Development of immunotherapeutic approaches to achieve xenophagy in macrophages infected with Rhodococcus equi

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Development of immunotherapeutic approaches to achieve xenophagy in macrophages infected with \textit{Rhodococcus equi}

by

Mahesh Bhandari

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Immunobiology

Program of Study Committee:
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ABSTRACT

Macrophages are the host cells for *Rhodococcus equi*. Activation of FcγRI can induce phagocytosis, antigen presentation, cytokine production, antibody-dependent cell mediated cytotoxicity, reactive oxygen species (ROS) production, and induction of autophagy machinery in macrophages. Here we observe enhanced macrophage bactericidal activity against intracellular *R. equi* concurrent with superoxide production and induction of autophagy. Following ligation of IFN-γ, TLR4 and FcγRI receptors, murine bone marrow-derived macrophages, equine pulmonary-alveolar macrophages and monocyte-derived macrophages were tested for superoxide production, induction of autophagy and bacterial load through multispectral imaging flow cytometry, western blot and confocal microscopy. Results show that ligation of these receptors alone or in combination enhanced superoxide production and induction of LC3II. Importantly, however, FcγRI activation with a synthetic homodimeric peptide resulted in a sustained effect leading to a profound reduction in bacterial load as compared with other receptor agonists. These data are consistent with the notion that production of superoxide enhances autophagy resulting in reduction of bacterial load in infected macrophages.
CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

Thesis Organization

This thesis comprises five chapters. Chapter 1 is a literature review that familiarizes the reader with background information about innate and adaptive immunity, macrophages as host cells of intracellular pathogens, FcγRI in macrophages, production of superoxide, autophagy in macrophages and the pathogen *Rhodococcus equi*. In addition, reasons why the research presented was performed are presented. Chapter 2 describes FcγRI mediated intracellular reduction of *Rhodococcus equi* load in infected macrophages using a homo dimeric peptide specific for FcγRI. Chapter 3 discusses the results of activation of FcγRI in the production of superoxide, induction of autophagy and reduction of bacterial load. Chapter 4 concludes this thesis and briefly highlights further investigations required to effectively activate macrophages in vivo to clear the pathogen using a homo dimeric peptide FCγRI binding strategy. References cited are listed at the end of the thesis.

Literature Review

Innate Immunity

The mammalian immune system is comprised of innate and adaptive arms. The innate immune system, also known as non-specific immune system, is the first line of defense against pathogenic microorganisms (25). Innate immune cells include a group of specialized hemopoietic cells including: phagocytes (neutrophils, monocytes,
macrophages, and myeloid dendritic cells (DCs)); innate lymphocytes (natural killer cells, NKT cells, γ/δT cells, and plasmacytoid DCs, along with mast cells, basophils, eosinophils, and nuocytes (60). In addition, nonprofessional phagocytic cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to innate immunity (70). Cells of innate immune system recognize pathogens with their conserved pattern-recognition receptors (PRRs). Antigen presenting cells like DCs and macrophage recognize pathogen associated molecular patterns (PAMPs) with the help of PRRs (1,53). The PAMPs recognition by PRRs in antigen presenting cells leads to their activation and subsequent elicitation of an adaptive immune response. There are various kinds of PRRs: TLRs, RIG-I–like receptors, Nod-like receptors (NLRs), and C-type lectin receptors (CLRs) (1,27,70). Collectively these cells and their respective ligands/receptors recognize and respond to invading viral, bacterial, fungal and parasitic invaders.

**Adaptive immunity**

The adaptive immune system, also known as the acquired immune response, is composed of highly specialized T and B-lymphocytes, which are involved in the process that eliminates pathogen or prevents/limits pathogen growth (25). In contrast to the pathogen recognition of the innate immune system, adaptive immune responses depend on receptors that are not encoded in the germ line but are generated de novo in each organism. The specific receptors are selected through a process of somatic recombination (gene rearrangement) of a large pool of gene segments and V(D)J recombination (25,1,4). This mechanism involves a small number of genes to generate a
tremendous number of different antigen receptors, which are then expressed on each individual lymphocyte capable of recognizing components of potential pathogens. In addition to specificity, immunological memory is another principal feature of the adaptive immune system. It involves a tightly regulated interaction between antigen-presenting cells and T and B-lymphocytes, which help in elimination of pathogens in the late phase of infection and generation of immunological memory \(1, 4\). After the first encounter with the pathogen, long-lived memory B cells and memory T cells are produced which are key in specific immunity. They have the capacity to get activated quickly and generate robust protective responses in case of re-exposure to same pathogen \(4,40,8\).

**Macrophage**

Macrophages are the immune cells which are involved in the primary response to pathogens, in the tissue homeostasis, coordination of the adaptive immune response, inflammation, resolution, and repair. \(44\). Macrophages originate as mononuclear phagocytes which represents a subgroup of leukocytes described as a population of bone marrow derived myeloid cells. \(27,48\). Mononuclear phagocytes are composed of monocytes, macrophages and dendritic cells. Macrophage precursors are released into the circulation as circulating peripheral-blood mononuclear cells (PBMCs) and they give rise to a variety of tissue-resident macrophages throughout the body. \(48,16,7\). Tissue resident macrophage includes lung (alveolar macrophage), bone (osteoclasts), central nervous system (microglial cells), connective tissue (histiocytes), liver (kuffer cells) \(46\). Several macrophage subsets with distinct functions and physiological roles have
been described which arise in response to different innate or adaptive immune signals. Broadly, three populations of macrophages have been proposed that can arise in response to cytokine stimuli from immune cells. Classically activated macrophages (M1 macrophages) arise in response to interferon- (IFN) produced by T helper 1 (TH1) cells, CD8+ T cells or natural killer (NK) cells or tumor-necrosis factor (TNF), produced by antigen-presenting cells (APCs); Wound-healing or alternatively activated macrophages (M2 Macrophages) in response to interleukin-4 (IL-4), produced by TH2 cells or granulocytes; Regulatory macrophages generated in response to various stimuli, including immune complexes, prostaglandins; G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells or IL-10. Each of these three populations has a distinct physiology. Classically activated macrophages have microbicidal activity, whereas regulatory macrophages produce high levels of IL-10 to suppress immune responses and wound-healing macrophages involved in tissue repair. (46, 48).

Macrophages are equipped with a broad range of receptors that make them efficient at recognition of pathogen, phagocytosis and induction of inflammatory cytokines. Pathogen recognition is followed by surface changes, uptake, signaling, and altered gene expression, contributing to homeostasis, host defense, innate effector mechanisms, and the induction of acquired immunity (71,16). Phagocytic surface receptors include non-opsonic receptors (e.g. C-type lectins and Scavenger Receptors) as well as opsonic receptors (e.g. complement receptor and Fc receptors). Toll-like receptors (TLRs) are pathogen-sensing receptors, some of which are expressed on the surface (e.g. TLR4), while others are vacuolar (e.g. TLR9). Sensing receptors are also
found in the cytoplasm, and these include the NOD-like receptors (NLRs), RIG-like receptors (RLRs), and DNA sensors (e.g. AIM2) (60,67). Macrophages are responsible for engulfing pathogens and killing them. However, some pathogens resist and subvert this process and continue to live inside the macrophage. In this way they also serve as a host for various intracellular pathogens like *M. tuberculosis, R. equi, Leishmania* and many viruses (27).

**CD64 (FCγRI) in Macrophage**

Fc receptors are the proteins found on the surface of macrophage and other phagocytes, which play a central role in immunity. On the surface of the macrophage, several Fc receptors are expressed which bind to the different classes of immunoglobulins (51). The family of Fc receptors binding to IgG (FcγR) belongs to the immunoglobulin superfamily and plays a crucial role in phagocytosis of pathogens (5). The FcγR family in mice and humans is composed of one high-affinity activating receptor, FcγRI (CD64); FcRs are with low to medium affinity for the antibody Fc fragment and inhibitory FcRs. In humans three different classes of FcγR are known and four in mice. FcγRI is the high affinity receptor that binds monomeric IgG2a in mice and IgG1 and IgG3 in humans (51). IgG are the most abundant and important isotype in serum which form IC (immune complexes) and are involved in the interaction with FcγRI expressed on the surface of macrophages. Activating FcγRI can induce phagocytosis, antigen presentation, the production of reactive oxygen species (ROS) and cytokines, and antibody-dependent cellular-mediated cytotoxicity (ADCC). FcγRI is expressed on most myeloid cells including monocytes, macrophages, and dendritic cells.
Moreover, granulocytes express FcγRI upon activation, and FcγRIIA can also be expressed on platelets and B cells. IFNγ is a potent stimulator of FcγRI expression (2, 51). In mice, FcγRI has similar high-affinity binding properties as its human ortholog. Although the extracellular structures are comparable, considerable differences in intracellular domains exist between mice and humans. Human FcγRIIA contains ITAM in its intracellular domain, which is not present in mouse FcγIII. Importantly, murine Fcγ RI and FcγRIV preferentially bind mIgG2a, and in contrast to FcγRI, FcγRIV binds this isotype with moderate affinity (51). Mouse models emphasized the role of various FcRs in normal immunity and in various disease models and Ab therapy. Moreover, identification and use of an Fc-mimic cyclic peptide family that works as IgG has been reported. With a strict specificity for FcγRI, it has been shown to be able to mimic IgG-Fc interaction and FcγR co-localization, which could be used for treatment of different disease conditions (3). In another study, antibody mediated host cell protection against intracellular pathogens which evade lysosomal fusion has been shown. Using specific antibodies, FcR are engaged and the intracellular pathogenic bacterium is targeted into the lysosomal compartment. The pathogen targeting to the lysosomal compartment is dependent on FcR signaling and is independent of FcR mediated phagocytosis (28).

**Superoxide Production in Macrophage**

Reactive oxygen species (ROS) are essential components of the innate immune response against intracellular bacteria (36). Phagocytic leukocytes like macrophages generate reactive oxygen species in response to appropriate external stimuli, such as FcγRI and TLR signaling. Cross-linking of FcγRI on macrophage triggers production of
superoxide anion ($O_2^-$). Superoxide is produced continuously over a long period at the subsaturating concentration of FcγRI ligands (58). FcγRI activation on human monocytic cells results in a transient activation of NADPH oxidase (59). Mitochondria and NADPH oxidase (NOX) are actively involved in production of reactive oxygen species (ROS) across the membrane of phagocytes. It has been shown by various sources that mitochondria are the main source for regulation of autophagy by ROS whereas NADPH oxidase is involved in the trigger of bacterial autophagy (23,24). NADPH oxidase, a multi-protein complex, exists in the dissociated state in inactive cells and after stimulation it assembles into the functional oxidase complex and then generates superoxide anions (64, 66). In phagocytes, NOX enzymes are assembled on the phagosomal membrane and produce superoxide by transferring electrons from cytosolic NADPH to oxygen in the phagosomal lumen. The oxidase complex consists of a membrane bound catalytic subunit gp91phox (NOX2) and regulatory cytosolic subunits p22phox, p47phox, p40phox, p67phox and the small GTPase RAC (62). The assembly of the cytosolic subunits with gp91phox to form an active complex regulates the activity of gp91phox. Studies have shown that superoxide production via phagocytosis is triggered by p40phox and binds to phosphatidylinositol-3-phosphate, a phosphoinositide found in the membranes of phagosomes. NADPH oxidase activation is the crucial downstream event of phagocytosis and plays a key role in clearance of the intracellular pathogens (23, 72).

**Autophagy in Macrophages**
Autophagy is a fundamental cellular homeostatic mechanism in which cytoplasmic material, damaged organelles, protein aggregates, or invading pathogens delivered to lysosomes for degradation involve formation of double-membrane vesicles, called autophagosomes which sequester cytoplasm (23, 39,33). Autophagy is emerging as an important innate defense mechanism against pathogenic infection, including bacteria, virus, and parasites. When autophagy results in the killing of intracellular pathogens it is referred to as xenophagy (47, 39). In addition to its role in pathogen degradation, it has also been shown to activate adaptive immune system. It also plays an important role in the destruction of intracellular pathogens as well as antigen processing and presentation (39,38).

Microtubule-associated protein chain 3 (LC3) is widely considered as an autophagy marker protein. Conversion of LC3 I to LC3 II is positively correlated with induction of autophagy and formation of autophagy vacuoles. Similarly, Beclin1 or autophagy related gene (Atg 6) plays an important role in initiation of autophagy in forming autophagy vacuoles (20). The autophagy system can target bacteria in phagosomes, stimulating phagosome maturation and preventing pathogen escape into the cytosol. In response to specific signaling events, such as FcγR, TLR and IFNγ signaling, the NOX2 NADPH oxidase is assembled on the formation of phagosomal membrane and generates superoxide via transfer of electrons from cytosolic NADPH to oxygen in the lumen of the phagosome. Recent research studies have provided evidence that NOX2-generated reactive oxygen species (ROS) are necessary to target LC3 to phagosomes. FcγR-mediated phagocytosis alone can induce LC3 recruitment to
phagosomes, revealing a coupling of superoxide production with induction of autophagy (23,65). Similarly, it has been shown that cytokines also control autophagy, such as T helper type 1 (Th1) cytokines like tumor necrosis factor (TNF)-α and interferon (IFN)-γ; inhibited by the Th2 cytokines - interleukin (IL)-4 and IL-13 and the anti-inflammatory cytokine IL-10 via the PI-3-kinase pathway (50, 56). Recent studies have demonstrated that specific induction of autophagy can overcome inhibition of phagosomal maturation and lead to suppression of intracellular M. tuberculosis load, indicating promising clinical outcomes by enhancement of innate mechanisms of xenophagy (50). Following autophagy induction, Beclin-1 and its binding partner class III phosphoinositide 3-kinase (PI3K) are necessary for initiation of autophagosome formation (39), inferred by detection of microtubule-associated protein light chain 3 (LC3), which exists as a cytosolic protein (LC3-I) until induction of autophagy whereupon a lipidated membrane bound protein is formed (LC3-II) (20). Similarly, recent research studies have shown Rubicon, a RUN domain protein as Beclin-1 interacting and cysteine-rich containing autophagy protein, as a positive regulator of NADPH oxidase. After microbial infection or ligation of TLR2, rubicon interacts with the p22phox subunit of the NADPH oxidase complex, accelerating its phagosomal trafficking to induce a burst of reactive oxygen species (ROS) and inflammatory cytokines. Rubicon's actions in autophagy and phagocytosis are shown to be functionally and genetically separable and outcome depends on the environmental stimuli (75,76).

Rhodococcus equi and immunity related with it
*Rhodococcus equi* is a Gram-positive, obligate aerobic, facultative intracellular coccobacillus capable of replicating in macrophages, including alveolar macrophages (11,21,49). It is closely related phylogenetically to *Mycobacterium tuberculosis* (45) and it causes severe pyogranulomatous pneumonia of disease in 6 weeks - 6 months old foals (61). It is a common causative agent of bacterial pneumonia in foals and immunocompromised adults. In addition, it has been shown to be an important zoonotic pathogen that causes tuberculosis-like symptoms in AIDS patients (61, 32). *R. equi* has evolved a mechanism to escape bactericidal activity in macrophages and instead it multiplies and finally kills macrophages by necrosis (42). Phagosomes containing virulent *R. equi* do not progress into endocytic organelles that fuse with lysosomes and thereby fail to acidify because of a lack of acquisition of the proton-pumping vacuolar ATPase (12). Replication and virulence of *R. equi* in host macrophages depends on the presence of unique and *R. equi*-specific family proteins, virulence-associated protein family (VAP family). There are six full-length VAP genes (VAP A, C, D, E, G, H) and 3-truncated VAP pseudo genes (VAP F, I and X). Among them, the VAP A plasmid that encodes virulent, temperature inducible and surface expressed lipoprotein, is shown to be critical for virulence of *R equi*. VAP A is critical for intracellular growth in macrophages and induction of disease in foals. (69,17,12,54).

**Innate Immunity**

Typically, foals are susceptible to *R. equi* pneumonia 6 weeks after birth to less than 6 month of age. Inhalation is the common route of infection in foals (22). After introduction into lungs, *R. equi* is engulfed by alveolar macrophages through a process
of receptor-mediated phagocytosis, which can be mediated by complement receptor (CR3 or Mac-1), macrophage mannose receptor, and TLR 2 (15). In addition, *R. equi* may use the mannose receptor for entry into the macrophage, which may recognize the lipoarabinomannan (LAM), one of the components of outer surface of bacteria. It may be either directly or through mannose binding protein or surfactant molecules attaching to LAM. (15,18). Once taken up by resident macrophages, virulent *R. equi* is able to modify the phagocytic vacuole to prevent acidification and subsequent fusion with lysosomes (12). The bacteria then alter its gene expression system to survive in the hostile environment. It increases its ability to uptake essential nutrients like iron to resist the reactive oxygen intermediates from the host (63). The macrophage dies by necrosis due to rapid growth of bacteria (42). *R. equi* opsonization with its specific antibody may promote it to be taken into macrophage via the Fc receptor and subsequent fusion of *R. equi* containing phagosome with lysosome and may lead to killing (10). Activating murine macrophages with IFNγ promotes the production of reactive oxygen and nitrogen intermediates. These two radicals alone can’t kill but when they combine to form peroxynitrite killing is achieved (9). The other cytokine TNF-α might play synergistic role with IFNγ to clear the pathogen (31). The early host defense is mediated by neutrophils, which clear the pathogen well when it is opsonized with specific antibody. It has been shown that macrophages from foal can’t clear the pathogen well whereas neutrophils from both foals and adult can clear it (43).

**Acquired Immunity**

**Cellular Immunity**
In mice, CD4+ T cells mediate the protective immune response against *R. equi*, however, the role of CD8+ T cell is not clear (26,17). IFNγ enhances the clearance of *R. equi* whereas biased production of IL-4 (Th2-biased) increases the risk of disease (26). Both CD4+ and CD8+ T cells are involved in the clearance of the organism in the horse (30). Foals that develop *R. equi* pneumonia have comparable CD4+ and CD8+ T cell distributions in peripheral blood with healthy foals (14). Moreover, T lymphocytes and plasma cells are virtually absent in lung tissues in the first week of life, and bronchus-associated lymphoid tissue (BALT) is only observed by 12 wk. of age (14). In addition, the availability of leukocytes in bronchoalveolar lavage fluid in foals less than 3 wk. of age is one-half the value for adult horses. The percentage of CD4+ T cells increases markedly in the first 3 wk. of life, reaching values comparable with adult horses by 8 wk. Therefore, the pulmonary leukocyte population is quite limited during the first several weeks of life, and it is unlikely that these cells would control substantial airway infection during this period. Adult horses and greater than 6-wk-old foals develop CTLs which efficiently kill *R. equi*-infected cells; however, this is not observed with CTLs from foals younger than 3 weeks of age (57). CD8+ T cells recognize and kill *R. equi* infected macrophages in a major histocompatibility complex class I (MHC class I) unrestricted fashion in adult horses (57). Foal monocyte-derived macrophages express lower levels of CD1 than adult horse cells, and *R. equi* infection trigger lower expression of CD1b on equine monocyte-derived macrophages. This may contribute to longer disease duration in the foal, which already has reduced CD1 expression (55).

*Humoral Immunity*
Antibodies play a crucial role as opsonins for efficient phagocytosis. In the foal, humoral protection would be useful during the initial exposure to the pathogen, before *R. equi* reaches the intracellular environment. Therefore, in the neonate, the passive transfer of immunoglobulin through colostrum or plasma transfusion could be helpful in providing the initial protection in the airways until its own cellular and humoral immunity are developed (6). Active immunization of mares, and the subsequent passive transfer of immunity to foals via colostrum ingestion, does not appear to consistently prevent foals from developing *R. equi* pneumonia. Studies have reported no significant reduction in *R. equi* pneumonia with the use of vaccines that contained killed or inactivated *R. equi* strains (14,18). There is also disparity in prevention of disease in foals using the *R. equi* specific transfusion of hyperimmune plasma. In studies by Caston et al., 2006, in equine neonates infected experimentally with *R. equi*, intravenous plasma transfusion containing specific antibodies against *R. equi* or VapA administered before infectious challenge, aided significantly in the recovery of disease compared with placebo.

In naturally infected foals and herd studies, intravenous administration of hyperimmune plasma showed a decrease in the incidence of pneumonia, a reduction in the risk of developing disease, or no significant effect, which suggests the participation of other risk factors (e.g., pathogen exposure or competence of different arms of the immune system) in the development of disease in natural conditions. It is possible that *R. equi* specific antibodies and other non-specific immunomodulators present in plasma may synergistically enhance bacterial removal and killing (18). The mechanism of
protection with hyperimmune plasma has not been fully described. The viability of intracellular bacteria may be affected by the opsonization, but results suggest the need for additional activation of macrophages to elicit effective bacterial killing (18,49).

**Conclusions**

*R. equi* infection in foals is a serious and complex problem. *R. equi* evolved resistance to escape mechanisms of bacterial killing. Naive foals are exposed to high numbers of virulent bacteria on farms endemic with *R. equi*. However, most foals do not develop disease, suggesting individual risk factors. Research findings suggest that susceptibility to *R. equi* infection and disease in the foal is not because of their inability to produce a Th1-type immune response, although this type of response requires time and effective signaling interaction between antigen-presenting cells and lymphocytes. The effect of colostrum or hyperimmune plasma derived *R. equi* specific antibodies in the neutralization and opsonization of the bacterium before it reaches the intracellular space is not clear. Passive transfer of immunoglobulin through intravenous plasma transfusion helps but does not prevent disease completely. Efforts have increased in recent years, with the ultimate aim of developing immunoprophylactic approaches (such as vaccines and hyperimmune plasma) or other control strategies to prevent disease. A number of immune factors predispose some foals to *R. equi* infection, such as deficient serum opsonic activity, limited TLR signaling and CD86 expression by dendritic cells, reduced MHC class II and CD1 expression on APC, and deficient IFN-γ signaling by CD4+ and CD8+ T cells. All these factors play a role in limiting innate immune mechanisms and subsequent development of an effective acquired cell-mediated immune
response to *R. equi* during the early stages of a foal’s life. It may be that in some foals, the developing TH1 response may not be adequate to combat a concentrated aerosol challenge. An effective immune response to *R. equi* infection consists of a mixed Th1/Th2 cell-mediated response. The potential vaccines/adjuvants to stimulate the limited innate immune response of foals that may be tried include FCγRI agonists, TLR agonists, MHC-II and CD1 activators to improve the presentation of protein, lipid and lipoarabinomannan antigens, characteristic of *R. equi* to T cells.
CHAPTER 2. FCYRI-MEDIATED INTRACELLULAR REDUCITON OF 
RHODOCOCCUS EQUI LOAD IN INFECTED MACROPHAGES

Abstract

Macrophages are the host cells for Rhodococcus equi. Activation of macrophage FcγRI can induce phagocytosis, antigen presentation, cytokine production, antibody-dependent cell mediated cytotoxicity, reactive oxygen species (ROS) production, and ROS-dependent induction of autophagy machinery. Here we demonstrate enhanced macrophage bactericidal activity against intracellular R. equi. Murine bone marrow-derived macrophages were tested for the reduction of bacterial load following ligation of FcγRI. Similarly, bacterial load in infected macrophages was analyzed with multispectral imaging flow cytometry and confocal microscopy. Results show that stimulation of FcγRI achieves significant reduction of the bacterial load in infected macrophages. The data are consistent with the notion that production of superoxide enhances autophagy resulting in reduction of bacterial load in infected macrophages.

Introduction

Rhodococcus equi is a facultative intracellular bacterium that can cause bronchopneumonia in foals. It is the most serious cause of pneumonia in foals 1-5 month of age; but adult horses, unless they are immunocompromised, are not susceptible. R. equi has also emerged as an opportunistic pathogen in immunosuppressed humans, especially those infected with HIV. R. equi is a gram-positive, facultative intracellular pathogen that is nearly ubiquitous in soil. Only certain types (Vap A, B, C)
are pathogenic. The actinobacteria \textit{R. equi} and human pathogen \textit{M. tuberculosis} are phylogenetically related. A variety of antimicrobials are active against \textit{R. equi} when evaluated in vitro. However, many of these antimicrobials are ineffective in vivo. \textit{R. equi} has been reported to develop progressive antimicrobial resistance causing difficulty in treating foals. The protection of hyper-immune plasma has not been consistent to save foals from high morbidity and mortality against this disease.

FcγRI is the high affinity IgG binding/activating receptor on the surface of macrophages and monocytes. After binding IgG, FcγRI interacts with an accessory chain known as the common γ chain (γ-common chain), which possesses an ITAM motif that is necessary for triggering cellular activation. Mouse models emphasized the role of various FcRs in normal immunity and in various disease models and antibody therapy. Moreover, identification and use of a cyclic peptide that works as IgG has been reported. With a strict specificity for FcγRI, it has been shown to be able to mimic IgG-Fc interaction and FcγR co-localization, which could be used for treatment of different disease conditions (3). In another study, antibody-mediated host cell protection against intracellular pathogens, which evade lysosomal fusion, has been shown using specific antibodies whereby FcR are engaged and the intracellular pathogenic bacterium is targeted into the lysosomal compartment. Targeting to the lysosomal compartment is dependent on FcR signaling and is independent of FcR mediated phagocytosis (26,27).

\textbf{Materials and Methods}

\textbf{Cells and cell culture}
Bone marrow cells were harvested from femurs and tibias of C3HeB/FeJ mice. Briefly, bone marrow was flushed out from the long bones of the limbs with complete cell culture medium (CTCM), with DMEM (Dulbecco’s modification of eagle’s medium) and supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, 0.05 µM 2-mercaptoethanol, 4.5 g/L Glucose and 25 mM HEPES. Cells were centrifuged at 250 g for 15 minutes at 4°C. The cell pellet was resuspended in bone marrow medium (30% L-cell conditioned medium, 20% fetal bovine serum (FBS), 50% DMEM, 2 mM L-glutamine, and 100 U penicillin per ml, 100 µg of streptomycin per ml and 1 mM sodium pyruvate). (20-25) X106 cells was seeded into 15 mm petriplate and incubated at 37°C in 5% CO2. On day 2 of culture, non-adherent cells were gently removed and fresh Bone marrow medium was added. At day 6, the adherent cell population was collected and after washing with PBS, trypan blue exclusion was used to count live cells, which were resuspended in complete tissue culture medium.

**Bacterial Preparation**

*R. equi* stock from -80°C was thawed and streaked onto an LB agar plate and was grown for 30 hours in 37°C. Preparation of inoculum *R. equi*, strain T194, was provided as a clinical isolate from a pneumonic foal by Dr. Ronald Griffith, Iowa State University. A single colony was seeded to 20 mL of brain, heart infusion (BHI) media for 15 hours in a shaking incubator at 200 rpm in 37°C to an O.D. of 0.25, which corresponds to 10^8 bacteria per ml. Bacteria were washed in sterile PBS and resuspended in the brain hearth infusion (BHI) broth spinning at 10,000 rpm for 5 mins.
**Confocal Microscopy**

BMDMs (0.5 million) were seeded in 24 well plates with tissue coverslips and infected with *R. equi* at MOI 3 for different time points. Later the cells were fixed, permeabilized and incubated with rabbit polyclonal *R. equi* antibodies (1:3000) for 1 hour as primary antibodies. The coverslips were incubated with goat anti-rabbit Alexa 488 (1:3000) as secondary antibodies for 30 minutes. Later the coverslips were mounted with prolong Gold antifade reagent with DAPI upon the slide and visualized under Olympus Confocal microscope.

**Quantification of intracellular bacterial replication and reduction in load**

To investigate intracellular bacterial quantification and reduction in load, 2 x 10^6 BMDMs were plated in flow tubes with CTCM medium. Cells were infected with MOI 3 *Rhodococcus equi* strain T194. After 2 hours the cells were washed with 1X PBS at 250 g for 10 min at 40°C to remove extracellular bacteria and added fresh CTCM containing 50µg/ml gentamicin. The macrophages were then activated with homodimeric peptide (as described in Bonetto et. al, 2009, fig 3.1) consisting of 6 µM/ml. At different time points (3hr, 6hr, 12hr, 24hr, 48hr, 72hr, 96hr) post-activation the cells were analyzed for reduction in the bacterial load. For analysis, 0.5 million cells from the original flow tube were taken and transferred into clear-view snap cap micro centrifuge tubes. The cells were washed by adding 1 ml of cold wash buffer (2% heat inactivated FBS, 0.1% sodium azide in 1XPBS) at 250 g for 10 min at 4 degree C. The supernatant was discarded and residual buffer removed by tapping the inverted micro centrifuge tube
on paper towel. The cell pellet resuspended by gently raking the micro centrifuge tube across a test tube rack. The cells were fixed by adding 100 µl 4% paraformaldehyde in PBS for 15 min at room temperature (RT). The cells were washed by adding 1 ml of wash buffer and centrifuged at 250 g for 10 min at 4 degree C. Following fixation, the cells were permeabilized with 0.1% Triton-X 100 for 4 min at room temperature and washed with wash buffer at 250 g for 10 min at 4°C. Blocking was done using blocking buffer (10% goat serum, 0.4% bovine serum albumin in 1X PBS). Primary and secondary antibodies were diluted 1:3000 in blocking buffer. To stain intracellular bacteria, cells were incubated with 200µl of primary antibody (rabbit polyclonal R. equi antibody as described in Ramos et. al, 2011) for 30 min at room temperature and washed with the wash buffer. The cells were then stained with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Invitrogen) for 30 min at room temperature and washed with wash buffer at 250 g for 10 min at 4°C. Cells then resuspended in 50µl of PBS containing 1% PFA and stored in the dark at 4°C until acquisition in Amnis-ImageStream x and Image analysis was performed using Amnis-IDEAS version 5.0 (Amnis Corporation, Seattle, WA).

Overview of Sample Acquisition and Image Analysis

Initially, the appropriate numbers of events to be acquired were determined along with the use of different types of fluorescent dyes. Similarly, cell classification criteria were determined to eliminate the unwanted events. Sample collecting channels were selected. A different single color sample was acquired for each fluorophore in the experiment to develop a compensation matrix. Test samples were acquired and saved for
the image analysis. For image analysis, the IDEAS program was launched and internalization wizard selected and the test sample was run. The compensation matrix is developed using single color controls in the experiment. Appropriate channels were selected for the respective populations. A single cell population and best-focused cells were selected for the data analysis. Proper gating strategy was applied and determined to better characterize the internalization of \( R. \text{equi} \). The IDEAS mask function, spot, peak and intensity data analysis options were used define the spot count in the cell. A statistics report was generated. Image analysis was performed using IDEAS software. For details see please see Appendix A and B.

**Results**

![Peptide Structure](image)

**Figure 1.** Structure of parallel homodimeric peptide. Peptide is depicted in single-letter amino acid code as described in Bonetto et al., 2009.
Figure 2. Internalization of R. equi in the bone marrow derived macrophages. The green are (AF488) R.equi and blue is DAPI nuclear stain. Monolayers of BMDM cells were infected with R. equi T194 and fixed at different time points post infection and stained to visualize the spatial relationship of the bacteria with the host cell. The images show the subsequent increase of bacterial load in the infected macrophages over the time. Bacteria were labeled with polyclonal R. equi as described in M&M. Scanning laser confocal microscopy was used to collect these images.
**Figure 3.** Schematic gating strategy applied to determine the internalization of *R. equi*. For details of gating strategy see Appendix A.

**Figure 3A.** To gate cells in focus, the IDEAS feature Gradient RMS of the bright field image is plotted in a histogram. The gradient RMS feature measures the sharpness quality of an image by detecting changes of pixel values in the image. A higher gradient RMS values indicates a more focused image.

**Figure 3B.** To analyze single cells only, it is important to get rid of multicellular events and debris. Single and multicellular aggregate events were separated using IDEAS features area and aspect ratio of bright field image. A gate was drawn to limit only the single cell events.
**Figure 3C.** To separate green fluorescent cells, a gate was drawn on the cells with high max pixel values and Intensity in the green fluorescence channel. The selected cells in the gate included the AF 488 positive cells.

**Figure 3D.** The cells in the internalized gate from figure 1C was further characterized based on the number of spots.
Figure 4. Cell images of internalized *R. equi*. Representative images of BMDM cells that internalized *R. equi*. The channel 01 (Ch01) is the bright field image; Channel 02 (Ch02) is AF488 and Last panel Ch1/Ch2 is the overlay of channel 1 and Channel 2 combined. The image is of 40X magnification and Multi Spectral imaging flow cytometer was used to collect these images.
Figure 5. Effect of different concentration of homodimeric peptide in reducing the bacterial load in the infected macrophage with *R. equi*. BMDM cells were infected with *R. equi* and different concentration of peptide were added 2 hour post infection and sample was run in MIFC 96 hour post infection. Final concentration of 6µM of peptide was effective in reducing the bacterial load significantly. Results represent means of samples in duplicates, and were confirmed by separate independent experiments.
Figure 6. Homodimeric peptides acts via FcγRI to reduce *R. equi* load (percent macrophages positive for bacteria) in the infected macrophages. BMDM cells were infected with *R. equi* and peptide concentration of 6µM was added 2 hrs post infection and sample from 72 hrs and 96 hrs post infection was run in MIFC. Percentage of bacterial internalization was calculated using IDEAS software. *R. equi* was significantly reduced in Peptide added macrophages as compared with the no peptide applied macrophages. (Student’s t test P values: ** P<0.01 and * P<0.05).
Figure 7. Effect of addition of Peptides on *R. equi* infected BMDM cells. Overlay of three different treatment groups (Pink- no peptide treated group; Green- Peptide treated group; Red- Non infected control). (A) Overlay of bacterial spot count and (B) overlay of spot intensity. Data is from 96-hour time point.
**Figure 8.** Comparison of three treatment groups using mean spot count. Untreated group, peptide used group and uninfected. The untreated group (no rx) shows the highest number of bacteria and treatment with peptide (pep) shows the reduction in bacterial load when compared with uninfected controls. Results represent means of samples in duplicates, and were confirmed by separate independent experiments.
Rhodococcus equi remains a great challenge to the health of young foals and immunocompromised adults. It is recognized as a major cause of pneumonia in foals (69). It remains a major concern for equine breeders worldwide (49). The incidence of *R. equi* pneumonia appears to be increasing possibly because of intensified management of equine breeding farms and climate change. This disease continues to be major challenge for clinicians and the equine industry in terms of epidemiological pattern and therapeutic control due to its complex host-pathogen interaction causing disease (69,49).

It appears that innate, humoral and cell mediated immune responses all play a role in susceptibility to *R. equi* pneumonia. It has been shown that immunocompromised AIDS people are susceptible to *R. equi*. AIDS patients get suppression of cell-mediated immunity as a result of selective depletion of CD4+ T cells, which supports the role of cellular immunity for protection against *R. equi* (61,32). Similarly, studies have shown an age-dependent increase in IFNγ production in foals. However, foals are shown to be able to produce IFNγ when activated with proper stimuli and mount Th1 response (26). In one study, a dose dependent correlation of Th1/Th2 responses has been demonstrated. Infection with low CFU of *R. equi* has shown to trigger a Th1 response whereas infection with higher CFU elicits a Th2 response (18). Similarly, antigen specific immunoglobulin is necessary in opsonization and phagocytosis of virulent *R. equi* by alveolar macrophage. Low circulating IgG titers in the foal may contribute to the susceptibility of *R. equi* pneumonia in foals (61,6,49). The use of hyperimmune plasma to prevent *R. equi* infection is also not well described (10).
R. equi is an intracellular coccobacillus that survives and replicates in macrophages, including alveolar macrophages (21). It interferes with phagosomal maturation following phagocytosis and suppresses acidification of the phagosome. Phagosomes containing virulent R. equi do not progress into an endocytic organelle that fuses with lysosomes. Intracellular replication leads to necrosis of the macrophage and a pyogranulomatous inflammatory response (42).

The objective of the work here was to assess the effect of activation of FcγRI on production of superoxide, induction of autophagy and the reduction of intracellular R. equi load. Macrophages were infected with R. equi strain T194 and allowed to internalize. The infected macrophages were treated (or incubated) with IgG-Fc mimicking homodimeric peptide. With strict specificity for FCγRI, it has been shown to be able to mimic IgG-Fc interaction and FcγRI colocalization as described in Bonetto et al, 2009. Similarly studies have shown that engaging FcR with specific antibodies can target intracellular pathogen such as M. bovis and legionella pneumophila to the lysosome for degradation. The lysosomal targeting of phagosomes is dependent on FcR signaling but is independent of FcR mediated phagocytosis (28). In our experiment we used synthetic peptide to target FcγRI. The peptide in monomeric (soluble) form binds and blocks the receptor. In contrast, the homodimeric peptide activates macrophages accompanied by a burst of superoxide, which is necessary to kill intracellular pathogen like R. equi. To our knowledge, the targeting of an intracellular pathogen for lysosomal degradation using the IgG-Fc mimicking synthetic peptide strategy has not been previously demonstrated. In principle, this strategy could be used in combination or
alone with other agonists to control infection by an intracellular pathogen. Moreover, the
efficacy and stability of the peptide in vivo needs to be tested. The result described here
is post infection when R. equi is already inside the macrophage and peptide is added to
the infected macrophage afterwards. In contrast, in the experiment by Joller et. al.,
macrophages were activated before the Legionella infection. Our results extend the work
by these investigators highlighting the role of Fc signaling in the control of intracellular
pathogens. In addition, this work suggests further that infected macrophages can be
activated and can reduce the burden of an intracellular pathogen. The exact mechanism
behind the reduction of the pathogen load needs to be elucidated. Intracellular replication
of R. equi starts between 6 and 12 hours of culture and reaches at peak level at 48 hour
of infection (21). In our study, up until 72-hour post infection there was no difference in
bacterial load as compared with control. However, at 96-hour post infection there was a
significant reduction in the intracellular bacterial load. Importantly, FcγRI activation
with a synthetic homodimeric peptide resulted in a sustained effect leading to a profound
reduction in bacterial load as compared with other receptor agonists.

There have been studies regarding control of intracellular R. equi in macrophage
using opsonization with R. equi specific antibody, but the results are not consistent and
have shown only to be active against bacteria in the extracellular environment (10).
Similarly, cytokine induced macrophage killing has also been shown, particularly with
IFNγ and TNF-α which may be important for in vivo killing as well. Studies have
demonstrated that by blocking IFNγ activity, mice fail to clear pulmonary R. equi
infection. Moreover, the exposure of R. equi infected macrophage to IFNγ enhances
microbicidal activity with production of reactive oxygen and nitrogen intermediates (9). In our study, we used *R. equi* specific polyclonal antibody to measure the bacterial internalization and sensitivity by measure of fluorescence as well as to minimize the inaccuracy of bacterial counts due to clumping with the CFU method. However, it may be of limited use for in vivo studies. Previously, we had bio-transformed *R. equi* with GFP and RFP but fluorescence signals from them were not sensitive enough for bacterial quantification.

In the present study, to detect and quantify internalized *R. equi*, we designed a high throughput quantitative experimental protocol that can detect bacteria as well as other internal markers associated with the bacteria. Until now, different independent techniques such as microscopy, flow cytometry, luciferase activity measurement have been the methods used to study the phagocytosis, internalization and quantification of *R. equi*. The techniques such as flow cytometry and luciferase activity measurement generate good statistical data but it is not able to accurately quantify internalization and progression into the phagosome. Similarly, microscopy generates images with better resolution but it is time consuming and involves small sample sizes. Overcoming the drawbacks of the independent techniques, multi-spectral imaging flow cytometry (MIFC) is a new technology that uses aspects of both microscopy and flow cytometry to perform multi-color spectral fluorescence and bright field imaging simultaneously through a laminar core. This helps to better analyze different intracellular fluorescent signal intensities and generate data at high speed. In contrast to the labor-intensive
counting of CFU, the use of MIFC requires no additional time for growth of the colonies.

The fact that intracellular reduction of *R. equi* load in infected macrophage-using a peptide strategy was effective, this observation suggests an important way to treat *R. equi* pneumonia in foals. If sufficient amount of macrophage activating agonists such as homodimeric peptide can be delivered through inhalation, enhanced intracellular bacterial killing may be achieved. Moreover, the efficacy of this method needs to be tested by in vivo experiments.

In the experiment we performed we were not able to demonstrate the pathway of internalization of *R. equi* in in macrophage. If the outcome of the efficacy of the homodimeric peptide depends on the pathway of internalization, further experiments need to be performed. In addition, we were not able to use randomly generated peptide as a control along with the monomeric peptide of the same sequence, which could be used as a control. The data obtained in the present study provide new insights into the pathogenesis of and immunity against *R. equi* infection that require further investigation.
CHAPTER 4. GENERAL CONCLUSIONS AND FUTURE WORK

General Conclusions

*Rhodococcus equi* is an intracellular pathogen that has the capacity to survive and replicate within the alveolar macrophage following inhalation. It causes pneumonia in young horses and immunocompromised humans. It replicates inside a specialized macrophage compartment, the *R. equi* containing vacuole (RCV) and arrests further phagosome maturation to fuse with lysosomes. The bacteria then replicate inside the vacuole and the host cell eventually dies by necrosis. The objective of this thesis was to explore a new therapeutic approach that can be used to treat rhodococcal pneumonia in foals. Despite advances in our understanding of the organism and its ecology, there are still important gaps in our understanding of the nature of disease due to *R. equi* and the nature of immunity in the foal. Why some foals are affected by these infections and others are not and why immunocompromised adults gets infection and adults do not remain unclear. At this point, we have little understanding of the role of immunogenetic factors predisposing foals to the disease. The discovery of specific innate host factors related to disease risk may help to clarify the pathogenesis and complex epidemiology of this disease. Because of the lack of therapeutic alternatives and rising concerns regarding antimicrobial resistance, there is a great need to develop alternative means for prevention and treatment of the disease. Finally, an effective therapeutic means remains elusive because of the complexity of the immune response to *R. equi* and the challenges posed by limitations of some elements of the immune response of young foals.
Overall my work demonstrated FcγRI activation with a synthetic homodimeric peptide results in a sustained effect leading to a profound reduction in bacterial load in the infected macrophages. Further in vivo testing of the peptide is needed to understand its efficacy for therapeutic use. This approach has also suggested alternatives to FcγRI activation that could be tested including approaches using multiple antigenic peptides (MAP).

**Future Works**

Given the reduction of bacterial load in the infected murine macrophage with the use of a homodimeric that specifically binds FCγRI peptide, it would be important to determine if the same is true for equine macrophages as well. In addition, further works regarding the mechanism of the bacterial killing and cellular signaling events associated with it need to be elucidated. Some of our preliminary works on ligation of FCγRI on murine bone marrow derived macrophages showed induction of Superoxide. In contrast, blocking with monomeric FcγRI peptides resulted in depressed superoxide production compared to controls. Further, we have observed differences in terms of superoxide production in foal and adult macrophages after ligation with the homodimeric peptide. This could result in differences of microbicidal activity between adult and foal monocyte-derived macrophages (MDMs) from blood and pulmonary-alveolar macrophages (PAMs) from bronchoalveolar lavage fluid (BALF). In the future it will be important to perform similar experiments to confirm the differences in the superoxide production and correlation with the reduction of bacterial load.
Within the past decade, autophagy of foreign microorganisms (xenophagy) has become increasingly recognized as an important innate defense mechanism, particularly with respect to intracellular bacterial pathogens (23,24). Recent studies have demonstrated that specific induction of autophagy can overcome inhibition of phagosomal maturation and lead to suppression of intracellular *M. tuberculosis* load, indicating promising clinical outcomes by enhancement of innate mechanisms of xenophagy (50). In the line of xenophagy, in another set of studies, we infected bone marrow derived macrophages with *R. equi* and ligated with the peptide as well. Our preliminary data suggest the induction of cellular autophagy machinery in macrophages after FcγRI ligation with the peptide as well as infection with *R.equi*. Further studies are needed to describe these mechanisms. Finally, understanding-signaling pathways involved in the regulation of autophagy will be important in developing new strategies to abrogate infection with *R.equi*. 
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APPENDIX A

SAMPLE ACQUISITION ON THE IMAGESTREAMX

1. Power up ImageStreamX and launch INSPIRE.

2. Initialize Fluidics. At the end of this script SpeedBeads should be running.

3. In the file menu, choose Load Default Template.

4. In the Image Gallery view menu, select ALL and Press Run Setup to start imaging the beads.

5. Adjust Core Tracking to center images laterally (if necessary).

6. Select the Brightfield (BF) channel and click Set Intensity.

7. Wait until the Flow Speed CV is consistently less than 0.2%.

8. Calibrate the instrument daily. In the ASSIST tab, click Start All to run calibrations and tests and verify that all have passed.

9. Press Flush Lock and Load (FLL) to load the first sample. Load the brightest sample in the experiment that fluoresces with each fluorochrome used. It's critical that you run this sample first to establish the instrument settings and then DO NOT change them for the entire experiment.

10. Turn on each laser used in the experiment and set the Laser Power so each fluorochrome has max pixel values between 100 and 4000 counts, as measured in the scatter plots.

11. Set Cell Classification criteria, to eliminate collection of unwanted objects. For collecting data from only the cells, select the Area Lower Limit in BF channel to
50 µm. Objects with area less than 50 µm will be considered debris and will not be acquired. Select channels to be collected.

12. Enter the File Name, Destination Folder, set Sequence # to 1 and the Number of Events to acquire.

13. Click Run Acquire to collect and save the first experiment data file.

14. Click FLL and run the next experimental sample. Repeat until all experimental samples have been collected.

15. Click Comp Settings (turns off brightfield and scatter laser and enables collection of all channels) and collect 500 positive cells from each single stained sample for each fluorophore in the experiment to develop a compensation matrix.
APPENDIX B

IMAGE ANALYSIS

1. Launch IDEAS and double-click on the Internalization wizard and load one of the test sample .rif files.

2. Create a compensation matrix by clicking on 'New Matrix' in step 2. The compensation wizard is launched. Add files for the single color controls in the experiment. Click next through the wizard following directions until the compensation matrix file is saved and loaded into the box in step 2 of the Internalization wizard.

3. Click Next and follow directions until a .daf file is generated.

4. Set image display properties by selecting the image channels used during acquisition. Click on Ch 2 (AF 488) (BF and SSC are default selected).

5. Select the image channel for making the cell boundary (CH01) and the channel in which bacteria were collected (CH02).

6. A scatter plot of Brightfield Area versus Brightfield Aspect Ratio of all of the cells is generated. Define the single cell population by clicking on individual dots and gating around singlets. Single cells have an aspect ratio of around 1 and doublets around 0.5.

7. A histogram of the Brightfield Gradient Root Mean Square (RMS) of the brightfield image is generated and the population view in the image gallery is set to selected bin. Click on the bins to determine where cells in best focus begin and
draw a line region to gate focused cells. The higher the Gradient RMS, the better focused. Skip the next step unless there are other stains you want to gate on.

8. A new scatter plot of Intensity of Channel 2 on the x-axis versus Max Pixel of Ch2 on the y-axis is generated. Click on the dots and view the images to help you draw the region around the cells that are positive for bacteria.

9. A histogram of the Internalization feature is generated with a region that begins at 0, which should be adjusted by observing images. The Internalization feature is a ratio of the intensity inside the cell to the intensity of the whole cell. It is scaled such that at a value of 0 about half of the intensity is inside. The wizard has created a region to designate the inside by making a mask that has used the cell image input from step 5 to find the cell surface and eroded this by 4 pixels. Note that this mask can be manually adjusted for different cell types when necessary.

10. Create a new histogram with the new Internalization feature based on the eroded Object mask. Draw a region to gate on internalized cells by viewing the imagery in selected bin mode.

11. Finally, to eliminate the cells with background labeling and to identify specific internalized bacteria, the IDEAS Spot Count feature was used. Spot count is a feature that counts the number of connected components or small masks in an image. The mask functions, Spot, Peak and Intensity were used to define the spots. The spot mask finds the bright details in an image that have a user specified radius and threshold above the local background, the mask is refined by
breaking apart high intensities into individual spots using the Peak function and then spots above an intensity of 200 counts were included. See the Amnis spot-masking guide for further information. A statistics report template was generated by including various features in the Reports menu.

12. This file was saved as template file to be used for batch analysis of all experimental files.

13. In the IDEAS software, click Tools→ Batch Data Files and input all the .rif files. Add the compensation matrix file (.ctm) and the template file (.ast) in the corresponding sections. Submit the batch for processing. After the processing step, all the .rif files are analyzed and .daf files are generated for each of the individual raw files. A final report file is generated with statistics for all the samples.