Establishment of a Long Term Cell Culture Model for Testing Anti-Infectives against *Mycobacterium avium* subsp. *paratuberculosis*

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for degree of

Doctor of Philosophy

in

Biomedical and Veterinary Sciences

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March 18th, 2015

Blacksburg, Virginia

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, retinoic acid, vitamin D, phorbol myristate acetate, J774A.1 murine macrophages, peptide nucleic acids

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Abstract

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a very slow growing bacterium that is the causative agent of Johne's disease (JD) in ruminants and has long been suggested to be associated with complications of Crohn's disease (CD) in humans. Although there is no direct evidence that MAP is the primary etiological agent for CD, most CD patients are found to have MAP in their intestinal tissues. The current control measures for JD in cattle, sheep, and goats have only been minimally effective, and there are only medications to treat the symptoms of mycobacterial infections associated with CD in humans.

Along with not being able to cure MAP infections, there is no established laboratory animal model for testing therapeutics. When mice are infected with MAP they develop systemic infection and do not mimic disease observed in ruminants. J774A.1 murine macrophages typically have a very short lifespan of about 4-6 days, however MAP infected cell cultures can survive up to about 10 days. Using a modified protocol of Estrella *et al.* (2011), we have been able to establish a 45-60 day long-term MAP infected J774A.1 murine macrophage cell culture model. With the addition of retinoic acid (RA), vitamin D (VD), and phorbol myristate acetate (PMA) in combination in cell culture, we were able to screen novel therapeutics before embarking on *in vivo* testing in animals. This is a significant step forward in Crohn's and Johne's disease treatment research. We

are not only able to test various drugs against specific strains of MAP to determine susceptibility, but we are also able to test a wide variety of drugs at the same time, with relatively minimal cost.

We have evaluated the efficacy of clarithromycin, azithromycin, isoniazid, amikacin, ethambutol, ciprofloxacin, levofloxacin, rifampicin, clofazimine, as well as a combination of clarithromycin, rifampicin, and clofazimine using our MAP infected macrophage cell culture model. We were able to determine the drugs' differential ability to kill intracellular MAP in the early stages of infection, versus chronic stages of infection, and against two different strains of MAP, 43015 and 19698 that affect humans and cattle respectively. The minimal inhibitory concentration (MIC) of each drug was determined as per NCCLS protocol *in vitro*, and the drugs were tested at the MIC value, along with one concentration above and below the MIC in our cell culture model. The antimicrobials were found to be effective at different stages of cell culture infection and in different strains of MAP. Some drugs were more effective at early stages of MAP infection, whereas others were more effective in chronic or latent stages of infections. It is important to note that although a drug may be effective at a certain stage of infection, it may not necessarily be effective against all strains of MAP. The most promising results were seen with a combination of clarithromycin, clofazimine, and rifampicin, which was effective at all stages of infection with both strains of MAP tested. This long term cell culture model will provide researchers with important screening tools for evaluating new therapeutics before embarking on costly *in vivo* testing, and allow the assessment of therapeutics at different stages of MAP infection but also against an array of intracellular pathogens.

Dedication

I would like to dedicate my dissertation to my parents, Mr. Yos and Mrs. Shatchada Kimsawatde. Without their love and support, this would not have been possible. In fact, nothing in my life would have been possible without them. They have pushed me to be stronger and braver than I ever thought I could be, and to do the best I could to succeed. This work is a product of their continued hope and faith in me that I hope will never run out.

Acknowledgements

I feel truly blessed to have met so many amazing people during my graduate experience at Virginia Tech. I have never been good with words, but would like to acknowledge the following people, who made this experience possible, either directly or indirectly.

First, I would like to thank Dr. Nammalwar Sriranganathan (better known as Dr. Nathan) for being such a great advisor. I could not have asked for a better mentor. He has taught me that all experiments, whether or not they work, teach us something. He allowed me to grow as an independent student, as well as a researcher. He taught me that you can do anything if you put your mind to it.

I would like to thank my committee members, Dr. Kevin Edgar, Dr. Kevin Lahmers, and Dr. Judy Riffle, for their input and constant guidance. They have always been helpful and offer a different perspective for me to think about and evaluate. Their difficult questions and points raised have shaped me into a stronger and more thoughtful researcher. I would like to thank them all for their support throughout my graduate experience.

I would like to thank all my lab mates in Lab 5, past and present. Dr. Ashish Ranjan and Dr. Neeta Jain were one of the main reasons I joined the lab. If I had never met them in Molecular Biology Lab, I'm not sure where I would have ended up. Dr. Neeta Jain and Samantha Casterlow, were not only my lab mates, but great friends as well. Dr. Ronald Tyler was always a humorous officemate and a great softball co-captain (#Kibz4Life). I'd like to acknowledge Dr. Eva Restis, Dr. Hamzeh Alquban, Garrett Smith, and Betsy Schroeder, for always keeping the lab lively, and being available to help troubleshoot any problems. I would like to mention Grant Waldrop who is new to the lab and always eager to help whenever needed, for which I am so thankful. I would

like to thank Catherine Carlson for not only being an amazing laboratory technician, but being our Lab Mom. She is always there when we need her help with our experiments, and always so helpful and caring. I could not have wished for a better person to train and shape the researcher I am today.

To everyone at the Center of Molecular Medicine and Infectious Diseases (CMMID), staff and students, thank you all for everything. I could not have asked for a better place to work, and the people in the building were all so friendly and energetic. A special thanks to everyone in glassware, as well as Nancy Tenpenny for always being so helpful whenever needed. I would like to thank Dr. Stephen M. Boyle for reviewing my dissertation and always providing great feedback and comments. A special thanks to Dr. Jeffry Alexander for all of his help with the Peptide Nucleic Acid (PNA) work, including designing the PNA and helping me setup the experiments; as well as being a great friend.

A big thanks to the Research and Graduate Studies (RGS) Department of the Virginia-Maryland College of Veterinary Medicine for their constant support and funding. I would especially like to mention Becky Jones for her constant helpfulness and always being there for her students. She is absolutely, hands down, one of the best graduate coordinators. Thank you to Dr. Roger Avery for being an amazing Dean and always being there for his students. Thank you to Terry Lawrence for his help in designing and printing of posters and always allowing me to use his pictures in my posters. Thank you to the Virginia Tech-Post Baccalaureate Research Education Program (VT-PREP) for giving me an opportunity to find myself and realize how much I love research. I would also like to acknowledge the Multicultural Academic Opportunities Program (MAOP) for their partial funding in my graduate experience.

I would like to mention everyone in The Unusual Suspects, especially Dr. Jacob Kocher, who is not only one of my best friends, but my brother from another mother. We've been through so much together, and I wouldn't have asked for it any other way. Mariah Weiss, Kim Allen, Josh Skroupa and Kurt Bleach, thanks for always keeping it interesting. I have enough conversations to last me a lifetime, and hope it never stops. I'm still hoping we keep in touch after we go our separate ways, so that we can force our children to be friends. James Budnick and Thomas Brickler, thanks for being great roommates, and for keeping me sane, and reminding me that there's always a brighter and funnier side in life. Dr. Vrushali Chavan, I'm not sure what graduate school would have been like if I never met you. From the moment I met you, I knew we'd be best friends, and I'm glad I wasn't wrong.

To The Thais, who have always been there for me through thick and thin, I would like to thank each and every one of you. To the Charoenvisals, thank you for being my family away from home and always being so caring. To Tita, Jew, Pilin, Ball, Point, BM, Unchalisa, and Kwang, thank you for the friendship and being a part of my life. You guys always made Blacksburg so entertaining, and thank you for being a part of #mybburgfam. You guys made this place home for me, and I cannot thank you enough for always being there for me. I would like to especially mention Unchalisa, my partner in crime, my sister from another mother, and my constant support system.

To all my friends in BMVS, please know that my graduate experience would not be the same without you. There are too many of you to name, and I'd be too scared to leave someone's name out as all of you had an equal part in making this experience so fulfilling. But please know that you have made my graduate experience amazing, and made Blacksburg a home away from

home. For those that I have met along the way, thank you for everything, and making this experience so great.

To the Phongs as well as Pat and Theo, thank you for giving me a place to call home in Virginia and for everything that you do for me. I'm so thankful for having such great relatives. And last, but not least, to my parents, and my sister, Gaan, thank you for your unconditional love, and constant support. You are my biggest fans, and could not have asked for a better family. You are truly my rock, and I'm not sure if I could have done this, or gone through life without all that you guys do for me. Thank for always being there for me, and having so much faith in me.

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List of Abbreviations

AMI	Amikacin
AZI	Azithromycin
CD	Crohn's Disease
CFU	Colony Forming Unit
CLA	Clarithromycin
CLO	Clofazimine
CIP	Ciprofloxacin
CPP	Cell-penetrating peptide
CWC	Cell wall competent
CWD	Cell wall deficient
DMEM	Dulbecco's Modified Eagle's Medium
DSS	Dextran sodium sulfate
ETH	Ethambutol
FBS	Fetal Bovine Serum
ISO	Isoniazid
JD	Johne's Disease
L	Liter
LEV	Levofloxacin
MΦ	Macrophage
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MBC	Minimal Bactericidal Concentration
mg	Milligram

mL	Milliliter
MIC	Minimal Inhibitory Concentration
mm	Millimeter
MOI	Multiplicity of Infection
Mtb	*Mycobacterium tuberculosis*
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
NOD2	Nucleotide binding and Oligomerization Domain 2
OD	Optical density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMA	Phorbol Myristate Acetate
PNA	Peptide Nucleic Acid
P/S	Penicillin/Streptomycin
RA	Retinoic Acid
RIF	Rifampicin
RPM	Revolutions Per Minute
SEM	Standard Error of the Mean
µg	Microgram
µL	Microliter
USDA	United States Department of Agriculture
VD	Vitamin D
[ ]	Concentration

Chapter 1
Literature Review

Mycobacterium avium subsp. *paratuberculosis*

There are more than 70 *Mycobacterium* ssp. found throughout the world [1, 2]. Mycobacteria are aerobic and non-motile bacteria. Although they are considered Gram-positive, they do not take up Gram's stain due to the high lipid content of the cell wall, and they are characteristically acid-fast. The genus *Mycobacterium* includes bacteria known to cause serious chronic diseases such as tuberculosis, leprosy, and Johne's disease in mammals [3]. Mycobacteria are able to colonize their host without the host showing any signs or symptoms of infection until the disease develops and then it becomes difficult to treat [4]. Their structural, physiological, and genetic characteristics are major contributors to their ability to cause chronic persistent infections and their slow growth may aid in their resistance to many antimicrobials.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an intracellular pathogenic bacterium. It is similar to but quite distinct from other mycobacterial species [5]. Although it is 99 percent genetically similar to *Mycobacterium avium* subsp. *avium* that causes tuberculosis in birds [6], MAP has many differentiating phenotypic features. It grows much slower than most other mycobacteria, requires the addition of an iron chelator and transport protein known as Mycobactin J for it to grow *in vitro*, infects mammals preferentially, and does not infect birds [7].

Culturing of MAP from feces and intestinal tissues is the common confirmation of infection [8]. Culturing in liquid media offers the greatest sensitivity for detection; however, the BD BACTEC 460 system commonly used has been discontinued due to lack of demand [8]. Therefore,

clinicians and researchers often use polymerase chain reaction (PCR) in addition to culturing in broth and on solid agar for confirmation of their tentative diagnosis [8, 9].

MAP is the causative agent of Johne's disease, which affects ruminants such as cattle, sheep, and goats, and has been associated with Crohn's disease in humans. There are many different strains of MAP [9], however two strains, both well-characterized, are the focus of this doctoral research effort. MAP strain ATCC 19698 is an isolate from the feces of a cow with naturally acquired Johne's disease. MAP strain ATCC 43015, also known as strain Linda, is an isolate from the ileum of a 15-year-old Rhode Island girl with Crohn's disease. Although these strains are genetically related, comprehensive analysis suggests that there are genetic differences between the MAP isolates based upon their origin either from tissue (MAP strain 43015) or from feces (MAP strain 19698) [10]. These two strains are of interest in this doctoral research effort as they are representatives of MAP isolated from a Johne's patient (MAP strain 19698) and MAP isolated from a Crohn's patient (MAP strain 43015).

MAP strain 43015 was originally isolated by Chiodini *et al.* in 1984 [11]. The bacterium was isolated from a Crohn's disease patient and found to be an acid-fast bacillus ranging in size from 1.8 to 2.3 μM by 0.3 μM. The primary culture took 15-72 weeks to grow in medium, while the subculture took about 4-8 weeks in the same medium. The generation time in liquid was found to be 15-20 hr and optimal at 37°C. MAP strain 19698 on the other hand, was isolated from the feces of a cow with naturally acquired paratuberculosis and is slightly faster growing than strain 43015 with subcultures taking 3-6 weeks to grow [12]. Although MAP strain 43015 and 19698 are similar in size, a distinguishable genetic phenotypic difference of some MAP cultures isolated from Crohn's patients is that they exist in cell wall deficient (CWD) forms [13]. MAP strain 43015 is an example of a CWD form. This is an important pathogenicity factor in the development of

human disease as well as susceptibility to cell wall active antimicrobials *in vivo*. CWD forms of MAP are extremely difficult to isolate, and difficult to detect as they lack a thick lipid-containing cell wall that makes it difficult to positively identify through acid-fast stain. They revert to cell wall competent (CWC) forms when cultured *in vitro* [13]. MAP strain 19698 is not a CWD form. Thus, these two strains of MAP were not only of interest in this doctoral research because they are well characterized, but also because they have different cell walls; CWD (MAP strain 43015) vs. CWC (MAP strain 19698). Because of the differences in their cell envelopes one would not be surprised to see differences in their susceptibility to antibiotics.

Johne's and Crohn's Diseases

Johne's disease was described by Heinrich A. Johne, a German bacteriologist and veterinarian, in 1905 and found to be caused by *Mycobacterium avium* subsp. *paratuberculosis* [14]. Johne's disease (JD), also referred to as paratuberculosis, is found worldwide and is a contagious, chronic, persistent, and sometimes fatal infection that primarily affects the small intestines of ruminants. It causes chronic granulomatous enteritis in ruminants, and is passed on from animal to animal through the fecal-oral route, as well as through the mother's colostrum to its young [6]. Although it primarily infects ruminants, it has also been known to infect non-ruminant species such as foxes and rabbits [15].

Although MAP infections create significant losses for cattle farms each year, controlling JD proves to be very difficult due to the variable progression of the disease from the sub-clinical to clinical stages, sometimes taking up to 5 years [16]. It is also difficult to diagnose JD in the early stages of infection. Most farms therefore incorporate a test and cull program to keep their

herds free from JD; however, this has been found to be ineffective in eradicating MAP from the herd [17]. The impact of JD on animal health and the farm's profitability has led to interest in controlling MAP infections in cattle.

MAP infections predominantly occur in calves at a young age, and calves of less than six months are found to be most susceptible [18]. It has been documented that the severity and rate of JD progression is dependent on factors such as exposure dose as well as the age of the animal at infection [19]. As stated previously, MAP infection usually occurs through the fecal-oral route, but it has also been found to be transmitted *in utero* [20]. Because it can be transmitted via the fecal-oral route, contamination of udders, as well as calving pens increases the chances of infection [21]. Colostrum and milk derived from infected animals are capable of infecting calves [22, 23]. Thus, farms that feed calves pooled colostrum from multiple cows have a higher risk of JD in their herds [22]. Therefore, incorporation of improved calf management and hygiene is becoming more important in controlling MAP infection in farms [24]. Other risk factors that increase chances of MAP transmission include group housing of cows [25], the use of group calving pens with the presence of more than one cow in each pen [26, 27], and not cleaning the pens between births [28].

Crohn's disease (CD), on the other hand, is a type of inflammatory bowel disease that can affect any part of the gastrointestinal tract of humans [29]. It causes a wide variety of symptoms, similar to Johne's disease, including chronic diarrhea and weight loss. The result of this inflammatory disease is primarily due to an autoimmune response attacking the gastrointestinal tract. The possibility that Crohn's disease may be caused by an infectious bacterium, MAP, has been postulated for years, however, the topic is still controversial. Additionally, many observations have been made that suggest MAP may be a trigger for the development of CD. These observations include lesions occurring in regions with high MAP bacterial concentrations,

recurrent ulcers occurring in the Peyer's patches, and granulomas containing MAP [5, 30-34]. The severity of the disease is correlated to the bacterial MAP density.

Although the etiological agent for CD is still uncertain, more evidence is accumulating to suggest that MAP is the infectious agent; however, the mechanism is still unclear. There is clinical evidence of MAP involvement in CD [35]. There seems to be a genetic association with the disease, primarily in the variations of the Nucleotide binding and Oligomerization Domain 2 (NOD2) gene and its proteins, which senses bacterial cell walls [36, 37]. Much research has been done on the polymorphism of the NOD2 gene, showing that it is a genetic predisposition factor for both CD and MAP infections [37-41]. Live MAP has been found in pasteurized cow's milk, suggesting that dairy and meat products could be playing a role in transmission of MAP to the human population [5]. Men and women are equally affected, and the disease can develop at any age, however symptoms normally start to present themselves during the teenage years and twenties [42]. There is no known pharmaceutical or surgical cure, and treatment options are restricted to controlling symptoms through palliative measures in hopes of maintaining remission and preventing frequent relapses as well as selective resection of affected segments of the gastrointestinal tract as a last resort.

Host immune responses against *M. paratuberculosis* infections

It has been shown that mycobacteria are capable of survival in human and animal macrophages [43-45]. Current models propose that upon ingestion of the bacteria, MAP is able to cross the intestinal barrier by internalization into microfold cells and into the immune cells [46, 47]. It is believed that there are specific physiological factors that aid MAP in their ability to

survive *in vitro* and *in vivo*. *Mycobacterium* spp. are representative of many highly successful pathogens that are able to evade innate immunity by manipulating the host to ensure long-term survival [44].

When a phagosome fuses with a lysosome, a membrane enclosed organelle forms within the phagocytic cell (i.e. macrophage), known as a phagolysosome that contains reactive molecules, lytic enzymes and antimicrobial peptides. This fusion typically occurs following phagocytosis of a foreign object, such as a bacterium. After this fusion, the pathogen within the phagolysosome is usually killed and digested by the reactive molecules and lytic enzymes combinations. However when mycobacteria are taken up, the macrophages are unable to digest/kill them [48], and the pathogen is able to multiply unchecked. Mycobacteria rely on multiple virulence factors that ensure their retention within phagosomes. The cell wall of the mycobacteria is one factor that prevents the fusion of the phagosome with the lysosome [49]. By preventing phagosome-lysosome fusion, the phagocytized mycobacteria are able to survive in the phagosome where they are able to persist and multiply without detection by the host immune system [50]. In some cases, where the fusion of phagosomes with lysosomes does occur, mycobacteria have been shown to be resistant to antimicrobial peptides and lytic enzymes; and some appear to replicate normally in the presence of the hydrolytic lysosomal compounds within the macrophages [51].

When mycobacteria are in phagosomes, they are able to selectively inhibit the proton-ATPase that is normally responsible for acidification along the endocytic pathway to lysosomes [48]. This limited acidification induced by the mycobacteria in the phagolysosome is a key mechanism for their intracellular survival, along with the fact that mycobacteria are able to withstand acidic conditions [45]. The mycobacteria also evade macrophage-killing by neutralizing reactive nitrogen intermediates [52, 53]. Reactive nitrogen intermediates have a bacteriostatic

effect on mycobacteria *in vitro*, and by neutralizing these intermediates they are able to survive within the macrophages [53, 54]. The low metabolic activity of the mycobacteria leads to persistent intracellular survival within macrophages; this state makes it very difficult to use short-term therapy effectively to treat mycobacterial diseases successfully.

Bacterial characteristics that aid resistance of *Mycobacterium* to antibiotics

Mycobacteria are known to be hardy, slow growing bacteria. Thus drug treatment times for mycobacterial infections are usually extremely long. Many species of mycobacteria become inactive for long periods of time, during which they are not found to be metabolizing. Since most antibiotics are only effective against bacteria that are metabolizing, they are unable to completely eradicate the mycobacteria [55]. Therefore, it is necessary to treat mycobacterial infections for an extended period of time to allow the therapeutics to act against both latent (dormant) and actively replicating bacteria [44].

Antibacterial drugs work in various ways to treat bacterial infections; affecting cell wall synthesis or disruption of plasma membrane, or inhibiting protein or nucleic acid synthesis. The structural and physiological characteristics of mycobacteria are major contributors to their resistance to many antimicrobials [44, 56]. Their unique lipid rich cell wall allows them to survive long exposure to acids and bases, detergents, oxidative bursts, lysis by complement, and many antibiotics [56]. All *Mycobacteria* share a characteristic cell wall that is much thicker than those of other bacteria. Their cell walls are hydrophobic and full of mycolic acids; the mycolates are in the outer membrane that is attached via arabinomannan to the peptidoglycan [43]. The cell wall of mycobacteria has very low permeability, a major factor in antibiotic resistance. This waxy,

non-fluid barrier restricts the passage of both hydrophobic and hydrophilic compounds [43], thus making it very difficult for drugs to penetrate the cell wall and kill the bacteria; hence they are resistant to many antibiotics [49].

Present vaccines and therapies

One of the current vaccines against JD is Mycopar®. It "is a whole cell bacterin containing inactivated *M. paratuberculosis* suspended in oil" and is used "for vaccination of calves as an aid in the control of clinical disease due to *M. paratuberculosis*" [57]. Although the vaccination provides protection against clinical disease, it does not necessarily prevent infection. Other vaccine candidates have been tested, and although some have been shown to reduce incidence of infection and fecal shedding, none were able to completely prevent MAP infection and eliminate fecal shedding [58]. Therefore, vaccination of cattle is controversial and not widely used as it interferes with diagnostic tools for paratuberculosis [59]. Besides cattle, it is not common to vaccinate other ruminants; the most cost-effective way to manage Johne's disease is prevention.

On a more encouraging note, a study published in 2015 evaluated a goat based 'indigenous' vaccine against bovine JD in endemically infected native cattle herds [60]. An 'indigenous-vaccine' was prepared from a native bio-type MAP strain S5 of goat origin and evaluated in a cattle herd. Response to the vaccination was evaluated based on health, productivity, immunological parameters, survival, bacteremia, seroconversion and status of shedding. The major findings were a reduction in MAP shedding and a disappearance of MAP from blood circulation in vaccinated cattle. This application of a goat based vaccine for therapeutic

management of bovine JD is the first of its kind. However, it needs more testing before it can be declared an effective vaccine and therapeutic for JD prevention and treatment.

It is less expensive to prevent introduction of JD into a herd/flock than it is to control or eradicate the infection [61]. It is recommended that animals be bought from test-negative herds to ensure against importing infected animals that may spread the disease [62]. In addition, practicing calving hygiene and avoiding the pooling of milk colostrum minimizes the chances of MAP transmission [22].

It has not been considered economical to treat cases of Johne's disease using antibiotics as the chances of curing an animal are very low, and the cost of the drugs is very high [62, 63]. Since MAP is very slow growing, it is time consuming to determine which antibiotics would work best with the particular strain of MAP infecting the animal. The treatment course takes many months and the meat and milk from these animals are not suitable for human consumption [62]. Rather than treating the animal, those with Johne's disease are therefore slaughtered to prevent spread of the disease. As a result of this practice, very little research has been done on MAP drug susceptibility.

A comprehensive evaluation of the *ex vivo* effects of antimicrobial drugs on both animal and human-origin MAP was published in 2009 by Krishnan *et al.* [64]. It was found that macrolide drugs (azithromycin and clarithromycin) have the highest *in vitro* efficacy against MAP. Other broad spectrum drugs (amikacin, ciprofloxacin, levofloxacin, and rifampicin) showed intermediate effect on some strains of MAP. Most first-line anti-tuberculosis (ethambutol and isoniazid) or anti-leprosy (dapsone and clofazimine) drugs were found to have no effect against MAP *in vitro*. However, rifampicin and rifabutin showed some efficacy [62]. Previous treatment with isoniazid at 20 mg/kg administered orally every 24 hours for the rest of the infected animal's life or

rifampicin at 20 mg/kg administered orally every 24 hours for animals with acute onset of diarrhea, for extended periods resulted only in relief from the symptoms rather than a definitive cure [65]. Long term administration of clarithromycin in conjunction with rifabutin and/or clofazimine has showed promise with two thirds of test CD patients entering remission, however a minority of the patients were found to have drug resistant MAP [66].

With this data, some rationale for choosing antibiotics effective in treating MAP infections is available. However, *in vitro* assays with mycobacteria are still poor predictors of treatment efficacy for *in vivo* mycobacterial infections [62]. This may be due to the fact that the drugs are only tested on acute infection of cells in culture as opposed to persistent infections of cells. This problem can be addressed with a long-term *in vitro* cell culture model that can mimic long-term infection *in vivo.*

Although it is not economical to treat animals with Johne's disease, there is a constant search for therapeutics for humans. At this time, there is no cure for Crohn's patients, and there is only medication to help with the symptoms associated with disease, rather than treating the bacterial infection itself. Johne's disease symptoms correlate with Crohn's disease, but not necessarily the same with respect to the pathogen.

Treatment for Crohn's disease has changed over the years, with new therapies targeting specific locations of infection in the gastrointestinal tract and targeting specific cytokines [67, 68]. Biologic anti-tumor necrosis factor (anti-TNF) agents have shown significant advances in maintenance of remission [69]. If medical therapy is found to be ineffective, surgery and resection of the inflamed area is performed [70, 71].

Phases of clinical research

According to the US Food and Drug Administration (FDA) (Silver Spring, MD, FDA.gov), there are multiple stages of clinical research in drug development and review in which scientists have to go through to design, discover, and test drugs in an attempt to find evidence for useful novel therapuetics. In the preclinical phase, drugs are tested in non-human subjects (*in vitro* and *in vivo*) to gather efficacy, toxicity, and pharmacokinetic information. The next step is Phase 1 of clinical trials, which involves testing of the drug on healthy volunteers for dose-ranging. This phase is used to determine whether the drug is safe to further check for potential efficacy. The drug is tested at escalating dosages to determine the best and safest dose. In Phase 2, drugs are tested on a small number of patients to assess efficacy and safety. This stage determines whether the drug has any biological activity or efficacy, but is not presumed to have a therapeutic effect quite yet. By Phase 3, which is testing of the drug on patients to assess efficacy and safety, the drug is presumed to have a therapeutic effect. Of 5,000-10,000 drugs discovered, only about 250 move on to the preclinical phase. Of the 250 that move on from the preclinical phase, only about 5 compounds make it to phase 1-3 trials. Lastly, of those 5 compounds, only 1 makes it past FDA review and sent into large scale production and distribution, where it will still be monitored for its efficacy and safety. As *in vitro* assays with MAP are still poor predictors of treatment efficacy for *in vivo* MAP infections [62, 64, 72], numerous drugs for MAP infections do not make it past preclinical phases. Therefore there is a need for an effective *in vitro* model for testing of potential therapeutics before progressing on to *in vivo* testing, as well as clinical trials. Thus a long-term murine cell culture model, could be an effective means to test therapeutics before advancing to *in vivo* testing in mice; bovine cell culture models to cattle testing; human cell culture models to human testing.

Present cell culture and animal models of paratuberculosis infection

Currently there is no established long-term cell culture system to test therapeutics against MAP infections. Although there is an established bovine monocyte cell culture model [73], it is not a long-term infection model [74]. For animal models, there are a few *in vivo* trials due to financial issues. The use of ruminants for MAP research is limited due to the fact that hosts need to be maintained for 2 to 3 years before they develop clinical paratuberculosis. Smaller animal models include chickens, guinea pigs, hamsters, mice and rabbits; however, they do not develop disease symptoms like ruminants [6]. St. Jean *et al.* attempted to treat bovine paratuberculosis, and it was "concluded that daily isoniazid (20 mg/kg) alone or in combination with rifampin at (20 mg/kg) for the duration of the animal's life can forestall progression of MAP infections but does not cure them i.e., when treatment stopped, the cows relapsed" [62, 75]. This treatment is prohibitively expensive, thus only animals of high genetic importance undergo antibiotic therapy.

One of the most promising cell culture models is one demonstrated by Estrella *et al.* [76]. They found that activation by retinoic acid (RA), a vitamin A precursor, and vitamin D3 (VD) prior to *M. tuberculosis* (Mtb) infection, increased mycobacterial uptake but inhibited the subsequent intracellular growth of the bacteria by inducing reactive oxygen species and autophagy. In their studies, THP-1 human monocytes were infected with Mtb. The activated THP-1 cells have marked phenotypical changes as THP-1's multiply adjacent to each other to form multinucleated giant cells (MNGCs). Typically MNGCs occur over 30 days of *in vitro* culture and contain non-replicating persisting Mtb for more than 60 days in culture [76]. Estrella *et al.* showed that they could maintain Mtb infected THP1 cells for more than 60 days. This raised the possibility that this activation could potentially provide a cell culture model for testing therapeutics against MAP.

MAP infected mice do not develop symptoms as seen in ruminants, thus have not shown promise as an effective animal model in the past. However, with combined dextran sodium sulfate (DSS)-induced colitis with MAP infection, mice were seen to have increased liver and spleen sizes, as well as increased number and size of MAP-induced liver granulomas and higher MAP counts in their tissues [77]. This is a promising murine model for the study of MAP human infections. However, before proceeding to *in vivo* testing in mice, drugs should still be tested *in vitro* beforehand.

Rationale and Significance:

According to the U.S. National Johne's Education Initiative, 1 out of 10 animals moving through livestock auction facilities is infected with Johne's disease. In 2007, the United States Department of Agriculture (USDA) found that 68% of U.S. dairy operations were infected with MAP [78]. The Johne's incidence in the USA has been steadily increasing, there is no cost effective treatment, and the current control measures have not been very successful. The best prevention is to buy cattle from herds that test negative, and conduct testing of the animals frequently to ensure that the herd has not become infected. In cattle, the main symptoms are persistent diarrhea and wasting. These symptoms usually do not appear until about 3-5 years of age. Once classic symptoms of Johne's disease appear, it is too late for effective control measures, let alone treatment. If cattle, by 5 years of age, do not show any signs of sickness, this usually means they are resistant to the disease, or has not been exposed.

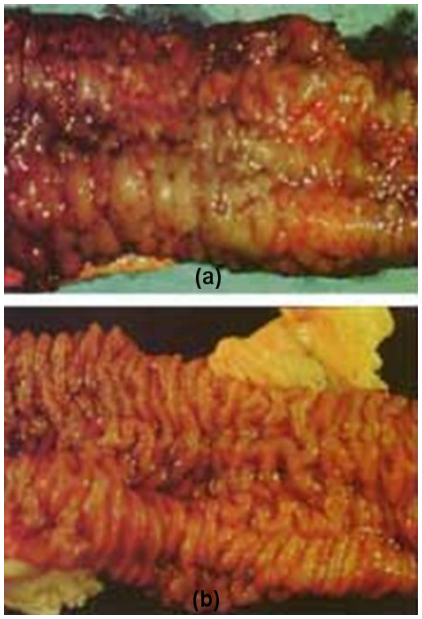

Figure 1. Section of the Intestinal Tissue from (a) Johne's and (b) Crohn's patients. *Regional enteritis with fibrosis and thickening of the ileum wall is shown. The lesions from the two diseases are very similar.*
http://www.crohns.org/map_food/photo.htm
(Used under fair use, 2015)

As previously mentioned, the prevalence of Johne's disease in the U.S. is fairly high. According to the USDA's National Animal Health Monitoring system, 5% of dairy cows may be infected with MAP [62]. This leads to loss in productivity estimated to be $200 to $250 million annually [61]. In addition, a small percentage of store bought high temperature short time (HTST for 15 second) pasteurized milk has been shown to contain viable MAP [79].

There appears to be a correlation between an increased prevalence of CD in humans and JD in cattle in the western world. CD is associated with a wide variety of symptoms, caused by the immune system attacking the GI tract and producing inflammation (autoimmune response). There is no cure for CD and the medication available can only treat the symptoms. The correlation between CD and MAP has been controversial since the first culturing of this bacterium from the intestinal tissues of several patients with CD by Chiodini *et al.* in 1984 [80]. Subsequently, MAP has become a candidate for the causative complicating factor of CD because of several observations: lesion similarity with JD [81]; its detection in the tissues and blood of CD patients at a greater frequency than those without CD; the detection of antibodies against MAP antigens compared to non-CD controls; and the detection of MAP in human breast milk from CD patients [82]. The controversy exists, in part, because it has also been reported that humans infected with MAP do not show a higher prevalence of CD. MAP has been isolated from humans without CD, and there is a lack of evidence that the consumption of food containing MAP causes CD [83]. Although there is no direct evidence that MAP is the primary etiological agent for CD, most CD patients have signs of an autoimmune inflammatory disease and are found to have MAP in their intestinal tissues (Figure 1). Although it not possible to fulfill Koch's postulates for proving that MAP causes CD, the overwhelming probability and public health risk favor the conclusion that MAP are pathogenic to humans and responsible for the CD complications but may not be the cause of CD.

Currently, there is no well-established long term *in vitro* infection model for testing therapeutics against MAP infections. When novel therapeutics are developed, the first step is to test them *in vitro.* Mice have been demonstrated to be an ineffective model for MAP infections without the chemical induction of colitis prior to MAP infection, as they do not reproduce lesions

seen in ruminants, even when the infection is systemic [84]. Few drugs are tested or used directly in infected large animals as it is prohibitively expensive. It takes MAP infected animals a few years to start showing symptoms of infection. Thus it would be much easier if there were an established *in vitro* model, where the results could correlate to the effect of treatment in animals. As current *in vitro* assays with MAP are still poor predictors of treatment efficacy for *in vivo* MAP infections due to the fact that the drugs are only tested on acute infection of cells in culture as opposed to persistent infections of cells, there is a real need for a long term *in vitro* MAP infection model for drug susceptibility testing.

Moreover the results from such tests could be used to develop correlates of treatment in animals allowing effective treatment of intracellular MAP infections in susceptible ruminants with JD. In addition the results could provide some guidelines for treatment of humans with CD complicated with MAP infection.

Objective of the Dissertation:

Establish a long term *in vitro* MAP infected cell culture model, in order to screen novel and conventional therapeutics and thereby develop some guidelines to begin *in vivo* testing in animals. This could potentially be the first significant step forward in Crohn's and Johne's disease treatment research. Assess a cell culture model to test specific drugs against specific strains of MAP to determine susceptibility, to test a wide variety of drugs at the same time with minimal cost. This long-term cell culture infection model may allow testing of the drugs' ability to kill intracellular MAP in the early acute, chronic as well as latent stages of infection.

This model may help determine the ability of different therapeutics to suppress growth as well as kill MAP at different stages of infection; this was not possible previously due to lack of an available MAP infected cell culture model which survived for extended periods. Furthermore, this insight into a new *in vitro* model could potentially eliminate unnecessary preliminary testing in mice and ruminants, as this cell culture model would be able to screen potential candidates at different stages of infection. This effort will provide researchers with important screening tools for new therapeutics before actually embarking on costly *in vivo* testing, and allow the assessment of therapeutics against an array of intracellular pathogens. This i*n vitro* cell culture MAP infection model may provide scientists with an efficient method to evaluate not only new as well as old therapeutics, but also those that showed promise, but were abandoned due economical and/or technical roadblocks of testing in animals.

Overall Goal:

Develop a MAP cell culture infection model for acute, chronic and persistent infection for testing conventional and novel therapeutics:

Evaluate the addition of vitamins A and D (RA and VD respectively), as well as phorbol myristate acetate (PMA), to J774 A.1 cells to establish a cell culture system that can possibly survive long-term, defined as 45 to 60 days. Test the cell culture system as a MAP infection model to see if it can represent acute, chronic and persistent infections. Determine the effective concentrations of RA (1nM to 1μM), VD (1nM to 1μM), and PMA (10ng/mL=16nM), in both the short term and long term studies. Determine the RAVD concentrations that allow successful extension of the lifespan of MAP infected J774A.1 murine macrophages. A MAP infected J774A.1 cell culture model can then be used to test therapeutics and develop a model that has the potential to be an alternative to testing of new therapeutics *in vivo*. This will potentially allow screening of large numbers of therapeutics before embarking on testing *in vivo* in animals.

Overall Hypothesis:

Certain antimicrobials are differentially effective in killing *Mycobacterium avium* subsp. *paratuberculosis* at various stages of infection (acute, chronic and latent infection).

Aim 1: Development of a long-term cell culture model to monitor *Mycobacterium avium* subsp. *paratuberculosis* infections.

Aim 2: Determination of minimal bactericidal concentrations of anti-infectives against intracellular *Mycobacterium avium* subsp. *paratuberculosis* in short term and long-term infections using an established cell culture model.

Aim 3: Evaluation of peptide nucleic acids (PNAs) and their effect on the growth of *Mycobacterium avium* subsp. *paratuberculosis* and their potential as a therapeutic against MAP infection.

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Chapter 2

Development of a Long Term Cell Culture Model for *Mycobacterium avium* subsp. *paratuberculosis* Infections

Abstract:

Mycobacterium avium spp. *paratuberculosis* (MAP) is a slow growing bacterium that is the causative agent of Johne's disease (JD) in ruminants and has long been suggested to be associated with Crohn's disease (CD) in humans. Although there is no direct evidence that MAP is the primary etiological agent for CD, up to 60% of CD patients are found to have MAP in their intestinal tissues [1].

JD is a contagious, chronic, and sometimes fatal infection that primarily infects the small intestines of ruminants. In humans, CD is considered an inflammatory autoimmune disease that can affect any part of the gastrointestinal tract and is complicated by MAP infection symptoms. There is no cost effective vaccine or treatment for JD or CD. The current control measures for JD have only been minimally successful, while there are only medications to treat the symptoms associated with CD.

Along with not being able to cure MAP infections, there is no laboratory animal model for testing novel therapeutics or vaccine candidates. When mice are infected with MAP, they develop systemic infection and do not mimic intestinal disease as seen in ruminants. Without a good animal model, it is impossible to study MAP infections and test novel therapeutics in a laboratory setting. Estrella *et al.* (2011) showed that phorbol myristate acetate increased the uptake of *M. tuberculosis* by THP-1 cells *in vitro*, and retinoic acid (vitamin A precursor) and vitamin D extended the

lifespan of these infected cells. Untreated MAP infected cells typically have a very short lifespan of about 4-6 days. This above protocol has been tested and a modified protocol has been developed, which demonstrated that RAVD and PMA in combination could extend the viability of MAP infected J774A.1 murine macrophages to 45-60 days. This new refinement of an *in vitro* model could potentially minimize or even eliminate therapeutic testing in animal models.

Introduction:

Mycobacterium avium subsp. *paratuberculosis* (MAP) is an intracellular pathogenic bacterium. It is similar to, but quite distinct from other mycobacterial species [2, 3]. Although it is 99 percent genetically similar to *Mycobacterium avium subsp. avium* [4], it has many differentiating phenotypic properties. It is the causative agent of Johne's disease, which affects ruminants such as cattle, sheep, and goats, and has been associated with complications of Crohn's disease in humans [2, 5-9]. MAP is different from most other bacterial pathogens as it is able to survive *in vivo* for years before the host shows signs of infection or symptoms of disease [10-12]. MAP is a very slow growing bacterium with a doubling time of 22-24 hr [13]. It has been found to survive in macrophages avoiding the host's immune system. Although it is able to survive for a long period of time *in vivo* [14], the available cell culture systems of infection are limited due to the short life span of macrophages.

Currently there is no long-term murine cell culture system to test therapeutics against MAP infections. Although there is an established bovine monocyte cell model [15], it is not a long-term infection model [16]. When mice are infected with MAP, they develop systemic infection, and do not mimic disease symptoms in ruminants. As for larger animal models, there have been few *in*

vivo trials. The use of ruminants for research is limited due to the fact that hosts need to be maintained for 2 to 3 years before they develop clinical paratuberculosis. Smaller animal models include chickens, guinea pigs, hamsters, mice and rabbits, however they all do not develop disease symptoms seen in ruminants [4]. St Jean *et al.* [17, 18] attempted to treat bovine paratuberculosis and he "concluded that daily isoniazid (20 mg/kg) alone or in combination with rifampin at (20 mg/kg) for the duration of the animal's life can forestall progression of MAP infections but does not cure them i.e., when treatment stopped, the cows relapsed". In addition, this long-term therapy is prohibitively expensive, and thus only animals of high genetic importance undergo this treatment.

One of the most promising cell culture models is one demonstrated by Estrella *et al.* [19]. In their studies, they used THP-1 cells, a human monocyte cell line, infected with *M. tuberculosis* (Mtb). They found that the activation of the cell line by retinoic acid (RA), a vitamin A precursor, and vitamin D3 (VD) prior to Mtb infection, increased mycobacterial uptake but inhibited the subsequent intracellular growth by inducing reactive oxygen species and autophagy. PMA is the most common chemical activator for THP-1 cells, and is used to induce adherence and make the cells more macrophage-like. The activated THP-1 cells showed marked phenotypic changes, as THP-1 cells adjacent to each other formed multinucleated giant cells (MNGCs). Typically MNGCs occur over 30 days of *in vitro* culture and contain non-replicating but persisting live Mtb for more than 60 days in culture [19]. They showed that they could maintain Mtb infected THP-1 cells for more than 60 days. This observation raised the possibility of developing a cell culture model for testing therapeutics against MAP in macrophages as well.

In this study, the effect of RAVD and PMA on longevity of MAP infected J774A.1 murine macrophages were examined. MAP infected cells typically have a very short lifespan of about 10-

14 days. The modified protocol, demonstrated RAVD and PMA in combination could extend the viability of MAP infected J774A.1 murine macrophages to 45-60 days. Initial evaluation of short-term infection of 24 days progressed to developing and testing a long-term infection model of 60 days. This refinement of an *in vitro* infection model could potentially minimize testing in animals, and allow the opportunity to screen novel therapeutics before actually embarking on *in vivo* testing in animals.

Materials and Methods:

Bacterial strains and cell lines:

Mycobacterium avium subsp. *paratuberculosis* strains 43015 (human) and 19698 (cattle) (American Type Culture Collection, ATCC, Manassas, VA) was routinely grown at 37°C in #1507 Middlebrook broth made up of 4.7 g Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ), 0.5 g Tween 80 (Sigma-Aldrich, St. Louis, MO), 2.0 mg Mycobactin J (Allied Monitor Inc., Fayette, MO), 900 mL distilled water. After autoclaving, the media was cooled to 60°C in a water bath, then 100 mL OADC enrichment (oleic acid, albumin, dextrose, catalase) (BD, Franklin Lanes, NJ) was added. This medium is an enhanced Middlebrook broth used specifically for the growth of MAP in culture. For preparing #1507 Middlebrook agar, the same components as #1507 Middlebrook broth were used with the addition of 20.0 g of Bacto agar (Difco). For determining cell cytotoxicty, J774A.1 murine macrophage cells (ATCC TIB-67) were used. The murine macrophage-like ATCC J774A.1 cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% heat-inactivated fetal bovine serum (FBS) (Corning,

Manassas, VA) and 1% penicillin-streptomycin (P/S) (Corning Inc.) in a humidified 5% CO_2 atmosphere at 37°C.

Activation of J774A.1 macrophages:

When needed, cells were expanded into individual 75 cm^2 flasks and activated with RA (1μM) and VD ((cholecalciferol version) 1μM), or PMA (10ng/mL=16nM) (Sigma-Aldrich, St. Louis, MO), or RAVD plus PMA for 3 days prior to infection with MAP. These concentrations of RA, VD and PMA were based on the optimal doses of RAVD and PMA determined by receptor expression studies in activated RAVD-THP-1 cells [19]. After three days, the cells were washed three times with 10 mL of phosphate buffered saline (PBS) (Corning Inc.) to remove traces of RAVD and/or PMA.

Infection of J774 A.1 with MAP and short-term and long-term growth curves:

MAP suspensions were subject to vortexing with 10 grams of 2 mm sterile glass beads and then matched to the McFarland standard #1 (3 x 10^8 bacteria) and spun at 1,000 x g for 2 min [19]. The supernatant containing single colony forming units (CFUs) of MAP without clumps were used for infection. Activated J774 A.1 cells were then infected with a multiplicity of infection (MOI) of 10 with MAP for 24 hr at 37°C and 5% CO_2 with gentle mixing to ensure uniform infection. Cell lines were then washed three times with 10 mL of sterile PBS to remove non-phagocytized MAP, then dispensed into 24-well plates (Corning Inc.) with 2 mL in each well for generation of growth curves or 4-well chamber slides (Corning Inc.) for qualitative analysis by Kinyoun acid fast staining. Multiple times during the growth curve assays, the macrophage growth medium was

changed. This was done to remove dead, non-adhering macrophages as well as remove spent medium. At the specified time points, cells were then washed three times with 1 mL of sterile (to further remove dead macrophages) PBS and lysed with 200 μL 0.1% Triton X-100™ (Sigma-Aldrich), every day for 10 days for short-term growth curves or every day for 4 days and then every week for 60 days for long-term growth curves. The lysates were plated using 8 ten-fold dilutions in PBS on #1507 agar to determine intracellular CFUs of MAP harvested at various time points.

Statistical analysis:

All statistical analyses were performed with Student's two-tailed *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA). *P*-values of ≤ 0.05 were considered significant.

Results:

Short Term Growth Curves

1) *Differential effects of RAVD and PMA on the uptake and survival of MAP strains 43015 and 19698 in J774A.1 macrophages*

J774A.1 MΦs were activated with RAVD or PMA for three days prior to infection and evaluated for uptake and growth of MAP; the results are graphically depicted in Figure 2.1. Although RAVD activated MΦs were unable to survive longer than the control MΦs (Figure 2.2), they were able to host more MAP between day 4 to day 18 post infection with MAP strains 43015 and 19698 (Table 2.1). However, PMA activated MΦs were unable to survive much past 10 days of infection (Figure

2.2). Figure 2.2 shows the number of live J774A.1 macrophages present at specific time points post infection with MAP. The actual CFUs/mL of intracellular MAP at each time point is shown in Table 2.1. The results from the short term growth curves with activation by RAVD or PMA alone suggested that perhaps the MΦs' ability to support MAP infection would be affected if RAVD and PMA were added together for activation prior to infection with MAP with either MAP strain. Figure 2.2 represents live cell counts of the macrophages at the specific time points at which the amount of intracellular MAP was determined. Intracellular MAP was determined from live cells only. For clarity purposes, the results are shown qualitatively in the figures, and followed by actual numbers (quantitatively) in the tables. Please refer to the tables for specific values at each specific time point, as well as an analysis of the significance.

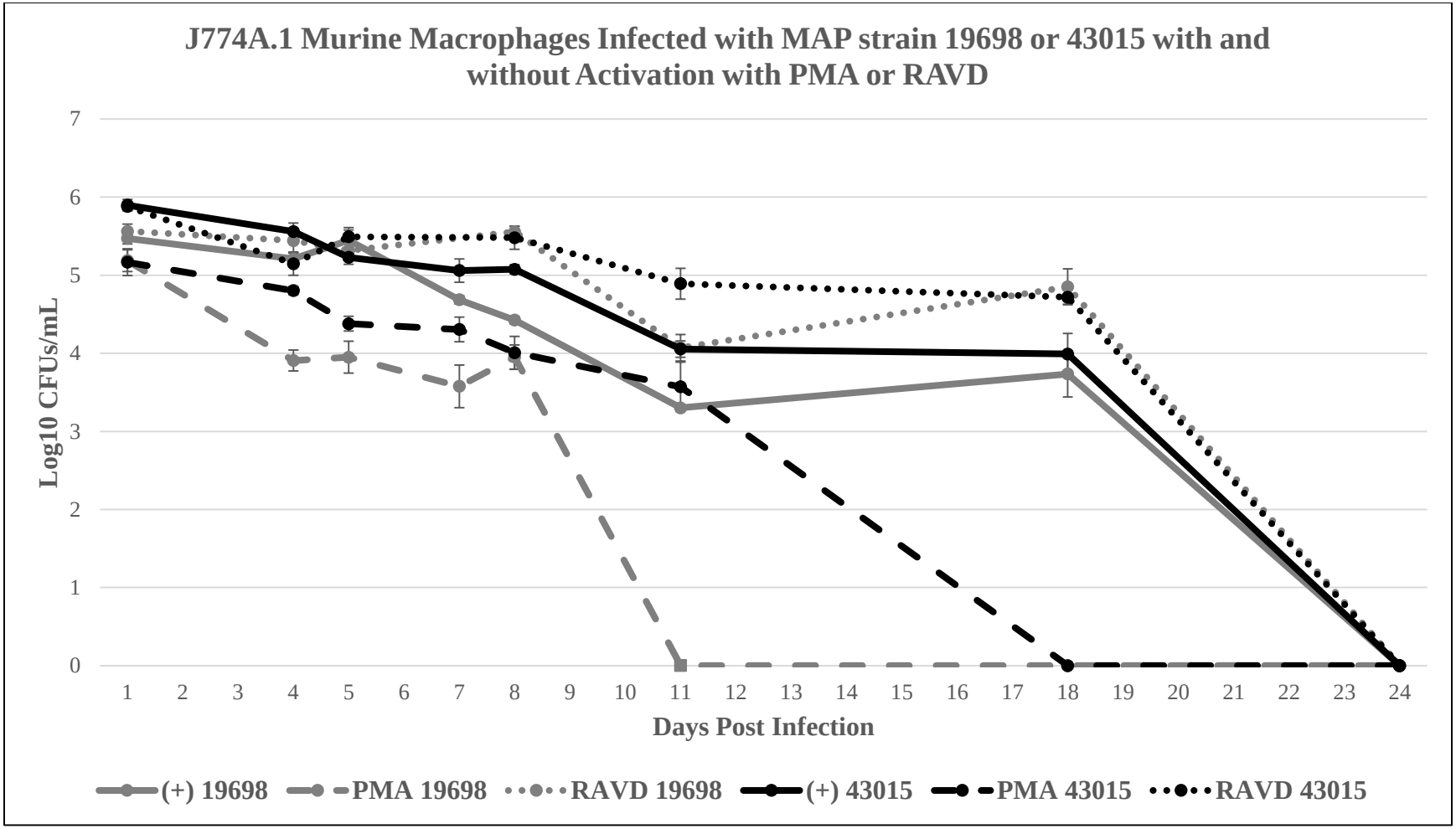


Figure 2.1 Short-term infection of J774A.1 murine MΦs with MAP strain 19698 or 43015, with or without activation with PMA or RAVD. None of the cells survived past 24 days of infection. Values are the mean ± standard error of the mean (SEM) of intracellular MAP at specific time points in at least three separate experiments.

Table 2.1 Short-term growth curve of J774A.1 macrophages with or without activation with RAVD or PMA. Effect of RAVD or PMA on uptake and survival of MAP strains 43015 and 19698 in J774A.1 macrophages. Values are log difference from the control at the specified time points.

MAP 43015					
Days Post Infection	**Control-MΦ Log_{10} CFUs/mL**	**PMA-MΦ Log_{10} CFUs/mL**	**PMA-MΦ CFUs/mL Log Difference**	**RAVD-MΦ Log_{10} CFUs/mL**	**RAVD-MΦ CFUs/mL Log Difference**
1	5.90 ± 0.07	5.17 ± 0.17	0.73* ↓	5.88 ± 0.05	0.02 ↓
4	5.56 ± 0.11	4.80 ± 0.06	0.76* ↓	5.15 ± 0.15	0.41* ↓
5	5.23 ± 0.09	4.38 ± 0.09	0.85* ↓	5.49 ± 0.09	0.26 ↑
7	5.06 ± 0.15	4.31 ± 0.16	0.75* ↓		
8	5.08 ± 0.06	4.01 ± 0.21	1.07* ↓	5.48 ± 0.15	0.40* ↑
11	4.05 ± 0.10	3.57 ± 0.32	0.48 ↓	4.89 ± 0.20	0.84* ↑
18	3.99 ± 0.26	0	3.99* ↓	4.72 ± 0.09	0.73* ↑
24	0	0	0	0	0
MAP 19698					
Days Post Infection	**Control-MΦ Log_{10} CFUs/mL**	**PMA-MΦ Log_{10} CFUs/mL**	**PMA-MΦ CFUs/mL Log Difference**	**RAVD-MΦ Log_{10} CFUs/mL**	**RAVD-MΦ CFUs/mL Log Difference**
1	5.47 ± 0.07	5.19 ± 0.14	0.29 ↓	5.56 ± 0.09	0.09 ↑
4	5.21 ± 0.08	3.91 ± 0.13	1.30* ↓	5.44 ± 0.16	0.23 ↑
5	5.45 ± 0.16	3.95 ± 0.20	1.50* ↓	5.32 ± 0.11	0.13 ↓
7	4.69 ± 0.05	3.58 ± 0.27	1.11* ↓		
8	4.43 ± 0.05	3.95 ± 0.16	0.47* ↓	5.56 ± 0.07	1.13* ↑
11	3.30 ± 0.01	0	3.30* ↓	4.07 ± 0.17	0.77* ↑
18	3.74 ± 0.30	0	3.37* ↓	4.85 ± 0.23	1.11* ↑
24	0	0	0	0	0

↓ Refers to the log value being lower than the control (i.e. non activated macrophages).
↑ Refers to the log value being higher than the control.
*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

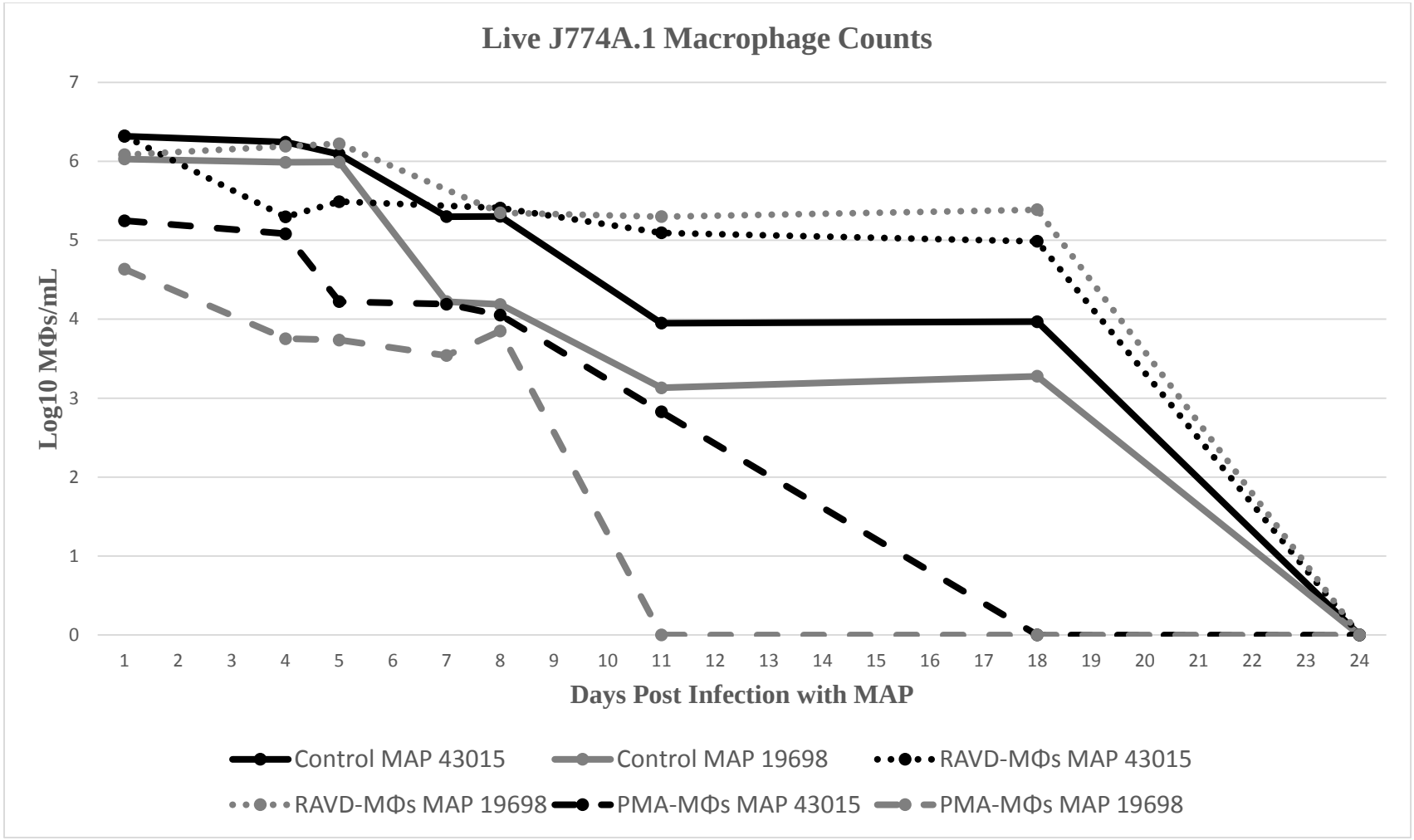


Figure 2.2 Live cell counts of J774A.1 murine MΦs infected with MAP strain 19698 or 43015 with and without activation with PMA or RAVD. Values are the average of triplicate wells at each time point post infection.

2) *Differential effects of RAVD plus PMA on the uptake and survival of MAP strain 19698 in J77A.1 macrophages*

J774A.1 MΦs were activated with RAVD plus PMA for three days prior to infection and evaluated for uptake and control of MAP infections. These results are depicted as a graph in Figure 2.3. This study was conducted with MAP strain 19698 to see if the results were any different from the previous study in which RAVD and PMA were used separately to activate MΦs. Although the initial uptake of MAP seemed less in the RAVD plus PMA activated MΦs than in the control MΦs (no activation), the differences were not significant (Table 2.2). Again, for clarity purposes, the qualitative results are shown in the figures followed by the quantitative results in the tables. The RAVD plus PMA activated MΦs were able to survive up to day 21 post infection, and possibly even longer as seen from live cell counts (Table 2.3). The control-MΦs were unable to survive much past day 10 post infection, and the macrophages appeared to be slowly dying off (Table 2.3). The log difference of MAP-CFUs/mL at each time point is shown in Table 2.2. Prior to sampling at each time point, the MAP infected cells were washed with PBS to remove dead cells. Thus the intracellular MAP determination was only from live cells. Previous experiments were done to ensure that the MAP determined was from intracellular MAP of live cells only. These results intrigued us and made us wonder how the MΦs would be effected if the infection had gone on for longer.

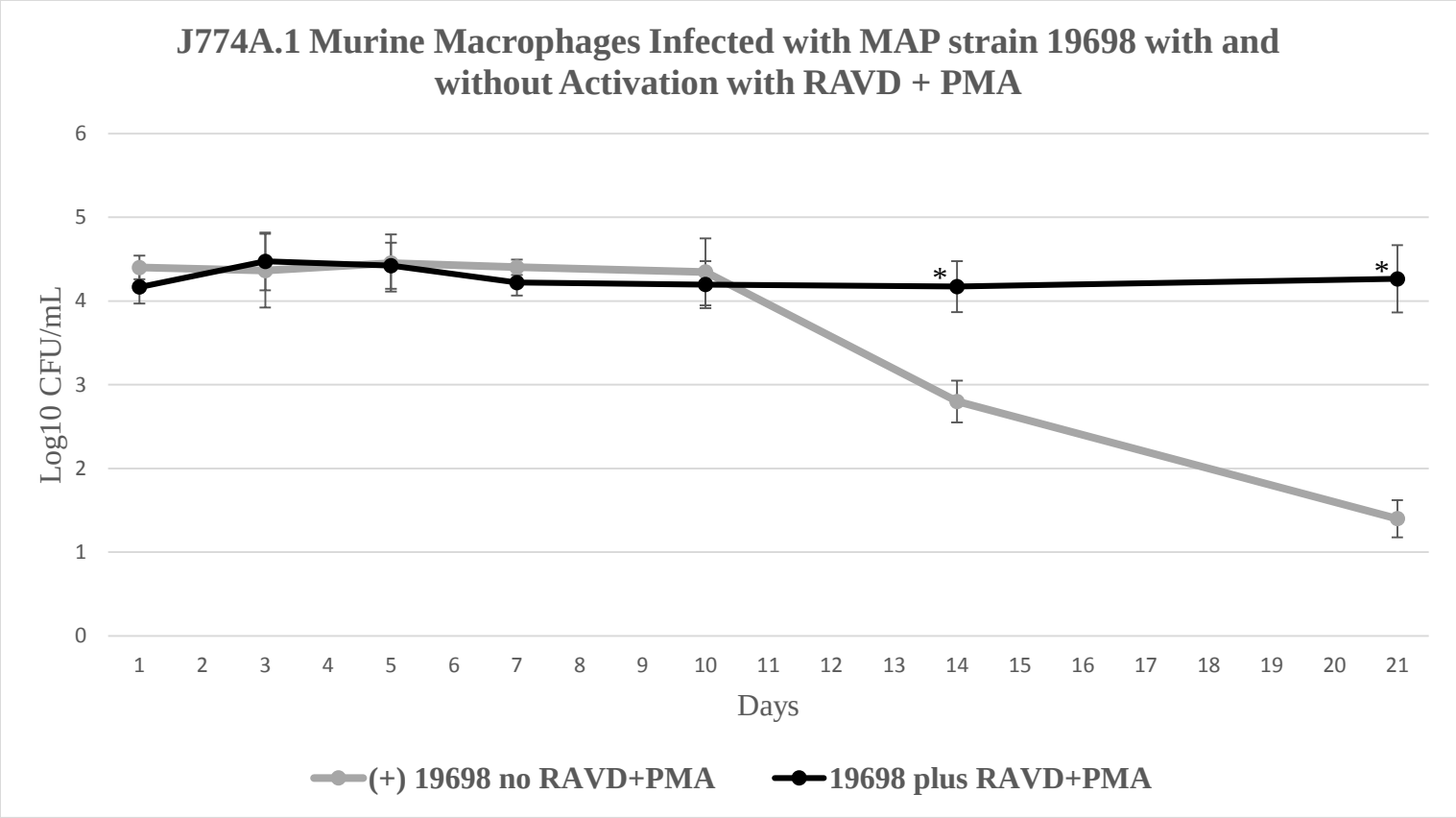


Figure 2.3 Short-term infection of J774A.1 murine MΦs with MAP strain 19698 with or without activation with RAVD plus PMA. Values are the mean ± SEM of intracellular MAP at specific time points in at least three separate experiments.

*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

Table 2.2 Short-term growth curve of MAP strain 19698 in J774A.1 macrophages with and without activation by RAVD plus PMA. Effect of RAVD plus PMA on uptake and survival of MAP strain 19698 in J774A.1 macrophages. Values are the log difference from the control at the specified time points.

Days Post Infection	RAVD&PMA-MΦs CFUs/mL Log Difference
1	0.23 ↓
3	0.11 ↑
5	0.03 ↑
7	0.18 ↑
10	0.15 ↑
14	1.37* ↑
21	2.86* ↑

↓ Refers to the log value being lower than the control.
↑ Refers to the log value being higher than the control.
*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

Table 2.3 Live cell counts of J774A.1 murine macrophages infected with MAP strain 19698 with and without activation with RAVD plus PMA. Values are the average of triplicate wells at each specific time point post infection.

Days Post Infection	Control MΦ cell count (Log MΦs/mL)	RAVD & PMA MΦ cell count (Log MΦs/mL)
1	4.12	3.98
3	4.56	4.87
5	4.34	4.99
7	4.12	4.75
10	4.02	4.78
14	3.01	4.56*
21	1.20	4.67*

*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

Long Term Growth Curve

Infected J774A.1 murine MΦs were evaluated for growth of MAP strains and the results are shown in Figure 2.4. The MAP strains in the RAVD plus PMA activated MΦs were able to survive up to 60 days post infection at which time the experiment was terminated. The control-MΦs were unable to survive much longer past day 10 post infection. According to the live cell counts of the J774A.1 macrophages, there were live activated cells even at day 60 post-infection of MAP strain 43015 whereas there were no live cells past day 38 for non-activated but infected macrophages. There were live activated macrophages at day 63 post-infection of MAP strain 19698 whereas there were no live cells found past day 49 for non-activated infected macrophages. These results are shown in Table 2.5. Log difference of CFUs/mL of MAP are shown in Table 2.4. Microscopic analysis of Kinyoun stained MAP (acid fast bacilli; AFB) in fixed RAVD plus PMA activated J774A.1 MΦs showed differential uptake of MAP (higher amounts of MAP in RAVD plus PMA activated macrophages than in control (non-activated) macrophages) as well as survival of the macrophages with intracellular MAP (Figure 2.5A-C). From these figures, it is noted that there are fewer macrophages in the later stages of infection (i.e. day 30 post-infection) in the non-activated control macrophages when compared to the RAVD plus PMA activated macrophages. The higher magnification (100x) of the cells is a representative of a non-infected macrophage vs. an infected macrophage.

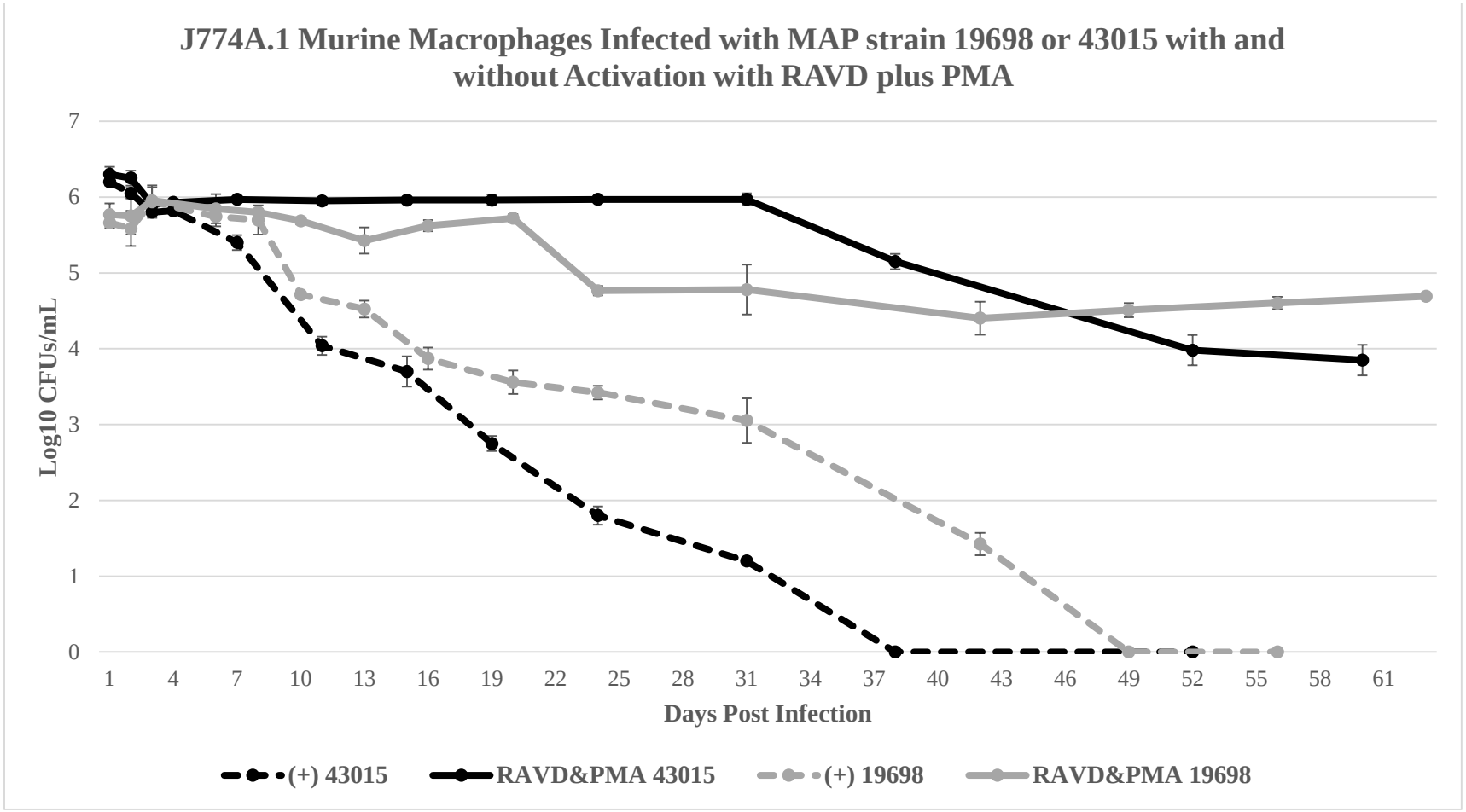


Figure 2.4 Long-term infection of J774A.1 murine MΦs with MAP strain 43015 or 19698, with or without activation with RAVD plus PMA. Cells activated with RAVD plus PMA were able to survive longer than cells that were not activated. Values are the mean ± SEM of intracellular MAP at specific time points in at least three separate experiments.

Table 2.4 Long-term growth curve of J774A.1 macrophages with or without activation with RAVD plus PMA. Effect of RAVD plus PMA on uptake and survival of MAP strains 43015 and 19698 in J774A.1 macrophages. Values are the log difference from the control at the specified time points.

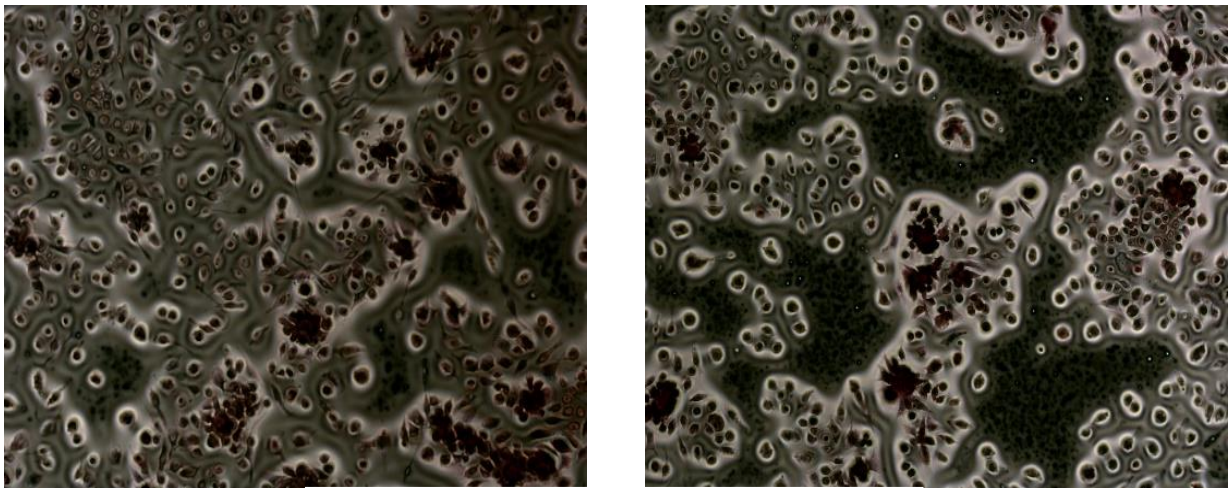
	MAP 43015	MAP 19698
Days Post Infection	**RAVD&PMA-MΦ CFUs/mL Log Difference**	**RAVD&PMA-MΦ CFUs/mL Log Difference**
1	0.1	0.11
2	0.2	0.16
3	0.1	0.01
4	0.11	
6		0.10
7	0.57*	
8		0.10
10		0.97*
11	1.91*	
13		0.90*
15	2.26*	
16		1.04*
19	3.21*	
20		1.16*
24	4.17*	0.34
31	4.77*	1.73*
38	5.15*	
42		2.98*
49		4.51*
52	3.98*	
56		4.60*
60	3.85*	
63		4.69*

All values had a log value, which was higher than the control.
*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

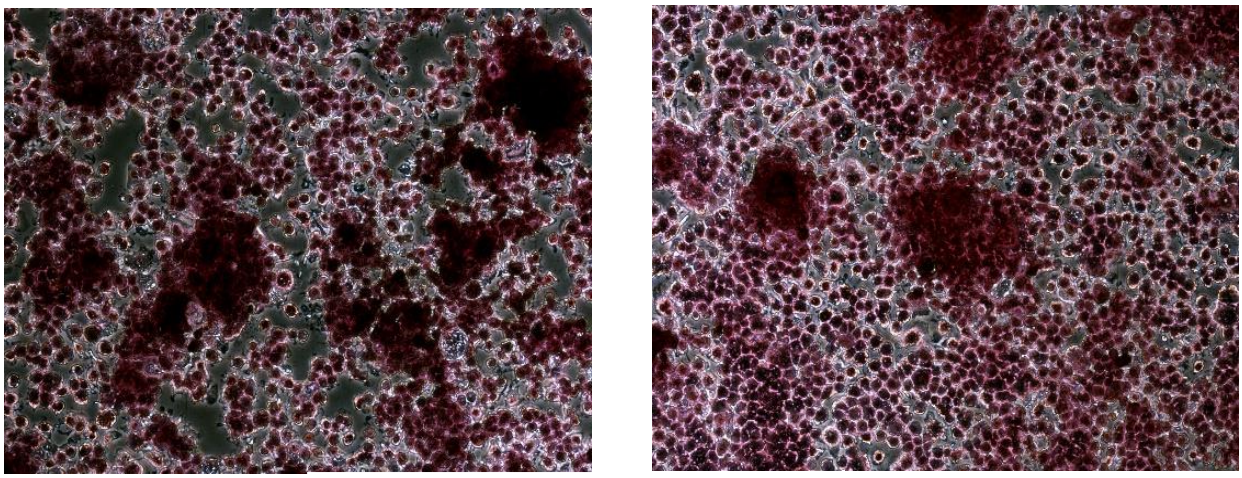
Table 2.5 Live cell counts of J774A.1 murine macrophages infected with MAP strain 43015 or 19698 with and without activation with RAVD plus PMA. Values are the average of triplicate wells at each specific time point post infection.

	MAP 43015		MAP 19698	
Days Post Infection	**Control MΦ cell count (Log MΦs/mL)**	**RAVD & PMA MΦ cell count (Log MΦs/mL)**	**Control MΦ cell count (Log MΦs/mL)**	**RAVD & PMA MΦ cell count (Log MΦs/mL)**
1	5.78	5.83	5.25	5.34
2	5.53	5.65	5.13	5.18
3	5.55	5.71	5.54	5.45
4	5.21	5.67		
6			5.42	5.54
7	5.02	5.75*		
8			5.01	5.66
10			4.97	5.78*
11	4.12	5.88*		
13			4.25	5.33*
15	3.25	5.77*		
16			4.03	5.45*
19	2.50	5.35*		
20			3.89	5.68*
24	1.11	5.45*	2.99	4.89*
31	0.98	5.28*	2.87	4.77*
38	0	4.97*		
42			1.24	4.65*
49			0	4.87*
52	0	3.95*		
56			0	4.90*
60	0	3.88*		
63			0	4.88*

*Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

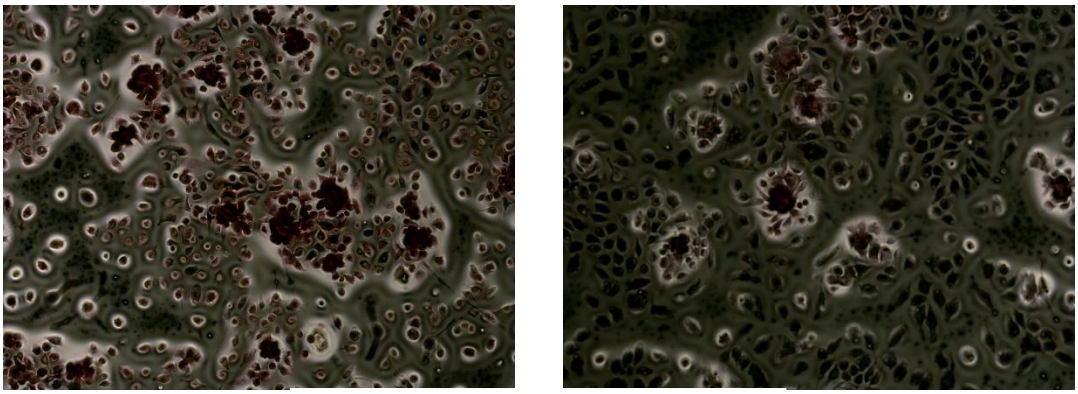


Day 1 Post Infection with MAP

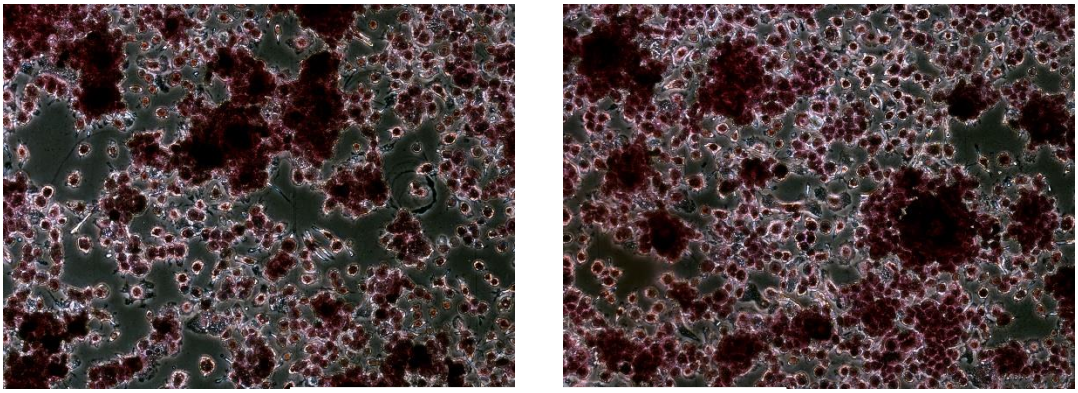


Day 10 Post Infection with MAP

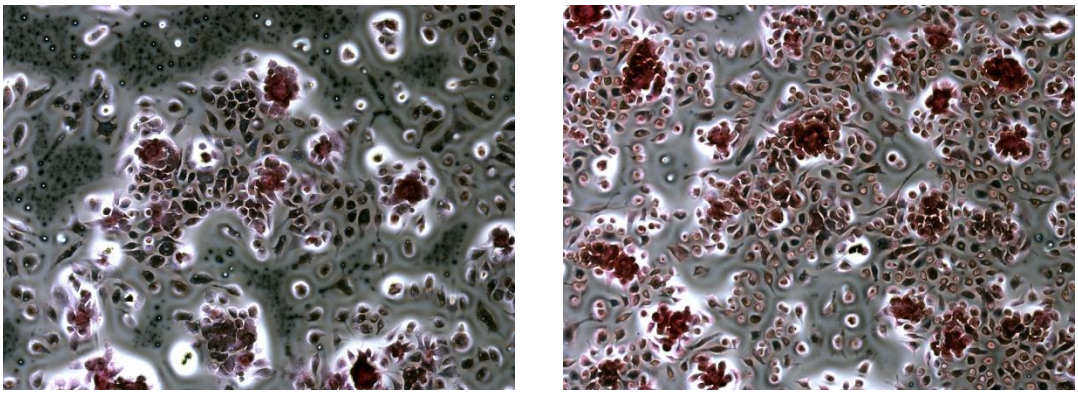
Figure 2.5A Representative microscopic view (40x magnification) of Kinyoun stained MAP strain 19698 (acid fast bacilli; AFB) in fixed RAVD plus PMA activated J774A.1 MΦs. MΦs showed differential uptake and survival of MAP in J774A.1 MΦs at representative times (i.e. 1 through 10 days post-infection). MAP strain 19698 in MΦs not activated (left) and activated (right) with RAVD plus PMA. Pink is acid-fast MAP.



Day 1 Post Infection with MAP



Day 10 Post Infection with MAP



Day 30 Post Infection with MAP

Figure 2.5B Representative microscopic view (40x magnification) of Kinyoun stained MAP strain 43015 (acid fast bacilli; AFB) in fixed RAVD plus PMA activated J774A.1 MΦs. MΦs showed differential uptake and survival of MAP in J774A.1 MΦs at representative times (i.e. 1 through 30 days post-infection). MAP strain 43015 in MΦs not activated (left) and activated (right) with RAVD plus PMA. Pink is acid-fast MAP.

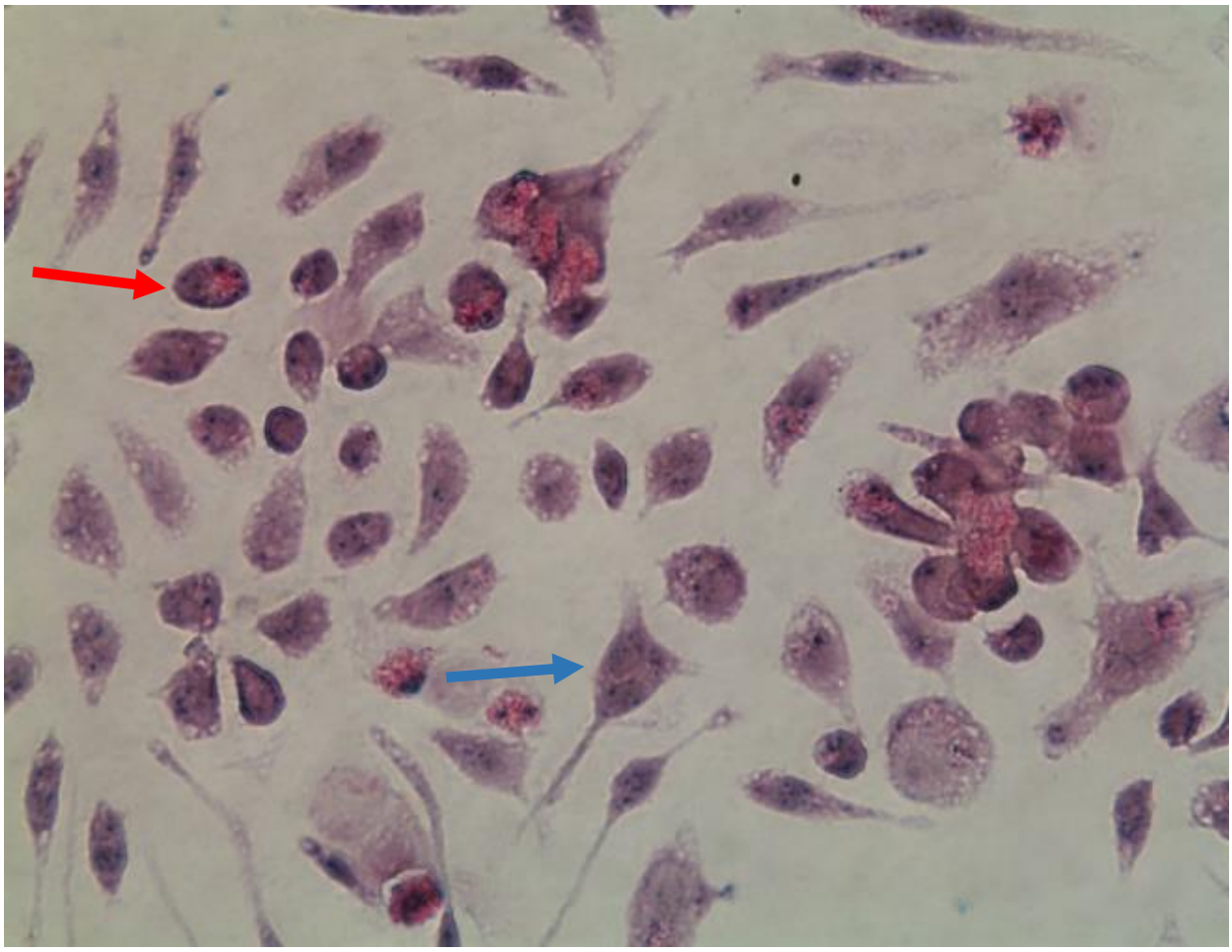


Figure 2.5C Higher magnification (100x) of what MAP looks like in the MΦs. (Blue arrow represents an uninfected macrophage; Red arrow represents a MAP infected macrophage; pink inside the macrophage is MAP)

Discussion:

Our initial studies indicated that neither RAVD nor PMA alone led to long living J774A.1 macrophages, however their combination was very effective. The short term growth curve with RAVD-activated MΦs did not show a big difference in the uptake of MAP, however the PMA-activated MΦs indicated less engulfment contrary to what has been reported in the literature [19]. However, without the activation of both RAVD and PMA, the infected macrophages were unable to survive longer than the control macrophages (no activation). From our data, it is clear that infected macrophages survive longer than uninfected non-activated macrophages. Intracellular pathogens including *Brucella* have been shown to extend the life of infected macrophages by preventing apoptosis, and this can also be seen in our experiments when comparing normal macrophages vs. infected but not RAVD plus PMA activated macrophages.

Interestingly, J774 A.1 macrophages with the activation by RAVD plus PMA, were able to not only survive short term infections, but were also able to survive long term infections as well. In our experiments, the amount of intracellular MAP was assessed as a means to prove that the macrophages were still viable at that specific time points. The cell culture medium of the macrophages was changed regularly, thus discarding the dead cells that lift off from the cell culture plate; this also removes any floating extracellular MAP as well. Prior to lysing the cells to determine intracellular MAP CFUs at a specific time point, the cells were washed three times with PBS. This again ensures that all dead cells were discarded, along with extracellular MAP. Thus, the evaluation of intracellular MAP was from viable adherent macrophages only.

Retinoic acid and vitamin D associated with nuclear receptor activation are basic essential components of a healthy culture medium [20-23], therefore it makes sense why they would help

keep the macrophages alive longer; however unlike RAVD, PMA T cell activators are not endogenously produced in most hosts *in vivo* [24]. Therefore it was very interesting to see the effects RAVD plus PMA in combination had on J774A.1 macrophages, as the additives have never been tested together to help longevity of any cells previously.

When J774A.1 macrophages die, they lift off of the surface of the growth chamber to which they were adherent. Therefore, by washing the macrophages prior to lysing, we were eliminating dead cells, and extracellular MAP therefore were only taking into account the intracellular MAP in the live macrophages. We previously confirmed this by serial dilution plating of the culture medium removed from the wells prior to lysis and found some extracellular MAP in the cell culture supernatant. From the same supernatant, we also diluted and plated medium that had 0.1% Triton X-100™ added to lyse the dead cells, to determine intracellular MAP within dead cells, and found slightly higher amounts of MAP compared to not lysed samples. Thus intracellular MAP helped us gauge how many macrophages were still alive over the course of the short term and long term infections. The qualitative analysis through Kinyoun staining of the acid-fast bacilli [25] inside the macrophages supported our assumption that there were live macrophages at the specified time points. Kinyoun stain was used as opposed to the typical Ziehl-Neelson stain, as the Kinyoun method does not require heating, thus macrophages intact preventing possibility of lysing the MΦs.

In an attempt to verify the results reported by Estrella *et al.* [19], experiments were done to test the longevity of THP-1 human monocyte cells (data not shown). Instead of verifying their results, we found that MAP infected THP-1 cells were able to survive with or without the activation of RAVD plus PMA, contradicting their findings. Previous observations were with THP-1 cells infected with *M. tuberculosis* compared to our findings with THP-1 cells infected with MAP. This suggested that THP-1 cells may be supportive of intracellular MAP regardless of activating agents.

This observation needs to be further tested in order to confirm the efficacy of THP-1 cells need or lack of need for activation before their use as an infection model for MAP.

From our experiments, a novel murine macrophage cell culture model was refined to study the long-term persistence of MAP. The availability of a cell culture model that reflects the natural niche of MAP *in vitro* is important for many reasons. This model can make it possible to study the dynamics of MAP persistence over time, as well as help devise methods to treat persistent MAP infected macrophages. This long-term cell culture model opens many doors to the therapeutic world of MAP infections, allowing researchers to test novel therapeutics before embarking on animal testing.

Citations:

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Chapter 3

Use of a Long Term Cell Culture Model to Determine Minimal Bactericidal Concentrations of Anti-Infectives against Intracellular *Mycobacterium avium* subsp. *paratuberculosis*

Abstract:

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of granulomatous enteritis in ruminants, better known as paratuberculosis or Johne's disease (JD). JD is prevalent in domestic ruminants worldwide, and creates an impact on the economy as many therapuetics are not cost effective and are unable to completely cure the infection. With the addition of retinoic acid and vitamin D (RAVD) and phorbol myristate acetate (PMA) in combination, a long term *in vitro* MAP infected J774A.1 murine macrophage cell culture model has been established in our laboratory.

The efficacy of clarithromycin, azithromycin, isoniazid, amikacin, ethambutol, ciprofloxacin, levofloxacin, rifampicin, clofazimine and a combination of clarithromycin, rifampicin, and clofazimine were tested using the above MAP infected macrophage cell culture model. The conventional drugs listed above were evaluated at the determined Minimal Inhibitory Concentrations (MICs) *in vitro,* along with one concentration above and one concentration below their MIC. The drugs' differential ability to kill intracellular MAP (strains 19698 and 43015) in the early stage of infection versus chronic stage of infection were determined. Some drugs were more effective at early stages of MAP infection (isoniazid), whereas others were more effective in chronic or late stage of infection (clarithromycin, rifampicin). Although a drug may be effective at a certain stage of infection, this does not necessarily mean it will be effective against both strains of MAP. Levofloxacin for example, was effective at the early and chronic stages of infection, but

only against MAP 43015. The most promising bactericidal results were seen with the combination of clarithromycin, clofazimine, and rifampicin; this combination was effective at all stages of infection with both strains of MAP. This cell culture model has the potential for the assessment of novel therapeutics at different stages of infection on many, if not all intracellular pathogens.

Introduction:

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow growing bacterium that is the causative agent of Johne's disease (JD) in ruminants and has long been suggested to be associated with complications of Crohn's disease (CD) in humans [1-3]. Although there is no direct evidence that MAP is the primary etiological agent for CD, most CD patients are found to have MAP in their intestinal tissues [2-11]. The current control measures for JD have only been minimally successful, while there are only medications to treat the symptoms of mycobacterial infections associated with CD [1, 18, 27].

According to the U.S. National Johne's Education Initiative (Colorado Springs, CO; johnesdisease.org), one out of 10 animals moving through livestock auction facilities are infected with Johne's disease. In 2007, the United States Department of Agriculture (USDA) found that 68% of U.S. dairy operations were infected with MAP [28]. The Johne's incidence in the USA has been growing; there is no effective treatment, and the current control measures have not been very successful.

There are about 10 different strains of MAP [32], however the strains that are the focus of this research are strains 19698 and 43015. MAP strain 19698 is an isolate from the feces of cow

with naturally acquired Johne's disease, while strain 43015, also known as strain Linda, is an isolate from the ileum of a 15-year-old girl from Rhode Island with Crohn's disease.

Mycobacteria are known to be hardy, slow growing bacteria, thus treatment times for mycobacterial infections is usually extremely long [3]. Many species of mycobacteria become inactive (dormant) for long periods of time and do not actively metabolize. Because most antibiotics are only effective against bacteria that are metabolically active, they are unable to completely eradicate the inactive bacteria [33]. Therefore it is necessary to treat mycobacterial infections for extended periods of time so that the antibiotics can act against bacteria that are latent as well as actively replicating [12].

A comprehensive evaluation of the effects of antimicrobial drugs *in vitro* on both animal and human-origin MAP was published in 2009 by Krishnan *et al*. [20]. Their study found that macrolide drugs (azithromycin and clarithromycin) have the highest *in vitro* efficacy against MAP. Other broad spectrum drugs (amikacin, ciprofloxacin, levofloxacin, and rifampicin) showed intermediate efficacy in some strains of MAP. Most first-line anti-tuberculosis (ethambutol and isoniazid) or anti-leprosy (dapsone and clofazimine) drugs were found to have no effect against MAP *in vitro*. However rifampicin and rifabutin showed some efficacy [20]. With this data, some rational guidance for choosing antibiotics effective in treating MAP infections has been provided.

There is no effective established laboratory animal model for testing therapeutics against MAP infections [13, 23, 24]. When mice are infected with MAP, they develop systemic infection that does not mimic the disease in ruminants. Without a cost effective animal model it is difficult to develop effective treatments for MAP infections and test novel therapeutics in a laboratory setting. Estrella *et al.* showed that vitamin A and D (RA and VD respectively) increased the uptake of *M. tuberculosis* by THP-1 human moncyte cell culture *in vitro* as well as extended the lifespan

of these infected cells [25]. Phorbol myristate acetate (PMA) was previously found as a chemical activator to induce adherence of THP-1 cells making them more macrophage-like [26, 28]. Previously, we have found that MAP infected J774A.1 macrophages typically have a very short lifespan of about 10 days. With a combination of RAVD plus PMA, we have been able to establish a J774A.1 macrophage cell culture model that is able to survive MAP infections for 45-60 days. In this study, we evaluate the susceptibility of J774A.1 macrophages infected with MAP strains 43015 and 19698 to free drugs vs. a combination of three drugs (clarithromycin, clofazimine, and rifampicin).

Materials and Methods:

Bacterial strains and cell lines:

For details about bacterial strains and cell lines used in the following experiments, please see Materials and Methods of Chapter 2.

Drugs:

The following drugs were tested: azithromycin and ciprofloxacin (Fluka; St Louis, MO, USA), amikacin, clarithromycin, clofazimine, ethambutol, levofloxacin, and rifampicin (Sigma-Aldrich, St Louis, MO, USA), and isoniazid (Aldrich Chemical Company, Milwaukee, WI, USA). Rifampicin was considered an alternative to rifabutin, as they are similar drugs and have been found to have comparable rates of cure and relapse [27].

Stock solutions of drugs (10 mg/mL) were prepared using the following solvent: Water for amikacin, ethambutol and isoniazid; 0.1 N sodium hydroxide for ciprofloxacin and levofloxacin; methanol for clofazimine, rifampicin and rifabutin; ethanol for azithromycin and clarithromycin. As microbial contamination of commercially manufactured drugs is extremely rare and the relatively high concentration of drugs in stock solutions, they were prepared aseptically but not filter sterilized. Stock solutions were aliquoted into small vials of 1 mL and were stored at -80°C until needed.

Macrophage cell viability and cytotoxicity assay:

To determine any possibility of cytotoxicity caused by the drugs and whether or not the drugs had any undesirable effect on macrophages, a tetrazolium compound-based cell viability assay was used [Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation MTS Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-caboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega Inc., Fitchburg, WI)]. Approximately 2 x 10^5 J774A.1 cells/well were resuspended in DMEM with 10% fetal bovine serum (FBS) (Corning Inc., Corning, NY) and 1% penicillin/streptomycin (P/S) (Corning Inc.) antibiotic, and seeded into a 96 well tissue culture plate (Mfr. No. 3585, Corning Inc.). After 24 hr of incubation, medium was removed and cells were washed twice with 100 µL of phosphate buffered saline (PBS) (Corning Inc.). The drugs were diluted in DMEM media containing 10% FBS at various concentrations and then added to the specified wells. Following 24 hr incubation the cells were washed twice with 100 µL of PBS to remove extracellular drugs. The number of viable cells was measured based on the amount of absorbance at 490 nm due to formazan product. Live and dead cells were determined by trypan blue exclusion assay and the number of viable and dead cells counted using a gridded haemocytometer.

Activation of J774A.1 macrophages (MΦs):

Murine J774A.1 MΦs were maintained at 37°C and 5% CO_2 in DMEM with 10% fetal bovine serum. As required, cells were expanded into individual 75 cm^2 flasks and activated with RA (1μM), VD (1μM), and PMA (10ng/mL=16nM) for 3 days prior to infection with MAP. These concentrations of RA, VD (cholecalciferol) and PMA were used as suggested as optimal doses of RAVD and PMA based on receptor expression in RAVD-THPs [24-26, 28] and also shown to be effective in our previous experiments (Chapter 2).

Preparation of bacterial cultures and Minimal Inhibitory Concentration (MIC) testing of drugs:

To test the efficacy of the different drugs, MAP was grown in pure culture following standard protocol [29-31]. A frozen stock culture of MAP was diluted in #1507 Middlebrook broth to 1.0 x 10^8 colony forming units/mL (CFUs/mL) and incubated with various concentrations of each drug in triplicates using 96-well plates at 37°C. The plates were incubated at 37°C for 3-4 weeks and visually inspected for growth. CFUs were determined by actual colony counts and corrected for dilution factor to obtain CFUs/mL to determine minimal bactericidal concentrations (MBC) of each drug.

Susceptibility testing by agar dilution method as per National Clinical Laboratory Standard [31]*:*

Drug dilutions were added to 10 mL of molten #1507 Middlebrook agar. Quadrant plates (Cat. No. 1-3163-100, Corning Inc.) were swirled gently to allow mixing of the drug with the medium before solidification of agar. The final concentrations in each quadrant were 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL. A MAP inoculum suspension equivalent in turbidity to that of No. 1 McFarland

standard was diluted to 10^{-2} and 10^{-4}. Drug-containing and control quadrants were inoculated with 0.1 mL of the 10^{-2} dilution while the 1:100 control (1% control) quadrants received 0.1 mL of the 10^{-4} dilution. Plates were sealed with Parafilm® and incubated at 37°C for 4-5 weeks. Drug susceptibility was determined by monitoring bacterial growth. If the MAP strain was susceptible to the drug concentration in a specific quadrant, there was no growth seen [30].

Minimal Bactericidal Concentration (MBC) Tests in Long Term Infected Macrophages:

MBC determinations in infected macrophages were performed using a modification of National Committee for Clinical Laboratory Standards Broth Microdilution Technique [30, 31]. MAP suspensions were subject to vortexing with 10 grams of 2 mm glass beads, matched to the McFarland standard #1 and centrifuged at 1,000 x g for 2 minutes. The supernatant containing single colony forming units (CFUs) of MAP (i.e. without clumps) was used for infection. Murine macrophage cells, J774A.1 (ATCC), were grown to a 90% confluent monolayer in $75cm^2$ flasks and were activated in the flasks with RAVD plus PMA for 3 days prior to infection (see previous method in Chapter 2 for activation of cells). The cells were harvested by scraping and then seeded at a density of ca. 2 x 10^6 cells/well in 24-well plates (Corning Inc.). The cells were grown in DMEM with 10% FBS and infected at multiplicity of infection: 10 (MOI = 1:10) with MAP. At 24 hr post infection, the cells were washed two times with 1 mL of DMEM with 10% FBS to remove any non-phagocytized MAP. Then 1 mL of DMEM with 10% FBS with drugs at various concentrations were added to the infected cells and incubated for a further 48 hr. Infected macrophages and treated infected macrophages were washed three times with 1 mL of PBS and lysed with 200 µL 0.1% Triton X-100™ (Sigma-Aldrich) (i.e. at day 1 post-infection, cells were

treated for a further 48 hr and lysed on day 3; at day 30 post-infection, cells were treated for 48 hr and lysed on day 32; and on day 45 post-infection, cells were treated for 48 hr and lysed on day 47). CFUs/mL of MAP in the lysates were determined by plating 10-fold serial dilutions onto #1507 Middlebrook agar plates and incubating the plates at 37°C for 3-4 weeks. The infected cells were treated and lysed in the early, mid, and late stages of infection (days 1, 30 and 45 post infection). Prior to lysing, the infected cells were treated for 48 hours with drugs at different concentrations to see at which concentration that stage of infected cells was susceptible. Cell culture medium was changed every 3-4 days to prevent acidification of DMEM as well as provide fresh medium for the cells to survive.

Comparison of a combination of clarithromycin, rifampicin and clofazimine:

MBCs were determined as described above. However, the efficacy of the individual drugs was tested in comparison to a combination of drugs at the following concentrations. The concentrations of the combination of drugs was chosen based off of the results of the MBC results of the individual drugs. The lowest concentration was one concentration higher than the MIC value, and the middle concentration was 2 concentrations higher than the MIC value, and the highest concentration was 3 concentrations higher than the MIC value. Therefore clarithromycin at 1, 2, and 4 µg/mL, clofazimine at 8, 16, and 32 µg/mL, and rifampicin at 4, 8 and 16 µg/mL were tested. A combination of the drugs at their lowest, middle, and highest concentrations were tested. The lowest combination of drugs included 1 µg/mL of clarithromycin, 8 µg/mL of clofazimine, and 4 µg/mL of rifampicin. The middle combination of drugs was 2, 16, and 8 µg/mL of clarithromycin, clofazimine, and rifampicin respectively. The highest combination of drugs was 4, 32, and 16 µg/mL of clarithromycin, clofazimine, and rifampicin respectively.

Results:

Macrophage culture – cytotoxicity assay:

The potential cytotoxicity of the drugs on J774A.1 macrophages was determined using a MTS assay. Figure 3.1 shows the results of the MTS assay and shows that all the drug concentrations tested from 1-32 µg/mL were found to be non-toxic. The drugs did not have an adverse effect on the cell viability in the range of concentrations studied. Cells treated with the drugs at various concentrations showed viability percentages as higher than 80% as compared to the control and were considered not toxic (according to the manufacturer's protocol and referenced publications). The macrophages maintained normal morphology at all the drug concentrations tested (data not shown).

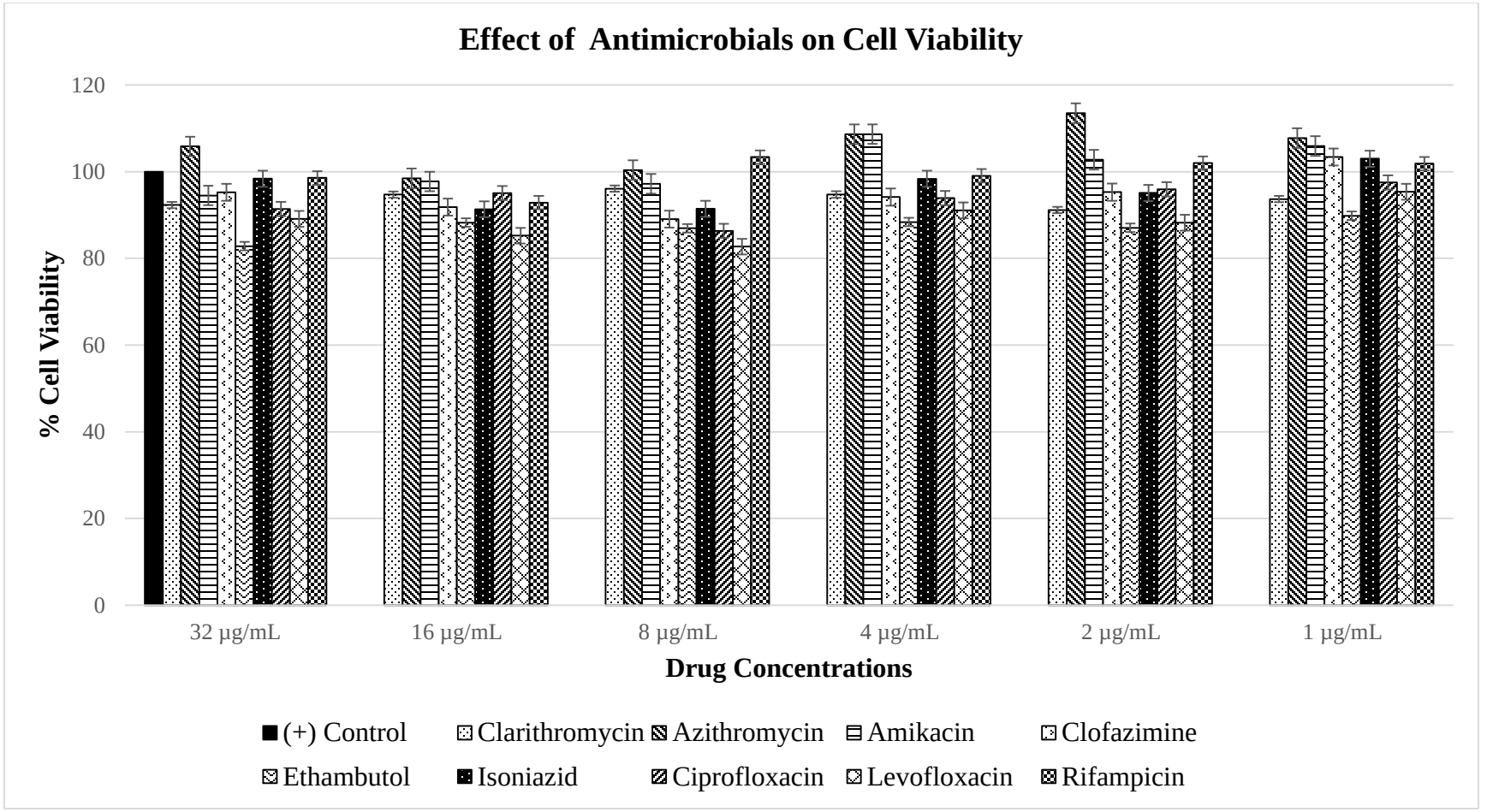

Figure 3.1 Antimicrobials *In vitro* Toxicity Testing.
J774A.1 macrophages were incubated with various antimicrobials in triplicate at 1-32 µg/mL, or with medium alone. After 24 hr of incubation, the viability of cells was measured using an MTS assay. The cell viability in the treated cells were calculated relative to the cell viability of the control group. The data represents the mean ± SEM of two independent experiments.

Minimal Inhibitory Concentration assays:

Table 3.1 summarizes the results of the MIC assays conducted at concentrations between 0.25 – 32 µg/mL of various drugs tested. MAP strains 43015 and 19698 were susceptible to clarithromycin at all concentrations tested (no growth seen at any concentration), whereas both strains were resistant to isoniazid at all concentrations. Both strains of MAP were susceptible to rifampicin at 1.95 µg/mL; clofazimine, azithromycin, ciprofloxacin, and levofloxacin at 3.9 µg/mL; and amikacin and ethambutol at 7.8 µg/mL.

Table 3.1 Minimal Inhibitory Concentration (MIC) assay results. An MIC assay was conducted at drug concentrations 0.25 – 32 µg/mL. A frozen stock culture of MAP was diluted in #1507 broth to 1.0 x 10^8 CFUs/mL and incubated with various concentrations of each drug in triplicates. The plates were incubated at 37°C for 3-4 weeks and visually inspected for growth. If MAP strains were susceptible to the drug concentration in a specific quadrant, there was no growth observed

Drug	MAP strain 19698	MAP strain 43015
Clarithromycin	Susceptible to all [ ]s	Susceptible to all [ ]s
Azithromycin	3.9 µg/mL	3.9 µg/mL
Amikacin	7.8 µg/mL	7.8 µg/mL
Ethambutol	7.8 µg/mL	7.8 µg/mL
Isoniazid	Resistant to all [ ]s	Resistant to all [ ] s
Levofloxacin	3.9 µg/mL	3.9 µg/mL
Ciprofloxacin	3.9 µg/mL	3.9 µg/mL
Clofazimine	3.9 µg/mL	3.9 µg/mL
Rifampicin	1.95 µg/mL	1.95 µg/mL

Susceptibility to drugs determined by agar dilution method:

The results from the agar dilution method were similar to the results seen in the MIC assays. However in this agar method, drug dilutions were added to 10 mL #1507 Middlebrook agar rather than added to #1507 Middlebrook broth. Table 3.2 summarizes the findings. Similar to results previously seen in the MIC determination, MAP strains 43015 and 19698 were susceptible to clarithromycin at all concentrations tested (no growth seen at any concentrations), whereas both strains were resistant to isoniazid at all concentrations. Both strains of MAP were susceptible to rifampicin at 2 mg/L; clofazimine, azithromycin, ciprofloxacin, and levofloxacin at 4 mg/L; and amikacin and ethambutol at 8 mg/L.

Table 3.2 Susceptibility testing by agar dilution method. The final concentrations in each quadrant were 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/mL. MAP was inoculated on each quadrant and plates were incubated at 37°C for 4-5 weeks. If MAP was susceptible to the drug concentration in a specific quadrant, there was no growth seen. Results are representative of triplicate quadrants on different agar plates.

Drug	MAP strain 19698	MAP strain 43015
Clarithromycin	Susceptible at all [ ]s	Susceptible at all [ ]s
Azithromycin	4 µg/mL and above	4 µg/mL and above
Amikacin	8 µg/mL and above	8 µg/mL and above
Ethambutol	8 µg/mL and above	8 µg/mL and above
Isoniazid	Resistant to all [ ]s	Resistant to all [ ]s
Ciprofloxacin	4 µg/mL and above	4 µg/mL and above
Levofloxacin	4 µg/mL and above	4 µg/mL and above
Clofazimine	4 µg/mL and above	4 µg/mL and above
Rifampicin	2 µg/mL and above	2 µg/mL and above

Minimal Bactericidal Concentration tests in long term MAP infected RAVD plus PMA activated J774A.1 macrophages:

The results of the MBC tests can be seen in Figures 3.2 – 3.10 and the results are summarized in Tables 3.3 – 3.11. Each Figure between 3.2 – 3.10 is sub labeled 'A' or 'B'. Those with sub-label 'A' are *in vitro* efficacy results of the specific drug against MAP strain 43015, while those with sub-label 'B' are *in vitro* efficacy results of the specific drug against MAP strain 19698. J774A.1 macrophages (with or without RAVD plus PMA activation) infected with MAP 19698 (MOI of 10) were treated with drugs at three different concentrations. These results are shown in the figures and explained further in the tables that follow. This is to help clarify the details of each specific drug concentration against the specific MAP strain at the specific times point post-infection. The three different drug concentrations chosen were based on the MIC value (previously found through the MIC assays and the MBC by agar dilution method), as well as using one concentration above and one concentration below the MIC. For example, intracellular MAP susceptibility to azithromycin was tested at concentrations 2, 4 (MIC value), and 8 μg/mL. The data represents the mean ± standard error of $\log_{10}$ MAP CFUs/mL of the treatments tested in triplicate in three different trials. Tables 3.3 – 3.12 summarize the susceptibility of long term RAVD plus PMA activated J774A.1 macrophage infected with MAP strain 43015 and 19698 and treatment with the different drugs over time compared to the control (no treatment).

Each drug was found to have a different MBC value. Clarithromycin was effective against intracellular MAP infection with strain 43015 at the early and latent stages of infection, but was found effective against intracellular MAP infection with strain 19698 at chronic and latent stages of infection. Azithromycin was effective against both strains of MAP in the early and latent stages of infection, but only effective against MAP 43015 at the chronic stage of infection. Amikacin

was effective against both strains of MAP at the chronic stage of infection, but only effective against MAP 43015 in the early stage and MAP 19698 in the latent stage of infection. Ethambutol was effective against both strains of MAP at the early stage of infection, but only effective against MAP strain 19698 at the chronic stage, and was not found to be effective against either strain in the latent stage of infection. Although MAP was found to be resistant to isoniazid at all concentrations tested *in vitro*, isoniazid was effective against both strains of MAP in the early stage of infection, however it did not show any effectiveness at the chronic or latent stages of infection. Ciprofloxacin was effective against both strains of MAP in the early and latent stages of infection, but only effective against MAP strain 19698 at the chronic stage of infection. Levofloxacin on the other hand, was only effective against MAP strain 43015, and only in the early and chronic stages of infection. Clofazimine was effective against both strains in the latent stage of infection, but only effective against MAP strain 19698 at the chronic stage of infection. Rifampicin was effective against both MAP strain 19698 and 43015 in the chronic stage of infection, but only effective against MAP strain 43015 in the early stage and MAP strain 19698 in the latent stage of infection.

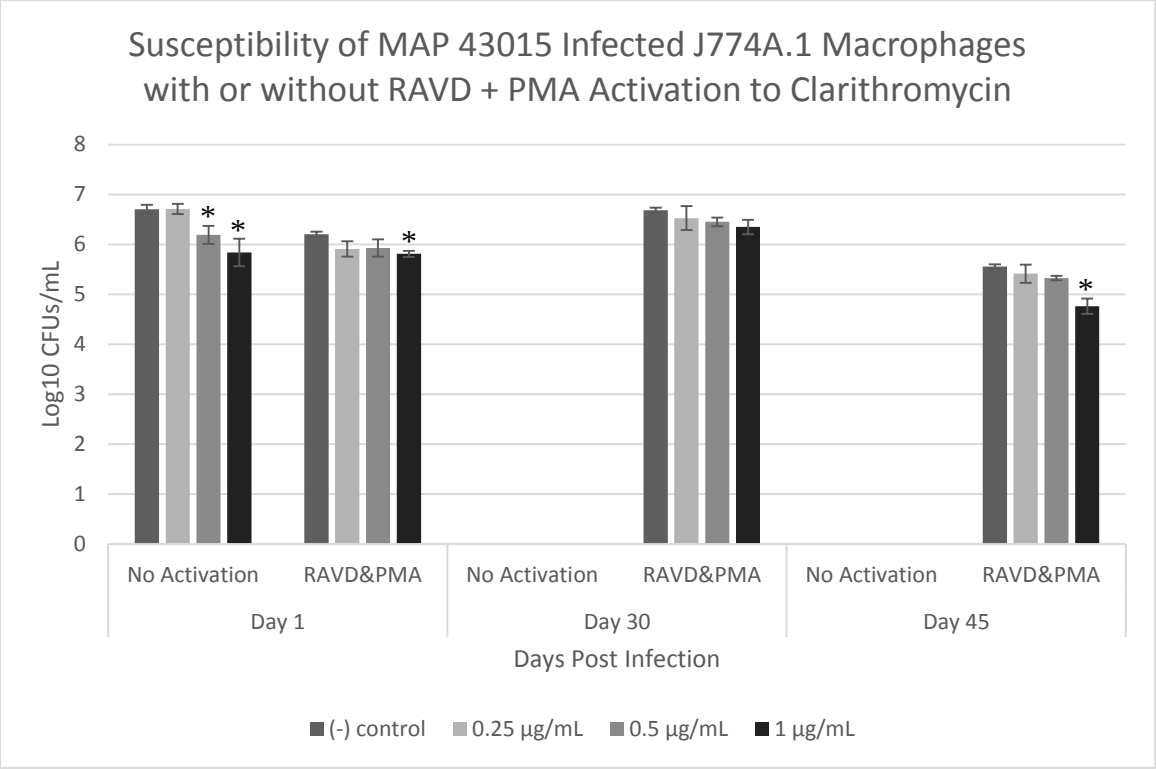

Figure 3.2A *In vitro* efficacy of clarithromycin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with clarithromycin at 0.25, 0.5 or 1 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

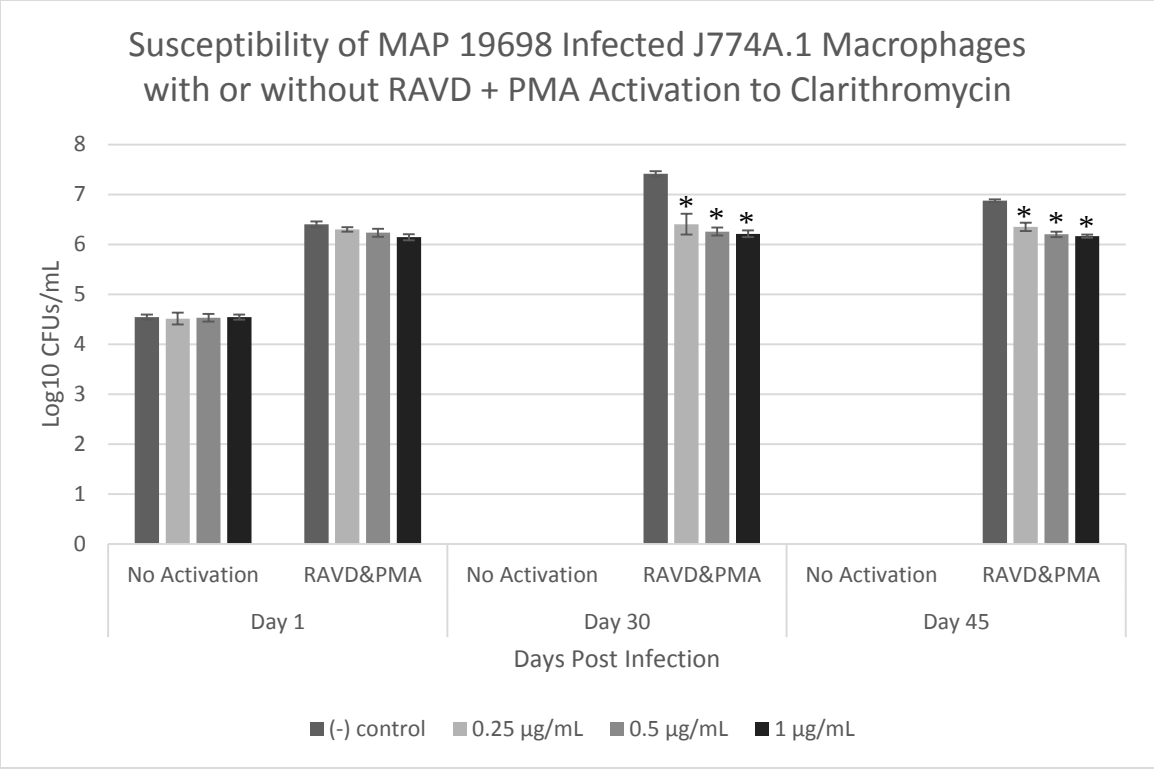

Figure 3.2B *In vitro* efficacy of clarithromycin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with clarithromycin at 0.25, 0.5 or 1 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.3 Clarithromycin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	6.20 ± 0.05		6.68 ± 0.05		5.55 ±0.05	
0.25	5.91 ± 0.15	0.29	6.53 ± 0.24	0.15	5.41 ± 0.18	0.14
0.5	5.93 ± 0.17	0.27	6.45 ± 0.09	0.23	5.33 ± 0.05	0.22
1	5.81 ± 0.06	0.39*	6.35 ± 0.14	0.33	4.76 ± 0.15	0.79*
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.40 ±0.06		7.41 ± 0.05		6.87 ± 0.03	
0.25	6.30 ± 0.04	0.10	6.40 ± 0.21	1.01*	6.35 ± 0.08	0.52*
0.5	6.23 ± 0.08	0.17	6.26 ± 0.08	1.15*	6.20 ± 0.05	0.67*
1	6.14 ± 0.06	0.26	6.21 ± 0.07	1.12*	6.17 ± 0.03	0.70*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

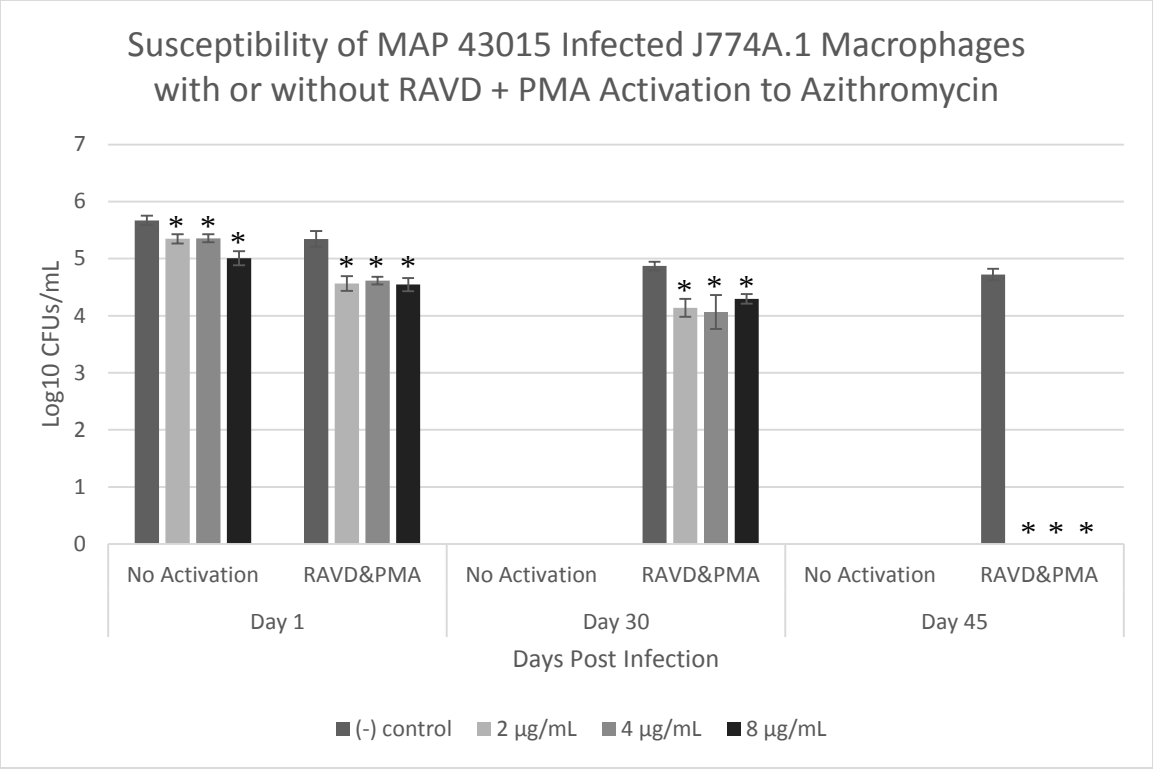

Figure 3.3A *In vitro* efficacy of azithromycin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with azithromycin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

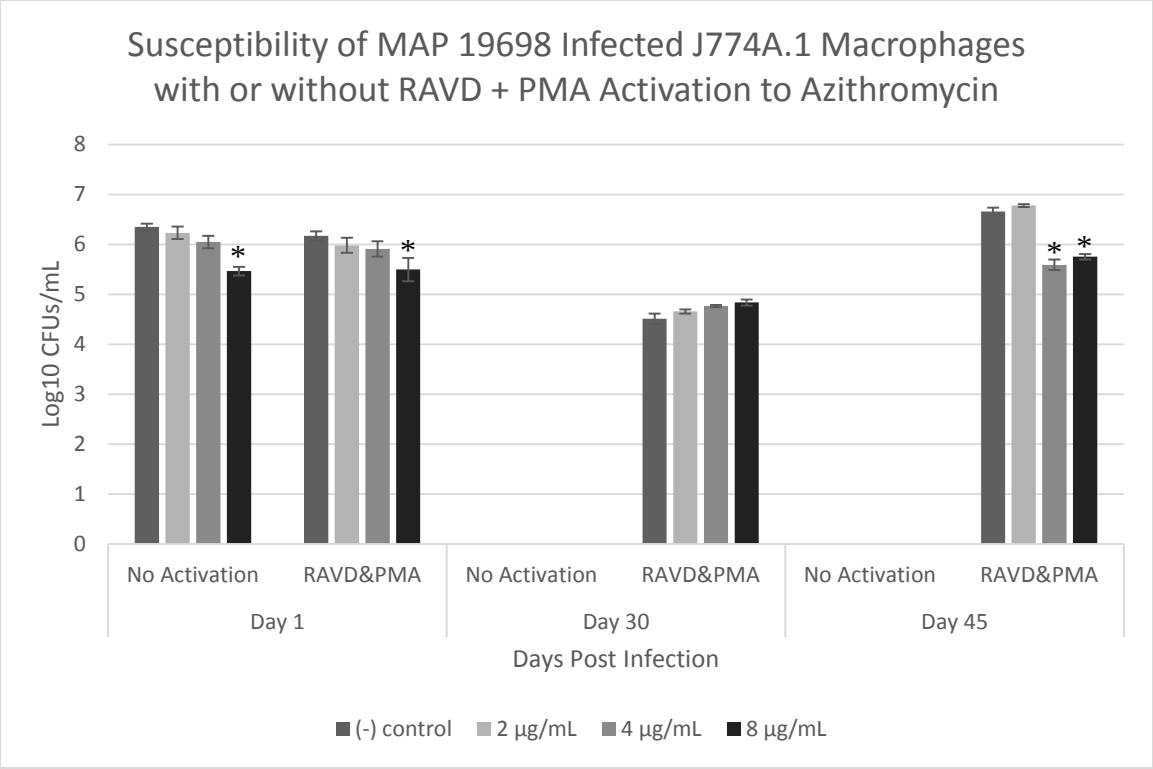

Figure 3.3B *In vitro* efficacy of azithromycin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with azithromycin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.4 Azithromycin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	5.35 ± 0.14		4.87 ± 0.08		4.72 ± 0.10	
2	4.57 ± 0.13	0.81*	4.14 ± 0.16	0.73*	0	4.72*
4	4.61 ± 0.07	0.74*	4.06 ± 0.30	0.81*	0	4.72*
8	4.54 ± 0.12	0.81*	4.29 ± 0.08	0.58*	0	4.72*
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.17 ± 0.09		4.51 ± 0.10		6.66 ± 0.08	
2	5.98 ± 0.15	0.19	4.66 ± 0.04	+ 0.15	6.78 ± 0.03	+ 0.12
4	5.91 ± 0.15	0.26	4.77 ± 0.02	+ 0.26	5.60 ± 0.11	1.06*
8	5.50 ±0.24	0.67*	4.84 ± 0.06	+ 0.33	5.75 ± 0.05	0.91*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

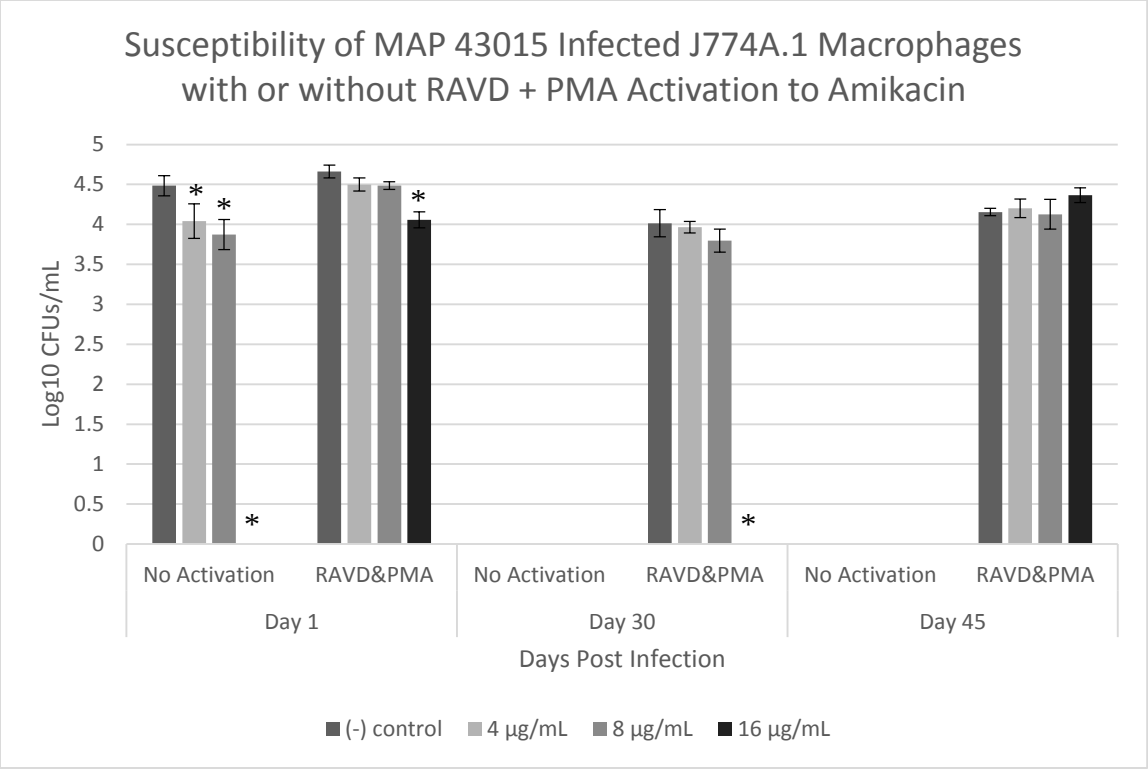

Figure 3.4A *In vitro* efficacy of amikacin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with amikacin at 4, 8, and 16 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

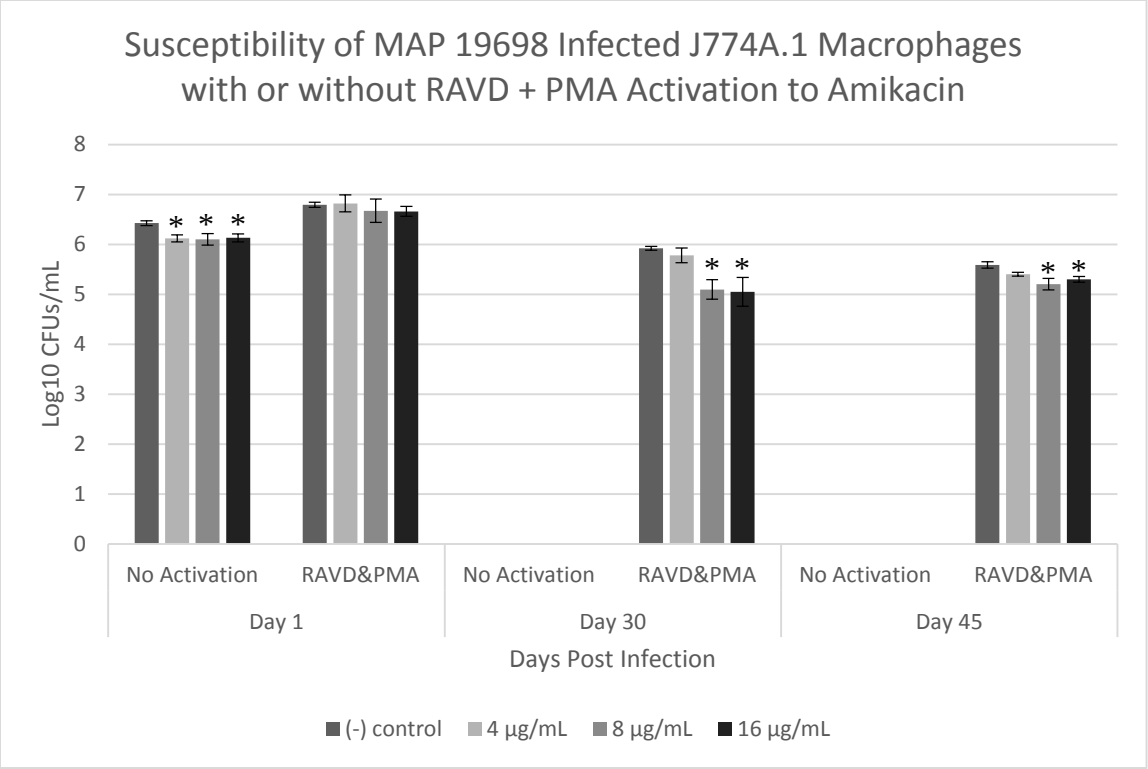

Figure 3.4B *In vitro* efficacy of amikacin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with amikacin at 4, 8, and 16 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.5 Amikacin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	4.66 ± 0.08		4.01 ± 0.17		4.15 ± 0.05	
4	4.50 ± 0.08	0.16	3.96 ± 0.07	0.05	4.20 ± 0.12	+ 0.05
8	4.48 ± 0.05	0.18	3.80 ± 0.14	0.21	4.13 ± 0.18	0.02
16	4.06 ± 0.10	0.60*	0	4.01*	4.37 ± 0.09	+0.22
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.79 ± 0.05		5.92 ± 0.04		5.59 ± 0.06	
4	6.82 ± 0.17	+ 0.03	5.78 ± 0.15	0.14	5.40 ± 0.04	0.19
8	6.67 ± 0.23	0.12	5.10 ± 0.19	0.82*	5.20 ± 0.11	0.39*
16	6.66 ± 0.10	0.13	5.05 ± 0.29	0.87*	5.30 ± 0.06	0.29*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

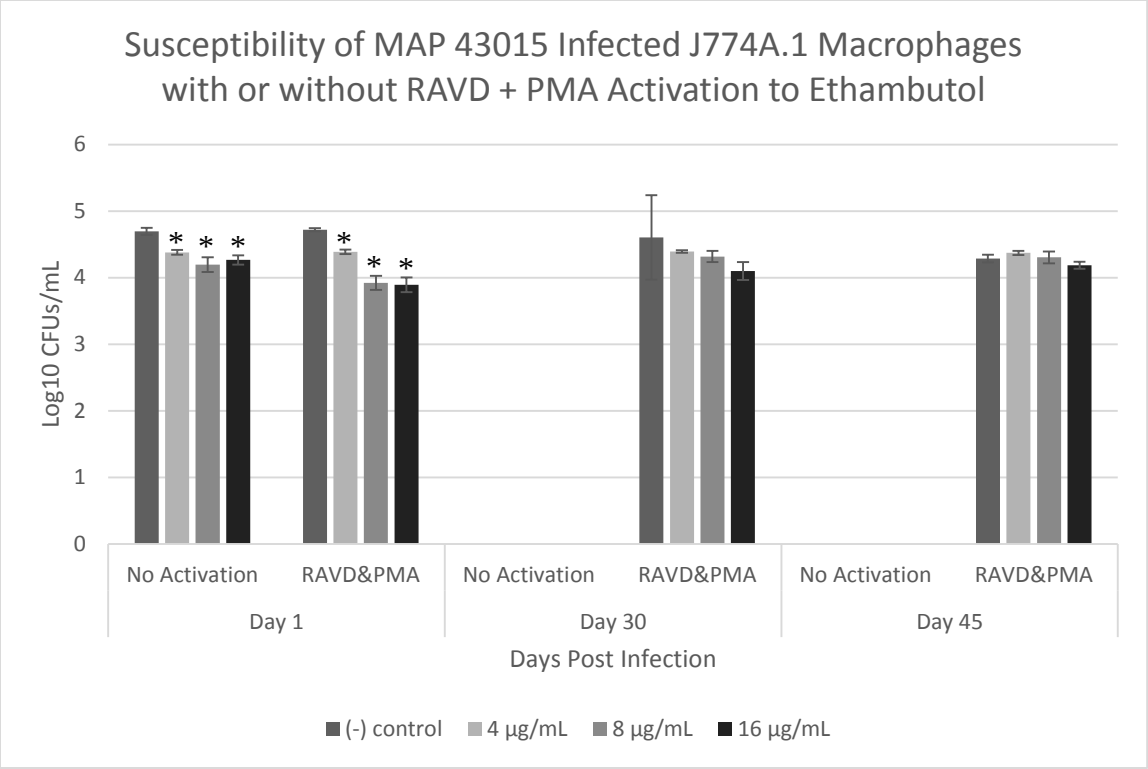

Figure 3.5A *In vitro* efficacy of ethambutol against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with ethambutol at 4, 8, and 16 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

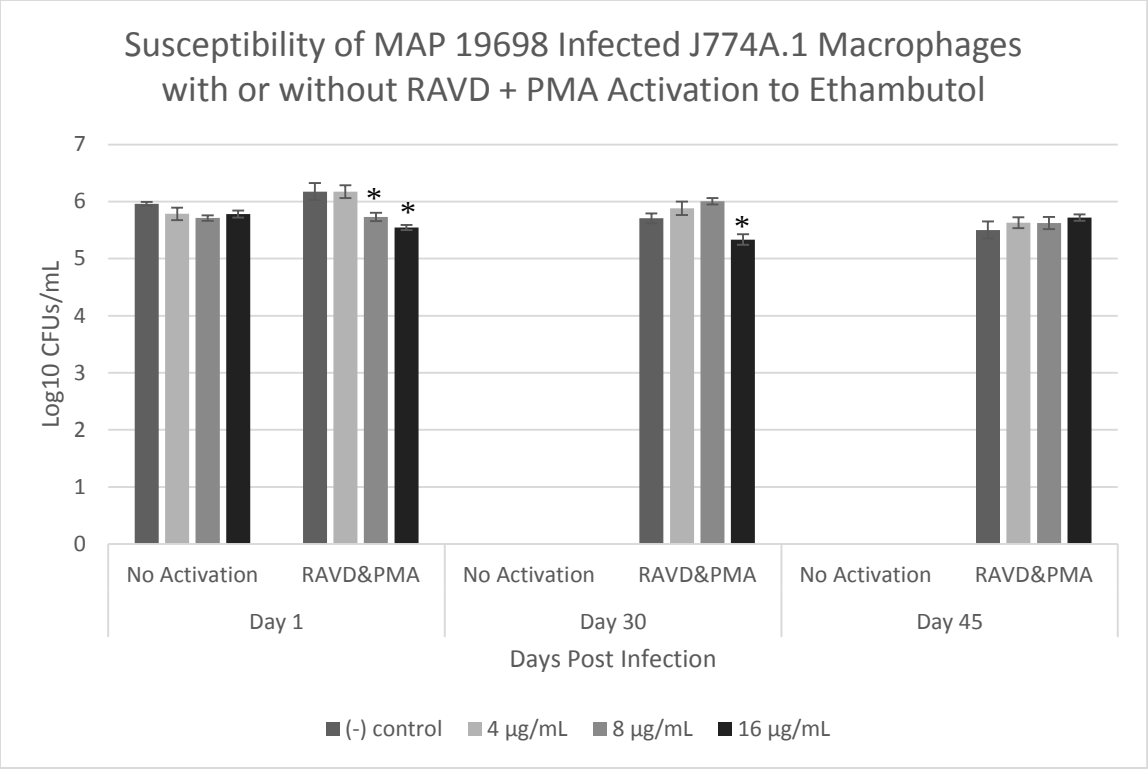

Figure 3.5B *In vitro* efficacy of ethambutol against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with ethambutol at 4, 8, and 16 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.6 Ethambutol susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	4.72 ± 0.02		4.60 ± 0.64		4.29 ± 0.06	
4	4.39 ± 0.03	0.35*	4.39 ± 0.02	0.21	4.37 ± 0.03	+ 0.08
8	3.92 ± 0.11	0.80*	4.32 ± 0.08	0.28	4.30 ± 0.09	+ 0.01
16	3.89 ± 0.11	0.83*	4.10 ± 0.13	0.50	4.19 ± 0.05	0.01
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.18 ± 0.15		5.71 ± 0.09		5.50 ± 0.15	
4	6.17 ± 0.11	0.01	5.88 ± 0.12	+ 0.17	5.63 ± 0.09	+ 0.13
8	5.73 ± 0.07	0.37*	6.01 ± 0.06	+ 0.30	5.62 ± 0.11	+ 0.12
16	5.54 ± 0.05	0.64*	5.33 ± 0.09	0.41*	5.72 ± 0.06	+ 0.22

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

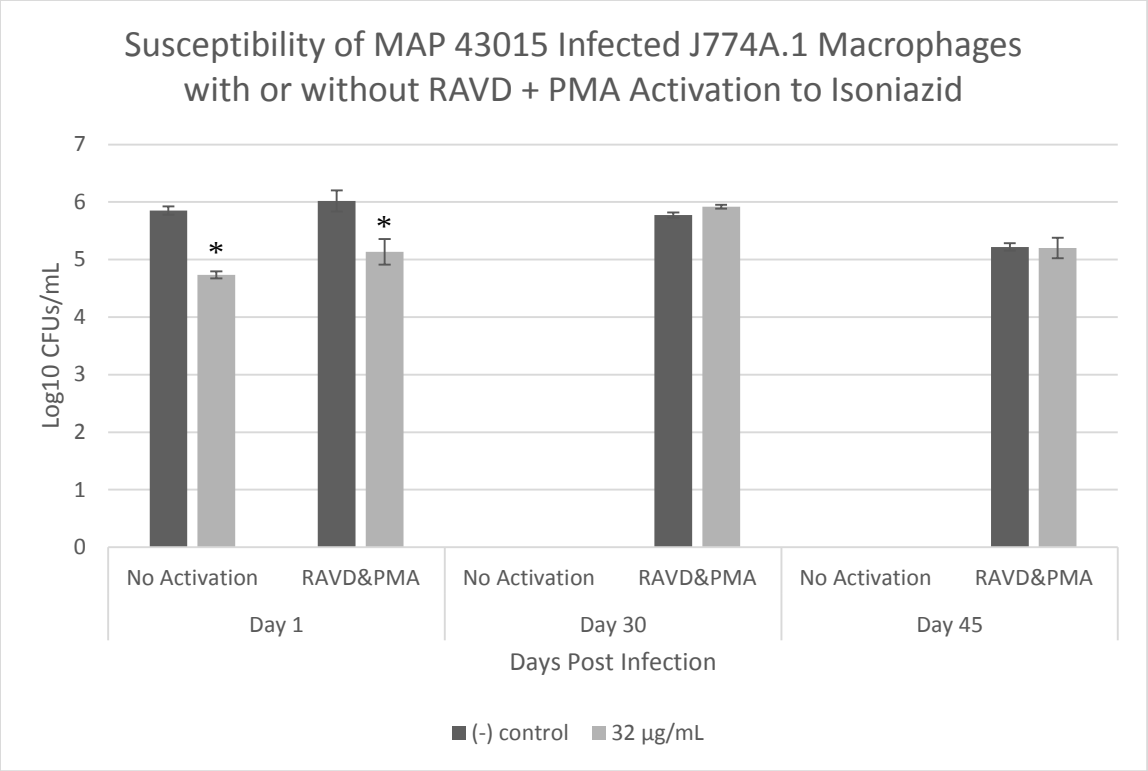

Figure 3.6A *In vitro* efficacy of isoniazid against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with isoniazid at 32 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicates, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

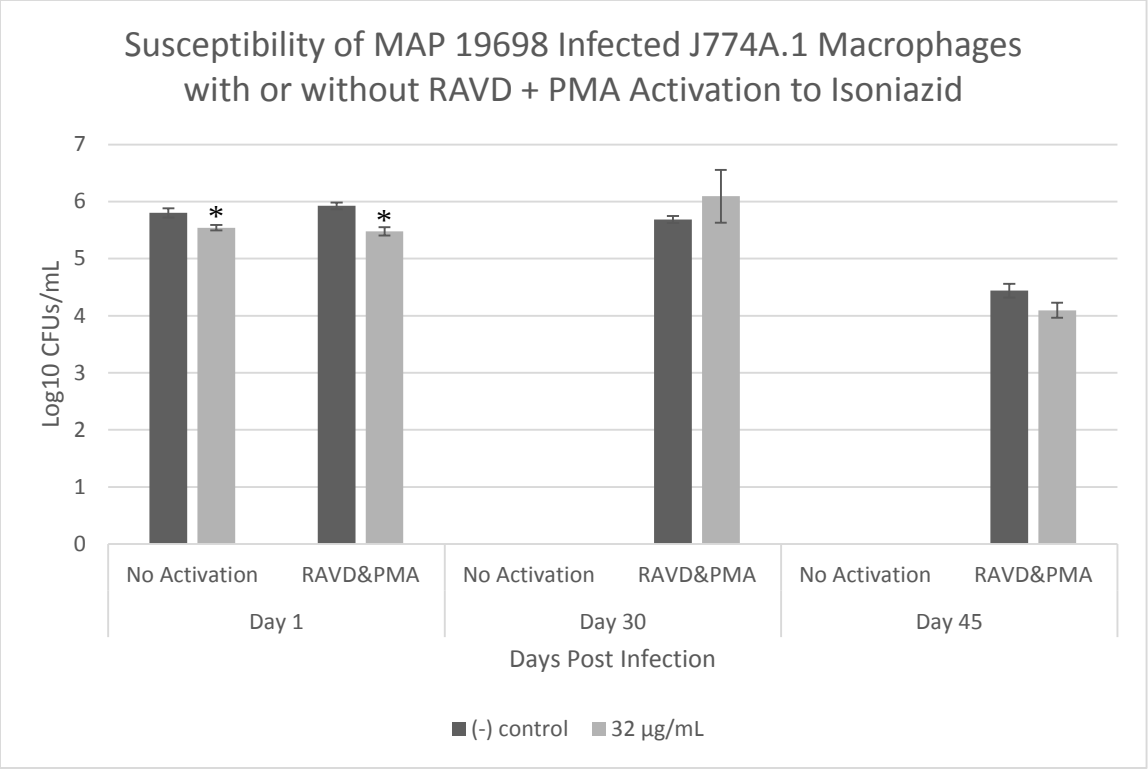

Figure 3.6B *In vitro* efficacy of isoniazid against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with isoniazid at 32 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.7 Isoniazid susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	6.02 ± 0.18		5.77 ± 0.04		5.22 ± 0.06	
32	5.13 ± 0.22	0.89*	5.92 ± 0.03	+ 0.15	5.20 ± 0.18	+ 0.02
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	5.92 ± 0.06		5.69 ± 0.06		4.44 ± 0.12	
32	5.48 ± 0.07	0.44*	6.09 ± 0.46	+ 0.40	4.10 ± 0.13	0.34

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

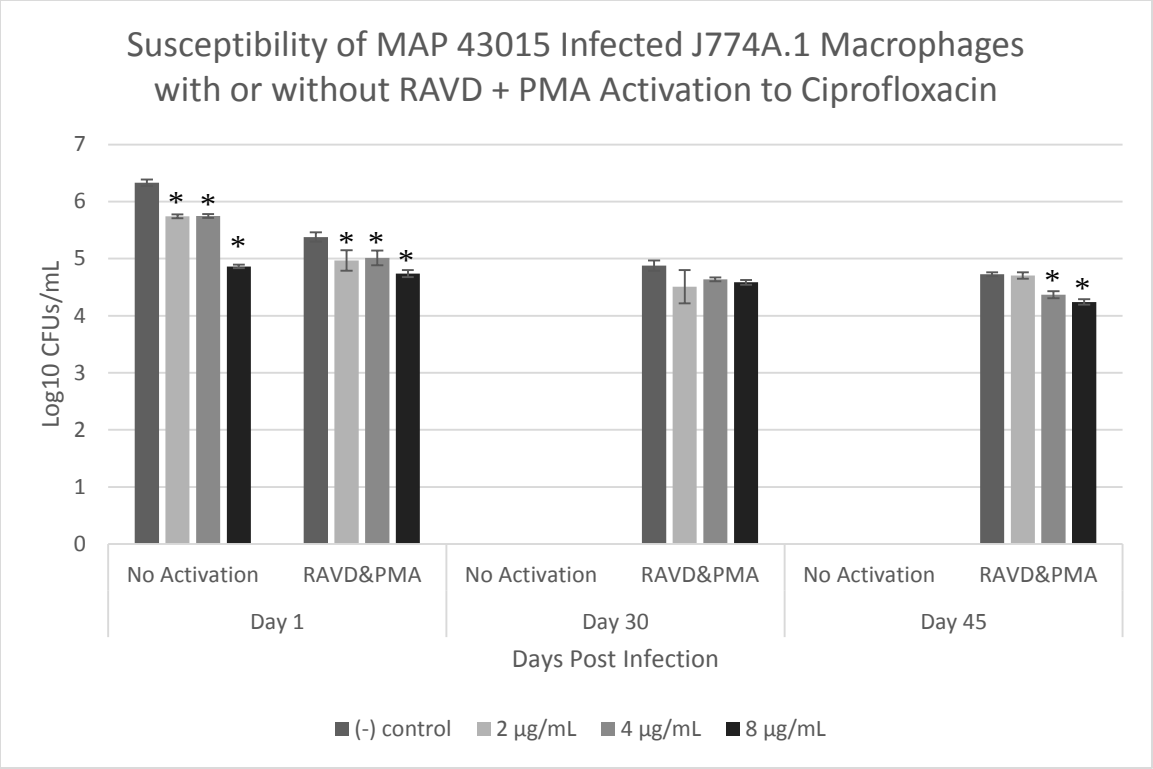

Figure 3.7A *In vitro* efficacy of ciprofloxacin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with ciprofloxacin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

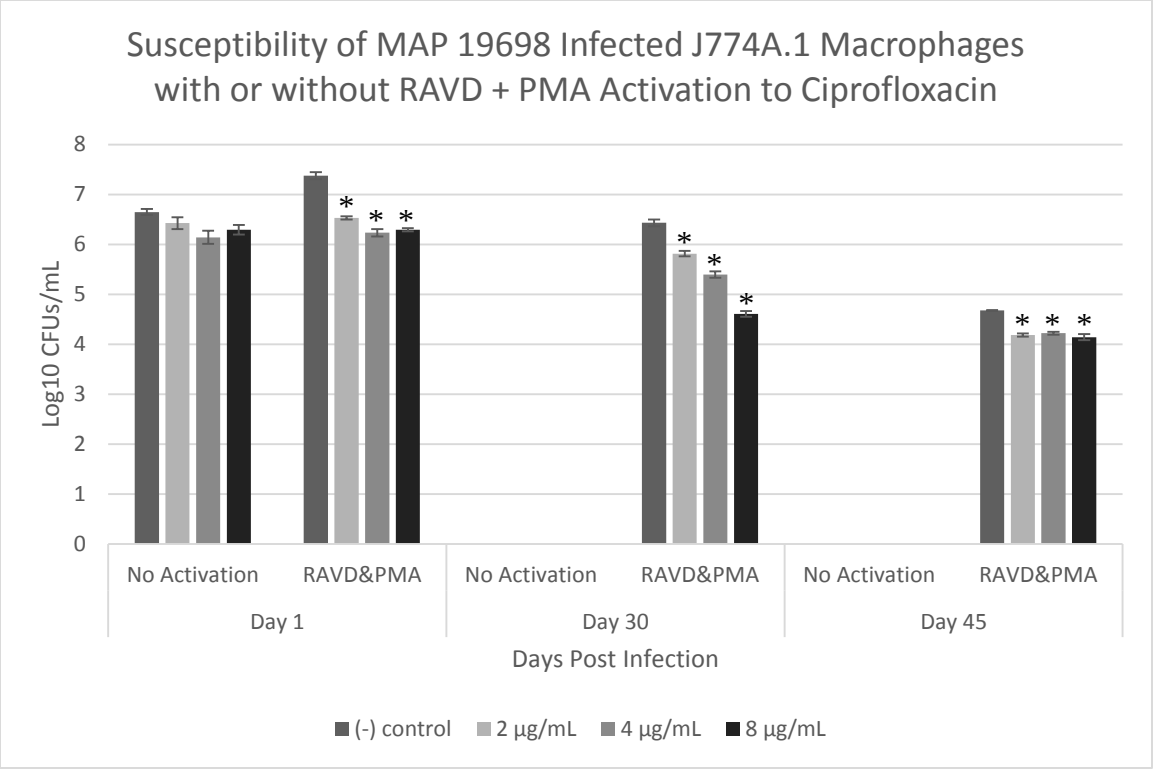

Figure 3.7B *In vitro* efficacy of ciprofloxacin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with ciprofloxacin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.8 Ciprofloxacin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	5.38 ± 0.08		4.88 ± 0.09		4.73 ± 0.03	
2	4.97 ± 0.18	0.41*	4.51 ± 0.29	0.37	4.70 ± 0.06	0.03
4	5.01 ± 0.13	0.37*	4.64 ± 0.03	0.24	4.37 ± 0.06	0.36*
8	4.74 ± 0.06	0.64*	4.58 ± 0.04	0.30	4.24 ± 0.05	0.49*
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	7.38 ± 0.07		6.42 ± 0.07		4.68 ± 0.01	
2	6.53 ± 0.03	0.85*	5.81 ± 0.06	0.61*	4.18 ± 0.03	0.50*
4	6.23 ± 0.07	1.15*	5.39 ± 0.06	1.03*	4.22 ± 0.03	0.46*
8	6.29 ± 0.03	1.09*	4.61 ± 0.06	1.81*	4.14 ± 0.06	0.54*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

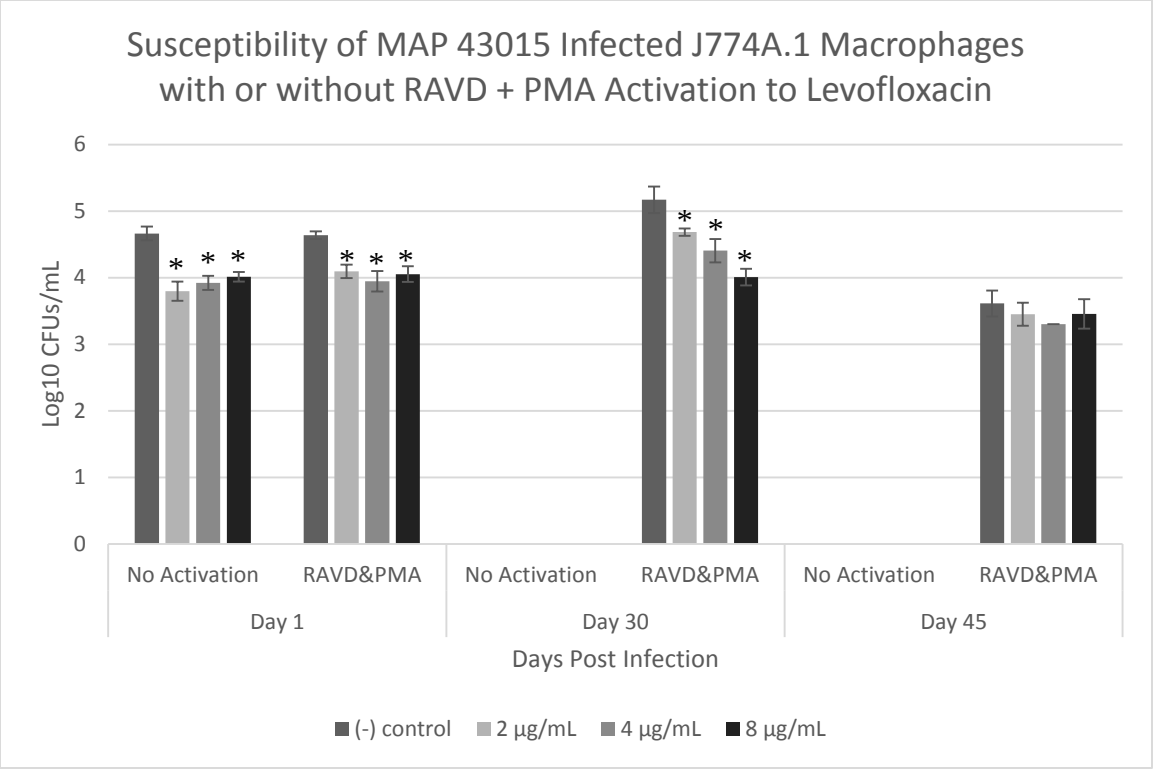

Figure 3.8A *In vitro* efficacy of levofloxacin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with levofloxacin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

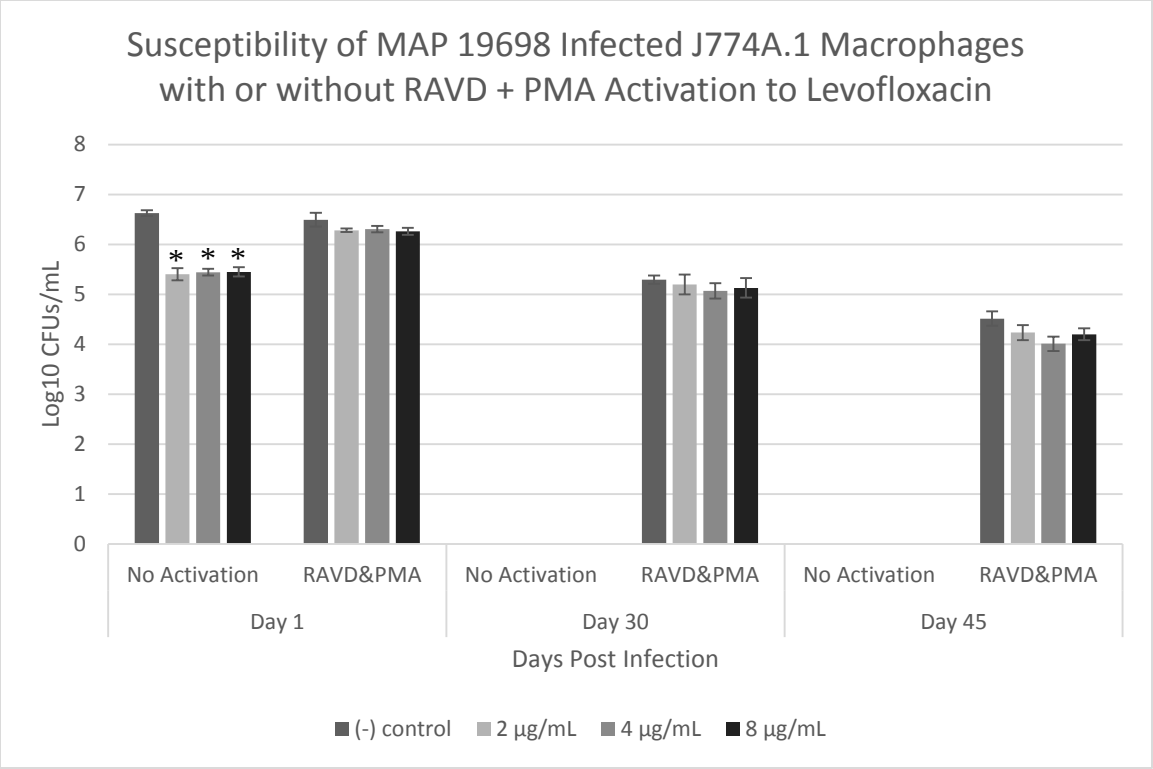

Figure 3.8B *In vitro* efficacy of levofloxacin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with levofloxacin at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.9 Levofloxacin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	4.64 ± 0.06		5.17 ± 0.20		3.61 ± 0.20	
2	4.09 ± 0.10	0.55*	4.68 ± 0.06	0.49*	3.45 ± 0.17	0.16
4	3.95 ± 0.15	0.69*	4.40 ± 0.17	0.77*	3.30 ± 0	0.31
8	4.05 ± 0.12	0.59*	4.01 ± 0.12	1.16*	3.46 ± 0.22	0.15
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.49 ± 0.14		5.28 ± 0.08		4.15 ± 0.15	
2	6.28 ± 0.03	0.20	5.20 ± 0.20	0.08	4.23 ± 0.15	+0.08
4	6.30 ± 0.06	0.19	5.07 ± 0.15	0.21	4.01 ± 0.15	0.14
8	6.26 ± 0.07	0.23	5.13 ± 0.20	0.15	4.20 ± 0.12	+0.05

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

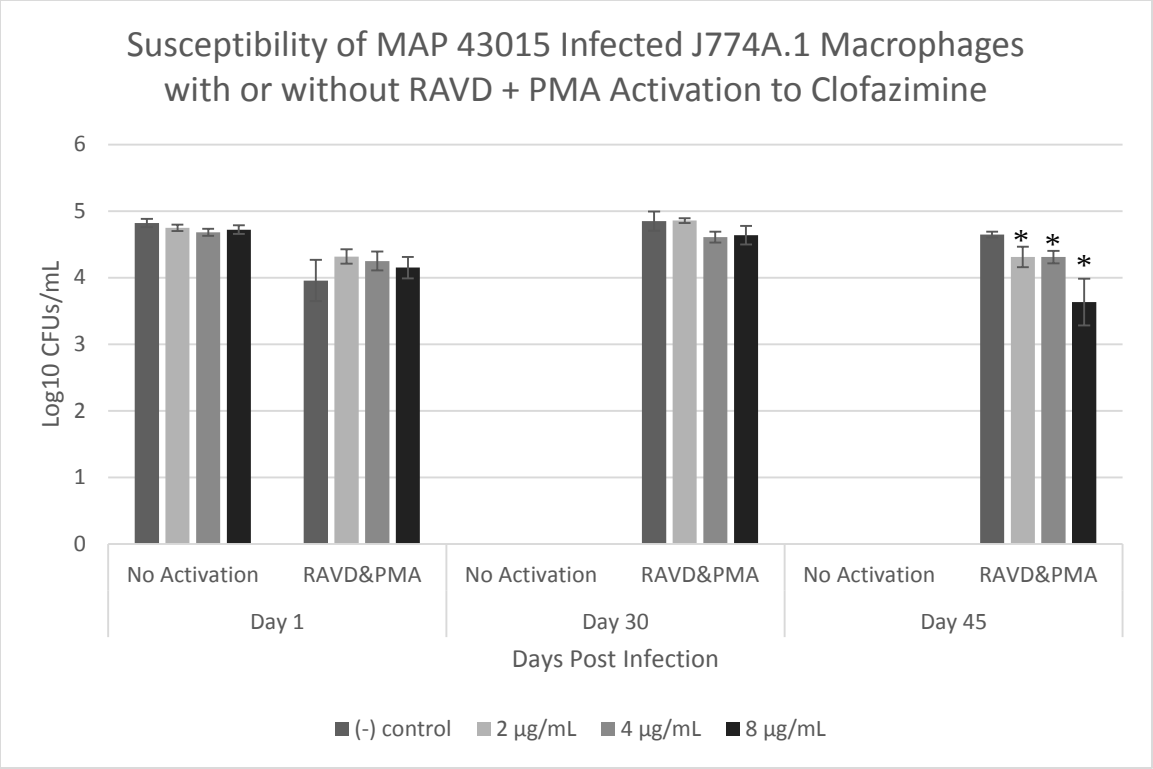

Figure 3.9A *In vitro* efficacy of clofazimine against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with clofazimine at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

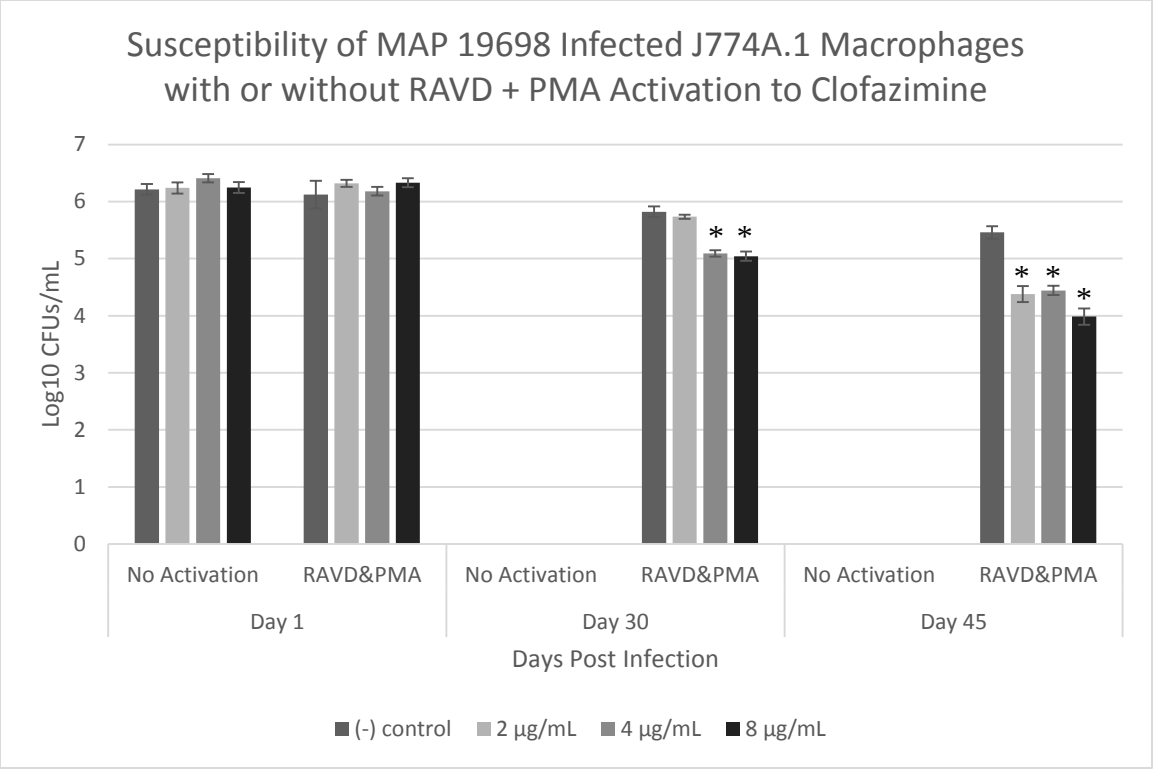

Figure 3.9B *In vitro* efficacy of clofazimine against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with clofazimine at 2, 4, and 8 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.10 Clofazimine susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	3.96 ± 0.31		4.85 ± 0.15		4.65 ± 0.04	
2	4.32 ± 0.11	+ 0.36	4.86± 0.04	+ 0.01	4.31 ±0.15	0.34*
4	4.25 ± 0.14	+ 0.29	4.61 ± 0.08	0.24	4.31 ± 0.09	0.34*
8	4.15 ± 0.16	+ 0.19	4.64 ± 0.14	0.21	3.63 ± 0.35	1.02*
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.12 ± 0.24		5.82 ± 0.09		5.46 ± 0.11	
2	6.32 ± 0.06	+ 0.2	5.73 ± 0.04	0.09	4.38 ± 0.14	1.08*
4	6.18 ± 0.08	+ 0.06	5.09 ± 0.06	0.73*	4.44 ± 0.08	1.02*
8	6.33 ± 0.08	+ 0.21	5.04 ± 0.08	0.78*	3.98 ± 0.14	4.48*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

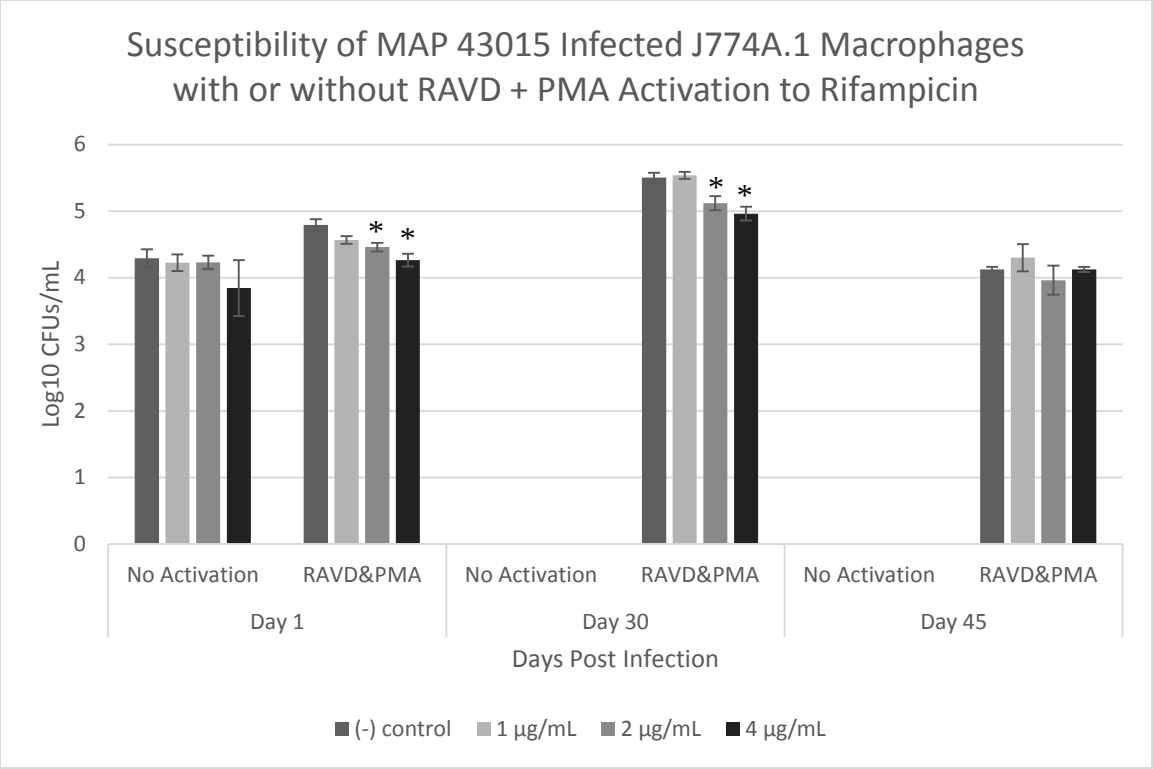

Figure 3.10A *In vitro* efficacy of rifampicin against MAP strain 43015. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with rifampicin at 1, 2, and 4 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

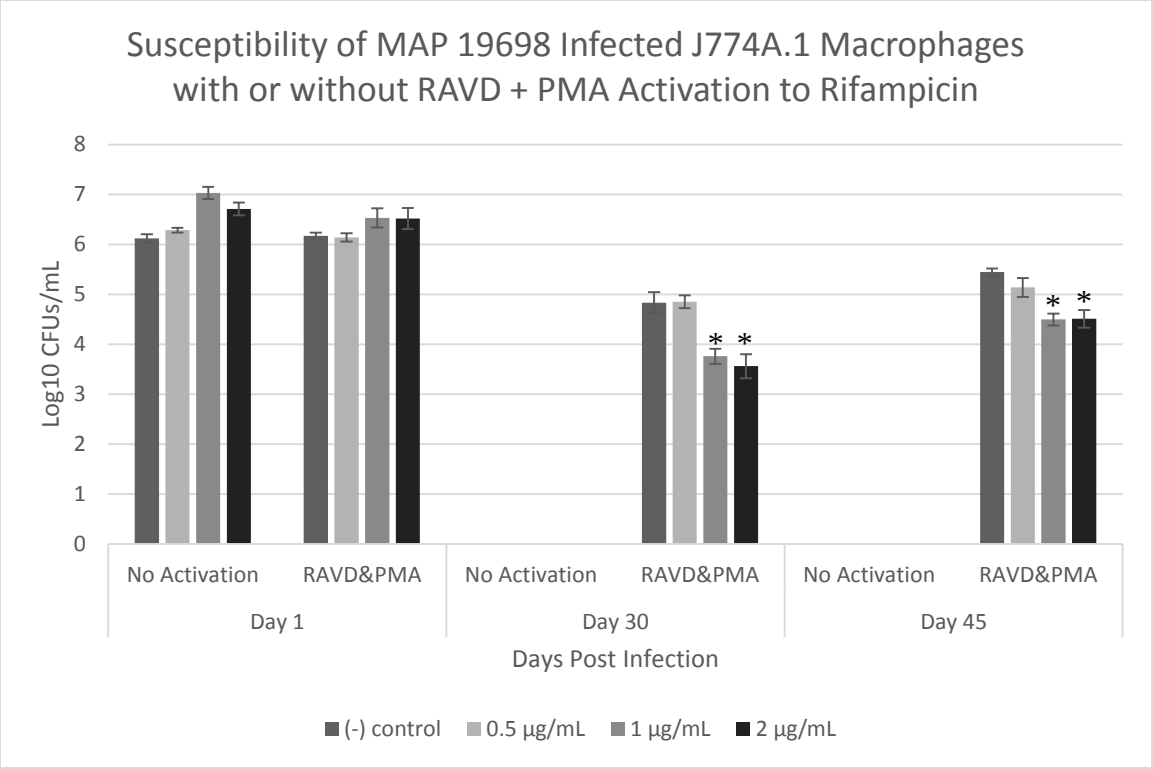

Figure 3.10B *In vitro* efficacy of rifampicin against MAP strain 19698. J774A.1 macrophages with or without RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with rifampicin at 1, 2, and 4 µg/mL at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate, three separate times. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 3.11 Rifampicin susceptibility of long term RAVD plus PMA activated J774A.1 macrophages infected with MAP strains 43015 and 19698.

MAP 43015	Days Post Infection					
	Day 1		Day 30		Day 45	
Drug Concentration (µg/mL)	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control	Log CFU/ml/well[a]	Log reduction compared to (-) control
(-) control	4.79 ± 0.09		5.50 ± 0.08		4.12 ± 0.04	
1	4.57 ± 0.06	0.22	5.54 ± 0.05	+ 0.04	4.30 ± 0.20	+ 0.18
2	4.46 ± 0.06	0.33*	5.12 ± 0.11	0.38*	3.96 ± 0.22	0.16
4	4.26 ± 0.10	0.53*	4.96 ± 0.10	0.54*	4.12 ± 0.04	0
MAP 19698	**Days Post Infection**					
	Day 1		**Day 30**		**Day 45**	
Drug Concentration (µg/mL)	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**	**Log CFU/ml/well[a]**	**Log reduction compared to (-) control**
(-) control	6.17 ± 0.06		4.84 ± 0.21		5.44 ± 0.07	
1	6.14 ± 0.08	0.03	4.85 ± 0.13	+ 0.01	5.14 ± 0.19	0.30
2	6.53 ± 0.19	+ 0.36	3.76 ± 0.15	1.08*	4.50 ± 0.12	0.94*
4	6.52 ± 0.21	+ 0.35	3.56 ± 0.24	1.28*	4.51 ± 0.17	0.93*

[a] Mean ± standard error in triplicate wells of 3 different experiments.

+ Log values are found to be higher than the log value of the (-) control

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

To further test the established cell culture model and to further evaluate the efficacy, a combination of drugs, clarithromycin, rifampicin, and clofazimine, were tested at the three different concentrations explained in the methods. The results are shown in Figures 3.11 A and B. The combination of drugs show efficacy against both strains of MAP, at all three stages of infection tested. The results are summarized in Tables 3.12. A summary of the all MBC results, free drugs as well as the combination of drugs, are shown in Table 3.13.

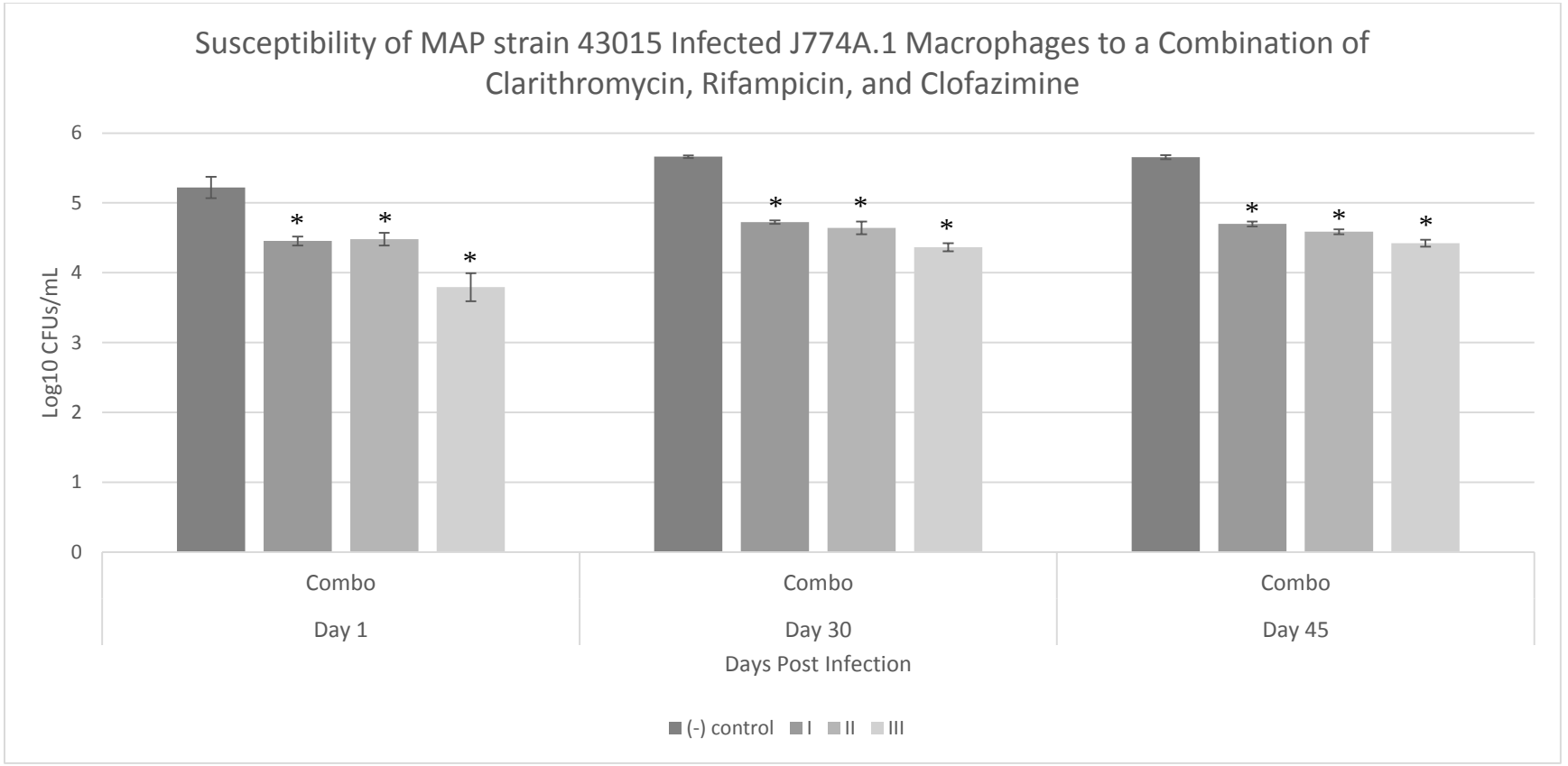

Figure 3.11A *In vitro* efficacy of clarithromycin (CLA), rifampicin (RIF), clofazimine (CLO) in combination (Combo). J774A.1 macrophages with RAVD plus PMA activation were infected with MAP strain 43015 (MOI of 10) and treated with a combination of CLA, RIF and CLO at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Drug concentrations (µg/mL):

	Combo
I	1 CLA, 4 RIF, 8 CLO
II	2 CLA, 8 RIF, 16 CLO
III	4 CLA, 16 RIF, 32 CLO

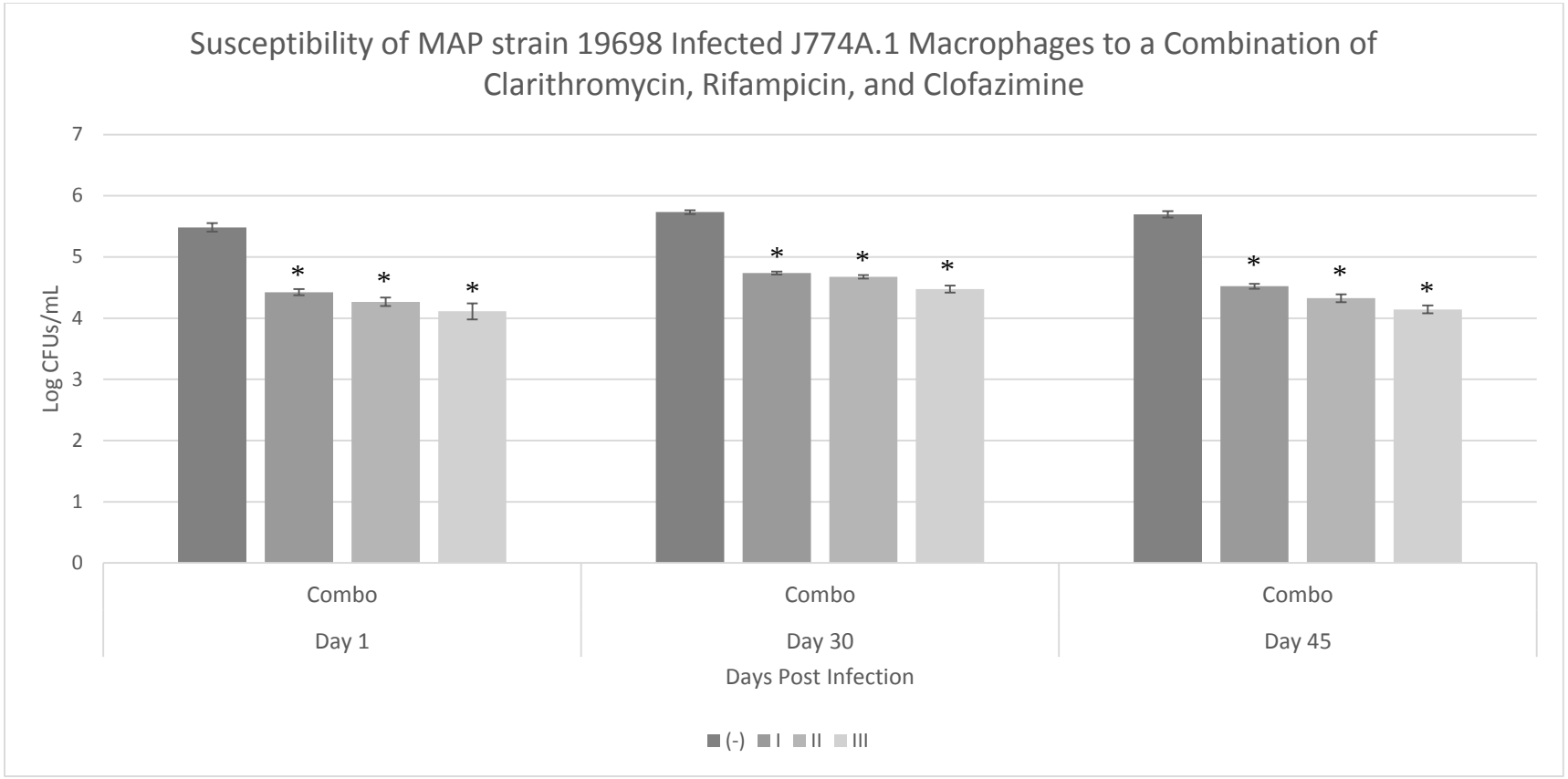

Figure 3.11B *In vitro* efficacy of clarithromycin (CLA), rifampicin (RIF), clofazimine (CLO) in combination (Combo). J774A.1 macrophages with RAVD plus PMA activation were infected with MAP strain 19698 (MOI of 10) and treated with a combination of CLA, RIF and CLO at day 1, day 30, and day 45 post infection. Non-treated cells were the positive control. All the treatments were tested in triplicate. The data represents the mean ± SE of $\log_{10}$ MAP CFUs/mL.

*Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Drug concentrations (µg/mL):

	Combo
I	1 CLA, 4 RIF, 8 CLO
II	2 CLA, 8 RIF, 16 CLO
III	4 CLA, 16 RIF, 32 CLO

Table 3.12 Susceptibility of MAP strains 43015 and 19698 to treatment with a combination of clarithromycin, clofazimine, and rifampicin (combination) as compared to the control (no treatment) on day 1, 30, and 45 post infection.

MAP 43015	Combination					
	Day 1		Day 30		Day 45	
Drug Concentration^	Log CFU/mL/well[a]	Log Reduction	Log CFU/mL/well[a]	Log Reduction	Log CFU/mL/well[a]	Log Reduction
(-) Control	5.22 ± 0.15		5.66 ± 0.02		5.66 ± 0.03	
I	4.45 ± 0.07	0.77*	4.73 ± 0.03	0.93*	4.70 ± 0.03	0.96*
II	4.48 ± 0.09	0.74*	4.64 ± 0.09	1.02*	4.59 ± 0.03	1.07*
III	3.79 ± 0.20	1.43*	4.37 ± 0.06	1.29*	4.42 ± 0.05	1.24*
MAP 19698	Combination					
	Day 1		Day 30		Day 45	
Drug Concentration^	Log CFU/mL/well[a]	Log Reduction	Log CFU/mL/well[a]	Log Reduction	Log CFU/mL/well[a]	Log Reduction
(-) Control	5.48 ± 0.07		5.73 ± 0.03		5.70 ± 0.05	
I	4.42 ± 0.05	1.06*	4.74 ± 0.02	0.99*	4.52 ± 0.04	1.18*
II	4.27 ± 0.07	1.21*	4.67 ± 0.03	1.06*	4.33 ± 0.64	1.37*
III	4.11 ± 0.13	1.37*	4.47 ± 0.06	1.26*	4.14 ± 0.06	1.56*

[a] Mean ± standard error in at least 3 different wells.

^ See drug concentration in table shown below Figures 3.11A and B respectively.

* Values found to be significantly different ($P \leq 0.05$) from the control by analysis using a two-tailed Student's *t*-test.

Table 3.13 Summary of drug efficacy at different stages of infection.
Susceptibility of long term RAVD plus PMA activated J774A.1 macrophage infected with MAP strain 43015 and 19698.

X – Represents susceptibility at that specific stage of infection in that specific strain of MAP; a blank box means that there was no susceptibility noted.

	Stage of Infection					
Drug	**Early**		**Chronic**		**Latent**	
	43015	**19698**	**43015**	**19698**	**43015**	**19698**
Clarithromycin	X			X	X	X
Azithromycin	X	X	X		X	X
Amikacin	X		X	X		X
Ethambutol	X	X		X		
Isoniazid	X	X				
Ciprofloxacin	X	X		X	X	X
Levofloxacin	X		X			
Clofazimine				X	X	X
Rifampicin	X		X	X		X
Combination	X	X	X	X	X	X

Discussion:

The results from this study were consistent with what was previously found and published about drug susceptibility of MAP strains [20]. However, because two different strains of MAP, 19698 and 43015 were used, a difference in susceptibility to each drug at different concentrations was observed. As expected, a combination of drugs was the most effective at treating MAP infected J774A.1 macrophages.

The various drugs tested in this chapter all have different mechanisms of action against MAP. Clarithromycin and azithromycin are both macrolide drugs, binding to the 50s subunit of the bacterial ribosome, interfering with protein synthesis and thus inhibiting translation. Amikacin binds to the 30s ribosomal unit, causing misreading of mRNA and thus leaving the bacterium unable to synthesize vital proteins required for growth. Ethambutol disrupts arabinogalactan synthesis by inhibiting enzyme arabinosyl transferase thus leading to increased permeability of the bacterium's cell wall. Isoniazid binds to the *inhA* gene and inhibits synthesis of mycolic acids. Ciprofloxacin and levofloxacin are both broad-spectrum antibiotics active against Gram-positive and Gram-negative bacteria. Ciprofloxacin inhibits DNA gyrase, thereby inhibiting cell division, while levofloxacin inhibits DNA gyrase and topoisomerase, components necessary for supercoiling DNA to fit into newly formed cells and to separate DNA that has been replicated respectively. Clofazimine binds to guanine bases of MAP bacterial DNA blocking template function of DNA and thus inhibiting bacterial proliferation. Rifampicin inhibits bacterial DNA dependent RNA synthesis by inhibiting RNA polymerase. All the drugs listed above, have various mechanisms to prevent proliferation of MAP.

As hypothesized, the combination of the three drugs (clarithromycin, clofazimine, and rifampicin) was significantly more effective in treating macrophages infected with MAP strain 19698 and 43015 in treatment assays, showing susceptibility at all concentrations tested. The drugs have possible potentiated/synergistic effects, with the effect of the drug(s) being enhanced by the other drug(s), than by itself alone. Further tests need to be done to verify the statement, however it is worth noting that clofazimine alone was not effective against either MAP strain in the early stage of infection, however the combination of the drugs with clofazimine showed effectiveness in the early stage of infection. It seems that the combination of drugs work in synergy, reinforcing drug interactions such that the joint effect of the drugs administered simultaneously is greater than the individual effects. This was seen in the fact that all three concentrations of the combination of drugs tested showed efficacy, in all three stages of infection, on both strains of MAP, whereas none of the individual drugs (clarithromycin, clofazimine, nor rifampicin) showed comparable efficacy at all stages of infection of cells.

The long-term infection and treatment assay results summarized in Tables 3.3 - 3.12 are the log reduction of each treatment group as compared to the control group, at day 1, 30 and 45 post infection. Significant bacterial reductions were noted in the specific Tables 3.3 - 3.12. For day 1 post infection, there are results from non-activated MAP *in vitro* infection and treatment with the various drugs (Figures 3.2 – 3.10). These results were compared with the RAVD plus PMA activated MAP *in vitro* infection and treatment. This comparison was an evaluation of the effect of drug uptake with and without RAVD plus PMA activation of macrophages prior to infection. The results of the non-activated and activated intracellular infection and treatment did not show a significant difference in the day 1 studies. The non-activated cells also served as a negative control, as by day 30 and 45 post infection, the cells were unable to survive without prior

activation with RAVD plus PMA even with frequent changes of the cell culture medium, therefore there are no results for non-activated cells at 30 and 45 day post-infection.

This *in vitro* model could potentially eliminate unnecessary preliminary testing in mice and ruminants. This effort will provide researchers with an important screening tool for new therapeutics before actually embarking on costly *in vivo* testing, and allow the assessment of therapeutics against many of the intracellular pathogens, at different stages of infection. This *in vitro* cell culture model may provide scientists with an efficient method to evaluate not only new treatments, but also those that showed promise, but were abandoned due economical and/or technical roadblocks of testing in animals. This study mainly focused on the effects of conventional drugs used to treat mycobaterial infections at different stages of infection. Their efficacies were evaluated to potentially provide insight into which drug(s) should be used to treat MAP infections at different stages of infection.

With the results found in this study, the next step should be to test other combinations of drugs, as well as individual drugs tested in this study, and the combination of the three drugs, against other strains of MAP, and even with other *Mycobacterium* species. With the activated macrophage infection cell culture model, multiple drugs were tested simultaneously and established the susceptibility of MAP strains as well as intracellular MAP in macrophages. We were able to test 10 different drugs, and their efficacy against MAP 19698 and 43015 long term *in vitro* cell culture infection. In addition, the common anti-mycobacterial drugs were tested for their effectiveness against MAP strains 43015 and 19698 at the early, chronic and latent stages of MAP infection in J774 A.1 cells.

Through our findings, we can conclude that none of the single drugs can be considered the "most effective", as each drug has differential effects against the two different strains of MAP, as

well as differential effectiveness in the stages of infection evaluated. The drug clarithromycin, to which MAP was found to be susceptible at all concentrations, did not have the greatest efficacy in the MAP infected long term cell culture model. Clarithromycin and ciprofloxacin were effective against both strains in late infection, and ciprofloxacin was also effective against both strains in the early stage of infection. However levofloxacin was only effective against MAP strain 43015 in the early and chronic stages of infection. Isoniazid, although previously found to be ineffective against MAP as determined by the MIC and MBC agar dilution methods, was effective against both strains of MAP in the early stage of infection in the MAP infected cell culture model. This was quite noteworthy, considering both strains of MAP were resistant to isoniazid at all concentrations as determined by *in vitro* tests. Our results could explain why previous clinical studies have used isoniazid as a treatment for MAP infections [32], as well as clarithromycin, rifabutin, and clofazimine together [33]. However, from our results azithromycin, which was effective at all stages of infection with MAP strain 43015, seems to be more effective in treatment of MAP strain 43015 infections than clarithromycin, and may be a better choice in future therapeutic combinations.

A combination of three drugs (clarithromycin, clofazimine, and rifampicin) provided the most promising results, as it was effective against both MAP strains 43015 and 19698 at the early, chronic and latent stage of infection in the cell culture model. Clarithromycin and clofazimine were effective against both strains of MAP in the late stage of infection, whereas rifampicin was effective against both strains of MAP in the chronic stage of infection. Therefore, added together, these three drugs combination was effective in treating MAP strains 43015 and 19698 infections in the early, chronic, and latent stages of infection.

Before, this dissertation research, there was no well-established long term *in vitro* cell culture infection model for testing therapeutics against MAP infections. Current research has characterized and established a long term J774A.1 murine macrophage MAP infection model for evaluating anti-infectives. This model provides researchers with important screening tools for new therapeutics before actually embarking on costly *in vivo* testing, and allow the assessment of therapeutics at different stages of infection on an array of intracellular pathogens. This has been shown to be a long term infection model that can be used to test combination therapeutics, against multiple strains of MAP, at the same time, thus saving money and time on the testing of novel therapeutics. Further studies are needed to test the efficacy of other drugs that may be effective against intracellular MAP, as well as to test the drugs evaluated in this study against other strains of MAP.

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Chapter 4

Peptide Nucleic Acids and its Effect on the Growth of *Mycobacterium avium* subsp. *paratuberculosis*: A pilot study *in vitro*.

Abstract:

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a very slow growing bacterium that is the causative agent of Johne's disease (JD) in ruminants and has long been associated with complications of Crohn's disease (CD) in humans. Because there are not many effective antibiotics to cure paratuberculosis infections, there is a great need for more effective therapeutics. Peptide nucleic acids (PNAs) are synthetic single stranded nucleic acid analogues with a peptide backbone instead of the sugar backbone. They are extremely nuclease resistant, protease resistant and bind to complementary strands of both deoxyribonucleic (DNA) and ribonucleic (mRNA) acid irreversibly. Thereby they are able to inhibit bacterial transcription and translation and are a very promising development for antisense therapies. In numerous bacterial species, PNAs have been shown to inhibit bacterial growth in both pure culture and in infected cell lines. The anti-*rpsl* PNA, which binds to the *rpsl* gene encoding ribosomal protein S12, was examined for its ability to inhibit MAP growth. This PNA significantly inhibited MAP bacterial growth throughout a 4-6 week period. This pilot study demonstrates the usefulness of PNA antisense constructs as a potential therapeutic regime for MAP infections.

Introduction:

According to the National Johne's Education Initiative (Colorado Springs, CO, johnesdiesase.org), one out of 10 animals moving through livestock auction facilities are infected with Johne's disease. In 2007, the United States Department of Agriculture (USDA) found that 68% of U.S. dairy operations were infected with MAP [1]. The Johne's incidence in the USA has been growing; there is no effective treatment, while the current control measures have not been very successful [2]. In recent years, targeting bacterial genes using antisense technology has gained interest as a treatment for bacterial infections [3, 4]. Numerous studies have shown that various PNAs have the ability to form strong bonds with complementary strands of bacterial gene(s), and are capable of inhibiting bacterial growth [5-9]. As there is no cure for MAP infections, and current therapeutics are either limited to the treatment of symptoms in humans or too expensive to be economical for the effective treatment of animals, a novel alternative therapeutic is of great interest [10]. Moreover, there was a published study demonstrating the ability of a number of specific PNAs to inhibit the growth of *Mycobacterium smegmatis*, a closely related bacterium in the same genus as MAP [7].

To determine whether or not PNAs have an effect on the growth of MAP, two essential genes were analyzed at the sequence level: the *inhA* gene that encodes for NADH-dependent enoyl-[acyl-carrier-protein] reductase InhA (NADH-dependent enoyl-ACP reductase) a target for isoniazid and ethionamide in *Mycobacterium tuberculosis* [11], as well as the *rpsL* gene that encodes ribosomal protein S12 involved in protein synthesis [12]. The *rpsl* gene seemed most promising as the DNA sequence was identical in almost all of the mycobacterial species examined. The synthetic anti-*rspl* PNA was therefore examined to determine whether it had bactericidal or bacteriostatic effects.

Materials and Methods:

Bacterial strains and cell lines:

For details about bacterial strains and cell lines used in the following experiments, please see Materials and Methods of Chapter 2.

Synthesis and characterization of PNA:

PNA (Table 4.1) was synthesized by Panagene Inc. (Daejeon, South Korea). PNA was conjugated with the cell-penetrating peptide (CPP) KFFKFFKFFK to facilitate its efficient uptake through the bacterial cell envelope [13]. The *rpsL* gene target shown in Table 4.2 was selected by a comparative analysis of the 5' regions adjacent to the start codon across multiple pathogenic *Mycobacterium* species. Sets that are colored similarly contain identical sequences.

Table 4.1 Cell-penetrating peptide-peptide nucleic acid (CPP-PNA) structure, gene target, and PNA bases.

Target gene	Description	CPP-PNA sequence[a]	PNA bases
rpsl	ribosomal protein S12 involved in protein synthesis	H-KFFKFFKFFK-O-ttgg**cat**ct-NH_2	9

[a] The synthetic PNA with CPP (KFF…), was linked by an ethylene glycol linker designated as 'O'; a glycol linker of nine atoms used to distance the hybridization portion of the molecule from the CPP was obtained from Panagene Inc., (Daejeon, South Korea). The bolded '**cat**' in the PNA indicates the complementary sequence to ATG start codon of the specified gene.

Table 4.2 Comparative analysis of the 5' regions adjacent to the start codon of two genes as potential targets for PNAs across multiple *Mycobacterium* species. Cells that are colored similarly contain identical sequence. Bold sequences indicate the 'start' codon. Sequences prepared by BLAST of the NCBI genome database.

***Mycobacterium* Genome**	***inh*A**	***rps*L (30s s12)**
smegmatis-model	ACCAA**ATG**AC	AC**ATG**CCAA
abscessus	GAATC**GTG**GC	AC**ATG**CCAA
avium	CGGAC**ATG**GC	AG**ATG**CCAA
paratuberculosis	CGGAC**ATG**GC	AG**ATG**CCAA
bovis Tokyo	CGCAC**ATG**AC	AG**ATG**CCAA
bovis Pasteur	CGCAC**ATG**AC	AG**ATG**CCAA
bovis AF2122/97	CGCAC**ATG**AC	AG**ATG**CCAA
tuberculosis KZN	CGCAC**ATG**AC	AG**ATG**CCAA
tuberculosis F11	CGCAC**ATG**AC	AG**ATG**CCAA
tuberculosis H37RA	CGCAC**ATG**AC	AG**ATG**CCAA
tuberculosis H37Rv	CGCAC**ATG**AC	AG**ATG**CCAA
tuberculosis CDC1551	CGCAC**ATG**AC	AG**ATG**CCAA
leprae Br4923	CGAAC**ATG**GC	AT**ATG**CCCA
leprae TN	CGAAC**ATG**GC	TG**ATG**CCAC

Inhibition of MAP growth with PNAs:

Determination of susceptibility of MAP to the *rpsl* PNA was assessed using a growth inhibitory assay in a microtiter plate. A frozen stock of MAP was diluted in #1507 Middlebrook broth to 1 x 10^5 CFU/mL and was incubated with indicated PNAs (volume = 20 µL) at the concentration of 20 µM in triplicates using a 96-well (total volume = 100 µL/well) low adhesion microtiter plate (cat# 3474; Corning). To prevent clumping of MAP, 3-2 mm sterile glass beads were added to each well. To reduce edge effects and evaporation, the entire perimeter of unused wells had 200 µL of #1507 Middlebrook broth added to help minimize the effect of evaporation. The plate was sealed with an adhesive lid (Microseal® B; Bio-Rad, Hercules, CA) and was incubated at 37^0C with constant shaking. Optical density at 550 nm (OD_{550}) was read once a week for 5 weeks. OD as a function of time was compared with untreated controls. At each time point post-treatment, the PNA-treated MAP was serially diluted and plated on #1507 Middlebrook agar plates. The plates were incubated at 37^0C for 3-4 weeks and CFUs were estimated by counting colonies and corrected for dilution to determine CFU/mL.

Statistical analysis:

All statistical analyses were performed with Student's two-tailed *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA). *P*-values of ≤ 0.05 were considered significant.

Results:

The anti-*rpsl* PNA complimentary to the start codon region encoded by the rpsL gene in MAP (Table 4.1) was evaluated. The anti-*rpsl* PNA was not toxic at 20 μM to J774A.1 murine macrophages (previously tested; data not shown).

The results of the PNA inhibition experiments on MAP strains cultured in microtiter plates are shown in Figure 4.1. The PNA treated wells showed significant inhibition of growth (measured by OD_{550} nm) as opposed to the untreated control wells. In Figures 4.2 and 4.3 the CFUs of both strains are plotted with and without the anti-*rpsl* PNA over the course of 5 weeks. These results are also summarized in Table 4.3. The figures represent a qualitative look at the results, while the table represents a quantitative look at the results. This is to clarify the specific values at each time point in the PNA treatment assays in the different strains of MAP. The anti-*rpsl* PNAs at 20 μM showed significant reduction of MAP growth in broth culture of both the MAP 43015 and 19698 strains after 1 week of treatment. In MAP strain 43015, there is about a log difference relative to the untreated culture at each time point, showing a bacterial inhibition of about 90%. However it is worth noting that at week 3, the log reduction is about 2, or close to 99% bacterial inhibition. In MAP strain 19698, there is significant bacterial reduction of about 1.5 logs, correlating to about 96% reduction, at all time points except in week 1. The negative controls (water alone) did not inhibit the growth of either strain of MAP nor did the CPP alone (data not shown).

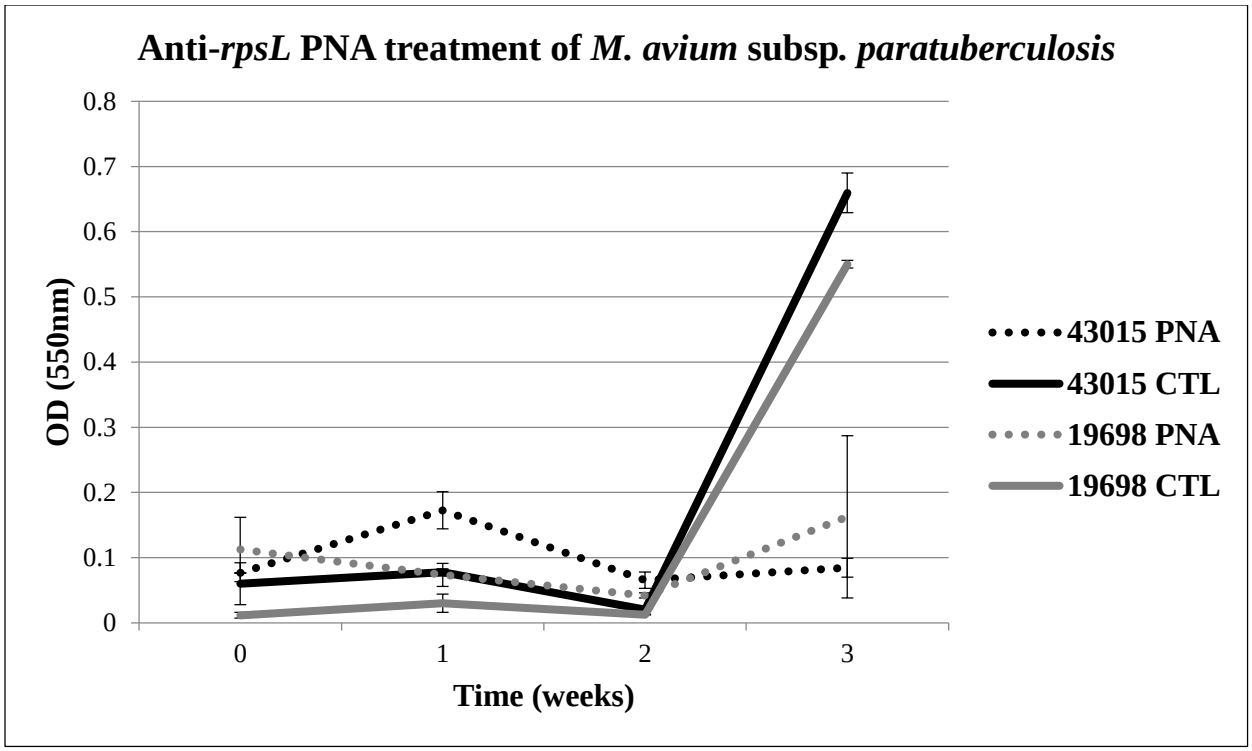

Figure 4.1 Anti-*rpsl* PNA treatment of *Mycobacterium avium* subsp. *paratuberculosis* strains 43015 and 19698. Grown at 37°C in #1507 Middlebrook medium enriched with 10% ODAC**.** Volume was 100 µL/well in a 96 well ultra-low binding microtiter plate. Plate was shaken before each read. Data shown is mean from duplicate cultures per treatment (20 µM PNA), error bars represent standard error of the mean.

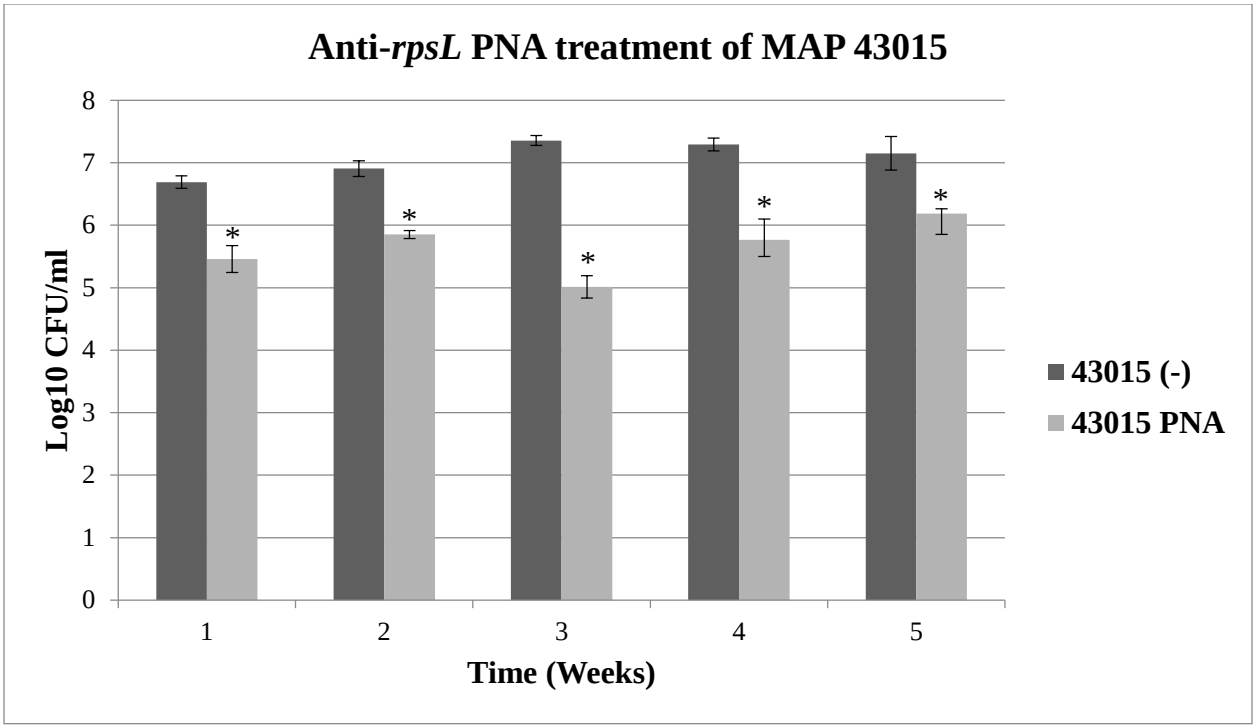

Figure 4.2 Anti-*rpsl* PNA treatment of MAP strain 43015. Grown at 37°C in #1507 Middlebrook medium enriched with 10% ODAC. 43015 (-) represents the no treatment control. Volume was 100µL in 96 well ultra-low binding microtiter plate, plate was continually shaken during growth. Data shown is mean from duplicate cultures per treatment (20µM PNA), error bars represent standard deviation (SD).

* Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

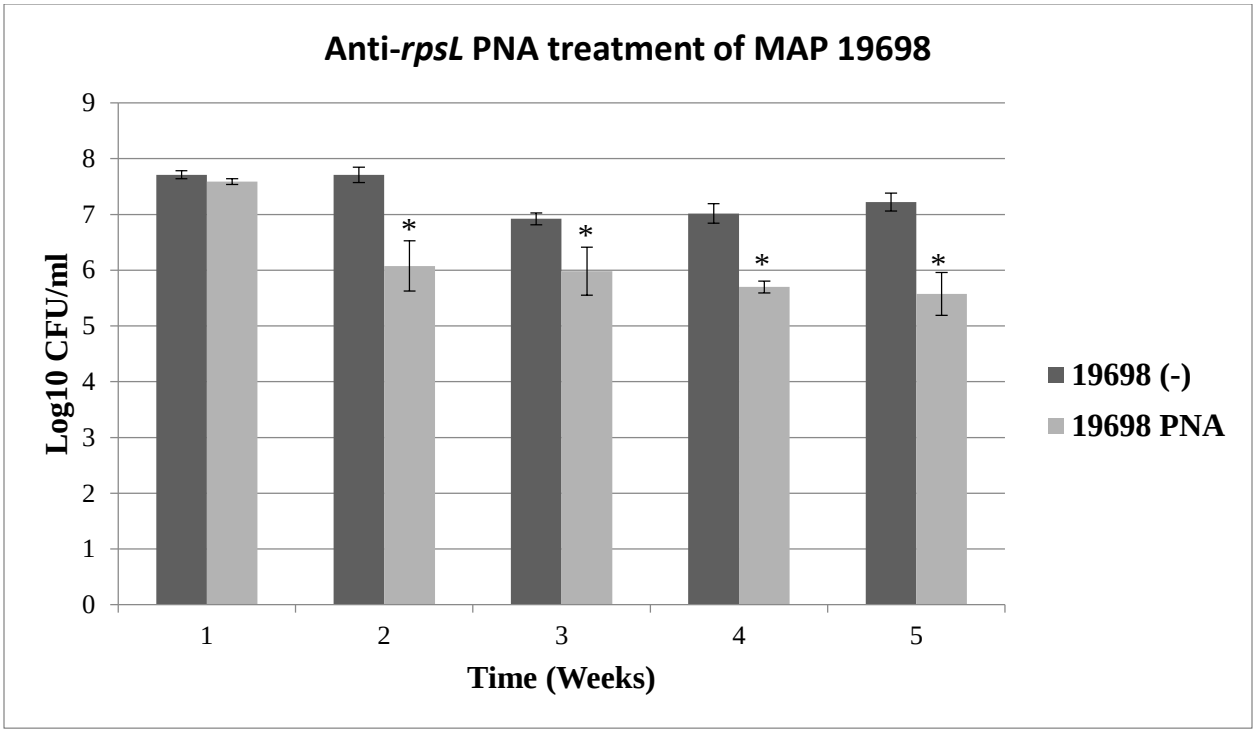

Figure 4.3 Anti-*rpsl* PNA treatment of MAP strain 19698. Grown at 37°C in Middlebrook #1507 medium enriched with 10% ODAC. 19698 (-) represents the no treatment control. Volume was 100µL in 96 well ultra-low binding microtiter plate; plate was continually shaken during growth. Data shown is mean from duplicate cultures per treatment (20µM PNA), error bars represent SD.

* Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Table 4.3 Susceptibility of MAP strains 43015 and 19698 to the anti-*rpsl* peptide nucleic acid (PNA) after 5 weeks in #1507 Middlebrook broth as compared to no treatment (No PNA, water alone).

	MAP 43015			MAP 19698		
Weeks Post Treatment	**Control (untreated) Log CFU/mL/well[a]**	**PNA (treated) Log CFU/mL/well[a]**	**Log reduction**	**Control (untreated) Log CFU/mL/well[a]**	**PNA (treated) Log CFU/mL/well[a]**	**Log Reduction**
1	6.69 ± 0.10	5.46 ± 0.22	1.23*	7.71 ± 0.07	7.59 ± 0.05	0.12
2	6.91 ± 0.12	5.85 ± 0.06	1.06*	7.71 ± 0.14	6.08 ± 0.45	1.63*
3	7.36 ± 0.08	5.02 ± 0.18	2.34*	6.92 ± 0.11	5.98 ± 0.43	0.94*
4	7.29 ± 0.10	5.77 ± 0.33	1.52*	7.02 ± 0.17	5.70 ± 0.11	1.32*
5	7.15 ±0.27	6.19 ± 0.07	0.96*	7.22 ± 0.16	5.58 ± 0.38	1.64*

[a] Mean ± standard error in at least 3 different wells.

* Values found to be significantly different ($P \leq 0.05$) from the control using a two-tailed Student's *t*-test.

Discussion:

Peptide nucleic acids (PNAs) designed to target specific genes create promising opportunities to control the growth of bacteria [3-7]. Since PNAs can be designed to target genes specifically involved in the synthesis of DNA, RNA, proteins, and lipid and carbohydrate metabolism, they represent a new means to target rate limiting metabolic steps. This pilot study demonstrated that the anti-*rpsL* PNA inhibits the growth of MAP in culture.

In this study, we focused on a PNA that plays a role in protein synthesis of MAP, the *rpsl* gene. It is worth noting, that although the anti-*rpsl* PNA was able to significantly reduce growth of MAP strain 43015 and MAP strain 19698 it was not able to completely inhibit growth (90%-99%). Based on growth measured indirectly from the OD values, there was a significant inhibition in growth of MAP strains. However when MAP cultures were serially diluted onto plates to determine CFUs, although there was a significant reduction in bacteria, there was still bacterial CFU. In MAP strain 43015 treatment with PNA, there was a reduction of about 2 logs at week 3; 99% bacterial reduction. However, by week 4, the CFU increased. Because the PNA was only added once, in the beginning of the experiment, there may not have been enough PNA to inhibit new MAP. This suggests that a higher concentration of the PNA may be needed for complete inhibition of bacterial growth over a long time frame (e.g. chronic infection), or re-addition of PNA in between the experiment's time course.

It is also important to note that treatment assays with MAP strain 19698, there was no significant reduction in week 1. This is different from the results observed in MAP strain 43015 infection and treatment with PNA at week 1. This is another example showing that although the

two MAP strains are genetically similar, there may be differences in uptake of the PNA or some other difference as yet to be defined.

As mentioned previously, MAP strain 43015 is a cell wall deficient (CWD) form of MAP [14]. Only *in vitro* has it been found to revert back to the cell wall containing (CWC) form. Because these PNA experiments were done in broth culture, and not intracellular, this could explain the difference in bacterial reduction and inhibition at week 1 between the two different strains of MAP. Due to the lack of cell wall in MAP strain 43015 the PNA may have been able to penetrate more easily as opposed to MAP strain 19698 with an intact cell wall. Thus a gene of interest for further PNA studies may be those involved in uptake or genes targeted towards cell wall synthesis.

An average bacterium contains about 3000-5000 genes. For example, MAP contains 4,350 genes [15], and its essential genes could be targeted for anti-sense therapy. The concentration examined in this study, 20 μM, required for inhibition of growth of MAP in culture was consistent with those reported for other bacterial species: *Mycobacterium smegmatis*, 10-40 μM [7]; and *Klebsiella pneumonia*, 10-40 μM [6].

The observation that a PNA was effective in limiting the growth of MAP in broth culture opens the possibility of treating intracellular infections of MAP within macrophages, and even treating MAP infections in animals. However, before one can move on to infection and treatment, further studies need to be done to check the potential of other PNAs in cultures against MAP strains 43015 and 19698, as well as with other strains of MAP. Once suitable PNAs are identified, the PNAs can be tested for their efficacy in the limitation of growth of MAP inside macrophages. Regardless of whether PNAs are discovered that are effective in limiting MAP growth *in vitro* and

in macrophages, there is still a need for development of a carrier system that will deliver the PNAs specifically to MAP-infected cells and tissue. This will not only decrease the amount of PNAs necessary for treatment, but also decrease the cost associated with treatment with PNAs. Because MAP primarily live and survive inside phagocytic cells, such as macrophages, targeting can be simplified [16]. Thus, a delivery system that enables targeting macrophages would allow for the greatest possibility of success in allowing the PNAs to deliver the therapeutic effect, as the CPP associated with the PNA is non-discriminatory as to the type of cell membrane it penetrates, whether it be mammalian or microbial [13].

Treatment with a PNA that has great potential in culture and *in vitro*, along with a delivery system that targets the area where the bacteria reside would potentially be the frontrunner in an alternative therapeutic regimen for inhibition of bacterial MAP growth, as well as serve as a treatment for MAP infections. However, anti-sense therapy is an expensive effort as it currently costs $800 to $1,000/100 nM of PNA from commercial sources. Although 100 nmoles was enough to perform this one single proof of concept experiment, it was not cost effective to proceed to treatment of MAP infected cell culture system and especially murine infection and treatment with the PNA. Further studies need to be conducted to determine the efficacy of this PNA at higher concentrations. With the cell culture model previously established, the anti-*rpsl* PNA may be tested in higher concentrations, as well as in long-term *in vitro* infection with MAP.

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Chapter 5

General Conclusions and Future Studies

Discussion and Conclusions:

The research presented in this dissertation focused on finding an effective treatment for early, chronic, and latent stages of MAP infection in a cell culture model. Paratuberculosis infections consist of three phases: acute, chronic, and latent [1, 2]. However, an acute infection in an animal can take years before it progresses to chronic infection where signs and symptoms are actually seen [3]. The incidence of Johne's disease in the USA has been growing and there is no effective vaccine or treatment and the current control measure have been minimally successful [4, 5]. As mentioned previously, the best prevention is to buy cattle from test negative herds, and to test your animals frequently to ensure that the herd is not infected. The prevalence of Johne's disease in dairy herds in the United States results in huge economic loss annually [6]. In addition to this, the human population is constantly exposed to *Mycobacterium avium* subsp. *paratuberculosis* (MAP), as a small percentage of MAP in milk of infected herds is able to survive pasteurization [7]. This could be a reason for the correlation with the increased incidence of Crohn's disease, however CD is still considered basically a disease associated with autoimmune disease [8-12].

Despite this public health importance, there is still no cure of MAP infections, and there was no effective laboratory cell culture model for testing therapeutics [4, 13]. The increasing prevalence of Johne's disease in cattle, sheep, and goats in the nation's food supply and potential linkage to the increased incidence of JD therefore has inspired efforts to explore alternative approaches to finding treatments for MAP infections [14]. In this research a long term cell culture

model to monitor MAP growth was developed. With this established cell culture model, the minimal bactericidal concentrations of anti-infectives against intracellular MAP in short term as well as long term studies were assessed.

Since MAP is an intracellular pathogen, there are challenges involved in research aimed towards treatment of Johne's and Crohn's disease [1]. There is no effective vaccine or treatment protocol and there is a lack of effective control measures as it is difficult to detect patients with MAP infections as clinical signs are slow in onset. All these challenges make it extremely difficult to test new therapeutics for such a chronic disease. Therefore, to date, only the symptoms associated with MAP infections are treated with medication [15-17]. The research conducted in this dissertation sought to provide a cell culture model to study MAP infections and thus provide insight into its potential application towards Johne's and Crohn's disease treatment.

Prior to the establishment of a long-term cell culture model, it was only possible to test the antibiotics clarithromycin, clofazimine, and rifampicin individually for their efficacy against intracellular MAP infections at the early stage of infection. In addition to testing the drugs individually, a combination of the drugs (clarithromycin, clofazimine, and rifampicin) were tested, along with other antibiotics including azithromycin, ethambutol, amikacin, isoniazid, ciprofloxacin, and levofloxacin. Not only were the individual drugs, as well as the combination of drugs, tested for their efficacy in treating MAP infections at the early stage of infection, their efficacies at different stages of infection were also examined. However, the combination of the three drugs was found to be more effective than free drugs alone at all stages of infection examined.

In addition to testing clarithromycin, clofazimine, and rifampicin separately, as well as in combination, a different therapeutic approach was tested as well. The efficacy of a peptide nucleic acid (PNA) against MAP was tested. Using a naturally occurring gene as a template, an anti-*rspl*

PNA that binds to the *rpsl* gene (ribosomal protein S12) found in MAP was synthesized commercially. The bacterial growth in broth study results showed that the anti-*rpsL* PNA was significantly effective in reducing MAP growth by 90-99% but unable to completely inhibit growth. Although PNAs could potentially be a different therapeutic approach to controlling MAP infections, further studies are needed to find a concentration where inhibition of MAP is achieved during long term infections. In addition, other PNAs need to be evaluated to determine if their efficacy is greater than that of *rpsL* for inhibition of MAP strains [22-28]. With the establishment of a long-term cell culture model, we are given the opportunity to test the drugs and PNAs in other stages of infection (acute, chronic, and late stages).

The development of this cell culture model is an important development as an alternative to complicated and expensive animal testing. This *in vitro* cell culture model could completely change the way therapeutics against paratuberculosis as well as other mycobacterial infections are tested. The cell culture model has the potential to speed up the process of testing therapeutics and make testing of new drugs less expensive. This model also has the potential as an alternative to animal testing.

From the dissertation results, the following conclusions can be made:

1. The lifespan of MAP infected J774A.1 macrophages can be extended from the typical 10-20 days to 45-60 days with the activation and supplementation of retinoic acid, vitamin D, and phorbol myristate acetate (RAVD plus PMA).
2. This cell culture model can be used to test therapeutics, individually as well as combinations of drugs, against MAP infections in different stages of infection, ranging from acute, chronic, and latent infection.
3. Peptide nucleic acids have the potential as effective therapeutics against MAP infections.

Future Directions:

The results of this dissertation research has provided a long term cell culture model that can be used to not only test other therapeutics, but can be used to reexamine drugs that have been previously tested but did not show effective results [16, 17]. Some drugs that were previously tested only in acute stages of infection because of macrophage viability, were not evaluated against chronic and latent infections with MAP. With this model, it may be possible to more accurately assess whether or not it is worth abandoning a specific drug, or if there is potential as a treatment at other stages of infection (i.e. rather than just acute infection). This cell culture model could be applied to other pathogens as well, particularly those defined as facultative intracellular pathogens (e.g. Mtb, Brucella spp). However further evaluation of the extended cell culture model will be needed to ensure that this model works with other bacterial species.

In our studies, a peptide nucleic acid was tested as a potential therapeutic against MAP infections. The anti-*rpsl* PNA was able to significantly inhibit growth of MAP in culture. To further test this PNA, further *in vitro* infection experiments are needed to be performed in our cell culture model. The established cell culture model could allow rapid assessment of PNAs, thus providing a more cost effective way to analyze the potential of these expensive PNAs. The examination of PNAs as a therapeutic approach, as opposed to conventional drugs, could potentially prevent the threat of and minimize the development of drug resistance as no resistance to PNAs have been found in bacteria so far. In addition, development of drug resistance can be minimized by a combination of PNAs targeting different essential genes at the same time.

Another application of this cell culture model is to assess Metadichol® as a bactericidal agent. In preliminary research conducted herein, the effectiveness of Metadichol® in increasing the longevity of cells was conducted (see Appendix 1). Although Metadichol® was able to significantly extend the lifespan of J774A.1 murine macrophages as compared to the control cells (no activation), the MAP load (amount of infected bacteria taken up by the macrophages) was less than that found in RAVD plus PMA activated MΦs. This suggested the possible efficacy of Metadichol® as a bactericidal agent. However, further tests will be needed to determine its bactericidal efficacy. So much more can be learned and discovered about potential alternatives to conventional and nonconventional therapeutics; the established cell culture model may be able to provide a means to do so without harming animals.

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Appendix 1

Effect of Metadichol® on the Longevity of J774A.1 Murine Macrophages Infected with *Mycobacterium avium* subsp. *paratuberculosis*

Abstract:

Metadichol® is a patented proprietary mixture of nanoparticles and nanoparticulate formulations of policosanol (NanoRx) whose overall dimensions are less than 100 nm in diameter and has been shown to have some very broad non-specific biological effects. With the addition of retinoic acid (RA), vitamin D (VD), and phorbol myristate acetate (PMA) in combination, the viability (lifespan) of MAP infected J774A.1 murine macrophages from the typical 10-20 days has been extended to 45-60 days. To test whether Metadichol's® broad non-specific biological effects had positive effects on longevity of a cell line, it was tested using J774A.1 macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Although Metadichol® was able to slightly extend the lifespan of the macrophages, there was less intracellular MAP (> 1 log) found in macrophages activated with Metadichol® than those activated with RAVD plus PMA. This reduction in intracellular MAP suggests that Metadichol® may have bactericidal effects and raises the possibility that Metachiol® could be used as a novel treatment of MAP infections.

Introduction:

M. avium spp. *paratuberculosis* (MAP) is a slow growing bacterium and is the causative agent of Johne's disease in ruminants and has been associated with Crohn's disease (CD) in humans [1-7]. Although most CD patients are found to have MAP in their intestinal tissues, the cause and effect issue is controversial as Koch's postulates have not been fulfilled to establish

MAP as the etiological agent for CD. There is currently no effective treatment for Johne's or Crohn's disease although there are medications to treat the symptoms of the bacterial infections.

Metadichol® is a trademarked mixture of nanoparticles and nanoparticulate formulations of policosanol less than 100 nm in overall dimension and shown to have some very broad non-specific biological effects (Patent: US 0215752 A1, US 8722093 B2). Metadichol® has been found to have an effect on the innate immune response. In addition, it has been shown to lower cholesterol as well as serum lipids with consequent blood pressure reduction. It has also been shown to have very broad effects on the host including its anti-oxidant effects, counteracting insulin resistance, raising vitamin C levels and regulating blood sugar levels [8]. It has also been shown to activate cells via Vitamin D receptors by activating many nuclear receptors [9]. As our laboratory had previously developed a long-term cell culture model using the addition of RAVD and PMA to activate macrophages. We wondered if Metadichol® would have any effect on the longevity of the macrophages as seen with the activation with RAVD plus PMA [10]. Metadichol® activated macrophages exhibited significantly less intracellular MAP over time. Instead of using Metadichol® as an activating agent for prolonging the lifespan of macrophages, it might be beneficial to examine Metadichol® as a therapeutic agent instead.

Materials and Methods:

Bacterial strains and cell lines:

For details about bacterial strains and cell lines used in the following experiments, please see Materials and Methods of Chapter 2.

Macrophage (MΦs) cell viability and cytotoxicity assay:

To determine any possibility of cell cytotoxicity caused by Metadichol® (NanoRX Inc., Chappaqua, NY), a tetrazolium compound-based cell viability assay was used [Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation MTS Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-caboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega Inc., Fitchburg, WI)]. Approximately 2 x 10^5 J774A.1 cells/well were resuspended in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) antibiotic, and seeded into a 96 well tissue culture plate. After 24 hr of incubation, medium was removed and cells were washed twice with 100 µL of phosphate buffered saline (PBS) (Corning Inc., Corning, NY). Metadichol® was diluted in DMEM medium containing 10% FBS at the concentrations of 5 mg/mL to 1 pg/mL, decreased by one half-fold, and then added to the specified wells. Following 24 hr of incubation, cells were washed twice with 100 µL of PBS to remove traces of Metadichol®. The number of viable cells was measured based on the amount of absorbance at 490 nm due to formazan product [11, 12]. Cell viability was also measured by trypan blue exclusion assay [13] and counting the number of viable and dead cells using a gridded haemocytometer.

Comparison of three different batches of Metadichol® (determining standardization of product):

To compare the three different batches of Metadichol® a cell viability and cytotoxicity assay was conducted on all three batches simultaneously. The same MTS assay protocol as stated above and in Chapter 3 was conducted.

Activation of J774A.1 macrophages:

Murine J774A.1 MΦs were maintained at 37°C and 5% CO_2 in DMEM with 10% fetal bovine serum. When needed, cells were expanded into individual 75 cm^2 flasks and activated with RAVD (1μM) (VD in cholecalciferol version) and PMA (10ng/mL=16nM) (Sigma-Aldrich, St. Louis, MO) [10] or Metadichol® (3 μg/mL) (NanoRx) for 3 days prior to infection with MAP. Metadichol® concentration used for activation was determined from the results based on the cytotoxicity assays: it was the highest concentration found not toxic.

Infection of MΦs with MAP for short-term and long-term growth curves:

For details about infection of MΦs with MAP for term growth curves, please see Materials and Methods of Chapter 2.

Statistical Analysis:

All statistical analyses were performed with Student's two-tailed *t*-test using Microsoft Excel (Microsoft Corp., Redmond, WA). *P*-values of ≤ 0.05 were considered significant.

Results:

The toxicity of Metadichol® on J774A.1 macrophages was measured using an MTS assay. Figure A1.1 shows that Metadichol® at 10 µg/mL and lower was not toxic. Values above 80% as compared to the control of 100% are considered non-toxic. It is important to note that at concentrations of 10 µg/mL down to 10 pg/mL, the cells were more metabolically active than the control cells. The cells that were found to be non-toxic maintained normal morphology (data not shown). Figure A1.2 shows that the production of Metadichol® is well standardized as the MTS assay results of three different batches showed similar results. Figure A1.2 also represents the cytotoxicity of Metadichol® in a range, from 100 µg/mL down to 1.56 µg/mL. Metadichol® showed no toxicity at concentrations 3.125 ug/mL and lower in all three batches.

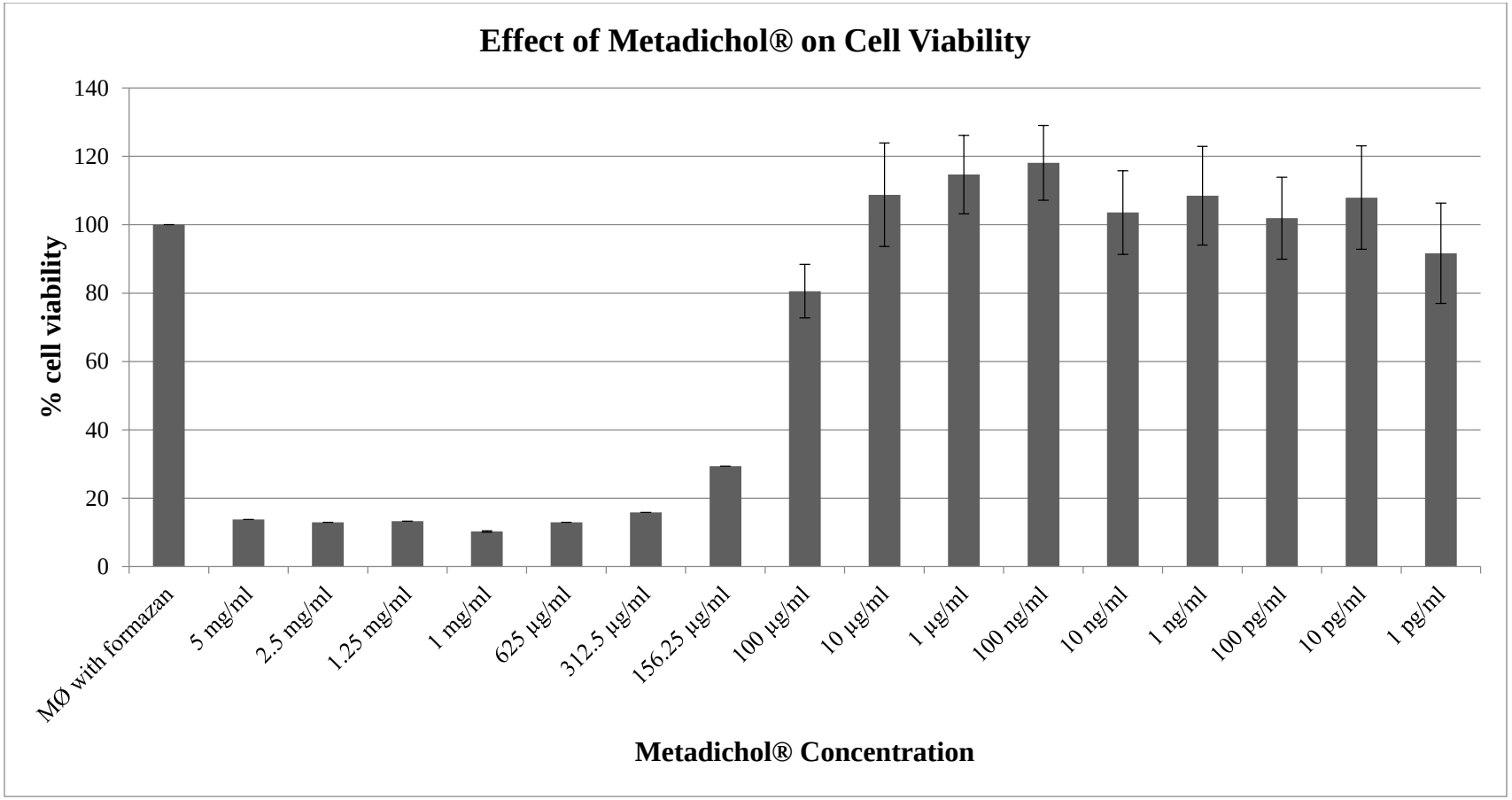

Figure A1.1 Cytotoxicity Assay of Metadichol®.
Metadichol® at concentrations ranging from 5 mg/mL down to 1 pg/mL, by a half-fold reduction were tested for cytotoxicity. The values are the mean ± SEM of triplicate wells.

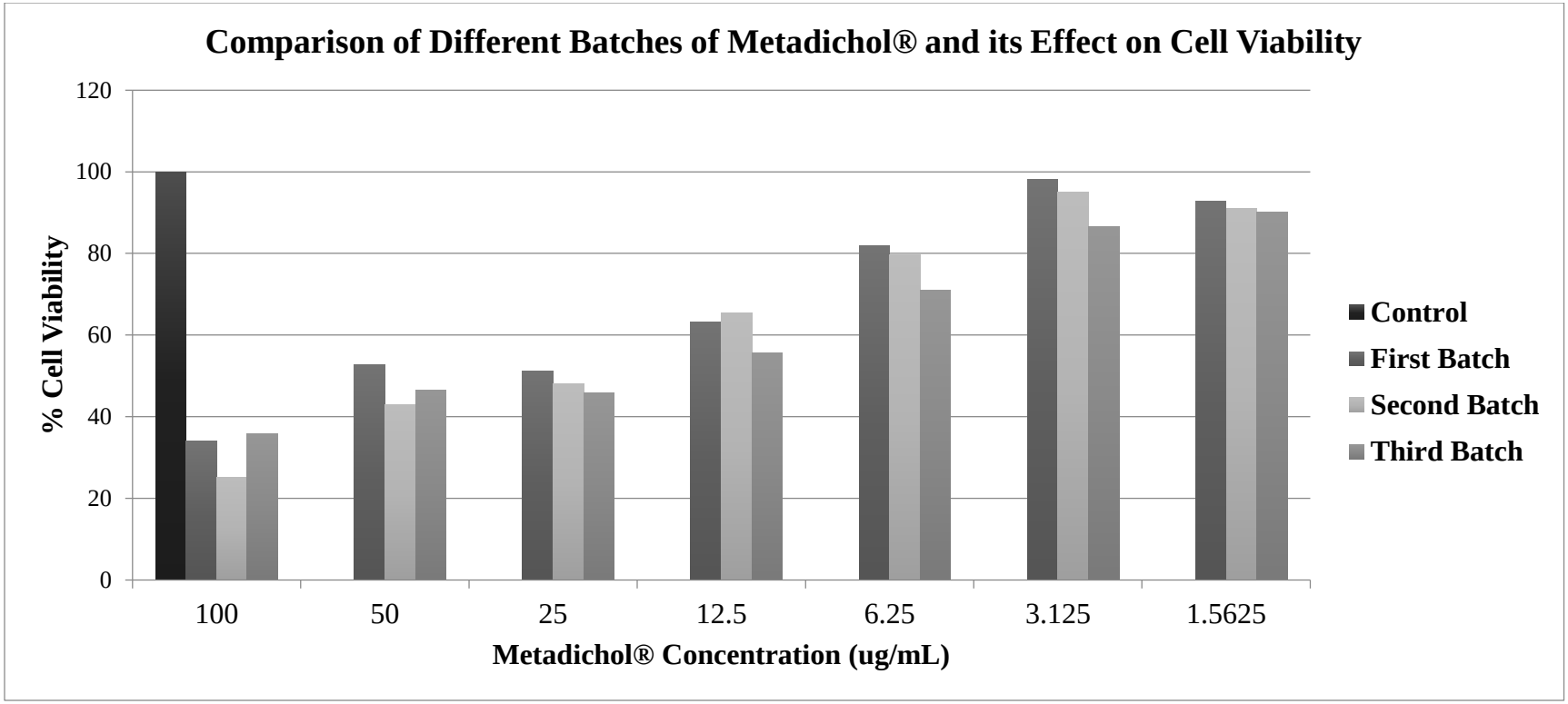

Figure A1.2 Cytotoxicity Assay for 3 Different Batches of Metadichol®
Metadichol® from three different batches was tested at concentrations ranging from 100 µg/mL down to 1.5625 µg/mL, by a half-fold reduction were tested for toxicity using the MTS assay. The values are the mean of triplicate wells.

Figure A1.3 demonstrates the effect of RAVD plus PMA and Metadichol® on MAP strain 19698 in infected J774A.1 murine macrophages. In this short-term growth curve, Metadichol® activated cells did not exhibit a significant increase in bacterial engulfment of MAP as compared to RAVD plus PMA activated cells. It was also noted that MAP strain 19698 was not able to survive in Metadichol® activated cells for as long as MAP strain 19698 in RAVD plus PMA activated cells. The results of this short-term growth curve, is also summarized in Table A1.1. The details of Figure A1.3 are shown in Table A1.1, to help clarify values and significance at each specific time point test.

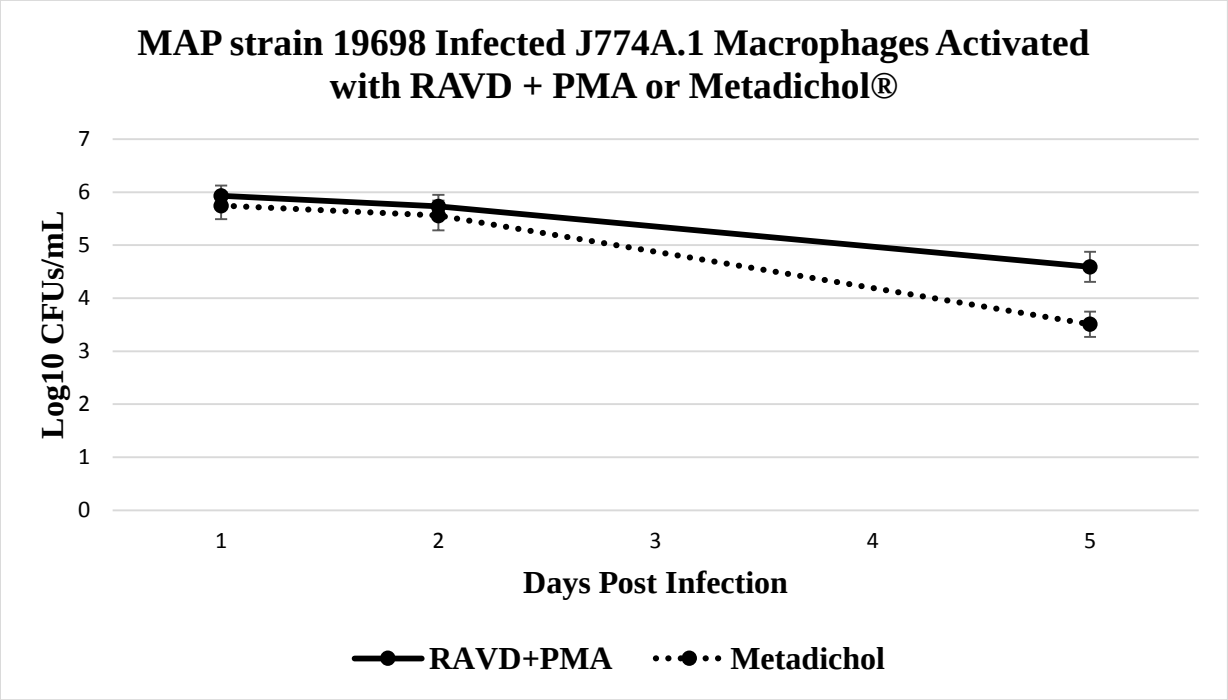

Figure A1.3 Effect of RAVD plus PMA or Metadichol® activation on MAP strain 19698 in J774A.1 murine macrophages.
Values are the mean ± SEM of three replicate wells.

Table A1.1 Survivability of intracellular MAP strain 19698 in macrophages activated with Metadichol® as compared to RAVD plus PMA in a short-term growth curve.

Days Post Infection	Log CFU/mL/well[a]	Log Reduction compared to RAVD plus PMA activated MΦs
1	5.75 ± 0.25	0.18
2	5.56 ± 0.28	0.17
3		
4		
5	3.51 ±0.24	1.98*

[a] Mean ± SEM in at least 3 wells.
*Values found to be significantly different ($P \leq 0.05$) from the RAVD activated cells using a two-tailed Student's *t*-test.

In Figure A1.4, the effect of RAVD plus PMA and Metadichol® on MAP strain 43015 in infected J774A.1 macrophages was evaluated. From this long-term growth curve, again Metadichol® did not show a significant increase in bacterial uptake as compared to RAVD plus PMA activated cells nor the control cells. Metadichol® activated cells were also noted to have lesser effect on the longevity of the macrophages. This is evidenced by the fact that there is less intracellular MAP, and therefore suggests less overall macrophages at a specific time point. Although not as many Metadichol® activated macrophages were able to survive for as long as RAVD plus PMA activated macrophages, there was still a significantly higher amount of macrophages than that compared to the control (no activation). Table A1.2 summarizes the findings of Figure A1.4 in a quantitative manner to better show the values at each specific time point, and compares intracellular MAP values between RAVD plus PMA activated cells against the control and against Metadichol® activated cells.

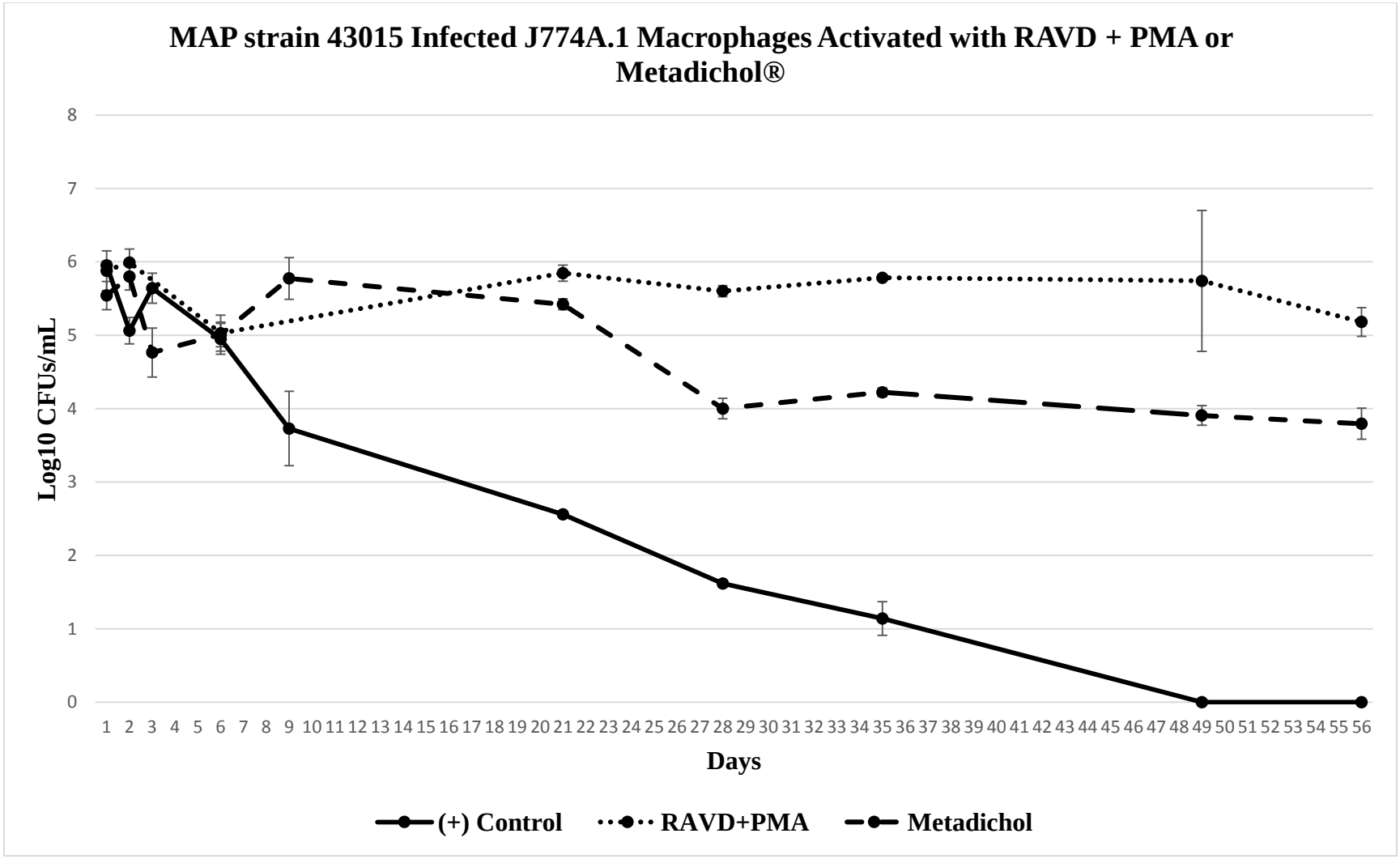

Figure A1.4 Effect of RAVD plus PMA or Metadichol® activation on J774A.1 murine macrophages infected with MAP strain 43015 as compared to the positive control (no activation).
Values are the mean ± SEM.

Table A1.2 Survivability of intracellular MAP strain 43015 in macrophages activated with Metadichol® as compared to RAVD plus PMA in a long-term growth.

The values that are shown in this table, are only those of specific time points where intracellular MAP was determined.

	RAVD plus PMA			Metadichol®	
Days Post Infection	**Log CFU/mL/well[a]**	**Log Difference from control**	**Log Difference from Metadichol®**	**Log CFU/mL/well[a]**	**Log Difference from control**
1	5.88 ± 0.27	- 0.07	+ 0.34	5.54 ± 0.19	- 0.41
2	5.99 ± 0.18	+ 0.93*	+ 0.19	5.80 ± 0.19	+ 0.74*
3				4.77 ± 0.33	- 0.87*
6	5.03 ± 0.25	+ 0.08	+ 0.02	5.01 ± 0.17	+ 0.06
9				5.77 ± 0.28	+ 2.04*
21	5.85 ± 0.11	+ 3.29*	+ 0.43*	5.42 ± 0.07	+ 2.86*
28	5.60 ± 0.08	+ 3.99*	+ 1.6*	4.00 ± 0.14	+ 2.39*
35	5.78 ± 0.03	+ 4.46*	+ 1.56*	4.22 ± 0.06	+ 3.08*
49	5.74 ± 0.96	+ 5.74*	+ 1.83*	3.91 ± 0.13	+ 3.91*
56	5.18 ± 0.20	+ 5.18*	+ 1.39*	3.79 ± 0.21	+ 3.79*

[a] Mean ± SEM in at least 3 wells.
+ Log value increase.
- Log value decrease.
*Values found to be significantly different ($P \leq 0.05$) from the control (not activated cells) using a two-tailed Student's *t*-test.

Discussion:

A long-term cell culture model has been established with the addition of RAVD plus PMA to murine macrophages as activating agents prior to infection with MAP. With the many effects Metadichol® has been shown to have, this study tested if it would be a good activating agent to increase the longevity of infected cells, as seen with RAVD plus PMA. However, the results suggest that Metadichol® may not be the most effective activating agent in enhancing the uptake of MAP into J774A.1 macrophages. This can be seen in both the short term and the long term growth curves (Figures A1.3 and A1.4 respectively). Metadichol® was found not toxic at concentrations of ≤ 10 μg/mL; further testing showed that it was not toxic at ≤ 3.125 μg/mL.

Metadichol® activated macrophages were able to survive significantly longer than the control cells (i.e. without activation). However specific live and dead cell counts were not determined. Due to the fact that there was less intracellular MAP over time, it is assumed that less cells were able to survive long-term. Future experiments need to be conducted to assure that our assumptions are correct. To check the actual bactericidal effects of Metadichol® on MAP, minimal inhibitory concentration assays, as well as minimal bactericidal concentration assays need to be conducted. Although Metadichol® did not seem to improve the longevity of the murine macrophage cells, it has potential as a therapeutic approach to treatment and control of MAP.

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