

Determination of Rickettsial Viability and Characterization of Macrophage Death in
Cultures of Gamma-Interferon-Treated Macrophages Infected with *Rickettsia prowazekii*

A Thesis submitted
to the Graduate School
Valdosta State University

in partial fulfillment of requirements
for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology
in the College of Arts and Sciences

December 2012

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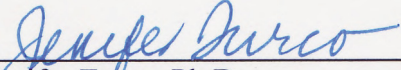
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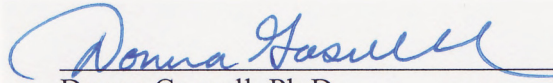
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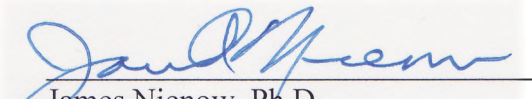


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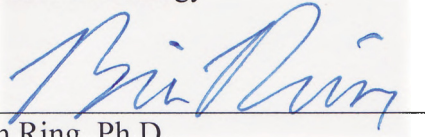
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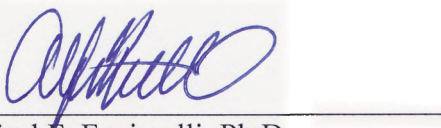


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ABSTRACT

Rickettsia prowazekii is an obligate intracellular, Gram-negative bacterium that is transmitted via body lice and is the causative agent of epidemic typhus. During an *R. prowazekii* infection, the bacteria target the endothelial cells lining small blood vessels, as well as macrophages. When cultured macrophage-like cells are pre-treated with gamma interferon (IFN- γ) and then infected with *R. prowazekii*, the macrophages die within a few hours (Turco & Winkler, 1984; Turco & Winkler, 1994). It is unknown if the rickettsiae are killed as the macrophages die and the mechanism of cell death seen in IFN- γ -treated, infected RAW264.7 cells has yet to be characterized. The research presented here had two objectives: to determine if the rickettsiae are viable after infection and death of IFN- γ -pre-treated, macrophage-like RAW264.7 cells and to determine if the osmotic lysis inhibitor polyethylene glycol (PEG) has the ability to decrease cell death in RAW264.7 cells treated with IFN- γ . Rickettsial viability was assessed by determining the ability of the bacteria to grow in untreated Vero cells after their release from dead IFN- γ -treated, infected RAW264.7 cells. The rickettsiae released from the dead IFN- γ -treated RAW264.7 cells were able to grow in Vero cells over 49 hours, indicating that they are viable after the death of their host cells. Sufficiently large PEGs can inhibit osmotic lysis, which contributes to macrophage death during oncosis and/or caspase-1- dependent pyroptosis. At 30 mM, PEGs \geq 2,000 daltons (nominal average molecular weight) have a protective effect against cell death in IFN- γ -treated, infected RAW264.7 cells, while PEGs \leq 1,450 daltons do not have an effect on RAW264.7 cell death.

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ACKNOWLEDGEMENTS

This research was supported in part by funds from Valdosta State University (VSU). It was conducted in a certified biosafety level 3 facility at VSU, with the approval of the VSU Institutional Biosafety Committee.

Many thanks to my committee members for their advice, guidance, patience, and encouragement throughout this process: Dr. Jenifer Turco, my committee chair; Dr. Donna Gosnell; Dr. James Nienow; and Dr. Brian Ring. I sincerely appreciate all of the help you have given me in becoming a better student and teacher. Thank you to Dr. Archana Bhasin and Dr. Robert Gannon for your guidance throughout my research process.

Thank you to all of my peers, especially Christine, J. Ryce, Matt, Lisa, and Amy, for challenging me to think outside of the “microbiology box”. To my parents, Bill and Marsha Huitt, thank you for all of your physical, emotional, and monetary support through this crazy journey. I could not have done this without you. To my brothers, Geoff and Kevin Huitt, thank you for teaching me how to be tough and how to love others without regret.

Finally, to Mark, thank you for accepting me as the “Bio nerd” that I am. I am so lucky to call you my husband. I could never thank you enough for being my shoulder to cry on, my person to bounce ideas off, my personal IT man, and the love of my life. I can only hope to support you in life the way you have supported me through this experience.

Chapter I

INTRODUCTION AND LITERATURE REVIEW

The genus *Rickettsia* includes several different species of bacteria, including the causative agents of epidemic typhus (*Rickettsia prowazekii*) and Rocky Mountain spotted fever (RMSF, *Rickettsia rickettsii*). These organisms are Gram-negative, rod-shaped bacteria (0.3-0.5 x 0.8-2 μm) with a complex life cycle that includes both arthropod and human hosts (Bechah et al., 2008; Rahman et al., 2010; Yu & Walker, 2005). *Rickettsia* spp. are obligate intracellular bacteria, so they must infect a eukaryotic host cell in order to survive and reproduce. The genus includes two groups of organisms: the spotted fever group and the typhus group. The spotted fever group includes *R. rickettsii*, *R. conorii*, and *R. akari*, while the typhus group includes *R. prowazekii* and *R. typhi*.

Rickettsia prowazekii preferentially targets the endothelial cells lining small blood vessels, but can also infect macrophages. *R. prowazekii* is the cause of epidemic typhus in humans, using the human body louse as its transmission vector. Symptoms associated with this disease are rapid onset of fever, headache, and rash (Bechah, Capo, Mege, & Raoult, 2008).

Some studies have shown that the proteins gamma-interferon (IFN- γ) and tumor necrosis factor- α (TNF- α) have anti-rickettsial properties and are very important in the clearance of rickettsial infections (Turco & Winkler, 1988; Feng, Popov, & Walker, 1994; Turco & Winkler, 1997; Walker, Olano, & Feng, 2001). IFN- γ and TNF- α are

proteins called cytokines, which allow immune cells to communicate with each other. These cytokines are also essential for innate and adaptive immunity against viruses and intracellular bacteria, including species of *Rickettsia* (Turco & Winkler, 1988; Feng, Popov, & Walker, 1994; Turco & Winkler, 1997; Walker, Olano, & Feng, 2001). When cultured macrophage-like RAW264.7 cells are pre-treated with IFN- γ 24 hours prior to infection with *R. prowazekii*, many of the RAW264.7 cells die within 4 hours after infection (Turco & Winkler, 1984; Turco & Winkler, 1988; Turco & Winkler, 1994). Currently, it is unknown what happens to *R. prowazekii* after the death of the IFN- γ -treated, host RAW264.7 cells. The mechanism of cell death in IFN- γ -treated, *R. prowazekii*-infected RAW264.7 cells is also unknown; understanding the mechanism will aid in understanding the disease pathology of epidemic typhus as well as interactions between *R. prowazekii* and its host cells.

This project had two purposes: to investigate the viability of *R. prowazekii* after the death of IFN- γ -treated, macrophage-like RAW264.7 cells and to establish the effect of polyethylene glycols (PEGs), osmotic lysis inhibitors, on the viability of IFN- γ -treated RAW264.7 cells after infection with *R. prowazekii*.

Purposes and Significance of Research

This study provides insight into how *R. prowazekii* damages macrophages and how it contends with the mammalian innate immune system. Death of mammalian cells has been intensively studied after infection with many pathogens, and several mechanisms of eukaryotic cell death, specifically apoptosis, autophagy, oncosis, and caspase-1-dependent cell death, are described below.

The first aim of this research was to determine whether the rickettsiae that infect

and kill IFN- γ -treated RAW264.7 cells remain alive after the death of the host cells. If the rickettsiae remain viable after the death of their macrophage-like host cells, it is possible that the rickettsiae are benefitting from the host cell death. By killing activated macrophages, the rickettsiae might have an increased ability to spread throughout the host organism. If the rickettsiae are not viable, there is a possibility that the macrophages are dying for the greater good of the host organism in order to prevent further spread of the bacteria. However, it is currently unknown if this particular form of macrophage cell death occurs *in vivo*.

The second part of this research focuses on efforts to prevent the death of IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* by using the osmotic lysis inhibitor PEG. In osmotic lysis, membrane damage results in an influx of water into the cell, causing the cell to burst. PEG has been shown to decrease cell death caused by both oncosis and pyroptosis (Sun et al., 2005; Pei, Turse, Wu, & Ficht, 2006; Liao & Mogridge, 2009). If the addition of PEG prevents the death of IFN- γ -treated, infected RAW264.7 cells, it is possible that osmotic lysis is contributing to cell death and that oncosis or pyroptosis is the mechanism of cell death occurring.

The remainder of this chapter includes a literature review with background information on the genus *Rickettsia*, including a discussion of the similarities and differences between the spotted fever group and the typhus group. *R. prowazekii* is discussed in detail, as well as effects of IFN- γ , TNF- α , and other cytokines on the viability of infected RAW264.7 cells. Four different types of cell death, including caspase-1-dependent cell death, are also described.

Literature Review

The Genus *Rickettsia*

The genus *Rickettsia*, a member of the α subgroup of Proteobacteria, includes two groups of disease-causing bacteria, the spotted fever group and the typhus group (Stothard, Clark, & Fuerst, 1994; Perlman, Hunter, & Zchori-Fein, 2006). These bacteria are members of the family *Rickettsiaceae*, which also includes the genus *Orientia* (Yu & Walker, 2005).

The members of both the spotted fever and typhus groups of the genus *Rickettsia* are transmitted to vertebrates via blood-sucking arthropods (Perlman et al., 2006). All members of this genus are obligate, intracellular Gram-negative bacteria. Some are believed to be potential biological weapons, including *R. prowazekii* and *R. rickettsii*. The spotted fever group includes the species *R. conorii*, *R. rickettsia*, and *R. akari* [the only member of the genus that preferentially infects and replicates in macrophages and monocytes (Kenyon & Pedersen, 1980)], while the typhus group includes *R. typhi* and *R. prowazekii*. There is also a third, ancestral group that includes *R. bellii* and *R. canadensis* (Yu & Walker, 2005), but this group shows early divergence in the genus.

In both epidemic typhus (*R. prowazekii*) and RMSF (*R. rickettsii*), the rickettsial pathogens multiply primarily in the endothelial cells of small blood vessels, but they can also infect and multiply in macrophages. Epidemic typhus is a louse-borne disease characterized by a rapid onset of a fever, headache, and rash (Bechah et al., 2008), while RMSF is a tick-borne disease that causes fever, headache, myalgias, and gastrointestinal symptoms; a rash will appear approximately three days after infection (Thorner, Walker, & Petri, 1998). Both chloramphenicol and tetracycline antibiotics are able to fight

epidemic typhus and RMSF infections.

Rickettsia Transport and Metabolism

Because rickettsiae are intracellular bacteria, they have evolved distinctive transport systems compared with free-living bacteria (Winkler, 1976; Winkler & Daugherty, 1984; Atkinson & Winkler, 1985; Winkler & Daugherty, 1986, Atkinson & Winkler, 1989; Winkler, Daugherty, & Hu, 1999; Tucker, Winkler, Driskell, & Wood, 2003; Audia & Winkler, 2006; Frohlich, Roberts, Housley, & Audia, 2010). These transport systems are specific for certain metabolites, including uridine 5'-diphosphoglucose, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), uridine monophosphate (UMP), guanosine monophosphate (GMP), adenosine monophosphate (AMP), S-adenosylmethionine, and dihydroxyacetone phosphate. These molecules must be transported across the membrane from the host cell to *Rickettsia* in order for the bacterium to complete certain metabolic pathways. The ability of rickettsiae to transport substrates from their host cell cytosol is likely related to the evolution of the small rickettsial genome, only 1.1 kilobase pairs and 834 open reading frames in *R. prowazekii* (Andersson et al., 1998; Audia & Winkler, 2006). The small genome of *R. prowazekii* is similar in size and function to mitochondrial DNA. Some of the genes that are similar between *R. prowazekii* and mitochondria involve transport of substrates across the membrane (Andersson et al., 1998).

Rickettsia species have two different transport systems for AMP and adenosine diphosphate (ADP)-ATP. The transport system used in *Rickettsia* to transport ADP and ATP has the typical characteristics seen in both prokaryotic and eukaryotic cells for carrier-mediated transport (Audia & Winkler, 2006; Dunbar & Winkler, 1997;

Schmitz-Esser et al., 2004; Winkler, 1976). This transport system is very specific and has an activation energy that is comparable to other exchange processes. The ADP/ATP exchange transport system seen in rickettsiae transports these nucleotides quickly, but exchange in one direction is dependent on exchange in the opposite direction (Winkler, 1976).

Species of *Rickettsia* have evolved the ability to transport AMP intact and phosphorylated, unlike other bacteria that have to dephosphorylate AMP prior to transport (Atkinson & Winkler, 1985). It is possible that the influx of AMP from the host cytoplasm is the source of adenine nucleotides used in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis in a rickettsial cell (Winkler, 1976; Atkinson & Winkler, 1985).

For rickettsiae, the prominent source of energy comes from the oxidation of glutamate, but glutamine and pyruvate are also used (Yu & Walker, 2005). Oxidation of glutamate drives electron transport, which is coupled with oxidative phosphorylation and the import of lysine and proline. *R. prowazekii* does not use glucose or glucose-6-phosphate, but it may use pyruvate similarly to mitochondria. Pyruvate dehydrogenase in *R. prowazekii* is dependent on coenzyme A, NAD, and thiamine pyrophosphate, similar to mitochondrial import of pyruvate from the cytoplasm (Yu & Walker, 2005).

Rickettsia prowazekii

Rickettsia prowazekii is part of the typhus group of the *Rickettsia* genus and causes epidemic typhus. *R. prowazekii* lacks intracellular mobility and it can multiply to great numbers without a large amount of damage to its host cell (Bechah et al., 2008). However, once the rickettsial burden becomes too much, lysis of the host cell membrane

will occur. If not controlled, epidemic typhus caused by *R. prowazekii* can result in massive damage to its host. Endothelial cell damage can lead to extensive vasculitis, edema, hypovolemia, and an increase in the inflammatory response of the host immune system as well as coagulation mechanisms (Bechah et al., 2008). These processes can potentially result in rashes on the skin, severe heart problems, brain lesions, seizures, coma, and, in some cases, death.

R. prowazekii has a complex lifecycle that involves both a human and an arthropod host (Bechah et al., 2008; Yu & Walker, 2005). First, an infected louse must bite and defecate onto an uninfected human. When the human host scratches the bite, the *R. prowazekii* in the fecal matter of the louse can penetrate the wound and begin to infect human host cells. The incubation period of epidemic typhus is 10-14 days, during which the bacteria will grow and proliferate in the cells they infect (Bechah et al., 2008). Once *R. prowazekii* gets into its human host, it induces potential host cells to engulf it via phagocytosis. The bacterium will then escape the phagosome created by phagocytosis and begin to divide in the cytoplasm. Once the rickettsial burden becomes too great for the host cell, it will burst and release rickettsiae to find new host cells, causing the symptoms associated with epidemic typhus. Uninfected lice will feed on an infected host, become infected and subsequently spread *R. prowazekii* to an uninfected host, leading to the spread of epidemic typhus. In addition to causing epidemic typhus, *R. prowazekii* kills 100% of the lice it infects, so the disease is not transmitted vertically from parent to offspring lice (Yu & Walker, 2005).

Two methods for the detection of *R. prowazekii* have been published that use Real-Time polymerase chain reaction (PCR) in order to detect specific genes (Jiang,

Temenak, & Richards, 2003; Stenos, Graves, & Unsworth, 2005). The basis for the first system is the gene *ompB*; this system is used for the detection of *R. prowazekii* but not other *Rickettsia* species (Jiang et al., 2003). OmpB is an outer-membrane protein and was first described in *R. prowazekii* (Roux & Raoult, 2000). In *R. prowazekii*, the OmpB surface protein represents about 10-15% of the total protein mass and it is the dominant surface protein antigen. The second, more recently described assay detects the *gltA* gene (Stenos et al., 2005). *GltA* is a citrate synthase gene that has been conserved in the *Rickettsia* genus. This system can be used to detect all *Rickettsia* species.

In the experiments described in the second chapter of this thesis, both the avirulent Madrid E and the virulent Breinl strains of *R. prowazekii* were used. Breinl and Madrid E have different abilities to grow in mouse macrophage-like RAW264.7 cells (Turco & Winkler, 1982; Turco & Winkler, 1994). The Breinl strain grows well in the macrophage-like cells, while the Madrid E strain shows restricted growth; this is similar to the growth pattern seen in human monocyte-derived macrophages (Gambrill & Wisseman, 1973). This similarity makes the RAW264.7 cells a good model system to investigate how *R. prowazekii* operates in the human innate immune system.

Ge et al. (2004) performed a comparative genome analysis between the Madrid E and Breinl strains. The genes *virB4* (virulence protein), *ftsK* (cell division protein), *lpxA* (UDP-N-acetylglucosamine acyltransferase), and *rfbE* (O-antigen ABC export system; ATP-binding protein) seem to be overexpressed in the Breinl strain. These genes are known to be virulence or virulence-related genes in other bacterial species, but their roles in *R. prowazekii* have yet to be determined. The results put forth by Ge et al. (2004) also indicate that there was a substantial deletion in the *rp084* gene in the Breinl strain. Upon

amplification of the *rp084* gene via PCR and reverse transcriptase-PCR, neither DNA nor RNA was present for this gene in the Breinl strain. When analyzing the sequence of the *rp084* gene, Ge et al. (2004) discovered that 1,812 base pairs were missing in the Breinl strain including part of the neighboring *rp083* gene, all of the *rp084* gene, and part of the non-coding regions between the two genes. The significance of this deletion is still unknown, but several other possible virulence genes were found that could account for the difference between the two strains. It is important that the differences between these strains are eventually understood in order to construct a better understanding of the virulence of *R. prowazekii*.

R. prowazekii may influence its host through ankyrin repeat-containing proteins, which have also been considered important proteins in other intracellular bacteria, including *Legionella pneumophila* and *Coxiella burnetii* (Pan et al., 2008; Bechah et al., 2010). An ankyrin repeat is a 33-residue motif consisting of two alpha helices separated by loops (Mosavi, Cammett, Desrosiers, & Peng, 2004). Ankyrin proteins are important for attachment of membrane proteins to a eukaryotic actin-based membrane skeleton. This linkage is very important for maintenance of plasma membrane integrity as well as anchorage of ion channels, exchangers, and transporters in prokaryotic and eukaryotic membranes. Though ankyrin repeats are found in all three domains of organisms (*Bacteria*, *Archaea*, and *Eukarya*), the majority of the ankyrin repeats are found in eukaryotic organisms (Mosavi, Cammett, Desrosiers, & Peng, 2004). Bechah et al. (2010), found that Rp22 and Breinl, virulent strains of *R. prowazekii*, had a split mutation in an ankyrin repeat-containing protein when compared to avirulent Madrid E and Erus strains, though the function of this mutation is unknown.

There is also evidence that post-translational modifications of proteins may play a role in *R. prowazekii* virulence, as they do in other parasite-host relationships, like *E. coli*'s relationship with its host cells (Andersson et al., 1998; Polevoda & Sherman, 2007; Turco & Winkler, 1994). Post-translational methylation of ribosomal proteins and translation factors may have an impact on virulence of *R. prowazekii* strains. In addition, the virulent strains of *R. prowazekii*, Breinl and Evir, have more methylated lysine than lysine on the surface of their cells than does the avirulent, Madrid E, strain, though the overall amount of lysine is similar among the three strains (Balayeva & Nikolskaya, 1973; Rodionov, Eremeeva, & Balayeva, 1991). There is a possibility that the amount of methylated lysine on the surface of the Evir and Breinl strains could affect their virulence when compared to the Madrid E strain. Through proteomic analysis, over-production of methyltransferase, particularly RP789 and RP527, was seen in virulent *R. prowazekii* strain Rp22 compared to avirulent strain Erus (Bechah et al., 2010).

Surface-exposed proteins may also play a role in virulence of *R. prowazekii* strains (Bechah et al., 2010). It seems as though a family of surface-exposed proteins, the Sca family, is critical in the interaction between *Rickettsia* and eukaryotic cells. The Sca family of proteins includes outer-membrane proteins rOmpA, rOmpB, and adhesin proteins Adr1 and Adr2. There is a possibility that nonsynonymous mutations, found by Bechah et al. (2010), in both virulent and avirulent strains of *R. prowazekii* in the *sca* family of genes (which code for surface proteins) may affect their function in the different strains, though their full function is still not clear.

Rickettsia prowazekii-Host Cell Interactions

Phagosomal Escape

One important characteristic of *Rickettsia* species is their ability to escape the phagosome upon entry into the host cell. The mechanism of this escape is currently unknown, but recent genetic work has highlighted four genes that are potentially responsible for membranolytic activity (Whitworth et al., 2005). Two of these genes, *pat1*, which codes for a patatin B1 precursor, and *tylA*, which codes for a hemolysin, were implicated in phagosomal escape, but are not expressed during peak phagosomal escape. Patatin is a glycoprotein found in plants that acts as a storage protein with lipase activity.

The phenotypes of the genes *pld* and *tylC* are detected in phospholipase D activity and hemolytic activity, respectively (Whitworth et al., 2005). Both *tylC* and *pld* are active during the time of phagosomal escape. Orthologs of *tylC* and *pld* have been found in *R. typhi*, *R. conorii*, and *R. rickettsii*; these findings could indicate evolutionary benefits of these genes. Although phospholipase D activity seems to be important for phagosomal escape by *R. prowazekii* as shown by Whitworth et al. (2005), a mutation in the *pld* gene that renders it nonfunctional does not affect the ability of *R. prowazekii* to escape the phagosome, as shown by Driskell et al. (2009). This is an indication that other factors are involved in phagosomal escape, possibly *tylC*, *tylA*, or *pat1*.

Phospholipase A₂ (PLA₂) is thought to mediate the entry of *R. prowazekii* into its host cells, as well as rickettsial escape from the phagosome (Winkler & Miller, 1982; Rahman et al., 2010; Housley, Winkler, & Audia, 2011). The role of PLA₂ is to release free fatty acids and lysophospholipids by hydrolyzing the *sn*-2 ester bond of phospholipids. There have been identified two open reading frames in the *R. prowazekii* genome with assumed patatin-like PLA₂ motifs (Andersson et al., 1998; Rahman et al.,

2010). Inhibition of PLA₂ activity by an irreversible inhibitor of both the calcium-dependent and calcium-independent PLA₂ leads to a decrease in PLA₂-mediated cytotoxicity by *R. prowazekii* and *R. typhi* in cultured Vero cells (Rahman et al., 2010).

Effect of Different Cytokines on the Host Cell-*R. prowazekii* Interaction

Cytokines are proteins secreted by cells of the immune system that allow cells to communicate with one another. Certain cytokines can change the interactions between *R. prowazekii* and cultured cells (Turco & Winkler, 1984; Turco & Winkler, 1994; Turco & Winkler, 1997). For example, IFN- γ and TNF- α have anti-rickettsial properties and prevent rickettsiae from growing in IFN- γ - and/or TNF- α -treated host cells (Turco & Winkler, 1988; Feng, Popov, & Walker, 1994; Turco & Winkler, 1997; Walker, Olano, & Feng, 2001). In addition, treatment of macrophage-like RAW264.7 cells with IFN- γ or with high concentrations of alpha/beta interferons (IFN- α/β) prepares the cells to be rapidly killed after infection with *R. prowazekii*. Though IFN- α/β (type I interferon) typically blocks the spread of viruses and prevents the growth of tumors, it does play other roles in the immune system. Type I interferon is also involved in clearance of bacterial infections and has been implicated in some autoimmune diseases such as lupus and arthritis (Trinchieri, 2010). IFN- α/β is structurally much different than IFN- γ (type II IFN) and it binds to a different receptor on the surface of its target cells (Schroder et al., 2004). Though the receptors for IFN- α/β and IFN- γ differ substantially, there is an overlap in the target genes and signaling pathways between the two types of IFN.

In vivo, macrophages are activated by IFN- γ secreted by activated T cells, but IFN- γ can be secreted by many different cells, including natural killer cells, B cells, and professional antigen-presenting cells (APCs) (Schroder et al., 2004; Janeway et al., 2005).

APCs are an integral part of the immune response due to their ability to ingest foreign bodies, break them up and present them, extracellularly, to passing T cells and B cells. Once a macrophage is activated, it produces nitric oxide (NO) and can kill ingested or intracellular bacteria through production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). IFN- γ also primes macrophages to respond to lipopolysaccharide (LPS), which is present in the cell walls of Gram-negative bacteria.

Death of IFN- γ -treated, macrophage-like RAW264.7 cells requires infection with viable rickettsiae and involves the loss of macrophage membrane integrity as evidenced by the permeability of the cells to trypan blue dye (Turco & Winkler, 1994). This is also true for RAW264.7 cells treated with high concentrations of IFN- α/β and infected with *R. prowazekii*. In addition, this cell death does not depend on the macrophage respiratory burst (Turco & Winkler, 1994; Turco, Keysary, & Winkler, 1989; Turco, Liu, Gottlieb, & Winkler, 1998). Respiratory burst in macrophages was not stimulated by infection with *R. prowazekii* in untreated or IFN- γ -treated RAW264.7 cells, based on the lack of macrophage production of superoxide and hydrogen peroxide (Turco & Winkler, 1989). This was further confirmed by measurement of glucose oxidation. Infection of untreated and IFN- γ -treated RAW264.7 cells with *R. prowazekii* did not increase the activity of the hexose monophosphate shunt, which is measured by the difference in carbon dioxide production from [1- 14 C]glucose and [6- 14 C]glucose. The killing of IFN- γ -treated RAW264.7 cells was inhibited in the presence of anti-rickettsial antibodies, added prior to infection. However, the cell death was not inhibited by antibiotics that had anti-RNA or anti-protein synthesis properties (Turco & Winkler, 1989).

Interestingly, if the macrophage-like cells are pre-treated with IFN- γ plus bacterial

LPS, there is a nitric oxide synthase (NOS)-dependent reduction in the number of RAW264.7 cells that are killed after infection with *R. prowazekii* and incubation for 8-10 hours (Turco & Winkler, 1994). Pre-treating the RAW264.7 cells with LPS and IFN- γ led to an increase in the production of nitrite [a degradation product of nitric oxide (NO)], along with a reduction in the number of cells killed after infection with *R. prowazekii*. When the NOS pathway was inhibited, RAW264.7 cell death increased again. This evidence showed that the NOS pathway is not responsible for IFN- γ -treated, infected macrophage death and further suggested that the NOS pathway might actually protect the macrophages by killing the rickettsiae. A later study determined that exposure of isolated *R. prowazekii* organisms to NO kills the rickettsiae and prevents them from infecting macrophage-like cells or fibroblast cells (Turco et al., 1998). When macrophage-like cells are pre-treated with IFN- γ and infected with *R. prowazekii*, the macrophages die, independent of NO production, within 4 hours after infection, but the mechanism of death is currently unknown. The purpose of this project is to investigate this killing activity.

There is also an increase in macrophage production of some proinflammatory cytokines, such as TNF- α , interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), in response to infection with *R. akari* and *R. typhi* (Radulovic et al., 2002). However, there remains no increase in detectable IFN- γ , interleukin-10 (IL-10), and interleukin-12 (IL-12).

Although Radulovic et al. (2002) used the macrophage-like P388D1 cell line, *R. akari*, and *R. typhi*, this study can provide insight about what is happening when macrophages are infected with *R. prowazekii*.

Proinflammatory cytokines increase the recruitment of other innate immune cells (dendritic cells and neutrophils) to the site of infection. This increase in immune cells can

aid in clearance of infections without the activation of the adaptive immune system.

When macrophages are infected with *R. prowazekii* they produce TNF- α , which could be important in the host defense against the rickettsial infection (Turco & Winkler, 1994). It is clear that TNF- α is a host anti-rickettsial defense because it restricts growth of *R. prowazekii* Madrid E and Breinl stains in mouse fibroblast L929 cells. Feng et al. (1994) showed that depletion of both IFN- γ and TNF- α (through neutralizing antibodies) led to overwhelming rickettsial disease and death in mice infected with *R. conorii*. Although TNF- α is produced upon infection of untreated and IFN- γ -treated RAW264.7 cells with *R. prowazekii*, neutralization of the TNF- α did not inhibit the killing of the IFN- γ -treated RAW264.7 cells (Turco & Winkler, 1994).

Treatment with IFN- γ restricts the growth of *R. prowazekii* in fibroblastic L929 cells and this restriction in growth is also seen in any surviving, IFN- γ -treated RAW264.7 cells (Turco & Winkler, 1984; Turco & Winkler, 1994). The rapid cell death seen in IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* is not observed in mouse fibroblast cells, although damage to fibroblast cells does occur at later times. The relationship between the mechanisms of host cell damage and restriction of rickettsial growth is currently unknown.

Although spotted fever rickettsiae (*R. rickettsii* and *R. conorii*) and typhus rickettsiae (*R. prowazekii* and *R. typhi*) differ, information about species in one group can provide insights about species in the other group. IL-1 β , TNF- α , and IFN- γ are involved in clearance of rickettsiae from the network of blood vessels they infect, and this clearance involves the production of nitric oxide by the infected endothelial cells (Feng & Walker, 1993; Jerrells, Li, & Walker, 1988; Li, Jerrells, Spitalny, & Walker, 1987).

When Swiss Webster mice were infected with *R. conorii* and injected with anti-IFN- γ antibody, the combination was lethal to the animals, which are typically resistant to this rickettsia (Li et al., 1987). These cytokines can also affect the permeability of endothelial monolayers, in the presence and absence of rickettsiae. In the absence of IL-1 β , TNF- α , and IFN- γ , *R. rickettsii* causes an increase in the permeability of the microvascular endothelium (Woods & Olano, 2008). However, in the presence of these cytokines, the permeability of a cultured endothelial monolayer is amplified. The cytokines alone, in low doses, also produce vascular permeability, which is greater than that observed with *R. rickettsii* infection alone. It is possible that the presence of these cytokines during *R. rickettsii* infection is of great benefit to the host because the cytokines aid the infected cells in eliminating the intracellular invader. However, the increased vascular permeability could be contributing to the pathology of RMSF due to the increased ability of *R. rickettsii* to circulate through its host.

Types of Cell Death

Some pathogens cause macrophages and/or other types of cells to die; cell death can occur by apoptosis, autophagy, oncosis, or caspase-1-dependent pyroptosis (Fink & Cookson, 2005). Additional forms of cell death such as mitotic catastrophe, anoikis, excitotoxicity, Wallerian degeneration, cornification, and others have also been described (Green, Ferguson, Zitvogel, & Kroemer, 2009; Kroemer et al., 2005; Melino, Knight, & Nicotera, 2005). Although cell death occurs in each instance, some of the processes that take place inside the cell differ substantially.

Apoptosis

Apoptosis is a common form of cell death. During this process, the cell begins to

disassemble itself through a series of proteases called caspases, although caspase-1 is not involved (Bergsbaken et al., 2009; Bergsbaken & Cookson, 2007; Fink & Cookson, 2005). Characteristics of apoptosis include DNA cleavage and condensation of the nucleus and cytoplasm; the plasma membrane remains intact. Apoptotic cells also package their contents into apoptotic bodies that can be engulfed by neighboring cells. The presence of the apoptotic bodies ensures that intracellular contents do not get released directly into the extracellular fluid. Because no cytokines are released from apoptotic cells, this mechanism is considered to be anti-inflammatory. *R. rickettsii* inhibits apoptosis in endothelial cells by activating the transcription factor NF- κ B (Clifton et al., 1998).

Autophagic Cell Death

Autophagic cell death, or type II cell death, has very different cellular characteristics than apoptosis (Fink & Cookson, 2005). Autophagy is an anti-inflammatory mechanism by which the cellular components are degraded within autophagic vacuoles called autophagosomes. Morphologically, autophagic cells have slightly condensed chromatin and vacuolization will occur. This is a highly regulated process that goes through a series of steps, beginning with the sequestering of cytoplasmic material in autophagosomes. In order for the contents to be degraded, the autophagosomes must fuse with lysosomes through the use of microtubules, which ensure fusion of the membranes. While a cell is undergoing autophagic cell death, it can be phagocytosed by neighboring cells before the mechanism is complete.

Autophagic events can occur during the development of an organism, as well as in times of starvation and in the event of disease or infection (Klionsky & Emr, 2000).

Once the autophagosomes begin to form, cytoplasmic components are sequestered and eventually degraded. Autophagy has been called a type II form of cell death because, in the presence of apoptosis inhibitors, autophagy can still cause cell death. It is not clear whether the complete degradation of cellular components, including the membrane, is the cause of the cell death or if autophagic cells are engulfed by neighboring cells before the process is complete.

Oncosis

Oncosis is a form of cellular death in which rapid nuclear and cellular swelling are observed, along with the disintegration of the membrane (Fernandez-Prada, Hoover, Tall, & Venkatesan, 1997; Sun et al., 2005). DNA fragmentation does not occur; a distinct feature that separates oncosis from apoptosis and autophagy. During oncosis, cellular membranes are disrupted; this causes increased permeability to ions and water. When more ions are able to enter the cell, more water enters the cell to compensate. This compensation is what causes the cellular swelling and will eventually cause the cell to burst. The effect of membrane pores that are formed during oncosis can be decreased using PEGs during infection with a microorganism that causes oncosis (Pei, Turse, Wu, & Ficht, 2006). PEGs are known osmotic lysis inhibitors that also have membrane repair and fusion capabilities (Ahkong, 1975; Davidson, 1977; Liao & Mogridge, 2009; Lentz, 1994; Lentz, 2007; Pei, Turse, Wu, & Ficht, 2006; Sun et al., 2005). In contrast, the presence of PEGs does not inhibit cell death resulting from high concentrations of hydrogen peroxide and staurosporine, both inducers of apoptosis (Sun et al., 2005). When J774.A1 macrophages are infected with *Brucella abortus*, oncosis of the macrophages can be seen with transmission electron microscopy (Pei, Turse, Wu, &

Ficht, 2006). When a 30 mM concentration of PEGs with molecular weights of 3,350 and 8,000 daltons are added during infection, the amount of cell death caused by the *B. abortus* infection decreases. This suggests that PEGs can be used in order to decrease cellular membrane disruption caused by formation of pores.

Oncosis not only leads to cellular and nuclear swelling, but depletion of the cellular energy stores also occurs (Fink & Cookson, 2005). This loss of energy stores can lead to failure of ionic plasma membrane pumps. Oncosis may also be regulated by an alteration of calcium levels inside the host cell. If calcium levels in the cell rise, a number of cysteine proteases can be activated that cause plasma membrane breakdown.

Oncosis is considered a necrotic type of cell death; some researchers use the term “necrosis” to refer to the morphology of the cell after it has died (Fink & Cookson, 2005). Necrosis can also be used to describe non-apoptotic cell death that tends to be accidental. In addition, it may be used to describe the presence of dead cells or tissues, but not necessarily the mechanism by which the death occurred.

Pyroptosis

The type of cell death known as pyroptosis is dependent on the activation of caspase-1 (Bergsbaken et al., 2009). This caspase-1-dependent form of cell death has been studied in macrophages infected with other bacteria such as *Salmonella*, *Shigella*, *Yersinia*, *Legionella*, and *Burkholderia* (Bergsbaken & Cookson, 2007; Bergsbaken, Fink, & Cookson, 2009; Brennan & Cookson, 2000; Fink & Cookson, 2006; Fink, Bergsbaken, & Cookson, 2008; Lilo, Zheng, & Bliska, 2008; Sun, Lu, Perviaz, Cao, & Gan, 2005; Suzuki et al., 2007). Caspase-1, also called interleukin converting enzyme, is a protease that cleaves the inactive forms of the cytokines interleukin-1 β (IL-1 β) and interleukin-18

(IL-18) in order to make them active.

Pyroptosis is considered pro-inflammatory since the activation and release of mature IL-1 β and IL-18 can recruit more immune cells, such as monocytes and neutrophils, to the site of infection (Bergsbaken et al., 2009). During caspase-1-dependent pyroptosis several distinct cellular features can be seen. Once IL-1 β and IL-18 are processed and activated, pores begin to form in the membrane of the macrophage. This pore formation has been correlated with the release of these cytokines (Bergsbaken et al., 2009; Fink et al., 2008; Lilo et al., 2008). The pores do not necessarily cause cell lysis, but lysis has been seen in cells in which caspase-1 has been activated (Fink & Cookson, 2006). At large enough sizes, PEGs can be used to decrease cell lysis that may be caused by any pores that have formed during pyroptosis (Liao & Mogridge, 2009; Sun et al., 2005). In the presence of 30 mM concentrations of these PEGs, damage to infected macrophages decreases, although IL-1 β is still released (Liao & Mogridge, 2009). IL-1 β is released to the environment as an inflammatory cytokine, which will recruit other immune cells to the site of infection and activate the secretion of other cytokines, like TNF- α (Netea, Simon, van de Veerdonk, Kullberg, Van der Meer, & Joosten, 2010). The processing of IL-1 β , however, does not play a role in caspase-1-dependent cell death (Liao & Mogridge, 2009).

Activation of caspase-1 is not completely understood, but there are a few details currently known. It is likely that the mechanism of activation of caspase molecules is conserved, so it is no surprise that the caspase recruitment domain (CARD) is involved in the activation of caspase-1 (Poyet, Srinivasula, Tnani, Razmara, Fernandes-Alnemri, & Alnemri, 2001; Razmara, Srinivasula, Wang, Poyet, Geddes, DiStefano, Bertin, &

Alnemri, 2002). Homologs of the CARD domain have been discovered in the nematode *Caenorhabditis elegans* as well as in mammalian species. A CARD domain is found in an adaptor molecule, Ipaf, that directly associates with the CARD domain of the caspase-1 precursor, procaspase-1. Ipaf is directly involved in the recruitment and activation of caspase-1 through a CARD-CARD interaction (Poyet et al., 2001; Srinivasula, Poyet, Razmara, Datta, Zhang, & Alnemri, 2002).

The CARD domain in the apoptosis-associated speck-like protein containing a CARD (ASC) recruits procaspase-1 to an inflammasome through a CARD-CARD interaction (Kanneganti, Lamkanfi, & Nunez, 2007). An inflammasome is a large complex of proteins containing NOD-like receptors (NLRs) (Franchi, Eigenbrod, Muñoz-Planilla, & Nuñez, 2010; Kanneganti, Lamkanfi, & Nuñez, 2007; Liao & Modridge, 2009). NLRs bind and recognize microbial structures called pathogen-associated molecular patterns (PAMPs) in the host cell cytosol. The NLRs will then induce assembly of the inflammasome. Once procaspase-1 is recruited to the inflammasome, a CARD-pyrin (PYD) protein bridges an association between procaspase-1 and the NLRs, allowing procaspase-1 to be activated into caspase-1 (Kanneganti, Lamkanfi, & Nuñez, 2007). Once caspase-1 is activated, it then cleaves pro-IL-1 β into the active form and IL-1 β is released from the cell.

DNA damage can also occur during caspase-1-dependent pyroptosis (Bergsbaken et al., 2009). DNA is cleaved by a caspase-1-activated nuclease that has yet to be identified. This cleavage, however, does not produce an “oligonucleosomal DNA fragmentation pattern” as is seen in apoptosis (Bergsbaken et al., 2009). Even though DNA damage occurs along with nuclear condensation, the nuclear membrane of the cell

remains intact. Destruction of the actin cytoskeleton of the cell also occurs during pyroptosis, although the mechanism has not been identified yet. Not only does activation of caspase-1 cause the previously mentioned cellular features, it also degrades cellular inhibitor of apoptosis protein (cIAP), and cleaves/inactivates metabolic enzymes including aldolase, triose-phosphate isomerase, glyceraldehydes-3-phosphate dehydrogenase, and α -enolase (Shoa, Yeretssian, Doiron, Hussain, & Saleh, 2007). Thus, the pro-forms of IL-1 β and IL-18 are not the only substrates of caspase-1.

Description of Thesis

The purpose of the present study was to investigate what is happening inside the host cell when *R. prowazekii* infects IFN- γ -pre-treated RAW264.7 cells. The first aim of this project was to determine if the rickettsiae were viable after the death of the IFN- γ -treated RAW264.7 cells. The second task was to determine if PEGs of various sizes could prevent death of the IFN- γ -pre-treated RAW264.7 cells infected with *R. prowazekii*. The materials and methods used to assess these two issues are described in detail in Chapter 2 and the results and conclusions drawn from those experiments are found in Chapters 3 and 4.

Chapter II

MATERIALS AND METHODS

Cell Cultures, Rickettsiae, and IFN- γ

The mouse macrophage-like RAW264.7 cell line and the mouse L929 cell line were both kindly provided by Jonathon Audia and Herbert Winkler (University of South Alabama, Mobile, AL). The Vero cell line was provided by Dallas Ingram (Tifton Veterinary Diagnostic and Investigational Laboratory, University of Georgia, Tifton, GA). RAW264.7 cells were grown in Dulbecco modified Eagle medium (DUL) plus 10% newborn bovine serum (NBS) (Life Technologies Corporation, Grand Island, NY). The Vero cells were grown in Eagle minimal essential medium (MEM) plus non-essential amino acids and 5% fetal bovine serum (FBS) (PAA Laboratories, Inc., Westborough, MA) and 5% NBS. Cells were grown in a humidified, CO₂ incubator at 35°C until they were ready to be harvested.

Rickettsia prowazekii strains Madrid E and Breinl were generously provided by Herbert Winkler and Jonathon Audia. Rickettsial suspensions were prepared from infected L929 cells. After harvest, the rickettsiae were suspended in sucrose-phosphate-glutamate (SPG) buffer (0.218 M sucrose, 3.76 mM KH₂PO₄, 7.1 mM K₂HPO₄, 4.9 mM potassium glutamate, pH 7.0) and samples were stored at -80°C (Bovarnick, Miller, & Snyder, 1950). Recombinant murine IFN- γ derived from *Escherichia coli* was a gift from Genentech Incorporated (South San Francisco, CA).

Preparation of Cell Cultures for Infection

RAW264.7 cells were scraped from the cell culture plate and centrifuged in a small amount of medium at $400 \times g$ for 7 minutes. Cells were resuspended in DUL with 5% NBS and counted in a Fuchs Rosenthal counting chamber at a 1/50 dilution.

RAW264.7 cell density was adjusted to 5.5×10^5 cells/mL for each experiment in DUL with 5% NBS. Vero cells were washed twice with a trypsin-EDTA solution (Life Technologies Corporation, Grand Island, NY) and incubated for several minutes in fresh solution until the cells were released from the cell culture plate. Cell suspensions were added to MEM with 5% FBS, and the mixture was then centrifuged at $400 \times g$ for 7 minutes. Cells were resuspended in MEM with 5% FBS and counted in a Fuchs Rosenthal counting chamber at a 1/10 dilution. Vero cell density was adjusted to 3.2×10^5 cells/mL for each experiment.

Cells were then planted in 24-well plates (0.4 mL per well) and incubated overnight. The 24-well plates had cover slips added to their wells prior to addition of the cells when the initial rickettsial infection or rickettsial growth was to be determined. Next, 0.1 mL of IFN- γ diluted in DUL with 5% FBS was added to the RAW264.7 cells as required. IFN- γ was used at a final concentration of 25 units/mL. DUL with 5% NBS or MEM with 5% FBS was added to all control RAW264.7 cells and Vero cells, respectively. After addition of IFN- γ , the cells were incubated for 22-25 hours in a CO₂ incubator at 35°C. After the allotted time, the cells were washed once with their respective medium (without IFN- γ) before infection or mock-infection.

Infection of Cultured Cells with *R. prowazekii* and Evaluation of Initial Infection and Rickettsial Growth

R. prowazekii was diluted in DUL with 5% NBS and 0.5 mL was added to each well for infection. Appropriate dilutions of each *R. prowazekii* preparation were determined prior to use in experiments as described in the next section. The 24-well plate was centrifuged at $500 \times g$ for 15 minutes at room temperature in order to increase the rate of infection, then it was incubated for 45 minutes at 35°C. To check the initial (1 hour) infection, some of the cover slips (which were predetermined) were then removed, air-dried, and stained by the Giménez method (Giménez, 1964). Forty-eight hours later, growth cover slips were removed and stained. The cells were examined with a light microscope (Giménez, 1964; Turco & Winkler, 1983). On each cover slip, the number of rickettsiae in each of 100 cells was counted and the percent infected cells was determined. The total number of rickettsiae in each of 100 counted cells was summed and divided by the percent infected cells to determine the number of rickettsiae per infected cell. If the total number of rickettsiae in a cell was more than 100, it was recorded as 100+, and a value of 100 was used in the calculations.

Determination of Rickettsial Dilutions

For each new preparation of rickettsiae used, an experiment was performed to evaluate its effectiveness in killing IFN- γ -treated RAW264.7 cells. Dilutions of each rickettsial preparation, such as 1/500, 1/1000, and 1/1500, were tested. The procedure for determining RAW264.7 cell death 4 hours after addition of *R. prowazekii* is described below. Initial infection (1 hour) and 49-hour growth were also evaluated for each dilution to determine which dilution would be appropriate for use in experiments.

Preparations were also checked for contamination in 49-hour growth wells. The highest dilution of rickettsiae that resulted in death of approximately 80% of the IFN- γ -treated RAW264.7 cells was generally used in subsequent experiments.

Giménez Staining Procedure

In preparation for staining, cells were fixed in 0.37% formaldehyde in 0.1 M sodium phosphate buffer (pH 6.8) for 20 minutes; then the cells were washed by sequentially transferring them to three Coplin jars of 0.1 M sodium phosphate buffer (pH 6.8) for several minutes per jar. Basic fuchsin stain (prepared by filtering a mixture of 4 mL basic fuchsin stock solution plus 10 mL 0.1 M sodium phosphate buffer, pH 7.45, through Whatman #1 paper) was then put on the cover slips. After 2 minutes, the slides were washed with deionized water and agitated in 0.8% malachite green oxalate for 20-30 seconds. The desired effect is bright red rickettsiae against a blue-green host cell background (Giménez, 1964; Wisseman, Waddell, & Walsh, 1974).

Determination of RAW264.7 Cell Death

Three hours after the initial cover slips were removed, macrophage death (cytotoxicity) was determined using trypan blue staining. For each well, 125 μ L of trypan blue stain was added to the existing medium and mixed. After incubation for 8-10 minutes, the medium plus trypan blue was removed and \sim 0.5 mL of Dulbecco's phosphate buffered saline solution without calcium or magnesium was added to the well to prevent drying. The cells that were stained blue by the dye were considered dead, while the clear cells were considered viable (Tennant, 1964). At least 100 cells were counted in each well (duplicates or triplicates, depending on the experiment) and the percent dead cells was calculated for each well.

Determination of Rickettsial Viability after RAW264.7 Cell Death

A sample of *R. prowazekii* Breinl in SPG was thawed and part of it was diluted and used to infect untreated and IFN- γ -treated RAW264.7 cells. The remainder was put on ice until needed as a control for later infection of Vero cells. Approximately 4 hours after addition of the rickettsiae to the RAW264.7 cells, macrophage death was checked in some wells. For other wells, the untreated (alive) and IFN- γ -treated (dead) RAW264.7 cells were scraped into the media from the bottoms of their respective wells and broken via bead-beating to release the rickettsiae. Two wells of either untreated or IFN- γ -treated cells were scraped and put into a 1-mL vial one-half full with 2.5-mm beads. The vial was then placed in the bead-beater and beaten at 4,800 rpm for 90 seconds (30 seconds beating, 30 seconds on ice, repeated 2 times). The RAW264.7 cell-rickettsia suspension was then used to replace the MEM with 5% FBS on some of the Vero cells. In addition, the *R. prowazekii* sample that had been held on ice was diluted and added to other Vero cells as a control. The Vero cells were centrifuged at $500 \times g$ for 15 minutes and incubated for 45 minutes after infection. Initial cover slips (removed 1 hour after addition of rickettsiae), as well as cover slips removed 48 hours later, were stained and evaluated for rickettsiae as described in the section above.

In some experiments, IFN- γ -treated, infected RAW264.7 cells were scraped into the media from the bottoms of their wells and vortexed in order to break up the cellular debris before infection of Vero cells. Two wells of IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* were put into a vial (same used for bead-beating) and the cell suspension was vortexed on a vortexer for a total of 90 seconds (30 seconds vortexing, 30 seconds ice, repeated 2 times). The rickettsiae-cell suspension was then added to Vero

cells, which were centrifuged and incubated as described previously. Initial cover slips (removed 1 hour after addition of rickettsiae), as well as cover slips removed 48 hours later, were stained and evaluated for rickettsiae as described previously.

PEG Protection Experiments

Untreated or IFN- γ -pre-treated RAW264.7 cells (described previously) were washed with DUL with 5% NBS. The RAW264.7 cells were then infected with *R. prowazekii* (either strain Madrid E or strain Breinl) in the presence of various concentrations of sucrose (control) or PEGs of different nominal average molecular weights (PEG 400, 1450, 2000, 4000, or 8000) (Sigma Chemical Co., St. Louis, MO; Alfa Aesar, Ward Hill, MA; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (described previously by Sun et al., 2005). Sucrose or PEGs and *R. prowazekii* were diluted in DUL with 5% NBS.

In some experiments, cover slips were removed and the cells were stained by the Giménez method 1 hour after addition of the rickettsiae. After incubation for an additional 3 hours, viability of the RAW264.7 cells was determined in some wells. In other wells, the cells may have been washed (depending on the experiment) and the appropriate medium was added to the washed wells. These cells were then either evaluated for viability after an additional 3 hours, or they were incubated for 45-48 hours and cover slips were removed and stained to determine the growth of *R. prowazekii*.

Statistical Analyses

Means and standard deviations (SD) were calculated using at least two determinations per experiment for at least two independent experiments, unless otherwise noted. For each set of experiments, a one-way analysis of variance (ANOVA) and

multiple comparisons were done using the Assistant in Minitab 16 Statistical Software (Minitab Inc., State College, PA; Minitab Inc., 2010). Differences between means were considered statistically significant if p was less than 0.01 or 0.1, depending on the data set.

Chapter III

RESULTS

Introduction

Rickettsial viability was determined by the bacteria's ability to grow in Vero cells after being released from untreated or IFN- γ -treated RAW264.7 cells. There were few or no rickettsiae present in the culture media either 1 hour or 4 hours after infection with *R. prowazekii* in both untreated or IFN- γ -treated, infected RAW264.7 cells, indicating that nearly all the bacteria were associated with the cells after one hour. The rickettsiae released from untreated and IFN- γ -treated RAW264.7 cells were able to grow in Vero cells, indicating that the bacteria were viable after the death of their host cells. Releasing the *R. prowazekii* from IFN- γ -treated RAW264.7 cells via vortexing or bead-beating made no difference in the amount of bacterial growth seen in Vero cells.

The second set of data suggests that PEG 2000 and PEG 4000 inhibit osmotic lysis in RAW264.7 cells that have been pre-treated with IFN- γ and infected with *R. prowazekii*. It was also established that the protection of PEG was concentration dependent and that PEGs $\leq 1,450$ daltons would protect against cell death at high enough concentrations. PEG must be present in the culture medium in order to provide protection for IFN- γ -treated RAW264.7 cells infected with *R. prowazekii*. The data collected with PEGs suggest that either oncosis or caspase-1-dependent pyroptosis is responsible for the death of IFN- γ -treated RAW264.7 cells infected with *R. prowazekii*.

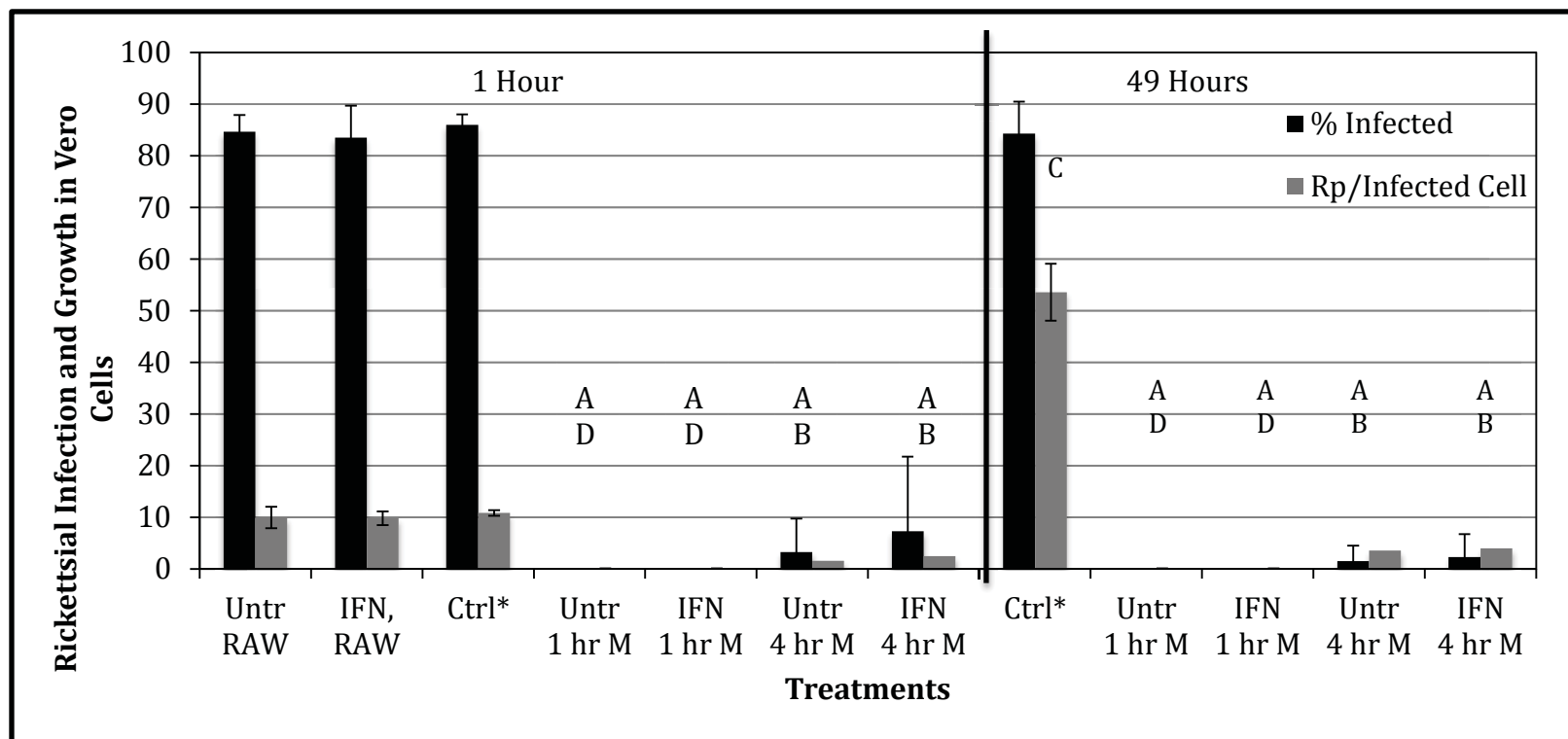
Rickettsial Viability After Death of IFN- γ -Pre-treated RAW264.7 Cells

In the first part of this thesis, it was important to establish whether the rickettsiae were being killed as they infected and subsequently killed the IFN- γ -pre-treated RAW264.7 cells. Whether any of the rickettsiae that had been added to the RAW264.7 were present in the culture medium after 1 hour or 4 hours was investigated. The culture media were removed from untreated and IFN- γ -treated, infected RAW264.7 cell cultures at each time and added to Vero cells. For the media samples collected at 1 hour, very few or no rickettsiae were observed in the initial (1 hour) Vero cell cover slips and no rickettsial growth was seen after 48 additional hours (Figure 1, Table 1). Percent infected cells in Vero cell cultures that were directly infected with rickettsiae that had been held on ice (controls) differed significantly from percent infected cells in Vero cell cultures that were incubated with media from infected RAW264.7 cells (Figure A2). These findings suggest that most of the rickettsiae were associated with the RAW264.7 cells when the culture medium was removed; however, the possibility that some rickettsiae might have remained in the culture medium after centrifugation and subsequently died during the 45-minute incubation period cannot be excluded.

Similar results were obtained for the media samples removed from the infected RAW264.7 cells at 4 hours (Figure 1, Table 1). To assess rickettsial growth, a one-way ANOVA was used to compare the numbers of rickettsiae per infected cell (Figure A1). The numbers of rickettsiae per infected cell that were observed in the control Vero cells at 1 hour (10.8) and 49 hours (~54) differed significantly; this finding indicates that the rickettsiae grew well in the control Vero cells. In contrast, for the few infected cells observed in the Vero cell cultures incubated with culture media from infected RAW264.7

cells, the numbers of rickettsiae per infected Vero cell were very low (6.2-9.8 at 1 hour and 14.0-15.9 at 49 hours) (Figure 1, Table 1). Overall, these findings suggested that even after the IFN- γ -treated, rickettsia-infected RAW264.7 cells died, most of the rickettsiae were associated with the RAW264.7 cells when the culture medium was removed. From these results, it was clear that it would be necessary to actually disrupt the infected RAW264.7 cells in order to release any viable rickettsiae.

Figure 1: Percent Infected and Rickettsiae per Infected Cell in Vero Cell Cultures Incubated with Culture Media Collected from *R. prowazekii* Breinl-Infected RAW264.7 Cell Cultures.



Details are given in the legend for Table 1. Untreated RAW264.7 cells (Untr RAW) and IFN- γ -treated RAW264.7 cells (IFN RAW) were stained 1 hour after addition of *R. prowazekii* Breinl. Ctrl*, Vero cells infected with *R. prowazekii* held on ice; M, culture media from RAW264.7 cells infected with *R. prowazekii* Breinl; Rp, *R. prowazekii*. One-way ANOVAs showed significant differences between treatments. ^A Denotes a significant difference between the percent infected in Vero cells incubated with the culture media and the Vero cells directly infected with *R. prowazekii* ($p < 0.01$). ^B Indicates that Rp/infected cell represents the value for one culture; no infected cells were observed in the remaining 3 cultures for the particular treatment. ^C Denotes a significant difference between the Rp/infected cell in Vero cells at 1 hour and 49 hours ($p < 0.01$). ^D Denotes cultures in which no infected cells were observed.

Table 1: Percent Infected and Rickettsiae per Infected Cell in Vero Cell Cultures Incubated with Culture Media Collected from *R. prowazekii* Breinl-Infected RAW264.7 Cell Cultures.

Sample Used for Infection	Time of Sample Collection	Cell Line Infected and Treatment	Time of Vero Cell Staining	% Infected		Rickettsiae/Infected Cell	
				Mean	SD	Mean	SD
<i>R.p.</i> ^a . Breinl	NA ^b	RAW ^c , untreated	NA	84.7	3.2	10.0	2.1
<i>R.p.</i> Breinl	NA	RAW + IFN- γ	NA	83.5	6.2	9.8	1.3
<i>R.p.</i> Breinl, Ice^d	NA	Vero, untreated	1 h PRV^e	86.0	2.0	10.8	0.5
Medium, untreated RAW	1 h PRR ^f	Vero, untreated	1 h PRV	0.0 ^A	0.0	NA	NA
Medium, RAW + IFN- γ	1 h PRR	Vero, untreated	1 h PRV	0.0 ^A	0.0	NA	NA
Medium, untreated RAW	4 h PRR	Vero, untreated	1 h PRV	3.3 ^A	6.5	6.2 ^B	NA
Medium, RAW + IFN- γ	4 h PRR	Vero, untreated	1 h PRV	7.3 ^A	14.5	9.8 ^B	NA
<i>R.p.</i> Breinl, Ice	NA	Vero, untreated	49 h PRV	84.3	6.1	53.6^C	5.5
Medium, untreated RAW	1 h PRR	Vero, untreated	49 h PRV	0.0 ^A	0.0	NA	NA
Medium, RAW + IFN- γ	1 h PRR	Vero, untreated	49 h PRV	0.0 ^A	0.0	NA	NA
Medium, untreated RAW	4 h PRR	Vero, untreated	49 h PRV	1.5 ^A	3.0	14.0 ^B	NA
Medium, RAW + IFN- γ	4 h PRR	Vero, untreated	49 h PRV	2.3 ^A	4.5	15.9 ^B	NA

A sample of rickettsiae was thawed and part of it was diluted and added to untreated and IFN- γ -treated RAW264.7 cells. The remaining portion was held on ice for later use. Some RAW264.7 (RAW) cells were stained one hour after addition of the rickettsiae (shaded rows). After incubation of some of the remaining RAW cells for about 3 additional hours, RAW cell viability was evaluated ($1 \pm 1\%$ and $83 \pm 4\%$ dead in the untreated and IFN- γ -treated cultures, respectively); and RAW cell culture media were removed and added to Vero cells. The rickettsiae that had been held on ice were also diluted and added to Vero cells. Vero cells were stained 1 hour and 49 hours after these additions. One-way ANOVAs showed significant differences between treatments. ^ADenotes a significant difference between the percent infected in Vero cells incubated with the culture media and the Vero cells directly infected with *R. prowazekii* ($p < 0.01$). ^B Indicates that Rp/infected cell represents the value for one culture; no infected cells were observed in the remaining 3 cultures for the particular treatment. ^C Denotes a difference between the Rp/infected cell in Vero cells at 1 hour and 49 hours ($p < 0.01$). ^a*R.p.*, *R. prowazekii*; ^bNA, not applicable; ^cRAW, RAW264.7 cells; ^dIce, Rickettsiae that were held on ice before dilution and addition to Vero cells; ^ePRV, after addition of rickettsiae or medium to Vero cells; ^fPRR, after addition of rickettsiae to RAW264.7 cells.

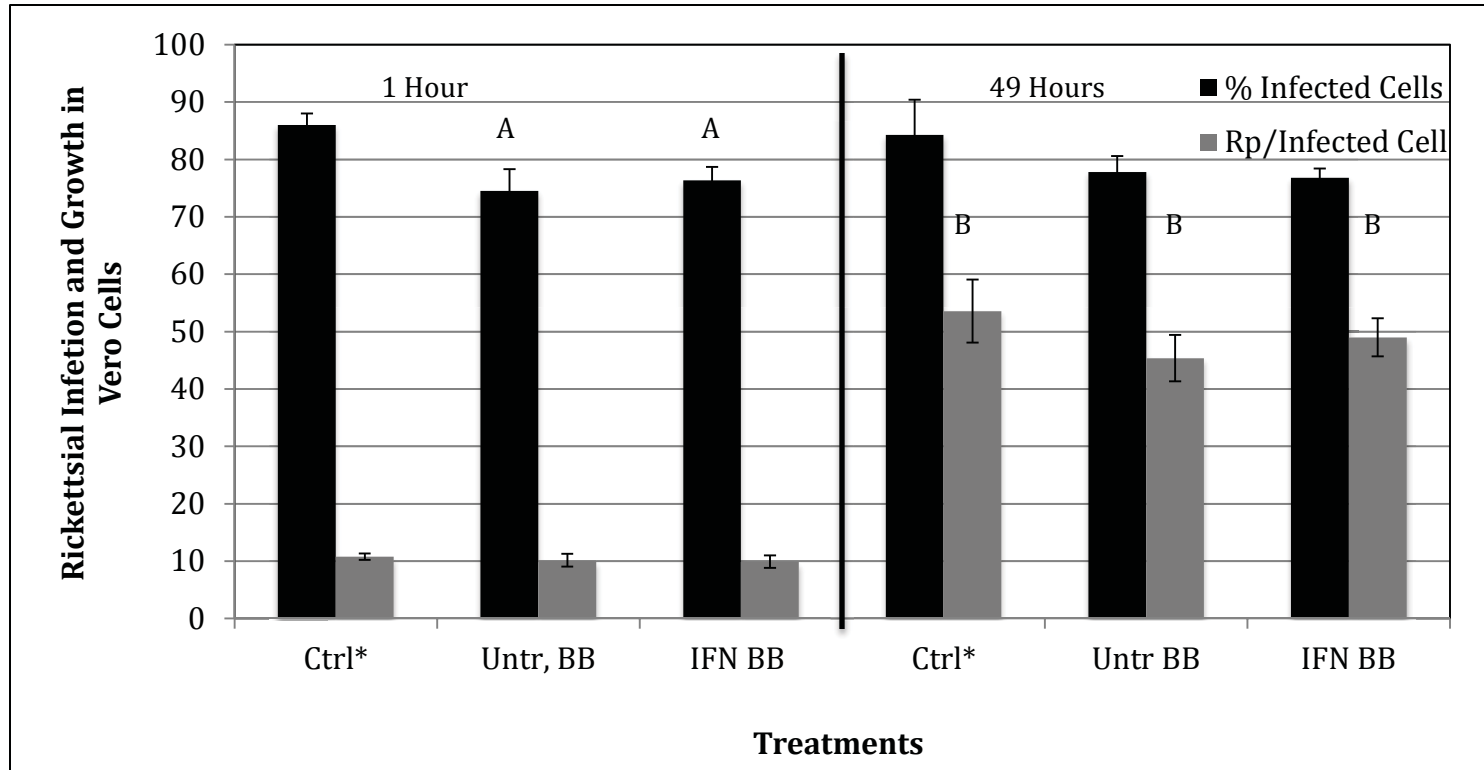
Since very few rickettsiae were present in the culture media at the 1-hour and 4-hour time points, the RAW264.7 cells were scraped off the bottoms of the their wells and combined with the media before testing for viable rickettsiae. Before being added to Vero cells, these samples were bead-beaten in order to break any intact macrophages. The rickettsiae were then added to Vero cells and allowed to grow over a 49-hour period. The rickettsiae recovered from both untreated and IFN- γ -treated RAW264.7 cells infected and grew well in Vero cells, increasing from ~10 rickettsiae per infected cell initially (1 hour) to ~45-49 rickettsiae per infected cell after 48 additional hours (Figure 2, Table 2). These data indicate the rickettsiae remain alive after the death of their IFN- γ -pre-treated RAW264.7 host cells. Vero cells were also directly infected with *R. prowazekii* as a positive control (Figure 2, Table 2); in these cultures the number of rickettsiae increased from ~11 at 1 hour to ~54 at 49 hours.

One-way ANOVAs and multiple comparisons were used to evaluate the numbers of rickettsiae per infected Vero cell and the percent infected cells for the different treatments (Figures A3 and A4). As expected, for each treatment, the rickettsiae per infected cell at 49 hours was significantly greater than the number at 1 hour; in addition, the numbers of rickettsiae per infected cell did not differ significantly between treatments at 1 hour or 49 hours (Figures 2 and A3, Table 2). However, the analysis did reveal a statistically significant difference between the percent infected Vero cells for the cultures that were directly infected (~86%) and the cultures that were infected with rickettsiae from bead-beaten RAW264.7 cells (~75-76%) at 1 hour after infection, but the size of the difference was small (Figures 2 and A4, Table 2). The results from similar, additional experiments (Table 3, Figure A6) showed a similar trend. At the 1-hour time point, the

percent infected Vero cells for cultures that were directly infected was ~82%, while the percent infected for cultures infected with rickettsiae from bead-beaten RAW264.7 cells was ~75% (Table 3, Figure A6). However, in this instance the difference was not statistically significant. The numbers of rickettsiae per infected cell showed no difference between these two treatments in either set of experiments.

No significant differences were observed at a given time point between Vero cells infected with rickettsiae released from bead-beaten, IFN- γ -treated macrophages and Vero cells infected with rickettsiae released from bead-beaten, untreated macrophages (Figures 2, A3, and A4; Table 2). Overall, the data indicate that the infectiveness and growth of the rickettsiae released from the IFN- γ -treated RAW264.7 cells were comparable to the infectiveness and growth of the rickettsiae released from untreated RAW264.7 cells. In addition, the rickettsiae from both sources grew similarly in Vero cells when compared to the control rickettsiae that had been held on ice.

Figure 2: Viability of *R. prowazekii* Breinl Harvested from Untreated and IFN- γ -Treated RAW264.7 Cells, As Determined by Their Subsequent Growth in Vero Cells.



Details are given in the legend for Table 2. One-way ANOVAs and multiple comparisons revealed significant differences between treatments. ^A Denotes a significant difference between the percent infected in Vero cell cultures incubated with Rp from bead-beaten RAW cells and percent infected in Vero cell cultures incubated with Rp Ctrl* at one hour ($p < 0.1$). ^B Denotes a statistically significant difference between a 49-hour value and the corresponding 1-hour value for rickettsiae per infected cell ($p < 0.01$). Rp, *R. prowazekii*; RAW, RAW264.7 cells; Ctrl*, rickettsiae that were held on ice before dilution and addition to Vero cells; Untr BB, rickettsiae from untreated, bead-beaten RAW cells; IFN BB, rickettsiae from gamma interferon-treated, bead-beaten RAW cells

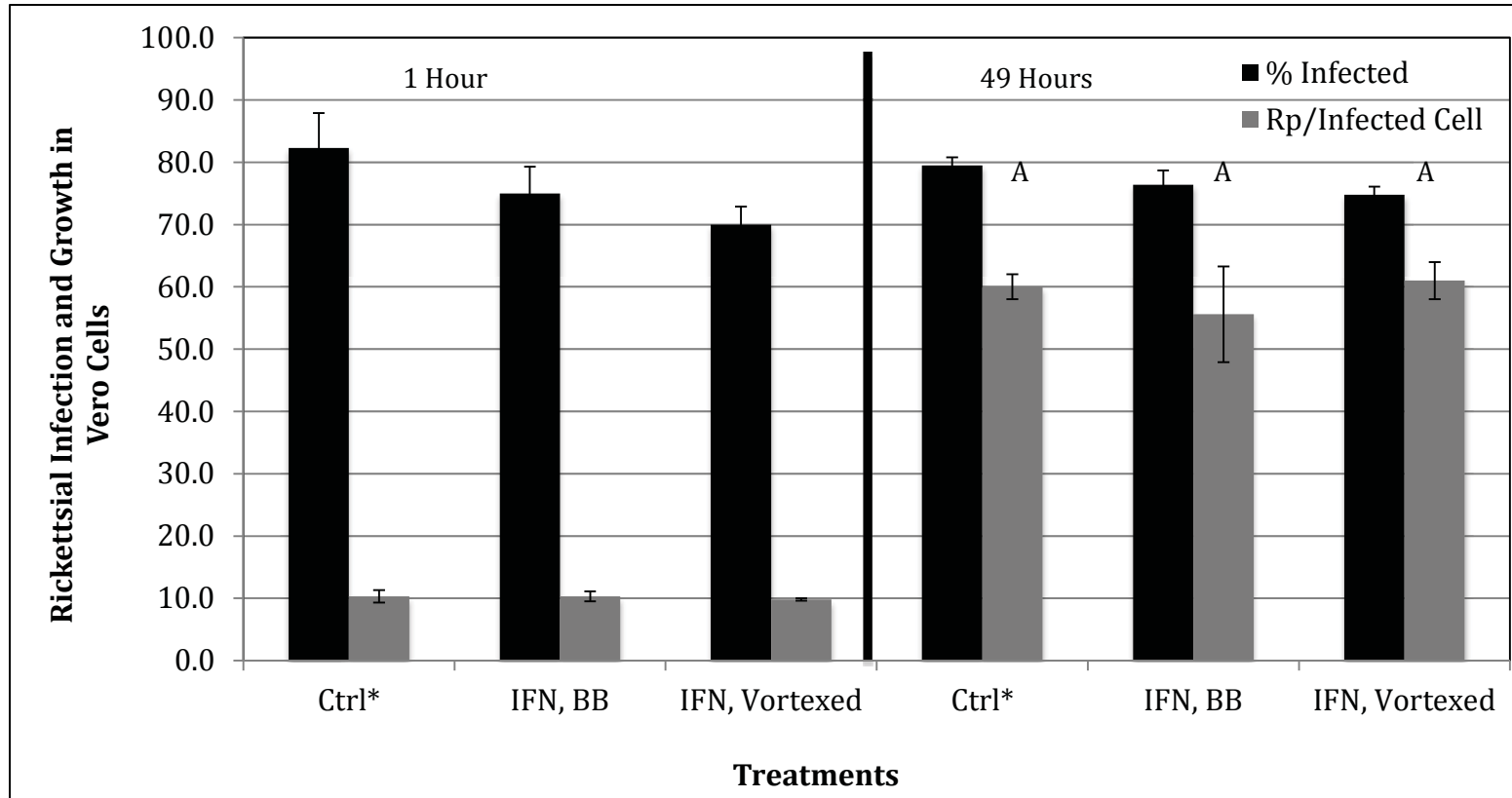
Table 2: Viability of *R. prowazekii* Breinl Harvested from Untreated and IFN- γ -Treated RAW264.7 Cells, As Determined by Their Subsequent Growth in Vero Cells.

Rp Sample Used for Infection	Host Cells	Time (h)	% Infected		Rp/Infected Cell		% Dead Cells
			Mean	SD	Mean	SD	Mean \pm SD
Rp	RAW, Untr	1	84.7	3.2	10.0	2.1	1 \pm 1
Rp	RAW + IFN	1	83.5	6.2	9.8	1.3	83 \pm 4
Rp, Ctrl*	Vero, Untr	1	86.0	2.0	10.8	0.5	-
RAW,Untr; Bead-Beaten	Vero, Untr	1	74.5 ^A	3.8	10.2	1.1	-
RAW+IFN, Bead-Beaten	Vero, Untr	1	76.3 ^A	2.3	9.9	1.1	-
Rp, Ctrl*	Vero, Untr	49	84.3	6.1	53.6^B	5.5	-
RAW, Untr; Bead-Beaten	Vero, Untr	49	77.8 ^A	2.8	45.4 ^B	4.0	-
RAW+IFN, Bead-Beaten	Vero, Untr	49	76.8 ^A	1.6	49.0 ^B	3.3	-

A sample of rickettsiae (Rp) was thawed and part of it was diluted and added to untreated (Untr) and gamma interferon (IFN)-treated RAW262.7 (RAW) cells. The remaining portion was held on ice for later use (Rp, Ctrl*). Some RAW cells were stained 1 hour after addition of the rickettsiae (% infected and Rp/infected cell in shaded rows). After incubation of other RAW cells for 3 additional hours, RAW cell viability was evaluated (% Dead Cells). The RAW cells in additional wells were scraped into the media and bead-beaten before being added to Vero cells. Rp, Ctrl* was also diluted and added to other Vero cells. Vero cells were stained at 1 and 49 hours after addition of rickettsial samples. One-way ANOVAs showed significant differences between treatments. ^A Denotes a significant difference between the percent infected in Vero cell cultures incubated with Rp from bead-beaten RAW cells and percent infected in Vero cell cultures incubated with Rp Ctrl* at 1 hour ($p < 0.1$). ^B Denotes a significant difference between a 49-hour value and the corresponding 1-hour value for Rp/infected cell in Vero cells ($p < 0.01$).

Next, it was necessary to determine whether the growth of rickettsiae released from IFN- γ -treated RAW264.7 cells resulted from the *R. prowazekii* released from the ~83% dead cells or the ~17% live cells that were broken during the bead-beating process. In two experiments, the cells scraped from some of the IFN- γ -treated, infected wells were vortexed instead of bead-beaten. This ensured that the debris from dead macrophages was broken apart and the rickettsiae were released, but there were no beads present to break any intact macrophages. The percent infected Vero cells and the rickettsiae per infected Vero cell, both initially and after 49 hours, did not differ significantly for the vortexed and bead-beaten treatments (Figures 3, A5, and A6; Table 3). These data indicated that the majority of the rickettsiae released from the bead-beaten, IFN- γ -treated RAW264.7 cell cultures must have come from the macrophages that had died.

Figure 3: Percent Infected and Rickettsiae per Infected Cell in Vero Cells Infected with *R. prowazekii* from Broken RAW264.7 Cells.



Details are given in the legend for Table 3. One-way ANOVAs showed significant differences between treatments for rickettsiae per infected cell but not for percent infected. ^A Denotes a statistically significant difference between a 49-hour value and the corresponding 1-hour value for rickettsiae per infected cell ($p < 0.01$). * Denotes rickettsiae held on ice for approximately 4 hours before dilution and addition to Vero cells.

Table 3: Percent Infected and Rickettsiae per Infected Cell in Vero cells Infected with *R. prowazekii* from Broken RAW264.7 cells.

Rp Sample Used for Infection	Host Cells	Time (h)	% Infected		Rp/Infected Cell		% Dead Cells
			Mean	SD	Mean	SD	Mean
Rp	RAW, Untr	1	75.0	1.8	9.3	0.1	0.5
Rp	RAW + IFN	1	75.0	0.8	9.8	0.5	83
Rp, Ctrl *	Vero, Untr	1	82.3	5.6	10.3	1.0	-
Bead-Beaten RAW + IFN	Vero, Untr	1	75.0	4.3	10.3	0.8	-
Vortexed RAW + IFN	Vero, Untr	1	70.0	2.9	9.8	0.3	-
Rp, Ctrl*	Vero, Untr	49	79.5	1.3	60.0^A	2.0	-
Bead-Beaten RAW + IFN	Vero, Untr	49	76.4	2.3	55.6 ^A	7.7	-
Vortexed RAW + IFN	Vero, Untr	49	74.8	1.3	61.0 ^A	3.0	-

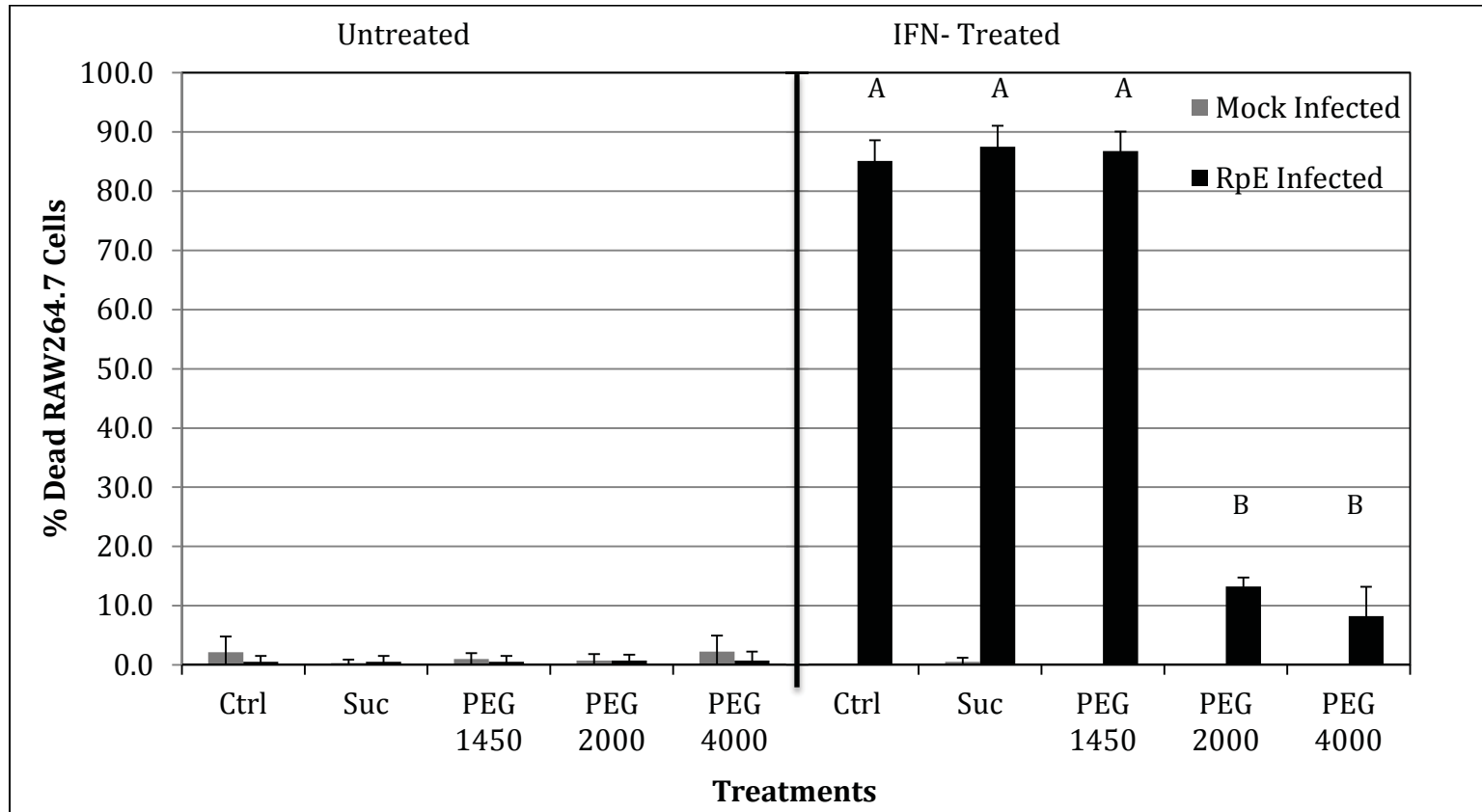
A sample of rickettsiae (Rp) was thawed and part of it was diluted and added to untreated (Untr) and gamma interferon (IFN)-treated RAW262.7 (RAW) cells. The remaining portion was held on ice for later use (Rp, Ctrl*). Some RAW cells were stained one hour after addition of the rickettsiae (shaded rows). After incubation of the remaining RAW cells for three additional hours, RAW cell viability was evaluated (% Dead Cells). The gamma-interferon (IFN)-treated RAW cells in some wells were scraped into the media and either bead-beaten or vortexed before being added to Vero cells. Rp, Ctrl* was also diluted and added to other Vero cells. Vero cells were stained at 1 and 49 hours after addition of rickettsial samples. One-way ANOVAs showed significant differences between treatments for Rp/infected cell but not for percent infected. ^A Denotes a difference between the Rp/infected cell in Vero cells at 1 hour and 49 hours ($p < 0.01$).

Effect of PEGs or Sucrose on Macrophage Death

In order to determine if osmotic lysis might be occurring in IFN- γ -pre-treated RAW264.7 cells, sucrose or PEGs of different molecular weights were added to the cells during infection with *R. prowazekii*. Each compound was initially tested at a concentration of 30 mM, as described by other researchers (Pei, Turse, Wu, & Ficht, 2006; Sun et al., 2005). PEGs 2000 and 4000 significantly decreased the macrophage death that occurred in IFN- γ -pre-treated RAW264.7 cultures infected with *R. prowazekii* Madrid E strain from ~85% to ~8-13%, but sucrose (342.3 grams/mole) and PEG 1450 had no effect on cell death (Figures 4 and A7). Similar results were obtained with IFN- γ -treated RAW264.7 cell cultures infected with *R. prowazekii* Breinl (Figures 5, 6, 7, A7, and A8). PEG 8000 was not soluble at 30 mM; therefore it was tested at a concentration of 15 mM. PEG 8000 at 15 mM had a protective effect very similar (~7% dead RAW264.7 cells) to that of PEG 4000 at 30 mM (Figure 6). In summary, the results indicate that PEGs 2000 and 4000 provided protection at 30 mM; whereas, PEG 1450 and sucrose (342.3 g/mole) at 30 mM did not. Cells were also mock-infected in the presence of PEG or sucrose to be certain that these compounds did not affect the viability of the RAW264.7 cells without the presence of *R. prowazekii* (Figures 4 and 5). In no instance did the PEGs or sucrose kill the mock-infected RAW264.7 cells.

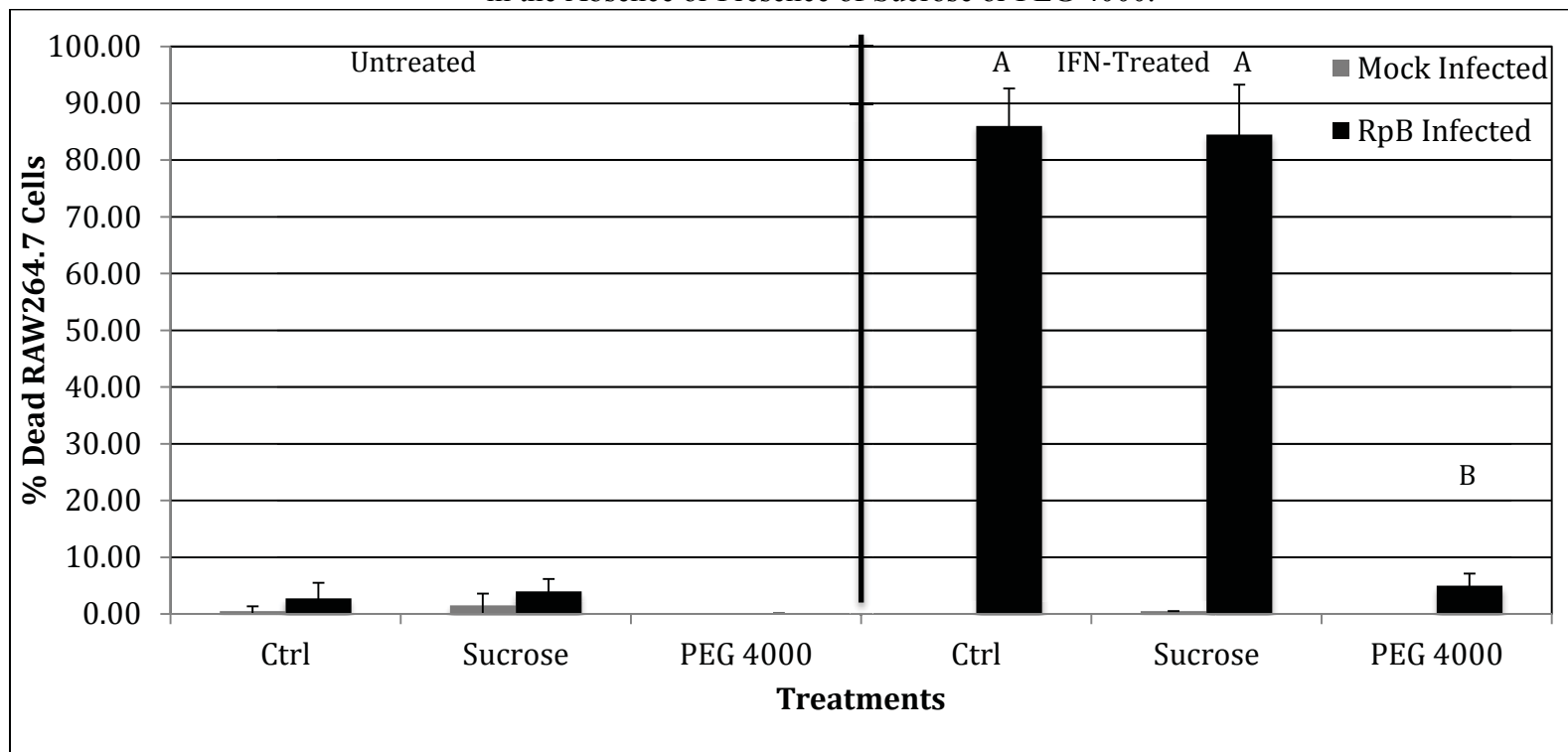
As indicated previously, for most of the experiments performed, the concentration of either sucrose or PEG was 30 mM. In two experiments, multiple concentrations of PEGs 400, 1450, 2000, 4000, and 8000 were tested (Figure 6). PEG 8000 was not soluble at 30 mM; therefore, it was tested at concentrations \leq 15 mM. PEGs 2000 and

Figure 4: Percent Dead RAW264.7 Cells in Cultures Mock-Infected or Infected with *R. prowazekii* Madrid E Strain in the Absence or Presence of Sucrose or PEGs.



Untreated or IFN- γ -treated RAW264.7 cells were mock-infected (grey bars) or infected (black bars) with *R. prowazekii* Madrid E strain in the presence or absence of 30 mM sucrose (Suc), PEG 1450, PEG 2000, or PEG 4000 for approximately 4 hours and then stained with trypan blue. Percent trypan blue positive cells was translated to percent dead cells. A one-way ANOVA revealed significant differences between treatments. ^A Denotes a statistically significant difference between IFN- γ -treated, infected RAW264.7 cell cultures and untreated, infected RAW264.7 cell cultures ($p < 0.01$). ^B Denotes a statistically significant difference between IFN- γ -treated, infected RAW264.7 cell cultures in the presence of PEG and in the absence of PEG ($p < 0.01$).

Figure 5: Percent Dead RAW264.7 Cells in Cultures Mock-Infected or Infected with *R. prowazekii* Breinl Strain in the Absence or Presence of Sucrose or PEG 4000.

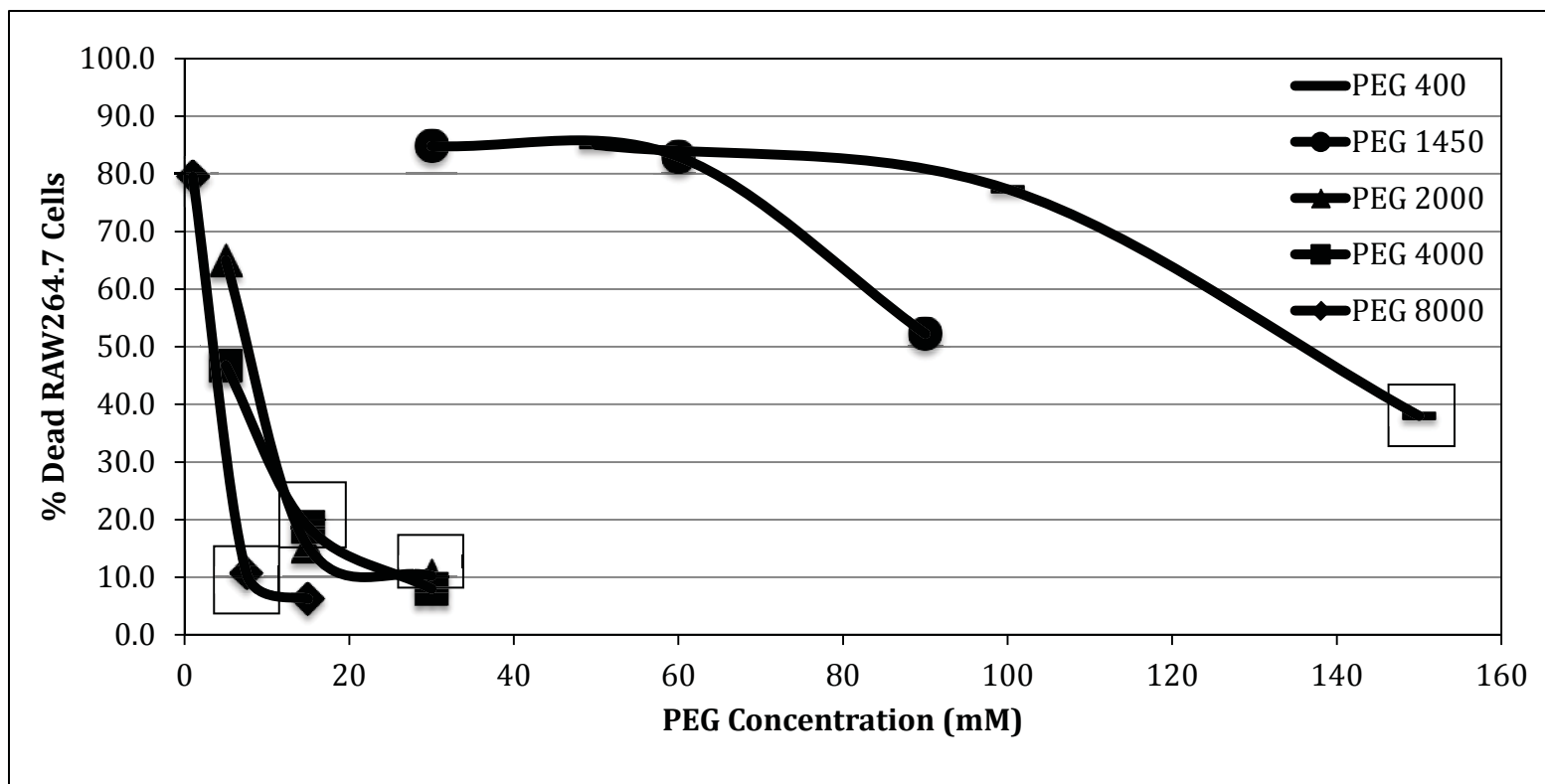


Untreated or IFN- γ -treated RAW264.7 cells were mock-infected or infected with *R. prowazekii* Breinl strain in the presence or absence of 30 mM sucrose or PEG 4000 (30 mM) for approximately 4 hours and then stained with trypan blue. Percent trypan blue positive cells was translated to percent dead cells. A one-way ANOVA showed significant differences between treatments. ^A Denotes a statistically significant difference between IFN- γ -treated, infected RAW264.7 cell cultures and untreated, infected RAW264.7 cell cultures. ^B Denotes a statistically significant difference between IFN- γ -treated, infected RAW264.7 cell cultures in the presence of PEG 4000 and in the absence of PEG 4000 ($p < 0.01$).

4000 (which protected the RAW264.7 cells at a concentration of 30 mM), were evaluated at concentrations ≤ 30 mM. PEGs 1450 and 400 (which were not protective at a concentration of 30 mM), were tested at concentrations ≥ 30 mM.

The curves in Figure 6 appear to fall into two groups that correlate with the protection provided by each PEG at 30 mM (PEG 8000, 15 mM). PEG 8000 showed a marked ability to protect the RAW264.7 cells at concentrations of 15 mM and 7.5 mM, but not at a concentration of 1 mM. Similarly, PEGs 4000 and 2000 dramatically protected the macrophages at concentrations of 30 mM and 15 mM, but not at a concentration of 5 mM. In contrast, PEG 1450 and PEG 400 failed to substantially protect the macrophages except when they were used at concentrations of 90 mM and 150 mM, respectively, when they provided only modest or moderate protection. The data indicated that molar concentration played a critical role in the protective effects of all molecular weights of PEG. However, data for the lower molecular weight PEGs (1,450 and 400 daltons) indicated that mass concentration was also important. For PEG 400, a concentration of 150 mM corresponds to 60 mg/mL, which is the same mass concentration as the highly protective solutions of 30 mM PEG 2000, 15 mM PEG 4000, and 7.5 mM PEG 8000 (Figure 6). Nevertheless, for PEG 400, increasing the mass concentration to 60 mg/mL provided only moderate protection that did not match the level of protection provided by the same mass concentrations of PEGs with molecular weights $\geq 2,000$ daltons.

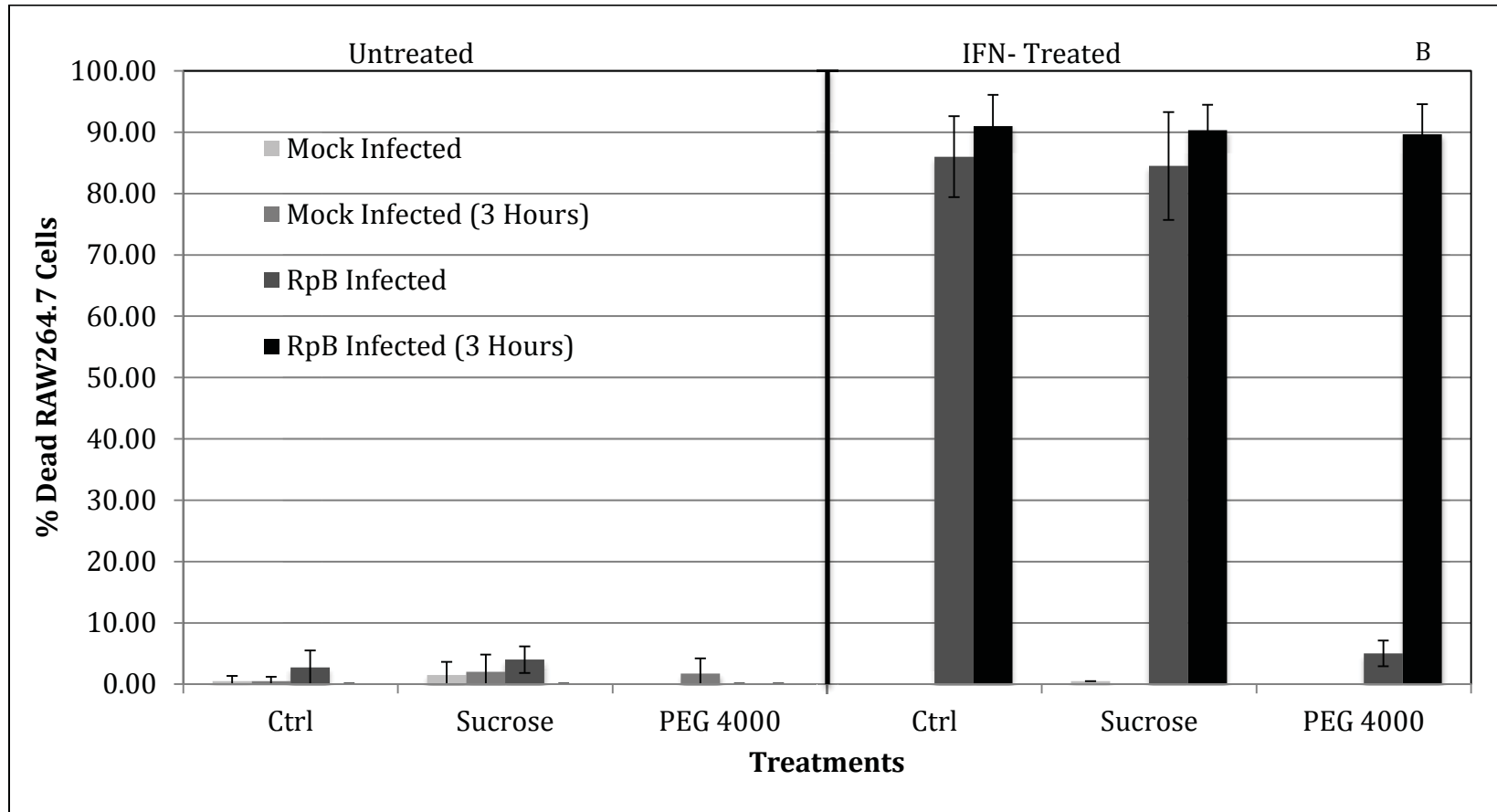
Figure 6: Percent Dead RAW264.7 Cells in Cultures Infected with *R. prowazekii* in the Presence of Different Concentrations of PEGs 400, 1450, 4000, or 8000.



IFN- γ -treated RAW264.7 cells were infected with *R. prowazekii* Breinl strain in the presence of PEGs 400, 1450, 2000, 4000, or 8000 for approximately 4 hours and then stained with trypan blue. Two determinations in two independent experiments were counted for PEGs 1450, 4000, and 8000 and three determinations in one experiment were counted for PEG 400 and 2000. Percent trypan blue positive cells was translated to percent dead cells. Boxed data points denote a concentration of 60 mg/mL.

Whether the protective effect of PEG 4000 on the IFN- γ -pre-treated RAW264.7 cells infected with *R. prowazekii* was permanent was examined next. After the initial 4-hour incubation period, the media containing sucrose or PEG 4000 was removed from two wells for each treatment and replaced with Dulbecco's medium + 5% NBS. The cells were then incubated for an additional 3 hours and stained with trypan blue. This part of the experiment revealed that without PEG 4000 in the media, RAW264.7 cell death was restored to values similar to those observed in the IFN- γ -pre-treated, infected cultures that had never been treated with PEG (Figures 7 and A8). The cell death for IFN- γ -pre-treated, *R. prowazekii* Breinl-infected RAW264.7 cells in the presence of PEG 4000 averaged approximately 5%, but once the PEG 4000 was taken away, cell death increased to an average of approximately 90%. Once again, mock-infected cells were not affected by addition of sucrose or PEG 4000, nor were they affected the change of media (Figure 7).

Figure 7: Percent Dead RAW264.7 cells in *R. prowazekii* Breinl Strain-Infected or Mock-Infected Cultures Three Hours After Removal of Sucrose or PEG 4000.



Untreated and IFN- γ -pre-treated cells were mock-infected or infected with *R. prowazekii* Breinl strain in the presence or absence of sucrose or PEG 4000 (30 mM) for approximately 4 hours, and then the sucrose or PEG 4000 was removed. Cells were incubated for an additional 3 hours and stained with trypan blue. Percent trypan blue positive cells was translated to percent dead cells. One-way ANOVAs showed significant differences between treatments. ^B Denotes a statistically significant difference between IFN- γ -treated RAW264.7 cell cultures infected with *R. prowazekii* Breinl strain in the presence of PEG 4000 and similarly treated cultures from which the PEG was removed ($p < 0.01$). Ctrl, cultures incubated without sucrose or PEG.

In a few experiments, the ability of PEG 4000 to protect IFN- γ -treated RAW264.7 cells after infection with *R. prowazekii* was determined. PEG 4000 was added in 1-hour increments after infection with *R. prowazekii* starting at 1 hour and ending 10 minutes before the addition of trypan blue. PEG 4000 was able to protect the IFN- γ -treated RAW264.7 cells from death at every time period except for 10 minutes prior to addition of trypan blue (data not shown). Possible explanations for these interesting results will be discussed in Chapter 4.

Not only was it important to determine the effects of sucrose and PEG 4000 on the cell death in RAW264.7 cell cultures pre-treated with IFN- γ and infected with *R. prowazekii*, but it was also necessary to know whether the presence of sucrose or PEG 4000 during the first four hours after addition of the rickettsiae to the RAW264.7 cells might influence the ability of *R. prowazekii* to grow inside the host cells. In order to investigate this matter, rickettsiae counts were completed at 1 hour and 49 hours. Cover slips from both untreated and IFN- γ -treated RAW264.7 cells were counted at 1 hour and only untreated RAW264.7 cells were counted at 49 hours. *R. prowazekii* Breinl strain was used for these experiments because of its ability to grow well in RAW264.7 cells compared to the Madrid E strain.

Presence of sucrose or PEG 4000 (30 mM) during the first four hours after the addition of rickettsiae did not appear to affect *R. prowazekii*'s ability to infect either the untreated or the IFN- γ -treated RAW264.7 cells. In addition, the sucrose and PEG 4000 did not appear to affect the ability of the rickettsiae to grow within the untreated RAW264.7 cells (Table 4). The average values for percent infected ranged from 87.3 to 90.3, and a one-way ANOVA and multiple comparisons revealed no significant

differences between treatments (Table 4 and Figure A10). The average values for rickettsiae per infected cell ranged from 13.8 to 18.7 initially (1 hour) and from 37.3 to 40.1 after 48 additional hours (Table 4 and Figure A9). These data suggested that comparable rickettsial growth had occurred in the control cultures (no addition to the rickettsiae during infection), the cultures exposed to sucrose, and the cultures exposed to PEG 4000. An ANOVA and multiple comparisons showed that the 49-hour value for rickettsiae per infected cell differed significantly from the corresponding 1-hour value only for the sucrose-treated RAW264.7 cells (Figure A9). This result is probably explained by the large standard deviations and small sample sizes (number of determinations varied between 3 and 6 for each treatment) for the different treatments at each time point.

Table 4: Percent Infected and Rickettsiae per Infected Cell in Untreated and IFN- γ -Pre-treated RAW264.7 Cells Infected with *R. prowazekii* Breinl in the Absence or Presence of Sucrose or PEG 4000.

RAW264.7 Cell Treatment	Addition during Infection	Time (h)	% Infected		Rp/Infected Cell	
			Mean	SD	Mean	SD
Untreated	None	1	90.3	6.4	18.7	9.2
Untreated	Sucrose	1	87.3	7.3	16.1	9.4
Untreated	PEG 4000	1	88.0	5.8	16.3	10.1
IFN	None	1	88.0	4.2	13.9	7.7
IFN	Sucrose	1	88.8	4.5	14.9	8.8
IFN	PEG 4000	1	87.0	4.7	13.8	7.4
Untreated	None	49	90.0	1.6	37.3	5.3
Untreated	Sucrose	49	90.0	1.4	37.9 ^A	5.6
Untreated	PEG 4000	49	88.8	1.3	40.1	7.4

Untreated and IFN- γ -pre-treated RAW264.7 cells were infected in the presence or absence of sucrose or PEG 4000 (30 mM). Cover slips were removed from some wells and stained 1 hour after addition of the rickettsiae. After incubation for 3 additional hours, the remaining wells were washed and fresh medium without sucrose or PEG was added. These cells were incubated for 45 additional hours; then cover slips were removed and stained. A one-way ANOVA showed significant differences between groups. ^A Denotes a statistically significant difference between Rp/infected cell at 49 hours and Rp/infected cell at 1 hour for the corresponding treatment/addition ($p < 0.1$).

Chapter IV

DISCUSSION AND CONCLUSION

The rapid cell death seen in IFN- γ -pre-treated RAW264.7 cells infected with *R. prowazekii* has yet to be characterized. This study set out to examine two main questions: whether or not the rickettsiae remain viable after the death of their IFN- γ -pre-treated RAW264.7 host cells, and if PEGs of certain molecular weights decrease the amount of cell death in IFN- γ -treated, infected RAW264.7 cell cultures. The results from the rickettsial viability experiments showed that the rickettsiae that infected IFN- γ -treated RAW264.7 cells are viable after the death of the host cells. In the PEG experiments, it was found that PEGs $\geq 2,000$ daltons will protect IFN- γ -treated, infected RAW264.7 cells from death. The effect that PEG has on cell death of IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* is concentration dependent and PEG must be present in the media to have a protective effect.

Viability of Rickettsiae after Death of RAW264.7 Cells

The first objective addressed in this study was to investigate whether *R. prowazekii* remained viable after the infection and death of IFN- γ -pre-treated, macrophage-like RAW264.7 cells. Although it is not known if this killing occurs *in vivo*, this question can give insight into whether the killing of the macrophages might be more beneficial to the host or to the pathogen. Determining whether the rickettsiae or the host organism benefits from this rapid cell death of macrophages can aid in the understanding

of the pathology of epidemic typhus. If the rickettsiae are alive after the death of IFN- γ -treated macrophages, they could take advantage of this death in order to spread through the host body more rapidly; in this case the pathogen would benefit. If the macrophage death were to kill the infecting rickettsiae, however, the cell death could prevent the rapid spread of rickettsiae to vulnerable cells, thus benefitting the host organism.

In order to determine the viability of the rickettsiae after death of the RAW264.7 cells, rickettsial growth was monitored in Vero cells for 49 hours. First, it was determined that there are very few or no viable rickettsiae still in the culture medium 1 hour or 4 hours after addition of *R. prowazekii* to the RAW264.7 cells. When the culture media from the untreated and IFN- γ -treated, infected RAW264.7 cell cultures were taken and put onto Vero cells, there was no apparent rickettsial growth after 49 hours for either condition. This indicates that all or most of the rickettsiae are associated with their host cells a mere one hour after addition to the cultures.

Once it was determined that there were few or no rickettsiae in the macrophage culture media at 1 or 4 hours after addition of the rickettsiae, untreated or IFN- γ -treated, infected RAW264.7 cells were scraped into their respective media and bead-beaten 4 hours after infection, in order to prepare rickettsial suspensions. In each instance, the numbers of rickettsiae per infected Vero cell were considerably higher (~45-53 rickettsiae per infected cell) at 49 hours than at one hour (~10 rickettsiae per infected cell). These findings indicate that the rickettsiae remain viable since they grow in cultured Vero cells after the death of IFN- γ -treated RAW264.7 cells. The fact that *R. prowazekii* remains viable in IFN- γ -treated macrophages implies that the death of the macrophage-like host

cells would be more advantageous to the rickettsiae than it would be to the host organism, due to the fact that IFN- γ is used to activate macrophages in the human body *in vivo*. However, *R. prowazekii* primarily infects endothelial cells, in addition to macrophages, and the effect of IFN- γ on these cells and their ability to survive infection with *R. prowazekii* is not yet understood. It has been shown that mouse fibroblasts (L929) differ from macrophages in their *in vitro* response to pre-treatment with IFN- γ and infection with *R. prowazekii* (Turco & Winkler, 1984; Turco & Winkler, 1988; Turco & Winkler 1998).

In one data set, there was a small, but significant difference in the percentage of Vero cells that were infected with *R. prowazekii* directly (control) when compared to the Vero cells infected with *R. prowazekii* harvested from untreated and IFN- γ -treated RAW264.7 cells. The difference between these treatments could be caused by a loss of rickettsiae during the bead-beating process. There is a possibility that macrophages, macrophage debris, and rickettsiae were left in the vial with the beads, stuck to the beads themselves, or that some of the rickettsiae were damaged in the bead-beating process. It is also possible that not all the rickettsiae that originally infected the RAW264.7 cells remained viable after the death of their host macrophages, although this seems very unlikely since infection of and growth within Vero cells did not differ significantly for rickettsiae harvested from untreated RAW264.7 cells and rickettsiae harvested from IFN- γ -treated RAW264.7 cells that had died.

Effect of PEGs of Different Molecular Weights on the Viability of RAW264.7 Cells

The second question that was addressed in this project concerned the effect that PEGs had on the viability of RAW264.7 cells treated with IFN- γ and infected with

R. prowazekii. PEGs are osmotic lysis inhibitors that have been shown to prevent cell death caused by oncosis or pyroptosis in macrophages infected with microorganisms other than *R. prowazekii*, such as *Legionella*, *Salmonella*, and *Shigella* (Sun et al., 2005; Pei, Turse, Wu, & Ficht, 2006; Liao & Mogridge, 2009). Such osmotic lysis presumably occurs after the cell membrane has sustained some initial damage. When 30 mM PEGs 2000 and 4000 are present during and after the addition of *R. prowazekii* to the macrophage cultures, the cell death in IFN- γ -treated RAW264.7 cells decreases dramatically and the RAW264.7 cells do not become permeable to trypan blue. Although PEGs 2000 and 4000 prevent osmotic lysis of the RAW264.7 cells, addition of 30 mM PEG 1450 or 30 mM sucrose during infection with *R. prowazekii* does not have any effect on cell death. This lack of inhibition of cell death with sucrose and PEG 1450 indicates that these molecules are too small to protect the IFN- γ -pre-treated, infected RAW264.7 cells from losing their membrane integrity at the 30 mM concentration. The ability of PEGs 2000 and 4000 to inhibit cell death implies that they are large enough to help the RAW264.7 cells treated with IFN- γ and infected with *R. prowazekii* maintain their membrane integrity, preventing the uptake of trypan blue.

The results from the PEG experiments give insight into the size of potential pores that might be formed in the membrane. The hydrodynamic radii (Hr) of PEGs 2000, 4000, and 8000 are 1.4 nm, 1.90 nm, and 3.2 nm, respectively (Scherrer & Gerhardt, 1971; Scherrer, Beaman, & Gerhardt, 1971). Because these PEGs prevented cell death in IFN- γ -treated, infected RAW264.7 cells, and PEG 1450 (Hr = 1.2 nm) did not, it suggests that if pores are being formed in the cell membrane and if PEG is interacting with these pores, their radii are smaller than 1.4 nm. If pores are being formed in the membrane of

the IFN- γ -treated RAW264.7 cells infected with *R. prowazekii*, PEGs between the molecular weights of 1,450 and 2,000 daltons could be used to better estimate the size of the radii.

Based on previous studies with PEG, there are a few possible explanations for how PEGs 2000, 4000, and 8000 prevent IFN- γ -treated, infected RAW264.7 cells from being stained with trypan blue. First, PEGs 2000 and 4000 could prevent the initial damage to the membranes (such as formation of pores in the cell membrane). Second, the large PEGs could either enter (Ternovsky, Okada, & Sabirov, 2004) or coat the surfaces of the pores (or other sites of membrane damage) once they are formed, thereby preventing other events (such as changes in the concentrations of intracellular and extracellular ions) that would eventually lead to entry of excess amounts of water into the cells. Third, PEGs 2000 and 4000 might be facilitating repair of the damaged membranes (Borgens & Shi, 2000) before lysis can occur. A fourth possibility was that PEGs 2000 and 4000 are able to bind trypan blue in the media and prevent it from crossing even a compromised cellular membrane. However, this explanation was eliminated because cell death was not prevented with the addition of PEG 4000 ten minutes before staining with trypan blue. Likewise, if PEG 4000 was added to some wells at the time that replicate wells showed macrophage death and allowed to incubate for one hour before staining with trypan blue, macrophage death was still apparent. A fifth possibility is that PEG changes the activity of water outside of the cell (Steuter, Mozafar, & Goodin, 1981; Ninni, Carmargo, & Meirelles, 1999). The findings of these experiments indicate that PEG 4000 does not prevent trypan blue from entering a cell with membrane damage prior to addition of the PEG. It is very difficult at this point to fully explain what is occurring

when PEGs 2000 and 4000 dramatically decrease cell death in RAW264.7 cells pretreated with IFN- γ and infected with *R. prowazekii*.

It was also necessary to know whether the effect of PEG on the death of IFN- γ -treated, infected RAW264.7 cells was only size dependent, or if there was also concentration dependence. A mass concentration of 60 mg/mL was used initially for PEGs 8000, 2000, and 400 (7.5 mM, 30 mM, and 150 mM, respectively). The results indicated that PEG 400 would protect at this high concentration, but the protection was mild to moderate. Sucrose (342.3 g/mole) was also used at a concentration of 60 mg/mL in one experiment. The results of this experiment suggested that 60 mg/mL of sucrose might be somewhat less protective than the same mass concentration of PEG 400, since the sucrose only decreased cell death approximately 20% (data not shown). Next, the effects of multiple concentrations of PEGs 400, 1450, 2000, 4000, and 8000 were examined.

From experiments with different concentrations of PEGs 400, 1450, 2000, 4000, and 8000, it was established that PEG concentration was a factor in its protective effect against cell death in IFN- γ -treated, infected RAW264.7 cell cultures. For PEGs $\geq 2,000$ daltons, the effect was diminished as the concentration decreased from 30 mM, while PEGs $\leq 1,450$ daltons (that show no effect at 30 mM) showed an increase in effect as the concentration increased, though the effect was not as strong as that seen with PEGs $\geq 2,000$ daltons. Previous investigations on changes in water activity and osmotic pressure created by different sizes of PEGs indicated that lower molecular weight PEGs could change the water activity of the external solution to the same extent as larger PEGs, but that higher concentrations of solute were required (Money, 1989).

By applying this information to the concentration data in this thesis, it is possible that a change in the external osmotic pressure could be responsible for the partial protective effect seen with high concentrations of low molecular weight PEGs.

After it was established that PEG 4000 had the ability to protect IFN- γ -treated RAW264.7 cells infected with *R. prowazekii*, it was of interest to investigate whether the PEG 4000 had to be continuously present or if the effect on the RAW264.7 cells was permanent. In several experiments, PEG 4000 was taken away from mock-infected or *R. prowazekii*-infected cell cultures 4 hours after addition of the rickettsiae and replaced with Dulbecco's medium and 5% NBS. After approximately 3 additional hours of incubation (after PEG removal), the cell death in the RAW264.7 cells was restored. This restoration of cell death indicates that PEGs must be present in the medium in order for osmotic lysis to be inhibited.

In another set of experiments, PEG 4000 was added to IFN- γ -treated RAW264.7 cells at 1-hour increments, after addition of *R. prowazekii*. Cell death was inhibited in all wells when PEG 4000 was added 1, 2, or 3 hours after addition of rickettsiae. However, if PEG 4000 was added a mere 10 minutes prior to staining with trypan blue (about 4 hours after addition of the rickettsiae), the cell death was not prevented. It is possible that membrane integrity does not become compromised until 3 or more hours after addition of *R. prowazekii* to IFN- γ -treated RAW264.7 cells.

Though it is not known exactly what is occurring when IFN- γ -treated RAW264.7 cells are infected with *R. prowazekii* in the presence of PEGs larger than 2,000 daltons, there have been many studies on the effects of PEG on membrane damage, repair, and fusion. PEG is a membrane fusogen; it can fuse cell membranes together (Ahkong, 1975;

Davidson, 1977; Lentz, 1994; Lentz, 2007). More recently, it has been shown that PEG can also repair membrane damage, specifically in spinal cords of guinea pigs (Borgens & Shi, 2000; Luo & Shi, 2007; Shi & Borgens, 2000). When PEG was injected into crushed spinal cords, the damage was repaired and the cords showed full recovery. Recently, there has been work done on PEG inhibition of apoptosis and the effects of PEGs on caspase-3, a marker for apoptotic cell death (Luo & Shi, 2007). This inhibition of apoptosis was seen after spinal cord injuries in guinea pigs because apoptosis is thought to be a major source of cell loss and neurological deficits following a spinal cord injury. The cell death after addition of PEG to the site of injury remained low 7 days after the injury. Addition of PEG also lowered caspase-3 activity in the injured area, and caspase-3 activity was significantly lower in the treated cords compared with the untreated injured cords 1 day after the spinal cord injury.

The *in vivo* work done by Luo & Shi (2007) contrasts with the *in vitro* work done by Sun et al. (2005), who determined that PEG did not inhibit apoptosis induced in cultured cells by staurosporine or hydrogen peroxide. The difference between the effects of PEG on apoptosis *in vivo* and *in vitro* implies that there are other factors possibly working with PEG *in vivo*.

Neither sucrose nor PEG 400 added during the first 4 hours after rickettsial addition to RAW264.7 cells had an effect on the ability of *R. prowazekii* Breinl strain to infect and grow within the untreated RAW264.7 cells. This was a concern because if these compounds were harming the rickettsiae or preventing them from infecting the RAW264.7 cells, these effects could explain the decrease in cell death, as opposed to

prevention of osmotic lysis. Previous work showed that viable rickettsiae are required in order for the death of IFN- γ -treated RAW264.7 cells to occur (Turco & Winkler, 1984).

Because of the results of the experiments with PEGs, it is apparent that osmotic lysis contributes to the death of the IFN- γ -treated, infected RAW264.7 cells. It can be concluded that when RAW264.7 cells are pre-treated with IFN- γ and infected with *R. prowazekii*, the membrane integrity of the RAW264.7 cells becomes compromised at some point approximately 3-4 hours after infection, and the cells burst from an influx of water and ions from the environment. It is still unknown if the membrane damage is rickettsia-induced or host cell induced, but it is clear that osmotic lysis is occurring in IFN- γ -treated RAW264.7 cells infected with *R. prowazekii*. Because PEG can be used to protect cells against death from oncosis and caspase-1-dependent pyroptosis and PEG has a protective effect against death of IFN- γ -treated RAW264.7 infected with *R. prowazekii*, the type of cell death occurring can be narrowed. Apoptosis cannot be completely ruled out, however, because PEG has been shown to prevent apoptosis *in vivo* and inhibit caspase-3 (Luo & Shi, 2007).

Summary and Conclusions

Rickettsia prowazekii causes epidemic typhus in humans and preferentially infects endothelial cells. It also has the ability to infect macrophages, and when it infects cultured, IFN- γ -treated macrophage-like RAW264.7 cells, many of these cells die within 4 hours after infection. It was imperative to understand whether or not the rickettsiae remained viable after infection and death of IFN- γ -treated RAW264.7 cells. Insight into this question allows conclusions to be made about whether *R. prowazekii* or the host organism it infects might profit more from the death of IFN- γ -treated, infected

macrophages. The rickettsiae that had infected both untreated and IFN- γ -treated RAW264.7 cells were able to grow comparably in Vero cells, and this information was used to conclude that the death of IFN- γ -treated, infected macrophages might increase the spread of rickettsial infection to other host cells.

In Madrid E strain infections and Breinl strain infections, addition of PEG 2000 or PEG 4000 during infection with *R. prowazekii* led to a significant decrease in the amount of cell death seen in IFN- γ -treated RAW264.7 cell cultures. Because of this decrease in cell death by known osmotic lysis inhibitors, it was concluded that the cell death seen in IFN- γ -treated RAW264.7 cells infected by *R. prowazekii* involves osmotic lysis of the cell membrane. There was also no effect of the PEGs or sucrose on the ability of *R. prowazekii* Breinl strain to grow in untreated RAW264.7 cell cultures.

A full characterization of what is happening in RAW264.7 cells pre-treated with IFN- γ and infected with *R. prowazekii* is closer. This is a complex system because of the intracellular nature of *R. prowazekii* and the numerous restrictions that have been placed on it. It is obvious that more research will need to be done in the future in order to fully describe the relationship between *R. prowazekii* and IFN- γ -treated RAW264.7 cells.

LITERATURE CITED

- Ahkong, Q. F., Howell, J. I., Lucy, J. A., Safwat, F., Davey, M. R., & Cocking, E. C. (1975) Fusion of Hen Erythrocytes with Yeast Protoplasts Induced by Polyethylene Glycols. *Nature*, 255, 66-67.
- Andersson, S. G. E., Zomorodipour, A., Andersson, J. O., Sicheritz-Ponten, T., Alsmark, U. C. M., Podowski, R. M., Naslund, A. K., Eriksson, A.-S., Winkler, H. H., & Kurland, C. G. (1998) The Genome Sequence of *Rickettsia prowazekii* and the Origin of Mitochondria. *Nature*, 396, 133-143.
- Atkinson, W. H., & Winkler, H. H. (1985) Transport of AMP by *Rickettsia prowazekii*. *Journal of Bacteriology*, 161(1), 32-38.
- Atkinson, W. H., & Winkler, H. H. (1989) Permeability of *Rickettsia prowazekii* to NAD. *Journal of Bacteriology*, 171, 761-766.
- Audia, J. P., & Winkler, H. H. (2006) Study of the Five *Rickettsia prowazekii* Proteins Annotated as ATP/ADP Translocases (Tlc): Only Tlc1 Transports ADP/ATP, While Tlc4 and Tlc5 Transport Other Ribonucleotides. *Journal of Bacteriology*, 188(17), 6261-6268.
- Balayeva, N. M., & Nikolskaya, V. N. (1973) Increased Virulence of the E Vaccine Strain of *Rickettsia prowazekii* Passaged in the Lungs of White Mice and Guinea Pigs. *Journal of Hygiene, Epidemiology, Microbiology and Epidemiology*, 17, 11-20.
- Bechah, Y., Capo, C., Mege, J.-L., & Raoult, D. (2008) Epidemic Typhus. *Lancet Infectious Diseases*, 8, 417-426.

- Bechah, Y., El Karkouri, K., Mediannikov, O., Leroy, Q., Pelletier, N., Robert, C., Medigue, C., Mege, J.-L., & Raoult, D. (2010) Genomic, Proteomic, and Transcriptomic Analysis of Virulent and Avirulent *Rickettsia prowazekii* Reveals its Adaptive Mutation Capabilities. *Genome Research*, 20, 655-663.
- Bergsbaken, T., & Cookson, B. T. (2007) Macrophage Activation Redirects *Yersinia*-infected Host Cell Death from Apoptosis to Caspase-1-Dependent Pyroptosis. *PLoS Pathogens*, 3(11), 1570-1582.
- Bergsbaken, T., Fink, S., & Cookson, B. T. (2009) Pyroptosis: Host Cell Death and Inflammation. *Nature Reviews: Microbiology*, 7, 99-109.
- Borgens, R. B., & Shi, R. (2000) Immediate Recovery from Spinal Cord Injury Through Molecular Repair of Nerve Membranes with Polyethylene Glycol. *The Journal of the Federation of American Societies for Experimental Biology*, 14, 27-35.
- Bovarnick, M. R., Miller, J. C., & Snyder, J. C. (1950) The Influence of Certain Salts, Amino Acids, Sugars and Proteins on the Stability of Rickettsiae. *Journal of Bacteriology*, 59(4), 509-522.
- Brennan, M. A., & Cookson, B. T. (2000) *Salmonella* Induces Macrophage Death by Caspase-1-Dependent Necrosis. *Molecular Microbiology*, 38(1), 31-40.
- Clifton, D. R., Goss, R. A., Shani, S. K., van Antwerp, D., Baggs, R. B., Marder, V. J., Silverman, D. J., & Sporn, L. A. (1998) NF- κ B-Dependent Inhibition of Apoptosis is Essential for Host Cell Survival During *Rickettsia rickettsii* Infection. *Proceedings of the National Academy of Sciences*, 95, 4646-4651.

- Davidson, R. L., & Gerald, P. S. (1977) Induction of Mammalian Somatic Cell Hybridization by Polyethylene Glycol, pgs. 325-338 in Wilson, L. & Matsudaira, P., *Methods in Cell Biology*, 15th ed. Academic Press, Inc., London, England.
- Driskell, L. O., Yu, X., Zhang, L., Liu, Y., Popov, V. L., Walker, D. H., Tucker, A. M., & Wood, D. O. (2009) Directed Mutagenesis of the *Rickettsia prowazekii* *pld* Gene Encoding Phospholipase D. *Infection and Immunity*, 77(8), 3244-3248.
- Dunbar, S. A., & Winkler, H. H. (1997) Increased and Controlled Expression of the *Rickettsia prowazekii* ATP/ADP Translocase and Analysis of Cysteine-less Mutant Translocase. *Microbiology*, 143, 3661-3669.
- Fernandes-Alnemri, T., Wu, J., Yu, J.-W., Datta, P., Miller, B., Jankowski, W., Rosenberg, S., Zhang, J., & Alnemri, E. S. (2007) The Pyroptosome: A Supramolecular Assembly of ASC Dimers Mediating Inflammatory Cell Death Via Caspase-1 Activation. *Cell Death and Differentiation*, 14, 1590-1604.
- Fernandez-Prada, C. M., Hoover, D. L., Tall, B. D., & Venkatesan, M. M. (1997) Human Monocyte-Derived Macrophages Infected with Virulent *Shigella flexneri* In Vitro Undergo a Rapid Cytolytic Event Similar to Oncosis but Not Apoptosis. *Infection and Immunity*, 65(4), 1486-1496.
- Feng, H. M., & Walker, D. H. (1993) Interferon-gamma and Tumor Necrosis Factor-Alpha Exert Their Antirickettsial Effect via Induction of Synthesis of Nitric Oxide. *American Journal of Pathology*, 143, 1016-1023.
- Feng, H. M., Popov, V. L., & Walker, D. H. (1994) Depletion of Gamma Interferon and Tumor Necrosis Factor Alpha in Mice with *Rickettsia conorii*-Infected Endothelium: Impairment of Rickettsicidal Nitric Oxide Production Resulting in

- Fatal, Overwhelming Rickettsial Disease. *Infection and Immunity*, 62(5), 1952-1960.
- Fink, S. L., & Cookson, B. T. (2005) Apoptosis, Pyroptosis, Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells. *Infection and Immunity*, 73(4), 1907-1916.
- Fink, S. L., & Cookson, B. T. (2006) Caspase-1-Dependent Pore Formation During Pyroptosis Leads to Osmotic Lysis of Infected Host Macrophages. *Cellular Microbiology*, 8(11), 1812-1825.
- Fink, S. L., Bergsbaken, T., & Cookson, B. T. (2008) Anthrax Lethal Toxin and *Salmonella* Elicit the Common Cell Death Pathway of Caspase-1-Dependent Pyroptosis via Distinct Mechanisms. *Proceedings of the National Academy of Sciences*, 105(11), 4312-4317.
- Frohlich, K. M., Roberts, R. A. W., Housley, N. A., & Audia, J. P. (2010) *Rickettsia prowazekii* Uses an *sn*-Glycerol-3-Phosphate Dehydrogenase and a Novel Dihydroxyacetone Phosphate Transport System To Supply Triose Phosphate for Phospholipid Biosynthesis. *Journal of Bacteriology*, 192(17), 4281-4288.
- Gambrill, M. R., & Wisseman, C. L. Jr. (1973) Mechanisms of Immunity in Typhus Infections. I. Multiplication of Typhus Rickettsiae in Human Macrophage Cell Cultures in the Nonimmune System: Influence of Virulence of Rickettsial Strains and of Chloramphenicol. *Infection and Immunity*, 8(4), 519-527.
- Ge, H., Chuang, Y.-Y. E., Zhao, S., Tong, M., Tsai, M.-H., Temenak, J. J., Richards A. L., & Ching, W.-M. (2004) Comparative Genomics of *Rickettsia prowazekii* Madrid E and Breinl Strains. *Journal of Bacteriology*, 186(2), 556-565.

- Giménez, D. F. (1964) Staining Rickettsia in Yolk-Sac Cultures. *Biotechnic & Histochemistry*, 39(3), 135-140.
- Green, D. R., Ferguson, T., Zitvogen, L., & Kroemer, G. (2009) Immunogenic and Tolerogenic Cell Death. *Nature Reviews: Immunology*, 9, 353-363.
- Housley, N. A., Winkler, H. H., & Audia, J. P. (2011) The *Rickettsia prowazekii* ExoU Homologue Possesses Phospholipase A₁ (PLA₁), PLA₂, and Lyso-PLA₂ Activities and Can Function in the Absence of Any Eukaryotic Cofactors *In Vitro*. *Journal of Bacteriology*, 193(18), 4634-4642.
- Janeway, Jr. C. A., Travers, P., Walport, M., & Shlomchik, M. J. (2005) Immunobiology: The Immune System in Health and Disease (6th Edition). Garland Science Publishing. New York, NY.
- Jerrells, T. R., Li, H., & Walker, D. H. (1988) *In Vivo* and *In Vitro* Role of Gamma Interferon in Immune Clearance of Rickettsial Species. *Advances in Experimental Medicine and Biology*, 239, 193-200.
- Jiang J., Temenak J. J., & Richards A. L. (2003) Real-Time Duplex Assay for *Rickettsia prowazekii* and *Borrelia recurrentis*. *Annals of the New York Academy of Sciences*, 990, 302–310.
- Kanneganti, T-D., Lamkanfi, M., & Nunez, G. (2007) Intracellular NOD-like Receptors in Host Defense and Disease. *Immunity*, 27, 549-559.
- Kenyon, R. H., & Pedersen Jr., C. E. (1980) Immune Responses to *Rickettsia akari* Infection in Congenitally Athymic Nude Mice. *Infection and Immunity*, 28(2), 310-313.

- Klionsky, D. J., & Emr, S. D. (2000) Autophagy as a Regulated Pathway of Cellular Degradation. *Science*, 290, 1717-1721.
- Kroemer, G., El-Deiry, W. S., Golstein, P., Peter, M. E., Vaux, D., Vandenabeele, P., Zhivotovsky, B., Blagosklonny, M. V., Malorni, W., Knight, R. A., Piacentini, M., Nagata, S., & Melina, G. (2005) Classification of Cell Death: Recommendations of Nomenclature Committee on Cell Death. *Cell Death and Differentiation*, 12, 1463-1467.
- Lentz, B. (1994) Polymer-Induced Membrane Fusion: Potential Mechanism and Relation to Cell Fusion Events. *Chemistry and Physics of Lipids*, 73, 91-106.
- Lentz, B. (2007) PEG as a Tool to Gain Insight into Membrane Fusion. *European Journal of Biophysics*, 36, 315-326.
- Li, H., Jerrells, T. R., Spitalny, G. L., & Walker, D. H. (1987) Gamma Interferon as a Crucial Host Defense Against *Rickettsia conorii* in Vivo. *Infection and Immunity*, 4, 47-57.
- Liao, K-C., & Mogridge, J. (2009) Expression of Nlrp1b Inflammasome Components in Human Fibroblasts Confers Susceptibility to Anthrax Lethal Toxin. *Infection and Immunity*, 77(10), 4455-4462.
- Lilo, S., Zheng, Y., & Bliska, J. B. (2008) Caspase-1 Activation in Macrophages Infected with *Yersinia pestis* KIM Requires the Type III Secretion System Effector YopJ. *Infection and Immunity*, 76(9), 3911-3923.
- Luo, J., & Shi, R. (2007) Polyethylene Glycol Inhibits Apoptotic Cell Death Following Traumatic Spinal Cord Injury. *Journal of Brain Research*, 1155, 10-16.

- Melino, G., Knight, R. A., & Nicotera, P. (2005) How Many Ways to Die? How Many Different Models of Cell Death? (Editorial). *Cell Death and Differentiation*, 12, 1457-1452.
- Minitab Inc. (2010) Minitab Assistant White Paper, One-Way ANOVA. Retrieved August, 2012, from http://www.minitab.com/en-CN/support/documentation/Answers/Assistant%20White%20Papers/OneWayANOVA_MtbAsstMenuWhitePaper.pdf.
- Mosavi, L. K., Cammett, T. J., Desrosiers, D. C., & Peng, Z-Y. (2004) The Ankyrin Repeat as Molecular Architecture for Protein Recognition. *Protein Science*, 13, 1435-1448.
- Netea, M. G., Simon, A., van de Veerdonk, F., Kullberg, B.-J., Van der Meer, J. W. M., & Joosten, L. A. B. (2010) IL-1 β Processing in Host Defense: Beyond the Inflammasomes. *PLoS Pathogens*, 6(2), e1000661.
- Ninni, L., Carmargo, M. S., & Meirelles, A. J. A. (1999) Water Activity in Poly(Ethylene Glycol) Aqueous Solutions. *Thermochimica Acta*, 328, 169-176.
- Pan, X., Luhrmann, A., Satoh, A., Laskowski-Arce, M. A., & Roy, C. R. (2008) Ankyrin Repeat Proteins Compromise a Diverse Family of Bacterial Type IV Effectors. *Science*, 520(5883), 1651-1654.
- Pei, J., Turse, J. E., Wu, Q., & Ficht, T. A. (2006) *Brucella abortus* Rough Mutants Induce Macrophage Oncosis that Requires Bacterial Protein Synthesis and Direct Contact with the Macrophage. *Infection and Immunity*, 74(5), 2667-2675.
- Perlman, S. J., Hunter, M. S., & Zchori-Fein, E. (2006) The Emerging Diversity of Rickettsia. *Proceedings of the Royal Society*, 273, 2097-2106.

- Polevoda, B., & Sherman, F. (2007) Methylation of Proteins Involved in Translation. *Molecular Microbiology*, 65(3), 590-606.
- Poyet, J.-L., Srinivasula, S., Tnani, M., Razmara, M., Fernandes-Alnemri, T., & Alnemri, E. S. (2001) Identification of Ipaf, a Human Caspase-1-activating Protein Related to Apaf-1. *The Journal of Biological Chemistry*, 276(30), 28309-28313.
- Radulovic, S., Price, P. W., Beier, M. S., Gaywee, J., Macaluso, J. A., & Azad, A. (2002) *Rickettsia*-Macrophage Interactions: Host Cell Responses to *Rickettsia akari* and *Rickettsia typhi*. *Infection and Immunity*, 70(5), 2576-2582.
- Rahman, M. S., Ammerman, N. C., Sears, K. T., Ceraul, S. M., & Azad, A. F. (2010) Functional Characterization of a Phospholipase A₂ Homolog from *Rickettsia typhi*. *Journal of Bacteriology*, 192(13), 3294-3303.
- Razmara, M., Srinivasula, S. M., Wang, L., Poyet, J.-L., Geddes, B. J., DiStefano, P. S., Bertin, J., & Alnemri, E. S. (2002) CARD-8 Protein, a New CARD Family Member that Regulates Caspase-1 Activation and Apoptosis. *Journal of Biological Chemistry*, 277(16), 13952-13958.
- Rodionov, A. V., Eremeeva, M. E., & Balayeva, N. M. (1991) Isolation and Partial Characterization of the M_r 100 kD Protein from *Rickettsia prowazekii* Strains of Difference Virulence. *Acta Virologica*, 35, 557-565.
- Roux, V., & Raoult, D. (2000) Phylogenetic Analysis of Members of the Genus *Rickettsia* Using the Gene Encoding the Outer Membrane Protein rOmpB (*ompB*). *International Journal of Systematic & Evolutionary Microbiology*, 50(4), 1449-1455.

- Scherrer, R., & Gerhardt, P. (1971) Molecular Sieving by the *Bacillus megaterium* Cell Wall and Protoplast. *Journal of Bacteriology*, 107(3), 718-735.
- Scherrer, R., Beaman, T. C., & Gerhardt, P. (1971) Macromolecular Sieving by Dormant Spore of *Bacillus cereus*. *Journal of Bacteriology*, 108(2), 868-873.
- Schmitz-Esser, S., Linka, N., Collingro, A., Beier, C. L., Neuhaus, H. E., Wagner, M., & Horn, M. (2004) ATP/ADP Translocases: a Common Feature of Obligate Intracellular Amoebal Symbionts Related to Chlamydiae and Rickettsiae. *Journal of Bacteriology*, 186(3), 683-691.
- Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004) Interferon- γ : An Overview of Signals, Mechanisms, and Functions. *Journal of Leukocyte Biology*, 73, 163-189.
- Shi, R., & Borgens, R. B. (2000) Anatomical Repair of Nerve Membranes in Crushed Mammalian Spinal Cord with Polyethylene Glycol. *Journal of Neurophysiology*, 29, 633-643.
- Shoa, W., Yeretssian, G., Doiron, K., Hussain, S. N., & Saleh, M. (2007) The Caspase-1 Digestome Identifies the Glycolysis Pathway as a Target During Infection and Septic Shock. *The Journal of Biological Chemistry*, 282(50), 36321-36329.
- Srinivasula, S. M., Poyet, J.-L., Razmara, M., Datta, P., Zhang, Z., & Alnemri, E. S. (2002) The PYRIN-CARD Protein ASC is an Activating Adaptor for Caspase-1. *Journal of Biological Chemistry*, 277(24), 21119-21122.
- Stenos, J., Graves, S. R., & Unsworth, N. B. (2005) A Highly Sensitive and Specific Real-Time PCR Assay for the Detection of the Spotted Fever and Typhus Group

- Rickettsiae. *American Journal of Tropical Medicine and Hygiene*, 72(6), 1083-1085.
- Steuter, A. A., Mozafar, A., & Goodin, J. R. (1981) Water Potential of Aqueous Polyethylene Glycol. *Plant Physiology*, 67, 64-67.
- Stothard, D. R., Clark, J. B., & Fuerst, P. A. (1994) Ancestral Divergence of *Rickettsia bellii* from the Spotted Fever and Typhus Groups of *Rickettsia* and Antiquity of the Genus *Rickettsia*. *International Journal of Systematic Bacteriology*, 44(4), 798-804.
- Sun, G. W., Lu, J., Pervaiz, S., Cao, W. P., & Gan, Y. (2005) Caspase-1-Dependent Macrophage Death Induced by *Burkholderia pseudomallei*. *Cellular Microbiology*, 7(10), 1447-1458.
- Suzuki, T., Franchi, L., Toma, C., Ashida, H., Ogawa, M., Yoshikawa, Y., Mimuro, H., Inohara, N., Sasakawa, C., & Nunez, G. (2007) Differential Regulation of Caspase-1 Activation, Pyroptosis, and Autophagy via Ipaf and ASC in *Shigella*-Infected Macrophages. *PLoS Pathogens*, 3(8), 1082-1091.
- Tennant, J. R. (1964) Evaluation of the Trypan Blue Technique for Determination of Cell Viability. *Transplantation*, 2(6), 685-815.
- Ternovsky, V. I., Okada, Y., & Sabirov, R. Z. (2004) Sizing the Pore of the Volume-Sensitive Anion Channel by Differential Polymer Partitioning. *FEBS Letters*, 576, 433-436.
- Thorner, A. R., Walker, D. H., & Petri, Jr., W. A. (1998) Rocky Mountain Spotted Fever. *Clinical Infectious Diseases*, 27(6), 1353-1359.

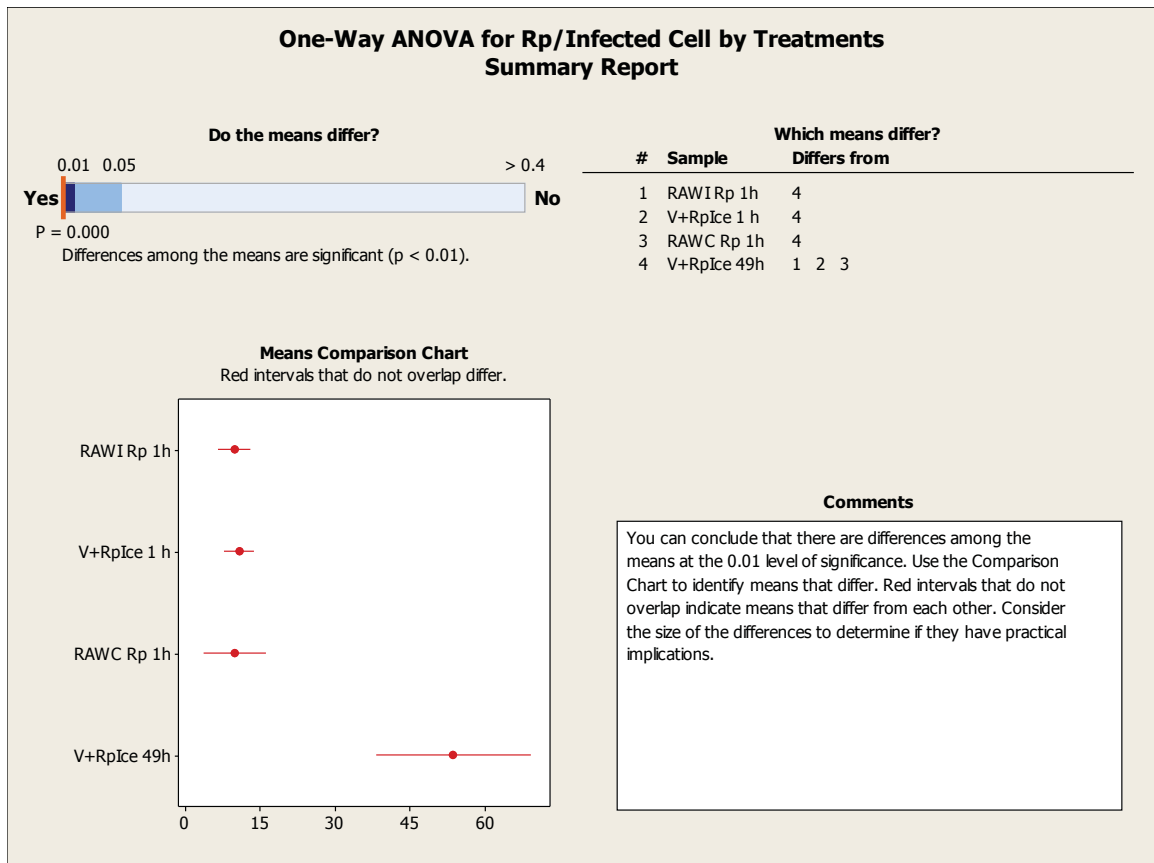
- Trinchieri, G. (2010) Type I Interferon: Friend or Foe? *Journal of Experimental Medicine*, 207(10), 2053-2063.
- Tucker, A. M., Winkler, H. H., Driskell, L. O., & Wood, D. O. (2003) S-Adenosylmethionine Transport in *Rickettsia prowazekii*. *Journal of Bacteriology*, 185(10), 3031-3035.
- Turco, J., & Winkler, H. H. (1982) Differentiation Between Virulent and Avirulent Strains of *Rickettsia prowazekii* by Macrophage-Like Cell Lines. *Infection and Immunity*, 35(3), 783-791.
- Turco, J., & Winkler, H. H. (1983) Cloned Mouse Interferon- γ Inhibits the Growth of *Rickettsia prowazekii* in Cultured Mouse Fibroblasts. *Journal of Experimental Medicine*, 158, 2183-2164.
- Turco, J., & Winkler, H. H. (1984) Effect of Mouse Lymphokines and Cloned Mouse Interferon- γ on the Interaction of *Rickettsia prowazekii* with Mouse Macrophage-Like RAW 264.7 Cells. *Infection and Immunity*, 45(2), 303-308.
- Turco, J., & Winkler, H. H. (1988) Interactions Between *Rickettsia prowazekii* and Cultured Host Cells: Alterations Induced by Gamma Interferon, pgs. 95-115 in G.I. Byrne and J. Turco (ed), *Interferon and Nonviral Pathogens*. Marcel Dekker, Inc., New York, NY.
- Turco, J., Keysary, A., & Winkler, H.H. (1989) Interferon- γ - and Rickettsia-Induced Killing of Macrophage-Like Cells Is Inhibited by Anti-Rickettsial Antibodies and Does Not Require Respiratory Burst. *Journal of Interferon Research*, 9, 615-629.

- Turco, J., & Winkler, H. H. (1991) Comparison of Properties of Virulent, Avirulent, and Interferon Resistant *Rickettsia prowazekii* Strains. *Infection and Immunity*, 59(5), 1647-1655.
- Turco, J., & Winkler, H. H. (1994) Relationship of Tumor Necrosis Factor Alpha, the Nitric Oxide Synthase Pathway, and Lipopolysaccharide to the Killing of Gamma Interferon-Treated Macrophagelike RAW264.7 Cells by *Rickettsia prowazekii*. *Infection and Immunity*, 62(6), 2568-2574.
- Turco, J., & Winkler, H. H. (1997) Cytokines Influencing Infections by *Rickettsia* species, pgs. 29-52 in B. Anderson, H. Friedman, & M. Bendinelli (ed.), *Rickettsial Infection and Immunity*. Plenum Publishing Corp., New York, NY.
- Turco, J., Liu, H., Gottlieb, S. F., & Winkler, H. H. (1998) Nitric Oxide-Mediated Inhibition of the Ability of *Rickettsia prowazekii* to Infect Mouse Fibroblasts and Mouse Macrophage-like Cells. *Infection and Immunity*, 66(2), 558-566.
- Walker, D. H., Olano, J. P., & Feng, H.-M. (2001) Critical Role of Cytotoxic T Lymphocytes in Immune Clearance of Rickettsial Infection. *Infection and Immunity*, 69(3), 1841-1846.
- Whitworth, T., Popov, V. L., Yu, X. J., Walker, D. H., & Bouyer, D. H. (2005) Expression of the *Rickettsia prowazekii* *pld* or *tlyC* Gene in *Salmonella enterica* Serovar Typhimurium Mediates Phagosomal Escape. *Infection and Immunity*, 73(10), 6668-6673.
- Winkler, H. H. (1976) Rickettsial permeability: an ADP-ATP Transport System. *Journal of Bacteriology*, 251, 389-396.

- Winkler, H. H., & Miller, E. T. (1982) Phospholipase A and the Interaction of *Rickettsia prowazekii* and Mouse Fibroblasts (L-929 Cells). *Infection and Immunity*, 38(1), 109-113.
- Winkler, H. H., & Daugherty, R. M. (1984) Proline Transport and Metabolism in *Rickettsia prowazekii*. *Journal of Bacteriology*, 158(2), 460-463.
- Winkler, H. H., & Daugherty, R. M. (1986) Acquisition of Glucose by *Rickettsia prowazekii* through the Nucleotide Intermediate Uridine 5'-Diphosphoglucose, *Journal of Bacteriology*, 167(3), 805-808.
- Winkler, H. H., Daugherty, R., & Hu, F. (1999) *Rickettsia prowazekii* Transports UMP and GMP, but not CMP, as Building Blocks for RNA Synthesis. *Journal of Bacteriology*, 181, 3238- 3241.
- Wisseman, C. L. Jr., Waddell, A. D., & Walsh, W. T. (1974) In Vitro Studies of the Action of Antibiotics on *Rickettsia prowazekii* by Two Basic Methods of Cell Culture. *Journal of Infectious Diseases*, 130(6), 564-574.
- Woods, M. E., & Olano, J. P. (2008) Host Defenses to *Rickettsia rickettsii* Infection Contribute to Increased Microvascular Permeability in Human Cerebral Endothelial Cells. *Journal of Clinical Immunology*, 28,174-185.
- Yu, X.-J., & Walker, D. H. (2005) Genus I. *Rickettsia* da Rocha-Lima 1916, 567^{AL}, pgs. 96-113 in D. J. Brenner, N. R. Krieg, & J. T. Staley (ed.), *Bergey's Manual of Systematic Bacteriology*. 2nd ed., Vol. 2, Springer, NE.

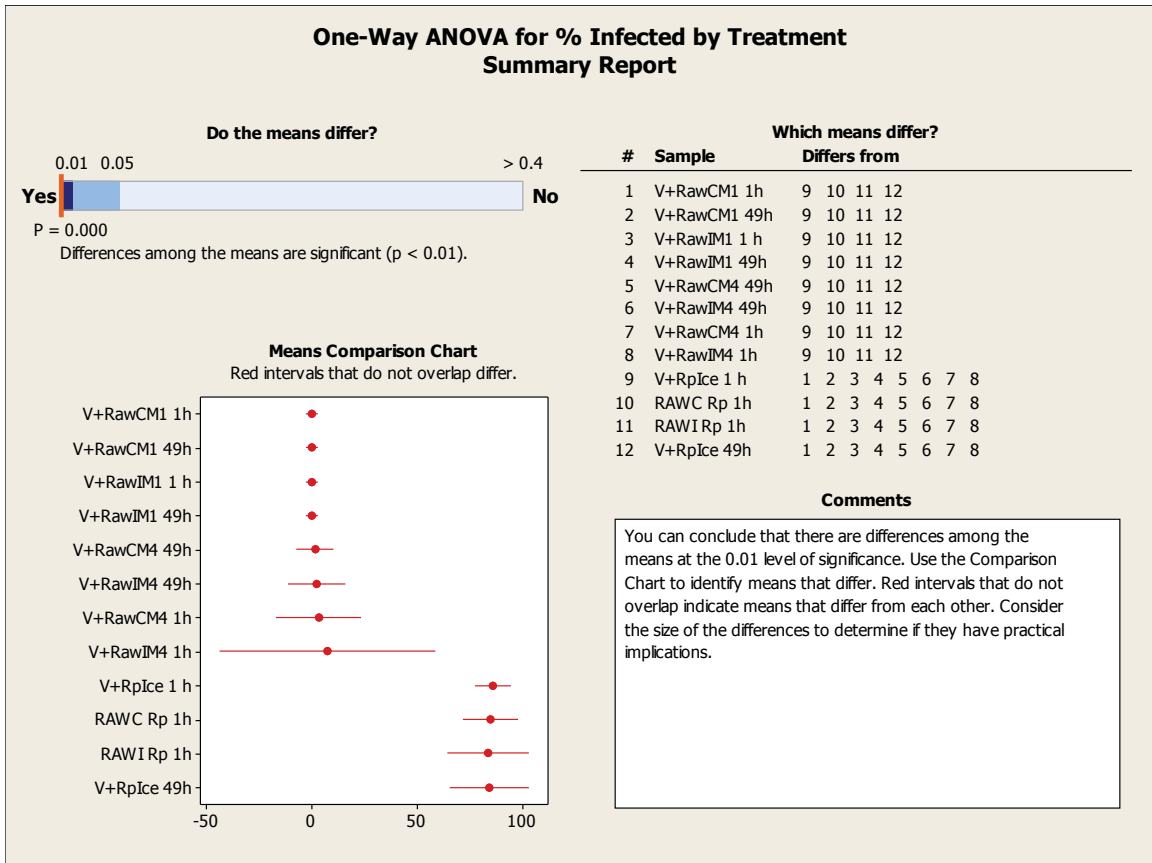
APPENDIX:
Statistical Analyses

Figure A1: One-Way Analysis of Variance (ANOVA) for Rp/Infected Cell by Treatments for Vero Cell Cultures Incubated with Culture Media Collected from *R. prowazekii* Breinl-Infected RAW264.7 Cell Cultures.



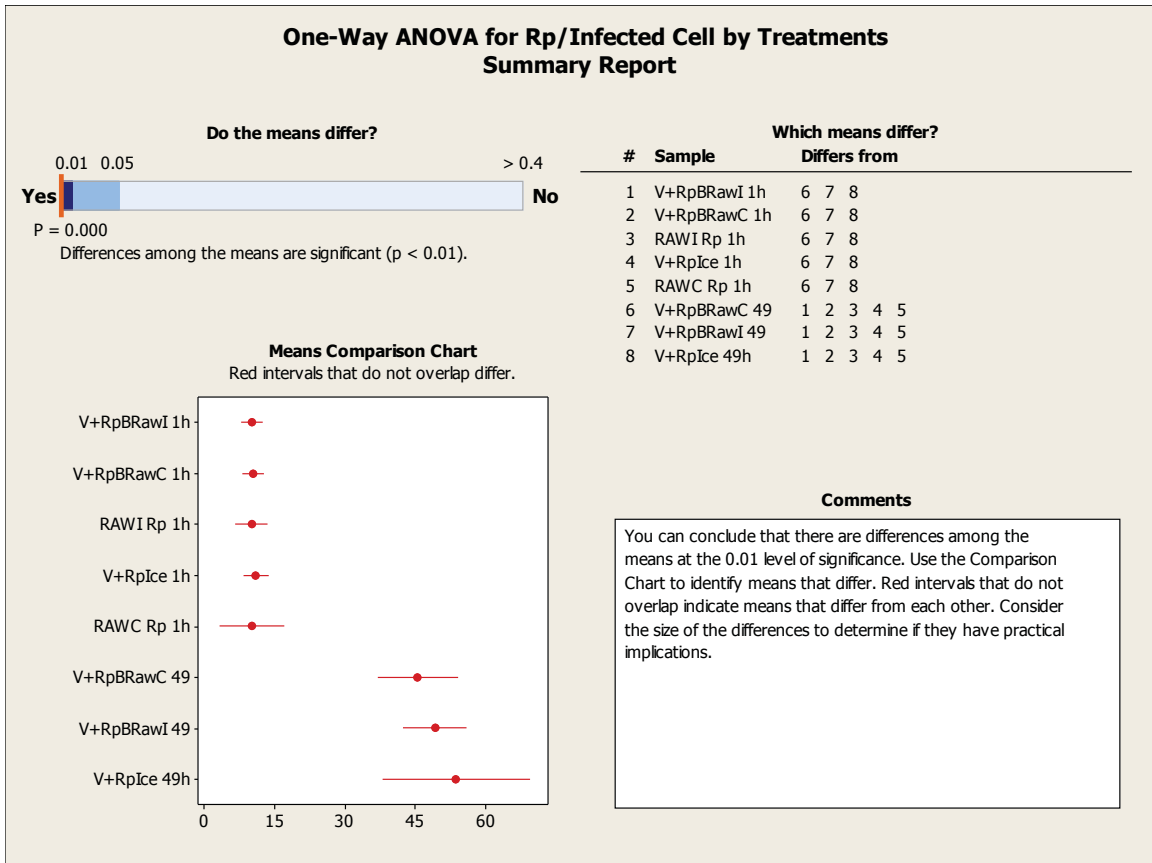
Details are given in the legend for Table 1. One-way ANOVA and multiple comparisons were done using the Assistant in Minitab 16. Rp, *R. prowazekii*; V, Vero cells; RAW, RAW264.7 cells; C, untreated control; I, IFN- γ ; RpIce, Rp that was held on ice before dilution and addition to Vero cells; 1h, 1 hour after additions to Vero cells (or RAW cells), 49h, 49 hours after additions to Vero cells.

Figure A2: One-Way ANOVA for Percent Infected Cells by Treatments for Vero Cell Cultures Incubated with Culture Media Collected from *R. prowazekii* Breinl-Infected RAW264.7 Cell Cultures.



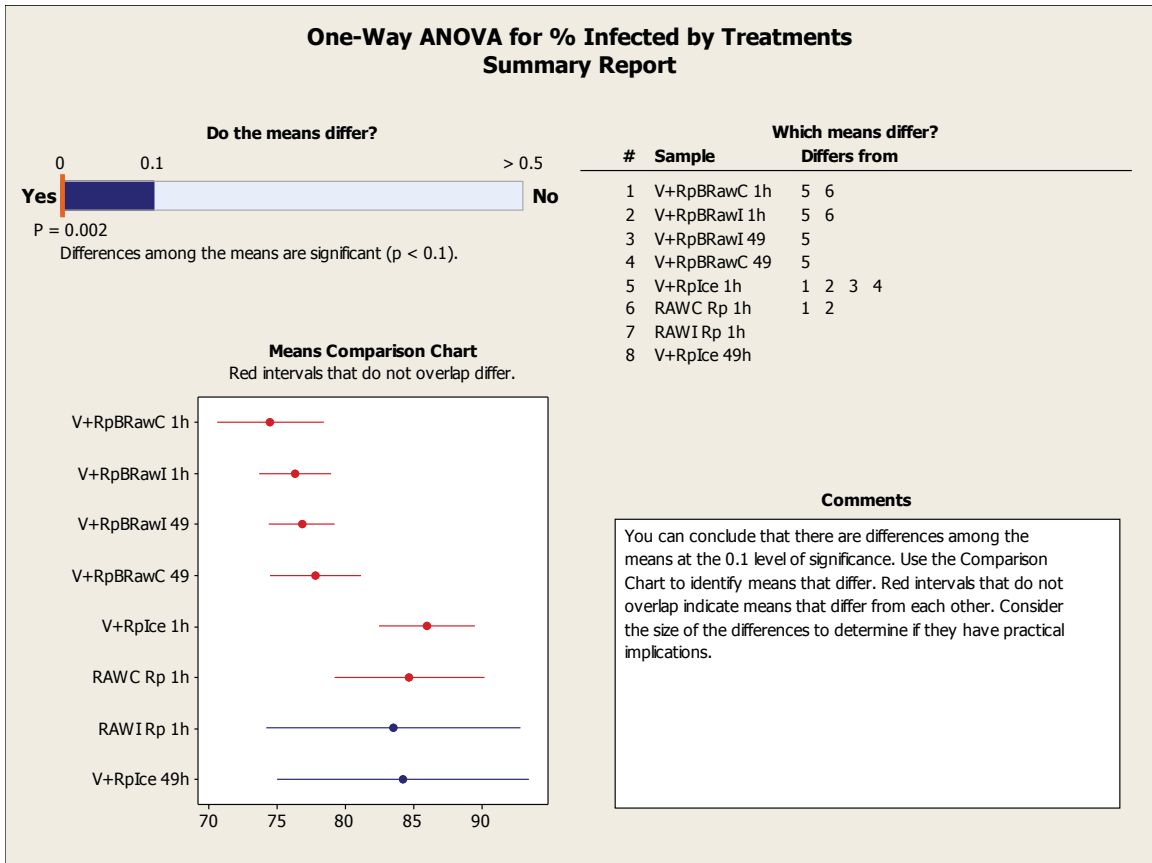
Details are given in the legend for Table 1. One-way ANOVA and multiple comparisons were done using the Assistant in Minitab 16. Rp, *R. prowazekii*; V, Vero cells; RAW, RAW264.7 cells; C, untreated control; I, IFN- γ ; RpIce, Rp that was held on ice before dilution and addition to Vero cells; RawM1 and RawM4, media removed from infected RAW cells 1 hour or 4 hours (respectively) after initial addition of Rp; 1h, 1 hour after additions to Vero cells (or RAW cells), 49h, 49 hours after additions to Vero cells.

Figure A3: One-Way ANOVA for Rp/Infected Cell by Treatments for Vero Cell Cultures Incubated with Bead-Beaten RAW264.7 Cells Infected With *R. prowazekii* Breinl Strain.



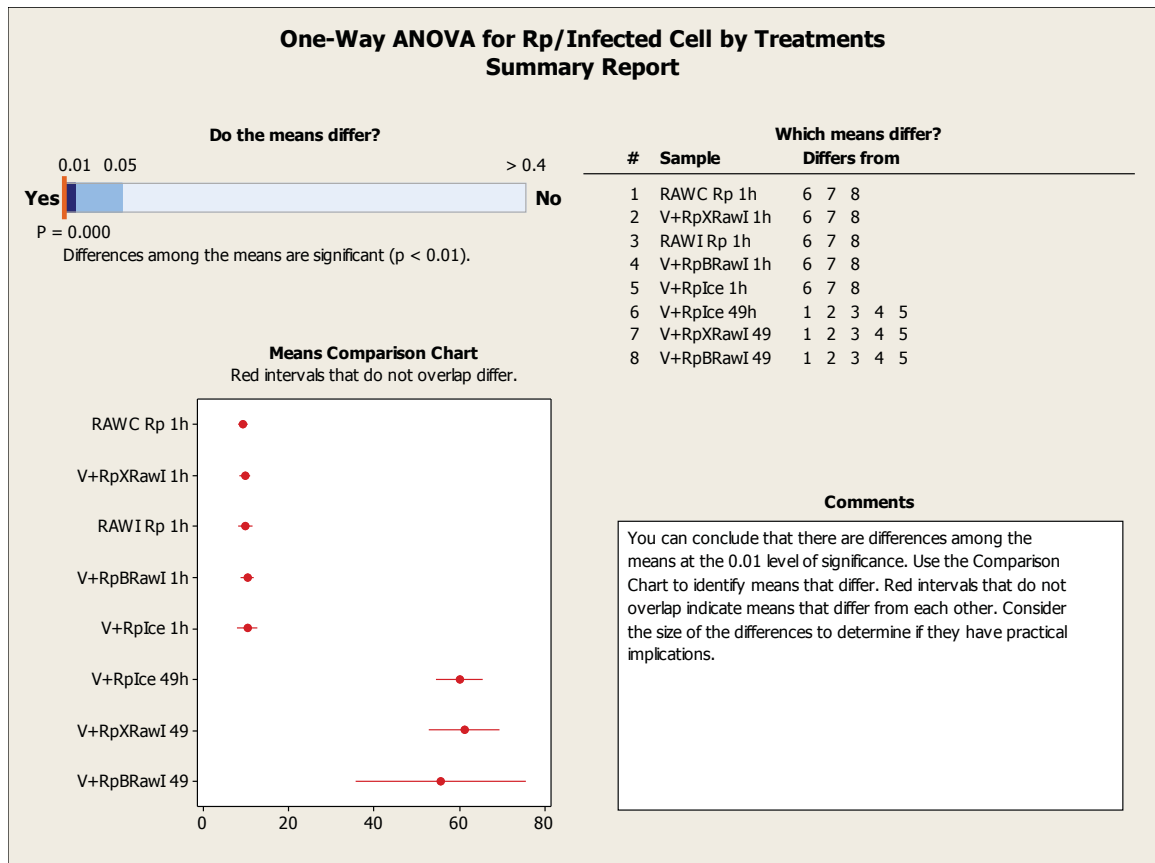
One-way ANOVA and multiple comparisons were done using the Assistant in Minitab 16. Rp, *R. prowazekii*; V, Vero cells; RAW, RAW264.7 cells; C, untreated control; I, IFN- γ , RpIce, Rp that was held on ice before dilution and addition to Vero cells; RpBRawC, Rp released from bead-beaten, untreated RAW cells; RpBRawI, Rp released from bead-beaten, IFN- γ -treated RAW cells; 1h, 1 hour after additions to Vero cells or RAW cells; 49h or 49, 49 hours after additions to Vero cells.

Figure A4: One-Way ANOVA for Percent Infected Cells by Treatments for Vero Cell Cultures Incubated with Bead-Beaten RAW264.7 Cells Infected With *R. prowazekii* Breinl Strain.



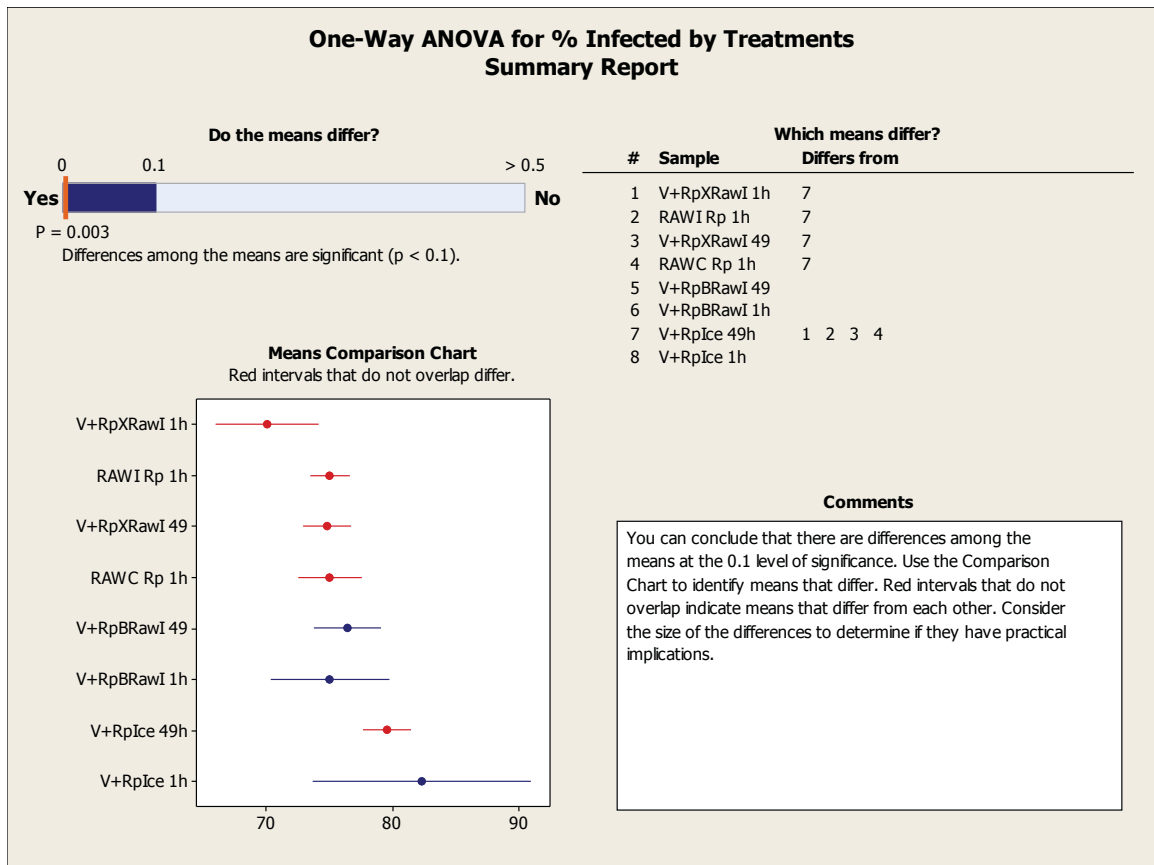
Details are given in the legend for Figure A3.

Figure A5: One-Way ANOVA for Rp/Infected Cell by Treatments for Vero Cell Cultures Incubated with Bead-Beaten or Vortexed RAW264.7 Cells Infected With *R. prowazekii* Breinl Strain.



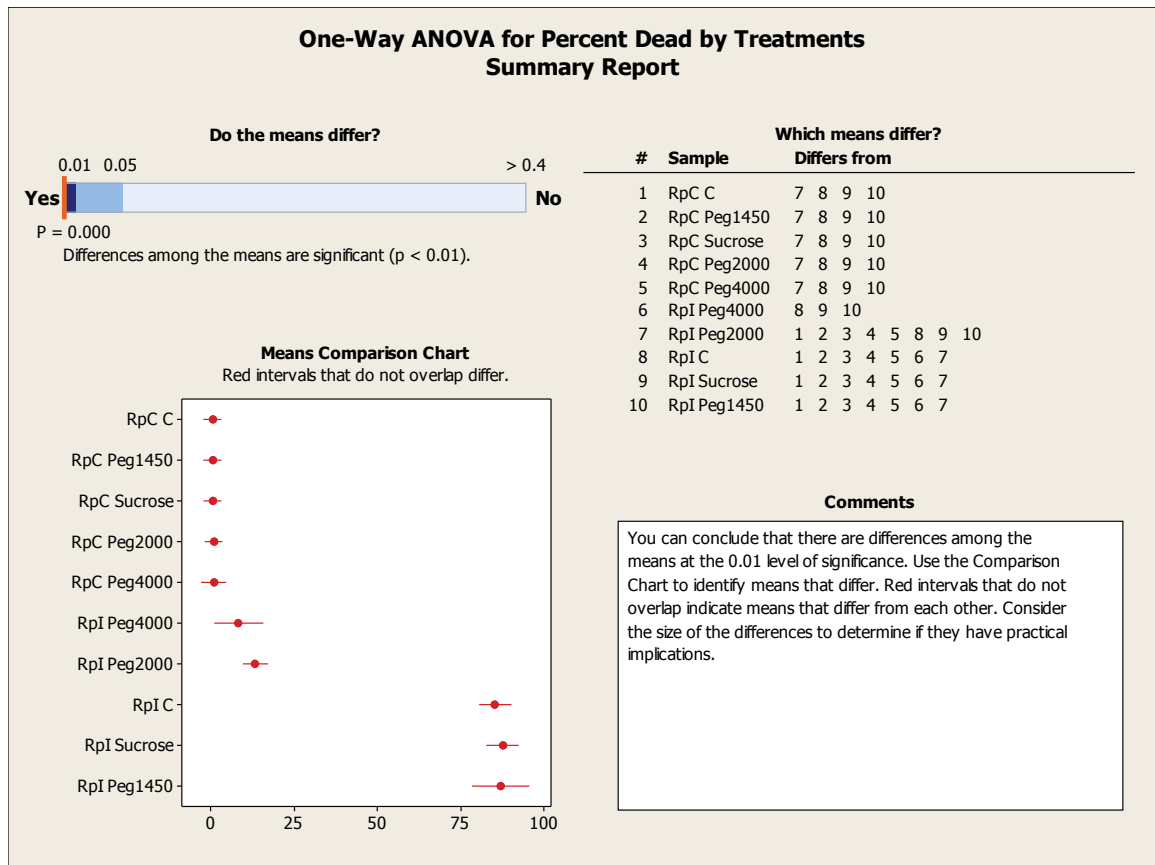
One-way ANOVA and multiple comparisons were done using Minitab 16. V, Vero cells; RAW, RAW264.7 cells; Rp, *R. prowazekii*; C, untreated control; I, IFN- γ ; RpIce, Rp that was held on ice before dilution and addition to Vero cells; RpBRawI, Rp released from IFN- γ -treated, bead-beaten RAW cells; RpXRawI, Rp released from IFN- γ -treated, vortexed RAW cells; 1h, 1 hour after additions to Vero cells or RAW cells; 49h or 49, 49 hours after additions to Vero cells.

Figure A6: One-Way ANOVA for Percent Infected Cells by Treatments for Vero Cell Cultures Incubated with Bead-Beaten or Vortexed RAW264.7 Cells Infected With *R. prowazekii* Breinl Strain.



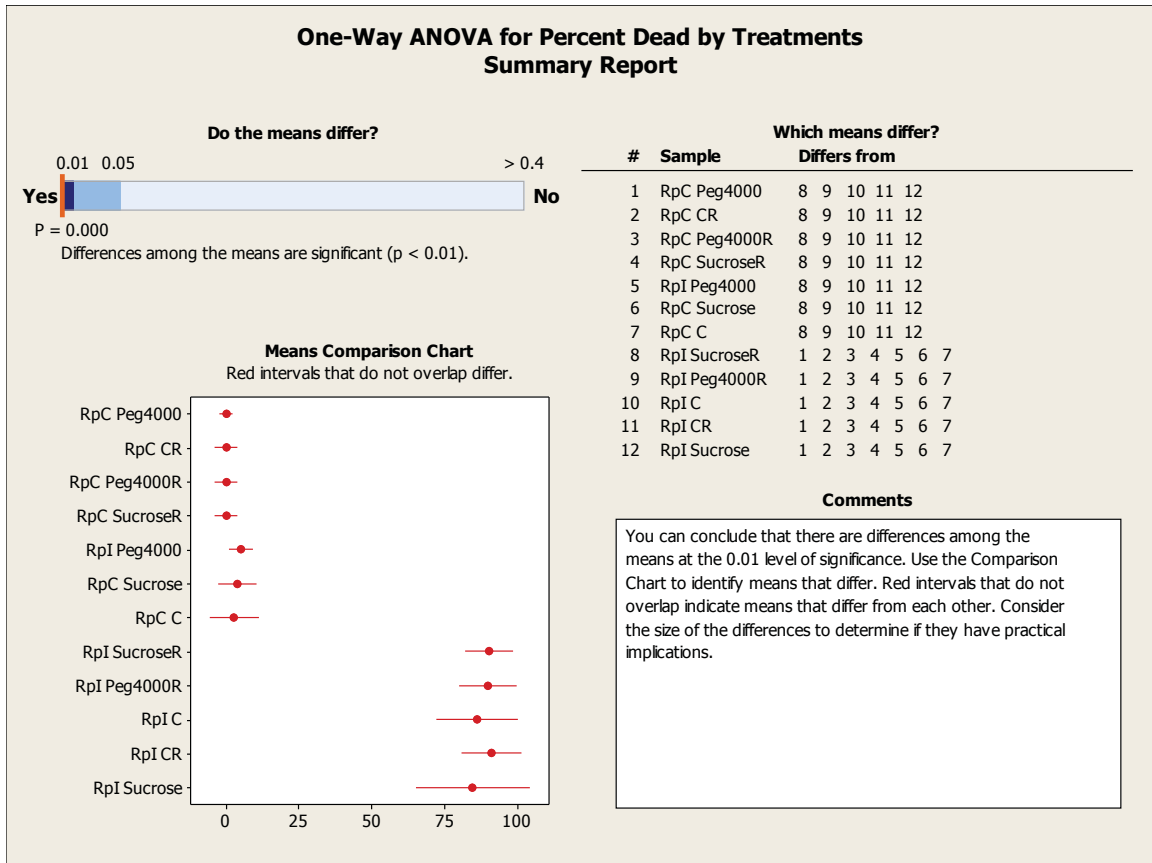
Details are given in the legend for Figure A5.

Figure A7: One-Way ANOVA for Percent Dead RAW264.7 Cells by Treatments for Cultures Infected with *R. prowazekii* Madrid E Strain in the Absence or Presence of Sucrose or PEGs of Different Molecular Weights.



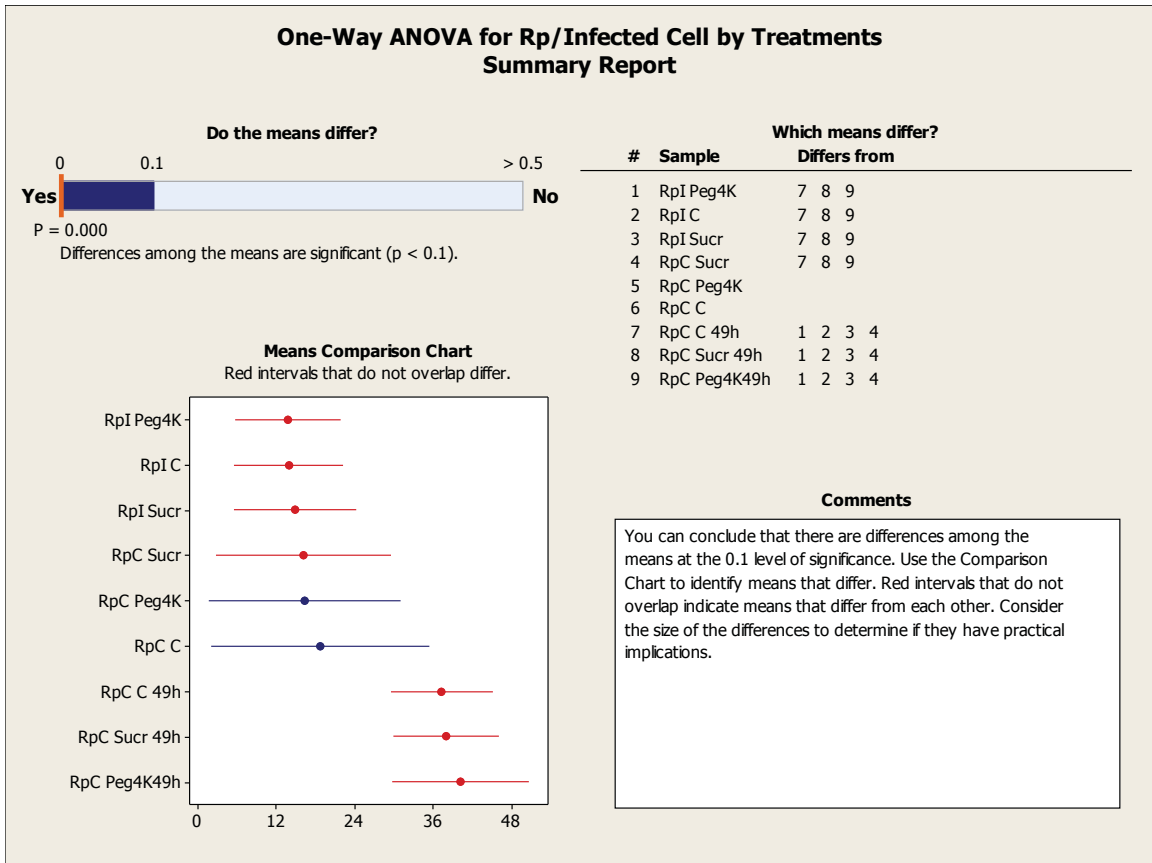
One-way ANOVA and multiple comparisons were done using Minitab 16. RpC, untreated RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of PEGs (Peg) or sucrose; RpI, IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of PEGs or sucrose.

Figure A8: One-Way ANOVA for Percent Dead RAW264.7 Cells by Treatments for Cultures Infected with *R. prowazekii* Breinl Strain in the Absence or Presence of Sucrose or PEG 4000.



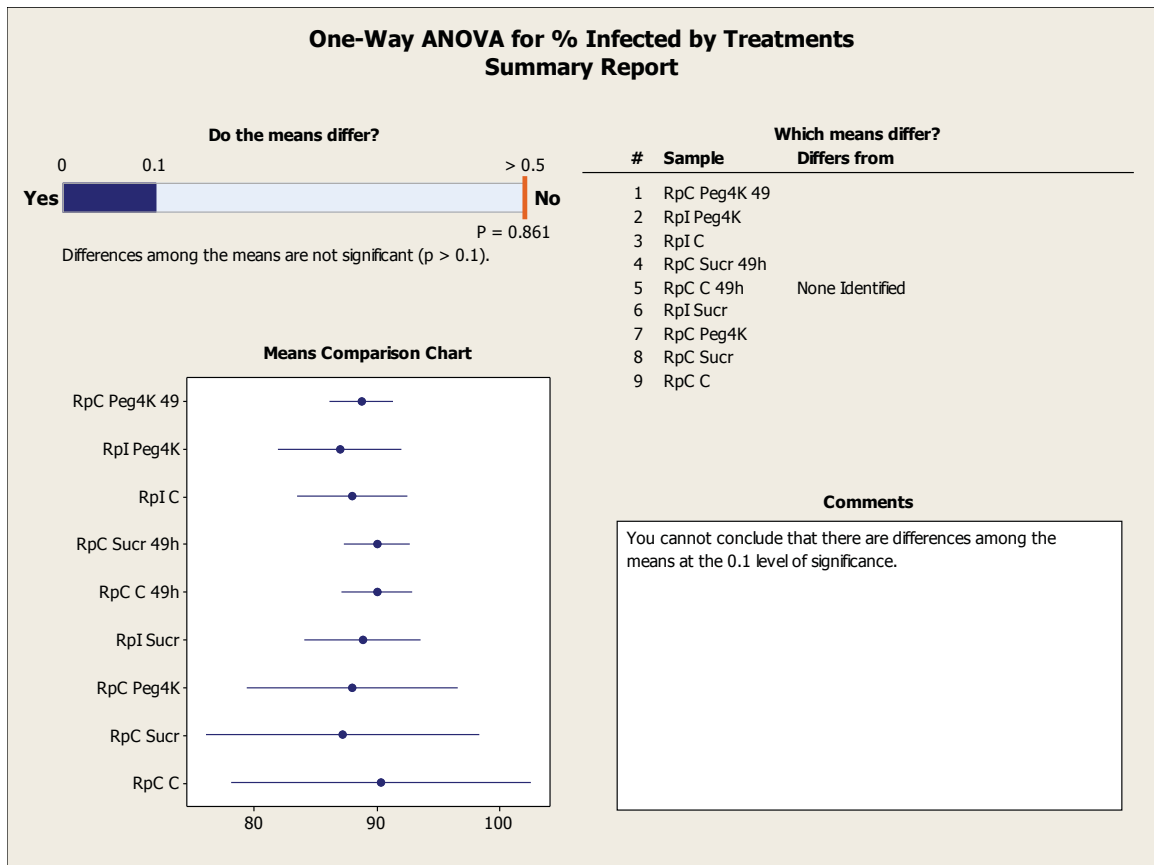
One-way ANOVA and multiple comparisons were done using Minitab 16. RpC, RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of PEG (Peg) or sucrose; RpI, IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of PEG or sucrose. R indicates that the medium was removed 4 hours after addition of rickettsiae and it was replaced with fresh medium without PEG or sucrose for an additional 3-hour incubation period.

Figure A9: One-Way ANOVA for Rp/Infected Cell by Treatments for RAW264.7 Cell Cultures Infected with *R. prowazekii* Breinl Strain in the Absence or Presence of Sucrose or PEG 4000.



One-way ANOVA and multiple comparisons were done using Minitab 16. RpC, RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of PEG 4000 (Peg4K) or sucrose (Sucr); RpI, IFN- γ -treated RAW264.7 cells infected with *R. prowazekii* in the absence (C) or presence of Peg4K or Sucr. For treatments labeled with 49h or 49, the medium was removed from the cells 4 hours after addition of the rickettsiae. The cells were then washed and given fresh medium without PEG or Sucr until staining was done at 49 hours. All remaining treatments represent cells stained 1 hour after addition of the rickettsiae.

Figure A10: One-Way ANOVA for Percent Infected RAW264.7 Cells by Treatments for Cultures Infected with *R. prowazekii* Breinl Strain in the Absence or Presence of Sucrose or PEG 4000.



Details are given in the legend for Figure A9.