STUDY OF DIVERSE HOST IMMUNE RESPONSES TO VIRAL AND BACTERIAL PATHOGENS

by

Jack Bruno Plewa

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TABLE OF CONTENTS

TABLE OF CONTENTS CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

ABSTRACT

Brucella abortus is the bacterium that causes brucellosis, an infection transmitted from cattle to people, often through consumption of raw milk and contact with aborted materials. With antibiotic resistance on the rise, phage therapy for bacterial infection may become a useful approach. The direct effects of phage on mammalian cells is important to understand, yet understudied. *In vivo* delivery of low phage MOI to the mouse lung was more effective at diminishing *Brucella* burden than higher doses of phage. In an *in vitro* model of intracellular *Brucella* infection, low phage MOI was capable of minimizing human THP-1 monocyte infection, but, unexpectedly, use of higher phage MOI diminished this effect. We hypothesized that recognition of these phage preparations may induce an antiviral immune suppressive response that may counteract their anti-bacterial effects. Indeed, when the type I IFN signaling pathway was disrupted in mice, phage treatment was more effective. However, when attempting to induce type I IFN *in vitro* using both human monocyte and mouse macrophage cell lines, we were unable to stimulate expression of type I IFN with *Brucella* phage, including in response to a combination of phage and bacteria. We then examined the effect of phage treatment on macrophage cell surface markers that are indicative of activation/differentiation. Interestingly, while *Brucella* LPS induced expression of CD71 and CD206, the addition of phage suppressed upregulation of these markers. Our discovery of immune suppressive effects of *Brucella* bacteriophage is an important consideration for using phage as a treatment

CHAPTER ONE

INTRODUCTION

Brucellosis

Human brucellosis is one of the most common zoonotic diseases (animal-to-human crossover) worldwide, with more than 500,000 new cases annually (1). It is associated with substantial residual disability and is an important cause of travel-associated morbidity (1). While endemic regions in the world have controlled spread of the disease, brucellosis has emerged in other places, like central Asia, and worsened in others, like Syria. In people, brucellosis is most commonly contracted orally by ingestion of infected material (such as unpasteurized dairy products), through inhalation, or through open wounds. Animal farm workers and veterinarians are often at risk of contracting the bacteria through contact with infected animals. In livestock, the disease primarily triggers spontaneous abortion, with expelled fetal material carrying large amounts of infectious bacteria (2). Animal farm workers and veterinarians who come into contact with infected expelled fetal material are at high risk of acquiring the infection. Human disease typically appears as short-term flu-like symptoms, such as undulant fever, headache, joint and back pain, and fatigue (2). Severe illness in humans can persist if untreated.

Brucella Bacteria

Brucellosis is caused by the Gram-negative facultative intracellular bacteria *Brucella abortus, B. melitensis, B. suis, B. ovis, B. neotomae,* and *B. canis. Brucella* can survive in the presence or absence of oxygen (3). This offers a selective advantage over other bacteria, allowing *Brucella* to predominate in many different environments because they can easily adapt

to changing conditions. In addition, Gram-negative bacteria have three protective layers: the outer membrane (which has phospholipids and lipopolysaccharide), peptidoglycan, and an inner membrane (which is a phospholipid bilayer; 4). *Brucella* bacteria are small coccobacilli, measuring about 0.6–1.5 μm (5). They are also non-spore forming and non-motile. Moreover, *Brucella* are facultative intracellular, meaning they replicate mainly inside cells, specifically macrophages, during infection (6), but can also replicate outside the cell. By replicating inside of macrophages, *Brucella* bacteria are able to prevent destruction by the immune system. They do this through VirB-dependent sustained interactions with the cell endoplasmic reticulum (6). The *virB* locus encodes a type IV secretion system (T4SS), which is necessary for intracellular survival. T4SSs are multiprotein nanomachines found in the cell envelope of many bacteria that exhibit crucial roles during human infection (7). One study showed that a fraction of the bacteria that survive initial macrophage killing proceed to replicate in a separate compartment from the endocytic pathway (3). The VirB system is then involved in controlling the maturation of *Brucella*-containing vacuoles into organelles that have sustained interactions and fusion with the endoplasmic reticulum, which allows for bacterial replication to occur.

B. abortus and *B. melitensis* are highly infectious to people via aerosol and are classified as select agents because of their potential for use in bioterrorism. *B. abortus* is primarily a bacterium that infects cattle, and *B. melitensis* is associated with disease in sheep and goats. Both species can infect other ruminants, such as bison and elk. This is problematic for protecting livestock herds and cattle from infection in the Greater Yellowstone Area (GYA) because of grazing land shared with wild bison and elk (2).

Animal Vaccines

Before the 1954 Brucellosis Eradication Program was developed, brucellosis had a major impact on the economy, with $400 million lost in 1952. With this program came the development of two *Brucella* vaccine strains (RB51 and S19) and surveillance programs, which have significantly reduced infection in animals (8). However, a considerable disadvantage of S19 is its smooth phenotype, due to a full-length O-chain lipopolysaccharide (LPS), which induces an indistinguishable antibody response from the one induced by natural infection. This makes it difficult to distinguish between vaccinated and naturally infected animals when running serological tests (8). Thus, this vaccine strain is no longer used in the U.S. The vaccine strain currently used in the U.S. contains the live attenuated *B. abortus* strain RB51. A drawback of this strain is that it is resistant to rifampin, one of the few antibiotics that can penetrate infected cells (2, 8). RB51 also requires boosting, is only 70-80% effective, and can be abortogenic (8). Furthermore, RB51 is infectious in humans and can cause outbreaks of brucellosis.

Despite the success of the eradication program, natural reservoirs of *B. abortus* still exist in elk and bison in areas such as the GYA surrounding Yellowstone and Grand Teton National Parks. This poses a huge risk to the status of local livestock herds, with the rest of the U.S. being mostly free of bovine brucellosis. A recent National Academy of Sciences report concluded that significant resources are needed to address the increasing incidence of brucellosis in cattle and domestic bison herds in the GYA in the past few decades due to transmission from elk (9). Without making changes and investing in this problem, brucellosis may spread beyond the GYA into other parts of the United States, leading to serious economic and potential public health issues (9).

Human Infection

Although human-to-human transmission is rare, transmission from infected animals to humans occurs frequently. In the fall of 2017, the CDC reported that people who drink raw milk in four states (Connecticut, New Jersey, New York, and Rhode Island) were at risk of infection with RB51 (10). That September, a patient in New Jersey got sick with RB51 after drinking raw milk from a company called Udder Milk. Vaccinated cattle can shed RB51 in their milk, which means the milk can be infectious to people if it is not pasteurized. Since RB51 is resistant to rifampin, people infected with RB51 wouldn't be able to be treated for the infection. This can result in long-term health issues such as heart problems, meningitis, and miscarriages in pregnant women. Other antibiotics may be used in combination treatment, like doxycycline and streptomycin, but are not always effective (2). Thus, there is clearly a need for better vaccines and novel treatment approaches. In 2022, researchers at the University of Florida developed a live mucosal vaccine that generated protective memory T cell responses against wildtype *B. melitensis* in a mouse model (11).

Brucellosis is currently treated in humans with long-term, high-dose antibiotics (2). Long-term treatment can allow the bacteria to adapt to the antibiotics and eventually become resistant to them. Moreover, long-term treatment can kill large quantities of the commensal bacteria in the human microbiome, resulting in what is called microbiota dysbiosis. To mitigate the possibility of antibiotic resistance and antibiotic-induced microbiota dysbiosis, it is important to pursue alternative treatment options.

Innate Immunity

Cellular innate immune mechanisms are critical for defending against *Brucella* infection (12). Understanding how the innate immune response works to control the bacterium is essential to understanding how to control and eliminate *Brucella*, such as *B. abortus*. The recognition of pathogens by the innate immune systems involves pattern recognition receptors (PRRs). Once a PRR recognizes a pathogen, it initiates a signal cascade inside the host cell that ends with the cell producing and releasing cell signaling molecules called cytokines (13). These cytokines then bind to other immune cells and trigger them to produce more cytokines, both the same and different ones, to initiate more defense pathways.

One type of PRR is the Toll-like receptor (TLR), which is a host cell membrane or endosomal receptor (14). Upon activation by foreign pathogens, TLRs transduce signals via MyD88 or TRIF, which are adaptor molecules (15). This leads to activation of the host cell. On the one hand, MyD88 is important for TLR-mediated activation of NF-κB (which plays a role in regulation of immune responses and inflammation) and the induction of proinflammatory cytokines (16, 17, 18). On the other hand, TRIF is important for TLR-mediated activation of type I interferons (IFNs), which are antiviral cytokines (19) with immunoregulatory effects.

Cells that express TLRs can also present antigen on their cell surface. Such cells, called antigen-presenting cells (APCs), include macrophages and dendritic cells (DCs). They will recognize pathogens, and then eat, or phagocytose, them. Once the pathogen has been phagocytosed, the APC will destroy the pathogen inside and then present a piece of it, known as an antigen, on the surface of the cell to be recognized by other immune system cells. Specifically, they will interact with T cells (20). In doing so, DCs act as a bridge between innate and adaptive immunity. In addition, DCs play distinct roles in shaping T cell development,

differentiation, and function. Without MyD88, DCs cannot fully mature and thus cannot produce TLR2, which is crucial in the production of the cytokine interleukin-12 (IL-12). IL-12 has been shown to play a role in rescuing host susceptibility in MyD88 knockout-infected animals and has a central role in T cell-mediated responses in inflammation (21). Understanding the role of different adapter molecules, TLR receptors, and interleukins can provide insight into ways to fight bacterial infection with novel therapeutics. Gomes *et al.* (2012) supports other literature in claiming that TLR9 is the most important single TLR when it comes to fighting *B. abortus* (22). TLR9 triggers immunity to intracellular pathogens by recognizing bacterial DNA. It does so by binding unmethylated CpG DNA, which is a site in the DNA sequence where a cytosine is followed by a guanine. CpG methylation is a regulatory mechanism in eukaryotes but not in prokaryotes; thus, unmethylated CpG DNA is seen as foreign to the eukaryotic immune system.

Type I IFNs typically induce an antiviral immune response. However, they have been shown to be produced in response to bacterial infection and have even been shown to lead to killing of bacteria by facilitating lysis of infected cells (22). After their induction via the downstream signaling cascade of TLRs, type I IFNs induce transcription of IFN-stimulated genes (ISGs) via the JAK-STAT pathway (23). This signaling cascade is initiated when IFNα and IFNβ bind the IFNα receptor (IFNAR) on another cell. This receptor is made up of IFNAR1 and IFNAR2 subunits (23). This binding activates the protein tyrosine kinases JAK1 and TYK2 (Janus kinase 1 and tyrosine kinase 2, respectively). Activated JAK1 and TYK2 phosphorylate STAT1 and STAT2 (signal transducer and activator of transcription 1 and 2), leading to their dimerization and translocation to the nucleus. Here, they form a complex with IFN-regulatory factor 9 (IRF9) and activate transcription of ISGs. ISGs can then work to block certain viral

pathways in the host. For example, Mx proteins prevent nuclear import of viral genetic material, and IFIT proteins prevent protein synthesis of viral genes (24).

Previous research has shown that *B. abortus* induces type I IFN production in mice, and this response promotes, rather than reduces, infection (25). Another study confirmed this and found that *B. abortus* induces type I IFN production in macrophages and splenocytes (26). Interestingly, IFNAR knockout mice infected with *Brucella* produce more IFNγ and nitric oxide than wildtype mice, leading to improved disease outcomes (26). Thus, the presence of type I IFNs reduces the antibacterial response, therefore enhancing bacterial survival. The antibacterial response includes the production of IL-12 and IFNγ, which helps reduce *Brucella* burdens in mouse spleens (27). Specifically, administering recombinant IL-12 to infected mice led to a significant reduction in splenic bacterial counts three weeks post-infection. When anti-IFNγ antibody was simultaneously administered with recombinant IL-12, bacterial counts increased, suggesting that IL-12 and IFNγ work together to reduce bacterial counts in the spleen. In the lungs of *Brucella*-infected mice, increased IL-12 and IFNγ were detected at day seven post-infection (28), and these cytokines help control *Brucella* infection in human patients as well (29). This is interesting, given that type I IFNs suppress IL-12 and IFNγ (30 and 31, respectively). Furthermore, type I IFNs have been shown to suppress IL-6 production, thus reducing induction of phagocytic macrophages and leading to increased bacterial survival (32).

Phage Therapy

Phage Overview.

For about 100 years, scientists have known that bacteria are susceptible to infection with bacteriophage, which are viruses that specifically infect bacteria (33). Bacteriophage, or phage for short, are now being intensively researched as therapeutic

alternatives to antibiotics for extracellular bacterial infection (34, 35). Lytic phage replicate inside bacteria and lyse the cell to release their progeny, thus killing the bacteria, while lysogenic phage incorporate their viral genome into the host genome to be replicated into bacterial progeny (36).

History. Around the time phage were discovered, scientists realized the clinical implications phage could have on treating bacterial infections, with the first publication on phage therapy coming out in 1921 (33). By injecting phage into sick patients, the bacterial infection could potentially be eradicated. However, with the discovery of antibiotics came a general abandonment of phage therapy, and so phage therapy research was pushed aside. But recently, with the rise of antibiotic-resistant bacteria, the idea of phage therapy has resurfaced as an alternative treatment option. This could potentially reduce the number of human deaths due to antibiotic-resistant bacterial infections. However, there is still much to learn about phage therapy before implementing it as a treatment.

Fortunately, there has been some success in using phage therapy in human clinical trials, although no treatments have yet been approved for regular clinical use. In 2018, The Center for Innovative Phage Applications and Therapeutics (IPATH) was founded at the UC San Diego (UCSD) School of Medicine. Their goal is to pursue phage therapy as a new treatment for combating antimicrobial resistant diseases. The Center has already treated patients with phage therapy through emergency approval by the FDA. UCSD researchers are continuously working hard on clinical trials to get non-emergency use of phage therapy approved.

In 2017, Schooley *et al.* used a phage cocktail to treat a patient with resistant *Acinetobacter baumannii* (37). The patient was 68 years old and diabetic, with necrotizing

pancreatitis complicated by the multidrug resistant bacteria. Multiple courses of antibiotics did nothing, and they continued to deteriorate over a four-month period. After intravenous and percutaneous administration of a phage cocktail that contained nine different phage with lytic activity for *A. baumannii*, the infection was cleared, and the patient returned to good health. However, the researchers did observe emergence of phage-resistant bacteria but quickly resolved the issue by using new phages. Clinical trials continue to study the use of phage therapy in humans in order to acquire regular use approval from the FDA. Interestingly, phage therapy is used as a routine medical practice in Georgia, Poland, and Russia (38).

Advantages. There are many potential advantages of phage therapy over antibiotics. For example, phage are highly specific to the bacterial species they infect because they can use bacterial-specific receptor binding proteins to bind and infect the cell (39). This bacterial host specificity of phage reduces concern about detrimental off-target effects on the microbiome in people and animals. Furthermore, phage are self-replicating in the presence of the bacterial host and will presumably be eliminated when the host is gone.

The combination of antibiotics with phage can be an effective treatment even despite antibiotic resistance. In one study, vancomycin-resistant *Enterococcus faecalis* (VRE) was treated with a combination of both anti-*E. faecalis* phage and vancomycin (40). The combination treatment was synergistically effective compared to treatment with phage or antibiotic alone. These results show that the phage greatly enhance the ability of antibiotics to treat antibiotic resistant bacteria. Similar results might be obtained with other bacteria, such as *Brucella*.

Surprisingly, in one study (41), evolution of bacteria exposed to antibiotics and phage increased bacterial sensitivity to antibiotics even though they became resistant to phage. This

was observed when a combined phage-antibiotic treatment was used to fight *Pseudomonas aeruginosa* prosthetic vascular graft infections (41). The antibiotics alone did not work to eliminate the infection due to antibiotic resistance. When the phage OMKO1 was used to target the bacteria, wild-type bacteria were killed by the phage, but some bacteria evolved to become phage-resistant, which led to increased antibiotic sensitivity in those bacteria. After a single treatment with both phage OMKO1 and the antibiotic ceftazidime, a chronic *P. aeruginosa* infection of an aortic Dacron graft was eradicated with no signs of recurrence. This specific phage binds to an outer membrane protein on the bacteria that is part of the multidrug efflux system. Hence, phage treatment exerts a selective pressure, favoring phage-resistant mutations that alter the efflux-pump proteins; some of these mutations inactivate the antibiotic-resistance pump. Thus, in this system, there is a trade-off between antibiotic resistance and phage resistance (41). These results may occur with other antibiotic-resistant bacteria if phage that utilize resistance proteins as receptors or in some other way can be identified. Also, if a cocktail of multiple phage is used, the chance of developing resistance to just one phage is highly reduced.

Disadvantages. There are several challenges that may arise with phage therapy. For example, it takes a large number of phage virions to eradicate a bacterial population (34). Once all the different challenges and problems are understood, phage therapy may be more effective at treating bacterial infections. Although phage therapy has been successfully applied in some agricultural, food-processing, and fishery industries, additional carefully controlled experiments need to be run to address potential drawbacks and ensure full safety and efficiency for use in humans and animals with any bacterial infections (35).

One major drawback is that the host immune system can produce immunity to the therapeutic agent. Anti-phage activity of phagocytes, antibodies, and serum were identified during phage treatment by high-resolution fluorescence microscopy (42). Innate immunity was boosted during bacterial infection, and the mammalian-host response to the bacteria facilitated removal of phage from the system. However, adaptive immunity plays a larger role in the removal of phage. One previous study showed that phage induced anti-phage IgM and IgG antibodies 5-10 days after being injected into mice (42). In pre-immunized mice, Hodyra-Stefaniak *et al.* (2015) found that phage concentrations in the blood of high IgM mice decreased much more rapidly than in control mice shortly after phage injection. In addition, no active phage were found in the blood of mice that developed a strong secondary response (IgG) to the phage. Therefore, it appears as though a second round of phage injection may be ineffective. Each therapeutic phage treatment might be limited to one use per patient. Another interesting finding is that serum complement was identified as a factor contributing to phage neutralization, despite the fact that phage are not harmful to mammalian cells. More research needs to be completed to further understand the mechanisms taking place during the innate and adaptive immune responses against the phage before phage therapy can be implemented as a treatment against bacterial infections.

Moreover, a direct innate response to phage by mammalian cells is rarely considered and is insufficiently studied. One paper concluded that lysogenic Pf phage specific to *Pseudomonas aeruginosa* triggered an antiviral immune response (43). With an immune response to the phage as well as the bacteria, this prevented the clearance of the bacterial infection and allowed the bacteria to thrive. Since the phage are lysogenic, they do not harm the bacteria unless they are

triggered to become lytic. In this case, the phage seem to be distracting the immune system from the bacterial infection, which allows the infection to get worse. However, it is important to note that certain triggers can cause the phage to switch from lysogeny to lysis, such as environmental damage to the host or a peak in host growth that allows for successful viral replication (44). So, the authors suggest that vaccination against the phage could be a potential treatment for bacterial infections since the presence of the phage actually helps the bacteria. The vaccine would provide immunity against the phage so that it cannot exacerbate the infection. This suggests that the innate response to any phage should be studied before use as an antibacterial therapeutic.

Similarly, another paper shows that lysogenic Pf phage promotes noninvasive infection *in vivo* (45). As the bacteria grow in biofilms, they dramatically increased the production of Pf phage. In a murine pneumonia model, Pf phage prevented the spread of *P. aeruginosa* from the lung. The phage promoted bacterial adhesion to mucin and inhibited bacterial invasion of airway epithelial cultures, suggesting that Pf phage traps *P. aeruginosa* inside the lung. Moreover, phage-infected *P. aeruginosa* were less prone to phagocytosis by macrophages than uninfected bacteria because Pf phage induce M2 macrophage phenotype, which is less effective at bacterial phagocytosis than M1 phenotype. Collectively, these data suggest that Pf phage promote bacterial infection to one specific area rather than reduce it. Therefore, phage for other bacteria must be carefully researched before considering phage therapy as a possible treatment against bacterial infections in humans, given the possibility that the phage could worsen the infection.

Phage readily bind extracellular bacteria, but they may not readily access bacteria that reside inside cells. As such, it is unclear whether there might be an effective treatment option for intracellular pathogens. To be successful for these types of pathogens, phage may need to be

delivered to the host by approaches that facilitate uptake by host cells, such as macrophages. For example, one approach under consideration is the use of liposomes (46). Phage could also be used in combination with other countermeasures, such as antibiotics or even immune modulators to treat intracellular pathogens (41).

Phage Therapy for *Brucella*

Phages that specifically lyse *Brucella* were isolated in the late 1950s. They were initially discovered by Russian workers who isolated them from manure and old cultures of *Brucella* (47). Fourteen phage were isolated from a collection of 200 *Brucella* strains. These phage only lyse *B. abortus*, and do not lyse *B. melitensis* or *B. suis*. The susceptibility of bacteria to phage lysis is very specific to surface antigens of taxonomic groups, species, or strains of bacteria (48). Sequence analysis of *Brucella* phage (49, 50) showed nucleotide variations between the same phage tested from different labs. These variations occurred in genes that are most likely involved in host specificity, which explains the differences in effect between the original reference phage and homonymous phage—supposedly identical phage of the same homology—used in other labs (49). As such, it is important to know the specificity and function of each phage if they are to be used in treating *Brucella* infections.

Brucella bacteria use the VirB system to replicate intracellular, as previously described (6). This could pose a problem with using *Brucella* phage as therapy because, like most antibiotics, phage may face difficulty penetrating mammalian cells. By understanding that *Brucella* is an intracellular pathogen, we can better approach how to create novel therapeutics that will eliminate it as opposed to most extracellular pathogens that are easier to target with

simple antibiotics. Thus, it is important to determine whether *Brucella*-specific phage can target bacteria inside of macrophages.

Results to Date. Because *Brucella* phage are so specific and lead to robust lysis, they could prove useful in clinical treatment of infections. One recent study was marginally successful at controlling *B. abortus* strain 544 infection in mice after treatment with an unspecified phage *in vivo* (51). Mice were treated with a *Brucella* phage dose of 10^6 plaque forming units (PFU)/ml by the intraperitoneal route 48 hours before and after infection. The spleens were aseptically collected 15 days after treatment, and any bacteria present were quantified by culturing via serial dilutions. The researchers found that *Brucella* phage significantly reduced bacterial counts in the spleen of infected mice compared to control-treated infected mice (up to an 80-fold reduction). Another study used the *Brucella* phage Tb to treat *B. abortus* strain 544 infection in guinea pigs *in vivo*. Guinea pigs were given a phage dose of 10^9 PFU/ml by the intracardiac route every other day for seven days. Unfortunately, this strain did not work well in chronic infections (52). Therefore, there is still a need for more animal studies to properly identify the optimal phage and route/interval of phage delivery to treat brucellosis.

CHAPTER TWO

LESS MAY BE MORE FOR *BRUCELLA* BACTERIOPHAGE THERAPY DUE TO IMMUNE SUPPRESSIVE EFFECTS

Contribution of Authors and Co-Authors

Manuscript in Chapter Two

Author: Jodi F. Hedges

Contributions: Designed, performed, and analyzed experiments, wrote manuscript

Co-Author: Deann T. Snyder

Contributions: Designed, performed, and analyzed experiments.

Co-Author: Amanda Robison

Contributions: Designed, performed, and analyzed experiments.

Co-Author: Jack Plewa

Contributions: Designed, performed, and analyzed experiments, wrote manuscript

Co-Author: Kaylee Evans

Contributions: Designed, performed, and analyzed experiments.

Co-Author: Klara Aspelin

Contributions: Designed, performed, and analyzed experiments.

Co-Author: Jory Baldridge

Contributions: Designed, performed, and analyzed experiments.

Co-Author: Agnieszka Rynda-Apple

Contributions: Provided murine BMDMs and methods for using them.

Co-Author: Abby Luu

Contributions: Provided murine BMDMs and methods for using them.

Co-Author: Blake Wiedenheft

Contributions: Collaborator advised on methods to study phage. Sequenced and helped characterize *Brucella* EF4 phage.

Co-Author: Mark A. Jutila

Contributions: PI, designed experiments, and assisted in writing.

Manuscript Information Page

Jodi F. Hedges, Deann T. Snyder, Amanda Robison, Jack Plewa, Kaylee Evans, Klara Aspelin, Jory Baldridge, Blake Wiedenheft, Mark A. Jutila

PHAGE: Therapy, Applications, and Research

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Jodi F. Hedges, Deann T. Snyder, Amanda Robison, Jack Plewa, Kaylee Evans, Klara Aspelin, Jory Baldridge, Blake Wiedenheft, Mark A. Jutila*

Department of Microbiology and Cell Biology, Montana State University, Bozeman, MT 59718

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Running title: High dose *Brucella* bacteriophage suppresses immune responses

*Corresponding Author:

Mark A. Jutila, Ph.D.

Regents Professor

Microbiology and Cell Biology

Montana State University

Health Sciences Building

2155 Analysis Dr.

Bozeman, MT 59718

mark.jutila@montana.edu

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Abstract

We are studying the applicability of *Brucella*- specific phage to counter brucellosis. With antibiotic resistance on the rise and new realizations concerning the detrimental effects of long-term antibiotic use, phage therapy for bacterial infection may become a useful approach. However, phage are frequently less effective at countering bacterial infection *in vivo* than is expected, considering their strong anti-bacterial effects *in vitro*. The direct effects of phage on mammalian cells is important to understand, yet understudied. *In vivo* delivery of low phage MOI to the mouse lung was more effective at diminishing *Brucella* burden than higher doses of phage. In an *in vitro* model of intracellular *Brucella* infection, low phage MOI was capable of reducing human THP-1 monocyte infection, but, unexpectedly, use of higher phage MOI diminished this effect. We hypothesized that recognition of these phage preparations may induce an antiviral immune suppressive response that may counteract their anti-bacterial effects. Indeed, when the type I IFN signaling pathway was disrupted in mice, phage treatment was more effective. However, when attempting to induce type I IFN *in vitro* using both human monocyte and mouse macrophage cell lines, we were unable to stimulate expression of type I IFN with *Brucella* phage. We then examined the effect of phage treatment on macrophage cell surface markers that are indicative of activation/differentiation in cultured cells. Interestingly, while *Brucella* LPS in culture for 24-96 hours led to increased expression of CD71 and CD206, the addition of phage suppressed upregulation of these markers. Our discovery of immune suppressive effects of *Brucella* bacteriophage is an important consideration for using phage as a treatment.

Introduction

Brucellosis is a zoonotic infection caused by gram-negative facultative intracellular bacteria, *B. abortus, B. melitensis, B. suis, B. ovis, B. neotomae,* and *B. canis. B. abortus* and *B. melitensis* are highly infectious to humans by the aerosol route. *Brucella* spp. are considered potential bioweapons, and there are no approved vaccines for use in humans. In livestock, primary disease presentation is spontaneous abortion, with expelled material carrying large amounts of infectious bacteria. Human disease typically presents with undulant fever, headache, joint and back pain, and fatigue (2). In humans, brucellosis is most often contracted orally via ingestion of infected material, through inhalation, or open wounds. Human brucellosis is one of the most common zoonotic diseases worldwide with more than 500,000 new cases annually, is associated with substantial residual disability, and is an important cause of travel-associated morbidity (1).

Prior to the development of the 1954 Brucellosis Eradication Program, brucellosis had a staggering impact on the economy with $400 million lost in 1952. With this program came the development of *Brucella* vaccine strains, RB51 and S19, and surveillance programs, which have dramatically reduced infection (8). The S19 vaccine strain is serologically identical to natural *B. abortus* infection, which convolutes surveillance, thus this vaccine strain is no longer used in the U.S. (8). The RB51 vaccine strain is both infectious in humans and resistant to rifampin, one of the primary antibiotics used to treat brucellosis (2, 8). In the fall of 2017, consumers of raw milk in 4 states were at risk for infection with RB51 (10). Furthermore, RB51 is only 70-80% effective, can be abortogenic, and animals must be re-vaccinated (8). Despite the success of this program, *B. abortus* still infects elk and bison in the Greater Yellowstone Area surrounding

Yellowstone and Grand Teton National Parks, posing a risk to the status of local livestock herds. Controlling infection in animals by vaccination is important for reducing economic impact and transmission to humans. The current treatment for brucellosis in humans is long term high dose antibiotics (2), which has potential negative outcomes, such as bacterial resistance and antibiotic-induced gut dysbiosis. Thus, the need for improved vaccines and novel alternative treatment options for *Brucella* and bacterial disease in general is warranted.

We have long known that bacteria are susceptible to infection with bacteriophage, viruses that specifically infect bacteria. Bacteriophage, or phage for short, are now under intense study as therapeutic alternatives to antibiotics for extracellular bacterial infection (34, 35). Phage that lyse *Brucella* were isolated in the late 1950s and have been used diagnostically to determine *Brucella* strains (47, 48). These phage have been extensively studied and sequenced (49, 50). Because they are specific to *Brucella* and lyse the bacteria, these phage could have therapeutic utility. This has been successfully attempted in guinea pigs *in vivo*, with the *Brucella* phage Tb and *B. abortus* strain 544. However, this strain had seemingly poor recovery *in vivo* (52). Another more recent study was marginally successful at controlling infection with *B. abortus* strain 544 in mice after treatment with an unspecified phage (51). There is a need for further animal studies to identify the optimal phage, route, and interval of phage delivery to counter *Brucella* infection.

B. abortus is a facultative intracellular pathogen and replicates inside macrophages during infection (4). This is a potential problem with the use of *Brucella* phage as therapy because, like most antibiotics, phage may face difficulty penetrating mammalian cells. Thus, it is important to assess whether phage can access bacteria inside macrophages. In our *in vivo*

infection mouse model, low phage MOIs could dampen bacterial replication, but adding more phage reversed this effect. Thus, we have discovered that higher numbers of phage appear to induce an immune dampening innate response. In an effort to identify the potential immune suppression induced by phage, we assessed the treatment regimen in the absence of type I IFN signaling. The results suggested that this pathway may contribute, at least in part, to the diminished effectiveness of phage treatment in the mouse model of brucellosis.

Given that *Brucella* mostly infects macrophages, we simulated our *in vivo* experiments in an *in vitro* infection model using cultured human macrophages. Similar to the *in vivo* effect, higher phage MOIs were not effective at reducing bacterial burdens *in vitro*. Next, we measured type I IFN transcripts to determine if macrophages are the source of type I IFNs. Surprisingly, we found no change in antiviral gene expression when cells were treated with phage compared to the control group, suggesting that macrophages may not account for the type I IFN response *in vivo*. To further address the effects of phage on monocytes/macrophages, we examined the effect of phage treatment on macrophage cell surface markers that are indicative of activation/differentiation. Interestingly, we found that *Brucella* phage suppressed LPS in culture-induced upregulation of CD71 and CD206. Additional studies are needed to determine the molecular basis for the selective effect of phage on these two surface markers, and whether suppression of either receptor contributes to the reduced *Brucella* killing.

Cellular innate immune mechanisms are critical to defense against *Brucella* infection (12, 53). We recently determined that enhanced innate immune responses better protect against bacterial infection (54) and synergize with antibiotics to increase protection, as we have recently demonstrated (55). Similarly, we combined stimulation of innate immunity with phage treatment

in attempt to overcome a potential immune dampening effect of phage *in vivo*. The results suggest that the intracellular nature of *Brucella* replication will be difficult to overcome with phage treatment. There are a number of intracellular bacteria that cause intractable infections of great concern such as *Chlamydia trachomatis, Mycobacterium tuberculosis,* and another select agent, *Coxiella burnetii*. Thus, these results are not only important for development of a novel treatment approach for brucellosis, but are also likely to have broad applications to other intracellular pathogens.

Material and Methods

Brucella LPS Extraction

Brucella LPS was extracted from an overnight culture of *Brucella* S19 using the Boca LPS extraction kit (Boca Scientific Inc., Dedham), according to manufacturer's instructions. The wet pellet of the overnight culture was weighed. The pellet was resuspended in 400 µl of 0.85% NaCl and stirred at 4°C for 30 min. An equal volume of butanol was added and stirred another 30 minutes. The slurry was centrifuged at 35,000 × *g* for 20 min. at 4°C. The aqueous layer with LPS was removed and stored at 4°C while the pellet was resuspended with 50 µl of saline for another 30 min. This suspension was centrifuged at 35,000 × *g* for 20 min. at 4°C and the pellet was discarded. The aqueous layers were combined and centrifuged at 35,000 × *g* for 20 min. at 4°C. The supernatant was retained and 400 µl of methanol were added and stirred at 4°C for 20 min. The precipitate was centrifuged at 13,000 × *g* for 20 min. at 4°C. The supernatant was discarded, and the pellet was dissolved in lysis buffer to break open the cells and was boiled for 5 min. Proteinase K (50 µg/10 mg protein) and DNase (50 µg/1 mg nucleic acid) were added and heated for 90 min. at 60°C followed by overnight incubation at 4°C. This was centrifuged at

13,000 × *g* for 20 min. at 4°C. The supernatant was discarded, and the pellet was suspended in ice-cold methanol and allowed to incubate on ice for 30 min. The precipitate was centrifuged at 13,000 × *g* for 20 min. at 4°C and washed two more times with ice-cold methanol. The pellet was dissolved in endotoxin-free water and centrifuged overnight at 150,000 × *g* at 4°C. The resulting pellet was suspended in endotoxin-free water.

To confirm that *Brucella* LPS had been extracted, we performed SDS-PAGE with silver staining, Western blot analysis using anti-*B. abortus* LPS monoclonal antibody (Santa Cruz, sc-58097), and an LAL (limulus amebocyte lysate; Associates of Cape Cod, East Falmouth) test to establish the Endotoxin Units (EU). SDS-PAGE was performed on a 12% acrylamide running gel with a 4% stacking gel. Silver staining was performed following the manufacturer's instructions (Bio-Rad, Hercules). Western blotting was performed by transferring to nitrocellulose at 0.01 Amps constant current overnight at 4°C, incubation with primary antibody diluted 1:500 in Blotto buffer (5% non-fat dry milk in PBS with 0.02% sodium azide) overnight at 4°C, and then washed three times in PBS (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4) with 0.5% Tween 20 (PBS/T). The secondary antibody (goat anti-mouse IgG) was diluted 1:1000 in Blotto, and then the blot was incubated for 45 min at room temperature followed by three washes with PBS/T. The LAL test was performed on *Brucella* LPS and phage preparations according to the manufacturer's instructions (Associates of Cape Cod) using *E. coli* LPS as a control.

B. abortus LPS is far less toxic than *E. coli* LPS and has been described as immune dampening. For example, *Brucella* LPS was 10,000-fold less potent in eliciting fever in rabbits and 400-fold less potent in inducing TNFα production than *E. coli* LPS (56). These results

suggest that *Brucella* LPS is much less likely than *E. coli* LPS to evoke endotoxic shock, thus making *Brucella* LPS a potential component of vaccines as a carrier so that it can stimulate human B cells. Since *Brucella* LPS does not cause inflammation, it made it useful as a negative control in our experiments.

Bacteriophage

Six lytic *Brucella* phage were obtained from Félix d'Hérelle Reference Center for Bacterial Viruses at the Université Laval (Québec, Canada) (Tb, Fz, S708, Wb, Bk, R/C; 50). Two phage were isolated from elk fecal samples from three different sites in the Greater Yellowstone Area. Fecal samples were homogenized, sterile filtered, mixed with the *B. abortus* S19 strain, and plated in soft agar. All phage were amplified by plate lysate and purified following standard protocols (57). A plate lysate assay is a concentrated liquid sample of phage. To get this, a plate of bacteria is infected with phage, which allows the phage to lyse the bacterial cells. Then, SM buffer is added to the plate surface to collect the phage. To get counts of phage, we serially diluted them out so that we could actually count individual plaques at a certain dilution. All phage were also assessed by the LAL assay as described above to measure the level of LPS contamination.

Cells

THP-1 Cell Differentiation and *In Vitro* Infection. THP-1 cells were acquired from the American Type Culture Collection (Manassas) and maintained in complete RPMI medium with 10% fetal bovine serum and 4.5 g/l total glucose (cRPMI). THP-1 cells were passaged for less than 4-5 weeks in order to maintain cell integrity. Cells were cultured at 1×10^6 cells/well in 24-well plates with 40 ng/ml PMA for 4 days to allow for development of adherent macrophages

followed by a one-day rest in cRPMI before initiating experiments. Cells were infected with *B. abortus* strain S19 (MOI of 25) from log phase cultures in 200μl THP-1 media and simultaneously treated with phage at MOIs of 1, 10 or 50 or vehicle. After 30 minutes, media was removed and replaced with media containing 100μg/ml gentamicin for another hour. Cells were washed once with PBS, lysed with 0.1% Triton-X in PBS and plated on potato infusion agar (PIA Fisher 251100) plates and colonies were counted after 5-6 days incubation.

Human Peripheral Blood Mononuclear Cells. Whole blood was drawn from adult human volunteers into sodium heparin tubes (Becton Dickinson VT6480). Histopaque 1077 (Sigma-Aldrich, St. Louis) was used to separate peripheral blood mononuclear cells (PBMCs) from whole blood, as previously described (58, 59). Cell preparations were plated in X-VIVO 15 medium (Lonza, Morrisville) at 1×10^6 cells/ml at 37°C, 5% CO_2. Cells were cultured with different phage (see above) at MOIs of 50 and 150, phage buffer (SM buffer), or *Brucella* LPS at equivalent concentrations as that contaminating each phage preparation. Cultures were incubated for 24, 48, 72, or 96 hours, and then stained with antibodies to be measured via flow cytometry (see below).

Murine Bone Marrow-Derived Macrophages. The immortalized murine bone marrow-derived macrophage (mBMDM) cell line (BEI Resources no. NR-9456) was derived from primary bone marrow cells from C57BI6/J wild-type mice immortalized by infection with the ecotropic-transforming replication-deficient retrovirus J2 [carrying v-myc and v-raf(mil) oncogenes] (60) using described techniques (61). Cells were maintained in complete DMEM with 10% fetal bovine serum (cDMEM). Cells were plated at 1×10^6 cells/ml at 37°C, 5% CO_2

with either media, phage buffer (SM buffer), WB or EF4 phage (MOI 500/1000 or 50/100, respectively), or polyIC (40 or 50 µg). Cells were incubated with treatments and controls for 2, 24, or 48 hours before cells were lysed and RNA was extracted to undergo RT-qPCR.

Mice and Infection

All animal studies were carried out in compliance with the Montana State University Institutional Animal Care and Use Committee. The mice (at least 5 mice per group) were 6- to 10-week-old C57BL/6, IFN-receptor-deficient ($IFNAR^{-/-}$), or IFNβ-deficient ($IFN\beta^{-/-}$) mice (the latter on a C57BL/6 background).

To assess *B. abortus* infection *in vivo*, mice were infected with 1×10^5 CFU of *B. abortus* strain 2308 by the intratracheal route under BSL-3 containment. Mice were treated with varying concentrations of phage by the intratracheal route between 1 to 10 days after infection. Some mice were also treated intraperitoneally with the IFNAR blocking antibody (clone MAR1-5B3) by administering 1.6mg/mouse on day 6 post-infection and 0.8mg/mouse on day 10 post-infection. Mice infected with *B. abortus* develop few symptoms and generally do not lose substantial mass (data not shown). Mice were monitored daily and euthanized on day 14 post-infection. Lungs and spleens were collected, homogenized, serially diluted, and plated on *Brucella* agar plates. The plates were incubated for 5-6 days at 37°C and colonies were quantified.

Mice were also treated with lipo-CRX, a TLR4 agonist contained in a liposome, which we recently showed enhanced *B. abortus* clearance in mice (55). The vehicle control for this experiment was a liposome preparation that did not have the TLR4 agonist or phage included. In another experiment, the phage EF4 was incorporated into the liposome preparation with or

without the addition of CRX to the liposome particles. We estimated that the amount of phage in the liposomes delivered to mice was ~ $3x10^5$ PFU/mouse in these experiments.

RNA Extraction and RT-qPCR

RNA was extracted from cells using the RNeasy Mini Kit from Qiagen following the manufacturer's protocol. RNA was reverse transcribed using the MyiQ Single Color Real-Time PCR Detection System. Cycle 1: Step 1 at 65℃ for 5 min, Cycle 2: Step 1 at 4℃ for 3 min, Step 2 at 25℃ for 5 min, Step 3 at 50℃ for 60 min, and Step 4 at 72℃ for 15 min. After that, cDNA was amplified using the 7500 Fast Real-Time PCR System. AzuraQuant 2x Green Fast qPCR Mix Lo Rox from Azura Genomics was used. Stage 1: 1 rep at 50℃ for 2 min, Stage 2: 1 rep at 95℃ for 10 min, Stage 3: 40 reps at 95℃ for 15 sec followed by a dissociation stage at 60℃ for 1 min, Stage 4: 1 rep at 95℃ for 15 sec followed by 60℃ for 1 min, then 95℃ for 15 sec again, ending at 60℃ for 15 sec. All primers were used at 5 µM. RPL37A was as the housekeeping gene primer, specific to both human and murine cells: FWD 5'-ATTGAAATCAGCCAGCACGC-3', REV 5'-AGGAACCACAGTGCCAGATCC-3'. Mouse IFNα consensus: FWD 5'-GCTAGGHYTRTGCTTTCCT-3', REV 5'-CACAGRGGCTGTGTTTCTTC-3', human IFNα consensus: FWD 5'-GACTCCATCTTGGCTGTGA-3', REV 5'-TGATTTCTGCTCTGACAACCT-3', human IFNβ: N124 5'-TGGAGAAGCACAACAGGAGAGC-3', P9 5'-ACTGCAACCTTTCGAAGCCTTT-3'.

Flow Cytometry

Cultured PBMCs were concentrated by centrifugation and then stained in FACS buffer (PBS plus 2% horse serum to block Fc receptor binding) containing the following antibodies:

CD16 APC Cy7 (BD Pharmingen Cat: 557758, Clone: 3G8), CD80 Alexa Fluor 647 (Biolegend Cat: 305216, Clone: 2D10), CD86 PE (Biolegend Cat: 305406, Clone: IT2.2), CD206 FITC (Invitrogen Cat: 53-2069-42, Clone: 19.2), CD71 APC Cy7 (Biolegend Cat: 334110, Clone: Cy1G4), CD71 PerCP EFluor (Invitrogen Cat: 46-0719-42, Clone: OKT9) and CD14 PE Cy5 (Invitrogen Cat: 15-0149-42, Clone: G1D3). Negative controls included unstained cells. Differences in the staining profiles of each antibody also confirmed specificity of the antibody stains. For each sample of 100ul, 2.5ul of antibody was added and then incubated at 4℃ for 30 minutes, followed by a wash in FACS buffer. Samples were resuspended in 0.1-1ml FACS buffer or PBS and analyzed on a SE520EON Stratedigm, BD Acurri C6, or BD FACS Calibur cytometer.

Statistics

Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov test (with Dallal-Wilkinson-Liliefor P value) was used to confirm that the data formed a Gaussian distribution. Data were analyzed with Students *t*-test when comparing only 2 groups, and one-way ANOVA with Bonferroni post-test when comparing multiple groups.

Results

Characterizing *Brucella* Bacteriophage

Six phage were obtained from Félix d'Hérelle Reference Center for Bacterial Viruses from the Université Laval in Québec, Canada (Tb, Fz, S708, Wb, Bk, R/C; 50). Each phage preparation induced differing levels of lysis in a standard plaque assay (Fig. 1A). We also

recently isolated new phage from elk fecal samples. Briefly, 15 elk fecal samples were collected from three different sites in the Greater Yellowstone Area. Fecal samples were homogenized, sterile filtered, and mixed with the *B. abortus* S19 strain and plated in soft agar. Most plates contained no plaques, but two plates had one plaque each (named EF4 and GV2). EF4 was selected for sequence analysis (62), and its structure was confirmed by transmission electron microscopy (Fig. 1B). Sequence analysis showed that EF4 was about 99.8% identical to the *Brucella* phage Tb, Fz, S708, Wb, Bk, and R/C (63), suggesting that these are all variants of the same phage. However, some of these phage infect different species of *Brucella* than others (50) and showed differences in their lytic potential (Fig. 1A). All phages, including these new isolates, were amplified by plate lysate and purified following standard protocols (57). Contaminating LPS was determined for each preparation by LAL assay.

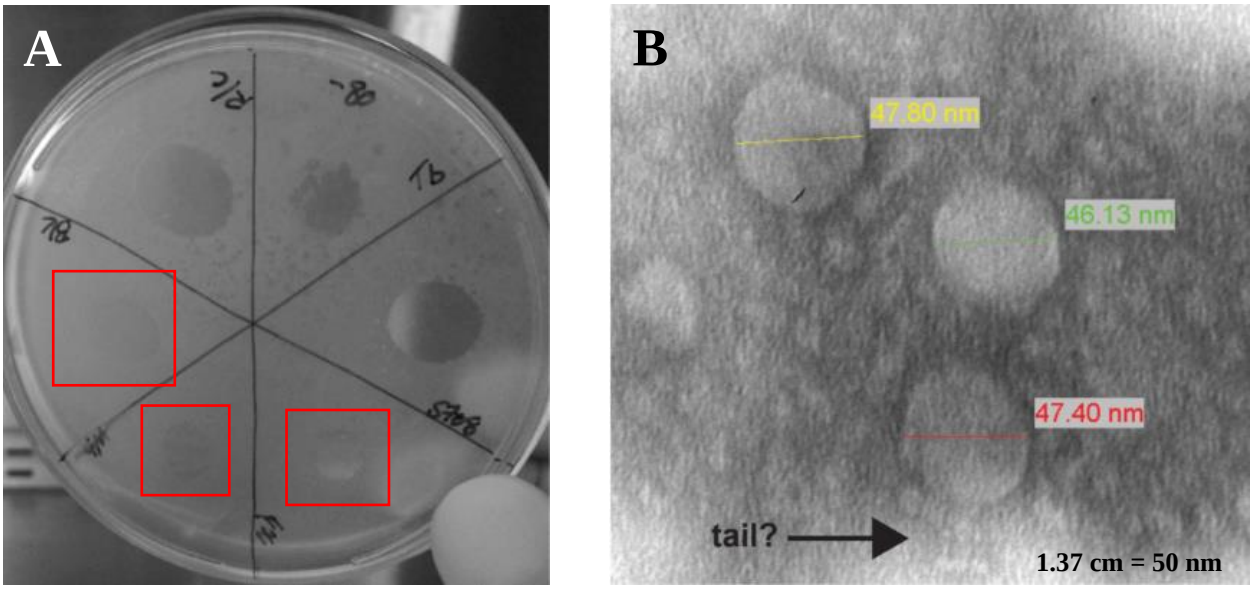

Figure 1. Characterizing *Brucella* Bacteriophage. (A) Phage plaques on a lawn of *B. abortus* S19. Red boxes show hard-to-see plaques. The spots are large zones of clearance of many plaques in the same place. (B) Electron microscope picture of EF4 phage, courtesy of the Blake Wiedenheft lab at Montana State University-Bozeman (62). Capsid diameter and a possible tail are indicated.

In Vivo Assays

We tested whether highly lytic preparations could reduce bacterial burden in the lungs and spleen of mice infected with *B. abortus*. We based the initial doses and intervals for phage treatments on existing studies with *Brucella* phage and phage in other models (64-68) and assumed high doses should lead to greater efficacy. Specifically, we started with phage doses of 10^7 to 10^8 PFU per mouse. Mice were infected intratracheally (i.t.) with 10^5 CFU of the virulent strain of *B. abortus* (2308) and then treated i.t. with either 10^7 or 10^8 phage PFUs on day 1 post infection. Surprisingly, this dose and interval of phage delivery did not have any effect on bacterial burdens in lungs or spleens collected at day 14 post infection (Fig. 2A and B).

We then tested a higher dose of two different phage (10^9), which were administered on either day 1 or day 7 post infection (Fig. 2C and D). Higher doses of phage delivered on day 1 post infection were not effective in countering infection and actually increased the bacterial burden in the lung, to a small degree for phage S708 and significantly in the case of EF4. In contrast, when the same dose of phage was delivered at 7 days post infection, there was a decrease in the bacterial burdens in the lungs for S708 phage, but not EF4 (Fig. 2C). With this high dose of phage, the bacterial burden increased slightly in the spleen, regardless of the interval of delivery.

Given that these higher doses of phage early in infection were not effective for protection from dissemination to the spleen, we opted to explore delivery of lower doses of phage to the lung. Figure 2E and F demonstrate that when phage was decreased from 10^7 PFU down to 10^3 PFU, administered on day 1 post infection, there was a subtle change in bacterial burden in the spleen (Fig. 2F), but not in the lungs (Fig. 2E). When we decreased the phage down to 10^3 PFU, it appeared that phage delivered to the lung slightly decreased the dissemination to the spleen in

this experiment, although the decrease was not significant. These results suggest that using less phage may have an effect on the dissemination of *Brucella* from the lung to the spleen. However, our data also indicated that using small amounts of phage are likely insufficient to overcome the infection, while using too much may promote infection, depending on treatment interval.

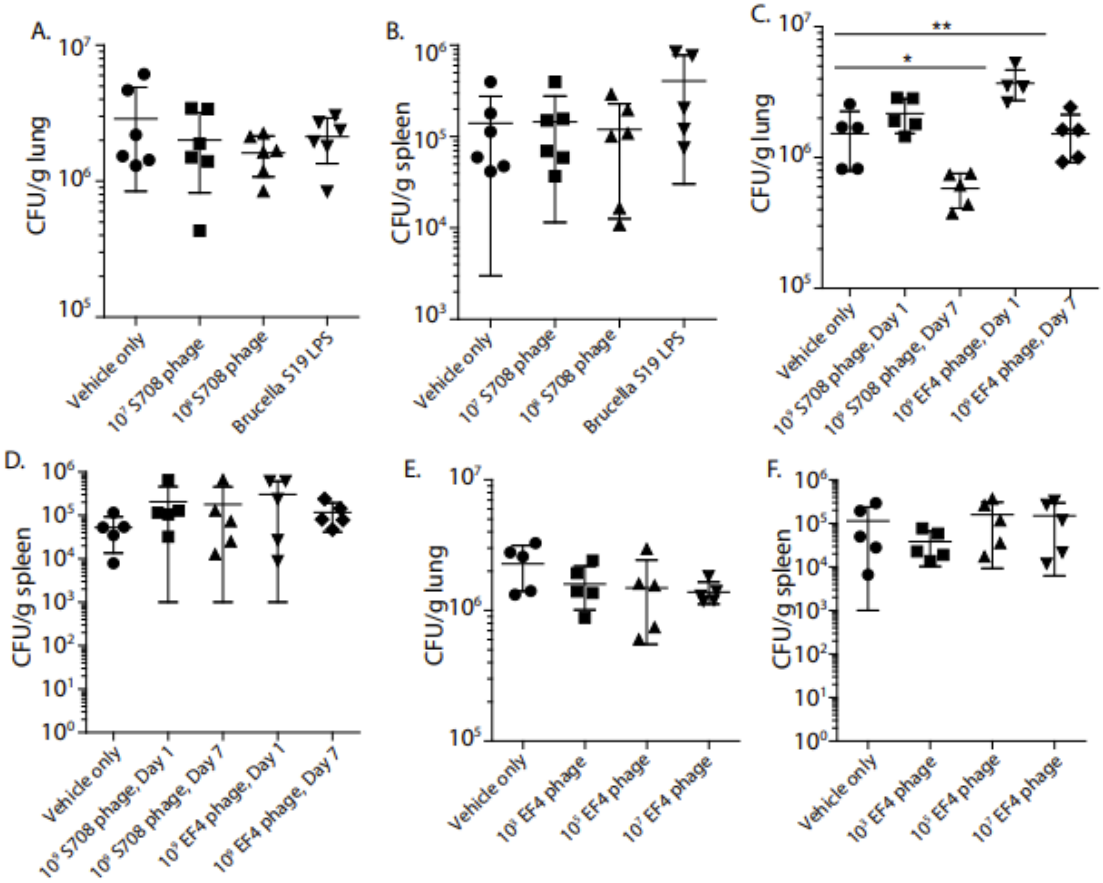

Figure 2. Varying doses and intervals of phage delivery *in vivo* only slightly affected *B. abortus* burdens in spleens and lungs in the mouse model of brucellosis. Mice treated with 10^7 or 10^8 S708 phage on day 1 post infection with 10^5 CFU *B. abortus* had no changes in bacterial burdens in (A) lungs and (B) spleens after a 14-day infection. When an even higher dose of 10^9 was delivered on day 1 post-infection, there was again no change in bacterial burden, but when delivered on day 7 post-infection there was a significant decrease in burdens in the lung after treatment with S708 phage (C), but not the spleen (D). *$p<0.05$, **$p<0.01$, as measured by the Student's t test for each paired comparison. (E) *B. abortus* burdens in the lung were slightly decreased following treatment with 10^7 phage on day 1 post infection, compared to lower doses. (F) The dose of 10^3 phage appeared to slightly decrease bacterial burdens in the spleen. For E and F, changes were not significant, but similar results were found in 2 independent experiments.

Type I IFN encourages *Brucella* replication *in vivo* and can suppress immune responses in macrophages (26). As such, we hypothesized that cells may detect viral patterns in phage following high dose delivery *in vivo*, leading to the expression of type I IFN. Such a response could be responsible for decreased effectiveness of phage when high doses were delivered. To address this possibility, we used two separate experiments to disrupt type I IFN signaling *in vivo* in an attempt to improve the outcome of phage treatment. In these tests, we delivered 10^7 PFU phage to the lungs on both days 6 and 10 post-infection to maintain the presence of phage later in the infection.

Figure 3A and B show that mice deficient in IFN signaling, either due to genetic deficiency of the receptor (IFNAR) or a primary antiviral cytokine (IFNβ), had slightly improved bacterial burdens in the spleen both compared to untreated wild type and following treatment with high dose phage. When compared to phage-treated wild type mice, which showed a slight increase in *B. abortus* burden over wild type saline-treated controls, phage treated IFNAR- or IFNβ-deficient mice had approximately 10 fold less CFUs in the spleen ($p=0.0029$ and 0.0042, respectively).

We also confirmed this finding by assessing treatment of the mice with the IFNAR-blocking antibody, MAR1-5G3, which blocks type I IFN temporarily though a different mechanism than gene deficiency. In this case, there was a significant reduction of *B. abortus* in the spleens of antibody- and phage-treated mice (Fig. 3C). As seen in the genetic deficient mouse experiments, IFNAR-blocking antibody did not affect lung bacterial burdens. Thus, disruption of type I IFN signaling slightly improved the outcome of phage treatment, suggesting that this

antiviral signaling pathway may be, at least in a minor way, responsible for the fact that high dose phage delivery does not affect *B. abortus* burdens.

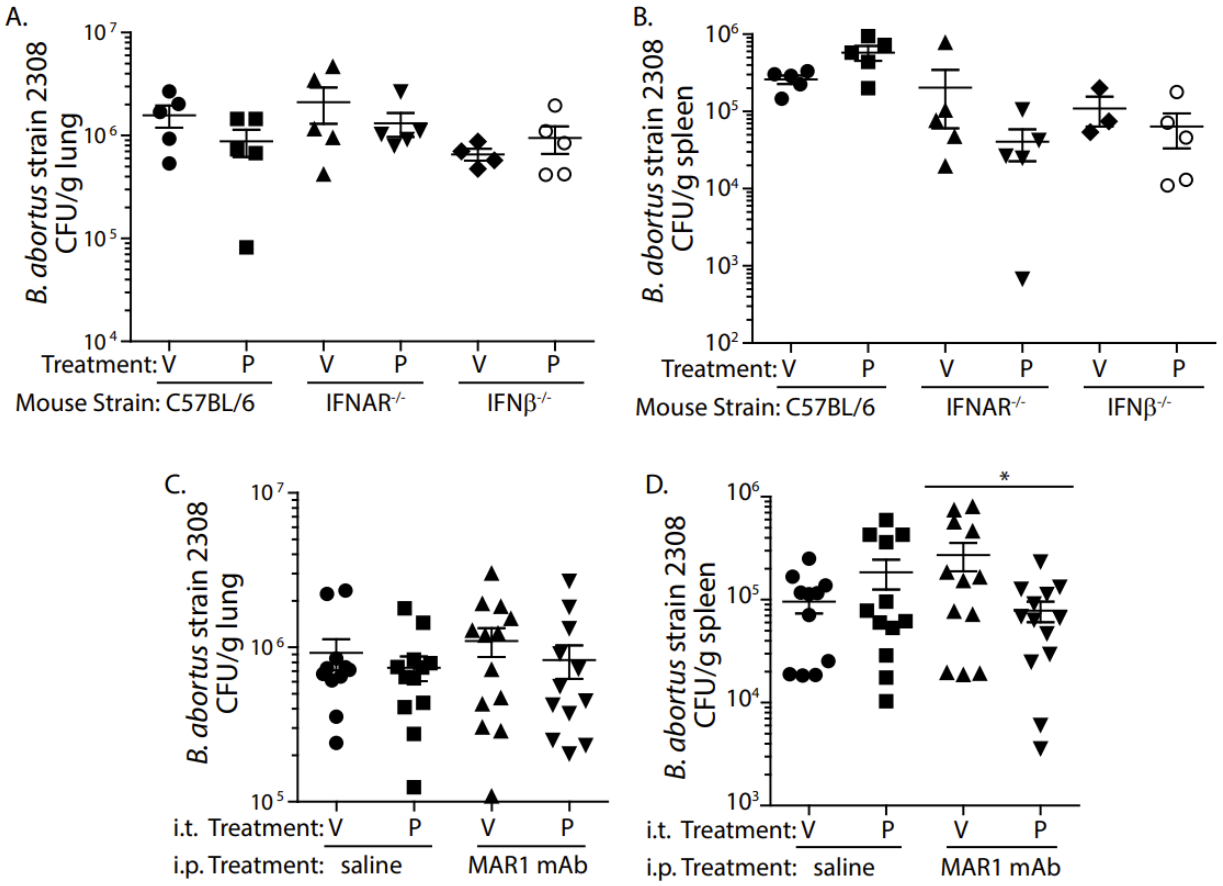

Figure 3. Type I IFN signaling *in vivo* is partially responsible for the fact that treatment with phage does not reduce *B. abortus* burdens. Mice deficient in genes expressing the type I IFN receptor (IFNAR$^{-/-}$) or IFN-β (IFN- β $^{-/-}$) or wild type mice were infected with *B. abortus* (10^5 CFU) and treated on day 6 and day 10 post-infection with 10^7 PFU EF4 phage (P) or vehicle (V). Bacterial loads in the spleens (A) and lungs (B) were assessed. Wild-type mice were also infected with *B. abortus* and then either injected i.p. with saline or the IFNAR-blocking antibody MAR1 and treated with either EF4 phage or vehicle on days 6 and 10-post infection. Bacterial loads in the spleens (C) and lungs (D) were assessed. C and D show two experiments combined. *$p<0.05$ as measured by the Student's t test for each paired comparison.

In all likelihood, phage therapy will be combined with a number of other types of antibacterial therapies in practice. Thus, in an effort to overcome an immune dampening pro-bacterial effect of phage treatment, we combined phage treatment with a liposome formulation of an innate TLR4 agonist called lipo-CRX, which we recently showed increased clearance of *B. abortus* in mice (55). The agonist was delivered either alone or with EF4 phage at 10^7 PFU/mouse on day 1 post-infection, and compared to that dose of phage or the agonist delivered alone. The agonist alone significantly reduced bacterial burdens in the spleens and lungs, but the phage alone did not (Fig. 4A, B). Combining the phage with lipo-CRX did not improve bacterial burdens compared to use of lipo-CRX alone, suggesting that lipo-CRX could not reverse the pro-bacterial, immune suppressive effect of phage.

In a final attempt to optimize phage delivery, we added the phage to the liposome formulation (lipo-phage), with or without the addition of CRX to the liposome particles. These liposomes were delivered to mice on days 6 and 11 post-infection. In this case, it appeared that the lipo-phage formulation improved bacterial burdens in a tissue-specific manner. The lower dose reduced spleen burdens only, while using 10-fold more per mouse improved lung but not spleen bacterial burdens (Fig. 4C and D). Addition of CRX into the lipo-phage formulation did not improve the outcomes compared to lipo-phage alone. Thus, our various attempts to provide a sterilizing treatment for *B. abortus* infection in mice, using phage alone, in combination with innate agonists, or encasing the phage in a liposome, failed. Naturally, we could not attempt every possible phage/agonist combination and interval for treatment in our studies. However, these results suggest that the intracellular nature of *Brucella* replication is not amenable to phage

therapeutic treatment and that the type I IFN response induced by phage is only part of the problem.

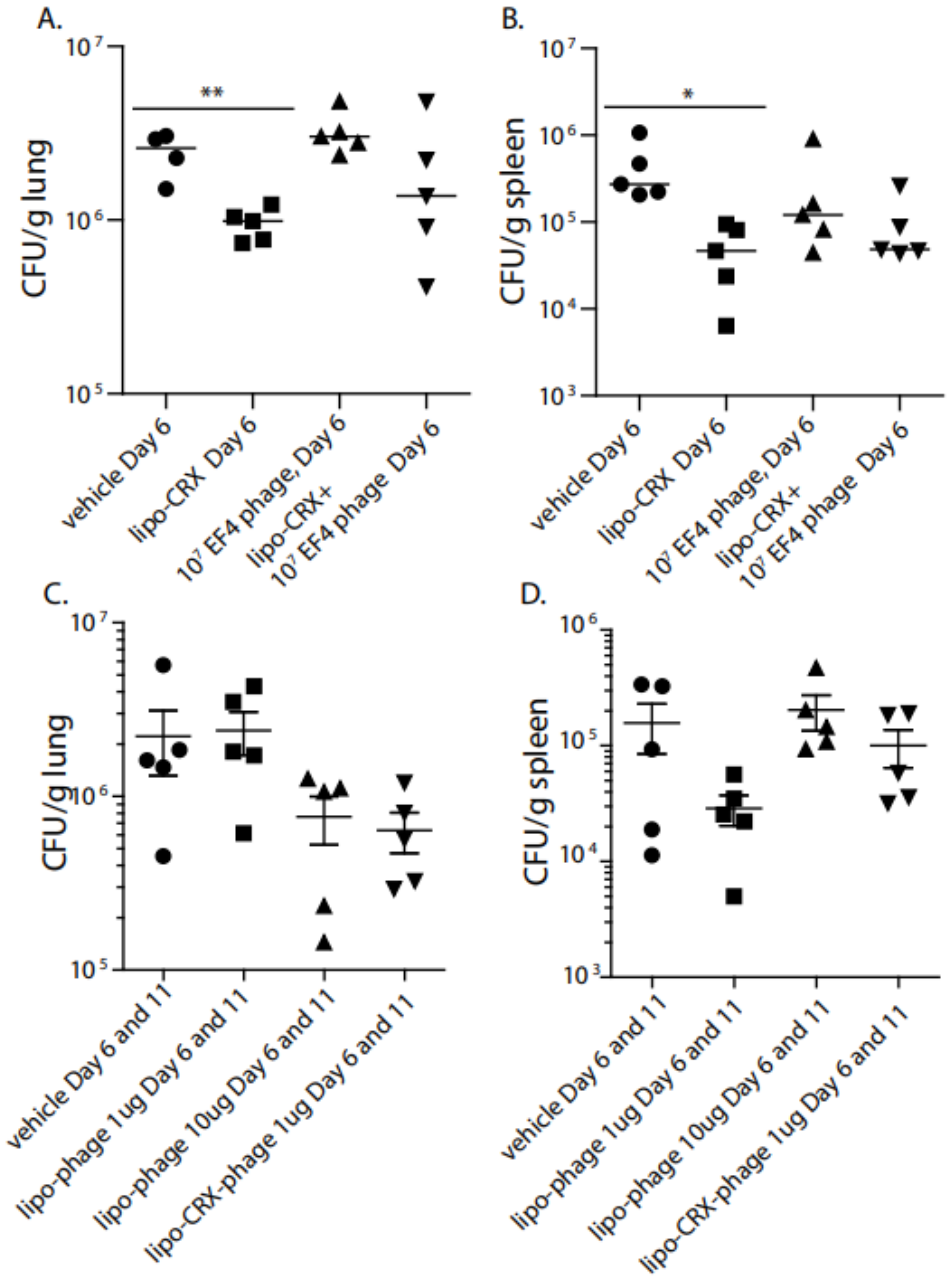

Figure 4. Combining innate immuno-stimulation with phage did not significantly alter *B. abortus* burdens beyond the effect of immuno-stimulation alone. Mice were infected with 10^5 CFU *B. abortus* and then treated with lipo-CRX, 10^7 phage, or a combination on day 6 post-infection. Only the innate agonist lipo-CRX decreased *B. abortus* burdens in the lungs (A) and spleens (B), and the addition of phage appeared to reduce this effect. In C and D, we treated mice with phage included in liposome formulations (lipo-phage) at two doses and with the addition of CRX into the liposome. (C) Both the higher dose of lipo-phage and the combination of lipo-CRX-phage slightly decreased lung burdens. (D) Only the lower dose of phage could decrease bacterial burdens in the lungs, but none of the changes in C or D were significant.

In Vitro Assays

We next wanted to establish an *in vitro* model to further exam the observed phage effect on *B. abortus*. Briefly, the human monocyte THP-1 cell line was differentiated by PMA then infected with *B. abortus* S19 strain as previously described (69). Cells were then simultaneously infected and treated with phage for 30 minutes, and then the inoculum was removed and medium containing gentamycin was added to eliminate any *B. abortus* left in the supernatant fluid. As a control, we treated THP-1 cells with equivalent amounts of purified *B. abortus* LPS found in our phage preparations to determine any phage-specific effects. As another control, we also treated THP-1 cells with *E. coli* LPS to activate the cells and increase their anti-bacterial response. After one hour, cells were rinsed and lysed, and intracellular *B. abortus* was measured by dilution and plating onto PIA plates.

As expected, addition of either *E. coli* or *Brucella* LPS caused reduction in intracellular *B. abortus*, likely due to activation of the THP-1 cells. Phage at MOIs of 1 and 10 were effective in countering *in vitro* infection, but increasing the amount of phage to an MOI of 50 could no longer decrease intracellular *B. abortus* (Fig. 5A-C), even though the phage preparation contained sufficient LPS to activate the THP-1 cells. This effect was consistent among all phage tested (S708, WB, EF4, Bk, Wb, R/C). In Figure 5D, we assessed the interaction between phage and bacteria in cRPMI medium, in the absence of THP-1 cells. This control experiment ensured that phage were capable of decreasing bacterial titers in the same conditions as in Figures 5A-C. Altogether, these results further support our *in vivo* findings that increasing the number of phage does not increase protection, but rather suppresses macrophage killing of the bacterium. This finding suggests that the phage may have a direct negative effect on innate immune cells.

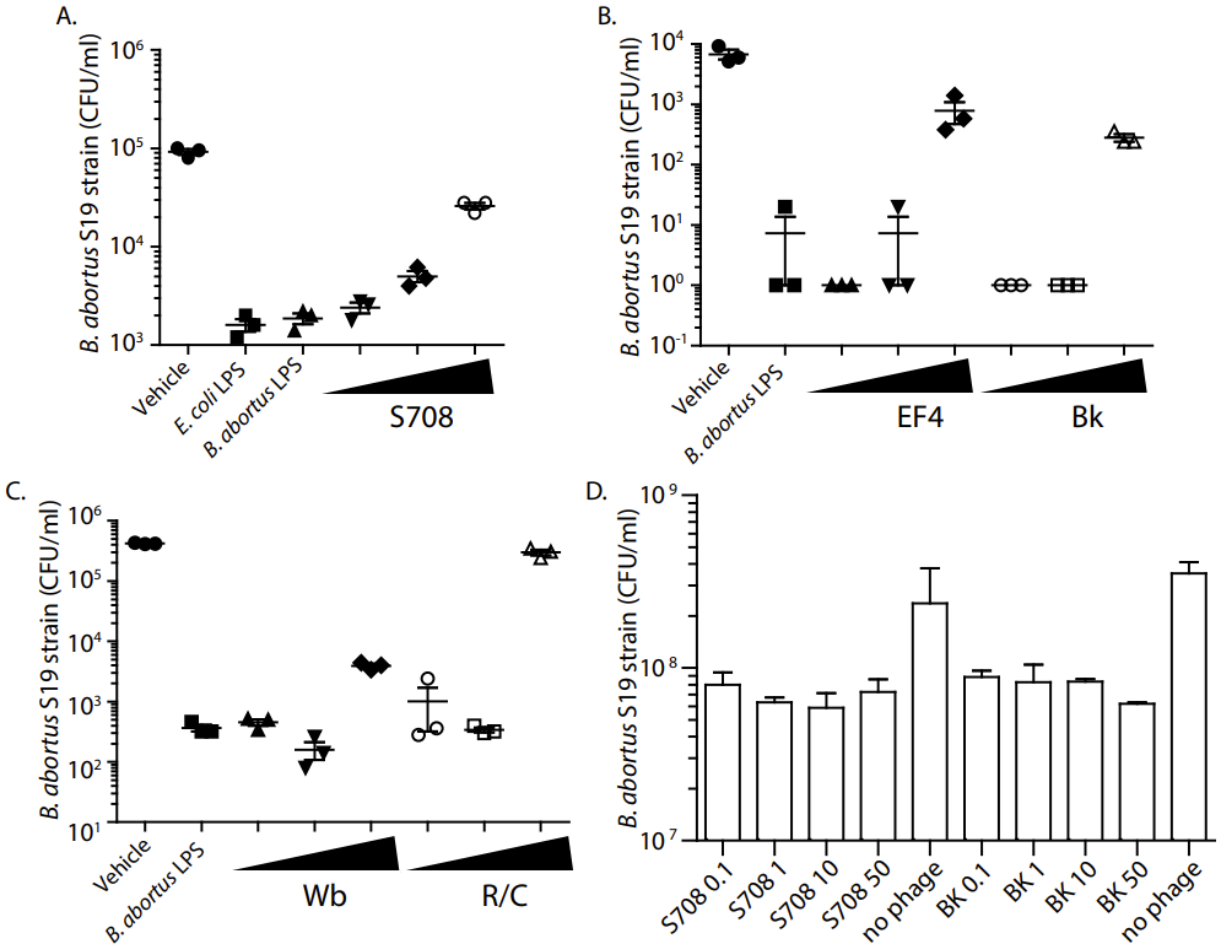

Figure 5. In an *in vitro* infection model using *B. abortus* S19 strain, increasing amounts of phage (MOI of 1, 10, or 50) reversed the anti-bacterial effect. THP-1 cells were differentiated by PMA, and then phage and bacteria were added concurrently. *B. abortus* inside the cells was measured. A-C represent separate experiments with varying levels of bacterial detection, but a similar overall conclusion, that using phage at an MOI of 1 and 10 was generally effective in countering *in vitro* infection, but increasing the amount of phage could no longer decrease intracellular *B. abortus*. (D) To ensure that the phage could be effective in the medium that supports THP-1 cells, a control experiment was performed in the same conditions, with just the phage and the bacteria but no THP-1 cells. These assays were performed multiple times in triplicate wells for each experiment. Statistical analyses were not applied to these technical replicates within experiments because the consistency and magnitude of the differences was evident.

Since we detected a contribution of type I IFN on reducing the effectiveness of phage *in vivo,* we tested whether type I IFN was induced in phage-treated THP-1 cells, and if so, did this response contribute to the phage-induced suppression of THP-1 killing of *B. abortus*. We also examined responses in an immortalized murine bone marrow-derived macrophage (BMDM) cell line to reflect our in vivo studies possibly better in mice. For these experiments, cells were treated with differing amounts of phage, and type I IFN transcripts were measured via RT-qPCR. Phage buffer (SM buffer) was used as a negative control, as well as *Brucella* LPS, given that the phage preparation always contained some level of LPS contamination. We found that *Brucella* phage did not induce type I IFN expression in either of these cell types (Fig. 6 and not shown). A summary of the different cell types, phage concentrations used, and incubation times can be found in Table 1. Our studies to date are limited by the restricted number of macrophage-type cells we have examined, but they suggest that macrophages might not account for the type I IFN response *in vivo*. Just as importantly, type I IFNs likely do not account for the suppressed THP-1 killing of *B. abortus* induced by phage treatment.

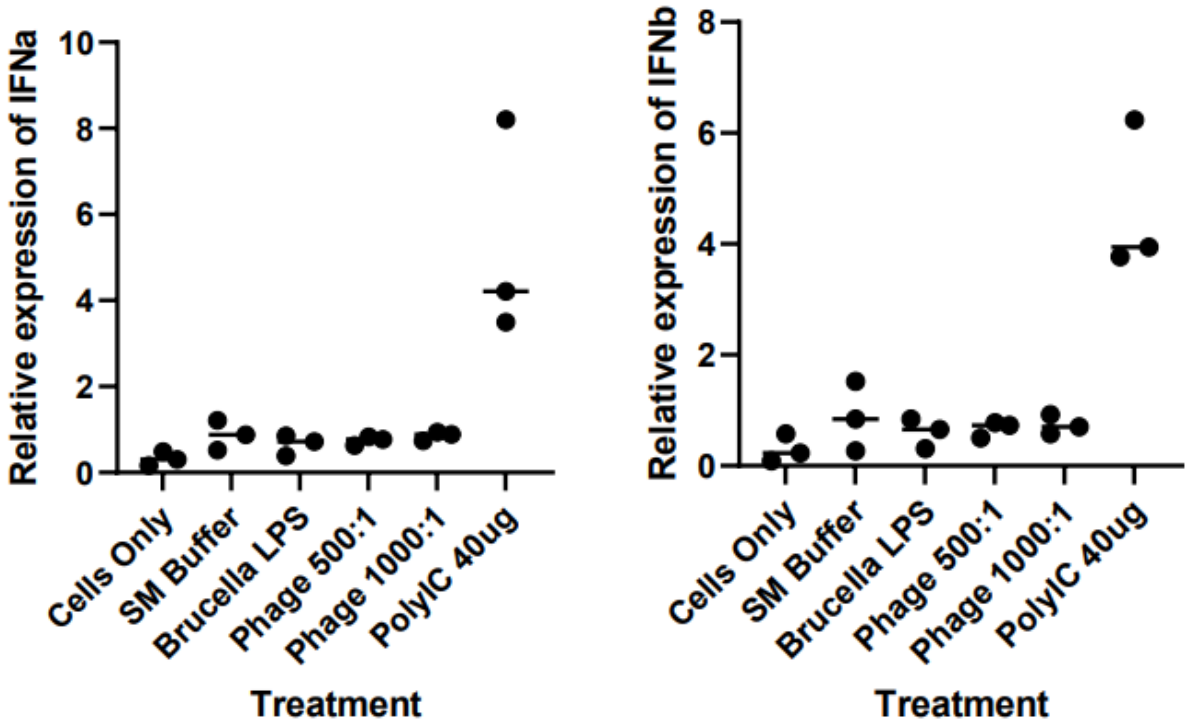

Figure 6. Representative data showing that *Brucella*-specific phage does not induce type I IFN *in vitro* in murine BMDMs. BMDMs were treated with the indicated number of phage for 48 hours, and cellular RNA was collected to measure IFNa/b transcripts via qRT-PCR. Comparisons were made to SM buffer and *Brucella* LPS as negative controls and poly I/C as a positive control. Data are representative of six experiments (Table 1).

Table 1. Different experimental conditions that failed to induce type I IFN.

Cell Type	Phage	Phage MOI	Bacteria	Bacteria MOI	Incubation Time
mBMDM	EF4	50	-	-	2
mBMDM	EF4	100	-	-	2
mBMDM	WB	500	-	-	24
mBMDM	WB	1000	-	-	24
mBMDM	WB	500	-	-	48
mBMDM	WB	1000	-	-	48
THP-1	S708	100	*Brucella*	10	4

To further address the effects of phage on monocytes/macrophages, we examined the effect of phage treatment on macrophage cell surface markers that are indicative of activation/differentiation. We used cultured human PBMCs for these experiments to avoid the confounding effects of the extensive proliferative capacity and activated state of the THP-1 and BMDM cell lines on the interpretation of *in vitro* activation/differentiation experiments. Here, the effects of EF4 phage on expression of CD14, CD16, CD206, CD71, CD80, and CD86 on human macrophages were determined by multi-color flow cytometry.

We first tested the effect of phage (150 phage per PBMC) on human PBMCs cultured for 48 hours in serum-free X-VIVO medium. We compared the effect of the phage to an equivalent amount of contaminating *Brucella* LPS in the phage preparation, as well as to the phage buffer alone. As expected, CD14, CD16, CD206, CD71, CD80, and CD86 were all expressed on cultured human monocytes, and LPS treatment induced modest to robust changes in expression of each of these markers (data not shown). Interestingly, even though the phage preparation contained the same amount of *Brucella* LPS used to treat the cells, upregulation of two markers, CD71 and CD206, was suppressed, whereas phage had no unique effect on the other markers (Fig. 7). Moreover, *Brucella* LPS significantly increased CD206 expression, but had no effect on CD71. Phage treatment did not cause increased cell death in comparison to control- or LPS-treated cells, as determined by light scatter analysis (data not shown). We next determined the kinetics of the response. The suppression of CD71 and CD206 upregulation was first detected at 24 hours and continued through 96 hours, with the greatest difference between the phage- and LPS-treated cells occurring at 48-72 hours (Fig. 8). Using the 72-hour timepoint, we then tested different doses of phage and found that 50 phage per PBMC still induced the effect (data not

shown). These results show that the EF4 phage preparation suppresses some responses in human macrophages.

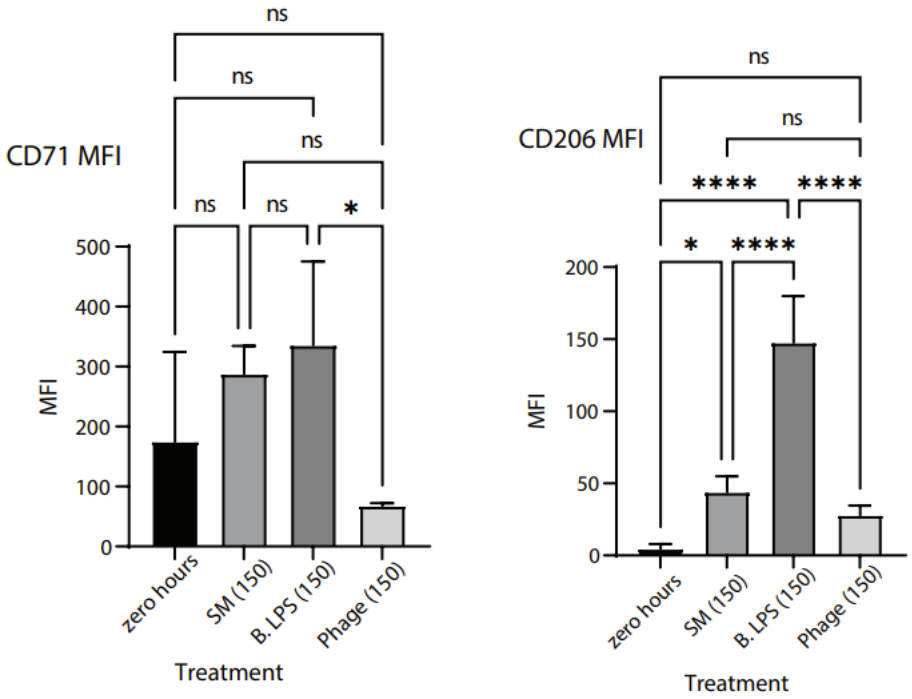

Figure 7. *Brucella*-specific phage suppress *Brucella* LPS-induced activation of the macrophage activation/differentiation markers CD71 and CD206. Phage were incubated with human PBMCs for 48 hours at 37°C. Cells were then collected and stained for flow cytometry. One-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

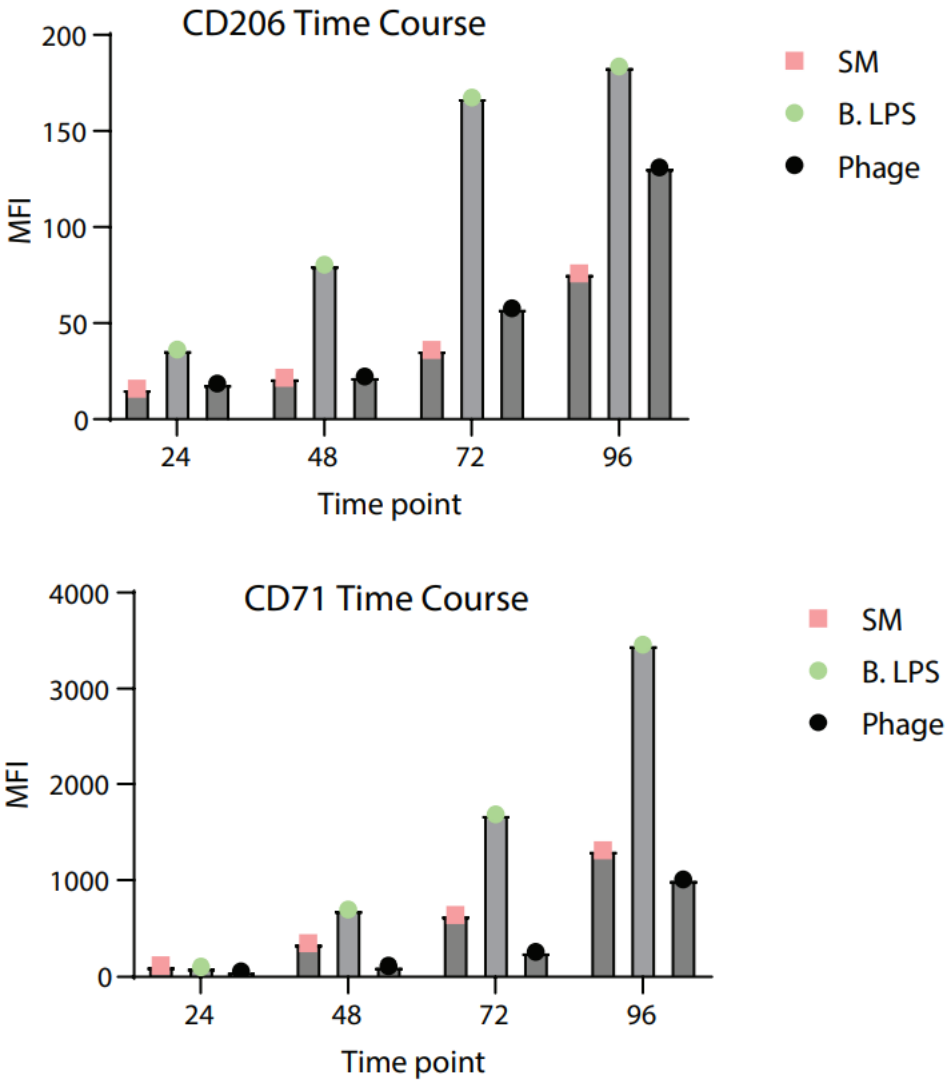

Figure 8. *Brucella*-specific phage suppresses CD206 and CD71 over time. Phage were incubated with human PBMCs for 24, 48, 72, or 96 hours at 37°C. Cells were then collected and stained for flow cytometry. These are results from a single experiment.

Discussion

Bacteriophage specifically infect and evolve with their host bacteria. While these *Brucella*-specific phage lyse bacteria in culture (50), a mutually beneficial relationship between phage and bacteria may have also evolved. In other words, our data suggest that these phage may have evolved to be immunosuppressive for the benefit of their bacterial host. Our findings

suggest an innate response of mammalian cells to high concentrations of these bacteriophage that promotes bacterial replication. This effect was seen using five different Brucella-specific lytic phages, isolated eithers decades ago or within the past 3 years, suggesting a highly conserved mechanism.

The potential suppressive effect of *Brucella* phage was detected when we tested their potential to counter *B. abortus* infection in mice. When high doses of phage were delivered to the lung early in infection, there was either no effect or surprisingly a slight increase in bacterial burdens. A lack of extracellular bacterial host for the phage to infect could explain the outcome of no difference in bacterial burdens at early intervals. For this and other intracellular bacteria, methods to deliver phage more efficiently to the intracellular compartment where the bacteria are replicating may be necessary. The slight increase in bacteria when phage is delivered at early intervals is consistent with an immune dampening response to high dose phage and warrants further characterization. This result led to curiosity about how lower phage doses may alter disease outcome.

We found that decreasing the amount of phage delivered *in vivo* early in infection actually was associated with slightly decreased bacterial burdens in the spleens. High doses of phage (10^9) had a substantial effect on bacterial burdens when delivered at day 7 post infection. This data indicates that phage is capable of affecting bacterial levels when delivered directly to a specific tissue, but when delivered early in infection has either no effect or can increase bacterial numbers. In the lung at day 7 post-infection, there may be a larger number of extracellular bacteria accessible for phage infection. Furthermore, at this later interval, any dampening of innate responses by phage may not be as important as earlier in infection. However, phage

treatment in the lung at day 7 post infection is clearly too late to affect bacterial burdens in the spleens, where infection is already established.

An immunosuppressive effect of a lysogenic, but not lytic, phage has been previously noted (43). This lysogenic phage appears to be inducing an innate antiviral response (type I IFN) that promotes infection by its target bacterium, *Pseudomonas aeruginosa*, by suppressing macrophage killing (45). We determined that type I IFN signaling may be responsible, at least in a minor way, for the fact that high dose phage fails to decrease *B. abortus* burdens *in vivo*. The effect was detected in mice deficient in type I IFN production or by inhibiting type I IFNs with specific antibody. This pathway has previously been shown to enhance infection for particular bacterial pathogens (70, 71), specifically *Brucella* (72). The production of type I IFN is detrimental to a host during *B. abortus* infection because it negatively impacts the antibacterial response, which favors the bacteria's survival (72). This may be evidence of the evolution of a mutually beneficial relationship between phage and Brucella bacteria.

Since phage-induced type I IFNs suppress macrophage killing of *P. aeruginosa*, we reasoned a similar response may contribute to the phage-induced suppression of macrophage killing of *B. abortus* noted *in vitro*. However, we were unable to show a phage-induced type I IFN response in either human or mouse macrophages. Changing the cell type, phage, phage concentration, and incubation times had no effect on this outcome. Further analysis is required to determine the cellular source of type I IFN *in vivo*, but these results strongly suggest that type I IFNs do not account for the inhibition of macrophage-killing of *Brucella* induced by phage *in vitro*.

In contrast to the lack of effect on type I IFN production, we did define a novel effect of phage on *in vitro* activation/differentiation of human macrophages. Phage suppressed expression of CD71 and CD206 on human macrophages, whereas minimal impact on CD14, CD16, CD80, and CD86 was detected. Thus, *Brucella* phage uniquely altered the phenotype of activated/differentiated macrophages. CD71 is a transferrin receptor that is upregulated on lung macrophages and used as a marker for these cells (73). The binding of iron to transferrin can lead to reduction of bacterial growth. CD206 is a C-type lectin, which serves as a PRR involved in uptake of viral, fungal, and bacterial pathogens through recognition of mannan glycoproteins involved in macrophage uptake of some pathogens (74). By suppressing these receptors, *Brucella*-specific phage may assist in bacterial survival. Additional studies are needed to determine the molecular basis for the selective effect of phage on these two surface markers, and whether suppression of either receptor contributes to the reduced *Brucella* killing.

We recently showed that stimulating macrophage activation with a non-toxic TLR4 agonist could enhance clearance of *B. abortus in vivo* (55). As such, we tested if such a treatment could overcome the lack of efficacy of high-dose *Brucella* phage *in vivo*, even though this wasn't seen *in vitro*. The TLR4 agonist is an aminoalkyl glucosaminide phosphate called CRX, which is incorporated into liposomes as an approach to minimize toxicity (called lipo-CRX). We treated mice with lipo-CRX with or without phage in the *Brucella* challenge model, which did not increase the efficacy of the phage treatment over lipo-CRX alone. As another approach, we incorporated phage within liposomes, with and without CRX. Again, CRX treatment did not affect the efficacy of the phage treatment. Interestingly, incorporation of phage into liposomes alone did show a modest benefit, perhaps due to more efficient delivery of phage into the

macrophage. Our studies suggest that perhaps liposome delivery of phage in combination with anti-type I IFNs and/or antibiotics might be considered in developing novel treatment approaches for brucellosis. Importantly, these results show that macrophage activation *in vivo* with a TLR4 agonist cannot overcome the lack of efficacy/suppressive effects of high-dose phage treatment *in vivo*.

Finally, in this study, we only delivered the phage to the lung. We reasoned that placing the phage in the same tissue as the infecting bacteria would allow maximum effect of the phage. It appeared that higher doses of phage later in infection could substantially decrease bacterial burdens in the lungs, whereas a lower dose early was necessary to alter dissemination of the infection to the spleen. Thus, using both of these intervals and doses of phage may be a more effective treatment. There is a clear need for alternatives to antibiotics as resistance is a major concern. Phage therapy offers great potential for some bacterial infection. However, whether they can be used for intracellular pathogens is unknown. There is still much to learn about the effects of phages, not only on the host bacteria *in vivo*, but also on the host immune response itself.

Future Directions

Moving forward, we would like to determine the mechanism by which phage induce suppression of macrophages and the innate immune response. Given that *Brucella* phage suppress the anti-bacterial effects of the macrophage during infection, there appears to be a beneficial co-evolutionary relationship between *Brucella* and its phage and thus should be further explored. Since the phage are enhancing *Brucella* survival by suppressing macrophage function, perhaps the phage is responsible for the possibility of secondary infections affecting the host. For

example, cases of brucellosis and COVID-19 co-infection have been reported (75). Co-infection studies would need to be completed to determine the role of *Brucella* phage in host susceptibility to COVID-19 and potentially other infections.

CHAPTER THREE

OTHER COLLABORATIVE STUDIES

Mycoplasma ovipneumoniae

Mycoplasma pneumoniae are the smallest self-replicating bacteria, consisting of a circular, double-stranded DNA molecule, with 500–1000 genes, one sixth of that found in *Escherichia coli* (76). *Mycoplasma* can be spherical or filamentous, and unlike all other prokaryotes, they have no cell walls, which has led them to be categorized as *Mollicutes*, meaning "soft skin" (76). *Mollicutes* are Gram-positive and include spiroplasma, mycoplasma, and acholeplasma (76). *Mycoplasma* is often compared to crabgrass given how persistent its infections are in cell culture and how difficult it is to detect and diagnose.

In 1972, *Mycoplasma* was identified as the infectious agent associated with pneumonia in domestic sheep (*Ovis aries*) in Queensland, Australia (77). This strain of bacteria became known as *Mycoplasma ovipneumoniae* (*M. ovi*). In the last decade, it has been identified in bighorn sheep (*Ovis canadensis*) in the Northwest United States (78).

Disease

When it was first discovered in domestic sheep in 1972, *M. ovi* was thought to lead to respiratory distress and pneumonia, characterized by lesions on the lungs, specifically in lambs aged 5 to 15 weeks (77). Morbidity in infected domestic sheep is considered modest, however, its introduction into naïve bighorn sheep is thought to be quite lethal, predisposing animals to polymicrobial secondary infections and causing up to 90% loss of adults in some cases (79, 80). In contrast, *M. ovi* causes overt clinical symptoms in bighorn sheep including lethargy, fever,

nasal discharge, ear paresis and headshaking, and coughing, and can be associated with high mortality rates (81). Coughing produces infected respiratory droplets which allows for easy transmission between sheep, especially when animals are in close contact.

Research

In the absence of effective countermeasures to eliminate infection within a flock, current management practices focus on restricting where domestic sheep can graze, causing hardships for the industry. Critical to the development of new treatments for *M. ovi* infection is an understanding of disease pathogenesis, bacterial persistence, and the role of the immune system in countering infection. Monoclonal antibodies (mAbs) provide powerful tools for such studies. For example, they can be used to detect bacteria in tissues and fluid samples, analyze bacteria in *in vitro* assays, and for analysis and purification of specific bacterial antigens. To our knowledge, there have been no reports of generation of specific monoclonal antibodies against *M. ovi*. Here we describe the generation of a new mouse monoclonal antibody that is useful for analysis of *M. ovi* by flow cytometry.

Materials and Methods

Immunization Protocol and Generation of Hybridomas. All animal studies were carried out in compliance with the Montana State University Institutional Animal Care and Use Committee, following the NIH Guide for Care and Use of Laboratory Animals, Eighth Edition. Briefly, one group of female Balb/c mice (Jackson Labs, 10-15 weeks old) were immunized with ~$6x10^6$ *M. ovi* bacteria, grown as described below, emulsified in Titermax adjuvant (Sigma Aldrich) and injected intraperitoneally (i.p.). At two weeks, mice were bled, and sera was

collected for analysis of anti-*M. ovi* antibodies. Mice were then boosted with the same dose of bacteria and adjuvant.

A second group of mice were immunized with $1x10^7$ *M. ovi* grown under conditions leading to biofilm formation (see below). Mice were immunized as in prior experiments. Three weeks later, mice were bled, and sera was collected. One week later, one mouse was euthanized, and terminal bleeds were done to collect large amounts of serum for analysis.

A fusion was done using a mouse from the first group of mice that was boosted i.p. with $6x10^6$ *M. ovi* in PBS alone. Four days after the boost, the spleen was collected and processed for fusion with SP2/0-Ag14 (SP2/0) cells. Briefly, 1/3 of the spleen cells was mixed with SP2/0 cells in a ratio of 3 spleen cells to 1 SP2/0 cell and centrifuged at 1,000 RPMs. The pellet was carefully resuspended into 1.5 ml of 50% 1,450 polyethylene glycol (PEG, ATCC) in RPMI1640 (MediaTech) plus 10% DMSO, incubated for 1 min at 37^{o}C, centrifuged at 1,000 RPMs. PEG was removed, and the cell pellet was slowly resuspended and plated in RPMI containing 20% fetal bovine serum (FBS, Hyclone), 50 µM 2-mercaptoethanol, and hypoxanthene, aminopterin and thymidine (HAT, Sigma Aldrich). Balb/c peritoneal lavage cells were added as feeders. Additional medium was added on days 3 and 6, and the resulting supernatant fluids were assayed for antibody against cultures of *M. ovi* by flow cytometry on days 9-21. Positive clones were passaged in 20% FBS RPMI containing only hypoxanthene and thymidine, and then eventually subcloned by limiting dilution. The isotype was determined by use of IsoStrip™ mouse monoclonal antibody isotyping kit (Sigma Aldrich).

ELISA. An ELISA plate was coated with 100ul of *M. ovi* antigen (0.5ug/ml) diluted in sodium bicarb buffer and incubated overnight at 4 °C. The next day, the coating solution was

removed, and the plate was washed 5 times in wash buffer (1X PBS + 0.1% Tween 20). The plate was blocked by adding 200ul of 1% milk in PBS + 0.1% Tween 20 at 37 °C for one hour. After incubation, the plate was washed. The primary antibody was diluted from 1:400 to 1:6400 and 100ul/well was added, followed by incubation at room temperature. After 2 hours, the plate was washed. HRP-linked goat anti-mouse antibody was diluted 1:10K and 100ul was added in wash buffer and incubated for 90 minutes at 37 °C. The secondary antibody was removed, and the plate was washed. Finally, 100ul of TMB (50/50 volume of both components) was added, incubated for 10 minutes at room temperature in the dark, and stopped with 100ul 2N H_2SO_4. The absorbance was read at 405 nm.

M. ovipneumoniae and Flow Cytometric Analysis. Planktonic *M. ovi* (Y98, ATCC) was grown at 37 °C in *Mycoplasma* broth, which was supplemented with dextrose (Thermo Fisher Scientific), l-cysteine hydrochloride monohydrate (Thermo Fisher Scientific), beta-NAD (Thermo Fisher Scientific), thallium acetate (Acros Organics), phenol red (Sigma Aldrich), porcine serum (Quad Five, Ryegate, MT), equine serum (Quad Five), and penicillin G potassium salt (Sigma Aldrich) under microaerophilic conditions and quantified as described (Johnson, T 2022 Vet. Micro). *M. ovi* was also grown as a biofilm. The biofilms were generated by inoculating *M. ovi* into *Mycoplasma* broth onto glass bottom 96-well plates and incubating for 48 hours, then media was removed and PBS was added to the wells. The well was scraped to collect the bacteria which were extensively washed and quantified by FACS as described (Robison, E. 2022, Viruses). Concentrated stocks were used for the immunizations (see above) and flow cytometry staining protocols. For a single-color flow cytometry protocol, $2x10^6$-$5x10^6$ bacteria were added to 1ml Eppendorf tubes and 100-200ul of either diluted (1:400 to 1:3200) pre-

immunization or post-immunization mouse sera, hybridoma culture supernatant fluids, or medium/buffer alone were added, and the cells incubated on ice for 30-45 minutes. The cells were then washed in FACS buffer (PBS plus 2% horse serum), pelleted at 17,000xg for 20 minutes, and then resuspended in 200ul of a 1:250 dilution of goat anti-mouse Ig (H&L chain) conjugated to phycoerythrin (PE; Southern Biotech) and then incubated again for 30 minutes on ice. The cells were washed in FACS buffer, pelleted, and then resuspended in 0.5-1.0ml FACS buffer and analyzed on either a BD FACSCalibur or SE520EON Stratedigm cytometer. Negative controls included medium/buffer alone, pre-immunization serum, or negative hybridoma supernatant fluids. Positive controls included dilutions of post-immunization serum confirmed to have high titer anti-*M. ovi* antibodies. A two color-flow cytometry protocol was developed to ensure reactivity with *M. ovi* and not components in the complex *Mycoplasma* culture medium. Here, SYBR green was added at a final dilution of 1:2000 to the antibody-stained preparations, described above, during the final 10-15 minute incubation of the second stage reagent. SYBR green has previously been shown to bind *M. ovi* DNA and fluoresces in the FL-1 channel, whereas FL-2 is used for the PE second stage.

Results and Discussion

Pre- and post-bleed sera from mice immunized two times with *M. ovi* were used to stain cultures of *M. ovi* by flow cytometry. Sera from all immunized mice showed reactivity with *M. ovi* cultures, with the serum from one mouse (mouse B) selected for further characterization and for an eventual fusion protocol (data not shown). A limited dose analysis of the pre- and post-bleed sera was done to confirm specific antibody reactivity. All tested dilutions of the post-bleed serum stained positive for *M. ovi*, while all dilutions of pre-bleed sera stained negative (Fig. 9).

M. ovi-specific antibodies were still evident at a 1:3200 dilution (Fig. 9). A fusion was then done on the spleen from mouse B, four days after an i.p. boost with bacteria, and resulting hybridomas were screened for specific antibody by flow cytometry. One antibody was selected, KJ01F1, that specifically stained a fraction of the cultures of *M. ovi* that were stained by mouse B anti-serum (Fig. 10A). This hybridoma was subcloned and the monoclonal antibody was typed as an IgM. KJ01F1 was further distinguished from the negative serum (Fig. 10B and C).

Upon characterization of the reactivity of KJ01F1, we determined that it reacted with medium components that were enriched during the concentration of *M. ovi,* which were not removed by repeated washing of the bacteria preparation. Furthermore, the KJ01F1 reactive medium components displayed FSS and SSC light scatter profiles that completely overlapped with the light scatter profiles of the bacterium, as determined by flow cytometry (data not shown). Using KJ01F, we developed a new flow cytometry assay to select for antibodies specific for the bacterium itself. Here we used published protocols (82) to stain *M. ovi* using SYBR green detected in the FITC (FL-1) channel, combined with specific antibody in the PE (FL-2) channel. As shown in Figure 11A, KJ01F1 mainly reacted with SYBR green negative particles in the cultures. Some staining was evident on a fraction of SYBR green positive cells, but these likely reflected medium components bound to the bacteria.

To eventually generate new bacteria-specific mAbs, we immunized four new mice with bacteria grown as a biofilm. Our goal here is to select for B cell clones producing antibodies specific to the biofilm prior to the fusion. Three weeks after immunization, mouse sera were collected and tested by ELISA for reactivity with *M. ovi* cultures. All four sera samples were positive (data not shown). We then collected terminal serum from one of the mice and tested it in

the two-color *M. ovi* specific flow cytometry assay. As shown in Figure 11B, specific antibody reactivity was detected with a 1:1000 dilution on gated SYBR green-positive *M. ovi*.

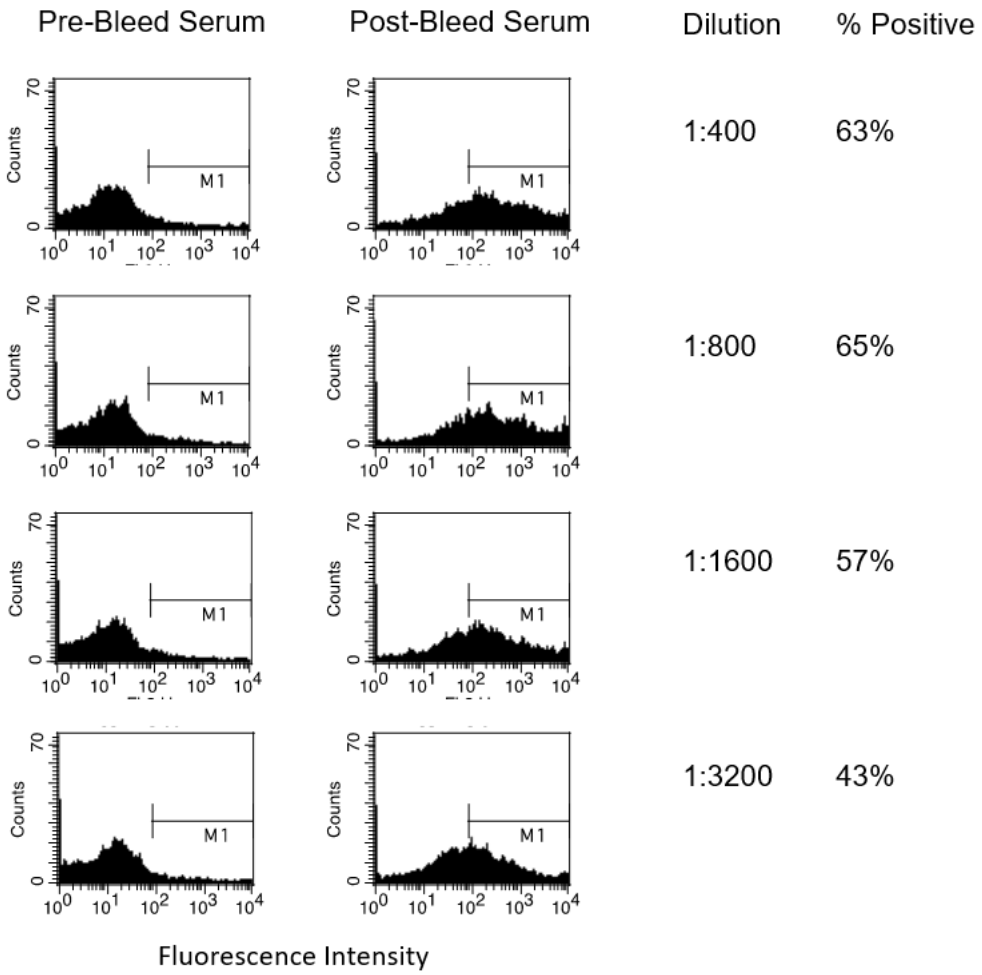

Figure 9. Serum from a *Mycoplasma ovipneumoniae* immunized mouse has high titer antibody against the bacteria as measured by flow cytometry. Serum collected prior to immunization and after two immunizations with *M. ovi* was diluted as indicated and used to stain and analyze the bacteria by flow cytometry. The marker (M1) region used to determine the percentage positive was based on staining with the second stage antibody alone.

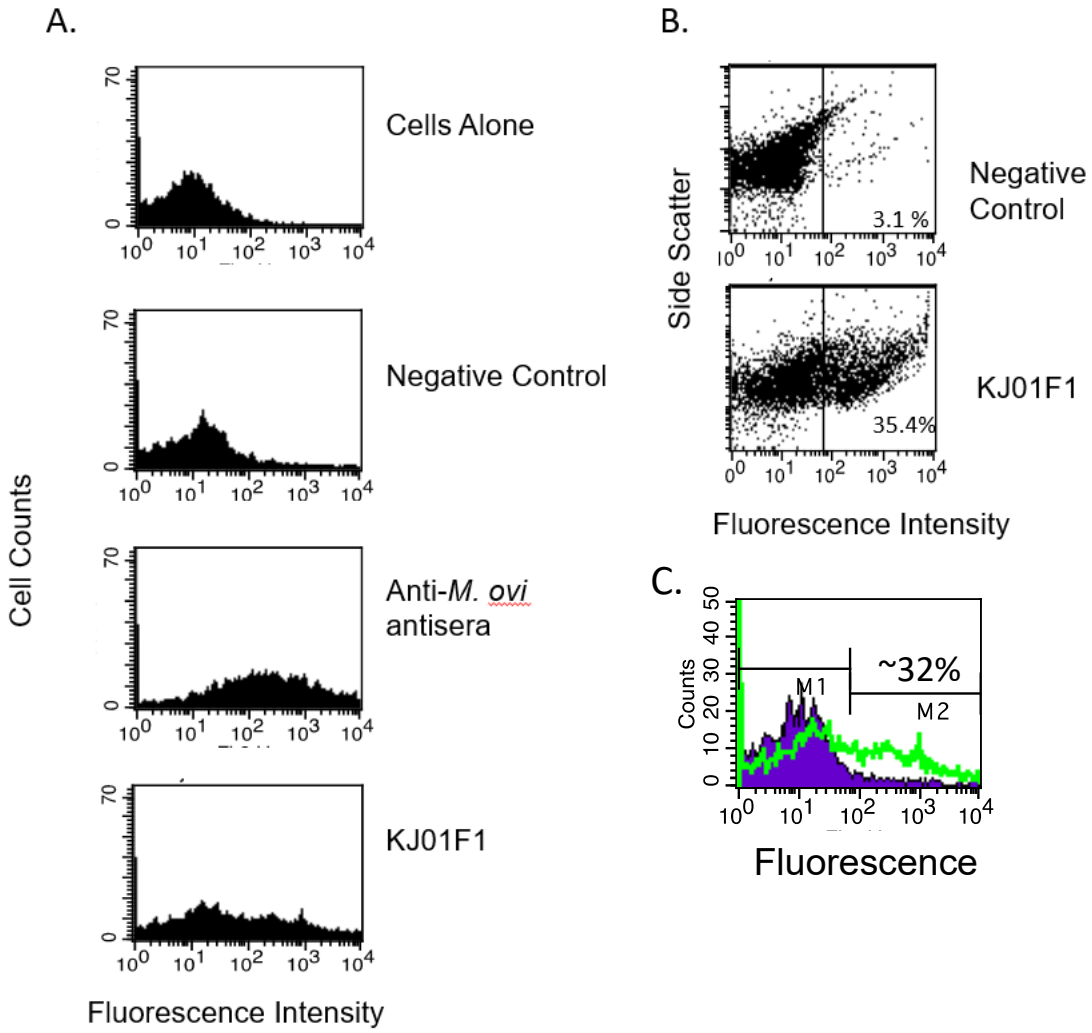

Figure 10. KJ01F1 stains a subset of *M. ovipneumoniae* (Y98) cultures. (A) *M. ovi* (Y98) was stained with a 1:400 dilution of antisera from a *M. ovi* immunized mouse as a positive control or KJ01F1 in a flow cytometry assay using PE-labeled goat anti-mouse Ig as a secondary. (B) Dot plots comparing side scatter versus fluorescence intensity of negative control and KJ01F1 staining are shown. Percent positive staining was determined using the indicated markers. (C) Histogram comparing counts versus fluorescence intensity of negative serum (purple) and KJ01F1 (green) is shown. The marker (M1) region used to determine the percentage positive was based on staining with the second stage antibody alone.

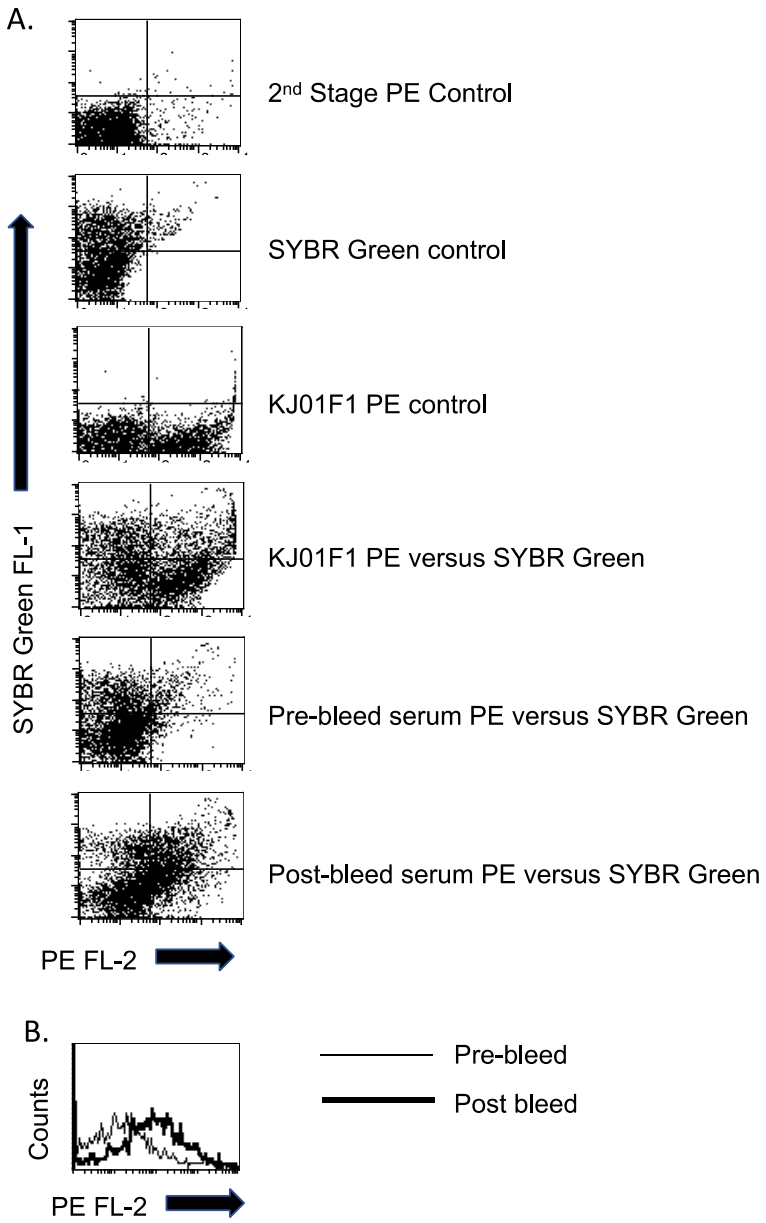

Figure 11. Comparison of SYBR green versus KJ01F1 or mouse sera staining of *M. ovipneumoniae* cultures by 2-color flow cytometry. Cultures of *M. ovi* were stained with KJ01F1, 1:1000 dilution of mouse sera obtained pre- or post-immunization with biofilm cultures of *M. ovi*, followed by PE-labeled goat anti-mouse Ig. SYBR green was added where indicated to label bacterial DNA. (A) 2-color dot plots are shown for the 2nd stage negative control, SYBR green label alone, KJ01F1 alone, or combined with SYBR green, pre-bleed mouse sera combined with SYBR green, and post-bleed mouse sera combined with SYBR green. (B) Histogram overlays of the staining with pre- and post-bleed mouse sera on gated SYBR green-positive *M. ovi*.

SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2, also known as SARS-CoV-2, is a human coronavirus that has recently caused a global pandemic (83). Coronaviruses are enveloped and contain positive-sense single-stranded RNA, capable of undergoing translation in the cytoplasm of its host immediately upon infection. Of all known RNA viruses, coronaviruses are the largest, with a genome of about 30 kilobases. SARS-CoV-2 is a β-coronavirus, meaning it can infect mammals. Of its four essential structural proteins (nucleocapsid, matrix, envelope, and spike), the spike protein on the surface of the virus can attach to the cellular receptor ACE2 on the surface of human cells. ACE2, or angiotensin-converting enzyme 2, is found in the lower respiratory tract of humans. The spike protein is the proverbial key that unlocks ACE2, allowing the virus to enter the cell, often causing infection and leading to the disease caused by SARS-CoV-2, COVID-19.

COVID-19

Coronavirus disease of 2019 (or COVID-19) is an acute respiratory infectious disease, spread by respiratory droplets (83). Disease is highly transmissible in people and causes severe health concerns in the elderly and immunocompromised. Common symptoms of infection include mild flu-like symptoms, like fever, malaise, and cough (84). However, some people have developed acute respiratory distress syndrome (ARDS), respiratory failure, multi-organ failure, and even death (85). Current vaccines used to prevent infection include the Pfizer/BioNTech and Moderna vaccines, which contain mRNA encoding the SARS-CoV-2 spike protein (86).

Models to Study

Simple ways to study SARS-CoV-2 include using cell culture models. Common human cell lines that scientists have used are Calu-3 (pulmonary), Caco-2 (intestinal), Huh-7 (hepatic) and HEK 293T (renal; 87). Another popular cell line is the Vero E6 cell, derived from an African green monkey kidney (87). Altogether, these cells can be used for isolating and producing SARS-CoV-2. In addition to cell lines, animals can be used to study infection and disease. A recent *Nature* methods paper highlighted several animal models used to facilitate *in vivo* research, including golden hamsters, mice, ferrets, and nonhuman primates (88).

Organoids

Human organoid culture models offer incredibly powerful approaches to study human pathogen-tissue interactions. Organoids are miniature organ models used *in vitro* and are derived from either stem cells or tissues extracted from patients, while maintaining a three-dimensional microenvironment (89). Organoids can self-organize and differentiate into functional cell types, simulating how organs function *in vivo*. Studying organoids can help to better understand human development, tissue/organ regeneration, and human disease (89). Previous studies have used brain organoid models to study Zika virus and found that the virus infects neural progenitor cells, leading to increased cell death (90). Recently, human organoids have been used to study SARS-CoV-2 infection. These include lung, intestinal, liver, kidney, brain, tonsil, and even retinal organoids (89). Given that COVID-19 can cause gastrointestinal symptoms, such as vomiting and diarrhea, use of intestinal organoids to study host-virus interactions is of particular interest. Previous research found that SARS-CoV-2 infection led to structural changes in small intestinal

organoids, along with overall organoid deterioration (91). Continued use of organoid models for studying SARS-CoV-2 can further our understanding of COVID-19.

Results

Given that COVID-19 can cause gastrointestinal symptoms, such as vomiting and diarrhea, intestinal organoids are of particular interest. Previous research found that SARS-CoV-2 infection led to structural changes in small intestinal organoids, along with overall organoid deterioration (91). We wanted to use sera from vaccinated individuals to block SARS-CoV-2 infection in organoids. Briefly, virus was mixed with serum at a final dilution of 1:256 and incubated with the organoids at 37°C for 1.5 hours. The organoids were then washed with DMEM, gently centrifuged for 3 minutes at ~150-200xg, supernatant was aspirated, and organoids were resuspended in Matrigel and plated. At 0 and 72 hours, supernatants were collected and later, viral plaques were quantified by plaque assay. As shown in Figure 12, sera from vaccinated individuals significantly reduced viral titers in the infected organoids compared to sera from unvaccinated individuals, thus having the therapeutic capability of reducing viral infection. This specific experiment was part of a much larger study being prepared for publication: Jutila, M.A., Loveday, E., Synder, D., Chang, C., Blackwell, K., Jenkins, B., Walk, S., Sebrell, T., Cherne, M., Bimczok, D., Plewa, J., Jerome, M., and Hedges, J. Use of human sera following COVID-19 infection or vaccination to interrogate SARS-CoV-2 infection in human epithelial cell and intestinal organoid cultures.

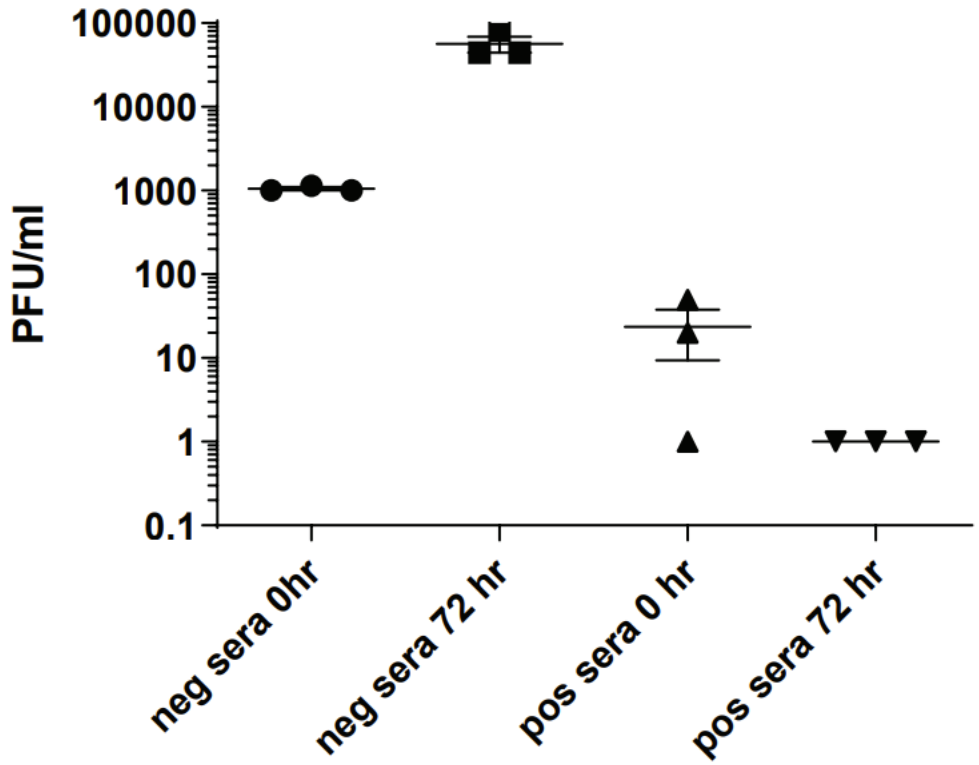

Figure 12. Infection of human intestinal organoids with SARS-CoV-2 was partially blocked via anti-sera from vaccinated individuals. Negative or positive sera for anti-SARS-CoV-2 antibodies were mixed with virus and then used to infect organoids for 0 or 72 hours. A plaque assay was performed on organoids supernatants to measure viral counts.

A final collaborative study that was part of the overall efforts in this Thesis involved the characterization of the liposomal TLR4 agonist preparation (Lipo-CRX). This preparation was used in our experiments to increase the efficacy of phage treatments in our mouse model of brucellosis presented in Chapter Two. We first confirmed the unique activity of lipo-CRX to enhance innate immune cell responses *in vitro* and *in vivo*, while inducing less toxicity than other TLR4 agonists, such as LPS. We then showed that lipo-CRX treatment *in vivo* enhanced clearance of *B. abortus* infection in mice (55).

REFERENCES CITED

1. Pappas, G., P. Papadimitriou, N. Akritidis, L. Christou, and E. V. Tsianos. 2006. The new global map of human brucellosis. Lancet Infect. Dis. **6**:91-99.

2. Corbel, M. J. 2006. *Brucellosis in humans and animals*, pp. 1, 4, 5, 7, 12, 13, 16, 36, 41. World Health Organization Press, Geneva.

3. Yamamoto, N., and M. L. Droffner. 1985. Mechanisms determining aerobic or anaerobic growth in the facultative anaerobe *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **82**:2077-2081.

4. Breijyeh, Z., B. Jubeh, and R. Karaman. 2020. Resistance of Gram-negative bacteria to current antibacterial agents and approaches to resolve it. Molecules **25**:1-23.

5. Głowacka, P., D. Żakowska, K. Naylor, M. Niemcewicz, and A. Bielawska-Drózd. 2018. *Brucella* - virulence factors, pathogenesis and treatment. Pol. J. Microbiol. **67**:151-161.

6. Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198**:545-556.

7. Ke, Y., Y. Wang, W. Li, and Z. Chen. 2015. Type IV secretion system of *Brucella* spp. and its effectors. Front. Cell. Infect. Microbiol. **5**:72.

8. Goodwin, Z. I., and D. W. Pascual. 2016. Brucellosis vaccines for livestock. Vet. Immunol. Immunopathol. **181**:51-58.

9. National Academies of Sciences, Engineering, and Medicine. 2017. *Revisiting Brucellosis in the Greater Yellowstone Area*, pp. 1-209. The National Academies Press, Washington, DC.

10. Centers for Disease Control and Prevention. 2017. People in four states may be drinking contaminated raw milk. https://www.cdc.gov/media/releases/2017/p1121-contaminated-raw-milk.html. Retrieved April 1, 2021.

11. Goodwin, Z. I., X. Yang, C. Hoffman, and D. W. Pascual. 2022. Live mucosal vaccination stimulates potent protection via varied CD4+ and CD8+ T cell subsets against wild-type *Brucella melitensis* 16M challenge. Front Immunol. **13**:1-16.

12. Pei, J., X. Ding, Y. Fan, A. Rice-Ficht, and T. A. Ficht. 2012. Toll-like receptors are critical for clearance of *Brucella* and play different roles in development of adaptive immunity following aerosol challenge in mice. Front. Cell. Infect. Microbiol. **2**:1-10.

13. Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. Cell **140**:805-820.

14. Takeda, K., T, Kaisho, and S. Akira. 2003. Toll-like receptors. Annu. Rev. Immunol. **21**:335-376.

15. Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell **2**:253-258.

16. Kawai, T., O. Adachi, T. Ogawa, K. Takeda, and S. Akira. 1999. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity **11**:115-122.

17. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. Nat. Rev. Immunol. **4**:499-511.

18. Dolcet, X., D. Llobet, J. Pallares, and X. Matias-Guiu. 2005. NF-kB in development and progression of human cancer. Virchows Arch. 446:475-482.

19. Ullah MO, Sweet MJ, Mansell A, Kellie S, Kobe B. TRIF-dependent TLR signaling, its functions in host defense and inflammation, and its potential as a therapeutic target. J Leukoc Biol. 2016 Jul;100(1):27-45. doi: 10.1189/jlb.2RI1115-531R. Epub 2016 May 9. PMID: 27162325.

20. Waisman, A., D. Lukas, B. E. Clausen, and N. Yogev. 2017. Dendritic cells as gatekeepers of tolerance. Semin. Immunopathol. **39**:153-163.

21. Teng, M. W. L., E. P. Bowman, J. J. McElwee, M. J. Smyth, J.-L. Casanova, A. M. Cooper, and D. J. Cua. 2015. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. Nat. Med. **21**:719-729.

22. Gomes, M. T., P. C. Campos, L. A. de Almeida, F. S. Oliveira, M. M. Costa, F. M. Marim, G. S. M. Pereira, and S. C. Oliveira. 2012. The role of innate immune signals in immunity to *Brucella abortus*. Front. Cell. Infect. Microbiol. **2**:1-9.

23. Ivashkiv LB, Donlin LT. Regulation of type I interferon responses. Nat Rev Immunol. 2014 Jan;14(1):36-49. doi: 10.1038/nri3581. PMID: 24362405; PMCID: PMC4084561.

24. Schoggins JW. Interferon-Stimulated Genes: What Do They All Do? Annu Rev Virol. 2019 Sep 29;6(1):567-584. doi: 10.1146/annurev-virology-092818-015756. Epub 2019 Jul 5. PMID: 31283436.

25. Huang LY, Ishii KJ, Akira S, Aliberti J, Golding B. Th1-like cytokine induction by heat-killed Brucella abortus is dependent on triggering of TLR9. J Immunol. 2005 Sep 15;175(6):3964-70. doi: 10.4049/jimmunol.175.6.3964. PMID: 16148144.

26. de Almeida, L. A., N. B. Carvalho, F. S. Oliveira, T. L. Lacerda, A. C. Vasconcelos, L. Nogueira, A. Bafica, A. M. Silva, and S. C. Oliveira. 2011. MyD88 and STING signaling pathways are required for IRF3-mediated IFN-β induction in response to *Brucella abortus* infection. PLoS One **6**:1-14.

27. Sathiyaseelan J, Goenka R, Parent M, et al. Treatment of Brucella-susceptible mice with IL-12 increases primary and secondary immunity. Cell Immunol 2006; 243: 1-9. 2006/12/23. DOI: 10.1016/j.cellimm.2006.10.003.

28. Hielpos MS, Ferrero MC, Fernández AG, et al. Btp Proteins from Brucella abortus Modulate the Lung Innate Immune Response to Infection by the Respiratory Route. Frontiers in Immunology 2017; 8. Original Research. DOI: 10.3389/fimmu.2017.01011.

29. Eskandari-Nasab E, Moghadampour M, Asadi-Saghandi A, et al. Levels of Interleukin-(IL)-12p40 are Markedly Increased in Brucellosis Among Patients with Specific IL-12B Genotypes. Scandinavian Journal of Immunology 2013; 78: 85-91. DOI: https://doi.org/10.1111/sji.12054.

30. Byrnes AA, Ma X, Cuomo P, Park K, Wahl L, Wolf SF, Zhou H, Trinchieri G, Karp CL. Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. Eur J Immunol. 2001 Jul;31(7):2026-34. doi: 10.1002/1521-4141(200107)31:7<2026::aid-immu2026>3.0.co;2-u. PMID: 11449355.

31. McNab, F., Mayer-Barber, K., Sher, A. *et al.* Type I interferons in infectious disease. *Nat Rev Immunol* **15**, 87–103 (2015). https://doi.org/10.1038/nri3787.

32. Lee AJ, Feng E, Chew MV, Balint E, Poznanski SM, Giles E, et al. (2022) Type I interferon regulates proteolysis by macrophages to prevent immunopathology following viral infection. PLoS Pathog 18(5): e1010471.

33. Cisek, A. A., I. Dąbrowska, K. P. Gregorczyk, and Z. Wyżewski. 2016. Phage therapy in bacterial infections treatment: one hundred years after the discovery of bacteriophages. Curr. Microbiol. **74**:277-283. bacteriophages

34. Abedon, S. T. 2018. Phage therapy: various perspectives on how to improve the art, pp. 113-127 in *Host-Pathogen Interactions: Methods and Protocols*, ed. C. Medina and F. J. López-Baena. Springer, New York.

35. Inal, J. M. 2003. Phage therapy: a reappraisal of bacteriophages as antibiotics. Arch. Immunol. Ther. Exp. (Warsz.) **51**:237-244.

36. Campbell, A. 2013. The future of bacteriophage biology. Nat. Rev. Gen. **4**:471-477.

37. Schooley, R.T., B. Biswas, J.J Gill, A. Hernandez-Morales, J. Lancaster, L. Lessor, J. J. Barr, S. L. Reed, F. Rohwer, S. Benler, A. M. Segall, R. Taplitz, D. M. Smith, K. Kerr, M. Kumaraswamy, V. Nizet, L. Lin, M. D. McCauley, S. A. Strathdee, C. A. Benson, R. K. Pope, B. M. Leroux, A. C. Picel, A. J. Mateczun, K. E. Cilwa, J. M. Regeimbal, L. A. Estrella, D. M. Wolfe, M. S. Henry, J. Quinones, S. Salka, K. A. Bishop-Lilly, R. Young, and T. Hamilton. 2017. Development and Use of Personalized Bacteriophage-Based Therapeutic Cocktails To Treat a Patient with a Disseminated Resistant Acinetobacter baumannii Infection. Antimicrob Agents Chemother. **61**:1-14.

38. Reardon, S. Phage therapy gets revitalized. *Nature* **510**, 15–16 (2014). https://doi.org/10.1038/510015a.

39. Dunne, M., B. Rupf, M. Tala, X. Qabrati, P. Ernst, Y. Shen. E. Sumrall, L. Heeb, A. Plückthun, M. J. Loessner, and S. Kilcher. 2019. Reprogramming bacteriophage host range through structure-guided design of chimeric receptor binding proteins. Cell Rep. **29**:1336-1350.

40. Shlezinger, M., S. Coppenhagen-Glazer, D. Gelman, N. Beyth, and R. Hazan. 2019. Eradication of vancomycin-resistant enterococci by combining phage and vancomycin. Viruses. **11**:1-15.

41. Chan, B. K., P. E. Turner, S. Kim, H. R. Mojibian, J. A. Elefteriades, and D. Narayan. 2018. Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. Evol. Med. Pub. Health **1**:60-66.

42. Hodyra-Stefaniak, K., P. Miernikiewicz, J. Drapała, M. Drab, E. Jończyk-Matysiak, D. Lecion, Z. Kaźmierczak, W. Beta, J. Majewska, M. Harhala, B. Bubak, A. Kłopot, A. Górski, and K. Dąbrowska. 2015. Mammalian host-versus-phage immune response determines phage fate *in vivo*. Sci. Rep. **5**:1-13.

43. Sweere, J. M., J. D. Van Belleghem, H. Ishak, M. S. Bach, M. Popescu, V. Sunkari, G. Kaber, R. Manasherob, G. A. Suh, X. Cao, C. R. de Vries, D. N. Lam, P. L. Marshall, M. Birukova, E. Katznelson, D. V. Lazzareschi, S. Balaji, S. G. Keswani, T. R. Hawn, P. R. Secor, and P. L. Bollyky. 2019. Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection. Science **363**:1-12.

44. Karen D. Weynberg. Chapter One - Viruses in Marine Ecosystems: From Open Waters to Coral Reefs. Editor: Carolyn M. Malmstrom. Advances in Virus Research, Academic Press, Volume 101, 2018, Pages 1-38.

45. Secor, P. R., L. A. Michaels, K. S. Smigiel, M. G. Rohani, L. K. Jennings, K. B. Hisert, A. Arrigoni, K. R. Braun, T. P. Birkland, Y. Lai, T. S. Hallstrand, P. L. Bollyky, P. K. Singh, and W. C. Parks. 2016. Filamentous bacteriophage produced by *Pseudomonas aeruginosa* alters the inflammatory response and promotes noninvasive infection *in vivo*. Infect. Immun. **85**:1-11.

46. Zhang R, Qin X, Kong F, Chen P, Pan G. Improving cellular uptake of therapeutic entities through interaction with components of cell membrane. Drug Deliv. 2019 Dec;26(1):328-342. doi: 10.1080/10717544.2019.1582730. PMID: 30905189; PMCID: PMC6442206.

47. Parnas, J., A. Fekttbiwski, and W. Bulikowski. 1958. Anti-*Brucella* phage. Nature **182**:1610-1611.

48. van Drimmelen, G. C. 1960. "Species" of *Brucella* characterized by phage lysis. Bull. World Health Organ. **23**:127-130.

49. Hammerl, J. A., C. Göllner, C. Jäckel, H. C. Scholz, K. Nöckler, J. Reetz, S. Al Dahouk, and S. Hertwig. 2017. Genetic diversity of *Brucella* reference and non-reference phages and its impact on *Brucella*-typing. Front. Microbiol. **8**:1-12.

50. Farlow, J., A. A. Filippov, K. V. Sergueev, J. Hang, A. Kotorashvili, and M. P. Nikolich. 2014. Comparative whole genome analysis of six diagnostic brucellaphages. Gene **541**:115-122.

51. Prajapati, A., D. Ramchandran, H. Verma, M. Abbas, and M. Rawat. 2014. Therapeutic efficacy of *Brucella* phage against *Brucella abortus* in mice model. Vet. World **7**:34-37.

52. Corbel, M. J. and J. A. Morris. 1980. Investigation of the effect of *Brucella*-phage on the course of experimental infection with *Brucella abortus*. Br. Vet. J. **136**:278-289.

53. Skyberg, J. A., T. Thornburg, M. Rollins, E. Huarte, M. A. Jutila, and D. W. Pascual. 2011. Murine and bovine γδ T cells enhance innate immunity against *Brucella abortus* infections. PLoS One **6**:1-12.

54. Hedges, J. F., A. M. Mitchell, K. Jones, E. Kimmel, A. G. Ramstead, D. T. Snyder, and M. A. Jutila. 2015. Amphotericin B stimulates γδ T and NK cells, and enhances protection from *Salmonella* infection. Innate Immun. **6**:598-608.

55. Hedges JF, Snyder DT, Robison A, et al. A TLR4 agonist liposome formulation effectively stimulates innate immunity and enhances protection from bacterial infection. Innate Immunity. 2023;0(0). doi:10.1177/17534259231168725.

56. Goldstein, J., T. Hoffman, C. Frasch, E. F. Lizzio, P. R. Beining, D. Hochstein, Y. L. Lee, R. D. Angus, and B. Golding. 1992. Lipopolysaccharide (LPS) from *Brucella abortus* is less toxic than that from *Escherichia coli*, suggesting the possible use of *B. abortus* or LPS from *B. abortus* as a carrier in vaccines. Infect. Immun. **60**:1385-1389.

57. Bonilla, N., M. I. Rojas, G. N. F. Cruz, S-H. Hung, F. Rohwer, and J. J. Barr. 2016. Phage on tap-a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. PeerJ. **4**:1-18.

58. Holderness, J., L. Jackiw, E. Kimmel, H. Kerns, M. Radke, J. F. Hedges, C. Petrie, P. McCurley, P. M. Glee, A. Palecanda, and M. A. Jutila. 2007. Select plant tannins induce IL-2Rα up-regulation and augment cell division in γδ T cells. J. Immunol. **179**:6468-6478.

59. Hedges, J. F., D. Cockrell, L. Jackiw, N. Meissner, and M. A. Jutila. 2003. Differential mRNA expression in circulating gammadelta T lymphocyte subsets defines unique tissue-specific functions. J. Leukoc. Biol. **73**:306-314.

60. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 2008 Aug;9(8):847-56. doi: 10.1038/ni.1631. Epub 2008 Jul 11. PMID: 18604214; PMCID: PMC2834784.

61. Roberson SM, Walker WS. Immortalization of cloned mouse splenic macrophages with a retrovirus containing the v-raf/mil and v-myc oncogenes. Cell Immunol. 1988 Oct 15;116(2):341-51. doi: 10.1016/0008-8749(88)90236-5. PMID: 2460250.

62. Cicha, C., J. Hedges, I. Novak, D. Snyder, M. Jutila, and B. Wiedenheft. 2020. Complete genome sequence of *Brucella abortus* phage EF4, determined using long-read sequencing. Microbiol. Resour. Announc. **9**:1-20.

63. Boratyn, G. M., J. Thierry-Mieg, D. Thierry-Mieg, B. Busby, and T. L. Madden. 2019. Magic-BLAST, an accurate RNA-seq aligner for long and short reads. BMC Bioinformatics. **20**:405.

64. Carmody, L. A., J. J. Gill, E. J. Summer, U. S. Sajjan, C. F. Gonzalez, R. F. Young RF, J. J. LiPuma. 2010. Efficacy of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection. J. Infect. Dis. **201**:264-271.

65. Pincus, N. B., J. D. Reckhow, D. Saleem, M. L. Jammeh, S. K. Datta, and I. A. Myles. 2015. Strain specific phage treatment for *Staphylococcus aureus* infection is influenced by host immunity and site of infection. PLoS One **10**:1-16.

66. Guang-Han, O., C. Leang-Chung, K. M. Vellasamy, V. Mariappan, C. Li-Yen, and J. Vadivelu. 2016. Experimental phage therapy for *Burkholderia pseudomallei* infection. PLoS One **11**:1-16.

67. Capparelli, R., N. Nocerino, M. Iannaccone, D. Ercolini, M. Parlato, M. Chiara, and D. Iannelli. 2010. Bacteriophage therapy of *Salmonella enterica*: a fresh appraisal of bacteriophage therapy. J. Infect. Dis. **201**:52-61.

68. Hua, Y., T. Luo, Y. Yang, D. Dong, R. Wang, Y. Wang, X. Mengsha, G. Xiaokui, H. Fupin, H. Ping. 2018. Phage therapy as a promising new treatment for lung infection caused by carbapenem-resistant *Acinetobacter baumannii* in mice. Front. Microbiol. **8**:1-11.

69. Czyż, D. M., J. W. Willett, and S. Crosson. 2017. *Brucella abortus* induces a Warburg shift in host metabolism that is linked to enhanced intracellular survival of the pathogen. J. Bacteriol. **199**:1-14.

70. Hedges, J. F., A. Robison, E. Kimmel, K. Christensen, E. Lucas, A. Ramstead, and M. A. Jutila. 2016. Type I IFN counters or promotes *Coxiella burnetii* replication dependent on tissue. Infect. Immun. **84**:1815-1825.

71. Kovarik, P., V. Castiglia, M. Ivin, and F. Ebner. 2016. Type I interferons in bacterial infections: a balancing act. Front. Immunol. **7**:1-8.

72. Snyder, D. T., J. F. Hedges, and M. A. Jutila. 2017. Getting "inside" type I IFNs: Type I IFNs in intracellular bacterial infections. J. Immunol. Res. **2017**:1-17.

73. Allden SJ, Ogger PP, Ghai P, McErlean P, Hewitt R, Toshner R, Walker SA, Saunders P, Kingston S, Molyneaux PL, Maher TM, Lloyd CM, Byrne AJ. The Transferrin Receptor CD71 Delineates Functionally Distinct Airway Macrophage Subsets during Idiopathic Pulmonary Fibrosis. Am J Respir Crit Care Med. 2019 Jul 15;200(2):209-219. doi: 10.1164/rccm.201809-1775OC. PMID: 31051082; PMCID: PMC6635794.

74. Cooper GE, Pounce ZC, Wallington JC, Bastidas-Legarda LY, Nicholas B, Chidomere C, et al. (2016) Viral Inhibition of Bacterial Phagocytosis by Human Macrophages: Redundant Role of CD36. PLoS ONE 11(10): e0163889. https://doi.org/10.1371/journal.pone.0163889.

75. Elzein F, Alsherbeeni N, Almatrafi K, Shosha D, Naoufel K. COVID-19 co-infection in a patient with brucella bacteremia. Respir Med Case Rep. 2020;31:101183. doi: 10.1016/j.rmcr.2020.101183. Epub 2020 Aug 6. PMID: 32834987; PMCID: PMC7409995.

76. Razin S. Mycoplasmas. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 37.

77. Carmichael LE, St George TD, Sullivan ND, Horsfall N. Isolation, propagation, and characterization studies of an ovine Mycoplasma responsible for proliferative interstitial pneumonia. Cornell Vet. 1972 Oct;62(4):654-79. PMID: 4672899.

78. Highland MA, Herndon DR, Bender SC, Hansen L, Gerlach RF, Beckmen KB. Mycoplasma ovipneumoniae in Wildlife Species beyond Subfamily Caprinae. Emerg Infect Dis. 2018 Dec;24(12):2384-2386. doi: 10.3201/eid2412.180632. PMID: 30457547; PMCID: PMC6256407.

79. Besser, T. E., E. Frances Cassirer, M. A. Highland, P. Wolff, A. Justice-Allen, K. Mansfield, M. A. Davis, and W. Foreyt. 2013. Bighorn sheep pneumonia: Sorting out the cause of a polymicrobial disease. Prev. Vet. Med. 108: 85-93.

80. Besser, T. E., E. F. Cassirer, K. A. Potter, J. VanderSchalie, A. Fischer, D. P. Knowles, D. R. Herndon, F. R. Rurangirwa, G. C. Weiser, and S. Srikumaran. 2008. Association of Mycoplasma ovipneumoniae Infection with Population-Limiting Respiratory Disease in Free-Ranging Rocky Mountain Bighorn Sheep (Ovis canadensis canadensis). Journal of Clinical Microbiology 46: 423-430.

81. Mycoplasma ovipneumoniae. USDA APHIS | Mycoplasma ovipneumoniae. (n.d.). Retrieved February 17, 2023, from https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/sa_animal_disease_information/sheep-goat/movi/mycoplasma-ovipneumoniae#:~:text=Clinical%20signs%20include%20lethargy%2C%20fever,ovipneumoniae.

82. Melissa G. Ackerman, David A. Schneider, Katherine N.K. Baker, Thomas E. Besser. Comparison of three methods of enumeration for Mycoplasma ovipneumoniae. Journal of Microbiological Methods. Volume 165, 2019, https://doi.org/10.1016/j.mimet.2019.105700.

83. Ludwig S, Zarbock A. Coronaviruses and SARS-CoV-2: A Brief Overview. Anesth Analg. 2020 Jul;131(1):93-96. doi: 10.1213/ANE.0000000000004845. PMID: 32243297; PMCID: PMC7173023.

84. Poutanen SM, Low DE, Henry B, Finkelstein S, Rose D, Green K, Tellier R, Draker R, Adachi D, Ayers M, Chan AK, Skowronski DM, Salit I, Simor AE, Slutsky AS, Doyle PW, Krajden M, Petric M, Brunham RC, McGeer AJ; National Microbiology Laboratory, Canada; Canadian Severe Acute Respiratory Syndrome Study Team. Identification of severe acute respiratory syndrome in Canada. N Engl J Med. 2003 May 15;348(20):1995-2005. doi: 10.1056/NEJMoa030634. Epub 2003 Mar 31. PMID: 12671061.

85. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020 Feb 15;395(10223):497-506. doi: 10.1016/S0140-6736(20)30183-5. Epub 2020 Jan 24. Erratum in: Lancet. 2020 Jan 30;: PMID: 31986264; PMCID: PMC7159299.

86. Hadj Hassine I. Covid-19 vaccines and variants of concern: A review. Rev Med Virol. 2022 Jul;32(4):e2313. doi: 10.1002/rmv.2313. Epub 2021 Nov 9. PMID: 34755408; PMCID: PMC8646685.

87. Pires De Souza GA, Le Bideau M, Boschi C, Wurtz N, Colson P, Aherfi S, Devaux C, La Scola B. Choosing a cellular model to study SARS-CoV-2. Front Cell Infect Microbiol. 2022 Oct 21;12:1003608. doi: 10.3389/fcimb.2022.1003608. PMID: 36339347; PMCID: PMC9634005.

88. Chu, H., Chan, J.FW. & Yuen, KY. Animal models in SARS-CoV-2 research. Nat Methods 19, 392–394 (2022). https://doi.org/10.1038/s41592-022-01447-w

89. Han, Y., Yang, L., Lacko, L.A. et al. Human organoid models to study SARS-CoV-2 infection. Nat Methods 19, 418–428 (2022). https://doi.org/10.1038/s41592-022-01453-y

90. Garcez, P. P. et al. Zika virus impairs growth in human neurospheres and brain organoids. Science 352, 816–818 (2016).

91. Kruger, J. et al. Drug inhibition of SARS-CoV-2 replication in human pluripotent stem cell-derived intestinal organoids. Cell Mol. Gastroenterol. Hepatol. 11, 935–948 (2021).