of MyD88^{-/-} joints at day 3 were significantly lower compared to infected WT mouse joints (Fig. 13B). Flow cytometry of infected joints also revealed reduced myeloid cell infiltration in MyD88^{-/-}, relative to WT joints early in infection (data not shown). Despite reduced inflammation at early time points, MyD88^{-/-} mice developed similar pathology by day 7 and equaled or surpassed WT inflammation by day 14 and 28 post-infection (data not shown).

MyD88 mediates inflammatory cytokine production in *B. melitensis*-infected mouse joints early in infection.

The production of inflammatory cytokines can induce inflammation and arthritis (197, 198). To determine what inflammatory cytokines are MyD88-dependent during *B. melitensis*-induced articular inflammation, cytokines were measured in ankle joint homogenates following footpad infection with 1x10⁶ CFUs of *B. melitensis* 16M. At day 1 post-infection, the concentrations of the pro-inflammatory cytokines: CXCL2, CXCL1, IL-1 β , TNF- α , CCL2, IL-6, CCL3 and IFN- γ were all significantly lower in MyD88^{-/-}, compared to WT joint homogenates (Fig. 14A-H). However, by day 3 post-infection, TNF- α and IFN- γ concentrations in MyD88^{-/-} joints increased to WT levels (Fig. 14D&H), while WT levels of CXCL1, CCL2 and IL-6 dropped and returned to similar levels as MyD88^{-/-} joints

Figure 13. MyD88 mediates early arthritis and musculoskeletal inflammation.

WT, C57BL/6, and MyD88^{-/-} mice were infected in each rear footpad with 1×10^6 *B. melitensis* 16M. Mice were euthanized 3 days post-infection and H&E staining was conducted on mouse joints. Representative images of joint sections from WT and MyD88^{-/-} mice are depicted with amplified boxed regions displayed beneath each image (**A**). Magnification is as follows: row one=40x, row two=100x, rows 3 and 4=400x. The black boxes indicate areas of arthritis or inflammation within and around the joint, and the white boxes indicate areas of musculoskeletal inflammation. Tissue architecture is indicated by: B=bone, SM=skeletal muscle and J=joint space. Areas of pathology are indicated by: I=inflammation, M=macrophages, N=neutrophils. Day 3 WT and MyD88^{-/-} ankle joint H&E staining was scored for total inflammation (**B**). Histology scores are from one of two independent experiments (5 mice/group) from which sections were obtained. *P<0.05 as compared to sections from WT mice.

Figure 14. MyD88^{-/-} joints have delayed cytokine production following footpad infection with *B. melitensis*. WT, C57BL/6, and MyD88^{-/-} mice (3-4 mice/group/time point) were infected in each rear footpad with 1×10^6 *B. melitensis* 16M and sacrificed at days 1, 3, 7, 14 and 28. Rear joints were homogenized and CXCL2 (A), CXCL1 (B), IL-1β (C), TNF-α (D), CCL2 (E), IL-6 (F), CCL3 (G) and IFN-γ (H) concentrations were quantified by Luminex and normalized to total protein via BCA. $P < 0.05$ as compared to infected WT mice. Error bars depict standard errors of the mean. Data is representative of one kinetic experiment.

(Fig. 14B&E&F). At day 3 post-infection IL-1 β , CCL3 and CXCL2 levels increased in MyD88^{-/-} joints, while WT levels decreased (Fig. 14A,C,&G). Eventually, all inflammatory cytokine concentrations became similar among groups by day 14 post-infection and at later time points.

MyD88 aids in systemic *Brucella* clearance and T cell production of IFN- γ

To determine if MyD88 is required for protection against systemic infection in this model, wild-type C57BL/6 (WT) and MyD88^{-/-} mice were infected in the footpad with 1x10⁶ CFUs of *B. melitensis* 16M. *Brucella* spleen colonization in MyD88^{-/-} mice was similar to WT mice until day 14; however, at 28 days post-infection MyD88^{-/-} mice had significantly increased splenic bacterial loads (Fig. 15A). To determine the role of MyD88 on systemic adaptive immune responses, CD4⁺ and CD8⁺ T cells from spleens of WT and MyD88^{-/-} mice were evaluated for IFN- γ production. While the proportion of CD4⁺ and CD8⁺ T cells amongst splenocytes did not differ between WT and MyD88^{-/-} mice (data not shown), at days 7 and 14 post-infection, the percentage of IFN- γ producing CD4⁺ and CD8⁺ T cells was significantly lower in MyD88^{-/-} mice compared to WT mice (Fig. 15B&C). By day 28 post-infection, IFN- γ production by T cells was similar between mouse strains (data not shown).

Figure 15. MyD88 aids in systemic *Brucella* clearance and T cell IFN- γ production.

WT, C57BL/6, and $\text{MyD88}^{-/-}$ mice were infected in each rear footpad with 1×10^6 *B. melitensis* 16M. At days 1, 3, 7, 14 and 28, mice were euthanized and bacterial loads were enumerated in the spleen (3-4 mice/group/timepoint) (A). At days 7 and 14 flow cytometry was performed to determine IFN- γ production by T cells from infected-mouse spleens (B). Representative plots showing IFN- γ production by gated CD4 $^{+}$ and CD8 $^{+}$ T cells from WT and $\text{MyD88}^{-/-}$ mice 14 days post-infection (C). $P < 0.05$ as compared to WT mice. Error bars depict standard errors of the mean. Data is representative of one kinetic experiment.

DISCUSSION

With over 500,000 human cases reported each year, brucellosis is a globally important zoonotic disease (8). Arthritis and musculoskeletal inflammation are the most common focal symptoms of human brucellosis; (7, 8) however, the study of these manifestations has been hampered due to the absence of relevant mouse models. Intraperitoneal, pulmonary, or oral infection of wild-type, inbred, mouse strains with conventional doses of *Brucella* does not induce arthritis and musculoskeletal inflammation. Others have demonstrated that intra-articular inoculation of heat killed *Brucella* or *Brucella* lipoproteins into the knee induces osteoclastogenesis and recruitment of monocytes and neutrophils (153, 187). Footpad inoculation with *Brucella* spp. has been used to study *Brucella* dissemination and efficacy of vaccines (199-202), but inflammation in the joint and surrounding tissue was not described. Here we show that wild-type C57BL/6 mice inoculated via the footpad with 10^3 - 10^6 CFUs of live *Brucella* develop arthritis and musculoskeletal inflammation. Similar to what has been described in human patients with articular brucellosis (63), and what we previously found in IFN- γ ^{-/-} mice, footpad infected mice also developed inflammation of musculoskeletal tissues surrounding the joint. To our knowledge this is the first description of a wild-type mouse strain developing arthritis and musculoskeletal inflammation when infected with a conventional dose of *Brucella*. We previously reported that IFN- γ ^{-/-} mice infected i.p. with *B. melitensis* developed exacerbated arthritis and joint colonization relative to IFN- γ ^{-/-} mice infected i.p. with *B. abortus* (58). However, here we found that following footpad inoculation, *B. melitensis*, *B.*

abortus, and *B. suis*-infected, mice all developed ankle swelling and exhibited similar joint bacterial loads and inflammation. Thus, the augmented arthritogenicity of *B. melitensis* observed in IFN- γ ^{-/-} mice could be due to an enhanced ability to disseminate to the joint, rather than an enhanced ability to cause inflammation upon reaching the joint. Similar to what we previously reported in i.p.-infected IFN- γ -deficient mice (70), here we found that adaptive immune responses are not required for joint swelling in footpad inoculated mice. However, we did find that B and/or T cells are requisite for resolution of joint infection and inflammation in this model. Thus, resolution of infection and inflammation in the footpad model could be due to adaptive immune cell production of IFN- γ .

Utilizing this footpad model of *Brucella* infection, we demonstrated MyD88 is important for eliciting early inflammatory responses within the first 3 days post-infection. Aspects of MyD88-dependent inflammation appear to be protective at later times, as MyD88^{-/-} mice had delayed resolution of inflammation. This may be due to increased bacterial loads as MyD88^{-/-} mice had significantly higher joint bacterial burdens by day 3 post-infection compared to wild-type, C57BL/6 mice. We also demonstrated that MyD88^{-/-} mice had delayed expression of TNF- α , IL-1 β , CCL2, IFN- γ , and CXCR2 ligands. This is of interest as these cytokines were found at elevated levels in the synovial fluid from a human brucellosis patient with arthritic manifestations (66). MyD88 has previously been shown to be important for controlling systemic *Brucella* infection in mice (203). Likewise, MyD88 signaling has been reported to drive dendritic cell maturation and in turn T cell secretion of IFN- γ , which is protective against systemic *Brucella* infection (141, 204). In this

model we demonstrated mice deficient in MyD88 have impaired CD8⁺ and CD4⁺ T cell production of IFN- γ in their spleens. This suggests the footpad model of *Brucella* infection may be a useful tool for analyzing systemic control of inflammation and *Brucella* clearance. Another *Brucella* infection model demonstrated macrophage and dendritic cell production of IL-12 and TNF- α are MyD88 dependent during systemic infection (204). TLR2 and TLR6 can recognize and protect against systemic brucellosis, and there is varied evidence that TLR4 may also be involved (108-111, 203, 205). TLR9 signaling can also increase the number of IFN- γ ⁺ cells after i.p. infection (205). Furthermore, IL-1 and IL-18 signaling can utilize MyD88 for downstream neutrophil recruitment, NK and T cell activation, and production of inflammatory cytokines (195, 196). Thus, the early inflammation and joint cytokine production mediated by MyD88 are likely due to TLR activation and/or IL-1R/IL-18R relay which contribute to *Brucella* clearance at later times.

Although MyD88 promotes inflammation early after infection, another mechanism independent of MyD88 induces inflammation by day 7. Joint levels of the inflammatory cytokines, TNF- α , CXCL2, IL-1 β , CCL3 and IFN- γ , in mice lacking MyD88 elevated similarly to control animal levels by day 7, and swelling in MyD88^{-/-} mouse joints was greater than that of control animals by day 6 post-infection. This increase in joint inflammation in MyD88^{-/-} mice may also be dependent on damage-associated molecular patterns induced by overwhelming bacterial burden, which could signal through MyD88 independent pathways (206). It is becoming increasingly clear that cytosolic signaling mechanisms may also

induce inflammation upon *Brucella* infection. Although the MyD88-independent inflammatory pathway has yet to be determined in this model, one study demonstrated an important role for inflammasomes that require the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) in the host response to *Brucella* infection (117). Additionally, neutrophils infected with *Brucella* have been shown to induce TAK1 and SYK kinase-dependent oxidative bursts (207). Thus, the inflammasome, TAK1 and/or SYK kinase-dependent pathways could induce inflammation in mice lacking MyD88 in our model.

Although this is the first time MyD88 has been shown to be important for *Brucella* clearance in the joints, MyD88 is critical for joint pathogen clearance in *Borrelia burgdorferi*-induced arthritis. In contrast with *Brucella* infection, MyD88 does not appear to play a role in Lyme arthritis development (208, 209). In mice inoculated with *E. coli* or *Streptococcus* cell wall fragments, MyD88 is critical for the induction of arthritis and is dependent on TLR2 and TLR4, respectively (115, 116). Similarly, TLR4 antagonism can suppress autoimmune arthritis models, such as collagen-induced arthritis (210). It is clear that MyD88 is responding to *Brucella* infection of the joints, but the nature of the reacting cell types remain unknown. Numerous cell types in the joint, including macrophages, dendritic cells, synovial fibroblasts, chondrocytes, endothelial cells and fibroblast-like synoviocytes, are capable of responding to exogenous and endogenous TLR ligands and could be mediating inflammation in our model (109, 129, 155, 211-213).

In summary, we have described a footpad model of *Brucella*-infection that results in reproducible joint swelling, arthritis, and musculoskeletal inflammation

following infection with *B. melitensis*, *B. abortus* or *B. suis*. One aspect of human infection that this model does not recapitulate is the dissemination of *Brucella* to the joint. However, local delivery of *Brucella* directly to the joint and surrounding tissue allows us to synchronize infection and inflammation. This synchronization allows us to investigate mechanisms of the host response that are specific to these tissues, whereas in a systemic infection, deficiencies in the host immune response could affect bacterial dissemination to the joint and thus make it difficult to determine if changes in joint inflammation are due to local immune responses, or due to a change in the dissemination of *Brucella* to the joint.

With this model we are also able to study *Brucella*-induced arthritis in the context of an intact IFN- γ response. In IFN- γ -deficient mice, *Brucella*-induced focal inflammation does not resolve and the animals eventually succumb to infection after 4-8 weeks. In this footpad infection model, mice do not display systemic symptoms of disease (beyond colonization of the spleen and liver) and are able to slowly resolve inflammation, an effect that requires adaptive immunity. Thus, this novel model allows us to study both the initiation and resolution of focal inflammation due to *Brucella* infection. Here we utilized this footpad model and determined MyD88-dependent pathways are responsible for inducing early arthritis and musculoskeletal inflammation; however, MyD88-deficiency also leads to an increase in joint bacterial loads, which may delay resolution of articular inflammation.

CHAPTER V

Inflammasomes confer protection via IL-18 and pyroptosis, and are negatively regulated by IFN- γ -dependent nitric oxide during *Brucella* infection

ABSTRACT

Brucellosis, caused by the intracellular bacterial pathogen *Brucella*, is a zoonotic disease for which arthritis is the most common focal complication in humans. Here we show caspase-1 and caspase-11 initiate *Brucella*-induced joint inflammation, but also restrict *Brucella* replication. In contrast, AIM2 and NLRP3 were dispensable for control of joint infection by *Brucella*. Both IL-1 and IL-18 promoted joint inflammation, however only IL-18 contributed to protection against *Brucella* infection of the joint. *In vitro* studies demonstrated caspase-1 and caspase-11 both induce pyroptosis which limited *Brucella* infection in macrophages. While early IFN- γ production required caspase-1/11 and IL-18, caspase-1/11-dependent clearance of *Brucella* was not entirely IFN- γ dependent. However, IFN- γ deficiency resulted in severe inflammation that was entirely inflammasome dependent and, in particular, reliant on NLRP3. IFN- γ was vital for induction of the nitric oxide (NO) producing enzyme, iNOS, in infected joints, and NO inhibited caspase-1 activation in *Brucella*-infected macrophages *in vitro*. During *Brucella* infection *in vivo*, exacerbated joint inflammation was seen in iNOS deficient mice along with an increase in joint IL-1 β compared to wild-type mice. Collectively these data demonstrate inflammasomes induce inflammation in an IL-18 and IL-1 dependent

manner, and inflammasome-dependent IL-18 and pyroptosis restrict *Brucella* infection. Moreover, IFN- γ reduces inflammation by inhibiting excessive caspase-1 activation through production of NO. Thus NO donors, in combination with antibiotics, may be an effective therapy for treating *Brucella*-induced inflammation.

INTRODUCTION

Identification of inflammatory responses contributing to articular brucellosis have been hindered by the lack of relevant mouse models. We have previously reported i.p. *Brucella* infection in IFN- γ -deficient mice results in arthritis and musculoskeletal inflammation of ankle joints as early as 15 days post-infection. Arthritis in this systemic model was partially dependent on IL-1R and CXCR2, while adaptive immune cells were not required for inflammation (70, 165). It is currently unknown if IFN- γ prevents arthritis by limiting *Brucella* dissemination to the joints, and/or if IFN- γ acts locally in the joint to restrict infection and inflammation. Previously we demonstrated that mice inoculated with *Brucella* locally in footpads resulted in synchronized infection and joint inflammation in wild-type (WT) mice, allowing us to investigate mechanisms of the host response that are specific to the joint (160).

Inflammasomes are multi-protein structures that recognize threats in the host cell cytosol. Canonical inflammasomes are equipped with sensors that detect intracellular antigens and damage associated molecular patterns (DAMPs) (120). This initiates a cascade of protein oligomerization and recruitment resulting in inflammasome assembly. Upon activation, canonical inflammasomes recruit pro-

caspase-1, a cysteine protease, and cleave it into its functional form, caspase-1 (120, 214). Caspase-1 can then activate pro-inflammatory cytokines as well as trigger a programmed inflammatory cell death known as pyroptosis (120, 215). Due to the inflammatory nature of inflammasomes they contribute to many models of inflammation including pathogen induced and sterile arthritis (216, 217).

Brucella infection in macrophages activates the canonical inflammasome cytosolic sensors: NLR family, pyrin domain containing 3 (NLRP3), and absent in melanoma 2 (AIM2). Studies have shown that the *Brucella* Type IV secretion system and mitochondrial damage can stimulate NLRP3, while *Brucella* DNA activates the AIM2 inflammasome pathway (117, 218, 219). Following stimulation of the NLRP3 or AIM2 sensors and subsequent cleavage of caspase-1, active caspase-1 proteolytically cleaves IL-1 β and IL-18 into their functional/secreted forms. IL-1R and IL-18 are known to restrict *Brucella* burdens during chronic *Brucella* infection but may also lead to immunopathology, as they play a pro-inflammatory role in rheumatoid arthritis (117, 136, 137, 219). While NLRP3, AIM2 and caspases-1/11 are protective against chronic systemic *Brucella* infection *in vivo* (117), there are multiple studies indicating NLRP3 and/or AIM2 contribute to pathology in autoreactive arthritis models (217, 220, 221). Thus, while inflammasomes contribute to restriction of systemic *Brucella* infection, they could also induce deleterious joint inflammation conferred by *Brucella*.

Unlike canonical inflammasomes, the non-canonical inflammasome, caspase-11, does not use additional cytosolic protein sensors to identify intracellular threats; rather, caspase-11 directly recognizes, and is activated by,

cytosolic bacterial LPS. Like caspase-1, caspase-11 can also induce pyroptosis which can aid in the clearance of gram-negative intracellular pathogens (215). There is limited knowledge on *Brucella*'s ability to activate caspase-11. In fact, one study showed the *Brucella* effector protein TcpB, can induce degradation of caspase-11 (122). However to our knowledge the role of caspase-11 in response to *Brucella in vivo* has not been directly examined. Here we describe a role for both caspase-1 and caspase-11 in *Brucella* induced arthritis and define the inflammasome effectors responsible for inflammation and control of infection. Additionally, we reveal IFN- γ induced NO is able to suppress inflammasome activation.

RESULTS

Inflammasomes have IL-1-independent effects on inflammation and control of infection during *Brucella*-induced arthritis

Following intraperitoneal *Brucella* infection, IL-1R contributes to the development of *Brucella* induced arthritis in IFN- γ -deficient mice (165). Thus, to examine if inflammasome dependent IL-1 contributes to joint inflammation during local *B. melitensis* infection we infected caspase-1/11^{-/-} and IL-1R^{-/-} mice in the rear footpads (Fig 16). Beyond day 6 post-infection, caspase-1/11^{-/-} mouse ankles displayed variable but slightly increased swelling compared to WT animals, a trend which was also evident when a higher dose of *Brucella* was used for infection (Fig

Figure 16. Caspase-1/11 induces inflammation and controls joint *Brucella* burden.

All mice were infected in both rear footpads with 1×10^5 *B. melitensis* 16M (A-H). WT and caspase-1/11^{-/-} mouse joint swelling was recorded over time (A). Mice were euthanized on day 34 and ankle joint and spleen bacterial loads were enumerated (n=5 mice/group). Data are representative of one experiment (B). Joint swelling was recorded over 7 days (4-5 mice/group) (C). Data representative of >4 experiments. Mice were euthanized on day 7 post-infection and H&E staining was conducted on the rear ankle joints and scored for inflammation severity (D&E) (6-8 mice/group). Images (40x) are depicted with amplified boxed regions (200x) displayed to the right of each image (D). WT and caspase-1/11^{-/-} mice were euthanized at days 1, 2 and 7 post-infection and joint bacterial burdens determined (3-5 mice/group), day 1 data are representative of one experiment, day 2 is representative of > 3 experiments and day 7 is representative of > 3 experiments (F). Joint swelling was measured in infected WT and IL-1R^{-/-} mice over time (G). Mice were euthanized and joint bacterial loads determined at day 7. Data is representative of 2 independent experiments (3-5 mice/group) (H). *, P<0.05 compared to WT mice. Error bars depict S.D. of the mean.

16A and Fig 17A). This long-term study revealed a minor role of caspase-1/11 in systemic clearance of *Brucella* from the spleen but only when a dose of 1×10^5 *B. melitensis* was used for infection (Fig 16B and Fig 17B). Interestingly, caspase-1/11^{-/-} mice exhibited delayed swelling with significantly reduced ankle diameters at days 2 and 3 compared to WT mice (Fig 16C). Consistent with our swelling data, H&E staining revealed decreased inflammation in caspase-1/11^{-/-} joints at day 2 post-infection (Fig 16D-E). Additionally, caspase-1/11^{-/-} mice exhibited lower levels of inflammatory cytokines in their joints compared to WT mice (Fig 18A-F). In contrast to the phenotype observed at days 2 and 3, caspase-1/11^{-/-} mice had increased swelling by days 4-5 when compared to WT animals (Fig 16C). To explain this escalation of swelling in caspase-1/11^{-/-} mice we hypothesized inflammasomes may be important for mediating bacterial clearance in the joints. When joint bacterial loads were examined, caspase-1/11^{-/-} mice had similar CFUs compared to WT at days 1 and 2 post-infection; however, by day 7 caspase-1/11^{-/-} animals had significantly increased *Brucella* loads (Fig 16F). To examine whether IL-1 was inducing this arthritis and musculoskeletal inflammation, swelling of WT and IL-1R^{-/-} mouse ankles was recorded over 7 days post-*Brucella* infection. Interestingly, IL-1R^{-/-} mice only displayed slightly reduced swelling at day 2, and *Brucella* loads at day 7 were similar among groups (Fig 16G&H). This disparity in swelling kinetics and joint *Brucella* burdens between IL-1R^{-/-} and caspase-1/11^{-/-} mice suggests inflammasomes have IL-1R independent effectors that contribute to *Brucella*-induced arthritis and control of infection.

Figure 17. Caspase1/11 deficiency results in delayed resolution of joint swelling.

WT and caspase-1/11^{-/-} mice were infected in each rear footpad with 1×10^6 *B. melitensis* 16M and swelling was monitored (**A**). Mice were euthanized on day 34 post-infection and spleen and joint *Brucella* burdens were enumerated (5 mice/group) (**B**). *, $P < 0.05$ compared to WT mice. Error bars depict S.D. of the mean. Data are representative of one experiment.

Figure 18. Inflammasomes mediate pro-inflammatory cytokine production in *Brucella*-infected joints.

WT and caspase-1/11^{-/-} mice were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. Mice were euthanized at day 2 post-infection and levels of joint cytokines were determined. Data are representative of two independent experiments. (n=3 mice/group) (A-F). *, P<0.05 compared to WT mice. Error bars depict the S.D. of the mean.

Caspase-11 contributes to *Brucella*-induced arthritis and restricts *Brucella* infection in the joint

Many studies utilize caspase-1/11^{-/-} mice to study the role of caspase-1 despite the fact that these animals also lack functional caspase-11. To determine if the non-canonical inflammasome, caspase-11, contributes to the initiation of inflammation and/or the clearance of *Brucella* from the joint, WT and caspase-11^{-/-} mice were infected in the rear footpads with *B. melitensis*. Indeed caspase-11^{-/-} mice displayed reduced swelling at day 2 post-infection, while day 2 joint bacterial loads remained similar to WT (Fig 19A and data not shown). The reduction of joint swelling in caspase-11^{-/-} mice relative to WT animals was not to the same magnitude as that seen in caspase-1/11^{-/-} mice, suggesting a partial role of caspase-11 in inflammasome dependent induction of swelling. Histopathology analysis at day 2 post-infection confirmed caspase-11's inflammatory role, as caspase-11^{-/-} joints had reduced inflammation compared to WT (Fig 19B&C). Additionally, IL-6 and TNF- α levels were significantly decreased in caspase-11^{-/-} joints at day 2 post-infection (Fig 20A-F). Caspase-11^{-/-} mice also displayed increased joint swelling by day 5 post-infection (Fig 19A). To examine the effects of caspase-11 on bacterial clearance, we measured joint bacterial burdens on day 7. Joint CFUs were slightly, but significantly, elevated in caspase-11^{-/-} joints compared to WT mice (Fig 19D). Similar to our swelling data, the difference in CFUs between caspase-11^{-/-} and WT mice was not to the same magnitude as the

Figure 19. Caspase-11 is inflammatory and limits *Brucella* infection in the joints.

All mice were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. Joint swelling was measured over time in WT and caspase-11^{-/-} mice. Data representative of 3 independent experiments (4-5 mice/group) (A). Mice were euthanized at day 2 post-infection and H&E staining was conducted on mouse ankles. Representative images of joint sections from WT and Caspase-11^{-/-} mice are depicted (100x) (B). Severity of pathology was then scored. Histology scores are from one of two independent experiments (4-5 mice/group) from which sections were obtained (C). Mice were euthanized on day 7 post-infection and joint *Brucella* loads were enumerated. Data are representative of 2 independent experiments (4-5 mice/group) (D). NLRP3^{-/-} and AIM2^{-/-} mice were evaluated for joint *Brucella* burdens at day 7 post-infection. Data are representative of 2 independent experiments (4-5 mice group) (E&F). *, $P < 0.05$ compared to WT mice. Error bars depict S.D. of the mean.

Figure 20. Caspase-11 contributes to inflammasome dependent pro-inflammatory cytokine production in *Brucella*-infected joints.

WT and caspase-11^{-/-} mice were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. Mice were euthanized at day 2 post-infection and levels of joint cytokines were determined. Data are representative of one experiments. (n=3 mice/group) (A-F). *, P<0.05 compared to WT mice. Error bars depict the S.D. of the mean.

difference between caspase-1/11^{-/-} and WT mice. To assay what canonical inflammasome could be involved in caspase-1 dependent control of *Brucella* infection, NLRP3^{-/-} and AIM2^{-/-} mice were infected and evaluated for *Brucella* burdens at day 7 post-infection. Interestingly, NLRP3^{-/-} nor AIM2^{-/-} mice displayed impaired ability to control joint *Brucella* burdens (Fig 19E&F).

Caspase1/11-dependent IL-18 mediates joint swelling and *Brucella* clearance from the joint

In addition to IL-1 β , inflammasomes also activate IL-18. To investigate if IL-18 is an inflammasome-dependent effector of *Brucella*-induced arthritis, IL-18 was neutralized in WT and caspase-1/11^{-/-} mice and the impact on arthritis assessed. Joint swelling 2 days post-infection was reduced in anti-IL-18-treated mice compared to WT animals, but IL-18 neutralization did not have as great of an effect as caspase-1/11^{-/-} deficiency. Additionally, the effects of IL-18 relied on caspase-1/11, as swelling in caspase-1/11^{-/-} mice was similar to caspase-1/11^{-/-}-anti-IL-18 treated animals (Fig 21A). Bacterial loads at day 2 post-infection were similar between both WT and caspase-1/11^{-/-} joints either sufficient or neutralized of IL-18 (Fig 21B). To examine if IL-18 contributes to swelling or joint bacterial restriction beyond the initial initiation of inflammation, swelling in IL-18^{-/-} mice was monitored over the course of 7 days. IL-18^{-/-}/IL-1R^{-/-} mice were also included in our study to further examine the role of IL-1R in our model. IL-18^{-/-} mice only had reduced joint swelling at day 2 post-infection while IL-18^{-/-}/IL-1R^{-/-} mice had significantly reduced

Figure 21. IL-18 contributes to inflammasome dependent joint inflammation and *Brucella* clearance.

WT or caspase-1/11^{-/-} mice were treated with an IgG isotype control or anti-IL-18 then infected in each rear footpad with 1×10^5 *B. melitensis* 16M (A-B). At day 2 post-infection ankle swelling was recorded (A) and joint CFUs were enumerated. Data are combined from 2 separate experiments 8-10 mice/group total (B). WT, IL-18^{-/-} and IL-1R/IL-18^{-/-} mice were infected in the rear footpad with 1×10^5 *B. melitensis* 16M and swelling was recorded over time (C). Mice were euthanized on day 7 post-infection and joint bacterial burdens determined. Data are representative of 2 independent experiments (4-5 mice/group) (D). Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

ankle widths at day 2 and 3 when compared to WT mice (Fig 21C). By day 7 post-infection, IL-18 deficiency resulted in increased joint *Brucella* burdens, however IL-1R deficiency in IL-18^{-/-} mice had no additive effect on control of joint infection (Fig 21D). These data indicate IL-18 and IL-1R are involved in the early induction of swelling during *Brucella*-induced arthritis, but IL-18 alone is required for restriction of *Brucella* infection in the joint. Relative to WT mice however, caspase-1/11 deficiency resulted in increased bacterial burdens and had a greater effect on joint swelling than IL-18/IL-1R deficiency. This suggests IL-18 and IL-1 are not the sole effectors of caspase-1/11.

Eicosanoids and perforin-mediated cytotoxicity are dispensable for control of *Brucella* infection in the joint

Although inflammasome activation of IL-1 β and IL-18 are the best characterized modes of caspase-1/11-dependent antimicrobial activity, inflammasomes can protect against infection via other means, including inducing eicosanoid storms (222). Thus, we treated *Brucella* infected mice with the eicosanoid inhibitor, indomethacin. Indomethacin treatment reduced joint swelling at day 2 post-infection, but no significant difference in joint *Brucella* loads was observed at day 7 post-infection relative to WT mice (Fig 22A&B). In fact, there was a trend of reduced *Brucella* burdens in indomethacin treated mice indicating eicosanoids are not bactericidal but actually may aid in *Brucella* colonization of the joint (Fig 22B). Additionally, IL-18 has recently been shown to activate perforin-mediated cytotoxicity as a mode of intracellular bacterial clearance (134). Therefore, the role of cytotoxicity was evaluated using perforin^{-/-} (perf^{-/-}) mice,

Figure 22. Eicosanoids and perforin are dispensable for control of *Brucella* infection in the joint.

Mice were either treated with indomethacin or a vehicle and were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. At day 2 post-infection ankle swelling was measured (A). Mice were euthanized at day 7 post-infection and joint CFUs were enumerated (5 mice/group) (B). *, $P < 0.05$ compared to vehicle-treated mice. Data are representative of 2 independent experiments. WT and perf^{-/-} mice were infected in each rear footpad with 1×10^6 *B. melitensis* 16M. Mice were euthanized at day 6 post-infection and joint *Brucella* burdens were evaluated (5 mice/group) (C). Data are representative of one experiment. Error bars depict S.D. of the mean.

but no notable changes in joint *Brucella* loads was observed (Fig 22C).

Caspase-1 and caspase-11 induce cell death in response to *Brucella* and restrict infection in macrophages.

Pyroptosis and subsequent efferocytosis of dead host cells containing bacteria can be an important antimicrobial mechanism of inflammasomes (215). To demonstrate if pyroptosis occurs during *Brucella* infection, BMDMs were infected with *B. melitensis* and evaluated for cell death at 24 and 48 hours post-infection. *Brucella* induced robust cell death in WT macrophages, while cell death was reduced in caspase-11^{-/-} macrophages and abolished in caspase-1/11^{-/-} cells at both 24 and 48 hours post-infection, indicating both caspase-1 and caspase-11 contribute to *Brucella*-induced macrophage death. Interestingly NLRP3^{-/-} and AIM2^{-/-} mice had similar amounts of cell death as WT macrophages, suggesting NLRP3 or AIM2 alone are not required to induce pyroptosis (Fig 23A&B). We also observed elevated *Brucella* levels in wells with caspase-1/11^{-/-} and caspase-11^{-/-} BMDMs at 24 and 48 hours post infection (Fig 23C&D). Similarly, joint cells from footpad infected caspase-1/11^{-/-} mice displayed reduced Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL), which identifies DNA fragmentation during late stage apoptosis or pyroptosis, compared to WT mice (223) (Fig 24A-B). To determine if caspase-11 could be directly activated by *Brucella* LPS, we treated peritoneal macrophages with purified *Brucella abortus* S19 LPS, which has a similar structure to *B. melitensis* LPS (224). We found that *Brucella* LPS was able to induce caspase-11-dependent cell death,

Figure 23. *Brucella* induces caspase-1 and caspase-11 dependent pyroptosis.

WT, caspase-1/11^{-/-}, caspase-11^{-/-}, NLRP3^{-/-} and AIM2^{-/-} BMDMs were infected with *B. melitensis* 16M at an MOI of 100 (A-D). Supernatants were harvested at 24 and 48 hours and % cell death was determined by LDH release (A&B). Remaining cells were lysed and *Brucella* loads determined (C&D). Data are representative of 2 independent experiments (4 wells/group). Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

Figure 24. *Brucella* infection induces caspase-1/11-dependent cell death *in vivo*, and transfection of *Brucella* LPS induces caspase-11 dependent cell death *in vitro*.

WT and caspase-1/11^{-/-} mice were infected in each rear footpad with 1x10⁵ *B. melitensis* 16M. Mice were euthanized at day 2 post-infection and joints were evaluated for TUNEL staining via flow cytometry. Depicted is a representative histogram of the proportion of TUNEL⁺ cells in WT versus caspase-1/11^{-/-} mice (**A**), and the percentage of TUNEL positive cells (n=3-5mice/group) in each mouse strain (**B**). *, P<0.05 compared to WT mice. Data are representative of one experiment. WT or caspase-11^{-/-} macrophages were treated with *Brucella* LPS alone, or *Brucella* LPS packaged in Fugene. After 9 hours of culture supernatant was harvested and assessed for cell death. Data are representative of one experiment (3 wells/group) (**C**). Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

and this phenotype was enhanced upon the addition of the transfection reagent Eugene, confirming cytosolic *Brucella* LPS is capable of inducing caspase-11-dependent cell death (Fig 24C). These data suggest caspase-11 and caspase-1 induced pyroptosis is protective against *Brucella* infection.

Inflammasomes do not restrain *Brucella* infection due to their ability to restrict host L-lactate production

Previous studies have demonstrated that *B. abortus* thrives in glycolytic cells via *B. abortus*' ability to utilize host lactate via the *Brucella* L-lactate dehydrogenase gene (*lldD*) (101, 225). However, inflammasome activation resulting in pyroptosis, releases host cell lactate dehydrogenase which can degrade L-lactate (226). Additionally, other studies suggest active caspase-1 can directly inhibit glycolysis and subsequent production of L-lactate via cleavage of glycolytic enzymes (227). To determine if caspase-1/11 was protective against *Brucella* via its ability to restrict L-lactate, WT and caspase-1/11^{-/-} bone marrow derived macrophages (BMDMs) were infected with *B. melitensis*, and L-lactate in cell supernatant was measured. L-lactate production was induced during *B. melitensis* infection in both WT and caspase-1/11^{-/-} macrophages; however, L-lactate levels in wells containing caspase-1/11^{-/-} cells were elevated compared to WT (Fig 25A). While WT and caspase-1/11^{-/-} cells had similar rates of *Brucella* phagocytosis (data not shown), caspase-1/11^{-/-} BMDMs had increased *Brucella* burden at 48 hours post-infection (Fig 25B). In order to determine if glycolysis and subsequent production of L-lactate aided in *Brucella* survival, we inhibited glycolysis using 2-deoxy-glucose (2-DG) in *B. melitensis* infected BMDMs. At

Figure 25. Increased lactate production by Caspase-1/11^{-/-} macrophages does not promote *Brucella* infection.

BMDM strains were infected for 6 hours with *B. melitensis* 16M at and MOI of 100 as described in Materials and Methods (A-F). At 48 hours post-infection L-lactate was measured in cell supernatant (A). Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. At 48 hours post-infection cells were lysed and *Brucella* loads were determined. Data representative of 2 independent experiments with 6-8 wells/group. *, P < 0.05 compared to WT infected BMDMs (B). Some WT wells were treated with 2-DG following infection. At 48 hours IL-1β was measured in cell supernatants (C) and *Brucella* loads were determined (D). Data are representative of 2 independent experiments (3-8 wells/group) *, P < 0.05 compared to WT as determined by ANOVA followed by Dunnett's test. WT BMDMs were infected with either *B. melitensis* or *B. melitensis* Δ*lIdD*. At 48 hours post-infection intracellular *Brucella* burdens was determined. Data are representative of one of two independent experiments (6 wells/group) *, P < 0.05 compared to WT *B. melitensis* (E). BMDMs were co-infected with a 1:1 ratio of Δ*lIdD* and an Δ*lIdD* complemented strain of *B. melitensis*. 48 hours post-infection cells were lysed and the competitive index was determined in each BMDM strain. Data are representative of two independent experiments 5 wells/group (F). Error bars depict S.D. of the mean.

1000 μ M, 2-DG reduced both intracellular *B. melitensis* and IL-1 β release, while 100 μ M 2-DG only inhibited IL-1 β release (Fig 25C&D). Thus, 2-DG may inhibit inflammasome-dependent IL-1 β production, and intracellular survival of *Brucella*. Interestingly, *Brucella* infected mice treated with 2-DG exhibited unaltered *Brucella* loads, but reduced swelling, relative to vehicle treated mice (Fig 26A&B), suggesting 2-DG may also inhibit inflammasome dependent inflammation *in vivo*.

As an alternative to targeting host cell metabolism, we made a *B. melitensis* L-lactate dehydrogenase mutant (Δ *lldD*), to inhibit *Brucella*'s ability to utilize free L-lactate (101). Δ *lldD* was attenuated in WT macrophages compared to WT *B. melitensis*, (Fig 25E). Therefore, we investigated whether *lldD* conferred a greater competitive advantage in caspase-1/11^{-/-} cells than in WT macrophages due to enhanced lactate production. Thus, we performed competition experiments between Δ *lldD* and a complemented Δ *lldD* strain in both WT and caspase1/11^{-/-} cells. We found that our complemented strain out-competed our mutant in both WT and caspase-1/11^{-/-} macrophages; however, the competitive index of the *lldD*-complemented strain compared to Δ *lldD* was not significantly different between macrophage strains. These data indicates caspases1/11 does not restrict *Brucella* survival via their ability to reduce L-lactate production (Fig 25F).

IFN- γ restrains Caspase-1/11-induced joint inflammation

We previously demonstrated IFN- γ prevents inflammation and joint infection in a systemic model of *Brucella*-induced arthritis (70). In addition, we have found that caspase-1 dependent IL-18 induces protective IFN- γ in response to

Figure 26. 2-DG prevents swelling during *Brucella* infection.

Mice were infected in both rear footpads with *B. melitensis* 16M and were treated i.p. with 2-DG on days 1 and 3 post-infection. Swelling was recorded over time (**A**) and joint *Brucella* burdens were measured at day 7 post-infection (**B**). Data is reported as the difference in swelling from the start of 2-dG treatment (day 1 post-infection). Data is representative of one experiment 4-5 mice/group. *, $P < 0.05$ compared to WT mice. Error bars depict S.D. of the mean.

Francisella tularensis (228). Therefore, the effects of inflammasomes on IFN- γ production in *Brucella*-induced arthritis were investigated. At day 2 post-*Brucella* infection, IFN- γ levels were elevated in WT joints compared to caspase-1/11^{-/-} mice (Fig 18B). IL-18 deficiency also resulted in significantly reduced joint IFN- γ levels, suggesting caspase-1/11-dependent IL-18 could control *Brucella* colonization in the joint (Fig 27). To investigate whether IFN- γ was protective during focal infection and if this protection was inflammasome dependent, we treated WT and caspase-1/11^{-/-} mice with anti-IFN- γ or an isotype control. IFN- γ neutralized WT mice displayed markedly enhanced joint swelling by day 3 post-infection and everyday thereafter compared to all other groups (Fig 28A). This severe joint swelling was associated with an increase in myeloid cell recruitment including a robust increase in neutrophils and macrophages (Fig 29A&B). In contrast, IFN- γ neutralization in caspase-1/11^{-/-} mice did not result in enhanced joint swelling; rather, it reduced swelling at days 3-4 post-infection. Histological analysis at day 7 post-infection demonstrated caspase-1/11^{-/-}-anti-IFN- γ treated mice had similar joint inflammation scores as WT and caspase-1/11^{-/-} IgG-treated mice. In contrast, WT-anti-IFN- γ treated mice had significantly greater inflammation scores compared to all other groups examined (Fig 28B&C). IL-1 β was also elevated in joint homogenates of WT-anti-IFN- γ treated mice compared to all other groups, possibly indicating increased caspase-1 activation in the absence of IFN- γ (Fig 30). At day 7 post infection, we found mice deficient in either IFN- γ or caspase-1/11 had increased *Brucella* burdens; however, caspase-1/11^{-/-} mice neutralized of IFN- γ had higher *Brucella* joint colonization than WT mice neutralized of IFN- γ .

Figure 27. IL-18 contributes to IFN- γ production in *Brucella* infected joints. WT mice were treated with anti-IL-18 or a vehicle control, then mice were infected in each rear footpad with *B. melitensis* 16M. Mice were euthanized at day 2 post-infection and joint IFN- γ was determined (n=5mice/group) Data are representative of one experiment. *, P<0.05 compared to vehicle-treated mice. Error bars depict S.D. of the mean.

Figure 28. IFN- γ deficiency results in severe caspase-1/11-dependent inflammation.

WT and caspase-1/11^{-/-} mice were treated with anti-IFN- γ or an isotype control, then infected in both rear footpads with 1×10^5 *B. melitensis* 16M. Joint swelling was measured throughout the experimental time course (**A**). Mice were euthanized at day 7 post-infection and H&E staining was conducted and slides were scored for inflammation (**B&C**). Depicted are representative images (100X) from each group with amplified boxed regions (400X) displayed to their right (**B**). Joint *Brucella* burdens were also enumerated 7 days post-infection (**D**). Data are representative of 3 independent experiments with 3-5 mice/group except for panel C, in which data was combined from 2 experiments with 10-13 mice/group. Means/medians with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test or ANOVA on ranks. Error bars depict S.D. of the mean.

Figure 29. IFN- γ deficiency leads to increased myeloid cell recruitment in *Brucella* infected joints.

Select mice were treated with an anti-IFN- γ depleting antibody. Then, mice were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. Mice were euthanized at 7 days post-infection and flow cytometry was conducted on joints from infected mice and naïve controls (**A&B**). Depicted are histograms of CD11b⁺ cells in individual mice (**A**), and bar charts indicating the average number of neutrophils or macrophages in each group (n=4-5 mice/group) (**B**). Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean. Data are representative of one experiment.

Figure 30. IFN- γ deficiency leads to increased inflammasome dependent IL-1 β *in vivo*.

WT and caspase-1/11^{-/-} mice were treated with anti-IFN- γ or an isotype control, then infected in both rear footpads with 1×10^5 *B. melitensis* 16M. Mice were euthanized at day 7 post-infection. IL-1 β was measured from joint homogenates (4-5 mice/group). Data are representative of 2 independent experiments. Means with the same letter are not statistically different from each other as determined via ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

This indicates that while IL-18 induced IFN- γ may be partially responsible for inflammasome-dependent protection, caspase-1/11 has additional IFN- γ independent means of restricting *Brucella* infection (Fig 28D).

Adaptive immune cell IFN- γ production contributes to *Brucella* clearance

We have previously demonstrated that T-cells make IFN- γ upon footpad infection with *Brucella*. Additionally, NK cells can be important producers of IFN- γ and $\gamma\delta$ T-cells have been reported to make IFN- γ upon *Brucella* infection (163, 229). In order to determine what cells were making protective IFN- γ in our model we neutralized NK cells or $\gamma\delta$ T-cells from WT mice (Fig 31A&B). Both NK-cell neutralized mice and $\gamma\delta$ T-cell neutralized mice displayed similar swelling and joint *Brucella* burdens at day 7 post-infection, indicating NK-cells or $\gamma\delta$ T-cell are not essential for protective IFN- γ in our model. Similarly, we compared control WT and Rag1^{-/-} mice to those neutralized of IFN- γ and evaluated joint CFUs at day 7 post-infection. Rag1^{-/-} mice only had an effect on joint *Brucella* burdens in mice sufficient in IFN- γ , indicating adaptive immune cell IFN- γ is important for joint *Brucella* clearance (Fig 31 C&D).

NLRP3 and Caspase-11 are partially responsible for inducing inflammation in IFN- γ deficient mice.

In order to identify what inflammasomes contribute to arthritis and if these phenotypes are altered in the absence of IFN- γ , we treated WT and NLRP3^{-/-} mice

Figure 31. Adaptive immune cell IFN- γ production is important for *Brucella* clearance.

Mice were treated i.p. with anti-NK-cell antibody or anti- $\gamma\delta$ T-cell antibody 1 day prior and 3 days after infection in both rear footpads with 1×10^5 *B. melitensis* 16M. Joint swelling (**A**) and CFUs (**B**) were evaluated 7 days post-infection. Data are representative of one experiment 5 mice/group. In a separate study WT and Rag1^{-/-} mice were treated with anti-IFN- γ or an isotype control, then infected in each rear footpad with 1×10^5 *B. melitensis* 16M. At 7 days post-infection mice were euthanized and joint CFUs were enumerated (**C&D**). Data are representative of 1 experiment 3-5 mice/group. *, $P < 0.05$ compared to WT mice. Error bars depict S.D. of the mean.

with anti-IFN- γ or an isotype control. Only a mild reduction in swelling was seen in the isotype-treated NLRP3^{-/-} mice compared to WT isotype controls at day 2 post infection. Interestingly, in the absence of IFN- γ , NLRP3 deficiency reduced joint swelling throughout infection (Fig 32A). We also examined AIM2's role in inducing joint inflammation; however, AIM2 appeared to be dispensable for the induction of swelling both in the presence and absence of IFN- γ (Fig 32B). Swelling in IFN- γ neutralized mice was partially attributed to the non-canonical inflammasome, as caspase-11^{-/-}-anti-IFN- γ treated mice had reduced joint swelling at days 5 and 7 compared to WT, IFN- γ neutralized mice (Fig 32C).

IFN- γ induces the upregulation of iNOS, and iNOS deficiency enhances joint inflammation

To explain why NLRP3 plays a major role in swelling only in the absence of IFN- γ and why inflammation in IFN- γ -deficient mice is caspase1/11-dependent, we considered IFN- γ may be inhibiting inflammasome activation. Others have found that IFN- γ induced nitric oxide (NO) can inhibit NLRP3 and caspase-1 (161, 230, 231). Thus, we hypothesized the NO producing enzyme, inducible nitric oxide synthase (iNOS), may be enhanced by IFN- γ during *Brucella* infection of the joint. Hence, we examined iNOS transcript in joints from infected mice treated with anti-IFN- γ or an isotype control. At day 2 post-infection, isotype control mice displayed increased iNOS transcript compared to IFN- γ -deficient mice (Fig 33A). IFN- γ was also critical for iNOS mRNA expression at later time points (data not shown). To determine if iNOS was protective against swelling, ankle widths of iNOS deficient

Figure 32. NLRP3 and Caspase-11 induce inflammation in the absence of IFN- γ .

WT and NLRP3^{-/-} mice were treated with anti-IFN- γ or an isotype control, then infected in their rear footpads with 1×10^5 *B. melitensis* 16M. Rear ankle joint swelling was measured overtime. Data are combined from 2 independent experiments (9-10 mice/group total) **(A)**. WT and AIM2^{-/-} mice were treated with anti-IFN- γ or sPBS, then infected in the rear footpads with 1×10^5 *B. melitensis* and ankle swelling was monitored overtime. Data are representative of 2 independent experiments (4-5 mice/group) **(B)**. WT and Caspase-11^{-/-} mice were both treated with anti-IFN- γ then infected with 1×10^5 *B. melitensis* 16M in both rear footpads. Ankle swelling was evaluated for 7 days. Data are combined from 3 separate experiments (13-18 mice/group total) **(C)**. Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

Figure 33. Nitric oxide inhibits *Brucella*-induced caspase-1 activation and IL-1 β production.

WT mice were treated with either anti-IFN- γ or an isotype control, then mice were infected in the rear footpads with 1×10^5 *B. melitensis* 16M. Mice were euthanized at day 2 post-infection along with naïve mice and RNA was isolated from the ankle joints and relative iNOS transcript was then determined. Data are representative of one experiment (3-4 mice/group). *, $P < 0.05$ compared to WT mice (**A**). In a separate experiment, WT mice received anti-IFN- γ while other WT mice along with NOS2^{-/-} mice received an isotype control. Mice were infected in the rear footpads with 1×10^5 *B. melitensis* 16M and monitored for swelling (**B**). Mice were euthanized at day 7 post-infection and joint CFUs and IL-1 β were enumerated (**C&D**). Data are representative of one experiment (4-5 mice/group). WT BMDMs were infected with *B. melitensis* 16M at an MOI of 100 for 6 hours. Media containing increasing doses of SNAP was added immediately after infection. 24 hours post-infection intracellular *Brucella* burdens were enumerated (**E**) and IL-1 β levels in cell supernatants were determined (**F**). Cell supernatants and cell lysates were also subjected to western blot analysis (**G**). Data is representative of 2 independent experiments with 3-5 wells/group. Means with the same letter are not statistically different from each other as determined by ANOVA followed by Tukey's test. Error bars depict S.D. of the mean.

mice (NOS2^{-/-}) were evaluated following *Brucella* infection. NOS2^{-/-} mice displayed increased swelling relative to WT-IgG mice, but reduced swelling compared to WT-anti-IFN- γ treated animals (Fig 33B). At day 7 post-infection, iNOS deficiency also resulted in increased joint *Brucella* burdens relative to WT mice, but this difference was not as pronounced as that observed with anti-IFN- γ treatment (Fig 33C). NOS2^{-/-} mice also had similarly elevated joint IL-1 β as WT IFN- γ neutralized mice (Fig 33D), despite bacterial loads nearly 100-fold lower, indicating NO could limit inflammation by mechanisms beyond controlling *Brucella* burdens. Similarly, mice that received the iNOS inhibitor, aminoguanidine hemisulfate salt (AGHS), in their drinking water had increased joint inflammation and *Brucella* burdens at day 18 post-infection relative to controls (Fig 34A&B). We also observed IL-1 β was upregulated in the joints of AGHS treated animals compared to animals receiving regular drinking water (Fig 34C).

Nitric oxide inhibits *Brucella*-induced caspase-1 activation

To determine if NO can directly inhibit inflammasome activation during *Brucella* infection, BMDMs were infected with *B. melitensis* then treated with increasing doses of the NO donor, S-nitroso-N-acetyl-penicillamine (SNAP). *In vitro* media conditions and infection times were optimized so that intracellular *Brucella* loads were similar in WT and SNAP treated wells at the time of cell lysate and supernatant harvest (Fig 33E). Analysis of IL-1 β in cell supernatants at 24 hours post-infection demonstrated reduced IL-1 β release upon administration of SNAP (Fig 33F). Western blot analysis confirmed that caspase-1 is activated by

Figure 34. *In vivo* inhibition of iNOS with AGHS results in enhanced joint inflammation.

Some mice were given drinking water containing 2.5% AGHS while others were given normal drinking water. All mice were infected in each rear footpad with 1×10^5 *B. melitensis* 16M. Animals were euthanized at day 18 post-infection and H&E staining was conducted. Representative images (100x) from mice receiving normal drinking water or AGHS are depicted (**A**). *Brucella* burdens (**B**) and IL-1 β (**C**) were also evaluated in joint homogenates (n=4-5mice/group). *, P<0.05 compared to mice receiving normal drinking water. Error bars depict S.D. of the mean. Data are representative of one experiment.

Brucella infection, as cleaved caspase-1 was found in the cell supernatant of infected but not uninfected macrophages. Furthermore, supernatants from *Brucella* infected cells treated with SNAP contained reduced activated caspase-1 compared to vehicle treated (Fig 33G).

DISCUSSION

Globally brucellosis is a prevalent zoonotic disease and *Brucella*-induced arthritis is the most common focal complication of human brucellosis, occurring in 40-80% of patients (56, 57). Here we investigated the role of inflammasomes in a murine model of *Brucella* induced arthritis. We show that inflammasomes initiate inflammation and arthritis in mouse ankle joints. Changes in articular inflammatory responses due to inflammasomes at day 2-post-infection were not dependent on joint *Brucella* burden, as all mouse genotypes examined in this study had similar joint *Brucella* loads at this time. Thus, day 2 was used to determine differences in initiation of joint inflammation.

Caspase-1 and caspase-11 both initiated a joint inflammatory response at day 2 post-*Brucella* footpad infection. Inflammasomes play a major role in inducing pathology in rheumatoid arthritis due to their ability to process IL-1 and IL-18 into their active forms (136, 217). Indeed, caspase-1/11 dependent inflammation was partially reliant on IL-18, while only modest inflammatory effects of IL-1 were observed. Although inflammasomes did not influence *Brucella* burdens early in infection we did uncover an antimicrobial role for caspase-1 and caspase-11 beyond day 6 post-infection. Caspase-1/11, ASC, AIM2 and NLRP3 have all been

shown to be protective against systemic *Brucella abortus* colonization 4 weeks following i.p. inoculation (117), but nothing is known about caspase-11's anti-microbial role. Here we demonstrated caspase-1/11 reduced joint *Brucella* loads by roughly one log and ~1/3 of this phenotype was attributed to caspase-11. Caspase-11 traditionally recognizes enterobacteria LPS with hexa or penta-acylated lipid A (232, 233). *Brucella*'s LPS structure is known to be highly immunoevasive in part due to its hepta-acylated lipid A, core oligosaccharide and its O-polysaccharide (224). Here we demonstrated caspase-11 contributes to joint inflammation, and both live *Brucella* and *Brucella* LPS induce caspase-11-dependent cell death. Similarly, *Acinetobacter baumannii*, another Gram-negative bacterium with hepta-acylated lipid A, has also been shown to activate caspase-11, suggesting caspase-11's repertoire of lipid A recognition may be more diverse than once thought (234, 235).

The bactericidal activity of caspase-1 and caspase-11 may be in part due to their ability to induce pyroptosis. This inflammatory form of cell death can damage intracellular bacteria and render them confined to pore-induced intracellular traps thereby allowing bacteria to be easily killed via efferocytosis (215). In *Brucella* infected macrophages, we observed caspase-1 and caspase-11 dependent cell death, suggesting infected macrophages die via pyroptosis. Wells with reduced cell death contained increased *Brucella* burdens per well, indicating pyroptosis may protect the host by eliminating macrophages as a reservoir of *Brucella* infection. This was surprising, as canonically *Brucella* is thought to inhibit the death of host cells (154). Additionally, we demonstrated that transfection of

macrophages with *B. abortus* S19 LPS results in induction of caspase-11 dependent cell death. To our knowledge this is the first report of caspase-11 recognizing *Brucella* LPS, although one study did demonstrate chemical inhibition of caspases 4 and 5 (human homologues of murine caspase-11) reduces *Brucella* LPS-induced upregulation of Annexin V on human neutrophils (236). DAMPs generated by caspase-11 dependent pyroptosis can activate NLRP3 and in turn caspase-1 (237). NLRP3 did not influence joint *Brucella* burdens, but caspase-11 was protective against *Brucella* colonization. Similarly, in our *in vitro* studies caspase-11 was required for inducing death of macrophages, while NLRP3 was dispensable, identifying caspase-11 has effects independent of the NLRP3-caspase-1 axis. Taken together these results suggest caspase-11 induces cell death and leads to restriction of *Brucella* infection.

Elevated lactate levels are observed in multiple forms of arthritis, including septic arthritis and rheumatoid arthritis (238). Patients with *Brucella* induced arthritis display similar synovial levels of lactate as patients with rheumatoid, or crystal-induced arthritis, but have higher lactate levels than osteoarthritis patients (239). Pyroptosis releases lactate dehydrogenase into the extracellular environment which can subsequently cause degradation of free L-lactate (240, 241). Alternatively, active caspase-1 can directly inhibit glycolysis and in turn L-lactate by cleaving important glycolytic enzymes such as α -enolase, aldolase and GAPDH (227). In an attempt to determine if inflammasomes restricted intracellular *Brucella* survival due to a low lactate environment, we inhibited glycolysis and subsequent lactate production using 2-DG in host macrophages. Treatment with

2-DG reduced *Brucella* macrophage burdens; however, glycolysis inhibition also hindered IL-1 β release, indicating 2-DG may impede inflammasome activity or IL-1 β mRNA expression. Glycolysis is important for NLRP3 inflammasome priming and has been shown to induce IL-1 β in myeloid cells in response to LPS (242-244). We demonstrated inhibiting glycolysis with 2-DG reduced swelling but did not alter joint *Brucella* loads in vivo. Further, experiments should determine if increasing doses of 2-DG or different glycolysis inhibitors could reduce *Brucella* burdens. Thus, glycolysis inhibitors could serve as potential therapeutics for *Brucella*-induced inflammation and infection due to their ability to inhibit excessive inflammasome-dependent inflammation while at the same time limiting lactate production and thus the survival of *Brucella*.

To address if inflammasomes restricted *Brucella* survival by reducing L-lactate, without potentially altering inflammasome priming or activation, we made a *Brucella* mutant defective in L-lactate dehydrogenase. Similar to another study using *B. abortus* (101), we found that *Brucella* L-lactate dehydrogenase was important for intracellular *B. melitensis* survival. However, inflammasomes do not appear to aid in *B. melitensis* restriction through their ability to limit L-lactate, as the Δ lIdD complemented strain did not outcompete Δ lIdD to a greater degree in caspase-1/11-deficient cells relative to WT BMDMs. During acute or focal infection, there may be a requisite of L-lactate for *Brucella* survival in the host, suggesting host factors that induce L-lactate production may aid in *Brucella*'s intracellular viability. Host metabolism is critical to *Brucella* infection, as the type of metabolism *Brucella* prefers may depend on the stage of infection (100, 101). One group

demonstrated that in a model of chronic infection *Brucella* shifts host metabolism to fatty acid oxidation in order to utilize free host glucose (100). Thus, *Brucella* may prefer a glycolytic environment during acute or focal infection and beta-oxidation conditions during chronic infection.

Protection conferred by inflammasomes is due in part to IL-18. At day 7 post-infection restriction of *Brucella* burdens in the joint was partially dependent on IL-18, but independent of IL-1R signaling in the presence or absence of IL-18. Additionally, IL-1R was not responsible for protection during chronic joint infection (data not shown). Caspase-1/11 contributed to bacterial resistance to a greater extent than IL-18, indicating there are other inflammasome dependent mechanisms such as pyroptosis that contribute to *Brucella* restriction. IL-18 can induce production of IFN- γ , which is a well-known mediator of protection against *Brucella* infection and chronic *Brucella*-induced arthritis (70, 160). Some pathogen induced models have demonstrated IFN- γ is secreted due to activation of inflammasomes and subsequent IL-18 production (228, 245). We found the bactericidal actions of caspase-1/11 and IFN- γ were additive indicating the antimicrobial activity of caspase-1/11 is not completely dependent on inflammasome production of IFN- γ . Interestingly, the abrogation of IFN- γ induced severe swelling and inflammation in WT mice starting at day 3 post-infection, and this inflammation was almost entirely dependent on caspase-1/11.

Prior to these studies, it was unclear if the protective effect of IFN- γ against *Brucella* induced arthritis following intraperitoneal infection was due to restricting hematogenous spread of *Brucella* to the joint or if IFN- γ acted locally to suppress

arthritis (70, 165). Here we show IFN- γ acts within the joint to reduce *Brucella* replication and inflammation, as footpad infected IFN- γ -deficient mice developed a more severe arthritis with increased joint *Brucella* burdens compared to WT mice. We previously demonstrated lymphocytes do not play a major role in the induction of arthritis in this model (160). Here we determined IFN- γ neutralization enhances *Brucella* burdens in both Rag1^{-/-} mice and WT mice. This indicates there are cells independent of traditional lymphocytes that produce IFN- γ . Additionally, adaptive immune cell production of IFN- γ is important for *Brucella* clearance, and NK cells or $\gamma\delta$ T-cells are not essential for protection against joint infection. Thus, the innate source of IFN- γ remains unknown in our model. Tissue-resident type 1 innate lymphoid cells are known for their fast-acting production of IFN- γ in response to pathogens and may be the protective IFN- γ producing cell type (246, 247).

The ASC-dependent inflammasomes, AIM2 and NLRP3, are known to recognize *Brucella* and restrict *B. abortus* splenic burdens during chronic infection (117, 219). Surprisingly, AIM2 and NLRP3 were dispensable for control of joint infection. NLRP3 or AIM2 can compensate for one another as has been described in pulmonary aspergillosis, which could explain why we did not observe altered *Brucella* joint burdens in mice only lacking NLRP3 or AIM2 (248). Alternatively, caspase-1 may have an AIM2/NLRP3 independent activator specific to control of joint tissue. In addition to being dispensable for joint *Brucella* clearance, AIM2 did not induce joint swelling in the presence or absence of IFN- γ . NLRP3 contributed modestly to joint swelling in the presence of IFN- γ , but only at day 2 post-infection.

In contrast, NLRP3 played a major role in joint swelling in IFN- γ neutralized mice throughout infection. Chronic human brucellosis patients develop an impaired IFN- γ response to *Brucella* compared to patients at the onset of disease (148). Thus, during chronic *Brucella* infection, attenuated IFN- γ production may lead to enhanced NLRP3 activation and deleterious inflammation.

IFN- γ can induce the NO producing enzyme iNOS. We found that iNOS expression in *Brucella*-infected mouse joints is IFN- γ -dependent. Although NO is generally thought to be inflammatory it can have immunosuppressive effects and is known to restrict *Brucella* replication in macrophages (143). Here we demonstrated iNOS deficiency increased arthritis and joint *Brucella* burden. Furthermore, iNOS deficiency enhanced production of IL-1 β in the joint, possibly indicating increased inflammasome activation. NO can modulate signaling of kinases and transcription factors via thiol nitrosylation (s-nitrosylation), the addition of an NO group to a protein cysteine residue (249). NO can inhibit NLRP3 and caspase-1 activation by s-nitrosylation of the proteins' active site (231, 250). This results in failed recruitment and activation of caspase-1, thus suppressing several key inflammatory processes (161, 230, 250). The immunosuppressive effect of NO is protective in diseases with severe immunopathology such as LPS-induced septic shock (250), and in a murine model of tuberculosis (161). Similarly, IFN- γ /NO-dependent inhibition of inflammasome activation controls *Brucella*-induced inflammation, as we found that the NO donor, SNAP, inhibits caspase-1 activation *in vitro*, and that IFN- γ is critical for controlling inflammasome-dependent pathology during *Brucella* infection *in vivo*. Unregulated inflammasome activation in the

absence of IFN- γ could also lead to mortality, as one study demonstrated T cell or IFN- γ deficiency resulted in similarly elevated *Brucella* burdens in the spleen, lung and liver following intranasal infection; however, IFN- γ deficient mice developed neutrophilia and succumbed to infection while T cell deficient mice do not (142). This suggests disease severity and mortality in IFN- γ deficient mice is due to overwhelming pathology, possibly caused by an inability to inhibit inflammasome induced inflammation. Human brucellosis patients are more likely to have polymorphisms associated with reduced IFN- γ (145, 251); thus, impaired IFN- γ production in these patients could lead to deficient NO production and inflammasome-dependent pathology. NO donors are well tolerated in humans (252). Additionally, NO is antimicrobial against *Brucella* (143) and can also potentially inhibit deleterious caspase-1 and NLRP3-dependent inflammation (161). Thus, NO donors, in combination with antibiotics, might be potential therapeutic candidates to treat brucellosis, particularly in patients with impaired IFN- γ production.

CHAPTER VI

Discussion

Human brucellosis is a systemic disease of which arthritis is the most common focal complication (56, 57). *Brucella* antigen in the joints causes inflammation which initiates an often prolonged arthritis in humans (57). There are limited animal models for the study of human brucellosis as rodents do not recapitulate human symptomology and/or susceptibility. Thus, *in vivo* and tissue specific mechanisms of *Brucella* infection remain largely unexplored, despite a growing need for new therapeutics aimed at treating chronic arthritis. In these studies, we used a murine systemic infection model, and characterized a new model of *Brucella*-induced arthritis to understand the inflammatory responses induced by *Brucella* in the joints.

Model Advantages and Disadvantages

These studies utilized a previously described *Brucella*-induced arthritis model in which IFN- γ deficient mice develop arthritis 2-4 weeks post systemic infection via inoculation by the intranasal, intraperitoneal or oral routes (165). We also defined and characterized a new model in which footpad infection of WT mice develop symptoms of arthritis as early as 1 day post-infection. While these models are the first reasonable murine systems for the study of *Brucella*-induced arthritis, both encompass several advantages and disadvantages. Humans generally

develop arthritis through oral or aerosol transmission, thus the systemic model, but not the footpad model, recapitulates the traditional dissemination of bacteria to the joint (16, 17, 165). In contrast, most humans have a sufficient IFN- γ response, making the systemic IFN- γ -deficient model less biologically relevant (165, 253). It is also difficult to experimentally measure pertinent immune responses in mice that lack IFN- γ signaling, as it is an important inducer of inflammation and bacterial clearance (140, 253, 254). Inflammation generally, but not always, correlates with *Brucella* burdens in our studies. Mice containing genetic deletions may have increased dissemination of bacteria rather than a defect in the joint immune response, which can result in differential inflammation in the systemic model. Thus, in systemically infected mice it can be difficult to discern which immune responses may be mediated by local inflammation versus those resulting from differential dissemination. Footpad infected mice develop arthritis much earlier than intraperitoneal infected IFN- γ -deficient mice, indicating inflammation in this model is due to innate responses. When arthritis is evident in the systemic model, adaptive immune responses presumably have already been established, and mice succumb to infection around 6 weeks post-infection. Thus, these models may be advantageous depending on what responses are being measured (Table 4).

While these models present with various advantages and disadvantages experimental murine *Brucella*-induced arthritis is a valuable tool for studying immune responses. In both models swelling correlated with inflammation allowing us to conduct longitudinal studies in which swelling was measured as an indicator of the inflammatory response. Interesting time points could then be more

IFN- γ deficient (systemic model)	Footpad (local model)
<ul style="list-style-type: none"> • Biologically relevant route of joint infection (dissemination to the joint) • Can study different routes of infection (e.g. oral, intranasal or intraperitoneal) 	<ul style="list-style-type: none"> • WT mice (immune competent mice) • Joint tissue specific immune responses • Innate immune responses in the absence of adaptive priming • Independent of bacterial dissemination • Can study IFN-γ responses • Can study initiation and resolution of inflammation • Can use murine infection models beyond 6 weeks post-infection

Table 4. Advantages of the murine models of *Brucella*-induced arthritis.

Comparisons of individual advantages of a systemic IFN- γ -deficient mouse model of *Brucella*-induced arthritis verse a local footpad model of *Brucella*-induced arthritis.

thoroughly analyzed via necropsy of the animal and commencement of H&E staining, CFU analysis, inflammatory cytokine quantification and other experimental assays. Similar to human arthritis both models exhibited joint inflammation independent of the *Brucella* species used to infect the animal (53-55, 165). With these new valuable models *Brucella*-induced arthritis, particularly footpad infected WT mice, are an invaluable system to not only study the immune response to *Brucella*, but to understand inflammatory processes within the joint.

Inflammatory Dichotomy in the Joint Versus the Systemic Periphery

Due to *Brucella*'s immune evasion strategy and the many anti-inflammatory proteins it employs (99, 107, 255), it is hypothesized that *Brucella* induces low-grade inflammation which does not result in a large enough immune response to induce bacterial clearance. Thus, this low-grade inflammatory state is the presumed mechanism underlying bacterial persistence within the host (92). In contrast to this idea, we found that upon footpad infection tissues within the joint mount a strong and early inflammatory response to *Brucella* and then resolve inflammation. Similarly, brucellae begin to reach the joint at 2 weeks post-infection in systemically infected IFN- γ -deficient mice, concomitant with development of arthritis (165). Thus, we speculate that a robust inflammatory response to *Brucella* is specific to joint tissue and may be the reason for arthritic sequelae often associated with this disease. There may be truth to the dogma suggesting that a low grade inflammatory state induces bacterial persistence, as footpad infected mice inoculated with 1×10^6 bacteria resolve joint inflammation and induce joint

Brucella clearance faster than mice infected with a dose of 1×10^3 . We suspect efficient innate and adaptive immune priming requires a large amount of *Brucella* antigen and results in more rapid clearance and resolution of both the pathogen and inflammation. This may be a consequence of a stronger macrophage response during acute infection as inflammatory macrophages, specifically IFN- γ activated macrophages contribute largely to *Brucella* clearance (140, 143). Therefore, footpad inoculation using large amounts of *Brucella* antigen and adjuvant may be an effective vaccine strategy as joint tissue induces an innate response that seems to prime adaptive immunity.

Previously it was found that following i.p. infection with *B. melitensis*, IL-1 contributes to clearance in the spleen of WT and IFN- γ deficient mice at day 56 post-infection (165). In contrast, we did not observe a role for IL-1 in the clearance of bacteria from the joint or spleen following footpad infection of WT mice at day 30 post-infection. This indicates IL-1 may not be important for inducing *Brucella* clearance in joint tissues, or IL-1 only restricts of *Brucella* infection at timepoints later than 30 days post-infection. We suspect variation in the IL-1 response between tissues could be due to the unique cellular subset, and how they respond to IL-1 present within the joint or periphery. Joints are composed of fibroblast-like synoviocytes (FLS), chondrocytes, osteocytes, immune cells and many other cell types (256). FLS are important for providing nutrients to the cartilage through their production of a lubricant called synovial fluid (257). FLS can also secrete a variety of pro-inflammatory cytokines during rheumatoid arthritis (258). Additionally, it is well known that synovial hyperplasia, or inability for FLS to undergo apoptosis, is

a common manifestation of rheumatoid arthritis, and *Brucella* infection of FLS enhances host cell viability (156, 257, 259, 260). FLS hyperplasia, and subsequent increase in inflammatory cytokine production, can also be induced by IL-1 signaling (261). Since *Brucella* can infect FLS, proliferation of these cells may allow for an enhanced reservoir in the joint compared to the periphery (155). Future studies should investigate whether synoviocyte proliferation aids in *Brucella* survival within the joint or if their robust production of cytokines allows for *Brucella* clearance in joint tissues.

Prolonged inflammation is known to induce bone destruction through the differentiation of monocytes into osteoclasts. These specialized cells typically absorb bone during bone remodeling, a process known as osteoclastogenesis. Bone resorption is greater than formation during *Brucella* infection, thus causing erosion (153, 262, 263). Inflammatory cytokines TNF- α , IL-1 β and IL-6 are known to induce bone resorption and are secreted upon FLS *B. abortus* infection, possibly contributing to the arthritic phenotype we observe (187, 264). Not much is known about chondrocytes during *Brucella* infection; however, they have been observed to secrete a variety of collagenases and proinflammatory cytokines such as IL-1 β and TNF- α in osteoarthritis patients (265). Although these cells were not directly studied in our investigation, they may be important mediators of inflammation or pathology and aid in the understanding of how the pathways described here are related.

We found that activated IL-18 induces inflammation upon *Brucella* infection. Additionally, we showed CXCR2 but not CCR2 initiated *Brucella*-induced arthritis.

These mechanisms may be joint specific as FLS can respond robustly to IL-18 which upregulates endothelial adhesion molecules and activates pro-inflammatory transcription factors (266). Additionally, human FLS from rheumatoid arthritis patients can produce the human CXCR2 ligand, CXCL8, which may result in enhanced neutrophil recruitment (267). IL-18 can also induce secretion of CXCR2 ligands but not CCR2 ligands in synovial fibroblasts isolated from rheumatoid arthritis patients (268). Thus, FLS may bridge the gap between inflammasome activated IL-18 and the recruitment of neutrophils via CXCR2 signaling. Alternatively, chondrocytes may inhibit synoviocyte IL-18 induced inflammation as they are strong producers of iNOS and subsequent NO production. NO manufactured by chondrocytes required IL-1 β and/or TNF- α priming which we showed to be highly upregulated in *Brucella* infected joints (269).

Innate and Adaptive IFN- γ production

Adaptive immune cells are partially responsible for arthritis resolution in IFN- γ sufficient mice, as Rag1^{-/-} mice displayed increased inflammation at day 30 post footpad infection. This response was largely due to $\alpha\beta$ T-cells as mice lacking these cells exhibited defects in arthritis resolution, while mice deficient in B-cells did not express this phenotype (data not shown). Additionally, we demonstrated systemically infected IFN- γ -deficient mice do not resolve joint swelling. These data demonstrate T-cells mediate inflammation resolution in an IFN- γ dependent manner. T-cells produce IFN- γ especially when primed by IL-12 and IL-18 signaling (270). In WT footpad infected mice, we observed an increase in IFN- γ producing

CD8 and CD4 T-cells at 14 days post-infection compared to those at 8 days post-infection. Furthermore, footpad infected mice that lack adaptive immune cells displayed enhanced joint *Brucella* burdens compared to WT mice, and this protective effect was completely dependent on IFN- γ . Thus, T-cell induction of IFN- γ is needed for resolution of arthritis and *Brucella* joint clearance. IFN- γ is also important for macrophage bactericidal activity and inflammatory macrophages play a key role in *Brucella* clearance (140, 143, 271). Additionally, we did not observe a difference in joint *Brucella* loads in WT IFN- γ -deficient mice compared to IFN- γ neutralized mice which lacked the monocyte recruitment receptor CCR2. Thus, we predict IFN- γ is needed for macrophage activation and in turn clearance of *Brucella* in the joint. Collectively we believe T-cell IFN- γ production and in turn macrophage activation are vital for joint *Brucella* clearance and arthritis resolution.

Activation of T-cells and their subsequent IFN- γ production generally has an onset of about 1-2 weeks (272). Footpad infected IFN- γ deficient mice however, develop robust inflammation, compared to infected WT-IgG mice, as early as 3 days post-infection. Furthermore, systemically infected Rag1^{-/-} mice neutralized of IFN- γ displayed reduced swelling compared to WT anti-IFN- γ treated mice, but they still exhibited a robust amount of swelling compared to WT-IgG treated mice. Additionally, footpad infected WT mice depleted of NK cells or $\gamma\delta$ T-cells did not display an increase in their inflammatory phenotype or *Brucella* joint clearance compared to WT animals. This indicates that innate IFN- γ also plays an important role in the clearance of *Brucella* from the joint, and that an unconventional cell type may induce IFN- γ . Systemic infection of the spleen begins to resolve inflammation

and clear *Brucella* at roughly 2 weeks post-infection, which follows the timeline of adaptive IFN- γ responses (165, 272). In contrast, *Brucella* clearance and swelling resolution are both generally evident by day 3 post-footpad infection, and leukocyte recruitment begins to dampen around day 7. Thus, this timely IFN- γ response appears to be native to the joint. ILCs may induce this early IFN- γ response however this does not explain the dichotomy of clearance in the joints versus the spleen as ILCs are not cells solely native to the joint (247, 273). Thus, specific joint resident cells such as FLS may also be vital sources of IFN- γ as cultured FLS spontaneously make IFN- γ and it is upregulated in patients with rheumatoid arthritis (257).

Inflammation v. Bacterial Clearance

Dogma suggests there is often a tradeoff for inflammation. While inflammation works to clear the pathogen, it also induces pathology (76). Furthermore, *Brucella* preferentially replicates in immune cells such as macrophages further confounding this paradox, as macrophages can control *Brucella* burden (143). We found that deletion of CXCR2 reduced inflammation but did not increase bacterial loads. This may indicate that there is “counterproductive inflammation”, or inflammation that contributes to pathology but not *Brucella* clearance. However, these experiments were conducted in an IFN- γ -deficient murine model. Thus, repeat studies in footpad infected WT and CXCR2^{-/-} mice could demonstrate if this inflammation is truly “counterproductive” or if CXCR2 signaling requires IFN- γ to restrict *Brucella* burdens. Unlike CXCR2 signaling,

MyD88, IL-18, caspase-1, and caspase-11 initiate early inflammation (~day 2) which appears to reduce *Brucella* loads at later time points (~day 7). MyD88^{-/-}, IL-18^{-/-}, caspase1/11^{-/-} and caspase-11^{-/-} mice all exhibited delayed inflammatory responses, as all developed increased swelling between days 3-7 post-infection. Delayed inflammation could be a result of increased recognition of *Brucella* PAMPs, but is more likely due to increased DAMPs induced by overwhelming *Brucella* infection. DAMPs can be powerful immune stimulants and are found in large abundance in the synovial tissue of patients with rheumatic disease (206). We found that pyroptosis was driven by *Brucella* infection, particularly *Brucella* LPS. This lytic form of cell death is known to release DAMPs (206). High mobility group box 1 (HMGB1) is a common DAMP that is released during an LPS-mediated endotoxin shock model. HMGB1 induced inflammation in LPS treated mice in an inflammasome dependent manner, but inflammation was independent of IL-18 and IL-1 β signaling (274). Thus, overwhelming CFUs and inflammasome activation may induce DAMPS which can lead to the inflammatory phenotype seen in *Brucella*-induced arthritis.

Neutrophils are Mediators of *Brucella*-induced Inflammation

Neutrophils were clearly one of the primary cell types recruited to the joint upon *Brucella*-induced inflammation. We demonstrated this recruitment was in large part CXCR2 dependent and CXCR2 ligands, CXCL1 and CXCL2 were substantially upregulated in infected mouse joints. Similarly, the human CXCR2 ligand, CXCL8, is elevated in humans with *Brucella* induced arthritis (66).

Neutrophil recruitment correlated with severity of arthritis. For instance, we observed enhanced neutrophil recruitment in the joints of footpad infected anti-IFN- γ treated mice compared to WT mice. Similarly, we observed a reduction in neutrophils in MyD88^{-/-} mice compared to WT at day 2 post *Brucella* footpad infection. Thus, depleting neutrophils, neutrophil recruitment or CXCR2 ligands may work as a sufficient therapy for treating excessive inflammation in the joint.

Possible Mechanisms of MyD88 Dependent Inflammation

MyD88 has long been known to protect mice from *Brucella* infection (203, 204); however, it remains controversial as to what initial receptors, or TLRs, are contributing to MyD88 activation as *Brucella* generally has weak immune recognition in the systemic periphery. Our footpad model exhibits robust MyD88-dependent “productive inflammation.” Thus, it is an excellent candidate for studying *Brucella* activation of TLRs, and understanding their role in inflammation and bacterial clearance. Additionally, DAMPs released during pyroptosis, such as HMGB1, may also signal through MyD88 via HMGB1 initiation of TLR4 and TLR2 signaling (275, 276). TLR4 signaling can also signal through TRIF allowing inflammatory signal transduction to occur even in the absence of MyD88 (105). Although one study revealed TRIF does not contribute to clearance of *Brucella* in the spleen and peritoneal cavity following i.p. *Brucella* infection (205), TRIF signaling has not been evaluated in the joints. Hence, this signaling event could lead to the inflammation seen in MyD88^{-/-} mice around day 7 post-infection.

MyD88 is essential for priming many inflammatory components including inflammasome proteins, (277) pro-IL-1 β , pro-IL-18 and other inflammatory cytokines and chemokines such as TNF- α and CXCR2 ligands (117, 278). We have demonstrated that MyD88 is critical for IL-1 β production in the joint. Similarly, others have described MyD88 is required for caspase-1 activation in *Brucella* infected BMDMs (117). Considering MyD88's extensive role in regulating a multitude of inflammatory responses, MyD88 is likely required for some inflammasome dependent inflammation in *Brucella* induced arthritis. MyD88 mediated inflammation is "productive" and works to clear bacteria in the joint. This may be due to MyD88/inflammasome dependent IL-18, as IL-18 can encourage IFN- γ production (279). However, MyD88 has protective effects beyond IFN- γ as i.p. infected MyD88^{-/-}-anti-IFN- γ treated mice succumb to infection sooner than WT-anti-IFN- γ treated animals (data not shown). Additionally, MyD88^{-/-} mice were defective in resolution of inflammation and bacterial clearance (day 28), which was similar to observations in mice lacking adaptive immune cells (Rag1^{-/-} mice). MyD88^{-/-} mice also exhibited reduced IFN- γ production by CD4 and CD8 T-cells, and displayed decreased anti-*Brucella* antibodies in sera compared to WT mice (data not shown). This indicates MyD88 is at least partially responsible for priming the adaptive immune response to *Brucella*. We suspect that MyD88 dependent IL-18R signaling in T cells is important for clearance of *Brucella* at later time points. In particular, we hypothesize T cells produce IFN- γ in response to IL-18 that aids in macrophage activation (140, 279, 280).

Metabolism's Role During *Brucella* Infection

Although we believe quick, robust, macrophage activation and, in turn, MyD88 signaling is important for clearing *Brucella*, MyD88 activation after the initial state of recognition may be a disadvantage to the host in certain cell types. Bacterial LPS can induce TLR-signaling, and presumably MyD88 signaling in macrophages, shifting their metabolism to a glycolytic state. (281). We and others have demonstrated that the byproduct of glycolysis, lactate, aids in *Brucella* survival (101), and preliminary studies indicate infected MyD88^{-/-} BMDMs do not produce lactate to the same magnitude as infected WT cells (data not shown). Although MyD88^{-/-} mice exhibit defects in *Brucella* clearance, MyD88 signaling in infected cells may be intrinsically detrimental to the host. We suggest MyD88 dependent cytokines are important for restricting *Brucella*, but MyD88 intrinsic induction of lactate aids *Brucella* in establishing its replicative niche. Indeed, we found that mice lacking MyD88 in myeloid cells make reduced TNF- α , which we found to be protective against *Brucella* infection, however MyD88-signaling in myeloid cells does not aid in *Brucella* clearance (data not shown). MyD88's production of lactate may contribute to *Brucella*'s preference of macrophages as their favored host. While *Brucella* replicates in alternatively activated macrophages which utilize fatty acid β -oxidation as their primary metabolism (100, 242), it is unclear if *Brucella* prefers glycolytic cells over β -oxidative cells during acute infection or if increased lactate aids *Brucella* in establishing its intracellular niche. Future studies should elucidate the role of metabolism in acute versus

chronic infection and determine if MyD88 signaling, and in turn glycolytic metabolism, aid in *Brucella* replication in specific cell types such as macrophages.

Enhanced MyD88-dependent glycolysis can also lead to the production of the pro-inflammatory cytokine IL-1 β (103, 242). We demonstrated the glycolysis inhibitor 2-dG restricts IL-1 β secretion and intracellular *Brucella* burdens in macrophages. This may be due to reduced production of MyD88 dependent pro-IL-1 β (242) or MyD88-dependent inflammasome activation/priming (117, 277), as glycolysis has been shown to induce inflammasome proteins such as NLRP3 (243, 244). Additionally, monocytes from rheumatoid arthritis patients produce increased NLRP3 dependent IL-1 β when given increasing doses of glucose (282). When we inhibited glycolysis with 2-dG in the footpad model of *Brucella* induced arthritis, mice exhibited reduced joint swelling without altering joint *Brucella* burdens. 2-dG treated BMDMs also displayed decreased levels of IL-1 β , indicating glycolysis is critical for IL-1 β priming and/or inflammasome priming/activation. Further studies need to investigate optimal dosage of 2-dG and if increasing the concentration of 2-dG can enhance or reduce *Brucella* clearance. Mechanisms for glycolysis dependent inflammation should additionally be studied in *Brucella* induced arthritis in order to determine the critical cell types involved in pathology and if glycolysis is truly MyD88-dependent in our model. Others have observed glucose metabolism in fibroblast-like synoviocytes can contribute to human rheumatoid arthritis (283). Taking these phenotypes into account 2-dG may be a complementary therapy for reducing *Brucella* induced arthritis.

IFN- γ Dependent Mechanisms for Controlling Inflammasome Dependent Inflammation

Inflammasomes contributed extensively to *Brucella*-induced arthritis particularly in the absence of IFN- γ . We demonstrated *in vitro* that NO inhibits caspase-1 activation, but it is unknown how and where in the inflammasome pathway NO is suppressive. Similar to other studies of inflammasome mediated pathology, further experiments should investigate if NO s-nitrosylates NLRP3 and/or caspase-1 (161, 231, 250). Additionally, to our knowledge no studies have determined if the caspase-11 pathway can be suppressed by NO via s-nitrosylation. Thus, future investigations should also determine if NO donors such as SNAP can limit pathology in the joint or even in the systemic periphery of IFN- γ -deficient mice. Intraperitoneal infected IFN- γ -deficient mice succumb to infection while T-cell deficient mice survive (142). Death in these mice is due to overwhelming neutrophilia, as both types of immunocompetent mice had similarly elevated bacterial loads. We suspect this phenotype is due to inflammasome activation and IL-18 release which can result in increased recruitment of neutrophils (284).

In vivo experiments demonstrated NOS2^{-/-} mice developed more robust swelling compared to WT mice, but this phenotype was not as severe as that seen IFN- γ neutralized mice. Additionally, IFN- γ deficient mice had enhanced joint *Brucella* loads compared to WT and NOS2^{-/-} mice. Thus, IFN- γ may also prevent inflammation by reducing *Brucella* loads and/or IFN- γ may have other roles in limiting inflammasome-induced inflammation. A regulatory role of IFN- γ has been observed in a murine rheumatoid arthritis model; however, suppression of

inflammation was only speculated to be due to IFN- γ inhibition of inflammasomes as IFN- γ R^{-/-} mice had reduced levels of activated caspase-1 compared to WT (285). Preliminary experiments show caspase-11 is primed and activated to a greater degree in IFN- γ -deficient mouse joints (data not shown). We demonstrated caspase-11 contributes to arthritis in the absence of IFN- γ , indicating inflammation inhibited by NO could potentially be due to caspase-11. Macrophages *in vitro* require IFN- γ or type I IFNs for caspase-11 priming (286). Hence, enhanced priming of caspase-11 may be due to type I IFNs and should be further investigated. ER stress can also induce priming of caspase-11 which has been shown to contribute to LPS induced pathology (287). *Brucella* is considered a model organism for activating host ER stress (288). Therefore, ER stress may lead to enhanced caspase-11 priming and in turn inflammation.

Mechanism of IL-18 induced Inflammation

We demonstrated inflammasome activation of IL-18 contributes to *Brucella*-induced arthritis development, but the mode in which IL-18 mediates inflammation is currently unknown in our model. IL-18 can activate neutrophils as human peripheral blood derived neutrophils constitutively express IL-18R. Neutrophils from rheumatoid arthritis patients respond to IL-18 by inducing enhanced production of inflammatory cytokine and chemokines. IL-18 activated neutrophils display an increase in granule release, increase respiratory bursts and secreted cytokine/chemokine including TNF- α and CXCL8 (284). Additionally, neutrophils are recruited to the peritoneal cavity upon i.p. administration of IL-18 in WT mice.

Similarly, treatment with IL-18 receptor antagonist (IL-18RA) reduced swelling and concentrations of TNF- α and myeloperoxidase in the joints of a mouse model of paw edema (284). IL-18 also contributes to a model of streptococcal cell wall induced arthritis in an IFN- γ independent manner (289). These studies along with our own underscore the possibility that IL-18R along with CXCR2 initiate the pathogenic recruitment of neutrophils in murine *Brucella*-induced arthritis. Moreover, CXCR2 antagonists in combination with IL-18RA may reduce pathology in human patients.

Cell Death

It is unclear what type of cell death is best for facilitating *Brucella* clearance. We demonstrated that *Brucella* LPS-induced cell death is mediated through pyroptosis. Additionally, caspase-1 and caspase-11 BMDMs contain less intracellular bacteria which was independent of NLRP3 or AIM2 signaling. We hypothesize caspase-1 and caspase-11 clear bacteria by eliminating its macrophage reservoir, but pyroptosis has also been demonstrated to clear bacteria by trapping them in PITs then promoting neutrophil efferocytosis of the pyroptotic cell (215, 290). *Brucella* can resist bactericidal killing inside the neutrophil environment (207, 291); however the process of undergoing pyroptosis also damages the bacteria making them more susceptible to microbial killing by ROS and antimicrobial peptides (215, 290). Although some intracellular pathogens are cleared by apoptosis (292), *Brucella* canonically inhibits cell death to promote its replication (154). *Brucella* LPS can also dissociate from smooth to rough in

order to encourage cell death, allowing release of the bacteria and further dissemination (126, 293). Considering pyroptosis eliminates the bacterial reservoir while possibly damaging the bacteria leading to effective neutrophil clearance, pyroptosis may be more advantageous to the host than apoptosis or other forms of cell death.

Guanylate Binding Proteins as Inducers of Inflammasome Activation

Recently it was shown that type I IFN dependent GBPs are important for canonical inflammasome recognition, and consequential caspase-1 activation and clearance of *Brucella* (118). GBP have also been described as essential for non-canonical inflammasome activation, as GBPs can expose LPS contained inside the pathogen containing vacuole to the cytosol. This exposure allows LPS to be recognized by caspase-11 (294). Other studies suggest GBPs can act as an LPS adaptor which recognizes LPS and permits caspase-11 activation (295, 296). Although we have demonstrated IFNAR partially limits swelling at day 3 post footpad infection (data not shown), the role of type 1 IFNs and GBPs in our model is not clear. We found that *Brucella*'s non-canonical hepta-acylated LPS activates caspase-11, but it is unknown if GBPs are required as adaptors for caspase-11 recognition and/or needed for LPS exposure to the cytosol. Future studies should identify if GBP, or caspase-11 in general, can recognize hepta-acylated LPS and other non-canonical bacterial LPS structures. Investigations should also analyze whether GBPs are needed for inflammasome induced inflammation/clearance and

whether GBPs directly recognize LPS and/or release *Brucella* antigen from the BCV.

Potential Therapeutics for the Treatment of *Brucella*-Induced Arthritis

In these investigations, we identified several candidate targets for the therapeutic treatment of *Brucella*-induced arthritis. The most favorable therapeutics are likely those inhibiting inflammatory mediators that lead to “counter productive inflammation”, but do not impact clearance of *Brucella*. Although extensive study of these potential treatments need to be investigated in several organ systems of WT mice, our most favorable candidates are CXCR2 antagonists, 2-dG and potentially NO donors. CXCR2 antagonists such as reparixin have been shown to reduce antigen induced arthritis in mice (192). CXCR2 antagonists have also been used in humans to decrease the growth of tumors and to treat pulmonary disease (297, 298). As numerous models of disease suggest a pathological role for CXCR2, extensive studies have been dedicated to safe and effective development of CXCR2 antagonists (190, 299-301). Additionally, 2-DG inhibits human monocyte chemotaxis as glycolysis can aid in myeloid cell mediated inflammation (302, 303). Similarly, studies have demonstrated 2-dG treatment can suppress neutrophil activity and 2-dG therapy is well tolerated in humans (304, 305). NO donors may also be effective therapies, as we demonstrated NO can inhibit inflammasome mediated inflammation and can be bactericidal against *Brucella* (143, 306). Further investigation on dosing and types of NO donors warrant deeper investigation. In addition, IL-1 was identified

as a contributor of joint inflammation. Although IL-1 did not confer *Brucella* clearance in the joint, it was protective in the spleen of WT mice. Thus, it may be more advantageous to design an IL-1 therapy that would remain locally within the joint. Extensive research is working to advance intraarticular injections and novel drug delivery systems to keep drugs contained within the joints in order to combat articular inflammatory diseases (307, 308).

Mediators that induced inflammation but also contribute to clearance of *Brucella* may likewise be useful therapy targets, as all treatments described here are presumed to be used in combination with antibiotics. Inflammasome inhibitors for the treatment of several inflammatory diseases have long been suggested and several small molecule inflammasome inhibitors exist (309, 310). To our knowledge, no human clinical trials have investigated the effects of specific inflammasome inhibitors on inflammatory disease. However, Colchicine, a recognized anti-inflammatory agent, was demonstrated to reduce IL-1 β , IL-18 and pro-caspase-1 mRNA and active caspase-1 in humans with acute coronary syndrome (311). MyD88 suppression may also be an effective therapy; however, due to MyD88's central role in inflammation and subsequent bacterial clearance, it may be our least likely therapeutic candidate. Further studies should differentiate the "productive" versus "counterproductive" inflammatory role downstream or upstream (TLRs) of MyD88 in order to further hone in on the deleterious inflammation initiating pathology.

Conclusion

This work represents substantial progress in understanding how *Brucella* activates the immune system and induces pathology. We demonstrated CXCR2 dependent neutrophil recruitment was largely pathogenic in the joints following i.p. *Brucella* infection in mice threated with anti-IFN- γ . Understanding the possible pitfalls of using IFN- γ -deficient mice we generated a new model of *Brucella* induced arthritis, giving researchers a valuable tool for understanding pathology in the joint following *Brucella* footpad infection. Pervious *Brucella* work has focused almost entirely on systemic responses which have weak innate immune recognition of *Brucella*. This new model has allowed us to investigate how joint specific tissues respond to infection independent of bacterial dissemination to the joint. This model also allows for longitudinal studies in mice that induce quick, robust, immune responses which we believe to be specific to joint tissue. We determined these vigorous immune responses initially rely on MyD88 signaling, which is important for subsequent clearance of *Brucella* and resolution of inflammation. This inflammatory state at least partly relies on glycolytic metabolism to initiate such responses. Finally, we demonstrated inflammasomes confer inflammation in an IL-18 and IL-1 dependent manner, and inflammasome-dependent IL-18 and pyroptosis restrict *Brucella* infection. Pyroptosis was shown to be caspase-11 dependent, a mechanism previously thought to be evaded by *Brucella* (122). IFN- γ is protective against *Brucella*-induced pathology partly due to its production of NO, which was shown to suppress inflammasome mediated inflammation. The inflammatory pathways described in this work are likely interconnected, are there

are likely many others that are currently unknown (Fig 35). Further research should consider what cells, particularly those specific to the joint, recognize *Brucella* and initiate robust articular inflammatory responses. Studies may also examine if MyD88 dependent glycolytic cell metabolism is advantageous to the host or the pathogen during acute *Brucella* infection. Finally, investigations should examine other pathways in which IFN- γ suppresses inflammasome mediated inflammation.

Figure 35. Possible mechanisms of inflammation during *Brucella* induced arthritis.

Brucella can be recognized by TLR signaling via MyD88 and inflammasomes. MyD88 can prime inflammasome components, IL-18, IL-1, CXCL1 and CXCL2. CXCL1 and CXCL2 signal through the neutrophil receptor CXCR2, then neutrophils are recruited to the joint tissue. Inflammasomes are required for IL-1 and IL-18 activation and secretion which all can enhance joint inflammation. IL-1 and IL-18 can signal through their respective receptors downstream to MyD88. IL-18 may signal through FLS which can induce the production of CXCL1 and CXCL2. Inflammasomes can induce pyroptosis which may also lead to enhanced joint inflammation. Finally, IFN- γ -dependent nitric oxide can inhibit inflammasome activation therefore inhibiting joint inflammation. Arrows represent pathways confirmed by us and others during *Brucella* infection, while dashed arrows indicate our hypothesis. $\vdash$ Indicates inhibition of a pathway.

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VITA

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